Parasite diversity within native and invasive terrapins: Implications for conservation

By

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THESIS

submitted in fulfillment of the requirements for the degree
Doctor of Philosophy
(Ph.D.)

in

ZOOLOGY

at the Potchefstroom Campus of the North-West University
and the University of Perpignan

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September 2014

“A grey head is a crown of glory, It is found in the way of righteousness.” – Proverbs 16:31

This thesis is dedicated to my grandmother, Maria Elizabeth Jacoba Meyer, who passed away during my studies while I was in France in June 2012. I was unable to attend her funeral and till today it is an unreal feeling when I realise that I cannot go and visit her and share a few laughs over a cup of rooibos tea. I truly miss her and know we will be reunited again. Until that day, every cup of rooibos tea I enjoy, I think of my ouma Moeder.

16 July 1916 – 12 June 2012

Acknowledgements

This project required the help of numerous people, institutions and game reserves. I am indebted to the following:

Firstly, to the God almighty who gave me this wonderful opportunity to work in the Nature that He has created. For guiding me through my studies and giving me the strength, knowledge and patience to succeed through all the tough and happy times.

To my supervisors, Prof. Louis du Preez and Prof. Olivier Verneau, who guided me with their knowledge and experience. Thank you for all the field trips we have done together and time spent in the field. It will always be remembered. Thank you for having patience to answer all my questions and to be strict when it was needed. Both have trusted me to be able to get this work done and helped me to strive for greatness. Thank you for not only being my supervisors, but also father figures and very good friends. I grew a lot as a researcher under your guidance and learnt a lot. A special thanks to Olivier for opening his home to me when I visited France and for really making a huge effort in making me feel welcome. Thank you for all the trips across France so that I could have experienced another country to great extent. This was truly an amazing experience. To Nacera Kaid from the University of Perpignan. Thank you for helping me with the registration and administrative duties at the University. It is much appreciated and also thank you for always having a smile and just brightening up the day and for all the dinner invites during my stay in France, they were all amazing.

I am indebted to Gael Simon and Laurent Heritier who helped me immensely in the field to collect samples over the three year study in France. You made the field trips a lot of fun. Thank you for becoming some of my best friends in France. You made my long visits a lot of fun, making the longing for home easier. Your friendship will always be cherished. A special thank you to Olivier, Laurent and Elodie Bonneau, for helping me with my molecular work in France. Thank you to Carmen Palacios, Elisabeth Faliex, Elsa Amilhat, Anne-leila Meistertzheim, who gave advice with any problems I had with my molecular work in France, it is much appreciated. Thank you to Olivier and Anne-leila for translating my abstract to French. I would also like to thank Jerome Boissier and Yves Desdevises for comments on my yearly reports. The comments you gave really helped me stay on track with my studies.

Thank you to the AACRG group for their assistance during my studies, especially to the secretaries, Leana Mostert and Leoné Hudson for booking rental cars for various trips as well as countless other administrative necessities. A special thanks to Donnavan Kruger, Ed

Netherlands and Courtney Cook. You guys have helped me immensely during my studies. Donnavan and Ed, you guys are some of the best people to do fieldwork with. Thank you for all the unforgettable trips and all the help you gave me throughout my studies. Ed and Courtney, thank you for helping me with some of my chapters, giving advice and helping me to get the message across. A special thank you is needed for Ed who spent a lot of time helping me with my molecular work in South Africa. It is much appreciated. I hope we can work together on more projects in the near future. Thank you to the molecular lab at the North West University, with special thanks to Ina van Niekerk, Karen Jordaan, Hermoine Venter, Bianca Peterson and Abraham Mahlatsi for always willing to give a helping hand.

For help on various fieldtrips I am indebted to James Harvey, Mathieu Badets, Vazi Latez, Marine Pezin and Maxine Theunissen. You guys always bring a smile to the trip and good work ethic.

For help with data analysis and GIS maps, I would like to thank Prof. Victor Wepener, Prof. Nico Smith, Donnavan Kruger, Gordon O'Brien and Mari du Toit.

As this project was conducted across South Africa, I am grateful to the following people for allowing me to work on their properties, reserves, parks and zoos: Mike Adams and Chris de Beer from the National Zoological Gardens in Pretoria, for Prof. Antoinette Kotze for always organizing accommodation for me on site; the late Ian Visser and Bret Garner at Johannesburg zoo; Donald Strydom and his team at Khamai Reptile Park, Ben Von Weillich at Blyde Wildlife estate, Mike Cowden at Zandspruit Estate and Patrick at Tshukudu Game Lodge, all in Hoedspruit; to Carl Westpal at Mitchell Park, John Dives at Bluff Nature Reserve, Wayne Matthews and Leonard at Tembe Elephant Park, Richard Penn-Sawers at Ndumu Game Reserve, all in KwaZulu Natal; Jack Seale at Hartebeespoort Reptile Park, Jenny at Sable Ranch in Brits and Clive McDowell who is a private owner of exotic terrapins in Cape Town, Hendrik Louw at World of Birds and Neil at Cango Crocodile Park, all in the Western Cape.

To my lovely wife Lourinda, family and friends who have encouraged me throughout this whole experience. Who always had an inspirational message for when I was tired or down. I will be forever grateful to Lourinda for all her love and support over the last couple of years, especially in times where I was in the field or in France for long periods of time. You stood strong and pursued this dream alongside me and I love you a lot. A special thanks to my inlaws for all the effort they put in to come and stay with Lourinda on the farm during my fieldtrips. I am truly grateful.

The NRF and CNRS has provided the majority of funding for this project. Thanks go to them and also to the NWU for funding towards student bursaries.

Research permits were provided by Ezemvelo KZN Wildlife (Permit No: OP 631/2011); Eastern Cape Department of Economic Development and Environmental Affairs (Permit nos: CRO 11/11CR and CRO 12/10CR); Free State Department of Economic Development, tourism and Environmental Affairs (Permit no: 01/8421); Mpumalanga Tourism and Parks Agency (Permit no: MPB. 5296); North West Department of Economic Development, Environment, Conservation and Tourism (Permit no: 028 NW-11). Each are thanked for allowing access to a number of their reserves. Ethical clearance was gained from the North West University to conduct this study on terrapins: Ethical clearance number: NWU – 00050 – 11 – A4.

I declare that this thesis is my own work unless specifically acknowledged in text. It has not been submitted before for any degree or examination at any other university.

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12 September 2014

Version française abrégée

Les tortues d'eau douce constituent aujourd'hui l'un des groupes de vertébrés les plus menacés au monde, avec plus de la moitié des 300 espèces en danger de disparition. Bien qu'une attention particulière ait été portée sur la conservation de nombreux groupes animaux, tels que les requins, les raies, les amphibiens, les oiseaux et les mammifères, les tortues d'eau semblent malheureusement avoir été en partie ignorées. Sur les 317 espèces actuellement connues, environ 10% sont considérés comme en danger critique sur la Liste rouge des espèces menacées de l'UICN et environ 42% des tortues d'eau douce sont considérés comme menacées. L'émyde lépreuse, *Mauremys leprosa* (Geomydidae), est l'une de ces espèces. Sa distribution s'étend du nord de l'Afrique (Tunisie, Algérie et Maroc) à l'Europe (Portugal, Espagne et sud de la France). Si le statut de cette dernière est considéré comme "moins préoccupant" en Afrique du Nord selon les critères de l'UICN, elle est classée comme «vulnérable» sur la liste rouge européenne des Reptiles et considérée comme «en danger» en France où elle n'est présente que dans la région Languedoc-Roussillon, plus précisément dans le département des Pyrénées Orientales. En Afrique du Sud, on compte cinq espèces de tortues d'eau douce toutes rattachées à la famille des Pelomedusidae, à savoir une espèce rattachée au genre *Pelomedusa*, en fait *Pelomedusa subrufa*, et quatre espèces rattachées au genre *Pelusios*, *Pelusios castanoides*, *P. rhodesianus*, *P. sinuatus* et *P. subniger*. Si toutes ces espèces ne semblent pas aujourd'hui menacées, il n'en demeure pas moins qu'une veille attentive demeure nécessaire.

L'une des menaces qui pèse actuellement sur l'ensemble des tortues d'eau douce au monde, plus particulièrement en Europe et en Asie, repose sur la présence de la trachémyde à tempes rouges, *Trachemys scripta elegans*, espèce d'origine nord américaine. Cette espèce a été introduite dans la plupart des régions du globe dès les années 70 suite au commerce international sur les animaux de compagnie. Relâchée depuis massivement dans les zones humides, elle constitue une réelle menace pour les espèces indigènes. Plus compétitive que les espèces locales, elle risque de déplacer certaines populations autochtones, voire conduire à terme à l'extinction de nombreuses espèces. Une autre menace sous jacente à l'introduction de *T. s. elegans* résulte de l'introduction de ses propres parasites et pathogènes qu'elle est susceptible de véhiculer et qui, une fois transmis aux espèces locales, peuvent s'avérer encore plus néfastes que l'espèce hôte elle-même.

Le travail de cette thèse pour l'essentiel a donc consisté à évaluer la diversité spécifique de deux groupes de parasites, métazoaires (Plathelminthes, Monogenea) et protozoaires (Apicomplexa), au sein de l'émyde lépreuse dans le sud de la France et nord de l'Espagne et à travers les différentes espèces de Pelomedusidae en Afrique du Sud. Cette recherche a été également entreprise sur l'espèce exotique *T. s. elegans* dans la mesure où cette dernière est

très abondante dans les hydrosystèmes fluviaux d'Europe et a été rapportée, mais ce de manière plus sporadique, en Afrique du Sud. Les objectifs étaient donc d'évaluer les risques potentiels d'introduction de parasites suite à l'introduction de l'espèce invasive dans les milieux naturels.

Le chapitre 1 est une introduction générale sur les reptiles et les chéloniens ainsi que sur l'importance des chéloniens, plus particulièrement des tortues d'eau douce, dans les écosystèmes aquatiques. Les diverses menaces qui pèsent sur les tortues, à savoir la dégradation et la perte des habitats naturels, la pollution, la surexploitation commerciale, les espèces envahissantes, leurs parasites et les maladies émergentes sont abordées. Enfin les caractéristiques biologiques des différentes espèces hôtes et parasites examinées dans le cadre de cette thèse sont également discutées.

Le Chapitre 2 documente l'étendue des invasions biologiques d'espèces parasites Polystomatidae au sein de différentes populations d'émydes lépreuses (*M. leprosa*) de France et d'Espagne, suite à l'introduction de *T. s. elegans*. A partir du séquençage de la cytochrome c oxydase I (COI) des parasites de *M. leprosa* échantillonnés en milieu naturel, l'arbre de Minimum Evolution résultant de l'analyse des séquences ainsi que la comparaison des distances p des différents haplotypes illustrés révèlent des richesses parasitaires beaucoup plus élevées que celles attendues, ce qui suggère que des transferts d'hôtes se sont opérés. Dans cette étude, huit espèces différentes de polystomes parasites ont été observées chez *M. leprosa* : quatre espèces ont été signalées dans la vessie urinaire, trois autres l'ont été dans la cavité pharyngale et une dernière dans les sacs conjonctivaux. Si deux de ces espèces sont connues pour être des espèces parasites naturelles de cet hôte, les autres infestent pour la plupart des tortues américaines des genres *Chrysemys* et *Graptemys* dans leur milieu naturel, ce qui témoigne de l'existence de transferts de parasites des tortues américaines vers l'espèce indigène. Afin d'expliquer ces résultats, deux hypothèses non exclusives ont été suggérées. *T. s. elegans*, qui est aussi porteur de ces parasites, pourrait être le vecteur d'introduction d'espèces exotiques de polystomes dans les milieux naturels dans la mesure où les autres espèces de tortues américaines ne sont pas présentes dans les biotopes où cohabitent *M. leprosa* et *T. s. elegans*. Une seconde hypothèse serait d'envisager que des individus de l'espèce *M. leprosa* aient été relâchés dans les milieux naturels après qu'ils aient séjourné en captivité en présence des autres tortues américaines. Quelle qu'en soit l'explication, la présence d'espèces parasites exotiques chez *M. leprosa* pose de nombreuses questions quant à l'impact d'agents pathogènes infectieux pouvant être transmis par l'espèce invasive *T. s. elegans*.

Le Chapitre 3 relate une expérience menée sur *M. leprosa* afin d'évaluer si la production d'œufs par les polystomes sont sous le contrôle des paramètres environnementaux. La

température étant l'un des facteurs abiotiques clés affectant la dynamique parasitaire, nous avons mesuré la production quotidienne d'œufs de parasites pendant 26 jours chez cinq tortues isolées et mises dans des conditions telles où elles pouvaient passer du temps non seulement dans l'eau mais aussi au soleil. Cette expérience a démontré que la température extérieure influence le rythme de ponte de *Neopolystoma sp*., un des parasites de *M. leprosa*, avec un décalage de deux jours suite à un changement de température de l'environnement extérieur. Nous en concluons que le parasite serait adapté aux conditions physiologiques internes de son hôte, hôte ectothermique qui nécessite des bains de soleil réguliers pour réguler sa propre température interne afin d'assurer ses fonctions physiologiques. Le décalage entre la production des œufs et l'augmentation de la température pourrait être attribué à la sécrétion d'hormones chez l'hôte qui, une fois secrétées, agiraient en tant que stimulateurs de la reproduction du parasite.

Le Chapitre 4 traite par l'approche « Relative Risk Model (RRM) » de la viabilité de l'espèce indigène *M. leprosa* dans une petite rivière du sud de la France, la Fosseille. Cette approche prend en compte non seulement les effets de l'espèce à tempes rouges mais également les menaces externes anthropiques et les conditions environnementales. Les risques pour l'espèce ont été estimés pour quatre scénarios différents (S1 à S4) : (S1) conditions « naturelles », c'est-à-dire celles observées actuellement sur la rivière ; (S2) augmentation du nombre de tortues invasives *T. s. elegans* dans l'écosystème aquatique ; (S3) retrait complet des tortues invasives ; (S4) déversement important d'eaux usées à partir de la station d'épuration située sur un des sites. En utilisant le paradigme d'évaluation des risques écologiques ainsi que la méthodologie RRM, il a été possible d'estimer la viabilité des tortues sur cette rivière divisée pour l'étude en six portions connectées les unes aux autres. Les résultats montrent une augmentation des risques au cours d'apports d'eaux usées plus importants ou suite à une augmentation des effectifs de l'espèce invasive, et ce en fonction des portions, des habitats et du point d'embouchure de la rivière situé au niveau de l'étang de Canet – St Nazaire. Les résultats de cette étude utilisant cette méthodologie démontrent que divers facteurs pourraient avoir un effet sur la viabilité des tortues *M. leprosa* dans la rivière de la Fosseille. Ils montrent aussi comment ces risques, provenant de différentes sources, sont répartis le long du réseau hydrographique. Validée uniquement sur la Fosseille, cette méthodologie pourrait être adaptée à d'autres systèmes fluviatiles de plus grande taille, à d'autres régions mais également pourrait inclure d'autres sources biologiques ou d'autres groupes taxonomiques. Cette méthode pourrait être aussi utilisée pour gérer efficacement les espèces envahissantes et la manière dont les mesures de gestion doivent être réparties sur les zones d'étude. Enfin elle pourrait également souligner les données supplémentaires à acquérir afin de renforcer les scénarios d'évaluation des risques. Dans certaines parties du sud de l'Europe où *M. leprosa* est présente, des

mesures de conservation ont été mises en place, et ce plus particulièrement dans les zones humides, par le contrôle des méthodes de pêche, la gestion des zones aquifères, la restauration de l'habitat, le contrôle de la vente des tortues exotiques et la régulation des populations sauvages de tortues à tempes rouges. La méthodologie RRM employée pourrait être mise en œuvre dans le cadre de ces mesures de conservation afin de prévoir et d'en améliorer les résultats futurs.

Le Chapitre 5 discute de la diversité des hémogrégarines (Protozoa: Apicomplexa: Haemogregarinidae) de tortues d'eau douce (*Pelomedusa subrufa*, *Pelusios castanoides*, *P. rhodesianus*, *P. sinuatus* et *P. subniger*) en Afrique du Sud. Cette étude a été réalisée à partir d'une double approche, morphologique et moléculaire. Sur la base des caractéristiques morphologiques, les hémogrégarines parasitant les espèces de tortues *P. sinuatus, P. subniger* et *P. castenoides* peuvent être considérées comme conspécifiques. Si les stades de développement, tels que les trophozoïtes, prémérontes et mérontes sont morphologiquement similaires au sein des hémogrégarines de toutes les espèces de tortues étudiées des deux genres, les stades gamontes diffèrent cependant entre les espèces des deux genres, étant beaucoup plus petits chez *P. subrufa*. Les divergences génétiques (18S p-distances) estimées entre des isolats obtenus à partir de *P. sinuatus* et *P. subrufa* (cette étude) et *P. subniger* et *P. williamsi* (données publiées par d'autres auteurs) sont si faibles (1 seule mutation entre toutes les séquences), que cela suggère que toutes ces hémogrégarines sont conspécifiques, ce qui contredit les données morphologiques. Cependant les différences morphologiques observées entre les isolats de *Pelusios* spp. et *Pelomedusa* pourraient s'expliquer par du polymorphisme intraspécifique, dans la mesure où il a été montré par d'autres auteurs que certaines hémogrégarines présentent un certain degré de variabilité morphologique intraspécifique durant différentes phases de l'infection. Des recherches complémentaires sont donc nécessaires pour déterminer si les mutations observées entre les différents isolats des deux espèces investiguées, *P. subrufa* et *P. sinuatus*, sont fixées ou non afin de conclure sur la spécificité de ces parasites.

Le chapitre 6 discute de l'ensemble des résultats obtenus dans le cadre de cette thèse et des risques que pose l'introduction d'espèces exotiques pour les espèces de tortues indigènes. De nouvelles pistes de recherche sont également discutées afin de valider certains résultats obtenus mais également d'évaluer l'impact d'agents exotiques sur les espèces de tortues dans leur environnement naturel.

Thesis Summary

Terrapins are one of the most endangered vertebrate groups, with almost half of the more than 300 extant species threatened with extinction. This study was conducted to investigate parasite host-switching from the invasive American Red-eared slider, *Trachemys scripta elegans,* to the native Mediterranean pond terrapin, *Mauremys leprosa* in natural environments in southern France and Spain. The study also included a risk assessment approach that was developed to assess the viability of the native *M. leprosa* terrapins in a small river of southern France. The diversity of haemogregarine parasites within South African terrapins was also explored. The thesis is structured as follows:

CHAPTER 1 gives a broad introduction to reptiles and chelonians as well as the importance of chelonians in ecosystems with emphasis to the threats that are driving terrapins to decline. The various terrapin species examined during this study as well as the parasite groups of interest (Monogenea and Apicomplexa) are also discussed in detail.

CHAPTER 2 documents the extent of platyhelminth invasions from *T. s. elegans* to natural *M. leprosa* populations in northern Spain and southern France. From DNA barcoding analysis based on the sequencing of the Cytochrome c Oxidase I gene, the inferred Minimum Evolution tree and p-distance comparisons of closely related haplotypes revealed a greater polystome richness within *M. leprosa* than expected, suggesting that host switching may take place in natural environments. *T. s. elegans* would serve as a carrier for a variety of polystomes that usually infest American turtles in their home range. These are transmitted to *M. leprosa* throughout the south of France, also suggesting that turtle polystomes are not strictly host-specific.

CHAPTER 3 investigates polystome egg production under changing environmental conditions. The experimental procedure that was conducted on *M. leprosa* showed that environmental temperature has an effect on the egg laying rhythm of its parasite, i.e., *Neopolystoma* sp., with a two day lag of egg production in response to environmental temperature change. Results suggest the adaptability of the parasite to the physiology of their chelonian hosts which are ecthothermic animals. They also show that eggs production may be attributable to the release of host factors like hormones that once secreted may act and stimulate parasite reproduction.

CHAPTER 4 relates risk assessment for the viability of the native Mediterranean pond terrapin (*M. leprosa*) in a natural environment by using the Relative Risk Model (RRM) method, taking into consideration various threats and environmental conditions that may impact this species.

CHAPTER 5 examines the diversity of South African terrapin haemogregarines (Protozoa: Apicomplexa: Haemogregarinidae) as well as their phylogenetic placement among haemogregarines based on molecular and morphological evidences.

CHAPTER 6 summarizes all findings of the study and explores new ways of Research.

Résumé de thèse

Les tortues forment l'un des groupes de vertébrés les plus menacés, avec à peu près la moitié des 300 espèces existantes en danger d'extinction suite en partie aux invasions biologiques. Cette étude a été réalisée pour évaluer les transferts de parasites de la trachémyde à tempes rouges, *Trachemys scripta elegans* espèce de tortue d'eau douce américaine invasive, vers l'émyde lépreuse *Mauremys leprosa* dans les environnements naturels du sud de l'Europe. L'étude comprend également une approche d'évaluation des risques qui a été développée pour estimer la viabilité de l'espèce indigène *M. leprosa* dans une petite rivière du sud de la France. La diversité des hémogrégarines (parasites protozoaires) des tortues d'Afrique du Sud a été également étudiée. La thèse est structurée en six chapitres:

Le CHAPITRE 1 est une introduction générale sur les reptiles et les chéloniens et sur l'importance de ces derniers dans les écosystèmes. Elle met également l'accent sur les menaces qui sont susceptibles à terme de conduire au déclin des tortues. Les différentes espèces de tortues étudiées (Pelomedusidae) dans le cadre de cette thèse ainsi que les parasites d'intérêt (Monogenea et Apicomplexa) sont également discutés.

Le CHAPITRE 2 décrit l'étendue des invasions biologiques de plathelminthes parasites de *T. s. elegans* vers les populations naturelles de *M. leprosa* dans le sud de la France et le nord de l'Espagne. A partir d'analyses du type « DNA barcoding » basées sur le séquençage de la cytochrome c oxydase I d'échantillons parasites collectés sur l'émyde lépreuse, l'arbre d'évolution minimum (ME tree) ainsi que la comparaison des distances p calculées entre les haplotypes les plus proches dans l'arbre révèlent une richesse spécifique chez *M. leprosa* beaucoup plus grande que celle attendue, ce qui suggère que des transferts d'hôtes ont probablement eu lieu en milieu naturel et que ces parasites ne sont pas strictement hôtes spécifiques. Les conclusions de cette étude indiquent que *T. s. elegans* servirait de vecteur pour une variété de polystomes d'origine américaine, qui seraient transmis à l'espèce indigène.

Le CHAPITRE 3 décrit une étude expérimentale réalisée sur *M. leprosa* dont les objectifs étaient de voir s'il existait une relation entre la production d'œufs de parasites et les conditions environnementales. Les résultats montrent que la température extérieure a un effet sur le rythme de ponte du parasite, c'est-à-dire *Neopolystoma* sp., et que la production d'œufs varie avec un décalage de deux jours en réponse à des changements de température. Ces résultats suggèrent une capacité d'adaptation du parasite à la physiologie de son hôte chélonien, qui est un animal ectotherme, mais aussi que la production d'oeufs pourrait être liée à la libération d'hormones sécrétées par l'hôte, qui une fois libérées stimuleraient la reproduction du parasite.

Le CHAPITRE 4 rapporte une étude sur l'évaluation des risques quant à la survie de l'émyde lépreuse dans son environnement naturel. Cette étude a été réalisée en s'appuyant sur l'approche RRM « Relative Risk Model », qui prend en compte diverses menaces pouvant influer sur la survie de espèce.

Le CHAPITRE 5 décrit la diversité des protozoaires hémogrégarines (Apicomplexa, Haemogregarinidae) de tortues sud africaines (*Pelomedusa subrufa*, *Pelusios castanoides*, *P. rhodesianus*, *P. sinuatus* et *P. subniger*) ainsi que leur position phylogénétique en s'appuyant sur des évidences morphologiques et moléculaires.

Le CHAPITRE 6 est une conclusion générale qui résume les résultats majeurs de cette thèse et qui explore de nouvelles pistes de recherche.

Verhandeling Opsomming

Varswaterskilpaaie is een van die mees bedreigde vertebraat groepe, met byna die helfte van die meer as 300 bestaande spesies bedreig. Hierdie studie is uitgevoer om parasiet-gasheer oordrag vanaf die indringer Amerikaanse Rooi-oor waterskilpad, *Trachemys scripta elegans*, na die inheemse Mediterreense damskilpad, *Mauremys leprosa* in natuurlike omgewings in die suide van Frankryk en Spanje te ondersoek. 'n Risiko-assessering analise om die lewensvatbaarheid van die inheemse *M. leprosa* varswaterskilpaaie in 'n klein riviersisteem in die suide van Frankryk te evalueer is ontwikkel. Die diversiteit van haemogregarien bloedparasiete in Suid-Afrikaanse varswaterskilpaaie is bestudeer. Die verhandeling word soos volg gestruktureer:

HOOFSTUK 1 gee 'n breë inleiding tot reptiele en skilpaaie en lê klem op die belangrikheid van skilpaaie in ekosisteme en die bedreigings verantwoordelik vir die afname in varswaterskilpadgetalle. Die verskillende skilpadspesies wat ondersoek word tydens hierdie studie asook die parasietgroepe van belang (Monogenea en Apicomplexa) word ook breedvoerig bespreek.

HOOFSTUK 2 dokumenteer die omvang van platwurm infesterings vanaf *T. s. elegans* na inheemse *M. leprosa* bevolkings in die noorde van Spanje en die suide van Frankryk. Vanaf DNA gekodeerde analises, gebaseer op nukleotiedvolgordes van die Sitokroom C Oksidasie I geen, het die verwysde Minimum Evolusie boom en p-afstande vergelykings vanaf nou verwante haplotipes 'n groter polistoom diversiteit binne *M. leprosa* as wat verwag is getoon. Dit dui daarop dat parasiet-gasheer wisseling in natuurlike omgewings kan plaasvind. *Trachemys s. elegans* dien as 'n draer vir 'n verskeidenheid van polistoom parasiete afkomstig vanaf Amerikaanse skilpaaie. Parasiete word ook regdeur die suide van Frankryk aan *M. leprosa* oorgedra. Dit dui daarop dat skilpad polistome waarskeinlik nie streng gasheerspesifiek is nie.

HOOFSTUK 3 ondersoek polistoom eierproduksie onder veranderende klimaatstoestande. Die eksperimentele prosedure met *M. leprosa* as proefdier het getoon dat omgewingstemperatuur 'n invloed het op die eierleggingritme van die polistoom parasiet *Neopolystoma* sp. Twee dae na die temperatuur gestyg het is ‘n toename in eirproduksie waargeneem en twee dae na ‘n temperatuurdaling is ‘n daling in eierproduksie waargeneem.Resultate dui daarop dat die parasiet aanpas by die fisiologiese veranderinge van hul ektotermiese gashere. Dit wys ook dat eierproduksie toegeskryf kan word aan die vrylating van hormone wat deur die gasheer afgeskei word en dan sodoende die parasiet kan stimuleer om voort te plant.

HOOFSTUK 4 verwys na ’n risiko-assessering raamwerk vir die lewensvatbaarheid van die inheemse Mediterreense damskilpad (*M. leprosa*) in 'n natuurlike omgewing met behulp van die relatiewe risiko model (RRM). Verskillende bedreigings en omgewings-toestande wat hierdie spesies beïnvloed is in ag geneem.

HOOFSTUK 5 ondersoek die diversiteit van die Suid-Afrikaanse varswaterskilpad haemogregariene (Protozoa: Apicomplexa: Haemogregarinidae) sowel as hul filogenetiese plasing gebaseer op molekulêre en morfologiese eienskappe.

HOOFSTUK 6 gee ’n opsomming van al die bevindinge van die studie en maak voorstelle vir verdere navorsing.

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Chapter 2 Results – Parasite host-switching from the invasive American Red-eared slider, *Trachemys scripta elegans,* to the native Mediterranean pond terrapin, *Mauremys leprosa* in natural environments

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Keywords

Chelonia, Terrapin, Invasive, Risk, Host-switching, Monogenea, Polystomoides, Neopolystoma, Apicomplexa, Haemogregarins

CHAPTER 1

General Introduction and Literature Review

"Behold the turtle. He makes progress only when he sticks his neck out."

- James Bryant Conant

1.1 Introduction to the study animal

1.1.1 Reptilia

Reptiles evolved from early amphibians during the Paleozoic Era some 250 million years ago (Hedges and Poling, 1999; Reisz *et al.,* 2011; van Tuinen and Hadly, 2004). The oldest reptile fossil discovered to date is that of *Hylonomus lyelli* (Dawson, 1863), dating back to a period at least 50 million years before the first dinosaurs appeared. Reptiles evolved into various forms including dinosaurs that dominated the earth for 150 million years. Reptiles are remnants from the past, and over the years new species have evolved and taken the place of the extinct dinosaurs (Branch, 1998; Burton, 1975).

During the Triassic and Jurassic periods, high rates of cladogenesis generated various groups of animals adapted to almost every terrestrial, freshwater and marine habitat throughout global temperate, tropical and desert environments (Vidal and Hedges, 2009). The horny dry skin that is covered in scales or scutes is an adaptation that allowed reptiles to make the transition from water to land. They are amniotes like mammals and birds, and thus have a foetal membrane surrounding the developing embryo inside the egg; however, egg development usually takes place without any parental care. Reptilian eggs can tolerate a larger range of environmental conditions than amphibian eggs during development which allows them to be less dependant on water. This feature allows reptiles to adapt to more arid environments and inhabit a variety of habitats across the world (Alexander and Marais, 2007; Branch, 1998; Jacobsen, 2005). Whereas birds and mammals generate heat through internal metabolism of 90% of their food into heat to maintain muscle and biochemical functions, reptiles are ectothermic animals that need to bask in the sun to gather heat energy. This enables birds and mammals to be active during times when reptiles cannot, but this requires a continuous intake of food which reptiles do not require as they become temporarily dormant and save energy in this manner (Branch, 1998).

According to cladistic systematics proposed by Willi Hennig in the 1950s, monophyletic branches, or better known as clades, can be determined on the basis of shared derived features alone (Hennig, 1999). Reptiles are known to be derived from sauropsidians by key features such as a ventral keel, the hypophysis, on the cervical vertebrae (Lecointre and Guyader, 2001). Sauropsidians evolved into two clades, anapsids and diapsids (Fig. 1.1). In the past, the absence of temporal openings in the skull has been used to place chelonians under anapsids, but virtually all molecular data up to date have grouped them with birds and crocodilians under diapsids together with squamates and sphenodonts (Nagashima and Savin, 2009; Hedges, 2012). Reptiles consist of four orders: (1) Rhynchocephalia Williston, 1925 (represented by the tuatara); (2) Squamata Oppel, 1811 (represented by snakes and lizards); (3) Crocodylia Owen, 1842 (represented by crocodiles, alligators and gavials) and (4) Chelonia Linnaeus, 1758 (represented by tortoises, terrapins and marine turtles) (Branch, 1998; Boycott and Bourquin, 2000).

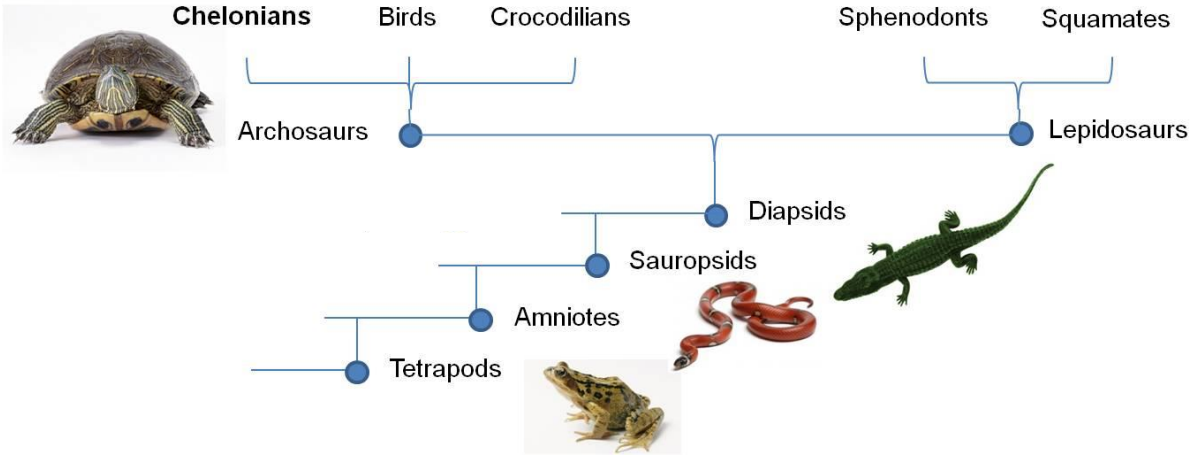

Figure 1.1: Cladogram showing the lineages of the various Sauropsids that evolved into two clades, turtles, birds and crocodilians on one side, sphenodonts and squamates on the other. Adapted from Bonin *et al.,* (2006).

These scaly animals play important ecological roles, as predators, prey, grazers, seed dispersers and commensal species; additionally, they also serve as bio-indicators for environmental health. Because they occur in fairly specific environments they provide excellent study models of the biological and evolutionary processes underlying speciation (Raxworthy *et al.*, 2008; Read, 1998). The distribution of reptiles is generally smaller than that of birds and mammals (Anderson, 1984; Anderson and Marcus, 1993), making them more susceptible to threats. The vulnerability makes reptiles a group of conservation concern. Regional assessments in Europe (Cox and Temple, 2009) and southern Africa (Bates *et al.*, in press.) indicate that one-fifth and one-tenth of European and African reptilian species, respectively, are

threatened with extinction. Threats that are at the origin of these declines and extinctions include habitat degradation and loss, diseases, climate changes, pollution, as well as unsustainable trade and invasive species (Cox and Temple, 2009; Gibbons *et al.*, 2000; Todd *et al.*, 2010).

1.1.2 Chelonians

Chelonians have existed on earth since the late Triassic period when dinosaurs roamed the earth. The first fossil with clear terrapin similarities is *Odontochelys semitestacea* (Li *et al.,* 2008) of China dated to about 220 million years ago (Li *et al.,* 2008; Reisz and Head, 2008). Today they consist of as many as 460 taxa (species and subspecies) that are found throughout the world inhabiting various environments including; aquatic, oceanic and terrestrial habitats in temperate and tropical regions (Iverson *et al.,* 2003; Fritz and Havas, 2007; Rhodin *et al.,* 2010).

Chelonians are distinguished by their unique armored shell, constituing a very successful body plan that has remained unchanged through evolution (Buhlmann *et al.,* 2009). The shell can be soft, leathery, hard, flat, knobbed or hinged (Jacobsen, 2005) and is an important characteristic in classifying and identifying various species (CITES, 2000). The order Chelonia (syn. Testudines) Linnaeus, 1758 includes tortoises (land), turtles (marine) and terrapins (freshwater) (Branch, 2008). Chelonians are divided in two groups: Cryptodira (Cope, 1868) and Pleurodira (Cope, 1864) regarding four main characteristics;

1) Head and neck. For Cryptodira, the head can be withdrawn vertically into the shell and is protected by the forelimbs, whereas specimens from the group Pleurodira can withdraw their head sideways into the shell, and in this manner cannot be protected by the forelimbs (Branch, 2008);

2) Plastron scutes. In specimens of Cryptodira, the horny scutes may be lost in some groups (soft shell terrapins) and the anterior gular scutes may fuse together, whereas an extra pair of anterior intergular horny scutes are present in specimens of Pleurodira (Branch, 2008);

3) Pelvic girdle. In specimens of Cryptodira the pelvic girdle is not fused to the shell, instead it is attached with ligaments, whereas in specimens of Pleurodira, the pelvic girdle is fused to the shell (Branch, 2008);

4) Skull. In specimens of Cryptodira, the skull is reinforced by the pterygoid bone, whereas in specimens of Pleurodira, the skull is reinforced by the quadrate bone (Branch, 2008).

Cryptodira is the largest group, containing eleven families: Carettochelydae Boulenger, 1887, Cheloniidae Oppel, 1811, Chelydridae Gray, 1831, Dermatemydidae Gray, 1870,

Dermochelyidae Fitzinger, 1843, Emydidae Rafinesque, 1815, Geoemydidae Theobald, 1868, Kinosternidae, Agassiz, 1857, Platusternidae Gray, 1869, Testudinidae Batsch, 1788, and Trionychidae Fitzinger, 1826. This group includes tortoises, sea turtles and some of the terrapins (Bonin *et al.,* 2006) and is distributed worldwide. The Pleurodira consists of only three families: Chelidae Gray, 1825, Pelomedusidae Cope 1868 and Podocnemididae Cope, 1868. These three families are restricted to the southern hemisphere and encompass only terrapins (Boycott and Bourgin, 2000; Jacobson, 2005).

Although terrapins were successful in surviving environmental changes in the past, several factors have made terrapins more vulnerable to threats posed by man (Turtle Conservation Coalition, 2011). These factors include delayed sexual maturity, high fecundity together with high juvenile mortality and the long lifespan with a high adult mortality. Terrapins today are one of the most endangered vertebrate groups in the world, with almost half of the more than 300 species threatened with extinction (Rhodin *et al.,* 2010; www.iucnredlist.org). Attention has been drawn to the conservation of other animal groups, but terrapins are in greater risk of looming extinction than birds, mammals, amphibians, sharks and rays, and other vertebrate groups such as primates (Hoffmann *et al.,* 2010; Rhodin *et al.,* 2010; www.iucnredlist.org). Of the 317 currently recognized species of terrapins, roughly 10% are considered to be critically endangered on the International Union for Conservation of Nature (IUCN) Red List of Threatened species (Buhlmann *et al.*, 2002, IUCN, 2008) and roughly 42% are considered threatened (IUCN,2008). Throughout the world there are various threats impacting terrapins that could lead to a decline in populations or even extinction. Exploitation as food and as pets is the main cause for their declines, especially in Asia, whereas habitat loss and degradation are other contributing factors to these declines (Van Dijk *et al.,* 2000).

1.2 Importance of terrapins

Terrapins play a major role in the ecosystems they inhabit, serving as a source species from which other animals and plants benefit (Turtle Conservation Coalition, 2011). Terrapin populations in an aquatic ecosystem have a high biomass and as a group, they are one of the most long-lived and slow-growing animals in the world (Iverson, 1982).

The shell of the terrapins can be seen as one of the most unique and specialized adaptations in the animal kingdom. The shell represents a large amount of the total body mass and this bony structure is composed of phosphorus and calcium. The shell can therefore be seen as a valuable nutrient sponge that retains and transports nutrients in aquatic ecosystems. The service of nutrient cycling is a critical part in ecosystems and is one of the factors that produces clean water in both natural and human dominated environments (Bouchard and Bjorndal, 2000; Bjorndal and Jackson, 2003). As human populations increase worldwide, the amount and types of nutrients that are produced, processed and stored within freshwater ecosystems have been altered (Millennium Ecosystem Assessment, 2005).

Given that the rate of biodiversity loss in aquatic ecosystems worldwide is increasing, aquatic ecosystem functions are undoubtedly changing and various natural ecosystem services provided by animals could be lost (Duffy, 2003) In particular large, long-lived terrapins that are extirpated from aquatic ecosystems would no longer be able to process, move, and store the various nutrients that such ecosystems need. Without terrapins, the ecosystems that are also critically important for human-welfare eco-services, would gradually undergo the loss of biodiversity and degrade in a manner that is not fully understood and is difficult to predict (Turtle Conservation Coalition, 2011). Terrapins can also be useful biological monitors and environmental indicators and as a consequence can be applied to a wide range of bio-detection of contaminants occurring at low levels (Burger and Gibbons, 1998). Gibbons and Greene (s.a.) found that terrapins amplify environmental signals through bio-magnification, making them very sensitive bio-monitors. They are valuable, long-term bio-accumulators and have the ability to serve as reservoirs of the chemical composition of an area by storing it in their bones, shells and eggshells (Gibbons and Greene, s.a.).

1.3 Threats to terrapins

1.3.1 Habitat loss and degradation

Through various anthropogenic activities humans contribute significantly to habitat degradation and loss. They alter landscapes for development of infrastructure, harvesting of natural resources, and agricultural use (Collins and Storfer, 2003). Due to the alterations to these environments, declines and local extinctions of species can occur (Findlay and Houlahan, 1997). This is especially true for habitats adjacent to wetlands, rivers and ponds. These habitats are of critical importance in protecting and conserving biodiversity (Findlay and Houlahan, 1997; Calhoun and Klemens, 2002). Terrapins are one of the many animal groups that rely on aquatic habitats for survival. Globally terrapin habitat is disappearing through the loss or degradation of land to benefit man, converting terrapin habitat in such a manner that it is unsuitable for habitation (Orenstein, 2001). Although the consequences of habitat loss to terrapins have only recently been studied (Kjoss and Litvaitis, 2001; Gibbs and Shriver, 2002), it was shown that the degradation and loss of habitats can result in abnormal population structures (Dodd, 1990; Reese and Welsh, 1998). This could even result in population declines or extinctions (Gibbons *et al.,* 2000). As habitats are altered, degraded and destroyed, the terrapins that are still occupying the remaining habitat became isolated, which in turn reduces movements between suitable sites and decreases the genetic variability among populations (Gray, 1995). Not only does wetland degradation have an effect on terrapin populations, but the degradation of the area around the wetland, such as road construction, may also affect terrapins. The life cycle of many terrapin species includes annual migration to nesting sites by females, migration of juveniles, and movement to more suitable habitats such as the migration of males to find mates (Gibbons, 1986).

Many terrapins are killed on roads (Goodman *et al.,* 1994; Ashley and Robinson, 1996) during these migration excursions (Haxton, 2000; Gibbs and Shriver, 2002). Various terrapin species move to nesting sites during early mornings and late afternoons (Legler, 1954; Ernst, 1986), which increases the risk of being run over by cars (Festin, 1996). The mortality rate is thus higher during these migration periods. Even though male terrapins do occasionally travel overland (Gibbons, 1986), it is mainly female terrapins that are killed on the roads by vehicles. Female terrapins of some species may even take multiple excursions overland before the nesting process commences (Reese and Welsh, 1998). Roads in this regard could also create isolated patches and may lead to smaller populations which are more vulnerable to stochastic environmental events that could lead to a decrease in genetic diversity among populations and also to a decline or even local extinction of species (Wilcox and Murphy 1985; Lande 1988).

1.3.2 Pollution

Multiple forms of contamination can occur in freshwater ecosystems. These include toxic chemicals from industrial processes, rivers containing excess nutrients from farms, trash from landfills blown into river systems and the sky covered in smoke from industrial activities (Heath, 1995). Pristine landscapes may also be affected by pollution far away upstream of the area. Pollution could cause areas to become muddy, it could poison soils and waterways and this in turn could kill various animals and plants that rely upon these polluted aquatic environments (Kazlauskiene, 2012). Pollution can occur at various degrees; for example, at local (e.g., pesticides and eutrophication), regional (e.g., acid rain) and global (e.g., carbon dioxide emissions and climate change) scales (Kiesecker, 2003). Aquatic ecosystems are impacted by eutrophication due to nutrient enrichment, sediments, acidic compounds, heavy metals and biocides (Cowan, 1995). Fertilizers for agriculture and sewage are two factors mainly causing eutrophication due to the high content of nitrogen and phosphorous. This in turn leads to blue-green algal blooms that interfere with nutrient cycling causing a collapse in the functioning of a wetland, via alterations of the original fauna and flora that inhabited the unaltered environment. Eutrophication could also lead to the extensive growth of invasive aquatic plants that could threaten the biodiversity of the aquatic system (Henderson, 2010).

Herbicides are widely used in agriculture. One such pesticide is atrazine, a broad leaf herbicide that is known to act as a pollutant of ground and surface water. It has been reported to be active and cause damage at low, ecological concentrations (Hayes *et al.,* 2006). It allegedly affects gonadal development, slows down growth, and causes immuno-suppression in terrapins rendering them more prone to pathogens (Polakiewicz and Goodman, 2013). Contaminants have also been linked to abnormalities in terrapin eggs and hatchlings (Bishop *et al.,* 1998; Bell *et al.*, 2006). Another pesticide, organochlorine is linked to a mycoplasmal respiratory infection within the eye, ear and nose in *Terrapene carolina Carolina,* the Eastern box turtle (Tangredi and Evans, 1997).

1.3.3 Commercial over-exploitation

Although habitat disturbance and habitat destruction are general problems, they are worsened by direct human exploitation of terrapins. Terrapins are harvested around the world for food and commerce, medicine, aphrodisiacs, research and as pets (Bonin *et al.,* 2006; Barrios-Garrido and Montiel-Villalobos, 2006). The commercial trade in terrapins exceeds sustainable levels such that the extinction of some species in the wild may occur within the next decade (Mockenhaupt, 1999, Gibbons *et al.,* 2000).

The abuse and exploitation of terrapins have occurred since ancient by a wide range of civilizations. They are slow moving animals and thus easy to capture to provide various products such as meat, oil, fat, bony shells and valuable scutes (Bonin *et al.,* 2006). Ancient civilizations such as the Chinese used terrapins for divination purposes (Allan, 1991). The increase in terrapin trade is due to globalization and this accelerates the process of exploitation (Bonin *et al.,* 2006). The Red-eared slider *Trachemys scripta elegans* (Wied, 1839), native to the United States of America (USA), is one of the most popular species in the pet trade. It is estimated that between 1989 and 1997 roughly 52 million specimens were exported worldwide (Bunnell 2005; Telecky, 2001). According to the United States Department of the Interior – Fish and Wildlife Service in the last five years, from January 2008 up to February 2013, approximately 18 million individuals have been exported from the USA (G. Townsend, personal communication).

Large quantities of terrapins are also shipped to Asia, mainly to China, which is the greatest consumer worldwide (Gong *et al.,* 2009). In China, terrapins are popular as food, in particular, the Asian taste for turtle soup is one of the main factors driving them to extinction (Altherr and Freyer, 2000). Uncontrolled harvesting of terrapins for the food industry contributes to the decline of populations in the wild in Vietnam, Bangladesh, Indonesia and even in parts of the USA where 'China Towns' within large cities serve turtle soup. As these terrapin populations subside, they are being acquired from neighbouring countries putting the terrapin populations from these countries also under immense pressure (Turtle Conservation Fund, 2002). Terrapin populations are distinguished by distinct characteristics including; delayed maturity, high annual survivorship of adults, and high natural levels of nest mortality (Congdon *et al.,* 1993, 1994, Heppell 1998) These characteristics may influence wild populations to decline if anthropogenic harvesting continues at the current rapid pace. Adult terrapins are usually captured, and this leaves mainly juvenile terrapins in the population which are not able to reproduce and replace the captured terrapins. It will take a long time before these juveniles will reach adulthood, and by that time, the possibility of them being captured is inevitable.

1.3.4 Invasive species

Humans have played a major part in introducing exotic species into new environments. They have spread species outside their native ranges both intentionally and accidentally. The list of established invasive species is growing rapidly, and many of them cause significant economic and ecological effects (Vitousek *et al.,* 1997a). Biological invasions are one of the major threats to biodiversity (Strayer *et al.*, 2006; Ricciardi, 2007). They could have a negative effect on native species through predation and competition for food, the spread of pathogens, alteration of ecosystem functioning and abiotic features of the environments (Strayer *et al*.,

2006; Ricciardi, 2007). Invasive plants and animals are not only agricultural pests, but they could also be hazardous to human health, have a negative effect on transportation by damaging roads, and impede conservation efforts by invading new areas of conservation concern (United States Congress, 1993). Biological invasions form a major part of global environmental changes; leading to major losses in the economic value, biological diversity and even in the function of the ecosystems invaded (Wittenberg and Cock, 2001).

Several invasive species have effectively established populations over large areas of Europe (Hulme, 2007) and global biodiversity scenarios show a potential increase in biological invasions in European ecosystems (Sala *et al.,* 2000). In various instances, biological invasions imposed a negative effect on the economy. Invasive pests and weeds are one of the invasions that have impacted the agricultural, forestry and aquacultural sectors (Williamson, 2002). Biological impacts include the destruction caused by Japanese knotweed (*Fallopia japonica*) to the defence of floods as well as the reduced foraging success of aquatic animals (Maerz *et al.*, 2005). Parasites have also become invasive, such as *Gyrodactylus salaris* (i.e. an ectoparasite of Atlantic salmon) and *Anguillicola crassus* (i.e. swimbladder nematode of eels) that caused major declines in fisheries' income in various Nordic countries (Riddington *et al.,* 2006; Wielgoss *et al.,* 2008). The muskrat (*Ondatra zibethicus*) and coypu (*Myocastor coypus*), both became invasive species in Europe through the fur industry, and caused major damage to river banks by digging holes which in turn caused detrimental effects during floods (D'adamo *et al.,* 2000; Skyriene and Paulaskas, 2012). This loss of river banks could decrease available basking sites of terrapins.

Throughout the world terrapins are popular as pets. As they grow older, they require bigger containers and are more difficult to maintain. They are often released into the natural environment where they may establish feral populations. This has been observed in many European countries (Warwick, 1991), South Africa (Newberry, 1984) and Asian countries (Warwick, 1991; Moll, 1995). The Red-eared slider is a good example of such a species and is considered one of the top 100 most invasive terrapin species in the world with detrimental effects on native species (Lowe *et al.,* 2004). It has established itself in more than 25 countries (see GISD, 2010) and in the majority of the continents (Cadi *et al.*, 2004). The European Union (EU) banned the import of the species *T. s. elegans* in December 1997 (Bringsøe, 2006), but this led to the import of other exotic species into Europe (Adrados *et al.,* 2002; Bringsøe, 2006). Other species that were being imported included *Graptemys pseudogeographica* (Gray, 1831)*, Pseudemys floridana* (LeConte, 1830)*, Chelydra serpentina* (Linnaeus, 1758)*,* among others, and they were also observed to be present in the wild (Martínez-Silvestre *et al.,* 2006). In South Africa the same thing happened in the 1980's when feral populations of Red-eared sliders were documented near Durban and Pretoria, where they likely contributed to the local extinction of

African native terrapin *Pelusios rhodesianus* (R. Boycott, personal communication). Near Pretoria the invasive Red-eared slider had been reported from localities including Six Mile Spruit, Moraletta Spruit, and the Hartebeespoort. Elsewhere on the highveld they have been found in Zoo Lake, Boksburg Lake, and in Germiston (Boycott and Bourquin, 2000).

Around 1970 large numbers of subadult terrapins of the genera *Chrysemys*, *Graptemys*, *Trachemys* and *Pseudemys* were imported into France as pets (Warwick, 1991). Large numbers of these terrapins were released and especially the Red-eared slider soon became an invasive threat to the threatened local species, the European pond terrapin, *Emys orbicularis* (Cadi and Joly, 2004). The Red-eared slider may indeed outcompete *E. orbicularis* for food, basking sites or breeding habitats (Cadi and Joly, 2003) and recent studies showed that competition is very likely to occur also between sliders and *M. leprosa* another indigenous terrapin of Southern Europe (Polo-Cavia *et al.,* 2008; Polo-Cavia *et al.*, 2009a,b; Polo-Cavia *et al.*, 2010). Polo-Cavia *et al.*, (2009) indeed showed that invasive sliders could add to the displacement of native terrapins in their natural environments.

The introduction of invasive terrapins into natural wetland systems may further lead to direct and indirect damage to plants and animals in natural environments. The omnivorous diet of Red-eared sliders may cause a direct adverse effect. In a small enclosed freshwater ecosystem small populations or even a single individual slider, could cause great damage to rare or endangered organisms such as threatened aquatic plants or amphibians (Semenov, 2010). Sliders may also prey on various invertebrate species, fish, egg clutches, tadpoles or adult frogs and even young of waterfowl. This in turn could cause a serious distortion within the composition of an ecosystem. Introduction of invasive species can therefore alter the organization and the functioning of local communities through assorted processes such as predation, competitive exclusion, parasite transfers and dispersal of new pathogens (Butterfield *et al.,* 1997; Dodd and Seigel, 1991; Manchester and Bullock, 2000; Vitousek *et al.*, 1997a; Williamson, 1996).

1.3.5 Parasites and emerging infectious diseases

Parasites and emerging infectious diseases influence terrapin survival in ways, such as influencing host genetic diversity and co-evolutionary processes to changing species composition in ecological ecosystems (Altizer et al., 2003). Because epidemics of parasites and diseases may cause disasters for plants and animals due to quick evolutionary adaptations, invasive pathogens are of big concerns in conservation biology (Daszak *et al.*, 2000; Harvell *et al.,* 1999; Lafferty and Gerber, 2002). Of all the various parasites occurring in aquatic environments, parasites that only require one host to complete development are most likely to establish in new environments (Hayward *et al.,* 2001). They have the ability to play an effective

role in determining the success of an invasion through their impact on host interactions (Hatcher *et al.*, 2006). Parasites have the ability to drive a species to extinction (usually a native species replaced by an invasive species) as a result of the effects of competition (de Castro and Bolker, 2005). Because invasive species establish themselves in new environments, they may lose their natural enemies, including their parasites, through stochastic processes. This could be an important factor that drives the success of invasive species (Torchin *et al.*, 2003; Torchin and Mitchell, 2004).

Terrapins are found to be infected with various types of ecto- and endoparasites (McAllister *et al.*, 2008; Zelmer and Platt, 2008) including: Digenea Carus, 1863; Monogenea Carus, 1863; Aspidogastrea Faust and Tang, 1936; Nematoda Cobb, 1932; Haematozoans (Danilewsky, 1886); Coccidia Leuckart, 1897 and leeches (Hirudinea Lamarck, 1818). As terrapins are being shipped around the globe they may carry with them their own parasites. When these terrapins are released and feral populations establish; then their parasites can be introduced into these new environments. Hays *et al.*, (1999) documented that *Emys marmorata* (Baird and Girard, 1852), an endangered terrapin endemic to North America, contracted a herpes-like virus from introduced captive individuals and this could have been the reason for some population declines. Red-eared sliders are carriers of various parasites that could be released into the environment and could potentially infect new hosts. Previous research has shown that the spread of parasites can occur from native European terrapins to introduced American terrapins in wild populations (Hidalgo-Vila *et al.,* 2009) and from exotic American terrapins to native European terrapins in captivity (Verneau *et al.*, 2011). Evolutionary old parasitic associations seldom have adverse effects on the host, but when host switching takes place and new parasitic associations are formed, infections often are severe with adverse effects. In this study the focus was mainly on two parasitic groups; Monogenea Carus, 1863 and Apicomplexa Levine, 1970. Monogeneans were selected for this study seeing that they are an evolutionary old group of parasites that evolved within sarcopterygian hosts, especially amphibians and chelonians (Verneau *et al.,* 2002). Furthermore, polystome host switching was documented between introduced and native chelonians in confined environments (Verneau *et al.,* 2011). Apicomplexans were also surveyed because it is known that they tend to have a negative impact on their hosts and thus are of major conservation concern.

1.4 Terrapin genera and species examined in the current study

1.4.1 South African terrapins

Family: Pelomedusidae, Cope, 1868

African terrapins are closely related and have been placed together in the family Pelomedusidae (Pleurodira). The most identifiable feature of this family is that the terrapins cannot retract their heads completely into the shell and thus retract it sideways underneath the carapace edge. The nuchal and supracaudal scutes are absent, whereas the plastron has an intergular scute. The nostrils of all species are situated at the tip of the snout for easy breathing at the surface. The forelimbs have strong claws that help with the shredding of food. Small tentacles are visible beneath the chin (Branch, 1999; Bonin *et al.,* 2006). They are only found in the Southern Hemisphere, mainly in Africa and Madagascar (Bonin *et al.,* 2006).

Genus: *Pelomedusa* Wagler, 1830 (Marsh terrapins)

The genus *Pelomedusa* contains only one species, i.e. *Pelomedusa subrufa* (Lacepède, 1788), that is widely distributed in sub-Saharan Africa (Branch, 1999; Bonin *et al.,* 2006). However, Vargas-Ramirez *et al.* (2010) suggested that it might be a complex of species.

Pelomedusa subrufa (Lacepède, 1788) (Common African helmeted terrapin) (Fig. 1.2)

This species has a wide distribution and is the most widely distributed African terrapin, occupying the entire continent south of the Sahara. This species also occurs in Madagascar and in Yemen and was most likely introduced to these localities by humans (Branch, 1999; Bonin *et al.,* 2006).

Pelomedusa subrufa can be distinguished from other African terrapins by the fact that it has a poorly ossified unhinged plastron and five claws on the forelimbs. This species is usually small in size (200 mm), but in rare cases specimens in South Africa have been found to be considerably larger (330 mm). The shell is fairly flat with a smooth oval profile ,and juveniles have slight serrations around the edge of the shell. The adults vary in color from beige to green and the head is wide and flat and capped by two large supraorbital scales together with two temporal scales and a single large frontal scale. These scales make the head look like it is covered by a helmet, hence the common name. The color of the head usually coincides with the carapace, with some light speckles on top of the head with a pale colored throat, with two small barbells situated on the chin. The plastron has a pale color that varies from yellow to light green.

The plastron is not concave and ends with a large anal notch. The feet are moderately webbed and contain two phalanges in each digit (Branch, 1999; Bonin *et al.,* 2006).

This species is found in various aquatic habitats that vary from lowland areas and up to altitudes of 3 100 m. These terrapins travel between aquatic environments during rainy seasons and when the rain and water dry up, they bury themselves deeply in the mud and estivate for very long periods. They can be observed basking on banks, as well as on rocks and trees emerging from the water. They have an omnivorous diet, but are by preference carnivorous. Breeding for this species occurs in spring or autumn according to latitude, and each female lays only one clutch per year containing up to 42 eggs (Branch, 1999; Bonin *et al.,* 2006).

Figure 1.2: Common African helmeted terrapin, *Pelomedusa subrufa,* from Sable Ranch, Brits in the North West province, South Africa.

Genus: *Pelusios* Wagler, 1830 (African Hinged terrapins)

The genus *Pelusios* is the most diverse genus of all African terrapins (Fritz *et al.,* 2011). The distinctiveness of all recognized *Pelusios* species was confirmed by molecular analyses; however all the species grouped within *Pelusios* do not form a clade (Fritz *et al.,* 2011; Fritz *et al.,* 2013). They are distinguished by a hinge in the front part of the plastron, hence the common name. The hinge runs along the pectoral abdominal suture and between the underlying hypoplastral and mesoplastral bones. This hinge can close by the use of a large muscle that is attached to the axillary buttress. The function of the hinged plastron is to protect the head and forelimbs when it closes (Branch, 1999; Bonin *et al.,* 2006).

Pelusios castanoides Hewitt, 1931 (East African yellow bellied hinged Terrapin) (Fig. 1.3)

Pelusios castanoides occurs in eastern Africa including, Malawi, Mozambique, Swaziland and in northern KwaZulu-Natal in South Africa. Some isolated populations of the species also occur in Madagascar and the Seychelles (Branch, 1999; Bonin *et al.,* 2006).

This is a medium sized terrapin (230 mm) and can weigh up to 900 g. The shell is elongated and oval in shape, usually smooth with the carapace domed. The marginal scutes are not serrated with the exception of the posterior marginals. The posterior plastron lobe is slightly constricted with a deep anal notch. The supracaudal scute is divided in two with no nuchal scute. The plastron is large and covers the entire area of the carapace. Axillary scutes are absent. The color of the carapace varies from olive, blackish-brown to yellow, with the plastron being yellow in color with faint black markings on the front sutures. The head is wide and medium in size and flattened on top with a blunt, rounded snout. The head is blackish-brown in color with fine yellow vermiculation. The skin of the neck and limbs is yellow-brown. Two barbells are present on the chin. Each limb contains feet that are slightly webbed with five claws. The posterior lobe of the plastron of females tends to be larger than that of males (Branch, 1999; Bonin *et al.,* 2006).

These terrapins prefer lakes, brackish water and ponds at low altitudes. They occur on the coast of southern Kenya, through central Mozambique to northern KwaZulu-Natal province in South Africa. Isolated populations also occur on Madagascar and the Seychelles. They usually bury themselves in the mud when water dries up, and only re-emerge after heavy rains. This species is carnivorous and mainly feeds on mollusks, invertebrates, fish and aquatic plants. Breeding occurs after the first heavy rains and clutch sizes vary from five to 25 eggs (Branch, 1999; Bonin *et al.,* 2006).

Figure 1.3: East African yellow-bellied terrapin, *Pelusios castanoides,* from Tembe Elephant Park, in the northern KwaZulu-Natal province, South Africa.

Pelusios rhodesianus Hewitt, 1927 (Variable hinged Terrapin) (Fig. 1.4)

Pelusios rhodesianus is widespread across central and eastern Africa, also from the southern part of the Democratic Republic of the Congo and Uganda, south to Angola and in the Okovango swamps in Botswana, down to central Zimbabwe and central Mozambique, with some populations in northern KwaZulu-Natal and around Durban in South Africa (Branch, 1999; Bonin *et al.,* 2006).

Pelusios rhodesianus is medium in size (250 mm) with the females being larger than males. The carapace has an oval shape, is smooth, elongated and domed in form with a weak keel being present towards the rear in some specimens. The posterior marginal scutes are not serrated and the nuchal scute is absent. The plastron is large and covers most of the carapace opening like in *P. castanoides.* The anterior lobe is broad, with the posterior lobe being smaller in size with an anal notch. Axillary scutes are absent and the supracaudal scutes are divided. The head is small and flat, with a small projecting snout. The upperjaw is bicuspid with two barbells present on the chin. The head is dark brown in color with black on top with

vermiculation on the head in populations found in the north, and with yellow lines down the side of the head in southern populations. The carapace and plastron are uniform dark brown to black. The skin is light brown to yellow (Branch, 1999; Bonin *et al.,* 2006).

Terrapins occupy calm waters in large river systems, marshes, lakes and artificial ponds. They are primarily carnivorous, but they may eat some aquatic plants. Breeding occurs after the first rain in spring with nesting periods that follow throughout summer. Clutch sizes vary from 10 up to 15 eggs (Branch, 1999; Bonin *et al.,* 2006).

Figure 1.4: Variable hinged terrapin, *Pelusios rhodesianus,* from Tembe Elephant Park, in the northern KwaZulu-Natal province, South Africa.

Pelusios sinuatus (Smith, 1838) (East African serrated hinged Terrapin) (Fig. 1.5)

This species is widespread across eastern parts of Africa. Their distribution extends from tropical East Africa, along the Zambezi River to Victoria Falls in Zimbabwe and then south to KwaZulu-Natal in South Africa and into Swaziland (Branch, 1999; Bonin *et al.,* 2006).

Out of all the *Pelusios* spp., this is the largest species with females weighing up to 9 kg and measuring up to 485 mm. The shell is elongated and oval, with a flattened top and sometimes depressed along the vertebral scutes. In some specimens, a light keel is often reduced to single points on vertebral scutes one to four. The carapace is widened to the posterior after the plastral hinge. The posterior marginals are serrated with the nuchal scute absent and the supracaudal divided. Plastron is slightly smaller than the carapace with a strong hinge on the front of the plastron. The posterior plastron lobe does not constrict, but a large anal notch is present, with the notch angled in males and rounded in females. An axillary scale is present at the front junction of the carapace and the plastron, with males having a slightly concaved plastron. The color of the carapace varies from grey to black, whereas the plastron is yellow to light orange with black borders. The head is broad and flattened, with a slightly pointed snout. The upper jaw is notched and often bicuspid with two barbells present on the chin. The color of the head is blackish brown, with yellow to brown vermiculation around the head. The skin, neck and limbs have a pale-olive color (Branch, 1999; Bonin *et al.,* 2006).

Pelusios sinuatus inhabits aquatic environments, from perennial rivers to permanent lakes and pans in coastal savannas at an altitude of roughly 1 500 m. Terrapins can be seen basking on river beds, emergent trees and branches and rocks during the day. Their diet is mainly carnivorous, with larger specimens eating freshwater mussels, fish and carrion and in some cases aquatic vegetation. Breeding and nesting usually takes place after the first summer rains and clutch sizes vary from seven up to 27 eggs (Branch, 1999; Bonin *et al.,* 2006).

Figure 1.5: East African serrated hinged terrapin, *Pelusios sinuatus,* from Ndumo game reserve, in the northern KwaZulu-Natal province, South Africa.

Pelusios subniger (Lacepède, 1788) (East African black hinged Terrapin) (Fig. 1.6)

Pelusios subniger has a very wide distribution stretching from southeastern Africa, from Burundi and Tanzania to eastern Democratic Republic of Congo, Zambia and northern Botswana. In southern Africa terrapins occur from Zimbabwe and northern Botswana up to the upper Limpopo River in to southern Mozambique and in to the northern regions of the Kruger National Park in South Africa. They also occur in eastern Madagascar and the Seychelles, although they were most likely introduced to these countries (Branch, 1999; Bonin *et al.,* 2006).

This species is fairly small in size (200 mm) with the females being larger than males. It has a smooth shell that lacks a keel and is unserrated. The supracaudal scute is divided and a nuchal scute is absent. The shell has an oval shape in males, but it is more rounded in females. All the vertebral scutes are broader than they are long and the plastron with the anterior lobe is much broader than the posterior lobe. A small plastral hinge is present at the anterior side of the plastron, with an anal notch present at the posterior end of the plastron. No axillary bud is present. The color of this species varies according to its habitat and ranges from dark brown to a grey color. The plastron completely closes the open area of the carapace and has a dark brown color to it. The head is wide and rounded with a short round snout. The head is uniformly brown and is not vermiculated on the sides. The jaw is yellow with the skin and neck being grey to black. Two barbells are present on the chin (Branch, 1999; Bonin *et al.,* 2006).

Pelusios subniger inhabits a variety of aquatic environments including, swamps, lakes, ponds, rivers and temporary wetlands. This species is mainly nocturnal and walks between habitats at night, whereas it basks during the day on river beds, rocks and fallen tree stumps. Their diet is mainly omnivorous and consists of crabs, worms, insects, amphibians, fish and carrion, and occasionally aquatic plants. Terrapins estivate during dry seasons under the hardened mud and only emerge after the first rains in spring where after breeding occurs. Eggs are laid in nests with clutch sizes that vary from eight to 12 eggs (Branch, 1999; Bonin *et al.,* 2006).

Figure 1.6: East African black hinged terrapin, *Pelusios subniger,* from Tembe Elephant Park, in the northern KwaZulu-Natal province, South Africa.

1.4.2 European terrapins

Family: Geoemydidae, Theobald, 1868

The family Geoemydidae is the largest turtle family in the world, accounting for approximately 25% of the total species-level diversity of turtles (Iverson, 1992). Terrapins are mainly freshwater aquatic and semi-aquatic with a wide distribution including Europe, North Africa, India, southern Russia, Indonesia, the Philippines, Mexico, Ecuador, Venezuela and Brazil (Ernst and Barbour, 1989; Iverson, 1992). These terrapins vary in size from 10 up to 80 cm in carapace length. They have webbed feet and the pelvic girdles articulate with their plastrons flexibility. Their heads are retracted vertically into the shell (Cryptodira) with the forelimbs protecting the head. The carapace contains 24 marginal scutes whereas the plastron contains 12 scutes with no mesoplastron (Rhodin *et al.,* 2010).

Genus: *Mauremys*, Gray, 1869

Mauremys has a patchy distribution that includes parts of the western Palearctic and parts of the Oriental and eastern Palearctic regions. There are six species known from this genus*; M. annamensis, M. caspica, M. japonica, M. leprosa, M. mutica* and *M. rivulata* (Branch, 1999; Bonin *et al.,* 2006), but only *M. leprosa* was surveyed.

Mauremys leprosa (Schweigger, 1812) (Mediterranean pond Terrapin) (Fig. 1.7)

Mauremys leprosa is present in the western part of the Mediterranean region, Spain, Portugal, the south of France and the north-western part of Africa, specifically; Morocco, Algeria, Tunisia and Libya (Branch, 1999; Bonin *et al.,* 2006).

Mauremys leprosa is one of the largest species in the *Mauremys* genus with a length up to 250 mm in females. The carapace in this species is oval in shape and rather flat with the median keel disappearing in adults. The marginal scutes are smooth without any serrated edges. The color of the carapace varies from red-brown to olive with orange and yellow marking on the scutes of juveniles. The plastron has a yellow color with dark lines running down the centre on occasions. The head is brown to blue in color with a grey background with yellow stripes running from the neck to behind the tympanum. In some specimens these yellow lines could extend to the rear of the snout, and can be completely missing in some individuals (Branch, 1999; Bonin *et al.,* 2006).

This is a cryptic species that often hides in the mud at the bottom of their aquatic environments. These terrapins occur in stagnant water bodies, lakes, ponds and in some cases, in brackish water. They can often be spotted basking on emergent branches. Terrapins that occur in the northern range may hibernate over winter, whereas terrapins from the southern range may estivate under dry leaves or mud. Their diet is primarily carnivorous, consisting of small fish, amphibians, gastropods and insects. Mating occurs in early spring and then nesting occurs during the summer. A female could nest up to three times in a season, with clutch sizes being between six to nine eggs (Branch, 1999; Bonin *et al.,* 2006).

Figure 1.7: Spanish terrapin, *Mauremys leprosa,* from the Baillaury River (Banyuls/Mer), in southern France.

1.4.3 Invasive terrapin

Family: Emydidae, Rafinesque, 1815

The family Emydidae with the exception of *Emys orbicularis* from the Mediterranean region is restricted to the Americas and is the most diverse in North America (Bonin *et al.,* 2006). The Emydidae is comprised of almost 50 species from 10 genera. Shells vary and range from domed to flattened and are very colorful in some species which makes these terrapins very popular in the pet trade (Bonin *et al.,* 2006). In some species, a prominent ridge may be visible from the front to the back of the carapace with a prominent bridge connecting the carapace and the plastron. Some members of the family have a hinge that separates the pectoral and abdominal segments. The skull is also very small in this family of terrapins (Rhodin *et al.,* 2010).

Genus: *Trachemys*, Agassiz, 1857 (Slider terrapins)

Trachemys is native to America and consists of 15 species; *T. adiutrix, T. callirostris, T. decorate, T. decussate, T. dorbigni, T. emolli, T. gaigeae, T. garyi, T. nebulosa, T. ornate, T. scripta, T. stejnegeri, T. taylori, T. terrapin, T. venusta* and *T. yaquia*. Only *T. scripta,* subspecies *elegans*, was used in this study (Branch, 1999; Bonin *et al.,* 2006).

Trachemys scripta elegans (Wied, 1839) (Red-eared slider Terrapin) (Fig. 1.8)

Trachemys scripta elegans originated from warm climates in the southeastern United States. Their natural distribution includes, Colorado, Virginia and Florida, but due to the pet trade, feral populations have established on various countries over the world (Branch, 1999; Bonin *et al.,* 2006).

This species reaches a size of 280 mm and has become “popular” all across the world for its ability to invade natural ecosystems. It is identified by a wide red band behind the eye, with narrow stripes on the chin and each costal has a transverse yellow band. The carapace is oval and fairly dome shaped. Its colour varies from brown to olive, with yellow to orange markings in between scutes. The plastron of *T. s. elegans* is commonly marked with black blotches, spots and ocelli. These markings could extend across the various scutes. The skin colour is brown to dark olive and in some cases even black, with bright yellow lines running from the base to the tip of each limb. The forelimbs of males contain very long second, third and fourth claws (Branch, 1999; Bonin *et al.,* 2006).

Trachemys scripta elegans is a very opportunistic animal and can adapt very easily to various aquatic environments although it is most common in calm waters with muddy bottoms with abundant vegetation. It also likes a habitat that contains a lot of basking spots such as emerging tree branches, rocks and river banks. Terrapins can remain active all year round in suitable environments, but they may estivate during very hot and dry months. These terrapins are diurnal and sleep during the night either at the bottom of the water or in some cases on the surface. They have an omnivorous diet consisting of snails, crayfish, amphibians, small fish and aquatic plants. The juveniles of this species are more carnivorous and as they grow older and become adult terrapins, they eat more plants. Breeding occurs in spring after the first rains and nesting occurs during the summer months and clutch sizes may vary drastically from two to 33 eggs (Branch, 1999; Bonin *et al.,* 2006).

Figure 1.8: Invasive Red-eared slider terrapin, *Trachemys scripta elegans,* from the Fosseille River, in southern France.

1.5 Parasite classes examined in this study

1.5.1 Monogenea

Monogeneans are mainly ectoparasites of vertebrates and are for the most part found on the gills and external surfaces of fish (Roberts and Janovy Jr., 2000), but are also endoparasitc in aquatic tetrapods such as the hippopotamus, anurans and chelonians. The polystomatid flatworms (Platyhelminthes, Monogenea, Polystomatidae) are found in all three suborders of the amphibians (see Verneau, 2004) and in freshwater chelonians (see Morrison and Du Preez, 2011). They form an evolutionary old group of parasites and are found in all parts of the world (Verneau *et al.,* 2002). Up to date, there are 23 genera which include over 200 species from various aquatic or sub-aquatic hosts (Du Preez *et al.,* 2010). They all have a direct life cycle with free swimming oncomiracidia, and are mostly host and site specific (Verneau, 2004). Three polystome genera *Polystomoides* Ward, 1917*, Polystomoidella* Price, 1939 and *Neopolystoma* Price, 1939, are found within terrapins in sites such as; the urinary bladder, conjunctival and pharyngeal cavities (Morrison and Du Preez, 2011). Terrapin polystomes have a reproductive strategy that is adapted to their hosts' primarily aquatic. They are distinguished from all other polystomes by (1) undiverticulated intestinal gut caecae of identical length that do not form any anastomoses, (2) a dense sphere-shaped testis that is located in the centre of the parasite, (3) skeletal elements in the suckers that improve the parasite's grip on the host and (4) a genital bulb that may have a great quantity of genital spines, with some species even having two rows of various sizes (Morrison and Du Preez, 2011).

1.5.2 Apicomplexa

The phylum Apicomplexa Levine, 1970 is probably the largest group of parasitic protists that differs from the other by a specific single plastid organelle, called the apicoplast that is surrounded by three or four membranes. The function of the apicoplast is lipid and heme biosynthesis, which is needed for survival. Antonie van Leeuwenhoek found the first apicomplexan protozoan in 1674. He saw an oocyst of *Eimeria stiedae* Lindemann, 1865, in the gall bladder of a rabbit (Lee *et al.,* 2000). However, *Gregarina ovata* was the first Apicomplexan to be named by Dufour (1828) in earwigs. There are about 4000 known species that are classified into multiple genera (Bush *et al.,* 2001). Apicomplexans parasitize vertebrates and invertebrates and can be either benign or pathogens such as *Plasmodium* that cause serious illnesses in humans and other animals. Other illnesses caused by apicomplexans are coccidiosis and toxoplasmosis in humans and various animals that are domesticated (Bush *et al.,* 2001). Apicomplexans are delineated as a group by various synapomorphies that include a conoid, a polar ring, apical rings, vesicles called rhoptries and micronemes that open at the

anterior of the cell (Siddell, 1995). They secrete enzymes that enable them to enter cells. The membrane for the rest of the cell is supported by vesicles called alveoli which form a semi-rigid pellicle, except for the mouth called the micropore (Lee *et al.,* 2000; Bush *et al.,* 2001).

The life cycle of apicomplexans is very complex and varies a lot among the apicomplexan groups but is very unique among eukaryotes when looked at their ontogenetic development (Siddell, 1995). Most protists have simple life histories that involve binary fission that could include an intervening cystic stage. When compared to metazoans, where each zygote produces one or only a few individuals of the species, apicomplexans undergo a proliferative ontogeny cycle (Siddell, 1995). Both sexual and asexual reproduction takes place in apicomplexans although some skip various stages (Lee *et al.,* 2000; Bush *et al.,* 2001). A host is typically parasitized by the parasite, which divides to produce sporozoites (sporogony) that enter the blood cells. In the blood cells the sporozoites or merozoites divide repeatedly and forms more merozoites through schizogony or merogony (Lee *et al.,* 2000; Bush *et al.,* 2001). The cell then ruptures and merozoites are released that could parasitize new surrounding cells. This step in the life cycle could occur several times, until gamonts are produced that are the sexually reproductive stages in the life cycle. The produced gamonts will form male and female gametes (Lee *et al.,* 2000) inside the invertebrate host where they fuse to form new cysts (Fujioka and Aikawa, 2002) to continue the life cycle. Inside the invertebrate host, the zygote undergoes sporogony, where an oocyst is formed. The oocyst will form sporozoites which will be transferred back to the vertebrate host (Lee *et al.,* 2000). There are variations in these stages and some apicomplexans even have more than one host. This in turn allowed for partial classification of apicomplexans, but the morphological traits, host associations and life cycles is not sufficient information for describing species in this group (Barta, 1997; Bush *et al.,* 2001). The phylum Apicomplexa makes out most of the Sporozoa group. This group is parasitic protozoans without flagella, silia, or pseudopods. However, most apicomplexans have the ability to move.

Blood parasites are common in terrapins and these infections can have adverse effects on their hosts. The more common blood parasites include *Haemoproteus, Haemogregarina, Trypanosoma.* In a study of Bornean river terrapins suffering from lethargy, ulcerations and caseous necrosis of the plastron, specimens were evaluated for haematology and plasma chemistry. Intra-erythrocytic haemogregarine parasites were found associated with anaemia, low haemoglobin, basophilia, eosinophilia, heterophilia and azurophilia (Knotkona *et al.,* 2005).

1.6 Project Aim

The broad aim of this study was to provide information on the diversity of parasites in invasive and native terrapin species in southern Europe (France and Spain) and South Africa and to evaluate the potential impacts of *T. s. elegans*, together with their parasites and other ecological factors, on the viability of the Mediterranean pond terrapin, *M. leprosa.*

Objectives:

1.6.1 Provide information on host-switching from the invasive American red-eared slider, T. s. elegans, to the native Mediterranean pond terrapin, M. leprosa in natural environments

Previous studies have shown that host switching of polystome parasites may occur from American to indigenous terrapins in confined habitats (Verneau *et al.,* 2011). Because no studies have documented the extent of host switching in wild populations, our objectives were to determine the diversity of polystome species within *M. leprosa* populations and to verify to what extent host-switching may take place between the introduced invasive Red-eared slider and the native Mediterranean pond terrapin in wild habitats of northern Spain and southern France.

1.6.2 Measure the effect of environmental temperatures on the parasitemia of terrapins in France

Global warming is changing climates all over the world and is affecting environmental conditions and mainly temperature (Botkin *et al.,* 2007). The objective was to investigate the influence of environmental pressures on polystome egg production of the terrapin bladder parasite *Neopolystoma* sp.

1.6.3 Assess the relative risk on the viability of the M. leprosa population along the Fosseilles River, Pyrénées Orientales region, France, using the relative risk method

Mauremys leprosa is considered to be endangered in France (IUCN France, MNHN and SHF, 2009). Various factors may lead to the loss of populations or even local extinction in France. Thus the objective was to assess the viability of the native Mediterranean pond turtle (*M. leprosa*) in a small river system close to Perpignan in the south of France, taking into consideration not only the effect of the invasive Red-eared Slider but other external anthropogenic threats and environmental conditions. Risk is calculated for four source scenarios: (1) current conditions as observed on site (2) increase of the number of the invasive *T. s. elegans* terrapins in the river system (3) complete removal of invasive terrapins from the river system (4) a sewage spill from a sewage plant situated upstream.

1.6.4 Determine the diversity of haemogregarines (Protozoa: Apicomplexa: Haemogregarinae) within South African terrapins based on morphological and molecular evidences

Very little research has been done on haemogregarine protozoans from freshwater terrapins and mainly studies that were conducted only focused on morphological characteristics for parasite descriptions. The following objectives were (1) to investigate the diversity of *Haemogregarina* (Apicomplexa: Haemogregarinidae) parasites from South African terrapins (*Pelomedusa subrufa*, *Pelusios castanoides, P. rhodesianus, P. sinuatus,* and *P. subniger*) by comparing the various morphological stages within the erythrocytes of the host species; (2) to assess the 18S phylogenetic relationships between haemogregarines of South African terrapins and other African ones to confirm their membership to the genus *Haemogregarina*.

CHAPTER 2

Parasite host-switching from the invasive American red-eared slider, *Trachemys scripta elegans,* to the native Mediterranean pond terrapin, *Mauremys leprosa* in natural environments

"To confront a living environment as parasites do is not only to colonize a new type of environment but to begin an entirely new lifetime adventure."

- Claude Combes

2.1. Introduction

Over time humans have changed in their behavior from being mainly agrarian to being urbanites. It reached a point where population growth in rural areas basically seized and future population growth shifted to urban communities (Wu, 2008). In an attempt to stay in contact with nature and for companionship many people living in urban areas keep pets, for animals have always reminded us of our animal origins (Phineas, 1974; Covert *et al.,* 1985). Humans have also adapted to a touristic lifestyle and travel more and further. With the increase of the global aviation network, much more goods, including animals are being traded between countries and thus the introduction of non-native species into new bio-geographic areas has been considerably accelerated over the past decades (Vitousek *et al.,* 1997b; Lowe *et al.,* 2000; Arena *et al.,* 2012). Because introduced species do not share a common evolutionary pathway with native species in their new environment, behavior, ecology and demographic characteristics of native species may be impacted upon (Huxel, 1999; Mooney and Cleland, 2001; Shea and Chesson, 2002). The organization and the functioning of local communities through assorted processes such as predation, competitive exclusion, parasite loss or parasite transfer may also be altered (Lodge, 1993; Williamson, 1996; Vitousek *et al.,* 1996; Hudson and Greenman, 1998; Holway and Suarez, 1999; Tompkins *et al.,* 2002; Clay, 2003; MacNeil *et al.,*

2003; Torchin *et al.,* 2003; Torchin and Mitchell, 2004; Smith *et al.*, 2006; Crowl *et al.,* 2008). In turn this may cause drastic and irreversible changes in ecosystems (Williamson, 1996; Vitousek *et al.,* 1997b; Chapin III *et al.,* 2000; McNeely, 2001; Mooney and Cleland, 2001; Daszak *et al.,* 2000; Ehrenfeld, 2010).

One such animal group that is globally very popular in the pet trade is terrapins. This is especially true for the red-eared slider, *Trachemys scripta elegans* (Wied, 1839), because of its striking colors, small body size of hatchlings and minimal husbandry requirements (Telecky, 2001). As a result various terrapin farms from the United States of America (USA) were involved in selling young red-eared sliders to mainly Europe (Warwick, 1991; Lutz, 2000; Telecky, 2001) and Asia (Goh and O'riordan, 2007; Ramsey *et al.,* 2007). Approximately 52 million individuals of *T. s. elegans* were exported from the USA between 1989 and 1997 (Telecky, 2001; Bunnell, 2005) and exportation of this species is still taking place. According to the United States Department of the Interior – Fish and Wildlife Service in the last five years, from January 2008 up to February 2013, approximately 18 million individuals have been exported from the USA (Pers. Comm. G. Townsend). However, because sliders grow rapidly and soon lose their striking juvenile color pattern, they become less attractive and are often released in the wild by owners without considering potential environmental implications. As a consequence, the red-eared slider has become the most widely invasive reptile species in the world (Kraus, 2009). Feral populations established in natural freshwater ecosystems in Canada (Harding, 1997), West Indies and Caribbean sea (Perry *et al.,* 2007), Japan (Itô *et al.,* 2000; Yasukawa, 2005), Korea (Lever, 2003; Ramsey *et al.,* 2007), Taiwan (Chen and Lue, 1998), Singapore (Lamar and Love, 1997; Goh and O'riordan, 2007), New Zealand (Dykes, 2007), Australia (Burgin, 2006; Edwards, 2007) and various countries in Europe (Cadi and Joly, 2004).

While habitat destruction and human pressure are the main factors in the decline of some terrapin species, competition between native and introduced terrapins could worsen the state of native terrapin populations (Da-Silva and Blasco, 1995; Pleguezuelos, 2002; Gibbons *et al.,* 2000; Cadi and Joly, 2003, 2004; Ficetola *et al.,* 2009; Polo-Cavia *et al.,* 2008, 2009a, 2010,

2011, 2012). It has been shown for instance that *T. s. elegans* may outcompete the native European pond terrapin, *Emys orbicularis* (Linnaeus, 1758), for food, basking sites or breeding habitats (Cadi and Joly, 2003, 2004). Similar recent studies suggested that competition is also very likely to occur between sliders and the Mediterranean pond terrapin, *Mauremys leprosa* (Schweigger, 1812) (Polo-Cavia *et al.,* 2008, 2009b, 2010, 2011, 2012). Chemical cues that are released from the invasive species could also adversely alter the behavior of *M. leprosa* leading to its displacement in natural environments (Polo-Cavia *et al.,* 2009a). Furthermore sliders are carriers of various parasites that could be released into new environments and potentially become established in novel new host species. Hays *et al.* (1999) documented that *Actinemys marmorata* (Baird and Girard, 1852), which is an endangered endemic terrapin to North America, contracted the herpes-like virus from introduced captive *T. s. elegans* individuals. This could possibly explain the decline observed in some *Actinemys* populations (Hays *et al.,* 1999). Whereas studies have shown that the spread of parasites can occur from native European to introduced American terrapins in wild populations (Hidalgo-Vila *et al.,* 2009), Verneau *et al.* (2011) showed horizontal parasite transfers from exotic American to native European terrapins in captivity.

Examples of such parasites include the polystomatid flatworms (Platyhelminthes, Monogenea, Polystomatidae), which are endoparasites in aquatic tetrapods but mainly infest anurans and freshwater chelonians (see Verneau, 2004). These parasites are found as adults in the urinary bladder, cloaca, pharyngeal cavity and conjunctival sacs of terrapins (see Morrison and Du Preez, 2011). They have a reproductive strategy that is well adapted to the ecology of their hosts that are primarily aquatic and they have a direct life cycle with free swimming larvae (oncomiracidia). They were assumed to be mostly host and site specific (see Verneau, 2004) until Verneau *et al.* (2011) reported host-switching in confined environments from introduced American terrapins to native ones, namely *E. orbicularis* and *M. leprosa*. The Mediterranean pond terrapin, which originated in the western region of North Africa according to Fritz *et al.* (2006), is mainly distributed in countries surrounding the Mediterranean Sea, namely Tunisia, Algeria and Morocco in North Africa, as well as Spain, Portugal and France in southern Europe

(Bonin *et al.,* 1998). While *M. leprosa* is considered as “Least Concern” in North Africa according to the IUCN criteria (Cox *et al.,* 2006), it is classified as “Vulnerable” in the European Red List of Reptiles and in the Spanish Red List (Da-Silva, 2002; Cox and Temple, 2009) and as “Endangered” in France (IUCN France, MNHN and SHF, 2009) where it occurs only in the Languedoc-Roussillon province, more specifically in the Pyrénées Orientales region. Understanding how *T. s. elegans* may impact native terrapin species is fundamentally important when considering conservation issues (Luiselli *et al.,* 1997; Cadi and Joly, 2004). Therefore our objectives were to determine the diversity of polystome species within *M. leprosa* and to what extent host-switching may take place between the introduced invasive terrapin species and the native Mediterranean pond terrapin in wild habitats of northern Spain and southern France.

2.2 Materials and methods

2.2.1 Host sampling (M. leprosa and T. s. elegans)

Traps were set in rivers, streams and ponds where terrapins were observed or within habitats that might be suitable for terrapins. Nine sites were investigated in natural water bodies of the Pyrénées Orientales region in southern France that correspond to the Agly, Baillaury, Basse, Fosseille, Têt (three distinct sites next to the villages of Canet, Bompas and Corneilla-la-Rivière) and Tech rivers, and to a small canal that flows to the brackish lagoon of Salses-Leucate next to the village of St. Hippolyte (Fig. 2.1). Six sites were explored along small rivers in the Catalonia province (Northeastern Spain), namely Anyet, Orlina, Merdanc, Llobregat, Reguerada and Riudarenes (Fig. 2.1). Another locality in the Aragon province (Northern Spain) was sampled in an oxbow of the Ebro River, namely La Alfranca. Finally, four other localities were also investigated in Algeria, namely Rouina, Réghaïa, El Amra and Oued Rhiou (Fig. 2.1). Crayfish traps were baited with pork liver, left overnight and removed the following day. Captured individuals of *M. leprosa* were then marked in the field by making marginal cuts on the carapace scutes following an international procedure for Capture-Mark-Recapture of terrapins. Marked terrapins were taken back to the experimental place for parasite screening over a period of usually three – four days. They were released at the exact locality where collected after the screening process. Invasive *T. s. elegans* were not released but euthanized by cardiac injection of 10% sodium pentabarbitone (Euthapent, Kryon Laboratories, South Africa) following French national rules for invasive species.

Figure 2.1: Map showing the sample sites in Algeria, northern Spain and southern France where *M. leprosa* was monitored for polystomes. The circles with continuous and dashed lines represent various infection sites within terrapins *M. leprosa* and *T. s. elegans*, respectively. Blue corresponds to polystomes from the pharyngeal cavity, red to polystomes from the urinary bladder and green to polystomes of the conjunctival sacs. No color indicates that no infection was detected for a specific infection site.

2.2.2 Parasite sampling (polystomatids)

Because *M. leprosa* is a protected species in Europe, we followed a non-invasive method for parasite screening (Verneau *et al.,* 2011). Terrapins were kept individually at room temperature for three consecutive days in plastic boxes containing water to a depth of about 30 mm. When infected with mature polystomes, parasite eggs are released and washed out from the infection sites, i.e., the pharyngeal cavity and/or conjunctival sacs, or excreted with urine from the bladder. Water was then filtered daily through a set of sieves of 500 µm and 100 µm, respectively. Polystome eggs are retained on the 100 µm sieve while the 500 µm sieve retain most of the debris that foul the water. The content of the 100 µm sieve was then rinsed into a

Petri dish using a wash bottle and eggs were searched for and collected using a dissecting microscope. They were identified by their orange-brown colour and pear (for parasites found either in the urinary bladder or the pharyngeal cavity) or fusiform shape (for parasites found in the conjunctival sacs) (Fig. 2.2) and preserved in 70% molecular grade ethanol pending DNA extraction. A few individuals of *M. leprosa* collected in Algeria as well as specimens of *T. s. elegans* were dissected and adult parasites preserved following the same procedure used for polystome eggs.

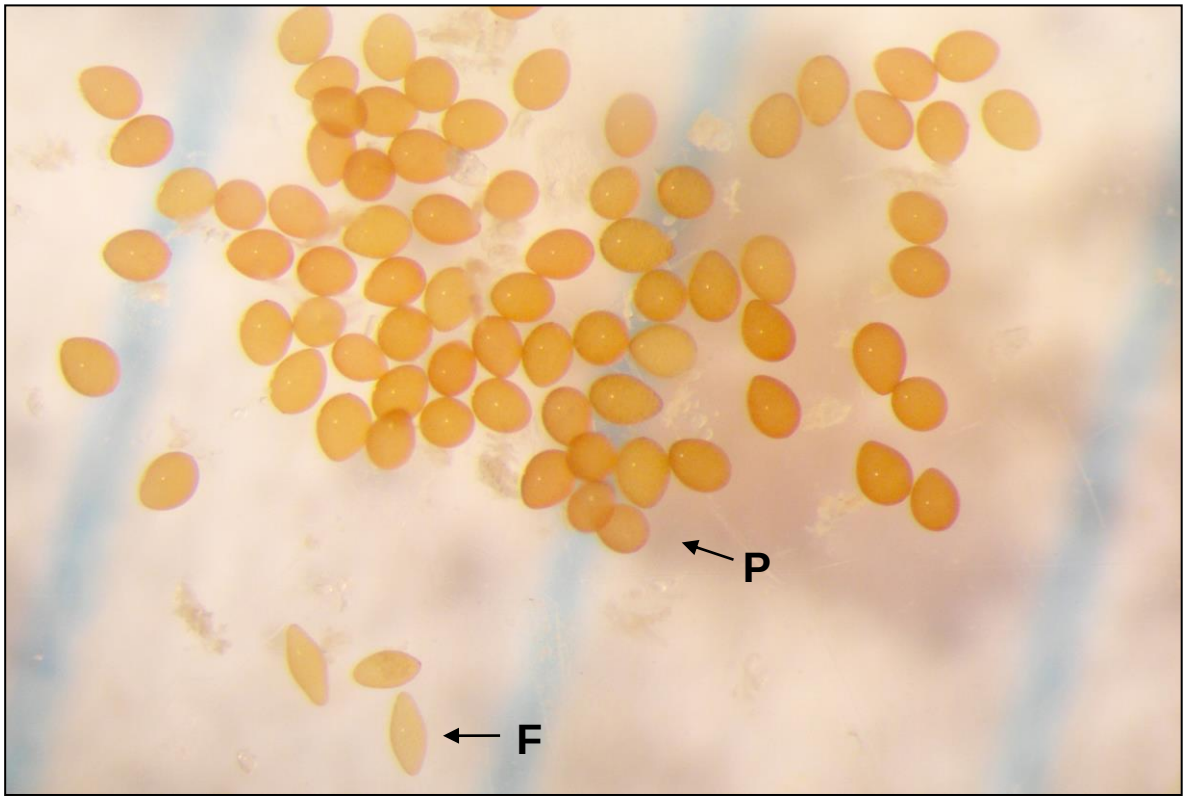

Figure 2.2: Polystome eggs were identified by their orange-brown colour and pear **(P)** (for parasites found either in the urinary bladder or the pharyngeal cavity) or fusiform **(F)** shape (for parasites found in the conjunctival sacs).

2.2.3 Molecular experiments

Polystome eggs and adult parasites were removed from ethanol and lyophilized by using a centrifugal evaporator (Universal Vacuum System Plus UVS400A). DNA extractions were carried out for 30 minutes at 55°C with 100 µL of 10% Chelex and 20 µL of proteinase K 10 mg mL^{-1}. Eggs were then grounded with a micro-pestle and DNA extractions were completed for 30

minutes at the same temperature. Enzymatic reaction was then stopped at 100°C for 15 minutes and DNA samples were stored at 4°C until used for PCR.

Amplification of the partial Cytochrome c Oxidase I (COI) gene and purification of PCR products were done according to the procedure developed in Verneau *et al.* (2009) and Du Preez *et al.* (2010), with the forward LCO1p (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') (Littlewood *et al.,* 1997) and reverse HCOX1R (5'-AACAACAAACCAAGAATCATG-3') primers. PCR followed one initial step of 5 minutes at 95°C for long denaturation, 30 cycles of 1 minute at 94°C for denaturation, 1 minute at 48°C for annealing, 1 minute at 72°C for elongation and one final step of 10 minutes at 72°C for terminal elongation, yielding a product of approximately 360 bp that was checked on a 1% agarose gel. Each DNA sample was amplified three times independently in 25 µL final volume and PCR products were sent to the Genoscreen Company (Lille, France) for purification and sequencing that was performed with either HCOX1R or both PCR primers.

2.2.4 Sequence analysis

Sequence chromatograms were first checked with the program Genious (Biomatters Ltd) and then edited using the MEGA version 5 software (Tamura *et al.,* 2011). New sequences were aligned with published sequences retrieved in Verneau *et al.,* (2011) using Clustal W (Thompson *et al.,* 1994). Two polystome species of amphibians were also included in the analyses for rooting the tree. A Minimum Evolution (ME) tree based on the Kimura-2 parameters distance was constructed with the MEGA software and a bootstrap test (1000 replications) was applied to assess for its robustness. Absolute differences (p-distance) between each pair of sequences were also measured for species identification, regarding the specific COI divergence threshold determined by Verneau *et al.,* (2011) on chelonian polystomes.

2.2.5 Naming of the various sequences

Chelonian polystomes can be identified to the genus level based on the number of hamuli, namely *Neopolystoma* with no hamuli, *Polystomoidella* with one pair and *Polystomoides* with

two pairs. When sequences were obtained from previously described adult parasites, they were named according to the systematic of the species followed by its haplotype number (for example *Polystomoides tunisiensis*H25). For undescribed species, only the genus name was given followed by the corresponding haplotype number (for example *Neopolystoma* sp6H21). On the opposite, when sequences were obtained from polystome eggs, the corresponding haplotype number was just assigned (for example X. spH39). However if those sequences were identical to sequences characterizing adult polystomes, they were named following the nomenclature given to adult parasites.

2.2.6 Estimate of species diversity

Verneau *et al.,* (2011) showed that polystomes occupying the same microhabitat within terrapins of the same species always had genetic divergence levels (p-distance) lower than 2% in the COI. On the other hand, genetic divergence levels between polystomes collected from the same biological niche within terrapins of distinct species were always higher than 2% in the COI, although terrapins occurred in sympatry. It was thus hypothesized that the divergence threshold for chelonian polystome delineation was about 2% (Verneau *et al.,* 2011). The diversity of polystome species infecting *M. leprosa* and *T. s. elegans* was therefore evaluated from the ME tree following p-distance comparisons of closely related haplotypes.

2.3 Results

2.3.1 Prevalence of infected hosts

Field investigation results are listed in Table 2.1. Among the 16 sampling sites where *M. leprosa* was surveyed in France and Spain, *T. s. elegans* was collected only at six of them. *M. leprosa* was found to be infected by polystomes almost everywhere except at the Basse and Têt (Corneilla-la-Rivière) rivers in France and at Reguerada and Riudarenes in Spain. Of the 389 *M. leprosa* specimens screened for parasite eggs in both countries, 144 (37%) were found to be infected as they released either pear or fusiform shaped eggs. Of the 28 *T. s. elegans* specimens screened, 14 (50%) were found to be infected, releasing only pear shaped eggs at three distinct sites, a canal next to St. Hippolyte, the Têt (Bompas) and Fosseille rivers.

Table 2.1: Sampling localities for *M. leprosa* (*M. l.*) and *T. s. elegans* (*T. s. e.*) in southern France (Fr), northern Spain (Sp) and Algeria (Alg), with GPS coordinates, number of individuals sampled for polystomes, number of infected hosts and prevalence.

Sampling localities	**GPS coordinates**	**No. of individuals sampled**	**No. of individuals infected with prevalence (%)**
St Hippolyte (Fr)	42°48'17.030"N / 2°58'15.09"E	12 (*M. l.*) 3 (*T. s. e.*)	10 (83.3%) 1 (33%)
Basse (Fr)	42°38'04.150"N / 2°46'30.28"E	12 (*M. l.*)	0
Agly (Fr)	42°45'38.020"N / 2°55'29.51"E	14 (*M. l.*) 1 (*T. s. e.*)	7 (50%) 0
Têt (Bompas) (Fr)	42°42'54.430"N / 2°56'02.48"E	16 (*M. l.*) 8 (*T. s. e.*)	7 (43,75%) 6 (75%)
Têt (Canet) (Fr)	42°42'34.690"N / 3°00'08.18"E	1 (*M. l.*)	1 (100%)
Têt (Corneilla-la-Rivière) (Fr)	42°41'25.400"N / 2°43'36.88"E	10 (*M. l.*)	0 (0%)
Tech (Fr)	42°35'07.490"N / 2°59'04.76"E	35 (*M. l.*) 6 (*T. s. e.*)	14 (40%) 0
Baillaury (Fr)	42°27'45.850"N / 3°05'27.16"E	154 (*M. l.*)	42 (27,3%)
Fosseille (Fr)	42°40'00.920"N / 2°58'00.80"E	45 (*M. l.*) 10 (*T. s. e.*)	28 (62,2%) 7 (70%)

Anyet (Sp)	42°21'39.261"N / 2°58'39.45"E	31 (*M. l.*)	12 (38,7%)
Orlina (Sp)	42°22'37.203"N / 3°01'50.56"E	23 (*M. l.*)	16 (69,6%)
Llobregat (Sp)	42°21'14.287"N / 2°54'08.70"E	10 (*M. l.*)	1 (10%)
Merdanc (Sp)	42°22'01.908"N / 3°00'26.68"E	13 (*M. l.*)	4 (30,7%)
Reguerada (Sp)	42°23'18.466"N / 3°03'08.98"E	7 (*M. l.*)	0 (0%)
Riudarenes (Sp)	41°49'18.600"N / 2°42'20.06"E	4 (*M. l.*)	0 (0%)
La Alfranca (Sp)	41°36'17.568"N / 0°45'43.72"E	2 (*M. l.*) 1 (*T. s. e.*)	2 (100%) 0
Rouina (Alg)	36°15'02.930"N / 1°49'00.45"E	16 (*M. l.*)	15 (93,75%)
Lake of Réghaïa (Alg)	36°46'16.700"N / 3°20'10.85"E	19 (*M. l.*)	3 (15,78%)
El Amra (Alg)	36°18'18.720"N / 1°50'31.81"E	10 (*M. l.*)	3 (30%)
Oued Rhiou (Alg)	35°58'24.010"N / 0°55'22.73"E	1 (*M. l.*)	1 (100%)

2.3.2 Haplotype diversity within polystomes of M. leprosa and T. s. elegans

A total of 253 new sequences were obtained from parasite eggs or adults, among which 218 from polystomes of *M. leprosa* and 35 from polystomes of *T. s. elegans*. They were subsequently aligned with COI nucleic acid sequences characterizing chelonian polystome species. A ME tree was first constructed from the analysis of all COI sequences (not shown). It helped to sort out groups of identical sequences and to collapse them into unique haplotypes (see Appendix A). A total of 13 new haplotypes not present in the database of Verneau *et al.,* (2011) were found (GenBank Accession numbers XXX to YYY), among which 11 characterized polystomes of *M. leprosa* (H57, H59, H69, H70, H78, H80, H82, H83, H85, H86 and H87) and two characterized polystomes of *T. s. elegans* (H77 and H81). Two other haplotypes (H55 and H88) were also identified from polystome eggs and adults collected from *T. s. elegans* in the Turtle farm of Sorède (France) and from *Kinosternon leucostomum* (Duméril and Bibron) of Costa Rica, respectively (GenBank Accession numbers XX and YY). At the end, 66 haplotypes were retained to analyze their phylogenetic relationships and to identify clades of interest (Fig. 2.3).

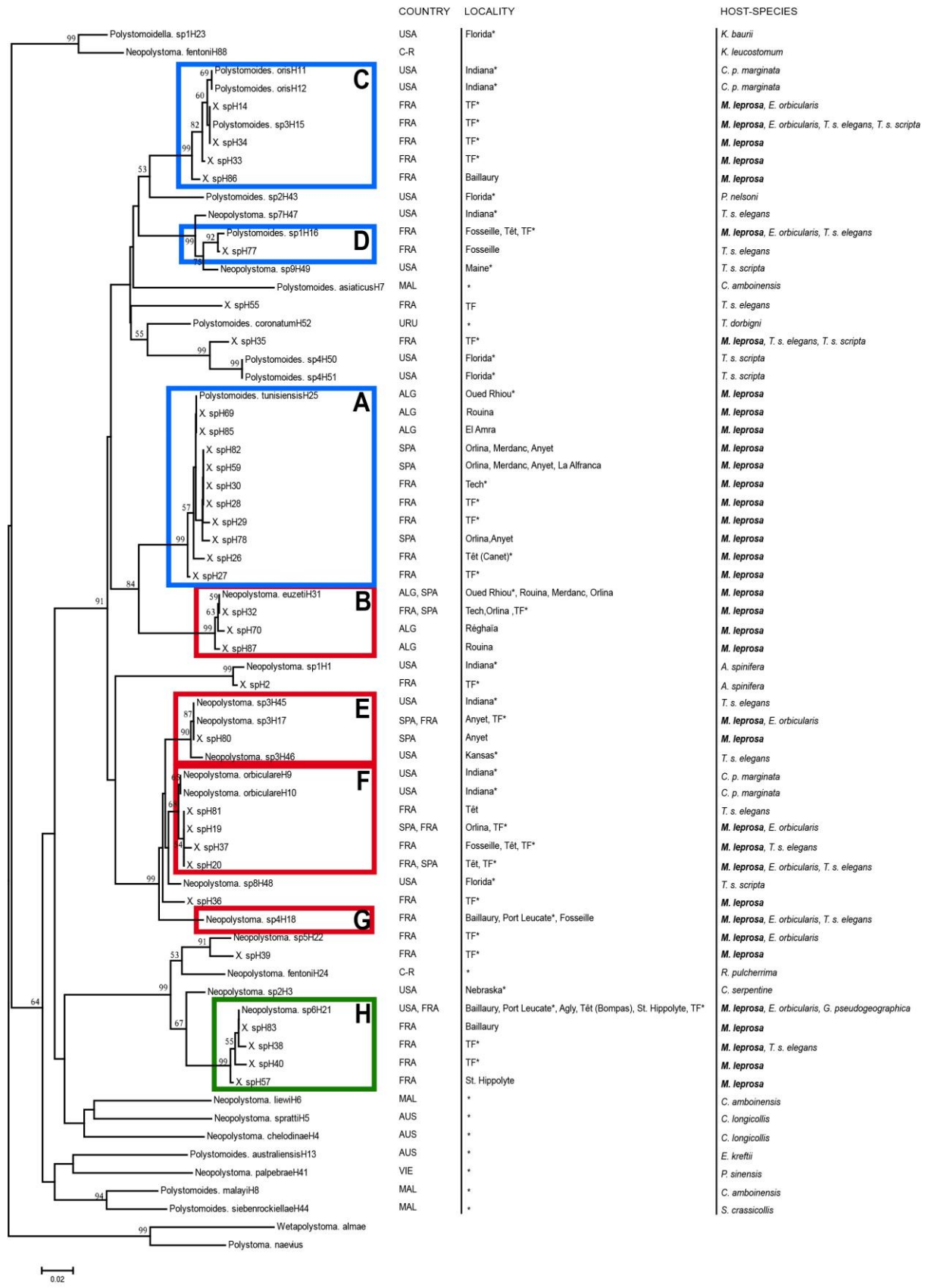
COUNTRY
LOCALITY
HOST-SPECIES
Polystomoidella. sp1H23 USA Florida* K. baurii
Neopolystoma. fentoniH88 C-R K. leucostomum
Polystomoides. orisH11 USA Indiana* C. p. marginata
Polystomoides. orisH12 USA Indiana* C. p. marginata
X. spH14 FRA TF* M. leprosa, E. orbicularis
Polystomoides. sp3H15 FRA TF* M. leprosa, E. orbicularis, T. s. elegans, T. s. scripta
X. spH34 FRA TF* M. leprosa
X. spH33 FRA TF* M. leprosa
X. spH86 FRA Baillaury M. leprosa
Polystomoides. sp2H43 USA Florida* P. nelsoni
Neopolystoma. sp7H47 USA Indiana* T. s. elegans
Polystomoides. sp1H16 FRA Fosseille, Tét, TF* M. leprosa, E. orbicularis, T. s. elegans
X. spH77 FRA Fosseille T. s. elegans
Neopolystoma. sp9H49 USA Maine* T. s. scripta
Polystomoides. asiaticusH7 MAL * C. amboinensis
X. spH55 FRA TF T. s. elegans
Polystomoides. coronatumH52 URU * T. dorbigni
X. spH35 FRA TF* M. leprosa, T. s. elegans, T. s. scripta
Polystomoides. sp4H50 USA Florida* T. s. scripta
Polystomoides. sp4H51 USA Florida* T. s. scripta
Polystomoides. tunisiensisH25 ALG Oued Rhiou* M. leprosa
X. spH69 ALG Rouina M. leprosa
X. spH85 ALG El Amra M. leprosa
X. spH82 SPA Orlina, Merdanc, Anyet M. leprosa
X. spH59 SPA Orlina, Merdanc, Anyet, La Alfranca M. leprosa
X. spH30 FRA Tech* M. leprosa
X. spH28 FRA TF* M. leprosa
X. spH29 FRA TF* M. leprosa
X. spH78 SPA Orlina,Anyet M. leprosa
X. spH26 FRA Tét (Canet)* M. leprosa
X. spH27 FRA TF* M. leprosa
Neopolystoma. euzetiH31 ALG, SPA Oued Rhiou*, Rouina, Merdanc, Orlina M. leprosa
X. spH32 FRA, SPA Tech,Orlina ,TF* M. leprosa
X. spH70 ALG Réghaïa M. leprosa
X. spH87 ALG Rouina M. leprosa
Neopolystoma. sp1H1 USA Indiana* A. spinifera
X. spH2 FRA TF* A. spinifera
Neopolystoma. sp3H45 USA Indiana* T. s. elegans
Neopolystoma. sp3H17 SPA, FRA Anyet, TF* M. leprosa, E. orbicularis
X. spH80 SPA Anyet M. leprosa
Neopolystoma. sp3H46 USA Kansas* T. s. elegans
Neopolystoma. orbicularеH9 USA Indiana* C. p. marginata
Neopolystoma. orbicularеH10 USA Indiana* C. p. marginata
X. spH81 FRA Tét T. s. elegans
X. spH19 SPA, FRA Orlina, TF* M. leprosa, E. orbicularis
X. spH37 FRA Fosseille, Tét, TF* M. leprosa, T. s. elegans
X. spH20 FRA, SPA Tét, TF* M. leprosa, E. orbicularis, T. s. elegans
Neopolystoma. sp8H48 USA Florida* T. s. scripta
X. spH36 FRA TF* M. leprosa
Neopolystoma. sp4H18 FRA Baillaury, Port Leucate*, Fosseille M. leprosa, E. orbicularis, T. s. elegans
Neopolystoma. sp5H22 FRA TF* M. leprosa, E. orbicularis
X. spH39 FRA TF* M. leprosa
Neopolystoma. fentoniH24 C-R * R. pulcherrima
Neopolystoma. sp2H3 USA Nebraska* C. serpentine
Neopolystoma. sp6H21 USA, FRA Baillaury, Port Leucate*, Agly, Tét (Bompas), St. Hippolyte, TF* M. leprosa, E. orbicularis, G. pseudogeographica
X. spH83 FRA Baillaury M. leprosa
X. spH38 FRA TF* M. leprosa, T. s. elegans
X. spH40 FRA TF* M. leprosa
X. spH57 FRA St. Hippolyte M. leprosa
Neopolystoma. liewiH6 MAL * C. amboinensis
Neopolystoma. sprattiH5 AUS * C. longicollis
Neopolystoma. chelodinaeH4 AUS * C. longicollis
Polystomoides. australiensisH13 AUS * E. kreftii
Neopolystoma. palpebraeH41 VIE * P. sinensis
Polystomoides. malayiH8 MAL * C. amboinensis
Polystomoides. siebenrockiellaeH44 MAL * S. crassicollis
Wetapolystoma. almae
Polystoma. naevius
A
B
C
D
E
F
G
H
0.02

Figure 2.3: Minimum Evolution tree resulting from the analysis of 66 nucleic acid sequences obtained from polystomes sampled from captive and wild terrapin populations. Values along branches indicate the bootstrap proportions resulting from 1,000 resampling. Polystome species boxed in blue (A, D and C) are from the pharyngeal cavity, in red (B, E, F and G) from the urinary bladder, in green (H) from the conjunctival sacs. The assigned abbreviations next to each species indicate the countries where parasites were collected: ALG – Algeria; AUS – Australia; CR - Costa-Rica; FRA – France; MAL – Malaysia; SPA – Spain; USA - United States of America; VN – Vietnam; URU - Uruguay. Abbreviations used for terrapin species names are from top to bottom: *K. baurii* = *Kinosternon baurii*; *K. leucostomum* = *Kinosternon leucostomun*; *C. p. marginata* = *Chrysemys picta marginata*; *M. leprosa* = *Mauremys leprosa*; *E. orbicularis* = *Emys orbicularis*; *T. s. elegans* = *Trachemys scripta elegans*; *T. s. scripta* = *Trachemys scripta scripta*; *P. nelsoni* = *Pseudemys nelsoni*; *C. amboinensis* = *Cuora amboinensis*; *T. dorbigni* = *Trachemys dorbigni*; *A. spinifera* = *Apalone spinifera*; *R. pulcherrima* = *Rhinoclemmys pulcherrima*; *C. serpentine* = *Chelidra serpentine*; G. pseudogeographica = *Graptemys pseudogeographica*; *C. longicollis* = *Chelodina longicollis*; *E. kreftii* = *Emydura kreftii*; *P. sinensis* = *Pelodiscus sinensis*; *S. crassicollis* = *Siebenrockiella crassicollis*

2.3.3 Polystome species diversity within M. leprosa

Haplotypes characterizing polystomes infesting wild *M. leprosa* were subdivided into eight clades (A to H) according to the ME tree (Fig. 2.3). Because p-distance estimates between haplotypes within each clade were less than 2%, but higher between haplotypes among the distinct clades, all clades were considered as distinct polystome species.

Clade A: Polystomoides tunisiensis Gonzales and Mishra, 1977

It comprises eleven distinct haplotypes (H25, H26, H27, H28, H29, H30, H59, H69, H78, H82 and H85) and is considered as *P. tunisienis* which was collected for the first time from the pharyngeal cavity of *M. leprosa* at Oued Rhiou in Algeria (Verneau *et al.,* 2011). H25 characterizes polystome eggs and adults collected from a single terrapin in Oued Rhiou, H26 characterizes polystome eggs collected from a single specimen of *M. leprosa* near to Canet on the Têt river, H27, H28 and H29 characterize polystome eggs collected from a single captive specimen of *M. leprosa* and H30 characterizes polystome eggs collected from nine distinct

specimens of *M. leprosa* sampled in the Tech river (see Verneau *et al.,* 2011 and Appendix A). H59 characterizes polystome eggs collected from nine distinct specimens of *M. leprosa* in Spain, namely in Anyet, Merdanc, La Alfranca and Orlina, H69 characterizes two polystomes collected from a single specimen of *M. leprosa* in Rouina, H78 characterizes polystome eggs collected from eight distinct specimens of *M. leprosa* in Orlina and Anyet, H82 characterizes polystome eggs collected from nine distinct specimens of *M. leprosa* in Anyet, Merdanc and Orlina, and finally H85 characterizes a polystome collected from a single specimen of *M. leprosa* in El Amra.

Clade B: Neopolystoma euzeti Combes and Ktari, 1976

It comprises four distinct haplotypes (H31, H32, H70 and H87) and is considered as *N. euzeti* which was collected for the first time from the urinary bladder of *M. leprosa* at Oued Rhiou in Algeria (Verneau *et al.,* 2011). H31 characterizes one polystome adult collected from a single terrapin in Oued Rhiou (Verneau *et al.,* 2011), but also another polystome adult sampled from the urinary bladder of a single specimen of *M. leprosa* in Rouina and polystome eggs collected from five distinct specimens of *M. leprosa* in Spain, namely in Merdanc and Orlina. H32 characterizes polystome eggs collected from a single captive specimen of *M. leprosa* (Verneau *et al.,* 2011), but also polystome eggs collected from five distinct specimens of *M. leprosa* in Orlina and in the Tech river. Finally H70 and H87 characterize two polystome adults collected from two specimens of *M. leprosa* in Réghaïa and Rouina, respectively.

Clade C: Polystomoides oris Paul, 1938

It comprises seven distinct haplotypes (H11, H12, H14, H15, H33, H34 and H86) and is considered as *P. oris* which was collected for the first time from the pharyngeal cavity of wild American painted terrapins *Chysemys picta marginata* Agassiz, 1857 (see Verneau *et al.,* 2011). H11 and H12 characterize polystome adults collected from *C. p. marginata* of the USA (Verneau *et al.,* 2011). H14 characterizes polystome eggs of captive *M. leprosa* and *E. orbicularis*, H15 characterizes polystome eggs of captive *M. leprosa*, *E. orbicularis* and *T. s.*

elegans while H33 and H34 characterize only polystome eggs of captive *M. leprosa* (Verneau *et al.,* 2011). Finally H86 characterizes polystome eggs collected from six distinct specimens of *M. leprosa* in the Baillaury river.

Clade D: Polystomoides sp1 (see Verneau *et al., 2011)*

It comprises two distinct haplotypes, namely H16 and H77. H16 was assigned to *Polystomoides* sp1 which was collected for the first time from the pharyngeal cavity of a single captive specimen of *E. orbicularis* (Verneau *et al.,* 2011). It also characterizes polystome eggs collected from three individuals of *M. leprosa* and five of *T. s. elegans* sampled at the Fosseille river and from one specimen of *M. leprosa* and seven of *T. s. elegans* sampled at the Têt river, next to Bompas. H77 characterizes a polystome egg collected from a single individual of *T. s. elegans* at the Fosseille river.

Clade E: Neopolystoma sp3 (see Verneau *et al., 2011)*

It comprises four distinct haplotypes (H17, H45, H46 and H80). H45 and H46 were assigned to *Neopolystoma* sp3 which was collected for the first time from the urinary bladder of two specimens of American *T. s. elegans* (Verneau *et al.,* 2011). H17 characterizes two polystome adults collected from the same ecological niche of captive *E. orbicularis* and *T. s. elegans*, but also polystome eggs collected from four specimens of *M. leprosa* in Anyet. Finally H80 characterizes polystome egg collected from a single specimen of *M. leprosa* in Anyet.

Clade F: Neopolystoma orbiculare (Stunkard, 1916)

It comprises six distinct haplotypes (H9, H10, H19, H20, H37 and H81) and is considered as *Neopolystoma orbiculare* which was collected for the first time from the urinary bladder of American *C. p. marginata* (Verneau *et al.,* 2011). H9 and H10 characterize seven polystome adults collected from two terrapins in the USA (Verneau *et al.,* 2011). H19 characterizes polystome eggs collected from three captive specimens of *M. leprosa* and from one captive specimen of *E. orbicularis*, H20 characterizes polystome eggs collected from four captive

specimens of *M. leprosa*, but also from three captive specimens of *E. orbicularis* and from two captive specimens of *T. s. elegans* and H37 characterizes polystome eggs collected from two captive specimens of *M. leprosa* (Verneau *et al.,* 2011). H19, H20 and H37 also characterize polystome eggs collected from wild specimens of *M. leprosa* in Orlina (H19), in the Têt (H20 and H37) and Fosseille (H37) rivers, but also polystome eggs and adults collected from wild specimens of *T. s. elegans* in the Têt (H20) and Fosseille (H37) rivers. Finally H81 characterizes polystome eggs collected from a single specimen of *T. s. elegans* in the Têt river.

Clade G: Neopolystoma sp4 (see Verneau *et al., 2011)*

It comprises a single haplotype, namely H18 that was previously assigned to *Neopolystoma* sp4 collected for the first time from the urinary bladder of wild *E. orbicularis* in France (Verneau *et al.,* 2011). This haplotype also characterizes polystome eggs that were collected from 16 specimens of *M. leprosa* and from one specimen of *T. s. elegans* in the Baillaury and Fosseille rivers, respectively.

Clade H: Neopolystoma sp6 (see Verneau *et al., 2011)*

It comprises five distinct haplotypes (H21, H38, H40, H57 and H83). H21 was assigned to *Neopolystoma* sp6 collected for the first time from the conjunctival sacs of one specimen of the American Missipi map terrapin *Graptemys pseudogeographica* (Gray, 1831) (see Verneau *et al.,* 2011). H21 also characterizes polystome eggs collected from two French specimens of wild *E. orbicularis* and captive *M. leprosa*, respectively (Verneau *et al.,* 2011), but also polystome eggs and adult parasites collected from four specimens of *M. leprosa* in the Baillaury and polystome eggs collected from two specimens of *M. leprosa* in the Agly river, from one specimen of *M. leprosa* in the Têt river and from five specimens of *M. leprosa* at St. Hippolyte. H38 characterizes polystome eggs of captive *M. leprosa* and *T. s. elegans* terrapins while H40 only characterizes polystome eggs of captive specimens of *M. leprosa* (Verneau *et al.,* 2011). Finally H57 and H83 characterize polystome eggs collected from two specimens of *M. leprosa* at St. Hippolyte and in the Baillaury river, respectively.

2.4 Discussion

2.4.1 Polystome diversity within M. leprosa

Due to limited interspecific differences in morphological traits used in identifying polystome species (Tinsley, 1973), it is often very complicated or even impossible to identify a polystome only on morphological characteristics. Thus, emphasis has been placed on host-specificity although Pichelin (1995) stated that host identity cannot be used as a reliable taxonomic character among terrapin polystomes. Whereas amphibian polystomes are only found in the urinary bladder of post-metamorphic frogs, terrapin polystomes are known from three distinct biological niches, namely the urinary bladder, pharyngeal cavity and conjunctival sacs. The Southeast Asian box terrapin *Cuora amboinensis* (Daudin, 1801), for example, harbours *Neopolystoma liewi* Du Preez and Lim, 2000 within conjunctival sacs, *Polystomoides asiaticus* Rohde, 1965 in the pharyngeal cavity, *Polystomoidella mayesi* Richardson and Brooks, 1987 and *Polystomoides malayi* Rohde, 1963 in the urinary bladder. This explains the relatively high diversity of chelonian polystome species, namely to about 50 species (see Verneau, 2004; Morrison and Du Preez, 2011), in comparison to the low diversity of freshwater chelonians, i.e. about 200 species (Bonin *et al.,* 1998). Because DNA barcoding has proven to be an excellent tool for exploring biodiversity the last two decades (Darling and Blum, 2007; Meusnier *et al.,* 2008; Valentini *et al.,* 2009), considering up to eight distinct polystome species within allopatric *M. leprosa* populations along its natural range, is not unrealistic. Among those parasites, four of them were recorded from the urinary bladder, i.e., *N. euzeti*, *N. orbiculare* and two undescribed species named for convenience *Neopolystoma* sp3 and *Neopolystoma* sp4 in Verneau *et al.* (2011). Three other species were reported from the pharyngeal cavity, i.e. *P. tunisiensis*, *P. oris* and an undescribed species named *Polystomoides* sp1 by Verneau *et al.* (2011). The last species was located in the conjunctival sacs and named *Neopolystoma* sp6 by Verneau *et al.* (2011).

Although we cannot rule out that some polystomes are not specific to their hosts in natural environments and that some host species may be infected by distinct polystome species within

the same microhabitat, misidentification of host and/or parasite species may lead to false interpretations about evolutionary processes that generated parasite diversity. *Polystomoides ocellatum* (Rudolphi, 1819), for instance, was described from the pharyngeal cavity of the European pond terrapin *E. orbicularis* and later reported from the same ecological niche within *M. leprosa* in Morocco (Combes and Thiery, 1983). We showed from a DNA barcoding survey (data not shown) that *P. ocellatum* is in fact specific to its host and has never been found on *M. leprosa*. Similarly *Neopolystoma fentoni* Platt, 2000 has been described and recovered from the conjunctival sacs of two distinct host species, namely *Rhinoclemmys pulcherrrima* (Gray, 1855) and *K. leucostomum* of Costa Rica and considered as a single species. According to COI p-distances (this study) and the ME tree (Fig. 2.3), *N. fentoni* (H24 and H88) should be split into two distinct species, each of them being specific to their respective host (Fig. 2.3). On the opposite, distinct polystomes species were reported from the same biological niche within the same host species, as is the case for instance for the two species *Polystomoides nabedei* Kulo, 1980 and *Polystomoides chabaudi* Euzet and Combes, 1965 that were described from the urinary bladder of their chelonian host species *Pelomedusa subrufa* (Lacépède, 1788), in Togo and Madagascar, respectively. A recent phylogeographic survey that was conducted on the widely distributed helmeted terrapin *P. subrufa* in Africa and Madagascar has shown this terrapin could be a complex of up to nine non-overlapping species (Vargas-Ramírez *et al.,* 2010). Therefore, we might also consider that the two polystomes *P. nabedei* and *P. chabaudi* infect the same ecological niche of two distinct host species. Concerning *M. leprosa*, it has been shown that this species originated in North Africa and dispersed to the Iberian Peninsula afterwards (Fritz *et al.,* 2006). Although two genetic lineages were identified from analysis of the cytochrome b suggesting the existence of two subspecies, i.e., *Mauremys leprosa leprosa* (Schweigger, 1812) which is confined in the Iberian Peninsula, France and northern Morocco, and *Mauremys leprosa saharica* Schleich, 1996 which is confined in southern Morocco, eastern Algeria and Tunisia (Fritz *et al.,* 2006), misinterpretations in systematics of *M. leprosa* and their parasites cannot explain the occurrence of eight distinct polystome species within host populations (Fig. 2.3). Thus our results suggest that *M. leprosa* may be infected by numerous

polystome species in natural environments and that polystome diversity may be greater than expected, at least within the Mediterranean pond terrapin.

2.4.2 Patterns and processes of polystome evolution within M. leprosa populations

In Algeria, *T. s. elegans* has never been recorded in natural environments, probably because the pet trade in North Africa has been less than in Europe and Asian countries. This could explain why only both natural *M. leprosa* polystome species, namely *P. tunisiensis* and *N. euzeti*, were sampled from wild Algerian terrapins (Figs. 2.1 and 2.3). *P. tunisiensis* is also present in all Spanish investigated populations while *N. euzeti* occurs only in Merdanc and Orlina. In France, these two polystome species are only found from the Tech river *M. leprosa* population. These results were expected as exotic American terrapin species, particularly *T. s. elegans*, were rarely observed at Spanish sites and are not very common in the Tech River. One may therefore consider at this stage parallel evolution between each of these two polystome species and their natural host species, namely *M. leprosa*. However molecular results should be investigated more in depth from analysis of genetic networks and after a larger parasite sampling in the Iberian Peninsula and in the Maghreb countries to conclude.

Although both natural polystome species were found within Spanish *M. leprosa* populations, two other species, namely *Neopolystoma* sp3 and *N. orbiculare* were found in Anyet and Orlina, respectively (Figs. 2.1 and 2.3). *Neopolystoma* sp3 is an undescribed species that infests the urinary bladder of wild American *T. s. elegans*, while *N. orbiculare* is known as the natural polystome species that infests the urinary bladder of *C. p. marginata* (Stunkard, 1916). Because no American terrapin was reported from both localities, one may question the occurrence of these two non-native parasite species in *M. leprosa*. One possibility is that each parasite was introduced in both sites by *T. s. elegans* and *C. p. marginata*, respectively, before they disappeared from the natural environments. If this hypothesis cannot be ruled out, it seems very unlikely because both terrapin species have never been documented at these sites. Furthermore *C. p. marginata* was also not exported as much as *T. s. elegans* that can be found in various natural environments across the world. The other possibility that may be considered

is that *M. leprosa* was infected at another site, or in captivity, before it was introduced into the wild. This hypothesis seems more likely due to the fact that non-native polystomes are able to infect a broad range of terrapins in captivity (Verneau *et al.,* 2011), and since many terrapin farms and people keep a variety of endemic and exotic terrapins such as *T. s. elegans* and *C. p. marginata* whereby native terrapins could be introduced at a later stage in natural environments.

The occurrence of the three polystome species, namely *P. oris*, *Neopolystoma* sp6 and *Neopolystoma* sp4, which are reported from the *M. leprosa* population of the Baillaury in France (Figs. 2.1 and 2.3), may be explained in the same way as above. Regarding the two former species, *P. oris* is a parasite that infests the pharyngeal cavity of wild American *C. p. marginata* (Paul, 1938) whereas *Neopolystoma* sp6 is an undescribed species that infests the conjunctivals sacs of wild American *G. pseudogeographica* (Verneau *et al.,* 2011). Because these two host species have never been observed in this river system, in spite of a very intensive sampling at this site since 2006, and since no program of reintroduction has taken place for *M. leprosa* in France, some native already infected terrapins may probably have been released into this natural environment. This hypothesis is supported by recent genetic analyses that show from the analysis of the cytochrome b mitochondrial marker that some *M. leprosa* terrapins of the subspecies *M. l. saharica* in the Baillaury would have been illegally translocated (Palacios *et al.,* submitted). The same hypothesis may be applied to interpret the occurrence of *Neopolystoma* sp6 within *M. leprosa* terrapins in the small canal next to the village of St. Hippolyte and in the Agly and Têt rivers. At last, regarding *Neopolystoma* sp4, it was previously reported from wild specimens of *E. orbicularis* in the pond of Port Leucate in France (Verneau *et al.,* 2011). The occurrence of that species within specimens of *M. leprosa* in the Baillaury river, but also within one individual of *T. s. elegans* in the Fosseille river, raises the same questions about that parasite species. However because *Neopolystoma* sp4 has not yet been documented from wild American terrapins in their native range, we cannot conclude.

In the Têt and Fosseille rivers, the two polystome species, i.e., *N. orbiculare* and *Polystomoides* sp1, were reported from both *M. leprosa* and *T. s. elegans* terrapins (Figs. 2.1

and 2.3). Because *N. orbiculare* is a natural parasite of *C. p. marginata*, another American terrapin, and because *M. leprosa* and *T. s. elegans* terrapins share the same polystome at the Têt (H20) and Fosseille (H37) rivers, the most likely hypothesis is to consider parasite transfer between host species in the wild. Therefore, we may hypothesize that *T. s. elegans* serves as a carrier for *N. orbiculare* parasites and transmit them to native terrapins in natural environments. Regarding the undescribed species, *Polystomoides* sp1, although that parasite has never been reported from the pharyngeal cavity of wild American terrapins in their home range, it is likely a non-native polystome species for *M. leprosa*. Therefore, the same scenario can be highlighted to explain the occurrence of H16 within *M. leprosa* and *T. s. elegans* populations in both rivers. Considering *T. s. elegans* as a carrier for exotic polystome species in the wild is the most plausible hypothesis at this stage as this species is very common in confined environments and generally in contact with some other American terrapins. Once released into the natural environments, this species could transmit some parasites to native terrapins. However we cannot reject the alternative hypothesis which considers that some native terrapins may also be released in the field after being kept as pets with some American terrapins in closed environments.

2.5 Conclusion

We have shown that parasite host-switching is of big concern with reference to native *M. leprosa* terrapins in natural environments of Southern France and Northern Spain. As a result invasion of *T. s. elegans* and all parasites it carries could be a key stressor to endemic terrapin species. In a framework of ecological risk assessment (Keller and Lodge, 2002; Sergeant, 2002; Andersen *et al.,* 2004; Keller and Lodge, 2007; Keller and Perrings, 2011; Ricciardi *et al.,* 2011), we therefore recommend to evaluate potential adverse effects of new pathogens on indigenous *M. leprosa* populations in order to have a better idea on how these parasites spread and establish in natural environments and on the threats of these parasite introductions may pose.

CHAPTER 3

Neopolystoma sp. (Monogenea: Polystomatidae) egg production influenced by environmental temperature

"It doesn't make a difference what temperature a room is,
it's always room temperature."
- Steven Wright

3.1. Introduction

Parasites are usually introduced into new geographical regions following host introduction which may have negative or even devastating implications for human health and biodiversity (Poulin and Mouillot, 2003). The impact that a newly established host-parasite association will have on the host is determined by various factors such as the environmental conditions, distribution of the parasite, and the behaviour of the parasites and its hosts (Dunn, 2009; Prenter *et al.,* 2004; Torchin *et al.,* 2003). Parasitic infection dynamics is influenced by environmental temperature (Marcogliese, 2008; Karvonen *et al.* 2010; Tinsley *et al.* 2011). The prediction is, that temperature change could have a significant effect on the capability of a parasite to establish in a new environment. For internal parasites of endothermic animals, it is only the stages in the life cycle that do not occur in the host that are severely affected by fluctuations in environmental temperatures. This in turn could affect the infective stages of these parasites (Gannicott and Tinsley, 1998; Tinsley *et al.* 2011). However, in the case of parasites found in ectothermic animals, it is not only certain stages of the life cycle that are affected, but all of them (Tinsley *et al.* 2011), since ectotherms rely heavily on external environment for temperature regulation. There may be some change in the effects due to the host's behavior, such as in hosts that seek shelter from very low temperatures could protect their parasites, as well as the basking sessions that, in this case, terrapins undertake which could increase the physiological processes of their parasites (Gannicott and Tinsley, 1998; Tinsley and Jackson, 2002; Tinsley *et al.* 2011).

Freshwater terrapins are ectothermic reptiles that need daily basking to regulate their internal temperatures (Sturbaum, 1982).When temperatures increase during spring and summer times, terrapins are much more active and spend more time in water. They are hosts for a variety of parasites (McAllister, Bursey and Trauth, 2008; Zelmer and Platt, 2008), among other

polystomatids (Platyhelminthes, Monogenea) that are classified into three genera based on the presence/absence and number of hamuli located within the haptor: *Polystomoides* Ward, 1917 with one pair, *Polystomoidella* Price, 1939 with two pairs and *Neopolystoma* Price, 1939 with none. Polystomes are mostly host and site specific (see Verneau, 2004) and may occur either in the urinary bladder, pharyngeal or eye cavities (see Morrison and du Preez, 2011). However with the increase of the pet trade, some terrapins have been exported worldwide, particularly the North American Red-eared slider *Trachemys scripta elegans* (Reed and Gibbons, 2003). Once released into the wild, *T. s. elegans* can invade wetlands and may constitute feral populations (Cadi *et al.,* 2004). It has even been illustrated by parasite host switching between the Red-eared slider and indigenous terrapins in natural environments of Southern Europe (Meyer *et al.*, submitted), suggesting that host specificity for chelonian polystomes may be ruled out following host introduction into new environments.

The Mediterranean pond terrapin *Mauremys leprosa* (Schweiger, 1812) is a terrapin that occur in the Mediterranean basin, more precisely in Northern Africa (Morocco, Algeria and Tunisia) and Southern Europe (Portugal, Spain and France). This species is usually active from the end of winter to mid-autumn, where after they hibernate. Two polystome species (*P. tunisiensis* Gonzales and Mishra, 1977, and *N. euzeti* Combes and Ktari, 1976, found in the pharyngeal cavity and the urinary bladder respectively) were originally described from natural populations of *M. leprosa* from Tunisia (Combes and Ktari, 1976; Gonzales and Mishra, 1977). Terrapin polystomes have a unique reproductive strategy that is adapted to the lifestyle of its aquatic host. Polystome eggs are produced continuously by the parasite when the terrapin is kept in water, but not in large numbers. The eggs are not retained in the small uterus, but are released as soon as the host enters the aquatic environment, where they hatch after a fairly long period of time (Morrison and du Preez, 2011). Because we showed (Meyer *et al.,* submitted) that *M. leprosa* is infected by a non native polystome species (*Neopolystoma* sp.) in Southern France, our objectives were to investigate the influence of environmental temperatures on polystome egg production of that particular parasite and explain if the behaviour of exotic parasites is the same than native parasite species.

3.2 Material and Methods

3.2.1 Experimental design

As part of a bigger study, five *M. leprosa* terrapins infected with *Neopolystoma* sp. (Fig. 3.1) were collected in June 2011 from crayfish traps in the Baillaury river system situated in Banyuls sur Mer in southern France and marked according to an international procedure for Capture-Mark-Recapture. They were transported back to the laboratory and maintained individually outside over a 28 day period in 60 cm diameter plastic containers filled with water to the depth of about 50 mm (Fig. 3.2). Terrapins were acclimated over a period of two days before starting the experiment and fed every second day with pork liver. During the acclimation time for the terrapins, the water was not changed and containers were not moved to keep the terrapins free from any stress.

3.2.2 Parasite egg collection

A non-invasive technique (see Verneau *et al.* 2011) was used to collect polystome eggs over a period of 26 days. On a daily basis starting at 09h00 the water from the containers was poured through a pair of sieves, 500 µm and 100 µm respectively. The 500 µm sieve was used to collect large debris out of the water, whereas the 100 µm sieve retained polystome eggs and some equal size debris particles. The content of the 100 µm sieve was then rinsed into a Petri dish and screened under a dissecting microscope. Eggs collected from each terrapin were counted every day all over the experimental period and daily egg production for all five terrapins was averaged.

3.2.3 Data analysis

Local climatological data were obtained from the University of Perpignan weather station (http://gim.iut.univ-perp.fr/meteo/meteo_accueil.html). To avoid overestimating the goodness of fit as measured by the Pearson coefficient, homogeneity of variance (homoscedasticity) was analysed and a R^2 value was obtained. Homogeneity of variance distribution of average egg production data was tested with non-transformed data with residual (ZRESID = y axis) and predicted values (ZPRED = x axis). Average egg production data were normalized using Log^{10} transformation. A linear regression ANOVA with a confidence level of 95% ($p<0.05$) and Pearson correlation were performed to test the effect of environmental temperature on egg production, with the first variable as the independent variable and the second as the dependant variable. All analyses were performed using SPSS version 21 (IBM Corp.SPSS Inc., Armonk,NY, USA).

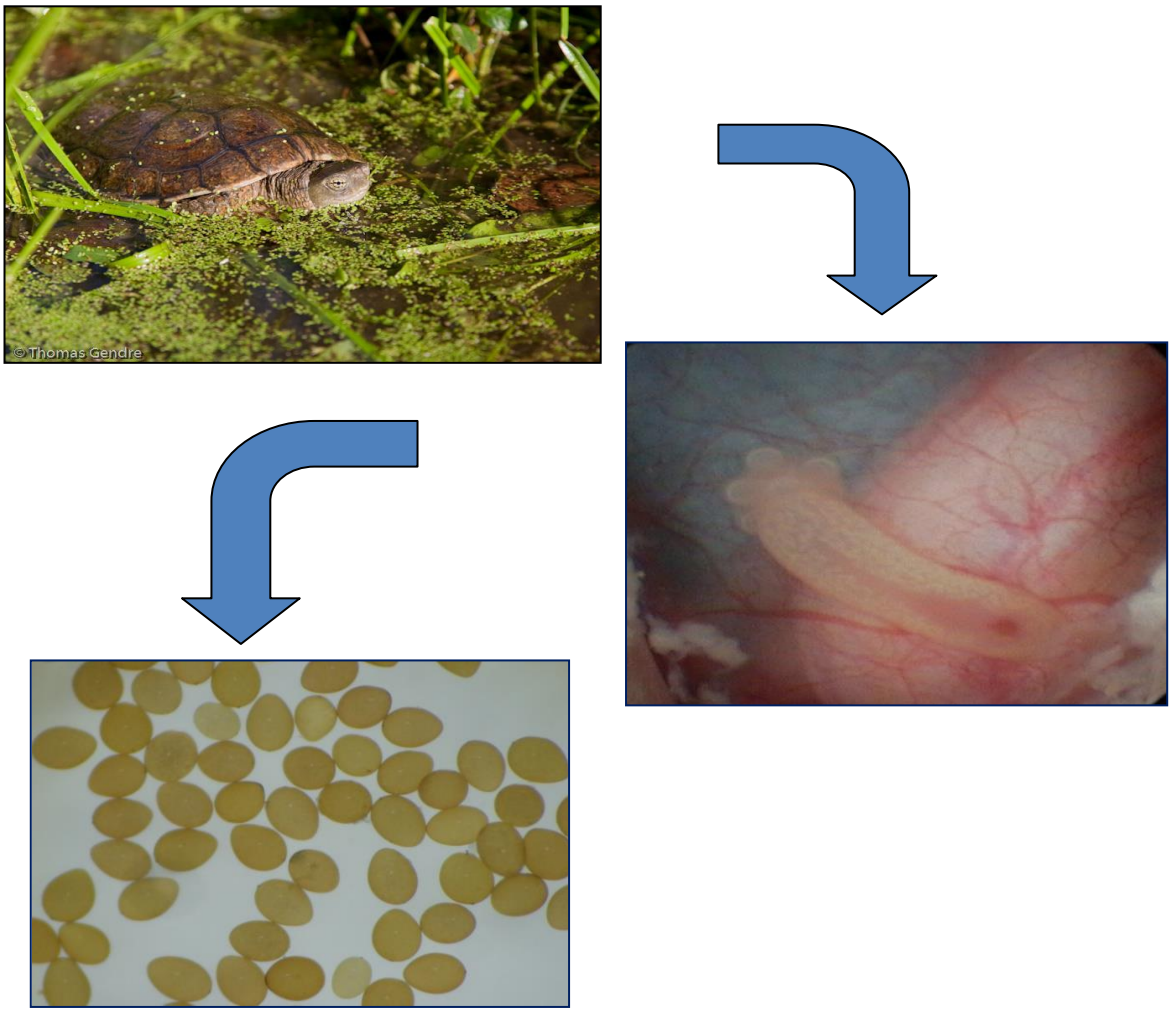

Figure 3.1: (a) The host species *Mauremys leprosa*. **(b)**. Mature *Neopolystoma* sp. located in the urinary bladder **(c)**. Polystome eggs.

Figure 3.2: *Mauremys leprosa* in a separate container. Tiles were placed to serve as basking spots after adding water.

3.3 Results

3.3.1 Parasite egg release

The regression standardized residual plot of the residuals versus predicted Y is shown in Figure 3.3. Its pattern indicates no uncertainty with the assumption that the residuals were normally distributed at each level of Y and constant in variance across levels of Y. Homogeneity of variance of average egg production data was tested with a residual scatter plot with standard regression in SPSS ($R^2 = 1.110^{-16}$). The mean number of polystome eggs collected daily for all 5 terrapins shows day to day variations ranging from 12.2 to 37.4 (Table 3.1).

Table 3.1: Total number of polystome eggs collected daily from each individual, with the mean number estimated from all 5 turtles.

Day	n Eggs harvested / day /turtle (Turtle no displayed below)					Ave. egg production	Ave. Tem	Min. Temp.	Max. Temp.
	177	260	313	330	336				
-2							17.5	15.4	23.3
-1							15.1	13.4	21.1
1	21	9	9	15	35	17.8	17.4	15.2	23.1
2	19	15	6	7	14	12.2	17.8	15.3	21.2
3	21	17	8	16	14	15.2	18.4	15.3	23
4	23	21	13	21	28	21.2	18.9	15.9	21.6
5	24	24	9	16	18	18.2	19.1	15.0	24.1
6	1	32	6	18	30	17.4	20.4	14.1	25.9
7	24	27	12	31	35	25.8	22.8	17.4	27.9
8	26	25	16	39	48	30.8	25.5	20.1	31.3
9	33	64	15	33	41	37.2	25.9	19.8	30.2
10	38	53	15	41	38	37	24.3	20.8	28.6
11	39	34	11	32	48	32.8	22.6	20.2	26.4
12	7	24	6	25	36	19.6	19.8	18.3	21.9
13	23	9	8	8	14	12.4	21.1	15.2	27.5
14	12	28	2	4	40	17.2	23.4	15.7	28.7
15	33	48	4	47	41	34.6	22.8	20.5	25.2
16	33	52	13	28	38	32.8	22.7	19.6	28.5
17	29	48	15	25	37	30.8	19.8	16.6	22.5
18	25	28	9	12	31	21	21.6	17.6	26.2
19	29	35	10	22	26	24.4	25.2	16.8	34.2
20	32	64	8	13	48	33	24.1	18.3	29.3
21	28	58	18	32	51	37.4	22.5	20.1	26.1
22	18	39	10	19	47	26.6	24.6	20.2	29.8
23	22	53	7	43	58	36.6	21.2	18.8	25.9
24	19	41	6	14	23	20.6	23.1	18.1	28.3
25	21	24	10	9	24	17.6	25.2	19.2	30.6
26	22	43	7	11	30	22.6	24.5	17.6	29.2

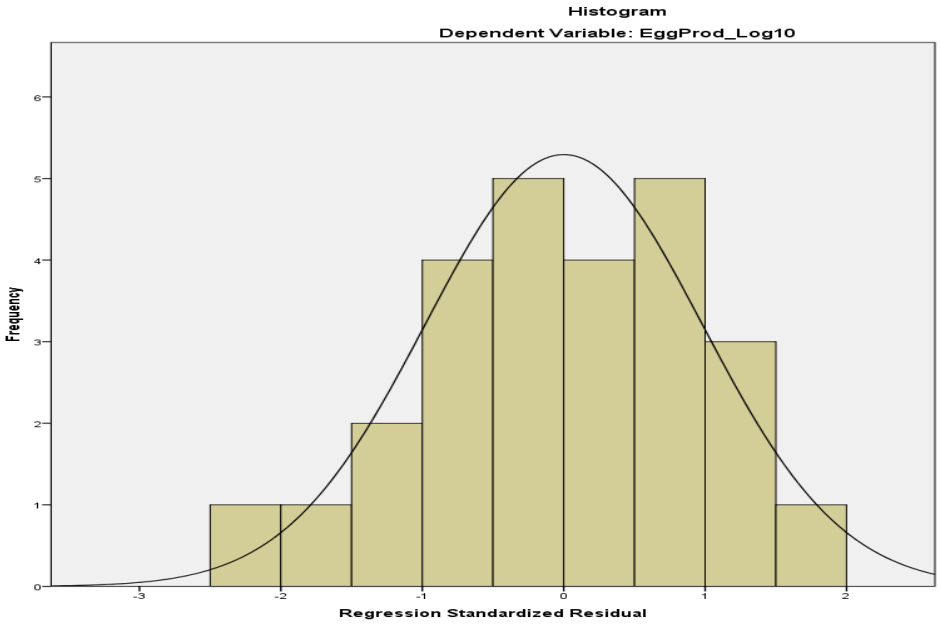

Figure 3.3: Regression standardized residual plot showing a normal distribution of average egg production data.

3.3.2 Correlation between environmental temperature and egg production

Days -1 and -2 represent temperature data two days prior to the onset of the experiment (Fig. 3.4 and table 1). Daily fluctuations in average environmental temperatures appear to be followed by fluctuations in egg outputs (Fig. 3.4). The Pearson's correlation indicates a moderate, positive correlation between average environmental temperature and average parasite egg production ($r = 0.531$, $P < 0.05$). Furthermore egg production decreases after a drop in temperature and increases after an increase in temperature with a two day lag pattern (Fig. 3.4).

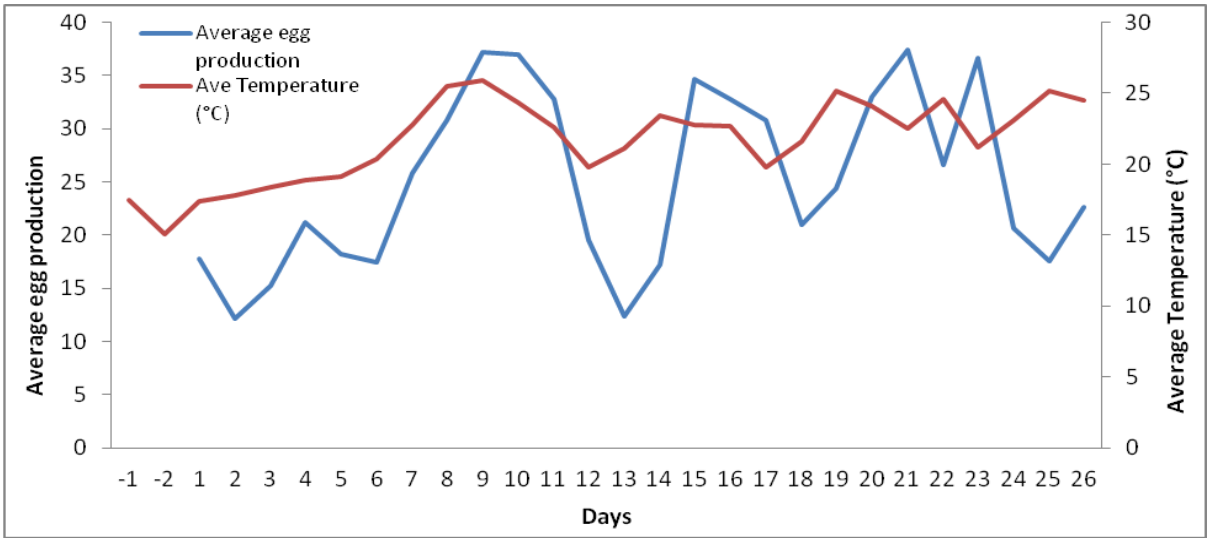

Figure 3.4: Correlation between environmental temperature and egg production over a 26 day period. The red line indicates average temperature and the blue line indicates average parasite egg release. Days -1 and -2 are indicative of temperature data two days prior to the start of the experiment.

3.4 Discussion

The influence of environmental temperatures and temperature fluctuations on the reproduction in monogeneans infecting ectothermic vertebrates has been studied by Kashkovskii (1982), Jackson and Tinsley (1988 a, b), Jackson and Tinsley (1998) and Gannicott and Tinsley (1998). In general, the maximum egg production by parasites takes place at environmentally relevant temperatures that are usually experienced by the host to be active. During the current study, an egg-laying rhythm was observed and continued both in warm (25.9 °C) and cooler (17.4 °C) temperatures. The increase of environmental temperatures resulted in the increase of polystome egg production, however, with a two day delay in response (Fig. 3.4). Terrapins function at their best within a range of optimum body temperatures that may vary, depending on the species (Bonin *et al.*, 2006). When temperatures increase during spring and summer times, terrapins bask during the day to raise their body temperature and accelerate their metabolism. As a result they are physiologically more “active” to mate and hunt in the waters. This indicates that parasites rely on behavioural and physiological traits of the terrapin host for their own reproduction. The two day lag between egg production and changes in environmental temperatures could be attributable to the release of host factors like hormones that once secreted may act and stimulate parasite reproduction. A few studies reported on the reproductive adaptability of monogenean species whereby egg production takes place at a broad range of temperatures depending on the species, for example: the parasite, *Pseudobenedenoides branchicola* Szidat, 1969 (Monogenea), found in the fish *Trematomus bernacchii* Boulenger, 1902, continues to produce eggs even at temperatures of -1 °C. The host and the parasite survive through these cold temperatures (Szidat, 1969). Kearn (1986) found that the eggs of most monogeneans hatch after 10 - 40 days in normal environmental temperatures (26 °C). However, the period it takes for an embryo to form in the egg decreases as the temperature increases, but the growth of the embryo is arrested at above and below a certain threshold temperature. The embryonation period of another monogenean parasite, *Benedenia seriolae* (Yamaguti, 1934), is also influenced by temperature. The embryonation period varied from 5 days for eggs incubated at temperatures of 24 – 28 °C up to 19 days for eggs incubated at a temperature of 14 °C. It was found that the maximum temperature for physiological tolerance of egg embryonation was 30 °C (Ernst *et al.* 2005). Other monogenean parasites lay their eggs in winter temperatures. This slows down or even seizes the development, these eggs only continuing to develop and hatching in early spring when the environment is at a preferred temperature (25 - 28 °C) (Paperna, 1963; Prost, 1963; Szidat, 1969). The overwintering eggs are the only source of infection in the new spring as the adult parasites do not survive the cold temperatures of the winter (Bychowsky, 1957). Bychowsky (1957) found the egg development period of *Dactylogyrus vastator,* Nybelin, 1924 (Monogenea) to be longer at low temperatures, discontinuing at temperatures below 4 °C. During autumn the adult parasites die and new infections in the spring are caused by the infective stages hatched

from eggs which survived the long cold months. However, there are other adult monogenean parasites that survive in their hosts throughout the winter and continue to produce eggs during the spring when the temperatures are favourable (Paperna, 1963; Prost, 1963; Szidat, 1969). The reproductive output of monogenean parasites could be dependent on various factors such as: environmental conditions, the age and size of the adult parasites, and the host itself according to the age, sex and health status of the host. Putting the differences between parasites aside, there are also variations in the egg production of individual parasites. Gannicott and Tinsley (1998) showed egg production by individual parasites of *Discocotyle sagittata* (Monogenea) (Leuckart, 1842) at constant temperatures can vary by 35 – 83 %. They suggested that the differences may be attributable to the host as an environment, host food availability and quality potentially influencing the hosts internal environment, or it could be simply the natural unpredictability of the parasites themselves.

Neopolystoma sp. showed a similar pattern to egg production in *Diplozoon* species. It showed a positive correlation with environmental temperature (see Macdonald and Jones, 1978). However, this is not the case for all poikilothermic hosts. Other polystomes, such as *P. integerrimum*, store gametes and vitelline cells during cold temperatures. This occurs when the host is inactive, with the monogenean parasite only producing and releasing eggs during warmer temperatures when the Pyrenean frog host, *R. temporaria*, enters the water during breeding season. This could be the same for the parasite and the terrapin host seeing that both amphibious animals have a similar internal system. In this case the highest temperature at which maximum egg production occurs varies between 4 – 8 °C (Combes, 1972). The low temperature whereby eggs were produced was of adaptive value seeing that the frogs breed during snow melt. Mills (1980), found that maximum egg production of *Transversotrema patialense* (Soparkae, 1924) (Digenea: Transversotrematidae), from the tropical fish host *Brachydanio rerio* (Hamilton-Buchanan), occurred at 29 °C. Egg production declined on both sides of this temperature and stalled at 35 and 17 °C respectively. Could this be the case in *Neopolystoma* sp.?

In this study, the optimum temperature for egg production could not be established since environmental temperature could not be regulated to maximum temperatures to determine at what maximum temperature egg production would stop, but the assumption can be made that maximum rates of egg production could occur at temperatures reached in the middle of summer due to higher temperatures. In turn, it can be said that as the terrapin hosts physiological processes slow down during winter, the same could happen for the parasite. So as the terrapin estivate, or in some cases even hibernate, the parasite could follow a same pattern by going into a resting phase over winter. Exotic parasites are also well adapted to new host species, and as a consequence may spread over the new host population and easily invade new species. Therefore it would be interesting to compare the rhythm of the same polystome species within its natural host species to conclude.

CHAPTER 4

Mediterranean pond terrapin *Mauremys leprosa* under threat? A relative risk method assessment study carried out along the Fosseille River, Pyrénées Orientales region, France

"Good risk management fosters vigilance in times of calm and instils discipline in times of crisis."

- Dr. Michael Ong

4.1. Introduction

Biodiversity is a very important non-renewable resource that once it is lost, we are unable to duplicate, substitute or restore it (Swanson, 1997). Together with the variability among living organisms at genetic, species and ecosystem level biodiversity is of critical importance to mankind (Bagri, McNeely and Vorhies, 1998). The widespread decline in biodiversity is taking place at an accelerating rate and became an important conservation issue (Glowka *et al.,* 1994; Novacek and Cleland, 2001; Sodhi *et al.*, 2004). The rapid decline in biodiversity is a result of the increase in disturbances such as; habitat loss or fragmentation; overexploitation of plant and animal species; pollution; climate change; agricultural, industrial and forestry developments and the introduction of invasive species (WRI, 1992; Novacek and Cleland, 2001; Sodhi *et al.*, 2004). Although species fluctuations and even extinctions is a natural process, it is as a result of human activities happening at an alarming rate (Pimm *et al.,* 1995; Leprieur *et al.,* 2008). With intercontinental flights in all directions, the world became a small place. A vast number of animals and plants are being transported all over the globe. As a result the integrity of the earth's biotic regions are being challenged (Vitousek *et al.* 1997; Kattenberg *et al.,* 1996). Human influences have caused many species to become under threat or even to be eliminated from their natural areas or habitats. Even in reserves, native species have been outcompeted or consumed by introduced species. Although introductions of invasive species could include species of economical importance for example; corn (*Zea mays*), domestic chicken (*Gallus* spp.) and even cattle (*Bos taurus*), roughly 10 – 30% of all the introductions become pests and cause serious environmental impacts (Primentel *et al.* 2001; Williamson and Fitter 1996). Various studies have been done on invasive species, but field studies have often been too distinctive to derive the general hypothesis of invasive species establishment and the effects it

could have on a community (Vermeij, 1996). In contrast laboratory setting, it is not always possible to replicate the external environmental factors that could have an influence in a natural environment (Doak *et al.,* 1998; Wardle, 2001). Some studies have been conducted to determine whether certain species have the potential to become invasive, cause impacts and in this manner, tried to design methods for prevention, detection, management and control efforts (Bomford *et al.,* 2005, 2009; Grosholz and Ruiz, 1996; Ricciardi and Rasmussen, 1998). Although it is possible to assess the risk of a species establishing itself in a new environment, the assumption is made by the investigator that any invasive species introduced into a new environment would have a negative effect (Hewitt and Hays, 2002). Another assessment of risk could be post introduction, where the negative effects must take place for the particular introduction to cause concern (Colnar and Landis, 2007). Invasive species could have a negative effect on native species in the environment, but is it the major risk factor that puts the native species at risk?

Wildlife experts often have the difficult task of analysing any shared ecological or life history characteristics of species that could have a risk on populations and even lead to declines or extinctions. Understanding these causes is of great importance to stop biodiversity loss. The highest risk of extinction involves species that have been classified as threatened in the World Conservation Union (IUCN) Red Lists. The causes of extinction risk of the threatened species may be detected when ecological characteristics of threatened and non-threatened species are compared (Rassi *et al.,* 2001). Analysing these risks can be difficult when only limited data are obtainable, and then still the experts need to provide a professional expert evaluation (Kotiaho *et al.*, 2005). A method used to predict these risks of impacts at a regional scale is Regional Scale Risk Assessment, using the Relative Risk Model (RRM) methodology (Landis and Wiegers 1997; Wiegers *et al.* 1998; Landis and Wiegers 2005) by combining scientific data with professional knowledge and experience. Although there is some debate about the use of RRM, it has been criticized (Cook *et al.,* 1999; Cormier *et al.*, 2000), authenticated and developed into the operational method presented by Landis (2004) and Colnar and Landis (2007). It has demonstrated its usefulness in evaluating and managing fauna and flora species in ecological systems (Cohen, 1988), water systems (O'Brien and Wepener, 2012), forests (Crome *et al.,* 1996), and other environmental research and management areas (Dixon and Ellison, 1996; Ellison, 1996; Wolfson *et al.,* 1996) as well as the possibility of species becoming invasive (Colnar and Landis, 2007). It is used in applied ecology to illustrate the logical or causal considerations of numerous sources of numerous stressors and the effect it has on numerous endpoints. This includes ecosystem dynamics and characteristics of the landscape that may affect the risk estimate (Landis and Wiegers, 1997).

The Mediterranean pond terrapin (*Mauremys leprosa*) is a terrapin species from the family Geoemydidae, which originated in the western region of North Africa (see Fritz *et al.*, 2006). It is mainly distributed in countries surrounding the Mediterranean Sea, namely Tunisia, Algeria and Morocco in North Africa, as well as Spain, Portugal and France in southern Europe (Bonin *et al.,* 1998). While *M. leprosa* is considered as “Least Concern” in North Africa according to the IUCN criteria (Cox *et al.*, 2006), it is classified as “Vulnerable” in the European Red List of Reptiles and in the Spanish Red List (Cox and Temple, 2009; Da-Silva, 2002) and as “Endangered” in France (IUCN France, MNHN and SHF, 2009) where it occurs only in the Languedoc-Roussillon province, more specifically in the Pyrénées Orientales region (Fig. 4.1). It inhabits nearly all accessible freshwater and brackish water bodies within its range, while preferring big, lasting water bodies (Keller and Busack, 2001). Some threats towards this species are habitat alteration, industrial and agricultural pollution, marsh drainage, aquifer water extraction, and fisheries by-catch. Competition from the invasive Red-eared slider (*Trachemys scripta elegans*) has been suggested as a major threat (Pleguezuelos, 2002).

The objective of this study was to use the RRM to assess the viability of the native Mediterranean pond terrapin (*M. leprosa*) in a small river system close to Perpignan in the south of France, taking into consideration not only the effect of the invasive Red-eared slider but other external human induced threats and environmental conditions. Risk is calculated for four source scenarios: (1) current conditions as observed on site, (2) increase of the number of the invasive *T. s. elegans* terrapins in the river system (3) complete removal of invasive terrapins from the river system (4) a sewage spill from a sewage plant situated upstream.

4.2. Material and Methods

4.2.1 Description of study area

The Fosseille River located close to Cabestany in the south of France (Pyrénées Orientales region), is a small river system that flows into the lagoon of Canet. The lagoon of Canet is connected to the ocean through a small water canal and as a result it is partially saline. The complete catchment of this river has been altered as a result of agriculture, urbanization, camping sites and roads. For the purpose of this study the river system was divided in to six sections, delineated by road bridges crossing the river (Fig. 4.1).

4.2.2 Risk Assessment Approach

Problem formulation

The approach to assess the viability of the endangered Mediterranean pond terrapin is classical in that it incorporates the problem formulation, conceptual model development, analysis and risk characterization phases. The RRM consists of 10 procedural steps that can be aligned with Ecological Risk Assessment frameworks (Landis and Wiegers,1997; Murray and Claassen, 1999; O'Brien and Wepener, 2012). The standardised terminology for RRM and definitions are listed in Table 4.1:

Table 4.1: A list of standardised terminology for RRM and definitions used within the context of this regional-scale risk assessment (adapted from O'Brien & Wepener, 2012)

Source	An entity, action or activity that releases to the environment or imposes on the environment a chemical, physical or biological stressor or stressors (USEPA, 1998).
Receptor	An ecological entity exposed to the stressor (USEPA, 1998).
Stressors	Any chemical, physical or biological entity that can induce an adverse response to the structure and function of an ecosystem (USEPA, 1998).
Habitats	Location where the receptor or group of receptors of the stressors assessed in the RRM lives. They are the physical ecosystem component/s that integrate the effects of stressors impacting on the system (Landis, 2005).
Assessment	Management goals, objectives or targets. An explicit expression of the environmental value that is to be protected, operationally defined by

endpoints	an ecological entity and its attributes (USEPA, 1998).
Ecological entity	A general term that may refer to a species, a group of species, an ecosystem function or characteristic, or a specific physical habitat. An ecological entity is one component of an assessment endpoint (USEPA, 1998).
Ranks	Unitless measures or scores assigned to source, stressor and/or habitats identified in an RRM according to a characterised ranking criteria unique to each entity that is usually based on a weighing factor. Ranks are then used in the calculation of risk in the RRM (O'Brien & Wepener, 2012; Landis, 2005).
Relative rankings	RRM method of assigning scores to individual source, habitat and/or endpoints in a comparable manner, i.e. the ranking of components of the RRM in a relative manner (O'Brien & Wepener, 2012).
Lines of evidence	Information derived from different sources or by different techniques that can be used to describe or interoperate risk estimates of endpoints (USEPA, 1998). Simply put, a line of evidence includes any useful set of data and/or associated analyses which can be used to provide information concerning the current state of an endpoint (Landis, 2005).
Risk rankings	Final risk score of risk regions within the RRM (O'Brien & Wepener, 2012).
Sensitivity	Refers to the robustness of the RRM assessment to withstand external influences (O'Brien & Wepener, 2012; Landis, 2005).
Uncertainty	Associated with the RRM analyses, uncertainty relates to there being a lack of sufficient knowledge within component/s of assessment to confidently accept the outcome of the assessment, in as much as the confidence of the outcome of the assessment should be considered in relation to the uncertainty of the assessment or components of the assessment (O' Brien & Wepener, 2012; Landis, 2005).

Ten steps for implementing the RRM are (O'Brien & Wepener, 2012):

Step 1: List key management goals for the area

The question to ask in this step was, what do you care about and where? The management goals are the first critical step of the risk assessment.

In this study, we wanted to assess the maintenance of the viability of the native Mediterranean pond terrapin (*M. leprosa*) populations in a small river system in the south of France, taking into consideration not only the effect of the invasive Red-eared slider (*T. s. elegans*) but other external manmade threats and environmental conditions.

Step 2: Create a map on which the possible sources and habitats applicable to the established management goals are indicated

First, the potential sources that were used for this study were identified. A detailed map of the area used in the study was constructed to establish the relationships between all the components of the RRM. The boundaries for the map were set according to the established management goals set in step 1. During this process all the possible variables that could have an influence on the endpoint was established.

Step 3: Divide the map into sections based on a combination of the management goals, sources and habitats

Combinations of the management goals, source information and habitat data were used to set up certain risk regions that were analyzed in a comparative way. The risk scores that were used during the RRM were based on these risk regions established in this step. The borders of the risk regions were determined after deliberation of the habitat segments and sources of stressors. The pathways of contact to these stressors also had to be taken into account. This ensured that the correct sources, stressors and habitats were incorporated into the various risk regions. We divided the study area into six risk regions using bridges that separate each section as boundaries. The regions include (Fig. 4.1):

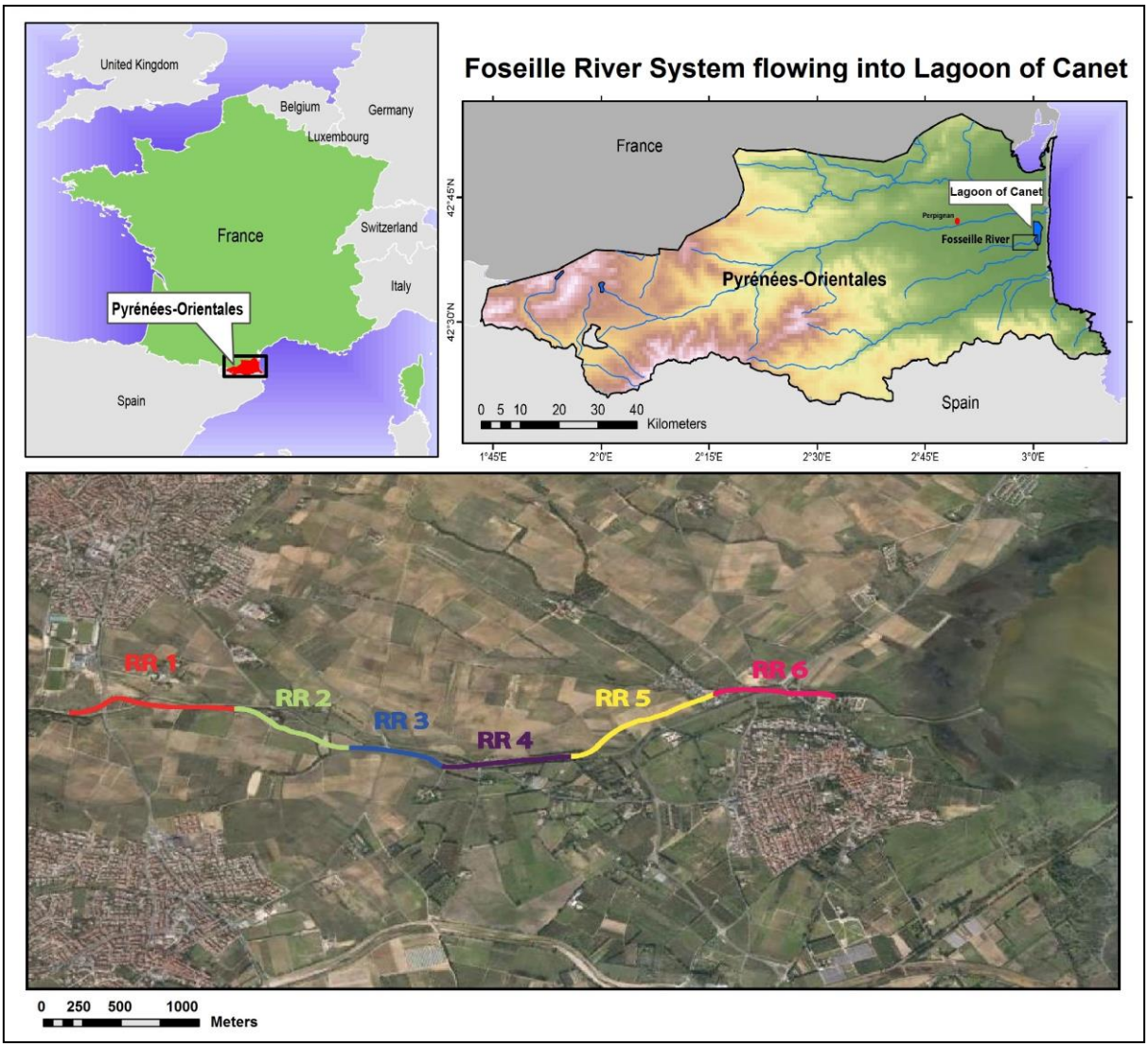

Figure 4.1: Generated map used in the RRM showing the six risk region (RR1 to RR6) in the Fosseille River system in the Pyrénées Orientales region in southern France.

1. Risk Region 1: The region stretches from the bridge over the Fosseille River between the towns of Saleilles and Cabestany and the first low water crossing, known as a “ford”.

2. Risk Region 2: Stretches from the first ford to the bridge across the Fosseille River between the towns of Cabestany and St-Nazaire.

3. Risk Region 3: This region stretches from the bridge where region 2 ended up to the second ford.

4. Risk Region 4: Region 4 stretches from the second ford down the river until a barrier constructed inside the Fosseille River system.

5. Risk Region 5: Section 5 runs from the barrier mentioned in region 4 down the river until the next bridge across the Fosseille River close to the town St-Nazaire.

6. Risk Region 6: Region six runs from the last bridge downstream next to a camping ground, and ends about 300 meters before the lagoon of Canet that consists of brackish water.

Step 4: Create a conceptual model that associates the sources of stressors to receptors and to the assessment endpoints

The conceptual model shows the potential relationships between the sources, stressors, habitats and endpoints that were used in the assessment of each risk region (Landis, 2005). A hypothetical model of a single relationship between a source, habitat and endpoint is shown in Figure 4.2. A conceptual model that is well constructed and gives sufficient information, acts as an extension of the basic framework of the RRM. The information that was used in step 2 was considered to construct the conceptual model and was collected from field sampling, databases, and previously published papers and reports (Fig. 4.3). Information based on literature, field analyses and professional opinion were used to identify nine possible sources that could influence the viability of *M. leprosa* terrapins in an aquatic system. This conceptual model allowed for the exploration of each potential pathway leading to impacts. The nine parent nodes included in the conceptual model were: man/disturbance to wildlife, invasive species, natural predators, substrate availability, water quality/conductivity, pathogens, parasites, food availability and population size of the native species. The justification for the use of these sources, are presented in Appendix B.

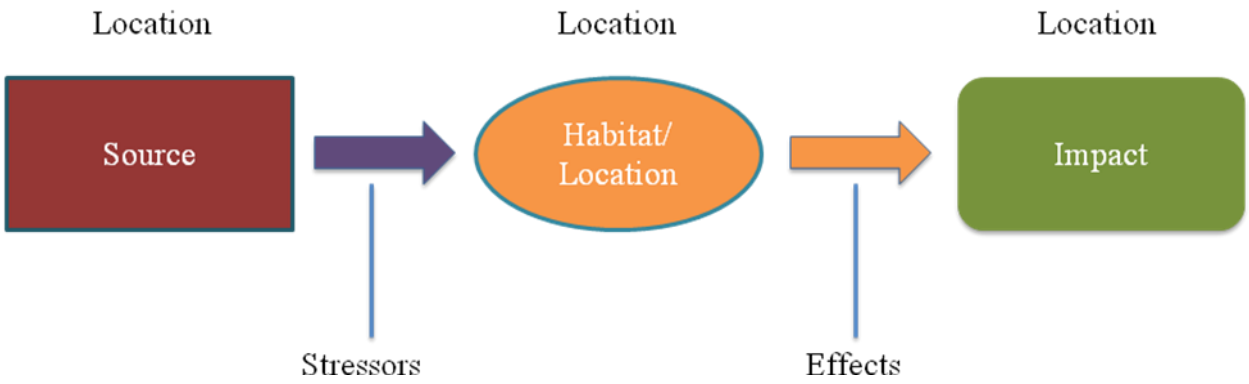

Figure 4.2: Hypothetical construction of the conceptual model presenting the possible relationships between identified sources, stressors, habitats and endpoints in the assessment (adapted from Landis and Wiegers 1997, 2005).

Step 5: Settle on a ranking system to calculate the relative risk to the assessment endpoints

Establishing a ranking system allowed for the calculation of relative risks to each assessment endpoint. This procedure involved the development of a ranking system for each source, stressor and habitat, this then contributed to the development of relative risks to each assessment endpoint (according to Landis, 2005). Data were transformed into non-dimensional ranks so that the effects of the various stressors on the various endpoints could be calculated

and compared. Ranks were assigned using criteria that were specific to the study area and were assigned according to the size and frequency of the sources and the availability of habitat. Rankings from a range of zero to six, with increments of two were assigned in the traditional RRM. Zero indicates no habitat or sources, while two, four and six indicates low, moderate and a high amount of habitat or source, respectively (Landis, 2005). The criteria for each ranking system were selected taking into consideration all of the obtainable information. Where adequate information about the concentration, response and fate of a stressor was available, ranks were assigned to an identified source. All this information obtained was used to establish the criteria for the ranking system as stated by Landis (2005). A ranking system for source and habitat variables at each risk region in this study is presented in Appendices A and B. Ranks were assigned to sources with the stressor relationship based on the presence of the source within a risk region and likely impacts linked with its location and successive downstream impacts.

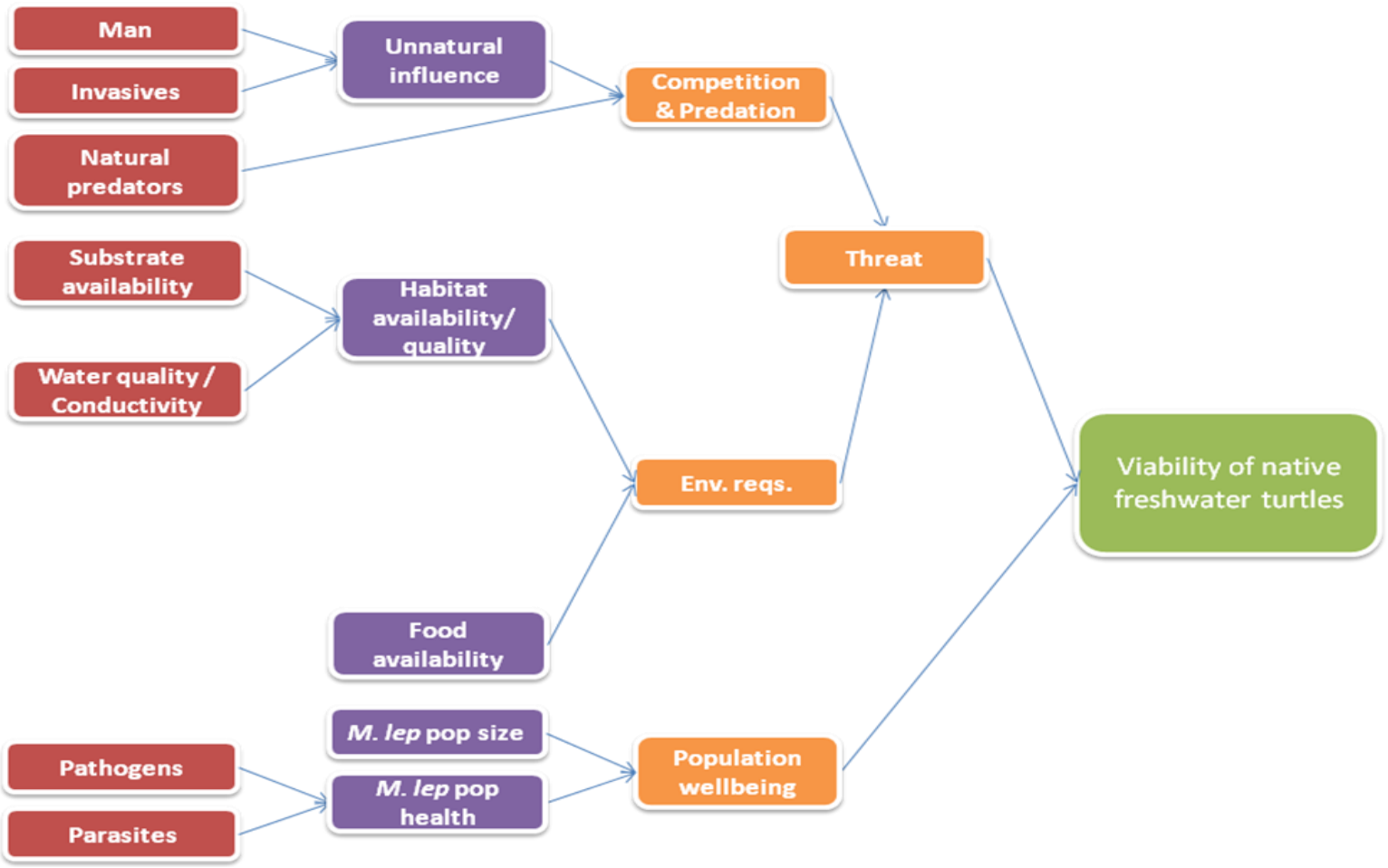

Figure 4.3: Conceptual model presenting the possible relationships between identified sources, stressors, habitats and the endpoint in this assessment study.

Step 6: Calculate the relative risks

During this step exposures and effects for the RRM were established with the addition of ranks to allow for the calculation of relative risks. In this step, ranks were used to determine the relationships between the risk components, including the source, habitat and impacts to assessment endpoints (according to Wiegers *et al.*, 1998). A Conditional Probability table was constructed where the exposure and effects were evaluated based on specific criteria and were assigned values which represented the specific state in each risk region. These values assigned in the Conditional Probability Table are displayed in Appendix C.

Bayesian Beliefs Networks (BBNs) were then used for the modelling purposes because it clearly displays the main influences on species population viability or the quality of the habitat together with their values and interactions. Also it combines categorical and continuous variables and it combines empirical data with expert judgment (Heckerman *et al.*, 1994). Predicted outcomes can be expressed as likelihoods as a basis for risk analysis and risk management (Marcot, 1998). BBNs are most useful when empirical data on population trends, demography and genetics are unavailable (Marcot *et al.,* 2001). The BBN modelling program Netica (Norsys) was used to perform standard belief updating which solves the network by finding the marginal posterior probability for each node. In BBN analysis, a conditional probability is the probability that some parameter will be in a certain state, such as the threats being high, given the input parameters, the conditional probabilities, and the rules governing how the probabilities combine (Marcot *et al.,* 2001). Netica assumes that conditional probabilities are independent and that previous probabilities are Dirichlet functions (Spiegelhalter *et al.,* 1993) meaning that probabilities are nonstop and delimited between 0 and 1 (Castillo *et al.*, 1997) and are a multi-state addition of the beta distribution.

The BBN model was constructed based on the expected influence of habitat alterations, abiotic and biotic factors on the viability of the native terrapin species. The relationship between the native terrapin species and the environment was determined by a list of qualitative, and where possible, quantitative ecological factors that could have influenced the distribution and abundance of the terrapin species. These data were collected from published literature, research reports, field data and professional opinion, but justification of the evidence used is required to evaluate uncertainty (Appendices A and B). During June 2013, water quality tests were conducted along the Fosseille River at various points in each risk region. Terrapins were collected in crayfish traps, marked, documented and released back into the environment at the same locality and basking terrapins were counted and documented for population estimates. Where data were insufficient, and literature was not available, the opinion of an expert in the field of study was applied for data analysis.

For each of the six risk regions a conditional probability table was constructed for the eight daughter nodes (Appendix C) to describe potential outcomes of interactions (demonstrated to have causal pathways, represented by arrows of variables based on given evidence. These tables represent the probability or frequency with which a node takes on each distinct condition, given the condition of any parent node that is connected to and interacts with it. These conditional probability tables were used to construct BBN for each one of the six risk regions in this study. A BBN for a reference condition was also constructed to determine a viable habitat and risks for the native terrapin species.

Step 7: Assess uncertainty and sensitivity analysis of the relative rankings

Uncertainty analysis

After the final risk scores were determined in the risk classification stage, the sources and amounts of uncertainty within each factor of the RRM were identified and analyzed using Monte Carlo analysis. This type of uncertainty analysis is a probabilistic approach that quantifies the change in model outputs or risk scores as a function of model inputs or ranks (Colnar and Landis, 2007). The inputs within the RRM are the ranks and filters and the outputs are the final risk scores. Using methodology similar to that of Hart Hayes and Landis (2004), we classified the uncertainty for each filter component and ranked as zero, low, moderate or high based on the amount of confidence within each assigned value according to available information. Thereafter we assigned discrete statistical distributions to represent the uncertainty within the ranks and filter components with medium and high classifications.

Once the uncertainty classifications were assigned, we ran the Monte Carlo simulations using Crystal Ball$^{(R)}$ 2000 software as a macro in Microsoft$^{(R)}$ 2007 Excel. The simulations were run using sufficient iterations (5000) to account for all variability in the model.

Sensitivity analysis

We then ran a sensitivity analysis using Crystal Ball$^{(R)}$ 2000 software as a macro in Microsoft$^{(R)}$ 2007 Excel. The sources of uncertainty that are influenced by either the model sensitivity or parameter uncertainty were examined by the sensitivity analysis (Goulet, 1995; Warren-Hicks and Moore, 1998).

Step 8: Generate testable scenarios for future field and laboratory investigations to decrease uncertainties and to verify the risk rankings

For the risk rankings of the RRM to be established and uncertainties to be reduced, appropriate hypotheses for field and laboratory investigations need to be established. For this, the outcomes of steps 6 and 7 need to be used. The RRM can then create predictions of patterns in the landscape and estimates of risk to the endpoint of the assessment (Landis, 2005). These predictions can then be tested by the various hypotheses that have been generated. The confidence of the risk assessors and decision makers could be increased by using this step to portray the risk outcomes for the environmental management (Landis, 2005).

Four scenarios were chosen to test the risk predictions:

Source scenario 1 — Current conditions

Source scenario 2 — Increase of invasive terrapin species establishing in the system

Source scenario 3 — Removal of invasive terrapin species from the system

Source scenario 4 — Sewage spill upstream (WQ, Salinity, Bacteria, Disease outbreaks)

Step 9: Test the scenarios that were generated in Step 8

A risk assessment should be able to provide predictions that can be tested using a variety of methods. Thus it is important to establish suitable hypotheses / scenarios for field and the laboratory investigations. This will help to reduce the uncertainty and to confirm the rankings of the RRM (O'Brien and Wepener, 2012). It may not be possible to perform landscape-scale experimental manipulations, but it is clearly possible to make predictions about patterns that should already exist. Being able to test and confirm at least part of the hypotheses generated by the risk assessment should increase the confidence of the risk assessors and decision makers in using the result for environmental management. By implementing the outcomes of steps six and seven, the RRM can generate predictions of patterns in the habitat and estimate the relative risk that it has on the endpoints of the specific assessment (Landis, 2005). For the four different scenarios RRM models were ran for each risk region to establish the risk status for each scenario.

Step 10: Present and communicate the results in a manner that effectively portrays the relative risk and uncertainty in response to the management goals

The results and outcomes of the RRM assessments, irrespective of the scientific validity, should be communicated to decision makers who commissioned the specific study. Communicating the outcomes to the decision makers must be done in such a manner to ensure that the relevant information is presented correctly and easily understood at all levels of complexity (O'Brien & Wepener, 2012).

4.3. Results

The risk characterisation step generated final risk scores for each risk region, source, habitat and endpoint (see Appendices A and B). Results of the risk assessment were the relative risk scores for the four scenarios as outlined risk scores given in parentheses for each risk region (Table 4.2 and refer to Appendix B for risk score distributions).

Table 4.2: Risk scores representing the various risks for the various scenarios for each risk region.

	Risk Score			
	Current Condition	**Increase in invasives**	**Invasives removed**	**Sewage spill upstream**
Risk Region 1	3.61	3.83	3.46	3.96
Risk Region 2	4.29	4.45	4.19	4.5
Risk Region 3	3.34	3.56	3.14	3.84
Risk Region 4	3.68	3.92	3.54	4.03
Risk Region 5	4.26	4.53	4.16	4.46
Risk Region 6	4.52	4.71	4.45	4.67

4.3.1 Scenario 1 - Current conditions

In the first source scenario risk regions two (4.29), five (4.26) and six (4.52) were the three regions at greatest risk, with risk region six having the highest risk. The source contributing mainly to the high risk in region six was population size, with disturbance to the environment / influence by man having a moderate to high risk score. Water quality and substrate availability contributed an almost zero to low risk score for all the six regions (Appendix B and Figure 4.4).

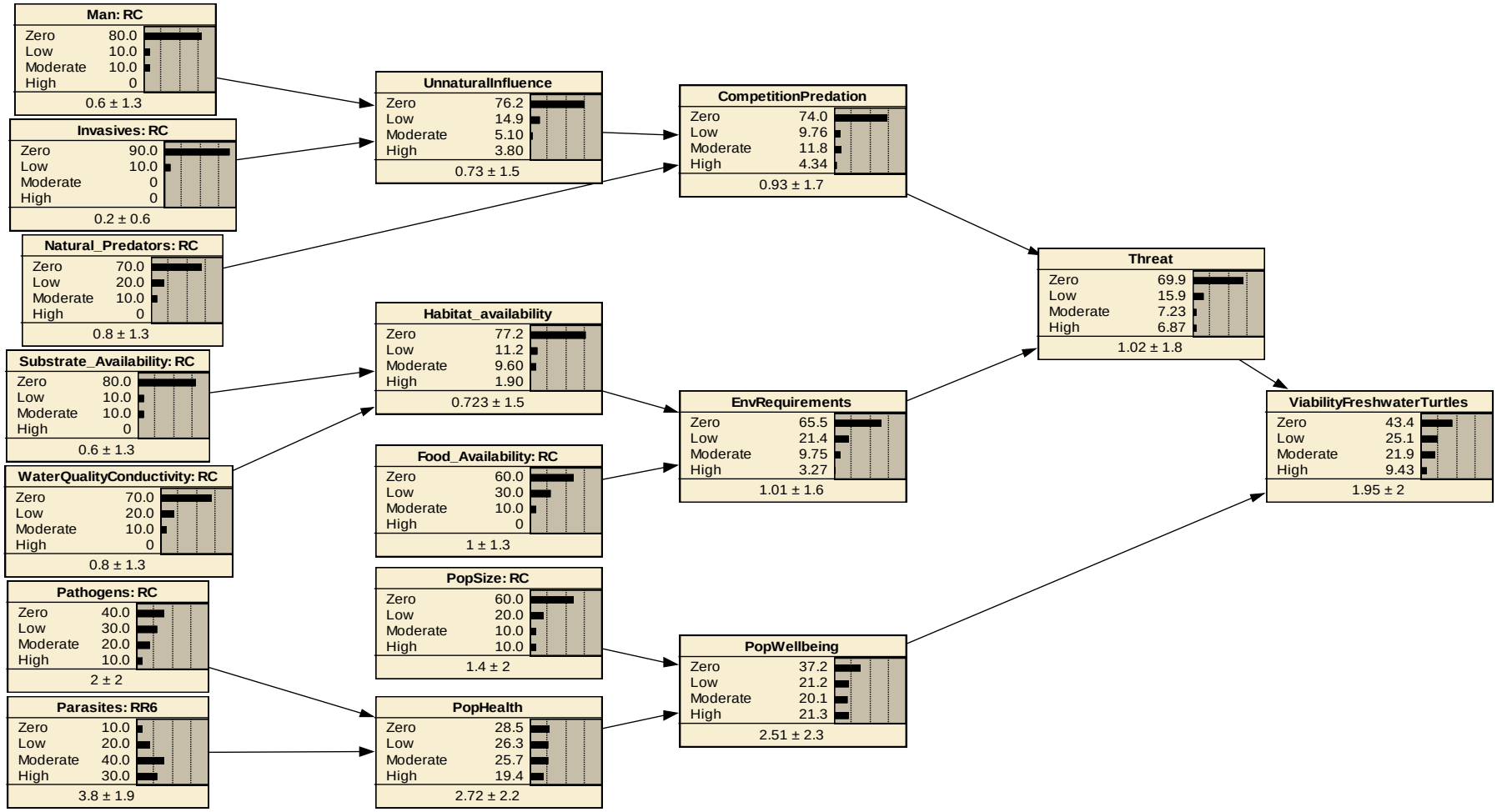

Figure 4.4: General structure of a Bayesian Belief Network (BBN) for evaluating the viability of the native terrapin specie outcome, showing nine parent nodes and eight daughter nodes. The condition of the daughter nodes can depict parameters as multiple discrete values or as continuous values.

Risk region 1

Disturbance to the environment / influence by man (5.4) posed the highest risk in this region followed by invasive species (3.5), pathogens (3), food availability (3.4) and *M. leprosa* population size (3.8) contributing to a moderate risk score, while natural predators (1.9) and parasites (2.2) have low risk. Substrate availability (0.8) and water quality almost had a zero risk score in risk region one (Fig. 4.5).

Risk region 2

Disturbance to the environment / influence by man (5.8) posed the highest risk in this region compared to the other regions. Population size (5.2) also showed a high risk score for this region, with pathogens (3), parasites, (3.9) and food availability (3.6) contributing to a moderate risk score. A low risk score was presented by invasive species (2.7) and by natural predators (1.9) while a almost zero risk score was presented by substrate availability (0.9) and water quality (0.4) (Fig. 4.5). This region is the region with the second highest risk score (Fig. 4.6).

Risk region 3

Risk region three had only one source with a high risk in the form of disturbance to the environment / influence by man (5). Invasive species (4), pathogens (3), parasites (3) and food availability all posed moderate risk with natural predators (1.6), water quality and population size (2.4) presenting a low risk. Substrate availability (0.6) had a zero - low risk score (Fig. 4.5). Risk region three is the risk region with the lowest risk out of all the six regions, although the region still had a moderate risk score (Fig. 4.6).

Risk region 4

Risk region four had only one source of high risk in the form of disturbance to the environment / influence by man (5.2). Pathogens (3), parasites (3), food availability (3.4) and population size (3.8) were sources with a moderate risk, while invasive species (2.9), natural predators (1.4) and water quality (2.2) were low risk sources. The only source with a very low risk score (0.6) was substrate availability (Fig. 4.5).

Risk region 5

In this region the source with the highest risk was population size (5.8) and disturbance to the environment / influence by man (3.2), pathogens (3) and food availability (3.7) all posing a moderate risk. Invasive species (2.7), natural predators (1.5), substrate availability (1.4), water quality (2) and parasites (1.4) contributed towards low risk scores. There were no sources in this region with a zero-low risk score (Fig. 4.5).

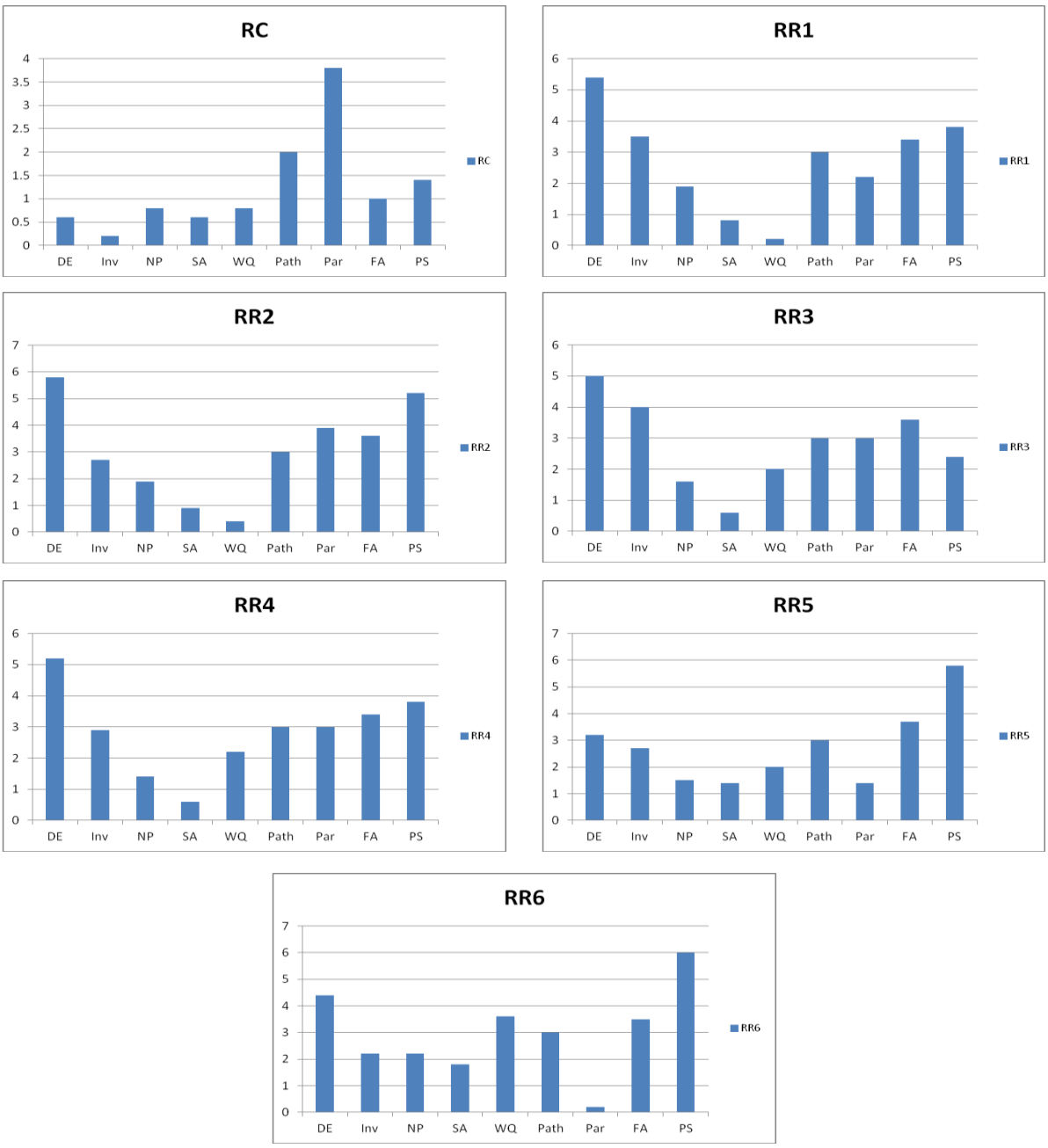

Figure 4.5: Relative contribution to risk from sources in the six risk regions (RR1 – RR6) together with the reference condition (RC). Y – axis is the relative risk score (0 = Zero, 2 = Low, 4 = Moderate and 6 = High). X – axis from left to right: DE = Disturbance to Environment, Inv = Invasive species, NP = Natural Predators, SA = Substrate Availability, WQ = Water Quality, Path = Pathogens, Par = Parasites, FA = Food Availability, and PS = Population Size.

Risk region 6

This region had the highest total risk score of the six regions (Fig. 4.6). Population size (6) was the only high risk source in this region, while disturbance to the environment / influence by man (4.4), water quality (3.6), pathogens (3) and food availability (3.5) had moderate risk scores. Invasive species (2.2), natural predators (2.2) and substrate availability (1.8) were the sources that would pose low risk with parasites (0.2) being the only source that had a risk score close to zero (Fig. 4.5).

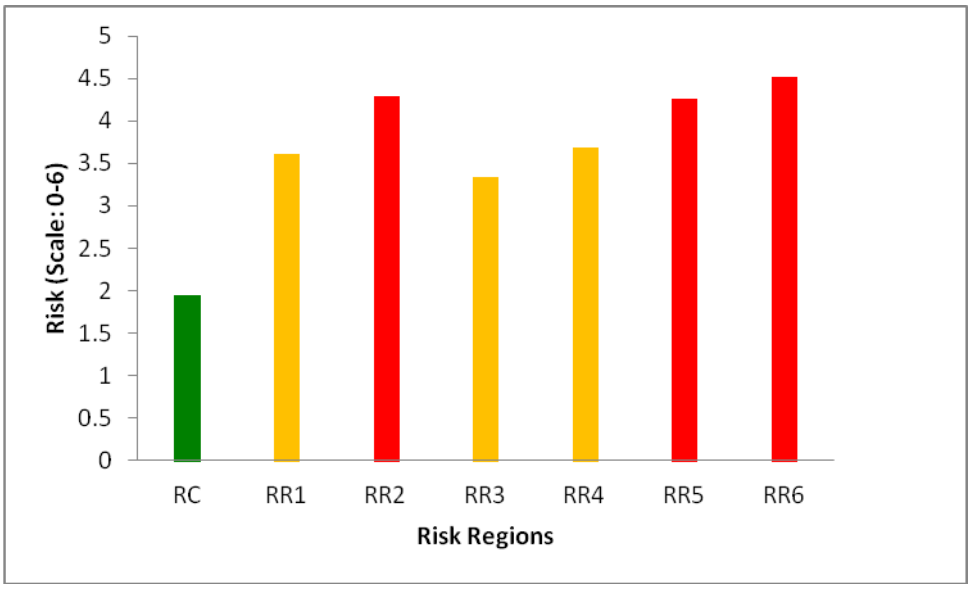

Figure 4.6: Graphical representation of the final risk scores obtained per region in the study area. Red bars present relatively high risk, yellow bars represent moderate relative risk and the green bar represents low relative risk.

4.3.2 Scenario 2 - Increase of invasive terrapin species establishing in the system

This scenario generated a higher risk score than for scenario one (Table 4.1). This scenario could also include invasive species reproducing new offspring in the river system. The regions with the highest risk score were risk regions two (4.45), five (4.53) and six (4.71) with risk region six having the highest risk score namely a score between moderate and high risk. Risk region three (3.56) had the lowest risk score, with the risk being close to moderate. The daughter node that was contributing to the rising of the risk score was “unnatural influences” and mainly “competition”.

4.3.3 Scenario 3 - Removal of invasive terrapin species from the river system

The risk score for all the risk regions decreased below the current condition risk scores (Table 4.1). Risk regions two (4.19), five (4.16) and six (4.45) were the regions with the highest risk scores, but had a moderate risk after the removal of invasive species. Risk region three had the lowest risk score with a low to moderate risk (Table 4.1). The main contributor to the lowering of the risk in this scenario besides the removal of the invasive species is the "reduction in competition" as a daughter node.

4.3.4 Scenario 4 - Sewage spill upstream

The influence from a hypothetical spillage was that the water quality could decrease and also pathogens could be introduced in to the river system. Risk regions two (4.5), five (4.46) and six (4.67) had moderately high risk scores with risk regions one (3.96), three (3.84) and four (4.03) displaying a moderate risk score. This was the scenario that resulted in the highest risk of all four scenarios with this scenario producing the highest risk at each region except region five and six (Fig. 4.7).

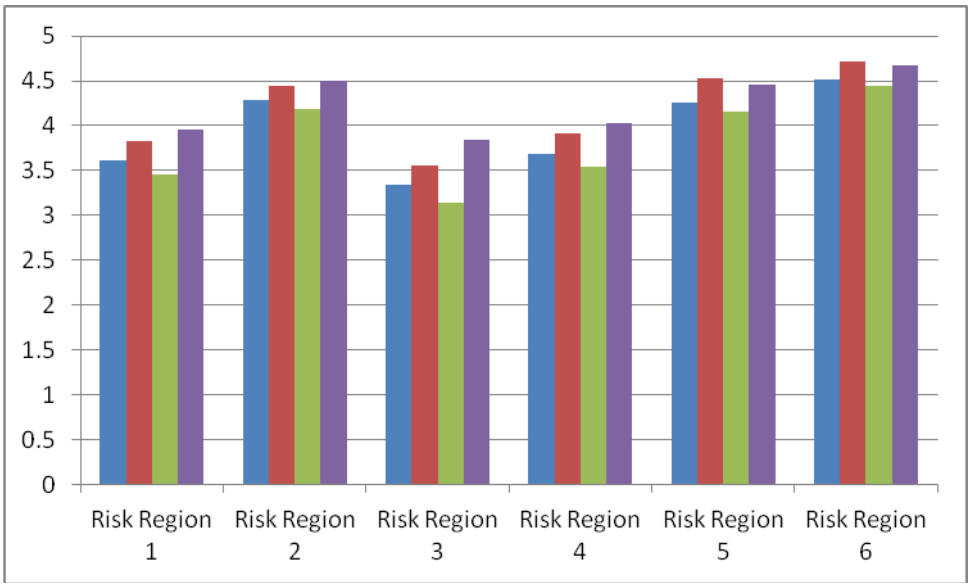

Figure 4.7: Graphical representation of the risk scores for the four scenarios used in the RRM. Blue bars present the current condition, red bars present the increase in invasive species, green bars present the removal of invasive species and the purple bars present a sewage spill from a sewage plant upstream from the six risk regions. X – axis = Risk regions, Y – axis = relative

4.3.5 Uncertainty analysis

The output was generated in the form of statistical distributions demonstrating the range of probable final risk estimates for each risk region, source, habitat and endpoint. The frequency chart for the uncertainty analysis showed a normal frequency distribution of data with a base case of 25.65 that corresponds to 50% (Fig. 4.8).

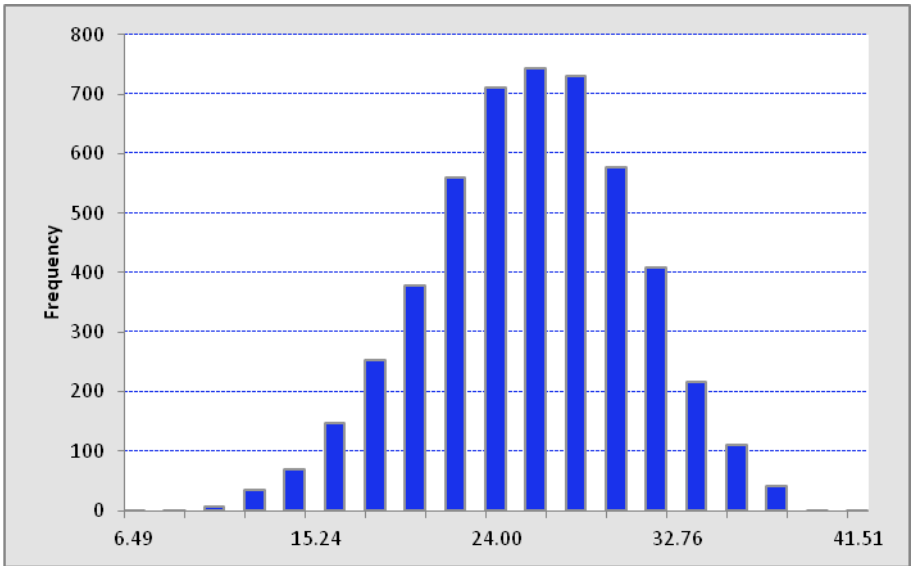

Figure 4.8: Frequency chart of uncertainty analysis showing a normal frequency distribution of data with a base case of 25.65 (50%). X – axis shows forecast values (6 – 42 = 0 – 100%) and Y – axis shows frequency values of risk data.

4.3.6 Sensitivity analysis

In the sensitivity analyses, correlation coefficients were generated to rank model parameters according to their input to prediction uncertainty. As a result, a high rank correlation indicates that the uncertainty within the model parameter has great significance in influencing the uncertainty within the model (Fig. 4.9)

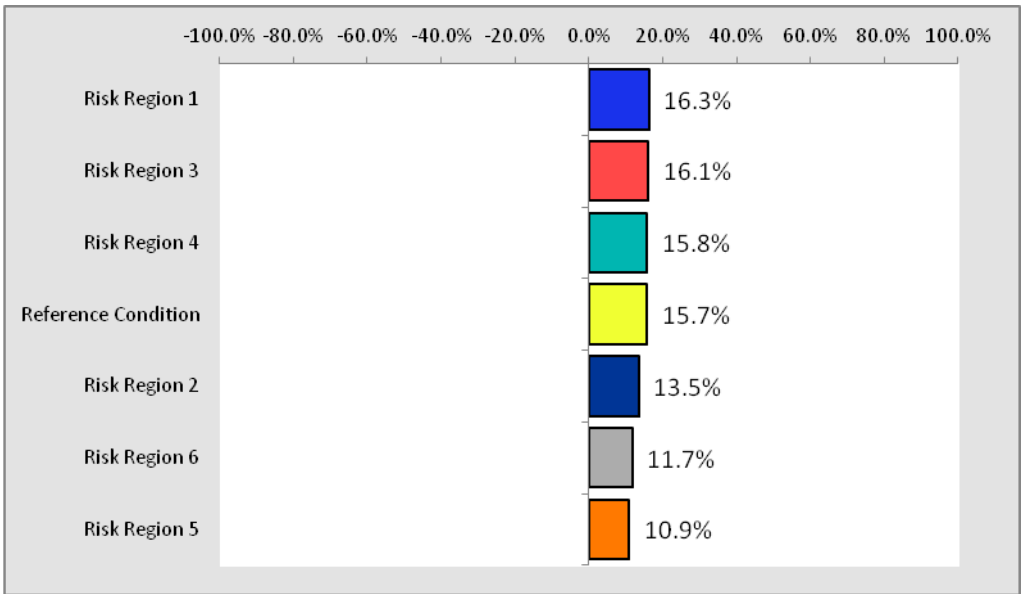

Figure 4.9: Relative risk to the various risk regions for Source scenario 1.

4.4 Discussion

In the first source scenario representing current conditions, out of all the sources that were included in the conceptual model, anthropogenic disturbance to the environment produced the highest risk score for the majority of the risk regions. The reason for this is that the majority of the land-use adjacent to the river system is agriculture, which has resulted in the removal of the natural habitat where terrapin species lay their eggs, or hibernate during winter. Further research must be conducted to determine whether the pesticides used in these agricultural regions, might pose a risk to the viability of the native terrapin species. Another source that contributed to a low to moderate risk, with the exception of risk region five where the risk was high, was *M. leprosa* population size. Population size is critical in maintaining the viability of the native species in the river system (Origgi and Jacobson, 2000). The high risk due to population size in risk region five and six is attributed to the few native terrapins that were collected in region five and that no terrapins were collected or cited in risk region six. However, this does not mean that the species is necessarily vulnerable in this region. The risk score for water quality in this region was high (Fig. 4.7) indicating that the salinity for this region could be above the preferred salinity (see Dunson and Mazzotti, 1989) for *M. leprosa* when compared to other risk regions upstream. Overall risk region six had the highest risk (Fig. 4.8) mainly due to the water quality / salinity in this region being too high for *M. leprosa*, and because we did not find or see any native terrapins in this region.

The second source scenario representing the increase of invasive terrapin species establishing in the system, resulted in the risk increasing from moderate to moderate-high. The risk scores increased for all the risk regions, but it was similar to that of scenario one. Invasive species resulted in a moderate risk score for risk region one and three (Fig. 4.7 and 4.8). In risk region three invasive species had the highest risk score and when considering population size of the native terrapin species (low risk), it could be interpreted that invasive species do not have a negative effect on the viability of *M. leprosa* (Fig. 4.7). However, a low risk score does not necessarily imply that a species may not need thorough intervention – it merely indicates that the species within the context of this risk assessment has a low risk.

For source scenario three all invasive species were removed from the river system, resulting in a reduction of risk scores for all six risk regions below the current condition (Table 4.2). The removal of the invasive species decreased the risk of competition on various aspects such as competition for food, basking spots, suitable habitat (Cadi and Joly, 2003; 2004; Polo-Cavia *et al.,* 2009; 2011). By removing the invasive species, parasite and pathogen transmission could also be decreased, seeing that the Red-eared slider is a carrier for various parasites and could transmit them to native terrapin species in the system (See chapter 2 on

polystome transmission in natural environments) and thus increasing the risk in population health of *M. leprosa*.

The fourth source scenario, represents a sewage spill upstream of all six risk regions, and resulted in the highest risk score of all the scenarios in this study (Fig. 4.9). Taking into account, during a sewage spill, various contaminants and pathogens could be released into the river system, oxygen depletion may take place and aquatic animals could die (Mallin *et al.,* 2007). This scenario increased the risk score of water quality and pathogens. The risk scores for risk regions one to four were higher than for any of the other scenarios. However, the risk score for risk regions five and six were lower than the risk scores for scenario two (increase in invasive species). The reason for this could be that a dilution effect may occur downstream.

We established that using the ecological risk assessment paradigm and the RRM methodology, it is possible to assess the viability of *M. leprosa* terrapins in a river system. This assessment indicated that there will be an increase in risk to various risk regions, habitats and the final endpoint, in the event of i) when a sewage spill occurs upstream of all the risk regions, and ii) when there is an increase in invasive species in the river system. In some parts of southern Europe where *M. leprosa* occurs, proposed conservation actions have been set in place and include wetland conservation, control of destructive fishing methods, aquifer management, control of illegal harvest, control of sale of exotic terrapins, habitat restoration, and control of feral populations of the Red-eared slider (Pleguezuelos, 2002). The RRM methodology could be implemented in these conservation management actions to improve and predict future outcomes.

The results in this study using the RRM showed that various factors could have an effect on the viability of *M. leprosa* terrapin in the Fosseille River system. It also showed how these risks of the various sources were spread out along the river system. While the scope was limited only to the Fosseille River, the methodology can be adapted to other bigger river systems, and can include other biological sources or other taxonomic groups. This model could also be used to manage invasive species effectively and how the management resources should be distributed over the study area and even the region. This method could also provide insight to what additional data needs to be gathered to strengthen the risk assessment. One main advantage of using this model is that one can assess the risks of various endangered species in different scenarios.

CHAPTER 5

Diversity of South African terrapin haemogregarines (Protozoa: Apicomplexa: Haemogregarinae) based on molecular and morphological evidences

"Every man has inside himself a parasitic being who is acting not at all to his advantage."

- William S. Burroughs

5.1 Introduction

Reptiles are hosts to various pathogens and parasites (Segade *et al.,* 2006; McAllister *et al.,* 2008, 2010; Zelmer and Platt, 2008, Davis and Sterrett, 2011), including flukes (Snyder, 2004), helminths (Aho, 1990), nematodes (Kuzmin, Tkach and Snyder, 2003) and intracellular haematozoans (Madsen, Ujvari and Olsson, 2005). Haematozoans may be found in various species, especially in wild animals and are represented by protozoans like plasmodiids, trypanosomatid flagellates and haemogregarines (Jacobson, 2010). Haematozoans from the haemogregarine group (Apicomplexa: Adeleorina) are of the most widely distributed haematozoans found in reptiles (Telford, 2009). Haemogregarines were first documented in the early 1900s, particularly by Robertson (1906), and Sambon and Seligmann (1907), these being some of the early examples of research done on snake and terrapin haemogregarines. About 400 species of haemogregarines have been described thus far (Telford, 2009), and are divided into three families and six genera: the Hepatozoidae Wenyon, 1926, with *Hepatozoon* Miller, 1908 and *Hemolivia* Petit, Landau, Baccam and Lainson, 1990; the Haemogregarinidae Neveu-Lemaire, 1901 with *Cyrilia* Lainson, 1981, *Desseria* Siddell, 1995 and *Haemogregarina* Danilewsky, 1885; the Karyolysidae Wenyon, 1926 with *Karyolysus* Labbé, 1894.

The majority of early descriptions were based on morphology of their erythrocytic stages but also on the precept, 'new host, new parasite' assuming a strict host-specificity, which resulted in many taxonomically incorrectly described species. This type of description was strongly discouraged, particularly by Ball (1967) (see Telford, 1984; Telford, 2009). Levine (1988) did a review of new parasites described and placed 300 species into the genus *Haemogregarina*. Siddell (1995) included only 19 species into the genus *Haemogregarina* (sensu stricto), all parasitizing chelonians and solely transmitted by leech vectors, and placed all

other haemogregarines parasitizing snakes, crocodilians, lizards and birds, in the genus *Haemogregarina* (sensu lato), suggesting that these, with further revision of life stage traits, rather be transferred to the genus *Hepatozoon*. Only one haemogregarine, parasitizing a terrestrial chelonian and for which the life cycle was elucidated, was an exception, it falling within the genus *Hepatozoon* (see Michel, 1973; Siddell, 1995; Smith, 1996) and later transferred to the genus *Hemolivia*, based on sporogonic stages (see Landau and Paperna, 1997). A year after Siddell's partial taxonomic review of the haemogregarines, the majority of reptilian haemogregarines were moved to the genus *Hepatozoon* by Smith (1996), with the exception of chelonian haemogregarines, which remained in *Haemogregarina* (s.s.) and one in *Hepatozoon* until its transfer into *Hemolivia*. Thereafter, during a redescription based on observations made on peripheral blood stages and identification of probable vectors (ticks and mosquitoes), two southern African terrestrial chelonian haemogregarines, *Haemogregarina fitzsimonsi* Dias, 1953 and *Haemogregarina parvula* Dias, 1953, were transferred to *Haemogregarina* (s.l.) (Cook *et al.*, 2009). Haemogregarines, such as *Hemolivia mauritanica* (Sergent and Sergent, 1904), parasitizing Palaearctic tortoises *Testudo marginata* Schoepff, 1792 and *Testudo graeca* Linnaeus, 1758, are transmitted by the tick *Hyalomma aegyptium* Linnaeus, 1758. The generic placement of this species through morphology was further supported by molecular analysis (see Harris *et al.*, 2013), emphasising the need to use both these descriptive tools together for a more accurate account of apicomplexan haematozoans' taxonomic and phylogenetic assignments as well as to elucidate haematozoan transmission and thus life cycles (Pers. Comm. Courtney Cook).

Haematozoans in all instances require invertebrate vectors, such as arthropods and annelids, these often being ticks and leeches respectively (Telford, 1984; Frye, 1991; Salakij *et al.*, 2002). Sexual reproduction of haematozoans, referred to as sporogony, occurs within vectors and serves to define the genus through different stages of sporogonic development (Telford, 2009). Without sporogonic knowledge the placement of most haemogregarines into one of the four genera found in reptiles was and is still very difficult, since the mature intraerythrocytic gamonts are almost morphologically indistinguishable (Telford, 2009). This again further emphasises the unsatisfactory nature of solely morphology-based gamont descriptions. Haemogregarines are often found intraerythrocytically and sometimes, but less commonly, free within the plasma. The gamonts of haemogregarines are mostly identifiable as elongated, sausage-shaped inclusions within the erythrocytes (Jacobson, 1983), the cytoplasm being pale to purple along with an eccentrically placed dark purple coloured nucleus when stained with Giemsa (Jacobson, 2010). Gamont parasitized blood cells may present with a laterally displaced nucleus or in some cases the gamont may completely encircle the host nucleus. This often results in host cells with an irregular shape and size (Lane and Mader, 1996).

There is very little information known about the biology, mode of transmission and various vectors of haemogregarines (Desser, 1993), and thus taxonomic conclusions are difficult. The genus *Haemogregarina* has a heteroxenous life cycle and parasitizes vertebrate hosts such as chelonians, fish and other ectothermic animals that are associated with aquatic habitats. The gamont stages are produced through merogony within the vertebrate host, either within the parenchymatous organs or including the peripheral blood of the vertebrate host, whereas the sporozoites are produced through sporogony within the haematophagous invertebrates, such as in leeches (Davies and Johnston, 2000). Currently the only described haemogregarines parasitizing terrapins are of the genus *Haemogregarina* (s.s.) as mentioned above. Only two complete life cycles of *Haemogregarina* species of terrapins have been described to date (Dvořáková *et al.,* 2014). The type species for the genus *Haemogregarina* namely *Haemogregarina stepanowi* (Danilewsky, 1885), was described from *Emys orbicularis* (Linnaeus, 1758) (European pond turtle), the life cycle of which was described by Reichenow (1910) and the second, *Haemogregarina balli* Paterson and Desser (1976), was described from *Chelydra serpentina* (Linnaeus, 1758) (Nearctic snapping terrapin) (see Siddell and Desser, 1990, 1992). The above life cycles are in sharp contrast to the only fully described haemogregarine, *Hemolivia mauritanica,* mentioned above, from terrestrial chelonians. The vector in the case of the terrestrial tortoise *Hemolivia* sp. is a tick, whereas it is a leech in the case of the aquatic chelonian *Haemogregarina* spp. Regarding transmission *Hemolivia* is transmitted through the ingestion of the infected tick vector by chance with the grass at resting sites by the chelonian intermediate host (see Široký *et al.*, 2007), whilst *Haemogregarina* spp. are transferred directly during the blood meal of the leech to the chelonian (see Paterson and Desser, 1976; Siddell and Desser, 1990, 1992). Again this stresses why accurate molecular-based phylogeny is instrumental in determining to what genus the haemogregarine belongs, which in turn provides clues to its transmission and life cycle.

From African terrapins, haemogregarines have been reported from the blood of *P. subniger* by Bouet (1909) in French West Africa and França (1912) in Guinea-Bissau (previously known as Portugese Guinea), and named *Haemogregarina sternotheri* França 1912. That species was also reported in Mozambique from *P. sinuatus zuluensis* (syn. *Sternotherus sinuatus*) (Smith, 1838) by Dias (1952) and from *P. sinuatus sinuatus* by Pienaar (1962). A significant contribution on reptile haematozoans in southern Africa was done by Pienaar (1962), describing several species from various reptiles. Pienaar (1962) described *Haemogregarina pelusiensi* from *P. s. sinuatus* of Mozambique. Paperna (1989) also found this haemogregarine species across other populations of *P. sinuatus* near Pietersburg in the Limpopo province of South Africa. Both studies only included peripheral blood morphometric measurements of *H. pelusiensi,* Pienaar (1962) himself taking measurements of meronts and gamont stages from only one specimen and Paperna (1989) measurements of trophozoites, meronts and gamonts from only three

specimens. South Africa is inhabited by five species of hard-shelled terrapins that belong to the family Pelomedusidae Cope, 1868: The common African helmeted terrapin, *Pelomedusa subrufa* (Lacepède, 1788), East African yellow-bellied mud terrapin, *Pelusios castanoides* Hewitt, 1931*,* Variable mud terrapin, *P. rhodesianus* Hewitt 1927, East African serrated mud terrapin, *P. sinuatus* (Smith, 1838) and the East African black mud terrapin, *P. subniger* (Lacepède, 1788) (Bonin *et al.*, 2006). While various haemogregarine species have been described within turtles, very little is known about haemogregarines parasitizing South African terrapins. Furthermore modern molecular genetic methods have not been used to describe *Haemogregarina* species in terrapins until recently within African hinged terrapins (see Barta *et al.,* 2012, Dvořáková *et al.,* 2014). By sampling all species of terrapins from South Africa over the wide distribution of the various species, the following objectives for this study were: (1) to investigate the diversity of *Haemogregarina* (Apicomplexa: Haemogregarinidae) by comparing the various morphological stages within the erythrocytes of the host species; (2) to confirm the systematic status of parasite species from comparisons of 18SrDNA sequences.

5.2 Materials and Methods

5.2.1 Blood sampling

Across South Africa, terrapins were captured in dams, ponds and river systems during summer from January 2011 until March 2013 (Tables 5.1 and 5.2). A total of 231 terrapins were collected using crayfish traps baited with chicken or ox liver. Blood samples were taken from the femoral artery in the hind leg or from the caudal vein in the tail using a 1ml fixed-needle insulin syringe. Thin blood smears were first prepared using a clean slide and a small drop of blood. The rest of blood was immediately fixed in 96% pure ethanol for molecular experiments. Slides were then air dried, fixed in absolute methanol for 5 min and stained with Giemsa-stain (FLUKA, Sigma-Aldrich, Germany) for 20 min following the method detailed by Cook *et al.* (2009; 2010). All the terrapins were finally released at the same location of capture.

Table 5.1. Investigated host species and collection sites; m - males, f - females, j - juv, nd - not determined. Abbreviations of the various provinces in brackets are as follows: EC - Eastern Cape, FS - Free State, GP - Gauteng, KZN - KwaZulu-Natal, Lim - Limpopo, MP - Mpumalanga, NW - North West and WC - Western Cape. NZG - National Zoological Gardens.

Host species	Sampling site	Number: m/f/juv/nd
Pelomedusa subrufa (Lacepède, 1788)	Vredefort Dome (NW), Brits (NW), Ikageng (NW), Hartebeespoort (NW), Koffiefontein (FS), Mitchells Park (KZN), Bluff (KZN), Ellisras (Lim), Jhb Zoo (GP), NZG (GP), Dwesa (EC), Plettenberg Bay (WC), Hout Bay (WC), Hoedspruit (Lim)	38/45/22/13
Pelusios castanoides Hewitt,1931	Tembe (KZN), Bonamanzi (KZN)	9/5/0/1
Pelusios rhodesianus Hewitt, 1927	Bluff (KZN)	0/3/0/0
Pelusios sinuatus (Smith, 1838)	Bonamanzi (KZN), Ndumo (KZN), NZG (GP), Hartebeespoort (NW), Hoedspruit (MP)	11/42/18/4
Pelusios subniger (Lacepède, 1788)	Tembe (KZN)	1/16/0/3

Table 5.2. Uninfected (first number) and infected hosts (second number) and prevalence in percentage (%) across eight provinces in South Africa.

Host species	Gauteng	North-West	Western Cape	Eastern Cape	KwaZulu-Natal	Free State	Limpopo	Mpumalanga	Total
Pelomedusa subrufa (Lacepède, 1788)	5/13 (38%)	36/53 (68%)	5/17 (29%)	12/12 (100%)	9/9 (100%)	8/11 (73%)	0/2 (0%)	1/1 (100)	76/118 (64%)
Pelusios castanoides Hewitt,1931	–	–	–	–	10/15 (67%)	–	–	–	10/15 (67%)
Pelusios rhodesianus Hewitt, 1927	–	–	–	–	0/3 (0%)	–	–	–	0/3 (0%)
Pelusios sinuatus (Smith, 1838)	0/2 (0%)	–	–	–	35/46 (76%)	–	–	24/27 (89%)	59/75 (79%)
Pelusios subniger (Lacepède, 1788)	–	–	–	–	18/20 (90%)	–	–	–	18/20 (90%)

5.2.2 Inspection of blood parasites

Blood smears were examined by using a 100x magnification objective lens, equipped with immersion oil on a Nikon Eclipse E800 compound microscope (Nikon, Netherlands). Images of certain intraerythrocytic forms were captured by a Nikon DXM1200 digital camera and measurements were taken using the Nikon NIS-Elements microscope imaging software program D3.2 (Nikon, Netherlands). All measurements were given in µm.

5.2.3 Molecular analyses and phylogenetic analyses

DNA was extracted from highly parasitized blood samples using the standard protocol for human or animal tissue and cultured cells as detailed in the NucleoSpin®Tissue Genomic DNA Tissue Kit (Macherey-Nagel, Germany). DNA concentration was quantified spectrophotometrically with a Nanodrop(R) Spectrophotometer ND-100 (ACTGene, USA) and DNA was stored at -20 °C until use. Amplification of *Haemogregarina*-like parasite 18S rDNA was undertaken in a Bio-Rad C1000 Touch™ Thermal Cycler using specific apicomplexan forward (EF: 5-'GAAACTGCGAATGGCTCATT-3') and reverse (ER: 5-'CTTGCGCCTACTAGGCATTC'-3) primers originally designed for *Eimeria* by Kvičerová *et al.* (2008) and validated for *Haemogregarina* spp. from terrapins (see Dvořáková *et al.*, 2014). PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles consisting of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds end extension at 72 °C for 60 seconds, and a final extension at 72 °C for 10 min. Amplification results were visualized using a Bio-Rad GelDoc Imaging System (Bio-Rad, UK). PCR products of about 1,150 kb were sent to the Inqaba Biotec Company (Pretoria, South Africa) for purification and sequencing in both directions with the same primers.

Chromatograms were edited and checked using MEGA5 (http://www.megasoftware.net). Sequences were further blasted using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast/) for possible host contaminations. New sequences obtained in the present study were compared to 21 existing haemogregarine 18S rDNA sequences, obtained from GenBank (see list of taxa and their accession numbers in Table 5.3) All sequences were aligned using CLUSTAL W software (Thompson *et al.*, 1994) implemented in MEGA5 (Tamura *et al.*, 2011). A Maximum Likelihood (ML) tree was finally constructed using MEGA5 (Tamura *et al.*, 2011) under the Tamura 3-parameter + Gamma model (T92+G) model (Tamura *et al.,* 2011) with *Cryptosporidium* used as the outgroup. Nodal support values were inferred following 1000 bootstrap replicates.

Table 5.3: GenBank accession numbers for sequences used along this study. * Is actually *Babesiosoma stableri* and not *Hemolivia mariare* as stated on GenBank (see Barta *et al.,* 2012).

Species	Acc. Numbers	References
Adelina dimidiata	DQ096835	Kopečná *et al.*(2006)
Cryptosporidium serpentis	AF093499	Xiao *et al.* (1999)
Dactylosoma ranarum	HQ224958	Barta *et al.* (2012)
Haemogregarina balli	HQ224959	Barta *et al.* (2012)
Haemogregarina sp. (*Pelusios marani,* Gabon)	KF257924	Dvořáková *et al.*(2014)
Haemogregarina sp. ex. (*Pelusios sinuatus*, South Africa)	To be assigned	This study
Haemogregarina sp. (*Pelusios subniger,* Mozambique)	KF257925	Dvořáková *et al.*(2013)
Haemogregarina sp. ex. (*Pelomedusa subrufa*, South Africa)	To be assigned	This study
Haemogregarina sp*.* (*Pelusios williamsi,* Kenya)	KF257923	Dvořáková *et al.*(2014)
Haemogregarina stepanowi (*Emys orbicularis*, Bulgaria)	KF257928	Dvořáková *et al.*(2014)
Haemogregarina stepanowi (*Mauremys caspica*, Iran)	KF257926	Dvořáková *et al.*(2014)
Haemogregarina stepanowi (*Mauremys leprosa*, Algeria)	KF257929	Dvořáková *et al.*(2014)
Haemogregarina stepanowi (*Mauremys rivulata*, Syria)	KF257927	Dvořáková *et al.*(2014)
**Hemolivia mariae/Babesiosoma stableri*	HQ224961	Barta *et al.* (2012)
Hepatozoon sp. *Boiga*	AF297085	Jakes *et al.* Unpublished
Hepatozoon americanum	AF176836	Mathew *et al.* (2000)
Hepatozoon ayorgbor	EF157822	Sloboda *et al.* (2007)
Hepatozoon canis	AY461378	Criado-Fornelio *et al.* Unpublished
Hepatozoon catesbianae	AF130361	Carreno *et al.* (1999)
Hepatozoon clamatae	HQ224962	Barta *et al.* (2012)
Hepatozoon felis	AY620232	Criado-Fornelio *et al.* (2006)
Hepatozoon sipedon	JN181157	Barta *et al.* (2012)
Hepatozoon ursi	EU041717	Kubo *et al.* (2008)

5.3 Results

5.3.1 General observations

Altogether, 163/231 (70.5%) of terrapins that were collected were parasitized by *Haemogregarina* spp. (Table 5.2). Of all the terrapins, the prevalence of infected *P. subrufa* was 64%, *P. castanoides* 67%, *P. sinuatus* 79% and *P. subniger* 90%. *Pelusious. rhodesianus* was not found to be parasitized by *Haemogregarina* spp. Four intraerythrocytic developmental stages of *Haemogregarina* were observed in this study. These included trophozoites, premeronts, meronts and mature gamonts. Trophozoites were the smallest stages found in the smears and had several small vacuoles found in the cytoplasm with a small nucleus at one pole of the parasite (Fig. 5.1 a, e, i, m). Premeronts were larger in size than trophozoites and had an elongated shape with a centrally placed, large, granular nucleus spanning the entire width of the parasite, with small vacuoles at the poles (Fig. 5.1 b, f, j, n). Meronts were larger in size and had 2 – 4 nuclei (Fig. 5.1 c, g, k, o). They were also the least commonly observed in the smears. Intraerythrocytic mature gamonts were the most frequently observed in the smears, which were elongated and slightly curved in form and were situated within a bean-shaped capsule or parasitophorous vacuole. They appeared to be folded over on themselves with the nucleus situated at the fold itself. The nucleus of mature gamonts was dense and was in most cases situated at one pole, the folding point of the parasite (Fig. 5.1 d, h, l, p). Parasitized erythrocytes were larger in size with the nucleus pushed to the side. Dehaemoglobinization of the host cell was prevalent during parasitisation (Fig. 5.1 l).

5.3.2 Microscopic description (Table 5.2) (n = indicates number of parasites measured)

Developmental stages of haemogregarines from *Pelomedusa subrufa*

Trophozoites: rare stages, elongated in form and tapering to one pole, with foamy vacuolated cytoplasm stained light purple, measuring 10.50 ± 0.81 (9.07 – 11.60) long by 4.02 ± 0.49 (3.41 – 5.14) wide (n = 10); nucleus often located at one pole with loosely arranged chromatin, staining dark purple, measuring 5.35 ± 0.45 (4.50 – 5.82) long by 3.52 ± 0.51 (2.85 – 4.63) wide (n = 10) (Fig. 5.1 m).

Premeronts: slightly bean-shaped and cytoplasm staining light purple, measuring 11.8 ± 0.87 (9.56 – 13.92) long by 5.18 ± 0.56 (3.91 – 6.14) wide (n = 20); a dark purple nucleus, located close to the centre of the parasite, spanning the width of the parasite, measuring 5.36 ± 0.86 (3.82 – 6.75) long by 4.56 ± 0.75 (3.17 – 6.01) wide (n = 20) (Fig. 5.1 n).

Meronts: rare stages with a whitish-purple stained cytoplasm, causing some degree of host cell hypertrophy and dehaemoglobinisation; measuring 13.63 ± 0.88 (12.10 – 14.81) long by

6.32 ± 0.83 (5.16 – 7.66) wide (n = 9); meronts contained 2-4 clusters of loosely arranged chromatin, staining dark bluish-purple (Fig. 5.1 o).

Mature gamonts (vermicular): slightly curved or bean-shaped, with a long recurved tail, with a light purple stained cytoplasm, causing frequent hypertrophy and dehaemoglobinisation of host cell (Figs. 5.1 p); measuring 21.59 ± 0.93 (20.12 – 23.93) long by 4.08 ± 0.46 (3.22 – 4.88) wide (n = 25); ovoid nucleus staining dark purple and located at the folding point of the gamont at one pole (taken in this case as the posterior pole), measuring 6.09 ± 0.66 (4.47 – 7.41) long by 3.36 ± 0.52 (2.41 – 4.49) wide (n = 25).

Developmental stages of haemogregarines from *Pelusios castanoides, P. sinuatus, and P. subniger*

Trophozoites: rare elongated stages frequently tapering to one end, foamy vacuoles often present at the poles, with a granular cytoplasm staining light purple (Fig. 5.1), measuring 11.59 ± 0.92 (9.76 – 12.81) long by 4.79 ± 0.76 (3.40 – 6.18) wide (n = 22) in *P. sinuatus* (Fig. 5.1 a), 11.10 ± 0.88 (9.45 – 12.29) long by 4.79 ± 0.44 (3.47 – 4.79) wide (n = 8) in *P. subniger* (Fig. 5.1 e), and 11.67 ± 0.88 (10.68 – 13.63) long by 4.06 ± 0.64 (3.09 – 4.83) wide (n = 9) in *P. castanoides* (Fig. 5.1 i), more or less centrally positioned nucleus, staining slightly darker purple compared to the cytoplasm, measuring, 5.59 ± 0.73 (4.40 – 7.17) long by 4.43 ± 0.92 (2.74 – 6.15) wide (n = 22) in *P. sinuatus* (Fig. 5.1 a), 5.59 ± 0.64 (3.09 – 4.92) long by 4.43 ± 0.50 (2.37 -3.79) wide (n = 8) in *P. subniger* (Fig. 5.1 e), and 4.98 (3.23 – 7.05) ± 1.02 long by 2.82 (2.00 – 4.03) ± 0.73 wide (n = 9) in *P. castanoides* (Fig. 5.1 i).

Premeronts: were lentiform to bean-shaped staining bluish-purple, measuring 11.8 ± 0.87 (9.56 – 13.92) long by 5.18 ± 0.56 (3.91 – 6.14) wide (n = 20) in *P. sinuatus* (Fig. 5.1 b), 12.64 (11.37 – 14.20) ± 0.83 long by 5.33 (4.28 – 6.52) ± 0.63 wide (n = 15) in *P. subniger* (Fig. 5.1 f), and 12.07 ± 0.73 (11.16 – 13.59) long by .85 ± 0.52 (4.01 – 6.09) wide (n = 18) in *P. castanoides* (Fig. 5.1 j); with a dark purple nucleus positioned centrally and frequently spanning the width of the parasite, measuring 5.36 ± 0.86 (3.82 – 6.75) long by 4.56 ± 0.75 (3.17 – 6.01) wide (n = 20) in *P. sinuatus* (Fig. 5.1 b), 4.15 ± 0.80 (3.13 – 5.89) long by 4.00 ± 0.91 (2.75 – 5.62) wide (n = 15) in *P. subniger* (Fig. 5.1 f), and 6.03 ± 0.63 (4.69 – 7.38) long by 3.42 ± 0.68 (2.43 – 4.62) wide (n = 18) in *P. castanoides* (Fig. 5.1 j).

Meronts: rare stages with a vacuolated cytoplasm, staining light purple causing some degree of host cell hypertrophy and dehaemoglobinisation (Fig. 5.1 c, g, k, o), measuring 13.51 ± 0.94 (12.01 – 14.60) long by 5.89 ± 0.36 (5.32 – 6.46) wide (n = 7) in *P. sinuatus* (Fig. 5.1 c), 15.22 ± 0.79 (14.21 – 16.55) long by 6.27 ± 0.34 (5.72 – 6.99) wide (n = 10) in *P. subniger* (Fig. 5.1 g), and 13.26 ± 0.74 (12.30 – 14.28) long by 5.43 ± 0.93 (4.56 – 7.14) wide (n = 11) in *P.*

castanoides (Fig. 5.1 k); meronts contained 2 – 4 clusters of loosely arranged chromatin, staining darker purple than the cytoplasm (n = 10).

Mature gamonts (vermicular): elongated and somewhat curved, with a long recurved tail, with the cytoplasm staining light purple, causing frequent hypertrophy and dehaemoglobinisation of the host cell (Figs. 5.1 d, h, l), gamont measuring 27.12 ± 0.98 (25.75 – 28.36) long by 3.62 ± 0.34 (3.07 – 4.57) wide (n = 25) in *P. sinuatus* (Fig. 5.1 d), 25.97 ± 0.94 (23.29 – 27.16) long by 4.49 ± 0.55 (3.24 – 5.26) wide (n = 25) in *P. subniger* (Fig. 5.1 h), and 26.29 ± 1.00 (24.21 – 27.93) long by 4.73 ± 0.53 (3.82 – 5.88) wide (n = 25) in *P. castanoides* (Fig. 5.1 l); ovoid to elliptical nucleus staining dark purple, located closer to the posterior pole of the parasite, measuring, 6.28 ± 0.77 (3.68 – 7.66) long by 3.33 ± 0.34 (2.80 – 4.23) wide (n = 25) in *P. sinuatus* (Fig. 5.1 d), 6.11 ± 0.35 (5.22 – 7.00) long by 3.71 ± 0.48 (2.81 – 4.53) wide (n = 25) in *P. subniger* (Fig. 5.1 h), and 5.75 ± 0.54 (4.90 – 6.79) long by 3.97 ± 0.39 (3.22 – 4.53) wide (n = 25) in *P. castanoides* (Fig. 5.1 l).

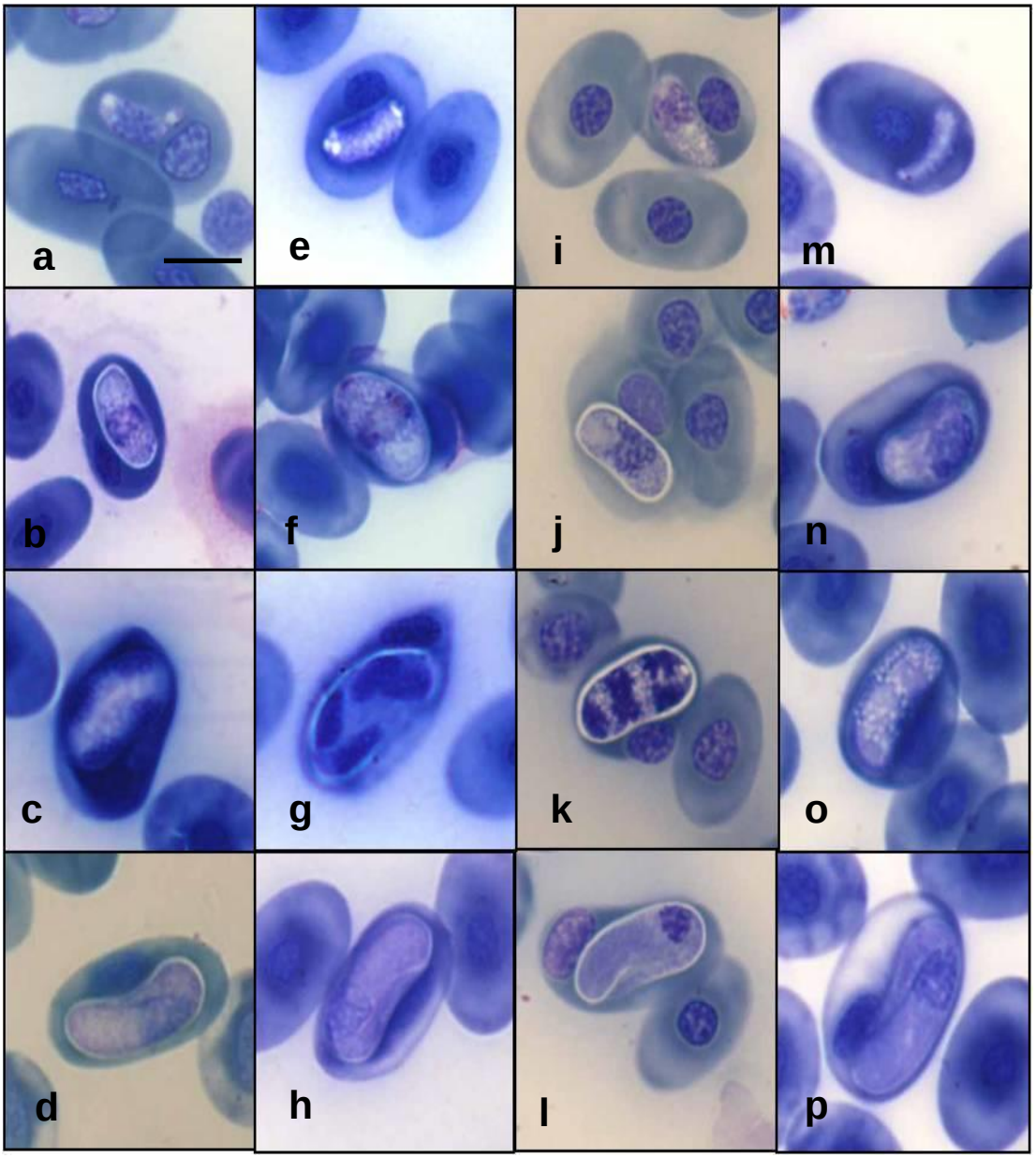

Figure 5.1: Micrographs of haemogregarines in the peripheral blood of *Pelomedusa subrufa* **(a-d)**, *Pelusios castanoides* **(e-h)**, *Pelusios sinuatus* **(i-l)** and *Pelusios. subniger* **(m-p)**. Scale bar: 10 *µ*m.

5.3.3 Phylogenetic analyses

From the 10 blood isolates investigated in molecular biology (five from *P. sinuatus*, one from *P. subniger*, one from *P. castanoides* and three from *P. subrufa*), only three gave 18S sequences typical of haemogregarines after the blast procedure. 18S sequences obtained from blood isolates of two specimens of *P. sinuatus* collected in Ndumo (KwaZulu Natal province) were identical to each other but differed by one single mutation from the 18S sequence obtained from the blood isolate of one single specimen of *P. subrufa* collected in the private reserve of Sable Ranch (North West Province). According to the ML tree (Fig. 5.2), parasites

may be considered as *Haemogregarina* spp. Within the *Haemogregarina* clade, two distinctive monophyletic groups are observed, a Palearctic/Nearctic clade and an Ethiopian clade, the former including *Haemogregarina stepanowi* and *Haemogregarina balli*, the latter including *Haemogregarina* spp. from *P. marani* (Gabon), *P. subniger* (Mozambique), *P. williamsi* (Kenya) and from South African terrapins, i.e. *P. sinuatus* and *P. subrufa*. *Haemogregarina* spp. isolated from *P. subniger*, *P. williamsi*, *P. sinuatus* and *P. subrufa* fall in the same group and all differ from each other by one single mutation.

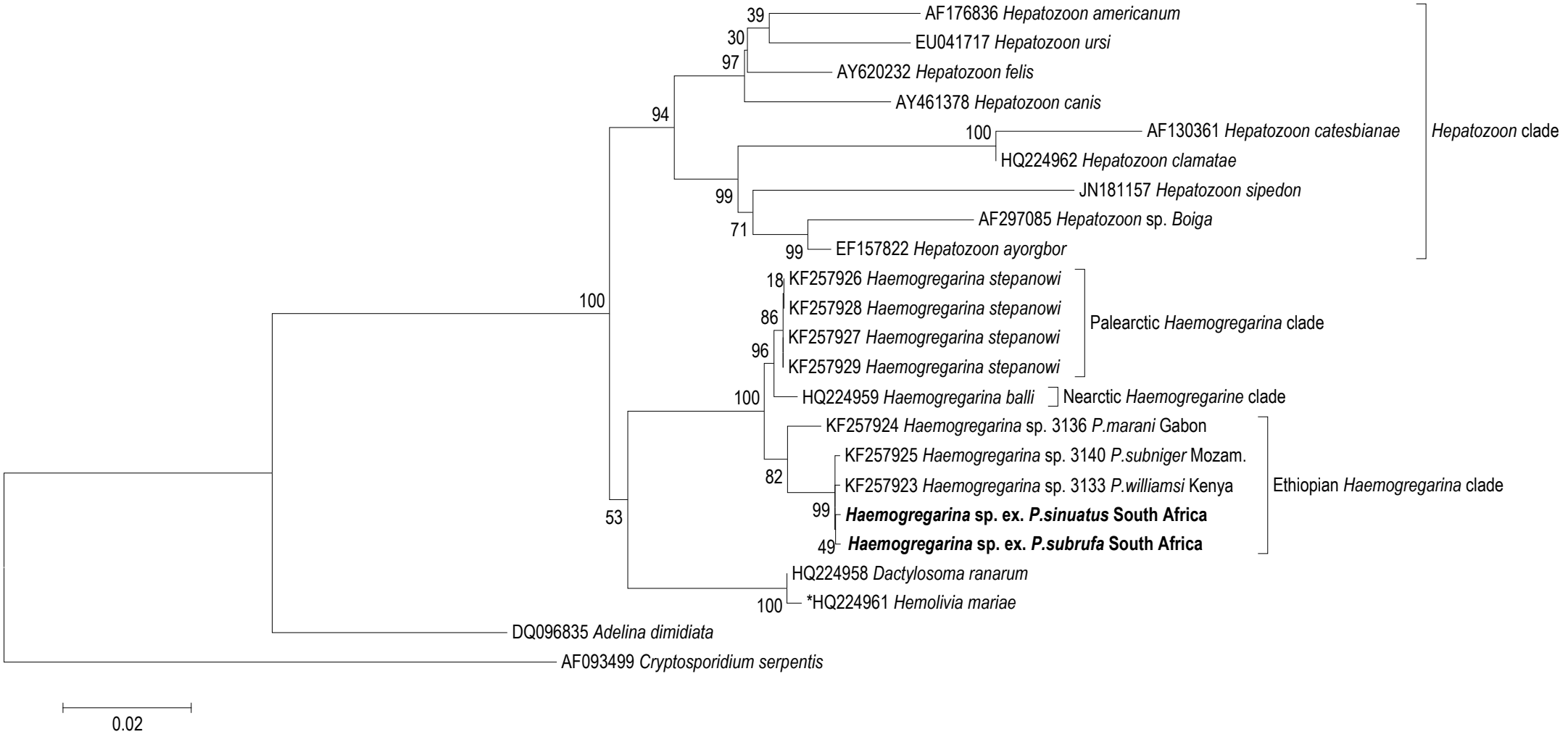

Figure 5.2: Maximum Likelihood tree of *Haemogregarina* species. *Haemogregarina* sp. ex. *P. sinuatus* and *Haemogregarina* sp. ex. *P. subrufa* appear in bold. *HQ224961 *Hemolivia mariae* on GenBank is in fact *Babesiosoma stableri* (see Barta *et al.* (2012).

5.4 Discussion

Based on morphological grounds, haemogregarines parasitizing *P. sinuatus, P. subniger* and *P. castenoides* may be considered as conspecific. Whereas developmental stages, like trophozoites, premeronts and meronts, are morphologically very similar within haemogregarines of all investigated host species (*Pelusios* spp and *P. subrufa*), they differ from that of *P. subrufa*, particularly in respect to gamonts, which are smaller for the latter. Therefore, the haemogregarines parasitizing all three *Pelusios* spp. may be considered as a separate species. In addition, they were very similar to a previously described *Haemogregarina* species, i.e., *H. pelusiensi* from *P. s. sinuatus*, first described by Pienaar (1962) from Mozambique and measuring 12.5 ± 3 x 3.4 µm (Pienaar (1962) only measured the length of the body and did not take into consideration the recurving of the intraerythrocytic parasite) and then studied by Paperna (1989) from South African terrapins who measured a body length of 13 µm and a total length of 24 µm with a width of 5 µm (Paperna (1989) measured the length of the body from the anterior up to where the body folds onto itself, thus the total length of the recurved body) (Table 5.4). However, another haemogregarine species, *H. maputensis* Dias and de Sousa (1950), was also described from a close relative of *P. s. sinuatus* in Mozambique, i.e. *P. s. zuluensis* Hewitt, 1927, which is now considered as *P. sinuatus* (Loveridge, 1941). All these findings suggest that *H. pelusiensi* and *H. maputensis* could be synonymous species. In addition Paperna (1989) described leeches, i.e., *Placobdella multistrigata* and *Placobdella costata* (Müller, 1846) species, as vectors for *H. pelusiensi*. Because the *Pesusios* spp and *P. subrufa* that were surveyed along this study were also parasitized by leeches located all around the cloaca, the neck and on the soft skin between limbs and the shell, we may therefore suggest, based on morphological grounds and the occurrence of leeches, that the haemogregarines found infecting *Pelusios* spp. from South Africa and those reported earlier by Dias and de Sousa (1950), Pienaar (1962) and Paperna (1989) from *P. s. sinuatus* are conspecific.

Over the years the phylum Apicomplexa has been mainly studied from morphological traits. It is only recently that the use of molecular methods introduced scientists to a new field that enabled them to sort out a bit of the confusion in the apicomplexan group. Dvořáková *et al.* (2014) focused on haemogregarines of Palearctic and Nearctic terrapins, with emphasis on *H. stepanowi* and *H. balli* species. From morphological and molecular evidences, he showed that *H. stepanowi*, which was originally described from *Emys orbicularis* (Reichenow, 1910), was also infesting *Mauremys leprosa*, *M. caspica* and *M. rivulata* in the same area. Blood isolates obtained from all four turtles showed indeed identical 18SrDNA sequences for all haemogregarines. Regarding the ML tree (Fig. 5.2), haemogregarines parasitising *Pelusios sinuatus* along with that parasitizing *Pelomedusa subrufa* fall into the Ethiopian clade with haemogregarines isolated from *P. marani*, *P. subniger* and *P. williamsi* by Dvořáková *et al.*

(2014). Genetic divergences (p-distances) between isolates obtained from *P. sinuatus*, *P. subrufa*, *P. subniger* and *P. williamsi* were so small (1 single mutation between all sequences), that one may first suggest they are all conspecific, thus contradicting morphological evidences. However some haemogregarines have demonstrated some degree of intraspecific morphological variability during different phases of infection and when parasitizing different host species (see Dvořáková *et al.,* 2014), which may explain some morphological differences between haemogregarines isolated from *P. sinuatus* and *P. subrufa*. Because Dvořáková *et al.* (2014) did not provide complete morphological measurements, a complete comparison of haemogregarines from the Ethiopian clade is not yet feasible from a morphological point of view. However, only four isolates have been currently sequenced, among which three from the same host species, i.e., *P. sinuatus* of the same area. To conclude about the systematic status of South African haemogregarines, it is now urgent to complete the sequencing for blood isolates obtained from several infected specimens of the same host species and all across their distribution to ascertain if observed mutations within the 18S are fixed or not within parasites infesting the same terrapin species.

Table 5.4. Measurements (*µ*m) of *Haemogregarina* species parasitizing freshwater terrapins from various geographical localities known from literature and host terrapins investigated along this study; na - data not available.

Locality	Parasite	Type host	Other hosts		Trophozoite		Premeront		Meront		Gamont		Reference
Ethiopian realm													
	Haemogregarina pelusiensi	*Pelusios sinuatus* (syn. *Pelusios sinuatus sinuatus*) (Pelomedusidae)	na	Body N	na		na		na		12.5±3 x 3.4 1		Pienaar, (1962)
	Haemogregarina pelusiensi	*Pelusios sinuatus* (syn. *Pelusios sinuatus sinuatus*) (Pelomedusidae)	na	Body N	na		na		15-17 x 12-13 3		13-24 x 5-9 3		Paperna, (1989)
	Haemogregarina pelusiensi	*Pelusios sinuatus* (syn. *Pelusios sinuatus sinuatus*) (Pelomedusidae)	*Pelusios subniger* (syn. *Sternotherus derbianus*); *Pelusios castanoides* (Pelomedusidae)	Body Nucleus N	11.45±0.89 4.35±0.61 4.91±0.80 3.40±0.72 39	x x 	12.17±0.81 5.12±0.57 5.18±0.76 3.99±0.78 53	x x 	14.00±0.82 5.86±0.54 n = 2-4 28	x 	26.46±0.97 4.28±0.47 6.05±0.55 3.67±0.40 75	x x 	This study
	Haemogregarina sp.	*Pelomedusa subrufa* (Pelomedusidae)	na	Body Nucleus N	10.50±0.81 4.02±0.49 5.35±0.45 3.52±0.51 10	x x 	11.35±0.53 4.96±0.38 5.37±0.66 3.29±0.33 20	x x 	13.63±0.88 6.32±0.83 n = 2-4 9	x 	21.59±0.93 4.08±0.46 6.09±0.66 3.36±0.52 25	x x 	This study
	Haemogregarina sternotheri	*Pelusios subniger/niger* (syn. *Sternotherus*	na	Body	na		na		na		12.6 x 1.8		Bouet, (1909); França, (1912)

		derbianus) (Pelomedusidae)							
Nearctic realm									
	Haemogregarina balli	*Chelydra serpentina* (syn. *Chelydra serpentina serpentina*) (Chelydridae)	*Chrysemys picta marginat, Glyptemys insculpta* (syn. *Clemmys insculpta*) (Emydidae)	Body Nucleus	na	na	18.6 (14.5-21) x 7.2 (5-10) n = 6-8	12.6 (8-14.5) x 5.3 (3-6.5)	Paterson and Desser, (1976)
	Haemogregarina macrochelysi	*Macrochelys temminkii* (Chelydrdae)	*Graptemys barbouri* (Emydidae)	Body Nucleus N	na na na	na na na	14.9±1.2 x 6.8±1.0 3-8 14	32.4±1.3 x 3.5±0.4 6.2±0.7 x 2.8±0.6 25	Telford, (2009)
Palearctic realm									
	Haemogregarina bagensis	*Mauremys leprosa* (syn. *Emys leprosa*) (Emydidae)	na	Body	na	8-10 x 2-3	11-20 x 10-14	25-30 x 4-5	Laveran and Pettit, (1909)
	Haemogregarina choudhuryi	*Lissemys punctata punctata* (Trionychidae)	na	Body N			10.0 x 5.5 10	8.0 x 2.5-3.5 10	Telford, (2009)
	Haemogregarina stepanowi	*Emys orbicularis* (Emydidae)	*Mauremys caspica* (syn. *Mauremys caspica caspica*) (Emydidae)	Body	na	na	16 x 6	na	Reichenow, (1910)
	Haemogregarina stepanowi	*Emys orbicularis* (Emydidae)	*Mauremys caspica* (syn. *Mauremys caspica caspica*) (Emydidae)	Body Nucleus	na	na	12.3 x 5.9 n = 2-4	15.7 x 5	Hahn, (1909)

Haemogregarina stepanowi	*Emys orbicularis* (Emydidae)	*Mauremys caspica* (syn. *Mauremys caspica caspica*) (Emydidae)	Body Nucleus	na	10.8±1.0 x 5.1±0.8 4.6±1.5 x 3.8±0.8	12-16 x 6-7, 15 x 11 n = 4-6, 8	32.0±1.9 x 4.2±0.7 5.5±0.8 x 3.5±0.5	Telford, (2009)
Haemogregarina stepanowi	*Emys orbicularis* (Emydidae)	*Mauremys caspica* (syn. *Mauremys caspica caspica*) (Emydidae)	Body Nucleus N	10.4±1.7 x 5.5±1.0 na 10	9.9±0.9 x 4.3±0.6 4.1±0.3 x 2.3±0.5 13	13 x 6 n = 6 1	32.3±2.1 x 3.2±0.3 5.9±0.7 x 3.1±0.5 30	Dvořáková *et al.,* (2014)
Haemogregarina stepanowi	*Mauremys caspica* (syn. *Mauremys caspica caspica*) (Emydidae)	*Emys orbicularis* (Emydidae)	Body Nucleus N	9.6±1.3 x 5.3±0.5 na 10	10.9±1.0 x 4.6±0.6 5.4±0.3 x 3.8±1.3 19	14 x 7 n = 4 1	32.5±1.3 x 3.3±0.4 5.5±0.7 x 3.9±0.9 17	Dvořáková *et al,.* (2014)
Haemogregarina stepanowi	*Mauremys leprosa* (syn. *Emys leprosa*) (Emydidae)	*Emys orbicularis* (Emydidae)	Body Nucleus N	12.0±0.0 x 5.0±0.0 na 2	11.8±0.8 x 5.4±0.6 5.6±0.9 x 3.9±0.7 20	na	34.3±1.4 x 3.0±0.2 6.6±1.4 x 2.4±0.5 16	Dvořáková *et al.,* (2014)
Haemogregarina stepanowi	*Mauremys rivulata* (syn. *Mauremys rivulata cretica*) (Emydidae)	*Emys orbicularis* (Emydidae)	Body Nucleus N	9.7±1.0 x 6.0±0.9 na 14	11.5±0.5 x 5.5±0.5 5.5±0.8 x 4.3±0.5 11	13 x 6, 19 x 11 n = 2, 8 2	32.2±1.9 x 3.6±0.4 6.0±1.0 x 4.5±0.8 29	Dvořáková *et al.,* (2014)

CHAPTER 6

General Discussion and Future Research

"In the end, we will save only what we love. We will love only what we know.
We only know what we are taught."
- Unknown

6.1 General Discussion

In this chapter the objective is to integrate the information gathered from the various parts of the study and to discuss the risks, control, and management of the global terrapin trade. The topics involved the apprehension of the introduction of invasive terrapins into new aquatic environments and the possible effects of parasite host switching. Studies detailed also focused on the effect of environmental temperatures on parasite adaptability within new host species. A relative risk method was developed to determine the viability of the native species *Mauremys leprosa* in its natural environment in France and the various threats that may influence its survival. This method could contribute to conservation strategies for terrapins globally. Lastly, a study was conducted to investigate the diversity of haemogregarine blood parasites in South African terrapins.

Declines of terrapin species have been reported globally. The causes are known in several instances, suspected in others, and unknown in some. Whereas some declines are local, others are more widespread. However, all of them can be detrimental to an ecosystem as discussed in Chapter 1. Ecosystems rely on terrapins to serve as a source species from which other animals and plants may benefit. As an agent for seed dispersal they are also very important for plants' dissemination around aquatic systems (see Liu, Platt and Borg, 2004). It is thus important to conserve these animals at least for the benefit they provide in all aquatic ecosystems they inhabit.

One of the major factors contributing to terrapin declines is their overexploitation (see Chapter 1). Throughout the world terrapins are popular as pets. Sub-adult terrapins are usually colourful and easy to keep and as they grow older they are less attractive and are thus less appealing as pets. They are often released in natural environments where they may establish feral populations. Invasive terrapins are able to adapt to a wide variety of habitats including lakes, ponds, swamps, streams and rivers. As mentioned in Chapter 2, one such species that had been introduced into various countries for food and pet trade and which succeeded in

adapting to new environments is the Red-eared slider (*Trachemys scripta elegans)*. The findings in Chapter 2 showed that this invasive terrapin is of great concern to *M. leprosa* in natural environments in southern France and northern Spain. Populations of *M. leprosa* are infected with invasive polystome species that switched from introduced *Trachemys* and *Graptemys* species. Those parasites have evolved and adapted to their new hosts and thus may survive in various habitats. The parasites could have adapted in such a way as does the host to go into a resting phase or hibernation during winter, as discussed in Chapter 3. The invasion of *T. s. elegans*, together with all the various parasites that it hosts, may be therefore a key stressor to native terrapins.

In South Africa, *T. s. elegans* was introduced in the 1980's and feral populations were documented near Durban and Pretoria and probably contributed to the local extinction of *Pelusios rhodesianus* (Richard Boycott, *Pers. Comm*.)*.* The invasive species has been reported from localities near Pretoria, which include Six Mile Spruit, Moraletta Spruit, and the Hartbeespoort. Elsewhere on the highveld, invasive terrapins have been found in Zoo Lake, Boksburg Lake and in Germiston. There are several reptile parks where that species is kept in the same ponds with local species. After consultation with the various conservation bodies it became clear that the current status of this invasive species in South Africa is not known and there are most likely many undetected populations (Warren Schmidt, Pers. Comm.). During the course of this study it was discovered that the pet trade of immature *T. s. elegans* was still very active in South Africa. The conservation bodies were notified about the situation; nonetheless, there are many loopholes whereby pet shop owners bypass the law. Although no polystomes were detected in any of the wild South African terrapins we surveyed, some American polystomes were found within specimens of the Red-eared slider at the National Zoological Gardens in Pretoria, South Africa, as well as within the Common African helmeted terrapin (*Pelomedusa subrufa*). This result indicates that these parasites could be introduced in the wild if more non-native sliders were to be released into aquatic environments in South Africa.

The European Union banned the importation of *T. s. elegans* and various other American terrapins such as *Graptemys* and *Pseudemys* species in 1997. However, this did not stop the terrapin trade into Europe and forced the focus of the trade to shift to other countries. Various African countries such as Mozambique, Tanzania, Nigeria, Benin and Togo (G. Townsend, Pers. Comm) have been now exporting terrapins to Europe. *P. subrufa* and *Pelusios sinuatus* terrapins are for instance exported from southern Africa to Europe, America, and Asia. As discussed in Chapter 5, terrapins in southern Africa are hosts for leeches that are vectors for various blood parasites (e.g.,*Trypanosoma* and *Hemogregarina*). Morphologically and phylogenetically, haemogregarines from *Pelusios* spp. of South Africa, Mozambique and Kenya are closely related and most likely conspecific to *Haemogregarina pelusiensi.* This would point

to a generalist parasite such as that of a terrestrial tortoise that had been found infecting five species of tortoises from a variety of biomes all over South Africa and Mozambique (Cook, 2012). Thus it is important now to determine specificity levels of *Hameogregarina* spp. within terrapins to adequately address the possible dangers of those parasites. When terrapins are exported to Europe, they could have the potential to become invasive and also transmit parasites to new host species that may have detrimental effects on the native terrapins. Although blood parasites are common in African terrapins, there were no infected terrapins among the two species *Emys orbicularis* and *M. leprosa* in northern Spain and southern France (personal unpublished data). However, we must remain cautious about the possibilities of zoonotic infections in countries where African terrapins are now introduced.

No animal species should be lost to extinction, as all of them are essential and significant. Over the years anthropogenic activities have endangered many terrapin species while driving others into extinction. At this point in time, we are facing a terrapin survival crisis unique in its severity and risk. Because the main causing factor is humans, we should therefore also be the solution. If we do not start to take conservation actions, many of the world's terrapins could become extinct within the next few decades.

A tool that will greatly benefit management strategies is the Relative Risk Method Model detailed in Chapter 5. This method can be applied to any animal species in threat, any area or region, and any threats influencing the animals in danger. The model can also be modified and adapted to assess the risk of various factors or species that needs to be assessed in a specific environment as shown in Chapter 5 and through this, talks can be given to the public to show the threats and their effects on terrapin species in nature. It is important to communicate with the public to aid awareness of these situations, and to get them involved in ecosystem conservation, not only for terrapins, but to benefit nature as a whole.

6.2 Future Research

- Evaluating potential adverse effects of new pathogens and exotic parasites in order to have a better idea on the threats they may pose on indigenous *M. leprosa* populations.

- Gathering more data across Europe to determine how wide the distribution of invasive species stretch and what environmental factors play a key role in the establishment of these species.

- Conducting a Regional-Scale Risk Assessment on a spatial scale taking into account multiple stressors affecting multiple endpoints. Local ecosystem dynamics and characteristics of the landscape across southern France should be also included in the assessment.

- Performing an in depth study of the diversity of haemogregarines within South African terrapins. Data are needed from terrapins of KwaZulu-Natal province to demonstrate parasite specificity of haemogregarines within *Pelusios* spp. and *P. subrufa.* Additional18S sequences from isolates of terrapins in all “infected” areas should contribute to understanding whether the observed mutations in the 18S between haemogregarines of *Pelusios* spp. and *P. subrufa* are fixed or not.

- Studying isolates of *P. subrufa* from all localities where infected terrapins occur to evaluate the status of *Haemogregarina* from this terrapin species. If it were to be a new parasite species, then its description will be conducted in detail including an investigation of the vector responsible for transmission.

- Conducting smear and squash techniques as well as standard histology on the blood collected from leeches found within infected terrapins in search of other parasite stages. In this manner we expect to elucidate the lifecycle of *Haemogregarina pelusiensi*.

CHAPTER 7
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Reference list formatted according to African Zoology guidelines.

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APPENDICES

Appendix A: List of hosts and parasites investigated, geographical origin, haplotype and source of polystomes, terrapin and DNA sample number.

Host species	Country - Locality	Haplotype - Polystome species	Terrapin #	Parasite DNA sample
Mauremys leprosa	France - Fosseille	H16 - *Polystomoides* sp1	20	MiAC484
		H16	34	MiAC480, MiAC481
		H16	47	MiAD2, MiAD307
		H37 - *Neopolystoma orbiculare*	3	MiAC472
		H37	13	MiAC486, MiAC487
		H37	35	MiAC482, MiAC483
		H37	43	MiAC488, MiAC489
		H37	47	MiAD1
		H37	55	MiAD306
		H37	88	MiAC473, MiAC474
		H37	152	MiAD316, MiAD317
Trachemys scripta elegans	France - Fosseille	H16 - *Polystomoides* sp1	18	MiAC602
		H16	22	MiAC600
		H16	28	MiAD314, MiAD315
		H16	30	MiAD8, MiAD9, MiAD10
		H16	40	MiAD305
		H77	20	MiAC599
		H37 - *Neopolystoma orbiculare*	40	MiAD311, MiAD312, MiAD313
		H18 - *Neopolystoma* sp4	15	MiAC597
Mauremys leprosa	France - Baillaury	H18 - *Neopolystoma* sp4	177	MiAC69, MiAC70, MiAC176
		H18	186	MiAC132, MiAC133, MiAC143, MiAC144
		H18	191	MiAC157, MiAC158
		H18	193	MiAC146
		H18	199	Mi771
		H18	202	Mi783, MiAC15, MiAC141, MiAC148

		H18	214	MiAC161, MiAC162
		H18	260	MiAC71, MiAC72, MiAC178
		H18	296	MiAC171
		H18	302	MiAC173, MiAC174
		H18	303	MiAC179, MiAC180
		H18	313	MiAC83, MiAC182
		H18	316-2	MiAC183
		H18	330	MiAC63
		H18	331	MiAC75, MiAC76
		H18	338	MiAC77
		H21 - *Neopolystoma* sp6	J2	Mi39
		H21	198	Mi983
		H21	213	Mi779
		H21	214	MiAC160
		H83	142	MiAC7
		H86 - *Polystomoides oris*	179	MiAC151, MiAC152
		H86	184	MiAC142
		H86	186	MiAC153, MiAC154
		H86	198	MiAC135, MiAC137
		H86	199	MiAC138, MiAC139
		H86	214	MiAC159
Mauremys leprosa	France - Têt_Bompas	H16 - *Polystomoides* sp1	79	MiAB602, MiAB622
		H20 - *Neopolystoma orbiculare*	93	MiAC119, MiAC228, MiAC490, MiAC491
		H20	89	MiAC128, MiAC224
		H20	92	MiAD325
		H20	103	MiAC494, MiAC495, MiAD320, MiAD322
		H37	76	MiAB605, MiAB606, MiAB610, MiAB628, MiAC218
		H37	92	MiAC114, MiAC492, MiAC493, MiAD324, MiAD327

		H37	103	MiAD321
		H21 - *Neopolystoma* sp6	89	MiAC113, MiAC469, MiAC470
Trachemys scripta elegans	France - Têt_Bompas	H16 - *Polystomoide*s sp1	1	MiAB629, MiAB639, MiAB642, MiAB643, MiAC583, MiAC350, MiAC351
		H16	2	MiAC593
		H16	24	MiAC360
		H16	3	MiAC585, MiAC352
		H16	4	MiAC591, MiAC592, MiAC354, MiAC355
		H16	5	MiAC356
		H16	7	MiAC590
		H20 - *Neopolystoma orbiculare*	3	MiAC353
		H20	7	MiAC589, MiAC357, MiAC358
		H81	3	MiAC586
Mauremys leprosa	France - Têt_Canet	H26 - *Polystomoides tunisiensis*	1	Mi-225*, Mi-226*, Mi-227*, Mi-228*, MiAC521, MiAC522, MiAC525, MiAC526
Mauremys leprosa	France - Agly	H21 - *Neopolystoma* sp6	69	MiAC467, MiAC468
		H21	83	MiAB615, MiAB631, MiAB632, MiAB635, MiAC479
Mauremys leprosa	France - Tech	H30 - *Polystomoides tunisiensis*	35	MiAC638
		H30	21	Mi657*, Mi658, Mi659, Mi660
		H30	22	Mi661, Mi662, Mi663, Mi664, Mi987*, Mi989*
		H30	24	Mi665, Mi667, Mi668
		H30	25	Mi669*
		H30	29	Mi670, Mi671, Mi672
		H30	30	Mi673, Mi676
		H32 - *Neopolystoma euzeti*	38	MiAC499
		H32	18	MiAB341, MiAB342, MiAB343
Mauremys leprosa	France - St. Hippolyte	H21 - *Neopolystoma* sp6	7	Mi244, Mi245, Mi246

		H21	8	Mi249
		H21	9	Mi243
		H21	10	Mi238, Mi239, Mi240
		H21	277	MiAC509
		H57	9	Mi241
Mauremys leprosa	Spain - Orlina	H19 - *Neopolystoma orbiculare*	15	MiAC543, MiAC544
		H31 - *Neopolystoma euzeti*	3	MiAC658, MiAD257
		H31	13	MiAC552
		H31	18	MiAC556
		H31	20	MiAD275
		H32	14	MiAC553, MiAC653, MiAD274
		H32	18	MiAC652, MiAD282, MiAD297, MiAD298
		H32	20	MiAC667
		H59 - *Polystomoides tunisiensis*	14	MiAC654, MiAD295
		H59	19	MiAC666
		H59	20	MiAD276, MiAD300, MiAD301, MiAD302
		H59	21	MiAC659
		H59	22	MiAC549, MiAC550, MiAC656
		H78	5	MiAC665, MiAD251, MiAD252
		H82	2	MiAC664
		H82	7	MiAC663, MiAD260, MiAD271, MiAD281
		H82	11	MiAC662, MiAD255, MiAD256
		H82	14	MiAD273
		H82	17	MiAD294
		H82	18	MiAD296, MiAD299
		H82	22	MiAD278
Mauremys leprosa	Spain - Anyet	H17 - *Neopolystoma* sp3	1	MiAC562
		H17	2	MiAD266
		H17	8	MiAD264, MiAD288, MiAD290, MiAD291

		H17	11	MiAC670, MiAD269, MiAD270, MiAD285, MiAD286
		H80	1	MiAC563
		H59 - *Polystomoides tunisiensis*	10	MiAC558, MiAC559
		H78	2	MiAD265
		H78	4	MiAC672
		H78	7	MiAD250
		H78	8	MiAD253
		H78	11	MiAC560, MiAC561
		H78	13	MiAC566
		H78	19	MiAC565
		H82	12	MiAC547
Mauremys leprosa	Spain - Merdanc	H31 - *Neopolystoma euzeti*	5	MiAC545, MiAC546
		H59 - *Polystomoides tunisiensis*	12	MiAC668, MiAD280
		H82	12	MiAC548
Mauremys leprosa	Spain - La Alfranca	H59 - *Polystomoides tunisiensis*	5009	MiAD347, MiAD348, MiAD349
		H59	5011	MiAD341, MiAD342, MiAD343, MiAD344, MiAD345, MiAD346
Mauremys leprosa	Algeria - Oued Rhiou	H25 - *Polystomoides tunisiensis*	1	Mi-110*, Mi-114*, Mi-116*, Mi-142*, Mi-143*, Mi-144*
		H31 - *Neopolystoma euzeti*	1	Mi-111*
Mauremys leprosa	Algeria - Rouina	H69 - *Polystomoides tunisiensis*	1	MiAB918, MiAB919
		H31 - *Neopolystoma euzeti*	1	MiAD330
		H87	2	MiAB920
Mauremys leprosa	Algeria - El Amra	H85 - *Polystomoides tunisiensis*	1	MiAD332
Mauremys leprosa	Algeria - Réghaïa	H70 - *Neopolystoma euzeti*	1	MiAB921

* Sequences reported in Verneau *et al.* (2011)

Appendix B: Overview of the ranking system and ranks assigned to source and habitat variables per risk region in this assessment study

DESCRIPTIONS	RANKS	SCORES	REFERENCE	REGIONS						MEASURE RANGES FOR RANKS	JUSTIFICATION	REFERENCES
Disturbance to wildlife	**Rank**	**Scores**	**Reference Condition**	**Region 1**	**Region 2**	**Region 3**	**Region 4**	**Region 5**	**Region 6**	**Distance of Buffer Zone**	**Justification**	**Reference**
Relative frequencies of habitat alterations by means of agriculture and land use next to river system	Zero	0	80	0	0	0	0	10	0	> 2000 m	Predictors of species persistence and local abundance for wetland reptiles at distances ranging from 250 to 2000 m from focal wetlands	Bonin, *et al.*, 2006; Burke and Gibbons, 1995 as reported in Roe and Georges, 2007
	Low	2	10	0	0	10	10	30	10	2000 - 1000 m		
	Moderate	4	10	30	10	30	20	50	60	1000 - 250 m		
	High	6	0	70	90	60	70	10	30	< 250m		
Invasives	**Rank**	**Score**	**Reference Condition**	**Region 1**	**Region 2**	**Region 3**	**Region 4**	**Region 5**	**Region 6**	**% Invasive present in system**	**Justification**	**Reference**
Absent	Zero	0	90	5	5	0	0	5	30	0%	Predictors of displacement of native populations species and competition among invasive and native species	Cadi and Joly, 2003; 2004; Polo-Cavia *et al.*, 2009; 2011
Low presence	Low	2	10	30	60	20	60	60	40	10%		
Present	Moderate	4	0	50	30	60	35	30	20	30%		
Reproducing	High	6	0	15	5	20	5	5	10	60%		
Natural Predators	**Rank**	**Score**	**Reference Condition**	**Region 1**	**Region 2**	**Region 3**	**Region 4**	**Region 5**	**Region 6**	**Diversity of predators present**	**Justification**	**Reference**
Absent	Zero	0	70	40	40	50	50	40	30	< 10 %	Interspecific differences in antipredator behaviour between *Trachemys scripta elegans* and *Mauremys leprosa*	Polo-Cavia *et al.*, 2008
Low presence	Low	2	20	30	30	25	35	50	40	10 % - 30 %		
Moderate presence	Moderate	4	10	25	25	20	10	5	20	30 % - 60 %		
High presence	High	6	0	5	5	5	5	5	10	> 60 %		
Substrate Availability	**Rank**	**Score**	**Reference Condition**	**Region 1**	**Region 2**	**Region 3**	**Region 4**	**Region 5**	**Region 6**	**% Reperian land available on riverbanks**	**Justification**	**Reference**
High basking	Zero	0	80	70	70	80	80	60	50	60 % Riperian land	Competition for	Cadi and Joly,

area											basking spots between native and invasive species	2003
Moderate basking area	Low	2	10	20	20	10	10	20	20	30 % Riperian land		
Low basking area	Moderate	4	10	10	5	10	10	10	20	10 % Riperian land		
No basking area	High	6	0	0	5	0	0	10	10	0 % Riperian land		
Water Quality / Conductivity	**Rank**	**Score**	**Reference Condition**	**Region 1**	**Region 2**	**Region 3**	**Region 4**	**Region 5**	**Region 6**	**Value of Conductivity in mS/m**	**Justification**	**Reference**
Very Low Conductivity	Zero	0	70	90	80	10	10	10	0	< 50 mS/m	Predictors of prefered conductivity whereby freshwater terrapins will enhabit an environment	Dunson and Mazzotti, 1989; Ficetola *et al.*, 2009
Low Conductivity	Low	2	20	10	20	80	70	80	30	50 - 500 mS/m		
Moderate Conductivity	Moderate	4	10	0	0	10	20	10	60	500 - 5000 mS/m		
High Conductivity	High	6	0	0	0	0	0	0	10	> 5000 mS/m		
Food Availability	**Rank**	**Score**	**Reference Condition**	**Region 1**	**Region 2**	**Region 3**	**Region 4**	**Region 5**	**Region 6**	**Diversity of food present in system**	**Justification**	**Reference**
High	Zero	0	60	10	10	10	10	10	10	> 70 % diversity	Predictors of high to low resource availability and quality	Mitchell, 1988; Cadi and Joly, 2003
Moderate	Low	2	30	30	15	15	30	20	25	70 - 50 % diversity		
Low	Moderate	4	10	40	60	60	40	45	45	50 - 20 % diversity		
None	High	6	0	20	15	15	20	25	20	< 20 % diversity		
***M. leprosa* Population Size**	**Rank**	**Score**	**Reference Condition**	**Region 1**	**Region 2**	**Region 3**	**Region 4**	**Region 5**	**Region 6**	**Amount of terrapins present**	**Justification**	**Reference**
High	Zero	0	60	0	0	10	10	0	0	n > 70	Professional Opinion	
Moderate	Low	2	20	20	10	60	10	0	0	70 - 40		
Low	Moderate	4	10	70	20	30	60	10	0	40 - 10		
None	High	6	10	10	70	0	20	90	100	n < 10		
Pathogens	**Rank**	**Score**	**Reference Condition**	**Region 1**	**Region 2**	**Region 3**	**Region 4**	**Region 5**	**Region 6**	**% of pathogen prevalence**	**Justification**	**Reference**
No infection	Zero	0	40	25	25	25	25	25	25	0%	Predictors of pathogen	Frye *et al.*, 1977; Hays *et*
Low infection	Low	2	30	25	25	25	25	25	25	10%		

Moderate infection	Moderate	4	20	25	25	25	25	25	25	30%	transmission between invasive species and native species	*al.,* 1999
High infection	High	6	10	25	25	25	25	25	25	60%		
Parasites	**Rank**	**Score**	**Reference Condition**	**Region 1**	**Region 2**	**Region 3**	**Region 4**	**Region 5**	**Region 6**	**% of parasite prevalence**	**Justification**	**Reference**
No infection	Zero	0	10	30	10	10	10	60	90	0%	Predictors of parasite transmission between invasive species and native species	Verneau *et al.,* 2011, Hidalgo-Vila *et al.,* 2009
Low infection	Low	2	20	40	10	40	40	20	10	10%		
Moderate infection	Medium	4	40	20	55	40	40	10	0	30%		
High infection	High	6	30	10	25	10	10	10	0	60%		

Appendix C: Conditional Probability Table was constructed and the exposure and effects were evaluated based on specific criteria and were assigned values which represented the specific state in each risk region

Conitional Probability table Unnatural Influence (DN)						Justification for CP's	
		Daughter node				JUSTIFICATION	REFERENCES
Disturbance to Wildlife (PN)	Invasives (PN)	Zero	Low	Moderate	High	Justification	References
Zero	Zero	90	10	0	0	Unnatural influences is low when the buffer zone for habitat alterations are > 2000 m from the focal water system and also when there are more connecte system to the main water system, and when invasive species are absent from the water system	Bonin *et al.*, 2006; Burke and Gibbons, 1995 as reported in Roe and Georges, 2007
Zero	Low	60	20	10	10		
Zero	Moderate	10	40	30	20		
Zero	High	0	10	40	50		
Low	Zero	60	20	10	10	Unnatural influences on freshwater terrapin species tend to increase slightly as the buffer sone are between 1000m - 2000m and as invasive species are establishing themselves in the water systems	Burke and Gibbons, 1995; Guzy, Price and Dorcas, 2013
Low	Low	30	60	10	0		
Low	Moderate	0	10	60	30		
Low	High	0	10	30	60		
Moderate	Zero	10	40	30	20	Buffer zone becomes smaller (250m - 1000m), and as invasive species tend to establish themselves more and could have the potential to displace native terrapin species from the river system, the risk becomes higher	Burke and Gibbons, 1995; Polo-Cavia *et al.* 2009
Moderate	Low	0	10	60	30		
Moderate	Moderate	10	40	40	10		
Moderate	High	0	0	30	70		
High	Zero	0	10	40	50	Buffer zone being below 250m from the riperian zone and invasive species that have established are reproducing in the water system could have a high risk on native terrapin species	Burke and Gibbons, 1995; Polo-Cavia *et al.* 2009; Cadi *et al.* 2004
High	Low	0	10	30	60		
High	Moderate	0	0	30	70		
High	High	0	0	10	90		

Note: (PN) Parent Node
(DN) Daughter Node

Disturbance to Wildlife (PN)	Invasives (PN)

Zero > 2000 m **Low** 2000 - 1000 m **Moderate** 1000 - 250 m **High** < 250m	**Zero** 0% **Low** 10% **Moderate** 30% **High** 60%

Conitional Probability table Competition and Predation (DN)						**Justification for CP's**	
		Daughter node				**JUSTIFICATION**	**REFERENCES**
Unnatural Influence (DN)	**Natural Predators (PN)**	**Zero**	**Low**	**Moderate**	**High**	**Justification**	**References**
Zero	Zero	100	0	0	0	Competition and predation is low when disturbance of wildlife and the presence of invasive species are absent or low and when the diversity of predators is less than 10% and interspecific differences in antipredator behaviour between *Trachemys scripta elegans* and *Mauremys leprosa* is similar	Polo-Cavia *et al.*, 2008; Burke and Gibbons, 1995; Guzy, Price and Dorcas, 2013
Zero	Low	75	15	10	0		
Zero	Moderate	5	25	60	10		
Zero	High	0	0	20	80		
Low	Zero	75	15	10	0	Risk of predation increases when the diversity of predators are between 10 - 30% of all the predators present in the river system and on the banks of the riperian area	Feinberg and Burke, 2003; Gibbons *et al.* 2000
Low	Low	20	70	10	0		
Low	Moderate	10	30	50	10		
Low	High	0	10	20	70		
Moderate	Zero	5	25	60	10	The more predators present >60 - 30% the higher the impact will be on nest predation and predation on new born and juvenile terrapins. Predators consist of crabs, mongooses, otters, seagulls and crows. The higher the effect from unnatural influences could also have a drastic impact on native terrapin species	Feinberg and Burke, 2003; Polo-Cavia *et al.*, 2008; Burke and Gibbons, 1995; Guzy, Price and Dorcas, 2013
Moderate	Low	10	30	50	10		
Moderate	Moderate	10	40	40	10		
Moderate	High	0	0	30	70		
High	Zero	0	0	20	80		
High	Low	0	10	20	70		
High	Moderate	0	0	30	70		
High	High	0	0	10	90		

Note: (PN) Parent Node
(DN) Daughter Node

Unnatural Influence (DN)	Natural Predators (PN)
Zero	**Zero** < 10 %
Low	**Low** 10 % - 30 %
Moderate	**Moderate** 30 % - 60 %
High	**High** > 60 %

Conitional Probability table Habitat availability / Quality (DN)						Justification for CP's	
		Daughter node				JUSTIFICATION	REFERENCES
Substrate Availability (PN)	**H2O quality/Conductivity (PN)**	**Zero**	**Low**	**Moderate**	**High**	**Justification**	**References**
Zero	Zero	100	0	0	0	Habitat suitability for species is high when riparian area ≥ 60 % and conductivity ≤ 50 mS/m according to Bodie, 2001	Cadi and Joly, 2003; Bodie, 2001
Zero	Low	80	20	0	0		
Zero	Moderate	10	30	50	10		
Zero	High	0	10	20	70		
Low	Zero	80	20	0	0	Habitat suitability for species decreases sharply according to Bodie, 2001 when conducticity increases ≤ 50 mS/m	Ficetola *et al.*, 2009; Bodie, 2001
Low	Low	30	60	10	0		
Low	Moderate	10	30	50	10		
Low	High	10	20	30	40		
Moderate	Zero	15	25	40	10	The tolerance levels of species is from 50 - 500 mS/m but condition is severly retarded between 500 mS/m and 5000 mS/m	Dunson and Mazzotti, 1989; Bodie, 2001
Moderate	Low	10	30	50	10		
Moderate	Moderate	10	40	40	10		
Moderate	High	0	10	30	60		
High	Zero	0	10	20	70	Salinity > 5000 mS/m limits the distribution of terrapins in a water system	Dunson and Mazzotti, 1989
High	Low	10	20	30	40		
High	Moderate	0	10	30	60		
High	High	0	0	10	90		
Note: (PN) Parent Node (DN) Daughter Node							

Substrate Availability (PN)	H2O quality/Conductivity (PN)
Zero 60 % Riperian land **Low** 30 % Riperian land **Moderate** 10 % Riperian land **High** 0 % Riperian land	**Zero** < 50 mS/m **Low** 50 - 500 mS/m **Moderate** 500 - 5000 mS/m **High** > 5000 mS/m

Conitional Probability table Environmental requirements (DN)						Justification for CP's	
		Daughter node				JUSTIFICATION	REFERENCES
Habitat Availability/Quality (DN)	Food Availability (DN)	Zero	Low	Moderate	High	Justification	References
Zero	Zero	90	10	0	0	Suitable habitat for native species is a clean water system with salinity of 0 - 500 mS/m and a riparian area suitable for basking. Food availability is best when a high diversity of food is present	Cadi and Joly, 2003; Bodie, 2001; Dunson, 1985; Dunson and Mazzotti, 1989
Zero	Low	70	30	0	0		
Zero	Moderate	10	30	50	10		
Zero	High	0	0	10	90		
Low	Zero	70	30	0	0	Diet of native species is: filamentous algae, insect larvae, earthworms, molluscs, amphibians, aquatic plants and carrion. As this diversity decreases, but water quality stays constant, the risk will increase slightly	Dunson, 1985; Dunson and Mazzotti, 1989; Palmer and Cordes, 1988
Low	Low	30	60	10	0		
Low	Moderate	10	30	50	10		
Low	High	10	20	20	50		
Moderate	Zero	10	30	50	10	When the riparian area decreases and basking spots become less but the salinity stays low, and there is a low diversity of food available, the risk will increase a bit	Dunson, 1985; Dunson and Mazzotti, 1989; Palmer and Cordes, 1988
Moderate	Low	10	30	50	10		
Moderate	Moderate	10	40	40	10		
Moderate	High	0	10	30	60		
High	Zero	0	0	10	90	When the riparian area is low where	Dunson, 1985;

High	Low	10	20	20	50	minimal basking areas is available and the salinity is high, with a low food diversity. The risk will increase drastically	Dunson and Mazzotti, 1989; Palmer and Cordes, 1988
High	Moderate	0	10	30	60		
High	High	0	0	20	80		

Note: (PN) Parent Node
(DN) Daughter Node

Habitat Availability/Quality (DN)	Food Availability (DN)
Zero Good riparian/salinity **Low** Good riparian/med. Salinity **Moderate** Low riparian/low salinity **High** Low riparian/high salinity	**Zero** High diversity **Low** Moderate diversity **Moderate** Low diversity **High** Very low diversity

Conitional Probability table *Mauremys leprosa*population health (DN)						Justification for CP's	
		Daughter node				JUSTIFICATION	REFERENCES
Pathogens (PN)	Parasites (PN)	Zero	Low	Moderate	High	Justification	References
Zero	Zero	90	10	0	0	Risk is low when a low diversity and prevalence of pathogens such as: *Aeromonas, Citrobacter, Enterobacter, Klebsiella, Proteus, Salmonella,* and *Serratia* together with low diversity and prevalence of parasites such as: Nematodes, Monogenea, Spirorchids, Bloodparasites are present in terrapins	Origgi and Jacobson, 2000; Frye *et al.*, 1977; Hays *et al.*, 1999; Merck Manual for pet health
Zero	Low	70	30	0	0		
Zero	Moderate	50	30	20	0		
Zero	High	10	20	30	40		
Low	Zero	70	30	0	0	As the prevalence in pathogens and parasites increase, the risk of bacterial infections in the mouth and intestinal	Origgi and Jacobson, 2000; Merck Manual
Low	Low	30	50	20	0		
Low	Moderate	20	30	40	10		

Low	High	10	15	35	40	regions could increase and could be fatal	for pet health
Moderate	Zero	50	30	20	0	Data is unavailable to demonstrate how the species responds to very high prevalences in pathogens and parasites above 50%, but from observations and specialist opinion it is assumed that very high prevalence levels of pathogens and parasites could be fatal according to Origgi and Jacobson, 2000	Origgi and Jacobson, 2000; Frye *et al.*, 1977; Hays *et al.*, 1999; Merck Manual for pet health
Moderate	Low	20	30	40	10		
Moderate	Moderate	10	40	40	10		
Moderate	High	0	20	30	50		
High	Zero	10	20	30	40		
High	Low	10	15	35	40		
High	Moderate	0	20	30	50		
High	High	0	0	20	80		

Note: (PN) Parent Node
(DN) Daughter Node

Pathogens (PN)	Parasites (PN)
Zero 0% Prevalence **Low** 20% Prevalence **Moderate** 30% Prevalence **High** 50% Prevalence	**Zero** 0% Prevalence **Low** 20% Prevalence **Moderate** 30% Prevalence **High** 50% Prevalence

Conitional Probability table Population well being (DN)						**Justification for CP's**	
		Daughter node				**JUSTIFICATION**	**REFERENCES**
***M. lep.* Pop Size (PN)**	***M. lep.* Pop Health (DN)**	**Zero**	**Low**	**Moderate**	**High**	**Justification**	**References**
Zero	Zero	90	10	0	0	Population well being is high when population size n>70 and Population health is 0% prevalence in pathogens and parasites according to	Origgi and Jacobson, 2000; Merck Manual for pet health
Zero	Low	70	30	0	0		
Zero	Moderate	20	30	40	10		
Zero	High	0	0	30	70		
Low	Zero	70	30	0	0	Population well being for species decreases as population size and health decreases	Origgi and Jacobson, 2000
Low	Low	40	50	10	0		
Low	Moderate	10	30	50	10		
Low	High	0	10	20	70		

Moderate	Zero	20	30	40	10	Data is unavailable to demonstrate how the species will respond to a reduction in population size < 40 but from specialist opinion it is assumed that as population health and population size both decrease, it will increase the sress levels on the population well being	
Moderate	Low	10	30	50	10		
Moderate	Moderate	10	40	40	10		
Moderate	High	0	20	30	50		
High	Zero	0	0	30	70		
High	Low	0	10	20	70		
High	Moderate	0	20	30	50		
High	High	0	0	20	80		

Note: (PN) Parent Node
(DN) Daughter Node

***M. lep.* Pop Size (PN)**	***M. lep.* Pop Health (DN)**
Zero n > 70 **Low** 70 - 40 **Moderate** 40 - 10 **High** n < 10	**Zero** 0% Prevalence **Low** 20% Prevalence **Moderate** 30% Prevalence **High** 50% Prevalence

Conitional Probability table Threats (DN)						**Justification for CP's**	
		Daughter node				**JUSTIFICATION**	**REFERENCES**
Competition and Predation (DN)	**Environmental Requirements (DN)**	**Zero**	**Low**	**Moderate**	**High**	**Justification**	**References**
Zero	Zero	100	0	0	0	Threats to native species is low when competition and predation is absent or low with good environmental requirements suitable for basking and nesting sites	Polo-Cavia, Lopez and Martin, 2008; Browne and Hecnar, 2007; Guzy, Price and Dorcas, 2013
Zero	Low	70	30	0	0		
Zero	Moderate	30	40	20	10		
Zero	High	0	10	20	70		
Low	Zero	70	30	0	0	Threats to native species will increase	Browne and

Low	Low	40	50	10	0	drastically as predator diversity increases and competition for food and basking and nesting spots increase	Hecnar, 2007; Guzy, Price and Dorcas, 2013
Low	Moderate	10	30	50	10		
Low	High	0	10	40	50		
Moderate	Zero	30	40	20	10	Threats to species will be high as predation, competition and invasive species increase with a decrease in habitat quality and water quality	Feinberg and Burke, 2003; Browne and Hecnar, 2007
Moderate	Low	10	30	50	10		
Moderate	Moderate	10	40	40	10		
Moderate	High	0	10	30	60		
High	Zero	0	10	20	70	Species may be at high risk as invasives reproduce and outcompete native species for food and habitat with a decrease in habitat quality	Cadi *et al.* 2004; Polo-Cavia, Lopez and Martin, 2008; Feinberg and Burke, 2003
High	Low	0	10	40	50		
High	Moderate	0	10	30	60		
High	High	0	0	20	80		

Note: (PN) Parent Node
(DN) Daughter Node

Competition and Predation (DN)	Environmental Requirements (DN)
Zero	**Zero**
Low	**Low**
Moderate	**Moderate**
High	**High**

Conitional Probability table Viability of native freshwater terrapins (DN)						Justification for CP's	
		Daughter node				JUSTIFICATION	REFERENCES
Population Wellbeing (DN)	Threats (DN)	Zero	Low	Moderate	High	Justification	References
Zero	Zero	90	10	0	0	Viability of species is high when population size and health is high and threats such as destruction of habitat, invasive species and predators are not present in a system	
Zero	Low	70	30	0	0		
Zero	Moderate	20	40	30	10		
Zero	High	0	30	60	10		

Low	Zero	70	30	0	0	As threats increase and habitat loss is of concern viability of species is sharply decreasing with the population well being also having an effect	
Low	Low	30	60	10	0		
Low	Moderate	10	20	70	0		
Low	High	10	10	20	60		
Moderate	Zero	20	40	30	10		
Moderate	Low	10	20	70	0		
Moderate	Moderate	10	40	40	10		
Moderate	High	0	10	30	60		
High	Zero	0	30	60	10		
High	Low	10	10	20	60		
High	Moderate	0	10	30	60		
High	High	0	0	20	80		

Note: (PN) Parent Node
(DN) Daughter Node

Population Well being (DN)	Threats (DN)
Zero	**Zero**
Low	**Low**
Moderate	**Moderate**
High	**High**