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Reptiles and Amphibians

Edited by David Ramiro Aguillón Gutiérrez

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Meet the editor

David Ramiro Aguillón-Gutiérrez was born in Mexico. He graduated from the Faculty of Veterinary Medicine and Animal Sciences, Autonomous University of Nuevo Leon, Mexico, in 2003. He obtained his MSc degree in Biological Sciences (specialist in Embryology) from the Lomonosov Moscow State University, Russia, in 2009 and his PhD degree in Biological Sciences (specialist in Embryology and Zoology) from the same university in 2012. From 2013 to 2014 he was a postdoctoral researcher in Biodiversity and Conservation at the Biological Science Center, Autonomous University of the State of Hidalgo, Mexico. Currently, he is a Professor at the Faculty of Biological Sciences, Juarez University of the State of Durango, Mexico.

Contents

Preface

It is a great honor for me to introduce this book entitled *Reptiles and Amphibians*. This project started in 2017 and reached its goal this year, 2018.

Reptiles and amphibians are vertebrate animals, and research on them is essential for an integral understanding of nature.

The book *Reptiles and Amphibians* will provide a view from various studies dealing with herpetology, all of them equally interesting and important.

In this book, diverse subjects on reptiles and amphibians are approached, among them the conservation, life histories, evolution, physiology, bioacoustics, monitoring and threats.

This book has eight chapters, with the participation of authors of six countries from all over the world (Brazil, France, México, Morocco, Scotland and Spain).

As an editor of this book, I would like to thank all the contributors, first to the IntechOpen publishing house, especially the Author Service Manager, Ms. Lada Bozic; second to the authors of the chapters, thank you for the effort and patience; and then to the readers of this book, we hope to fulfill their expectations.

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Chapter 1

Introductory Chapter: General Features of Reptiles and Amphibians

David Ramiro Aguillón Gutiérrez

Additional information is available at the end of the chapter

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1. Introduction

Reptiles and amphibians are ectothermic vertebrate animals with a wide distribution on the planet [1]. Although there are similarities between these biological groups, they also have many differences; however, both are studied under the same science, herpetology.

The reasons why herpetology studies these two groups (reptiles and amphibians) are not only exclusively based on taxonomy or phylogenetics (reptiles certainly originated from amphibians), but also in ecology, as the methods to study both reptiles and amphibians are in many cases similar [2].

Reptiles (Class Reptilia or Sauropsida) are organisms that have scales on the skin, are amniotes, which have colonized almost all terrestrial and marine environments, originated in the Carboniferous from the amphibians, they have a tricameral or tetracameral (crocodiles) heart, are phylogenetically related to birds (these arose from reptiles), ectotherms, and are characterized by being very efficient in the use of energy. Currently, there are four orders of reptiles with living representatives: Testudines (turtles), Squamata (snakes and lizards), Crocodilia (crocodiles, alligators, and gharials), and Sphenodontia or Rhynchocephalia (tuataras), of which there are only two species in New Zealand [3].

Amphibians (Class Amphibia), are characterized by having a naked skin, without scales, hair, or feathers, are ectotherms, anamniotas, have a tricameral heart, with three possible types of breathing (pulmonary, branchial, and cutaneous), and which are the only group of vertebrates that undergo metamorphosis, besides presenting some neotenic organisms. They originated in the Devonian from fish with lobed fins (Sarcopterygii). They have colonized almost all terrestrial (except Antarctic) and freshwater environments. Currently, there are three orders of amphibians with living representatives: Anura or Salientia (frogs and toads), Urodela or

Caudata (tritons and salamanders), and Gymnophiona or Apoda (caecilians). It is the group of vertebrates that proportionally has the highest number of endangered species [3–7].

Currently, both amphibians and reptiles face serious threats that place them at risk of disappearing. These threats include habitat loss, climate change, pollution, invasive species, illegal trade of species, emerging diseases, myths, and cultural factors [3, 8–10].

Conservation strategies for amphibians and reptiles, as for other biological groups, are very diverse and will depend on the particular threats that each site or species has. Among the most important strategies for the conservation of herpetofauna, we can mention the conservation of habitat, decree-protected natural areas and biological corridors, an increase of knowledge about herpetofaunal diversity through listings or inventories, deepening of research on their ecology of populations and communities, participation in trophic networks, and interactions with other organisms. Other strategies are to carry out reintroduction, introduction and increase programs, avoid or minimize pollution, monitor diseases, control the illegal trafficking of species, avoid the introduction of exotic species, and environmental education where respect and care of these living beings are promoted [3, 11].

Reptiles and amphibians, like other organisms, are bioindicators of environmental health, because when there are diseases of an epidemic nature, anomalies in more than 5% of the population, population declines or extinction of species, both locally and globally, we must consider that probably some anthropogenic factor is causing these effects, affecting not only amphibians and reptiles but the environment in general [12, 13].

Another important aspect is the use of reptiles and amphibians as models of research in experimental biology and biomedicine, since areas such as embryology (developmental biology), biology and regenerative medicine, ecotoxicology, endocrinology, and physiology have been greatly favored by the use of these animals in the laboratory [14, 15].

Also, reptiles and amphibians have been used as a source of food (frog legs, crocodile, turtle, snake, and iguana meat), as a source for making accessories or clothing (crocodile and snake skin, tortoiseshell), and as a source of medicines (bufotoxin, bufotenin, and antivenom).

As we can see, reptiles and amphibians have always been of great importance to society, so it is our responsibility to conserve them today and to ensure that these biological groups continue to exist in the future.

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Chapter 2

Speciation Routes of Anurans

Maria Luiza Beçak

Additional information is available at the end of the chapter

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Abstract

Genome duplication was long been reported by our laboratory in Brazilian anurans belonging to the Leptodactylidae and Hylidae families. Thanks to other investigators, several polyploid species with related diploid forms were also found in diverse regions of South America, Africa and Australia. Our cytogenetic, enzymatic and molecular experiments performed in the Brazilian polyploids showed a higher level of variability resulting from tetrasomic gene expression according to the binomial equation $(p + q)^4$. These results were supported by the classical Ohno's theory of vertebrate evolution via genome duplication. Later, we suggested that the epigenetic mutations could have a role in the expansion of the tetraploid animals exploring different regions. Here, we provide an overview of the data on polyploid anurans in light of the speciation process. Some biogeographic events of Mesozoic and Cenozoic Eras probably related to anurans evolution were also focused.

Keywords: anurans evolution, genome duplication, epigenesis, species diversification, geographic speciation concept

1. Speciation rhythms: slow × fast concepts

The current neo-Darwinism concept of evolution stated that speciation is a slow accumulation of random mutations and selection of favored phenotypes. Abrupt mass extinctions of the species and the appearance of new forms occurred in the Earth due to tectonisms, volcanisms, meteors and global warming.

The neo-Darwinism theory explains that the environmental alterations are associated with DNA modifications as changes of the amount of DNA content, point mutations, differences of either gene number, order and position. Up to the classical concept, reproductive isolation is created when allopatric populations separated by geographic barriers accumulate a differential pool of genetic mutations that prevent interbreeding. The idea that speciation is a slow process was recently supported by paleontological data indicating that some animal groups like dinosaurs and birds evolved gradually and not rapidly over long timescales [1].

Alternatively, a growing body of recent evidences suggests that new species can diversify rapidly in response to ecological alterations. An example of this hypothesis is the speciation of the killer whales demonstrated to occur in sympatric populations without geographic barriers but impelled by the search of new ecological niches [2]. Also, it has been recommended that fast speciation may also occur by epigenetic mutations affecting the mechanisms of gene regulation and being transgenerationally inherited [3–5]. Also, previous molecular results in studies with synthetic polyploids of *Brassica* suggested that fast genome changes may occur in new polyploids. This fact would favor the diversifications of both plants and animals [6].

2. Epigenetic traits in the evolution of polyploidy anurans

It is well established that each species contains a specific genome with distinct DNA sequences coding for proteins as well as an epigenome with other DNA sequences involved in gene regulation. The mechanisms of gene regulation include processes of methylation of DNA, alterations of histones by methylation or acetylation as well as noncoding DNA [7]. These mechanisms play important functions in cell differentiation during embryo development. The epigenetic traits remain in all adult life through mitosis. At gametogenesis, a new programmed epigenetic architecture is established in embryo development. Some alterations of gene expression produced by changes of these regulatory sequences without alterations of DNA sequences may create epigenetic mutations. It was suggested that this type of mutation can be transgenerationally inherited, as indicated in experiments with *Arabidopsis thaliana* [8].

In anurans, it was questioned whether epigenetic mechanisms besides polyploidy may have contributed creating diversity in this animal group [4]. This conclusion is based on about five findings reported in the tetraploid *Odontophrynus americanus*: (1) that the amount of RNA content in 4n animals is not increased [9], although they have double amount of 18S and 28S ribosomal genes [10]; (2) that a higher variability of isozymes and other proteins in 4n species is caused by the tetrasomic expression $(p + q)^4$ [11–15]. While in diploid species, the population equilibrium may be translated by the equation $(p + q)^2$, thus $p^2 + 2pq + q^2$ in tetraploid populations, it is translated by the equation $(p + q)^4$, thus $p4 + 4p^3q + 6p^2q^2 + 4pq^3 + q^4$. The geographical expansion showed by heterozygotic population is amplified, meaning increased quantitative and qualitative diversity. Although tetraploid species present double number of chromosomes, only genes of two chromosomes of the four homologous chromosomes are expressed. This fact was shown in the expression of albumin, G6PD and LDH genes in the electrophoresis analysis of tetraploid species [11]. Duplication of genes allows that although variability of the species is maintained by two genes of each allele, the two remaining homologous genes are free to mutate and eventually be positively selected leading to advantageous diversification and expansion of niches occupied by the tetraploid species [16]. These results are coherent with the concept of evolution by gene duplication [17]; (3) that there is a lower hemoglobin content in the 4n as to the 2n animals [18]; (4) that the reduced gene expression in the 4n animals is caused by the repression of half of the rDNA cistrons [19]; (5) that there is an amphiplasty configuration characterized by diphasic stages of cell cycle revealed in the two halves of the 4n genome. It may point differences in the DNA replication time probably caused by methylation [4, 20].

Based on these data, we reasoned whether the epigenetic regulation may have contributed to the wide expansion of the polyploid species through an epigenetic transgenerational inheritance. This idea would imply that the natural selection of non-Mendelian epigenetic mutations may operate in the evolution.

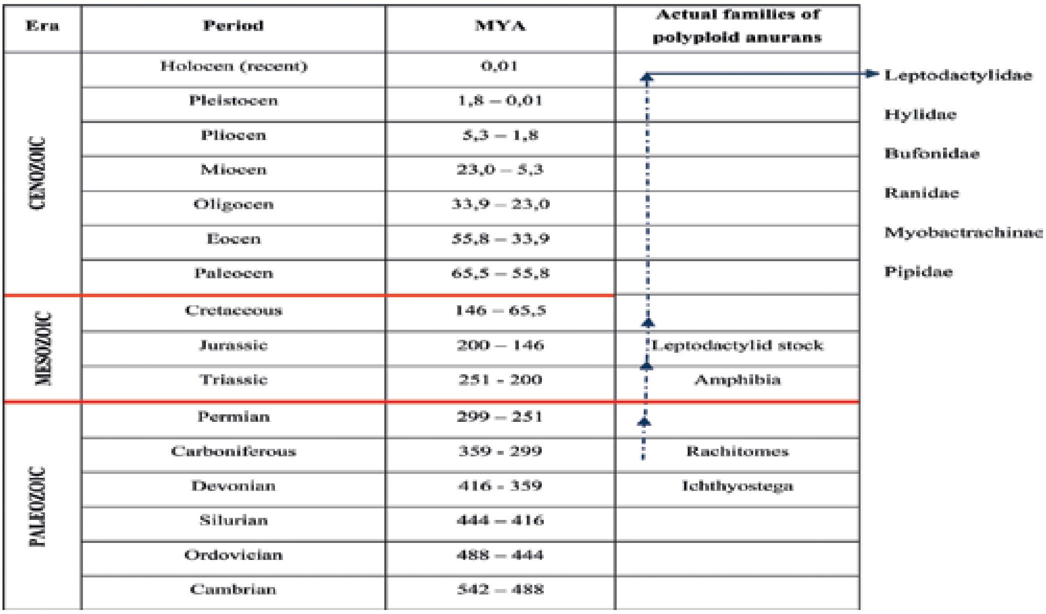
3. Leptodactylids evolution and correlated geobiological events

To get a better understanding on the mechanisms that accounted for Leptodactylids evolution, we also focused on some geobiological events of the Mesozoic/Cenozoic eras that drastically altered climate and ecological environments.

The knowledge of anurans evolution is greatly impaired by the scarcity of fossil records. The extinct Ichthyostega group was found to be the most primitive ancestral of living amphibians. These animals that lived in the Devonian period of the Paleozoic era (about 400 mya) evolved to the Rachitomes in the Carboniferous period (about 300 mya). During the Triassic period of Mesozoic era, the Rachitomes evolved to the Amphibia (200 mya) [17] (**Table 1**).

Systematics and cytogenetic data reviewed by Morescalchi [21] showed that the actual anurans are reminiscent of a Leptodactylid stock in the Jurassic period (about 150 mya) and diversified since the Cretaceous before the final breakage of the supercontinent Gondwan. Probably before the Cenozoic, some forms spread across parts of Gondwanaland and perhaps oriental parts. These anurans include Leptodactylidae, Hylidae, Bufonidae and Ranidae. The diversification of these forms is also extended to the Tertiary period from Paleocene to Miocene/Pliocene (65.5–1.8 mya). The origin of the Myobatrachidae is questioned whether it is related to the Bufonidae before Gondwan breakages [21] (**Tables 1** and **2**). The family Pipidae is one of the oldest families (early Jurassic) with forms specialized in early Cretaceous [29] and probably derived from Ascaphid forms [30] or from a pro-Anura stock [31, 32]. A more recent origin (Eocene/Miocene) was indicated to Brachicephalidae, Centrolenidae and Pseudidae [21]. The genus *Odontophrynus* classified in the Leptodactylidae family [33] was recently reclassified in the family Odontophrynidae (Anura: Neobatrachia) [28].

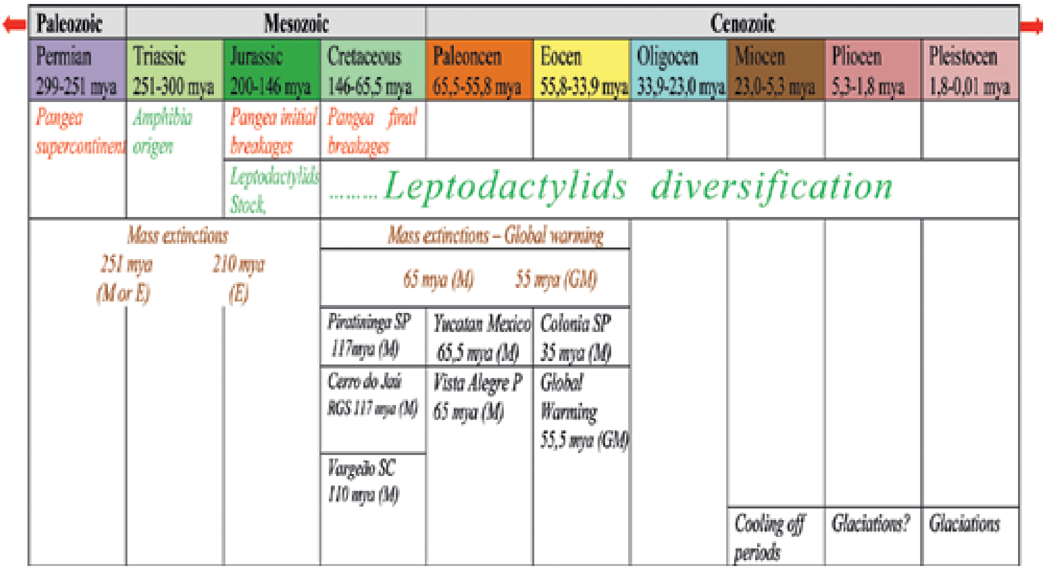
During evolution, these anurans faced several earth alterations caused by diverse events as Pangea fragmentation, impact of meteors, volcanisms and glaciations. Some of these alterations caused mass extinctions of most plant and animal species (**Table 2**; **Figure 1A** and **B**). Among these events, Pangea splitted forming Eurasia and Gondwan supercontinents in the Jurassic period (**Figure 1A** and **B**). After new breakages in the Cretaceous (about 65 mya), Eurasia separated in North America, Europe and Asia, and Gondwan splitted in South



Era	Period	MYA	Actual families of polyploid anurans
CENOZOIC	Holocen (recent)	0,01	
	Pleistocen	1,8 – 0,01	
	Pliocen	5,3 – 1,8	
	Miocen	23,0 – 5,3	
	Oligocen	33,9 – 23,0	
	Eocen	55,8 – 33,9	
	Paleocen	65,5 – 55,8	
MESOZOIC	Cretaceous	146 – 65,5	
	Jurassic	200 – 146	Leptodactylid stock
	Triassic	251 - 200	Amphibia
PALEOZOIC	Permian	299 – 251	
	Carboniferous	359 - 299	Rachitomes
	Devonian	416 - 359	Ichthyostega
	Silurian	444 – 416	
	Ordovician	488 – 444	
	Cambrian	542 – 488	

Leptodactylidae
Hylidae
Bufonidae
Ranidae
Myobactrachinae
Pipidae

Table 1. Evolution of actual polyploid anurans derived from a Leptodactylid stock: phylogenetic tree based on Ohno [17] and Morescalchi [21]; geologic periods from Teixeira et al. [22], mya = millions years ago.



Paleozoic	Mesozoic			Cenozoic					
Permian 299-251 mya	Triassic 251-300 mya	Jurassic 200-146 mya	Cretaceous 146-65,5 mya	Paleocen 65,5-55,8 mya	Eocen 55,8-33,9 mya	Oligocen 33,9-23,0 mya	Miocen 23,0-5,3 mya	Pliocen 5,3-1,8 mya	Pleistocen 1,8-0,01 mya
Pangea supercontinent	Amphibia origen	Pangea initial breakages	Pangea final breakages						
		Leptodactylids Stock,	.... Leptodactylids diversification						
Mass extinctions			Mass extinctions – Global warming						
251 mya (M or E)	210 mya (E)			65 mya (M)	55 mya (GM)				
			Piratininga SP 117mya (M)	Yucatan Mexico 65,5 mya (M)	Colonia SP 35 mya (M)				
			Cerro do Jaú RGS 117 mya (M)	Vista Alegre P 65 mya (M)	Global Warming 55,5 mya (GM)				
			Vargeão SC 110 mya (M)						
							Cooling off periods	Glaciations?	Glaciations

Table 2. Evolution of Leptodactylids (based on Morescalchi) [21] and some geobiological events during Mesozoic/ Cenozoic periods: continental drift [23]; glaciations and mass extinction [24, 25]; geologic times [22]; meteors [26], mya = million years ago, M = meteor, E = eruption, polyploidy was assumed to have happened in the Eocene in ***Xenopus*** [27] and in the ***Odontophrynus*** [28].

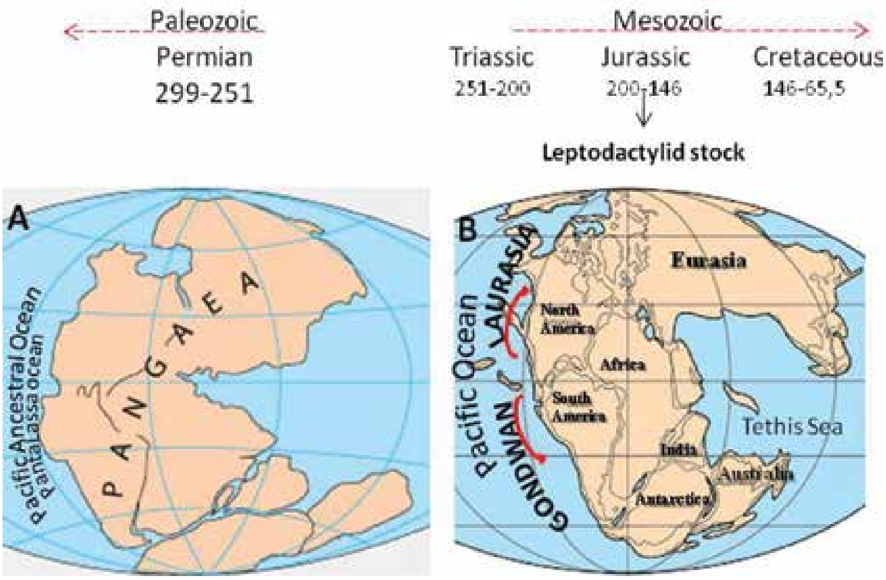


Figure 1. A schematic drawing showing the Pangea supercontinent in the Permian period (A) and its initial fragmentation in Laurasia and Gondwana during Jurassic/cretaceous (about 150 mya) followed by new fragmentation at the final of cretaceous (about 65 mya) that led to the actual configurations of the continents (B). Figures based on data by Grotzingert and Jordan [24], Gaeta Tassinari et al. [23] and geology.com/articles/supercontinent.shtml. The Leptodactylid stock occurred in the Jurassic period (about 150 mya) according to Morescalchi [21].

America, Africa, Australia and India [23] (**Figure 2**). As it is known, the movements and breakages of the ancient continents are explained by the theory of collision of tectonic plates [23, 34]. This theory confirmed the continental drift hypothesis of Wegener's (1912, 1915) on the breakages of the single earth supercontinent that led to current configuration [35].

In the case of southeastern Brazil, several meteors fell in diverse states during the Cretaceous and Cenozoic (**Table 2**; **Figure 3**). Some of these events caused mass extinction by alterations of temperature, earthquakes and volcanisms.

Another type of mass extinction occurred in the Paleocene/Eocene (about 55 mya) being caused by a global warming produced by the liberations of gas methane (**Table 2**) [25].

Several species were also eliminated by the glaciations in the Pleistocene and new species appeared. It was suggested that these glaciations could be extended to Pliocene (5.3–1.8 mya) and the cooling probably preceded the Miocene (23.0–5.3 mya). Also, it was proposed that this cooling may be related to the continental drift process [24]. Interestingly, this estimate includes the time of diversification of the Leptodactylid families from Paleocene to Miocene/Pliocene (65.5–1.8 mya).

Mass extinction also occurred in the Cretaceous probably caused by meteors (**Table 2**). In the final of Cretaceous (about 65 mya), a meteor fell on the region that today corresponds to Mexico and killed 75% of the species including the dinosaurs. It is known that the extinction of the reptiles favored mammalian expansion and evolution [26]. Another hypothesis is changing the explanation

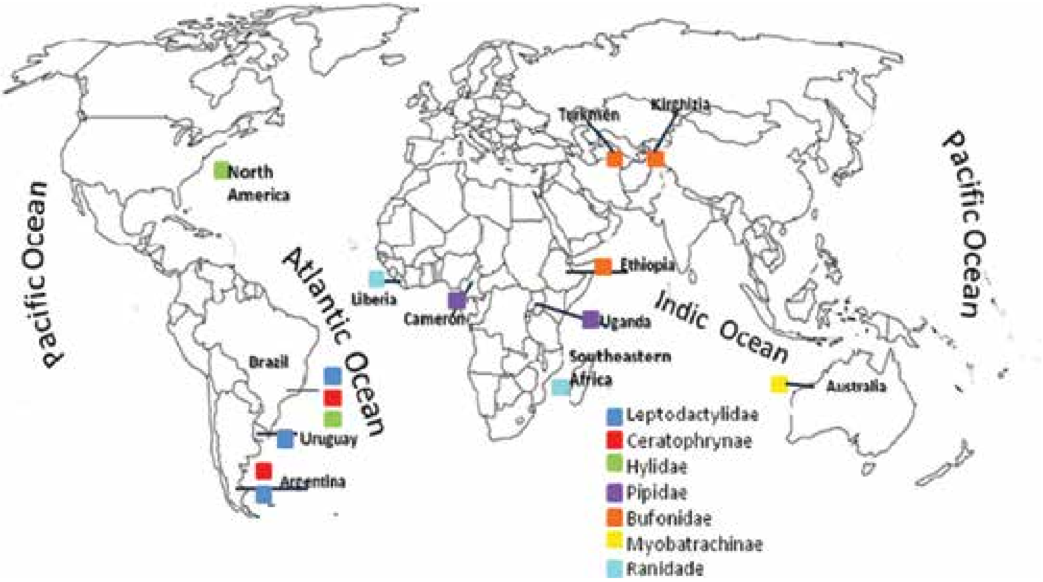


Figure 2. A schematic drawing of the present day continents with living polyploid and pos-polyploid species of anurans belonging to Gondwanian families (continental map is based on https://www.estadosecapitaisdobrasil.com/wp-content/uploads/2015/07/mapa-mundi-colorir-paises-2.pdf).

Figure 3. Localization of craters caused by meteors in southeastern Brazil during cretaceous, Paleocene and Eocene. The bigger crater in the central Brazil fell during the Triassic (based on Fairchild [26]).

that mass extinction of dinosaurs was caused only by the meteor. The data obtained in a metric called morphological disparity showed that the extinction of the carnivorous dinosaurs may also be related to the decline of the herbivorous reptiles before the striking of the meteor [36]. Also, the mass extinction of the Cretaceous was attributed to eruptions in Siberia [37].

4. Genome duplication driving Anura diversification

An extensive populational cytogenetic and molecular study was previously performed in our laboratory using Leptodactylids species derived from the Gondwanian stock. The results obtained in the genus *Odontophrynus* indicated that autopolyploidy was accounted for the diversification of the species through Brazil and other South American countries [38, 40, 51] (**Figure 4**). Other polyploid species with related 2n species were also described by several investigators in anurans from South America, Africa and Australia [41, 42] (**Tables 3** and **4**).

To verify the level of reproductive isolation between 2n and 4n species, several artificial hybridizations were performed. The mating chosen of ♀ *O. americanus* (4n = 44) × ♂ *O. cultripes* (2n = 22) and of ♀ *O. americanus* (4n = 44) × ♂ *O. americanus* (2n = 22) gave F_1 triploid hybrids (3n = 33). Some F_1 3n from ♀ *O. americanus* (4n = 44) × ♂ *O. cultripes* (2n = 22) exhibited *aneuploid* and *normal euploid* gametes. This indicated that 3n hybrids could produce descendent with higher levels of ploidy as F_2 6n animals [48, 71].

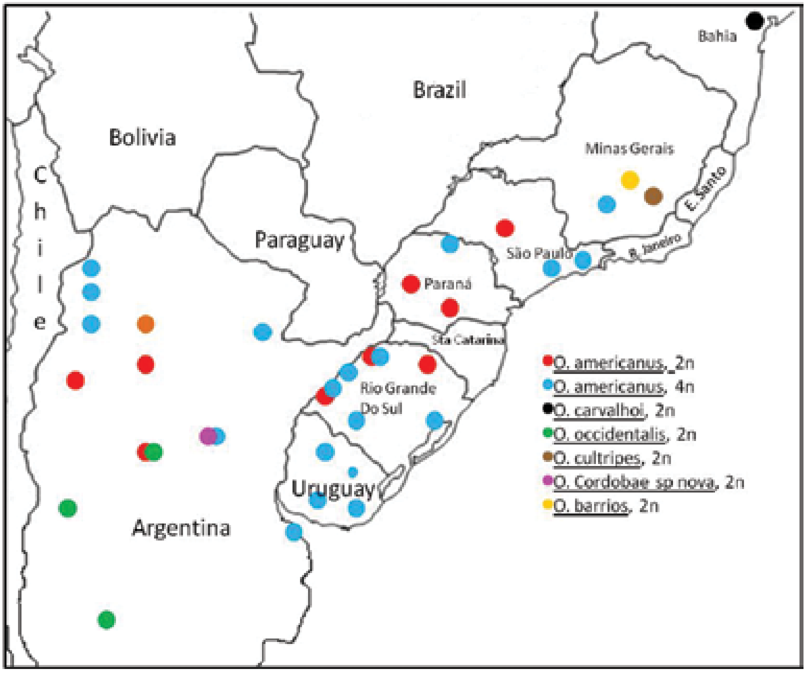


Figure 4. A schematic drawing showing the geographic distribution of diplo and tetraploid species of *O. americanus* in South American countries (see **Tables 3** and **4** for author's names) (based on Beçak [4]).

Families/species	Ploidy level	Ancestral number	References/localities
Leptodactylidae			
Odontophrynus americanus	4n = 44	n = 11	Beçak et al. [38]; Beçak [46]; Beçak et al. [39]; Beçak et al. [47, 48]; Beçak and Beçak [20]; Beçak and Kobashi [49]; Beçak [4]; Bogart [42]; Barrio and Pistol de Rubel [39]/Brazil and Argentina
Odontophrynus cordobae	4n = 44	n = 11	Martino and Sinsch [50]/Cordoba
Ceratophrys dorsata[*] (= *C. aurita*)	8n = 104	n = 13	Beçak et al. [38]; Beçak [46]/Brazil
Ceratophrys ornate	8n = 104	n = 13	Bogart [42]; Barrio and Chieri [52, 53]; Bogart and Wasserman [54]/Argentina
Eleuterodactylus binotatus[***] (pos-polyploid)	2n = 22	n = 11	Beçak and Beçak [55]/Brazil
Pleurodema bibroni (*P. darwinii*)	4n = 44	n = 11	Barrio and Chieri [52, 53]/Argentina
Pleurodema kriegi[**]	4n = 44	n = 11	Barrio and Chieri [52, 53]/Argentina
Pleurodema cordobae sp. nov	8n = 88	n = 11	Valetti et al. [56]; Salas et al. [57]/Argentina
Myobatrachidae			
Neobatractus sudelli[**]	4n = 48	n = 12	Ptacek et al. [58]/Australia
Neobatractus sutor[**]	4n = 48	n = 12	Mable and Roberts [59]/Australia
Hylidae			
Hyla versicolor[+]	4n = 48	n = 12	Wasserman [60]; Bogart and Wasserman [54]/North America
Phylomedusa burmeisteri[*] (P. tetraploid)	4n = 52	n = 13	Beçak et al. [47]/Brazil
Pipidae			
Xenopus vestitus[**]	4n = 72	n = 18	Tymowska et al. [62]/Uganda
Xenopus ruwenzoriensis[++,**]	6n = 108	n = 18	Tymowska and Fischberg [63]/Uganda
Xenopus sp. *wittei*[**]	4n = 72	n = 18	Fischberg and Kobel [64]; Tinsley et al. [65]/Uganda
Xenopus amieti[++]	4n = 72	n = 18	Kobel et al. [45]/Cameron
Bufonidae			
Bufo danatensis	4n = 44	n = 11	Pisanetz [66]; Kawamura [93]/Turkmen
Bufo viridis	4n = 44	n = 11	Mazik et al.[67]/Kirghizia/Russia
Bufo sp.	4n = 40	n = 10	Bogart and Tandy [68]/Ethiopia
Bufo armarae	4n = 40	n = 10	Tandy et al. [70]/Ethiopia

Families/species	Ploidy level	Ancestral number	References/localities
Ranidae			
Dicroglossus occipitalis	4n = 52	n = 13	Bogart and Tandy [68]/Liberia
Pyxicephalus delalandii	4n = 52	n = 13	Bogart and Tandy [68]/Southeastern Africa

[*] Meiotic multivalent.

[**] Few or absence of multivalent.

[***] Meiotic multivalent rings by multiple translocations pos-polyploidy.

[+] Considered to be an allopolyploid that arose from hybridization between eastern and western populations of *H. chrysosceles* [44].

[++] It is not known whether they are auto or allopolyploid species [45] based on Beçak [4] with additions.

Table 3. Polyploid species of anurans (authors/localities are indicated).

Families/species	2n	References/localities
Leptodactylidae		
Odontophrynus americanus[*]	22	Beçak et al.[47, 48]; Barrio and Pistol de Rubel [39]; Bogart and Wasserman [54]; Beçak and Beçak [41]/Brazil
Odontophrynus cultripes	22	Saez and Brum-Zorrilla [69]; Beçak [46]; Beçak et al. [39]; Beçak and Beçak [71]; Beçak and Beçak [40]; Beçak and Kobashi [49]/Brazil
Odontophrynus carvalhoi	22	Beçak et al. [47]; Beçak and Beçak [41]/Brazil
Odontophrynus barrio	22	Cei et al. [72]/Argentina
Odontophrynus occidentalis	22	Saez and Brum-Zorrilla [69]; Beçak and Beçak [71]; Beçak and Beçak [41]; Ruiz et al. [73]/Argentina
Odontophrynus cordobae[*] *sp. nov*	22	Martino and Sinsch [50]/Argentina
Ceratophrys ornata[*]	26	Barrio and de Chieri [52, 53]/Argentina
Hylidae		
Phyllomedusa burmeisteri[*]	26	Batistic et al. [74]/Brazil
Hyla chrysoscelis[*]	24	Bogart and Wasserman [54]; Ralim and Selander [44]/North America
Hyla andersoni	24	Wasserman [60]/North America
Pipidae		
Xenopus laevis laevis	2n = 36	Tymowska and Fischberg [62]
X. laevis petersi	2n = 36	Tymowska and Fischberg [62]
X. laevis victorianus	2n = 36	Tymowska and Fischberg [62]
X. (laevis) borealis	2n = 36	Tymowska and Fischberg [62]
X. gilli	2n = 36	Tymowska and Fischberg [62]
X. muelleri	2n = 36	Tymowska and Fischberg [62]
X. tropicalus	2n = 20	Tymowska [75]; Tymowska and Fischberg [63]
X. fraseri	2n = 36	Tymowska and Fischberg [63]; Kobel et al. [45]

Families/species	2n	References/localities
Bufonidae		
*Bufo viridis**	22	Mazik et al. [67]/Kirghizia—Russia
Bufo sp.	20	Bogart and Tandy [68]/Ethiopia
Ranidae		
*Dicroglossus occipitalis**	24	Bogart and Tandy [68]/Liberia
Pyxicephalus dilalandii	24	Bogart and Tandy [68]/Southeastern Africa

Table 4. Diploid species of anurans with related polyploid species* (references and localities are indicated) based on Beçak [4] with additions.

Besides autopolyploidy, it was reported that centric fusions also played a role in the evolution of anurans. In Leptodactylidae, the number of chromosomes found are 2n = 18, 2n = 20, 2n = 22 and 2n = 26. In Hylidae, we found 2n = 22, 2n = 24, 2n = 26 and 2n = 30 [43]. The reduction of the chromosome number was previously explained by centric fusions by Wickbom [76]. Alterations of secondary constrictions and of *C* and *NOR* bands were also found in species of *Odontophrynus* in South America [41, 77–80].

The mechanism of genome duplication was pointed to be an important process during vertebrate diversification by creating new mutations. This idea postulated that a new gene copy could change without disturbing the function of the old one [17]. When exposed to natural selection, the modified gene copies have an opportunity to help the species to explore new niches. According to this 2R–model, vertebrates evolved through two rounds of genome duplication. The first event in chordate evolution occurred possibly in the Cambrian period of the Paleozoic era. Later, a second round might have occurred in the Devonian [17]. A third duplication was described in fish genomes in the Devonian period after the radiation of ray-finned fish (Actinopterygian and Sarcopterygia lineages) [81].

According to Clarke [82], gene duplication does not explain diversification in teleosts, which present a great diversity of body plans.

5. Conclusions

A fundamental and complex problem in the study of evolution is to understand the mechanisms leading to speciation. Considering Anura evolution, the main questions to which this discussion is addressed are as follows: (1) When polyploidy occurred in the Leptodactylids? (2) Genome duplication increases variability? (3) Is speciation of polyploid anurans orchestrated by epigenetics events? (4) Were geographic barriers required for diversification of these anurans?

About 21 living polyploid and pos-polyploid species of Anura are known. According to Morescalchi [21], the actual families of Anura descend of a same Leptodactylid stock of the Jurassic period that dispersed during Jurassic/Cretaceous periods, 'prior to the final breaking-up of the Gondwanian supercontinent'. The diversification of these families extended to Cenozoic period (**Table 1**; **Figure 2**). It is unknown when genome duplication occurred in these anurans. The elucidation of this question is a challenging biological problem. Based on our cytogenetic results, we reasoned that the process of ploidy in *O. americanus* (4n), *Phyllomedusa burmeisteri* (4n) and *Ceratophrys dorsata* (8n) from Brazil [38, 40, 46] was a more recent event when compared with other polyploid events from Africa [4]. This conclusion is based on the fact that the whole genomes of the Brazilian anurans present multivalents at meiosis. These configurations indicate that homology is still high in each group of homologs. This observation agrees with the suggestion by Morescalchi [21] that some actual tropical species of Anura present intense karyological alterations by polyploidy correlated with the diversification process. Pos-polyploid alterations were described in the Brazilian anurans [55] and interpreted as signal of diploidizations [17]. An example of this process was reported in *Eleutherodactylus binotatus,* a diploid species (2n = 22) with a fourfold DNA content and exhibition of a dodecavalent ring in diplotene [55].

Besides *O. americanus,* other diplo and tetraploid species were studied (**Tables 3** and **4**). *Xenopus* genus of the family Pipidae, which is one of the oldest families of the Anura, probably emerged in early Jurassic [21]. *X. tropicalis* (2n = 20) is considered the most ancient extant species of this genus [83]. Experiments with *X. laevis* indicated that this species contains two pairs of loci for vitellogenin [84] and two genes for serum albumin [85]. Moreover, the finding that *X. laevis* (2n = 36) has two clusters of α- and β-globin genes while *B. tropicalis* (2n = 20) has only one cluster pointed that *X. leavis* is a probable tetraploid [86, 87]. Further studies of these genes led to the suggestion that the diversification of *X. laevis* and *X. tropicalis* happened in the Cretaceous period of the Mesozoic era at 110–120 mya. Later, it was suggested that the polyploidy of *X. laevis* happened in the Eocene period of the Cenozoic era at 40–60 mya [27]. In the case of *Odontophrynus*, it was suggested that polyploidy emerged by allopolyploidy from hybridization of ancestral diploid species during the Eocene when Earth faced several geological events [28].

Considering the level of variability of these tetraploid anurans, it was indicated that they have a tetrasomic gene expression according to the binomial equation $(p + q)^4$. This high variability probably facilitated the expansion of the 4n forms to different ecosystems.

Regarding the epigenetic events, it was reported that in the 4n specimens, gene expression is repressed by the methylation of half rDNA cistrons. This result was obtained using *in situ* hybridization experiments and NOR-banding. Epigenetic event was also observed with the detection of amphiplasty in 4n cells during mitosis. This fact indicated differences of chromatin packing between half genomes.

Yet, as to the mechanisms that cause reproductive isolation in these anurans, it remains an open question. The fact that genome duplication was a more recent event in the Brazilian anurans than in Africa indicates that polyploidy may have occurred in different times and that diversity may benefit from alterations of climate and from ecological impacts regardless of geographic barriers. This idea supports several facts as follows:

a. that polymorphisms of NORs and C-bands occur in allopatric diploid cryptic populations of *O. occidentalis* from Argentina [73];

b. that tetraploid populations which arose sympatrically from diploid ones maintain reproductive isolation bio-acoustic [54];

c. that in killer whale populations, geographic barrier is not a prerequisite for speciation [2];

d. that in members of the Poeciliidae fishes living in toxic sulphide waters speciation independent of geographical barriers [88];

e. that speciation may occur without geographic barriers [89];

f. that the hemoglobin patterns of allopatric populations of Brazilian 2n and 4n *O. americanus* indicate that speciation does not require geographic barrier [61];

g. That sympatry of both 2n and 4n specimens of *O. americanus* was reported in Santa Barbara do Sul, Brazil [90].

The argument that speciation operates without a previous geographic isolation leads us also to revive the suggestion by Lima de Faria [91]. He stated that modern species already carry molecular information of divergence from ancestral lineages before natural selection. This idea came from his analysis showing that although isolated about 50 mya in Australia, the marsupials exhibit similar phenotypic aspects to those of placental mammals. This implies that both groups evolved via similar mechanisms independent of the different habitats.

The data reviewed indicate that the anurans diversified by polyploidy and epigenetic events. Moreover, several results raise questions as to whether the process of speciation may occur without geographic barriers. These ideas sound as an alternative suggestion to the neo-Darwinism concept, establishing that the mechanism of speciation is a slow accumulation of natural mutations and selection of favored phenotypes in geographically isolated populations.

The studies here reviewed were addressed to the analysis of the role of polyploidy in anurans evolution. It is well known that polyploidy also occurs in some mammalian including human cells in organs such as in liver, heart, bone marrow and in embryos. The question that remains to be elucidated is the real role of polyploidy in mammalian cells [92].

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Chapter 3

Sea Turtle Beach Monitoring Program in Brazil

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Abstract

Beach monitoring programs provide important information on spatial and temporal patterns of occurrence, mortality, age structure, sex ratio, and variations associated with climatic and anthropogenic events as well as for the assessment of the health of marine organisms. The purpose of the Santos Basin Beach Monitoring Project is to evaluate the possible effects of oil and gas production and transport activities at Santos Basin on marine turtles, birds, and mammals by monitoring beaches and veterinary care facilities for live and dead animals. Five species of sea turtles occur in Brazil: the green turtle (*Chelonia mydas*), loggerhead turtle (*Caretta caretta*), hawksbill turtle (*Eretmochelys imbricata*), olive turtle (*Lepidochelys olivacea*), and leatherback turtle (*Dermochelys coriacea*), all of which are endangered and are fragile organisms that suffer from the impact of human activities during their long lifecycle. This chapter reports monitoring strategy activities and preliminary results after 1 year since the implementation the monitoring project to provide an important overview of sea turtles found in the Santos Basin.

Keywords: beach monitoring, Brazil, Cheloniidae, Dermochelyidae, sea turtles

1. Introduction

Sea turtles belong to the group Testudines, considered one of the most primitive Order of reptiles, and the oldest specimen found dating back 120 million years to the lower Cretaceous period [1]. These organisms belong to the order Testudines, which includes chelonians with the body encompassed by a bony carapace formed by the fusion of ribs and vertebrae [2].

Modern sea turtles form a monophyletic group, as all derive from a common ancestor from the suborder Cryptodira [3]. There are currently seven species distributed in two families. The family Dermochelyidae has only one species: the leatherback sea turtle (*Dermochelys coriacea*). The family Cheloniidae has six species: the green turtle (*Chelonia mydas*), loggerhead turtle (*Caretta caretta*), hawksbill turtle (*Eretmochelys imbricata*), olive ridley turtle (*Lepidochelys olivacea*), Kemp's ridley turtle (*Lepidochelys kempii*), and flatback turtle (*Natator depressus*), the latter two of which are the only species that do not occur in Brazil [3, 4]. Sea turtles have broad geographic distribution, occurring in all oceanic basins, with representatives found from the Arctic to Tasmania [3]. However, the reproduction of most individuals occurs in tropical and subtropical regions [4].

Knowledge on the biology of sea turtles and their relationships with the surrounding environment is fundamental to outlining effective conservation strategies [5, 6]. Scientific research involving sea turtles began to gain momentum in the mid twentieth century, before which targeted fishing efforts and the consumption of turtle meat and eggs were common practices throughout the world, along with the intensive degradation of coastal areas, and consequent loss of habitats [7, 8]. As sea turtles have a complex lifecycle, late sexual maturity, and broad geographic distribution, many gaps in knowledge remain, despite the advances in recent decades [6].

In Brazil, there were no laws protecting sea turtles until the 1980s and the consumption of meat and eggs and the slaughter of females in coastal areas were common events [9]. Due to international pressure for nations to work together based on the argument that sea turtles are migratory animals that visit various countries throughout their lifetimes, the Brazilian government created the National Sea Turtle Conservation Program, known as the TAMAR project. In 1989, sea turtles were officially considered endangered species that merited protection [IBAMA (Brazilian Environmental Protection Agency) ordinance n°. 1522 from December 19, 1989] [9]. Since its establishment, the TAMAR project has been working in eight Brazilian states and oceanic islands at feeding and nesting areas of these animals [10]. Moreover, there has been a growing number of researchers at higher education institutions, non-governmental organizations, and other institutes that study sea turtles and are dedicated to the protection of reproduction sites and the monitoring of non-reproductive occurrence/stranding in locations beyond the reach of the TAMAR project [11].

1.1. Marine turtles distribution in Brazil

1.1.1. Green turtle (Chelonia mydas)

The green turtle has one pair of prefrontal scales and four pairs of postorbital scales on the head and four pairs of overlapping lateral scutes on the carapace [10]. Carapace coloration varies, but normally has radial striae on each horny plate with a greenish-brown color and the plastron is white; hatchlings have a black dorsum [12]. Adults have a mean curvilinear carapace length of 115.6 cm and can weigh up to approximately 230 kg [4, 13]. The primary spawning areas are located on oceanic islands, such as the Rocas Atoll (pertaining to the state of Rio Grande do Norte), Fernando de Noronha Archipelago (pertaining to the state of Pernambuco), and Trindade Island (pertaining to the state of Espírito Santo), but nests are also found on mainland beaches, especially in the northern portion of the state of Bahia) [10, 14]. Juveniles (smaller than 30 cm) have an omnivorous diet and migrate from the pelagic oceanic environment to the coastal zone when reaching 30–40 cm, changing to a predominantly herbivorous diet. Green sea turtles feed mainly on algae and sea grass, but can also consume animal material as a secondary diet, such as jellyfish, salps, and sponges, with regional variations [14, 15]. This species has coastal habits, feeds along the entire coast of Brazil, and can even use the estuaries of rivers and lakes during its development [10, 16].

1.1.2. Loggerhead turtle (Caretta caretta)

The loggerhead turtle has two pairs of prefrontal scales and three pairs of postorbital scales on the head and five pairs of overlapping lateral scutes on the carapace, the first pair of which is smaller [10]. The carapace has a yellowish-brown color and the underside is a light yellow in adults [12]. The head is triangular and proportionally large in relation to the body [4]. Adult females have a mean curvilinear carapace length of 103 cm and weigh between 100 and 180 kg [10]. The primary nesting areas are located in the state of Sergipe and the northern portions of the states of Bahia, Espírito Santo, and Rio de Janeiro. Secondary nesting areas are found in the southern portions of the states of Espírito Santo and Bahia [10]. The loggerhead sea turtle has carnivorous, opportunistic eating habits, feeding on a broad variety of organisms, such as crustaceans, mollusks, and fish [15, 17]. This species feeds in neritic and oceanic environments and adults are often caught incidentally in industrial fishing operations in these areas [10, 18].

1.1.3. Hawksbill turtle (Eretmochelys imbricata)

The hawksbill turtle has two pairs of prefrontal scales and three pairs of postorbital scales on the head and four pairs of imbricated lateral scutes on the carapace; the margin of the shell is serrated and the beak resembles that of a bird [12]. The carapace ranges in color from light to dark brown with black spots and amber striae; the underside ranges in color from light yellow to white [4, 12]. Adult females have a mean curvilinear carapace length of 97.4 cm and weigh around 80 kg [12, 19]. The primary nesting areas are located in the states of Rio Grande do Norte and Sergipe as well as the northern portion of the state of Bahia. Secondary nesting areas are found in the state of Paraíba and the southern portion of the state of Bahia [19, 20]. Juveniles and adults feed mainly on crustaceans, sea urchins, mollusks, algae, bryozoans, coelenterates, and sponges, the latter two of which are considered the preferred food items by adults [21]. Due to its feeding preferences, the hawksbill sea turtle customarily inhabits sites with hard substrates, such as coral reefs. The main known feeding grounds are the Fernando de Noronha Archipelago, Rocas Atoll, Trindade Island, Abrolhos Archipelago (state of Bahia), Saint Peter and Saint Paul Archipelagos, Arvoredo Island (state of Santa Catarina), and Cagarras Islands (state of Rio de Janeiro) [19].

1.1.4. Olive ridley turtle (Lepidochelys olivacea)

The olive ridley turtle has two pairs of prefrontal scales and three pairs of postorbital scales on the head and an irregular number (five to nine) of lateral scutes on the shell. The carapace is rounded and elevated in the anterior portion [12], with an olive green to gray color; the underside is light yellow. Adults have a mean curvilinear carapace length of 73.1 cm and weigh around 50 kg, making it the smallest sea turtle occurring in the country [10]. The primary nesting areas are located in the states of Alagoas and Sergipe and the northern portion of the state of Bahia. The secondary nesting area is located in the state of Espírito Santo [10]. The olive ridley sea turtle feeds mainly on crustaceans, mollusks, fish, and bryozoans [15]. This species feeds in neritic and oceanic environments and is often caught incidentally during bottom trawl fishing and pelagic longline fishing operations [18, 22].

1.1.5. Leatherback turtle (Dermochelys coriacea)

The leatherback turtle has a carapace formed by leathering skin covered with osteoderms (no plates or scutes) and seven longitudinal keels [12, 23]. Its color ranges from black to bluish with white to light blue spots on the entire body [12]. Female adults have a mean curvilinear carapace length of 159 cm, but can reach as much as 182 cm and mean weight is 500 kg, making it the biggest of all sea turtles in the world [10]. The only known regular nesting area in Brazil is located in the northern portion of the state of Espírito Santo, whereas occasional nesting has been recorded in the states of Piauí, Rio Grande do Norte, Bahia, Rio de Janeiro, Santa Catarina, and Rio Grande do Sul [10]. The leatherback sea turtle forages from the ocean surface to considerable depths, preferably inhabiting the oceanic region. Its diet is composed of gelatinous zooplankton, such as scyphozoans, ctenophores, hydrozoans, and cubozoans [24]. Records of incidental catches during oceanic fishing operations are common on the Brazilian coast [22, 25].

1.2. Main threats

Sea turtles are subject to diverse threats throughout their lifecycle, mainly due to anthropogenic factors [26]. The history of exploitation for the consumption of meat and eggs, together with the long lifecycle and late maturity (at around 25–30 years) and the degradation of their natural habitats, has led to a decline in populations around the world, placing sea turtles as risk of extinction [26, 27]. Indeed, all sea turtle species are currently on the Red List of endangered species of the International Union for Conservation of Nature [28] and the Brazilian Environment Ministry (ordinance n° 444/2014 and n° 445/2014) (**Table 1**).

Currently, the main threats to sea turtles are coastal development, incidental catches during fishing operations, human consumption of meat, climate change, pollution, and exposure to pathogens [10]. Many human activities exert direct and indirect impacts on sea turtles. Coastal development leads to the use of areas that are important for these animals in terms of foraging and reproduction [26]. Examples of anthropogenic interferences considered threats to sea turtles include activities such as oceanfront housing, vehicular traffic, and artificial lighting on beaches in nesting areas, real estate development, the discarding of solid waste and chemical pollutants into the sea, the construction of ports, and, especially, fishing operations [29–36].

The consumption of meat and eggs [26] and incidental catches of sea turtles by fishing gear are the human actions with the greatest impact on sea turtle populations around the world [27, 37]. Several countries are working together in search of mitigating solutions aimed at reducing the number of incidental catches in different fisheries [38]. The main fisheries that affect sea turtles in Brazil are gillnetting, seining, traps and longline (surface and bottom) operations, with the highest mortality rates attributed to gillnetting and longline operations [39].

Espécies	**IUCN**	**MMA**
Dermochelys coriacea	VU	CR
Chelonia mydas	EN	VU
Caretta caretta	VU	*
Eretmochelys imbricata	CR	CR
Lepidochelys olivacea	VU	EN

*Species included in Normative Instruction N° 3 (May 27, 2003), which describes critically endangered species of Brazilian fauna.

Table 1. Endangered status of sea turtles occurring in Brazil according to Red List of International Union for Conservation of Nature and Brazilian Environment Ministry (DD, deficient data; VU, vulnerable, EN, endangered, CR, critically endangered).

In Brazil, loggerhead and olive ridley sea turtles are more frequently caught by pelagic longline and bottom seining operations [18, 22], whereas leatherback turtles are affected mainly by pelagic longline fishing [22, 40, 41]. Green and hawksbill sea turtles are more frequently caught by coastal artisanal gillnetting and seining operations due to the more coastal habits of these species, but are also caught as bycatch in oceanic fishing gillnetting and longline operations [22, 42–44].

1.3. Beach monitoring projects

Mitigation measures, such as beach monitoring projects (BMPs), have been implemented around the world with the aim of evaluating and minimizing environmental impacts on the marine biota caused by human activities. With the increase in public pressure and the sense of corporative responsibility, some companies have gone beyond the mitigation of these impacts and have invested in enterprises that include providing financial resources for the management of protected areas as well as support for scientific research and governmental training. In countries where the capacity and resources for protecting the environment are very limited, such actions are very important as an efficient manner to conserve biodiversity in areas affected by human intervention [45].

Specific, effective protocols for the recovery and protection of marine fauna in areas under the influence of these enterprises are scarce. Thus, marine animals have been one of the focuses of environmental licensing processes due to the recognized potential for causing lethal and sub-lethal disturbances to species. Sub-lethal effects stemming from metabolic exhaustion can be as devastating as lethal impacts and provoke adverse behavioral reactions, such as panic and the loss of foraging and defense capacities, which facilitates the occurrence of incidental catches, collisions with boat and stranding, as known in cetaceans [46].

The oil and gas industry has long adopted more effective environmental practices and increased awareness with regard to the social and environmental responsibility of its operations. For industrial activities to occur in a sustainable manner, it is fundamental to conduct multidisciplinary studies that furnish reference data on the environment in which such operations are developed [47]. In this context, the BMP is a condition for environmental licensing stipulated by the General Petroleum and Gas Coordination of *Instituto de Meio Ambiente e dos Recursos Renováveis* [IBAMA (Brazilian Institute of the Environment and Renewable Resources)] for situations with unclear environmental impacts that are inherent of the industrial oil production process.

The majority of studies on stranded marine animals are conducted with mammals [48–52] and seabirds [53–56]. However, researchers have become more engaged in the monitoring of stranded sea turtles in coastal regions throughout the world, including Brazil, which is an effective approach to investigating the ecology and epidemiology of these animals [29, 57–62]. Thus, the aim of this chapter is to describe the strategies of the BMP in the state of Rio de Janeiro, Brazil, and present the results of these strategies in the first year since its implementation with marine chelonian.

2. Methods

2.1. Study area: Santos Basin

The Santos Basin has an area of 276,900 km^2 and is bordered by the Campos Basin to the north off the municipality of Cabo Frio in the state of Rio de Janeiro and the Pelotas Basin to the south off the city of Florianópolis (state of Santa Catarina). In a region with very deep waters, the development of carbonate reservoirs occurs below a layer of salt in what is known as the pre-salt layer, characterizing one of the largest petroleum provinces in the world, with accumulations of heavy oil, light oil, and non-associated gas.

The pre-salt layer is a rock formation located in the subsoil of the Brazilian coastline that extends between the states of Santa Catarina and Espírito Santo, covering an area of approximately 800 km in length and 200 km in width. This group of rocks with the potential for the formation of petroleum is located under an extensive layer of salt reaching as much as 2 km in thickness. Such formations are found at distances of approximately 300 km from the coastline at depths of approximately 5000 m, of which the water column accounts for 2000 m, sediment accounts for 1000 m, and salt accounts for the last 2000 m. The pre-salt layer of the Santos Basin, which covers an area of 149,000 km^2, has one of the largest petroleum reserves in the country. The first production in this region occurred in the "Lula" field on May 1, 2009.

2.2. Beach monitoring project

The Brazilian oil company PETROBRAS (Petróleo Brasileiro S.A) has been conducting BMPs along the following oceanic basins: Potiguar (states of Rio Grande do Norte and Ceará in the northeastern region of the country), Sergipe-Alagoas (states of Sergipe and Alagoas in the northeastern region), Campos-Espírito Santo (states of Rio de Janeiro and Espírito Santo in the southeastern region), and Santos (states of Rio de Janeiro and São Paulo in the southeastern region and states of Paraná and Santa Catarina in the southern region). The main goal of a BMP is to record the occurrence of stranded marine animals, especially chelonians, mammals, and seabirds, and determine whether there is a relationship between the stranding of these animals and the oil exploration and production activities conducted by PETROBRAS on the coast of Brazil. Discontinuous stretches of beach have been actively monitored by land and/or sea through routinely traveling the entire length of beaches in search of carcasses and debilitated animals as well as through information provided by third parties, such as the local population, tourists, and public agencies. Information is collected about the environment and stranded organisms each monitoring day and necropsies are performed of dead animals, whenever possible, in search of evidence of anthropogenic interaction and the establishment of a possible cause of death.

BMPs can be conducted by universities, institutions, or environmental consulting firms in partnership with organizations recognized by the environmental authority in Brazil (IBAMA). The aim of shared execution is to avoid the overlap of sampling efforts in areas where groups have performed their services and circumvent the "dispute for carcasses," ensuring permanent access to the data for all parties involved.

2.3. Beach monitoring project in the state of Rio de Janeiro

This chapter addresses the implementation and data obtained from the BMP conducted in the Santos Basin (BMP-SB) in coastal municipalities located between the southern limit of the municipality of Paraty and the northern limit of the municipality of Saquarema in the state of Rio de Janeiro (**Figure 1**). Approximately 985 km of beaches are located on the mainland and nearby islands, involving a variety of environments, different beach morphologies, and different degrees of land use and occupation as well as land and marine environmental areas

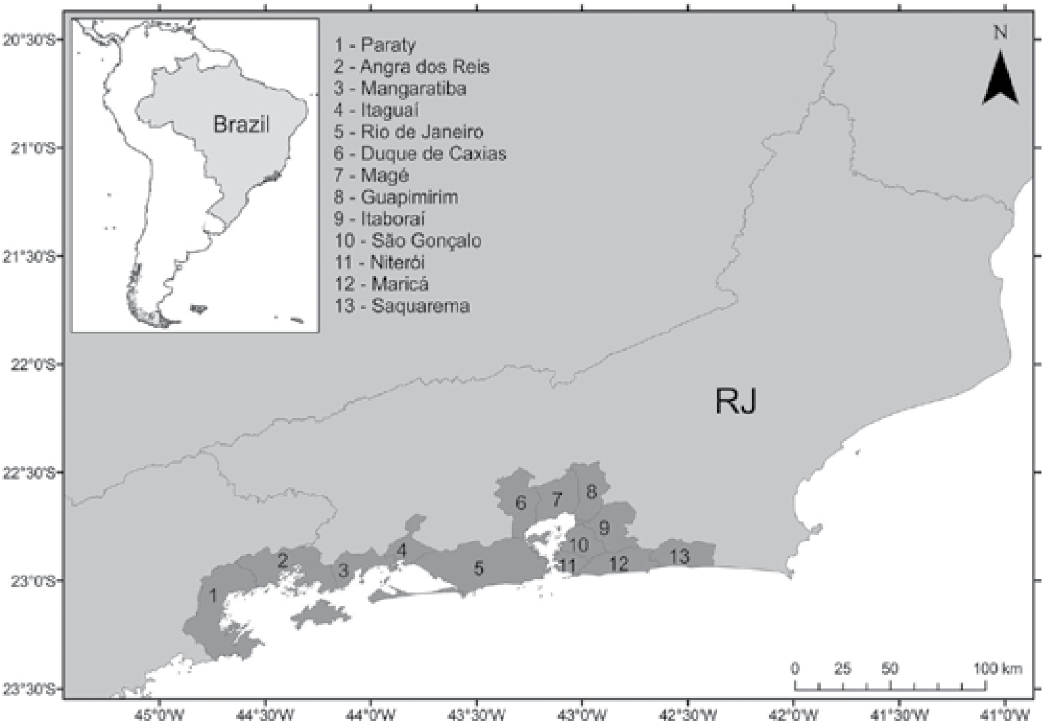

Figure 1. Area of the beach monitoring project in the state of Rio de Janeiro.

protected by state (Rio de Janeiro State Environmental Institute) and federal (Chico Mendes Institute for the Conservation of Biodiversity) institutions.

Prior to the onset of the activities, an *in situ* study was conducted to define the monitoring strategies, locations for the installation of veterinary facilities, and logistic support and determine the profile of the team needed for the execution of the intended actions. The area of coverage was then divided into five sectors, denominated "stretches": Stretch 1 corresponded to the municipalities of Saquarema and Niterói); Stretch 2 corresponded to the city of Rio de Janeiro and Guanabara Bay; Stretch 3 corresponded to the municipality of Mangaratiba; Stretch 4 corresponded to the municipality of Angra dos Reis; and Stretch 5 corresponded to the municipality of Paraty (**Figure 2**). The frequency (daily, weekly, and bi-weekly) and monitoring modality (active by land, active by boat, or notification from the network of collaborators) were based mainly on the geographical particularities of each stretch.

Three veterinary structures and four logistic support points were installed for the treatment of living (rehabilitation) and dead (necropsy) animals. The veterinary structures were installed at locations based on the premise that a debilitated animal could not be transported a distance greater than 150 km or for more than 2 h. The composition of the teams allocated to each stretch varied in accordance with the type of monitoring employed and the presence of a veterinary base. The teams included monitors (residents of local communities), field technicians (biologists, oceanographers, and scientists from similar fields), boatmen, environmental educators, communication assistants, veterinarians, veterinary assistants, general service assistants, administrative assistants, managers, and coordinators.

All field and veterinary teams received specific training prior to the initiation of the BMP-SB activities. The training involved the content of previously established protocols, which considered all actions envisaged for the project in an effort to ensure the collection of harmonious, standardized data. Eight guiding documents were drafted: (1) field activity protocol for beach monitoring by land and boat; (2) veterinary care protocol for living animals (rehabilitation, release, and destination of rehabilitated animals); (3) euthanasia protocol; (4) veterinary care protocol for dead animals (necropsies); (5) gastrointestinal content sorting protocol; (6) protocol for age and sexual maturity estimates; (7) protocol for collecting, storing, and sending samples for histopathological analysis; and (8) protocol for the collection of samples for the analysis of contaminants and biomarkers. Whenever changes are needed due to the dynamics of the activities, the protocols are revised and made available based on the functions each team exercises.

2.4. Monitoring strategies

Four monitoring modalities were defined: (1) active by land; (2) active by boat; (3) active by partners; and (4) notification from network of collaborators. Each stretch of the BMP-SB could involve one or more types of monitoring, which could be practiced at the same time (**Table 2**).

Active monitoring by land: Daily patrolling of beach by monitors and field technicians, preferably in pairs, trained to observe and record occurrences of stranded marine animals. Monitoring could be performed on foot, on a bicycle or traction vehicle (e.g., quadricycle) on 88 beaches, totaling 118.5 km/day.

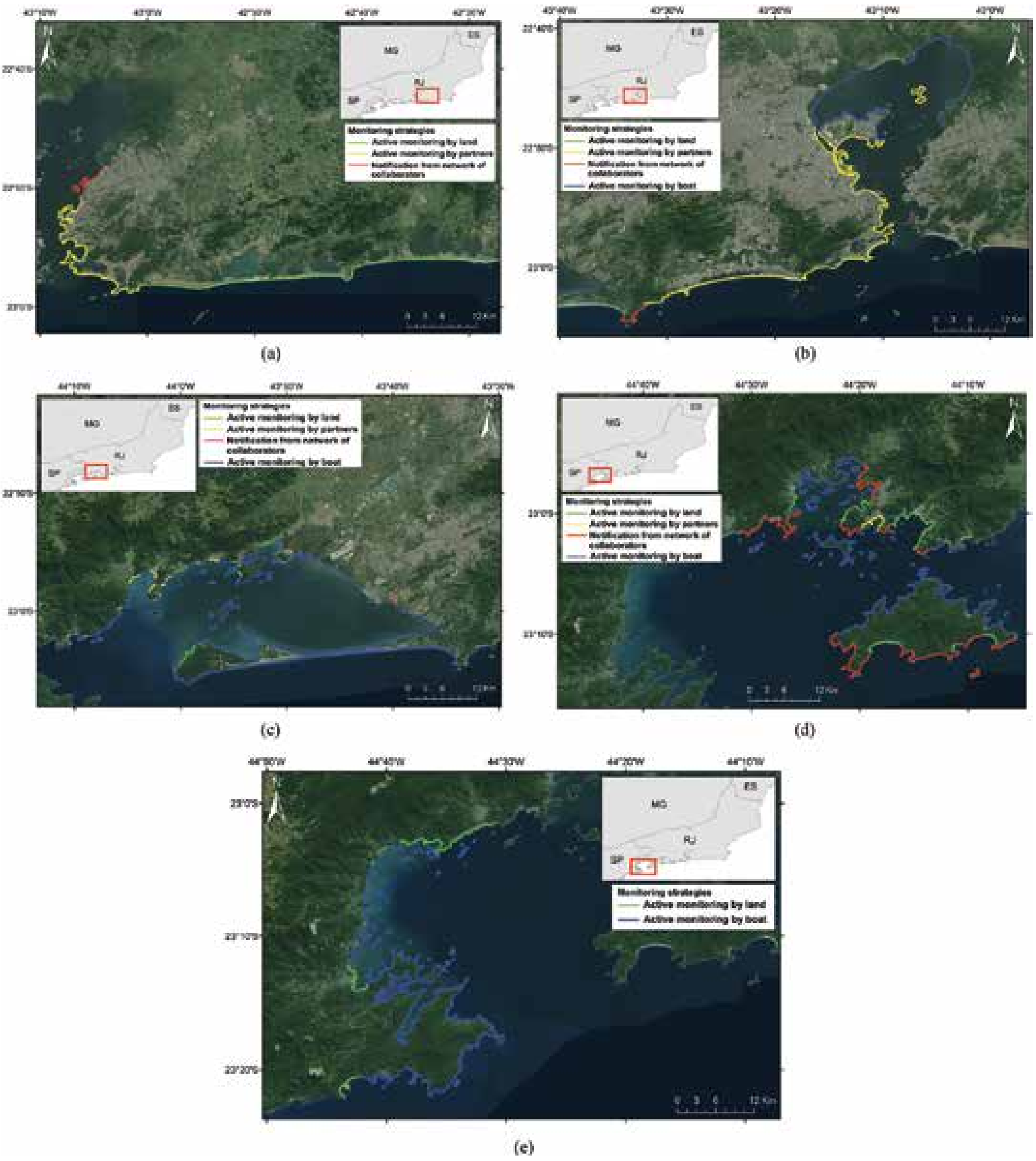

Figure 2. Sectorization of BMP-SB area. Colors indicate different monitoring modalities: green = active by land; blue = active by boat; yellow = active by partners; red = notification from network of collaborators. (A) Stretch 1: Saquarema to Niterói; (B) Stretch 2: Rio de Janeiro and Guanabara Bay; (C) Stretch 3: Mangaratiba; (D) Stretch 4: Angra dos Reis; and (E) Stretch 5: Paraty.

Active monitoring by boat: Patrolling of beaches with no access by land located on the mainland or islands performed by teams composed of at least a boatman and field technician searching for stranded or drifting animals. This modality is practiced either weekly or bi-weekly, depending on the navigation rules established by the regulating agency (Port Captaincy of the Brazilian Navy), totaling approximately 742 km/weeks.

Stretches	Monitoring efforts				
	Active by land	Active by boat	Active by partners	Notification from network collaborators	Total (Km)
1-Saquarema to Niterói	52.30	—	14.74	9.70	76.74
2-Rio de Janeiro and Guanabara Bay	6.65	62.00	51.24	1.52	121.41
3-Sepetiba to Mangaratiba	18.10	234.65	9.65	1.90	264.30
4-Angra dos Reis	22.48	219.75	4.83	30.55	277.61
5-Paraty	18.97	225.42	—	0.08	244.47
Total (Km)	**118.50**	**741.82**	**80.46**	**43.75**	**984.53**
Total (%)	**12.04**	**75.35**	**8.17**	**4.44**	**100**

Table 2. Total monitored area according to strategy employed for Santos Basin beach monitoring program in state of Rio de Janeiro, Brazil.

Active monitoring by partners: Monitoring performed indirectly on urban beaches and beaches located in private condominiums that have daily clean-up activities and/or the presence of lifeguards. When a marine animal is found by these partners, the team of the project is contacted to collect the animal.

Notification from network of collaborators: Monitoring performed by third parties. Contact could be made through the 800 number (free telephone call) or directly to any member of the project team. Permanent dissemination of the activities is performed in the communities as well as with fishermen associations, city halls, commercial establishments, and coastal enterprises.

2.5. Veterinary structure and monitoring support

Three veterinary bases were installed for the rehabilitation and necropsy of marine animals, located at Araruama, Rio de Janeiro, and Angra dos Reis municipalities; and four logistic support points were established, located at Maricá, Mangaratiba, Angra dos Reis (Ilha Grande), and Paraty municipalities, to give support to the monitoring activities. The veterinary bases were divided into two categories: rehabilitation center and stabilization unit, which were implemented following the guidelines available in IBAMA Normative Instruction N° 7 (from April 30, 2015), which stipulates use and management categories for wildlife in captivity and defines the procedures for the established categories. All structures have licenses from the environmental agency and professional class entity.

The rehabilitation center has the capacity to treat at least 70 marine animals per month, a minimum area of 700 m^2 and a structure composed of administrative, maintenance/services, and veterinary sectors as well as a specific room for environmental education activities. The veterinary sector is composed of a laboratory, clinic, infirmary with intensive care unit, stabilization, washing and drying room for oil-covered animals, animal kitchen, necropsy room, room for storing carcasses and samples, animal enclosure, and dressing rooms.

The stabilization unit is a simpler structure where debilitated animals receive first aid until strong enough to be transported to a rehabilitation center. This unit has the capacity to treat at least 20 animals per month and an area of 500 m^2. It is composed of an administrative sector and veterinary sector, which has a necropsy room, room for storing carcasses and samples, clinic, infirmary, kitchen, and animal enclosure.

The support point is a structure that provides support to the monitoring and administrative activities, containing an office, kitchen, and restrooms for the staff, enclosure for the temporary storage of carcasses, field material, and vehicles.

2.6. Field records

During the monitoring activities, the field teams collected information on the, when monitoring itself, sea turtles found either stranded or drifting in the water. A set of data was defined for each monitoring strategy to ensure standardization and the tracking of the effort performed on a given day. For such, field charts were always filled out at the beginning and end of each activity, including the date, time of the day, geographic coordinates, and environmental conditions (sky conditions, wind direction and intensity, ocean conditions, and tide). Global positioning system (GPS) equipment was also used throughout the entire course of each monitoring event. For monitoring strategies in which stranded animals were reported by third parties, the basic data (date, time when the call was received), means of notification (telephone, e-mail), state of the animal (alive or dead), size of the animal, location (name of beach and reference points), as well as the name and telephone number of the person who made the notification were recorded. This information enabled the field team to determine the best way to reach the location and the most adequate materials for the treatment of the animal.

When a sea turtle was found stranded, the field team collected a set of data on both the animal and the surrounding environment. A field chart was filled out containing the following information: date, time of day, characteristics of the environment (water, sand, mangrove, or rocks), species (to the lowest possible taxonomic level), sex, artificial markings (tags or other identification), state of the animal (alive or dead), condition of carcass (fresh, evident decomposition, advanced decomposition, or mummified) (**Table 3**), body condition (poor, intermediate, or good, based on condition of pectoral musculature, see [63]), development phase (pup, juvenile, or adult), evidence of anthropogenic interaction, presence of fibropapillomas, samples collected, and destination of the animal.

The photographic records of all sea turtles found followed a standardized guide, with obligatory images of the entire body (dorsal and ventral views), head (side and dorsal view), marks (natural and scars), tags (if present), abnormalities on the dermal scales of the head (central, lateral, and postorbital), and bodily deformations. For animals found alive, the ventral photograph was not taken by the field team in order to avoid greater harm and was performed by the veterinarian during rehabilitation or after death.

All measurements were linear, except for the carapace, which was a curvilinear measurement. A total of nine biometric variables were measured. For mummified carcasses or those with separated body parts due to decomposition or the action of predators, no measurements were made.

Code	Class	Description
2	Optimal condition (fresh animal)	Fresh normal appearance, with few or no lacerations caused by other animals, clear eyes, body firm and not inflamed, no discoloration, viscera intact and well defined.
3	Evident decomposition	Decomposed, but organs basically intact. Carcass intact, may be slightly bloated; may have missing skin in some places; may have internal or external signs of predation by necrophagous animals, but the organs are maintained.
4	Advanced decomposition	Carcass may or may not be intact; epidermis completely missing, numerous lacerations caused by necrophagous animals; strong odor; muscles with no consistency and frail, easily detached from bones; viscera missing or identifiable, but with coloration and appearance of intensive autolysis; brain reddish black.
5	Mummified state	Carcass or skin, when present, may be covering remaining skeleton; any remaining tissues are unidentifiable.

Table 3. Classification of decomposition stage of sea turtle carcasses; adapted from [64].

2.7. Rescue and transport of sea turtles

2.7.1. Living sea turtles

The rescue and transport of living sea turtles requires quickness and some basic procedures to increase the possibility of successful rehabilitation. The following were the main guidelines for rescuing living sea turtles: use gloves when handling the animal; keep the animal in a calm, shaded place in ventral decubitus; support it on foam rubber, cloth, or sand; and place wet cloth or towels on the carapace to reduce thermal stress and dehydration at temperatures above 25°C).

For transport to the veterinary base, the sea turtle was placed in a plastic box (appropriate for its size) with no sharp edges and lined with foam rubber (ideally, protective mats of smooth, impermeable material) or cloths to diminish stress and facilitate the subsequent cleaning of the recipients. The sea turtles were always transported in ventral decubitus and never in tanks with water due to the risk of drowning. Transport was executed in closed vehicles to avoid extreme temperature of heat or cold.

2.7.2. Dead sea turtles

Sea turtle carcasses found on the beach were retrieved following the criteria listed in **Table 4**, which are based on knowledge acquired from other projects and surveys, which identified that more than 90% of stranded individuals are juvenile green sea turtles (curvilinear carapace length between 30 and 50 cm). Those that did not fulfill the criteria and would therefore not be submitted to necropsy could be buried on the beach in a location above the water line of the spring tide, at a sufficient distance from urban areas and at a sufficient depth, as stipulated in the Procedures for the Monitoring of Stranded Sea Turtles in Feeding Areas and in accordance with the Management Plans for Environmental Protection Areas, or could be sent to a veterinary base to be discarded as biological waste. Burying the carcasses enables retrieval by scientific institutions that have the interest in and capacity to store the material.

	Code 2 (fresh)	Code 3 (evident decomposition)	Code 4 (advanced decomposition)	Code 5 (mummified)
Sea turtles covered in oil	All	All	All	All
Chelonia mydas	All	All	<30 cm >50 cm	None
Caretta caretta; Eretmochelys imbricata; Lepidochelys olivacea; Dermochelys coriacea	All	All	All	None

Table 4. Criteria established for retrieval of carcasses of five species of sea turtle and shipping to veterinary bases.

Sea turtle carcasses in adequate condition for necropsy were transported to the veterinary bases. The transport was planned in such a way as to optimize the travel time and avoid further decomposition. The animals were placed in specific transport cases (polystyrene chests) and kept cool with ice to slow down the decomposition process. After necropsy, the material was discarded as biological waste and collected by specialized firms. During the execution of the carcass procedures, individualized protection equipment was used to avoid contact of the skin and mucous membranes of the technicians with the biological waste of the animals.

2.8. Sea turtle care and necropsy

All living, debilitated sea turtles found stranded or drifting in the water were sent to the closest rehabilitation center or to a stabilization unit where they were given veterinary care. Prior to release back into the environment, all sea turtles were tagged using standard rings provided by the environmental agency in charge (TAMAR Center/Chico Mendes Institute for the Conservation of Biodiversity). The data on the tagged animals were entered in the national registry (TAMAR Project Information System (www.sitamar.tamar.org.br), verified, and validated by environmental analysts of the TAMAR Center/Chico Mendes Institute for the Conservation of Biodiversity, with both public access and restricted access modules.

All sea turtles found dead (fulfilling the criteria listed in **Table 4**) and those that died during the treatment were submitted to necropsy and the collection of biological material for macroscopic analysis, sorting of gastrointestinal contents, parasite taxonomic analysis, histopathological analysis and osteology in an attempt to determine the cause of death and whether there was an association with PETROBRAS activities. For recently dead animals that were not submitted to veterinary treatment, tissue samples were collected for the analysis of contaminants (polycyclic aromatic hydrocarbons and heavy metals) as well as chemical and molecular.

2.9. Aquatic biota monitoring information system

All data collected within the scope of the project are available through the *Sistema de Informação de Monitoramento da Biota* [SIMBA (Aquatic Biota Monitoring Information System), which has both private and public access modules (pmp.acad.univali.br/simba)]. The records in the system include information on the monitoring effort, records of sea turtles, biometrics, veterinary treatment, necropsy, and examinations performed. In the field, the monitoring teams used the SIMBA Mobile application to record data in real time and synchronize with SIMBA Web.

3. Results

During 1 year of activities (September 2016 to September 2017) of the BMP-SB in the area between the southern limit of the municipality of Paraty and the northern limit of the municipality of Saquarema in the state of Rio de Janeiro, 1138 sea turtles were recorded, of which 10.9% were found alive (n = 124) (**Table 5**). More than half of the specimens were in an advanced stage of decomposition (n = 599), and only 10.7% (n = 122) were fresh individuals (having died less than 24 h earlier) (**Table 5**).

All five species of sea turtle with known occurrence on the coast of Brazil were recorded. Green sea turtles accounted for 92.7% of the stranding events (1055 occurrences) (**Table 5**). Green turtles have cosmopolitan distribution from the tropics to temperate regions and exhibit more coastal habits, including the use of river and lake estuaries [14]. Hence, its predominance among the stranding events may be related to its living habits and geographic distribution.

High numbers of *C. mydas* have been recorded in other BMPs conducted in Brazil. The Campos-Espírito Santo BMP (southeastern Brazil) registered a total of 18,488 stranding events of green sea turtles in a 5-year period (2010–2015). The same monitoring project was conducted in the states of São Paulo, Paraná, and Santa Catarina by another institution recorded a total of 5221 stranding events involving the species in a 1-year period (2015–2016).

The highest number of sea turtle occurrences (n = 421; 37%) was recorded in Stretch 1 (municipalities of Saquarema and Niterói), which is the northernmost region of the study area,

	Alive	**Code 2**	**Code 3**	**Code 4**	**Code 5**	**Deaths***	**Total**	**%**
Caretta caretta	2	3	5	19	6	33	35	3.1
Chelonia mydas	120	118	150	553	114	935	1055	92.7
Dermochelys coriacea	0	0	1	7	0	8	8	0.7
Eretmochelys imbricata	1	0	0	2	1	3	4	0.4
Lepidochelys olivacea	1	1	1	12	2	16	17	1.7
Undetermined	0	0	1	6	12	19	19	1.5
Total	**124**	**122**	**158**	**599**	**135**	**1014**	**1138**	—
%	**10.9**	**10.7**	**13.9**	**52.6**	**11.9**	**89.1**	**100**	—

*Deaths = total of all dead individuals in different stages of carcass decomposition.

Table 5. Sea turtle species recorded according to carcass condition.

followed by Stretch 3 (municipality of Mangaratiba) (n = 347; 30.5%), Stretch 4 (municipality of Angra dos Reis) (n = 134; 11.8%), Stretch 2 (city of Rio de Janeiro and Guanabara Bay) (n = 129; 11.3%), and Stretch 5 (municipality of Paraty) (n = 107; 9.4%) (**Table 6**). Intrinsic characteristics of each stretch, such as a predominance of exposed beaches or sheltered beaches, geographical barriers (islands and cliffs of different sizes), development of fishing activities involving drum nets/seines, type of monitoring strategy, and proximity to reproductive areas, are factors that may exert an influence on the numbers of sea turtles found on each stretch.

Only green and loggerhead sea turtles were found stranded throughout the entire study area (**Table 6**). Olive ridley, leatherback, and hawksbill sea turtles were only found in areas in the more northern portion of the state of Rio de Janeiro (Stretches 1 and 2) (**Table 6**).

Loggerhead and olive ridley turtles were found in all seasons, particularly in spring (**Table 7**). There were no records of the hawksbill sea turtle in winter (**Table 7**). With the exception of one occurrence of the leatherback sea turtle in summer, all other stranding events of the species occurred in autumn on a single beach located in the municipality

	Stretch 1	Stretch 2	Stretch 3	Stretch 4	Stretch 5	Total	%
Caretta caretta	22	6	2	4	1	35	3.1
Chelonia mydas	375	115	336	123	106	1055	92.7
Dermochelys coriacea	7	1	0	0	0	8	0.7
Eretmochelys imbricata	4	0	0	0	0	4	0.4
Lepidochelys olivacea	11	6	0	0	0	17	1.5
Undetermined	2	1	9	7	0	19	1.7
Total	**421**	**129**	**347**	**134**	**107**	**1138**	—
%	**37.0**	**11.3**	**30.5**	**11.8**	**9.4**	**100**	—

Table 6. Sea turtle species recorded per stretch of BMP-SB area of coverage.

	Spring	Summer	Fall	Winter	Total	%
Caretta caretta	13	6	8	8	35	3.1
Chelonia mydas	189	229	285	352	1055	92.7
Dermochelys coriacea	0	1	7	0	8	0.7
Eretmochelys imbricata	1	2	1	0	4	0.4
Lepidochelys olivacea	7	4	4	2	17	1.5
Undetermined	11	3	0	5	19	1.7
Total	**221**	**245**	**305**	**367**	**1138**	—
%	**19.4**	**21.5**	**26.8**	**32.2**	**100**	—

Table 7. Sea turtle species recorded per season between September 2016 and September 2017.

	Notification by Network of Collaborators	**Active through partners**	**Active by land**	**Active by boat**	**Total**
Caretta caretta	11	5	17	2	35
Chelonia mydas	315	113	558	70	1056
Dermochelys coriacea	1	3	4	—	8
Eretmochelys imbricata	1	—	3	—	4
Lepidochelys olivacea	3	4	9	—	16
undetermined	—	1	18	—	19
Total	**331**	**126**	**609**	**72**	**1138**
%	**29.1**	**11.1**	**53.5**	**6.3**	**100**

Table 8. Sea turtle species recorded by BMP-SB using different monitoring strategies between September 2016 and September 2017.

of Maricá (Stretch 1) (**Table 7**). The period in which the leatherback turtles were found coincides with the industrial fishing season in the region, during which trawlers from different states perform gillnetting activities. Incidental catches of *D. coriacea* in gillnets

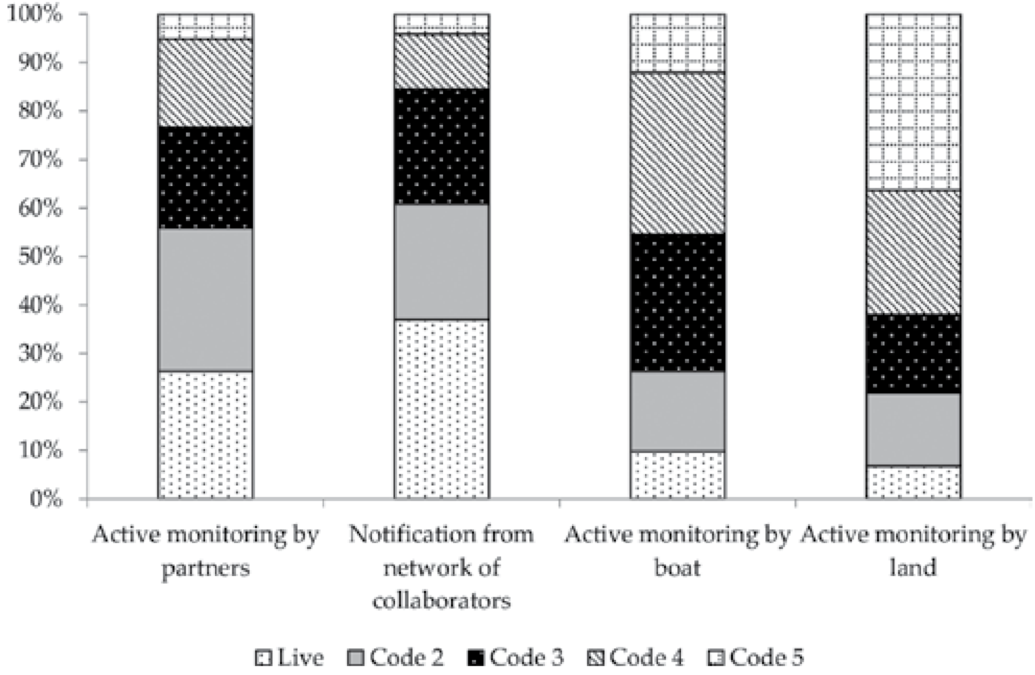

Figure 3. Proportion of sea turtles recorded with different monitoring strategies employed in BMP-SB according to condition of the carcass. Code 2 = fresh; Code 3 = evident decomposition; Code 4 = advanced decomposition; and Code 5 = mummified.

	Stretch 1		Stretch 2		Stretch 3		Stretch 4		Stretch 5		Total	%
	N	%	N	%	N	%	N	%	N	%		
Active by land	273	64.7	7	5.5	208	59.9	68	50.7	53	49.5	609	53.5
Active by boat	—	—	1	0.8	33	9.5	16	11.9	22	20.6	72	6.3
Active by partners	45	10.7	74	57.8	2	0.6	5	3.7	—	—	126	11.1
Notification by network of collaborators	104	24.6	46	35.9	104	30	45	33.6	32	29.9	331	29.1
Total	**422**	**100**	**128**	**100**	**347**	**100**	**134**	**100**	**107**	**100**	**1138**	**100**

Table 9. Total number (N) and relative frequency (%) of stranded sea turtles according to monitoring strategy and stretch recorded by BMP-SB between September 2016 and September 2017.

on the coast of the states of Rio de Janeiro and Espírito Santo have been described by other authors [65].

Considering the monitoring modalities in the BMP-SB, daily active efforts by land accounted for 609 of the total number of stranded sea turtles (53.5%) (**Table 8**). Retrieval following notifications accounted for 457 events (40.2%), 331 of which were notified by the network of collaborators and 126 through active partners, which was the modality most responsible for the retrieval of living individuals (**Figure 3**). It is hoped that the population that uses the communication channels of the BMP-SB is sensitized principally due to the debility of living animals. Carcasses in an advanced stage of decomposition or mummified were recorded largely during daily active monitoring by land (**Figure 3**). Boat monitoring was less effective, accounting for only 72 records (6.3%) (**Table 8**).

Daily active monitoring was the main form of records of stranded sea turtles in Stretches 1 and 3, whereas notifications were the main form in Stretch 2. The two forms (daily active monitoring by land and notifications) achieved similar results in Stretch 4. Boat monitoring had a better performance in Stretch 5, but still was not as effective as the other modalities (**Table 9**).

4. Discussion

In the state of Hawaii, USA, the US National Marine Fisheries Service (Pacific Islands Fisheries Science Center, Honolulu) has maintained a monitoring program since 1982, in which the community reports the occurrence of stranded sea turtles on beaches of the Hawaiian islands (Kauai, Oahu, Maui, and Hawaii) to the service by telephone and the animals are sent for either treatment or necropsy [59]. The program has led to the rehabilitation of 1000 sea turtles since its inception, demonstrating the effectiveness and success of this method [66].

In the state of California, USA, a BMP denominated "Beach Combers" was created in 1997, which occurs in the form of collaboration between the Moss Landing Marine Laboratories, Monterey Bay National Marine Sanctuary as well as other state and research institutions, including the

California Department of Fish and Wildlife and the US Geological Survey. Trained volunteers travel all beaches in Monterey Bay in search of stranded marine birds and mammals. The aim of this monitoring is to determine the health index of the sanctuary [67].

In Queensland, Australia, the Department of Environment and Heritage Protection maintains the StrandNet monitoring system. The waters are in the state marine parks, such as the Moreton Bay and Great Sandy Marine Parks in the southern portion of Queensland and the Great Barrier Reef, all of which are protected by laws that target marine fauna in these regions [67, 68]. Most stranding events are communicated by the staff of the governmental departments or park rangers of the Great Barrier Reef Marine Park Authority. Notifications are also received from the public. For such, a direct telephone line is available for use by the population. The stranding events are filed at StrandNet by registered users through a web-based interface and each record receives a unique identification number, differentiated by a single letter in the case of sea turtles. When the information of the record is inconsistent (time, location, or class of animal), the datum is entered as a non-confirmed record. After verification by a trained professional, the coordinates of the stranding, details of the location, date, sex, life stage, size, condition of the animal, and destination of the animal or carcass are recorded. When available, photographs are also uploaded [57]. Thus, the databank provides sufficient information for the development of studies that contribute to the management of endangered species and the unique ecosystem of the Queensland marine parks.

Programs have also been created to restore areas affected by accidents related to petroleum and gas activities, such as the oil spill that occurred in the Gulf of Mexico in 2010 on the Deepwater Horizon offshore drilling rig, which is considered the worst American environmental disaster [69]. Such actions are led by the Marine Fisheries Service of the US National Oceanic and Atmospheric Administration and the US Fish and Wildlife Service, with shared jurisdiction for the recovery and conservation of sea turtles listed in the U.S. Endangered Species Act [70].

In Brazil, sea turtles have been on the endangered species list since the end of the 1970s, but there was no marine fauna conservation program in the country at the time. The TAMAR project initiated its first continual monitoring efforts of the Brazilian coast with the aim of accompanying sea turtle stranding events and recognizing nesting sites [9]. Since then, different studies with the participation of Brazilian universities have been developed to identify events that impact the five sea turtle species that occur in Brazil, such as coastal development [26], fishing activities [18, 71], climate change [72, 73], pollution, and disease [29, 74–76].

The implementation of the BMPs on the coast of Brazil to evaluate the impact of activities related to the petroleum and gas industry on marine fauna occurred in 2010, with programs executed in the Potiguar Basin off the state of Rio Grande do Norte (336 km) and the Sergipe-Alagoas Basin off the states of Sergipe and Alagoas (254 km) in the northeastern region, the Campos Basin off the state of Espírito Santo (763 km) in the southeastern region, and the Santos Basin (more than 1500 km) off the states of Rio de Janeiro, São Paulo, Santa Catarina e Paraná in the southeastern and south region, totaling approximately 2853 km of coastline monitored by land and/or boat as well as notifications from 800 (free of charge) numbers.

As a complement to BMP activities, environmental education programs are offered to local populations. These programs involve recreational activities, awareness of the issue, and pertinent training of the target public in accordance with the age group. This aspect is of the utmost importance to the dissemination of the BMP, as it increases the network of collaborators and results in the awareness of the responsibility of the local population.

The BMP enables the analysis of scenarios of stranded marine animals, which, in turn, enables a clear view of possible long-term effects of petroleum activities. and generating long-term data on the mortality of these animals as well as information on threats, areas of use, age groups, migratory movements, feeding habits, etc., contributing knowledge on the biology of the monitored groups (marine birds, mammals, and chelonians) that can be used in the planning of policies directed at the conservation of species.

5. Conclusion

Beach monitoring is currently one of the main sources of information on the occurrence, diversity and biology of species of marine chelonians, especially in regions where research on these animals is incipient. Determining the exact cause of a stranding is a difficult task due to the combined influences of environmental, biological, and anthropogenic factors, which often act in a synergic manner. Moreover, symptoms and diseases can become obscured by the decomposition stage of the carcass. Stranding events can be caused by environmental factors (associated with oceanographic and climatic conditions) together with factors related to the health of the animal. Human activities developed and intensified in coastal regions are potential triggers of stranding events, such as becoming entangled in fishing nets, collisions with boats, and contact with environmental pollution, which makes populations more susceptible to infections and other health problems.

The determination of the anthropogenic impacts on fauna involves diverse difficulties that imply uncertainties and often impede coming to reasonable conclusions, especially in the short term. Such difficulties are linked to the need for a minimum monitoring time as well as fluctuations in natural and anthropogenic factors, which exert influences on the behavior of living animals and the stranding itself. Giving continuity to the acquisition of data will increase knowledge on species of marine chelonians in the state of Rio de Janeiro and the analysis of the data collected could enable the identification of the cause of death of stranded animals.

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Monitoring Physiological Responses in Giant Toad (*Rhinella marina*) from Coatzacoalcos, Mexico: A Comparative Study after 8 Years

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Abstract

The objective of this chapter is to present the results of a monitoring study carried out with physiological responses (biomarkers) in *Rhinella marina* (giant toad) for two different years, inhabiting the low basin of Coatzacoalcos river, one of the most contaminated regions in Mexico. A decrease in delta aminolevulinic acid dehydratase (δ-ALAD) (considered inhibition) and in the condition factor (1.2–1.5 times) found in toads of the industrial zone compared with reference organisms, each year. As for the hematological parameters, the mean corpuscular hemoglobin concentrations (MCHC), in the amphibians of industrial zone of the first sampling year show a decrease, while for the second sampling year, show an increment of 1.5 times than organisms of reference site. These effects could be associated with exposure to pollutants such as heavy metals (mainly Pb), which have been registered in different studies. This study demonstrates the usefulness of giant toads as biomonitors of contaminated sites.

Keywords: monitoring, physiological biomarkers, giant toads, pollution

1. Introduction

Amphibians are vertebrates that represent the link between life in the aquatic environment and adaptation to terrestrial life. They have important characteristics; such as its ectothermic physiology, metabolism, and highly permeable skin, which makes them sensitive to disturbances in the ecosystem such as changes in water conditions, as well as the presence of pollutants and certain diseases [1–3]. This complex life cycle makes them susceptible to different routes of exposure to environmental pollutants, which is why they have been considered as bioindicators of environmental quality [4]. Currently, environmental pollution is considered one of the main factors in the worldwide decline of amphibians that has occurred since 1990 [5]. Some species of amphibians have all the characteristics of a bioindicator or biomonitor, other species can satisfy only some of the criteria and are less suitable as study animals [2, 6].

The giant toad or cane toad (*R. marina*), **Figure 1** is a species of anuran, omnivorous, and opportunistic amphibian [7], with high somatic and hepatosomatic index [8], high biomass, and a voracious appetite [9], which makes it susceptible to the bioaccumulation of organic and inorganic pollutants and their toxicological effects [10, 11]. This amphibian is native to South America, Central America, and Mexico [12]. In Mexico, it is not listed in the species with special protection or in danger of extinction [13]. It has been used as a bioindicator in the evaluation of air pollution [14], infectious diseases [15], organochlorine pesticides [16], endocrine disrupters [17], pollution by lead [11], and other persistent organic pollutants (POPs) [10].

In some of the previous research, different physiological responses have been used, from general to specific responses of pollutants from the study sites. These responses are called biomarkers, where all biochemical, physiological, histological, morphological, and behavioral measurements are quantifiable in tissue or biological fluids from different organisms, including amphibians, like the *R. marina* [4, 18, 19]. An advantage of biomarkers is that variations that arise can be related by the influence of stressors and can serve as an early indication of major effects on the organism or the population of these [20]. Also, with this, it is possible to compare values of biomarkers of a resident population to contaminated sites with another reference population, as well as monitoring by seasons or years, to indicate the influence of exposure to pollutants in the general health status of organisms [21].

Moreover, in Mexico, there are several types of environmental pollution scenarios that have not been assessed from the point of view of the effects to the biota living these sites. In addition to this, there are very little environmental regulations as to some types of pollutants, such as: persistent organic compounds (POPs), polycyclic aromatic hydrocarbons (PAHs), and

Figure 1. Giant or cane toad (*R. marina*).

heavy metals. One of the sites has a higher record environmental pollution is the region of the lower Coatzacoalcos River Basin (Veracruz); region highly impacted due to industrial activities and urbanization that have contributed to the deterioration of ecosystems since the 1960s. Currently, Coatzacoalcos is considered one of the most polluted regions in Mexico [22, 23], to such a degree that, in environmental matrices such as water, soil, air, or sediment, and even in fauna, the presence of POPs, volatile organic compounds (VOCs), dioxins, heavy metals, among others, has been detected [11, 24–27]. Some of these pollutants have been associated with genotoxic or enzymatic effects in aquatic and terrestrial organisms at this site [10, 11, 28, 29]. In this context, the objective of this chapter is to present and compare the results of a monitoring of physiological responses (biomarkers) in *R. marina* carried out in the lower basin of the Coatzacoalcos river in different years as a follow-up of the environmental quality of the region.

2. Materials and methods

2.1. Study site

The lower basin of the Coatzacoalcos river is in the southeastern state of Veracruz, Mexico (18° 08′56″ N; 94°24′41″ W). It comprises 21 municipalities, which house petrochemical, industrial, urban complexes, and agricultural land areas. Pollution has been historic since oil exploration and refining works began at the beginning of the twentieth century up to industrialization, agricultural development, and urban growth at present, causing a rapid deterioration of the ecosystems found there.

The sampling stations were established along the basin according to the degree of contamination found. Thus, they were grouped by two zones: industrial and rural. In May 2008, the first sampling of monitoring study was carried out, while the second was carried out in February 2016 (**Figure 2**).

The industrial zone (I) was formed by the following sampling stations (red oval, **Figure 2**):

- Ejido Cangrejera: site adjacent to the petrochemical complexes of Pajaritos and Cangrejera, where various derivatives of chlorine and ethylene compounds are produced. In addition, the presence of POPs in both environmental and biological matrices has already been evidenced in this area, as well as the effects that these could be causing in terrestrial organisms [24, 28, 29].
- Estero del Pantano: site located on the banks of the Calzadas River. According to [11] the river receives discharges of sewage and industrial waters. Also, the presence of POPs and lead (Pb) has been demonstrated, as well as the effects of these compounds on terrestrial and aquatic organisms [10, 11, 30].

While the rural area (R) (yellow oval, **Figure 2**) was formed by:

- Ejido Limonta: Located upstream of the Coatzacoalcos river, in the municipality of Hidalgotitlán. It presents scarce urbanization and ecosystems still well preserved. At present, concentrations of pollutants have not been reported in this sampling station.

- San Carlos: Located next to the Uxpana river, upstream and that ends at the Coatzacoalcos river. It has well-preserved ecosystems. Like the previous one, no contaminant concentrations have been reported in this site.

The sampling stations of the rural area selected are characterized by semi-preserved ecosystems and minor impact by agricultural production, being susceptible areas where organophosphorus, organochlorine, or carbamate pesticides can be used to control pests or vectors.

As a reference, giant toads (seven organisms) kept in the laboratory for 1 year under conditions of feeding, humidity, and controlled temperature were selected, collected in a site outside the study area (Huasteca Potosina, San Luis Potosí) and free of exposure to pollutants.

2.2. Biological sampling

Adult male giant toads were collected per site by night transects and hand capture. In the first sampling (May 2008), 40 toads were collected, while in the second sampling (February, 2016), 30 toads were collected. The organisms were transported in containers to the laboratory (Facultad de Química-Universidad Veracruzana-Campus Coatzacoalcos). The body weight and snout-venth length (SVL) were taken. Subsequently, a blood sample (3–5 mL) was taken with heparinized syringes (following the guidelines established for amphibians and reptiles [31]). An aliquot

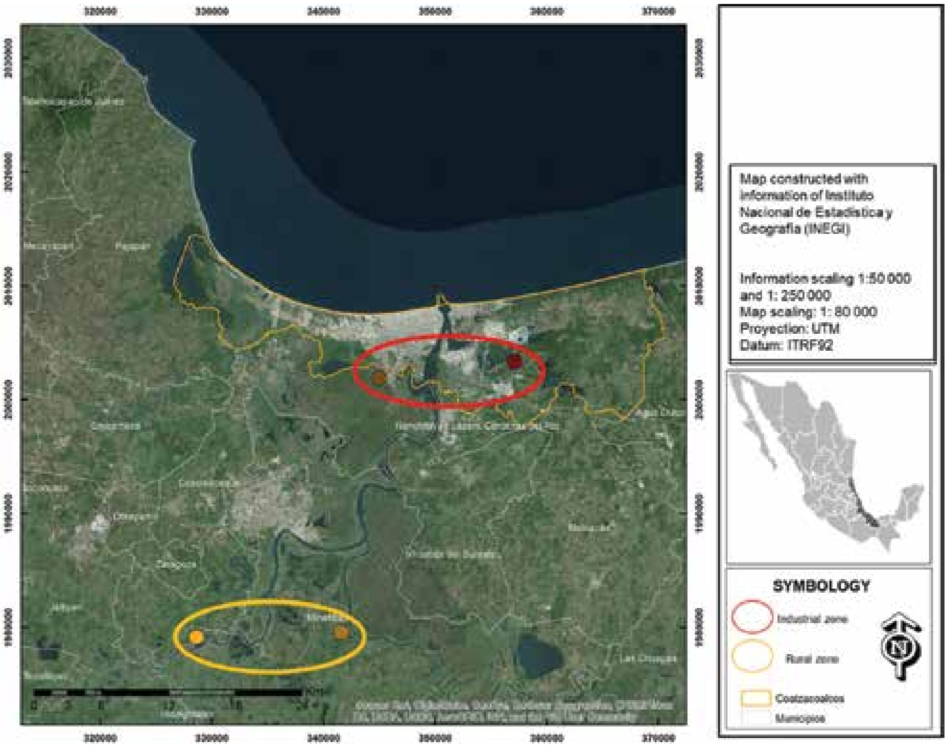

Figure 2. Zones of sampling in low basin of Coatzacoalcos (source: Google Earth, 2016).

of whole blood was stored in liquid nitrogen (−186°C) for enzymatic analysis. While with the rest the hematological parameters were analyzed. The toads were released in their respective habitat. The toads were collected under a scientific collection permit (SGPA/DGVS/09731/15) issued by the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT).

2.3. Evaluation of physiological responses (biomarkers)

2.3.1. Delta aminolevulinic acid dehydratase

The inhibition of the activity of the enzyme δ-ALAD is a biomarker that has shown in several studies a response to a specific contaminant. This enzyme is very sensitive to the effects of increased blood lead, even at very low levels [32]. In the biosynthesis of the heme group, the prosthetic group of hemoglobin (Hb) is selectively damaged by the binding of lead, thus affecting important steps of biosynthesis, inhibiting the activity of δ-ALAD (necessary for the synthesis of the heme group), and affecting the synthesis of protoporphyrin IX and hemoglobin [33, 34].

The determination of δ-ALAD was based on the method of [35] following modifications [11]. The whole blood samples (0.05 mL) were diluted with deionized water (1:35). Then 1 mL of 10 mM aminolevulinic acid (in phosphate buffer pH 6.4) was added. The samples were incubated at 38°C for 60 min in a water bath in complete darkness. The reaction was stopped by placing 1 mL of trichloroacetic acid (10%), then the samples were centrifuged for 10 min at 2000 rpm (Thermo Scientific® Sorvall Legend X1R). The supernatant was mixed with 1 mL of the Erich's solution. After 10 min, the absorbance was measured at 555 nm in a UV–Visible spectrophotometer (Thermo Scientific® Genesys 10S). Units of enzymatic activity were expressed as micromole per min per liter of red blood cells (RBC), which were calculated using the following formula (Eq. 1):

$$\delta\text{-ALAD} = \frac{\text{Abs} \times 100 \times D \times F}{\text{HT} \times 60 \times 0.062} \tag{1}$$

where Abs = absorbance of the sample, F = 2 (porphobilinogen conversion factor to δ-ALA), D = 35 (dilution factor), HT = hematocrit (%), 60 = incubation time (min), and 0.062 = molar extinction coefficient (L/μmol × cm).

2.3.2. Hematological parameters

The chemical and morphological parameters of the blood can provide a wide range of biomarkers; its use has increased because the sampling can be very fast and non-destructive [36]. The hematological parameters can provide evidence of pathology including anemia, dehydration, infectious processes, parasitism, or poisoning [37]. These parameters in turn may be related to pollution and its effects [38]. The volume of the cell pack or hematocrit (HT) is the percentage of the total volume of blood composed of red blood cells. The mean corpuscular hemoglobin concentration (MCHC) is the range of the weight of hemoglobin by the volume of the erythrocyte. An iron deficiency, increased immature erythrocytes (reticulocytosis) and methemoglobin can result in a decrease in MCHC values [32]. Some toxic compounds can alter the functioning of the hematological system through interference with cellular production in the bone marrow or in the synthesis of the heme group, by direct cytotoxicity to the cells, or by injuries in other tissues resulting in a loss of blood cells [32, 39].

Therefore, the hemoglobin (Hb), hematocrit (HT), and the mean corpuscular hemoglobin concentration (MCHC) were determined. The hemoglobin content (g/dL) was measured using the kit HemoCue Hb 201^{+} (microcuvettes and HemoCueHb 201^{+} Analyzer). The determination was made following the protocol of the commercial distributor [40]. The hematocrit was determined with the globular sedimentation method with the aid of the hematocrit chart (Critocaps™ tube reader). The MCHC was calculated integrating the two previous parameters following equation.

$$\mathrm{MCHC} = \frac{\mathrm{Hb}\ (\mathrm{g/dL}) \times 100}{\mathrm{HT}\ (\%)} \tag{2}$$

2.3.3. Condition factor (CF)

Condition indices are potentially attractive biomarkers because they are simple to implement and provide information on the use of energy as well as the general health status of the organism [41]. The morphometric index most used is the condition factor (CF) expressed as the weight (g)/length (cm). Pollutants can produce rapid and marked changes in condition indices.

To calculate the CF, the snout-vent length (SVL) of the toads was taken with a vernier caliper (mm) and its body weight (BW) (g) with an electronic scale. Subsequently, these two parameters were integrated and used the following formula (Eq. (3)) to calculate the CF (%).

$$\mathrm{CF} = \frac{\mathrm{BW}(\mathrm{g}) \times 100}{\mathrm{SVL}\ (\mathrm{mm})} \tag{3}$$

2.4. Statistical analysis

The statistical analysis was carried out with the GrapPhad Prism 6.0 software (for Windows, La Jolla California USA, www.graphpad.com). The results are reported in media ± standard error. A comparison analysis of means (Mann-Whitney test) was carried out to evaluate the differences between the biomarkers per years, with a level of significance of 1 and 5%. To evaluate the difference in δ-ALAD between years, zones, and the laboratory, the Kruskall-Wallis test was used. A correlation between δ-ALAD and hematologic parameters (Log-transformed) was realized with Spearman's test.

3. Results and discussion

3.1. δ-ALAD activity

The results of the δ-ALAD activity in blood of *R. marina* per year and per sampling area are shown in **Figure 3A**. When the results were compared per sampling area for each year, it was obtained that in 2008 the toads of the industrial zone (70.2 ± 3.3 μM × min × mL RBC) had lower activity of δ-ALAD compared with the organisms residing in the rural area (15.4 ± 2.3 μM ×

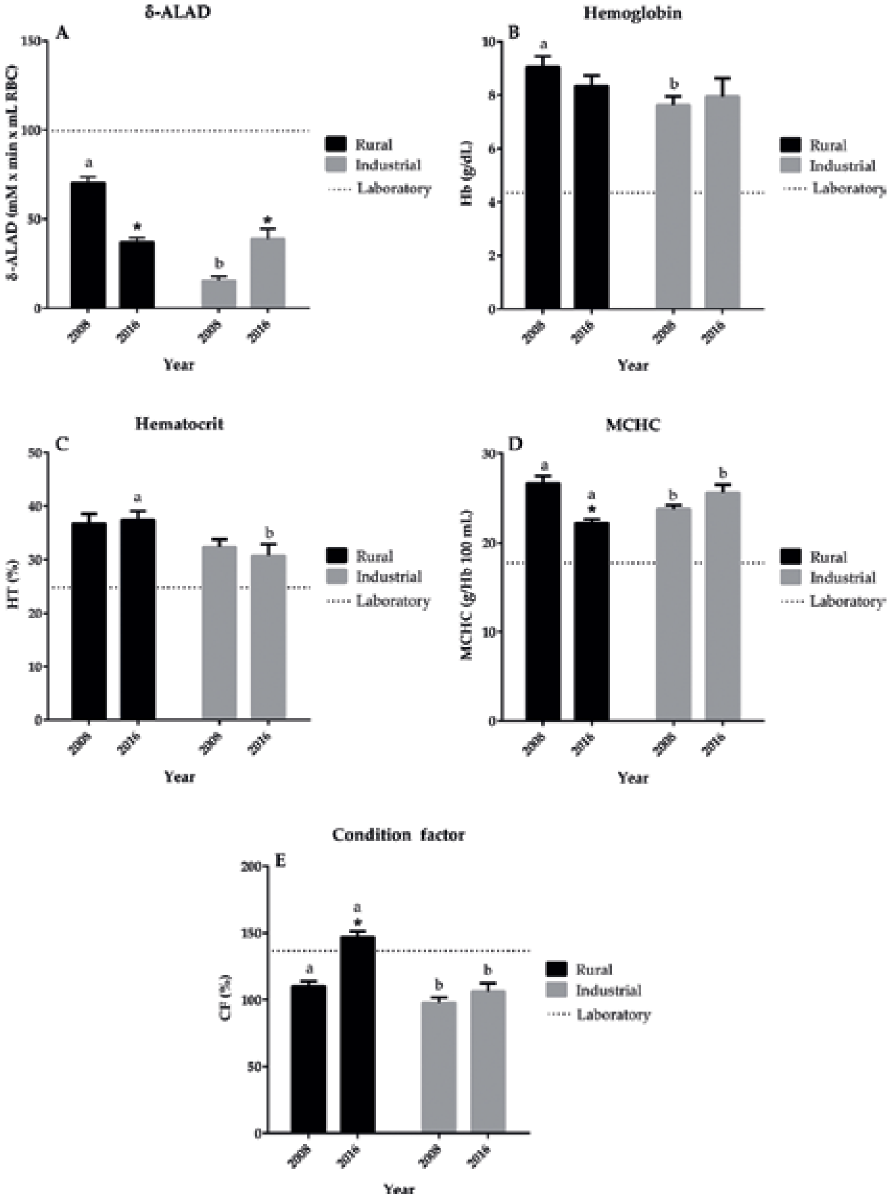

Figure 3. Comparison of biological responses (biomarkers) in blood of giant toad (*R. marina*) per year and per zone). *denotes a statistical differences between years per zone (MW-U test, $p < 0.001$), letters denotes a statistical differences between zones per each year (MW-U, $p < 0.05$, $p < 0.001$), 2008: R(n = 20), I(n = 20); 2016: R(n = 19), I(n = 11).

min × mL RBC) ($MW\text{-}U_{20,20} = 0.0$, $p < 0.001$). But, for 2016, the levels of this enzyme were found similar for the toads of both zones (36.9 ± 2.5 and 38.6 ± 5.9 μM × min × mL RBC, respectively). When comparing areas by year, for 2016, in the toads of the rural area, there was a statistically significant decrease in the δ-ALAD activity, compared with those of 2008 ($MW\text{-}U_{20,19} = 8.0$, $p < 0.001$). In contrast, with the organisms of the industrial zone, a significant increase was observed for the year 2016 ($MW\text{-}U_{20,11} = 26.0$, $p < 0.001$) (**Figure 3A**). On the other hand, the levels of total δ-ALAD, per year and per sampling area in *R. marina* of Coatzacoalcos were 35–60% lower than in the laboratory (99.6 ± 5.6 μM × min × mL RBC) ($KW\text{-}H_{2,77} = 16.5$, $p < 0.05$), which were not exposed to any pollutants.

The δ-ALAD activity has been shown to be a specific biomarker for evidence of lead exposure in organisms such as birds, amphibians, and mammals, both in the field and in the laboratory [42–44]. Our results show that, in 2008, toads from the industrial zone showed an inhibition of the δ-ALAD enzyme compared to those from the rural zone, which can be consistent and expected if this area is taken as a reference. This inhibition can be attributed to concentrations of lead found in the blood of giant toads, where residents of the industrial zone contained higher concentrations of this metal [11]. However, for 2016, the activity of δ-ALAD was similar for both areas. Refs. [33, 43] demonstrated in laboratory studies that exposure to lead in adult toads (*R. arenarum*) inhibited the δ-ALAD enzymatic activity, as concentrations of this metal increased. Likewise, it has been demonstrated the inhibition of δ-ALAD in birds, reptiles, and mammals resident in mining or industrial areas, which are associated with exposure to Pb [45, 46]. In this sense, intense industrialization in the lower basin of the Coatzacoalcos river is associated with the increase of heavy metals such as lead [47, 48]. Different studies in this region have reported the presence of lead in organisms such as fish, turtles, toads, and even humans, associated with neighboring industrial complexes [11, 30, 49]. Therefore, these could be factors that led to exposure to lead in the toads of the industrial zone in both 2008 and 2016. However, as mentioned above, in 2016, the levels of δ-ALAD decreased in the toads of the rural area and were found to be similar to those of the industrial zone. This could be due to two factors. The first is that the rural area is close to Minatitlán, municipality of Veracruz where industries are found, especially refining and petrochemical, which could be sources of non-point pollution of lead, and in recent years can also be considered to have been increased emissions (or possible sources), and therefore increased exposure to lead in organisms. On the other hand, a second factor that could be causing this exposure would be the extraordinary meteorological phenomena that have occurred in this area, for example, recent floods. It has been documented that some heavy metals, such as lead, and organic compounds can be mobilized and affect their bioavailability due to the removal of sediments when extreme weather events such as floods occur [27], so possibly an event like this has increased the exposure to lead in the organisms in the area.

On the other hand, the δ-ALAD activity in the laboratory toads was greater for those of Coatzacoalcos; this would also confirm an enzymatic inhibition in the organisms of this region, increasing in recent years. Finally, it could be said that the δ-ALAD activity could reflect a chronic exposure to lead, because the erythrocytes that carry out the transport of hemoglobin have a half-life of between 700 and 1400 days in *R. marina* [50].

3.2. Hematological parameters

The concentrations of hemoglobin and the percentage of hematocrit are presented in **Figure 3**. No statistical differences were found per year between zones (MW-U, $p > 0.05$). The levels of both parameters were similar in the toads resident in the rural area in 2008 (Hb = 9.05 ± 0.4 g/dL, HT = 34.6 ± 1.9%) and 2016 (Hb = 8.3 ± 0.4 g/dL, HT = 37.4 ± 1.6%) as well as those of the industrial zone in both years (Hb = 7.6 ± 0.3, HT = 32.3 ± 1.4; Hb = 7.9 ± 0.7 g/dL, HT = 30.7 ± 2.2%, respectively) (**Figure 3B** and **C**). However, when comparing by area in each year, a statistically significant decrease in the hemoglobin concentration of *R. marina* of the industrial zone was obtained in the sampling of 2008 ($\text{MW-U}_{20,20} = 86.5$, $p < 0.05$). While for 2016, Hb levels were similar ($p > 0.05$) (**Figure 3B**). Otherwise, with the percentage of HT, the levels in 2008 were similar in both areas. However, for 2016, a significant decrease was observed in the toads of the industrial zone ($\text{MW-U}_{19,11} = 54.0$, $p < 0.05$) (**Figure 3C**). When both parameters were integrated in MCHC (**Figure 3D**), a statistically significant decrease was observed in the toads of the rural area in 2016 (22.1 ± 0.4 g/Hb 100 mL)($\text{MW-U}_{20,19} = 50.5$, $p < 0.001$). When comparing areas for each year, a statistically significant decrease was obtained for organisms in the industrial zone (23.7 ± 0.4 g/Hb 100 mL) compared to those in the rural area (26.6 ± 0.7 g/Hb 100 mL) in the 2008 sampling ($\text{MW-U}_{20,20} = 118.0$, $p < 0.05$). While in the 2016 sampling, toads from the rural area showed a significant decrease compared to those in the industrial zone (25.6 ± 0.9 g/Hb 100 mL)($\text{MW-U}_{19,11} = 37.5$, $p < 0.05$). In turn, it is also observed that Coatzacoalcos toads showed higher levels (1.2–2.1 times) in the hematological parameters compared to those maintained in the laboratory (17.7 ± 1.1 g/Hb 100 mL).

Very few studies, in relation to pollutants and hematological parameters, have been carried out with amphibians. [51] used these parameters to evaluate the effects of agroecosystems on the health of amphibians; however, no statistically significant differences were found between the reference site and the agroecosystems. The results obtained in our study are contrary to those obtained in [52] where they showed a decrease in hematological parameters according to the presence of lead in tissues of the Egyptian toad *Amietophrynus regularis*.

We consider that the hematological parameters of Coatzacoalcos toads are altered (increases or decreases) when comparing areas in both years. But not so, if they are compared per year between zones, because similar values are obtained in Hb and HT, only one alteration was found in the MCHC in the organisms of the rural area in 2016. The increase of some parameters, such as Hb, could be related to a response of organisms to a decrease in the transport of oxygen in the blood (hypoxia), derived from anemia (anemic hypoxia) [53]. Hypoxia in some vertebrate organisms (fish, amphibians) has been related to the natural and anthropogenic increase of ammonium, sulfides, or organic matter, as well as the presence of pollutants (organic or heavy metals—lead) and excess nutrients [54, 55]. In the case of MCHC, the decrease in organisms residing in industrial zones, in the sampling of 2008, can be attributed to contamination in these places, being associated with a microcytic anemia, which could have been caused by exposure to pollutants such as heavy metals (e.g. lead) [11]. But for 2016, MCHC concentrations in *R. marina* decreased in the rural area and increased in the industrial zone. The above could support the hypothesis of the increase of pollutant concentrations in the rural area which is affecting the resident organisms of this area. On the other hand, the fact of finding parameters of greater hematological parameters in the Coatzacoalcos organisms compared with the laboratory ones could support the hypothesis proposed by [11], where they point out that one of the mechanisms of response of vertebrates to an exposure could be the increase in the number of erythrocytes (polycythemia) increasing the capacity of oxygen transport, and therefore, some hematological parameters. Suggesting this phenomenon as a compensation mechanism against anemic hypoxia.

3.3. Condition factor (CF)

The results of the condition factor of *R. marina* are shown in **Figure 3**. As observed, only a significant increase in CF of the toads of the rural area was found in 2016 (146.9 ± 4.0%) ($MW\text{-}U_{19,11} = 21.0$, $p < 0.001$). When comparing between areas for both years, a significant decrease of 11.3 and 27% in CF of the industrial zone organisms was observed, compared with those of rural areas in both years ($2008 = p < 0.05$, $2016 = p < 0.001$) (**Figure 3E**). When the CF of the Coatzacoalcos toads are compared with the ones of the laboratory (136.5 ± 8.8%), it is observed, only similar in the toads of the rural area sampled in 2016, the others show the pattern of decrease (11–27%).

The estimation of the condition factor in amphibians is important to evaluate if they are under environmental stress [56]. This biomarker is commonly used to assess the general health status of aquatic and terrestrial organisms, because it is considered a non-destructive biomarker of energy reserves [57]. This is very important given that energy reserves can be used for the maintenance, development, or reproduction of amphibians. Commonly, a greater reserve of energy in organisms gives them greater resistance without food, greater survival, and better reproductive performance compared to individuals with lower reserves [58].

There is very little information on the use of the condition factor in amphibians in contaminated sites. Some researchers [58] reported a decrease in the condition factor and enzymatic alterations in semi-aquatic and terrestrial frogs resident in agricultural sites, associating it with a possible activation of compensatory or detoxification systems in amphibians in the face of environmental stress or a decrease in their prey (mosquitoes) by the application of insecticides. In other organisms, such as fish and birds, the decrease in the condition factor after exposure to heavy metals and organochlorine compounds, respectively, has been reported [59, 60]. Exposure of organisms to pollutants can increase energy requirements, decrease the metabolic or nutrient assimilation rate, and even alter digestion enzymes [41, 54, 61]. This could explain the decrease in the condition factor of the resident toads of the industrial zone in our studies (both samplings), given that, as already mentioned, a greater presence of pollutants has been demonstrated in this area. Regarding the similarity found between the toads resident in the rural area of 2016 and the laboratory ones and their increase in comparison of the organisms of the rural (2008) and industrial sites (2016), another factor that could be influencing these differences could be the scarce availability of food, caused either by natural conditions or by anthropogenic conditions.

3.4. Relationship between δ-ALAD and MCHC

The results of the correlations between the δ-ALAD enzymatic activity and the MCHC are shown in **Figure 4**. As observed in this figure, for the organisms of the 2008 sampling, in the rural area, there is an association between these physiological responses. This same pattern is observed considering both (total) sites in this sampling year (**Figure 4A** and **C**—2008). As previously mentioned, the relationship of the activity of this biomarker and the hematological parameters is found in the fact that the former is part of the metabolic pathway of hemoglobin, which is congruent with these results. However, when making the correlations for the toads of the 2016 sampling, only a statistically significant correlation ($p < 0.05$) between both biomarkers is observed for the industrial zone (**Figure** 4B-2016). It is important to mention that for the laboratory toads, no correlations were found between the physiological responses, besides that the values of δ-ALAD in these were found above those of Coatzacoalcos, and the hematological

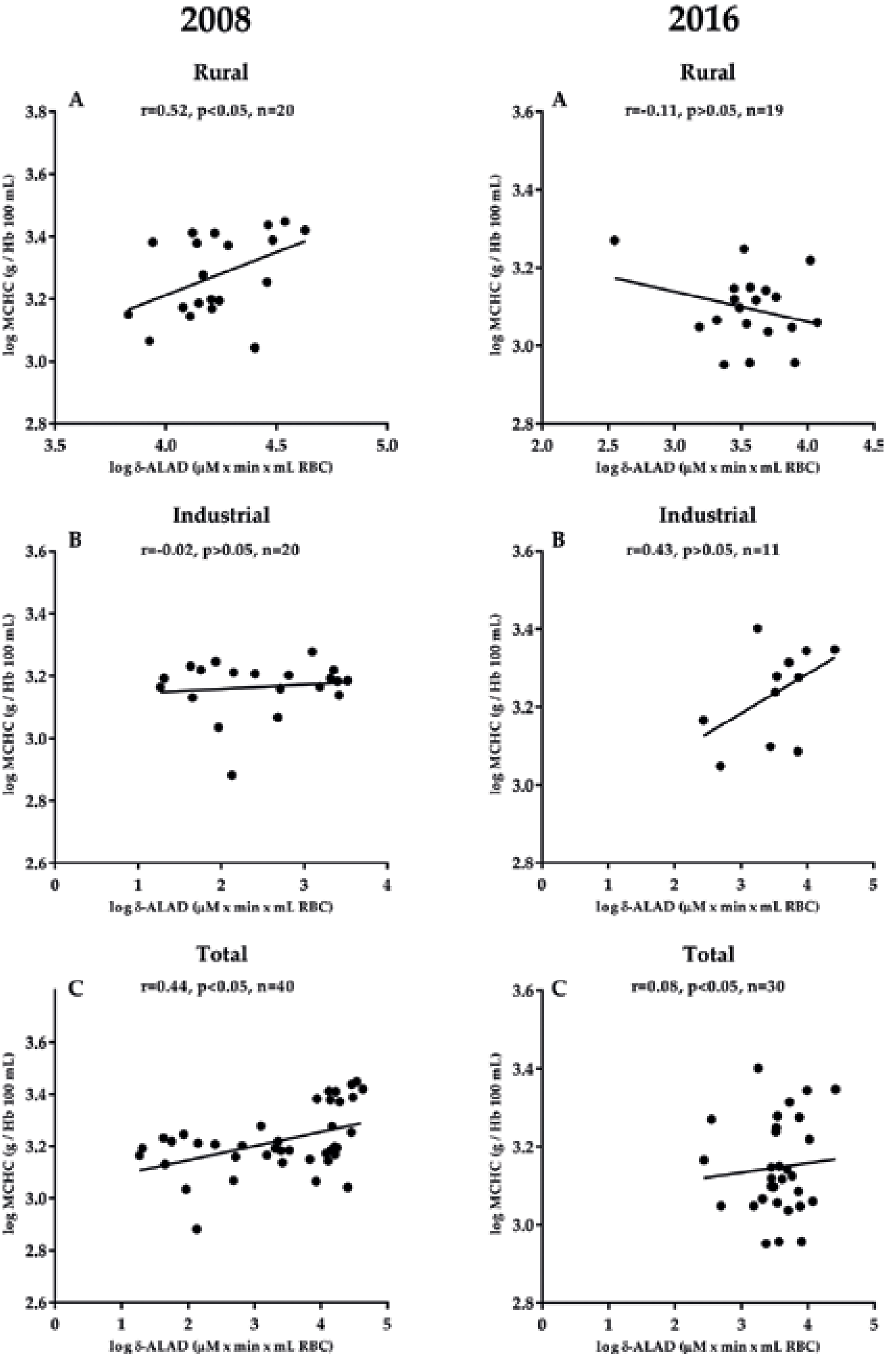

Figure 4. Correlations between δ-ALAD and hematologic parameters per site and per year (total) in blood of *R. marina* (Spearman test); A= Rural, B= Industrial, C= Total.

parameters in smaller quantity. This could support the hypothesis that organisms from this site could be producing more Hb and HT (MCHC) to defend against the effects caused by pollutants (heavy metals such as lead) found in the area, which is evident in the organisms of the rural area of the 2008 sampling. However, for the 2016 sampling, the rural area shows a decrease in the MCHC. Therefore, the hypothesis could be supported that the concentrations of lead have increased in this site and consequently the exposure, thus affecting the compensation system of the organisms in this area. In addition to confirming a chronic exposure to this pollutant.

It is important to mention that several routes and sources of exposure to lead can be found in the toads of Coatzacoalcos due to their complex life cycle. One of these can occur when the tongue of this organism catches its prey, because this organ can be in contact with the soil or sediments (which can be exposed by dredging that takes place in the area), and, as a consequence, ingest particles in which lead may be present [62]. Also, the exposure in this case could be increased by consuming preys that have been exposed to this metal and that contain it; in this case, the presence of lead has been demonstrated in insects that inhabited industrial sites [63] that could be part of the diet of the giant toad in Coatzacoalcos due to their voracious appetite [9]. Likewise, it has been demonstrated that the consumption of prey in some organisms can increase the concentrations of lead and manifest the toxic effects [64]. Therefore, this would complement the possible explanation of the decrease or increase in some physiological responses in organisms of the 2016 sampling.

Finally, it should be mentioned that the physiological responses quantified in *R. marina* are considered non-destructive biomarkers, because they are evaluated in the organism without sacrificing it. The above turns out to be important, because in this way, organisms could be monitored by seasons, years, or by places. Moreover, these studies would be relevant in *R. marina* because this amphibian has a life expectancy of 10–15 years, which could be complemented with a tagging of these. It should be noted that in this study no labeling of organisms was used.

4. Conclusion

The δ-ALAD activity, the hematological parameters, and the condition factor can be considered as biomarkers of exposure and/or effect, non-destructive, in giant toads in monitoring studies in sites contaminated by heavy metals or other pollutants. Especially δ-ALAD, which could reflect a chronic exposure in organisms. On the other hand, the results found with the organisms of the lower basin of the Coatzacoalcos river could lead to the need to make a new monitoring with emphasis on the rural regions to affirm or discard out if there is an increase in the concentration of pollutants, especially Pb, its possible causes and, if this may be affecting other organisms and even the human settlements that are there. New studies carried out in this region should take into account this type of physiological responses in a battery of biomarkers that reflect the response to other pollutants already registered on the site.

Once again, it was confirmed that the giant toad (*R. marina*) is a good biomonitor of contaminated sites and can be useful for the evaluation of exposure and effects by pollutants in different scenarios in Mexico due to its ease of capture, wide distribution, and, as already demonstrated, to its susceptibility to xenobiotics. Likewise, it is recommended to create new lines of research with this amphibian to be able to elucidate the effects or compensation mechanisms that may arise from natural or anthropogenic activities.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this chapter.

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Chapter 5

A Methodology Based on Bioacoustic Information for Automatic Identification of Reptiles and Anurans

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Additional information is available at the end of the chapter

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Abstract

Nowadays, human activity is considered one of the main risk factors for the life of reptiles and amphibians. The presence of these living beings represents a good biological indicator of an excellent environmental quality. Because of their behavior and size, most of these species are complicated to recognize in their living environment with image devices. Nevertheless, the use of bioacoustic information to identify animal species is an efficient way to sample populations and control the conservation of these living beings in large and remote areas where environmental conditions and visibility are limited. In this chapter, a novel methodology for the identification of different reptile and anuran species based on the fusion of Mel and Linear Frequency Cepstral Coefficients, MFCC and LFCC, is presented. The proposed methodology has been validated using public databases, and experimental results yielded an accuracy above 95% showing the efficiency of the proposal.

Keywords: acoustic data fusion, bioacoustic processing, biological acoustic analysis, anurans identification, reptiles identification, pattern recognition, cepstral coefficients

1. Introduction

The technological advances open the door to develop and implement tools in different and wide fields of science. In particular, the use of specific devices to acquire sound, the use of big computational load, the implementation on programming languages of feature extraction algorithms, and machine learning systems give the option to develop a novel approach to identify different kinds of animal species from their sounds. This type of tool will do easier Biologist's task on their studies about the environment and the behavior of those animal species.

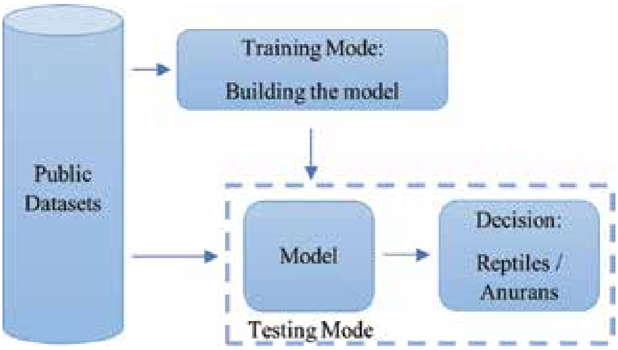

Figure 1. Block diagram of the recognition system based on reptiles and anurans.

Many features can be used for these tools, but it can be closed according to the kind of species to analyze. The main features are from videos, images, or sounds. For this work, the idea is to propose a methodology to identify reptile and anuran species; therefore, all previous features can be applied. Nevertheless, the main activity of these species is during the night. Hence, the sound is the most useful feature to know the daily activity and to carry out the species identification.

In this chapter, the bioacoustic information will be the feature used to this development; and a robust and novel proposal based on the fusion of MFCC and LFCC for the identification of different reptile and anuran species is presented. The proposed approach has been validated according to **Figure 1** using public datasets, and experimental results show the efficiency of the proposal. Based on a supervised classification system, this approach is composed by two modes, training and testing modes. This methodology follows a hold-out cross validation method.

In addition, a feature extraction technique with the highest classification capacity and minimal computation complexity is implemented. To face this challenge, a set of experiments allowing the comparison between the performances of the different feature extraction techniques to apply are shown. The goal is to specify which features are the most effective, obtaining the bioacoustic characteristics and the identification of reptile and anuran species.

The rest of this chapter is organized as follows: Section 2 shows related works. Section 3 describes the methods for automatic identification of reptile and anuran species. In Section 4, the experimental methodology and the results are described. Finally, in Section 5, the conclusions derived from this work are summarized.

2. Related works

There are numerous previous studies on the spectral-temporal characteristics of the acoustic emissions produced by animals, which attempt to analyze the frequency and time parameters of these emissions to identify patterns in their communications and social iterations [1, 2].

In recent years, various efforts have been made to automate the processing of acoustic information using intelligent systems. However, most of the studies conducted in this field have focused their research on a single animal group and, in most cases, these studies have been carried out on just a few species. For instance, one of the first attempts to automatically recognize animal species can be found in [3], where neural networks were used to classify the vocalizations of two false killer whales.

The sounds of insects have also been studied. As an example of this, in [4], their emissions were characterized by using LFCC, their fundamental harmonic and the distance of each call. Authors achieved an 86.3% identification result at species level using Gaussian mixture model (GMM) as a classifier. On the other hand, in [5], the identification of 14 species of birds using two different sets of parameters was proposed. The first data set represented sounds using Mel coefficients and the second consisted of a set of signal parameters such as frequency range, spectral flow, and Wiener's entropy. For the classification of the vocalizations, authors proposed a decision tree (DT) where each node of the tree was formed by a support vector machine. In their experiments, MFCC achieved the best results but separated the species into two sets of data.

Many other methods have been applied to other groups of animals such as primates [6, 7], bats [8], fishes [9, 10], elephants [11], dolphins [12], but birds [13–16] have been especially studied for their wide variety of vocalizations. Recently, however, the acoustic characteristics of the anurans have managed to attract the attention of the scientific community, due to their relatively simple vocalizations and abundant sound production, which make them ideal test subjects for automatic recognition. Therefore, several studies have been carried out with varying degrees of success extracting different types of acoustic signal parameters to characterize the amphibian vocalizations.

One example of this can be found in [17], where four anuran species were classified using neural networks (NN), applying a discrete wavelet transform (DTW) to get the main features of each frog call and Fisher's optimization criterion of reducing the data dimensionality. This method was able to identify the species with a success rate of 71%, but it required a high computational cost. Instead, in [18], five frog species were analyzed, computing the threshold-crossing rate, signal bandwidth, and spectral centroid. With these features, they achieved an accuracy of 89.05% by using K-Nearest Neighbors (KNN) and 90.30% by applying support vector machine (SVM). A different approach was proposed by Han et al. in [19] that combined three types of entropy (Shannon, Rényi, and Tsallis) to recognize nine Microhylidae frog species. This method managed to correctly identify only seven of the nine frog species due to the similar entropy values among these species.

Low-level acoustic attributes have been also used to discriminate frog vocalizations at genus level with a significant rate of success [20]. Coefficient of variation of root-mean-square energy, dominant frequency, and spectral flux were computed for short-time frames to distinguish between the advertising calls of four genera, *Bufo*, *Hyla*, *Leptodactylus*, and *Rana*.

On the other hand, MFCC have been widely used in the recognition of anurans and reptiles in combination with a variety of pattern recognition techniques, due to their noise robustness and computational efficiency. For instance, an interesting approach was developed by [21], that achieved the classification of 30 frogs and 19 cricket species with success rates above 96% with a large standard deviation. For this they split the acoustic signal into frames and calculated the average of the MFCC to train a linear discriminant analysis (LDA) algorithm. Another example can be found in [22], where the MFCC were tested in three algorithms: Local Mean KNN with Fuzzy Distance Weighting (LMkNN-FDW), sparse representation classifier (SRC), and SVM. LMkNN-FDW outperformed SRC and SVM, obtaining the highest-performance results on 20 frog species.

At present, deep learning techniques are being employed in frog acoustics classification [23–25], applying convolutional neural networks (CNN). However, most of these works also use MFCC as parameters, relying on the discriminatory capacity of the classifier without looking for a better representation of the acoustic signal information. **Table 1** summarizes some different techniques and algorithms that have been used in the recognition of anurans.

Lastly, the class *Reptilia*, however, has received little attention due to its limited sound production. In fact, to the best of our knowledge, the acoustic signals for reptile's automatic identification have been poorly considered in literature, being this work one of the first research to address this approach. Although only a few species such as crocodiles present an important repertory of calls. In this research, the sound emitted by reptiles has been intensively studied to verify the capacity of their calls for inter-species identification.

References	Parametrization	Classifier
Yen and Fu [17]	Discrete wavelet transform (DTW)	NN
Lee et al. [21]	MFCC	LDA
Brandes [43]	Peak frequencies and bandwidth	HMM
Acevedo et al. [41]	Call length, maximum and minimum frequencies, maximum power, and the frequency of maximum power	SVM, DT and LDA
Huang et al. [18]	Spectral centroid, signal bandwidth, and threshold-crossing rate	KNN and SVM
Han et al. [19]	Shannon, Rényi, and Tsallis entropies	KNN
Yuan et al. [27]	MFCC and linear predictive coding (LPC)	KNN
Bedoya et al. [42]	MFCC	Learning algorithm for multivariate data analysis (LAMDA)
Chen et al. [44]	Length of the segmented syllables	Multi stage average spectrum (MSAS)
Xie et al. [28]	Dominant frequency, syllable duration, frequency modulation, oscillation rate, and energy modulation	PCA and KNN
Hassan et al. [25]	MFCC	Convolutional neural networks (CNN)

Table 1. Summary of amphibian acoustic identification methods proposed by literature.

3. Methods

The proposed methodology in this chapter is illustrated in **Figure 2** and is composed by the following methods. First, both reptile and anuran audio recordings are processed by a segmentation algorithm to separate the acoustic signal in syllables. Next, the cepstral feature parameters, MFCC and LFCC, are extracted and fused in a vector standing for the main characteristics for each syllable. Then, these vectors are used as inputs in the classification phase for training and testing a classifier implemented by a machine learning algorithm. Next, a detailed description of each method is defined.

3.1. Signal segmentation

In order to obtain useful features for the automatic identification, the audio recordings are split into as many syllables as possible. This process is based on the work of Härmä [26] for acoustic signal segmentation. The algorithm makes use of the signal spectrogram to detect each sound and separate it into syllables. The spectrogram was determined by short-time Fourier transform (STFT) with the following Hamming window sizes which have been heuristically computed: 256 samples and 33% overlap for reptiles, and 512 samples and 25% overlap for anurans corpus. As a result, the matrix $H(f,t)$ represents the computed signal spectrum where f is the frequency and t the time. The segmentation procedure performs the following steps repeatedly until the end of the spectrogram is reached:

1. Find t_n and f_n such that $|H(f_n,t_n)| \geq |H(f,t)|$ computing the amplitude in t_n as $\Upsilon_n(0) = 20\log_{10}(|\mathrm{H}(\mathrm{f}_n,\mathrm{t}_n)|)$.
2. From t_n, seek the highest peak between $t > t_n$ and $t < t_n$ until $\Upsilon_n(t - t_n) < \Upsilon_n(0) - \beta\, dB$, where β is the stopping criteria. For reptile and anuran sounds, β has been set to 25 and 20 dB, respectively. The time interval $[t_n - t_s, t_n + t_e]$ represents the limits of the syllable.
3. This trajectory which represents a syllable is stored and then, is deleted from the matrix. The index n is updated to $n + 1$.

3.2. Features extraction and fusion

After carrying out the segmentation process, frequency domain characteristics are computed to gather useful information for the automatic classification. MFCC and LFCC have been applied in

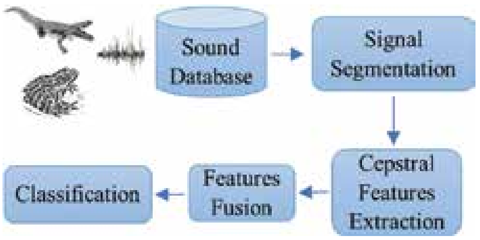

Figure 2. The proposed methodology for automatic acoustic identification of reptiles and anurans.

animal bioacoustic classification [4, 22, 27, 28], because they have a low computational cost and their implementations are easy. On the other hand, low frequency sounds are emitted by most species of reptiles and anurans, that is, in a 0–20 kHz interval, such as human auditory range. Thus, to reinforce the low frequency range, MFCC have been considered. Nevertheless, both corpuses can produce sounds above 20 kHz, and hence, to get a characterization in high frequency ranges, LFCC have also been used [29]. Thus, both cepstral coefficients are computed to parametrize the audio signal, because they contain information of lower and higher frequency.

These features are computed via STFT using 25 milliseconds Hamming window overlapping at 50%. In order to get this value, a set of experiments were carried out where the window size was modified from 10 ms to 1 s. After that, the discrete Fourier transform (DFT) is computed in each signal frame, and a bank of 40 and 26 triangular band pass filters for reptiles and anurans, respectively, are wrapped to the resultant spectrum. The MFCC are obtained by applying the discrete cosine transform (DCT) to log-magnitude filter outputs, $\log|\Upsilon_i|$, and taking the lowest values. MFCC features are calculated as follows, Eq. (1):

$$MFCC_j = \sum_{i=1}^{B} (\log|\Upsilon_i| \cos[j(i-0.5)]\pi/B), \qquad 1 \leq j \leq N \tag{1}$$

where j indicates the MFCC index, B is the number of triangular filters, and N is the MFCC to calculate.

On the other hand, LFCC are calculated using Eq. (2), where K is the number of DFT magnitude coefficients X_i.

$$LFCC_j = \sum_{i=1}^{K} \left(\log|X_i| \cos\left[\frac{ji\pi}{K}\right]\right), \qquad 1 \leq j \leq N \tag{2}$$

For both features, the coefficients number has been obtained by carrying out a set of experiments to achieve the highest accuracy in the classification phase. Thus, 18 coefficients have been taken for both MFCC and LFCC.

Finally, the cepstral coefficients are fused, concatenating the features as in Eq. (3), where each syllable is represented by a row. Hence, each row contains 36 coefficients, and the full matrix represents the coefficients extracted for all syllables of a species. Thus, a broad spectral representation of a call is used as input to the classification phase.

$$Features = \begin{pmatrix} MFCC_1 & LFCC_1 \\ \dots & \dots \\ MFCC_n & LFCC_n \end{pmatrix} \tag{3}$$

3.3. Classification

To validate the robustness of the proposed methodology based on cepstral coefficients fusion, three machine learning algorithms have been evaluated in the classification stage: K-nearest neighbor, random forest, and support vector machine.

3.3.1. K-nearest neighbor

KNN was proposed by Cover [30], and infers the new data classification based on the closest training samples. The machine learning algorithm considers the K-nearest point distances to the observation to predict which class is similar. Then, to calculate the class prediction, simple majority of neighbors is used. In this chapter, the number of nearest neighbors has been fixed to $k = \sqrt{N},$ where N denotes the length of the cepstral coefficients.

3.3.2. Random forest

RF is a machine learning algorithm presented by Breiman [31]. It is able to model non-lineal input variables, and in addition, it is robust to outliers in the training dataset. RF is an ensemble of decision trees. The generalization error converges to a limit when the number of trees in the forest becomes large. An average of the output votes from all the trees in the forest is computed for the prediction of the classes, Eq. (4). In this study, a value of K = 200 trees was utilized because it returns better results, with predictor variables $m = \sqrt{N},$ where N is the length of the cepstral coefficients.

$$Prediction = \frac{1}{K}\sum_{i=1}^{K} y_i, \quad where\ y_n\ is\ the\ nth\ tree\ response \tag{4}$$

3.3.3. Support vector machine

SVM [32] is a robust supervised learning technique and has been used to resolve the acoustic signal classification. The aim is to create non-overlapping partitions mapping the data as elements of a higher-dimensional space. SVM computes the classification of geometric parameters getting the optimal hyperplane from the training data which separates the data perfectly into two classes. Nevertheless, sometimes the training data cannot be separated lineally. In those cases, and in order to divide the classes, a non-linear kernel function is used to project the data into a higher dimensional space. In this chapter, an implementation based on LIBSVM library [33] was used implementing a C-Support Vector Classification (C-SVC) [34], which uses a decision function as showed in Eq. (5), where K is a radial basis function (RBF) kernel, $k(x, x') = e^{\left(-c\|x-x'\|^2\right)}$ $k(x, x') = e^{\left(-\gamma\|x-x'\|^2\right)}$. In order to carry out the multiclass classification, the strategy "one-versus-one" is performed generating one SVM for each pair of classes. Thus, for N different classes, N(N − 1)/2 classifiers are necessary to identify the samples.

$$f(x) = \mathrm{sign}\left(\sum_{i=1}^{l} y_i \alpha_i K(x, x') + b\right), \quad y_i \in \{1, -1\} \tag{5}$$

Lastly, a grid-search was implemented to adjust the SVM parameters ($\gamma = 2^{-12}, 2^{-11}, \ldots, 2^{2}$; $C = 2^{-2}, 2^{-1}, \ldots, 2^{10}$) using cross-validation to find the optimum kernel gamma parameter, γ, and the value of the penalty parameter of the error term (C). The values obtained for the kernel gamma were 0.45 and 1.45 for reptile and anuran corpus, respectively. For the penalty error term, the values were 30 and 20.

4. Experimental methodology and results

In this section, the datasets and the experimental results obtained from experiments carried out to evaluate the effectiveness of the proposed methodology are described and discussed. Experiments were focused onto comparing accuracy using the following different features: MFCC, LFCC, and MFCC/LFCC fusion. The syllables generated by the segmentation phase have been randomly rearranged and split in half—one for training the model and the rest for testing (k-fold cross-validation with k = 2). For each class, the accuracy has been evaluated as in Eq. (6), and then the results have been averaged. Using the feature with the best accuracy results, experiments varying the training size were also carried out, from 5 to 50% of the full dataset. The aim is to validate the performance and the robustness of proposed methodology.

In order to validate the experimental results, and to ensure statistical independence, all experiments have been repeated 100 times. The acoustic classification system was implemented in Matlab, and two classifiers were used for each dataset: KNN and SVM classifiers for reptile identification, and RF and SVM classifiers for anuran identification. The experiments were run in a non-dedicated Windows machine based on an Intel Core i7 4510 with a clock speed of 2 GHz, and 16 GB of RAM.

$$Accuracy = \frac{Syllables\ Correctly\ Identified}{Total\ Number\ of\ Syllabes} \times 100 \tag{6}$$

4.1. Datasets

Two different datasets have been built to validate the proposed methodology in this chapter. Each dataset contains audio content of anurans and reptiles, respectively.

4.1.1. Anurans dataset

The following three databases of anurans have been used to build the anurans dataset: the AmphibiaWeb database [35], a compilation of audio recordings of the amphibians of Cuba [36] and a sound guide of frogs and toads from southern Brazil and Uruguay [37]. AmphibiaWeb was created by the University of California (Berkley), where on-line information related to amphibian conservation and biology is stored. The recordings contain significant background noise and were mainly gathered in their own habitats. In addition, the signals were recorded with different sample formats and rates. From this database, a total of 41 anurans of several taxonomy families were selected, where most of them are anurans from previous literature studies [27, 28]. On the other hand, the collection of amphibians of Cuba contains 99 recordings of several types of advertisement and alert calls of 58 species, most of them endemic. Finally, the sound guide from Brazil and Uruguay is composed by 109 frogs and toads. From them, nine species have been rejected because they do not have enough samples to fit and test the model. Hence, a total of 199 species compose the whole anurans dataset. **Table 2** shows the number of segmented syllables grouped by taxonomic family.

Dataset	Family	Number of Species	Number of syllables
AmphibiaWeb	Bufonidae	6	270
	Dendrobatidae	2	36
	Hemiphractidae	1	34
	Hylidae	9	309
	Hyperoliidae	2	84
	Leptodactylidae	3	110
	Mantellidae	7	241
	Microhylidae	2	52
	Myobatrachidae	6	239
	Ranidae	1	19
	Scaphiopodidae	2	170
Cuba	Bufonidae	10	1141
	Eleutherodactylidae	42	2951
	Hylidae	4	737
	Ranidae	2	372
Brazil and Uruguay	Alsodidae	1	210
	Bufonidae	10	1500
	Brachycephalidae	2	124
	Centrolenidae	1	33
	Cycloramphidae	2	46
	Hemiphractidae	1	32
	Hylidae	49	4633
	Hylodidae	5	353
	Leptodactylidae	23	2971
	Microhylidae	1	54
	Odontophrynidae	5	914

Table 2. Anurans dataset.

4.1.2. Reptiles dataset

Sound repositories of reptiles is quite limited, because they have not been acoustically and exhaustively analyzed. Thus, reptile recordings form three Internet sound collections have been extracted to build the dataset. The Animal Sound Archive at the Museum für Naturkunde in Berlin [38] was the principal source of reptile audio recordings. It stores 120,000 tracks of diverse species which are freely available from their database. The second collection used was California Herps [39] which contains some Squamata sounds. Finally, a small number of tortoise vocalizations from the California Tortoise Club [40] collection was added to the dataset. Therefore, the whole dataset used in this work is formed by 1895 samples matching to 27 different reptile species and six family groups. **Table 3** shows the number of segmented syllables grouped by taxonomic family.

4.2. Analysis of accuracy

4.2.1. Anurans results

Table 4 indicates the accuracy results for each set of features and the computation time for training and testing by iteration. As it can be observed, Mel coefficients perform better results than LFCC when the number of anurans is small. Nevertheless, when it is increased, LFCC shows a superior performance because higher frequencies are better characterized. Hence, a MFCC and LFCC fusion is proposed to characterize the anuran sounds in lower as well as higher frequencies. The experiments reinforce that this approach improves the classification rate on all databases and the aggregate dataset. As it can be appreciated, RF is clearly outperformed by SVM in all experiments. Furthermore, a successful classification with an accuracy above 95% using the aggregate dataset was achieved. Regarding the training time, RF takes more computation time than SVM. Nevertheless, RF is clearly faster when testing is carried out. It is more noticeable when the species number increases.

A detailed analysis indicates that an accuracy of 98.70% was achieved for AmphibiaWeb database, outperforming other research in terms of number of species identified and accuracy [21, 22, 27, 28, 41–44]. Furthermore, 100% accuracy was reached for 24 anurans. On the other hand, the Cuba database stores some species with a reduced number of syllables, but even in this situation, the features fusion achieved a successful classification improving the accuracy about 5%. An accuracy of 84.90% was the worst result obtained, and a 100% classification rate was reached by 10 species. The mean total accuracy was 96.40% in 58 frog species. Regarding the Brazil–Uruguay dataset, the MFCC and LFCC fusion yielded an identification rate of 95.30% over 100 anurans, where only 16 species achieved an accuracy below 90%. Finally, a success rate of 95.29% was successfully achieved using the aggregate dataset. To the best of our knowledge, it is the largest number of toads and frogs identified using acoustic signals. The proposed methodology in this chapter was compared with some research in literature, **Table 5**. As can be seen, this approach is more robust than other research reaching a higher success rate.

Family	Number of species	Number of syllables
Alligatoridae	3	28
Gekkonidae	2	215
Helodermatidae	1	383
Viperidae	12	950
Elapidae	1	10
Testudinidae	8	309

Table 3. Reptiles dataset.

Database	Features	Classifier	Training Time(s)	Testing Time (s)	Accuracy ± std
AmphibiaWeb (41anurans)	MFCC	RF	0.68	0.04	96.10% ± 5.69
		SVM	0.11	0.08	97.82% ± 3.21
	LFCC	RF	0.69	0.04	95.83% ± 6.61
		SVM	0.11	0.09	96.81% ± 4.36
	MFCC+LFCC	RF	1.03	0.05	98.00% ± 3.92
		SVM	0.15	0.09	98.70% ± 2.58
Cuba (58 frogs)	MFCC	RF	3.37	0.08	86.08% ± 16.76
		SVM	0.49	0.51	91.64% ± 8.85
	LFCC	RF	3.19	0.08	90.69% ± 10.59
		SVM	0.47	0.49	90.92% ± 10.02
	MFCC+LFCC	RF	4.94	0.08	92.54% ± 9.33
		SVM	0.81	0.57	96.40% ± 4.03
Brazil and Uruguay (100 anurans)	MFCC	RF	10.13	0.17	84.74% ± 15.28
		SVM	1.73	4.33	90.53% ± 9.57
	LFCC	RF	10.48	0.18	88.03% ± 11.23
		SVM	1.64	4.51	91.69% ± 9,18
	MFCC+LFCC	RF	15.54	0.17	91.18% ± 10.70
		SVM	4.86	5.97	95.30% ± 5.28
AmphibiaWeb+Cuba+Brazil-Uruguay (199 anurans)	MFCC+LFCC	RF	69.78	0.42	90.29% ± 12.85
		SVM	56.4	38.95	95.29% ± 5.63

Table 4. Accuracy results for anurans dataset.

Furthermore, in this work, three public datasets were used, and therefore, this approach can be validated and contrasted.

4.2.2. Reptiles results

Table 6 shows the accuracy results for each set of features and the computation time for training and testing by iteration. As it can be observed, both MFCC and LFCC features obtain similar results. As is known, most of the reptile sounds are from 0.1 to 4 kHz. Therefore, Mel coefficients reinforce the lowest frequencies because those spectrum regions are enhanced. Nevertheless, some reptiles, such as lizards, emit high-frequency components even into the ultrasound range (>20 kHz). MFCC features contain poor information at these frequencies, because the area under the Mel-filter bank grows at higher frequencies. Hence, LFCC are more appropriate to parametrize those reptile sounds. Thus, in some experiments, LFCC surpasses MFCC when the best classifier is used, SVM. The experiments confirm that the MFCC/LFCC data fusion enhances the identification rate. As it can be appreciated, SVM slightly outperforms

Reference	Dataset	Features	Classifier	Accuracy (%)
Lee et al. [21]	30 frogs and 19 crickets	MFCC	LDA	96.8 and 98.1
Acevedo et al. [41]	9 frogs and 3 birds from Puerto Rico	Call duration/max. and min. Frequency/max. power/frequency of max. power	SVM	94.95
Chen et al. [44]	18 frogs	Syllable length/MSAS	Template based	94.3
Yuan et al. [27]	8 frogs (AmphibiaWeb)	MFCC	KNN	98.1
Xie et al. [28]	16 frogs from Australia	MFCC	KNN	90.5
In this work	41 anurans (AmphibiaWeb)	MFCC/LFCC	SVM	98.7
	58 frogs from Cuba			96.4
	100 anurans from Brazil-Uruguay			95.3
	199 species from all datasets			95.29

Table 5. State of the art comparison.

Features	Classifier	Training time (s)	Testing time (s)	Accuracy
MFCC	KNN	0.08	0.03	96.00% ± 7.20
	SVM	0.13	0.06	95.84% ± 7.74
LFCC	KNN	0.07	0.03	92.98% ± 9.95
	SVM	0.15	0.05	96.15% ± 5.35
MFCC+LFCC	KNN	0.12	0.05	97.78% ± 3.33
	SVM	0.23	0.06	98.52% ± 3.22

Table 6. Accuracy results for reptiles dataset.

KNN in all experiments. Furthermore, this approach yielded a successful classification with an accuracy above 98%. Regarding the training time, both KNN and SVM take similar computational cost time because the number of reptile species is small.

A detailed analysis reveals that a 100% accuracy was reached in 9 species, regardless of the cepstral coefficients employed. It is due to the spectral distribution of calls in those reptile species is clearly different from others. Nevertheless, the best classification results were achieved by using MFCC/LFCC feature fusion, outperforming both MFCC and LFCC, and independently of the used classifier. Thus, it is confirmed that this methodology achieves a better parametrization of the reptile sounds by keeping in account important information of low- and high-frequency zones. It allows to increase the system accuracy. Finally, it should be noted that the MFCC/LFCC fusion identified 13 species with an accuracy of 100%.

4.3. Analysis of training dataset size

In order to validate the robustness of this methodology, the efficiency of the system was tested by varying the training dataset size from 5 to 50%. All experiments were carried out by using the MFCC/LFCC features fusion and SVM as classifier.

4.3.1. Anurans results

Table 7 shows the experimental results varying the training size of the whole dataset of anurans, 199 species. As can be seen, larger training datasets are useful to improve the performance of this approach. In addition, an accuracy above 90% is yielded using only a 20% training size. It should be noted that the recordings of some species have very few syllables, even with only three samples. Therefore, when training size is considerably reduced, the classifier is modeled with only one sample. Precision, recall, and F-Measure measurements have been also computed by varying the training dataset size. As shown, these measurements follow a similar behavior related with accuracy, increasing when the training size does, and keeping values above 0.9 using only a 20% training size and close to 0.95 using a 40% of training size. Thus, small training datasets allow to make less the time needed and the computational cost to calculate the classifier model. This evidence that the fusion of both MFCC and LFCC features is efficient for modeling the discriminant information in the anurans sounds. Furthermore, the data fusion allows to obtain classification results above 80% in all cases, demonstrating the robustness of the feature fusion method.

4.3.2. Reptiles results

Table 8 shows the experimental results varying the training size of the whole dataset of reptiles, 27 species. As can be seen, the system accuracy increases when the training size does, and the proposed methodology can obtain good results with a low number of training samples. Thus, when the number of samples is close to 5%, this approach decreases in effectiveness, but even in these circumstances, the system yields an accuracy above 85%, keeping in mind that only one syllable characterizes most of the reptile species. For other training cases, the accuracy is above 90%. Furthermore, precision, recall, and F-Measure measurements also follow a similar behavior related with accuracy, that is, when the training size increases, the measurements also do, keeping values close to 0.9 using only a 5% training size and close to

Training size (%)	Accuracy (%) ± std	Precision	Recall	F-Measure
5	80.01% ± 18.05	0.86	0.80	0.83
10	86.94% ± 12.94	0.91	0.87	0.89
20	91.38% ± 8.71	0.94	0.91	0.92
30	93.53% ± 7.14	0.95	0.93	0.94
40	94.48% ± 6.72	0.96	0.94	0.95
50	95.29% ± 0.16	0.96	0.95	0.96

Table 7. Classifier performance with different training size for anurans dataset.

Training size (%)	Accuracy (%) ± std	Precision	Recall	F-Measure
5	85.50% ± 20.06	0.91	0.85	0.88
10	91.03% ± 14.06	0.94	0.91	0.92
20	94.81% ± 8.01	0.96	0.94	0.95
30	96.86% ± 5.39	0.97	0.96	0.97
40	97.88% ± 3.76	0.98	0.97	0.98
50	98.52% ± 3.26	0.98	0.98	0.98

Table 8. Classifier performance with different training size for reptiles dataset.

0.97 using a 30% of training size. Furthermore, a lower training dataset size offers savings in computational cost and time needed to compute the classifier model. This evidences that the fusion of both cepstral coefficients can be used in an effective way for discerning important information in the reptile sounds. Hence, the data fusion achieves the classification results above 85% in all cases, validating the robustness of the MFCC/LFCC features fusion.

5. Conclusions

Automatic species identification based on bioacoustic information has become an attractive research topic due to growing interest among biologists for sampling populations and controlling the conservation of these living beings in large and remote areas where environmental conditions and visibility are limited. In this chapter, a methodology based on the fusion of cepstral coefficients, MFCC and LFCC, was proposed and validated using public datasets of reptile and anuran species. This data fusion allows to characterize the acoustic signal with both low- and high-frequency components, being more robust against noise and increasing the classification rate. The results of the proposed methodology are encouraging with a mean accuracy of 95.29 and 98.52% for anurans and reptiles, respectively.

Regarding the anurans identification, the proposed methodology was collated with some research of literature, being more robust and identifying more species than the other techniques. Furthermore, public databases have been used, and therefore, this approach can be validated and contrasted. On the other hand, as far as authors know, the anurans dataset contains the largest number of toads and frogs automatically identified by acoustic characteristics. For reptile identification, the authors are not keeping in mind about other researches that have considered the use of reptile acoustic signals for species classification. Even so, the experimental results have demonstrated that the MFCC/LFCC feature fusion achieves a broad characterization of the acoustic signal, yielding a high identification rate.

Finally, the proposed methodology described in this chapter has been analyzed using scenarios with reduced training dataset, validating the robustness of the system. It declines in effectiveness when the training dataset size decreases, but even so, with only a 5% of the samples for training, this approach yields an accuracy above 80%, keeping in mind that many species are only characterized by only one syllable.

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Chapter 6

From Effects of Linear Transport Infrastructures on Amphibians to Mitigation Measures

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Additional information is available at the end of the chapter

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Abstract

Linear transport infrastructures (e.g., roads, highways, railways) are affecting biodiversity by habitat loss and fragmentation, degraded or suppressed connectivity, and direct and indirect mortality. In response, planners try to propose mitigation or compensatory measures. Amphibians are particularly impacted by these infrastructures, in terms of habitat loss but also because their obligatory migration to breeding sites exposed them to the barrier effect of infrastructure (direct mortality and loss of connection among subpopulations). Several compensatory (e.g., creation of new ponds) and mitigation measures (construction of wildlife passage) have been proposed specifically for amphibians. This chapter aims to describe measures implemented for amphibian populations and tries to evaluate their efficiency in terms of frequentation (wildlife passage) and population persistence.

Keywords: roads, railways, wildlife passage, mortality, amphibians, habitat fragmentation, population persistence

1. Introduction

The construction of linear transport infrastructure (LTI) such as roads and railways is one of the major anthropogenic alterations to the planet's ecosystems (e.g. see [1]). The effects of LTI such as roads (42 million km of roads around the world) and railways on wildlife were identified as early as the end of the nineteenth century [2]. Throughout the twentieth century, data on the effects of LTIs have accumulated, with a strong increase over the last 20 years [3, 4].

At the same time, planners are trying to use knowledge on species biology and the types of impacts identified to propose mitigation or compensatory measures. These actions have led to

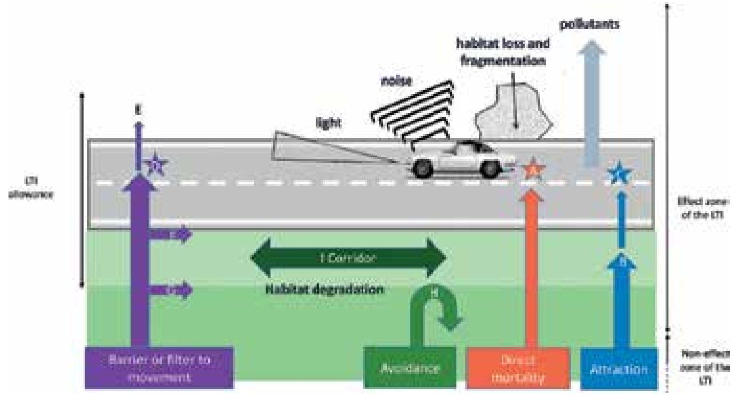

Figure 1. Schematic view of environmental effects of a linear transport infrastructure. The building of the LTI and the traffic induced a set of direct and indirect impacts on the biodiversity, in the effect zone of the LTI. (A) Direct mortality via collision with vehicles; (B) attraction (e.g. scavengers for crushed fauna); (C) potential mortality of attracted animals; (D) direct mortality of crossing individuals; (E) successful crossing; (F) avoidance of the LTI; (G) avoidance of the LTI allowance degraded habitat; (H) avoidance of the LTI allowance; (I) corridor effect (adapted from [22, 37]).

the emergence of a theme called "road ecology" [5], which focuses on impacts (roads, but can be extended to all transport infrastructures) and mitigation measures [3, 5].

LTIs have different effects on biodiversity. The animal component of this biodiversity, and in particular the amphibian community, will be considered here, even if the other components are concerned, for example, by habitat loss. The main impacts of LTIs are habitat loss, habitat fragmentation, loss of connectivity, and direct and indirect mortality (**Figure 1**). Historically, amphibians were among the first animals reported as being heavily impacted by roads [6].

While railways represent ~1 million km and are expected to increase by 45% by 2050 [1], their impact in comparison with other infrastructures such as roads and highways is poorly studied [3, 7]. Railways are expected to cause the same types of effects as roads [8], that is, habitat loss, landscape fragmentation, direct mortality [9] and indirect disturbances such as noise pollution and light pollution [10, 11]. Two hundred and fifty-nine studies have been published in relation to road networks, compared with 17 studies about rail networks (of which 3 concern amphibians) in 2014 [3].

2. Habitat loss and fragmentation

In amphibians, habitats destroyed by LTIs are breeding habitats (e.g., aquatic sites where egg and larvae development occur) and terrestrial habitats (transitional habitats for migration to and from aquatic sites, dispersal of juveniles, and growth/hibernation for juvenile and adult terrestrial phases). LTIs can also have a barrier effect, preventing colonization (dispersion) or movements between breeding sites and terrestrial habitat (migration). Habitat loss and the barrier effect (i.e., landscape fragmentation) are difficult to quantify separately because the composition and structuring of landscape elements affect the dynamics of the animal populations that frequent them [12].

Habitat loss and fragmentation can have measurable effects in terms of genetic richness (within the same species) or of specific richness. The genetic structure of a salamander species (*Plethodon cinereus*) is altered near a motorway, but not near secondary roads [13]. Habitat fragmentation leads to an increase in inbreeding, loss of heterozygosity, a decrease in adult numbers, loss of allelic richness and a greater differentiation among 'subpopulations', the agile frog *Rana dalmatina* [14, 15]. Roads are barriers to gene flow in the common frog *R. temporaria* in Germany and France, where genetic differentiation between populations is highest in the densest road network regions [16] or with a higher traffic [17]. Amphibian-specific richness is greater when moving away from roads [9, 18–20], but it is difficult to separate the effect of roads and the effect of landscape co-variables (e.g., forest cover, crops, urbanized area) [21].

The cumulative effects of ILTs delineate an 'impact zone' of various widths. The distance of effect can vary from 40 m to 1.5 km with an average of 500 m, depending on the species considered [22–26].

3. Direct mortality

LTIs are a major source of vertebrate mortality, with consequences to local population dynamics [27]. Due to user safety issues or exploitation (traffic loss), LTI studies are primarily concerned with large mammals (e.g., ungulates and bears, [3]). Vehicle collision mortality can affect the demographics of many vertebrate and invertebrate species, among them are amphibians [25, 28, 29].

As with roads, the direct mortality of wildlife crossing railway tracks depends on species agility and their ability to cross the railway. Mammals, birds, reptiles and amphibians suffer railway deaths [3, 7, 30, 31]. The railway track has been noted to trap small vertebrates such as terrestrial turtles (*Terrapene carolina*) between the tracks [32].

The persistence of crashed small vertebrate bodies can be very short due to scavengers. Their identification can also be difficult because of the body degradation. Both effects can affect species' detectability, degrading the accuracy of the mortality effect evaluation (but see [33] and the use of genetic tracks to identify crushed animal carcasses).

Direct mortality may cumulate with the barrier effect by decreasing the abundance, occurrence and specific richness of amphibian communities [12, 18, 21, 26, 34–36].

4. Mitigation measures

Increased knowledge of the effects of LTIs on biodiversity, biodiversity protection and legislation has led to the implementation of mitigation measures. The aim of these measures is to enable long-term population viability near the LTI [24, 35, 37]. The effect of habitat destruction is most often offset by measures to restore or create new habitats. A typical example is the creation of new amphibian-breeding ponds [38].

To reduce the barrier effect of LTIs, wildlife passages-devoted to a specific fauna of small vertebrates (including amphibians)-began to be implemented in the 1960s in Europe [39]. Wildlife passages (above or below the LTIs) aim to limit direct mortality and to maintain a level of connectivity, allowing gene flow between sub-populations on either side of the LTI [40], a key factor for the viability of amphibian metapopulation [9, 17].

Several approaches have been implemented to reduce direct amphibian mortality on LTIs during movements (migration and dispersal): the first one is to act on traffic, that is, vehicle speed reduction, signaling and temporary closure of roads [9, 25, 41]. The second one is by changing the main amphibian migration routes, for example, by creating breeding sites on the LTI side that avoids most breeding individuals having to cross it [38]. In Germany, 99% of the individual of a common toad *Bufo bufo* breeding population visited the new site the year after its creation [42].

The classical approach is to subtract migrating/dispersing individuals from the risk of crossing the LTIs, thanks to barrier systems (either permanent or temporary) and buckets that trap individuals, which are captured and released on the other side of the LTI.

In many cases, the direct mortality and barrier effects are reduced, thanks to a combination of devices that limit the access to the LTIs and allow its crossing. Wildlife passages above ILTs (viaducts) and barrier systems channel amphibians in their direction, drastically reducing mortality between wetlands but at a high construction cost [22, 43]. These viaducts are highly developed in different species of (large) vertebrates, but their attractiveness and efficiency for amphibians remain poorly documented [9, 44].

In most cases, the wildlife passage designed for amphibians is built to allow crossing below the LTI. It is a round or a square underpass (tunnel) of variable size (e.g., from 0.50 to 1.5 m in height). The entrances are connected to barriers (e.g., plastic or metallic fences). These barriers must be extended by at least 100 m on both sides of the tunnel and at least 0.6 m in height (e.g., [9, 28]).

In addition to the passage designed for wildlife, it is often possible to adapt the many hydraulic systems to make them usable by small vertebrates such as amphibians [22].

An important issue for wildlife passage efficiency is their location, that is, selected in order to maintain the main natural movements (e.g., migratory and dispersal of amphibians in terrestrial phases) [25, 28]. The choice can be dictated by the knowledge of migration routes (assessed by the distribution of crushing zone or the description of a landscape structure) to determine how the amphibian community functions in the landscapes impacted by the LTI. The location of high-crushing zone should not be used as the only indicator for placing wildlife passage: a crashed amphibian number can reflect local density, which can be strongly reduced near roads [28].

If the wildlife passages are effective in reducing adult-breeding mortality, they are often not suitable to animals returning from breeding sites such as juveniles [9]. This failure to account juvenile mortality could explain the declines and even the extinction of amphibian populations where adult mortality (*B. bufo*) has been strongly decreased, thanks to wildlife passages [9, 45].

On the other hand, a review of studies on 123 wildlife passages shows that they provide little information on the fact that they have decreased the barrier effects of the LTI on population connectivity [46].

5. Factors influencing the use of wildlife passage by amphibians

There is little information on the behavior of amphibians nearby and inside wildlife passages (tunnels). Some experiments exist with amphibians, to test the size of the tunnel, the type of substrate, light and humidity.

5.1. Type and size of wildlife passage

Three European anuran species (*B. bufo*, *R. esculenta* and *R. dalmatina*) were tested in an experimental design allowing counting the number of individual entering or not an artificial tunnel 2 m long. The proportion of crossing varied from 66, 65 and 27% in these three species [47]. The north American spotted salamander *Ambystoma maculatum* was tested with several tunnels varying in width (from 0.3 to 0.8 m) and used them whatever their width was [48].

5.2. Substrate type

A tunnel with an earthy substrate is more clearly used than a tunnel with a bare concrete substrate in anuran (*R. esculenta, R. dalmatina, R. clamitans, R. pipiens*) [47, 49]. A marginal effect was observed with the spotted salamander (*A. maculatum*) and the common toad *B. bufo* where the two substrates were used indifferently [47, 48]. In this experimental tunnel device, the choice was also offered to stay on a grassy substrate, or to use one of the tunnels with earthy or bare concrete substrates [47]. The water frog *R. esculenta* and the common toad *B. bufo* used both tunnels, while the agile frog *R. dalmatina* was more frequently found outside the tunnel on the grassy substrate [47]. The use of a natural substrate in wildlife passage can provide habitat continuity and encourage animals to cross [25], and bare concrete, with high alkalinity, could be a repellent to amphibians [47].

5.3. Other factors

Substrate moisture, air temperature, tunnel brightness and noise can influence the use of wildlife passages. A temperature difference between the outside and the inside of a tunnel can deter amphibians [25]. The presence of an opening on top of the tunnel, allowing ventilation and light entry, increased the crossing speed of the spotted salamander *A. maculatum* (cited in [25]). The green frog *R. clamitans* and the leopard frog *R. pipiens* prefer using artificial tunnel with some light entrance [49].

Microclimate (temperature, relative humidity, mean light) in the tunnel was recorded during movement and orientation of Australian frogs (striped marsh frog *Limnodynastes peronii*, the Golden bell frog *Litoria aurea* and the broad-palmed frog *L. latopalmata*) [52]. The tunnel usage was not related to air temperature, humidity or light level recorded inside the tunnel (**Figure 2**).

Figure 2. Experimental setup to study the tunnel-crossing behavior of amphibians. Experimental tunnel (ACO climate tunnel model KT 500) used to record amphibian-crossing movements, Photographs by Andrew Hamer (from ([52]). (a) Global view of the tunnel; (b) a view through the tunnel from the entrance arena; (c) view of the tunnel entrance with the infrared camera on the left and the temperature data logger next to the camera. The black plastic bucket is the raised acclimation chamber used for tested animals.

The soil moisture of the surrounding habitat influences the use of tunnels in the north American salamanders (the Santa Cruz long-toed salamander *A. macrodactylum* [51]).

The influence of environmental factors inside and in the surrounding habitats of tunnels on their use by amphibians definitively needs more experimental studies.

Amphibians use various information when migrating and dispersing. Anurans can use the reproductive call of their conspecific to locate the breeding site. This attraction was used to encourage the use of artificial tunnels [47, 52]. European newt species (the palmate newt, *Lissotriton helveticus*, the marbled newt *Triturus marmoratus*, the great crested newt *T. cristatus* and the smooth newt *L. vulgaris*), which do not have reproductive calls, can use the call of sympatric anuran species (the Perez's frog *Pelophylax perezi*, the common toad *B. bufo*, the green toad *Pseudoepidalea viridis*, and the Natterjack toad *Epidalea calamita*), to locate the breeding site [53, 61–63]. It is likely that many amphibians use this cue to orientate, but more studies are definitively needed, especially on the potential of improving the use of wildlife passages. Olfaction to detect the breeding site, thanks to its particular bench of odors, is well known in amphibians. It is also possible that amphibians use smelling traces (conspecific or other species) on the substrate to orientate [47, 54], but to our knowledge, this behavior has not been tested in a wildlife passage context.

6. Evaluating mitigation measures

Mitigation measures to reduce the negative effects of LTIs and traffic on amphibians have varying degrees of success [40, 47, 52].

It is crucial to carry out the evaluation of these mitigation measures and to clearly distinguish the (simple) counting of individual using a wildlife passage and the (complex) global evaluation of the mitigating measures. A combination of methods is often required to achieve this goal.

6.1. Compensatory pond and translocation

Compensatory measures in response to the destruction of aquatic sites include the creation of new ponds and possibly the translocation of the amphibian community that colonized the destroyed ponds [55]. Evaluation of these actions is very rare, most often without an estimate of the success of mitigation [38, 56]. New ponds can lead to an increase in amphibian biodiversity in highly altered habitats [27]. A return time of an amphibian community was 2–3 years in a compensatory pond, that is, the time to observe the same species as in the destroyed ponds [28].

Denton et al. [57] proposed several criteria to assess the success of mitigation based on the creation of compensatory ponds and translocation in the Natterjack toad *B. calamita*. The translocation was the transfer of spawns (5–6000 eggs) during two successive years. The first criterion is the initial success, that is, the emergence of metamorphosed individuals at least one of the 2 years of the transfer. The second criterion is the intermediate success, that is, the return of adults for reproduction before the third year after the transfer (age of sexual maturity is 3 years in this species). The third criteria is the complete success, that is, when reproduction continues in the new site for at least 5 years, with the number of adults remaining stable or increasing, and the production of a second generation of toadlets. The experiment has failed if no return or reproduction is observed for 10 years after the first transfer. These criteria can be adapted to different species to assess the success of mitigation measures. The assessment of compensatory pond measures should also take the maintenance of the quality of the newly created aquatic sites into account.

Evaluating the establishment of populations in translocated ponds remains a difficult task [38], due to large natural variations in amphibian populations and/or the long generation time, requiring long-term monitoring to verify the ‘real’ success of such operations.

6.2. Ecopasses and barriers to dispersion

Many amphibian species are known to use wildlife passages (tunnels), sometimes with a high abundance: Up to 3000 common toad *B. bufo* are counted each year in a wildlife passage in France [58], and the monitoring of a tunnel for 12 years in Switzerland shows an increase of its use by the common toad *B. bufo* and the common frog *R. temporaria* [59].

The most effective devices—without real comparative studies having been carried out—consist of a guide barrier and funnel underpasses [27, 40, 49, 50] (**Figure 3**). Barriers and walls are

Figure 3. Ecopassage designed for the migration of amphibians along the road RD657 in NE France. This ecopassage allowed the migration of amphibians such as the common frog *Rana temporaria* and the common toad *Bufo bufo*. A concrete tunnel, with the floor covered by the soil, allows the animal crossing. A metallic barrier prevents the animals to access the road and directs them toward the tunnel entrance. This ecopassage is located below the local road joining Novéant-sur-Moselle to Arry in Northeastern France (photograph by Alain Morand, CEREMA-EST).

effective in reducing amphibian mortality around LTIs when they are sufficiently high and well installed [27, 39, 40]. They effectively reduce collision mortality but lead to landscape fragmentation [25], making migration and/or dispersion impossible, thereby isolating populations [27]. This ability to control the movement of amphibians by physical barriers (e.g. fences) is used to channel them toward the inlet of the tunnel, thus preventing their penetration on the LTI [25, 40], and reducing direct mortality [40, 51]. However, arboreal species such as *Hyla* sp. suffered direct mortality as they can easily cross fences [40] and their use of tunnel is rare [9]. The fence, with a 0.60-m high, blocked 100% of the individuals in the Green frog *R. clamitans* and the Leopard frog *R. pipiens* [49].

Assessments of mitigation measures often lack scientific quality (e.g. no or poor data before the implementation, no replication of situations and no experimental assessment of wildlife passage use). In a review of 22 studies describing LTIs wildlife passage, only three evaluate the effectiveness (positive in the three studies) [27, 38, 40, 41]. The proportion of individuals entering a wildlife passage (its use) was 13%, while only 5% crossed it completely (effectiveness). This difference may be due to the tunnel characteristics (e.g. tunnel length) and/or the exploratory behavior of adults and juveniles [52].

There are many methods to measure the use of wildlife passage by large mammals. With smaller animals such as small mammals and amphibians, fingerprint plates, pitfall traps and fences, and camera traps have been used [27, 40, 50, 51]. Capture-marking-recapture provides information on the use of, for example, tunnels [50], but also on the dynamics of the monitored populations [60].

Nonfunctional wildlife passage is also observed [49], mostly due to their bad location, poor designs and/or behavior of target species [49]. It should also be noted that wildlife passage can potentially function as an ecological trap: Predators may use them as hunting grounds (e.g. carnivorous small mammal [9, 25, 41] and snake [29]).

7. Conclusion

The effectiveness of wildlife passages varies by species, and its evaluation will need several steps: The first and very important one is to evaluate the potential effects of the planned LTI on the population functioning (e.g. habitat destruction and connectivity) for the targeted animal community. This will allow classifying the different projects of wildlife passage in terms of objectives (which can differ from one wildlife passage to another). The monitoring (if no data are already available) has to be performed in the proposed locations of wildlife passages and in control areas (outside the site of, e.g., tunnel construction and in areas without the influence of the LTI). These monitoring has to be carried out post construction on the same locations using the same methods. In amphibian species, a combination of methods can be proposed to allow this monitoring, such as population genetics, capture-marking-recapture and occupancy modeling.

The assessment of mitigation measures (barrier/wildlife passage) focusing on amphibian community thus needs to take into account (1) the spatial aspect, that is, the effect distance of the LTI in the landscape (the 'impact zone'), the functioning of this community in the area where mitigation measures are implemented and the functioning of the community in the surrounding landscape; (2) the temporal aspect, that is, biological characteristics of each species (e.g., demographic traits) and of the landscape elements (e.g., natural evolution of compensatory ponds, of tunnel substrate and surroundings); and (3) the knowledge of behavioral determinants and movement capacities of each species that lead to the use or avoidance of wildlife passages.

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Chapter 7

Amphibian and Reptile Conservation in Scotland: Focus on the Great Crested Newt *Triturus cristatus* and the European Adder *Vipera berus*

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Additional information is available at the end of the chapter

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Abstract

Scotland, due to its geographical location and climate, has a relatively limited number of species of amphibians and reptiles. However, many of these are found widely across the country. As representatives of amphibians and reptiles in Scotland, this chapter will focus on the great crested newt *Triturus cristatus* and the European adder *Vipera berus*. For both species it will describe their distribution, habitat requirements, life cycles, population estimates and trends, legal protection and threats. Furthermore, it will outline recent conservation projects in Scotland showing how these creatures can live both in close proximity to humans and in developed landscapes relevant for their conservation elsewhere.

Keywords: Scotland, great crested newt, *Triturus cristatus*, common European adder, *Vipera berus*, biology, conservation

1. Introduction

Amphibian and reptile populations of numerous species around the world have been experiencing declines in numbers and contraction of ranges for many years. Such trends are of great concern because these animals make an important contribution to ecosystems and biodiversity wherever they are present. In many cases humans have caused the changes either directly through persecution and unregulated harvesting, or indirectly through habitat destruction, pollution and the transmission of diseases. This chapter describes two species, the great crested newt and the European adder, explaining their distribution and biology in

Scotland. Emphasis is placed on the interaction of these two animals with humans with descriptions of conservation projects that have protected populations following developments.

2. Great crested newt

2.1. Scottish distribution

The great crested newt is uncommon in Scotland with a restricted, fragmented range found predominately in low-lying areas on the mainland [1] (**Figure 1**). Globally this monotypic species is present in Western Europe from Britain and southern France to southern Fennoscandia and western Russia, with Scotland at the north-west edge of its world range.

In Scotland two populations are present with largest numbers in southern parts of the country and a smaller, separate population further north in the Highlands. The species is absent from Scottish islands with the exception of a single site on Arran and an unsuccessful artificial introduction on Skye. Highest numbers are found in the south of the country from south Fife, Lothian, south Stirlingshire and Clackmannanshire through to North Lanarkshire, Glasgow and West Dunbartonshire. Other populations are found in the south-west in Dumfries & Galloway with small isolated numbers present in Perth & Kinross, Scottish Borders, Argyll & Bute and Ayrshire. The smaller Highland population with about forty breeding ponds is located around Inverness [2]. Great crested newts have also been introduced to one site further north in Highland in Caithness.

2.2. Habitat requirements

In Scotland the great crested newt is found at low altitudes in two broad habitat types [1] (**Figure 2**). Many of the southern sites are similar to those in England and southern continental Europe with ponds in deciduous woodland, grassland, arable areas, and post-industrial sites containing flooded pits dug for clay extraction and quarries [3–5]. However, sites in the Highlands, Perth & Kinross and some in Dumfries & Galloway and Lothian are more similar to those used by the species in northern Europe having instead native conifers, birch *Betula* spp., heather *Calluna vulgaris* and blaeberry *Vaccinium myrtillus* [6, 7].

Great crested newts populations are most productive in ponds with a combination of open water and submerged and emergent aquatic vegetation, as this allows males to find and display to prospective females during the breeding season, offers protection from predators and provides egg-laying sites [8, 9] (**Figure 3**). Breeding sites in Scotland range from small shallow ponds to some very large water bodies such as Loch Flemington in the Highlands. However, in the larger lochs newts only breed in the shallow margins where they avoid fish, which consume their eggs and larvae. The species often exists in metapopulations with breeding occurring in several ponds in close proximity joined by suitable terrestrial habitat.

In breeding ponds aquatic flora is important as eggs are laid on submerged vegetation such as willowherb *Epilobium* spp., floating sweet-grass *Glyceria fluitans*, watercress *Nasturtium officinale*, forget-me-not *Myosotis* spp. or marsh cinquefoil *Potentilla palustris* [1]. However, eggs can also be found on decomposed vegetation and even discarded refuse such as plastic bags.

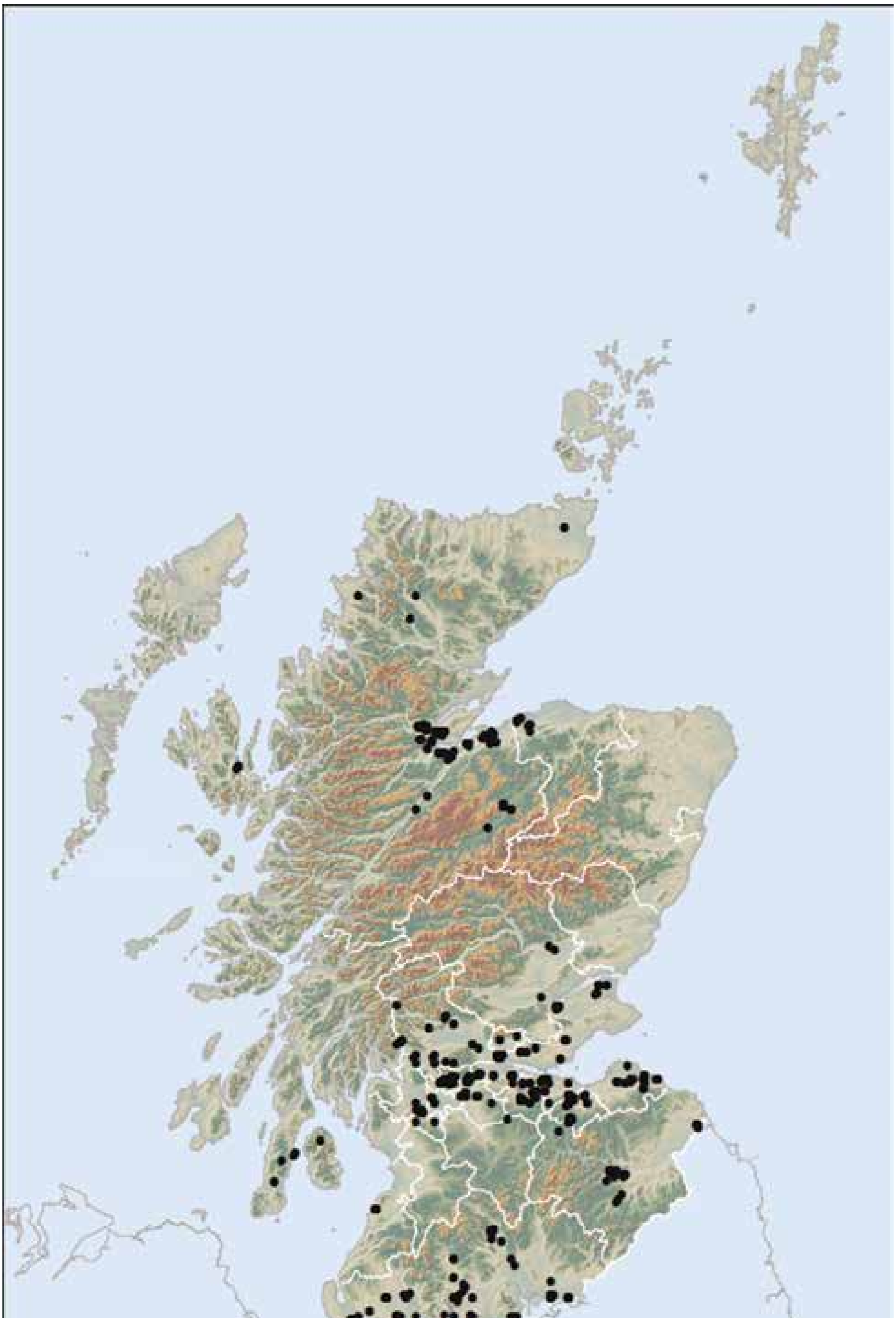

Figure 1. Distribution of the great crested newt *Triturus cristatus* in Scotland based on records 2000–2016. Derived from [1].

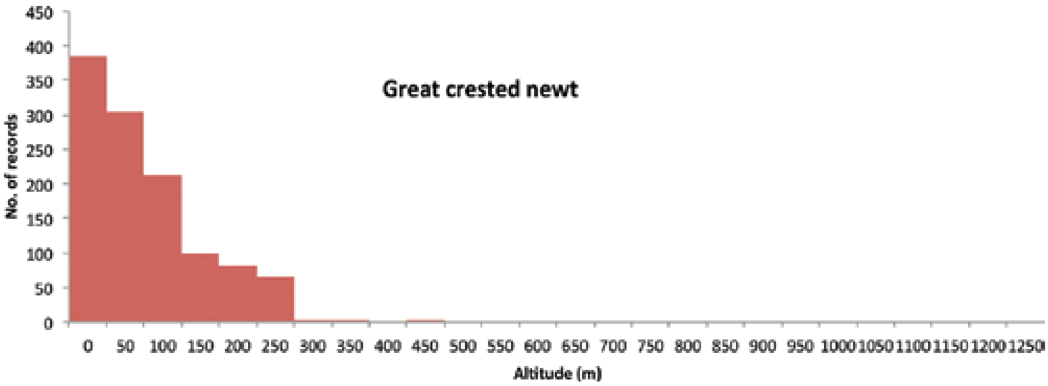

Figure 2. Incidence by altitude of great crested newts *Triturus cristatus* in Scotland based on records 2000–2016. Derived from [1].

Figure 3. A pond used by great crested newts *Triturus cristatus* for breeding at Gartcosh, North Lanarkshire, Scotland showing a combination of open water and emergent and submerged aquatic vegetation. This is one of a number of ponds created at the site to mitigate an industrial development that together hold the largest metapopulation of the species in Scotland.

The pH of water in pools and ponds influences the distribution of the great crested newt as it is usually found breeding at sites with a near neutral pH of 7.0–8.0 [4, 5]. However, in the Highlands breeding sites can have a pH as low as 4.9 but as larvae cannot develop below pH 4.5 the species is usually absent in more acidic water bodies [10].

During the aquatic phase juvenile and adult great crested newts feed on small invertebrates and larval amphibians, with tadpoles, molluscs and leeches preferred [11]. Larvae feed on plankton, particularly crustaceans. The terrestrial diet ranges from mites and springtails for juveniles, to worms, a wider range of arthropods and molluscs for adults.

2.3. Life cycle

The behaviour of the great crested newt during its annual life cycle has been studied and described [1, 5, 12].

Great crested newts hibernate through the winter usually on land with individuals hibernating under logs or rocks. They first emerge in spring from March, although most appear in April when night-time air temperatures reach about 5°C. Adults, which can live up to 15 years, are philopatric returning to ponds where they were born, mainly on warm wet nights with peak movements after midnight (**Figure 4A** and **B**). A variety of auditory cues are thought to be used to find breeding ponds such as the calls of common frogs *Rana temporaria*, common toads *Bufo bufo* and possibly wetland birds.

Males often arrive before females at ponds and occupy areas of open shallow water. Rival males defend territories mostly through visual display, so-called 'lekking', with physical contact between newts rare. The courtship by males of females involves whipping and fanning their tails, and a distinctive back arching known as the 'cat-buckle stance'. As well as visual cues the display involves the release by males of pheromones with the tail fanning transporting these to females. Females only mate after less than 10% of courtships, which is completed when a male deposits a gelatinous sperm package, called a spermatophore, with the female picking this up with her cloaca.

Fertilised eggs are then laid by the female with up to 300 eggs produced during a breeding season. Each egg is wrapped in the leaf of an aquatic plant, although eggs have also been found on discarded plastic and other materials in water. Females may use the same plant leaf to conceal more than one egg, leading to a 'concertina' effect; sometimes these are mixed with eggs of the smooth newt *Lissotriton vulgaris* or the palmate newt *L. helveticus*. The leaf wrapping both camouflages the eggs from predators and importantly shields them from ultra-violet light. Exposed eggs are vulnerable to ultra-violet light as the embryos lack the protective pigment of common frog or common toad eggs. Furthermore, up to 50% of newt embryos fail

Figure 4. Male (A) and female (B) great crested newts *Triturus cristatus* approaching a Scottish breeding pond. During the breeding season the male is distinguished by developing a high ragged dorsal crest that gives the species its name. The crest runs along the back ending at the base of the tail, with the tail having a separate, slightly smoother crest, which continues to the tip.

to fully develop due to developmental arrest syndrome, a genetic abnormality that affects all newt species in the genus *Triturus* [13].

The eggs hatch into larvae after 15–40 days with the length of development influenced by water temperature. Subsequent larval growth is dependent on food availability; other factors such as pollutants can retard larval growth [5]. Larvae usually metamorphose into juveniles during August or September. However, in some cooler or nutrient poor ponds larvae can overwinter in small numbers, although neotenous or paedomorphic great crested newts, where adults retain juvenile or larval traits, are very rare in Scotland [1].

After breeding many newts remain close to their breeding pond throughout the summer [14]. Adults and juveniles can be found foraging away from the breeding ponds in other shallow pools and puddles, but also on land under logs and rocks, and in vegetation [15]. Adults and juveniles can also travel further, enabling them to colonise new ponds, although the distances are limited, usually less than 1 km [16–18].

In autumn, newts hibernate underground when night-time temperatures drop below 5°C, typically from September, not reappearing until the following spring [15]. Metamorphosed juvenile newts have been shown to follow the scent trails of adults, possibly helping them to find suitable hibernation sites.

2.4. Scottish population and conservation

2.4.1. Scottish population numbers

The Scottish population has been estimated at between 3500 and 11,250 adults in the early twenty-first century [1, 19]. Around 200 occupied sites have been counted across the country suggesting that less than 1% of Scottish ponds are suitable for the species, with most only holding small populations [20]. However, numbers in ponds can range from fewer than ten adults to thousands as present at Gartcosh, North Lanarkshire, where the largest population in Scotland is found [21] (**Figure 3**). During a project to conserve this population, prompted by industrial development, 1012 adults were translocated in 2006, estimated to represent 9–29% of the national population at the time [19]. Subsequently, estimates of the Gartcosh population fluctuated from 100 to 515 adults with the peak count in 2015, of which 382 were males and 133 females [21]. Surveys over the period revealed a ratio of males to females ranging from 2.3:1.0 to 4.2:1.0. As the survey methods used revealed minimum estimates (6–23%) of the total population size [22] this implied that the 2015 Gartcosh population was in the range of 2239–8583 adults [21]. However, it is important to emphasise that great crested newt numbers in ponds can vary from year-to-year due to natural fluctuations as populations are thought to cycle every four years [5, 17, 23]. It has been suggested that such annual variations may be even greater in Scotland at the edge of the species' world range [24].

2.4.2. Legal protection, population trends, threats and conservation

The great crested newt is a species of international importance listed in Appendix II of the Bern Convention, Annexes II and IV of the EC Habitats Directive and classified by the International

Union for Conservation of Nature (IUCN) Red List of Threatened Species as 'Least Concern' due to a widespread distribution, although with a decreasing population [1, 5, 25, 26]. In Britain it has shown the highest rate of decline in recent years amongst native amphibians. This trend is shared in Scotland and as a species at the edge of its world range the great crested newt might be especially vulnerable [24]. Populations are protected by both European and UK legislation [1]. In the UK legislation states that it is illegal to injure, kill, disturb, capture, keep or sell great crested newts. It is also illegal to damage or destroy the habitat in which they live. This means that where land developments threaten populations dedicated surveys are required to determine their numbers and distribution. If these surveys reveal animals, developers are then obliged to propose mitigation for newts and their habitat to qualify for a license from a government regulatory agency such as Scottish Natural Heritage (SNH) before being allowed to proceed with construction.

As the species is found predominately in low-lying areas this has brought it into conflict with urbanization, agricultural changes and industrial developments [25, 26]. For example a study in the Glasgow area found that 35% of ponds had disappeared, mainly through landfilling with waste, with 48% of those remaining vulnerable [27]. Developments can also lead to habitat fragmentation. Isolated populations are at risk of extinction and there is evidence of reduced genetic diversity at such sites in Scotland [28, 29]. However, the loss of ponds and habitat is not always caused by humans: the natural succession of vegetation can result in ponds gradually being overgrown by bushes and trees, turning habitat into wet woodland and eventually drying out. This means that at many sites habitat management is required to prevent natural succession and maintain pond suitability.

That the great crested newt often exists in metapopulations, with breeding occurring in several close ponds, is important from a conservation perspective. It suggests that the species is less likely to become locally extinct as breeding success is not dependent on a single pond [30]. However, where an assemblage of ponds exists they all require protection with the management of habitat between them equally important. An example of this is the industrial development at Scotland's largest great crested newt site at Gartcosh and efforts to mitigate for this loss by the creation of a nature reserve containing multiple ponds to where newts were translocated [21, 31]. Encouragingly, the population at Gartcosh appears to have increased following the translocation suggesting that the newly created ponds and habitat are suitable. Translocation may therefore be an effective conservation mitigation strategy for the species [17, 32].

Where agricultural chemicals are used this is likely to cause the pollution of ponds [1]. The extraction of shale gas by so-called 'fracking', where large volumes of water are injected into the ground, may lead to contamination of water bodies.

Introduced invasive non-native species are a serious problem across the species' global range. Introduced fish eat newt larvae and reduce the variety and numbers of plants and invertebrates, which are important for newts [26, 33]. Furthermore fish can inhibit newt embryonic development and spread disease [34]. There is a further risk from other non-native species such as the signal crayfish *Pacifastacus leniusculus* and American mink *Neovison vison* that predate adult and larval newts.

The chytrid fungus *Batrachochytrium dendrobatidis* has had devastating effects on amphibian populations through the world, with evidence suggesting that the disease is spread by the international trade in these animals [35, 36]. Although great crested newts do not show symptoms or increased mortality when infected by chytrid fungus they can act as carriers [37]. However, the discovery of a related *B. salamandrivorans* in fire salamanders *Salamandra salamandra* in the Netherlands is very concerning as it caused 100% mortality of infected great crested newts in laboratory tests [36].

3. European adder

3.1. Scottish distribution

The European adder is found throughout the Scottish mainland and on a few west coast islands of the Inner Hebrides, although with a fragmented distribution [1] (**Figure 5**). Globally the species is found in central and northern Europe and Asia from Britain east to the Pacific coast of Russia, with Scotland at the north-west edge of its world range. Three subspecies are recognised with the nominate subspecies *V. b. berus* present in Scotland.

In Scotland the distribution is patchy with, on the mainland, concentrations across in the north of the country from Sutherland and Caithness to Wester and Easter Ross, Aberdeenshire, Angus and Perth & Kinross; and further to the south in Argyll & Bute, West Dunbartonshire, Stirlingshire, East Lothian, Scottish Borders, south Ayrshire and Dumfries & Galloway. On islands it is present on Skye, Mull, Islay, Jura and Arran with on some islands large populations.

3.2. Habitat requirements

Adders are found in a wide range of habitats in Scotland with no apparent relationship between distribution and landscape, suggesting that the species is versatile in its requirements [1] (**Figure 6**). Some are found on lowland mosses, flows and upland moors. But they are also present in coastal areas and islands, at cliff sites, on escarpments, slopes and in forest clearings. Snakes are also found in more developed areas, sometimes in close proximity to humans, such as on golf courses and the embankments of roads, railways and canals, and can be very tolerant of human activity [38–40].

Habitat features used by adders in Scotland have been studied [38]. Hibernation sites, known as hibernacula, where snakes spend the winter underground, have an open southerly or westerly aspect, usually on a slope which receives large amounts of sunlight (**Figure 7**). They can also be on flat ground, if the area is well drained and free from flooding. In mosses and flows this means that they are usually on higher ground amongst rocks and the roots of trees; and on upland moors gully slopes of streams are used. Dry-stone walls can also be exploited for hibernation.

Hibernation sites are usually associated with thicker vegetation such as bramble *Rubus fruticosus* agg. or gorse *Ulex* spp. and with stands of bracken *Pteridium aquilinum,* along with

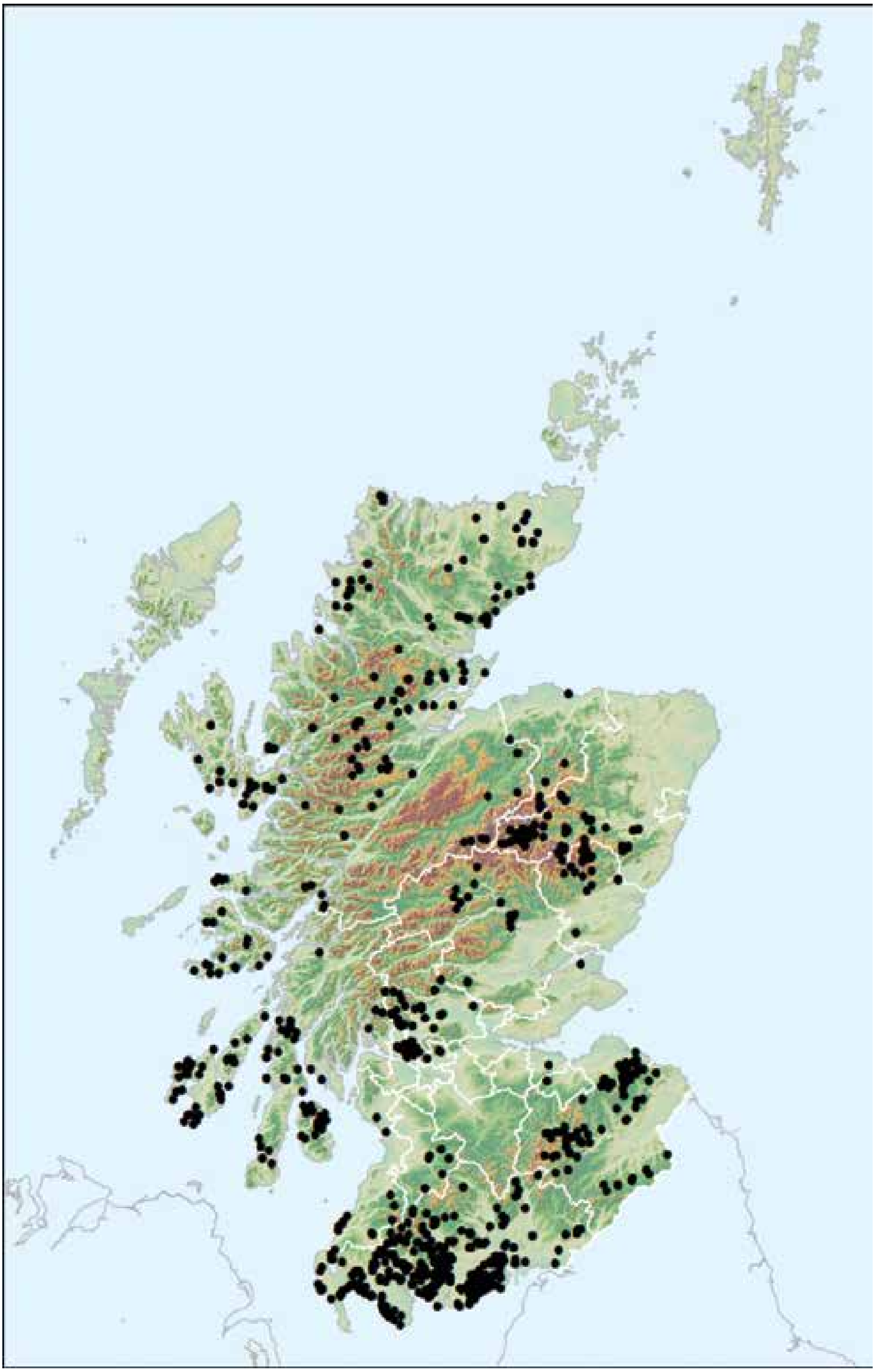

Figure 5. Distribution of the European adder *Vipera berus* in Scotland based on records 2000–2016. Derived from [1].

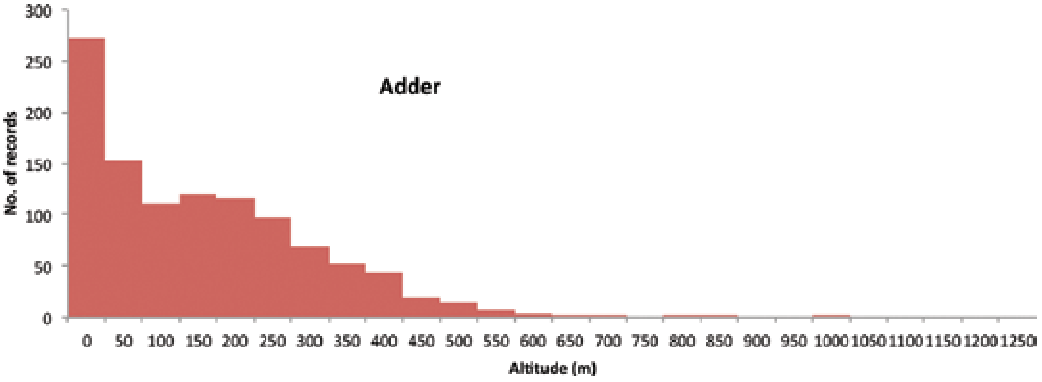

Figure 6. Incidence by altitude of European adders *Vipera berus* in Scotland based on records 2000–2016. Derived from [1].

Figure 7. European adder *Vipera berus* hibernation site at Loch Lomond, Stirlingshire, Scotland. The hibernaculum, where snakes spend the winter underground, has an open southerly aspect on a slope with patches of moss and thicker vegetation such as gorse *Ulex* spp. and stands of bracken *Pteridium aquilinum*. These provide areas for adders to bask in the sun and the opportunity to retreat to safety after disturbance.

patches of moss and grass. Snakes often bask in the sun on moss or grass and use the thorns and spikes of nearby bramble and gorse as protection under which to retreat when disturbed. Bracken appears to be an important habitat component in Scotland as adders are often found near it, with the bracken providing hibernation sites, protection and a warm microclimate [1, 40]. In winter, flattened bracken forms mats that provide insulation to hibernating snakes from cool temperatures. In summer, stands of bracken create sheltered, sunny areas. Adders are very well camouflaged when inhabiting bracken and the association of the species with bracken may explain its zigzag markings, which look very similar to dark brown bracken leaves. Similarly, the chestnut brown colour of juvenile adders is almost identical to bracken leaves in late summer, autumn and early spring. However, an alternative explanation for the zigzag is that it is a warning sign to discourage predators [41, 42].

Habitat near to hibernation areas is important, often containing wet or marshy ground, ponds and streams. This may be because such wet areas are a source of food items. Adders consume a range of prey including adult and young amphibians, reptiles, birds and small mammals [1, 43–47].

3.3. Life cycle

The behaviour of the adder during its annual life cycle has been studied and described [1, 48–50]. In Scotland a population of adders has been monitored on the shores of Loch Lomond, Stirlingshire with seasonal changes in behaviour recorded similar to those observed elsewhere in the UK and continental Europe [40, 51].

Adders hibernate through the winter at underground sites, males first emerging on sunny days in mid-February, with more males and females appearing in March. At Loch Lomond many snakes hibernate by themselves, although more rarely groups of up to four have been observed [40, 51]. Adders remain near to hibernacula, emerging and basking for extended periods in the cooler temperatures until mid-April when, in warmer conditions, they shed skins (ecdysis) and initiate courtship and mating (**Figure 8A** and **B**).

Adders can use the same hibernation and basking sites both within years and between years, and even throughout their lifetime, which can be up to 30 years [1, 50]. Individuals usually first emerge to bask in the morning sun, but later if conditions are not favourable. They then bask for 1–2 hours, often on sunny patches of moss or grass next to areas of bracken, gorse or bramble, before moving to cover, having warmed up. If disturbed they retreat to cover either underground or into the bracken, gorse or bramble, typically reappearing after a few minutes. Individuals sometimes 'mosaic bask' whereby they expose only parts of their body to sunlight to warm up while remaining camouflaged and thus hidden from predators.

Figure 8. Male (A) and female (B) European adders *Vipera berus* recovering in the Scottish spring after hibernation, having shed their skins. The male is distinguished by having a grey background skin colour and black edges to snout scales, whereas instead the female is brown with pale edges to snout scales.

In Scotland adders will attempt to bask on cloudy days, when the sun shines only for short periods, even if the air temperature is only just above freezing or when frost or snow is present. On first emergence they lie fully extended flattening their bodies, probably to maximise absorbance of solar energy. If conditions cool, snakes will coil to retain body heat, eventually retreating underground, only re-emerging if conditions improve. On such inclement days, basking by snakes can be interrupted and so last many hours.

Courtship and mating occur from middle April to early May when air temperatures increase and after females shed their skins. Recently shed males locate females by following their pheromone trails. If a male encounters another male who is also searching for a female, they can participate in a wrestling match described as the 'dance of the adders'. This can last from a few seconds to many minutes with the dominant male chasing off the rival. When a male finds a receptive female he initiates courtship by coiling, head-bobbing and licking, which lasts 1–2 hours. Mating follows a distinctive joint tail arching and also lasts 1–2 hours. After mating, males usually guard females to prevent them associating with other partners. Even so, females have been shown to mate with multiple partners in the same breeding season [52, 53].

Following the mating period males and unmated females move to other areas to feed [54]. Mated gravid females instead remain near to their hibernaculum, or find another sunny area nearby, where they bask for extended periods as this accelerates the growth of the developing young [55]. For the same reason they are sometimes found basking coiled with other female adders or with gravid slow-worms *Anguis fragilis*, likely to share body heat [40, 49]. Adders are viviparous giving birth to up to 13 live young, although the usual range is 4–10. Live-bearing reptiles are more common in colder climates, such as Scotland, where the young require the mother's warmth to develop. In contrast, many reptile species which inhabit warmer climates are able to lay eggs because ambient temperatures are high and constant enough for egg development. Gravid females are not thought to eat while carrying developing young and reproduction is physiologically stressful. This results in females usually not breeding every year [56, 57].

In Scotland young adders first appear from early August, but can be found up to late October and also sometimes in early spring immediately after hibernation. Young receive no parental support, surviving on a yolk supply within their body until prey is found which, if they are born late in the year, might not be until the following spring after hibernation. Female adders, after giving birth, feed and recover before hibernation; even so, mortality is higher for females in winters following birth [56, 57].

Adders undergo ecdysis shedding their skin from first emergence in early spring to late autumn, although it is most noticeable in April and May when snakes with fresh skin show the most vivid colours in preparation for mating (**Figure 8A** and **B**). Ecdysis in spring, late summer and early autumn appears to encourage males to follow females, but it is not known if successful mating occurs at later times of the year.

Adders return to hibernation sites from September and can bask until late October or even early November when hibernation begins. Animals occasionally emerge through the winter if it is sunny and mild. Adders have been noted hibernating underground alongside slow-worms, common toads and common lizards.

Adders have been observed showing striking synchronicity of behaviour at different times of the year both between individuals at a single site and between individuals at different nearby sites. For example, emergence from hibernation, skin-shedding, courtship and mating have each been noted to occur on the same day at different sites around Loch Lomond [1].

3.4. Scottish population and conservation

3.4.1. Scottish population numbers

Adder population numbers across Scotland have not been estimated although in the early twenty-first century some parts of the country hold high densities in suitable habitat where the species is not persecuted [1].

A few local Scottish population estimates have been made. In the late nineteenth century '40 per acre', equivalent to about 100 per hectare, were recorded on a lowland moss near Dumfries with a total population of 2400 [43]. More recently at a Loch Lomond site, which consists largely of native replanted forest, over 200 individuals were recorded in an area of six hectares over four years: during this period estimated population densities varied from 62 per hectare in 2012, to 236 per hectare in 2014 [40]. Furthermore, based on counts of individuals, the ratio of males to females varied between years, from 1.0:1.6 to 1.3:1.0, with an average during the period of 1.0:1.1, suggesting that similar numbers of the two genders were present [40]. At another site in Scotland, a lowland golf course, over 54 individuals were counted within four hectares over five years [58].

3.4.2. Legal protection, population trends, threats and conservation

The European adder is a species of international importance listed in Appendix III of the Bern Convention and classified by the IUCN Red List of Threatened Species as 'Least Concern' due to its widespread distribution, although with a decreasing population [1]. In Britain populations have shown declines in recent years [59]. Adders are protected by both European and UK legislation [1]. In the UK legislation states that it is illegal to injure, kill or sell animals.

In Scotland man is the most serious threat to the adder, both directly through persecution and indirectly by habitat loss [59–61]. Venomous snakes, such as the adder, have a long history of being misunderstood, feared and mistreated. To this day, despite legal protection, animals are still mistreated and killed.

Habitat loss is a problem, although many seemingly suitable areas remain in the country [1, 38, 62]. Adders have disappeared from many sites as a result of management practices such as drainage, muir-burning and afforestation. For example the conversion of moors to commercial forests is likely to have reduced adder numbers and distribution as this creates less suitable habitat for these reptiles. Similarly, the restoration of mosses, flows and peatlands can have negative impacts through the loss of hibernation sites if water levels are raised and appropriate mitigation is not put in place.

On a more positive note adders can be very tolerant of humans and human developments, if they are considered and suitable protection and precautions are put in place. This is illustrated by the site at Loch Lomond where high densities of snakes are present, some very close to human habitation [40]. Here a small hydroelectric scheme was installed with the development crossing through an area with the highest reptile densities [39]. The implementation of a mitigation plan to both move and protect the reptiles, the monitoring of adder numbers before and after the development, and the observation of snakes mating and giving birth close to the site during construction, together suggested that the population was unaffected by the development. Similarly, adders have been shown to prosper on a Scottish golf course where the habitat and management regime has allowed them to co-exist with golfers [58].

4. Conclusions

This chapter has described the distribution and life cycle of two species of amphibian and reptile in Scotland, the great crested newt and the European adder. In both cases examples of conservation projects are outlined that mitigated the effects of human development projects on local populations which appear to have been successful. Such work demonstrates that amphibians, reptiles and humans can co-exist to the benefit of both. This suggests that a wider understanding and appreciation of these wonderful creatures will encourage and increase this relationship in the future.

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Biology, Life History Traits and Conservation of the Vulnerable Souss Valley Tortoise in Arid Areas of West Central Morocco

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Abstract

The Souss Valley tortoise, *Testudo graeca soussensis*, is endemic to the semi-arid and arid low quality habitats of west central Morocco. Populations of this area are among the best-investigated populations in western Mediterranean. The long-term conservation of these declining populations is of particular management concern for this endemic and threatened subspecies. Here we present a data review on biological and ecological aspects of this subspecies dealing with morphology, distribution range and climatic niche, habitat use, food habits, activity cycle, and thermal responses to arid hot conditions, post-hatching growth, sexual maturity, sexual dimorphism, breeding ecology and demographics. After an analysis of the major threats on populations, we proposed management actions required to improve the conservation outcomes for this endangered tortoise in arid areas of west central Morocco. Conservation and restoration potentials of the declining populations and their habitat are also described, including habitat restoration and a captive breeding program.

Keywords: *Testudo graeca soussensis*, arid environments, west-central Morocco, taxonomic status, biology, ecology, life history, threatened subspecies, conservation

1. Introduction

The Spur-thighed Mediterranean (or Greek/Moorish) tortoise, *Testudo graeca* L., 1758, is the unique land chelonian species in Northwest Africa north of the Sahara desert [1]. It occurs along an altitudinal gradient from coastal to mountainous (up to 2090 m a.s.l.) and pre-Saharan areas covering various habitats and bioclimates ranging from xeric (116 mm) to mesic (1092 mm) types [2]. Most research works on its distribution, ecology, and life history were carried out in Morocco (e.g., [3–10]). In North Africa, the species comprises five distinct subspecies, two of which are endemic to Morocco. Among these, the Souss Valley tortoise, *T. graeca soussensis*, occupies the southernmost part of the species distribution range in Southwestern Morocco. This mostly arid and highly populated area of the country constitutes one of the most touristic regions with an important land use for agriculture and livestock pasture. As reported in the 1970s and 1980s in Morocco [3], very few populations can still be considered abundant and free from human disturbance in this part of the country. Three main populations, especially one in the central Jbilet mountains, west-central Morocco, were intensively studied during the last two decades. The first ecological investigations on *T. g. soussensis*, were conducted in the Admine Forest located in the Souss Valley [5] where the subspecies was originally described by Pieh [11]. All these populations are threatened due to increasing impacts of human activities (i.e., urbanization, agriculture, overgrazing) and climate change resulting in frequent droughts. So, conservation and restoration of the subspecies and its habitat in west-central Morocco are required. In this chapter, we provide a summarized literature review on the biology, ecology and life history of this subspecies in west-central Morocco. We will also report on the main threats and conservation and restoration potentials of declining populations.

2. Description of the subspecies

The subspecies was originally described, based on morphology, as *T. g. soussensis s* sensu stricto by Pieh [11]. It can be distinguished from all known *T. graeca* subspecies tortoises by lacking thigh tubercles, a larger carapace size in adults, striped carapace markings, and markings that reach over the supporting parts of the plastron. With a few exceptions, the tortoises of the Souss Valley possess strikingly long pectoral scales. The length of the pectoral scales middle seam is usually more than 2/3 of the length of the femoral scales middle seam. The Middle seam length of the pectoral scales in the majority of nominated form. In contrast, animals are less than 2/3 of the femoral scale mid-suture length. The third vertebral is relative to adult carapace length male *T. g. soussensis* shorter than in male *T. g. graeca* Sensu stricto.

In their detailed morphometric study, Carretero et al. [6] questioned the validity of the taxon *soussensis* and pointed out that the diagnostic characteristics of the Souss Valley Tortoise provided by Pieh [11] could be confirmed only to a minor extent for the population of the Admine Forest, with only 23% of the examined specimens having no thigh spurs, only 7% exhibiting a divided anal scute, and the dimensions of the third vertebral scute not differing from those in specimens from the surroundings of Essaouira. In this conjunction, it needs to be noted though that the population from Essaouira, as well as the one from the Jbilets Mts, north of Marrakech, are nevertheless currently referred to the subspecies *T. g. soussensis* [6].

2.1. Morphological variation and sexual dimorphism

Using 41 carapace dimensions, Carretero et al. [6] found a significant morphological differentiation among tortoises from Admine Forest, Essaouira and Jbilet Mts, west-central Morocco

(**Figure 1**). The population from Jbilet was the most differentiated (no overlap), those from Essaouira on the coast and from Admine showed limited overlap (5%). Tortoises from Jbilet living under harsher conditions (low precipitation, high thermal amplitude, low plant cover) were smaller, lighter, more flattened and less dimorphic in shape than the others. This morphological variation contrasted with the non-significant genetic difference based on 12S rRNA mtDNA among these three populations [12]. The observed morphological differences either have arisen very recently or can be attributed to phenotypic plasticity.

Indeed, *Testudo g. soussensis* exhibits high phenotypic plasticity in both size and shell shape [6, 13]. In arid environments, in the central Jbilet, it is smaller, with a more flattened shape and a less marked sexual dimorphism than in the populations of the coastal zone of Essaouira and the Admine Forest in the Souss Valley. In the latter, tortoises have a body size (carapace length, CL) reaching maximum values of 184 and 226 mm corresponding to body weights of 1200 and 1996 g, respectively in males and females [6, 13]. In addition, turtles in the coastal zone of Essaouira show a relatively pronounced melanism [6].

As *T. g. graeca*, *T. g. soussensis* are easily sexable; sexual size dimorphism with females larger than males, remained constant between sites. Sexual shape dimorphism is prominent in all populations, but the degree of dimorphism differs for some characters when corrected for size [6]. Females are distinguished by greater abdominal volume than males in order to increase the ability to carry eggs, while males develop larger openings in the carapace to increase their mobility in search of sexual partners. The males have a concave plastron, a more convex supra-caudal, and a longer, thicker tail, whereas the females have a flat or slightly domed plastron and a flattened, rounded-edge supra-caudal and a short obtuse tail [3, 14]. Moreover, Ben Kaddour et al. [14] tested the hypothesis according to which in chelonians a reduction of the openings in the shell improves protection against predation but also constrains the abdominal volume and limits the space available to move the limbs. As expected, these authors found in the Souss Valley tortoises from central Jbilet, that the shell was shown to provide a larger

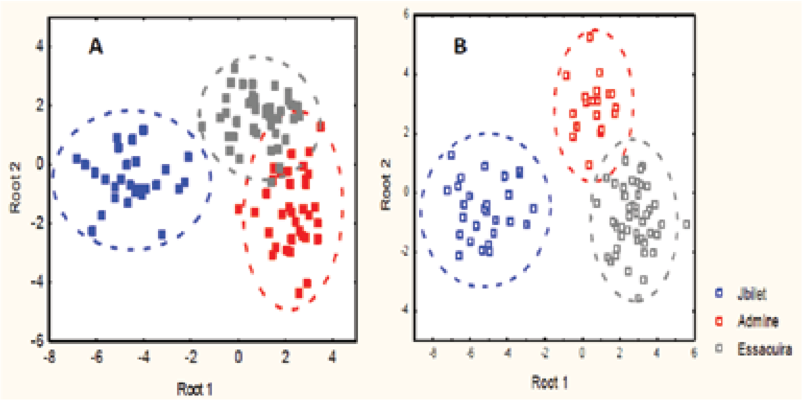


Figure 1. Results of the canonical analyses of morphometric variables (size-corrected) of *Testudo graeca soussensis* from West-central Morocco, performed separately for each sex (A: males and B: females) (modified after Carretero et al. [6]).

abdominal volume relative to tortoise size in females than in males. In males, deep notches in the shell and a reduction of several plastron plates offer more freedom to the limbs and to the tail; these characteristics presumably enhance mating success.

3. Taxonomy

As for the other populations of Northwest Africa, all Moroccan populations were put together with *T. g. graeca*, excepting the questionable revalidation of *T. whitei* by Highfield and Martin [15] (see [17]) and the long-debated description of *Furculachelys nabeulensis* Highfield [16]. While the former was practically omitted from the scientific literature, the latter taxon was eventually confirmed as a subspecies of *T. graeca* [17] eventually confirmed. Using mitochondrial cytochrome b gene and part of the adjacent tRNA-Thr gene, Fritz et al. [17] identified five distinct lineages within the western Mediterranean clade of *T. graeca* in North Africa. These lineages show a clear allo- or parapatric distribution from east Libya to west Morocco that matches well with previously morphologically defined subspecies (*T. g. cyrenaica, T. g. graeca, T. g. soussensis, T. g. marokkensis, T. g. nabeulensis*). Two of these are endemic to Morocco: *T. g. marokkensis*, and *T. g. soussensis* (**Figure 2**). As above-mentioned, this latter was described for the first time as a new subspecies by Pieh [11] on the basis of external morphology from the type locality near Agadir city (30°28′N, 9°55′W) in the lower Souss valley. The Souss valley is the alluvial basin of the Oued (river) Souss located in mid-southern Morocco, southwest of the Western High Atlas range and separated from the Sahara desert by Anti-Atlas Mountains.

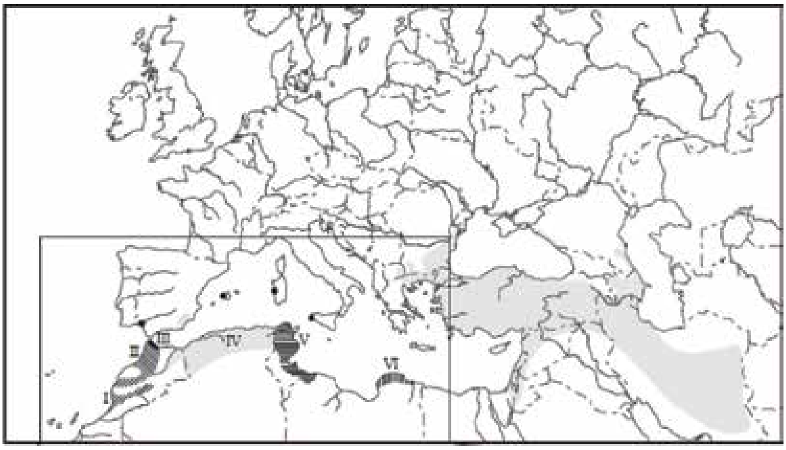


Figure 2. Geographic distribution of *Testudo graeca* subspecies currently recognized in the western Mediterranean: I—*Testudo graeca soussensis* Pieh, 2001; II—*T. g. marokkensis* Pieh and Perälä, 2004; III—*T. g. Lamberti* Pieh and Perälä, 2004; IV—*T. g. graeca* Linnaeus, 1758; V—*T. g. nabeulensis* (Highfield, 1990); VI—*T. g. cyrénaïque* Pieh and Perälä, 2002 (modified according to Fritz et al. [17]).

4. Current distribution and quaternary range dynamics

The distribution range of *T. g. soussensis* was restricted to the Souss valley and the few "records" from the surroundings of Ouarzazate [18, 19] likely to be referable to this subspecies as well [11]. Few years later, Pieh and Perälä [20] extended the distribution of this subspecies to the southern areas of the Central Meseta, i.e., to the northern parts of the High Atlas and the adjacent forelands (**Figure 3**).

Testudo g. soussensis occurs in three areas of west-central Morocco: the Souss Valley throughout the coastal line southwest to Anti-Atlas, semi-arid and arid lands north of High Atlas to the mid-Atlantic coastal line around Essaouira, and the area in the vicinity of Ouarzazate. According to the maps of Lambert [4] and the distribution map of Bons and Geniez [19], there is an occurrence gap for *T. graeca* in Morocco range of the ridges of the High Atlas and the Atlantic coast near Tamanar (see Lambert [3]). It occurs along an altitudinal gradient from 5 m on the Atlantic coast (Essaouira area) to 1600 m in the Anti-Atlas range (W of Aoulouz), under semi-arid to pre-Saharan bioclimates. South of the Anti-Atlas and High Atlas, the probability of occurrence drops considerably due to the strong aridity gradient; no tortoises have been recorded where annual rainfall is lower than 139 mm, recorded 40 km south-west of Tiznit in the southwest coast region of Morocco [4].

Following a purportedly tortoise-free zone (from the Atlantic coast around Safi via the Chemaia Plateau east to where the Middle and High Atlas merge [20], the northern parts of

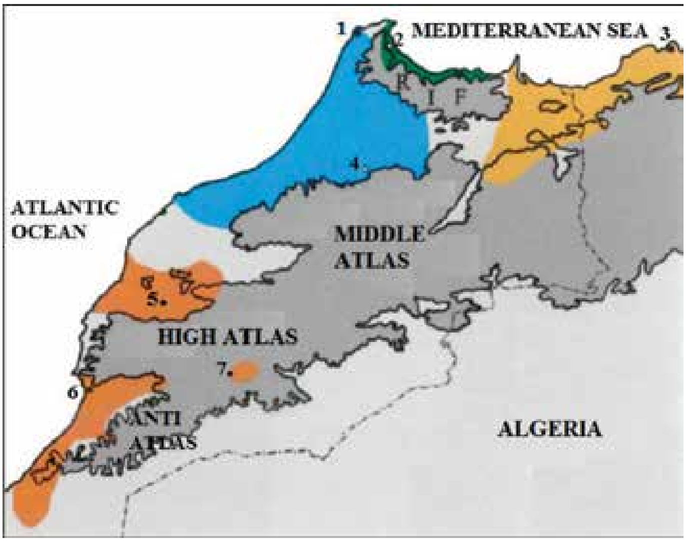


Figure 3. Distribution of *Testudo graeca soussensis* (*sensu stricto* and *sensu lato*) in western Morocco (orange); *Testudo graeca marokkensis* (light blue + *Testudo graeca lamberti* (green)) in northwestern Morocco, and *Testudo graeca graeca* in northeastern Morocco (ocher). The numbers refer to the cities Tangiers (1), Tetuan (2), Oran (Algeria [3]), Oulmes (4), Marrakech (5), Agadir (6) and Ouarzazate (7) (modified after Pieh and Perälä [20]).

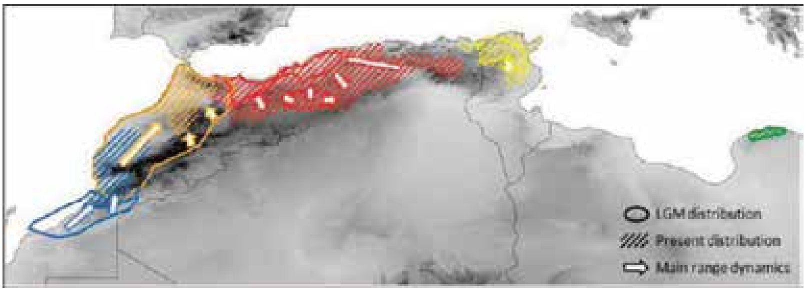


Figure 4. Schematic representation of the post-glacial range dynamics of the five subspecies of *T. graeca* in north Africa based on ENMs: *T. g. cyrenaica* (green), *T. g. nabeulensis* (yellow), *T. g. graeca* (red), *T. g. marokkensis* (orange) and *T. g. soussensis* (blue) (After Anadón et al. [21]).

the High Atlas are where the distribution range of *T. g. soussensis sensu lato* begins. It is once more separated by a tortoise-free zone along the western slopes of the High Atlas from that of the actual Souss Valley tortoises. Their occurrence extends from the Souss Valley and along the Atlantic coast to the level of the southernmost foothills of the Anti-Atlas.

Using ecological niche modeling, Anadón et al. [21] showed a clear niche differentiation among the five subspecies of *T. graeca* in North Africa, in response to past climate changes, particularly in relation to rainfall, covering a range from semiarid to humid. Since the Last Glacial Maxima, the range of *T. g. soussensis* has shifted northwards while those of the other subspecies have either expanded (*T. g. graeca*), contracted (*T. g. marokkensis*) or remained stable (*T. g. cyrenaica* and *T. g. nabeulensis*) (**Figure 4**). Molecular data supported this biogeographic dynamics that would have been strongly affected by precipitation changes during glacial periods. Rainfall plays a primary role in shaping the distribution of *T. graeca* [2, 4]. Rainfall in the coldest quarter (i.e., winter) is the strongest limiting factor for the five subspecies, supporting the key role of available primary productivity in spring for the energy budget and the population dynamics of the species. While *T. g. marokkensis*, *T. g. nabeulensis* and *T. g. cyrenaica* were qualified as 'humid' subspecies with a maximal probability of occurrence at a rainfall range of 800–1200 mm, *T. g. graeca and T. g. soussensis*, were defined as semiarid taxa since their probability of presence increases rapidly slightly below 200 and presents an optimum in semiarid conditions (200–400 mm). However, whereas *T. g. graeca* can be described as 'semiarid but generalist' taxa, able to cope with humid conditions, *T. g. soussensis* is a 'strict semiarid' taxon since the probability of presence plums between 300 and 400 mm.

5. Ecology and behavior

5.1. Habitat

Comparatively to the other North African subspecies, *T. g. soussensis* occurs in lower quality habitats with low rainfall and high thermal seasonality in semi-arid and arid zones of the central-western and southeastern parts of the Morocco [2]. Within the Admine Forest, in the

Souss Valley, there are pockets of agricultural land surrounding the villages, which are predominantly composed of small fields of 0.1–2 ha that are surrounded by thorny hedges. The most widespread hedging material is *Opuntia ficus indica*, a cactus species introduced from the Americas, whilst the endemic Jujube species *Ziziphus lotus* is the next most abundant. Both species provide the major present day habitat for *T. g. soussensis*: *O. f. indica* has created an important new habitat providing good protection from predators and humans, a food source, and good nesting, basking, aestivation and scrape sites (the use of individual sites depending on the aspect of the bank and season of the year). It is interesting to note that this introduced cactus, much maligned in Australia for its effect upon indigenous species, should now be such an important refuge for tortoises in Morocco.

In the arid-steppe lands of the Haouz plain and central Jbilet Mountains, tortoises were found to depend on the occurrence of large (taller than 1.25 m) and relatively abundant Jujube bushes (*Z. lotus*) to escape lethal overheating during the hot and dry periods [8]. In the absence of such relatively large bushes, the tortoises are under serious threat of lethal overheating, indeed ambient and estimated body temperatures largely exceeded 40°C for prolonged periods in all other microhabitats. Therefore, the short-term (hours in summer), survival of the tortoises depends closely on the presence of relatively large bushes; all individuals use this specific microhabitat intensively all year round, and exclusively in summer [5, 8].

5.1.1. Habitat use

The Souss tortoises do not appear most of the time in places where the vegetation overtops the height of cereal fields almost exclusively in or near Jujube bushes (*Z. lotus*). These largely impenetrable thorny bushes provide shelter for larger animals, and in their shadows grow herbaceous plants that feed the tortoises. Likewise, tortoises were found on stone wall field boundaries where wind and evaporation protection allow a rich vegetation flourishing. They are also encountered on the mid-Atlantic coastal area in sand dunes and within Argan forests, among scattered shrubs around cultivated fields [8, 9].

5.1.2. Tortoise scrapes and burrows

In the Souss Valley, tortoises use scrapes and burrows to avoid the mid-day heat, for shelter at night and for prolonged aestivation periods. These scrapes and burrows vary considerably in opening size and depth. If some relatively small ones can be produced by tortoises, others clearly surpass their digging abilities and shows signs of previous occupation by mammals such as honey badger (*Mellivora capensis*) and Geoffroy's ground squirrel (*Xerus erythropus*). Shallow scrapes are often observed at the base of *O. f. indica* hedging, where the presence of roots stabilizes the otherwise loose and structureless sandy soil. Larger burrows are also found in these hedges, but are more frequently located in fossilized sand dunes and in the banks of dry rivers. Burrow depth at these sites varied from 40 cm to more than 1.5 m. Several aestivating tortoises were located in a series of deep burrows in a fossil sand dune. Similarly, in the arid-steppes on the central Jbilet mountains, shallow scrapes were encountered under Jujube/Acacia bushes; tortoises can also shelter in large entrances of Bibron's Agama (*Agama impalearis*) burrows, under stones (shales) or within under-road/rail tunnels [5, 8, 9, 22].

During the hottest and driest periods (late May to early September) in the central Jbilet mountains [9], radio-tracked tortoises were all found sheltered for a long period under large bushes. Most of them (~90%) were immobile and partly buried. During cooler periods, notably spring and autumn, the tortoises were more frequently under the bushes, but moving regularly [9]. Tortoises used mostly jujube bushes that were higher than 3 m, and in few cases, oleanders (*Nerium oleander*) taller than 2 m. They were rarely observed in the open, moving relatively rapidly between shelters. Average home ranges (minimum convex polygon) represented less than 0.3 ha (range: 0.4–8.9 ha). The tortoises travelled 2.5 km on average (range: 1.3–3.9 m). Males travel over longer distances than females (3.2 versus 1.9 km) correlatively to their larger mean home range (4.7 versus 0.7 m^2) [8, 9].

5.2. Activity cycle and time-activity budget

5.2.1. *Annual activity*

According to Bayley and Highfield [5], brumation (i.e., a hibernation-like state that cold-blooded animals utilize during very cold weather) does not occur in the Admine Forest, Souss Valley, where average monthly mean temperatures in the coolest part of the year (December and January) do not fall below 14.8 and 13.6°C respectively. Average daily temperatures in winter are typically 23.8°C in November, 20.8°C in December and 31.1°C in January. Air temperatures at sites where active tortoises were observed in mid-December 1994 ranged from 24 to 27°C between 10.00 and 14.00 h. The temperature recorded in scrapes and shallow burrows occupied by tortoises was typically 24–26°C and the relative humidity there ranged from 48 to 60% compared to 20% or less in exposed areas.

Aestivation typically begins in June and ends in September. The precise timing is affected by prevailing temperature and, especially, by precipitation. In drought years aestivation may be considerably extended. Tortoises are invariably aestivating during July and August where average mean monthly temperatures are 22.2 and 22.5°C, respectively. Peak daily temperatures during these months can exceed 48°C [5, 8, 9].

Utilizing automatic acceleration recorders, Lagarde et al. [8] characterized and monitored long time behavioral sequences of free-ranging tortoises in central Jbilet Mountains. They recorded and compared the activity budget of males and females under natural conditions during the spring (**Figure 5**). Tortoises monitored during 24 consecutive hours, remained inactive at night. The average daily activity time was 7650 min. Under unfavorable climatic conditions (strong wind combined with an ambient temperature <15°C), some individuals were mostly inactive with a total activity of less than 5 min/day and few others remained motionless. The duration of the total activity time in active animals was on average less than 2 h/day. The mean daily active time (including the inactive animals) was significantly longer in females compared to males (9455 min versus 6039 min, respectively) [8, 9].

The tortoises devoted most of their activity time to walking and foraging behaviors (40 and 36% of the total activity time, respectively). Sexual and fighting behaviors were very infrequent representing less than 2% of the total activity time, in both sexes. Burrowing and walking behaviors lasted respectively less than 25 and 40 min per day without difference between sexes. Females spent more time foraging than males, but these were more frequently immobile (**Table 1**) [9].

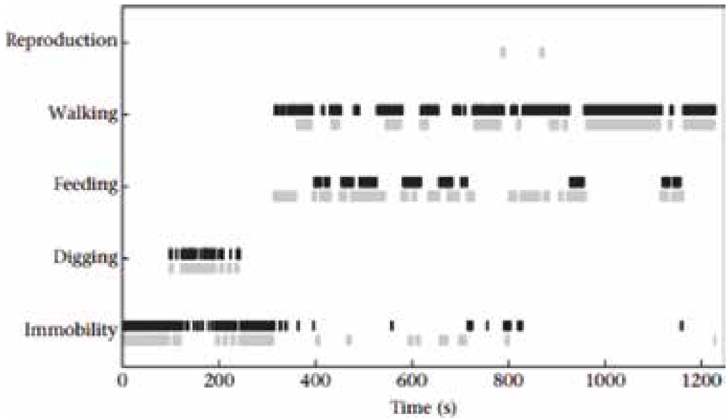
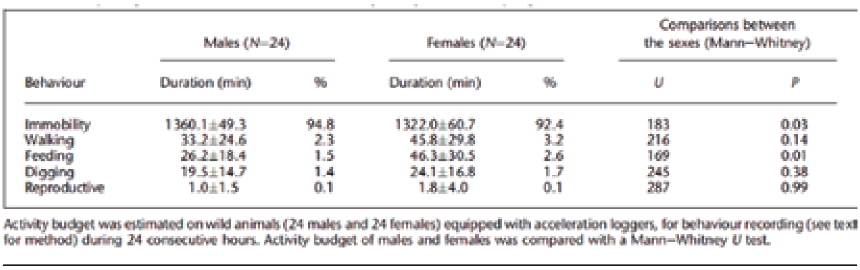


Figure 5. Observed (black bars) and reconstituted (grey bars) behaviors of *Testudo graeca soussensis* in the spring, in the Central Jbilets, Morocco (modified after Ben Kaddour et al., 2008).



	Males ($N=24$)		Females ($N=24$)		Comparisons between the sexes (Mann–Whitney)	
Behaviour	Duration (min)	%	Duration (min)	%	U	P
Immobility	1360.1±49.3	94.8	1322.0±60.7	92.4	183	0.03
Walking	33.2±24.6	2.3	45.8±29.8	3.2	216	0.14
Feeding	26.2±18.4	1.5	46.3±30.5	2.6	169	0.01
Digging	19.5±14.7	1.4	24.1±16.8	1.7	245	0.38
Reproductive	1.0±1.5	0.1	1.8±4.0	0.1	287	0.99

Activity budget was estimated on wild animals (24 males and 24 females) equipped with acceleration loggers, for behaviour recording (see text for method) during 24 consecutive hours. Activity budget of males and females was compared with a Mann–Whitney *U* test.

Table 1. Activity budget of free-ranging male and female *Testudo graeca soussensis* in spring, in the Central Jbilets, west-central Morocco (Ben Kaddour et al., 2008).

5.3. Thermal ecology

Like most animals, the Souss Valley tortoise must navigate between microhabitats to find essential feeding resources and thermal refuges. Combining radio-tracking of free-ranging individuals, microhabitat temperature monitoring and transects in a range of habitats, in central Jbilet Mts, Lagarde et al. [8] found tortoises to depend on the occurrence of large and relatively abundant Jujube bushes (*Z. lotus*) to escape lethal overheating (**Figure 6**).

As suggested by the null models, both the air temperature and the body temperature (Tb) of tortoises do not reach 40°C during cool/cloudy days. During sunny days, the tortoises are exposed to potential overheating (Tb > 40°C) and solar radiation for a minimum of 1 h. Large bushes provide thermally buffered conditions while other habitats are potentially risky. Females are less subject to overheating, owing to their much larger size (mean adult body mass: 1.0 versus 0.46 kg) [8, 9].

In central Jbilet Mts, during hot periods, the tortoises could safely travel around 900 m in the open in an average time of nearly 3 h. However, these values vary according to the time of emergence. The estimated safe distance rapidly decreased from 8:00 to 13:00, and then tended to stabilize after 15:30; reflecting the reducing intensity of solar radiation in the afternoon and

Figure 6. A pair of tortoises (left: male; right: female) active in the shade of a Jujube bush (*Zizyphus lotus*) in the central Jbilet Mountains, west-central Morocco (photo T. Louzizi).

concomitant decreasing overheating risk. However, individuals must shelter before night; tortoises leaving the bushes after 17:00 could not travel far as the activity stops around 19:00–19:30. In the afternoon the body temperature of the sheltered tortoises was already elevated, thereby improving locomotors performance compared to individuals leaving their refuge in the morning and that must bask in the sun prior movements (night temperatures were often low), but this also accelerated overheating risk [8, 9].

The estimated maximal Tb (recorded between 16:00 and 19:36) of the free-ranging tortoises, walking in the open was 34.7°C for males and 33.6°C for females. Males exhibited higher maximum Tb than females (33.1 ± 0.8°C versus 31.3 ± 0.7°C). The large body size of the females provided a buffering advantage and they were able to travel farther than males. For example, during the hottest periods (at 11:00), the estimated theoretical maximum distance, a tortoise can move is 494 m for males and 673 m for females [8, 9].

5.4. Food habits

The dietary constituents of the species in Admine forest (Souss Valley), included, in addition to various wild plant species, several cultivated species [5]. In this area, displacement of tortoises from the Argan forest because of overgrazing, loss of ground cover and drought has led to their concentration along the margins of cultivated fields. In these situations a high proportion of their diet now consists of *O. f. indica* cacti and agricultural crops (French and broad beans, lucerne, potato leaves, tomatoes, lettuce, zucchini, cauliflower and cabbage). In the Jbilet Mts, *T. g. soussensis* fed only on a small proportion of the available plant species during the spring season, but at the same time did not discriminate against species that would be toxic to mammals [7]. The diet composition, obtained by fecal analysis and compared to plant assemblages seems rather a specialist herbivore. The five main plant species found in fecal samples (*Leontodon saxatilis* (Asteraceae); *Malva parviflora* (Malvaceae); *Astragalus cruciatus*, *Medicago hispida* and *Lotus arenarius* (Fabaceae)) represent 70% of the identified material and were highly preferred whereas several other plant species, common in the field (e.g., *Eryngium ilicifolium* (Ombellifereae); *Emex spinosus* (Polygonaceae); *Spergula flaccida* Caryophyllaceae)) were actively avoided. Fabaceae made up 27% of the diet and may be important forbs in the diet of terrestrial tortoise owing to their high nutritional value. The diet composition of *T. g. soussensis* suggests that diet overlap may occur between domestic ungulates and tortoises in overgrazed landscape and could generate a competition context. However, *T. g. soussensis* seems to focus its foraging effort mostly under the spiny Jujube shrubs where the impact of overgrazing is strongly attenuated [7, 22].

The spring specific richness of plants in the Jbilet habitat was reduced during drier years (2011 and 2012) compared to that reported in a previous above-normal rainfall year [22] (44 and 27 versus 88 plant species). The dry plant biomass density differed greatly between under and out of the Jujube. The mean plant species richness and the total dry biomass density were higher in under than in out Jujube. Tortoises tended to consume only about 5–6 and almost the same species, out of all the available annual forbs and grasses. The total number of plant species that were counted as being within the top 5 for all tortoises included more than 8–10 species. Many other species, abundant in the habitat, were rather avoided. It was suggested that the few herbaceous plants that represented ~90% of tortoise diet, might be important in sustaining tortoise populations during droughts. These results underline the tortoises' vulnerability to global change on the availability of their preferred food plants [22].

The diet composition showed significant differences among seasons, either qualitatively or quantitatively [22]. The spring diet was more diverse with 16 plant species consumed against only 5 and 7 species in summer and autumn-winter respectively, with 5 shared species (*Cynodon dactylon, Salvia aegyptiaca, Leontodon saxatilis, Lotus arenarius* and *Medicago hispida*) during the all seasons.

5.5. Growth and sexual maturity

Both male and female tortoises from the central Jbilet Mountains, the Admine Forest, and near Essaouira, grew relatively quickly for 10–12 year in all three populations, after which their growth rates decreased markedly. Females in all three populations had greater asymptotic sizes than males, and CL and log mass differed significantly between sexes by age 9 year for the three populations, except for log mass of tortoises from Essaouira, which differed by age 6. Mean CLs and log masses were significantly greater in Admine and Essaouira tortoises than in tortoises from Jbilet [13].

The growth period T, equivalent to the age at which 90% of the asymptotic CL is reached, was delayed by approximately 3 year in females relative to males in tortoises from Jbilet (males 14.9 year; females 11.8 year) and Admine (males 12.0 year; females 9.0 year), but not in Essaouira (males 11.4 year; females 11.8 year). Growth was relatively fast for the first 7–10 year of life for tortoises in all three populations whether based on CL or log mass. Growth decreased rapidly after about 10–12 year. Based on calculated CL and calculated mass, tortoises from Admine became larger than tortoises from Jbilet between 3 and 6 year and tortoises from Essaouira exceeded the size of tortoises from Jbilet between 1 and 3 year. A significant sexual size dimorphism occurred by 9 year of age for the three populations based on calculated CL. Based on calculated mass, male and female tortoises from Essaouira diverged in size by 6 year of age, whereas there was no divergence between sexes in mass until 9 year of age for tortoises from Jbilet and Admine. Growth patterns differ significantly between sexes at an age below 8 years, but not between 1 and 5 years of age. From an age of 5 years, a decrease in growth rates of males is observed, whereas those in females increased until 6–7 years of age and then strongly decrease afterward. There was no significant difference in growth patterns between sexes after the age of 8 years [13].

The age at sexual maturity, estimated using large carapace growth annuli, varies from 6 to 9 years for males and from 8 to 11 years for females (7 versus 9 years in average) [13, 22]. The males reached their sexual maturity at a lower mean minimal size (90.7 ± 10 mm, range: 91.4–131.4 mm) than females (146.2 ± 16.5, range: 71.8–114.6 mm). More than 60% of males attained the sexual maturity between 6 and 7 years with a carapace length of 100–120 mm, while around 60% of females are mature between 9 and 10 years with a carapace length ranging from 140 to 170 mm. The estimated minimal size at maturity, based on the sexual behaviors observed in the wild (courtship, mating) is 104.8 and 152 mm, respectively for males and females [13].

Sexual size dimorphism index (SDI) calculated by dividing the size (mean CL) of the females by the size males and subtracting one [23], is almost constant in the three populations and averaged 0.17. Mass of all tortoises (all age classes and sexes combined) from the three populations is highly correlated to CL.

5.6. Reproductive ecology

Only limited data are available on breeding cycle and clutch characteristics in *T. g. soussensis* in west-central Morocco [5, 10].

Mating activity in the Souss Valley tortoises from Admine Forest occurs bimodally in the early spring and in September, immediately following emergence from aestivation. In semi-captive Souss Valley tortoises from central Jbilet Mts, courtship and mating activities in occurred in early spring, from March to early May, and again in mid-Autumn, for a shorter period from late October to early November [10].

Serreau et al. [25] reported low plasma testosterone levels that were associated with mating periods in spring and to a lesser extent in autumn whereas two peaks, one in winter and one in summer, corresponded to periods of inactivity. Strong contrasts in plasma hormone levels induced by experimental treatments did not result in changes in activity budget or space use, both in the short-term or more than one month after the beginning of the hormonal treatment. It was concluded that testosterone levels did not affect directly behavioral activity in *T. g. soussensis*, either immediately or after a one-month time delay.

The egg-laying season occurs in May and June in the Souss Valley [5] and from late May to early July in central Jbilet Mts [10]. Nesting sites in the Souss Valley are usually located in sandy banks on the periphery of cultivated fields and at the base of *O. f. indica* hedging [5].

Mean clutch size in *T. g. soussensis* varies from 3.44 to 3.80 eggs with a range of 1–7 within a clutch [5, 10]. As indicated by their shape index, eggs in *T. graeca soussensis* from central Jbilet are rather elliptical-oval in shape. However, sub-spherical eggs with a shape index close to 1 were found in Admine Forest, Souss Valley, Morocco [5] and North Essaouira, west-central

Morocco (unpublished data) (**Table 2**). The elongated eggs in *T. g. soussensis* from Jbilet may be attributed to their smaller body size and more flattened carapace compared to those from other populations of west-central Morocco [6]. The mean relative egg mass and relative clutch mass are 1.70 ± 0.28% and 4.06 ± 1.32%, respectively. No egg variable was significantly correlated with the female body size (carapace length and body weight) [10].

Average incubation duration at 31°C in *T. g. soussensis* in Jbilet, was 70 days ranging from 62 to 78 days [10]. Krüger [24] found comparable values of 72 and 81 days at 28°C (6 h) to 33°C (18 h) in two artificially incubated clutches from semi-captive females from Southwestern Morocco. On the other hand, in a successful near-natural incubation experiment in Germany, at temperatures varying from 15 to 41°C, the incubation duration was much longer, ranging from 82 to 140 days [24].

Emergence occurs in September. Mean hatchling body mass and carapace length in central Jbilet were 12.8 g and 35.7 mm, respectively (**Figure 7**). In semi-captive *T. g. soussensis*, Krüger [24] reported a lower mean hatching body mass of 11.7 g. The carapace in hatchlings is subcircular and more domed compared to that of adults with a more elongated and flattened carapace. The more domed shell of the hatchlings would reduce surface area relative to volume, probably to compensate for high evaporative water loss from the leathery soft shell compared to the hardened thick carapace of adults [10].

5.7. Population structure and dynamics

In the early 1990s, the Admine population showed a highly unbalanced sex-ratio of 2.25 (69.2% of males) and only 27.6% of immature individuals. A predominance of young males was encountered with 66% of the males or 41.4% of the total population. These males were aged of 11–25 years compared with 75% of the females or 20.7% of the total population [5].

In spring-summer 2001, Znari et al. [13] reported significant differences in size-structure, but not age-structure among three populations (the central Jbilet Mts, Admine forest and Near Essaouira) of west-central Morocco (**Figure 8**). No tortoises older than 16–18 years were found at any site. Most populations of tortoises suffered over-collecting for the tourist trade

Origin	Clutch number	Clutch size	Egg mass (g)	Egg length (mm)	Egg width (mm)	Shape index	Reference
Admin forest	-	(5-7)	-	30	27.5	1.09	Bayley & Highfield 1996
Southern Essaouira	10	3.8 (1-5)	-	-	-	-	Barje et al. (2005)
Southwest Morocco	2	(5-6)	-	-	-	-	Krüger (2007)
Northern Essaouira	7	3.6 (1-5)	17.28 (14.30 - 18.80)	32.06 (29.70- 35.40)	28.95 (27.80-30.20)	1.11	Unpublished data
Jbilet Mts, Morocco	6	3.44 (1-5)	19.34 (16.8-23.6)	36.24 (34.3-41.8)	29.99 (28-31.5)	1.21	Hichami et al. (2016)

Table 2. Clutch size and egg characteristics of *Testudo graeca soussensis* from different areas of west-central Morocco. Values are means with their standard deviations and/or ranges (between brackets) when available (modified after Hichami et al. [10]).

Figure 7. An unhatched egg and a newly-hatched Souss valley tortoise (*Testudo graeca soussensis*) obtained under artificial incubation at 31°C (photo M. Znari).

in the past, and some collecting still continues, which probably explains the lack of older tortoises. Juveniles (age < 7 year) represented 21 and 23% of the population for Jbilet and Essaouira, respectively, but only 14% for Admine. Young adults (7–12 year) were less frequently encountered in Essaouira (47%) than in Jbilet (61%) and Admine (68%). Conversely adults older than 12 year were more frequently encountered in Essaouira (28%) than in Jbilet and Admine (18% each). Sex-ratios were not significantly different from 1 (or 1:1) for both Jbilet and Essaouira populations, whereas for Admine, the sex-ratio was male biased (1.69 or 1:0.59) [13].

In the central Jbilet Mts, the apparent age-structure of tortoises captured during the activity season (mid-February to late May 2003) was characterized by the lack of juveniles aged from 2 to 5 years old. Most of the individuals (74%) were larger than 100 mm in carapace length and 72% more than 7 years old [14]. The age distribution was not significantly different between males and females [22]. In spring 2012, the apparent frequency distribution of the captured tortoises was characterized by the scarcity of juveniles aged 1–5 years, which accounted only for 5% of the total population. Most individuals observed (95%) were adults with a carapace length > 100 mm. The age-class structure among male and female subpopulations was significantly different. The adult sex ratio was male-biased, estimated to 1.2 (55% of males) [22] and 1.57 (61% of males), respectively in spring 2003 [7] and 2012 [22]. Females were significantly distributed in larger classes, the largest adult female and male reached the respective sizes of 190 and 148 mm.

The estimated population size in spring 2003 was 192 ± 20 corresponding to a 5.9 ind./ha population density [7]. Male and female subpopulation size estimates were respectively 121 ± 19 and 100 ± 18. In spring 2012, the estimated population size declined to an average of 88 ± 4 ind. leading to a mean population density of 2.7 ind./ha [22]. This indicates a considerable decline of 54% in less than 10 years. Mean annual survival rates for adult male and female tortoises, estimated during the period of 2008–2012, were respectively 0.86 ± 0.12 (confidence interval range: 0.743–0.978) and 0.81 ± 0.18 (confidence interval range: 0.634–0.987) [22].

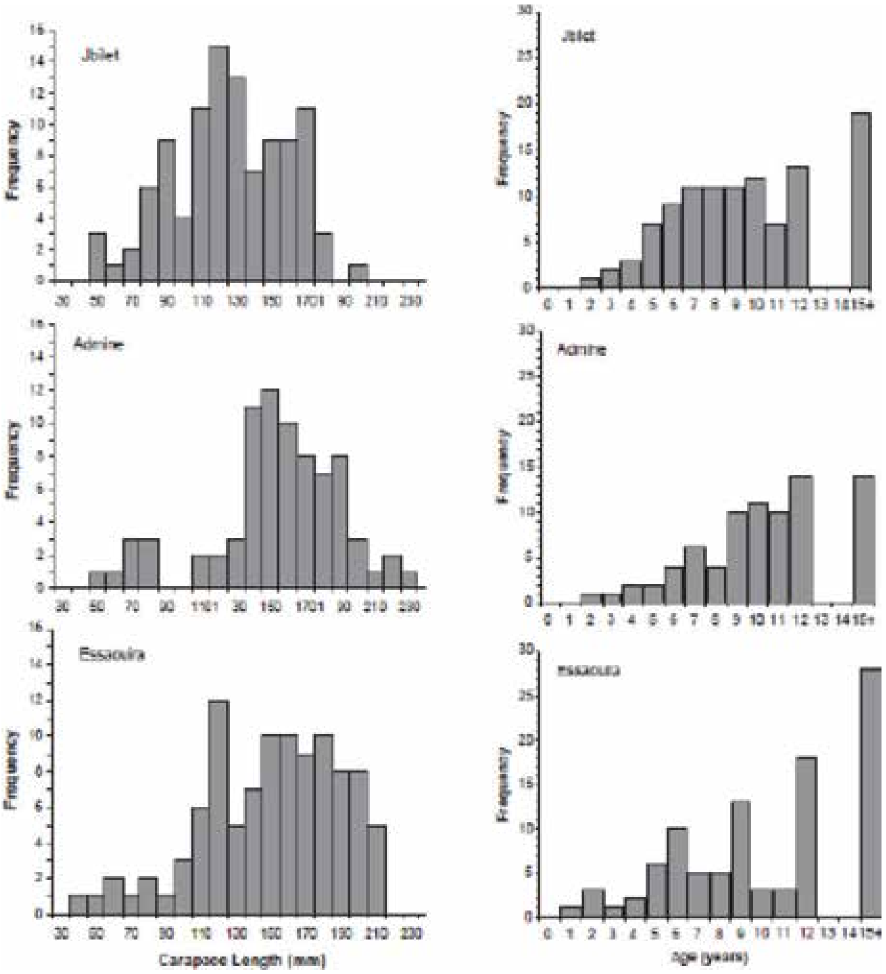


Figure 8. Population structure based on carapace length (left) and age (right) of *Testudo graeca soussensis* from the Jbilet Mountains (top), Admine Forest (middle), and near Essaouira (bottom), Morocco.

5.8. Predation and parasites

5.8.1. Predation

Predation, while almost exclusively a threat to eggs, hatchlings, and small juvenile tortoises, continues to be an ever-present threat. Nevertheless, in hatchlings, and to a lesser extent, in juveniles the shell is soft and/or not solid enough to resist to many predators such as canids, or birds for example (e.g., [26, 27]). In an open landscape, the predation on these fragile tortoises can be severe; sometimes leaving strong marks on the population dynamics [27]. In the Admine forest (Souss Valley), common predators of hatchlings observed in this region include hedgehogs, rats and birds of prey [5]. In an Argan (*Argania spinosa*) Forest, south of Essaouira, hatchlings of *T. g. soussensis* were found impaled by the Grey Shrike *Lanius* *meridionalis*, on spiny shrubs in both Essaouira region (31°23 N, 9°42 W) and the central Jbilet, near Marrakech [28]. All the impaled tortoises were located on scattered shrubs around the cultivated zones that possess abundant food resources and a relatively deep and loose soil; they may be attractive for females before laying. The mean carapace length in Essaouira was 28 ± 3 (range: 25–35 mm). Such a cryptic behavior may well be extremely pronounced in the soft hatchlings. Perhaps that the shrikes prospect areas where the concentration of juveniles is potentially high, typically around laying sites after emergence. The unlucky victims had not been discovered right in an Argan forest, though, but rather on solitary trees growing in areas used for agriculture.

5.8.2. Parasitism

Most of tortoises in central Jbilet Mts are infested, at various degrees, with ticks (with up to 10 ticks on the same tortoise) [21]. The infesting ticks, mostly *Hyalomma aegyptium*, the Family Ixodidae, were found to carry various microorganisms [29]. According to these authors, and in addition to various specific infections, the ticks can act as mechanical or biological vectors of different bacterial pathogens in both tortoises and humans. The most important bacteria, are *Salmonella* species bacteria that were found in almost 90% of tortoises from central Jbilet Mts [30]. In terrestrial chelonians, *Salmonella* species are considered part of the intestinal flora, and do not cause any clinical signs. However, in immune-compromised animals, they may cause salmonellosis, an important reptilian zoonosis [30].

6. Threats and conservation status

As the other Mediterranean populations, North African populations of *T. graeca* are considered as threatened, even though the true conservation status is not well known. In fact, these populations are declining in the majority of their habitats, particularly in Morocco and northwestern Algeria [31]. Since the 1980s, Lambert [3] had stated that only a few Moroccan populations of *T. graeca* can be considered abundant and free from human disturbance. Population decline is partly due to extensive collection for the pet and tortoise-derived souvenir trade to tourists [5], and also due to habitat losses resulting from human population growth and climate change which have promoted desertification, urbanization and the intensification of agriculture [5]. Because of this, *T. g. soussensis* is officially considered to be globally threatened, and is listed in Annex II of the Washington Convention and considered 'vulnerable' by the CITES. Surprisingly, in a recent national Red List assessment of Morocco's herpetofauna, Pleguezuelos et al. [32] considered *T. graeca* rather as a non-threatened species and is categorized Least Concern (LC). However, *T. graeca*, like all reptiles native to Morocco, is protected by law since 1978, banning domestic and international trade. Despite these designations, large numbers of Moorish tortoises are still being removed from the wild in Morocco every year [33], mainly for European pet shops. Besides international traffic, *T. graeca* in Morocco, is threatened mostly by collecting for household pets and 'medicinal' use, for manufacture of tourist souvenirs.

Znari et al. [13] reported on having counted not less than 692 *T. graeca* on offer in the souks (traditional markets) of Marrakech only in the period of July–August 2001. According to a more a recent survey conducted in 2013–2014 [33], 40% of shops selling tortoises throughout Morocco are found in Agadir (9%) and Marrakech (31%). Given that tortoises were reported to have been sourced near the cities in which they are sold; the subspecies most likely to be present in Marrakesh and Agadir is *T. g. soussensis*. The shops in these two cities sell around 16% (520/3270) of the total number of tortoises offered in 12 cities in Morocco; with an average number of 47–95 individuals per shop, respectively in Agadir and Marrakech; the global average being 82 tortoises. The annual turnover range is 195–782 tortoises with a monetary value of USD 1800–7200. Of about 900 tortoises measured, two-thirds measured <100 mm (<2–8 years of age) and <3% could comprise first-year individuals. Znari et al. [13] reported a similar percentage of 65.5% for <100 mm small-sized tortoises from the tourist-trade shops in Marrakech while the corresponding percentages from the natural populations ranged from only 8 to 23% in west-central Morocco (Marrakech, Essaouira and Agadir areas) [6, 13].

Tortoises are long-lived animals with high adult survival rates, late sexual maturity, low hatching and juvenile survival rates and a specialized diet. Because of this, increases in adult mortality due to overexploitation of adult specimens in a tortoise population will have long-term impacts on the population viability, making them very sensitive to the pet trade ([7, 18, 22, 33]. Bayley and Highfield [5] indicated that the population of tortoises observed in the lower Souss Valley near Agadir was not sustainable; the age sex structure showed a marked predominance of males and a notably small percentage of juveniles. This could be the same case for the population of the central Jbilet [22] and maybe other populations in west-central Morocco. The lower Souss Valley is not currently subject to collecting, although it was a collecting area in the past [4]. However, the current population structure has also been adversely affected by the severe environmental degradation, this area has suffered as a result of the collection of wood, overgrazing and cultivation resulting in deforestation and soil erosion both of which result in a serious reduction in availability of plant fodder which forms the natural diet of *T. graeca*. The acute loss of the endemic Argan tree in this region is a major factor in the loss of wild fodder plants as the forest canopy provided shade and permitted seedlings to survive [5].

In other parts of Morocco, like the Jbilet Mts, the jujube, *Z. lotus*, suffers from officially promoted programs of removal. *Testudo g. soussensis* is threatened by such habitat loss, as tortoises must navigate between microhabitats to find essential feeding resources and thermal refuges. They depend on the occurrence of large and relatively abundant bushes to escape lethal overheating [8] and to find food resources [7].

7. Conservation and restoration potentials

An analysis of the conservation and restoration potentials of *T. g. soussensis* and its degraded habitat in arid areas of west central Morocco, revealed great opportunities of measures and actions to be carried out in favor of the safeguard of the declining populations of the Souss

Valley tortoise [22]. Thus, an experimental captive breeding essay including a survey of reproduction, natural and artificial incubations of eggs and rearing of young captive-born tortoises, were carried out. Newly hatched tortoises, collected in the natural habitat, have been also hand-reared in the framework of a Head Starting operation, and were released in the wild at an age of 3–5 years for the reinforcement of remnant populations where the tortoises disappeared. Furthermore, actions of habitat restoration relying on replanting jujube bushes are proposed and consist, according to the level of environment deterioration, of an improved resource management or the reintroduction of the Jujube shrub and tortoises, which depend on. Finally, creating a tortoise sanctuary in the central Jbilet, reinforcing and connecting populations through replanting, are proposed. Protection of jujube bushes could considerably improve local habitats and biodiversity in arid steppe-lands of west-central Morocco (see also [8]).

8. Conclusion

The Souss Valley tortoise, *T. g. soussensis*, a morphologically-based subspecies, is an endemic to west-central Morocco. Its taxonomic status was confirmed by recent mitochondrial DNA data. Geographically-distant populations of this subspecies are morphologically, but not genetically differentiated. These tortoises occur in semi-arid to arid low quality habitats and their distribution, which is sensitive to precipitations, would have been shifted northwards since the Last Glacial Maxima. They are strictly herbivorous relying mostly on few fodder plants. They spend most of their time under bushes (especially larger ones) to avoid overheating and for more available fodder plants. Growth rate is relatively high in juveniles and then slows down in both sexes after sexual maturity. Adult females are larger than males with a relatively delayed sexual maturity. As in other subspecies, mating occurs in spring and early autumn and fecundity is relatively low as indicated by the small average clutch size. Population structure is characterized by very low proportions of juveniles and old adults along with a male-biased sex ratio. Most of populations are declining mainly due to over-collecting for pet trade as well as other threats related to human disturbances. Due to their soft carapace, hatchlings are particularly exposed to a high predation pressure. Most of tortoises are infested by ixodid ticks and *Salmonella*. Actions required for improving the conservation status for this threatened tortoise in arid areas of west-central Morocco are proposed, including habitat restoration and captive breeding for population reinforcement.

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Conflict of interest

There is no conflict of interest between the two authors about the content of the present chapter.

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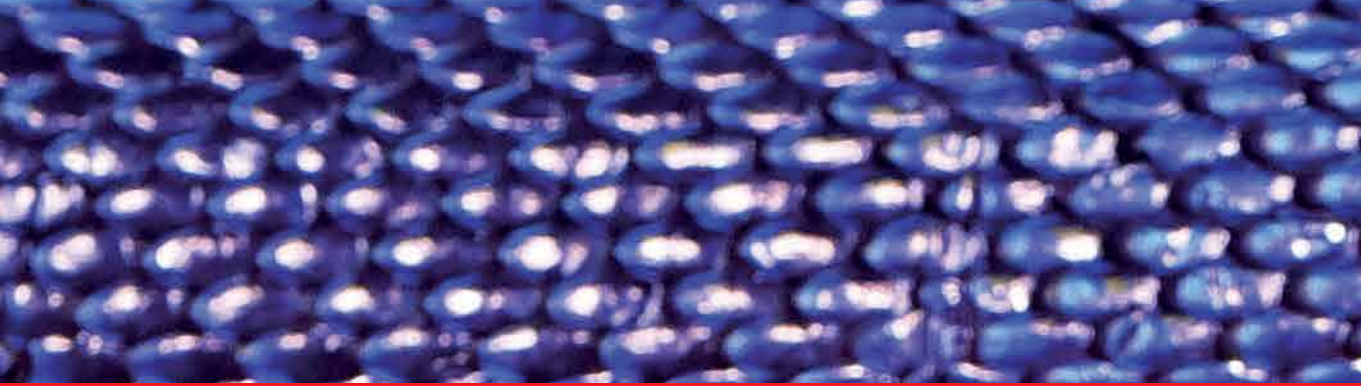

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The book *Reptiles and Amphibians* is a compilation of the current trends in herpetology, focusing on evolution, physiology, monitoring, bioacoustics, threats, and conservation biology. All the chapters present an interesting aspect of the biology of reptiles and amphibians, encompassing different groups of these animals such as frogs, toads, newts, chelonians and snakes from various parts of the world. Without a doubt, this book will help to keep updated on the current problems that arise in this interesting biological group.

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