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# Changements environnementaux et menaces sur la biodiversité des écosystèmes aquatiques

Laurent Héritier

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# THÈSE

Pour obtenir le grade de  
**Docteur**

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Environmental Sciences and Management (NWU)

Spécialité : **Biologie**

Présentée par **Laurent Héritier**

**Changements environnementaux et  
menaces sur la biodiversité des  
écosystèmes aquatiques**

**Environmental changes and threats on  
freshwater ecosystems and biodiversity**

Soutenue le 13 décembre 2016 devant le jury composé de

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# Sommaire

Chapitre I : Introduction .....	7
I. Contexte scientifique .....	7
1. L’empreinte anthropique sur les écosystèmes.....	7
A. Menaces abiotiques .....	8
B. Menaces abiotiques.....	8
II. Objectifs de la thèse.....	10
III. Les menaces biotiques et abiotiques des sites d’études .....	11
1. Le glyphosate et l’AMPA .....	11
2. La tortue de Floride à tempes rouges .....	12
A. Compétition avec les espèces natives.....	12
B. Transferts de parasites .....	14
3. Les polystomes .....	15
IV. Modèles d’études .....	17
1. La cistude d’Europe.....	17
2. L’émyde lépreuse.....	19
V. Principaux résultats .....	21
1. Etude de la diversité parasitaire de la cistude d’Europe <i>Emys orbicularis</i> .....	21
2. Application d’un outil moléculaire novateur pour la discrimination des espèces de parasites.....	22

3. Description des deux nouvelles espèces invasives de polystomes identifiées sur des tortues d'eau douce indigènes et exotiques dans les écosystèmes français .....	23
4. Description de trois nouvelles espèces de polystomes identifiées sur des tortues d'eau douce natives d'Amérique du Sud .....	25
5. Développement d'un outil permettant d'évaluer l'état de santé des populations de tortues d'eau douce et sa validation en conditions expérimentales .....	26
6. Application des biomarqueurs validés sur des populations de tortues d'eau douce en milieux naturels .....	28
VII. Synthèse et perspectives .....	29
Chapitre II. Introduction and invasion of the red-eared slider and its parasites in freshwater ecosystems of Southern Europe: risk assessment for the European pond turtle in wild environments .....	71
Chapitre III. The High Resolution Melting analysis (HRM) as a molecular tool for monitoring parasites of the wildlife .....	118
Chapitre IV. Demonstrating the value and importance of combining DNA barcodes and discriminant morphological characters for polystome taxonomy (Platyhelminthes, Monogenea).....	138
Chapitre V. First report of <i>Neopolystoma</i> (Monogenea, Polystomatidae) species in freshwater turtles from South America .....	162
Chapitre VI. Oxidative stress induced by Glyphosate-based herbicide on freshwater turtles	188
Chapitre VII. Oxidative stress biomarkers in the Mediterranean pond turtle ( <i>Mauremys leprosa</i> ) reveal stressful aquatic ecosystems in Southern France .....	211

## Liste des figures

Figure 1. <i>T. s. elegans</i> dans un biotope en France (©P. Fita)	14
Figure 2. <i>T. s. elegans</i> et <i>M. leprosa</i> dans le même environnement en France (©P. Fita)	14
Figure 3. Cycle de vie des polystomes	16
Figure 4. La cistude d'Europe dans un écosystème en France (©P. Fita)	18
Figure 5. L'émyde lépreuse dans un écosystème en France (©P. Fita)	20

# CHAPITRE I: INTRODUCTION

## I. Contexte scientifique

### 1. L'empreinte anthropique sur les écosystèmes

L'extinction massive des espèces est un phénomène qui a eu lieu à plusieurs reprises dans l'histoire de la vie (Fastovsky, Sheehan 2005; Elewa, Joseph 2009; Archibald et al. 2010; Schulte et al. 2010). Bien que les causes naturelles, telles que les changements climatiques et géologiques, ont été largement mentionnées pour expliquer les extinctions historiques, la sixième vague d'extinction actuelle découle des actions anthropiques (Chapin III et al. 2000) et est considérée comme la plus rapide et dévastatrice (Ceballos et al. 2010; Barnosky et al. 2011). L'empreinte humaine sur la Terre est en réalité si profonde qu'elle a entraîné des changements environnementaux qui affecte et modifie le fonctionnement des écosystèmes (Vitousek 1994; Vitousek et al. 1996; 1997a; 1997b; Mack, D'antonio 1998; Sala et al. 2000; Steffen et al. 2011). Parmi les biotopes naturels, les écosystèmes d'eau douce sont des habitats remarquables offrant de multiples services écologiques via les propriétés hydrologiques, les ressources en eau et la régulation du climat. Ainsi, ils ont une grande valeur économique et contribuent au bien-être des humains. Ils abritent également une grande diversité d'espèces, à savoir pas moins de 100.000, ce qui correspond à environ 9,5% de toutes les espèces décrites (Dudgeon et al. 2006; Balian et al. 2008). En raison des activités anthropiques, les écosystèmes d'eau douce subissent une forte pression, conduisant à des menaces croissantes sur la biodiversité (Revenga, Mock 2000; Dudgeon et al. 2006; Vaughn 2010; Strayer, Dudgeon 2010). En effet, ils sont considérés comme plus en péril que les environnements terrestres et marins (Dudgeon et al. 2006; Strayer, Dudgeon 2010). Si les recherches sur les écosystèmes aquatiques continentaux sont considérées comme moins attrayantes que celles conduites sur environnements terrestres ou marins, les trajectoires de perte d'espèces en font sans doute les plus urgentes (Abell 2002). Il est donc essentiel de poursuivre et de mener des études approfondies sur les écosystèmes d'eau douce pour protéger et gérer la biodiversité aquatique. La dégradation des eaux est actuellement omniprésente dans les cours d'eau à travers le monde, en raison de l'augmentation des activités anthropiques (Sala et al. 2000; Albiac et al. 2010; Bhuiyan et al. 2013). Les principales causes de cette perte de qualité de



l'eau et de la perturbation des écosystèmes sont l'extraction de l'eau, le redressement des cours d'eau, la construction de barrages et de la pollution de l'eau, qui sont des facteurs abiotiques, et l'introduction d'espèces exotiques, au titre de facteur biotique (Malmqvist, Rundle 2002; Sabater, Barceló 2010).

#### A. Menaces abiotiques : la pollution des eaux

De nombreux polluants tels que les gaz toxiques, les métaux lourds, les composés organiques et les bio-toxiques sont libérés dans les milieux naturels (Wang et al. 2013). Il a été démontré que la pollution des eaux est essentiellement due aux activités anthropiques comme les rejets des usines de traitement des eaux usées et des activités agricoles et industrielles (Schwarzenbach et al. 2006; Trujillo-Reyes et al. 2014). En ce qui concerne les pratiques agricoles, l'utilisation d'engrais, d'hormones, d'antibiotiques et de pesticides pourrait être des sources importantes de pollution (Trujillo-Reyes et al. 2014). En effet, des résidus de pesticides sont accumulés dans les systèmes fluviaux à travers le monde (Gill, Garg 2014). En France, 270.000 kilomètres de cours d'eau courent sur tout le territoire. Différents pesticides sont détectés à la fois dans les eaux superficielles et souterraines du pays (Institut Français de l'Environnement 2007; Commissariat Général au Développement Durable-Service de l'observation et des statistiques 2015). En effet, parce que 36% des eaux de surface présentent une qualité de l'eau allant de modérée à moyenne en 2005, et parce que 92% des points de contrôle ont révélé la présence de pesticides en 2013, les niveaux de contamination sont considérables (Commissariat Général au Développement Durable-Service de l'Observation et des Statistiques 2015).

#### B. Menaces biotiques : les invasions biologiques

Les invasions biologiques impliquent le transport et la persistance d'organismes dans de nouveaux environnements (Mack, D'antonio 1998) et sont considérés comme une composante majeure des changements environnementaux mondiaux (Vitousek et al. 1997a). Le processus d'invasion est divisé en trois étapes, à savoir l'introduction, l'établissement et la propagation (Engel et al. 2011). L'introduction est le résultat du transport et de la libération accidentelle ou intentionnelle d'organismes vivants, liés aux activités humaines et à la mondialisation (voir Lockwood et al. 2013). L'établissement et la propagation de ces espèces peuvent être

également influencés ces dernières, notamment à cause des changements climatiques (Engel et al. 2011). En conséquence, des espèces exotiques sont reportées au sein de nouveaux environnements partout dans le monde et en particulier en Asie (Ding et al. 2008), aux États-Unis d'Amérique (Lodge et al. 2006) et en Europe (Hulme et al. 2009). Lorsque ces espèces exotiques s'adaptent à de nouveaux biotopes, elles peuvent directement rentrer en compétition avec les espèces indigènes et entraîner des effets néfastes sur les populations. Les invasions biologiques sont donc considérées comme une menace majeure pour la biodiversité (Mack, D'antonio 1998; Daszak et al. 2000; Mooney, Cleland 2001; Pimentel et al. 2005; Simberloff 2013; Bellard et al. 2016). Plusieurs types de compétitions ont été reportés suite à la présence dans les biotopes d'espèces non-natives et sont mis directement en cause dans le déclin de populations d'espèces natives. En effet, l'introduction de l'écrevisse de Louisiane *Procambarus clarkii* s'est traduit par une compétition avec les espèces d'écrevisses natives pour les ressources et l'habitat et a eu un impact négatif sur les populations de ces dernières (Gherardi and Daniels, 2004). De même pour l'introduction de la grenouille taureau d'Amérique du nord *Lithobates catesbeianus* qui a entraîné une forte diminution des populations natives d'amphibiens suite à de la compétition par prédation (Teixeira et al. 2009). Le déplacement d'espèces peut également entraîner des déséquilibres écologiques par isolement génétique et baisse de la diversité allélique chez les espèces natives. C'est le cas sur les îles Bahamas où des rats ont été introduits et ont prédaté une espèce de lézard locale, *Anolis sagrei*, ce qui a conduit à un isolement des populations de sauriens et donc à une baisse de la diversité génétique, menaçant ainsi ces dernières (Gasc et al. 2010).

Un autre cas de compétition auquel nous nous sommes particulièrement intéressés est celui de l'introduction de parasites. En effet, ces derniers peuvent également être introduits avec leurs hôtes, bien que leur impact soit moins étudié que celui de ces derniers (Lowry et al. 2013). Une fois transmis aux espèces indigènes, les parasites peuvent affecter les interactions écologiques et la dynamique des populations de leurs hôtes (Peeler et al. 2011; Lymbery et al. 2014). En conséquence, le déplacement, l'installation et la reproduction d'espèces en dehors de leur environnement d'origine peut conduire à la prolifération d'espèces envahissantes et de leurs parasites, ce qui contribue à la perte de la biodiversité à l'échelle mondiale (Hulme 2007; Clavero et al. 2009). Plusieurs formes d'impact sur les populations naturelles ont été révélées concernant ces introductions d'hôtes et de parasites exotiques. En effet, de nombreuses études ont montré que les espèces de parasites invasives pouvaient infecter les espèces hôtes natives et constituer une menace pour ces dernières, que ce soit chez les invertébrés, par exemple chez les insectes (Meeus et al. 2011), ou chez les vertébrés tels que

les poissons (Gozlan et al. 2005; Barry et al. 2014), les amphibiens (voir Bucciarelli et al. 2014), les oiseaux (Wikelski et al. 2004; Miller et al. 2016) ou encore les tortues d'eau douce (Hays et al. 1999; Iglesias et al. 2015). Ce processus invasif est appelé « spill over » et reste le plus fréquemment mis en évidence (Kelly et al. 2009). Cependant, d'autres concepts ont également été évoqués. En effet, les espèces invasives hôtes peuvent perdre leurs parasites lors d'introductions dans de nouveaux milieux (Torchin et al. 2003) et peuvent être infectés par des parasites natifs de la faune locale (Poulin and Mouillot 2003). De ce fait, les hôtes exotiques peuvent ainsi constituer des réservoirs de parasites naturels et entraîner des modifications au niveau de la relation hôtes-parasites natifs, phénomène appelé « spill back » (Kelly et al. 2009). L'acquisition de parasites natifs par des hôtes invasifs peut entraîner une amplification de la dynamique parasitaire et par conséquent des prévalences plus élevées et une augmentation des risques de maladies dans le cas de parasites pathogènes (Kelly et al. 2009 ; Lymbery et al. 2014 ; Sherrard-Smith et al. 2014). Cependant, si les hôtes exotiques peuvent être infectés par les phases infestantes des parasites natifs mais que ces derniers ne peuvent se développer et se reproduire dans ce nouvel environnement hôte, alors ils peuvent jouer le rôle de puits de parasites et ainsi réduire les niveaux d'infestations chez les hôtes natifs, ce phénomène étant appelé « dilution effect » (Keesing et al. 2006 ; Kelly et al. 2009).

## II. Objectifs de cette thèse

De par la grande variété des habitats qu'il comprend, le bassin Méditerranéen fait partie des hotspots mondiaux de biodiversité (Cuttelod et al. 2008). En effet, cette zone comprend de nombreux habitats terrestres, montagnes, déserts et plaines, mais aussi des biotopes marins et aquatiques d'eau douce. Les espèces rencontrées dans le bassin Méditerranéen subissent également des pressions dues aux activités anthropiques. L'objectif principal de cette thèse était d'étudier les effets des modifications environnementales sur la biodiversité. Cependant, pour répondre à cette problématique, il a fallu lever plusieurs verrous scientifiques. Dans un premier temps, afin d'évaluer l'importance des menaces biotiques, il convenait de réaliser une étude de la diversité parasitaire sur une espèce modèle pour mettre en évidence d'éventuels transferts de parasites exotiques en milieu naturel. De même, le développement d'un outil d'identification des espèces de parasites s'est avéré primordial pour réaliser des suivis efficaces sur des espèces natives potentiellement parasitées. Enfin, le travail le plus important de cette thèse s'est orienté vers le développement d'un outil permettant de mesurer l'état de

santé de populations d'une espèce modèle. Nous avons choisi de réaliser ce travail au sein du bassin Méditerranéen et particulièrement dans la région Occitanie, située dans le sud de la France, avec un intérêt tout particulier pour le département des Pyrénées-Orientales.

### III. Les menaces biotiques et abiotiques des sites d'étude

Dans la région Occitanie et particulièrement dans le département des Pyrénées-Orientales, les activités agricoles sont encore intenses et principalement basées sur les cultures fruitières et les vignobles. En conséquence, des concentrations élevées de pesticides ont été détectées dans certains cours d'eau de ce département, ce qui a entraîné un déclassement de ces derniers avec une mauvaise qualité des eaux. De plus, d'importantes populations sauvages de *T. s. elegans* sont établies dans de nombreux écosystèmes au sein des Pyrénées-Orientales.

#### 1. Le glyphosate et l'AMPA

En 2003, 2004 et 2005, les principaux composés incriminés dans la mauvaise qualité des eaux étaient l'acide aminométhylphosphonique (AMPA), le glyphosate et le diuron (Institut Français de l'Environnement 2007). Depuis 2009, les principaux composés sont l'AMPA, le glyphosate et l'atrazine, un herbicide dont la vente est interdite en France depuis dix ans (Commissariat général au Développement durable Service de l'observation et des statistiques 2015). Les propriétés herbicides du glyphosate ont été découvertes et brevetées par la société Monsanto en 1974. Il a été vendu sous le nom de Roundup et est aujourd'hui l'herbicide le plus utilisé dans le monde entier (Franz et al. 1997). Le glyphosate est un acide organique faible ( $C_3H_8NO_5P$ ) utilisé sous forme de sels ( $C_6H_{17}N_2O_5P$ ) dans les formulations commerciales afin d'augmenter sa solubilité dans l'eau (Giesy et al. 2000). Les herbicides à base de glyphosate sont principalement composés de sel d'isopropylamine de glyphosate et un agent tensioactif, en général le polyoxyéthylène amine (POEA) (Giesy et al. 2000). Le glyphosate inhibe l'enzyme synthase 3-énolpyruvyl-shikimat-3-phosphate, qui est impliquée dans la synthèse des acides aminés aromatiques (Battaglin et al. 2005). Le rôle de l'agent tensioactif est de favoriser la pénétration du glyphosate dans les feuilles des plantes (Battaglin et al. 2005). Le premier composé produit lors de la dégradation du glyphosate par des micro-organismes est l'AMPA (Kolpin et al. 2006). De nombreuses bactéries peuvent dégrader le glyphosate, à savoir *Pseudomonas*, *Enterobacter*, *Rahnella*, *Serratia* et des cyanobactéries

(Forlani et al. 2008; Arunakumara et al. 2013; Benslama, Boulahrouf 2013). Si le glyphosate et l'AMPA sont détectés dans le même site, il est alors considéré que l'AMPA provient de la dégradation de ce dernier (Skark et al. 1998; Kolpin et al. 2006; Botta et al. 2009). Parce que sa durée de demi-vie est plus longue, l'AMPA est plus souvent détectée dans les cours d'eau que le glyphosate (Giesy et al. 2000). Ces deux composants sont détectés dans la plupart des zones humides de nombreux départements français, à l'exception des zones sans activités agricoles intenses ou localisées proches de reliefs (Commissariat général au Développement durable Service de l'observation et des statistiques 2015). La présence de ces composants dans les écosystèmes d'eau douce français pourrait devenir une menace sérieuse pour la biodiversité (Gill, Garg 2014).

La toxicité des formulations commerciales à base de glyphosate, ainsi que la toxicité des composés actifs isolés, c'est-à-dire le glyphosate, le POEA et le produit de dégradation l'AMPA, ont été étudiées chez les vertébrés. Il a été démontré dans des conditions expérimentales que les herbicides à base de glyphosate sont plus toxiques que le glyphosate testé seul (Howe et al. 2004; Peixoto 2005; Richard et al. 2005; Gasnier et al. 2009). En conséquence, ces formulations ont été testées par la suite chez les poissons, les amphibiens et les reptiles et se sont été révélées toxiques pour ces organismes (Relyea 2005a, b; Glusczak et al. 2006; Glusczak et al. 2007; Cavalcante et al. 2008; Costa et al. 2008; Do Carmo Langiano, Martinez 2008; Poletta et al. 2009; Modesto, Martinez 2010).

## 2. La tortue de Floride à tempes rouges

### A. Compétition avec les espèces natives

La tortue de Floride à tempes rouges est une tortue d'eau douce originaire de l'est des États-Unis et du nord du Mexique (Van Dijk et al. 2014). Parce que les juvéniles sont attrayants et très colorés, cette espèce a été largement exportée dans les années quatre-vingt-dix des États-Unis vers l'Europe (Lutz 2000) pour être vendue en tant qu'animal de compagnie, environ 52 millions d'individus entre 1989 et 1997 (Telecki 2001). Néanmoins, *T. s. elegans* présente une croissance rapide, devient agressive et beaucoup moins attrayante à l'âge adulte. De plus, sa taille relativement grande, de l'ordre de 20-30 cm de longueur de carapace, nécessite des équipements vastes et coûteux pour son maintien. En conséquence, de nombreux propriétaires ont relâché ces animaux devenus indésirables dans les milieux naturels. Parce que cette tortue

d'eau douce est rencontrée dans une vaste gamme d'écosystèmes aquatiques dans son habitat naturel et est de ce fait considérée comme généraliste (Bringsøe 2006), elle s'est adaptée à son nouvel environnement dans lequel elle a pu se reproduire et établir des populations. De ce fait, la tortue de Floride à tempes rouges figure dans la liste mondiale des 100 espèces envahissantes les plus néfastes du monde (Lowe et al. 2000). En effet, *T. s. elegans* est de nos jours rencontrée à l'état sauvage sur presque tous les continents, comme par exemple en Asie (Ramsay et al. 2007) ou en Europe dans des pays comme la Bulgarie (Tzankov et al. 2015), l'Italie (Ficetola et al. 2003; Fattizzo 2004; Ficetola et al. 2009), l'Espagne (Izquierdo et al. 2010) et la France (Servan, Arvy 1997; Arvy, Servan 1998; Meyer et al. 2015) (Figure 1). Elle peut ainsi fréquenter les mêmes milieux que d'autres espèces de tortues indigènes (figure 2). La tortue de Floride à tempes rouges présente des caractéristiques biologiques et écologiques particulières et peut constituer une réelle menace pour les espèces de tortues d'eau douce natives. En effet, c'est un omnivore opportuniste qui se nourrit d'une grande variété de plantes et d'animaux (Prévot-Julliard et al. 2007), présente une grande taille et un taux de fécondité élevé (Arvy et Servan, 1998; Bringsøe 2006). Il a été montré que *T. s. elegans* est entré en compétition avec la tortue à ventre rouge *Pseudemys rubiventris* (Le Conte, 1830) aux Etats-Unis (Pearson 2013) et est soupçonné d'être responsable du déplacement de la tortue casqué africaine *Pelomedusa subrufa* (Bonnaterre 1789) en Afrique du Sud (Ramsay et al. 2007). Il a également été démontré dans des conditions expérimentales que la tortue de Floride à tempes rouges pouvait rivaliser avec les deux tortues d'eau douce indigènes de France, la cistude d'Europe *Emys orbicularis* (Linnaeus 1758) pour les sites d'ensoleillement (Cadi, Joly, 2003) et les ressources alimentaires (Cadi, Joly 2004), ainsi qu'avec l'émyde lepreuse *Mauremys leprosa* (Schweigger 1812) pour les sites d'ensoleillement, les ressources alimentaires et le territoire (Polo-Cavia et al. 2008, 2009a, b, 2010, 2011, 2012).



Figure 1. *T. s. elegans* dans un biotope en France.



Figure 2. *T. s. elegans* et *M. leprosa* dans le même environnement en France.

## B. Transferts de parasites

Plusieurs études ont montré que *T. s. elegans* a été introduit dans les environnements naturels hors de son aire de distribution avec ses parasites. En Amérique du Nord, un cas de déclin des populations d'une espèce indigène, la tortue de l'ouest *Emys marmoratus* (Baird et Girard 1852) a été rapporté suite à la transmission de virus ou de mycoplasmes (Hays et al. 1999). De même en Espagne, une population de cistudes d'Europe a fortement diminué suite à

l'introduction d'hémoparasites (Iglesias et al. 2015). Dans le sud de l'Europe, en France et en Espagne, l'émyde lépreuse partagent souvent son environnement naturel avec la tortue de Floride à tempes rouges. Parce que Verneau et al. (2011) ont montré des transferts de parasites des tortues américaines vers les tortues indigènes en France en captivité, Meyer et al. (2015) ont mené une étude, principalement dans le sud de la France et dans le nord de l'Espagne, consistant à réaliser un inventaire de la diversité des parasites retrouvés chez *M. leprosa* en environnements naturels. Si ces auteurs ont montré la présence de parasites indigènes, un nombre inattendu d'espèces de parasites exotiques a été identifié à la fois chez l'espèce native et chez l'espèce invasive, laissant penser que *T. s. elegans* pourrait être un vecteur de parasites.

### 3. Les polystomes

Dans le phylum des plathelminthes, la classe des Polystomatoinea ne comprend qu'une seule famille, les Polystomatidés (Sinnappah et al. 2001). Cette dernière est représentée par 25 genres qui infectent les branchies ou la cavité buccale du dispneuste (Pichelin et al. 1991), la vessie des amphibiens en phase adulte (voir Prudhoe, Bray 1982), la vessie, les cavités palpébrales et le pharynx des chéloniens d'eau douce (voir Morrison, du Preez 2011) et la paupière de l'hippopotame africain (Thurston, Lois 1965). Parce que *Concinnocotyla australensis* (Reichenbach-Klinke 1966) occupe une position basale dans l'arbre phylogénétique des polystomes, comme son hôte Dipnoi au sein des Sarcoptérygiens, une origine très ancienne a été supposée pour les Polystomatidés (Verneau et al. 2002). Elle pourrait être liée à la divergence entre les Actinoptérygiens et les Sarcoptérygiens, il y aurait environ 425 millions d'années selon les synthèses paléontologiques de Janvier (1988) et Ahlberg (1999). Verneau et al. (2002) illustre également une relation phylogénétique de groupes frères entre les parasites des Batrachia (anoures et urodèles) et des chéloniens d'eau douce, qui pourrait être corrélée à la séparation entre les amphibiens et les amniotes. Enfin, Héritier et al. (2015) ont évoqué la possibilité d'un transfert de parasites de céciliens, ordre des Gymnophiones, vers les tortues d'eau douce, il y aurait environ 174 millions d'années, lorsque certaines tortues primitives auraient déjà été adaptée à un style de vie aquatique.

Les polystomes sont considérés comme hôte et site spécifiques (Verneau 2004). Parce que ces parasites ont un cycle de vie direct comprenant une phase larvaire infectante (oncomiracidium), ils constituent un bon modèle pour étudier les invasions biologiques,



comme cela a été montré dans les études menées par Verneau et al. (2011) et Meyer et al. (2015) sur les tortues d'eau douce. Les polystomes infectant les tortues d'eau douce sont divisés en trois genres, à savoir *Polystomoides*, *Polystomoidella* et *Neopolystoma*, en fonction du nombre d'hamuli situés entre la paire de ventouses postérieures du haptère, à savoir deux paires, une paire et aucune, respectivement. Selon Verneau 2004, environ cinquante espèces de polystomes infectant les tortues d'eau douce sont identifiées dans le monde entier.

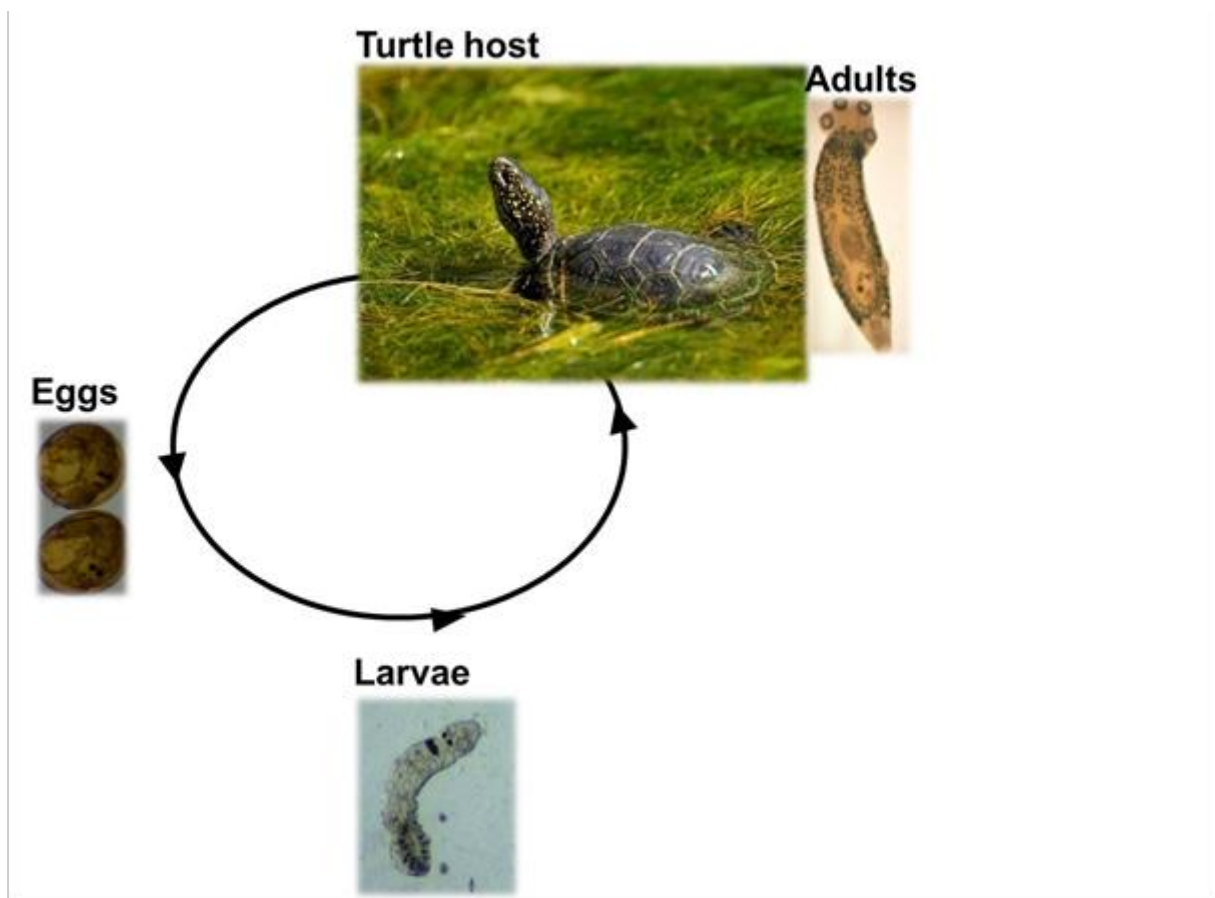


Figure 3. Cycle de vie des polystomes.

## IV. Les modèles d'étude

Nous avons choisi comme modèles d'étude les deux espèces de tortues indigènes de France et rencontrées dans les Pyrénées-Orientales, la cistude d'Europe *Emys orbicularis* et l'émyde lépreuse *Mauremys leprosa* pour plusieurs raisons essentielles. En effet, ces animaux peuvent être confrontés aux stress biotiques et abiotiques étant donné que ce sont des tortues aquatiques d'une part, et donc directement en contact avec les stress abiotiques, et que la tortue de Floride à tempes rouges est également un chélonien aquatique d'autre part, fréquentant les mêmes sites que ces deux dernières. De plus, les tortues sont sensibles aux polluants mais relativement résistantes ce qui permet de mesurer un effet de stress et de suivre l'évolution des cours d'eau en terme de qualité pour les espèces, ce qui n'aurait pas été possible avec les amphibiens qui sont beaucoup plus sensibles et disparaissent des cours d'eau si les concentrations en polluants sont trop élevées (Sparling et al. 2001; Howe et al. 2004). Enfin, *E. orbicularis* et *M. leprosa* sont des espèces protégées en France et bénéficient de plans nationaux d'actions qui intègrent l'impact des modifications environnementales sur ces deux espèces.

### 1. La cistude d'Europe *Emys orbicularis*

La cistude d'Europe est une tortue d'eau douce considérée comme une espèce phare du patrimoine naturel européen (Figure 3). Elle est la seule représentante européenne des Emydidae, qui sont principalement distribués dans la région néarctique avec 53 espèces (Van Dijk et al. 2014), avec *Emys trinacris* (Fritz et al. 2005b). La cistude d'Europe atteint environ 20cm à l'âge adulte et se caractérise par des taches jaunes sur le corps et la carapace, de longues griffes et une longue queue effilée. Cette espèce est particulièrement attachée aux milieux aquatiques (Dall'Antonia et al. 2001), où elle se nourrit de mollusques, de poissons ou d'amphibiens morts, d'insectes aquatiques et de têtards (Kotenko 2000). *E. orbicularis* présente une large aire de répartition, allant de la côte atlantique de l'Afrique du Nord à l'Ouest à la mer d'Aral, et de la région de Moscou jusqu'au sud de la Turquie (voir Fritz et al. 2009). Sa répartition géographique actuelle aurait été principalement façonnée par des événements climatiques successifs qui ont eu lieu durant le Quaternaire. Parce que la France est une zone de contact entre les populations des Balkans et celles des pays d'Europe du Sud comme l'Espagne et l'Italie, la présence de la cistude d'Europe dans les écosystèmes

aquatiques français reflète probablement plusieurs vagues de recolonisation des refuges du Sud durant l'Holocène (Lenk et al. 1999; Fritz et al. 2005a; Sommer et al. 2009; Pedall et al. 2011). La cistude d'Europe peut être divisée en au moins dix haploclades ou sous-espèces (Fritz et al. 2009; Stuckas et al. 2014), cette différenciation génétique ayant débuté il y a 3.0-4,1 millions d'années, à savoir au début du Pliocène moyen (Lenk et al. 1999; Fritz et al. 2009).

Malgré une baisse globale des populations en raison de rituels ancestraux, tels que sa consommation des temps préhistoriques jusqu'au milieu du XIXe siècle (Cheylan, Courtin 1976; Cheylan, Poitevin 1998; Cheylan, Poitevin 2003) et son utilisation en pharmacologie dans le XIXe siècle (voir Cheylan, Poitevin 1998), des populations d'*E. orbicularis* existent encore en France, en particulier dans la moitié sud du pays. Cependant, cette espèce était probablement plus répandue à l'époque préhistorique, comme en témoigne sa présence sur les sites archéologiques (Cheylan, Poitevin 1998; Hervet, Salotti 2000; Sommer et al. 2009). Basé sur la taille et la distribution géographique des populations, cette espèce a été longtemps considérée comme « quasi-menacée » en France (UICN France, MNHN et SHF, 2009), mais aussi dans le bassin méditerranéen (Cox et al. 2006) et en Europe (Cox, Temple 2009b). Son statut en France est maintenant de "préoccupation mineure" (UICN France, MNHN et SHF 2015), ce qui indique que le risque d'extinction peut être moins important que prévu. En Espagne, cependant, l'espèce est toujours considérée comme «vulnérable» (Pleguezuelos et al. 2004).



Figure 4. La cistude d'Europe dans un écosystème en France

## 2. L'émyde lépreuse *Mauremys leprosa*

L'émyde lépreuse est une tortue d'eau douce également représentative de la faune européenne. Cette tortue atteint environ 25 cm à l'âge adulte et se caractérise par une carapace ovale dont la couleur est généralement brun-rouge (Figure 4). Cette espèce se rencontre dans les ruisseaux et les étangs riches en végétaux (Da Silva, Blasco 2002) et pourrait être plus généraliste que la cistude d'Europe concernant le milieu de vie (Segurado, Araújo 2004). Son régime alimentaire est similaire à celui d'*E. orbicularis*. L'émyde lépreuse est originaire des régions occidentales d'Afrique du Nord selon les données fossiles (De Lapparent de Broin 2000). Sa présence sur la péninsule Ibérique remonterait au Pléistocène, ou même encore au Pliocène (De Lapparent De Broin 2001) et cette expansion démographique a été confirmée par l'identification génétique des deux lignées principales, *Mauremys leprosa leprosa* (Péninsule ibérique et le nord du Maroc) et *Mauremys leprosa saharica* (Sud du Maroc, Algérie et Tunisie) (Fritz et al. 2006). Son aire de distribution comprend les pays riverains de la mer Méditerranée, à savoir la Tunisie, l'Algérie et le Maroc en Afrique du Nord, ainsi que l'Espagne, le Portugal et la France en Europe du Sud (Bonin et al. 1998). Alors que le statut de *M. leprosa* a été considéré de "préoccupation mineure" en Afrique du Nord (Cox et al. 2006), cette tortue a été classée comme «vulnérable» dans la liste rouge européenne des reptiles (Da-Silva 2002; Cox, Temple 2009a) et comme «menacée d'extinction» en France (UICN France, MNHN et SHF, 2009). L'émyde lépreuse est aujourd'hui classée comme «vulnérable» en France (UICN France, MNHN et SHF, 2015), en partie à cause de la présence de petites populations endémiques du département des Pyrénées-Orientales, dans la région Occitanie (voir Palacios et al. 2015).



Figure 5. L'émyde lépreuse dans un écosystème en France

## V. Principaux résultats

### 1. Etude de la diversité parasitaire de la cistude d'Europe *E. orbicularis*

Cette étude fait l'objet d'un article en révision dans la revue Biodiversity and Conservation et correspond au chapitre II du présent document. Verneau et al. (2011) ont montré des transferts de parasites des tortues exotiques vers les deux espèces en captivité et Meyer et al. (2015) de *T. s. elegans* vers *M. leprosa* en milieux naturels. Devant un tel constat, une étude de la diversité des parasites de la cistude d'Europe a été menée en France, en Espagne et en Afrique du Nord, afin de vérifier si les parasites exotiques pourraient être identifiés sur cette tortue dans les écosystèmes d'eau douce.

Basé sur l'étude du gène codant pour la Cytochrome c Oxydase (COI), utilisé comme marqueur génétique pour la discrimination des espèces, cette étude met en évidence la présence de six espèces de parasites distinctes sur *E. orbicularis* dans les zones d'étude. Si l'espèce de parasite naturelle de la Cistude d'Europe a été identifiée en France, en Espagne et en Algérie, les autres espèces étaient soit des parasites exotiques décrites sur leurs hôtes dans les écosystèmes américains, soit des espèces non décrites mais considérées comme exotiques parce que rencontrées sur *E. orbicularis* et *T. s. elegans*. Ainsi, cette étude révèle des transferts de polystomes de l'espèce hôte exotique vers l'espèce native et illustre un autre cas d'invasion parasitaire sur un nouvel hôte tortue en Europe du sud, comme l'étude de Meyer et al. (2015) sur *M. leprosa*.

De nombreuses études ont confirmé que l'introduction de parasites non natifs pouvait constituer une menace pour les populations d'hôtes naturelles (Wikelski et al. 2004; Gozlan et al. 2005; Barry et al. 2014; Bucciarelli et al. 2014; Miller et al. 2016). Étant donné qu'aucune étude n'a été menée sur la pathogénicité de ces parasites, il pourrait être intéressant dans un contexte de conservation des espèces de développer une procédure dans le but d'illustrer si la santé des tortues d'eau douce française est impactée par la présence de ces parasites exotiques. De plus, le parasite naturel de la cistude d'Europe n'a été identifié que sur deux sites naturels en France. Dans tous les autres écosystèmes étudiés, *E. orbicularis* n'est infectée que par des espèces de polystomes invasives. Ainsi, les parasites naturels pourraient être également menacés par les espèces invasives. Il était prévu au cours de la thèse de réaliser des

infestations de tortues d'eau douce en conditions expérimentales avec des parasites naturels et exotiques afin de vérifier si une concurrence entre les espèces de parasites pouvait s'établir. Parce que cette expérimentation demandait des moyens techniques importants, elle n'a pas pu être effectuée. Cependant, cette expérience reste intéressante à prévoir dans l'avenir.

## 2. Application d'un outil moléculaire novateur pour la discrimination des espèces de parasites

Cette étude fait l'objet d'un article publié dans la revue *Parasitology* et correspond au chapitre III du présent document. L'étude de la biodiversité des polystomes au sein des populations de tortues d'eau douce implique le génotypage des échantillons, réalisé par l'amplification du gène COI par Polymerase Chain Reaction (PCR) et le séquençage. Parce que les espèces natives bénéficient d'un statut de protection, la plupart des échantillons analysés étaient des œufs de polystomes, collectés à l'aide d'une méthode non-invasive (Verneau et al. 2011). Etant donné que cette approche moléculaire implique la séparation des échantillons, effectuer des analyses à partir d'œufs de parasites la rendent longue et coûteuse. L'outil moléculaire High Resolution Melting (HRM) permet la détection des variations entre les séquences polymorphes grâce aux propriétés de fusion de l'ADN double brin amplifié. Cette approche présente plusieurs avantages, en effet les résultats peuvent se lire directement sans séquençage et plusieurs ADN mélangés peuvent être analysés dans un même échantillon. Les résultats ont montré que, pour chaque espèce parasite, lorsqu'un seul ADN a été utilisé dans les analyses, une température de dénaturation spécifique du gène cible a été obtenue, permettant la discrimination de chaque espèce. Ainsi, cette technique moléculaire s'est avérée très intéressante pour les analyses de mélange d'œufs de tortues infectées par une seule espèce de polystome. Parce que Meyer et al. (2015) ont également montré que dans la majorité des cas, l'émyle lépreuse est infectée par deux espèces de polystome en milieu naturel, l'une du pharynx et l'autre de la vessie, l'approche moléculaire HRM a été utilisée sur des mélanges d'ADN de deux espèces parasites, dans des proportions égales et différentes. Bien que jamais plus de deux espèces de polystomes du pharynx et de la vessie n'aient été identifiées au sein d'une population hôte, un mélange contenant l'ADN de trois espèces parasites a été également analysés. Les résultats ont montré que l'analyse HRM a permis la détection d'une double infection avec identification des espèces parasites et proportion de chaque espèce parasite infectant l'hôte. Cependant, cet outil moléculaire a montré des limites dans le cas

d'une triple infection, mais a tout de même permis d'obtenir des informations sur le nombre d'espèces polystome infectant une population de tortues d'eau douce sans pouvoir identifier avec précision les espèces.

Globalement, l'approche moléculaire HRM a été efficace pour différencier les différentes espèces de polystomes sélectionnées, en particulier dans les cas de simples et doubles infections. Dans le cas d'une population de tortues infectée par une ou deux espèces de polystome, cette technique permettrait de réaliser des économies de temps considérables et reviendrait nettement moins cher que l'outil moléculaire impliquant l'amplification génique par PCR et séquençage. En effet, de nombreux œufs provenant de différentes tortues peuvent être analysés dans un même échantillon et ce qui peut apporter rapidement des informations sur la diversité parasitaire d'une population hôte. La limite de cette méthode serait atteinte avec trois différentes espèces de parasites infectant une population de tortues. Les analyses ont permis de détecter la présence de plus de deux espèces de parasites, mais pas d'identifier les espèces. Cependant, il peut être utilisé pour une étude préliminaire de la diversité du parasite au sein d'une population hôte. Cette étude peut être poursuivie en incluant les autres espèces de polystome identifiées en France sur d'autres tortues hôtes, à savoir *E. orbicularis* et *T. s. elegans*, afin établir des suivis plus performants d'invasions parasitaires dans les écosystèmes aquatiques continentaux.

### 3. Description de deux nouvelles espèces invasives de polystomes identifiées sur des tortues d'eau douce indigènes et exotiques dans les écosystèmes français

Cette étude fait l'objet d'un article en révision dans la revue *Parasitology International* et correspond au chapitre IV du présent document. Des études moléculaires (Verneau et al. 2011; Meyer et al. 2015; Héritier et al. submitted) ont montré la présence de deux espèces de polystome infectant le pharynx des tortues exotiques et natives dans des espaces confinés ou naturels en France. Parce que ces deux espèces de parasites ont été également identifiées sur *Trachemys scripta* dans les écosystèmes aquatiques américains et parce que *E. orbicularis* et *M. leprosa* n'ont jamais été observées dans ces écosystèmes, elles ont été considérées comme des espèces parasites invasives en France, introduites par *T. s. elegans*. De ce fait, il s'est avéré primordial de les décrire dans un contexte de surveillance des invasions parasitaires dans les écosystèmes français.



L'étude des caractères morphologiques peut permettre la distinction d'espèces de polystome au sein d'un même genre, mais la plupart de ces caractères s'avèrent non discriminants pour la distinction d'espèces appartenant à deux genres différents. En conséquence, d'autres critères ont été utilisés pour la création de nouvelles espèces, telles que la spécificité de l'hôte reconnue depuis plusieurs années pour les polystomes (Du Preez, Kok 1997). Cependant, les études moléculaires menées sur ces parasites ont montré qu'en captivité comme en milieux naturels, les polystomes ne sont pas systématiquement spécifiques (Verneau et al. 2011; Meyer et al. 2015; Héritier et al. submitted). Parce que les deux nouvelles espèces de polystome sont issues du même genre, à savoir *Polystomoides*, et parce que des études moléculaires ont montré que ces deux espèces de parasites sont retrouvées sur plusieurs espèces de tortues hôtes, les descriptions ont été réalisées en combinant des analyses morphologiques avec des caractères discriminants sélectionnés et des analyses moléculaires.

La comparaison des séquences COI des deux nouvelles espèces a montré une divergence génétique nettement supérieure au seuil fixé par Héritier et al. (submitted), pour la discrimination des espèces de polystomes. Suite à une formation sur la morphologie et la taxonomie des polystomes en Afrique du Sud (août 2016) sous la direction de Louis du Preez, le nombre d'épines génitales et la forme des testicules sont apparus comme des caractères discriminants. De ce fait, ces caractères morphologiques ont été comparés chez les deux nouvelles espèces de parasites et avec une autre espèce de polystome américaine du pharynx auparavant décrite, nommée *Polystomoides oris* (Paul, 1938). Les résultats de cette analyse ont montré que ces deux caractères morphologiques étaient significativement discriminants, permettant ainsi la description de deux espèces de polystomes distinctes, en accord également avec les données moléculaires.

Ce travail a définitivement montré l'importance de combiner des caractères morphologiques et moléculaires pour la description de parasites. Il serait très utile qu'à l'avenir tous les systématiciens travaillant sur les polystomes puissent déposer sur une base de données des images de bonne qualité, en particulier du bulbe génital et du testicule, pour effectuer des comparaisons morphologiques. En ce qui concerne les comparaisons moléculaires, notre base de données comprend environ 100 séquences COI caractérisant plus de 30 espèces de polystome distinctes. Nous envisageons de créer un accès libre en ligne et de proposer une assistance moléculaire pour les personnes incapables d'effectuer ces travaux. Dans ces conditions, une révision de la taxonomie des polystomes pourrait être envisagée.

#### 4. Description de trois nouvelles espèces de polystomes identifiées sur des tortues d'eau douce native d'Amérique du Sud

Cette étude fait l'objet d'un article publié dans la revue *Parasites and Vectors* et correspond au chapitre V du présent document. Dans ce chapitre, trois nouvelles espèces de polystome du genre *Neopolystoma* sont identifiées et décrites en utilisant des caractères morphologiques, à savoir la taille du corps, le nombre d'épines génitales, la forme et la taille des testicules et des analyses moléculaires. Ces parasites ont été prélevés sur des tortues d'eau douce dans des milieux naturels de Guyane, deux espèces parasites sur la tortue forestière peinte *Rhinoclemmys punctularia* (Daudin, 1801), une dans les cavités palpébrales et l'autre dans la vessie, et la troisième espèce dans les cavités palpébrales de la tortue scorpion *Kinosternon scorpioides* (Linnaeus, 1766).

Les résultats de ce travail de thèse ont confirmé l'efficacité de la combinaison de caractères morphologiques et moléculaires dans la description des espèces parasites. De plus, ce travail a montré l'intérêt de décrire de nouvelles espèces de parasites chez des hôtes dans les écosystèmes sauvages. Parce que l'introduction d'espèces de parasites non indigènes peut contribuer à la perte de biodiversité (Hulme 2007; Clavero et al 2009), il est impératif de d'étudier la diversité parasitaire sur les populations hôtes indigènes. En effet, les études de Meyer et al. (2015) et Héritier et al. (submitted) ont montré que *T. s. elegans* pouvait transmettre ses parasites aux espèces de tortues indigènes. Cependant, certaines espèces de polystomes infectant les tortues indigènes étaient des espèces parasitaires non décrites (voir Chapitre III). Etant donné que la vente de la tortue de Floride à tempes rouge a été interdite en France, d'autres tortues d'eau douce sont désormais proposées dans le commerce des animaux. En effet, il est encore possible d'acheter des tortues d'eau douce dans des magasins spécialisés comme "La Ferme Tropicale" (Paris) ou directement à partir sur sites Web, comme par exemple Reptimania (<http://www.reptimania.com>), où des tortues natives des Etats-Unis, d'Amérique Centrale, d'Asie ou encore d'Afrique sont en vente. Toutes ces espèces sont donc devenues des envahisseurs potentiels des écosystèmes en France. De ce fait, avant que leurs parasites ne soient identifiés sur les tortues natives, il est primordial d'illustrer la diversité des polystomes au travers du monde.

## 5. Développement d'un outil permettant d'évaluer l'état de santé des populations de tortues d'eau douce et sa validation en conditions expérimentales

Cette étude fait l'objet d'un article en révision dans la revue *Environmental Toxicology and Chemistry* et correspond au chapitre VI du présent document. Dans le sud de la France, et en particulier dans le département des Pyrénées-Orientales, des activités agricoles intensives existent encore impliquant l'utilisation de pesticides et d'herbicides. L'étude d'un rapport sur la qualité des eaux non publié fournit par le Conseil Général des Pyrénées-Orientales a montré que des pesticides ont été détectés dans beaucoup de cours d'eau du département. Notre attention a été retenue par deux rivières, la Fosseille et l'Agouille de la Mar, qui ont été classées dans la pire des cinq catégories utilisées pour évaluer la qualité de l'eau en raison de la détection de fortes concentrations de glyphosate et de l'AMPA.

La toxicité des herbicides à base de glyphosate, ainsi que la toxicité des composés actifs isolés, à savoir le glyphosate, le POEA et le produit de dégradation l'AMPA a été étudiée chez les vertébrés. Il a été démontré en conditions expérimentales que les herbicides à base de glyphosate sont plus toxiques que le glyphosate testé seul (Howe et al. 2004; Peixoto 2005 ; Richard et al. 2005; Gasnier et al. 2009). En conséquence, ces formulations ont été testées et principalement sur les poissons, les amphibiens et les reptiles (Relyea 2005a, b; Glusczak et al. 2006; Glusczak et al. 2007; Cavalcante et al. 2008; Costa et al. 2008; Do Carmo Langiano, Martinez 2008; Poletta et al. 2009; Modesto, Martinez 2010) en utilisant des biomarqueurs tels que l'activité d'enzymes impliquées dans la lutte physiologique contre le stress oxydatif, la mesure de paramètres métaboliques ou encore l'étude des dommages cellulaires. Dans tous les cas, les formulations commerciales de glyphosate ont été jugées toxiques pour les organismes expérimentés. La biodiversité serait-elle ainsi affectée par la mauvaise qualité des deux rivières dégradées, la Fosseille et l'Agouille de la Mar ?

L'émyde lépreuse figure parmi les espèces rencontrées sur ces deux écosystèmes aquatiques, en dépit de la dégradation des eaux. La première étape de ce travail a consisté à développer des biomarqueurs pour évaluer l'impact des modifications environnementales sur les tortues. Une étude bibliographique préliminaire a montré l'intérêt de mesurer les variations de l'expression de gènes du stress, c'est-à-dire de gènes codant pour des protéines impliquées dans la défense physiologique contre le stress. En effet, ce biomarqueur a répondu chez des tortues d'eau douce soumises à des chocs thermiques ou un manque d'oxygène (Storey 2006;

Krivoruchko, Storey 2010; Stecyk et al. 2012; Krivoruchko, Storey 2013). Cependant, après l'étude de la toxicité des herbicides sur divers organismes, un biomarqueur a attiré notre attention. Les espèces réactives de l'oxygène (ROS), tels que l'anion superoxyde ( $O_2^-$ ) et le peroxyde d'hydrogène ( $H_2O_2$ ) sont produites de manière constitutive chez les organismes vivants en aérobie. Face à une situation de stress, leur production augmente pour provoquer une réaction physiologique de défense. En effet, ces ROS peuvent dégrader toute substance intrusive dans l'hôte et se lier à des facteurs de transcription conduisant l'activation de gènes codant pour des protéines impliquées dans les réponses physiologiques. Cependant, ces ROS doivent être par la suite neutralisés car ils peuvent également causer des dommages sur les parois lipidiques des cellules, sur les protéines et l'ADN. Ainsi, l'activité d'enzymes intervenant dans cette détoxification oxydative, telles que la catalase (CAT) et la superoxyde dismutase (SOD) peut être modifiée chez un organisme confronté à un stress environnemental (Hermes-Lima, Storey, 1993; Van Der Oost et al. 2003; Hermes-Lima 2004; Halliwell 2005). Ce biomarqueur n'a été, à notre connaissance, utilisé que dans une seule étude sur la tortue marine verte *Chelonia mydas* (Linnaeus 1758) (Labrada-martagon et al. 2011). Parce que l'utilisation de ce biomarqueur seul est insuffisante pour expliquer les réactions physiologiques (Do Carmo Langiano, Martinez 2008; Modesto, Martinez 2010), nous avons choisi de développer chez les tortues d'eau douce deux biomarqueurs, à savoir l'activité des enzymes anti-oxydantes et l'expression du gène codant pour ces dernières.

*Mauremys leprosa* étant considérée comme «vulnérable» depuis 2015 en France (UICN France, MNHN et SHF, 2015), il est inconcevable de sacrifier des spécimens pour une étude en milieu naturel. De même, il est impossible de mener une expérience impliquant la confrontation de ces animaux à un stress environnemental. Pour ces raisons, les biomarqueurs sélectionnés ont été développés à partir d'échantillons sanguins et les expérimentations conduites sur des juvéniles de *T. s. elegans*. Pour effectuer ce travail, une salle destinée aux travaux pratique nous a été attribuée et transformée en animalerie durant cette thèse. Elle a été équipée avec des bacs en plastiques, des lampes UV et des chauffages pour aquariums. Des juvéniles de *T. s. elegans*, 250 au total, ont été achetés à la société "la Ferme Tropicale" en Avril 2015, provenant de centres d'élevage aux États-Unis. Cependant, après cinq semaines d'acclimatation, toutes les tortues sont mortes pour des raisons inexplicables. Nous avons alors contacté "la Ferme des Tortues de Sorède" et nous avons pu récupérer trente juvéniles de *T. s. elegans*, ce qui a permis le lancement de la phase expérimentale.

Les résultats de cette expérience ont montré que les profils d'expression de gènes et les activités enzymatiques de la CAT et SOD ont pu être quantifiés à partir d'échantillons de sang

de tortues, comme dans l'étude de Labrada-Martagón et al. (2011). De ce fait, cette approche peut être appliquée pour des études d'écotoxicologie en environnements naturels, et en particulier sur des espèces menacées d'extinction. De plus, des variations dans l'expression et l'activité des deux enzymes anti-oxydantes ont été obtenues, ce qui a confirmé que ces biomarqueurs permettent d'évaluer le stress physiologique chez ces tortues face à des modifications environnementales. Cette étude a également montré que le glyphosate induit un stress chez les tortues, tout comme l'eau recueillie dans la rivière de la Fosseille. De ce fait, une étude sur les populations d'émyde lépreuse en milieu naturel dans le département des Pyrénées-Orientales a montré un intérêt tout particulier, sachant que le niveau de pollution des écosystèmes peut être ainsi estimé par l'état de santé des populations sauvages.

## 6. Application des biomarqueurs validés sur les populations de tortues d'eau douce en milieux naturels

Cette étude fait l'objet d'un article en révision dans la revue *Chemosphere* et correspond au chapitre VII de ce document. Suite au développement de biomarqueurs chez les tortues d'eau douce et leur validation en conditions expérimentales, le travail final de cette thèse fut d'appliquer cet outil sur les populations de *M. leprosa* sauvages, à savoir celles de la Fosseille et de l'Agouille de la Mar. Ne disposant pas de témoins comme en mésocosme, une comparaison avec une population retrouvée dans un écosystème contrasté, à savoir la rivière de la Baillaury, petit ruisseau courant dans le département entouré de cultures viticoles comme les deux précédentes rivières mais présentant une bonne qualité des eaux, a également été envisagée. Un travail de terrain a ensuite été réalisé pour recueillir le sang des tortues dans tous ces cours d'eau avant d'effectuer les analyses. Etant donné que des eaux traitées provenant de stations d'épuration (STEP) alimentent la Fosseille et l'Agouille de la Mar, les tortues échantillonnées dans ces deux cours d'eau ont été divisées en plusieurs groupes, à savoir en amont et en aval des STEP pour les analyses.

L'analyse de la réponse des biomarqueurs dans les populations sauvages de tortues a montré des variations significatives, pour l'expression des gènes comme pour les activités enzymatiques de la CAT et de la SOD. Cependant, la plupart des comparaisons n'ont révélé aucune différence significative. En effet, la variabilité obtenue dans les réponses physiologiques au sein des tortues pourrait expliquer l'absence de résultats significatifs. Parce qu'il est extrêmement difficile d'évaluer tous les paramètres du cycle de vie de spécimens en

milieux naturels, contrairement aux études menées en conditions expérimentales où tous les paramètres ont été fixés, certaines différences, bien que non significatives, ont été considérées comme des tendances dans la réponse au stress oxydatif chez ces animaux sauvages.

Les résultats obtenus lors de cette étude ont montré que la Fosseille et l'Agouille de la Mar représentent des environnements stressants pour l'émyde lépreuse, ce qui était attendu vu les niveaux de pollutions mesurés dans ces deux cours d'eau. En revanche, la Baillaury pourrait être l'écosystème le moins stressant, résultat qui était également attendu en raison de sa qualité des eaux. Cependant, dans les deux rivières dégradées, les animaux échantillonnés en amont des STEP sont moins stressés que ceux situés en aval. Parce que les activités agricoles sont similaires le long des deux cours d'eau, ces dernières ne peuvent pas être considérées comme le principal facteur impliqué dans le stress des populations sauvages. Ce résultat suggère que les eaux rejetées par les STEP pourraient impacter considérablement la biodiversité.

Le développement de cette approche au cours de cette thèse s'est avéré très efficace pour évaluer l'état de santé des populations de tortues d'eau douce en milieu naturel, ce qui peut ainsi refléter la qualité des habitats. Parce que diverses sources de contamination, biotiques comme l'introduction de *T. s. elegans* et ses parasites, et abiotiques avec la présence de glyphosate et l'AMPA, ont été identifiées dans la Fosseille et l'Agouille de la Mar, il est impossible de déconvoluer les causes de stress sur ces animaux sauvages. Cette approche pourrait être ainsi appliquée sur d'autres populations d'émydes lépreuses du département, et notamment lorsqu'une STEP déverse ces eaux traitées dans les cours d'eau.

## VI. Synthèse et perspectives

Ce travail de thèse a permis d'aborder une question fondamentale qui est l'impact des contaminants biotiques et abiotiques sur les écosystèmes aquatiques.

Dans un 1er temps, notre étude s'est portée sur l'introduction et les transferts de parasites non-natifs (Monogènes, Polystomatidae) en milieux naturels, de la tortue de Floride à tempes rouges *T. s. elegans*, espèce invasive, vers deux espèces modèles de tortues aquatiques natives du sud de l'Europe : l'émyde lépreuse et la cistude d'Europe. Suite aux études menées par Verneau et al (2011) en milieu confiné et Meyer et al (2015) en environnements naturels sur l'émyde lépreuse, nous avons recherché dans un premier temps si la cistude d'Europe était également infectée dans le sud de l'Europe par des polystomes introduits via *T. s. elegans*.

Répondre à cette question demeurerait essentiel afin de vérifier si nos deux espèces modèles étaient bien confrontées aux stress biotiques relatifs à l'introduction d'hôtes exotiques et de leurs parasites. Nous avons montré dans cette étude que la cistude d'Europe était infectée par cinq espèces de polystomes exotiques importés avec la tortue de Floride à tempes rouges, résultats similaires à ceux obtenus par Meyer et al. (2015) sur l'émyde lépreuse. Nous avons ainsi pu montrer que ces deux espèces de tortues aquatiques subissaient en plus des stress abiotiques liés à la pollution des eaux, des stress biotiques.

Une fois ce verrou scientifique levé, le projet initial de la thèse impliquait le développement d'un outil permettant de mesurer l'état de santé des tortues en milieu naturel. Nous avons choisi d'étudier les variations de l'activité d'enzymes impliqués dans la réponse face au stress oxydatif, à savoir la superoxyde dismutase et la catalase, ainsi que les variations de l'expression des gènes codant pour ces dernières. Avant d'appliquer cet outil sur des populations de tortues échantillonnées en milieu naturel, nous avons cherché à le valider en conditions expérimentales, afin de vérifier si les biomarqueurs envisagés ici étaient pertinents (réponse détectable) et s'ils répondaient à partir d'échantillons sanguins, dans la mesure où, nos deux espèces modèles natives étant des animaux protégés, il n'était pas possible de prélever des tissus ou organes. Cette validation de l'outil s'avérait nécessaire afin de savoir si les différents stress abiotiques et biotiques identifiés dans les zones d'étude provoquaient bien des réponses de nos biomarqueurs, permettant ainsi de mesurer un stress chez nos espèces modèles. Etant donné que le glyphosate est le principal composé qui décline les eaux de certains de nos sites sélectionnés, nous avons confronté en conditions expérimentales des juvéniles de *T. scripta* à une concentration élevée de glyphosate provenant d'une formulation commerciale, ainsi qu'à de l'eau de la Fosseille, rivière localisée aux abords de Perpignan et dont les eaux ont été estimées de mauvaise qualité suite à la détection de glyphosate. Nous avons obtenu une réponse des biomarqueurs dans les deux dispositifs expérimentaux permettant de valider notre outil de mesure du stress et qui plus est dans le sang des tortues. Il était ensuite prévu de réaliser des infestations contrôlées en utilisant nos espèces de parasites modèles (polystomes) sur des tortues juvéniles, afin de rechercher l'existence d'une potentielle réponse des biomarqueurs choisis suite à des infestations parasitaires. Enfin, nous avons également envisagé de tester nos biomarqueurs dans le cadre d'un effet synergique de facteurs de stress biotique et abiotique sur des tortues artificiellement infestées par des polystomes, et maintenues dans de l'eau contenant du glyphosate. Nous n'avons pas pu réaliser ces deux dernières expérimentations pour plusieurs raisons. Mesurer l'impact d'infestations par des parasites exotiques impliquait d'expérimenter sur des cistudes et des

émydes lépreuses juvéniles, ce qui s'avérait impossible devant la législation imposée pour ces espèces. Néanmoins, cette manipulation aurait pu être possible en demandant un certificat de capacité et l'autorisation d'expérimenter sur ces espèces, processus très longs et fastidieux qui aurait pris trop de temps dans le cadre de cette thèse. De plus, une fois toutes les autorisations obtenues, il aurait été nécessaire de rentrer en contact avec un centre spécialisé dans l'élevage de ces deux espèces pour disposer de juvéniles nés en captivité. Enfin, le cycle de vie des polystomes n'est pas complètement maîtrisé en conditions expérimentales. En effet, nous avons pu constater que les cycles de pontes ne sont pas toujours synchronisés, ce qui rend difficile l'obtention de larves en quantité suffisante pour effectuer des infestations contrôlées. Pour toutes ces raisons, et devant le temps de travail pris pour lever les autres verrous scientifiques, cette expérimentation s'est révélée impossible durant cette thèse. Néanmoins, de telles expérimentations, impliquant au préalable une étude approfondie sur le cycle de vie des polystomes, restent primordiales à développer à l'avenir afin d'appréhender les niveaux de stress engendrés d'une part, par la présence de parasites non-natifs et d'autre part, par la combinaison de facteurs de stress biotiques et abiotiques agissant en synergie.

Enfin, l'utilisation de notre outil de mesure de stress sur des populations d'émyde lépreuse en milieu naturel sur différents sites contrastés, nous a permis d'obtenir des résultats intéressants montrant que les animaux échantillonnés dans les sites les plus pollués sont les plus stressés. De plus, nous avons montré que les stations d'épurations localisées sur les deux cours d'eau les plus chargés en contaminants pourraient jouer un rôle important dans le stress subi par les organismes. En effet et contre toute attente, ce sont les tortues échantillonnées après les stations d'épurations qui sont les plus stressées, ce qui suggère que les eaux rejetées par les STEP pourraient avoir un impact négatif sur la biodiversité. Par manque de temps et devant l'investissement financier que représente la mesure des variations des activités enzymatiques, nous avons choisi dans ce travail de thèse de nous focaliser sur l'émyde lépreuse. Il serait intéressant à l'avenir d'appliquer cet outil sur les populations de cistudes, voire même sur d'autres espèces modèles en danger.



# CHAPITRE I: INTRODUCTION

## I. State of the art

### 1. Anthropogenic imprint on the ecosystems

Mass species extinction is a phenomenon that occurred repeatedly in the history of life (Fastovsky, Sheehan 2005; Elewa, Joseph 2009; Archibald et al. 2010; Schulte et al. 2010). While natural causes, such as climatic and geological shifts, have been largely mentioned to explain historic extinctions, the current sixth extinction wave derives from anthropogenic actions (Chapin III et al. 2000) and is considered as the most rapid and devastating (Ceballos et al. 2010; Barnosky et al. 2011). Human imprint on Earth is actually so profound leading global environmental changes that affects and modifies the functioning of ecosystems (Vitousek 1994; Vitousek et al. 1996; 1997a; 1997b; Mack, D'antonio 1998; Sala et al. 2000; Steffen et al. 2011). Among the natural biomes, freshwater ecosystems are remarkable habitats providing multiple ecological services via hydrological properties, water resource and climate regulation. As such, they have a significant economic value and contribute to human well-being. They also comprise a great species diversity, *i.e.* no less than 100,000 species which corresponds to approximately 9.5% of all described species (Dudgeon et al. 2006; Balian et al. 2008). As a result of anthropogenic activities, freshwater ecosystems face strong pressure, leading to growing threats on biodiversity (Revenga, Mock 2000; Dudgeon et al. 2006; Vaughn 2010; Strayer, Dudgeon 2010). They are likewise considered more imperiled than terrestrial and marine habitats (Dudgeon et al. 2006; Strayer, Dudgeon 2010). If researches are considered less appealing in freshwater ecosystems than that in the terrestrial or marine realms, the trajectories of species loss make it arguably the most urgent (Abell 2002). It is therefore crucial to pursue and undertake intensive surveys on freshwater ecosystems to protect and manage aquatic biodiversity. Water degradation is currently pervasive in worldwide watercourses, due to increasing anthropogenic activities (Sala et al. 2000; Albiac et al. 2010; Bhuiyan et al. 2013). The main causes of this loss of water quality and disruption of freshwater ecosystems includes water extraction, straightening of watercourses, dam constructions, and water pollution, as abiotic factors, and the introduction of alien species, as a biotic factor (Malmqvist, Rundle 2002; Sabater, Barceló 2010).

## A. Abiotic threat: the water pollution

Regarding pollution, numerous pollutants such as toxic gases, heavy metals, organics and biotoxics are released into natural environments (Wang et al. 2013). It has been shown that in a majority of cases, water pollution involved anthropogenic causes, namely wastewater treatment plants, industrial and intense agriculture activities (Schwarzenbach et al. 2006; Trujillo-Reyes et al. 2014). Concerning agriculture practices, the utilization of fertilizers, hormones, antibiotics and pesticides could be important sources of pollution (Trujillo-Reyes et al. 2014). Pesticides (insecticides and herbicides) residues are accumulated in worldwide freshwater bodies (Gill, Garg 2014). In France, 270.000 kilometers of watercourses run through the territory. Various pesticides are detected in both surface and ground waters of the country (Institut Français de l'Environnement 2007; Commissariat Général au développement durable-Service de l'observation et des statistiques 2015). Indeed, because 36 % of surface freshwaters present a moderate to poor quality of waters in 2005, and because 92 % of control points showed the presence of pesticides in 2013, contamination levels are considerable (Commissariat Général au développement durable-Service de l'observation et des statistiques 2015). In 2003, 2004 and 2005, the major compounds incriminated in the poor quality of freshwaters were the aminomethylphosphonic acid (AMPA), the glyphosate and the diuron (Institut Français de l'Environnement 2007). Since 2009, the major compounds are the AMPA, the glyphosate and the atrazine, an herbicide which the sale is banned for ten years (Commissariat général au développement durable-Service de l'observation et des statistiques 2015).

## B. Biotic threats: the biological invasions

Biological invasions implies the transport and persistence of organisms in novel habitats (Mack, D'antonio 1998) and are considered as a major component of global environmental changes (Vitousek et al. 1997a). The invasion process is divided in three stages, namely the introduction, the establishment and the spread (Engel et al. 2011). Introduction result from the transport and the accidental or intentional release of organisms following human activities and globalization (see Lockwood et al. 2013). Human mediation may also influence remaining two steps, the establishment and the spread, by the current climate change for instance (Engel et al. 2011). As a consequence, alien species are documented in new areas all over the world

and especially in Asia (Ding et al. 2008), in United States of America (Lodge et al. 2006) and in Europe (Hulme et al. 2009). When invasive species established in novel areas, they may directly compete with native species and cause strong adverse effects in native populations. Biological invasions are thus considered a major threat to biodiversity (Mack, D'antonio 1998; Daszak et al. 2000; Mooney, Cleland 2001; Pimentel et al. 2005; Simberloff 2013; Bellard et al. 2016). Several types of competitions have been recorded following the presence of non-native species in natural environments and are directly implicated in the decline of populations of native species. Indeed, the introduction of the Louisiana crayfish *Procambarus clarkii* resulted in competition with native crayfish species for resources and habitat and had a negative impact on the populations of the latter (Gherardi and Daniels, 2004). Similarly for the introduction of the North American bull frog *Lithobates catesbeianus* which resulted in a decrease of native amphibian populations following predation competition (Teixeira et al., 2009). Displacement of species can also lead to ecological imbalances through genetic isolation and allelic diversity decline in native species. This is the case in the Bahamas, where rats were introduced and preyed on a species of local lizard, *Anolis sagrei*, which led to isolation of the saurian populations and thus a decrease in genetic diversity, threatening the latter (Gasc et al., 2010).

Another case of competition to which we are particularly interested is that resulting from the introduction of parasites. Indeed, they can likewise be introduced along with their hosts, even though their impact has been the least studied (Lowry et al. 2013). Once transmitted to indigenous species, parasites may affect ecological interactions and population dynamics of their hosts (Peeler et al. 2011; Lymbery et al. 2014). As a consequence, displacement, installation and reproduction of non-native taxa outside their home range can result in the proliferation of potential invasive species and their parasites contributing to the global worldwide loss of biodiversity (Hulme 2007; Clavero et al. 2009). Indeed, numerous studies have showed that invasive parasites can be a threat for host natural populations, whether in invertebrates, for instance in insects (Meeus et al. 2011), or in vertebrates such as fishes (Gozlan et al. 2005; Barry et al. 2014), amphibians (see Bucciarelli et al. 2014), birds (Wikelski et al. 2004; Miller et al. 2016) and freshwater turtles (Hays et al. 1999; Iglesias et al. 2015). This invasive process is called "spill over" and remains the most frequently highlighted (Kelly et al. 2009). However, other concepts have also been mentioned. Invasive host species may lose parasites when introduced into new environments (Torchin et al. 2003) and may be infected with native parasites of local wildlife (Poulin and Mouillot 2003). As a result, exotic hosts can constitute reservoirs of natural parasites and lead to changes in the

native host-parasite relationship, a phenomenon known as "spill back" (Kelly et al. 2009). The acquisition of native parasites by invasive hosts may lead to increased parasite dynamics and consequently higher prevalences and an increased risk of disease in the case of pathogenic parasites (Kelly et al., 2009; Lymbery et al. 2014; Sherrard-Smith et al., 2014). However, if exotic hosts can be infected by the infesting phases of native parasites, but the latter cannot grow and reproduce in this new host environment, then they can act as a sink of parasites and then decrease the infestation levels in native hosts, this phenomenon being called a dilution effect (Keesing et al., 2006; Kelly et al., 2009).

## II. Objectives of the thesis

Because of the wide variety of habitats it includes, the Mediterranean basin is one of the world's biodiversity hotspots (Cuttelod et al., 2008). Indeed, this area comprises many terrestrial habitats, mountains, deserts and plains, but also marine and aquatic freshwater biotopes. Species encountered in the Mediterranean basin are also under pressure due to anthropogenic activities. The main objective of this thesis was to study the impacts of environmental modifications on the biodiversity. However, to address this issue, several protocols had to be developed. First, to the assessment of the importance of biotic threats, the design of a study of parasite diversity on a model species to identify possible transfers of invasive parasites in a natural environment was conducted. Similarly, the development of a tool for the identification of parasite species has proved to be essential for effective monitoring of potentially infested native species. Finally, the most important work of this thesis has focused on the development of a tool for measuring the health status of populations of a model species. We selected the Mediterranean area to perform this work, and especially in the Occitanie province, located in the south of France, with a special interest for the department of Pyrénées-Orientales.

## III. Biotic and abiotic threats from study sites

In the Occitanie province and particularly in the department of the Pyrénées-Orientales, agricultural activities are still intense and mainly based on fruit crops and vineyards. As a result, high concentrations of pesticides have been detected in some watercourses in this department, leading to a declassification of the latter with poor water quality. In addition,

large wild populations of *T. s. Elegans* are established in many ecosystems within the Pyrénées-Orientales.

## 1. Glyphosate and AMPA

In 2003, 2004 and 2005, aminomethylphosphonic acid (AMPA), glyphosate and diuron were the main contributors to poor water quality (Institut Français de l'Environnement 2007). Since 2009, the main products are AMPA, glyphosate and atrazine, a herbicide whose sale has been banned in France for ten years (Commissariat général pour le développement durable). The herbicide properties of the glyphosate were discovered and patented by Monsanto factory in 1974. It was sold under the name of Round up<sup>®</sup> and is nowadays the most used herbicide over the world (Franz et al. 1997). The glyphosate is initially a weak organic acid ( $C_3H_8NO_5P$ ) encountered as a salt formulation ( $C_6H_{17}N_2O_5P$ ) in commercial herbicides products in order to increase its solubility in water (Giesy et al. 2000). Glyphosate-based herbicides are principally composed of isopropylamine salt of glyphosate and a surfactant, generally the polyethoxylated tallowamine (POEA) (Giesy et al. 2000). Glyphosate works by inhibiting the enzyme 3-enolpyruvyl-shikimat-3-phosphate synthase, which is involved in the synthesis of aromatic amino acids (Battaglin et al. 2005). The role of the surfactant is to facilitate the penetration of the glyphosate into plant leaves (Battaglin et al. 2005). The first product of the degradation of the glyphosate by microorganisms is the AMPA (Kolpin et al. 2006). Numerous bacteria can degrade the glyphosate, such as *Pseudomonas*, *Enterobacter*, *Rahnella*, *Serratia* and cyanobacteria for instance (Forlani et al. 2008; Arunakumara et al. 2013; Benslama, Boulahrouf 2013). If glyphosate and AMPA are detected in the same site, then it is considered that AMPA degraded from the glyphosate (Skark et al., 1998; Kolpin et al. 2006; Botta et al., 2009). Because its term of half-life is longer, AMPA is more often detected and measured than the glyphosate (Giesy et al. 2000). These two components are detected in most watercourses of numerous French departments, except areas with no intense agricultural activities or localized near reliefs (Commissariat général au développement durable-Service de l'observation et des statistiques 2015). The presence of these components in French freshwater ecosystems could become a serious threat to biological communities (Gill, Garg 2014).

The toxicity of the glyphosate-based herbicides, as well as the toxicity of the active compounds alone, *i.e.* glyphosate, POEA and the degraded product AMPA, has been studied

across vertebrates. It has been shown under experimental conditions that glyphosate-based herbicides are more toxic than the glyphosate tested alone (Howe et al. 2004; Peixoto 2005; Richard et al. 2005; Gasnier et al. 2009). As a result, glyphosate-based herbicide formulations were tested in fishes, amphibians and reptiles and were revealed toxic for these organisms (Relyea 2005a, b; Glusczak et al. 2006; Glusczak et al. 2007; Cavalcante et al. 2008; Costa et al. 2008; Do Carmo Langiano, Martinez 2008; Poletta et al. 2009; Modesto, Martinez 2010).

## 2. The red-eared slider

### A. Competition with native species

The red-eared slider is a freshwater turtle native to the Eastern United States and the Northern parts of Mexico (Van Dijk et al. 2014). Because juveniles are attractive, they were exported in vast numbers in the eighties and nineties from the USA to Europe (Lutz 2000) to be sold as pets, about 52 million between 1989 and 1997 according to Telecky (2001). Nonetheless, the red-eared slider grows rapidly and becomes aggressive and less attractive as adult. In addition, its relatively large size in adulthood, of the order of 20-30 cm carapace length, requires large and expensive equipment for holding facility. As a result, numerous owners release these unwanted pets in natural environments. Since this freshwater turtle is encountered in a large range of aquatic ecosystems in its natural habitat and is considered as a habitat generalist (Bringsøe 2006), it has adapted to new environments where it reproduce and establish feral populations. As a consequence, it is nowadays considered among the world list of top 100 worst-invasive alien species (Lowe et al. 2000). Indeed, the red-eared slider is now encountered in the wild on nearly every continent including USA (Van Dick et al. 2014), Asia (Ramsay et al. 2007), Europe in country such as Bulgaria (Tzankov et al. 2015), Italy (Ficetola et al. 2003; Fattizzo 2004; Ficetola et al. 2009), Spain (Izquierdo et al. 2010) and southern France (Servan, Arvy 1997; Arvy, Servan 1998; Meyer et al. 2015) (Figure 1). In these areas, it can share habitats with native freshwater turtle species (Figure 2).

Because the red-eared slider presents particular biological and ecological characteristics, this terrapin could constitute a real threat to the native freshwater turtle species in the wild. Indeed, it is an opportunistic omnivore that feed on a wide variety of plants and animals (Prévot-Julliard et al. 2007), with a large body size and a high fertility (Arvy and Servan, 1998; Bringsøe 2006). As a result, *T. s. elegans* could indeed compete with the red-

bellied turtle *Pseudemys rubiventris* (Le Conte, 1830) in USA (Pearson 2013), and is suspected to be through competition responsible for the displacement of the African helmeted turtle *Pelomedusa subrufa* (Bonnaterre 1789) in South Africa (Ramsay et al. 2007). It also has been shown under experimental conditions that the red eared-slider could compete with the two French native freshwater turtles, the European pond turtle *Emys orbicularis* (Linnaeus 1758) for basking sites (Cadi, Joly 2003) and food resources (Cadi, Joly 2004), as well as with the Mediterranean pond turtle *Mauremys leprosa* (Schweigger 1812), for basking, feeding and territory (Polo-Cavia et al. 2008, 2009a, b, 2010, 2011, 2012).



Figure 1. The red-eared slider in natural environments in France



Figure 2. The red-eared slider and the native Mediterranean pond turtle in French ecosystem



## B. Parasites switching

Several studies have shown that *T. s. elegans* was introduced in wild environments out of its range of distribution with its parasites. In North America, a case of decline of native populations of the western pond turtle *Emys marmoratus* (Baird and Girard 1852) following the transmission of virus or mycoplasma carried by the red-eared slider was documented (Hays et al. 1999). Similarly in Spain, a population of *E. orbicularis* has severely decreased following the introduction of spirorchiids haemoparasites (Iglesias et al. 2015). In the Southern Europe, the Mediterranean pond turtle often share its natural environment with the red-eared slider. Because Verneau et al. (2011) showed horizontal parasite switching from American to the both French native freshwater turtles, i.e. *M. leprosa* and *E. orbicularis*, Meyer et al. (2015) conducted a study, principally in Southern France and in Northern Spain, that aimed to determine the parasite diversity within the Mediterranean pond turtle in wild habitats. If these authors showed the presence of native parasites within *M. leprosa*, a great unexpected numbers of invasive parasite species were identified within native and invasive freshwater turtles in wild environment, suggesting that the red-eared slider could be considered as a vector of parasites.

## 3. The Polystomatidae

Within the phylum of the Platyhelminthes, the Polystomatoinea is a classes which include one single family, the Polystomatidae (Sinnappah et al. 2001). This family is represented by 25 genera infecting gills or oral cavity of the Dipnoi (Pichelin et al. 1991), the bladder of amphibian adult hosts (see Prudhoe, Bray 1982), the bladder, conjunctival sacs and pharyngeal cavity of freshwater chelonians (see Morrison, Du Preez 2011) and the eyelid of the common hippopotamus (Thurston, Laws 1965). Because *Concinnocotyla australensis*, occupies a basal position within the phylogenetic tree of polystomes, like its host the Dipnoi within sarcopterygians, a very ancient origin was assumed for the Polystomatidae (Verneau et al. 2002). It could be related to the divergence between actinopterygians and sarcopterygians, estimated at about 425 Million years ago (Mya) based on the palaeontological syntheses of Janvier (1988) and Ahlberg (1999). Verneau et al. (2002) also illustrated a sister group relationship between parasites of the Batrachia (frogs and salamanders) and freshwater chelonians, that was well correlated to the separation of amphibians from amniotes. Finally,

Héritier et al. (2015) demonstrated a possible switch from caecilians to freshwater turtles at about 174 Mya, when some primitive turtles could have already been adapted to an aquatic life style.

Polystomes are considered as host and site specific (Verneau 2004). Because these parasites have a direct life cycle with free swimming infective larvae (oncomiracidium), they constitute a good model to survey the biological invasions, as has been shown in the studies conducted by Verneau et al. (2011) and Meyer et al. (2015) on freshwater turtles. Polystomes infecting terrapins are divided in three genera, *i.e.* *Polystomoides*, *Polystomoidella* and *Neopolystoma*, depending of the number of hamuli located between the posterior pair of suckers on the haptor, two pairs, one pair and none, respectively. According to Verneau 2004, about fifty polystome species infecting freshwater turtles are recorded around the world.

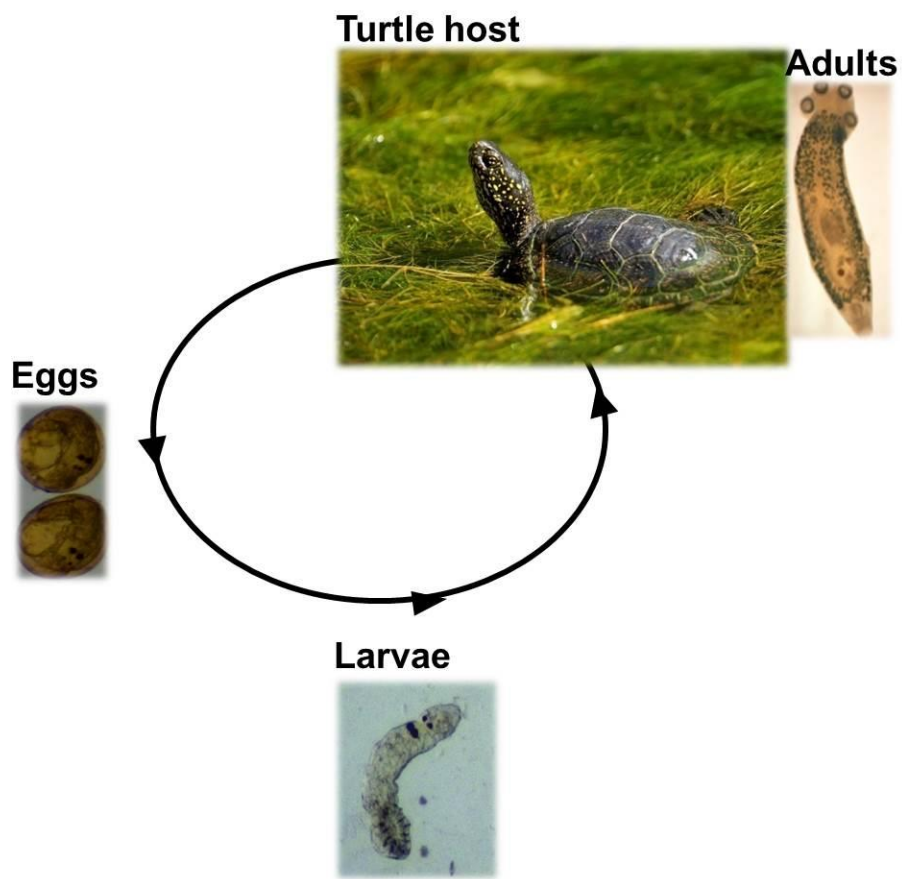


Figure 3. Illustration of the direct life cycle of polystome parasites

## IV. The model species of the study

We chose as study models the two native freshwater turtles in France and encountered in the Pyrénées-Orientales, the European pond turtle *Emys orbicularis* and the Mediterranean turtle *Mauremys leprosa* for several essential reasons. Indeed, these animals can be confronted with biotic and abiotic stresses since they are aquatic turtles in one hand, and thus directly in contact with abiotic stresses, and that the red-eared slider is also an aquatic chelonian in the other, encountered in the same sites as the latter two. In addition, turtles are sensitive to pollutants but relatively resistant, which makes it possible to measure a stress effect and monitor the evolution of rivers in terms of quality for species, which would not have been possible with amphibians that are much more sensitive and disappear from streams if concentrations and pollutants are too high (Sparling et al. 2001). Finally, *E. orbicularis* and *M. leprosa* are protected species in France and benefit from national action plans that integrate the impact of environmental changes on these two species.

### 1. The European pond turtle *Emys orbicularis*

The European pond turtle is a freshwater turtle considered as a flagship species of the European natural heritage (Figure 3). It is the only European representative of the Emydidae, which is mainly distributed in the Nearctic Region with 53 species (Van Dijk et al. 2014), with *E. trinacris* (Fritz et al. 2005b). The European pond turtle reaches about 20cm in the adulthood and is characterized by yellow spots on the body and the carapace, long claws and a long tapering tail. This terrapin is particularly attached to aquatic ecosystems (Dall'antonia et al. 2001), where it feeds on mollusks, dead fishes or amphibians, aquatic insects and tadpoles (Kotenko 2000). *Emys orbicularis* has a home range of about 12 millions square kilometers, extending from the Atlantic coasts of North-Africa in the West to Aral Sea in the East, and from Moscow region in the north to Turkey in the south (see Fritz et al. 2009). Its current geographical distribution would have been primarily shaped by successive climatic events that happened in the Quaternary period. Because France is a contact zone between populations from the Balkans and Southern European countries like Spain and Italy, the occurrence of *E. orbicularis* in French freshwater ecosystems probably reflects several waves of recolonization from Southern refuges in the Holocene period (Lenk et al. 1999; Fritz et al. 2005a; Sommer et al. 2009; Pedall et al. 2011). The European pond turtle may be subdivided in at least ten

haploclades or subspecies (Fritz et al. 2009; Stuckas et al. 2014) that started genetic differentiation 3.0 – 4.1 Million years ago, i.e. in the Early to Middle Pliocene period (Lenk et al. 1999; Fritz et al. 2009).

Despite an overall decline of populations due to ancestral rituals, such as its consumption from prehistoric times to the middle of the XIX<sup>e</sup> century (Cheylan, Courtin 1976; Cheylan, Poitevin 1998; Cheylan, Poitevin 2003) and its use in pharmacology in the XIX<sup>e</sup> century (see Cheylan, Poitevin 1998), populations of *E. orbicularis* still exist in France, especially in southern parts of the country. However, it was probably more widespread during prehistoric times, as evidenced by its presence in archaeological sites (Cheylan, Poitevin 1998; Hervet, Salotti 2000; Sommer et al. 2009). Based on its geographical distribution and populations size, it has been for a long time considered near-threatened in France (UICN France, MNHN & SHF, 2009), like in the Mediterranean basin (Cox et al. 2006) and in Europe (Cox, Temple 2009b). Its IUCN status in France is now listed as “Least Concern” (UICN France, MNHN & SHF 2015), indicating that the risk of extinction may be less than previously thought in this country. In Spain, however, the species is still considered as “Vulnerable” according to the National Red Book (Pleguezuelos et al. 2004).



Figure 4. The European pond turtle in French ecosystem

## 2. The Mediterranean pond turtle *Mauremys leprosa*

The Mediterranean pond turtle *Mauremys leprosa* (Schweigger 1812) is a freshwater turtle also representative of the European wildlife. This terrapin reaches about 25 cm in the adulthood and is characterized by an oval carapace which the color is generally red-brown (Figure 4). The Mediterranean pond turtle is encountered in streams and ponds with riparian vegetation (Da Silva, Blasco 2002) and could be more generalist regarding habitat than the European pond turtle (Segurado, Araújo 2004). However, the diet of both freshwater turtle species is similar. *M. leprosa* is originated to the western region of North Africa according to fossil records (De Lapparent De Broin 2000). Its occurrence in the Iberica Peninsula dates back the Pleistocene or even the Pleiocene (De Lapparent De Broin 2001) and this demographic expansion was confirmed by the genetic identification of two main lineages, *Mauremys leprosa leprosa* (Iberica Peninsula and Northern Morocco) and *Mauremys leprosa saharica* (Southern Morocco, Algeria and Tunisia) (Fritz et al. 2006). It's 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* was considered as “Least Concern” in North Africa according to the IUCN criteria (Cox et al. 2006), it was classified as “Vulnerable” in the European Red List of Reptiles and in the Spanish Red List (Da-Silva 2002; Cox, Temple 2009a) and as “Endangered” in France (IUCN France, MNHN and SHF, 2009). This freshwater turtle is nowadays classified as “Vulnerable” in France (UICN France, MNHN and SHF, 2015), partly because of small and isolated endemic populations occurring in the province of Languedoc-Roussillon, solely in the Pyrénées-Orientales department (see Palacios et al. 2015). In this area, the Mediterranean pond turtle often share its habitat with the red-eared slider, where it was showed parasite host-switching from the invasive to the native freshwater turtle species.



Figure 5. The Mediterranean pond turtle in French ecosystem

## V. Main results

### 1. Parasite diversity within the European pond turtle *E. orbicularis*

This study is the subject of an article in revision in the journal Biodiversity and Conservation and corresponding to the Chapter II of this document.

Verneau et al. (2011) showed parasite host-switching from exotic to both native French freshwater turtles in captivity and Meyer et al. (2015) illustrated transmission of parasites from the red-eared slider to the Mediterranean pond turtle in natural environments. Before such a finding, a study of the parasite diversity of the European pond turtle was investigated in France, Spain and North Africa, to verify if exotic parasites could be identified in this turtle in freshwater ecosystems.

Based on the use of the Cytochrome c Oxidase (COI) gene as a genetic marker for the discrimination of species, this study reveals the occurrence of six distinct parasite species in *E. orbicularis* in the investigated area. If the natural polystome species of the European pond turtle was identified in France, Spain and Algeria, the other polystome species were either exotic parasites described on their hosts in American ecosystems or either undescribed species considered as exotic ones because identified from *E. orbicularis* and *T. s. elegans* in French and Spanish natural environments. Thereby, this study reveal parasite host-switching from invasive to native freshwater turtles in the wild and illustrated also a case of parasitic invasion in a new turtle host, as it was showed from *M. leprosa* by Meyer et al. (2015).

Numerous studies have confirmed that introduction of non-native parasite could threat natural host populations (Wikelski et al. 2004; Gozlan et al. 2005; Barry et al. 2014; Bucciarelli et al. 2014; Miller et al. 2016). Since no studies have been conducted on the pathogenicity of these parasites, it could be interesting in a context of species conservation to develop a procedure in the aim to illustrate if the health of native French freshwater turtle is impacted by the occurrence of these exotic parasites. Furthermore, the natural parasite of the European pond turtle was only identified from two natural sites in France. In all other the ecosystems investigated, *E. orbicularis* was infected by invasive polystomes. Thereby, once may wonder if exotic polystomes could compete with the native ones and then threatened also



the natural parasites. It was intended during the thesis to performed freshwater turtle's infestations under experimental conditions with natural and exotic parasites to verify if competition between parasites species could be established. Because this experimentation involved too important technical resources, it could not be conducted. However, this experience remains interesting to perform in the future.

## 2. Application of an innovative molecular tool for parasite species discrimination

This study is the subject of an article published in the journal *Parasitology* and corresponds to the Chapter III of this document.

The study of the biodiversity of polystomes within freshwater turtle populations implies the genotyping of the parasite samples following COI gene amplification by Polymerase Chain Reaction (PCR) and sequencing. Because the French native freshwater turtles benefit of status of protection, most of the samples analyzed were polystomes eggs, collected using a non-invasive method (see Verneau et al. 2011). Since this molecular technic implies the separation of the samples, perform analyses from parasite eggs make it time-consuming and expensive with PCR molecular tool. The molecular tool High Resolution Melting (HRM) allows the detection of the variations between polymorphic sequences though the melting properties of double-stranded amplified DNA. This approach has several advantages, such as the direct reading of results without sequencing and the ability to analyze mixtures of samples.

Meyer et al. (2015) showed that the Mediterranean pond turtle was infected by six polystomes species in France, in the Pyrénées-Orientales department. These parasite species were selected to adapt and apply the HRM molecular tool for the identification of polystome species. Results showed that for each parasite species, when a single DNA was used in the analyses, a specific temperature of denaturation of the target gene was obtained, allowing the discrimination of each species. Thereby, this molecular technic proved very interesting for the analyses of mix of eggs from a turtle infected by one single polystome species. Because Meyer et al. (2015) also illustrated that in the majority of the case, the Mediterranean pond turtle could be infected by two polystome species in natural environments, one of the pharynx

and the other in the bladder, HRM molecular approach was applied on mix of DNAs of two species, in equal and different proportions. Although it was never shown that more than two polystome species could be identified in a turtle population, mix of DNAs of three species was also analyzed. The work of this thesis showed that the HRM molecular approach allowed the discrimination of a double infection, and furthermore can give information about the proportion of each parasite species infecting the host. However, this molecular tool has limitations for the identification of a triple infection, but can give information of the number of polystome species infecting a freshwater turtle population.

Overall, the molecular approach HRM was efficient to discriminate polystome species, particularly in the case of single and double infections. In the case of a turtle population infected by a single or two species of polystome, this technique provides considerable time savings and comes significantly cheaper than the molecular tool involving gene amplification by PCR and sequencing. Indeed, numerous eggs of different turtles can be analysed in the same pool to determine which parasite species infect the population in ecosystems. The limit of this method is achieved with three different species of parasites infecting a turtle population. The analyses allowed to detect the presence of more than two species of parasites but not to identify the species. However, it can be used for a preliminary study of parasite diversity within a host population. This study can be continued by including the other polystome species encountered in France on other turtle hosts, such as the European pond turtle or the red-eared slider, for a monitoring of parasite invasion in freshwater ecosystems.

### 3. Description of two new invasive polystome species identified from native and exotic freshwater turtles in French ecosystems

This study is the subject of an article in revision in the journal *Parasitology International* and corresponds to the Chapter IV of this document.

Molecular studies (Verneau et al. 2011; Meyer et al. 2015; Héritier et al. Submitted) showed the occurrence of two polystome species of the pharyngeal cavity, infecting European and American turtles in confined or natural areas in France. Because these two parasite species were also identified on *Trachemys scripta* in freshwater ecosystems in USA and

because *E. orbicularis* and *M. leprosa* were never observed in American wetlands, they were considered as invasive parasite species in France, carried by *T. s. elegans*. Thereby, it became interesting to describe them in a context of monitoring of invasive parasites in French ecosystems.

The study of morphological characters can be useful for distinguish polystome species from different genera, but can become not really discriminant for parasites of the same genera. As a consequence, others criterions were used to create new species, such as the host-specificity recognized for a long time for polystomes (Du Preez, Kok 1997). However, molecular investigations showed that in captivity as in natural environments, polystomes are not systematically host-specific (Verneau et al. 2011; Meyer et al. 2015; Héritier et al. Submitted). Because the two new polystome species are from the same genera, namely *Polystomoides*, and because molecular studies showed that these parasite species are not host-specific, descriptions by combining selected discriminant characters and molecular analysis were performed.

Comparisons of COI sequences of the two new species illustrated a genetic divergence clearly higher than the threshold fixed in Héritier et al. (Submitted) used for the discrimination of polystome species. Following a formation on the morphology and taxonomy of polystomes conducted in South Africa (august 2016) under the direction of Louis du Preez, it appears that the number of genital spines and the shape of the testis could be discriminant characters. Thereby, these morphological characters were compared between the two new species and another described American polystome, *Polystomoides oris* (Paul, 1938). The results of this analysis showed that these two morphological characters were significantly discriminant and that both undescribed polystome species were new distinct species, in accordance with the molecular data.

This work definitively showed the importance to combine morphological and molecular characters for parasite description. It could be very useful for the future that all the systematics working on polystome create a database comprising good quality pictures, especially of the genital bulb and the testis, for doing morphological comparisons. Regarding molecular comparisons, our database comprises about 100 COI sequences characterizing more than 30 distinct polystome species. We plan to create an online open access and to propose a molecular assistance for people unable to perform molecular works. Under these conditions, a revision of the taxonomy of the polystome could be considered.

## 4. Description of three polystome species in native freshwater turtles from South America

This study is the subject of an article published in the journal *Parasites and Vectors* and corresponds to the Chapter V of this document.

In this chapter, three new polystome species of the genera *Neopolystoma* were distinguished from other species and described using morphological characters, namely body size, number of genital spines, shape and size of the testis, and molecular analyses. These parasites were collected from freshwater turtles in natural environments in Guyane, two species from the spot-legged turtle *Rhinoclemmys punctularia* (Daudin, 1801), one in the conjunctival sac and the other in the bladder, and the third one from the scorpion mud turtle *Kinosternon scorpioides* (Linnaeus, 1766), in the conjunctival sacs.

The results of this thesis work have confirmed the efficacy of the combination of morphological characters and molecular in the description of parasitic species. Furthermore, this work showed the interest to describe new parasite species from hosts in wild ecosystems. Because introduction of non-native parasite species can contribute to the loss of biodiversity (Hulme 2007; Clavero et al. 2009), it is imperative to surveys native host populations. In the studies of Meyer et al. (2015) and Héritier et al. (Submitted), it was showed that the red-eared slider could transmit its parasite to the native turtle species. However, some polystome species infecting indigenous and invasive turtles were undescribed parasite species. This was the case for the two species described in the chapter 3 of this thesis. Since the sale of the red-eared slider was banned, others freshwater turtles were identified in the pet trade. Indeed, it is still possible to buy freshwater turtles in French society such as “La Ferme Tropicale”, Paris, or directly from websites, like for instance Reptimania (<http://www.reptimania.com>), where turtles from USA, Central America, South America, Asia and Africa are for sale. Regarding the case of the red-eared slider, all these freshwater turtles could become news invaders in French ecosystems. Before their parasites are identified on French native freshwater turtles, it is primordial to describe the polystome diversity in worldwide turtles.

## 5. Development of a tool to evaluate the health status of freshwater turtle populations and validation under experimental conditions

This study is the subject of an article submitted in the journal *Environmental Toxicology and Chemistry* and corresponds to the Chapter VI of this document.

In Southern France, and especially in the Pyrénées-Orientales department, intensive agricultural activities still exist with the use of pesticides and herbicides. After the study of an unpublished report of the Conseil Général of the Pyrénées-Orientales, it appears that pesticides were detected in almost the watercourses of this area, and especially in two minor streams. Indeed, two rivers namely the Fosseille and the Agouille de la Mar rivers were classified in the worst of the five listed categories that are used to evaluate the water quality because of the detection high concentrations of glyphosate and AMPA.

The toxicity of the glyphosate-based herbicides, as well as the toxicity of the active compounds alone, namely glyphosate, POEA and the degraded product AMPA, has been studied across vertebrates. It has been shown under experimental conditions that glyphosate-based herbicides are more toxic than the glyphosate tested alone (Howe et al. 2004; Peixoto 2005; Richard et al. 2005; Gasnier et al. 2009). As a result, glyphosate-based herbicide formulations were tested in fishes, amphibians and reptiles (Relyea 2005a, b; Gluszcak et al. 2006; Gluszcak et al. 2007; Cavalcante et al. 2008; Costa et al. 2008; Do Carmo Langiano, Martinez 2008; Poletta et al. 2009; Modesto, Martinez 2010) following studies of biomarkers such as anti-oxidative stress enzyme activities, metabolic parameters measures and examination of cellular damages. In all the case, glyphosate commercial formulations have proven toxics for the tested species. From this statement, once may wonder if the biodiversity was impacted by the poor quality of the two degraded rivers, namely the Fosseille and the Agouille de la Mar.

The Mediterranean pond turtle *M. leprosa* occurring among the species reported in the Fosseille and Agouille de la Mar rivers, despite the degradation of the waters. The first step was to develop biomarkers to assess the impact of the stressful environments on animals. A preliminary bibliographic investigation oriented us on the study of the variations in stress gene expression, i.e. genes coding for proteins implies in physiological responses, in animals

facing to stress conditions. Indeed, this biomarker responded within freshwater turtles face thermal shock or lack of oxygen (Storey 2006; Krivoruchko, Storey 2010; Stecyk et al. 2012; Krivoruchko, Storey 2013). However, after the study of the toxicity of the glyphosate-based herbicides on various organisms, another stress biomarker drew our attention. Reactive oxygen species (ROS), such as superoxide anion ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) are constitutively produced in organisms living in aerobic conditions. Facing to a stress situation, their production increasing to cause a physiological defense response. Indeed, ROS can attack all intrusive substance in the host and bind transcription factors leading the activation of genes coding for proteins implies in physiological responses. However, ROS may be after neutralized because they also able to cause damage to lipid walls of the cells, proteins and DNA. Thereby, the activity of enzymes involved in the oxidative detoxification, such as the catalase (CAT) and the superoxide dismutase (SOD), could be modified in organisms face to environmental stress (Hermes-Lima, Storey 1993; Van Der Oost et al. 2003; Hermes-Lima 2004; Halliwell 2005). Although this biomarker was only used in a single study on the Eastern Pacific green-sea turtle *Chelonia mydas* (Linnaeus 1758) (Labrada-Martagón et al. 2011) and because the single use of this biomarker is insufficient to explain physiological responses (Do Carmo Langiano, Martinez 2008; Modesto, Martinez 2010), we choose to develop for freshwater turtles two biomarkers, i.e. the activity of anti-oxidant enzymes and the expression of gene coding for these latter.

The Mediterranean pond turtle is considered as “vulnerable” since 2015 (UICN France, MNHN and SHF, 2015). As a result, it is inconceivable to sacrifice turtle specimens in a study in natural environment. Similarly, it is impossible to conduct an experiment involving animals faced to an environmental stress. For these reasons, the biomarkers selected were investigated from turtle blood samples and juveniles of the red-eared slider were used for the experiments. To perform this work, a former classroom for practice work was converted into a pet room, equipped with plastic tank waters, UV lamps and heating for aquariums. Juveniles red-eared sliders, 250 in total, were purchased at the French society “la Ferme Tropicale” in April 2015, from breeding centers of the USA. However, after five weeks of acclimation, all the turtles died for unexplained reasons. We then contacted “la Ferme des Tortues de Sorede” and we recovered thirty juveniles *T. s. elegans*, which helped to launch the experimental phase.

The results of this experiment performed under controlled conditions showed that gene expression profiles and enzyme activities of CAT and SOD could be quantified from fresh water turtle blood samples, like in the study of Labrada-Martagón et al. (2011) on the Eastern Pacific green sea turtle. Thereby, this approach can be applied for environmental ecotoxicology studies, especially on species that are endangered of extinction. Furthermore, results showed variations in gene expression and activities of both anti-oxidant enzymes, which demonstrated that these biomarkers allowed to measure physiological stress within turtles face to environmental modifications. This study also illustrated that the glyphosate at the concentration used induce a stress within turtles, as the water collected in the Fosseille River. Thereby, the investigation of the Mediterranean pond turtles in natural environments in the PO department proved a particular interest, as the pollution levels of the ecosystems could be assessed through the state of health of wild populations.

## 6. Application of validated biomarkers on freshwater turtle populations in natural environments

This study is the subject of an article in revision in the journal *Chemosphere* and corresponds to the Chapter VII of this document.

Following the development of the biomarkers within freshwater turtles and their validation under experimental conditions, the final work of this thesis was to apply this tool on wild turtle populations, i.e. *M. leprosa* populations of the Fosseille and the Agouille de la Mar rivers. To conduct a comparison with a contrasted ecosystem, the *M. leprosa* population of the Baillaury River, a small stream in the PO department surrounded by vineyards like the two latter rivers but with a good quality of waters, was also investigated. A fieldwork was then performed to collect turtle blood in all these rivers before running analyses. Since wastewater treatment plant (WTP) discharge processed waters in the Fosseille and Agouille de la Mar rivers, turtles sampled in these both streams were divided in several groups, i.e. upstream and downstream the WTP for analyses.

The analysis of the biomarkers response in wild population of turtles showed significant variations, for the gene expression and for the activities of CAT and SOD enzymes. However, most of the comparisons revealed no significant differences. Indeed,

variabilities in the physiological responses of turtles could explain the lack of significant results. Because it is extremely difficult to assess to all the specimens life style in natural environments, unlike the study conducted under experimental conditions, where all the parameters were fixed, some differences, even not significant, were considered as trends in the anti-oxidative stress response within animals in wild environments.

The results of this study showed that the Fosseille and the Agouille de la Mar rivers represent stressful environments for freshwater turtles, that was expected. The Baillaury River could be the less stressful ecosystem, probably due to the quality of the water. However, in the two degraded rivers, animals located upstream the WTPs are less stressed than those located downstream. Because agricultural activities are similar along both streams, they cannot be solely as the main factor involved in turtle's stress. This result suggests that processed waters discharged by the WTPs could considerably impact the biodiversity.

The development of this approach during this thesis proved very useful to evaluate the health of the freshwater turtle populations, reflecting the quality of the habitats. Because various pollutants, biotic such as the red-eared slider and its parasites, and abiotic such as the presence of glyphosate and AMPA principally, are identified in the Fosseille and Agouille de la Mar rivers, it is impossible to determine the causes of the stress on wild animals. However, it is really useful to evaluate the quality of these ecosystems. This approach will be used in the future within another *M. leprosa* populations of watercourses in the PO, especially where a WTP discharge processed waters.

## VI. Synthesis and perspectives

This work allowed us to address a fundamental issue that is the impact of biotic and abiotic contaminants on aquatic ecosystems. First, our study focused on the introduction and switching of non-native parasites (Monogenes, Polystomatidae) into natural habitats, from the red-eared slider *T. s. elegans*, an invasive species, to two model species of native freshwater turtles in southern Europe: the European pond turtle *Emys orbicularis* and the Mediterranean pond turtle *Mauremys leprosa*. Following the studies carried out by Verneau et al. (2011) in confined environments and Meyer et al. (2015) in natural environments on *M. leprosa*, we first investigated whether *E. orbicularis* was also infected in Southern Europe by polystomes introduced via *T. s. Elegans*.



Responding to this question remained essential to determine whether our two model species were confronted with the biotic stresses associated with the introduction of exotic hosts and their parasites. We showed in this study that the European pond turtle was infected by five species of exotic polystomes imported with the red-eared slider, results similar to those obtained by Meyer et al. (2015) on the Mediterranean pond turtle. We thus showed that these two species of freshwater turtles were confronted to biotic stresses, in addition to abiotic stresses due to the water pollution.

Once this scientific question has been resolved, the initial project of the thesis involved the development of a tool to measure the health status of turtles in natural environments. We have chosen to study the variations in the activity of enzymes involved in the response to oxidative stress, namely superoxide dismutase and catalase, as well as the variations in the expression of the genes encoding them. Before applying this tool to populations of turtle sampled in natural environments, we planned to validate it under experimental conditions in order to verify whether the biomarkers considered here were relevant (detectable response) and whether they responded from blood samples. Indeed, since our two native model species were protected animals, it was not possible to remove tissues or organs. This validation of the tool was necessary in order to know if the various abiotic and biotic stresses identified in the study zones caused many responses of our biomarkers, thus allowing measuring a stress in our model species. Because the glyphosate is the main compound that downgrades the waters of some of our selected sites, we have confronted in experimental conditions juveniles of *T. scripta* to a high concentration of glyphosate from a commercial formulation, as well as to the water of the Fosseille, a river located on the outskirts of Perpignan and whose waters were estimated to be of poor quality following the detection of glyphosate. We obtained a response of the biomarkers in the two experimental devices to validate our tool of measurement of the stress and more from turtle blood samples. Controlled infestations were then planned using our model parasite species (polystomes) on juvenile turtles to investigate the potential response of biomarkers selected following parasite infestations. Finally, we also considered testing our biomarkers as part of a synergistic effect of biotic and abiotic stressors on turtles artificially infested with polystomes and kept in water containing glyphosate. We have not been able to carry out these last two experiments for several reasons. Measuring the impact of infestations by exotic parasites involved experimenting with juvenile European freshwater turtles, which was impossible under the

legislation imposed for these species. Nevertheless, this manipulation could have been possible by requesting a certificate of capacity and authorization to experiment on these species, a very long and tedious process that would have taken too long in the framework of this thesis. Furthermore, once all the authorizations have been obtained, it would have been necessary to contact a center specialized in the breeding of these two species in order to have juveniles born in captivity. Finally, the life cycle of polystomes is not completely known under experimental conditions. Indeed, we have observed that the laying cycles are not always synchronized, which makes it difficult to obtain larvae in sufficient quantity to carry out controlled infestations. For all these reasons, and in view of the time taken to remove the other scientific locks, this experiment proved impossible during this thesis. Nevertheless, these experiments, involving a thorough study of the life cycle of polystomes, remain essential to develop in the future in order to understand the stress levels generated on the one hand by the presence of non-native parasites and on the other by the combination of biotic and abiotic stress factors acting in synergy.

Finally, the use of our stress measurement tool on *M. leprosa* populations from different and contrasted natural environments has enabled us to obtain interesting results showing that the animals sampled in the most polluted sites are the most stressed. Furthermore, we showed that the wastewater treatment plants located on the two streams with the most contaminants could play an important role in the stress suffered by the organisms. Indeed and contrary to expectations, it is the turtles sampled after the purification stations that are the most stressed, suggesting that the water discharged by the wastewater treatment plants could have a negative impact on biodiversity. Because of the lack of time and the financial investment involved in measuring variations in enzymatic activities, we have chosen to focus on the Mediterranean pond turtle in this thesis. It would be interesting in the future to apply this tool to populations of the European pond turtle, or even to other endangered model species.

## Références

- Abell R (2002) Conservation biology for the biodiversity crisis: a freshwater follow-up. *Conservation Biology* 16:1435-1437
- Ahlberg PE (1999) Palaeontology: Something fishy in the family tree. *Nature* 397:564-565
- Albiac J, Calvo E, Tapia J, et al. (2010) Water quality and nonpoint pollution: Comparative global analysis. *Re-thinking Water and Food Security*:251
- Archibald JD, Clemens W, Padian K, et al. (2010) Cretaceous extinctions: multiple causes. *Science* 328:973-973
- Arunakumara K, Walpola BC, Yoon M-H (2013) Metabolism and degradation of glyphosate in aquatic cyanobacteria: A review. *African Journal of Microbiology Research* 7:4084-4090
- Arvy C, Servan J (1998) Imminent competition between *Trachemys scripta* and *Emys orbicularis* in France. *Mertensiella* 10:33-40
- Balian E, Segers H, Lévêque C, et al. (2008) The freshwater animal diversity assessment: an overview of the results. *Hydrobiologia* 595:627-637
- Barnosky AD, Matzke N, Tomiya S, et al. (2011) Has the Earth's sixth mass extinction already arrived? *Nature* 471:51-57
- Barry J, Mcleish J, Dodd J, et al. (2014) Introduced parasite *Anguillicola crassus* infection significantly impedes swim bladder function in the European eel *Anguilla anguilla* (L.). *Journal of fish diseases* 37:921-924
- Battaglin WA, Kolpin DW, Scribner EA, et al. (2005) Glyphosate, other herbicides, and transformation products in midwestern streams, 20021. Wiley Online Library,
- Bellard C, Genovesi P, Jeschke J (2016) Global patterns in threats to vertebrates by biological invasions. *The Royal Society*, pp. 2015-2454
- Benslama O, Boulahrouf A (2013) Isolation and characterization of glyphosate-degrading bacteria from different soils of Algeria. *African Journal of Microbiology Research* 7:5587-5595
- Bhuiyan AB, Mokhtar MB, Toriman ME, et al. (2013) The environmental risk and water pollution: a review from the river basins around the world. *American-Eurasian Journal of Sustainable Agriculture* 7:126-136
- Boeger WA, Kritsky DC (2001) Phylogenetic relationships of the Monogenoidea. In: Littlewood DTJ and Bray RA (eds) *Interrelationships of the Platyhelminthes*. Taylor & Francis, London, pp. 92-102

- Bonin F, Devaux B, Dupré A (1998) Toutes les Tortues du Monde. Delachaux and Niestlé, Lausanne
- Bringsøe H (2006) NOBANIS–Invasive alien species fact sheet–*Trachemys scripta*. From: Online Database of the North European and Baltic Network on Invasive Alien Species–NOBANIS [www.nobanis.org](http://www.nobanis.org), Last view 1:2008
- Bucciarelli GM, Blaustein AR, Garcia TS, et al. (2014) Invasion complexities: the diverse impacts of nonnative species on amphibians. *Copeia* 2014:611-632
- Buhlmann KA, Akre TS, Iverson JB, et al. (2009) A global analysis of tortoise and freshwater turtle distributions with identification of priority conservation areas. *Chelonian Conservation and Biology* 8:116-149
- Cadi A, Joly P (2003) Competition for basking places between the endangered European pond turtle (*Emys orbicularis galloitalica*) and the introduced red-eared slider (*Trachemys scripta elegans*). *Canadian Journal of Zoology* 81:1392-1398
- Cadi A, Joly P (2004) Impact of the introduction of the red-eared slider (*Trachemys scripta elegans*) on survival rates of the European pond turtle (*Emys orbicularis*). *Biodiversity & Conservation* 13:2511-2518
- Cavalcante D, Martinez C, Sofia S (2008) Genotoxic effects of Roundup® on the fish *Prochilodus lineatus*. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 655:41-46
- Ceballos G, García A, Ehrlich PR (2010) The sixth extinction crisis: loss of animal populations and species. *Journal of Cosmology* 8:31
- Chapin III FS, Zavaleta ES, Eviner VT, et al. (2000) Consequences of changing biodiversity. *Nature* 405:234-242
- Cheylan M, Courtin J (1976) La consommation de la tortue cistude *Emys orbicularis* (L.) au post-glaciaire dans la Grotte de Fontbregoua (Salernes-Var). *Bulletin du Museum d'Histoire Naturelle de Marseille* 36:41-46
- Cheylan M, Poitevin F (1998) Impact of fire on a population of European pond turtles (*Emys orbicularis*) in southeastern France. *Mertensiella* 10:67-82
- Cheylan M, Poitevin F (2003) Les tortues du site de Lattara (IVe s. av. n. è.-IIe s. de n. è.): Intérêt archéozoologique et biologique. *Lattara* 16:137-145
- Clavero M, Brotons L, Pons P, et al. (2009) Prominent role of invasive species in avian biodiversity loss. *Biological Conservation* 142:2043-2049
- Costa MJ, Monteiro DA, Oliveira-Neto AL, et al. (2008) Oxidative stress biomarkers and heart function in bullfrog tadpoles exposed to Roundup Original®. *Ecotoxicology* 17:153-163

- Cox N, Chanson J, Stuart S (2006) The status and distribution of reptiles and amphibians of the Mediterranean Basin. IUCN
- Cox NA, Temple HJ (2009a) European red list of reptiles. Office for Official Publications of the European Communities, United Kingdom
- Cox NA, Temple HJ (2009b) European red list of reptiles. IUCN, Gland (Suiza)
- Cuttelod A, García N, Abdul Malak D, Temple H, Katariya V (2008) The Mediterranean: a biodiversity hotspot under threat. In: J.-C. Vié, C. Hilton-Taylor and S.N. Stuart (eds). The 2008 Review of The IUCN Red List of Threatened Species. IUCN Gland, Switzerland.
- Da-Silva E (2002) *Mauremys leprosa*. Atlas y Libro Rojo de los Anfibios y Reptiles de España Dirección General de Conservación de la Naturaleza-Asociación Herpetológica Española. Madrid 2:143-146
- Da Silva E, Blasco M (2002) *Mauremys leprosa*. Atlas y Libro Rojo de Los Anfibios y Reptiles de España:143-146
- Da Silva ET, Dos Reis EP, Feio RN, Filho OPR (2009) Diet of the invasive frog *Lithobates catesbeianus* (Shaw, 1802) (Anura: Ranidae) in Vicosa, Minas Gerais State, Brazil. South American Journal of Herpetology 4:286-294
- Dall'antonia L, Lebboroni M, Benvenuti S, et al. (2001) Data loggers to monitor activity in wild freshwater turtles. Ethology Ecology & Evolution 13:81-88
- Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife--threats to biodiversity and human health. Science 287:443-449
- De Lapparent De Broin F (2000) African chelonians from the Jurassic to the present: phases of development and preliminary catalogue of the fossil record.
- De Lapparent De Broin F (2001) The European turtle fauna from the Triassic to the Present. Dumerilia 4:155-217
- Ding J, Mack RN, Lu P, et al. (2008) China's booming economy is sparking and accelerating biological invasions. Bioscience 58:317-324
- Do Carmo Langiano V, Martinez CB (2008) Toxicity and effects of a glyphosate-based herbicide on the Neotropical fish *Prochilodus lineatus*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 147:222-231
- Du Preez LH, Kok DJ (1997) Supporting experimental evidence of host specificity among southern African polystomes (Polystomatidae: Monogenea). Parasitology Research 83:558-562
- Dudgeon D, Arthington AH, Gessner MO, et al. (2006) Freshwater biodiversity: importance, threats, status and conservation challenges. Biological reviews 81:163-182

- Elewa AM, Joseph R (2009) The history, origins, and causes of mass extinctions. *Journal of Cosmology* 2:201-220
- Engel K, Tollrian R, Jeschke JM (2011) Integrating biological invasions, climate change and phenotypic plasticity. *Communicative & integrative biology* 4:247-250
- Fastovsky DE, Sheehan PM (2005) The extinction of the dinosaurs in North America. *Gsa Today* 15:4-10
- Fattizzo T (2004) Distribution and conservational problems of *Emys orbicularis* in Salento (South Apulia, Italy). *Biologia* 59:13-18
- Ferronato BO, Marques TS, Guardia I, et al. (2009) The turtle *Trachemys scripta elegans* (Testudines, Emydidae) as an invasive species in a polluted stream of southeastern Brazil. *Herpetological Bulletin* 109:29-34
- Ficetola G, Monti A, Padoa-Schioppa E (2003) First record of reproduction of *Trachemys scripta* in the Po Delta. *Annali del Museo civico di Storia naturale di Ferrara* 5:125-128
- Ficetola GF, Thuiller W, Padoa Schioppa E (2009) From introduction to the establishment of alien species: bioclimatic differences between presence and reproduction localities in the slider turtle. *Diversity and Distributions* 15:108-116
- Forlani G, Pavan M, Gramek M, et al. (2008) Biochemical bases for a widespread tolerance of cyanobacteria to the phosphonate herbicide glyphosate. *Plant and cell physiology* 49:443-456
- Franz JE, Mao MK, Sikorski JA (1997) Glyphosate: a unique global herbicide. *American Chemical Society*
- Fritz U, Ayaz D, Hundsdörfer AK, et al. (2009) Mitochondrial diversity of European pond turtles (*Emys orbicularis*) in Anatolia and the Ponto-Caspian Region: Multiple old refuges, hotspot of extant diversification and critically endangered endemics. *Organisms Diversity & Evolution* 9:100-114
- Fritz U, Barata M, Busack SD, et al. (2006) Impact of mountain chains, sea straits and peripheral populations on genetic and taxonomic structure of a freshwater turtle, *Mauremys leprosa* (Reptilia, Testudines, Geoemydidae). *Zoologica Scripta* 35:97-108
- Fritz U, Cadi A, Cheylan M, et al. (2005a) Distribution of mtDNA haplotypes (cyt b) of *Emys orbicularis* in France and implications for postglacial recolonization. *Amphibia-Reptilia* 26:231-238
- Fritz U, Fattizzo T, Guicking D, et al. (2005b) A new cryptic species of pond turtle from southern Italy, the hottest spot in the range of the genus *Emys* (Reptilia, Testudines, Emydidae). *Zoologica Scripta* 34:351-371

- Gasc A, Duryea MC, Cox RM, Kern A, Calsbeek R (2010) Invasive Predators Deplete Genetic Diversity of Island Lizards. PLoS ONE 5(8): e12061.doi:10.1371/journal.pone.0012061
- Gasnier C, Dumont C, Benachour N, et al. (2009) Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology 262:184-191
- Gherardi F, Daniels WH (2004) Agonism and shelter competition between invasive and indigenous crayfish species. Canadian Journal of Zoology 82:1923-1932
- Gibbons JW, Scott DE, Ryan TJ, et al. (2000) The Global Decline of Reptiles, Déjà Vu Amphibians Reptile species are declining on a global scale. Six significant threats to reptile populations are habitat loss and degradation, introduced invasive species, environmental pollution, disease, unsustainable use, and global climate change. Bioscience 50:653-666
- Giesy JP, Dobson S, Solomon KR (2000) Ecotoxicological Risk Assessment for Roundup® Herbicide. In: Ware GW (ed) Reviews of Environmental Contamination and Toxicology: Continuation of Residue Reviews. Springer New York, New York, NY, pp. 35-120
- Gill HK, Garg H (2014) Pesticides: environmental impacts and management strategies. Pesticides—toxic aspects. InTech:188-230
- Gluszczak L, Dos Santos Miron D, Crestani M, et al. (2006) Effect of glyphosate herbicide on acetylcholinesterase activity and metabolic and hematological parameters in piava (*Leporinus obtusidens*). Ecotoxicology and environmental safety 65:237-241
- Gluszczak L, Dos Santos Miron D, Moraes BS, et al. (2007) Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 146:519-524
- Gozlan RE, St-Hilaire S, Feist SW, et al. (2005) Biodiversity: disease threat to European fish. Nature 435:1046-1046
- Halliwell B (2005) Free radicals and other reactive species in disease. eLS
- Hays DW, Mcallister KR, Richardson SA, et al. (1999) Washington State recovery plan for the western pond turtle. Washington Department of Fish and Wildlife, Olympia, WA
- Héritier L, Badets M, Du Preez LH, et al. (2015) Evolutionary processes involved in the diversification of chelonian and mammal polystomatid parasites (Platyhelminthes, Monogenea, Polystomatidae) revealed by palaeoecology of their hosts. Molecular Phylogenetics and Evolution 92:1-10
- Héritier L, Valdeón A, Sadaoui A, et al. (Submitted) Introduction and invasion of the red-eared slider and its parasites in freshwater ecosystems of Southern Europe: risk

- assessment for the European pond turtle in wild environment. *Biodiversity and Conservation*
- Hermes-Lima M (2004) Oxygen in biology and biochemistry: role of free radicals. *Functional metabolism: Regulation and adaptation* 1:319-66
- Hermes-Lima M, Storey KB (1993) Antioxidant defenses in the tolerance of freezing and anoxia by garter snakes. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 265:R646-R652
- Hervet S, Salotti M (2000) Les tortues pléistocènes de Castiglione (Oletta, Haute-Corse) et la preuve de leur indigénat en Corse. *Comptes Rendus de l'Académie des Sciences-Series IIA-Earth and Planetary Science* 330:645-651
- Howe CM, Berrill M, Pauli BD, et al. (2004) Toxicity of glyphosate based pesticides to four North American frog species. *Environmental Toxicology and Chemistry* 23:1928-1938
- Hulme PE (2007) Biological invasions in Europe: drivers, pressures, states, impacts and responses. *Biodiversity under threat* 25:56-80
- Hulme PE, Pyšek P, Nentwig W, et al. (2009) Will threat of biological invasions unite the European Union. *Science* 324:40-41
- Hupfer M, Kleeberg A (2007) State and pollution of freshwater ecosystems—warning signals of a changing environment. *Climate Change: Enough Water for all*:126-132
- Iglesias R, García-Estévez JM, Ayres C, et al. (2015) First reported outbreak of severe spirorchidiasis in *Emys orbicularis*, probably resulting from a parasite spillover event. *Diseases of aquatic organisms* 113:75-80
- Izquierdo GA, Del Cueto FF, Rodríguez-Pereira A, et al. (2010) Distribution records of non-native terrapins in Castilla and Leon region (Central Spain). *Aquatic invasions* 5:303-308
- Janvier P (1988) Forerunners of four legs. *Nature* 395:748-749
- Jiraungkoorskul W, Upatham ES, Kruatrachue M, et al. (2002) Histopathological effects of Roundup, a glyphosate herbicide, on Nile tilapia (*Oreochromis niloticus*). *Science Asia* 28:121-127
- Keesing F, Hotl RD, Ostfeld RS (2006). Effects of species diversity on disease risk. *Ecology letters* 9:485-498
- Kelly DW, Paterson RA, Townsend CR, Poulin R, Tompkins DM (2009) Parasite spillback: A neglected concept in invasion ecology? *Ecology* 90:2047-2056
- Kolpin DW, Thurman EM, Lee EA, et al. (2006) Urban contributions of glyphosate and its degradate AMPA to streams in the United States. *Science of the Total Environment* 354:191-197



- Kotenko T (2000) The European pond turtle *Emys orbicularis* (L.) in the steppe zone of the Ukraine. na
- Krivoruchko A, Storey KB (2010) Regulation of the heat shock response under anoxia in the turtle, *Trachemys scripta elegans*. *Journal of Comparative Physiology B* 180:403-414
- Krivoruchko A, Storey KB (2013) Anoxia-responsive regulation of the FoxO transcription factors in freshwater turtles, *Trachemys scripta elegans*. *Biochimica et Biophysica Acta (BBA)-General Subjects* 1830:4990-4998
- Labrada-Martagón V, Rodríguez PaT, Méndez-Rodríguez LC, et al. (2011) Oxidative stress indicators and chemical contaminants in East Pacific green turtles (*Chelonia mydas*) inhabiting two foraging coastal lagoons in the Baja California peninsula. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 154:65-75
- Lenk P, Fritz U, Joger U, et al. (1999) Mitochondrial phylogeography of the European pond turtle, *Emys orbicularis* (Linnaeus 1758). *Molecular ecology* 8:1911-1922
- Littlewood D, Rohde K, Bray R, et al. (1999) Phylogeny of the Platyhelminthes and the evolution of parasitism. *Biological Journal of the Linnean Society* 68:257-287
- Lockwood JL, Hoopes MF, Marchetti MP (2013) *Invasion ecology*. John Wiley & Sons
- Lodge DM, Williams S, Macisaac HJ, et al. (2006) Biological invasions: recommendations for US policy and management. *Ecological applications* 16:2035-2054
- Lowe S, Browne M, Boudjelas S, et al. (2000) 100 of the world's worst invasive alien species: a selection from the global invasive species database.
- Lutz CG (2000) *Pet turtle production*. Southern Regional Aquaculture Center
- Lymbery AJ, Morine M, Kanani HG, et al. (2014) Co-invaders: The effects of alien parasites on native hosts. *International Journal for Parasitology: Parasites and Wildlife* 3:171-177
- Mack MC, D'antonio CM (1998) Impacts of biological invasions on disturbance regimes. *Trends in Ecology & Evolution* 13:195-198
- Malmqvist B, Rundle S (2002) Threats to the running water ecosystems of the world. *Environmental conservation* 29:134-153
- Mann R, Bidwell J (1999) The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs. *Archives of Environmental Contamination and Toxicology* 36:193-199
- Meeus I, Brown MJ, De Graaf DC, et al. (2011) Effects of invasive parasites on bumble bee declines. *Conservation Biology* 25:662-671

- Meyer L, Du Preez L, Bonneau E, et al. (2015) Parasite host-switching from the invasive American red-eared slider, *Trachemys scripta elegans*, to the native Mediterranean pond turtle, *Mauremys leprosa*, in natural environments. *Aquatic Invasions* 10:79-91
- Miller PS, Lacy RC, Medina-Miranda R, et al. (2016) Confronting the invasive species crisis with metamodel analysis: An explicit, two-species demographic assessment of an endangered bird and its brood parasite in Puerto Rico. *Biological Conservation* 196:124-132
- Modesto KA, Martinez CB (2010) Roundup® causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus*. *Chemosphere* 78:294-299
- Mooney HA, Cleland EE (2001) The evolutionary impact of invasive species. *Proceedings of the National Academy of Sciences* 98:5446-5451
- Morick D, Baneth G, Avidor B, et al. (2009) Detection of *Bartonella* spp. in wild rodents in Israel using HRM real-time PCR. *Veterinary Microbiology* 139:293-297
- Morrison C, Du Preez L (2011) Turtle polystomes of the world
- Naeem S, Chapin III F, Costanza R, et al. (1999) Biodiversity and ecosystem functioning: maintaining natural life support processes. *Issues in Ecology* 4
- Ngui R, Lim YA, Chua KH (2012) Rapid detection and identification of human hookworm infections through high resolution melting (HRM) analysis. *PloS one* 7:e41996
- Nishizawa H, Tabata R, Hori T, et al. (2014) Feeding kinematics of freshwater turtles: what advantage do invasive species possess? *Zoology* 117:315-318
- Palacios C, Urrutia C, Knapp N, et al. (2015) Demographic structure and genetic diversity of *Mauremys leprosa* in its northern range reveal new populations and a mixed origin. *Salamandra* 51:221-230
- Pangasa A, Jex AR, Campbell BE, et al. (2009) High resolution melting-curve (HRM) analysis for the diagnosis of cryptosporidiosis in humans. *Molecular and Cellular Probes* 23:10-15
- Pearson SH (2013) The Potential for Competition Between the Red-bellied Turtle (*Pseudemys rubriventris*) and the Red-eared Slider Turtle (*Trachemys scripta elegans*). Drexel University,
- Pedall I, Fritz U, Stuckas H, et al. (2011) Gene flow across secondary contact zones of the *Emys orbicularis* complex in the Western Mediterranean and evidence for extinction and re-introduction of pond turtles on Corsica and Sardinia (Testudines: Emydidae). *Journal of Zoological Systematics and Evolutionary Research* 49:44-57
- Peeler EJ, Oidtmann BC, Midtlyng PJ, et al. (2011) Non-native aquatic animal introductions have driven disease emergence in Europe. *Biological Invasions* 13:1291-1303

- Peixoto F (2005) Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. *Chemosphere* 61:1115-1122
- Pichelin S, Whittington I, Pearson J (1991) *Concinnocotyla* (Monogenea: Polystomatidae), a new genus for the polystome from the Australian lungfish *Neoceratodus forsteri*. *Systematic Parasitology* 18:81-93
- Pimentel D, Zuniga R, Morrison D (2005) Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics* 52:273-288
- Pleguezuelos JM, Márquez R, Lizana M (2004) Atlas y libro rojo de los anfibios y reptiles de España. Organismo Autónomo de Parques Nacionales
- Poletta G, Larriera A, Kleinsorge E, et al. (2009) Genotoxicity of the herbicide formulation Roundup®(glyphosate) in broad-snouted caiman (*Caiman latirostris*) evidenced by the Comet assay and the Micronucleus test. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 672:95-102
- Polo-Cavia N, López P, Martín J (2009a) Interspecific differences in chemosensory responses of freshwater turtles: consequences for competition between native and invasive species. *Biological Invasions* 11:431-440
- Polo-Cavia N, López P, Martín J (2009b) Interspecific differences in heat exchange rates may affect competition between introduced and native freshwater turtles. *Biological Invasions* 11:1755-1765
- Polo-Cavia N, López P, Martín J (2010) Competitive interactions during basking between native and invasive freshwater turtle species. *Biological Invasions* 12:2141-2152
- Polo-Cavia N, López P, Martín J (2011) Aggressive interactions during feeding between native and invasive freshwater turtles. *Biological Invasions* 13:1387-1396
- Polo-Cavia N, López P, Martín J (2012) Feeding status and basking requirements of freshwater turtles in an invasion context. *Physiology & behavior* 105:1208-1213
- Polo-Cavia N, Lopez P, Martín J (2008) Interspecific differences in responses to predation risk may confer competitive advantages to invasive freshwater turtle species. *Ethology* 114:115-123
- Poulin R, Mouillot D (2003) Host introductions and the geography of parasite taxonomic diversity. *Journal of Biogeography* 30:837-845
- Prévot-Julliard A-C, Gousset E, Archinard C, et al. (2007) Pets and invasion risks: is the Slider turtle strictly carnivorous? *Amphibia-Reptilia* 28:139-143
- Prudhoe S, Bray RA (1982) *Platyhelminth parasites of the Amphibia*. Oxford University Press, London

- Ramsay NF, Ng PKA, O'riordan RM et al. (2007) The red-eared slider (*Trachemys scripta elegans*) in Asia: a review. Biological invaders in inland waters: profiles, distribution, and threats. Springer, pp. 161-174
- Relyea RA (2005a) The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecological applications* 15:618-627
- Relyea RA (2005b) The lethal impacts of Roundup and predatory stress on six species of North American tadpoles. *Archives of Environmental Contamination and Toxicology* 48:351-357
- Revenge C, Mock G (2000) Freshwater biodiversity in crisis. *Earth Trends World Resources Institute*:1-4
- Richard S, Moslemi S, Sipahutar H, et al. (2005) Differential effects of glyphosate and roundup on human placental cells and aromatase. *Environmental health perspectives*:716-720
- Robertson T, Bibby S, O'rourke D, et al. (2010) Identification of chlamydial species in crocodiles and chickens by PCR-HRM curve analysis. *Veterinary microbiology* 145:373-379
- Rohde K (1994) The origins of parasitism in the Platyhelminthes. *International journal for parasitology* 24:1099-1115
- Sabater S, Barceló D (2010) Water scarcity in the Mediterranean: Perspectives under global change. Springer
- Sala OE, Chapin FS, Armesto JJ, et al. (2000) Global biodiversity scenarios for the year 2100. *Science* 287:1770-1774
- Schulte P, Alegret L, Arenillas I, et al. (2010) The Chicxulub asteroid impact and mass extinction at the Cretaceous-Paleogene boundary. *Science* 327:1214-1218
- Schwarzenbach RP, Escher BI, Fenner K, et al. (2006) The challenge of micropollutants in aquatic systems. *Science* 313:1072-1077
- Segurado P, Araújo APR (2004) Coexistence of *Emys orbicularis* and *Mauremys leprosa* in Portugal at two spatial scales: Is there evidence of spatial segregation. *Biologia* 59:61-72
- Servan J, Arvy C (1997) Introduction de la tortue de Floride *Trachemys scripta* en France. Un nouveau compétiteur pour les espèces de tortues d'eau douce européennes. *Bulletin Français de la Pêche et de la Pisciculture*:173-177
- Sherrard-Smith E, Elizabeth A, Chadwick A, Cable J (2014) The impact of introduced hosts on parasite transmission: Opisthorchiid infections in American mink (*Neovison vison*). *Biological invasions* DOI 10.1007/s10530-014-0709-y

- Simberloff D (2013) Invasive species: what everyone needs to know. Oxford University Press
- Sinnappah ND, Lim LHS, Rohde K, et al. (2001) A paedomorphic parasite associated with a neotenic amphibian host: Phylogenetic evidence suggests a revised systematic position for Sphyrnauridae within anuran and turtle Polystomatoineans. *Molecular Phylogenetics and Evolution* 18:189-201
- Sommer RS, Lindqvist C, Persson A, et al. (2009) Unexpected early extinction of the European pond turtle (*Emys orbicularis*) in Sweden and climatic impact on its Holocene range. *Molecular ecology* 18:1252-1262
- Souza FL, Abe AS (2000) Feeding ecology, density and biomass of the freshwater turtle, *Phrynops geoffroanus*, inhabiting a polluted urban river in south-eastern Brazil. *Journal of Zoology* 252:437-446
- Sparling DW, Fellers GM, McConnell LL (2001) Pesticides and amphibian population declines in California, USA. *Environmental Toxicology and Chemistry* 20:1591-1595
- Stecyk JA, Couturier CS, Fagernes CE, et al. (2012) Quantification of heat shock protein mRNA expression in warm and cold anoxic turtles (*Trachemys scripta*) using an external RNA control for normalization. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* 7:59-72
- Steffen W, Persson Å, Deutsch L, et al. (2011) The Anthropocene: From global change to planetary stewardship. *Ambio* 40:739-761
- Storey KB (2006) Reptile freeze tolerance: metabolism and gene expression. *Cryobiology* 52:1-16
- Strayer DL, Dudgeon D (2010) Freshwater biodiversity conservation: recent progress and future challenges. *Journal of the North American Benthological Society* 29:344-358
- Stuckas H, Velo-Antón G, Fahd S, et al. (2014) Where are you from, stranger? The enigmatic biogeography of North African pond turtles (*Emys orbicularis*). *Organisms Diversity & Evolution* 14:295-306
- Telecky T (2001) United States import and export of live turtles and tortoises. *Turtle and tortoise newsletter* 4:8-13
- Thurston JP, Laws RM (1965) *Oculotrema hippopotami* (Trematoda: Monogenea) in Uganda. *Nature* 205:1127
- Tong SY, Giffard PM (2012) Microbiological applications of high-resolution melting analysis. *Journal of clinical microbiology* 50:3418-3421

- Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ, Kuris AM (2003) Introduced species and their missing parasites. *Nature* 421:628-630
- Trujillo-Reyes J, Peralta-Videa J, Gardea-Torresdey J (2014) Supported and unsupported nanomaterials for water and soil remediation: are they a useful solution for worldwide pollution? *Journal of hazardous materials* 280:487-503
- Tzankov N, Popgeorgiev G, Kornilev Y, et al. (2015) First survey on the invasive Pond slider (*Trachemys scripta*) in Bulgaria: historic development and current situation. *HYLA-Herpetološki bilten* 2015:18-27
- Van Der Oost R, Beyer J, Vermeulen NP (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental toxicology and pharmacology* 13:57-149
- Van Dijk PP, Iverson JB, Rhodin AG, et al. (2014) *Turtles of the world, 7th edition: annotated checklist of taxonomy, synonymy, distribution with maps and conservation status*. *Chelonian research monographs* 5:329-479
- Vaughn CC (2010) Biodiversity losses and ecosystem function in freshwaters: emerging conclusions and research directions. *Bioscience* 60:25-35
- Verneau O (2004) Origine et évolution des monogènes Polystomatidae, parasites d'amphibiens et de chéloniens d'eau douce. HDR thesis, Université de Perpignan Via Domitia, Perpignan
- Verneau O, Bentz S, Sinnappah ND, et al. (2002) A view of early vertebrate evolution inferred from the phylogeny of polystome parasites (Monogenea: Polystomatidae). *Proceedings of the Royal Society of London B: Biological Sciences* 269:535-543
- Verneau O, Palacios C, Platt T, et al. (2011) Invasive species threat: parasite phylogenetics reveals patterns and processes of host-switching between non-native and native captive freshwater turtles. *Parasitology* 138:1778-1792
- Vitousek PM (1994) Beyond global warming: ecology and global change. *Ecology* 75:1861-1876
- Vitousek PM, Antonio CM, Loope LL, et al. (1996) Biological invasions as global environmental change. *American Scientist* 84:468
- Vitousek PM, D'antonio CM, Loope LL, et al. (1997a) Introduced species: a significant component of human-caused global change. *New Zealand Journal of Ecology*:1-16
- Vitousek PM, Mooney HA, Lubchenco J, et al. (1997b) Human domination of Earth's ecosystems. *Science* 277:494-499

- Wang S, Sun H, Ang HM, et al. (2013) Adsorptive remediation of environmental pollutants using novel graphene-based nanomaterials. *Chemical Engineering Journal* 226:336-347
- Wikelski M, Foufopoulos J, Vargas H, et al. (2004) Galápagos birds and diseases: invasive pathogens as threats for island species. *Ecology and Society* 9:5

**CHAPITRE II. INTRODUCTION AND INVASION OF  
THE RED-EARED SLIDER AND ITS PARASITES IN  
FRESHWATER ECOSYSTEMS OF SOUTHERN EUROPE:  
RISK ASSESSMENT FOR THE EUROPEAN POND  
TURTLE IN WILD ENVIRONMENTS**



## Présentation du Chapitre II

Les invasions parasitaires peuvent affecter la dynamique des populations des espèces hôtes (Peeler et al. 2011; Lymbery et al. 2014). En France, des transferts de parasites ont été illustrés en captivité de tortues américaines des genres *Trachemys*, *Chrysemys* and *Graptemys*, vers les espèces de tortues d'eau douces naturelles, c'est-à-dire la cistude d'Europe et l'émyde lépreuse (Verneau et al. 2011). Les parasites utilisés dans cette étude en tant que modèles sont les polystomes (Plathelminthes, Monogènes). Parce qu'ils présentent un cycle de vie direct sans hôtes intermédiaires, ils constituent un bon modèle pour illustrer des transferts de parasites chez de nouveaux hôtes. Devant ce constat et parce que *T. s. elegans* se rencontre de nos jours en France dans les milieux naturels, et notamment dans le sud, Meyer et al. (2015) ont conduit une étude visant à inventorier la diversité parasitaire de l'émyde lépreuse en Europe du sud. En effet, cette espèce native partage son biotope naturel avec la tortue de Floride à tempes rouges dans de nombreuses zones humides en France et en Espagne. Ces auteurs ont mis en évidence des transferts de parasites des tortues invasives vers l'espèce naturelle, menaçant potentiellement les populations sauvages.

L'émyde lépreuse et la cistude d'Europe bénéficie d'un statut de protection en France, ce qui signifie que ces deux espèces de tortues patrimoniales pourraient être rapidement menacées en cas d'invasions de parasites exotiques. Tout comme *M. leprosa*, *E. orbicularis* évolue dans des environnements naturels au sein desquels *T. s. elegans* est également répertoriée en France et en Espagne. Devant les résultats obtenus par Meyer et al. (2015) concernant l'émyde lépreuse, cette étude propose la réalisation d'un inventaire de la diversité parasitaire de la cistude d'Europe, en France, en Espagne et en Afrique du Nord, afin de mettre en évidence des transferts de parasites exotiques dans un contexte de conservation des espèces natives face à aux invasions biologiques.

## Title

Introduction and invasion of the red-eared slider and its parasites in freshwater ecosystems of Southern Europe: risk assessment for the European pond turtle in wild environments

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## **Abstract**

The North American red-eared slider *Trachemys scripta elegans*, which is nowadays considered among the world's worst invasive species, could constitute a real threat to native freshwater turtles. Because this species shares the same habitats of the European pond turtle *Emys orbicularis* since its introduction in the European wetlands, we surveyed populations of both alien and indigenous species in France and Spain in order to determine the diversity of platyhelminth parasites (Monogenea, Polystomatidae) in natural environments and to evaluate the levels of risks associated with parasitism. DNA barcoding procedure based on partial cytochrome c oxidase I sequences revealed ten monogenean parasite species within *E. orbicularis* populations and/or captive animals among which nine species could be considered as introduced parasites from American freshwater turtles. Results indicated that host switching could have occurred either in natural environments, or in captivity, when indigenous turtles were kept with exotic species, demonstrating that captive turtles could act as reservoirs of parasites. The presence of non-native parasite species within wild populations of *E. orbicularis* in the European freshwater ecosystems also highlighted the risks that these parasites may pose on the survival of natural turtle's populations and on the dynamics of native parasites.

## **Keywords**

Freshwater ecosystems – Biological Invasions – DNA barcoding – Parasites – *Emys orbicularis* – *Trachemys scripta elegans*

## Introduction

While climatic and geological shifts have been largely invoked to explain historical extinctions, the current sixth extinction wave, which derives from anthropogenic actions (Chapin III et al. 2000), is considered as the most rapid and devastating (Ceballos et al. 2010; Barnosky et al. 2011). Human imprint on Earth is actually so profound that we are now facing global environmental changes affecting, among others, landscape structure and interactions between species within terrestrial and aquatic ecosystems (Vitousek 1994; Vitousek et al. 1996, 1997a, 1997b; Mack and D'Antonio 1998; Sala et al. 2000; Steffen et al. 2011). Biological invasions, for instance, can lead to direct competition between alien and native species causing strong negative effects in the latter ones (Daszak et al. 2000; Mooney and Cleland 2001; Pimentel et al. 2005; Simberloff 2013). Parasites, which can likewise be introduced along with their hosts, may also affect ecological interactions and population dynamics of indigenous species (Gozlan et al. 2005; Peeler et al. 2011; Barry et al. 2014; LyMBERY et al. 2014), following a process defined as “spillover effect” (Kelly et al. 2009). Nonetheless exotic species, when introduced in their new geographical range, can lose their parasites (Torchin et al. 2003). In that specific case, invasive host species can be infected with new species, then constituting host reservoirs for parasite transmission (Poulin and Mouillot 2003). This process, which is referred as “spillback effect”, can also impact native host-parasite interactions (Kelly et al. 2009; LyMBERY et al. 2014; Sherrard-Smith et al. 2014). As a consequence, regardless the processes behind the proliferation of non-native taxa outside their home range, successful invasions always result in global loss of biodiversity (Clavero and Garcia-Berthou 2005; Hulme 2007; Butchart et al. 2010; Clavero et al. 2009).

The red-eared slider, *Trachemys scripta elegans*, is a freshwater turtle native of the Eastern United States and northern parts of Mexico (Van Dijk et al. 2014). It was exported massively in the 1980's and 1990's from the USA to Europe to be sold as pets (Lutz 2000; Telecki 2001). Because this terrapin grows rapidly and becomes less attractive as adult, owners often released these unwanted animals in natural environments where they reproduced and established feral populations, as was the case in Italy (Ficetola et al. 2002, 2009; Fattizzo 2004) and in Southern France (Servan and Arvy 1997; Arvy and Servan 1998; Gendre et al. 2013; Meyer et al. 2015; present study). It is nowadays considered among the top 100 of the world's worst invasive alien species (Lowe et al. 2000). Given the biological and ecological characteristics of the red-eared slider, such as fecundity (Arvy and Servan 1998), feeding habits (Prévot-Julliard et al. 2007) and ability to spread (Nishisawa et al. 2014), this species could constitute a real threat to native turtles in the wild. It has been shown under experimental conditions that *T. s. elegans* could indeed compete with the European pond turtle *Emys orbicularis* for basking

sites (Cadi and Joly 2003) and food resources (Cadi and Joly 2004), as well as with the Mediterranean pond turtle *Mauremys leprosa*, for basking, feeding and use of space (Polo-Cavia et al. 2008, 2009a, 2009b, 2010, 2011, 2012). Besides the introduction of *T. s. elegans* in European freshwater ecosystems, some of its parasites were also reported among terrapins of Southern Europe (Verneau et al. 2011; Iglesias et al. 2015; Meyer et al. 2015). Regarding the distribution of the red-eared slider in Europe, Meyer et al. (2015) conducted a parasitological survey among distinct populations of the Mediterranean pond turtle in Southern France and Northern Spain to investigate parasite invasion. Several cases of host switching from *T. s. elegans* to *M. leprosa* in natural environments were documented for platyhelminth monogeneans of the family Polystomatidae, which were usually assumed to be host- and site-specific (see Verneau 2004). Although nothing was reported about the pathogenicity of polystomes, their presence could denote an additional and important threat for chelonian species. Indeed, it was suggested that the transmission of exotic parasites could explain the population decline of native turtle species, such as the western pond turtle *Actinemys marmorata* in North America following transmission of viruses or mycoplasmas (Hays et al. 1999), and also the European pond turtle in Spain following transmission of spirorchiids (Iglesias et al. 2015).

The European pond turtle is a flagship species of the European natural heritage. It has a home range of about 12 millions square kilometers, extending from Atlantic coasts of North-Africa in the West to Aral Sea in the East, and from Moscow region on the north to Turkey in the south (see Fritz et al. 2009). Despite an overall decline of populations due to ancestral rituals, such as its consumption from prehistoric times to the middle of the XIX<sup>e</sup> century (Cheylan and Courtin 1976; Cheylan 1998; Cheylan and Poitevin 2003) and its use in pharmacology in the XIX<sup>e</sup> century (see Cheylan 1998), populations of *E. orbicularis* still exist in France, especially in central, south-eastern and south-western parts of the country. Based on geographical distribution and population's size, it was for a long time considered near-threatened in France (UICN France, MNHN & SHF 2009), as in the Mediterranean basin (Cox et al. 2006) and in Europe (Cox and Temple 2009). It is now listed as "Least Concern" (UICN France, MNHN & SHF 2015). In Spain, however, the species is still considered as "Vulnerable" according to the National Red Book (Pleguezuelos et al. 2002). Because *T. s. elegans* now shares the same habitats as *E. orbicularis* through its geographical range, we may expect that polystomes of the red-eared slider also switched to the indigenous turtle. Therefore, the risk of extinction of *E. orbicularis* could be higher than believed if no conservation measures are planned in the near future, regarding particularly management and disposal of invasive species. In this study, we surveyed mainly populations of the European

pond turtle and the red-eared slider in Southern Europe in order to (i) determine the diversity of platyhelminth parasites and to (ii) evaluate the levels of risks associated with parasitism.

## **Materials and methods**

### **Host sampling**

Along this study, four freshwater turtle species were investigated, i.e. *E. orbicularis*, *T. s. elegans*, *M. leprosa* and *C. picta*. We studied mainly aquatic environments where *E. orbicularis* and *T. s. elegans* had been previously observed, sometimes in sympatry, and, occasionally, habitats that showed ecological characteristics suitable for both species. Fieldwork investigations were conducted from 2010 to 2015 during the spring and summer seasons in wetlands of France, Spain and Algeria (Fig. 1 and Table 1). We also sampled *E. orbicularis* in two natural areas of France where it was reintroduced and in turtle farms where it was kept with *T. s. elegans* for exposition purposes. Turtles were captured using catfish, floating crayfish or turtles traps set in pools, ponds, lakes or rivers for periods extending from one day to two weeks. Traps were firmly attached with a rope to the shore or vegetation, baited with fish or pork liver and set in such a way that trapped animals were able to breathe. Traps were checked daily and collected turtles were marked with marginal cuts on the carapace (Cagle 1939), following standard operating procedures for Capture-Mark-Recapture (CMR) of chelonians in France, or the code of Holland (1991) in the Ebro basin in Spain. Some turtles were placed individually for two to three days in small containers filled with about 25mm of water for polystome eggs investigation. Two other species, i.e. *M. leprosa* in Morocco and Algeria and the painted turtle *Chrysemys picta* in the USA, were also surveyed in their natural home range for their parasites (see Table 1). Turtles were finally released at the exact place of capture, with the exception of red-eared sliders that were euthanized according to the French national rules for invasive species and several specimens of *E. orbicularis* and *M. leprosa* on the one hand and *C. picta* on the other, that were collected in Algeria and in the USA, respectively.

### **Parasite sampling**

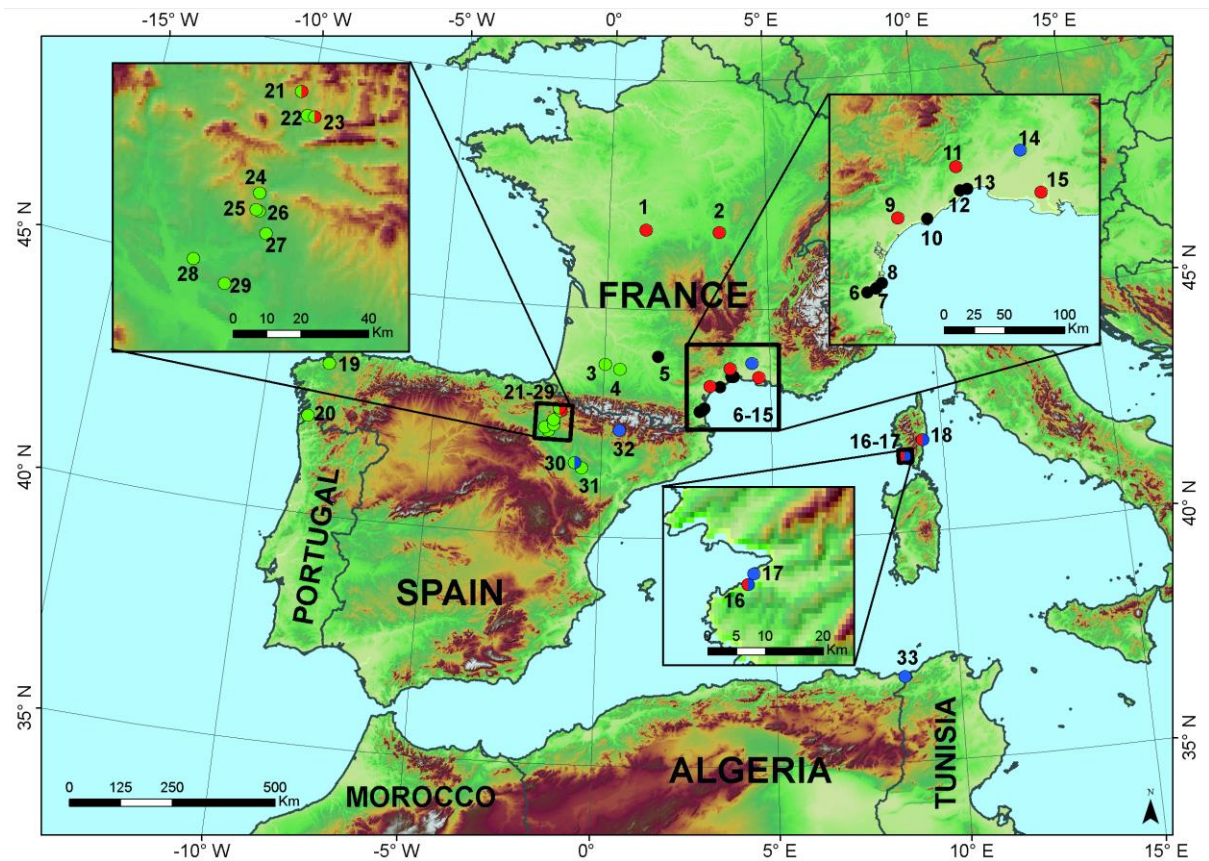
Because *E. orbicularis* is a protected species in France and Spain, we used a non-destructive approach to collect polystome eggs from alive specimens (Verneau et al. 2011; Meyer et al. 2015). The water of tanks in which turtles were kept was filtered using two sieves of 500 and 100 microns, respectively. Debris retained by the second sieve were observed under a dissecting microscope and, when present, polystome eggs of size of about 120-150 microns were isolated and preserved in 70% molecular grade ethanol until use for DNA extraction.

Table 1. List of the freshwater environments where turtles were surveyed along this study were found infected with polystomes. Numbered localities (1 to 33) are shown in Figure 1. Abbreviations used: Al = Algeria; Fr = France; Mo = Morocco; Sp = Spain; USA = United States of America; E. o. = *Emys orbicularis*; M. l. = *Mauremys leprosa*; T. s. = *Trachemys scripta*; C. p. = *Chrysemys picta*. Asterisks indicate sites where strong evidence exists about introduction, exposition or accidental release of indigenous turtle species.

Geographic area	GPS coordinates	Species
1. Ponds of the Natural Regional Park of La Brenne, Rosnay (Fr)	46°43'N / 1°10'E	E. o.
2. Pond of Grands Georgats, Lucenay-lès-Aix (Fr)	46°41'N / 3°29'E	E. o.
3. Ponds of Soulès, Cravencères (Fr)	43°46'N / 0°01'E	E. o.
4. Pond of En Cassagne, Ordan-Larroque (Fr)	43°40'N / 0°28'E	E. o.
5. Private owner at « Les Curbilles », Sauziere Saint Jean (Fr)*	43°58'N / 1°37'E	E. o., M. l. and T. s.
6. Agly river, Rivesaltes (Fr)	42°46'N / 2°53'E	E. o. and T. s.
7. Small canal close of the lagoon of Salses-Leucate, Saint-Hippolyte (Fr)	42°48'N / 2°58'E	E. o.
8. Pond of the pine forest, Port Leucate (Fr)	42°50'N / 3°02'E	E. o.
9. Private owner, Béziers (Fr)*	43°19'N / 3°11'E	E. o. and M. l.
10. Reserve of Bagnas, Agde (Fr)*	43°19'N / 3°30'E	E. o.
11. Ponds of Roquet, Saint-Gély-du-Fesc (Fr)	43°42'N / 3°47'E	E. o.
12. Reserve of Estagnol, Villeneuve-lès-Maguelone (Fr)*	43°31'N / 3°50'E	E. o.
13. Lake Méjean, Lattes (Fr)	43°32'N / 3°54'E	E. o.
14. Wastewater clarifier, Manduel (Fr)*	43°49'N / 4°27'E	E. o.
15. Wetlands of La Tour du Valat, Arles (Fr)	43°30'N / 4°40'E	E. o.
16. Rizzanese river, Portigliolo, Corsica (Fr)	41°38'N / 8°52'E	E. o.
17. Rizzanese river, Propiano, Corsica (Fr)	41°39'N / 8°52'E	E. o.
18. Pond of Gradugine, Prunelli-di-Fiumorbo, Corsica (Fr)	41°58'N / 9°25'E	E. o.
19. Betanzos, Galice (Sp)*	43°16'N / 8°12'W	E. o.
20. Porriño, Galice (Sp)	42° 05'N / 8°37'W	E. o.



21. Sastoya, Navarre (Sp)	42°46'N / 1°17'W	E. o.
22. Areta River, Arieiz, Navarre (Sp)	42°42'N / 1°16'W	E. o.
23. Areta River, Berroya, Navarre (Sp)	42°42'N / 1°14'W	E. o.
24. Gallipienzo, Navarre (Sp)	42°30'N / 1°26'W	E. o.
25. Ujué (1), Navarre (Sp)	42°28'N / 1°27'W	E. o.
26. Ujué (2), Navarre (Sp)	42°27'N / 1°26'W	E. o.
27. Murillo El Fruto, Navarre (Sp)	42°24'N / 1°25'W	E. o.
28. Caparroso, Navarre (Sp)	42°20'N / 1°41'W	E. o.
29. Bardenas, Navarre (Sp)	42°16'N / 1°34'W	E. o.
30. La Alfranca, Aragon (Sp)	41°36'N / 0°45'W	E. o.
31. Pina de Ebro, Aragon (Sp)	41°29'N / 0°33'W	E. o.
32. Estanya, Huesca (Sp)	42°1'N / 0°31'E	E. o.
33. Lake Oubeira, El Kala (Al)	36°51'N / 8°23'E	E. o.
Ornamental pond, Barbotan les Thermes (Fr)	43°57'N / 0°2'W	T. s.
Têt river, Bompas (Fr)	42°42'N / 2°56'E	T. s.
Fosseille river, Cabestany (Fr)	42°40'N / 2°57'E	T. s.
Lake of Salagou, Clermont-l'Hérault (Fr)	43°38'N / 3°21'E	T. s.
Pond of Or, Mauguio (Fr)	43°34'N / 4°00'E	T. s.
Turtle's farm « La vallée des tortues », Sorède (Fr)	42°30'N / 2°57'E	T. s.
Le Pigné, Ceyras (Fr)*	43°38'N / 3°27'E	M. l.
Oued Rhimel, Constantine (Al)	36°28'N / 6°26'E	M. l.
Pool of water, El Amra (Al)	36°21'N / 1°51'E	M. l.
Lake Tonga, El Kala (Al)	36°51'N / 8°30'E	M. l.
Oued Chlef, Rouina (Al)	36°15'N / 1°49'E	M. l.
Oum Er-Rbia river, Ahl Souss (Mo)	32°25'N / 6°30'W	M. l.
Tamesna dam, Ben Ahmed (Mo)	33°12'N / 7°05'W	M. l.
Connecticut (USA)	41°48'N / 72°05'W	C. p.
New York (USA)	41°27'N / 73°55'W	C. p.
North Carolina (USA)	35°30'N / 80°51'W	C. p.



**Fig. 1** Sampling localities of *E. orbicularis* in France, Spain, Algeria (See Table 1 for GPS coordinates and numbering). Blue, green and red circles indicate the occurrence of *P. ocellatum*, *Polystomoides* sp7 and *P. oris*, respectively. Black circles refer to some other polystomes (See Table 2 for species found).

Since polystomes may infect internal cavities, such as the conjunctival sacs, bladder or pharyngeal cavity of the same host, and because they are site-specific, parasite species recognition and infestation site were inferred from both DNA sequencing of the partial cytochrome c oxidase I (COI) gene and the shape of polystome eggs (see Verneau et al. 2011). We also removed, when possible, polystome adults from the pharyngeal cavity of *E. orbicularis* using long buccal swabs (see Valdeón et al. 2013). Finally, we extracted a few adult worms following the dissection of the bladder and pharyngeal cavity of three specimens of *E. orbicularis* and six of *M. leprosa* that were sampled in five distinct areas of Algeria and five specimens of *C. picta* that were collected in four distinct areas of the USA. Worms were also preserved in 70% molecular grade ethanol until use in molecular biology.

### **DNA extraction, amplification and sequencing**

Polystome eggs and adults were removed from ethanol, lyophilized with a centrifugal evaporator (Universal Vacuum System Plus UVS400A) to eliminate all traces of alcohol and then crushed with the help of a pestle. About 150µl of Chelex 10% and 20µl of proteinase K at 10mg.mL<sup>-1</sup> were added for tissue digestion, which was performed at 55°C for one hour. Enzymatic reaction was stopped at 100°C for 15min and DNA extracts were stored at 4°C. Amplification of the COI was conducted by Polymerase Chain Reaction (PCR) using the forward L-CO1p (5'-TTTTTGGGCATCCTGAGGTTTAT-3') and the reverse H-Cox1p2 (5'-TAAAGAAAGAACATAATGAAAATG-3') primers (Littlewood et al. 1997). When PCR failed to work, another reverse primer HCOX1R (5'-AACAAACAACCAAGAATCATG-3') (Meyer et al. 2015) was used in combination with L-CO1p. The amplification procedure started with an initial denaturation step of 5min at 95°C, followed by 30 cycles of 1min at 94°C for denaturation, 1min at 48°C for annealing and 2min at 72°C for elongation. A final step of 10min at 72°C was done for terminal elongation. Success of PCR was checked in 1% agarose gels that were stained with ethidium bromide. Amplifications were repeated twice for each sample and PCR products, of about 360bp, were sent to the Genoscreen Company (Lille, France) for purification and sequencing, which was completed with both forward and reverse amplification primers.

### **Phylogenetic analyses**

Chromatograms were first visually inspected using the software SeqScape v2.5 (Applied BioSystems). Edited sequences were subsequently exported and aligned with chelonian polystome sequences retrieved from Verneau et al. (2011) and Meyer et al. (2015) using Clustal W (Thompson et al. 1994) implemented in MEGA6 (Tamura et al. 2013). Two extra sequences corresponding to polystome species of amphibians, namely *Wetapolystoma almae* and *Polystoma naevius*, were used as outgroups. A Minimum Evolution tree was first constructed using MEGA6 to bring out identical sequences and integrate them into unique haplotypes for deciphering their phylogenetic relationships. A GTR + I +  $\Gamma$  model was then selected by the Akaike Information Criterion with Modeltest 3.06 (Posada and Crandall 1998). Three partitions were defined according to codon positions 1, 2 and 3, and model parameters were estimated independently for each partition. A Bayesian analysis was finally run using MrBayes software (Huelsenbeck and Ronquist 2001), with four chains of ten million generations each, sampled every 100 cycles. Bayesian posterior probabilities were estimated after removing the first 10000 trees as the burn-in-phase.

### **Genetic divergence threshold for species identification**

Our strategy for species identification involved a DNA barcoding procedure based on the comparison and divergences of mitochondrial COI sequences. We therefore used COI sequences of three polystome species to delimit the genetic divergence threshold following the procedure developed by Ferri et al. (2009). These species, which are morphologically well characterized, were *Polystomoides oris* of the pharyngeal cavity of *C. picta*, *Polystomoides tunisiensis* and *Neopolystoma euzeti* of the pharyngeal cavity and the bladder of *M. leprosa*, respectively. Parasites were collected from their specific hosts sampled in their home range and further processed for COI as described above. Intra and interspecific Kimura 2-parameters (K2P) distances and their standard relative errors were calculated with MEGA6. The cumulative error plot based on intra and interspecific K2P corrected distances was then obtained from R 3.1.3 (R Development Core Team 2015) to determine the genetic divergence optimum threshold within polystomes. Subsequently all haplotypes obtained from the sequencing of polystome eggs or adult worms examined during this study were assigned to known or undescribed species according to that optimum threshold.

## **Results**

### **Polystome sampling**

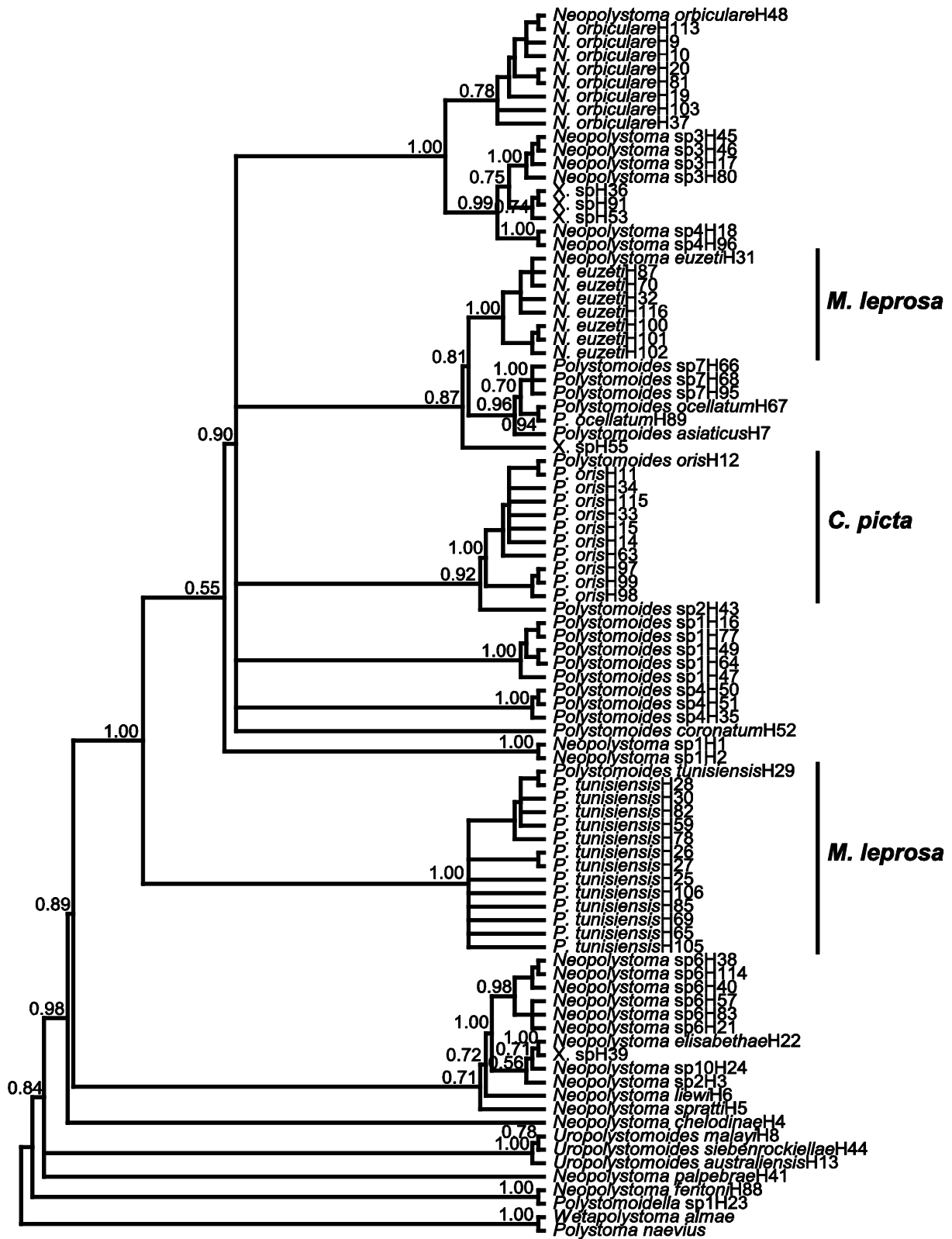
It has been noticed that infected turtles do not necessarily release polystome eggs. Parasite eggs production depends, among other factors, on the physiological conditions of the chelonian host, which may vary according to the seasonal period and environmental temperatures (see Meyer 2014). Because our strategy of polystome sampling mainly involved the collection of polystome eggs, we may have sometimes missed some infected turtles. For this reason, we only reported areas where infected turtles were collected: (i) Polystomes of *E. orbicularis* were detected from fifteen natural water bodies, in one artificial water area and in outdoor turtle enclosures of two turtle farms of France, from 13 natural water bodies in Spain and another area where one specimen was likely released and from a single location in Algeria (Table 1); (ii) Polystomes of *T. s. elegans* were detected from five natural water bodies, in one artificial water area and in outdoor turtle enclosures of two turtle farms of France (Table 1); (iii) Polystomes of *M. leprosa* were detected from outdoor turtle enclosures in France and another location where one specimen was likely released, from four natural water bodies in Algeria and from two others in Morocco (Table 1); (iv) Polystomes of *C. picta* were detected from three natural water bodies in the USA (Table 1).

### **COI haplotype diversity within polystomes**

The 394 new sequences that were obtained during this study were deposited in GenBank under accession numbers XXX to YYY (see Table S1 in Supplemental Data): (i) 208 sequences were from parasite eggs or adult worms collected from *E. orbicularis*, among which 110, 88 and three were respectively from parasites infecting the European pond turtle within the French, Spanish and Algerian freshwater ecosystems, and seven from parasites infecting captive or presumably released turtles; (ii) 143 sequences were from polystome eggs collected from the red-eared slider, among which 136 were from parasites infecting *T. s. elegans* within the French freshwater ecosystems and seven from parasites infecting captive animals; (iii) 16 sequences were from polystome eggs or adult worms collected from *C. picta* sampled in natural areas of the USA; (iv) 27 sequences were from polystome eggs or adult worms collected from *M. leprosa*, among which 22 were from parasites infecting *M. leprosa* within wetlands of Algeria and Morocco and five from parasites infecting captive or presumably released animals. All these sequences, added to the sequences extracted from Genbank, i.e. 30 from polystomes infecting *E. orbicularis*, 66 from polystomes infecting *T. scripta*, 262 from polystomes infecting *M. leprosa*, nine from polystomes infecting *C. picta* and 17 from polystomes infecting other chelonian species sampled from diverse parts of the world, allowed to classify them in 90 distinct haplotypes, among which 20 characterized specimens of *E. orbicularis* with haplotypes H14 to H22, H36, H53, H63, H66 to H68, H89, H91, H95, H96 and H113, 23 characterized specimens of *T. scripta* with haplotypes H15 to H18, H20, H21, H35, H37, H45 to H51, H53, H55, H64, H77, H81, H91, H114 and H115, 43 characterized specimens of *M. leprosa* with haplotypes H14 to H22, H25 to H40, H57, H59, H65, H69, H70, H78, H80, H82, H83, H85 to H87, H100 to H102, H105, H106 and H116 and nine characterized specimens of *C. picta* with haplotypes H9 to H12, H22, H97 to H99 and H103. The Bayesian tree (Fig. 2) depicts the phylogenetic relationships between 89 of the 90 haplotypes; H86 was indeed excluded because its sequence contained several undetermined nucleotides.

### **Genetic divergence threshold within polystomes**

Intra and interspecific genetic divergences were estimated from five haplotypes characterizing specimens of *P. oris* infecting *C. picta* of the USA (H11, H12 and H97 to H99), from 11 haplotypes characterizing specimens of *P. tunisiensis* infecting *M. leprosa* from France (H26 and H30), Spain (H59, H78 and H82), Algeria (H25, H69, H85, H105 and H106) and Morocco (H65) and from seven haplotypes characterizing specimens of *N. euzeti* infecting *M. leprosa* from France (H32), Spain (H31 and H32), Algeria (H70 and H87) and Morocco (H100 to H102). Haplotypes characterizing parasites infecting only captive turtles (H27 to H29 and H116) were excluded



**Fig. 2** Bayesian tree inferred from the analysis of 89 haplotypes characterizing chelonian polystomes. Values along branches indicate Bayesian Posterior Probabilities. *Mauremys leprosa* and *C. picta* correspond to the host species from which parasites, namely *N. euzeti*, *P. tunisiensis* and *P. oris*, were used for the determination of the genetic divergence threshold.

from analyses, to the exception of H65 that characterized a specimen infecting a turtle presumably released in the city of Ceyras in France, but also specimens infecting wild turtles in Morocco. The cumulative error plot of K2P distances is given in Fig. 3. It showed a large gap between intra and interspecific genetic divergences because the three selected polystome species were phylogenetically quite distant from each other. If we assume an optimum threshold of 3.4% of genetic divergence, then the probability of committing type I errors is less than 0.05 for co-specific specimens (see Ferri et al. 2009).

### **Polystome diversity within *E. orbicularis***

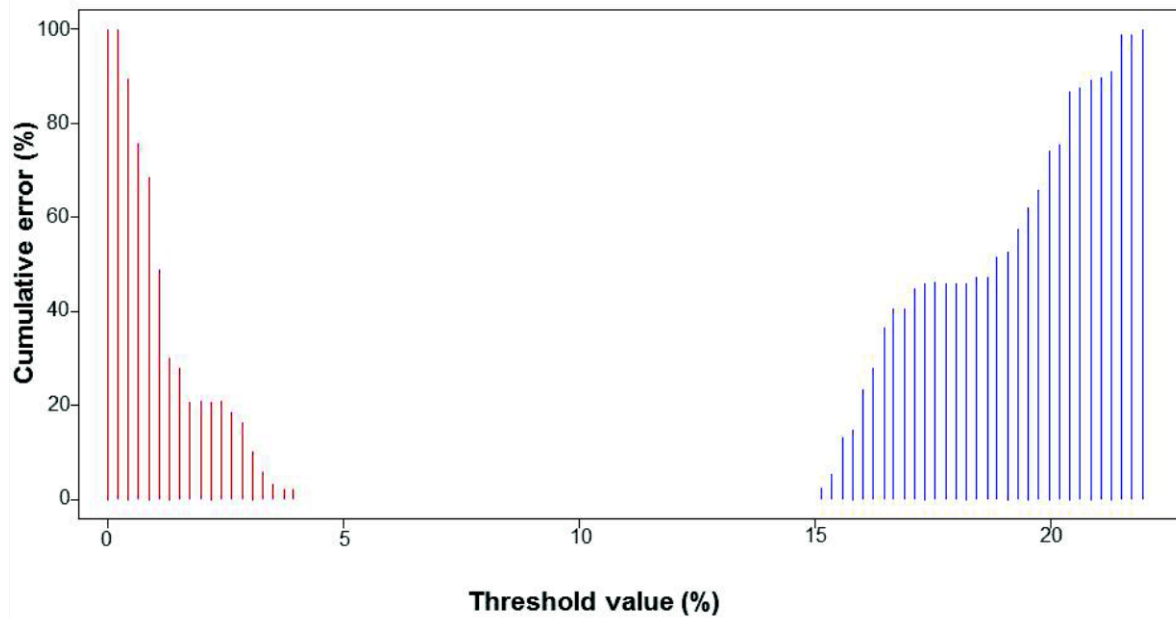
Haplotypes nested into particular monophyletic groups (see Fig. 2) and differing from each other by less than 3.4% of genetic divergence were collapsed into a single species (Fig. 4). As a result, six distinct polystome species within wild *E. orbicularis* could be here considered (See Table 2 and Fig. 4), some of them being able to infest several host species:

(i) *Polystomoides ocellatum* (see clade A, fig. 4), with two distinct haplotypes, was found in one location of Algeria (H89), in three areas of Corsica (France, H67), in two localities of Spain (H67) and in one artificial site (H67). That species, which infests the pharyngeal cavity, was the first polystome species being reported and described from *E. orbicularis* (Rudolphi 1819).

(ii) *Polystomoides* sp7 (see clade A, fig. 4), with three distinct haplotypes, was found in two locations of France (H68), in 12 areas of Spain (H66 and H95) and in one non-natural site (H95). It also infests the pharyngeal cavity. *Polystomoides ocellatum* and *Polystomoides* sp7, which are sister species, were never found within chelonian host species other than *E. orbicularis* (Fig. 2).

(iii) *Polystomoides oris* (see clade B, fig. 4), with twelve distinct haplotypes, was reported here from six localities of France (H15 and H63), among which two in Corsica, and in two areas of Spain (H15). It was also documented earlier in outdoor turtle enclosures (H14 and H15) (Verneau et al. 2011). This species, which infests the pharyngeal cavity, is actually the specific parasite of *C. picta* of the USA (Paul 1938). Although *P. oris* has never been found within introduced chelonian species in European freshwater ecosystems, it was reported within *T. s. elegans* in outdoor turtle enclosures (H15 and H115) (Verneau et al. 2011 and present study) and also within *M. leprosa* in the wild (H86) (Meyer et al. 2015) and outdoor turtle enclosures (H14, H15, H33 and H34) (Verneau et al. 2011).

(iv) *Neopolystoma* sp4 (see clade C, fig. 4), with two distinct haplotypes, was reported twice in freshwater ecosystems, i.e. in the locality of Port Leucate in France (H18) (Verneau et al. 2011 and present study) and



**Fig. 3** Cumulative error plot based on intra and interspecific Kimura 2-parameters (K2P) distances estimated from R 3.1.3 (R Core Team 2015). Type I (red) and type II (blue) errors were obtained with different thresholds for sequences of three polystome species.



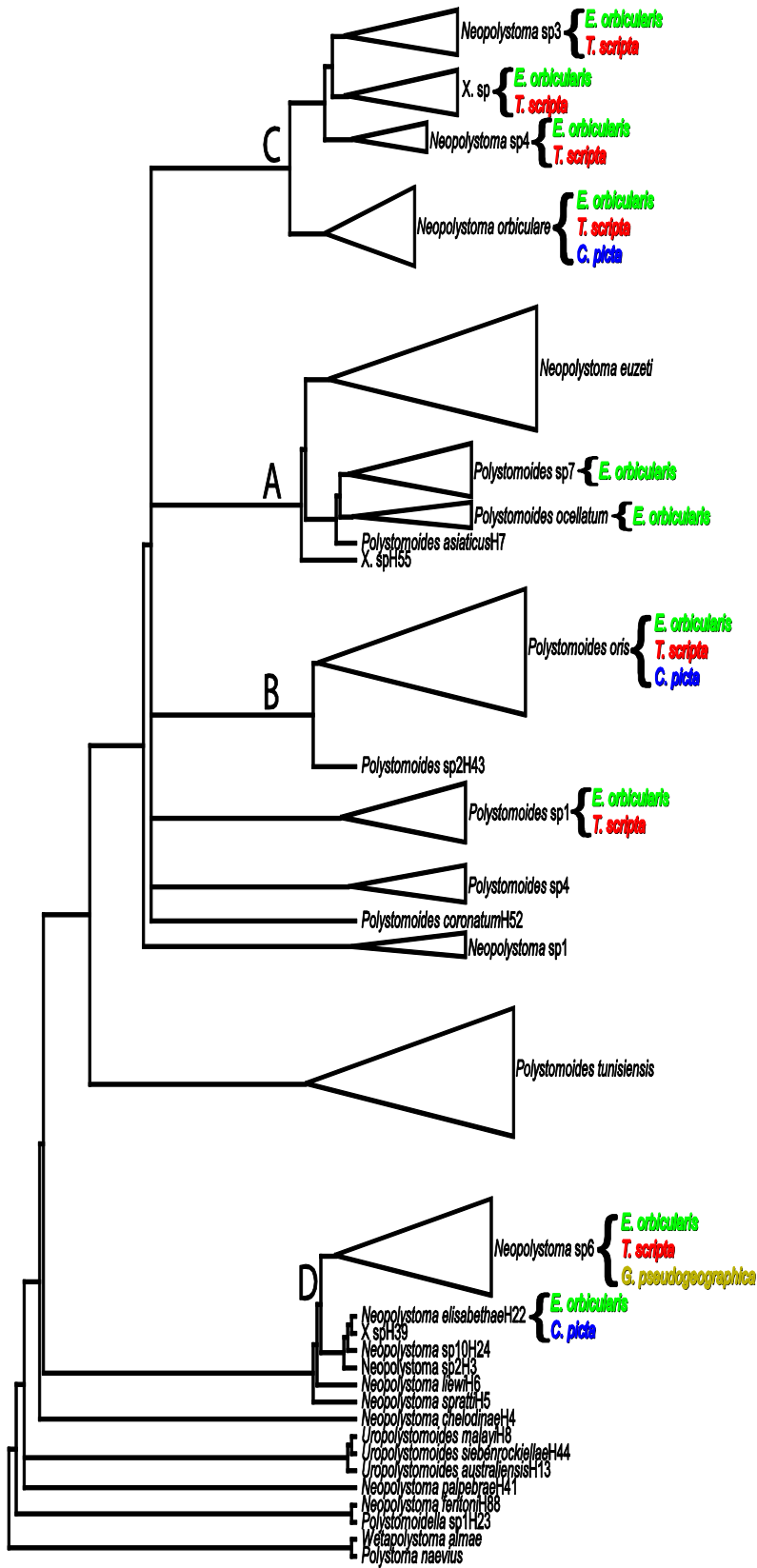


Fig. 4 Species tree derived from figure 2 after collapsing haplotypes characterizing the same polystome species.

A, B, C and D correspond to clades of interest.

Corsica (H18). It was also documented here in an artificial area (H96). This species, which infests the bladder, was also reported within introduced *T. s. elegans* in two natural wetlands of France (H18) (Meyer et al. 2015 and present study) and within *M. leprosa* in natural environments (H18) (Meyer et al. 2015).

(v) *X. sp* (unknown genus; see clade C, fig. 4), with three distinct haplotypes, was found here in five localities of France (H36, H53 and H91), among which two corresponded to water bodies where *E. orbicularis* was reintroduced. No genus name was assigned to this undescribed species as it was actually only characterized from polystome eggs. It has been here also reported within introduced *T. scripta* in two natural French freshwater ecosystems (H53 and H91) and one ornamental pond (H91). It was also documented earlier within *M. leprosa* in outdoor turtle enclosures (H36) (Verneau et al., 2011).

(vi) *Neopolystoma sp6* (see clade D, fig. 4), with six distinct haplotypes, was documented earlier in one locality of France (H21) and in outdoor turtle enclosures (H21) (Verneau et al. 2011). It was found here in another natural area of France (H21). This undescribed species, which infests the conjunctival sacs, could be actually the specific parasite of the Mississippi map turtle *Graptemys pseudogeographica* of the USA (H21) (Verneau et al. 2011). It was here also reported within introduced *T. s. elegans* in one natural French water body (H21) and outdoor turtle enclosures (H114). It was also documented earlier within *M. leprosa* in natural environments (H21, H57 and H83) and outdoor turtle enclosures (H21, H38 and H40) (Verneau et al. 2011; Meyer et al. 2015).

Four other polystome species were found only from captive animals (see Table 2 and Fig. 4):

(vii) *Neopolystoma sp3* (see clade C, fig. 4), with four distinct haplotypes, was reported in outdoor turtle enclosures (H17) (Verneau et al. 2011 and present study). This species, which infests the bladder, was also documented earlier within *T. s. elegans* in freshwater ecosystems of the USA (H45 and H46) (Verneau et al. 2011) and in outdoor turtle enclosures of France (H17) and within *M. leprosa* in wild environments (H17 and H80) (Meyer et al. 2015).

(viii) *Neopolystoma orbiculare* (see clade C, fig. 4), with nine distinct haplotypes, was reported in outdoor turtle enclosures (H19, H20 and H113) (Verneau et al. 2011 and present study). This species was also documented within *T. scripta* in one location of the USA (H48) (Verneau et al. 2011), in two French freshwater ecosystems (H20, H37 and H81) (Meyer et al. 2015 and present study) and in outdoor turtle enclosures (H20) (Verneau et al. 2011). This is the natural species from the bladder of *C. picta* of the USA (Stunkard 1916; Harwood 1931). It was also found earlier within *M. leprosa* in natural environments and outdoor turtle enclosures (H19, H20 and H37) (Verneau et al. 2011; Meyer et al. 2015).

Table 2. COI haplotype diversity of chelonian polystomes collected from *E. orbicularis*, *M. leprosa*, *T. scripta* and *C. picta* in natural freshwater environments and outdoor turtle enclosures. Asterisks indicate localities where sequences were reported either completely (\*) or partially (\*\*) in Verneau et al. (2011) and/or Meyer et al. (2015). Abbreviations used: Al = Algeria; Fr = France; Mo = Morocco; Sp = Spain; USA = United States of America.

Host species	Sampling locality	Number of samples	Type of samples		Number of infected hosts	Haplotype	Parasite species
			Worms	Eggs			
<i>E. orbicularis</i>	Agde (Fr)	1	0	1	1	H36	X. sp.
	Arles (Fr)	8	0	8	3	H15	<i>P. oris</i>
	Béziers (Fr)	4	0	4	3	H14	<i>P. oris</i>
	Cravencères (Fr)	1	0	1	1	H68	<i>Polystomoides</i> sp7
	Lattes (Fr)	2	0	2	2	H53	X. sp.
	Lucenay-lès-Aix (Fr)	1	0	1	1	H15	<i>P. oris</i>
	Manduel (Fr)	3	0	3	2	H96	<i>Neopolystoma</i> sp4
		1	0	1	1	H67	<i>P. ocellatum</i>
	Ordan Larroque (Fr)	4	0	4	3	H68	<i>Polystomoides</i> sp7
	Portigliolo (Fr)	5	0	5	4	H67	<i>P. ocellatum</i>
		5	0	5	5	H15	<i>P. oris</i>
	Port Leucate (Fr)**	27	0	27	13	H18	<i>Neopolystoma</i> sp4
		1	0	1	1	H21	<i>Neopolystoma</i> sp6
	Propriano (Fr)	7	0	7	3	H67	<i>P. ocellatum</i>
	Prunelli-di-Fiumorbo (Fr)	11	0	11	11	H15	<i>P. oris</i>
		2	0	3	2	H96	<i>Neopolystoma</i> sp4
		1	0	1	1	H18	<i>Neopolystoma</i> sp4
		1	0	1	1	H67	<i>P. ocellatum</i>
	Rivesaltes (Fr)	5	0	5	1	H91	X. sp.
	Rosnay (Fr)	6	0	6	4	H15	<i>P. oris</i>

Saint-Gély-du-Fesc (Fr)	16	0	16	6	H63	<i>P. oris</i>
	1	0	1	1	H36	X. sp.
Saint-Hippolyte (Fr)	3	0	3	1	H21	<i>Neopolystoma</i> sp6
Sauziere Saint Jean (Fr)	1	0	1	1	H17	<i>Neopolystoma</i> sp3
	1	0	1	1	H113	<i>N. orbiculare</i>
Sorède (Fr)*	3	0	3	2	H14	<i>P. oris</i>
	1	0	1	1	H15	<i>P. oris</i>
	1	1	0	1	H17	<i>Neopolystoma</i> sp3
	1	1	0	1	H16	<i>Polystomoides</i> sp1
	1	0	1	1	H19	<i>N. orbiculare</i>
	5	0	5	2	H20	<i>N. orbiculare</i>
	1	0	1	1	H21	<i>Neopolystoma</i> sp6
	2	2	0	1	H22	<i>N. elisabethae</i>
Villeneuve-lès-Maguelones (Fr)	12	0	12	6	H36	X. sp.
Arielz (Sp)	1	1	0	1	H66	<i>Polystomoides</i> sp7
Bardenas (Sp)	20	20	0	at least 9	H66	<i>Polystomoides</i> sp7
Betanzos (Sp)	1	1	0	1	H95	<i>Polystomoides</i> sp7
Berroya (Sp)	4	0	4	2	H66	<i>Polystomoides</i> sp7
	3	0	3	3	H15	<i>P. oris</i>
Caparroso (Sp)	1	1	0	1	H66	<i>Polystomoides</i> sp7
Estanya (Sp)	30	30	0	at least 7	H67	<i>P. ocellatum</i>
Gallipienzo (Sp)	1	1	0	1	H66	<i>Polystomoides</i> sp7
La Al Franca (Sp)	2	0	2	2	H67	<i>P. ocellatum</i>
	6	0	6	4	H66	<i>Polystomoides</i> sp7
Murillo El Fruto (Sp)	3	3	0	at least 2	H66	<i>Polystomoides</i> sp7
Pina de Ebro (Sp)	1	1	0	1	H66	<i>Polystomoides</i> sp7
Porriño (Sp)	8	8	0	4	H95	<i>Polystomoides</i> sp7
Sastoya (Sp)	1	0	1	1	H66	<i>Polystomoides</i> sp7
	1	0	1	1	H15	<i>P. oris</i>
Ujué 1 (Sp)	3	3	0	at least 2	H66	<i>Polystomoides</i> sp7
Ujué 2 (Sp)	3	3	0	at least 1	H66	<i>Polystomoides</i> sp7
El Kala (Al)	3	3	0	3	H89	<i>P. ocellatum</i>

<i>M. leprosa</i>	Banyuls/Mer (Fr)*	32	0	32	16	H18	<i>Neopolystoma</i> sp4
		10	0	10	6	H86	<i>P. oris</i>
		4	0	4	4	H21	<i>Neopolystoma</i> sp6
		1	0	1	1	H83	<i>Neopolystoma</i> sp6
	Béziers (Fr)	2	0	2	1	H116	<i>N. euzeti</i>
	Bompas (Fr)*	11	0	11	3	H37	<i>N. orbiculare</i>
		11	0	11	4	H20	<i>N. orbiculare</i>
		2	0	2	1	H16	<i>Polystomoides</i> sp1
		3	0	3	1	H21	<i>Neopolystoma</i> sp6
	Cabestany (Fr)*	13	0	13	8	H37	<i>N. orbiculare</i>
		5	0	5	3	H16	<i>Polystomoides</i> sp1
	Canet (Fr)*	8	0	8	1	H26	<i>P. tunisiensis</i>
	Ceyras (Fr)	1	0	1	1	H65	<i>P. tunisiensis</i>
	Claira (Fr)*	7	0	7	2	H21	<i>Neopolystoma</i> sp6
	Le Boulou (Fr)*	19	0	19	7	H30	<i>P. tunisiensis</i>
		4	0	4	2	H32	<i>N. euzeti</i>
		9	0	9	5	H21	<i>Neopolystoma</i> sp6
	Saint Hippolyte (Fr)*	1	0	1	1	H57	<i>Neopolystoma</i> sp6
		2	0	2	1	H59	<i>P. tunisiensis</i>
	Sauziere Saint Jean (Fr)	1	0	1	1	H14	<i>P. oris</i>
	Sorède (Fr)*	2	0	2	1	H15	<i>P. oris</i>
		1	0	1	1	H33	<i>P. oris</i>
		1	0	1	1	H34	<i>P. oris</i>
		5	0	5	3	H19	<i>N. orbiculare</i>
		4	0	4	4	H20	<i>N. orbiculare</i>
		2	0	2	2	H37	<i>N. orbiculare</i>
		1	0	1	1	H21	<i>Neopolystoma</i> sp6
1		0	1	1	H38	<i>Neopolystoma</i> sp6	
1		0	1	1	H40	<i>Neopolystoma</i> sp6	
1		0	1	1	H22	<i>N. elisabethae</i>	
1		0	1	1	H27	<i>P. tunisiensis</i>	
1		0	1	1	H28	<i>P. tunisiensis</i>	

	1	0	1	1	H29	<i>P. tunisiensis</i>
	2	0	2	1	H32	<i>N. euzeti</i>
	3	0	3	3	H35	<i>Polystomoides</i> sp4
	1	0	1	1	H36	<i>X. sp.</i>
	1	0	1	1	H39	<i>X. sp.</i>
Anyet (Sp)*	8	0	8	7	H78	<i>P. tunisiensis</i>
	2	0	2	1	H59	<i>P. tunisiensis</i>
	1	0	1	1	H82	<i>P. tunisiensis</i>
	11	0	11	4	H17	<i>Neopolystoma</i> sp3
	1	0	1	1	H80	<i>Neopolystoma</i> sp3
La Al Franca (Sp)*	9	0	9	2	H59	<i>P. tunisiensis</i>
Merdanc (Sp)*	2	0	2	1	H59	<i>P. tunisiensis</i>
	1	0	1	1	H82	<i>P. tunisiensis</i>
	2	0	2	1	H31	<i>N. euzeti</i>
Orlina (Sp)*	13	0	13	7	H82	<i>P. tunisiensis</i>
	11	0	11	5	H59	<i>P. tunisiensis</i>
	3	0	3	1	H78	<i>P. tunisiensis</i>
	5	0	5	4	H31	<i>N. euzeti</i>
	8	0	8	3	H32	<i>N. euzeti</i>
	2	0	2	1	H19	<i>N. orbiculare</i>
Constantine (Al)	2	2	0	1	H106	<i>P. tunisiensis</i>
El Amra (Al)**	1	1	0	1	H85	<i>P. tunisiensis</i>
	1	1	0	1	H31	<i>N. euzeti</i>
El Kala (Al)	1	1	0	1	H105	<i>P. tunisiensis</i>
Oued Rhiou (Al)*	6	1	5	1	H25	<i>P. tunisiensis</i>
	1	1	0	1	H31	<i>N. euzeti</i>
Réghaïa (Al)*	1	1	0	1	H70	<i>N. euzeti</i>
Rouina (Al)**	3	3	0	2	H69	<i>P. tunisiensis</i>
	1	1	0	1	H25	<i>P. tunisiensis</i>
	2	2	0	2	H31	<i>N. euzeti</i>
	1	1	0	1	H87	<i>N. euzeti</i>
Ahl Souss (Mo)	2	0	2	1	H100	<i>N. euzeti</i>

<i>T. scripta</i>	Ben Ahmed (Mo)	9	0	9	4	H65	<i>P. tunisiensis</i>
		2	0	2	1	H101	<i>N. euzeti</i>
		2	0	2	1	H102	<i>N. euzeti</i>
	Barbotan les Thermes (Fr)	4	0	4	2	H91	X. sp.
	Bompas (Fr)**	37	0	37	9	H16	<i>Polystomoides</i> sp1
		3	0	3	1	H77	<i>Polystomoides</i> sp1
		4	0	4	2	H20	<i>N. orbiculare</i>
		1	0	1	1	H81	<i>N. orbiculare</i>
	Cabestany (Fr)**	1	0	1	1	H18	<i>Neopolystoma</i> sp4
		78	0	78	21	H16	<i>Polystomoides</i> sp1
		6	0	6	3	H77	<i>Polystomoides</i> sp1
		8	0	8	4	H37	<i>N. orbiculare</i>
	Clermont-l'Hérault (Fr)	8	0	8	2	H64	<i>Polystomoides</i> sp1
	Mauguio (Fr)	1	0	1	1	H53	X. sp.
	Rivesaltes (Fr)	18	0	18	2	H91	X. sp.
		5	0	5	1	H18	<i>Neopolystoma</i> sp4
		1	0	1	1	H21	<i>Neopolystoma</i> sp6
	Sauziere Saint Jean (Fr)	2	0	2	1	H115	<i>P. oris</i>
	Sorède (Ts)**	4	3	1	2	H15	<i>P. oris</i>
		3	1	2	2	H17	<i>Neopolystoma</i> sp3
		10	0	10	5	H35	<i>Polystomoides</i> sp4
		1	0	1	1	H55	X. sp.
		1	0	1	1	H114	<i>Neopolystoma</i> sp6
	5	0	5	2	H20	<i>N. orbiculare</i>	
Florida (USA)*	1	1	0	1	H48	<i>N. orbiculare</i>	
	1	1	0	1	H50	<i>Polystomoides</i> sp4	
	2	2	0	2	H51	<i>Polystomoides</i> sp4	
Indiana (USA)*	1	1	0	1	H45	<i>Neopolystoma</i> sp3	
	1	1	0	1	H47	<i>Polystomoides</i> sp1	
Kansas (USA)*	1	1	0	1	H46	<i>Neopolystoma</i> sp3	
Maine (USA)*	1	1	0	1	H49	<i>Polystomoides</i> sp1	

<i>C. picta</i>	Connecticut (USA)	3	1	2	1	H97	<i>P. oris</i>
		1	0	1	1	H22	<i>N. elizabethae</i>
	Indiana (USA)*	1	1	0	1	H11	<i>P. oris</i>
		1	1	0	1	H12	<i>P. oris</i>
		1	1	0	1	H9	<i>N. orbiculare</i>
		6	6	0	2	H10	<i>N. orbiculare</i>
	New York (USA)	2	1	1	1	H97	<i>P. oris</i>
		2	1	1	2	H98	<i>P. oris</i>
	North Carolina (USA)	4	1	3	1	H98	<i>P. oris</i>
		2	1	1	1	H99	<i>P. oris</i>
		2	2	0	1	H103	<i>N. orbiculare</i>



(ix) *Neopolystoma elizabethae* (see clade D, fig. 4), with one haplotype (H22), was reported earlier from outdoor turtle enclosures (Verneau et al. 2011). This species is actually the specific parasite of the conjunctival sacs of the painted turtle *C. picta* of the USA (Platt 2000). It was also documented earlier within *M. leprosa* in outdoor turtle enclosures (H22) (Verneau et al. 2011).

(x) *Polystomoides* sp1, with five distinct haplotypes, was reported earlier in outdoor turtle enclosures (H16) (Verneau et al. 2011). This species, which infests the pharyngeal cavity, was also documented within *T. scripta* along ecosystems of the USA (H47 and H49) (Verneau et al. 2011), in three French water bodies (H16, H77 and H64) (Meyer et al. 2015 and present study) and within *M. leprosa* in wild environments (H16) (Meyer et al. 2015).

#### **Polystome diversity within *T. scripta***

Besides the parasite species listed above, another species has to date only been found within *T. scripta* and *M. leprosa*. *Polystomoides* sp4, with three distinct haplotypes, was reported earlier from the pharyngeal cavity of *T. scripta* in freshwater ecosystems of the USA (H50 and H51) (Verneau et al. 2011), in outdoor turtle enclosures (H35) and within *M. leprosa* in outdoor turtle enclosures (H35) (Verneau et al. 2011).

### **Discussion**

#### **Polystome species diversity within wild populations of *E. orbicularis***

Chelonian polystomes can be found within three distinct ecological niches, i.e. the pharyngeal cavity, the bladder and the conjunctival sacs of their host. Though distinction between species is based mainly on morphology and morphometry of haptor sclerified parts, host and site specificity was also regarded as a reliable guide to polystome identification (see Verneau 2004; Du Preez and Maritz 2006). Meyer et al. (2015) reported the occurrence of three distinct polystome species from the pharyngeal cavity and four others from the bladder of Mediterranean pond turtles in the wild, among which two were recognized as native and five as exotic, thus suggesting host-switching in Southern European freshwater ecosystems. According to the species tree in Fig. 4, ten distinct polystome species can be found within wild and/or captive *E. orbicularis* among which eight can also parasitize American chelonian species in the USA, in outdoor turtle enclosures and/or in French freshwater ecosystems. Among the two polystome species that were only recorded from *E. orbicularis*, one was regarded as *P. ocellatum* because specimens collected from Algeria had between 30 to 34 genital spines, which was very similar to the genital spine number (29 to 34) reported by Knoepffler and Combes (1977) for *P. ocellatum* of

Corsica, and because *T. s. elegans* had never been documented in North Africa, which rendered host switching very unlikely. On the opposite, the second parasite species, i.e. *Polystomoides* sp7, was considered as a non-native polystome of *E. orbicularis*, although this deserved to be proven. Actually, it could be that *Polystomoides* sp7 has never been observed within alien chelonian species, only because specimens of *T. s. elegans* have not yet been examined for polystomes in the surveyed areas of Spain where it mainly occurs. Indeed, *N. orbiculare*, for instance, which was reported from *M. leprosa* in Spanish Catalonia (Meyer et al. 2015), had until then only been found within *T. s. elegans* in French Catalonia (Meyer et al. 2015) and in the Eastern part of Spain (Domènech et al. 2015) where specimens of the red-eared slider were inspected for parasites.

Seven of the eight other species that were recorded from wild and/or captive European pond turtles, namely *Neopolystoma* sp3, *Neopolystoma* sp4, *Neopolystoma* sp6, *N. orbiculare*, *P. oris*, *Polystomoides* sp1 and *X. sp.*, were also documented in wild and/or captive introduced red-eared sliders and in Mediterranean pond turtles. If there was some evidence about the presence of *T. scripta* in French natural environments in the 1970's (Servan and Arvy 1997), *N. orbiculare* and *P. oris* were described from *C. picta* in the first half of last century (Stunkard 1916; Paul 1938), so well before the chelonian trade from the USA to Europe peaked up in the 1980's and 1990's (Warwick 1991; Lutz 2000; Telecky 2001). These two parasite species can therefore be considered with high confidence as originating from the USA, particularly because any report actually indicates the export of *E. orbicularis* from European countries to the USA. Consequently, these two parasites are likely alien parasite species in French freshwater ecosystems like all other non-specific parasites found within *E. orbicularis*, including *Polystomoides* sp7. The first mention of polystome host switching was by Verneau et al. (2011) from American to both indigenous European turtles in outdoor turtle enclosures. Soon after, Meyer et al. (2015) reported host switching from *T. s. elegans* to *M. leprosa* in natural environments of Southern Europe. To explain the great polystome diversity within the Mediterranean pond turtle, Meyer et al. (2015) argued that parasite transfers could have occurred in the wild when indigenous and invasive turtles shared the same habitats. However they also invoked several non-exclusive hypotheses to explain the presence of alien parasite species within *M. leprosa* when *T. s. elegans* was not recorded from the same water bodies. They suggested that direct transmission might have occurred from the invasive to the native species before alien turtles were removed or have disappeared from natural environments. They also considered that indigenous turtles might have been infected in outdoor turtle enclosures before being released or translocated in the field. Finally, they pointed out that *T. s. elegans* could serve as a carrier for exotic polystomes in natural environments, thus enhancing parasite transmission to native turtles in aquatic ecosystems. Regarding the polystome diversity within *E. orbicularis*,

both in the wild and in outdoor turtle enclosures, the same explanations as suggested for *M. leprosa* may be given. Some host transfers may have occurred directly in natural environments from invasive to native species, as illustrated for the species we named for convenience X. sp. along the Agly River beside Rivesaltes (France), or following the release of indigenous turtles kept in captivity, as illustrated for *Neopolystoma* sp4 and *Neopolystoma* sp6 in the pond of Port Leucate. As a consequence, turtle farms could act as reservoirs of parasites, as exemplified by the presence of no less than six polystome species within *E. orbicularis* and within *T. s. elegans*.

### **Impact of parasite introductions on host and biodiversity**

It is now well documented that chelonians, particularly freshwater turtles, represent one of the most endangered groups of animals (Buhlmann et al. 2009; Böhm et al. 2013). Six main categories of threats, which were reviewed in Gibbons et al. (2000) and Todd et al. (2010), are usually suspected to be associated with reptile declines, such as introduced invasive species (Cadi and Joly 2003; Polo-Cavia et al. 2010; Pearson et al. 2015), disease and parasitism (Stacy et al. 2008; Flint et al. 2009; Jones et al. 2016). Besides usual threats, chelonians may also suffer from new diseases involving non-native parasites that may be translocated in natural environments following invasion of alien host species. Whereas the literature regarding main emerging infectious diseases among wild reptiles, comprising chelonians, is quite abundant (see Schumacher 2006; Gibbons and Steffes 2013), there are very few accounts correlating the presence of invasive parasites and their effects on wild chelonians. The most documented case study concerns the interaction between *E. orbicularis* and *T. s. elegans* in North Western Spain. Iglesias et al. (2015) reported the outbreak of severe spirorchidiasis in *E. orbicularis*, probably resulting from a parasite spillover event after the introduction of *T. s. elegans* in freshwater environments. Although instances of diseases and/or mortalities among freshwater turtles that may be linked to polystome infection have not yet been reported, Henke et al. (1990) showed that aside from the presence of monogenean adults of *N. orbiculare* in the bladder of its host, which is probably the most common polystome in *T. s. elegans*, there were histological lesions associated with the deposition of eggs in the bladder wall. Therefore, we can assume that the presence of non-native polystomes within wild populations of *E. orbicularis* of the European freshwater ecosystems is probably not without consequences for animal health, though Miller (1998) reported no antemortem signs among infected turtles with *N. orbiculare*.

Although it is quite difficult to evaluate the biological disorders caused by the introduction of *T. s. elegans* and its parasites in wetlands of Europe, collection of baseline information on the distribution and

population dynamics of wildlife pathogens should be a priority in order to develop methods for predicting host shifts and evolution of alien pathogens (see Roy 2016). Freshwater turtles are among the most traded reptiles (Masin et al. 2014), especially the red-eared slider that was exported massively from the USA in the 1980's and 1990's (see Telecky 2001; Schlaepfer et al. 2005). *Trachemys s. elegans* has since invaded many new freshwater environments and a negative interaction with European indigeneous freshwater turtles has been well documented (Cadi and Joly 2003, 2004; Polo-Cavia et al. 2008, 2009a, b, 2010, 2011, 2012). However, no study has yet attempted to evaluate its impact on the biodiversity as a whole. When looking at the polystome diversity within wild populations of *E. orbicularis* in Southern European wetlands, six polystome species were clearly identified, among which five at least were supposed to be non-native species. Considering *Polystomoides* sp7 as an alien species, which has not yet been formally demonstrated, the single native polystome species, i.e. *P. ocellatum*, was documentend only in three natural areas of Corsica (France) (symbols 16 to 18 of Fig. 1), in two of Spain (symbols 30 and 32 of Fig. 1), and in another artificial site where turtles were probably released (symbol 14 of Fig. 1). Surprisingly it has never been found within *T. s. elegans*, while some nematodes from *E. orbicularis* and *M. leprosa* were identified in the invasive species in wild environments of Spain (Hidalgo-Vila et al. 2009). *Polystomoides ocellatum* can be hence considered as a rare species in natural European freshwater environments in comparison to the most common parasite species encountered, namely *P. oris* and *Polystomoides* sp7. It is thus alarming to note that the natural polystome species of the European pond turtle could be rapidly lost and replaced by non native parasite species. Because *T. s. elegans* is now distributed almost worldwide, we should be all concerned about the disastrous effects of the naturalization of its parasites within the local herpetofauna. Three alien helminths to Japan were indeed reported in specimens of *T. s. elegans* in Japan (Oi et al. 2012) and, similarly, American polystomes were found within *T. s. elegans* from Eastern Spain (Domènech et al. 2015) and South Africa (unpublished data). If we assume that native polystome species were replaced by alien parasite species in European freshwater ecosystems, which has not yet been established, ecological changes caused by host switching could have profound effects on the biodiversity as a whole, as illustrated in free living organisms following invasion (Novaro et al. 2000; Lambertucci et al. 2009). This issue should be therefore investigated more in depth in natural environments following monitoring of indigenou and exotic turtle species, which would enable the evaluation of native parasite extinctions and then ecosystem transformations.

## References

- Arvy C, Servan J (1998) Iminent competition between *Trachemys scripta* and *Emys orbicularis* in France. *Mertensiella* 10:33-40
- Barnosky AD, Matzke N, Tomiya S, Wogan GOU, Swartz B, Quental TB, Marshall C, McGuire JL, Lindsey EL, Maguire KC, Mersey B, Ferrer EA (2011) Has the Earth's sixth mass extinction already arrived? *Nature* 471:51-57
- Barry J, McLeish J, Dodd JA, Turnbull JF, Boylan P, Adams CE (2014) Introduced parasite *Anguillicola crassus* infection significantly impedes swim bladder function in the European eel *Anguilla anguilla* (L.). *J Fish Dis* 37:921-924
- Böhm M, Collen B, Baillie JEM et al (2013) The conservation status of the world's reptiles. *Biol Conserv* 157:372-385
- Buhlmann KA, Akre TSB, Iverson JB, Karapatakis D, Mittermeier RA, Georges A, Rhodin AGJ, Van Dijk PP, Gibbons JW (2009) A global analysis of tortoise and freshwater turtle distributions with identification of priority conservation areas. *Chelonian Conserv Biol* 8:116-149
- Butchart SHM, Walpole M, Collen B et al (2010) Global Biodiversity: Indicators of Recent Declines. *Science* 328:1164-1168
- Cadi A, Joly P (2003) Competition for basking places between the endangered European pond turtle (*Emys orbicularis galloitalica*) and the introduced red-eared slider (*Trachemys scripta elegans*). *Can J Zool* 81:1392-1398
- Cadi A, Joly P (2004) Impact of the introduction of the red-eared slider (*Trachemys scripta elegans*) on survival rates of the European pond turtle (*Emys orbicularis*). *Biodivers Conserv* 13:2511-2518
- Cagle FR (1939) A system of marking turtles for future identification. *Copeia* 3:170-173
- Ceballos G, García A, Ehrlich PR (2010) The sixth extinction crisis: loss of animal populations and species. *J Cosmol* 8:1821-1831
- Chapin III FS, Zavaleta ES, Eviner VT, Naylor RL, Vitousek PM, Reynolds HL, Hooper DU, Lavorel S, Sala OE, Hobbie SE, Mack MC, Díaz S (2000) Consequences of changing biodiversity. *Nature* 405:234-242
- Cheylan M (1998) Evolution of the distribution of the European pond turtle in the French Mediterranean area since the Post-Glacial. *Mertensiella* 10:47-65
- Cheylan M, Courtin J (1976) La consommation de la tortue cistude *Emys orbicularis* (L.) au post-glaciaire dans la grotte de Fontbregoua (Salernes-Var). *Bull Mus Hist nat Marseille* 36:41-46

- Cheylan M, Poitevin F (2003) Les tortues du site de Lattara (IV<sup>e</sup> s. av. n. è.- II<sup>e</sup> s. de n. è.): Intérêt archéozoologique et biologique. *Lattara* 16:137-145
- Clavero M, Garcíaberthou E (2005) Invasive species are a leading cause of animal extinctions. *Trends Ecol Evol* 20:110-110
- Clavero M, Brotons L, Pons P, Sol D (2009) Prominent role of invasive species in avian biodiversity loss. *Biol Cons* 142:2043-2049
- Cox NA, Chanson J, Stuart S (2006) The status and distribution of reptiles and amphibians of the Mediterranean Basin–IUCN. Gland, Switzerland and Cambridge, UK
- Cox NA, Temple HJ (2009) European red list of reptiles. Office for official publications of the European communities, Luxembourg
- Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife-threats to biodiversity and human health. *Science* 287:443-449
- Domènech F, Marquina R, Soler L, Valls L, Aznar FJ, Fernández M, Navarro P, Lluch J (2015) Helminth fauna of the invasive American red-eared slider *Trachemys scripta* in eastern Spain: potential implications for the conservation of native terrapins. *J Nat Hist* DOI:10.1080/00222933.2015.1062931
- Du Preez LH, Maritz MF (2006) Demonstrating morphometric protocols using polystome marginal hooklet measurements. *Syst Parasitol* 63:1-15
- Fattizzo T (2004) Distribution and conservational problems of *Emys orbicularis* in Salento (South Apulia, Italy). *Biologia* 59:13-18
- Ferri F, Barbuto M, Bain O, Galimberti A, Uni S, Guerrero R, Ferté H, Bandi C, Martin C, Casiraghi M (2009) Integrated taxonomy: traditional approach and DNA barcoding for the identification of filarioid worms and related parasites (Nematoda). *Front Zool* 6:1-12.
- Ficelota GF, Monti A, Padoa-Schioppa E (2002) First record of reproduction of *Trachemys scripta* in the Po Delta. *Ann Mus Civ St Nat Ferrara* 5:125-128
- Ficetola GF, Thuiller W, Padoa-Schioppa E (2009) From introduction to the establishment of alien species: bioclimatic differences between presence and reproduction localities in the slider turtle. *Divers Distrib* 15:108-116
- Flint M, Patterson-Kane JC, Limpus CJ, Work TM, Blair D, Mills PC (2009) Postmortem diagnostic investigation of disease in free-ranging marine turtle populations: a review of common pathologic findings and protocols. *J Vet Diagn Invest* 21:733-759

- Fritz U, Cadi A, Cheylan M, Coïc C, Détaint M, Olivier A, Rosecchi E, Guicking D, Lenk P, Joger U, Wink M (2005) Distribution of mtDNA haplotypes (cyt b) of *Emys orbicularis* in France and implications for postglacial recolonization. *Amphib-Reptil* 26:231-238
- Fritz U, Ayaz D, Hundsdörfer AK, Kotenko T, Guicking D, Wink M, Tok CV, Çiçek K, Buschbom J (2009) Mitochondrial diversity of European pond turtles (*Emys orbicularis*) in Anatolia and the Ponto-Caspian Region: Multiple old refuges, hotspot of extant diversification and critically endangered endemics. *Org Divers Evol* 9:100-114
- Gendre T, Verneau O, Palacios C (2013) Projet « Florida ». Interactions entre une espèce envahissante, la tortue de Floride, et deux tortues patrimoniales en Languedoc-Roussillon, la cistude d'Europe (*Emys orbicularis*) et l'émyde lépreuse (*Mauremys leprosa*). Conservatoire d'espaces naturels Languedoc-Roussillon
- Gibbons JW, Scott DE, Ryan TJ, Buhlmann KA, Tuberville TD, Metts BS, Greene JL, Mills T, Leiden Y, Poppy S, Winne CT (2000) The global decline of reptiles, déjà vu Amphibians. *BioScience* 50:653-666
- Gibbons PM, Steffes ZJ (2013) Emerging infectious diseases of chelonians. *Vet Clin Exot Anim* 16:303-317
- Gozlan R, St-Hilaire S, Feist SW, Martin P, Kent ML (2005) Disease threat to European fish. *Nature* 435:1046
- Harwood PD (1931) Some parasites from Oklahoma turtles. *J Parasitol* 18:98-101
- Hays DW, McAllister KR, Richardson SA, Stinson DW (1999) Washington State recovery plan for the western pond turtle. Wash Dept Fish and Wild, Olympia, Washington
- Henke SE, Pence DB, Rue MT (1990) Urinary bladders of freshwater turtles as a renal physiology model potentially biased by monogenean infections. *Lab Anim Sci* 40:172-177
- Hidalgo-Vila J, Díaz-Paniagua C, Ribas A, Florencio M, Pérez-Santigosa N, Casanova JC (2009) Helminth communities of the exotic introduced turtle, *Trachemys scripta elegans* in southwestern Spain: Transmission from native turtles. *Res Vet Sci* 86:463-465
- Holland DC (1991) A synopsis of the ecology and current status of the western pond turtle (*Clemmys marmorata*) in 1991. Report to USDIFish and Wildlife Service, National Ecology Research Center, San Simeon, California
- Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754-755
- Hulme PE (2007) Biological invasions in Europe: drivers, pressures, states, impacts and responses. *Environ Sci Technol* 25:56-80

- Iglesias R, García-Estévez JM, Ayres C, Acuña A, Cordero-Rivera A (2015) First reported outbreak of severe spirorchiidiasis in *Emys orbicularis*, probably resulting from a parasite spillover event. *Dis Aquat Organ* 113:75-80
- Jones K, Ariel E, Burgess G, Read M (2016) A review of fibropapillomatosis in green turtles (*Chelonia mydas*). *Vet J* 212:48-57
- Kelly DW, Paterson RA, Townsend CR, Poulin R, Tompkins DM (2009) Parasite spillback: A neglected concept in invasion ecology? *Ecology* 90:2047-2056
- Knoepffler L-P, Combes C (1977) Présence en Corse de *Polystomoides ocellatum* (Rudolphi, 1819) chez *Emys orbicularis* (L., 1758) (Chelonia, Emydidae). Considérations sur la répartition mondiale du genre *Polystomoides*. *Vie Milieu* 27:221-230
- Lambertucci SA, Trejo A, Di Martino S, Sánchez-Zapata JA, Donázar JA, Hiraldo F (2009) Spatial and temporal patterns in the diet of the Andean condor: ecological replacement of native fauna by exotic species. *Anim Conserv* 12:338-345.
- Lenk P, Fritz U, Joger U, Wink M (1999) Mitochondrial phylogeography of the European pond turtle, *Emys orbicularis* (Linnaeus 1758). *Mol Ecol* 8:1911-1922
- Littlewood DTJ, Rohde K, Clough KA (1997) Parasite speciation within or between host species? Phylogenetic evidence from site-specific polystome monogeneans. *Int J Parasitol* 27:1289-1297
- Lowe S, Browne M, Boudjelas S, De Poorter M (2000) 100 of the world's worst invasive alien species. A selection from the global invasive species database. Hollands Printing Ltd, New Zealand
- Lutz CG (2000) Pet turtle production. *SRAC Publication (Mississippi State Univ)* 439:1-4
- Lymbery AJ, Morine M, Kanani HG, Beatty SJ, Morgan DL (2014) Co-invaders: The effects of alien parasites on native hosts. *Int J Parasitol Parasites Wildl* 3:171-177
- Mack M, D'Antonio CM (1998) Impacts of biological invasions on disturbance regimes. *TREE* 13:195-198
- Masin S, Bonardi A, Padoa-Schioppa E, Bottoni L, Ficetola GF (2014) Risk of invasion by frequently traded freshwater turtles. *Biol Invasions* 16:217-231
- Meyer (2014) Parasite diversity within native and invasive terrapins: Implications for conservation. Thesis dissertation, North West University and University of Perpignan Via Domitia
- Meyer L, Du Preez L, Bonneau E, Héritier L, Quintana, MF, Valdeón A, Sadaoui A, Kechemir-Issad N, Palacios C, Verneau O (2015) Parasite host-switching from the invasive American red-eared slider, *Trachemys*



- scripta elegans*, to the native Mediterranean pond turtle, *Mauremys leprosa*, in natural environments. *Aquat Invasions* 10:79-91.
- Miller HA (1998) Urinary diseases of reptiles: Pathophysiology and diagnosis. *Semin Avian Exot Pet Med* 7:93-103
- Mooney HA, Cleland E (2001) The evolutionary impact of invasive species. *Proc Natl Acad Sci USA* 98:446-451
- Nishizawa H, Tabata R, Hori T, Mitamura H, Arai N (2014) Feeding kinematics of freshwater turtles: what advantage do invasive species possess? *Zoology* 117:315-318
- Novaro AJ, Funes MC, Walker RS (2000) Ecological extinction of native prey of a carnivore assemblage in Argentine Patagonia. *Biol Conserv* 92:25-33
- Oi M, Araki J, Matsumoto J, Nogami S (2012) Helminth fauna of a turtle species introduced in Japan, the red-eared slider turtle (*Trachemys scripta elegans*). *Res Vet Sci* 93:826-830
- Paul AA (1938) Life history studies of North American freshwater polystomes. *J Parasitol* 24:489-510
- Pearson SH, Avery HW, Spotila JR (2015) Juvenile invasive red-eared slider turtles negatively impact the growth of native turtles: Implications for global freshwater turtle populations. *Biol Conserv* 186:115-121
- Peeler EJ, Oidtmann BC, Midtlyng PJ, Miossec L, Gozlan RE (2011) Non-native aquatic animals introductions have driven disease emergence in Europe. *Biol Invasions* 13:1291-1303
- Pimentel D, Zuniga R, Morrison D (2005) Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecol Econ* 52:273-288
- Platt TR (2000) *Neopolystoma fentoni* n. sp. (Monogenea: Polystomatidae) a parasite of the conjunctival sac of freshwater turtles in Costa Rica. *Mem Inst Oswaldo Cruz* 95:833-837
- Pleguezuelos JM, Márquez R, Lizana M (2002) Atlas y libro rojo de los anfibios y reptiles de España. Dirección General de Conservación de la Naturaleza-Asociación Herpetologica Española (2<sup>nd</sup> printing), Madrid, 587 pp
- Polo-Cavia N, López P, Martin J (2008) Interspecific differences in responses to predation risk may confer competitive advantages to invasive freshwater turtle species. *Ethology* 114:115-123
- Polo-Cavia N, López P, Martin J (2009a) Interspecific differences in chemosensory responses of freshwater turtles: consequences for competition between native and invasive species. *Biol Invasions* 11:431-440
- Polo-Cavia N, López P, Martin J (2009b) Interspecific differences in heat exchange rates may affect competition between introduced and native freshwater turtles. *Biol Invasions* 11:1755-1765

- Polo-Cavia N, López P, Martin J (2010) Competitive interactions during basking between native and invasive freshwater turtle species. *Biol Invasions* 12:2141-2152
- Polo-Cavia N, López P, Martin J (2011) Aggressive interactions during feeding between native and invasive freshwater turtles. *Biol Invasions* 13:1387-1396
- Polo-Cavia N, López P, Martin J (2012) Feeding status and basking requirements of freshwater turtles in an invasion context. *Physiol Behav* 105:1208-1213
- Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14:817-818
- Poulin R, Mouillot D (2003) Host introductions and the geography of parasite taxonomic diversity. *J Biogeogr* 30:837-845
- Prévot-Julliard AC, Gousset E, Archinard C, Cadi A, Girondot M (2007) Pets and invasion risks: is the slider turtle strictly carnivorous? *Amphib-Reptil* 28:139-143
- R Development Core Team (2015) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria
- Roy H (2016) Control wildlife pathogen too. *Nature* 530:281
- Rudolphi CA (1819) Entozoorum synopsis cui accedunt mantissa duplex et indices locupletissimi. Berolini: 811 pp.
- Sala OE, Chapin III FS, Armesto JJ (2000) Global biodiversity scenarios for the year 2100. *Science* 287:1770-1774
- Servan J, Arvy C (1997) Introduction de la tortue de Floride *Trachemys scripta* en France. Un nouveau compétiteur pour les espèces de tortues d'eau douce européennes. *Bull Fr Pêche Piscic* 344/345:173-177
- Schlaepfer MA, Hoover C, Dodd KJR (2005) Challenges in evaluating the impact of the trade in amphibians and reptiles on wild populations. *BioScience* 55:256-264
- Schumacher J (2006) Selected infectious diseases of wild reptiles and amphibians. *J Exot Pet Med* 15:18-24
- Sherrard-Smith E, Elizabeth A, Chadwick A, Cable J (2014) The impact of introduced hosts on parasite transmission: Opisthorchiid infections in American mink (*Neovison vison*). *Biol Invasions* 17:115-122
- Simberloff D (2013) Biological invasions: Prospects for slowing a major global change. *Elem Sci Anth* 1:1-4
- Stacy BA, Wellehan JFX, Foley AM, Coberley SS, Herbst LH, Manire CA, Garner MM, Brookins MD, Childress AL, Jacobson ER (2008) Two herpesviruses associated with disease in wild Atlantic loggerhead sea turtles (*Caretta caretta*). *Vet Microbiol* 126:63-73

- Steffen W, Grinevald J, Crutzen P, McNeill J (2011) The Anthropocene: conceptual and historical perspectives. *Phil Trans R Soc A* 369:842-867
- Stuckas H, Velo-Antón G, Fahd S, Kalboussi M, Rouag R, Arculeo M, Marrone F, Sacco F, Vamberger M, Fritz U (2014) Where are you from, stranger? The enigmatic biogeography of North African pond turtles (*Emys orbicularis*). *Org Divers Evol* 14:295-306
- Stunkard HW (1916) On the anatomy and relationships of some North American trematodes. *J Parasitol* 3:21-27
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30:2725-2729
- Telecky TM (2001) United States import and export of live turtles and tortoises. *Turtle & Tortoise Newsl.* 14:8-13
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673-4680
- Todd BD, Willson JD, Gibbons JW (2010) The global status of reptiles and causes of their decline. In: Sparling DW, Linder G, Bishop CA, Krest SK (eds) *Ecotoxicology of amphibians and reptiles*, second edition. CRC Press, Taylor & Francis Group, Boca Raton, London, New York, pp 47-68
- Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ, Kuris AM (2003) Introduced species and their missing parasites. *Nature* 421:628-630
- UICN France, MNHN & SHF (2009) *La liste rouge des espèces menacées en France – Chapitre reptiles et amphibiens de France métropolitaine*. Paris, France
- UICN France, MNHN & SHF (2015) *La liste rouge des espèces menacées en France – Chapitre reptiles et amphibiens de France métropolitaine*. Paris, France
- Valdeón A, Rada V, Ayres C, Iglesias R, Longares LA, Lázaro R, Sancho V (2013) Distribution of *Polystomoides ocellatum* (Monogenea: Polystomatidae) in Spain parasitizing the European pond turtle (*Emys orbicularis*). 17th European Congress of Herpetology, Veszprém, Hungary
- Van Dijk PP, Iverson JB, Rhodin AGJ, Shaffer HB, Bour R (2014) *Turtles of the world, 7th edition: Annotated checklist of taxonomy, synonymy, distribution with maps, and conservation status*. *Chelonian Res Monogr* 5:329-479
- Verneau O (2004) *Origine et évolution des monogènes Polystomatidae, parasites d'amphibiens et de chéloniens d'eau douce*. HDR dissertation, Université de Perpignan Via Domitia

- Verneau O, Palacios C, Platt T, Alday M, Billard E, Allienne J, Basso C, Du Preez L (2011) Invasive species threat: parasite phylogenetics reveals patterns and processes of hostswitching between non-native and native captive freshwater turtles. *Parasitology* 138:1778-1792
- Vitousek PM (1994) Beyond global warming: Ecology and global changes. *Ecology* 75:1861-1876
- Vitousek PM, D'Antonio CM, Loope LL, Westbrooks R (1996) Biological invasions as global environmental change. *Am Sci* 84:468-478
- Vitousek PM, Mooney HA, Lubchenco J, Melillo JM (1997a) Human domination of earth's ecosystems. *Science* 277:494-499
- Vitousek PM, D'Antonio CM, Loope LL, Rejmanek M, Westbrooks R (1997b) Introduced species: a significant component of human-caused global change. *N Z J Ecol* 21:1-16
- Warwick C (1991) Conservation of red-eared terrapins, *Trachemys scripta elegans*: threats from international pet and culinary markets. *Testudo* 3:34-44

Table S1. COI haplotype diversity of polystomes from chelonians in natural environments and/or confined areas. Lines in purple indicate captive specimens. « a » means reported in Verneau et al. 2011 ; « b » means reported in Meyer et al. 2015 ; « c » means reported in Badets and Verneau 2009 ; « e » means reported in Littlewood et al. 1997. « \* » means explored in earlier studies. « # 2 turtles » means that polystomes were collected from two turtles and mixed.

Host species	Locality / Province / Country	Host number	Parasite sample	Polystome DNA number	COI Haplotype	Parasite species	Infection site	Genbank Accession COI number
<i>Emys orbicularis</i>	Agde / Languedoc - Roussillon / France	2	1 egg	Mi651	H36	<i>X. sp.</i>	Unknown	New x 1
<i>Emys orbicularis</i>	Arles / PACA / France	90	4 eggs	MiAD93, MiAD94, MiAD95, MiAD97	H15	<i>P. oris</i>	Pharyngeal cavity	New x 4
		716	1 egg	MiAD96	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1
		1024	3 eggs	MiAD90, MiAD91, MiAD92	H15	<i>P. oris</i>	Pharyngeal cavity	New x 3
<i>Emys orbicularis</i>	Béziers / Languedoc - Roussillon / France	Eo1	1 egg	Mi637	H14	<i>P. oris</i>	Pharyngeal cavity	New x 1
		Eo3	1 egg	Mi635	H14	<i>P. oris</i>	Pharyngeal cavity	New x 1
		Eo 56928	2 eggs	Mi632, Mi633	H14	<i>P. oris</i>	Pharyngeal cavity	New x 2
<i>Emys orbicularis</i>	Cravencères / Midi - Pyrénées / France	40	1 egg	MiAD221	H68	<i>Polystomoides sp?</i>	Pharyngeal cavity	New x 1
<i>Emys orbicularis</i>	Lattes / Languedoc - Roussillon / France	3	1 egg	MiAB823	H53	<i>X. sp.</i>	Unknown	New x 1
		29	1 egg	MiAB426	H53	<i>X. sp.</i>	Unknown	New x 1
<i>Emys orbicularis</i>	Lucenay-lès-Aix / Bourgogne / France	149	1 egg	MiAD98	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1
<i>Emys orbicularis</i>	Manduel / Languedoc - Roussillon / France	2	1 egg	MiAD75	H96	<i>Neopolystoma sp4</i>	Urinary bladder	New x 1
		2	1 egg	MiAD76	H67	<i>P. ocellatum</i>	Pharyngeal cavity	New x 1
		7	2 eggs	MiAD79, MiAD80	H96	<i>Neopolystoma sp4</i>	Urinary bladder	New x 2
<i>Emys orbicularis</i>	Ordan Larroque / Midi - Pyrénées / France	14	2 eggs	MiAB821, MiAB923	H68	<i>Polystomoides sp?</i>	Pharyngeal cavity	New x 2
		54	1 egg	MiAD220	H68	<i>Polystomoides sp?</i>	Pharyngeal cavity	New x 1
		56	1 egg	MiAD222	H68	<i>Polystomoides sp?</i>	Pharyngeal cavity	New x 1
<i>Emys orbicularis</i>	Portigliolo / Corsica / France	103	1 egg	MiAD35	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1
		142	1 egg	MiAB828	H67	<i>P. ocellatum</i>	Pharyngeal cavity	New x 1
		153	1 egg	MiAB814	H67	<i>P. ocellatum</i>	Pharyngeal cavity	New x 1
		209	1 egg	MiAD36	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1
		245	1 egg	MiAD34	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1
		278	1 egg	MiAD37	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1
		342	1 egg	MiAD38	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1
		356	1 egg	MiAB802	H67	<i>P. ocellatum</i>	Pharyngeal cavity	New x 1
		360	2 eggs	MiAB797, MiAB798	H67	<i>P. ocellatum</i>	Pharyngeal cavity	New x 2

<i>Emys orbicularis</i>	Port Leucate / Languedoc Roussillon / France	5	4 eggs	Mi96*, Mi97*, Mi98*, Mi119*	H18	<i>Neopolystoma</i> sp4	Urinary bladder	FR822543-46a		
		9	1 egg	MiAB759	H18	<i>Neopolystoma</i> sp4	Urinary bladder	New x 1		
		22	3 eggs	986*, 987*, MiAB761	H18	<i>Neopolystoma</i> sp4	Urinary bladder	FR822547-48a, New x 1		
		34	5 eggs	Mi95*, Mi102*, Mi103*, Mi118*, Mi145*	H18	<i>Neopolystoma</i> sp4	Urinary bladder	FR822549-52a, FR828367a		
		53	1 egg	Mi121*	H18	<i>Neopolystoma</i> sp4	Urinary bladder	FR828368a		
		87	2 eggs	Mi122*, Mi123*	H18	<i>Neopolystoma</i> sp4	Urinary bladder	FR828369-70a		
		92	1 egg	Mi84*	H21	<i>Neopolystoma</i> sp6	Conjunctival sacs	FR828375a		
		109	1 egg	MiAB38	H18	<i>Neopolystoma</i> sp4	Urinary bladder	New x 1		
		117	3 eggs	MiAB48, MiAB50, MiAB51	H18	<i>Neopolystoma</i> sp4	Urinary bladder	New x 3		
		144	3 eggs	MiAB55, MiAB56, MiAB57	H18	<i>Neopolystoma</i> sp4	Urinary bladder	New x 3		
		182	1 egg	MiAB846	H18	<i>Neopolystoma</i> sp4	Urinary bladder	New x 1		
		201	1 egg	MiAB762	H18	<i>Neopolystoma</i> sp4	Urinary bladder	New x 1		
		258	1 egg	MiAB753	H18	<i>Neopolystoma</i> sp4	Urinary bladder	New x 1		
		262	1 egg	MiAB755	H18	<i>Neopolystoma</i> sp4	Urinary bladder	New x 1		
		<i>Emys orbicularis</i>	Propriano / Corsica / France	21	4 eggs	MiAB29, MiAB31, MiAB33, MiAB830	H67	<i>P. ocellatum</i>	Pharyngeal	New x 4
				32	2 eggs	MiAB27, MiAB28	H67	<i>P. ocellatum</i>	Pharyngeal cavity	New x 2
				349	1 egg	MiAB813	H67	<i>P. ocellatum</i>	Pharyngeal cavity	New x 1
<i>Emys orbicularis</i>	Prunelli-di-Fiumorbo / Corsica / France	5	1 egg	MiAD19	H15	<i>P. oris</i>	Pharyngeal	New x 1		
		7	1 egg	MiAD18	H96	<i>Neopolystoma</i> sp4	Urinary bladder	New x 1		
		8	1 egg	MiAD12	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1		
		9	1 egg	MiAD16	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1		
		10	1 egg	MiAD17	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1		
		11	1 egg	MiAD21	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1		
		11	1 egg	MiAB779	H18	<i>Neopolystoma</i> sp4	Urinary bladder	New x 1		
		14	1 egg	MiAD20	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1		
		14	2 eggs	MiAB774, MiAD643	H96	<i>Neopolystoma</i> sp4	Urinary bladder	New x 2		
		19	1 egg	MiAD15	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1		
		20	1 egg	MiAD14	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1		
		23	1 egg	MiAD11	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1		
		25	1 egg	MiAD13	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1		
		26	1 egg	MiAD22	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1		
		210	1 egg	MiAB776	H67	<i>P. ocellatum</i>	Pharyngeal cavity	New x 1		
<i>Emys orbicularis</i>	Rivesaltes / Languedoc - Roussillon / France	8	5 eggs	MiAD625, MiAD626, MiAD627, MiAD628, MiAD629	H91	<i>X. sp.</i>	Unknown	New x 5		
<i>Emys orbicularis</i>	Rosnay / Centre / France	1701	1 egg	MiAD33	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1		
		1707	3 eggs	MiAD28, MiAD29, MiAD30	H15	<i>P. oris</i>	Pharyngeal cavity	New x 3		
		1709	1 egg	MiAD31	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1		
		#1 turtle	1 egg	MiAD32	H15	<i>P. oris</i>	Pharyngeal cavity	New x 1		
<i>Emys orbicularis</i>	Saint-Gély-du-Fesc / Languedoc - Roussillon / France	3	3 eggs	MiAB391, MiAB392, MiAB393	H63	<i>Polystomoides oris</i>	Pharyngeal	New x 3		
		5	2 eggs	MiAB382, MiAB383	H63	<i>P. oris</i>	Pharyngeal cavity	New x 2		
		7	2 eggs	MiAB404, MiAB406	H63	<i>P. oris</i>	Pharyngeal cavity	New x 2		
		8	4 eggs	MiAB399, MiAB400, MiAB401, MiAB402	H63	<i>P. oris</i>	Pharyngeal cavity	New x 4		
		9	3 eggs	MiAB395, MiAB397, MiAB398	H63	<i>P. oris</i>	Pharyngeal cavity	New x 3		
		13	2 eggs	MiAB386, MiAB388	H63	<i>P. oris</i>	Pharyngeal cavity	New x 2		
		49	1 egg	MiAB429	H36	<i>X. sp.</i>	Unknown	New x 1		
<i>Emys orbicularis</i>	Saint-Hippolyte / Languedoc - Roussillon / France	364	3 eggs	MiAD638, MiAD639, MiAD640	H21	<i>Neopolystoma</i> sp6	Conjunctival sacs	New x 3		

<i>Emys orbicularis</i>	Sauziere Saint Jean / Midi - Pyrénées / France	5 6	1 egg 1 egg	Mi642 Mi648	H17 H113	<i>Neopolystoma</i> sp3 <i>N. orbiculare</i>	Urinary bladder Urinary bladder	New x 1 New x 1
<i>Emys orbicularis</i>	Sorède / Languedoc - Roussillon / France	Eos6 Eos2 Eos9 Eos10 Eos10 Eos23 #1 turtle #1 turtle #1 turtle #1 turtle	2 eggs 1 egg 3 eggs 1 egg 1 egg 1 egg 1 worm (PL060528D1) 1 worm (PL060528E1) 2 eggs 2 worms (PL060529A1, 29A2)	Mi692*, Mi693* Mi687* Mi702*, Mi704*, Mi705* Mi709* Mi707* Mi932* Mi3* (Podes spD) Mi4* (Podes spE) Mi125*, Mi126* Mi2* (Podes spC), Mi162*	H14 H21 H20 H14 H19 H15 H17 H16 H20 H22	<i>P. oris</i> <i>Neopolystoma</i> sp6 <i>N. orbiculare</i> <i>P. oris</i> <i>N. orbiculare</i> <i>P. oris</i> <i>Neopolystoma</i> sp3 <i>Polystomoides</i> sp1 <i>N. orbiculare</i> <i>N. elizabethae</i>	Pharyngeal Conjunctival Urinary bladder Pharyngeal Urinary bladder Pharyngeal Urinary bladder Pharyngeal Urinary bladder Conjunctival	FR822535 <sub>Δ</sub> , FR828365 <sub>Δ</sub> FR828376 <sub>Δ</sub> FR828372 <sub>Δ</sub> to FR828374 <sub>Δ</sub> FR828366 <sub>Δ</sub> FR846488 <sub>Δ</sub> FR846486 <sub>Δ</sub> FR846487 <sub>Δ</sub> FR822537 <sub>Δ</sub> FR846489 <sub>Δ</sub> , FR828371 <sub>Δ</sub> FR828377 <sub>Δ</sub> , FR828378 <sub>Δ</sub>
<i>Emys orbicularis</i>	Villeneuve-lès-Maguelone / Languedoc - Roussillon / France	5 7 12 16 20 34	1 egg 1 egg 2 eggs 5 eggs 2 eggs 1 egg	Mi794 MiAB419 MiAB794, MiAB795 Mi795, Mi796, Mi797, MiAB421, MiAB790 Mi801, Mi806 MiAB783	H36 H36 H36 H36 H36 H36	X. sp. X. sp. X. sp. X. sp. X. sp. X. sp.	Unknown Unknown Unknown Unknown Unknown Unknown	New x 1 New x 1 New x 2 New x 5 New x 2 New x 1
<i>Emys orbicularis</i>	Arietz / Navarre / Spain	2457	1 worm	MiAD863	H66	<i>Polystomoides</i> sp7	Pharyngeal cavity	New x 1
<i>Emys orbicularis</i>	Bardenas / Navarre / Spain	2164858a 2164858b 1128 #1 turtle #2 turtles #2 turtles #2 turtles #Several turtles #Several turtles	1 worm 1 worm 1 worm 1 worm 2 worms 3 worms 3 worms 2 worms 6 worms	MiAB22 MiAB23 MiAD865 MiAD778 MiAD686, MiAD687 MiAD688, MiAD689, MiAD690 MiAD774, MiAD775, MiAD776 MiAD684, MiAD685 MiAD678, MiAD679, MiAD680, MiAD681, MiAD682, MiAD683	H66 H66 H66 H66 H66 H66 H66 H66 H66	<i>Polystomoides</i> sp7 <i>Polystomoides</i> sp7 <i>Polystomoides</i> sp7 <i>Polystomoides</i> sp7 <i>Polystomoides</i> sp7 <i>Polystomoides</i> sp7 <i>Polystomoides</i> sp7 <i>Polystomoides</i> sp7 <i>Polystomoides</i> sp7	Pharyngeal cavity Pharyngeal cavity Pharyngeal cavity Pharyngeal cavity Pharyngeal cavity Pharyngeal cavity Pharyngeal cavity Pharyngeal cavity Pharyngeal cavity	New x 1 New x 1 New x 1 New x 1 New x 2 New x 3 New x 3 New x 2 New x 6
<i>Emys orbicularis</i>	Berroya / Navarre / Spain	2403 2403 2409 2423 2431	1 egg 1 egg 1 egg 3 eggs 1 egg	MiAD103 MiAD104 MiAD99 MiAD100, MiAD101, MiAD102 MiAD107	H15 H66 H15 H66 H15	<i>P. oris</i> <i>Polystomoides</i> sp7 <i>P. oris</i> <i>Polystomoides</i> sp7 <i>P. oris</i>	Pharyngeal cavity Pharyngeal cavity Pharyngeal cavity Pharyngeal cavity	New x 1 New x 1 New x 1 New x 3 New x 1
<i>Emys orbicularis</i>	Betanzos / Galicia /	#1 turtle	1 worm	MiAD859	H95	<i>Polystomoides</i> sp7	Pharyngeal	New x 1
<i>Emys orbicularis</i>	Caparroso / Galicia /	695b	1 worm	MiAD861	H66	<i>Polystomoides</i> sp7	Pharyngeal	New x 1
<i>Emys orbicularis</i>	Estanya / Huesca / Spain	#1 turtle #4 turtles #5 turtles #X turtles #X turtles #X turtles #X turtles	1 worm 5 worms 4 worms 14 worms 2 worms 2 worms 2 worms	MiAD773 MiAD705, MiAD707, MiAD708, MiAD709, MiAD710 MiAD780, MiAD781, MiAD782, MiAD783 MiAD693, MiAD694, MiAD695, MiAD696, MiAD697, MiAD698, MiAD742, MiAD743, MiAD744, MiAD745, MiAD746, MiAD747, MiAD749, MiAD750 MiAD810, MiAD811 MiAD812, MiAD813 MiAD814, MiAD815	H67 H67 H67 H67 H67 H67 H67	<i>P. ocellatum</i> <i>P. ocellatum</i> <i>P. ocellatum</i> <i>P. ocellatum</i> <i>P. ocellatum</i> <i>P. ocellatum</i> <i>P. ocellatum</i>	Pharyngeal Pharyngeal Pharyngeal Pharyngeal Pharyngeal Pharyngeal Pharyngeal	New x 1 New x 5 New x 4 New x 14 New x 2 New x 2 New x 2

<i>Emys orbicularis</i>	Gallipienzo / Navarre /	#1 turtle	1 worm	MIAD739	H66	<i>Folystomoides</i> sp7	Pharyngeal	New x 1
<i>Emys orbicularis</i>	La Alfranca / Aragon / Spain	4195	1 egg	MIAB803	H66	<i>Folystomoides</i> sp7	Pharyngeal	New x 1
		4195	1 egg	MIAB804	H67	<i>F. ocellatum</i>	Pharyngeal cavity	New x 1
		4203	2 eggs	MIAB805, MIAB806	H66	<i>Folystomoides</i> sp7	Pharyngeal cavity	New x 2
		5003	2 eggs	MIAB807, MIAB808	H66	<i>Folystomoides</i> sp7	Pharyngeal cavity	New x 2
		5005	1 egg	MIAB816	H67	<i>F. ocellatum</i>	Pharyngeal cavity	New x 1
		5010	1 egg	MIAB817	H66	<i>Folystomoides</i> sp7	Pharyngeal cavity	New x 1
<i>Emys orbicularis</i>	Murillo El Fruto / Navarre / Spain	#1 turtle	1 worm	MIAD769	H66	<i>Folystomoides</i> sp7	Pharyngeal cavity	New x 1
		#2 turtles	2 worms	MIAD753, MIAD754	H66	<i>Folystomoides</i> sp7	Pharyngeal cavity	New x 2
<i>Emys orbicularis</i>	Pina de Ebro / Aragon / Spain	#1 turtle	1 worm	MIAD740	H66	<i>Folystomoides</i> sp7	Pharyngeal cavity	New x 1
<i>Emys orbicularis</i>	Porriño / Galicia / Spain	#1 turtle	2 worms	MIAD691, MIAD692	H95	<i>Folystomoides</i> sp7	Pharyngeal cavity	New x 2
		#1 turtle	3 worms	MIAD699, MIAD700, MIAD701	H95	<i>Folystomoides</i> sp7	Pharyngeal cavity	New x 3
		#1 turtle	2 worms	MIAD765, MIAD766	H95	<i>Folystomoides</i> sp7	Pharyngeal cavity	New x 2
		#1 turtle	1 worm	MIAD768	H95	<i>Folystomoides</i> sp7	Pharyngeal cavity	New x 1
<i>Emys orbicularis</i>	Sastoya / Navarre / Spain	2422	1 egg	MIAD105	H66	<i>Folystomoides</i> sp7	Pharyngeal cavity	New x 1
		2422	1 egg	MIAD106	H15	<i>F. oris</i>	Pharyngeal cavity	New x 1
<i>Emys orbicularis</i>	Ujué (1) / Navarre / Spain	#1 turtle	1 worm	MIAD777	H66	<i>Folystomoides</i> sp7	Pharyngeal cavity	New x 1
		#2 turtles	2 worms	MIAD751, MIAD752	H66	<i>Folystomoides</i> sp7	Pharyngeal cavity	New x 2
<i>Emys orbicularis</i>	Ujué (2) / Navarre / Spain	#3 turtles	3 worms	MIAD762, MIAD763, MIAD764	H66	<i>Folystomoides</i> sp7	Pharyngeal cavity	New x 3
<i>Emys orbicularis</i>	El Kala / El Tarf / Algeria	1	1 worm	MIAD450	H89	<i>F. ocellatum</i>	Pharyngeal cavity	New x 1
		2	1 worm	MIAD720	H89	<i>F. ocellatum</i>	Pharyngeal cavity	New x 1
		3	1 worm	MIAD721	H89	<i>F. ocellatum</i>	Pharyngeal cavity	New x 1



Mauremys leprosa	Banyuls-Mer / Languedoc - Roussillon / France	142	1 egg	MiAC7*	H83	Neopolystoma sp6	Conjunctival sacs	h		
		177	3 eggs	MiAC69*, MiAC70*, MiAC176*	H18	Neopolystoma sp4	Urinary bladder	h		
		179	2 eggs	MiAC151*, MiAC152*	H86	<i>P. oris</i>	Pharyngeal cavity	h		
		184	1 egg	MiAC142*	H86	<i>P. oris</i>	Pharyngeal cavity	h		
		186	4 eggs	MiAC132*, MiAC133*, MiAC143*, MiAC144*	H18	Neopolystoma sp4	Urinary bladder	h		
		186	2 eggs	MiAC153*, MiAC154*	H86	<i>P. oris</i>	Pharyngeal cavity	h		
		191	2 eggs	MiAC157*, MiAC158*	H18	Neopolystoma sp4	Urinary bladder	h		
		193	1 egg	MiAC146*	H18	Neopolystoma sp4	Urinary bladder	h		
		198	1 egg	Mi983*	H21	Neopolystoma sp6	Conjunctival sacs	h		
		198	2 eggs	MiAC135*, MiAC137*	H86	<i>P. oris</i>	Pharyngeal cavity	h		
		199	1 egg	Mi771*	H18	Neopolystoma sp4	Urinary bladder	h		
		199	2 eggs	MiAC138*, MiAC139*	H86	<i>P. oris</i>	Pharyngeal cavity	h		
		202	4 eggs	Mi783*, MiAC15*, MiAC141*, MiAC148*	H18	Neopolystoma sp4	Urinary bladder	h		
		213	1 egg	Mi779*	H21	Neopolystoma sp6	Conjunctival sacs	h		
		214	2 eggs	MiAC161*, MiAC162*	H18	Neopolystoma sp4	Urinary bladder	h		
		214	1 egg	MiAC160*	H21	Neopolystoma sp6	Conjunctival sacs	h		
		214	1 egg	MiAC159*	H86	<i>P. oris</i>	Pharyngeal cavity	h		
		260	3 eggs	MiAC71*, MiAC72*, MiAC178*	H18	Neopolystoma sp4	Urinary bladder	h		
		296	1 egg	MiAC171*	H18	Neopolystoma sp4	Urinary bladder	h		
		302	2 eggs	MiAC173*, MiAC174*	H18	Neopolystoma sp4	Urinary bladder	h		
		303	2 eggs	MiAC179*, MiAC180*	H18	Neopolystoma sp4	Urinary bladder	h		
		313	2 eggs	MiAC83*, MiAC182*	H18	Neopolystoma sp4	Urinary bladder	h		
		316-2	1 egg	MiAC183*	H18	Neopolystoma sp4	Urinary bladder	h		
		330	1 egg	MiAC63*	H18	Neopolystoma sp4	Urinary bladder	h		
		331	2 eggs	MiAC75*, MiAC76*	H18	Neopolystoma sp4	Urinary bladder	h		
		338	1 egg	MiAC77*	H18	Neopolystoma sp4	Urinary bladder	h		
		J2	1 worm	Mi39*	H21	Neopolystoma sp6	Conjunctival sacs	h		
		Mauremys leprosa	Béziers / Languedoc - Roussillon / France	#1 turtle	2 eggs	Mi654, Mi655	H116	<i>N. euzeti</i>	Urinary bladder	New x 2
		Mauremys leprosa	Bompas / Languedoc - Roussillon / France	76	5 eggs	MiAB605*, MiAB606*, MiAB610*, MiAB629*, MiAC218*	H37	<i>N. orbiculare</i>	Urinary bladder	h
				79	2 eggs	MiAB602*, MiAB622*	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	h
				89	2 eggs	MiAC128*, MiAC224*	H20	<i>N. orbiculare</i>	Urinary bladder	h
				89	3 eggs	MiAC113*, MiAC469*, MiAC470*	H21	Neopolystoma sp6	Conjunctival sacs	h
				92	1 egg	MiAD325*	H20	<i>N. orbiculare</i>	Urinary bladder	h
92	5 eggs			MiAC114*, MiAC492*, MiAC493*, MiAD324*, MiAD327*	H37	<i>N. orbiculare</i>	Urinary bladder	h		
93	4 eggs			MiAC119*, MiAC228*, MiAC490*, MiAC491*	H20	<i>N. orbiculare</i>	Urinary bladder	h		
103	4 eggs			MiAC494*, MiAC495*, MiAD320*, MiAD322*	H20	<i>N. orbiculare</i>	Urinary bladder	h		
103	1 egg			MiAD321*	H37	<i>N. orbiculare</i>	Urinary bladder	h		
Mauremys leprosa	Cabestany / Languedoc - Roussillon / France			3	1 egg	MiAC472*	H37	<i>N. orbiculare</i>	Urinary bladder	h
		13	2 eggs	MiAC486*, MiAC487*	H37	<i>N. orbiculare</i>	Urinary bladder	h		
		20	1 egg	MiAC484*	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	h		
		34	2 eggs	MiAC480*, MiAC481*	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	h		
		35	2 eggs	MiAC482*, MiAC483*	H37	<i>N. orbiculare</i>	Urinary bladder	h		
		43	1 egg	MiAC488*, MiAC489*	H37	<i>N. orbiculare</i>	Urinary bladder	h		
		47	1 egg	MiAD1*	H37	<i>N. orbiculare</i>	Urinary bladder	h		
		47	2 eggs	MiAD2*, MiAD307*	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	h		
		55	1 egg	MiAD306*	H37	<i>N. orbiculare</i>	Urinary bladder	h		
		88	2 eggs	MiAC473*, MiAC474*	H37	<i>N. orbiculare</i>	Urinary bladder	h		
		152	2 eggs	MiAD316*, MiAD317*	H37	<i>N. orbiculare</i>	Urinary bladder	h		
		Mauremys leprosa	Canet / Languedoc - Roussillon / France	1	8 eggs	Mi225*, Mi226*, Mi227*, Mi228*, MiAC521*, MiAC522*, MiAC525*, MiAC526*	H26	<i>Polystomoides tunisiensis</i>	Pharyngeal cavity	FR822576-79a and b
Mauremys leprosa	Ceyras / Languedoc - Roussillon / France	#1 turtle	1 egg	MiAB434	H65	<i>P. tunisiensis</i>	Pharyngeal cavity	New x 1		

Mauremys leprosa	Claira / Languedoc - Roussillon / France	69	2 eggs	MiAC467*, MiAC468*	H21	Neopolystoma sp6	Conjunctival sacs	h
		83	5 eggs	MiAB615*, MiAB631*, MiAB632*, MiAB635*, MiAC479*	H21	Neopolystoma sp6	Conjunctival sacs	h
Mauremys leprosa	Le Boulou / Languedoc - Roussillon / France	18	3 eggs	MiAB341*, MiAB342*, MiAB343*	H32	Neopolystoma euzeti	Urinary bladder	h
		21	4 eggs	Mi657*, Mi658*, Mi659*, Mi660*	H30	P. tunisiensis	Pharyngeal cavity	FR822583a and b
		22	5 eggs	Mi987*, Mi989*, Mi661*, Mi662*, Mi663*	H30	P. tunisiensis	Pharyngeal cavity	FR822584-85a and b
		24	3 eggs	Mi665*, Mi667*, Mi668*	H30	P. tunisiensis	Pharyngeal cavity	h
		25	1 egg	Mi669*	H30	P. tunisiensis	Pharyngeal cavity	FR822586a
		29	3 eggs	Mi670*, Mi671*, Mi672*	H30	P. tunisiensis	Pharyngeal cavity	h
		30	2 eggs	Mi673*, Mi676*	H30	P. tunisiensis	Pharyngeal cavity	h
		35	1 egg	MiAC638*	H30	P. tunisiensis	Pharyngeal cavity	h
		38	1 egg	MiAC499*	H32	N. euzeti	Urinary bladder	h
Mauremys leprosa	Saint-Hippolyte / Languedoc - Roussillon / France	7	3 eggs	Mi244*, Mi245*, Mi246*	H21	Neopolystoma sp6	Conjunctival sacs	h
		8	1 egg	Mi249*	H21	Neopolystoma sp6	Conjunctival sacs	h
		9	1 egg	Mi243*	H21	Neopolystoma sp6	Conjunctival sacs	h
		9	1 egg	Mi241*	H57	Neopolystoma sp6	Conjunctival sacs	KM258884h
		10	3 eggs	Mi238*, Mi239*, Mi240*	H21	Neopolystoma sp6	Conjunctival sacs	h
		277	1 egg	MiAC509*	H21	Neopolystoma sp6	Conjunctival sacs	h
Mauremys leprosa	Sauziere Saint Jean / Midi - Pyrénées / France	#1 turtle	2 eggs	Mi235, Mi236	H59	P. tunisiensis	Pharyngeal cavity	New x 2
Mauremys leprosa	Sorède / Languedoc - Roussillon / France	Mls1	2 eggs	Mi711*, Mi713*	H15	P. oris	Pharyngeal	FR822536a, FR822537a
		Mls1	1 egg	Mi712*	H34	P. oris	Pharyngeal cavity	FR822591a
		Mls2	2 eggs	Mi715*, Mi716*	H32	N. euzeti	Urinary bladder	FR822588a, FR822589a
		Mls4	1 egg	Mi719*	H36	X. sp.		FR822595a
		Mls5	1 egg	Mi721*	H20	N. orbiculare	Urinary bladder	FR822565a
		Mls5	1 egg	Mi720*	H35	Polystomoides sp4	Pharyngeal cavity	FR822592a
		Mls6	1 egg	Mi725*	H14	P. oris	Pharyngeal cavity	FR822556a
		Mls6	1 egg	Mi939*	H22	N. elizabethae	Conjunctival sacs	FR822569a
		Mls6	1 egg	Mi724*	H35	Polystomoides sp4	Pharyngeal cavity	FR822593a
		Mls6	1 egg	Mi728*	H37	N. orbiculare	Urinary bladder	FR822597a
		Mls6	1 egg	MiAB9*	H38	Neopolystoma sp6	Conjunctival sacs	FR822598a
		Mls10	3 eggs	Mi732*, Mi733*, Mi734*	H19	N. orbiculare	Urinary bladder	FR822559a to FR822561a
		Mls10	1 egg	Mi735*	H20	N. orbiculare	Urinary bladder	FR822566a
		Mls10	1 egg	Mi740*	H35	Polystomoides sp4	Pharyngeal cavity	FR822594a
		Mls18	1 egg	MiAB12*	H39	X. sp.	Conjunctival sacs	FR822599a
		Mls18	1 egg	MiAB13*	H40	Neopolystoma sp6	Conjunctival sacs	FR822600a
		Mls20	1 egg	Mi905*	H20	N. orbiculare	Urinary bladder	FR822567a
		Mls21	1 egg	Mi908*	H19	N. orbiculare	Urinary bladder	FR822562a
		Mls24	1 egg	Mi916*	H19	N. orbiculare	Urinary bladder	FR822563a
		Mls24	1 egg	Mi914*	H27	P. tunisiensis	Pharyngeal cavity	FR822580a
Mls24	1 egg	Mi915*	H28	P. tunisiensis	Pharyngeal cavity	FR822581a		
Mls24	1 egg	Mi917*	H29	P. tunisiensis	Pharyngeal cavity	FR822582a		
Mls28	1 egg	MiAB10*	H21	Neopolystoma sp6	Conjunctival sacs	FR822568a		
#1 turtle	1 egg	Mi135*	H20	N. orbiculare	Urinary bladder	FR822564a		
#1 turtle	1 egg	Mi137*	H33	P. oris	Pharyngeal cavity	FR822590a		
#1 turtle	1 egg	Mi85* (Podes spT)	H37	N. orbiculare	Urinary bladder	FR822596a		

Mauremys leprosa	Anyet / Catalonia / Spain	1	1 egg	MIAC562*	H17	Neopolystoma sp3	Urinary bladder	h
		1	1 egg	MIAC563*	H80	Neopolystoma sp3	Urinary bladder	h
		2	1 egg	MIAD266*	H17	Neopolystoma sp3	Urinary bladder	h
		2	1 egg	MIAD265*	H78	F. tunisiensis	Pharyngeal cavity	h
		4	1 egg	MIAC672*	H78	F. tunisiensis	Pharyngeal cavity	h
		7	1 egg	MIAD250*	H78	F. tunisiensis	Pharyngeal cavity	h
		8	4 eggs	MIAD264*, MIAD288*, MIAD290*, MIAD291*	H17	Neopolystoma sp3	Urinary bladder	h
		8	1 egg	MIAD253*	H78	F. tunisiensis	Pharyngeal cavity	h
		10	2 eggs	MIAC558*, MIAC559*	H59	F. tunisiensis	Pharyngeal cavity	h
		11	5 eggs	MIAC670*, MIAD269*, MIAD270*, MIAD285*, MIAD286*	H17	Neopolystoma sp3	Urinary bladder	h
		11	2 eggs	MIAC560*, MIAC561*	H78	F. tunisiensis	Pharyngeal cavity	h
		12	1 egg	MIAC547*	H82	F. tunisiensis	Pharyngeal cavity	h
		13	1 egg	MIAC566*	H78	F. tunisiensis	Pharyngeal cavity	h
		19	1 egg	MIAC565*	H78	F. tunisiensis	Pharyngeal cavity	h
Mauremys leprosa	La Alfranca / Aragon / Spain	5009	3 eggs	MIAD347*, MIAD348*, MIAD349*	H59	F. tunisiensis	Pharyngeal cavity	h
		5011	6 eggs	MIAD341*, MIAD342*, MIAD343*, MIAD344*, MIAD345*, MIAD346*	H59	F. tunisiensis	Pharyngeal cavity	h
Mauremys leprosa	Merdanc / Catalonia / Spain	5	2 eggs	MIAC545*, MIAC546*	H31	N. euzeti	Urinary bladder	h
		12	2 eggs	MIAC668*, MIAD280*	H59	F. tunisiensis	Pharyngeal cavity	h
		12	1 egg	MIAC548*	H82	F. tunisiensis	Pharyngeal cavity	h
Mauremys leprosa	Orlina / Catalonia / Spain	2	1 egg	MIAC664*	H82	F. tunisiensis	Pharyngeal cavity	KM258890h
		3	2 eggs	MIAC658*, MIAD257*	H31	N. euzeti	Urinary bladder	h
		5	3 eggs	MIAC665*, MIAD251*, MIAD252*	H78	F. tunisiensis	Pharyngeal cavity	KM258888h
		7	4 eggs	MIAC663*, MIAD260*, MIAD271*, MIAD281*	H82	F. tunisiensis	Pharyngeal cavity	h
		11	3 eggs	MIAC662*, MIAD255*, MIAD256*	H82	F. tunisiensis	Pharyngeal cavity	h
		13	1 egg	MIAC552*	H31	N. euzeti	Urinary bladder	h
		14	3 eggs	MIAC553*, MIAC653*, MIAD274*	H32	N. euzeti	Urinary bladder	h
		14	2 eggs	MIAC654*, MIAD295*	H59	F. tunisiensis	Pharyngeal cavity	KM258885h
		14	1 egg	MIAD273*	H82	F. tunisiensis	Pharyngeal cavity	h
		15	2 eggs	MIAC543*, MIAC544*	H19	N. orbiculare	Urinary bladder	h
		17	1 egg	MIAD294*	H82	F. tunisiensis	Pharyngeal cavity	h
		18	1 egg	MIAC656*	H31	N. euzeti	Urinary bladder	h
		18	4 eggs	MIAC652*, MIAD282*, MIAD297*, MIAD298*	H32	N. euzeti	Urinary bladder	h
		18	2 eggs	MIAD296*, MIAD299*	H82	F. tunisiensis	Pharyngeal cavity	h
		19	1 egg	MIAC666*	H59	F. tunisiensis	Pharyngeal cavity	h
		20	1 egg	MIAD275*	H31	N. euzeti	Urinary bladder	h
		20	1 egg	MIAC667*	H32	N. euzeti	Urinary bladder	h
		20	4 eggs	MIAD276*, MIAD300*, MIAD301*, MIAD302*	H59	F. tunisiensis	Pharyngeal cavity	h
		21	1 egg	MIAC659*	H59	F. tunisiensis	Pharyngeal cavity	h
		22	3 eggs	MIAC549*, MIAC550*, MIAC656*	H59	F. tunisiensis	Pharyngeal cavity	h
		22	1 egg	MIAD278*	H82	F. tunisiensis	Pharyngeal cavity	h

<i>Mauremys leprosa</i>	Constantine / Algeria	#1 turtle	2 worms	MiAD715, MiAD716	H106	<i>P. tunisiensis</i>	Pharyngeal cavity	New x 2
<i>Mauremys leprosa</i>	El Amra / Algeria	1	1 worm	MiAD332*	H85	<i>P. tunisiensis</i>	Pharyngeal cavity	KM258892 <sup>h</sup>
		#1 turtle	1 worm	MiAD717	H31	<i>N. euzeti</i>	Urinary bladder	New x 1
<i>Mauremys leprosa</i>	El Kala / Algeria	#1 turtle	1 worm	MiAD719	H105	<i>P. tunisiensis</i>	Pharyngeal cavity	New x 1
<i>Mauremys leprosa</i>	Oued Rhiou / Algeria	1	1 worm, 5 eggs	Mi110*, Mi114*, Mi116*, Mi142*, Mi143*, Mi144*	H25	<i>P. tunisiensis</i>	Pharyngeal cavity	FR822570-75 <sup>h</sup>
		1	1 worm	Mi111*	H31	<i>N. euzeti</i>	Urinary bladder	FR822587 <sup>h</sup>
<i>Mauremys leprosa</i>	Réghaia / Algeria	1	1 worm	MiAB921*	H70	<i>N. euzeti</i>	Urinary bladder	KM258887 <sup>h</sup>
<i>Mauremys leprosa</i>	Rouina / Algeria	1	2 worms	MiAB918*, MiAB919*	H69	<i>P. tunisiensis</i>	Pharyngeal cavity	KM258886 <sup>h</sup>
		1	1 worm	MiAD330*	H31	<i>N. euzeti</i>	Urinary bladder	<sup>h</sup>
		2	1 worm	MiAB920*	H87	<i>N. euzeti</i>	Urinary bladder	KM258894 <sup>h</sup>
		#1 turtle	1 worm	MiAD714	H31	<i>N. euzeti</i>	Urinary bladder	New x 1
		#1 turtle	1 worm	MiAD723	H69	<i>P. tunisiensis</i>	Pharyngeal cavity	New x 1
		#1 turtle	1 worm	MiAD718	H25	<i>P. tunisiensis</i>	Pharyngeal cavity	New x 1
<i>Mauremys leprosa</i>	Ahl Souss/ Morocco	7	2 eggs	MiAD364, MiAD365	H100	<i>N. euzeti</i>	Urinary bladder	New x 2
<i>Mauremys leprosa</i>	Ben Ahmed / Morocco	4	2 eggs	MiAD784, MiAD785	H65	<i>P. tunisiensis</i>	Pharyngeal cavity	New x 2
		5	2 eggs	MiAD793, MiAD796	H101	<i>N. euzeti</i>	Urinary bladder	New x 2
		5	3 eggs	MiAD792, MiAD794, MiAD795	H65	<i>P. tunisiensis</i>	Pharyngeal cavity	New x 3
		6	2 eggs	MiAD790, MiAD791	H102	<i>N. euzeti</i>	Urinary bladder	New x 2
		8	2 eggs	MiAD786, MiAD787	H65	<i>P. tunisiensis</i>	Pharyngeal cavity	New x 2
		9	2 eggs	MiAD788, MiAD789	H65	<i>P. tunisiensis</i>	Pharyngeal cavity	New x 2
<i>Trachemys scripta elegans</i>	Barbotan les Thermes / Midi - Pyrénées / France	#1 turtle	1 egg	MiAD657	H91	<i>X. sp.</i>	Unknown	New x 1
		#1 turtle	3 eggs	MiAD659, MiAD660, MiAD661	H91	<i>X. sp.</i>	Unknown	New x 3
<i>Trachemys scripta elegans</i>	Bompas / Languedoc - Roussillon / France	1	7 eggs	MiAB629*, MiAB639*, MiAB642*, MiAB643*, MiAC350*, MiAC351*, MiAC583*	H16	<i>Polystomoides sp1</i>	Pharyngeal cavity	<sup>h</sup>
		2	1 egg	MiAC593*	H16	<i>Polystomoides sp1</i>	Pharyngeal cavity	<sup>h</sup>
		3	2 eggs	MiAC352*, MiAC585*	H16	<i>Polystomoides sp1</i>	Pharyngeal cavity	<sup>h</sup>
		3	1 egg	MiAC353*	H20	<i>N. orbiculare</i>	Urinary bladder	<sup>h</sup>
		3	1 egg	MiAC586*	H81	<i>N. orbiculare</i>	Urinary bladder	KM258896 <sup>h</sup>
		4	4 eggs	MiAC354*, MiAC355*, MiAC591*, MiAC592*	H16	<i>Polystomoides sp1</i>	Pharyngeal cavity	<sup>h</sup>
		5	1 egg	MiAC356*	H16	<i>Polystomoides sp1</i>	Pharyngeal cavity	<sup>h</sup>
		7	1 egg	MiAC590*	H16	<i>Polystomoides sp1</i>	Pharyngeal cavity	<sup>h</sup>
		7	3 eggs	MiAC357*, MiAC358*, MiAC589*	H20	<i>N. orbiculare</i>	Urinary bladder	<sup>h</sup>
		24	1 egg	MiAC360*	H16	<i>Polystomoides sp1</i>	Pharyngeal cavity	<sup>h</sup>
		45	7 eggs	MiAD443, MiAD444, MiAD445, MiAD446, MiAD447, MiAD457, MiAD482	H16	<i>Polystomoides sp1</i>	Pharyngeal cavity	New x 7
		45	3 eggs	MiAD456, MiAD481, MiAD483	H77	<i>Polystomoides sp1</i>	Pharyngeal cavity	New x 3
		46	13 eggs	MiAD438, MiAD439, MiAD440, MiAD441, MiAD442, MiAD458, MiAD459, MiAD484, MiAD485, MiAD486, MiAD508, MiAD509, MiAD510	H16	<i>Polystomoides sp1</i>	Pharyngeal cavity	New x 13

<i>Trachemys scripta elegans</i>	Cabestany / Languedoc - Roussillon / France	15	1 egg	MIAC597*	H18	<i>Neopolystoma</i> sp4	Urinary bladder	h
		18	1 egg	MIAC602*	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	h
		20	1 egg	MIAC599*	H77	<i>Polystomoides</i> sp1	Pharyngeal cavity	KM258895h
		22	1 egg	MIAC600*	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	h
		28	2 eggs	MIAD314*, MIAD315*	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	h
		29	2 eggs	MIAD418, MIAD419	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 2
		30	3 eggs	MIAD8*, MIAD9*, MIAD10*	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	h
		40	1 egg	MIAD305*	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	h
		40	3 eggs	MIAD311*, MIAD312*, MIAD313*	H37	<i>N. orbiculare</i>	Urinary bladder	h
		88	1 egg	MIAD389	H77	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 1
		88	3 eggs	MIAD390, MIAD391, MIAD392	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 3
		88	1 egg	MIAD393	H37	<i>N. orbiculare</i>	Urinary bladder	New x 1
		89	2 eggs	MIAD424, MIAD425	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 2
		92	2 eggs	MIAD401, MIAD402	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 2
		93	2 eggs	MIAD414, MIAD415	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 2
		94	2 eggs	MIAD426, MIAD427	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 2
		95	11 eggs	MIAD422, MIAD423, MIAD454, MIAD455, MIAD476, MIAD477, MIAD478, MIAD479, MIAD480, MIAD511, MIAD512	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 11
		96	5 eggs	MIAD408, MIAD409, MIAD410, MIAD411, MIAD412	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 5
		97	2 eggs	MIAD399, MIAD400	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 2
		98	2 eggs	MIAD434, MIAD435	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 2
		108	2 eggs	MIAD420, MIAD421	H37	<i>N. orbiculare</i>	Urinary bladder	New x 2
		108	6 eggs	MIAD462, MIAD463, MIAD466, MIAD493, MIAD494, MIAD506	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 6
		108	4 eggs	MIAD464, MIAD465, MIAD495, MIAD507	H77	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 4
		109	8 eggs	MIAD394, MIAD395, MIAD396, MIAD397, MIAD398, MIAD487, MIAD488, MIAD489	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 8
		110	5 eggs	MIAD416, MIAD417, MIAD490, MIAD491, MIAD492	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 5
		111	2 eggs	MIAD448, MIAD449	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 2
		113	7 eggs	MIAD403, MIAD404, MIAD405, MIAD406, MIAD407, MIAD460, MIAD461	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 7
122	2 eggs	MIAD436, MIAD502	H37	<i>N. orbiculare</i>	Urinary bladder	New x 2		
122	9 eggs	MIAD437, MIAD496, MIAD497, MIAD498, MIAD499, MIAD500, MIAD501, MIAD504, MIAD505	H16	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 9		
<i>Trachemys scripta elegans</i>	Clermont-l'Hérault / Languedoc - Roussillon / France	13	3 ggs	MIAB415, MIAB416, MIAB417	H64	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 3
		20	5 eggs	MIAB408, MIAB409, MIAB410, MIAB411, MIAB412	H64	<i>Polystomoides</i> sp1	Pharyngeal cavity	New x 5
<i>Trachemys scripta elegans</i>	Mauguio / Languedoc - Roussillon / France	5	1 egg	MI650	H53	X. sp.	Unknown	New x 1
<i>Trachemys scripta elegans</i>	Rivesaltes / Languedoc - Roussillon / France	100	8 eggs	MIAD429, MIAD538, MIAD539, MIAD540, MIAD541, MIAD542, MIAD543, MIAD544	H91	X. sp.	Unknown	New x 8
		102	5 eggs	MIAD553, MIAD554, MIAD555, MIAD556, MIAD557	H18	<i>Neopolystoma</i> sp4	Urinary bladder	New x 5
		103	1 egg	MIAD467	H21	<i>Neopolystoma</i> sp6	Conjunctival sacs	New x 1
		123	10 eggs	MIAD469, MIAD470, MIAD471, MIAD546, MIAD547, MIAD548, MIAD549, MIAD550, MIAD551, MIAD552	H91	X. sp.	Unknown	New x 10

<i>Trachemys scripta</i>	Sauziere Saint Jean / Midi - Pyrénées / France	#1 turtle	2 eggs	Mi231, Mi232	H115	<i>P. oris</i>	Pharyngeal cavity	New x 2
<i>Trachemys scripta</i>	Sorède / Languedoc - Roussillon / France	Tses2	3 eggs	Mi744*, Mi745*, Mi746*	H35	<i>Polystomoides</i> sp4	Pharyngeal	FR828344a to FR828346a
		Tses5	2 eggs	Mi747*, Mi748*	H20	<i>N. orbiculare</i>	Urinary bladder	FR822610a, FR822611a
		Tses6	1 egg	Mi751*	H35	<i>Polystomoides</i> sp4	Pharyngeal cavity	FR828347a
		Tses9	1 egg	Mi841*	H55	<i>X. sp.</i>		KM258897a
		Tses10	2 eggs	Mi762*, Mi765*	H17	<i>Neopolystoma</i> sp3	Urinary bladder	FR822608a, FR822609a
		Tses10	1 egg	Mi950	H114	<i>Neopolystoma</i> sp6	Conjunctival sacs	New x 1
		Tses15	3 eggs	Mi890*, Mi891*, Mi892*	H20	<i>N. orbiculare</i>	Urinary bladder	FR822612a to FR822614a
		Tses19	2 eggs	Mi894*, Mi895*	H35	<i>Polystomoides</i> sp4	Pharyngeal cavity	FR828348a, FR828349a
		Tses46	3 eggs	Mi898*, Mi899*, Mi901*	H35	<i>Polystomoides</i> sp4	Pharyngeal cavity	FR828350a to FR828351a
		Tsss55	1 egg	Mi925*	H15	<i>P. oris</i>	Pharyngeal cavity	FR828357a
		Tsss55	1 egg	Mi922*	H35	<i>Polystomoides</i> sp4	Pharyngeal cavity	FR828358a
		#1 turtle	3 worms (PL090522B1 to B3)	Mi848*, Mi849*, Mi850*	H15	<i>P. oris</i>	Pharyngeal cavity	FR822558a, FR822605a, FR822606a
#1 turtle	1 worm (PL090522A1)	Mi767*	H17	<i>Neopolystoma</i> sp3	Urinary bladder	FR822607a		
<i>Trachemys scripta scripta</i>	Florida / USA	RL04023A2	1 worm (PL040602A1 = TIS340)	Mi198* (= 806)	H48	<i>N. orbiculare</i>	Urinary bladder	FR828359a
		RL040422A13	1 worm (PL040604A5 = TIS334)	Mi196*	H50	<i>Polystomoides</i> sp4	Pharyngeal	FR828361a
		RL040422B4	1 worm (PL040505A2 = TIS333)	Mi195*	H51	<i>Polystomoides</i> sp4	Pharyngeal	FR828362a
		RL04022A9	1 worm (PL040602D2 = TIS336)	Mi199* (= 802)	H51	<i>Polystomoides</i> sp4	Pharyngeal	FR828363a
<i>Trachemys scripta elegans</i>	Indiana / USA	TRP-2002//7-5	1 worm	721*	H45	<i>Neopolystoma</i> sp3	Urinary bladder	FR828354a
		TRP-2002//7-1	1 worm	720*	H47	<i>Polystomoides</i> sp1	Pharyngeal cavity	FR828356a
<i>Trachemys scripta elegans</i>	Kansas / USA	MTS-0007	1 worm	222*	H46	<i>Neopolystoma</i> sp3	Urinary bladder	FR828355a
<i>Trachemys scripta scripta</i>	Maine / USA	#1 turtle	1 worm	724*	H49	<i>Polystomoides</i> sp1	Pharyngeal cavity	FR828360a
<i>Chrysemys picta</i>	Connecticut / USA	RL150718A2	1 worm, 2 eggs	MiAD800, MiAD801, MiAD802	H97	<i>P. oris</i>	Pharyngeal cavity	New x 3
		RL150719C1	1 egg	MiAD833	H22	<i>N. elizabethae</i>	Conjunctival sacs	New x 1
<i>Chrysemys picta</i>	Indiana / USA	P-00-4/2	1 worm	219*	H11	<i>P. oris</i>	Pharyngeal cavity	FR822533a
		TRP-2002//7-1	1 worm	719*	H12	<i>P. oris</i>	Pharyngeal cavity	FR822534a
		P-00-4/4	1 worm	221*	H9	<i>N. orbiculare</i>	Urinary bladder	FR822531a
		P-00-4/3	1 worm	Mi188*	H10	<i>N. orbiculare</i>	Urinary bladder	FR822532a
		P-00-4/4	5 worms	Mi189*, Mi190*, Mi191*, Mi192*, Mi193*	H10	<i>N. orbiculare</i>	Urinary bladder	FR822538a to FR822542a
<i>Chrysemys picta</i>	New York / USA	RL150715E2	1 worm, 1 egg	MiAD797, MiAD798	H97	<i>P. oris</i>	Pharyngeal cavity	New x 2
		RL150715E2	1 egg	MiAD799	H98	<i>P. oris</i>	Pharyngeal cavity	New x 1
		RL150716A1	1 worm	PL150718A1	H98	<i>P. oris</i>	Pharyngeal cavity	New x 1
<i>Chrysemys picta</i>	North Carolina / USA	RL150726A2	1 worm, 1 egg	MiAD803, MiAD804	H99	<i>P. oris</i>	Pharyngeal cavity	New x 2
		RL150728C1	1 worm, 3 eggs	MiAD806, MiAD807, MiAD808, MiAD809	H98	<i>P. oris</i>	Pharyngeal cavity	New x 4
		RL150728C1	2 worms	MiAD818, MiAD854	H103	<i>N. orbiculare</i>	Urinary bladder	New x 2

**CHAPITRE III. THE HIGH RESOLUTION MELTING  
ANALYSIS (HRM) AS A MOLECULAR TOOL FOR  
MONITORING PARASITES OF THE WILDLIFE**

## Présentation du Chapitre III

Les inventaires réalisés sur la diversité parasitaire dans les études de Verneau et al. (2011), Meyer et al. (2015) et Héritier et al. (Submitted) sur l'émyde lépreuse et la cistude d'Europe ont été conduits en réalisant le génotypage du gène codant pour la Cytochrome c Oxidase et en analysant les divergences génétiques. Cette approche moléculaire implique l'amplification du gène cible par Polymerase Chain Reaction (PCR) et le séquençage des produits amplifiés pour chaque échantillon. Etant donné que ces deux espèces patrimoniales bénéficient d'un statut de protection, les échantillons analysés étaient dans la majorité des cas des œufs de parasites collectés de manière non-invasive (Verneau et al. 2011). Ainsi, les analyses par PCR et séquençage des produits demandent beaucoup de temps de manipulation et sont onéreuses. De ce fait, réaliser un inventaire de la diversité parasitaire au sein d'une population sauvage de tortues d'eau douce peut s'avérer laborieuse et imprécise vu le nombre d'œufs pondus par un polystome.

L'approche moléculaire High Resolution Melting (HRM) permet d'illustrer les divergences nucléotidiques entre les séquences COI par les propriétés de fusion de l'ADN du gène cible après amplification. Cette approche présente plusieurs avantages, en effet les résultats peuvent se lire directement sans séquençage et plusieurs ADN mélangés peuvent être analysés dans un même échantillon, permettant ainsi l'analyse de mélanges d'œufs de parasites de différentes espèces. Meyer et al. (2015) ont montré que l'émyde lépreuse pouvait être parasitée par six espèces de polystomes différentes. Si les deux espèces de parasites naturelles de cette tortue ont été identifiées, les quatre autres sont soit des parasites invasifs auparavant décrit sur des tortues dans des écosystèmes américains, soit des espèces de parasites non décrites mais considérées comme invasives car retrouvées sur plusieurs hôtes tortues différents, en captivité et en milieu naturels. Dans ce chapitre, il est proposé d'adapter l'approche moléculaire HRM pour la discrimination des espèces de polystomes identifiées sur *M. leprosa* afin de vérifier si cette technique permettrait de réaliser des inventaires plus performants au sein de population de tortues natives.



## The High Resolution Melting analysis (HRM) as a molecular tool for monitoring parasites of the wildlife

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### Keywords

HRM, 12S rRNA gene, Polystome, *Mauremys leprosa*, *Trachemys scripta elegans*, endangered wildlife.

## SUMMARY

In an interconnected world, the international pet trade on wild animals is becoming increasingly important. As a consequence, non-native parasite species are introduced, which affect the health of wildlife and contribute to the loss of biodiversity. Because the investigation of parasite diversity within vulnerable host species implies the molecular identification of large samples of parasite eggs, the sequencing of DNA barcodes is time-consuming and costly. Thereby, the objectives of our study were to apply the High Resolution Melting (HRM) approach for species determination from pools of parasite eggs. Molecular assays were validated on flatworm parasites (polystomes) infecting the Mediterranean pond turtle *Mauremys leprosa* and the invasive red-eared slider *Trachemys scripta elegans* in French natural environments. HRM analysis results indicated that double or multiple parasitic infections could be detected from wild animal populations. They also showed that the cycle of parasite eggs production was not regular over time and may depend on several factors, among which the ecological niche and the target species. Thereby, monitoring parasites from wild endangered animals implies periodic parasitological surveys to avoid false negative diagnostics based solely on eggs production.

## KEY FINDINGS

- HRM analysis was used for the monitoring of parasites within wild host populations
- Single and multiple parasite infections were revealed in experimental conditions
- This molecular method was validated on egg parasites from wild hosts
- This approach is appropriate for the study of parasite diversity among endangered wildlife populations

## INTRODUCTION

Global biodiversity is increasingly threatened by anthropogenic activities that contribute to alter the structure and functioning of ecosystems (Vitousek *et al.* 1997; Sala *et al.* 2000). The causes of species extinction mainly involve habitat loss, land cover change, pollution, overexploitation and introduction of alien species (Groom, 2005). Regarding biological invasions, if direct competition between invaders and native species has been well documented as a source of species decline (see Simberloff, 2013), the introduction of exotic parasites, which can affect the health of wildlife, also contributes to the loss of biodiversity (Gozlan *et al.* 2005; Barry *et al.* 2014; Iglesias *et al.* 2015). Thereby, surveying parasite diversity for conservation practices should not be neglected (Gómez and Nichols, 2013).

Parasites, which are commonly organisms with small body size, are mostly poor in morphological discriminant characters (Besansky *et al.* 2003). As a consequence, the “DNA barcoding” procedure (Hebert *et al.* 2003) is increasingly used for species identification (Eszterbauer *et al.* 2005; Berthier *et al.* 2014). In practice, a DNA sequence of the marker region, i.e. the DNA barcode, is obtained from unknown species and compared with sequences that are referenced in genomic databases. Because investigation of parasite biodiversity within vulnerable host species usually implies the molecular identification of large samples of parasite eggs, the sequencing of DNA barcodes can be time-consuming and costly. This has been the case when studying the parasite diversity of endangered freshwater turtle species in Southern Europe (Meyer *et al.* 2015; Héritier *et al.* submitted).

The molecular approach High Resolution Melting (HRM), which was introduced by Ririe *et al.* (1997) and Lay and Wittwer (1997), was originally used as a diagnostic tool for genotyping and mutation scanning in humans (Wittwer *et al.* 2003; Gundry *et al.* 2003). Variations between polymorphic sequences are detected through the melting properties of double-stranded amplified DNA (dsDNA). The melting profiles of each amplicon, which depend of the melting temperature ( $T_m$ ), are monitored through the release of a saturating fluorescent dsDNA binding dye as temperature increases. This approach was subsequently applied in a wide range of surveys (see Tong and Giffard, 2012), such as the identification of pathogenic parasites for medical and veterinary purposes (Pangasa *et al.* 2009; Roberston *et al.* 2010; Ngui *et al.* 2012) and the prevention of zoonoses (Morick *et al.* 2009). It has to date never been used for the monitoring of parasites within native wild host populations in a framework of biological invasions.

The Mediterranean pond turtle *Mauremys leprosa* (Schweigger, 1812) is a freshwater chelonian encountered in Northern Africa and South-Western Europe (Van Dijk *et al.* 2014). Though this taxon has not yet

been assessed for the IUCN red list, it has been classified as “Vulnerable” in France (UICN France, MNHN and SHF, 2015), partly because of small and isolated endemic populations occurring solely in the province of Languedoc – Roussillon (see Palacios *et al.* 2015). In this area, this species co-occurs with the invasive red-eared slider *Trachemys scripta elegans* (Wied, 1839) that may compete for basking, territory and feeding resources (Polo-Cavia *et al.* 2009, 2010, 2011). Meyer *et al.* (2015) also showed that non-native parasite species (Platyhelminthes, Monogenea, Polystomatidae) carried by *T. s. elegans* were transmitted to *M. leprosa* in natural environments of Southern Europe. Polystome diversity was assessed from a non-invasive approach involving the collection of polystome eggs from both turtles and DNA barcoding. Because one turtle can accommodate up to three distinct polystome species, each of them being specific of distinct ecological niches within the host, i.e. the pharyngeal cavity, the bladder and the conjunctival sacs, the molecular tool HRM was applied here after for the analysis of parasite diversity from pools of eggs. Our objectives were first to obtain reference melting curves for all documented parasite species in *M. leprosa* (see Meyer *et al.* 2015) and to develop cross DNA experiments from parasites of interest in order to illustrate cases of multiple infestations. Because *T. s. elegans* is considered as an invasive species in French natural environments, we also performed HRM analyses on polystome eggs and adults collected from this species in the field for validation *in situ*. In the context of conservation of threatened species, this approach should help stakeholders of environmental management for a more accurate development of parasitological monitoring.

## MATERIALS AND METHODS

### *Polystome selection for melting curves references*

Six of the polystome species that were documented within populations of *M. leprosa* in natural environments (Meyer *et al.* 2015) were selected to obtain reference melting curves: (i) *Polystomoides tunisiensis* Gonzales and Mishra (1977) and (ii) *Neopolystoma euzeti* Combes and Ktari (1976) were originally described from the pharyngeal cavity and the bladder of *M. leprosa*, respectively; (iii) *Polystomoides oris* (Paul, 1938) and (iv) *Neopolystoma orbiculare* (Stunkard, 1916) were originally described from the pharyngeal cavity and the bladder of the American painted turtle *Chrysemys picta marginata* (Agassiz, 1857), respectively; two undescribed species, namely (v) *Polystomoides* sp. from the pharyngeal cavity and (vi) *Neopolystoma* sp. from the bladder were reported from *T. s. elegans* and *M. leprosa* in French natural environments and considered as non-native species for the native freshwater turtle (see Meyer *et al.* 2015). All the DNAs used were from our DNA collection.

### *Sampling of polystome eggs and adults from wild T. s. elegans*

Red-eared sliders were captured using catfish traps that were set from two to three days along the watercourse La Fosseille in Southern France (see Meyer *et al.* 2015 for GPS coordinates). Traps were baited with pork liver and firmly attached to the vegetation. They were checked every day. Captured turtles were kept in the laboratory and maintained separately in small containers equipped with UVB lights and filled partially with water heated to 26°C. Polystome eggs were collected from four turtles following the procedure of Verneau *et al.* (2011) at two distinct periods, the first period extending from day one to day seven after capture, the second period extending from day 37 to day 44 after capture. Eggs that were gathered were pooled and separated into eight samples, depending on the infected turtle and sampling period, and stored at -20°C until molecular assays. Finally, the four specimens of *T. s. elegans* were euthanized by cardiac injection of 10% sodium pentobarbitone following French national rules for invasive species (Euthapent, Kryon Laboratories, South Africa) and dissected to collect parasites of the pharyngeal cavity and the bladder. Worms were stored at -20°C until use.

### *DNA extraction*

Adult worms and egg samples were crushed with the help of a pestle in 300µl of ultra-pure water. Then, 300µl of Lysis Buffer and 25µl of 10mg/ml proteinase K were added and mixtures were heated at 56°C for one hour. The DNAs were extracted using the DNA extraction Maxwell LEV Simply DNA Tissue Kit (Promega) following the manufacturer's instructions and eluted in 30µl of Sigma water. Their quality was evaluated by electrophoresis migration on a 1% agarose gel and their concentration assessed by spectrophotometry at 260nm (Nanodrop ND-1000, Thermo Fisher scientific Inc., MA, USA).

### *Cross DNA experiments*

DNAs from species recovered from the pharyngeal cavity (*P. tunisiensis*, *P. oris* and *Polystomoides* sp.) were crossed by two in equal proportions with the DNAs of species recovered from the bladder (*N. euzeti*, *N. orbiculare* and *Neopolystoma* sp.) to simulate double infections. DNA from *Polystomoides* sp. was subsequently crossed with DNA from *N. orbiculare* in different proportions, respectively 1/10, 1/4, 1/2, 3/4 and 9/10, to simulate double infections with different parasite loads. Finally, DNAs from three polystome species, namely *Polystomoides* sp., *N. orbiculare* and *Neopolystoma* sp., were crossed to illustrate cases of multiple infestations.

### *Primers design and genetic divergences*

Partial 12S rRNA sequences of *Polystomoides* sp. (XXX) and *Neopolystoma* sp. (YYY) were obtained following the procedure described in H eritier *et al.* (2015). Complete alignment was performed using MEGA6 (Tamura *et al.* 2013) with the two former sequences and the four others extracted from Genbank, namely *P. tunisiensis* (KR856116), *P. oris* (KR856115), *N. euzeti* (KR856101) and *N. orbiculare* (KR856103). The HRM primers were designed from the two most conserved regions flanking a variable portion of the 12S rRNA gene (Figure 1), using Primer Express v2.0 software (Applied Biosystems). The resulting primers, 12SF (5'-TGTTTAAACCCAGGTCGATATGC-3') and 12SR (5'-AATGACGGGCGATGTGTACC-3') allowed the amplification of fragments of about 121 bp, depending of the species. Genetic divergences between each pair of sequences (Indels, transversions and transitions) were estimated with MEGA6 and reported in Table 1.

### *qPCR-HRM Assays*

Real time PCR were run in a 384-well plate on a LightCycler® 480 Instrument (Roche), in a final volume of 10µl with MgCl<sub>2</sub> at 3mM, each primer at 0.5µM, 1X Master Mix Resolight® dye and 15ng of genomic DNA. Amplification consisted of 10min of pre-incubation at 95°C followed by 40 cycles consisting in denaturation at 95°C for 15s (ramping rate, 4.4°C/s), touchdown annealing from 65°C to 53°C for 15s (ramping rate, 2.2°C/s) and extension at 72°C for 20s (ramping rate, 4.4°C/s). After the amplification, the HRM conditions consisted of a denaturing step at 95°C for 1min followed by a hybridization step at 40°C for 1min. Melting curves were then generated by ramping from 65°C to 95°C at 0.02°C/s, taking 25 acquisitions per each degree centigrade. A negative 'no-template' control was included in the qPCR-HRM assay to detect potential DNA contamination. Reactions were conducted in duplicate and experiments were repeated twice for validation of the results. HRM products obtained for reference species were subsequently sequenced using both HRM primers by the company Genoscreen (Lille, France) to confirm species identity.

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P.oris TGTTTTAACCCAGGTCGATATGCTGCTTATTAAAAGGTA-TACAATGCAATACATTAAAA
Polystomoides sp. ....-GTT.....
N.orbiculare .....G...G-AGT.G.....T..
Neopolystoma sp. ....GTAG..G.....T..
P.tunisiensis .....A.....T.....ATT..A...--TT.....GT..
N.euzeti .....A.....T.....ATT.....--TT...T.....T..

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P.oris ATTTTTTAGATTTAAATTTTAATAAATTGAGGACTTAGAAGTAATATAAAATGGTAATGT
Polystomoides sp. ...GA...A.....G.....G.T...A.....G...A.....
N.orbiculare .G....G.....T...A..-TG...A.....C.....A.....
Neopolystoma sp. .GG...G.....T...AN.ATG...A.....C.....A.....
P.tunisiensis .A.AA.ATAT....TA.A.TT.-T..A.....A.GG....
N.euzeti .AA.A.A.AT...T..AAA.TT.GTT..A.....A.....

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P.oris TTATGTGAAGTTTAGGTTTAGTTGAGGTACACATCGCCCGTCATT
Polystomoides sp. ....A..A.....
N.orbiculare .....A..A.....
Neopolystoma sp. ....A.C.....
P.tunisiensis ..G.....C.-.....A..AG.....
N.euzeti .....-.....A.....

```

Figure 1: Alignment 5'-3' of 12S rRNA amplicons within polystomes. Identical nucleotides are represented by '.' and indels by '-'. Areas used for the design of primers are shaded.

Table 1. Number of genetic changes between 12S rRNA amplicons for six polystome species. Indels = Insertions / Deletions; Tv = Tranversions; Ts = Transitions.

	<i>P. oris</i>	<i>Polystomoides</i> sp.	<i>N. orbiculare</i>	<i>Neopolystoma</i> sp.	<i>P. tunisiensis</i>
<i>Polystomoides</i> sp.	Indels = 0 Tv = 5; Ts = 9				
<i>N. orbiculare</i>	Indels = 1 Tv = 6; Ts = 12	Indels = 1 Tv = 7; Ts = 10			
<i>Neopolystoma</i> sp.	Indels = 1 Tv = 8; Ts = 10	Indels = 1 Tv = 9; Ts = 12	Indels = 2 Tv = 1; Ts = 4		
<i>P. tunisiensis</i>	Indels = 3 Tv = 20; Ts = 12	Indels = 2 Tv = 17; Ts = 8	Indels = 4 Tv = 18; Ts = 12	Indels = 4 Tv = 19; Ts = 14	
<i>N. euzeti</i>	Indels = 2 Tv = 21; Ts = 6	Indels = 2 Tv = 21; Ts = 5	Indels = 3 Tv = 22; Ts = 10	Indels = 3 Tv = 21; Ts = 13	Indels = 1 Tv = 7; Ts = 10



### *Data analyses and interpretation*

Data were analyzed using the HRM analysis tools implemented in the LightCycler® 480 Software v.1.5.0 (Roche, USA). Only samples successfully amplified with a Ct lower than 30 were considered for further analysis. Curve shapes differences between samples were discriminated with the Gene Scanning module of the LightCycler 480 v.1.5.0 Software (Roche, USA). Melting curve data were adjusted manually and normalized fluorescence conditions were established and adjusted, with the threshold set up at 0 and the sensitivity at 0.26. Two melting peaks differing by a  $T_m > 0.5^\circ\text{C}$  were considered as different. Similarities or differences in the melting curves and reproducibility of HRM results were assessed by comparing the root-mean square error (RMSE) values. RMSE values were calculated using the normalized fluorescence data from comparisons of intraspecific and interspecific inter-runs duplicata, as previously recommended by Naue *et al.* (2014).

## RESULTS

### *Genetic divergences within polystomes and melting curve references*

The number of genetic changes between 12S rRNA amplicons extended from 7 up to 37 (Table 1). The development of the HRM assay associated with this molecular marker allowed the discrimination of the six polystome species recovered from *M. leprosa*,  $T_m$  temperatures ranging from  $73.5^\circ\text{C}$  to  $76.5^\circ\text{C}$  (Figures 2A and 2B). Regardless of the species investigated, replicate reactions and experiments gave Ct values within each species that never varied more than a factor of 0.5 cycle (results not shown). The highest Ct values were observed for the two native species, i.e. *P. tunisiensis* and *N. euzeti*, probably because of the occurrence of two nucleotide mutations within the forward primer. Overall these results indicated good amplification efficiencies for all species. To the exception of *P. tunisiensis*, whose shape of the melting curve may reflect the presence of several melting domains (not shown), the melting curves of all the other species had a shape indicating the presence of a single melting domain. RMSE values inferred from intraspecific inter-runs were compared to RMSE values inferred from interspecific inter-runs and presented graphically (Figure 2C). These values showed significant differences between melting curves of each species.

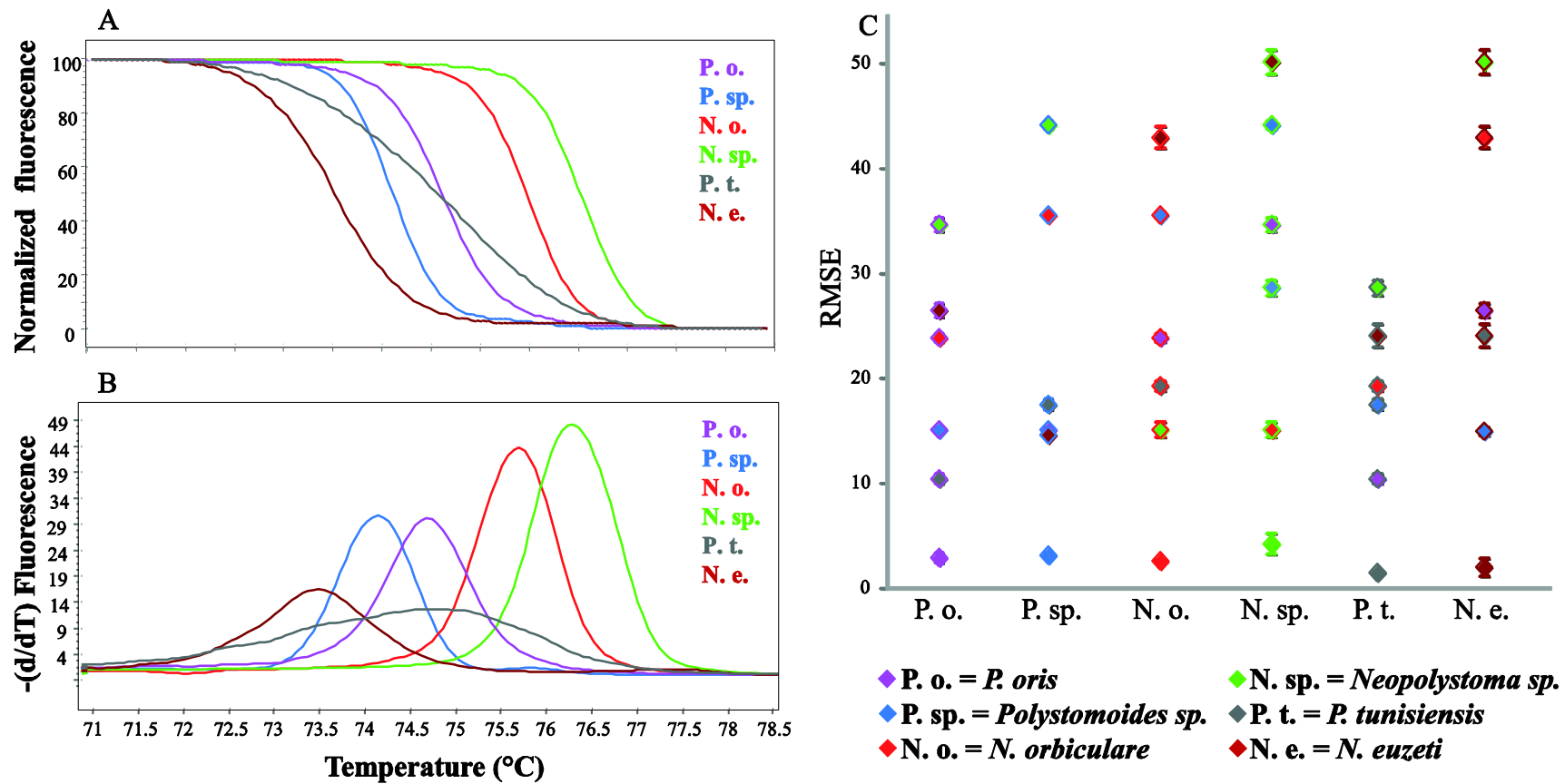


Figure 2 A: Normalized melting curves for the six polystome species. 2B. Melting peaks derived from normalized melting curves shown in A. 2C. RMSE values calculated from normalized fluorescence data of intraspecific and interspecific inter-runs duplicata.

### *Cross DNA experiments*

When DNAs from the exotic species of the pharyngeal cavity were crossed by two, in equal proportions, with DNAs from the exotic species of the bladder, two melting peaks were revealed, indicating the presence of the two distinct DNAs (see figure 3). A similar result was obtained when crossing by two the DNAs from the two native species, i.e. *P. tunisiensis* of the pharyngeal cavity and *N. euzeti* of the bladder. On the opposite, when DNAs from the exotic species of the pharyngeal cavity were crossed with DNA from *N. euzeti* on the one hand, and when DNAs from the exotic species of the bladder were crossed with DNA from *P. tunisiensis* on the other, a single peak characterizing the exotic species was systematically revealed for each crossing experiment. Overall these results indicated less efficient amplification levels in both native species. Furthermore when DNA of *Polystomoides* sp. was crossed with that of *N. orbiculare* in the proportions 1/4, 1/2 and 3/4, two peaks were revealed whose fluorescence intensity corresponded to the relative amounts of each DNA used in the experiment. However, when DNAs of the same species were crossed together in the proportions 1/10 and 9/10, respectively, one single peak was revealed corresponding to the DNA present in the highest amount. Finally, when DNAs from three distinct species were crossed in the same proportions, several peaks were revealed indicating the presence of several DNAs (Figure 3).

### *Parasite sampling from wild *T. s. elegans* and HRM assay on polystome eggs*

The number of polystome eggs collected from wild specimens of the red-eared slider during the two sampling periods and the number of parasite worms recovered after inspection of the bladder and pharyngeal cavity, are summarized in Table 2. For all the turtles examined, specimens of two polystome species were gathered and identified from DNA barcoding, namely *N. orbiculare* from the bladder and *Polystomoides* sp. from the pharyngeal cavity, which is in agreement with Meyer *et al.* (2015). HRM analyses showed contrasted results regarding surveyed turtles and polystome eggs samples. On *T. s. elegans* number 1 (Tse 1), regardless of the sampling period, one single species was revealed, i.e. *Polystomoides* sp. On Tse 2, the two species were revealed, however the fluorescence intensity was higher for *N. orbiculare* than *Polystomoides* sp. during the first sampling period. Inversely, it was lower for *N. orbiculare* than *Polystomoides* sp. during the second sampling period. On Tse 3, although the two species were revealed during the first sampling period, the fluorescence intensity was higher for *N. orbiculare* than *Polystomoides* sp. During the second sampling period, one single species was revealed, i.e. *Polystomoides* sp. On Tse 4, *N. orbiculare* was the single species revealed during the first sampling period, while *Polystomoides* sp. was the single species detected during the second sampling period (Table 3)

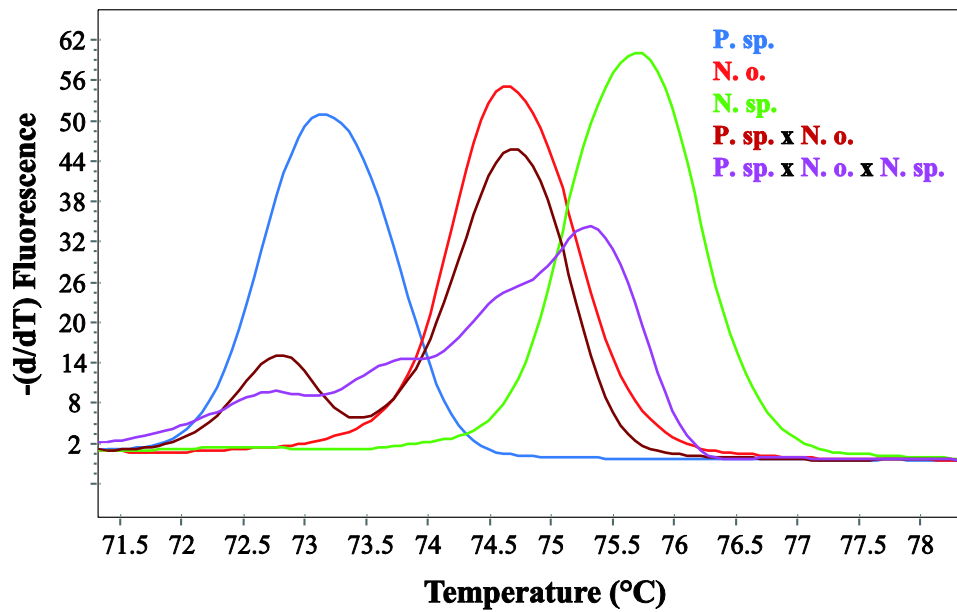


Figure 3: Melting peaks for the reference species (*Polystomoides* sp., *N. orbiculare* and *Neopolystoma* sp.), for a mixture of two DNAs (*Polystomoides* sp. x *N. orbiculare*) and for a mixture of three DNAs (*Polystomoides* sp. x *N. orbiculare* x *Neopolystoma* sp.).

Table 2. Polystome eggs and worms collected from distinct specimens of *T. s. elegans* (Tse 1 to Tse 4). Eggs were sampled at two distinct periods of time and worms were subsequently recovered following turtles dissection.

	<u>Number of eggs</u>		<u>Number of worms</u>	
	First sampling	Second sampling	Pharyngeal cavity	bladder
Tse 1	32	50	5	3
Tse 2	50	50	9	6
Tse 3	48	50	5	5
Tse 4	3	30	9	9

Table 3. Parasite species detected by HRM analysis on polystome eggs collected from Tse 1 to Tse 4 during first and second sampling periods. \* indicates the species for which the melting peak had the highest fluorescence intensity.

	First sampling	Second sampling
Tse 1	<i>Polystomoides</i> sp.	<i>Polystomoides</i> sp.
Tse 2	<i>Polystomoides</i> sp. + <i>N. orbiculare</i> *	<i>Polystomoides</i> sp.* + <i>N. orbiculare</i>
Tse 3	<i>Polystomoides</i> sp. + <i>N. orbiculare</i> *	<i>Polystomoides</i> sp.
Tse 4	<i>N. orbiculare</i>	<i>Polystomoides</i> sp.

## DISCUSSION

### *Experimental HRM assays for identification of parasites from endangered wildlife*

The first steps in assessing global biodiversity embrace specimens' collection and species recognition. Whereas the sampling of free living organisms can be an increasingly difficult task regarding the conservation status of some species, surveying endoparasites without host sacrifice can be even more difficult, or impossible. Therefore, the best way to assess internal parasites of threatened species involves parasite eggs detection. However, if the description of parasite species from the study of adult worms can be a difficult challenge regarding the paucity of good apomorphic morphological characters, species identification from the study of eggs morphology has never been completed. Consequently molecular technics are still the best suitable approaches to carry out taxonomic research in parasitology. Nonetheless, analyzing a great number of parasite eggs using DNA barcodes, which entails the sequencing of large DNA samples, can be time-consuming and costly. For this reason, the HRM molecular method was applied for the study of parasite diversity from pools of eggs. This approach relies on the determination of reference melting curves that derive from target sequences. Those ones must be sufficiently variable to get species-specific T<sub>m</sub> temperatures, but rather conserved for the design of amplification primers. The selected gene along this study, namely the mitochondrial 12S rRNA gene, was well appropriate for species recognition, since it allowed obtaining species-specific T<sub>m</sub> temperatures for all six investigated polystome species.

Because co-infections of wildlife are the rule (Bordes and Morand, 2011), eggs usually reflect the production of several distinct parasite species. We therefore performed cross DNA experiments in order to simulate double infections within hosts. When DNA from one exotic polystome species was crossed in equal

proportion with DNA of another exotic species, the two DNAs were systematically revealed demonstrating the usefulness of the HRM tool for the detection of double infections. If a similar outcome was obtained when crossing the DNAs of both native parasite species, inversely, a single DNA was revealed when crossing the DNAs of native with exotic polystome species, suggesting the presence of a single parasite species, i.e. the exotic species. Because the consensus sequence used for the design of the forward HRM primer differed from the nucleotide sequence of the two native target parasite species by two mutations (Figure 1), it could explain the decrease of the amplification efficiency of both species. This explanation holds as Ct values of the exotic species were lower than Ct values of the native ones. Similarly, when DNA from one exotic polystome species was crossed with DNA of another exotic species at a different concentration, the two DNAs were not systematically revealed. These results would indicate that double infections can be detected from HRM analysis only if parasite intensities of the less abundant species represent at least 1/4 of the total number of parasites. Finally, the HRM pattern resulting from the crossing of three distinct DNAs would indicate that multiple infections could be also detected, though the number of parasite species infecting host is difficult to evaluate. Overall, if the HRM molecular tool can be usefulness for characterizing double or multiple parasitic infections within host, some limitations were highlighted. HRM primers, first of all, must be 100% identical to the target gene sequence of all species if one may evidence all the species involved in the infection. Secondly, if one species is less abundant than another one, it may not be detected from an HRM analysis conducted on parasite eggs released by hosts. In conclusion, this approach may be well appropriate for preliminary studies based on parasite diversity among endangered wildlife as it gives a global overview of the parasite richness.

#### *HRM assays for parasite identification in wild environments*

Regarding the limitations of the HRM molecular approach discussed above, we explored the polystome diversity of the read-eared slider from French natural environments. This study was performed on pools of polystome eggs that were collected from four specimens of turtles co-infected with *Polystomoides* sp. and *N. orbiculare*. Though Tse 1 was infected with the two polystome species in the proportions of about 2/3 of *Polystomoides* sp. and 1/3 of *N. orbiculare*, only *Polystomoides* sp. was detected from HRM analysis, regardless of the sampling period. On the opposite, both polystome species were detected from Tse 2 during sampling periods 1 and 2, whereas it was also infected by the two parasite species in the same proportions as those reported for Tse 1. Tse 3 and Tse 4, which were infected by the two parasite species in the same proportions, i.e. 1/2 of *Polystomoides* sp. and 1/2 of *N. orbiculare*, also showed contrasted results. If the two polystome species were detected in Tse 3 during the first sampling period, a single polystome species, i.e. *Polystomoides* sp., was detected during the second

sampling period. Within Tse 4, two polystome species were also detected, however only *N. orbiculare* was identified during the first sampling period, and only *Polystomoides* sp. was identified during the second sampling period. Overall, these results were unpredicted regarding infection levels of both parasite species (see Table 2). We expected to reveal the presence of both species within each turtle regardless of the sampling period, which was actually not the case. In fact, the most predominant parasite species that was revealed during the first sampling period was *N. orbiculare*, while it was *Polystomoides* sp. during the second sampling period. This suggests that the cycle of eggs production is not regular over time and may depend on several factors, among which the target parasite species and the ecological niche where parasites reproduce sexually. Badets *et al.* (2010) showed from field studies that eggs production of polystomes infecting the stripeless tree frog, *Hyla meridionalis* Boettger (1874), was intimately related to the frogs' ecology and physiology. Badets (unpublished data) also showed from the same host-parasite interaction that the patterns of polystome eggs releases were different regarding the preference of parasites towards their hosts, male or female, suggesting that host physiology was indeed an important factor influencing parasite eggs production. Thereby, monitoring parasites from wild endangered animal populations, but also from captive specimens used in breeding programs, must imply periodic parasitological surveys to avoid false negative diagnostics based solely on eggs production. This is also very important in a veterinary framework, especially because the international pet trade on wild animals can lead to the introduction of non-native parasite species and to the emergence of new infectious diseases.

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## REFERENCES

- Badets, M., Morrison, C. and Verneau, O.** (2010). Alternative parasite development in transmission strategies: how time flies! *Journal of Evolutionary Biology* **23**, 2151-2162.
- Barry, J., McLeish, J., Dodd, J.A., Turnbull, J.F., Boylan, P. and Adams, C.E.** (2014). Introduced parasite *Anguillicola crassus* infection significantly impedes swim bladder function in the European eel *Anguilla anguilla* (L.). *Journal of Fish Diseases* **37**, 921-924.
- Berthier, P., Du Preez, L.H., Raharivololoniana, L., Vences, M. and Verneau, O.** (2014). Two new species of polystomes (Monogenea: Polystomatidae) from the anuran host *Guibemantis liber*. *Parasitology International* **63**, 108-119.
- Besansky, N.J., Severson, D.W. and Ferdig, M.T.** (2003). DNA barcoding of parasites and invertebrate disease vectors: what you don't know can hurt you. *Trends in Parasitology* **19**, 545-546.
- Bordes, F. and Morand, S.** (2010). The impact of multiple infections on wild animal hosts: a review. *Infection Ecology and Epidemiology* **1**, 7346.
- Eszterbauer, E., Marton, S., Rácz, O.Z., Letenyei, M. and Molnár, K.** (2006). Morphological and genetic differences among actinosporean stages of fish-parasitic myxosporeans (Myxozoa): difficulties of species identification. *Systematic Parasitology* **65**, 97-114.
- Gómez, A. and Nichols, E.** (2013). Neglected wild life: Parasitic biodiversity as a conservation target. *International Journal for Parasitology: Parasites and Wildlife* **2**, 222-227.
- Gozlan, R., St-Hilaire, S., Feist, S.W., Martin, P. and Kent, M.L.** (2005). Disease threat to European fish. *Nature* **435**, 1046.
- Groom, M.J.** (2005). Threats to biodiversity. Sinauer Associates, Inc.
- Gundry, C.N., Vandersteen, J.G., Reed, G.H., Pryor, R.J., Chen, J. and Wittwer, C.T.** (2003). Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. *Clinical Chemistry* **49**, 396-406.
- Hebert, P.D.N., Cywinska, A., Ball, S.L. and DeWaard, J.R.** (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B: Biological Sciences* **270**, 313-321.
- Héritier, L., Badets, M., Du Preez, L.H., Aisien, M.S.O., Lixian, F., Combes, C. and Verneau, O.** (2015). Evolutionary processes involved in the diversification of chelonian and mammal polystomatid parasites (Platyhelminthes, Monogenea, Polystomatidae) revealed by palaeoecology of their hosts. *Molecular Phylogenetics and Evolution* **92**, 1-10.



- Héritier, L., Valdeón, A., Sadaoui, A., Gendre, T., Ficheux, S., Bouamer, S., Kechemir-Issad, N., Du Preez, L.H., Palacios, C. and Verneau, O.** (2016). Introduction and invasion of the red-eared slider and its parasites in freshwater ecosystems of Southern Europe: risk assessment for the European pond turtle in wild environments. Submitted to *Biological Invasions*.
- Iglesias, R., García-Estévez, J., Ayres, C., Acuña, A. and Cordero-Rivera, A.** (2015). First reported outbreak of severe *spirorchidiasis* in *Emys orbicularis*, probably resulting from a parasite spillover event. *Diseases of Aquatic Organisms* **113**, 75-80.
- Lay, M.J. and Wittwer, C.T.** (1997). Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR. *Clinical Chemistry* **43**, 2262-2267.
- Meyer, L., Du Preez, L.H., Bonneau, E., Héritier, L., Quintana, M., Valdeón, A., Sadaoui, A., Kechemir-Issad, N., Palacios, C. and Verneau, O.** (2015). Parasite host-switching from the invasive American red-eared slider, *Trachemys scripta elegans*, to the native Mediterranean pond turtle, *Mauremys leprosa*, in natural environments. *Aquatic Invasions* **10**, 79-91.
- Morick, D., Baneth, G., Avidor, B., Kosoy, M.Y., Mumcuoglu, K.Y., Mintz, D., Eyal, O., Goethe, R., Mietze, A., Shpigel, N. and Harrus, S.** (2009). Detection of *Bartonella* spp. in wild rodents in Israel using HRM real-time PCR. *Veterinary Microbiology* **139**, 293-297.
- Naue, J., Hansmann, T. and Schmidt, U.** (2014). High-Resolution Melting of 12S rRNA and Cytochrome b DNA sequences for discrimination of species within distinct European animals families. *PLoS ONE* **9**, e115575.
- Ngui, R., Lim, Y.A.L. and Chua, K.H.** (2012). Rapid detection and identification of human hookworm infections through high resolution melting (HRM) analysis. *PLoS ONE* **7**, e41996.
- Palacios, C., Urrutia, C., Knapp, N., Quintana, M.F., Bertolero, A., Simon, G., Du Preez, L.H. and Verneau, O.** (2015). Demographic structure and genetic diversity of *Mauremys leprosa* in its northern range reveal new populations and a mixed origin. *Salamandra* **51**, 221-230.
- Pangasa, A., Jex, A.R., Campbell, B.E., Bott, N.J., Whipp, M., Hogg, G., Stevens, M.A. and Gasser, R.B.** (2009). High resolution melting-curve (HRM) analysis for the diagnosis of cryptosporidiosis in humans. *Molecular and Cellular Probes* **23**, 10-15.
- Polo-Cavia, N., Lopez, P. and Martin, J.** (2009). Interspecific differences in chemosensory responses of freshwater turtles: consequences for competition between native and invasive species. *Biological Invasions* **11**, 431-440.

- Polo-Cavia, N., Lopez, P. and Martin, J.** (2010). Competitive interactions during basking between native and invasive freshwater turtle species. *Biological Invasions* **12**, 2141-2152.
- Polo-Cavia, N., Lopez, P. and Martin, J.** (2011). Aggressive interactions during feeding between native and invasive freshwater turtle species. *Biological Invasions* **13**, 1387-1396.
- Ririe, K.M., Rasmussen, R.P. and Wittwer, C.T.** (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical Biochemistry* **245**, 154-160.
- Robertson, T., Bibby, S., O'Rourke, D., Belfiore, T., Agnew-Crumpton, R. and Noormohammadi, A.H.** (2010). Identification of chlamydial species in crocodiles and chickens by PCR-HRM curve analysis. *Veterinary Microbiology* **145**, 373-379.
- Sala, O.E., Chapin III, F.S. and Armesto, J.J.** (2000). Global biodiversity scenarios for the year 2100. *Science* **287**, 1770-1774.
- Simberloff, D.** (2013). Biological invasions: Prospects for slowing a major global change. *Elementa* **1**, 000008.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S.** (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725-2729.
- Tong, S.Y.C. and Giffard, P.M.** (2012). Microbiological applications of high-resolution melting analysis. *Journal of Clinical Microbiology* **50**, 3418-3421.
- UICN France, MNHN & SHF** (2015). La liste rouge des espèces menacées en France - Chapitre reptiles et amphibiens de France métropolitaine. Paris, France.
- Van Dijk, P.P., Iverson, J.B., Rhodin, A.G.J., Shaffer, H.B. and Bour, R.** (2014). Turtles of the world, 7<sup>th</sup> edition: Annotated checklist of taxonomy, synonymy, distribution, and conservation status. *Chelonian Research Monographs* **5**, 329-479.
- Verneau, O., Palacios, C., Platt, T., Alday, M., Billard, E., Allienne, J.F., Basso, C. and Du Preez, L.H.** (2011). Invasive species threat: parasite phylogenetics reveals patterns and processes of host-switching between non-native and native captive freshwater turtles. *Parasitology* **138**, 1778-1792.
- Vitousek, P.M., Mooney, H.A., Lubchenco, J. and Melillo, J.M.** (1997). Human domination of Earth's ecosystems. *Science* **277**, 494-499.
- Wittwer, C.T., Reed, G.H., Gundry, C.N., Vandersteen, J.G. and Pryor, R.J.** (2003). High-resolution genotyping by amplicon melting analysis using LCGreen. *Clinical Chemistry* **49**, 853-860.

**CHAPITRE IV. DEMONSTRATING THE VALUE AND  
IMPORTANCE OF COMBINING DNA BARCODES  
AND DISCRIMINANT MORPHOLOGICAL  
CHARACTERS FOR POLYSTOME TAXONOMY  
(PLATYHELMINTHES, MONOGENEA)**

## Présentation du Chapitre IV

Des études moléculaires (Verneau et al. 2011; Meyer et al. 2015; Héritier et al. Submitted) ont montré la présence de deux espèces de polystome infectant le pharynx des tortues exotiques et natives dans des espaces confinés ou naturels en France. Parce que ces deux espèces de parasites ont été également identifiées sur *Trachemys scripta* dans les écosystèmes aquatiques américains et parce que *E. orbicularis* et *M. leprosa* n'ont jamais été observées dans ces écosystèmes, elles ont été considérées comme des espèces parasites invasives en France, introduites par *T. s. elegans*. De ce fait, il s'est avéré primordial de les décrire dans un contexte de surveillance des invasions parasitaires dans les écosystèmes français.

La description d'espèces implique l'analyse de caractères morphologiques. Cependant, chez les parasites, beaucoup de caractères ne sont pas réellement discriminants, surtout concernant les comparaisons d'espèces de polystomes au sein d'un même genre. Si d'autres critères comme la spécificité de l'hôte, longtemps reconnue chez les polystomes (Du Preez and Kok 1997) furent utilisés pour justifier la description de nouvelles espèces, des études moléculaires plus récentes ont montré des transferts d'hôtes de ces parasites en milieux confinés et naturels (Verneau et al. 2011; Meyer et al. 2015; Héritier et al. Submitted).

Les comparaisons des séquences du gène COI chez ces deux nouvelles espèces parasites ont illustré une divergence génétique nettement supérieure au seuil fixé dans l'étude d'Héritier et al. (Submitted), utilisé pour la discrimination d'espèces chez les polystomes. Après une formation sur la morphologie et la taxonomie des polystomes suivie en Afrique du Sud sous la direction de Louis du Preez, il s'est avéré que le nombre d'épines génitales et la forme du testicule pourraient être des caractères discriminants. Ainsi, cette étude propose la distinction et la description de deux nouvelles espèces en utilisant des caractères morphologiques discriminants et moléculaires.

**Title:** Demonstrating the value and importance of combining DNA barcodes and discriminant morphological characters for polystome taxonomy (Platyhelminthes, Monogenea)

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## **Abstract**

Polystomes are monogenean parasites that infest mainly semi aquatic vertebrates, such as amphibians and chelonians. Owing to the lack of discriminative morphological characters and because polystomes are considered to be strictly host- and site-specific, host identity is often used as an additional character for parasite identification. Recent genetic studies, however, show that polystomes infecting freshwater turtles in outdoor turtle enclosures and natural environments, are not strictly host-specific. Therefore, we proposed a new procedure for turtle polystome taxonomy based on the combination of Cytochrome c Oxydase I sequences and two discriminant morphological characters, namely the number of genital spines and the testis shape. We tested the validity of this procedure with *Polystomoides oris*, which was collected from the pharyngeal cavity of the American painted turtle *Chrysemys picta* and two undescribed species, both collected from the pharyngeal cavity of the American slider *Trachemys scripta* and two other European turtles, namely the European pond turtle *Emys orbicularis* and the Mediterranean turtle *Mauremys leprosa*. A Principal Component Analysis based on both morphological characters allowed the separation of all specimens in three morphological groups, which matched well with the molecular data. As a result, we describe two new polystome species, i.e., *Polystomoides soledensis* n. sp. and *Polystomoides scriptanus* n. sp.

## 1. Introduction

The Polystomatidae *sensu* Sinnappah et al. [1] includes 25 genera that parasitize mainly semi-aquatic sarcopterygian hosts such as the Australian lungfish, amphibians, freshwater turtles and the African hippopotamus. With less than 200 species reported [2], the Polystomatidae can be considered a relictual family whose origins date back to the transition between strictly aquatic to semi-aquatic vertebrates, i.e., to about 425 Million years ago (Mya) [3]. In this scenario, polystomes would have primarily diversified within amphibians and secondarily switched from caecilians to chelonians at about 180 Mya [4]. Polystomes have a direct life cycle involving free swimming infective larvae, known as oncomiracidia. Whereas polystomes are recovered mostly from the bladder of amphibian hosts, they can be found in the bladder, pharyngeal cavity or conjunctival sacs of chelonian hosts. To date, 19 genera of polystomes are recognized as infecting amphibians, while only four are known parasites of freshwater turtles, i.e. *Polystomoides* Ward, 1917, *Polystomoidella* Price, 1939, *Neopolystoma*, Price, 1939 and *Uropolystomoides* Tinsley and Tinsley, 2016. From a taxonomic point of view, while polystome genera are mostly distinguished by morphological autapomorphies, species discrimination within genera is more subjective as it relies mainly on morphological characters that are not discriminative. Therefore, host species is often used as an additional character to describe new species [5, 6], under the assumption that polystomes are strictly host- [7] and site-specific [2].

Regarding the polystome database of Verneau [2], about 100 polystome species were reported within amphibians while only about 50 were described from chelonians. Although many species of polystomes can be viewed globally as host-specific, some species infecting amphibians were reported from more than a single host species, among which *Polystoma africanum* (Szidat 1932), which was described from *Sclerophrys regularis* (Reuss 1833) in Liberia and further reported within *Ptychadena mascareniensis* (Duméril and Bibron 1841) in Uganda. Conversely, some amphibians were found infected by more than a single polystome species, like *S. regularis*, which was found infected by *Eupolystoma alluaudi* (de Beauchamp 1913) in Togo, Tanzania, Cameroon and Nigeria and by *P. africanum* in Togo, Liberia, Sierra Leone and Ethiopia. Similarly, some parasite species infecting turtles were also reported from more than a single host species. This is the case of *Polystomoides coronatum* (Leydi 1888), which was described from the pharyngeal cavity of *Trachemys dorbigni* (Duméril and Bibron 1835) and later reported from the same ecological niche of *Actinemys marmorata* (Baird and Girard 1852), *Chrysemys picta* (Schneider 1783) and *Apalone spinifera* (Lesueur 1827), from the pharyngeal cavity and the bladder of *Trachemys scripta elegans* (Wied 1839), and from

the conjunctival sacs of *Apalone ferox* (Schneider 1783) and *Graptemys pseudogeographica* (Gray 1831). Finally, whereas polystomes of chelonians are mostly considered as host- and site-specific, instances of parasite infection within the same ecological habitat of the host are also reported. This was illustrated with *Polystomoides uruguayensis* [8] and *Polystomoides fuquesi* [9] that were both described from the pharyngeal cavity of a single host species, i.e., *Phrynops hilarii* (Duméril and Bibron 1835).

Recent genetic surveys that were performed among polystomes sampled from turtles in outdoor turtle enclosures and natural environments revealed that these parasites were always site-specific [10-12]. Likewise, never more than a single polystome species was found within an ecological niche of the same turtle. However, polystomes are not universally host-specific. This is highlighted by two European native turtle species that were found infected by non-native polystome species, following translocation of American turtles into Southern European freshwater ecosystems [11, 12]. These results thus challenged conventional wisdom, with the occurrence of the same polystome species in distinct ecological microhabitats of discrete host species on the one hand, and the presence of more than one polystome species infecting the same habitat of the same host species, on the other [2]. If host- and site-specificity can no longer be assumed, one may question the validity of numerous morphological characters that have been used in the discrimination of polystome species.

In the present study, we propose a new procedure for turtle polystome taxonomy, which is based on a combination of Cytochrome c Oxydase I (COI) sequences and discriminant morphological characters. From a global barcoding analysis, which includes hundreds of haplotypes characterizing more than 30 distinct polystome species infecting freshwater chelonians, we studied polystomes of the pharyngeal cavity of distinct host species, among which *Polystomoides oris* Paul 1938 of the painted turtle *C. picta* and undescribed specimens collected from the slider *Trachemys scripta*, the European pond turtle *Emys orbicularis* (Linnaeus 1758) and the Mediterranean turtle *Mauremys leprosa* (Schweigger 1812), to test the validity of our approach. Our objectives were to test the taxonomic approach of describing new polystome species based on molecular data and polystome photographs for objective comparisons.



## 2. Materials and Methods

### 2.1. Parasite sampling

Polystome eggs and/or worms were collected from three distinct freshwater turtle species, namely *E. orbicularis*, *T. scripta* and *C. picta*. Polystome samples from *E. orbicularis* were collected from a single turtle trapped in an outdoor turtle enclosure at Sorède (France) in May 2006. Polystome samples from *T. scripta* were collected from two turtles captured in natural environments of Florida (USA) in May 2004 and from a single turtle also captured in natural environments of North Carolina (USA) in July 2015. Polystome samples from *C. picta* were collected from a single turtle of freshwater ecosystems of North Carolina (USA) in July 2015.

### 2.2. Molecular and Phylogenetic analysis

Almost all COI sequences that were used for the phylogenetic analysis of chelonian polystomes came from the study of Héritier et al. [12]. We completed the data set with new sequences that were obtained from polystome eggs and adult worms, following the molecular procedure described in Héritier et al. [12]. Sequence alignment was performed with Clustal W [13] implemented in MEGA6 [14]. A GTR + I +  $\Gamma$  model of evolution was selected from the Akaike Information Criterion (AIC) with Modeltest 3.06 [15]. Three partitions were defined according to codon positions 1, 2 and 3, and model parameters were estimated independently for each partition. The phylogenetic tree was then inferred from a Bayesian analysis using MrBayes software [16], with four chains of ten million generations each, sampled every 100 cycles. Bayesian posterior probabilities were estimated after removing the first 10000 trees as the burn-in-phase.

### 2.3. Genetic divergence threshold for species identification

The threshold of 3.4% determined by Héritier et al. [12] from the cumulative error plot based on Kimura 2-parameters (K2P) distances was used for polystome discrimination.

### 2.4. Morphological and morphometrical analyses

Polystomes under investigation were washed free of fixative, stained overnight in a weak solution of acetocarmine, dehydrated, cleared in xylene and mounted in Canada balsam. Specimens were observed and photographed using Nikon Eclipse E800 compound and Nikon AZ100 microscopes. The number of hamuli and the location of polystomes in the host helped for genus determination. Length and width of testis were measured and the ratio determined. Genital spines, which are located within the copulatory bulb, were counted. These two

variables were finally used in an unconstrained Principal Component Analysis (PCA) using Canoco software v.5. A discriminant analysis using Wilks' Lambda was performed with SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA) to test for statistical significance of the results. Additional body morphometrics were also measured using Nikon NIS elements program to complement the description. Drawings based on photographs taken from type-materials were done with Adobe Illustrator software.

### 3. Results

#### 3.1. COI haplotype diversity and polystome species delineation

Five adult worms and 15 polystome eggs, which were collected from five distinct sliders, were processed for COI sequencing. Four haplotypes were identified: H64, H104, H107 and H118 (Table 1). According to the phylogenetic tree (Fig. 1) and the genetic threshold defined by Héritier et al. [12], three distinct polystome species, namely *N. orbiculare* (H104) and two undescribed species named here provisionally and for convenience *Polystomoides* sp1 (H64) and *Polystomoides* sp4 (H107 and H118) were characterized within that host.

The three polystome species under morphological investigation, i.e., *P. oris*, *Polystomoides* sp1 and *Polystomoides* sp4, comprised several distinct haplotypes. Concerning *P. oris*, parasite samples used for the molecular analysis were collected from *C. picta* in American freshwater ecosystems (H11, H12, H97 to H99), from *E. orbicularis* in Southern European freshwater environments (H15 and H63) and in outdoor turtle enclosures (H14 and H15), from *M. leprosa* in outdoor turtle enclosures (H14, H15, H33 and H34) and from *T. scripta* in outdoor turtle enclosures (H15 and H115) [10-12]. Concerning *Polystomoides* sp1, parasite samples were collected from *T. scripta* in American (H47 and H49) and French freshwater ecosystems (H16, H64 and H77), from *E. orbicularis* in outdoor turtle enclosures (H16) and from *M. leprosa* in French freshwater environments (H16) [10-12]. Finally, concerning *Polystomoides* sp4, parasite samples were collected from *T. scripta* in natural American environments (H50 and H51) and in outdoor turtle enclosures (H35) and from *M. leprosa* in outdoor turtle enclosures (H35) [10-12]

Table 1. COI haplotype diversity of polystomes sampled from <i>Trachemys scripta</i> in natural environments of North Carolina (USA)								
Host species	Locality / Province / Country	Host number	Parasite sample	Polystome DNA number	COI Haplotype	Parasite species	Infection site	Genbank Accession number
<i>Trachemys scripta</i>	North Carolina / USA	RL150727F3	7 eggs	MiAD849, MiAD850, MiAD852, MiAD853, MiAD871, MiAD873, MiAD875	H64	<i>Polystomoides</i> sp1	Pharyngeal cavity	KY569080- KY569086
		RL150727F3	1 egg	MiAD872	H104	<i>Neopolystoma orbiculare</i>	Bladder	KY569087
		RL150721B1	1 worm	MiAD823	H104	<i>N. orbiculare</i>	Bladder	KY569088
		RL150727F4	1 worm, 2 eggs	MiAD824, MiAD869, MiAD870	H104	<i>N. orbiculare</i>	Bladder	KY569089- KY569091
		RL150723F1	1 worm	MiAD822	H107	<i>Polystomoides</i> sp4	Pharyngeal cavity	KY569092
		RL150723F1	2 worms	MiAD855, MiAD856	H118	<i>Polystomoides</i> sp4	Pharyngeal cavity	KY569093, KY569094
		RL150725C1	5 eggs	MiAD843, MiAD844, MiAD845, MiAD846, MiAD848	H118	<i>Polystomoides</i> sp4	Pharyngeal cavity	KY569095- KY569099

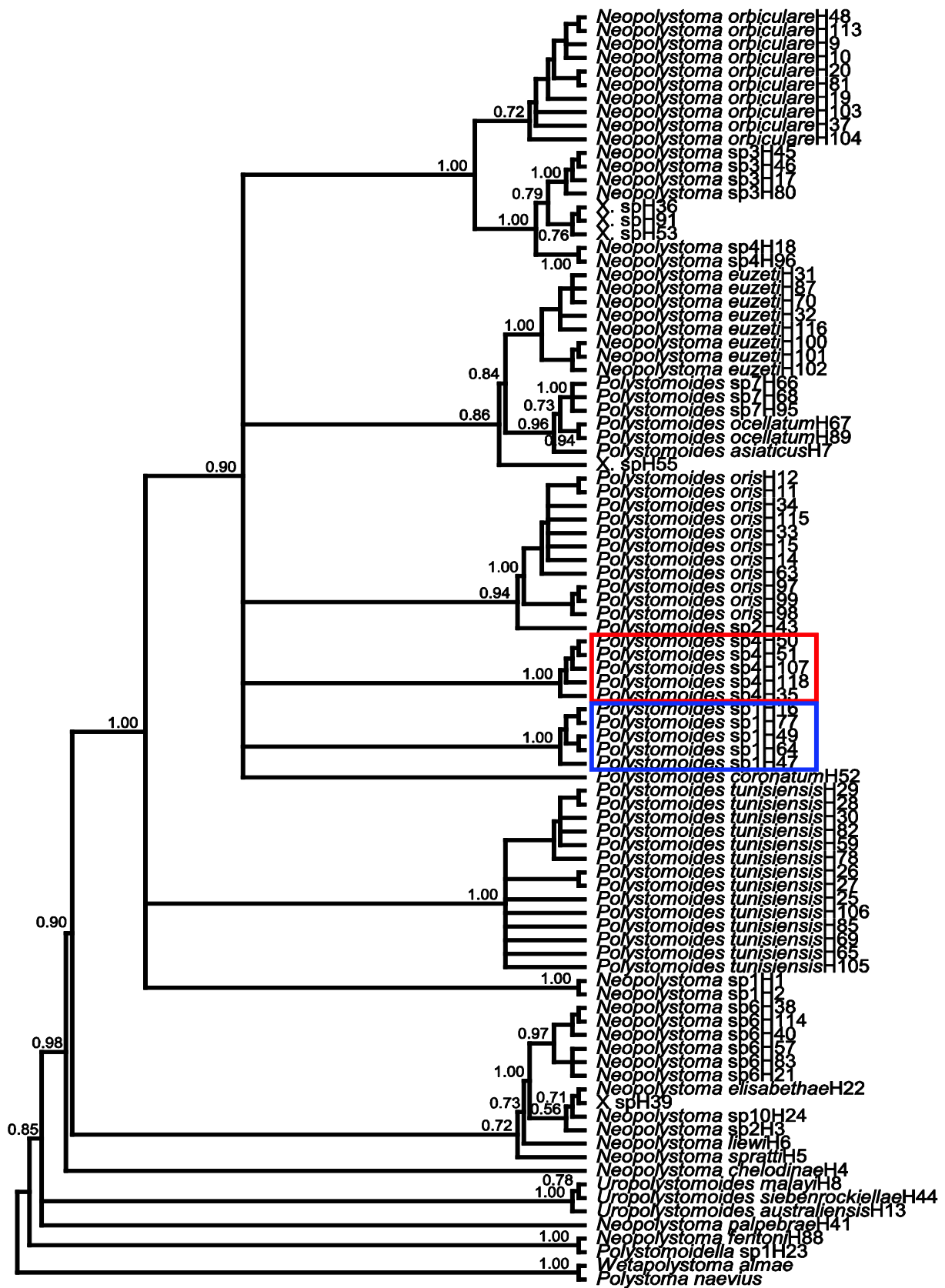


Figure 1. Bayesian tree inferred from the analysis of COI. Numbers at nodes correspond to Bayesian Posterior Probabilities. Species under investigation are framed in blue and in red.

### 3.2. Morphological species identification

Because more than a single polystome species has never been found in the same ecological niche of the same host [10-12], all specimens that were selected for morphology, with the exception of a single specimen that was processed in both molecular and morphological assays, came from turtles in which polystomes were previously investigated for molecular analysis. We studied three specimens of *P. oris* that were collected from *C. picta* (Fig. 2 and table 2), seven specimens of *Polystomoides* sp1 that were collected from *E. orbicularis* (Figs. 3 and 4 and table 2) and five specimens of *Polystomoides* sp4 that were collected from *T. scripta* (Figs. 5 and 6 and table 2). Specimen MNHNb3 was excluded from the PCA since the testis was obscured and could not be measured accurately. The presence of two pairs of hamuli and polystome location in the pharyngeal cavity indicated that all specimens belonged to *Polystomoides*. The PCA based on the genital spine number and the testis shape allowed the separation of all specimens in three morphological groups (Fig. 7). The P value of 0.013 from the Wilks' Lambda test indicated that the selected characters were significantly discriminant. Morphological groups identified using these methods were consistent with species discrimination based on the phylogenetic analysis of haplotypes. We therefore describe the two undescribed species (see below).

*Polystomoides soledensis* n. sp. (ex *Polystomoides* sp1, Figs. 3 and 4 and table 2)

**Specimens studied:** Seven specimens were studied morphologically. The holotype (MNHNal) and one paratype (MNHNal2) were deposited at the National Museum of Natural History, Paris, France and five paratypes (NMB P430-434) were deposited in the Parasitic Worm Collection, National Museum, Aliwal Street, Bloemfontein, South Africa.

**Type host:** *Emys orbicularis*.

**Type locality:** Outdoor turtle enclosures of the "Vallée des Tortues" at Sorède (France).

**Additional hosts and localities:** *Mauremys leprosa* in French aquatic ecosystems (Pyrénées Orientales); *Trachemys scripta* in French (Pyrénées Orientales) and American aquatic ecosystems (Indiana, Maine and North Carolina).

**Site:** Pharyngeal cavity.

**Etymology:** This parasite was named after the Turtle farm of Sorède in France where it was originally collected from *E. orbicularis*.

**Genetic diversity:** H16, H47, H49, H64, H77.

**Description:** Six haptorial suckers; two pairs of hamuli; marginal hooklets; vitellarium spread throughout the body length except in the genital, anterior and posterior parts of the parasite;

single testis; two lateral vaginae; a single pear shaped egg; number of genital spines ranged between 35 and 37; pharynx muscular.

**Additional body morphometrics:** See table 3.

*Polystomoides scriptanus* n. sp. (ex *Polystomoides* sp4, Figs. 5 and 6 and table 2)

**Specimens studied:** Five specimens were studied morphologically. The holotype (MNHNb1) and two paratypes (MNHNb2-3) were deposited at the National Museum of Natural History, Paris, France and two paratypes (NMB P435-436) were deposited in the Parasitic Worm Collection, National Museum, Aliwal Street, Bloemfontein, South Africa.

**Type host:** *Trachemys scripta*.

**Type locality:** Gainesville, Florida (USA).

**Additional hosts and localities:** *T. scripta* in American aquatic ecosystems (North Carolina) and in outdoor turtle enclosures (France); *Mauremys leprosa* in outdoor turtle enclosures (France).

**Site:** Pharyngeal cavity.

**Etymology:** This parasite was named after its host *T. scripta*, which was assumed to be the original host species.

**Genetic diversity:** H35, H50, H51, H107, H118.

**Description:** Six haptorial suckers; two pairs of hamuli; marginal hooklets; vitellarium spread throughout the body length except in the genital, anterior and posterior parts of the parasite; single testis; two lateral vaginae; a single pear shaped egg; number of genital spines ranged between 33 and 34; pharynx muscular.

**Additional body morphometrics:** See table 3.

Host species (host number)	<i>Polystomoides</i> species under investigation	Parasite number used in morphology	Official National Museum Number	Parasite number used in genetics	Locality	Pairs of hamuli	Number of genital spines	Testis length (µm)	Testis width (µm)	Testis ratio (Length/Width)
<i>C. picta</i> (RL150728C1)	<i>Polystomoides oris</i>	PL150729D2			North Carolina (USA)	2	27	199	188	1.05
<i>C. picta</i> (RL150728C1)	<i>Polystomoides oris</i>	PL150729D3			North Carolina (USA)	2	25	280	234	1.20
<i>C. picta</i> (RL150728C1)	<i>Polystomoides oris</i>	PL150729D4			North Carolina (USA)	2	26	250	262	0.95
<i>C. picta</i> (RL150728C1)	<i>Polystomoides oris</i>			PL150729D1	North Carolina (USA)					
<i>E. orbicularis</i> (Sor1)	<i>Polystomoides</i> sp1	PL060528E1	MNHNa2	PL060528E1	Turtle's farm (France)	2	35	684	658	1.04
<i>E. orbicularis</i> (Sor1)	<i>Polystomoides</i> sp1	PL060528E3	NMB P431		Turtle's farm (France)	2	35	565	607	0.93
<i>E. orbicularis</i> (Sor1)	<i>Polystomoides</i> sp1	PL060528E4	NMB P432		Turtle's farm (France)	2	37	606	588	1.03
<i>E. orbicularis</i> (Sor1)	<i>Polystomoides</i> sp1	PL060528E6	NMB P433		Turtle's farm (France)	2	36	549	536	1.02
<i>E. orbicularis</i> (Sor1)	<i>Polystomoides</i> sp1	PL060528E7	MNHNa1 (holotype)		Turtle's farm (France)	2	36	528	674	0.78
<i>E. orbicularis</i> (Sor1)	<i>Polystomoides</i> sp1	PL060528E8	NMB P434		Turtle's farm (France)	2	36	373	453	0.82
<i>E. orbicularis</i> (Sor1)	<i>Polystomoides</i> sp1	PL060528E10	NMB P435		Turtle's farm (France)	2	35	627	629	1.00
<i>T. scripta</i> (Flo1)	<i>Polystomoides</i> sp4	PL040602D1	MNHNb1 (holotype)		Florida (USA)	2	33	467	601	0.78
<i>T. scripta</i> (Flo1)	<i>Polystomoides</i> sp4			PL040602D2	Florida (USA)					
<i>T. scripta</i> (Flo 2)	<i>Polystomoides</i> sp4	PL040504A1	MNHNb2		Florida (USA)	2	34	168	335	0.50
<i>T. scripta</i> (Flo 2)	<i>Polystomoides</i> sp4			PL040505A2	Florida (USA)					
<i>T. scripta</i> (RL150723F1)	<i>Polystomoides</i> sp4	PL150728B2	NMB P438		North Carolina (USA)	2	34	226	330	0.68
<i>T. scripta</i> (RL150723F1)	<i>Polystomoides</i> sp4	PL150728B3	NMB P439		North Carolina (USA)	2	34	252	287	0.88
<i>T. scripta</i> (RL150723F1)	<i>Polystomoides</i> sp4	PL150728B4	MNHNb3		North Carolina (USA)					
<i>T. scripta</i> (RL150723F1)	<i>Polystomoides</i> sp4			PL150728B5	North Carolina (USA)					
<i>T. scripta</i> (RL150723F1)	<i>Polystomoides</i> sp4			PL150728B6	North Carolina (USA)					
<i>T. scripta</i> (RL150723F1)	<i>Polystomoides</i> sp4			PL150728B7	North Carolina (USA)					

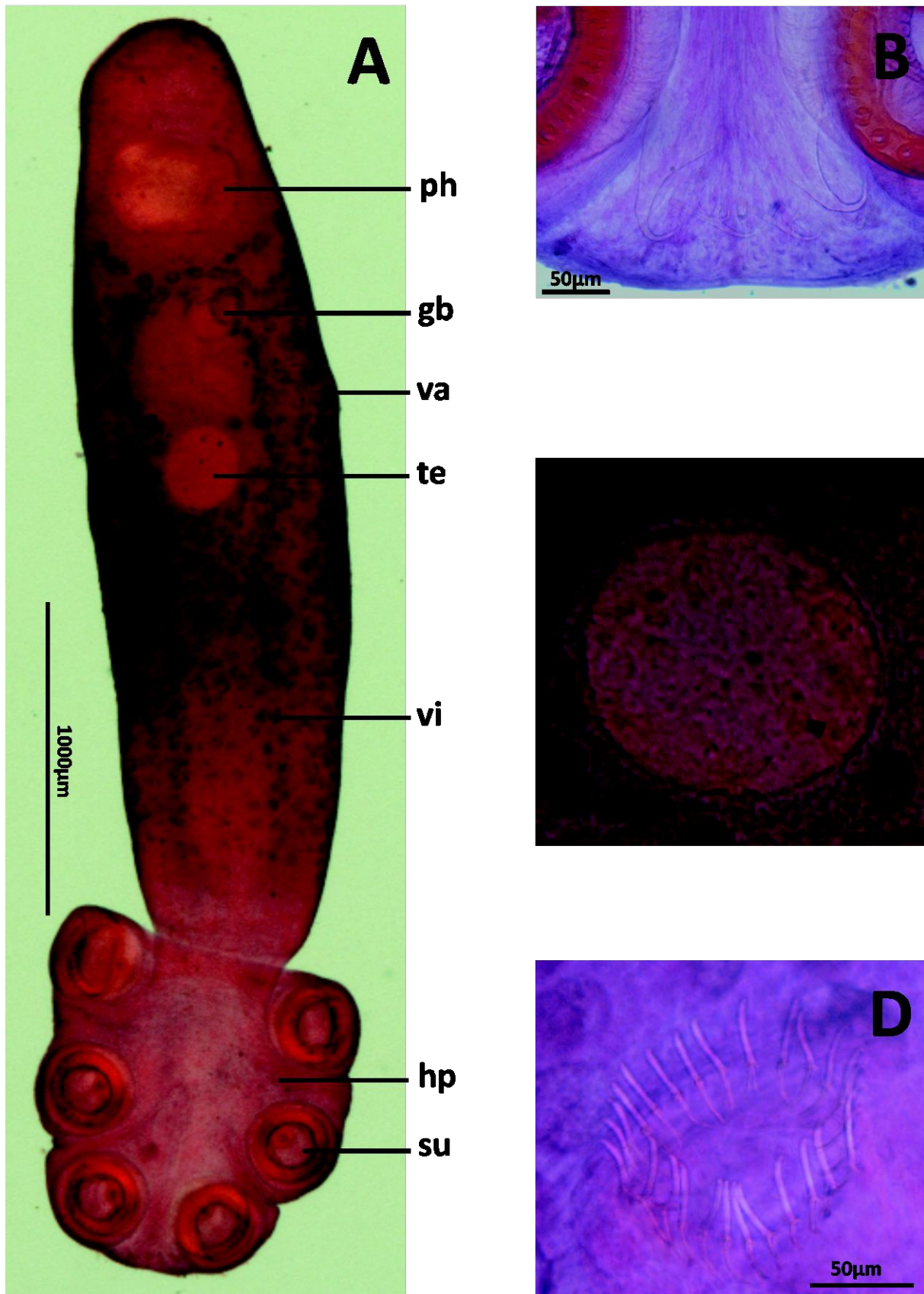


Figure 2. *Polystomoides oris*. A, ventral view; B, hamuli; C, testis; D, genital bulb. Abbreviations: gb, genital bulb; hp, haptor; ph, pharynx; su, sucker; te, testis; va, vagina; vi, vitellaria.



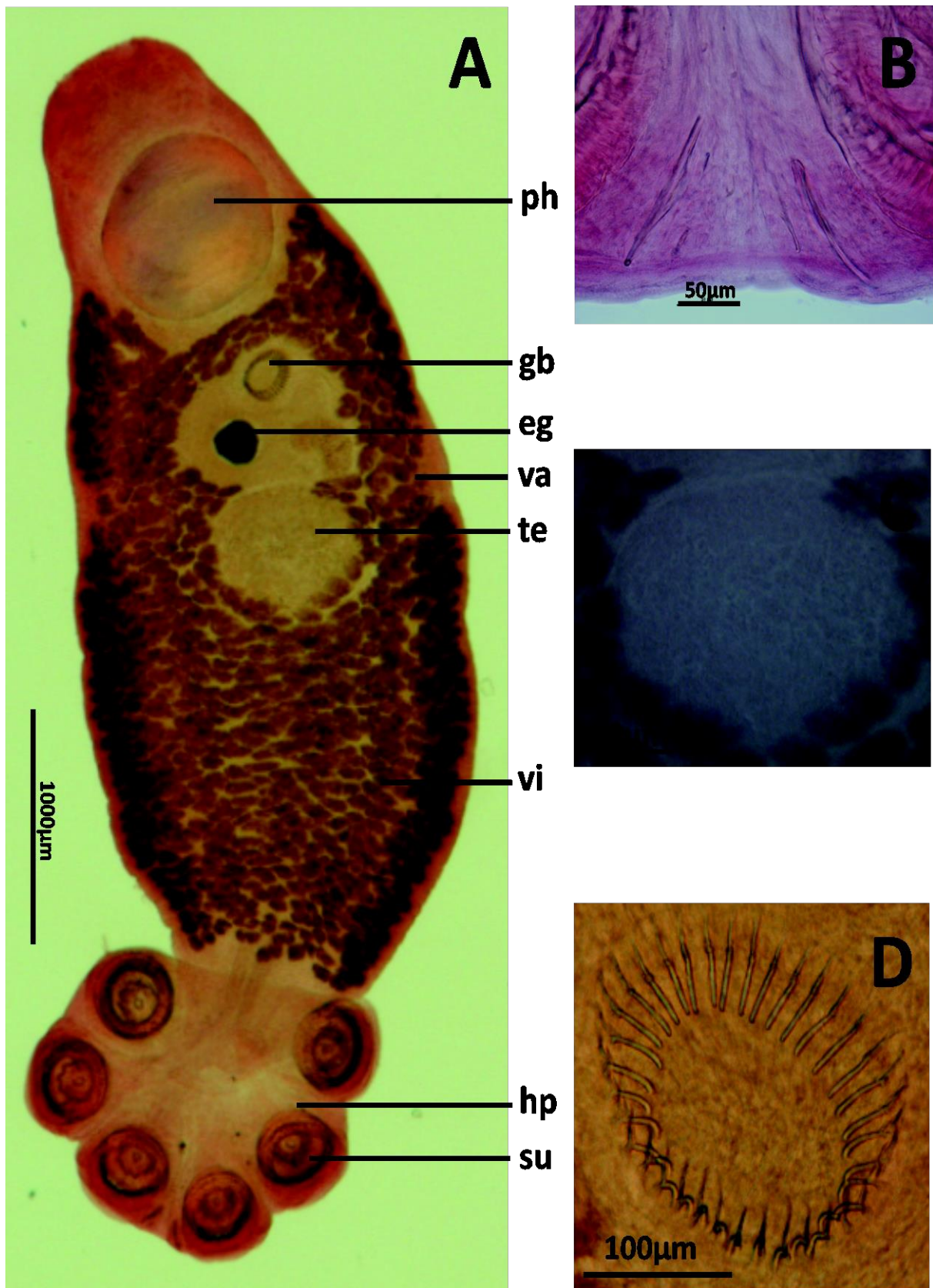


Figure 3. *Polystomoides soledensis* n. sp. A, ventral view; B, hamuli; C, testis; D, genital bulb. Abbreviations: eg, egg; gb, genital bulb; hp, haptor; ph, pharynx; su, sucker; te, testis; va, vagina; vi, vitellaria.

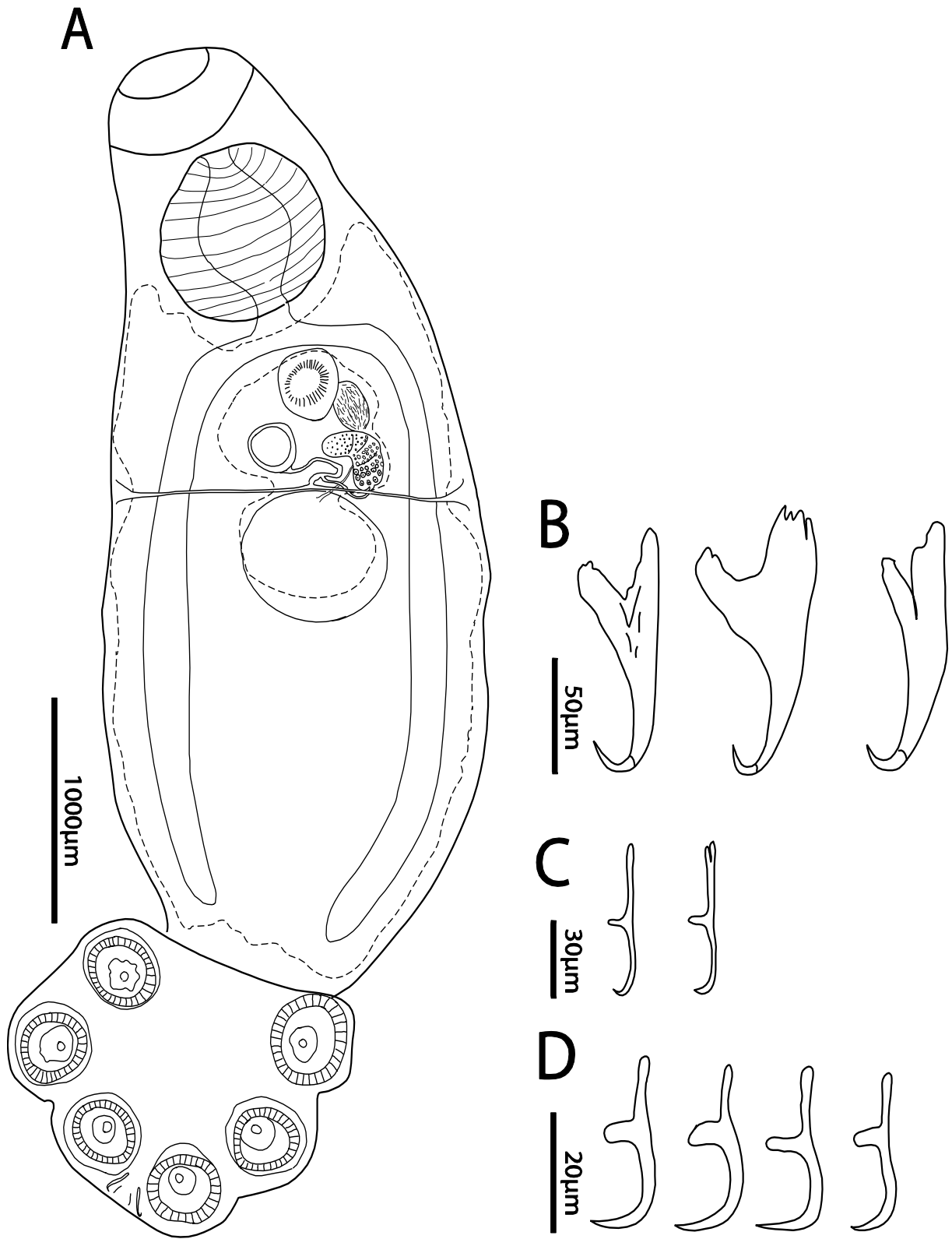


Figure 4. Drawing of *Polystomoides soledensis* n. sp. A, holotype; B, big hamuli drawn from holotype and paratypes; C, small hamuli drawn from holotype and paratypes; D, marginal hooklet 1 drawn from holotype and paratypes.

Table 3. Body morphometrics of *Polystomoides* spp.

	<i>P. soledensis</i> n. sp.	<i>P. scriptanus</i> n. sp.	<i>P. oris</i>
Body length	3033-5286 (4390)	2789-4954 (3745)	3622-4792 (4140)
Greatest width	1051-1875 (1412)	633-1491 (993)	821-1522 (1095)
Haptor length	756-1119 (931)	876-884 (880)	954-1183 (1070)
Haptor width	1133-1706 (1459)	1198-1250 (1224)	1073-1379 (1229)
Width at vagina	1019-1754 (1352)	608-1420 (912)	815-1332 (1007)
Oral sucker width	433-687 (545)	214-512 (315)	494-619 (536)
Pharynx length	415-757 (532)	214-390 (302)	311-672 (546)
Pharynx width	474-815 (604)	232-420 (326)	423-808 (558)
Ovary length	354-544 (439)	333-376 (354)	176-270 (213)
Ovary width	90-148 (123)	98-114 (106)	81-106 (95)
Testis length	438-667 (574)	197-276 (236)	270-272 (271)
Testis width	419-690 (598)	292-334 (313)	249-273 (261)
Genital bulbous width	329-408 (374)	303-312 (307)	129-211 (167)
Number of genital spines	34-36	31-34	26
Genital spine length	39-43 (41)	29-33 (31)	30-34 (32)
Number of eggs <i>in utero</i>	1	1	1
Egg length	214-238 (226)	218-249 (232)	265-280 (272)
Egg width	148-151 (149)	167-184 (172)	142-162 (152)
Haptoral sucker width	277-380 (312)	74-418 (207)	314-375 (344)
Haptor length: Body length ratio	0.19-0.25 (0.22)	0.23-0.38 (0.29)	0.25-0.27 (0.26)
Marginal hooklet 1 length	27.7-30.4 (28.8)	26.4-28.8 (27.3)	24.2-25.2 (24.6)

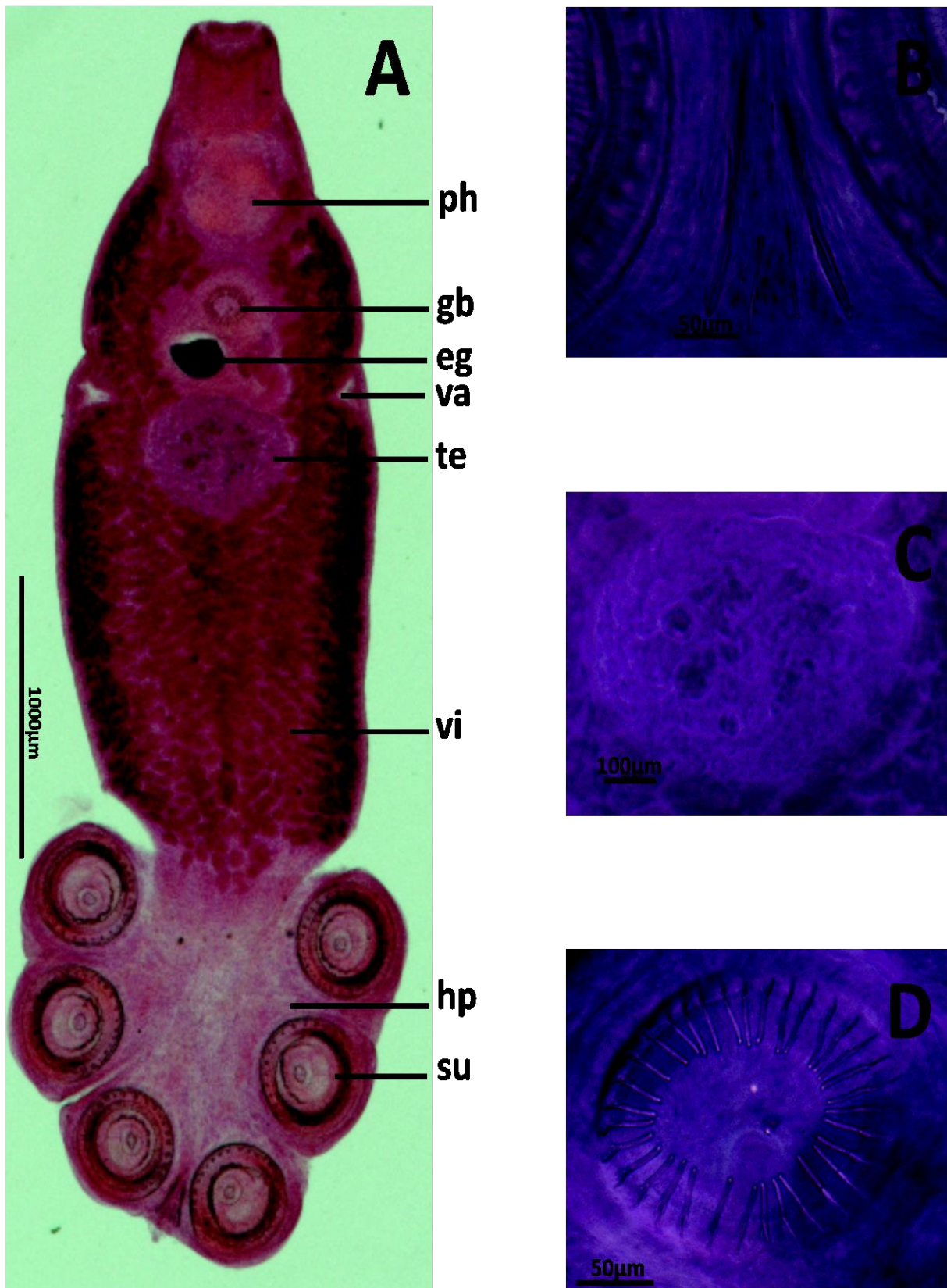


Figure 5. *Polystomoides scriptanus* n. sp. A, ventral view; B, hamuli; C, testis; D, genital bulb. Abbreviations: eg, egg; gb, genital bulb; hp, haptor; ph, pharynx; su, sucker; te, testis; va, vagina; vi, vitellaria.

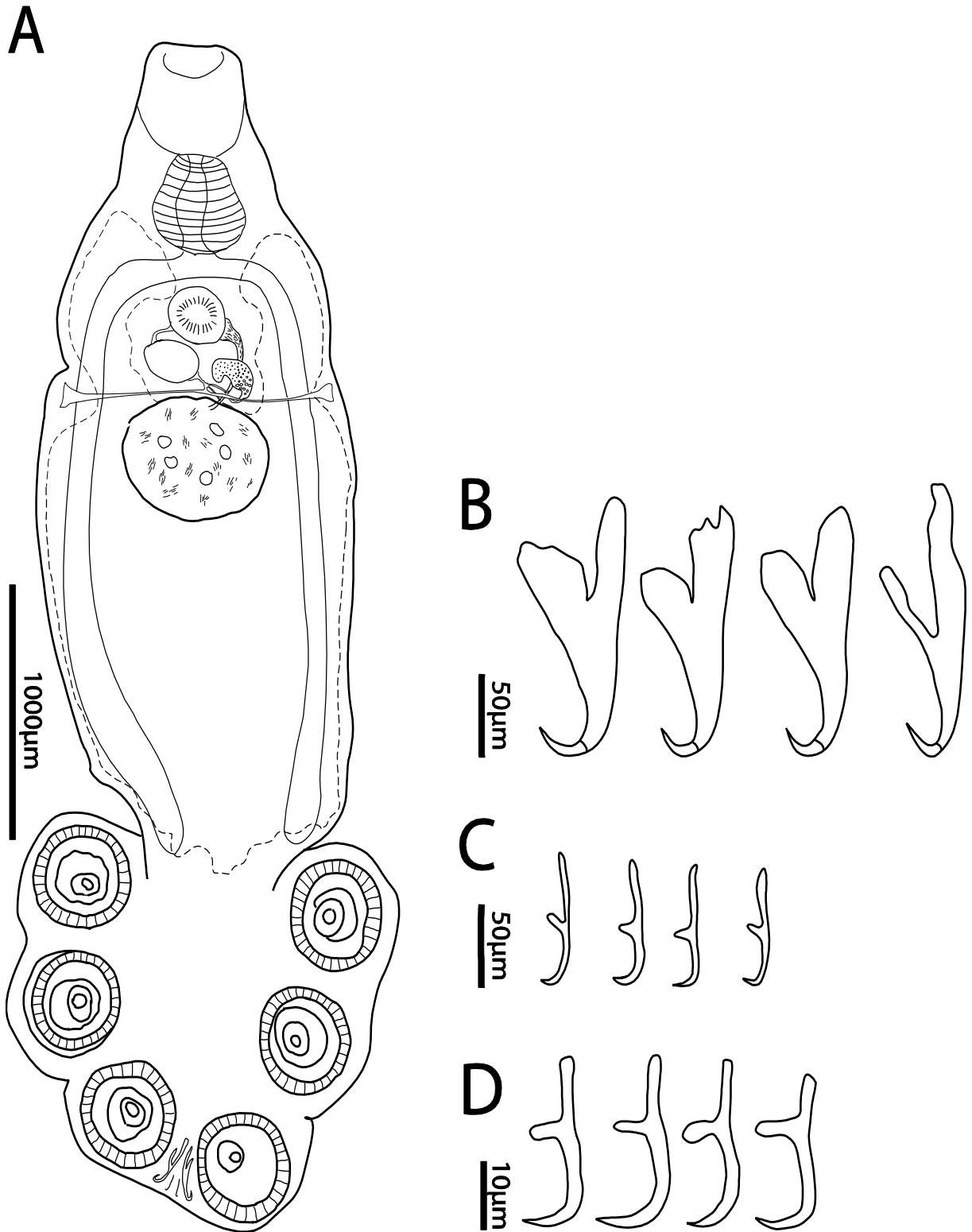


Figure 6. Drawing of *Polystomoides scriptanus* n. sp. A, holotype; B, big hamuli drawn from holotype and paratypes; C, small hamuli drawn from holotype and paratypes; D, marginal hooklet 1 drawn from holotype and paratypes.

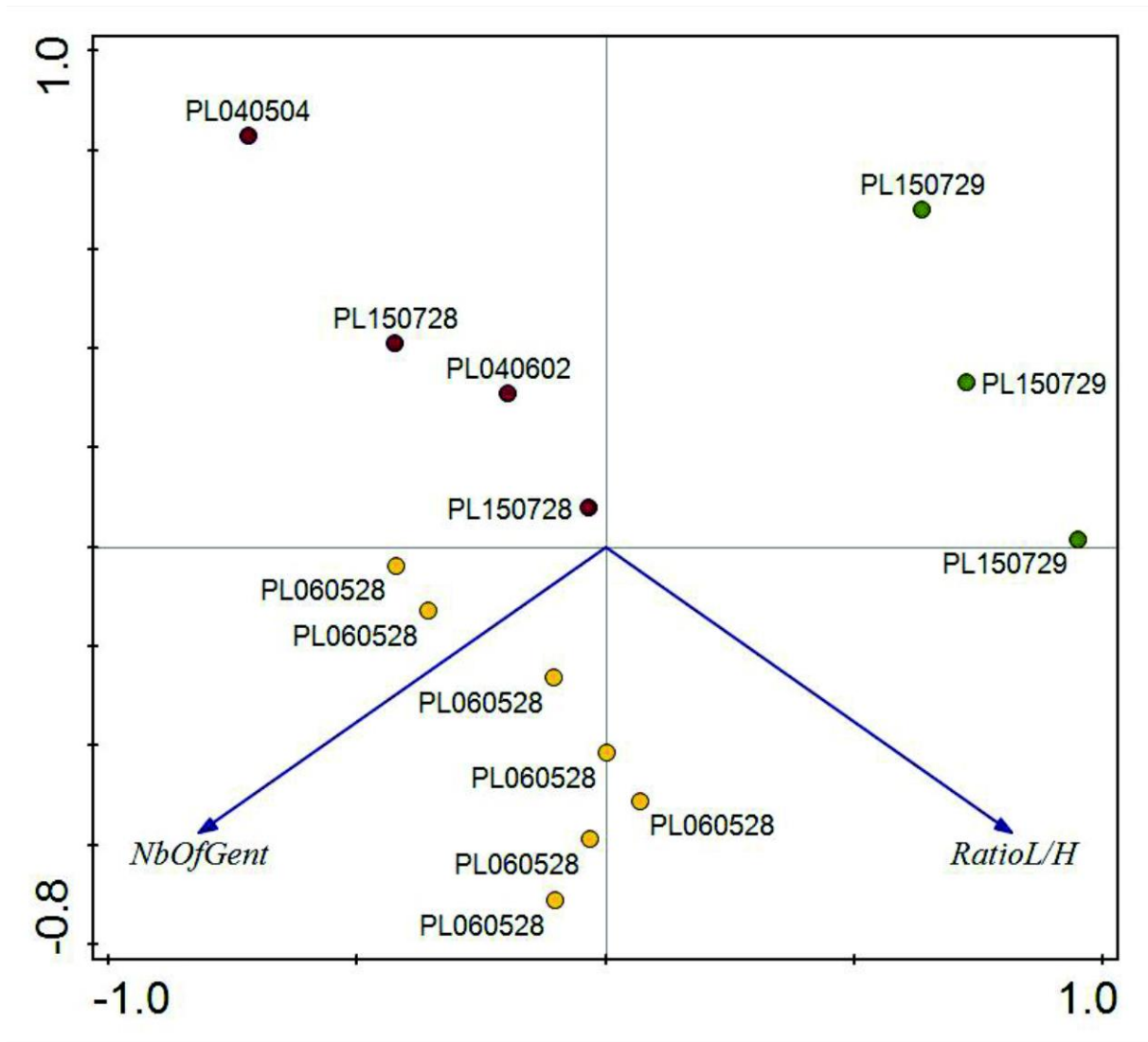


Figure 7. Descriptive analysis of polystome specimens collected from *C. picta* (green dots), *E. orbicularis* (yellow dots) and *T. scripta* (pink dots). Extra information regarding polystome morphology and morphometrics is given in table2. NbOfGent means number of genital spines; RatioL/H means testis length/testis width.

## 4. Discussion

### 4.1. Species validity

The first step in the description process involves a review of the literature. Regarding the database of Verneau [2], which compiled all references of papers based on polystome description, we first compared the morphology of our specimens to polystome species previously recorded from the pharyngeal cavity of freshwater turtles. Because the two new polystome species were found within wild North American turtles, i.e., *T. scripta*, and within captive and wild European turtles, namely *E. orbicularis* and *M. leprosa*, comparisons were done with American *Polystomoides* spp., namely *Polystomoides multifalx* (Stunkard 1924) and *P. oris*, and with European *Polystomoides* spp., namely *Polystomoides ocellatum* (Rudolphi 1819) and *Polystomoides tunisiensis* (Gonzales and Mishra 1977), the last two species infecting *E. orbicularis* and *M. leprosa*, respectively. Whereas the overall morphology was very similar for all these *Polystomoides* spp. and the two undescribed polystome species under investigation, the genital spine number differed suggesting the existence of two new species. The multivariate analysis, which was based on the genital spine number and testis shape, and the phylogenetic analysis, together, provide strong evidence for the presence of two definitively distinct species (Figs. 1 and 7). Because *T. scripta* was introduced following the pet trade from the USA to Europe [17, 18], Meyer et al. [11] concluded that it could be a carrier for American polystomes in European wild environments. Knowing that *E. orbicularis* and *M. leprosa* have never been observed in American wetlands, it is indeed very unlikely that these two turtle species could be vectors of parasites in the USA. Thereby *T. scripta* was considered as the original host species for both new polystome species, i.e., *P. soledensis* n. sp. and *P. scriptanus* n. sp..

### 4.2. The value to combine discriminant morphological characters and DNA barcodes

Stunkard [19] and MacCallum [20] described three and two polystome species from distinct freshwater chelonians collected in natural freshwater environments as well as in outdoor turtle enclosures of North America. These were further considered by Price (1939) as synonyms of *P. coronatum*. Similarly, Mañé-Garzón and Gil [8, 9] described two polystome species from the pharyngeal cavity of a single host species in natural freshwater ecosystems of Uruguay. Regarding the database of polystome drawings by Morrison and Du Preez [21], it is extremely difficult to differentiate chelonian polystomes from their morphology only, especially polystome species of a same genus. Morphometric measurements, such as body length, hamuli and hooklet size, are also in most cases not informative for species description.

This suggests that numerous polystome species could be synonymous [2]. Therefore, we suggest that almost all polystome species should be reexamined in light of the discriminant morphological characters we used along this study, namely the number of genital spines and the testis shape that can be inferred from polystome photographs (Figs. 2, 3 and 5). We thus propose that all researchers concerned about systematics of polystomatids use photographs of specimens for description. These pictures could be submitted to a database for further morphological comparisons. We also encourage authors to get at least partial COI sequences for molecular comparisons. To advance this goal, we plan to create an online open access of COI sequences, and, for scientists unable to perform molecular biology, we will provide molecular assistance.

#### 4.3. *The dogma of host specificity: myth or reality?*

Regarding the database of Verneau [2] and molecular surveys of Verneau et al. [10], Meyer et al. [11] and Héritier et al. [12], polystomes infecting freshwater turtles appeared site-specific but not necessarily host-specific. In this context, the dogma of host specificity is inappropriate as a basis for the definition of new polystome species, particularly in outdoor turtle enclosures and in natural freshwater ecosystems where invasive turtles are known. *Polystomoides soledensis* n. sp., for instance, was reported along this study from three distinct turtle species. If host specificity would have been considered here, three new species would have been erroneously described. We therefore recommend scientists to label parasite specimens during fieldwork according to the host species and sampling locality. We also advise them to collect host tissue for further molecular identification, which will provide a more accurate determination of host taxonomy. Before processing parasites for the morphological study, we also suggest the genotyping of at least two worms as a first stage in the description procedure.

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## References

- [1] Sinnappah ND, Lim LHS, Rohde K, Tinsley R, Combes C, Verneau, O. A paedomorphic parasite associated with a neotenic amphibian host: Phylogenetic evidence suggests a revised systematic position for Sphyrnauridae within anuran and turtle Polystomatoineans. *Mol Phylogenet Evol* 2001;18:189-201.
- [2] Verneau O. Origine et évolution des monogènes Polystomatidae, parasites d'amphibiens et de chéloniens d'eau douce. HDR thesis, Université de Perpignan Via Domitia 2004.
- [3] Verneau O, Bentz S, Sinnappah ND, Du Preez LH, Whittington I, Combes C. A view of early vertebrate evolution inferred from the phylogeny of polystome parasites (Monogenea: Polystomatidae). *Proc R Soc Lond [Biol]* 2002;269:535-543.
- [4] Héritier L, Badets M, Du Preez LH, Aisien MSO, Lixian F, Combes C, Verneau O. Evolutionary processes involved in the diversification of chelonian and mammal polystomatid parasites (Platyhelminthes, Monogenea, Polystomatidae) revealed by palaeoecology of their hosts. *Mol Phylogenet Evol* 2015;92:1-10.
- [5] Biserkov VY, Yildirimhan HS, Buchvarov G, Ugurtas IH. *Polystoma macrocnemis* n. sp. (Monogenea: Polystomatidae) from the Iranian longlegged wood frog *Rana macrocnemis* (Ranidae) in Turkey. *Syst Parasitol* 2001;48:61-66.
- [6] Du Preez LH, Tinsley R, de Sá R. Polystomatidae (Monogenea) of Southern African Anura: *Eupolystoma vanasi* n. sp. parasitic in *Schismaderma carens* (Smith). *Syst Parasitol* 2003;54:71-79.
- [7] Du Preez LH, Kok DJ. Supporting experimental evidence of host specificity among Southern African polystomes (Polystomatidae: Monogenea). *Parasitol Res* 1997;83:558-562.
- [8] Mañé-Garzon F, Gil O. Trematodos de las Tortugas del Uruguay, I. Una nueva especie del genero *Polystomoides* Ward 1917, de la cavidad bucal de *Phrynops geoffroana hillarii* (D. & B.). *Comun Zool Mus Hist Nat Montev* 1961;5:1-4.
- [9] Mañé-Garzon F, Gil O. Trematodos de las tortugas del Uruguay. V. Sobre un nuevo Polystomatidae de la faringe de *Phrynops geoffroana hillarii* (D. & B.) *Comun Zool Mus Hist Nat Montev* 1962;7:1-6.
- [10] Verneau O, Palacios C, Platt T, Alday M, Billard E, Allienne JF, Basso C, Du Preez LH. Invasive species threat: parasite phylogenetics reveals patterns and processes of hostswitching between non-native and native captive freshwater turtles. *Parasitology* 2011;138:1778-1792.

- [11] Meyer L, Du Preez LH, Bonneau E, Héritier L, Quintana MF, Valdeón A, Sadaoui A, Kechemir-Issad N, Palacios C, Verneau O. Parasite host-switching from the invasive American red-eared slider, *Trachemys scripta elegans*, to the native Mediterranean pond turtle, *Mauremys leprosa*, in natural environments. *Aquat Invasions* 2015;10:79-91.
- [12] Héritier L, Valdeón A, Sadaoui A, Gendre T, Ficheux S, Bouamer S, Kechemir-Issad N, Du Preez LH, Palacios C, Verneau O. Introduction and invasion of the red-eared slider and its parasites in freshwater ecosystems of Southern Europe: risk assessment for the European pond turtle in wild environments. Submitted to *Biol Cons*.
- [13] Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673-4680.
- [14] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biol Evol* 2013;30:2725-2729.
- [15] Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 1998;14: 817-818.
- [16] Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 2001;17:754-755.
- [17] Lutz CG. Pet turtle production. SRAC Publication (Mississippi State Univ) 2000;439:1-4.
- [18] Telecky TM. United States import and export of live turtles and tortoises. *Turtle & Tortoise Newsl* 2001;14:8-13.
- [19] Stunkard HW. On the anatomy and relationships of some North American trematodes. *J Parasitol* 1916;3:21-27.
- [20] MacCallum GA. Studies on the Polystomatidae. *Zoopathologica* 1918;1:103-120.
- [21] Morrison C, Du Preez LH. Turtle polystomes of the world. *Neopolystoma, Polystomoidella & Polystomoides*, VDM Verlag Dr. Muller, Saarbrücken 2011.

**CHAPITRE V. FIRST REPORT OF *NEOPOLYSTOMA*  
(MONOGENEA, POLYSTOMATIDAE) SPECIES IN  
FRESHWATER TURTLES FROM SOUTH AMERICA**

## Présentation du Chapitre V

Le chapitre précédent présente la description de deux nouvelles espèces de polystomes. Ces parasites ont été identifiés sur *Trachemys scripta* dans des écosystèmes aquatiques américains mais également sur *E. orbicularis* et *M. leprosa* dans les environnements naturels en France. Ces parasites invasifs étaient donc deux espèces non décrites transmises sur les espèces de tortues natives par *T. s. elegans* (Meyer et al. 2015; Héritier et al. Submitted). Parce que l'introduction d'espèces non natives de parasites peut contribuer à la perte de la biodiversité, il est impératif de surveiller ces invasions biologiques au sein des populations de tortues indigènes.

Ce chapitre présente la description de trois nouvelles espèces de polystomes collectées sur des tortues aquatiques sauvages de Guyane Française, à savoir la tortue forestière peinte *Rhinoclemmys punctularia* et la tortue scorpion *Kinosternon scorpioides*. Depuis que la vente de la tortue de Floride à tempes rouges a été interdite en France, les magasins spécialisés dans le commerce animalier se sont tournés vers d'autres espèces de tortues aquatiques en provenance d'Asie, d'Afrique, d'Amérique Centrale et d'Amérique du Sud. En effet, de nombreuses tortues sont disponibles à la vente et notamment les deux espèces citées précédemment. De ce fait, ces nouvelles espèces de tortues peuvent devenir de potentiels envahisseurs si un phénomène d'introduction en milieu naturel se reproduit, comme cela a été le cas pour la tortue de Floride à tempes rouges. Ainsi, avant que leurs parasites ne soient identifiés dans les écosystèmes aquatiques en France, il est primordial de les décrire sur leurs hôtes naturels pour une surveillance des invasions biologiques plus performante.

**Tracking platyhelminth parasite diversity from freshwater turtles in French Guiana: First report of *Neopolystoma* (Monogenea, Polystomatidae) with the description of three new species**

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## Abstract

**Background:** Polystomatid flatworms in chelonians are divided into three genera, i.e. *Polystomoides* Ward, 1917, *Polystomoidella* Price, 1939 and *Neopolystoma* Price, 1939, according to the number of haptorial hooks. Among the about 55 polystome species that are known to date from the 327 modern living chelonians, only four species of *Polystomoides* are currently recognised within the 45 South American freshwater turtles.

**Methods:** During 2012, several sites in the vicinity of the cities Cayenne and Kaw in French Guiana were investigated for freshwater turtles. Turtles were collected at six sites and the presence of polystomatid flatworms was assessed from the presence of polystome eggs released by infected specimens.

**Results:** Among the three turtle species that were collected, no polystomes were found in the gibba turtle *Mesoclemmys gibba* (Schweigger, 1812). The spot-legged turtle *Rhinoclemmys punctularia* (Daudin, 1801) was infected with two species of *Neopolystoma* Price, 1939, one in the conjunctival sacs and the other in the urinary bladder, while the scorpion mud turtle *Kinosternon scorpioides* (Linnaeus, 1766) was found to be infected with a single *Neopolystoma* species in the conjunctival sacs. These parasites could be distinguished from known species of *Neopolystoma* by a combination of morphological characteristics including body size, number and length of genital spines, shape and size of the testis. They were also differentiated at the molecular level using the *cox1* gene marker. Based on morphological and genetic evidences, three new species are described herein, namely *Neopolystoma cayensis* n. sp. and *Neopolystoma guianensis* n. sp. from the bladder and the conjunctival sacs of *R. punctularia*, respectively, and *Neopolystoma scorpioides* n. sp. from the conjunctival sacs of *K. scorpioides*. However the monophyly of *Polystomoides* and *Neopolystoma* is still questioned regarding their phylogeny based on a dataset comprising four concatenated genes, namely, 18S, 28S and 12S rRNA genes and *cox1*.

**Conclusions:** In addition to these being the first chelonian polystomes to be reported and described from French Guiana, they represent the first polystomes from the hosts *K. scorpioides* and *R. punctularia* and the first representatives of *Neopolystoma* from South America. Chelonian polystomes now require an in-depth morphological study to reconcile the taxonomy of the genera with species evolution.

**Keywords:** French Guiana, Freshwater turtle, *Kinosternon scorpioides*, *Mesoclemmys gibba*, *Rhinoclemmys punctularia*, Polystomatidae, *Neopolystoma*.

## Background

While freshwater makes up less than 1% of the earth's surface, it supports at least 100,000 animal species, approximately 9.5% of the animal diversity [1, 2]. As a starting point for conservation of the freshwater biodiversity, a comprehensive map grounded mainly on fish taxonomic studies was developed to design world's freshwater ecoregions [3]. Based on fish species richness and endemism, several areas were identified as being of significant importance, including large portions of the Amazon basin, among which the Guianas ecoregion [3]. Besides its exceptional richness in freshwater fishes [3], this ecoregion also exhibits a great diversity of freshwater turtles with ten species known to date [4]. These include the matamata turtle *Chelus fimbriata* (Schneider, 1783), the scorpion mud turtle *Kinosternon scorpioides* (Linnaeus, 1766), the gibba turtle *Mesoclemmys gibba* (Schweigger, 1812), the Guyanan toad-headed turtle *Mesoclemmys nasuta* (Schweigger, 1812), the big-headed sideneck turtle *Peltocephalus dumerilianus* (Schweigger, 1812), the Guianan shield side-necked turtle *Phrynops tuberosus* (Peters, 1870), the twist-necked turtle *Platemys platycephala* (Schneider, 1792), the giant South American river turtle *Podocnemis expansa* (Schweigger, 1812), the yellow-spotted river turtle *Podocnemis unifilis* Troschel, 1848 and the spot-legged turtle *Rhinoclemmys punctularia* (Daudin, 1801).

Globally turtles are known to be very sensitive to modifications to their environment. They are often among the first vertebrates to disappear from impacted or altered habitats [5] and, as a result, they represent one of the most endangered groups of animals [6, 7]. Of the 327 recognised species of extant chelonians (turtles and tortoises) that are distributed throughout the most hospitable ecoregions [4], 63% are considered threatened [6]. The main reasons for the decline is degradation and loss of habitat, but over-exploitation for the food market [8], pet trade [9], biological invasions [10-12] and diseases [13, 14] are also contributing causes. Freshwater turtles are indeed hosts to a wide variety of pathogens and parasites representing all major parasitic groups, including viruses [15], bacteria [16], blood parasites [17] and helminths [18, 19]. Among them, the Polystomatidae (Platyhelminthes: Monogenea) comprises 25 genera of which three, i.e. *Polystomoides* Ward, 1917, *Polystomoidella* Price, 1939 and *Neopolystoma* Price, 1939 infect freshwater turtles [20]. Only about 55 polystome species are known to date within chelonians, which represents approximately 25% of all known extant polystomes. Chelonian polystomes are worldwide distributed and form a clade that might have originated 178 million years ago, following a switch from caecilians to primitive freshwater turtles [21]. They would have secondarily diversified within hosts as a consequence of plate tectonics, co-divergences, turtle dispersal followed by host switching, intra-host speciation or a mixture of all these events [21]. Regardless of the scenarios involved in host-parasite diversification, these

parasites are mostly host- and site-specific [22]. However, they appear to be less specific following host releases, which has been demonstrated in confined and natural environments after translocation of American freshwater turtles, especially red-eared sliders *Trachemys scripta elegans* (Wied, 1939) [23-25].

Since the trade of sliders was banned in Europe in 1997 [9], an increasing number of disparate freshwater turtles globally appeared in the pet trade, providing a pool of new species susceptible to be introduced into natural environments and pose a high risk for European wetlands due to climate matching [9]. As the pet trade is growing, the list of traded turtles may change rapidly in response to supply and demand [9]. Hence, there is an urgent need to assess the parasite diversity of freshwater turtles in their area of origin before new species of parasites are introduced with their native hosts in novel environments. Due to the high diversity of turtles in the Guianas that could be potentially sold as pets in Europe and some other non-European countries, our first survey of platyhelminth parasites took place in this ecoregion. We first concentrated in French Guiana freshwater environments to collect and examine freshwater turtles and their platyhelminth parasites. We report and describe hereafter three yet unknown chelonian polystomes from two host species, *K. scorpioides* and *R. punctularia*.

## **Methods**

### **Host sampling**

During the period 9–23 April 2012, several swampy areas and ponds near Cayenne and Kaw in French Guiana were investigated for freshwater chelonians. Site A (4.89205N, 52.34643W), site B (4.90149N, 52.35767W), site C (4.82301N, 52.34115W), site D (4.66997N, 52.30560W) and site E (4.87901N, 52.25644W) were all water bodies in and around Cayenne, while site F (4.87082N, 52.33678W) was a forested pond on the road to the town of Kaw. Crayfish traps baited with pork or ox liver were anchored to the vegetation to ensure they did not roll into the deeper water. They were left out overnight and placed in such a way as to allow turtles to surface and breathe when trapped. Captured animals were removed from traps the following day and placed individually in plastic tubs containing water about 50 mm deep and covered with shelving to keep tubs cool and dark and to prevent turtles from escaping.

### **Parasite sampling**

The day after turtle's isolation, the water was poured through two plankton sieves with respective mesh size of 500 and 100  $\mu\text{m}$ . The 500  $\mu\text{m}$  sieve retained coarse debris and most of the faeces while the 100  $\mu\text{m}$  sieve retained parasite eggs. The content of the 100  $\mu\text{m}$  sieve was then washed into a Petri dish and inspected for the presence of polystome eggs using a Nikon SMZ 645



dissecting microscope. Turtles for which no parasite eggs were detected were screened on a second and third day and, if no polystome eggs were detected, animals were released at the point of collection. Turtles to be dissected were killed by means of a lethal injection of 0.5 ml Uthapent (sodium pentobarbitone) diluted with 4.5 ml water. The urinary bladder and all reproductive and excretory ducts, as well as the oral and nasal cavities and conjunctival sacs under the eyelids, were examined for polystomes. All visible parasites were removed, after which the host tissue was placed in hot 70% ethanol and vigorously shaken to detach any immature parasites that might have been overlooked. Live parasites were immediately placed in a drop of water on a specimen glass slide and briefly heated from below with a butane lighter until they relaxed and stopped moving. They were fixed in 10% neutral buffered formalin under very gentle coverslip pressure and permanently mounted. Some polystome eggs were also placed in dechlorinated water in cryo vials but failed to develop. Finally, sub-adult and mature specimens earmarked for molecular studies were fixed in molecular grade 70% ethanol.

### **Whole mount preparation, morphology and morphometry**

Polystomes were washed free of fixative and stained overnight in a weak solution of acetocarmine, dehydrated, cleared in xylene and mounted in Canada balsam. Specimens were examined using a Nikon Eclipse E800 compound microscope as well as a Nikon AZ100 (Nikon, Netherlands). Body and organs were measured using the Nikon NIS elements software program as well as marginal hooklets that were located in mature and immature parasites. All measurements are given in micrometres as the range followed by the mean in parentheses. Drawings, based on photographs taken of type-material, were prepared using Adobe Illustrator software.

### **Molecular methods**

Polystome specimens were dried, crushed and incubated at 55 °C for one hour in 150 µl of 10% suspension Chelex 100 sodium (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France) and proteinase K at a final concentration of 1 mg/ml. The enzymatic reaction was stopped at 100 °C for 15 min and DNA was kept at 4 °C until PCR amplification. Four molecular markers were investigated for genetic analyses: (i) the complete 18S rRNA gene was amplified in two overlapping fragments of about 1kb each, with the primers forward F18 (5'-ACC TGG TTG ATC CTG CCA GTA G-3') and reverse 18RG (5'-CTC TCT TAA CCA TTA CTT CGG-3') on the one hand and with the primers forward 18F3 (5'-GGA CGG CAT GTT TAC TTT GA-3') and reverse IR5 (5'-TAC GGA AAC CTT GTT ACG AC-3') on the other; (ii) partial nuclear 28S rRNA gene was also amplified in two overlapping fragments of about 1 kb and 500 bp each, with the primers

forward LSU5' (5'-TAG GTC GAC CCG CTG AAY TTA AGC A-3') and reverse IR16 (5'-ATT CAC ACC CAT TGA CTC GCG-3') on the one hand and with the primers forward IF15 (5'-GTC TGT GGC GTA GTG GTA GAC-3') and reverse LSU3' (5'-TAG AAG CTT CCT GAG GGA AAC TTC GG-3') on the other; (iii) a portion of the mitochondrial 12S rRNA gene of about 470 bp was amplified with the primers forward 12SpolF1 (5'-YVG TGM CAG CMR YCG CGG YYA-3') and reverse 12SpolR1 (5'-TAC CRT GTT ACG ACT TRH CTC-3'); (iv) a fragment of the mitochondrial cytochrome *c* oxydase subunit 1 (*cox1*) gene of about 350 bp was amplified with the primers forward L-CO1p (5'-TTT TTT GGG CAT CCT GAG GTT TAT-3') and reverse H-Cox1R (5'-AAC AAC AAA CCA AGA ATC ATG-3'). PCR amplifications were conducted following the procedure described in [21]. All PCR products were purified using the kit Wizard SV Gel and PCR Clean-Up System (Promega, Charbonnières-les-Bains, France) and sent to the company Genoscreen (Lille, France) for sequencing with the PCR primers. Chromatograms were finally examined with the software Geneious (Saint Joseph, Missouri, USA) to verify all substitutions along sequences.

### **Sequence alignments and phylogenetic analyses**

18S and 28S sequences were aligned regarding the secondary structure of nuclear rRNAs of chelonian polystomes and subsequent alignments defined by Héritier et al. [21]. Inversely, 12S and *cox1* sequences were aligned using Clustal W [26] implemented in the program MEGA, version 5 [27], under default parameters. In order to explore the relationships of the chelonian polystomes collected during the present study, sequences reported in [21, 24, 28-30] (GenBank accession numbers 18S: AJ228788, AJ228792, FM992696–FM992699, KR856126–KR856139; 28S: AF382065, FM992702–FM992706, KR856145–KR856158; 12S: KR856100–KR856119; *cox1*: Z83005, Z83007, Z83009, Z83011, FR822527, FR822529–FR822531, FR822534, FR822553, FR822555, FR822570, FR822587, FR822601, FR822603, FR822604, FR828360, KR856175–KR856177), as well as the newly generated sequences reported here (see below), were included in a global phylogenetic analysis that was performed following the procedure of Héritier et al. [21]. A global alignment was first inferred with the concatenated sequences recovered from 23 fully described or undescribed species. Regions not sequenced, which concerned particularly the 18S rRNA gene and the 500 bp fragment of the 28S rRNA gene of the polystome collected from the bladder of *K. scorpioides*, were treated as missing data. Ribosomal nuclear sequences were partitioned into two discrete regions, i.e. stems and loops, while 12S and *cox1* sequences were considered as two other distinct partitions. A doublet model was considered for the stem partition and a GTR + I + G for the loops. A GTR + I + G was also selected independently for the two last partitions following the Akaike Information Criterion (AIC)

implemented in Modeltest 3.06 [31]. A Bayesian analysis was run using MrBayes 3.04b [32], with four chains running for one million generations and sampled every 100 cycles. The consensus tree was then drawn after removing the first 1,000 trees (10%) as the 'burn-in' phase to obtain the Bayesian posterior probability for each association. *Nanopolystoma tinsleyi*, which was recognised as the sister group of chelonian polystomes after phylogenetic analysis [21], was used as the outgroup (GenBank accession numbers 18S: KR856124; 28S: KR856142; 12S: KR856077; *cox1*:KR856164).

### **Genetic divergences within chelonian polystomes**

Kimura 2-parameters (K2P) distances were estimated from the whole *cox1* dataset using MEGA, version 5, to assess genetic divergences among chelonian polystomes. Results were discussed in the light of the 3.4% *cox1* genetic divergence threshold defined by Héritier et al. [23] on chelonian polystomes.

## **Results**

### **Turtles and parasites collected**

The spot-legged turtle *R. punctularia* was found at all the sites except site A. Of the total sample, 23 specimens were collected at site B, three at site C, one at site D, three at site E and 10 at site F. The scorpion mud turtle *K. scorpioides* was found at all the sites except site E. Of the total sample, two specimens were collected at site A, one each at sites B and C, 13 at site D and four at site F. Of the total sample of the gibba turtle *M. gibba*, four specimens were collected at site D and three at site E.

Among the 68 turtles that were collected, two chelonian species were found to be infected with polystomes, i.e. *R. punctularia* and *K. scorpioides*, whereas *M. gibba* was uninfected. Based on the release of polystome eggs, infected turtles were euthanized and dissected to recover all polystomes. Of the 40 *R. punctularia*, infected animals were found at sites B and F. At site B, two out of 23 turtles released round polystome eggs, while another one released round and fusiform eggs. At site F, one of the ten turtles released both round and fusiform polystome eggs while a second one released only fusiform eggs. Of the 21 *K. scorpioides*, a single turtle out of the 13 collected at site D released fusiform eggs. Based on the absence of hamuli, all parasites found in the bladder and in the conjunctival sacs of their host could be considered at that stage as belonging to *Neopolystoma*.

**Class Monogenea (van Beneden, 1858)**

**Order Polystomatidea Lebedev, 1988**

**Family Polystomatidae Gamble, 1896**

***Neopolystoma cayensis* n. sp.**

***Type-host:*** *Rhinoclemmys punctularia* (Daudin, 1801) (Geoemydidae Theobald, 1868).

***Type-locality:*** Pond on the outskirts of Cayenne, French Guiana (4.87082N, 52.33678W).

***Site in host:*** Urinary bladder.

***Type-material:*** Morphological description based on 22 sexually mature worms. The holotype (NMB P394) and nine paratypes (NMB P395–P403) were deposited in the Parasitic Worm Collection, National Museum, Aliwal Street, Bloemfontein, South Africa.

***Voucher material:*** Remainder of specimens were deposited in the polystome collection of the North-West University, Potchefstroom, South Africa.

***Prevalence and intensity:***

Site B: Prevalence 13.0%; Mean intensity 12.7. Site F: Prevalence 10.0%; Mean intensity 176

***Representative DNA sequences:*** GenBank accession numbers: KY200986 (18S), KY200988 (28S), KY200991 (12S) and KY200994 (*cox1*).

***ZooBank registration:*** To comply with the regulations set out in article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN) [33], details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:B55369A7-5F88-4A72-94F2-E0CE354484A1. The LSID for the new name *Neopolystoma cayensis* n. sp. is urn:lsid:zoobank.org:act:A4E27E97-052E-4709-84AD-B262A1A625D4.

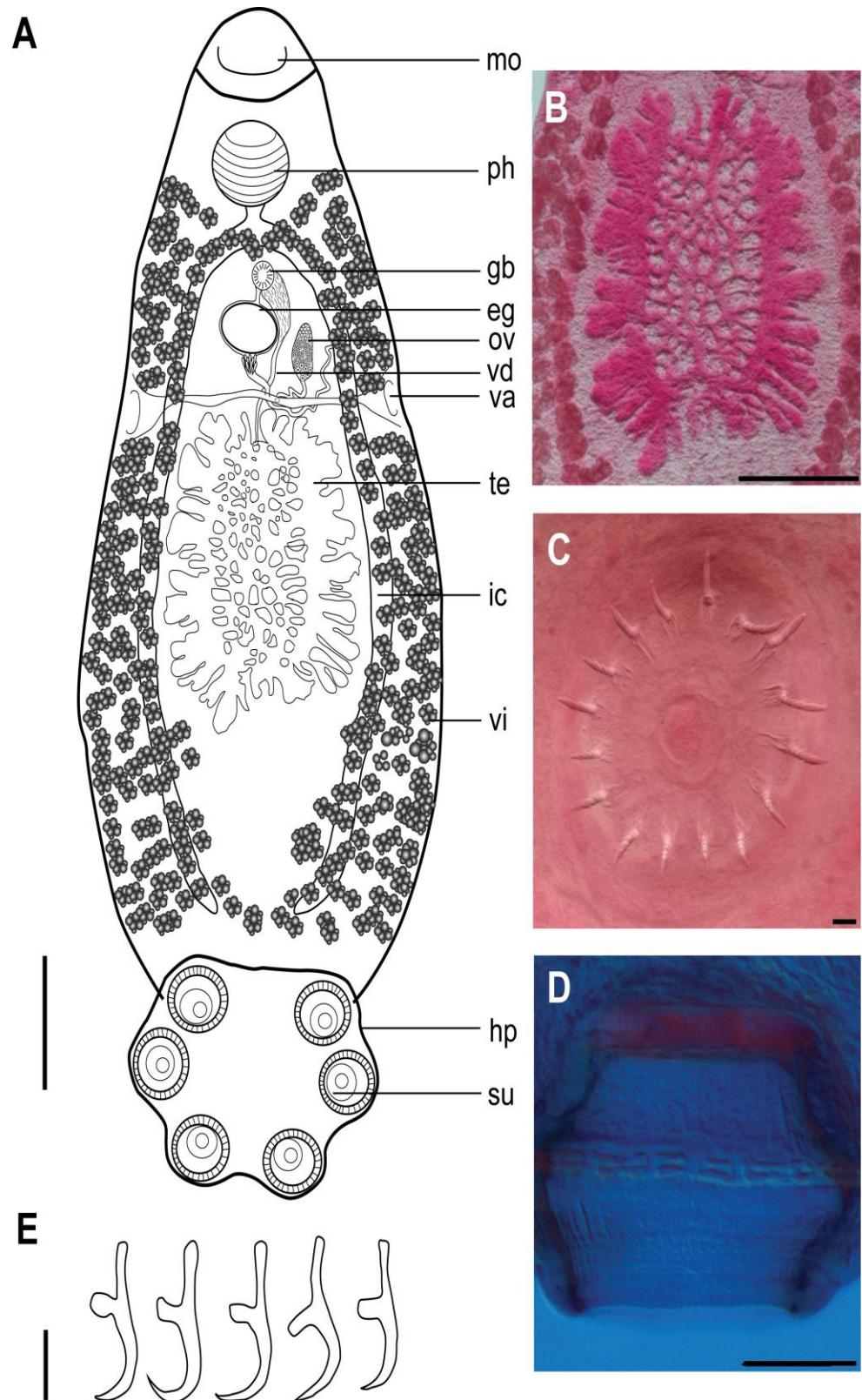
***Etymology:*** This parasite is named after Cayenne, the capital of French Guiana, where the parasite was found.

## **Description**

[Based on 22 egg-producing adults (see Table 1 and Fig. 1a-e). No larval measurements or characters are given as eggs collected from the host failed to develop.] Body elongate, pear shaped with the widest point about two-thirds from the anterior extremity (Fig. 1a), total length 3,342–5,575 (4,139), greatest width 1,353–1,961 (1,753). Haptor length 759–1,139 (912), haptor width 1,095–1,459 (1,249), haptor length to body length ratio 0.20–0.25 (0.22). Mouth subterminal, ventral. False oral sucker 130–356 (189) wide. Pharynx muscular, 293–401 × 276–457 (330 × 357). Intestine bifurcates without diverticula; caeca narrow, lacking anastomoses, run laterally, not confluent posteriorly, not extending into haptor. Testis single, mid-ventral, medial, posterior to ovary, flattened, lobed along outer margin with reticulated network of ducts in

**Table 1** Body morphometrics for the known species of *Neopolystoma* from the Neotropical realm

	<i>N. cayensis</i> n. sp.	<i>N. guianensis</i> n. sp.	<i>N. scorpioides</i> n. sp.	<i>N. domitilae</i>	<i>N. fentoni</i>
Body length	3,342–5,575 (4,139)	2,284–3,342 (2,737)	1,512–1,770 (1,658)	4,039–4,057	1,500–2,450 (1,985)
Body maximum width	1,353–1,961 (1,753)	830–1,164 (1,037)	868–907 (894)	1,320–1,722	426–760 (568)
Haptor length	759–1,139 (912)	634–852 (755)	445–511 (485)	1,046–1,067	449–690 (571)
Haptor width	1,095–1,459 (1,249)	784–1,014 (910)	719–765 (735)	1,416–1,851	550–850 (683)
Width at vagina	1,015–1,504 (1,281)	792–1,091 (989)	837–876 (863)		
Oral sucker width	130–356 (189)	189–391 (290)	240–245 (242)	370–450 (547)	240–496 (370)
Pharynx length	293–401 (330)	189–260 (213)	170–184 (177)	305–322	156–257 (216)
Pharynx width	276–457 (357)	226–283 (252)	226–231 (228)	328	185–367 (278)
Ovary length	140–357 (195)	149–243 (197)	136–187 (153)	322–402	80–245 (103)
Ovary width	62–118 (88)	71–120 (97)	66–71 (69)	112–127	55–169 (105)
Testis length	560–983 (762)	364–541 (437)	120–142 (127)	450–664	98–367 (225)
Testis width	468–1,283 (933)	380–527 (477)	246–301 (271)	289–338	78–251 (181)
Genital bulb width	73–129 (99)	49	45	241–273	30–83 (60)
Number of genital spines	16–17	8	8	20–21	8
Genital spine length	19–26 (23)	8.4–9.8 (8.9)	5.5–8.7 (7.7)	55–71	11
Number of eggs <i>in utero</i>	1	1	1	1	1
Egg length	209–289 (246)	280–344 (317)	253–279 (265)	305	245–332 (286)
Egg width	155–187 (165)	127–163 (148)	133–156 (143)	177	122–146 (136)
Haptoral sucker width	227–297 (261)	208–242 (226)	133–279 (220)	305–483	210–326 (265)
Haptor length: Body length ratio	0.22	0.28	0.29	0.26	0.29
Marginal hooklet 1 length	19.6–21.8 (21.2)	13.3–15.1 (14.3)	16.4–17.2 (16.8)	24.6	11.8–12.7 (12.5)



**Fig. 1** *Neopolystoma cayensis* n. sp. Holotype. **a** Ventral view. **b** Testis. **c** Genital spines. **d** Haptor showing a ring of skeletal elements. **e** Marginal hooklets. *Abbreviations:* eg, egg; gb, genital bulb; hp, haptor; ic, intestinal caecum; mo, mouth; ov, ovary; ph, pharynx; su, sucker; te, testis; va, vagina; vd, vas deferens; vi, vitellaria. *Scale-bars:* **a**, 1,000  $\mu\text{m}$ ; **b**, 500  $\mu\text{m}$ ; **c**, 10  $\mu\text{m}$ ; **d**, 100  $\mu\text{m}$ ; **e**, 10  $\mu\text{m}$

central area (Fig. 1a, b), 560–983 × 468–1,283 (762 × 933); large amounts of sperm observed in reticulated network. Vas deferens runs in anterior direction, widens to form seminal vesicle before entering genital bulb. Genital bulb median, ventral, posterior to intestinal bifurcation, 73–129 (99) in diameter, armed with single ring of 16–17 spines (Fig. 1c), 19–26 (23) long, tip of spine curved outwards. Ovary dextral, at 15% of body length from anterior extremity, small, 140–357 × 62–118 (195 × 88). Uterus short, tubular, anterior to ovary, containing single oval egg; egg capsule 209–289 × 155–187 (246 × 165). No intra-uterine development, egg operculate. Vaginae present, located about 1/3 from anterior extremity of body proper; body width at level of vagina 1,015–1,504 (1,281); body slightly indented at level of vaginae. Vitellarium in two broad lateral fields along full length of body proper except for anterior area including mouth and pharynx and posteriormost section of the body (Fig. 1a). Vitelline follicles in two lateral fields, joining in narrow strip between genital bulb and pharynx and posteriad, at level of caeca tips. Genito-intestinal canal prominent, dextral, joining intestinal caecum at level of ovary (Fig. 1a). Haptoral suckers 6, muscular, with well-developed skeletal structure inside (Fig. 1d), mean diameter 227–297 (261). Hamuli absent. Marginal hooklets (Fig. 1e) retained in mature parasites. Posteriormost marginal hooklet 1 19.6–21.8 (21.2) long; hooklets 2–8 19.4–22.3 (20.9) long.

***Neopolystoma guianensis* n. sp.**

**Type-host:** *Rhinoclemmys punctularia* (Daudin, 1801) (Geoemydidae Theobald, 1868).

**Type-locality:** Pond on the outskirts of the capital Cayenne, French Guiana (4.87082N, 52.33678W).

**Site in host:** Conjunctival sacs of the eye.

**Type-material:** Eleven sexually mature worms. The holotype (NMB P404) and two paratypes (NMB P405–P406) were deposited in the Parasitic Worm Collection, National Museum, Aliwal Street, Bloemfontein, South Africa.

**Voucher material:** Remainder of specimens were deposited in the polystome collection of the North-West University, Potchefstroom, South Africa.

**Prevalence and intensity:**

Site B: Prevalence 4.34%; Mean intensity 2.0. Site F: Prevalence 20.0%; Mean intensity 11.0.

**Representative DNA sequences:** GenBank accession numbers: KY200987 (18S), KY200989 (28S), KY200992 (12S) and KY200995 (*cox1*).

**ZooBank registration:** To comply with the regulations set out in article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN) [33], details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) of the article is

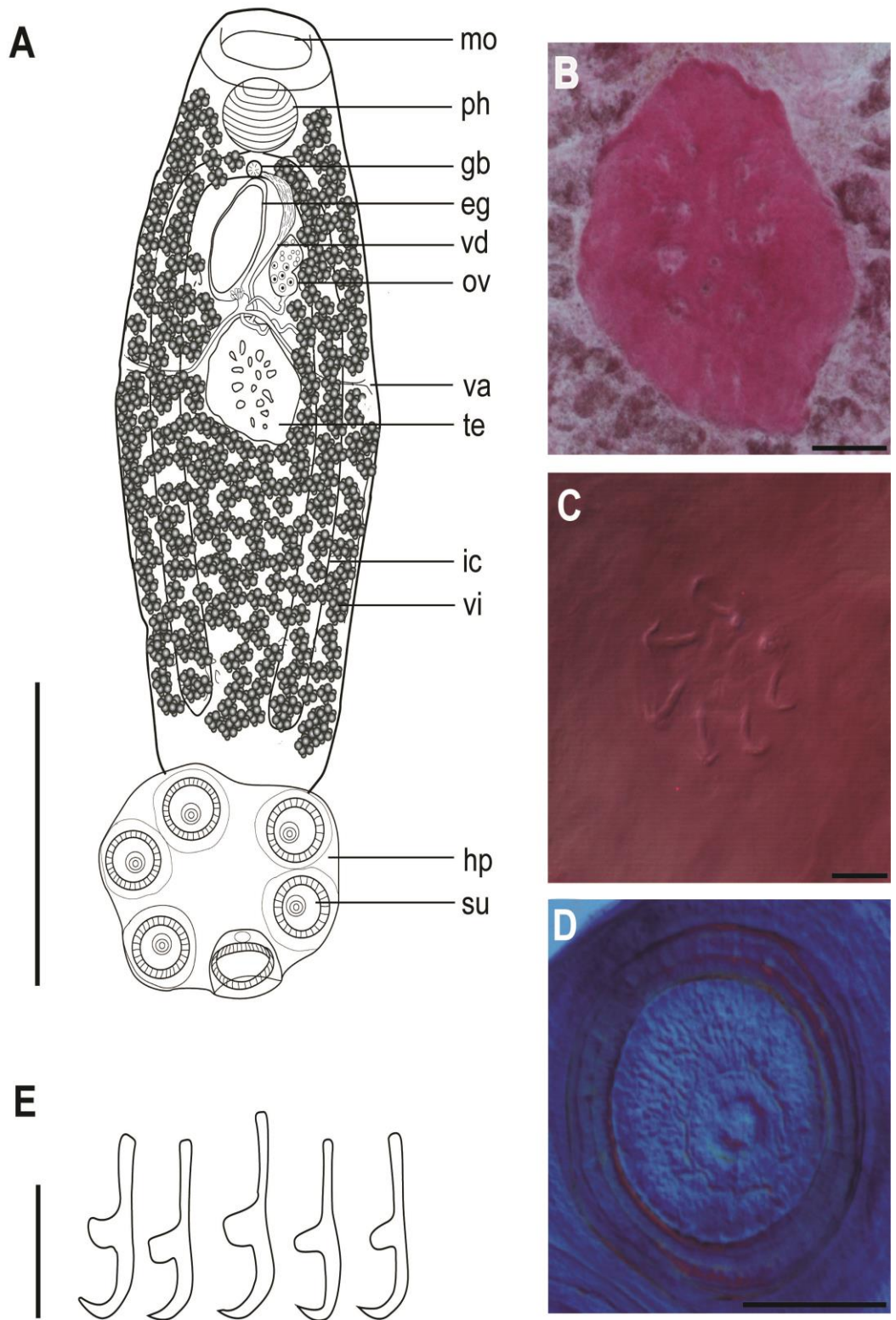
urn:lsid:zoobank.org:pub:B55369A7-5F88-4A72-94F2-E0CE354484A1. The LSID for the new name *Neopolystoma guianensis* n. sp. is urn:lsid:zoobank.org:act:1BDAA38D-D657-4DCC-9539-F5BDEFB463B8.

**Etymology:** This parasite is named after French Guiana.

### **Description**

[Based on 11 egg-producing adults (see Table 1 and Fig. 2a-e). No larval measurements or characters are given as eggs collected from the host failed to develop.] Body elongate, spindle shaped, with widest point in middle of body proper, total length 2,284–3,342 (2,737), greatest width 830–1,164 (1,037). Haptor length 634–852 (755), haptor width 784–1014 (910), haptor length to body length ratio 0.24–0.31 (0.28). Mouth subterminal, ventral. False oral sucker prominent, 189–391 (290) wide. Pharynx 189–260 × 226–283 (213 × 252). Intestine bifurcates without diverticula; caeca narrow, lacking anastomoses, run laterally, not confluent posteriorly, not extending into haptor. Testis single, spherical, compact (Fig. 2b), mid-ventral, medial, posterior to ovary (Fig. 2a), 364–541 × 380–527 (437 × 477). Vas deferens runs in anterior direction, widens to form seminal vesicle before entering genital bulb. Genital atrium median, ventral, posterior to intestinal bifurcation, 49 in length, with 8 spines, 8.4–9.8 (8.9) long (Fig. 2c); spines slightly curved. Ovary about 1/3 from the anterior extremity, dextral, small, 149–243 × 71–120 (197 × 97). Uterus short, tubular, anterior to ovary, contained one fusiform egg *in utero* (all specimens studied); egg capsule 280–344 × 127–163 (317 × 148). No intrauterine development observed, eggs operculate. Vaginae present, located nearly in middle of body proper; body width at the level of vagina 792–1,091 (989). Vitellarium extends from just behind pharynx throughout most of body proper except for area around gonads and posteriormost part of body proper. Genito-intestinal canal obscured by testis, dextral, joining intestinal caecum posterior to ovary (Fig. 2a). Live specimens were extremely flexible and able to extend the body proper to nearly double its length. Posterior section of body proper appears to be especially very flexible and elastic. Haptoral suckers 6, muscular, with well-developed skeletal structure inside (Fig. 2d), mean diameter 208–242 (226). Hamuli absent. Marginal hooklets retained in adult parasites (Fig. 2e). Posteriormost marginal hooklet 1 13.3–15.1 (14.3) long; hooklets 2–8 12.9–14.3 (13.9) long.





**Fig. 2** *Neopolystoma guianensis* n. sp. Holotype. **a**, Ventral view. **b**, testis. **c** genital spines. **d** haptoral sucker showing a ring of skeletal elements. **e** marginal hooklets. *Abbreviations:* eg, egg; gb, genital bulb; hp, haptor; ic, intestinal caecum; mo, mouth; ov, ovary; ph, pharynx; su, sucker; te, testis; va, vagina; vd, vas deferens; vi, vitellaria. *Scale-bars:* **a**, 1,000  $\mu\text{m}$ ; **b**, 100  $\mu\text{m}$ ; **c**, 10  $\mu\text{m}$ ; **d**, 100  $\mu\text{m}$ ; **e**, 10  $\mu\text{m}$

***Neopolystoma scorpioides* n. sp.**

**Type-host:** *Kinosternon scorpioides* (Linnaeus, 1766) (Kinosternidae Agassiz, 1857).

**Type-locality:** Forest pond along the Cayenne-Kaw road south of the town of Roura, French Guiana (4.66997N, 52.30560W).

**Site in host:** Conjunctival sacs of the eye.

**Type-material:** Three sexually mature worms. The holotype (NMB P407) and two paratypes (NMB P408–P409) were deposited in the Parasitic Worm Collection, National Museum, Aliwal Street, Bloemfontein, South Africa.

**Prevalence and intensity:**

Site D: Prevalence 7.7%; Mean intensity 4.0.

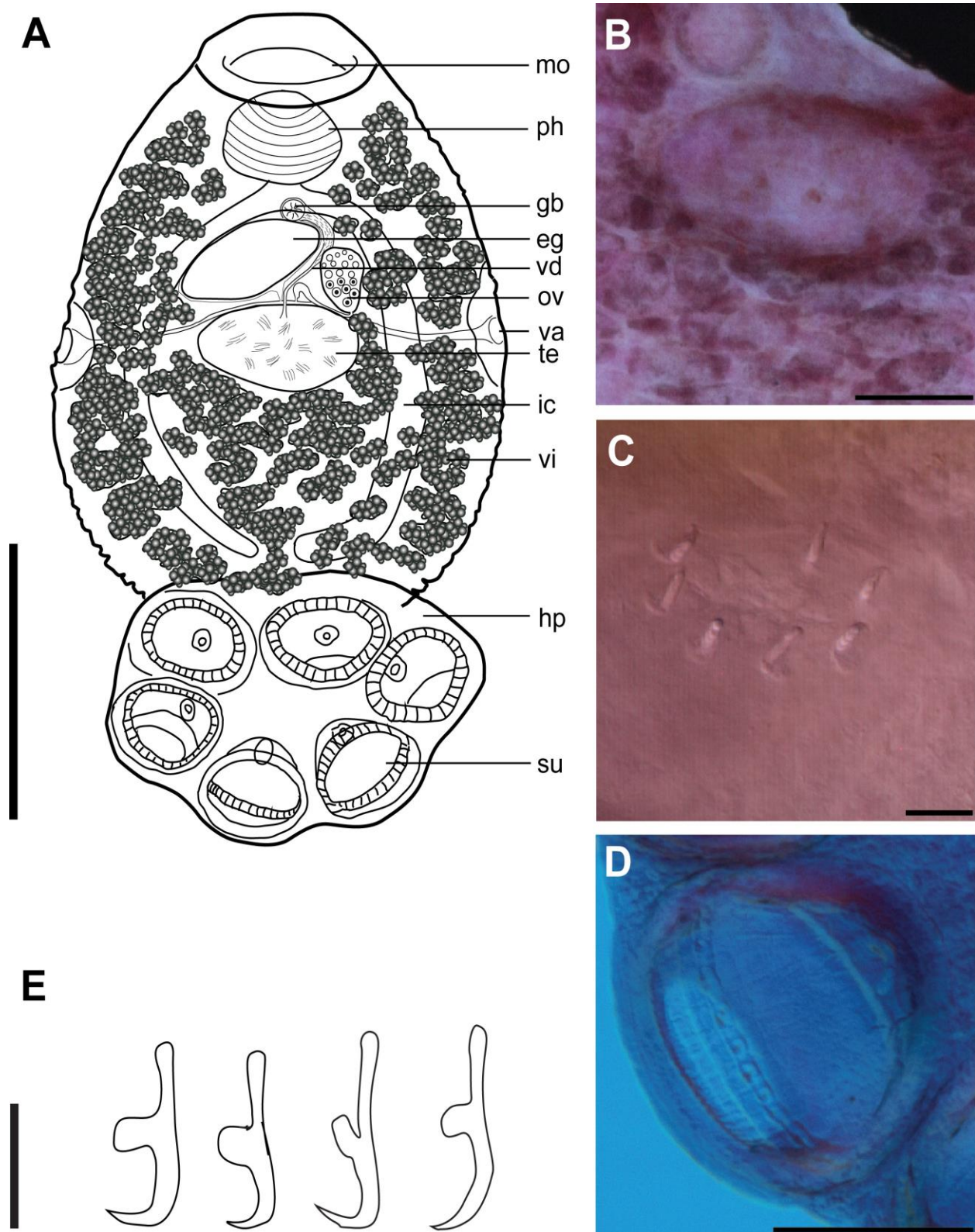
**Representative DNA sequences:** GenBank accession numbers: KY200990 (28S), KY200993 (12S) and KY200996 (*cox1*).

**ZooBank registration:** To comply with the regulations set out in article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN) [33], details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:B55369A7-5F88-4A72-94F2-E0CE354484A1. The LSID for the new name *N. scorpioides* n. sp. is urn:lsid:zoobank.org:act:7852C6EA-FDEC-435F-9319-5379747F7099.

**Etymology:** This parasite is named after the host *K. scorpioides*.

**Description**

[Based on three egg-producing adults (see Table 1 and Fig. 3a-e). No larval measurements or characters are given as eggs collected from the host failed to develop.] Body oval, total length 1,512–1,770 (1,658), greatest width 868–907 (894), width at vagina 837–876 (863). Haptor length 445–511 (485), haptor width 719–765 (735), haptor length to body length ratio 0.29. Mouth subterminal, ventral. False oral sucker prominent, 240–245 (242) wide. Pharynx 170–184 × 226–231 (177 × 228). Intestine bifurcates without diverticula; caeca lacking anastomoses, not confluent posteriorly, not extending into haptor. Testis single, compact, oval (Fig. 3b), mid-ventral, medial and posterior to ovary, 120–142 × 246–301 (127 × 271). Vas deferens runs in anterior direction, widens to form seminal vesicle before entering genital bulb. Genital atrium median, ventral, posterior to intestinal bifurcation, 45 in diameter, with eight straight spines with curved tips (Fig. 3c), 5.5–8.7 (7.7) long. Ovary dextral, at 44% of body length from anterior extremity, 136–187 × 66–71 (153 × 69). Uterus short, tubular, anterior to ovary, containing only one fusiform egg; egg capsule 253–279 × 133–156 (265 × 143). No intrauterine development, eggs operculate. Vitellarium extends from just behind pharynx throughout most of body proper

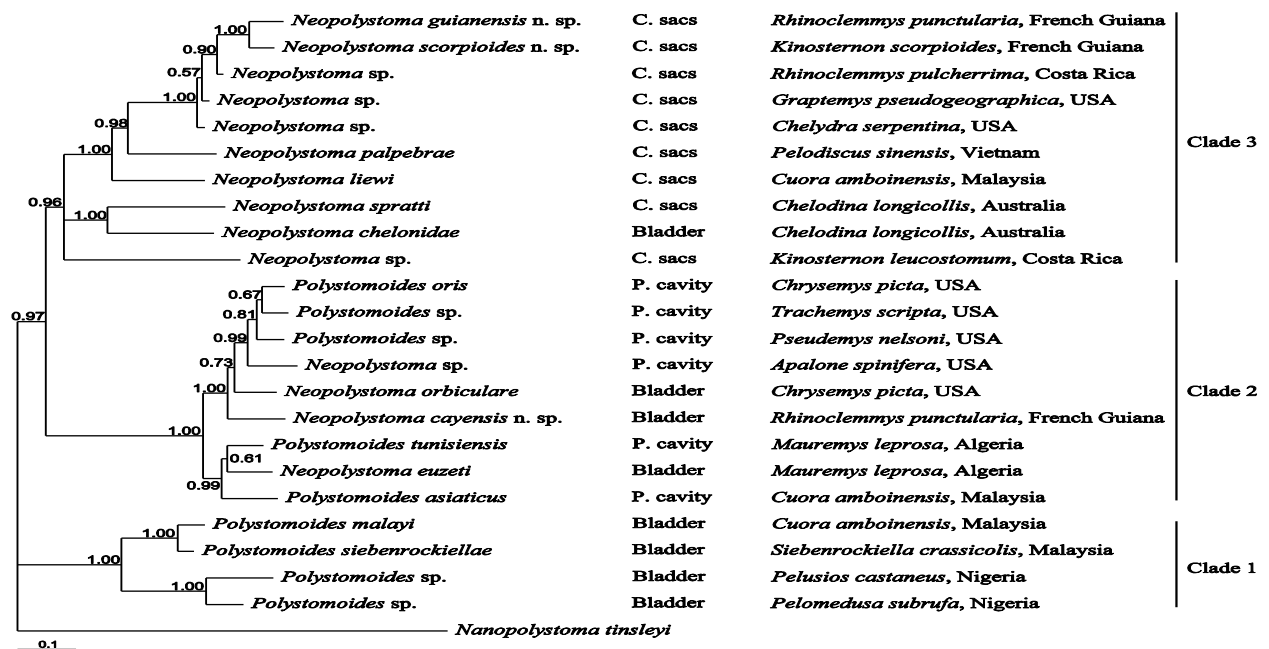


**Fig. 3** *Neopolystoma scorpioides* n. sp. Holotype. **a** Ventral view. **b** testis of holotype. **c** genital spines. **d** haptor sucker showing a ring of skeletal elements. **e** marginal hooklets. *Abbreviations:* eg, egg; gb, genital bulb; hp, haptor; ic, intestinal caecum; mo, mouth; ov, ovary; ph, pharynx; su, sucker; te, testis; va, vagina; vd, vas deferens; vi, vitellaria. *Scale-bars:* **a**, 1,000  $\mu\text{m}$ ; **b**, 100  $\mu\text{m}$ ; **c**, 10  $\mu\text{m}$ ; **d**, 100  $\mu\text{m}$ ; **e**, 10  $\mu\text{m}$

except for area around gonads and posteriormost part of body proper. Genito-intestinal canal obscured by testis, dextral, joining intestinal caecum posterior to ovary (Fig. 3a). Haptoral suckers 6, muscular, with well-developed skeletal structure inside (Fig. 3d), mean diameter 133–279 (220). Hamuli absent. Marginal hooklets (Fig. 3e) retained in adult parasites. Posteriormost marginal hooklet 1 16.4–17.2 (16.8) long; hooklets 2–8 13.4–14.8 (14.2) long.

### Molecular, phylogenetic and genetic divergence analyses

One worm of each of *N. cayensis* n. sp., *N. guianensis* n. sp. and *N. scorpioides* n. sp. were used for molecular studies. With the exception of the 18S rRNA and a portion of the 28S rRNA genes that failed to amplify for *N. scorpioides* n. sp., we obtained sequences for all three polystomes. The Bayesian tree showed with high confidence values the phylogenetic relationships within chelonian polystomes (Fig. 4). *N. guianensis* n. sp. and *N. scorpioides* n. sp. were sister species and nested in Clade 3, according to Héritier et al. [21]. Estimates of *cox1* genetic divergences between these two polystomes were about 15.5%, which was well beyond the threshold of 3.4% defined for chelonian polystomes by Héritier et al. [23]. These two polystomes thus belonged to separate species. Conversely, *N. cayensis* n. sp. was nested in Clade 2 according to Héritier et al. [21]. Estimates of *cox1* genetic divergences between this polystome and the former two species were 22.2 and 26.1%, respectively, and ranged from 16.9 to 20.3% with the most closely related species. These results also indicated that this polystome belonged to another species.



**Fig. 4** Bayesian tree inferred from the analysis of four concatenated genes. Numbers at nodes correspond to Bayesian posterior probabilities. *Abbreviations*: C. sacs, conjunctival sacs; P. cavity, pharyngeal cavity

## Discussion

### Diagnosis

Whereas two *Neopolystoma* species were known from Central America and so in the Neotropical realm, namely *Neopolystoma fentoni* Platt, 2000 from Costa Rica and *Neopolystoma domitilae* (Caballero, 1938) from Mexico, the three *Neopolystoma* species described herein are the first members of the genus recorded from South America. *Neopolystoma cayensis* n. sp. differs from other members of the genus reported in the Neotropical realm by a combination of characters. Based on the body length, *N. cayensis* n. sp., with a length of 3,342–5,575 (4,139)  $\mu\text{m}$ , is significantly longer than *N. guianensis* n. sp. with a length of 2,284–3,342 (2,737)  $\mu\text{m}$ , *N. scorpioides* n. sp. with a length of 1,512–1,770 (1,658)  $\mu\text{m}$  and *N. fentoni* with a length of 1,500–2,450 (1,985)  $\mu\text{m}$ . If it is not significantly longer than *N. domitilae* (4,039–4,057  $\mu\text{m}$ ), the latter is significantly longer than *N. guianensis* n. sp. and *N. scorpioides* n. sp. Based on the genital spine number, *N. cayensis* n. sp. with 16–17 spines differs from *N. domitilae* with 20–21 spines and from the three polystomes of the conjunctival sacs, namely *N. guianensis* n. sp., *N. scorpioides* n. sp. and *N. fentoni*, which all have eight spines. Based on the genital spine length, *N. cayensis* n. sp. with spine length of 19–26 (23)  $\mu\text{m}$  also differs from *N. domitilae* with spine length of 55–71  $\mu\text{m}$ , *N. fentoni* with spine length of 11  $\mu\text{m}$ , *N. guianensis* n. sp. with spine length of 8.4–9.8 (8.9)  $\mu\text{m}$  and *N. scorpioides* n. sp. with spine length of 5.5–8.7 (7.7)  $\mu\text{m}$ . Concerning testis length, it varies between all *Neopolystoma* species. *Neopolystoma cayensis* n. sp. has a testis length of 560–983 (762)  $\mu\text{m}$ , *N. guianensis* n. sp. a testis length of 364–541 (437)  $\mu\text{m}$ , *N. domitilae* a testis length of 450–664  $\mu\text{m}$ , *N. fentoni* a testis length of 98–367 (225)  $\mu\text{m}$  and *N. scorpioides* n. sp. a testis length of 120–142 (127)  $\mu\text{m}$ .

### Diversity of chelonian polystomes in South America

In spite of the considerable diversity of South American amphibians [34] and chelonians [4], relatively few polystomes were reported from South America [20, 22]. Currently, 16 polystomes are known from anurans, three from caecilians and six from chelonians. Turtle polystomes include *Polystomoides coronatum* (Leydi, 1888) from *Trachemys dorbigni* (Duméril & Bibron, 1835); *Polystomoides uruguayensis* Mané-Garzon & Gil, 1961 from *Phrynops hilarii* (Duméril & Bibron, 1835); *Polystomoides fuquesi* Mané-Garzon & Gil, 1962 from *P. hilarii*; *Polystomoides rohdei* Mané-Garzon & Holcman-Spector, 1968 from *T. dorbigni*; *Polystomoides brasiliensis* Vieira, Novelli, Souza & de SouzaLima, 2008 from *Hydromedusa maximiliani* (Mikan, 1820) and *Phrynops geoffroanus* (Schweigger, 1812); and

*Polystomoides magdalenensis* Lenis & Garcia-Prieto, 2009 from *Trachemys callirostris* (Gray, 1856). All of these were reported from the pharyngeal cavity of their host. If we assume, as stated by Verneau [22], that chelonian polystomes are host- and site-specific in natural environments on the one hand and that turtle species are infected by no more than three polystome species depending of their ecological niche on the other, it is likely that a single polystome species rather than two really infect each of the two freshwater turtles *T. dorbigni* and *P. hilarii*, respectively. We should therefore consider only four *Polystomoides* species occurring within South American chelonian hosts. However, this must be further confirmed from genetic analyses of the *cox1* marker. The description of three new species in this study, namely *N. cayensis* n. sp., *N. guianensis* n. sp. and *N. scorpioides* n. sp. is a novelty as it is the first statement of polystomes from the bladder and conjunctival sacs of South American turtles. Although Avila et al. [35] reported the presence of a *Neopolystoma* species from *Mesoclemmys vanderhaegei* (Bour, 1973) in Brazil, nothing was mentioned about the ecological niche of this undescribed parasite. We can, therefore, consider that *Neopolystoma* is here accounted for the first time in South America. Knowing that we surveyed in just two weeks 68 specimens of freshwater turtles belonging to three distinct species and in the light of the rich diversity of chelonians in South America [4], it is very likely that vast numbers of polystomes remain undescribed.

### **Status of *Neopolystoma* and *Polystomoides***

Whereas the three chelonian polystome genera *Neopolystoma*, *Polystomoides* and *Polystomoidella* are distinguished on the basis of the presence of hamuli, i.e. *Neopolystoma* has no hamuli, *Polystomoidella* has a single pair of hamuli and *Polystomoides* has two pairs, it is now well established that *Neopolystoma* and *Polystomoides*, at least, are not monophyletic [21, 36]. The phylogenetic study by H eritier et al. [21] showed that the absence of hamuli on the one hand and the presence of two pairs of hamuli on the other were not true morphological synapomorphies for *Neopolystoma* and *Polystomoides*, respectively. Whereas *Neopolystoma* and *Polystomoides* are known from the conjunctival sacs, bladder and pharyngeal cavity of their host [22], *Polystomoidella* is known only from the bladder of its host. Regarding our phylogenetic tree, all *Neopolystoma* spp. from the conjunctival sacs could form a distinct clade, i.e. Clade 3 (Fig. 4), if the possibility is considered of an accidental switch of vials or a mislabelled tissue when sampling *Neopolystoma chelodinae* (MacCallum, 1918), which infects the bladder of *Chelodina longicollis* (Shaw, 1794) from Australia and which is nested within that clade. Based on morphology, *Neopolystoma* spp. from the conjunctival sacs do not

differ significantly from any other chelonian polystomes from the bladder and pharyngeal cavity. However, parasites of the conjunctival sacs are the only chelonian polystomes that do not lay spherical eggs. They indeed produce fusiform or spindle-shaped eggs [24]. We can therefore hypothesise that this particular shape of egg could be a synapomorphy for the group of polystomes infecting the conjunctival sacs. However, this needs to be further validated after a more in-depth study of polystomes collected from Australian turtles, particularly *N. chelodinae* from *C. longicollis*. Clade 1 of the Bayesian tree contains only polystomes with two pairs of hamuli, i.e. *Polystomoides* spp. that infect the bladder of their host. Although species of *Polystomoides* can be also found within Clade 2, all of them infect the pharyngeal cavity of their host. Hence, this suggests that polystomes with two pairs of hamuli occurring in the bladder of their host belong to a separate lineage (Clade 1). On the opposite, Clade 2 of the Bayesian tree contains species of both genera, with *Polystomoides* spp. infecting the pharyngeal cavity and *Neopolystoma* spp. infecting either the pharyngeal cavity or the bladder. Because there is no trend in the number of hamuli and the ecological niche of the parasites nested in Clade 2, the morphology of these species should be revisited in order to establish whether morphological synapomorphies for that particular clade really exist.

### **The need for the study of helminth diversity**

In the pursuit of conservation efforts in any vertebrate group, it is important to understand the broad biological context of that group, including its parasite diversity. While some parasites are harmless to chelonian hosts, others may adversely affect host behaviour, general fitness and chances of survival [13, 14]. Furthermore, in a changing world where animals are sold for pets, some species may invade new environments and bring with them a great number of parasite species. Concerning turtles, Meyer et al. [25] and H eritier et al. [23] investigated the polystome richness among two European freshwater turtles, *Mauremys leprosa* (Schweigger, 1812) and *Emys orbicularis* (Linnaeus, 1758), respectively. They documented a greater polystome diversity than expected assuming a high degree of host- and site-specificity [22]. Surprisingly, a few polystome species reported within populations of *M. leprosa* and *E. orbicularis* were also found in American hosts that had never been recorded in the European freshwater environments of investigation. Meyer et al. [25] and H eritier et al. [23] showed that these parasite species were in fact introduced into natural wetlands following the introduction of the American red-eared slider *T. s. elegans*. Because that turtle is among the most common exotic turtle species in captivity, it would act as a reservoir of numerous parasite species [24], and, once released into freshwater habitats, it would have served as a

carrier of parasites, as for instance *Neopolystoma* sp. 4 (haplotype H18), *Polystomoides* sp. 1 (haplotype H16) and X. sp (haplotype H36), which would have been transmitted to native turtles across European freshwater environments [23, 25]. Since *T. s. elegans* was banned from the commercial trade in Europe, some other turtle species were identified in pet markets. Among the 17 chelonian species that were offered for sale by the French society “La Ferme Tropicale” on July 2016 07th (<https://www.lafermetropicale.com>), 13 freshwater species were from the USA, Central America, Asia and Africa. A similar situation was found on the online Reptimania society (<http://www.reptimania.com>). On July 2016 07th, numerous exotic chelonian species were indeed offered for sale, among which *R. pulcherrima*. Hence, this demonstrates the need to document and describe as fast as possible the helminth diversity of freshwater turtles in their home range before their parasites invade novel freshwater environments following the introduction and possible release of new exotic chelonian species.

### **Acknowledgments**

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### **Ethics approval**

This study was conducted under ethical clearance no 01D04 (Taxonomy and biology of polystomatid flatworms) issued by the ethical commission of the North-West University, Potchefstroom, South Africa.

### **Consent for publication**

Not applicable.

### **Availability of data and material**

Sequences for all three polystomes were deposited in the GenBank database under accession numbers KY200986 (18S), KY200988 (28S), KY200991 (12S) and KY200994 (*cox1*) for *N. cayensis* n. sp., KY200987 (18S), KY200989 (28S), KY200992 (12S) and KY200995 (*cox1*) for *N. guianensis* n. sp. and KY200990 (28S), KY200993 (12S) and KY200996 (*cox1*) for *N. scorpioides* n. sp. The type-specimens were deposited in the Parasitic Worm Collection, National Museum, Aliwal Street, Bloemfontein, South Africa under accession numbers (NMB



P394–P403) for *N. cayensis* n. sp., (NMB P404–P406) for *N. guianensis* n. sp. and (NMB P407–P409) for *N. scorpioides* n. sp.

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### **Competing interests**

The authors declare that they have no competing interests.

### **Authors' contributions**

MB initiated the research visit to French Guiana, obtained the collection permits and played an important role during the fieldwork with LDP and OV. LH played a key role in conducting the molecular and phylogenetic analyses under the supervision of OV. LDP performed the morphological analyses and, finally, LDP and OV took the leadership in writing the manuscript. All authors read and approved the final manuscript.

### **References**

1. Balian EV, Segers H, Lévêque C, Martens K. The freshwater animal diversity assessment: an overview of the results. *Hydrobiologia*. 2008;595:627-637.
2. Dudgeon D, Arthington AH, Gessner MO, Kawabata ZI, Knowler DJ, Lévêque C, et al. Freshwater biodiversity: importance, threats, status and conservation challenges. *Biol Rev*. 2006;81:163-182.
3. Abell R, Thieme ML, Revenga C, Bryer M, Kottelat M, Bogutskaya N, et al. Freshwater ecoregions of the world: A new map of biogeographic units for freshwater biodiversity conservation. *BioScience*. 2008;58:403-414.
4. Van Dijk PP, Iverson JB, Rhodin AGJ, Shaffer HB, Bour R. Turtles of the world, 7th edition: Annotated checklist of taxonomy, synonymy, distribution with maps, and conservation status. *Chelonian Res Monogr*. 2014;5:329-479.
5. Bour R. Global diversity of turtles (Chelonii; Reptilia) in freshwater. *Hydrobiologia*. 2008;595:593-598.

6. Buhlmann KA, Akre TSB, Iverson JB, Karapatakis D, Mittermeier RA, Georges A, et al. A global analysis of tortoise and freshwater turtle distributions with identification of priority conservation areas. *Chelonian Conserv Biol.* 2009;8:116-149.
7. Böhm M, Collen B, Baillie JE, Bowles P, Chanson J, Cox N, et al. The conservation status of the world's reptiles. *Biol Conserv.* 2013;157:372-385.
8. Turtle Conservation Fund. A global action plan for conservation of tortoises and freshwater turtles. Strategy and Funding Prospectus 2002–2007. Washington DC: Conservation International and Chelonian Research Foundation; 2002.
9. Masin S, Bonardi A, Padoa-Schioppa E, Bottoni L, Ficetola GF. Risk of invasion by frequently traded freshwater turtles. *Biol Invasions.* 2014;16:217-231.
10. Cadi A, Joly P. Competition for basking places between the endangered European pond turtle (*Emys orbicularis galloitalica*) and the introduced red-eared slider (*Trachemys scripta elegans*). *Can J Zool.* 2003;81:1392-1398.
11. Polo-Cavia N, López P, Martin J. Competitive interactions during basking between native and invasive freshwater turtle species. *Biol Invasions.* 2010;12:2141-2152.
12. Pearson SH, Avery HW, Spotila JR. Juvenile invasive red-eared slider turtles negatively impact the growth of native turtles: Implications for global freshwater turtle populations. *Biol Conserv.* 2015;186:115-121.
13. Schumacher J. Selected infectious diseases of wild reptiles and amphibians. *J Exot Pet Med.* 2006;15:18-24.
14. Gibbons PM, Steffes ZJ. Emerging infectious diseases of chelonians. *Vet Clin Exot Anim.* 2013;16:303-317.
15. Jungwirth N, Bodewes R, Osterhaus ADME, Baumgärtner W, Wohlsein P. First report of a new alphaherpesvirus in a freshwater turtle (*Pseudemys concinna concinna*) kept in Germany. *Vet Microbiol.* 2014;170:403-407.
16. Readell AM, Phillips CA, Goldberg TL. Prevalence of *Salmonella* in intestinal mucosal samples from free-ranging red-eared sliders (*Trachemys scripta elegans*) in Illinois. *Herpetol Conserv Biol.* 2010;5:207-213.
17. Telford SRJr, Norton TM, Moler PE, Jensen JB. A new *Haemogregarina* species of the alligator snapping turtle, *Macrochelys temminckii* (Testudines: Chelydridae), in Georgia and Florida that produces macromeronts in circulating erythrocytes. *J Parasitol.* 2009;95:208-214.
18. McAllister CT, Barger MA, Stuart JN. *Neoechinorhynchus emyditoides* Fisher, 1960 (Acanthocephala: Neoechinorhynchidae) from the Mexican plateau slider, *Trachemys*

- gaigeae* (Testudines: Emydidae) in New Mexico, U.S.A. *Comp Parasitol.* 2008;75:135-137.
19. Zelmer DA, Platt TR. Structure and similarity of helminth communities of six species of Australian turtles. *J Parasitol.* 2008;94:781-787.
  20. Morrison C, Du Preez LH. Turtle polystomes of the world: *Neopolystoma*, *Polystomoidella* & *Polystomoides*. Saarbrücken, Germany: VDM Verlag Dr. Müller; 2011.
  21. Héritier L, Badets M, Du Preez LH, Aisien MS, Lixian F, Combes C, Verneau O. Evolutionary processes involved in the diversification of chelonian and mammal polystomatid parasites (Platyhelminthes, Monogenea, Polystomatidae) revealed by palaeoecology of their hosts. *Mol Phylogenet Evol.* 2015;92:1-10.
  22. Verneau O. Origine et évolution des monogènes Polystomatidae, parasites d'amphibiens et de chéloniens d'eau douce. HDR dissertation, Université de Perpignan Via Domitia; 2004.
  23. Héritier L, Valdeón A, Sadaoui A, Gendre T, Ficheux S, Bouamer S, et al. Introduction and invasion of the red-eared slider and its parasites in freshwater ecosystems of southern Europe: risk assessment for the European pond turtle in wild environments. In press to *Biodiversity and Conservation*.
  24. Verneau O, Palacios C, Platt T, Alday M, Billard E, Allienne JF, Basso C, Du Preez LH. Invasive parasite threat: parasite phylogenetics reveals patterns and processes of host-switching between non-native and native captive freshwater turtles. *Parasitology.* 2011;138:1778-1792.
  25. Meyer L, Du Preez L, Bonneau E, Héritier L, Quintana, MF, Valdeón A, et al. Parasite host-switching from the invasive American red-eared slider, *Trachemys scripta elegans*, to the native Mediterranean pond turtle, *Mauremys leprosa*, in natural environments. *Aquat Invasions.* 2015;10:79-91.
  26. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl Acids Res.* 1994;22:4673-4680.
  27. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol.* 2011;28:2731-2739.
  28. Littlewood DTJ, Rohde K, Clough KA. Parasite speciation within or between host species? Phylogenetic evidence from site-specific polystome monogeneans. *Int J Parasitol.* 1997;27:1289-1297.

29. Littlewood DTJ, Rohde K, Clough KA. The phylogenetic position of *Udonella* (Platyhelminthes). *Int J Parasitol.* 1998;28:1241-1250.
30. Badets M, Verneau O. Origin and evolution of alternative developmental strategies in amphibious sarcopterygian parasites (Platyhelminthes, Monogenea, Polystomatidae). *Org Divers Evol.* 2009;9:155-164.
31. Posada D, Crandall KA. Modeltest: testing the model of DNA substitution. *Bioinformatics* 1998;14:817-818.
32. Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 2001;17:754-755.
33. International Commission on Zoological Nomenclature. Amendment of articles 8, 9, 10, 21 and 78 of the International Code of Zoological Nomenclature to expand and refine methods of publication. *Zootaxa.* 2012;3450:1-7.
34. Frost DR. Amphibian Species of the World: an Online Reference. Version 6.0 Electronic Database accessible at <http://research.amnh.org/herpetology/amphibia/index.html>. American Museum of Natural History, New York, USA. Assessed 07 July 2016.
35. Avila RW, Brito ES, Barrella TH, Strussmann C, Silva RJ. Endoparasites new to the Neotropical freshwater turtle, *Mesoclemmys vanderhaegei* (Bour 1973) (Testudines, Chelidae), from central Brazil. *Panam J Aquat Sci.* 2010;5:478-480.
36. Verneau O, Bentz S, Sinnappah ND, Du Preez LH, Whittington I, Combes C. A view of early vertebrate evolution inferred from the phylogeny of polystome parasites (Monogenea: Polystomatidae). *Proc R Soc B.* 2002;269:535-543.

**CHAPITRE VI. OXIDATIVE STRESS INDUCED BY  
GLYPHOSATE-BASED HERBICIDE ON  
FRESHWATER TURTLES**

## Présentation du Chapitre VI

La seconde partie de ce travail de thèse a constitué à développer un outil permettant d'évaluer l'état de santé des populations sauvages de tortues. En effet, après la mise en évidence d'invasions biologiques sur les espèces de tortues natives au sein de la zone d'investigation, l'étude d'un rapport fourni par le Conseil Général des Pyrénées-Orientales sur la qualité des eaux des rivières du département a révélé la présence de pesticides dans la plupart de ces dernières, comme le glyphosate et son produit de dégradation l'AMPA qui sont mesurés dans les plus hautes concentrations. Des études ont montré la toxicité des herbicides à base de glyphosate sur divers organismes comme les poissons, les amphibiens et les reptiles. Ainsi, entre la présence d'espèces invasives et de contaminants chimiques dans les cours d'eau des Pyrénées-Orientales, la biodiversité serait-elle impactée par cette dégradation des environnements naturels?

Afin de pouvoir répondre, la première étape a été de développer des biomarqueurs permettant d'illustrer une réponse face au stress chez les tortues d'eau douce. Si les variations dans l'expression des gènes de stress, c'est-à-dire des gènes codant pour des protéines impliquées dans la défense physiologique contre le stress, ont été sélectionnées comme biomarqueurs dans un premier temps, un autre biomarqueur a également attiré notre attention. Face à une modification environnementale, la production d'espèces réactives de l'oxygène augmente pour engendrer une réponse physiologique de lutte contre le stress. Si ces composés peuvent s'attaquer à toutes substances intrusives dans l'hôte, ils doivent être par la suite neutralisés car également capables de dégrader les parois lipidiques de cellules, les protéines et l'ADN. Ainsi, l'activité d'enzymes impliquées dans cette détoxification oxydative, telles que la Catalase et la Superoxyde dismutase, peut être modifiée en cas de stress environnemental.

Ce Chapitre propose une étude sur la mise au point de ces deux biomarqueurs, à savoir la mesure de l'activité enzymatique de la Catalase et de la Superoxyde dismutase et de l'expression des gènes codant pour ces dernières, sur les tortues d'eau douce. Parce que la cistude d'Europe et l'émyde lépreuse bénéficient de statut de protection, le développement de ces biomarqueurs sera effectué à partir d'échantillons sanguins après expérimentations sur des juvéniles de *T. s. elegans*.

## **Oxidative stress induced by Glyphosate-based herbicide on freshwater turtles**

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### **Keywords:**

*Trachemys scripta elegans*, *Mauremys leprosa*, Oxidative stress, Glyphosate, Catalase, Superoxide dismutase, Gene expression, Enzyme activity.

## **Abstract**

Freshwater ecosystems face very strong anthropogenic pressures, among which over-exploitation, habitat degradation, flow modification, species invasion and water pollution that lead to growing threats on biodiversity. Urbanization through wastewater treatment, industry through the release of inorganic and organic chemicals, and agriculture through the use of pesticides and herbicides, are the main factors involved in water pollution. In France, more precisely in the Pyrénées-Orientales department, the poor quality of the watercourses is mainly due to the use of glyphosate-based herbicides used in agricultural activities. Because these chemicals can impact local biodiversity, we thereby investigated under experimental conditions the physiological response of animals face to abiotic contaminants. We used as a model, juveniles of freshwater turtles, i.e., the red-eared sliders *Trachemys scripta elegans*. We measured the enzymatic activity and gene expression of oxidative stress biomarkers, namely catalase and superoxide dismutase, as well as the lipid peroxidation levels and the acetylcholinesterase activity in animals challenged with high concentrations of glyphosate-based herbicide on one hand and with degraded waters collected from a local water course on the other. We showed through variation in enzyme activities and gene expression that a glyphosate commercial formulation induced a stress in turtles. A similar outcome was obtained when turtles faced to degraded waters. These results indicate that the poor quality of regional waters may be a real threat for biodiversity, especially for the native endangered freshwater turtle, namely the Mediterranean pond turtle *Mauremys leprosa*. Furthermore, because turtles are globally less sensitive to contaminants than sentinel species, they could also constitute an excellent model to follow evolution of water quality through the study of oxidative stress biomarkers.



## INTRODUCTION

Freshwater ecosystems are remarkable natural habitats providing multiple ecological services via hydrological properties, water resource and climate regulation. As such, they have a significant economic value and then contribute to human well-being. Though they represent only less than 1% of the earth's surface, they also comprise a great diversity of species with no less than 100,000 species known to date, which corresponds to approximately 9.5% of all described species (Dudgeon et al., 2006; Balian et al., 2008). However, freshwater ecosystems face strong pressures following human activities, leading to growing threats on biodiversity (Strayer and Dudgeon, 2010). They are likewise considered more imperiled than their terrestrial and marine counterparts (Dudgeon et al., 2006; Strayer and Dudgeon, 2010). If freshwater research is indeed regarded as less sexy than that in terrestrial or marine realms, trajectories of species loss make it arguably the most urgent (Abell, 2002). It is therefore crucial to pursue and undertake intensive surveys on freshwater ecosystems to protect and manage aquatic biodiversity.

Among the many factors initiating the loss of freshwater biodiversity, the most important remain over-exploitation, habitat degradation, flow modification, species invasion and water pollution (Revenge and Mock, 2000; Baron et al., 2003; Dudgeon et al., 2006; Vaughn, 2010). Regarding pollution of fresh waters, different causes can be invoked either natural, like spring flood, fair weather periods and biogenetic agents, or anthropogenic like chemical and biological means that constitute the primary sources of environmental contamination (Hupfer and Kleeberg, 2007). The latter are usually the effect of urbanization through wastewater treatment, industry through the release of inorganic and organic chemicals, and agriculture through the use of pesticides and herbicides. The most common pollutants that are typically found within fresh waters then depend of the source of contamination, but also of the climate and of the geographical area (Villeneuve et al., 2011; Reoyo-Prats et al., submitted). In France, where intensive agricultural activities still exist, numerous pesticides and herbicides are then used. As a consequence, some chemicals are released in freshwater ecosystems, among others glyphosate, atrazine, diuron and imidaclopride (Commissariat général au développement durable – Service de l'observation et des statistiques, 2015: <http://www.developpement-durable.gouv.fr/IMG/pdf/CS697.pdf>).

Consequently, active ingredients of herbicides and their degradation products, particularly glyphosate and AMPA (Aminomethylphosphonic Acid) respectively, as well as pesticide active molecules which are quite common in aquatic ecosystems, may impact local biodiversity. Glyphosate-based herbicides are composed by a mixture of isopropylamine salt

of glyphosate, POEA (polyethoxylated tallowamine) which is the predominant non-ionic surfactant, and water (Giesy et al., 2000). Their toxicity, as well as the toxicity of their active compounds alone, has been indeed well studied across vertebrates, especially fish, amphibian and mammal. It has been shown under experimental conditions that commercial formulations of glyphosate-based herbicides are actually more toxic than the glyphosate only. This was deduced through studies of gene expression, mortality and teratology in tadpoles (Howe et al., 2004), through studies of mitochondrial bioenergetic functions in rats (Peixoto et al., 2005) and through studies of cellular damage and endocrine disruption in human cells (Richard et al., 2005; Gasnier et al., 2009). Commercial formulations of glyphosate-based herbicides were subsequently tested in fish (Gluszczak et al., 2006; Gluszczak et al., 2007; Cavalcante et al., 2008; Langiano and Martinez, 2008; Modesto and Martinez 2010) and in amphibian (Relyea et al., 2005a, 2005b; Costa et al., 2008) following studies of enzyme activity of acetylcholinesterase and oxidative stress enzymes, measures of metabolic parameters and examination of physiological cellular damages.

In Southern France and, more specifically, in the Pyrénées-Orientales (PO) department, agricultural activities are mainly based on fruit crops and vineyards. These can explain the detection of herbicide active ingredients in almost all main water courses, like the Tech (Unpublished report of the Conseil Général des Pyrénées-Orientales) and Têt (Reoyo-Prats et al., submitted) rivers, but also in some minor rivers, like the Agouille de la Mar, Baillaury and Fosseille rivers (Unpublished report of the Conseil Général des Pyrénées-Orientales). The relatively high concentrations of glyphosate (G) and AMPA in the Agouille de la Mar (G =  $0.420\mu\text{gL}^{-1}$  and AMPA =  $8.6\mu\text{gL}^{-1}$  in September 2011) and in the Fosseille (G =  $0.8\mu\text{gL}^{-1}$  in July 2011 and AMPA =  $13\mu\text{gL}^{-1}$  in September 2011) rivers have led to downgrade these two streams. As a result, both were classified in the worst of the five listed categories that are used to evaluate water quality. To the exception of amphibians that have never been recorded in these two watercourses, fishes such as roaches (*Rutilus rutilus*), gudgeons (*Gobio gobio*) and pumpkinseed sunfishes (*Lepomis gibbosus*), mammals such as coypus (*Myocastor coypus*) and rats (*Rattus norvegicus*), birds such as common kingfishers (*Alcedo atthis*) and little egrets (*Egretta garzetta*) and reptiles such as viperine snakes (*Natrix maura*) and Mediterranean pond turtles (*Mauremys leprosa*) are nonetheless frequently observed.

Among the species occurring in both degraded rivers, the Mediterranean pond turtle is probably the most threatened species. This species, which occurs almost exclusively in the PO department in France (see Palacios et al., 2015), has been indeed classified as “Vulnerable” in

this country (UICN France, MNHN and SHF, 2015). Surprisingly, the Fosseille turtle population, which is about 250 individuals (unpublished data), is considered as the second greatest population of the PO after the Baillaury population whose size was estimated to 314 individuals (Vazquez, 2012). Whereas two main threats were previously identified as a risk for *M. leprosa*, namely the invasive red-eared slider *Trachemys scripta elegans* that occurs in the same area and that can compete for basking, territory and feeding resources (Polo-Cavia et al., 2009, 2010, 2011), and its parasites that are transmitted to the indigenous turtle in natural environments (Meyer et al., 2015), the presence of glyphosate and AMPA in the Agouille de la Mar and Fosseille rivers could constitute another threat to the native freshwater turtle.

Regarding the poor quality of the two mentioned watercourses, which is likely due to the use of glyphosate-based herbicides for agriculture activities in the PO, one may wonder if these chemical can impact physiological parameters of the Mediterranean pond turtle. Because this species is endangered in France and benefits of very strict rules of protection, experimental design on that species to investigate stress-induced by glyphosate formulations was inconceivable. Therefore juveniles of captive *M. leprosa* were first used to measure the enzymatic activity of oxidative stress biomarkers, namely Catalase (CAT) and Superoxide dismutase (SOD), as well as gene expression of the same markers in turtle blood samples. Secondly, juveniles of captive *T. s. elegans* were selected to measure the response of CAT and SOD as well as two other biomarkers, namely the lipid peroxidation (LPO) and the acetylcholinesterase (AChE) activity face to high concentrations of glyphosate-based herbicide and water collected from the Fosseille river, respectively.

## **MATERIALS AND METHODS**

### **Selecting experimental animals**

#### *Mauremys leprosa*

Eleven juveniles of about 6 months old were maintained altogether in an aquarium filled with about 250 liters of water at the Zoodyssée Zoological Park of Chizé (Deux-Sèvres department, France). They were born in captivity at the same place. Size of their carapax ranged from 2,5cm to 3,5cm while weight was comprised between 5g and 10g.

#### *Trachemys scripta elegans*

Thirty juveniles of about 1 month old were maintained in captivity at the Turtles Farm of Sorède (PO department, France) where they were born. They were subsequently kept for two

months at the lab in 20 liter water tanks preheated at 25°C (15 turtles per tank), equipped with UV lamps (5.0 UVB Reptiles, Exo Terra<sup>®</sup>) and basking sites for acclimation. Turtles were fed every two days with commercial freshwater turtle food (Aquatic turtle Food, ZooMed<sup>®</sup>) until 48h before the beginning of experimentations. Size of their carapax ranged from 3cm to 4cm while weight was comprised between 10g and 15g.

## **Experimental design**

### *Mauremys leprosa*

Two blood samples of 0.15ml each were collected per individual at the Zoodyssée Zoological Park. Blood was drawn from the subcarapacial sinus using insulin syringes and immediately frozen in liquid nitrogen. Samples were subsequently preserved at -80°C until biochemical and molecular experiments.

### *Trachemys scripta elegans*

After an acclimation period of two months, turtles were divided into six groups of five individuals each in containers that were filled with 5cm of water without basking sites. As the response in enzyme activities is usually recorded between 6h and 96h after exposure (see Langiano and Martinez, 2008; Modesto and Martinez, 2010; Lushchak et al., 2009), three of the experimental groups of animals were analyzed 12h after exposure and the remaining three groups 96h after exposure. Two groups among the six were maintained in tap water which served as the control groups, namely Control-12h and Control-96h groups. Because the LC50 at 96h is 36.8mgL<sup>-1</sup> of Roundup for adults of the Nile tilapia (*Oreochromis niloticus*) (Jiraungkoorskul et al., 2002), and because the LC50 at 48h is 49.4mgL<sup>-1</sup> of Roundup for adults of the sign-bearing froglet (*Crinia insignifera*) (Mann and Bidwell, 1999), two groups were exposed to a formulation of glyphosate-based herbicide (Clinic<sup>®</sup>, 360g glyphosate L<sup>-1</sup>, Nufarm S.A.S., France) at a sub-lethal final glyphosate concentration of 30mgL<sup>-1</sup>, named further Glyphosate-12h and Glyphosate-96h groups for convenience. Finally, two groups were exposed to water collected from the Fosseille River in September 2015, named further Fosseille-12h and Fosseille-96h groups for convenience. Two blood samples of 0.15ml each were systematically collected per individual by the end of experiments. Blood was drawn from the occipital vein using insulin syringes and immediately frozen in liquid nitrogen. Samples were subsequently preserved at -80°C until biochemical and molecular experiments. Finally red-eared sliders were euthanized by cardiac injection of 10% sodium pentobarbitone (Euthapent, Kryon Laboratories, South Africa). Turtles were dissected and livers were

collected for each individual. Organs were frozen in liquid nitrogen and subsequently preserved at -80°C.

### **Gene expression of CAT and SOD**

#### *RNA extraction and reverse transcriptase*

Total RNA extractions were performed from blood samples of *M. leprosa* and from host liver of *T. s. elegans*, using Trizol Reagent (Life Technologies) following the manufacturer's protocol. RNA quality and concentrations were assessed by spectrophotometry at 280nm (Nanodrop ND-1000, Thermo Fisher scientific Inc., MA, USA). One microgram of total RNA (5µl at 200ng.µl<sup>-1</sup>) was reverse transcribed using the Maxima H Minus first strand cDNA synthesis kit (Thermo Scientific). cDNAs were diluted at 1/20 until use for real time PCR.

#### *Real time PCR*

The Light Cycler Probe Design<sup>TM</sup> Software version 1.0 was first used to design specific primers from partial *T. s. elegans* CDS CAT and SOD sequences extracted from Genbank (Accession numbers JX508630 and FJ834442, respectively) and from the complete 18SrRNA gene (Accession number M59398), the latter being used as the reference. The following Forward and Reverse primers, 18SQTseF: 5'-CCAGTAAGTGCGGGTCATAAGC-3', 18SQTseR: 5'-GACCTCACTAAACCATCCAATCG-3', CATQTseF: 5'-TCGCTGGAGAGTCTGGTTCA-3', CATQTseR: 5'-ACAAGATCCCAGATAACCCTCTTCTG-3', SODQTseF: 5'-CGGCCTTGGTCCTGGTTAA-3', SODQTseR: 5'-CGGCAGTATCGGTTGGTGTT-3' were used for real time PCR in both species, i.e. *T. s. elegans* and *M. leprosa*. Amplification experiments were performed with a Light cycler apparatus (Roche Molecular Biochemicals, Germany) in a final volume of 10µl containing LightCycler<sup>®</sup> 480 SYBR Green I Master Mix 1x final concentration, primers at 0.4µM and 2µl of cDNA. Cycling conditions were one cycle of 14' of preincubation at 95°C, followed by 45 cycles of 11s of denaturation at 95°C, 11s of annealing at 60°C and 19s of elongation at 72°C. The specificity of all primers was tested by the presence of a single peak observed within the melting curves. The 2<sup>-ΔΔCT</sup> method was used for analyzing relative changes in gene expression (Livak and Schmittgen, 2001).

## **Enzyme activity of CAT, SOD and AChE**

### *CAT activity*

Five microliters of blood were diluted in 200 $\mu$ l of cold assay buffer from the Catalase Activity Colorimetric/Fluorometric Assay Kit (BioVision). The mixture was centrifuged at 4°C during 15 min at 10,000g. Forty microliters of the supernatant was subsequently used for measuring enzyme activity at 570nm following the manufacturer recommendations. CAT activity was expressed in mU.mg of protein<sup>-1</sup>. The biochemical procedure was performed from blood samples for both turtle species.

### *SOD activity*

Five microliters of blood were diluted in 995 $\mu$ l of distilled deionized ultra-pure water. The mixture was centrifuged at 4°C during 15 min at 10,000g. Twenty microliters of the supernatant was diluted in 200 $\mu$ l of WST working solution from the Superoxide Dismutase (SOD) Activity Assay Kit (BioVision) and used for measuring enzyme activity at 450nm following the manufacturer recommendations. SOD activity was expressed in U.mg of protein<sup>-1</sup>. The biochemical procedure was performed from blood samples for both turtle species.

### *AChE activity*

Five microliters of blood were used in combination with the components from the Acetylcholinesterase Activity Colorimetric Assay Kit (Biovision) for measuring enzyme activity at 570nm following the manufacturer recommendations. AChE activity was expressed in mU.mg of protein<sup>-1</sup>. The biochemical procedure was performed from blood samples of *T. s. elegans* only.

## **Quantification of LPO**

Twenty microliters of blood were diluted in 300 $\mu$ l of MDA lysis buffer from the Lipid Peroxidation (MDA) Colorimetric/Fluorometric Assay Kit (BioVision). The mixture was centrifuged at 4°C, 15 min at 10,000g. Two hundred microliters of the supernatant was used for the quantification of LPO at 532nm following the manufacturer recommendations. Levels of LPO were expressed in nmol.mg of protein<sup>-1</sup>. The biochemical procedure was performed from blood samples of *T. s. elegans* only. The quantification for total proteins in all samples was done following the method of Lowry et al. (1951).

## **Statistical analyses**

Non-parametric tests, i.e. Kruskal-Wallis test (KW) by ranks followed by Mann-Whitney U test (MW), were conducted to assess variations in mRNA gene expression and enzyme activities using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA). The statistical significance was accepted at  $P < 0.05$ .

## **RESULTS**

### **Gene expression of CAT and SOD within *M. leprosa***

Ct values obtained from CAT and SOD gene expression among all specimens of *M. leprosa* were higher than those obtained from 18S gene expression among the same specimens. Ct values for the two targeted CAT and SOD genes were lower than 37 cycles and melting curves for both genes were similar to those obtained following preliminary tests conducted on mRNAs extracted from liver of captive adult red-eared sliders (data not shown).

### **Enzyme activity of CAT and SOD within *M. leprosa***

Measures of CAT and SOD enzyme activity from blood samples were similar to those obtained following preliminary tests conducted on blood extracts of captive adult red-eared sliders (data not shown).

### **Gene expression of CAT and SOD within *T. s. elegans***

Variations in the CAT gene expression between the six groups of turtles (Control-12h and -96h, Glyphosate-12h and -96h and Fosseille-12h and -96h) were observed (KW,  $P = 0.018$ ). No significant variation was observed between the three groups sampled 12h after exposure (Figure 1A). On the opposite, the CAT gene expression profile within the Glyphosate-96h group was 2.8 fold higher than that of the Control-96h group (MW,  $P = 0.008$ ), while the Fosseille-96h group showed a gene expression profile 2.1 fold higher than that of the Control-96h group (MW,  $P = 0.032$ ). No significant variation was observed between the Glyphosate-96h and Fosseille-96h groups (Figure 1B).

Variations in the SOD gene expression profile between the six groups of turtles were observed (KW,  $P = 0.014$ ). No significant variation was observed between the three groups sampled 12h after exposure (Figure 2A). On the opposite, the Glyphosate-96h group showed a gene expression profile 3.4 fold higher than that of the Control-96h group (MW,  $P = 0.008$ ) while the Fosseille-96h group showed a gene expression profile 2.2 fold higher than that of the Control-96h group (MW,  $P = 0.008$ ). However the Glyphosate-96h showed a gene

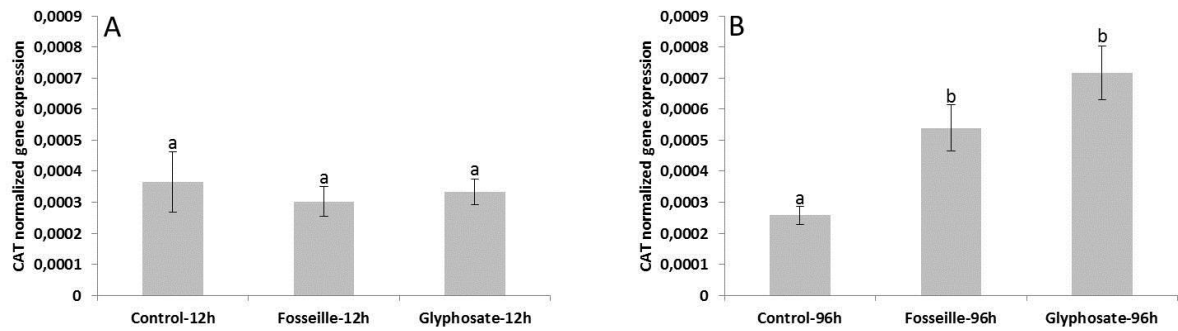


Figure 1: Comparison of CAT gene expression between Control, Fosseille and Glyphosate groups after 12h (A) and 96h (B) of exposure. Data sets are normalized to the 18S rRNA gene reference. Statistically significant differences ( $P < 0.05$ ) within experimental groups are illustrated with lowercase letters. Values are means  $\pm$  SEM.  $N = 5$  per exposure groups.

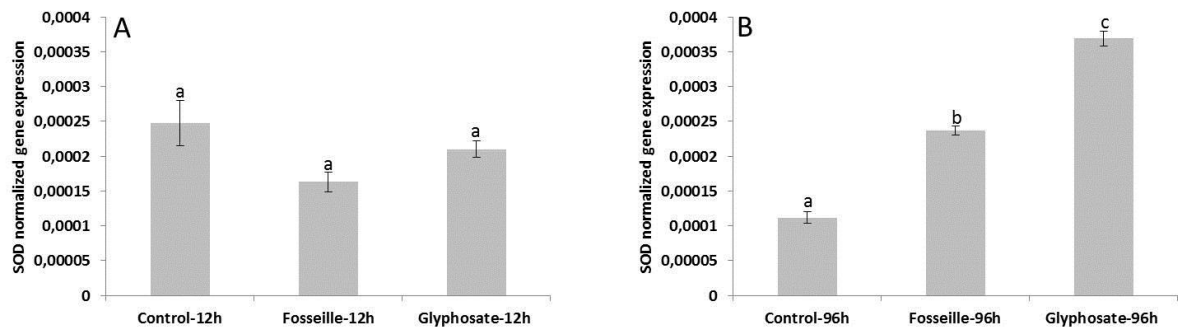


Figure 2: Comparison of SOD gene expression between Control, Fosseille and Glyphosate groups after 12h (A) and 96h (B) of exposure. Data sets are normalized to the 18S rRNA gene reference. Statistically significant differences ( $P < 0.05$ ) within experimental groups are illustrated with lowercase letters. Values are means  $\pm$  SEM.  $N = 5$  per exposure groups.



expression profile 1.5 fold higher than that of the Fosseille-96h group (MW,  $P = 0.008$ ) (Figure 2B).

### **Enzyme activity of CAT, SOD and AChE within *T. s. elegans***

Variations in the CAT activity between the six groups of turtles (Control-12h and -96h, Glyphosate-12h and -96h and Fosseille-12h and -96h) were observed (KW,  $P = 0.001$ ). The Glyphosate-12h group showed an enzyme activity profile 1.3 fold lower than that of the Control-12h (MW,  $P = 0.01$ ) and Fosseille-12h (MW,  $P = 0.008$ ) groups. No significant difference was observed between the Control-12h and Fosseille-12h groups (Figure 3A). Additionally, the Glyphosate-96h group showed an enzyme activity profile 1.5 fold higher than that of the Control-96h group (MW,  $P = 0.016$ ). The Fosseille-96h group also showed an enzyme activity profile 1.9 fold higher than that of the Control-96h group (MW,  $P = 0.016$ ). No significant difference was observed between the Glyphosate-96h and Fosseille-96h groups (Figure 3B). Surprisingly, the Control-12h group showed an enzyme activity profile 1.7 fold higher than that of the Control-96h group (MW,  $P = 0.02$ ).

Variations in the SOD activity between the six groups of turtles were observed (KW,  $P = 0.013$ ). No significant variation was observed between the Control-12h, Glyphosate-12h and Fosseille-12h groups (Figure 4A). Additionally, the Glyphosate-96h group showed an enzyme activity profile 1.6 fold lower than that of the Control-96h group (MW,  $P = 0.008$ ). No significant difference was observed between the Control-96h and Fosseille-96h groups, but also between the Glyphosate-96h and Fosseille-96h groups (Figure 4B). Surprisingly, the Control-12h group showed an enzyme activity profile 1.4 fold lower than that of the Control-96h group (MW,  $P = 0.01$ ).

Variations in the AChE activity between the six groups of turtles were observed (KW,  $P = 0.04$ ). No significant variation was observed between the Control-12h, Glyphosate-12h and Fosseille-12h groups (Figure 5A). Additionally, the Glyphosate-96h group showed an enzyme activity profile 1.8 fold lower than that of the Control-96h group (MW,  $P = 0.008$ ). The Glyphosate-96h group also showed an enzyme activity profile 2.1 fold lower than that of the Fosseille-96h group (MW,  $P = 0.008$ ). No significant difference was observed between the Control-96h and Fosseille-96h groups (Figure 5B).

### **Quantification of LPO within *T. s. elegans***

Variations in the levels of LPO between the six groups of turtles were observed (KW,  $P = 0.002$ ). The Fosseille-12h group showed LPO levels 1.2 fold lower than that of the Control-

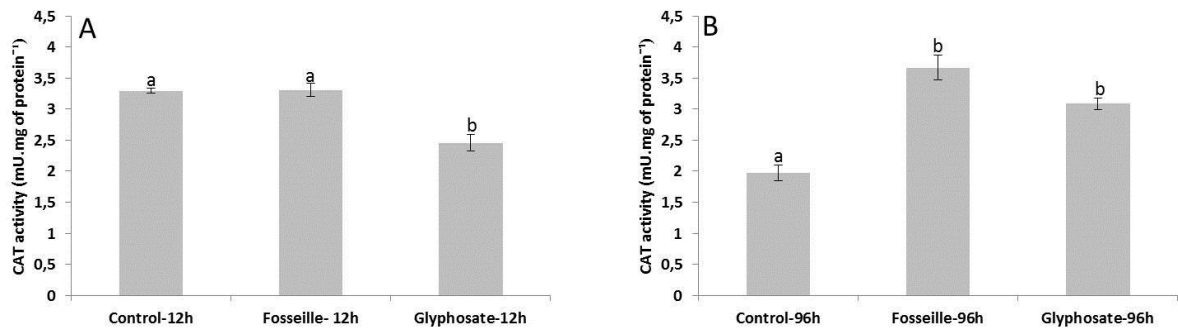


Figure 3: Comparison of CAT activity between Control, Fosseille and Glyphosate groups after 12h (A) and 96h (B) of exposure. Statistically significant differences ( $P < 0.05$ ) within experimental groups are illustrated with lowercase letters. Values are means  $\pm$  SEM.  $N = 5$  per exposure groups.

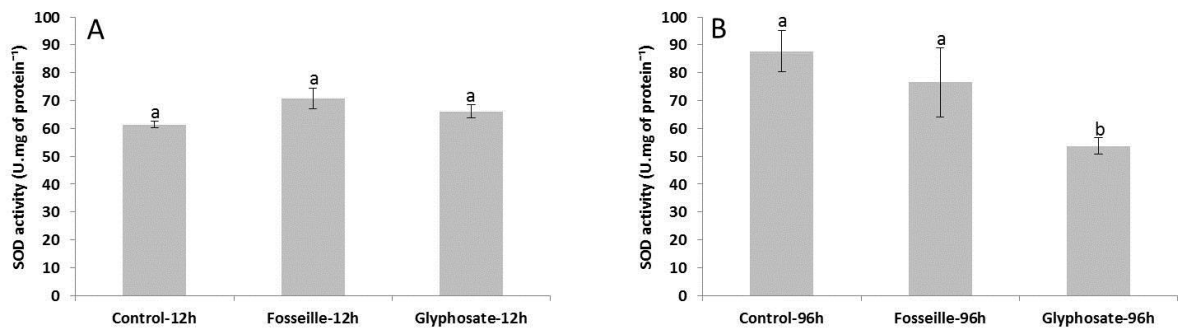


Figure 4: Comparison of SOD activity between Control, Fosseille and Glyphosate groups after 12h (A) and 96h (B) of exposure. Statistically significant differences ( $P < 0.05$ ) within experimental groups are illustrated with lowercase letters. Values are means  $\pm$  SEM.  $N = 5$  per exposure groups.

12h group (MW,  $P = 0.032$ ). No significant variation was observed between the Control-12h and Glyphosate-12h groups and between the Glyphosate-12h and Fosseille-12h groups (Figure 6A). Similarly, the Fosseille-96h group showed LPO levels 1.7 fold lower than that of the Control-96h group (MW,  $P = 0.029$ ). No significant variation was observed between the Control-96h and Glyphosate-96h groups and between the Glyphosate-96h and Fosseille-96h groups (Figure 6B). Surprisingly, the Control-12h group showed LPO levels 1.7 fold higher than that of the Control-96h group (MW,  $P = 0.016$ ).

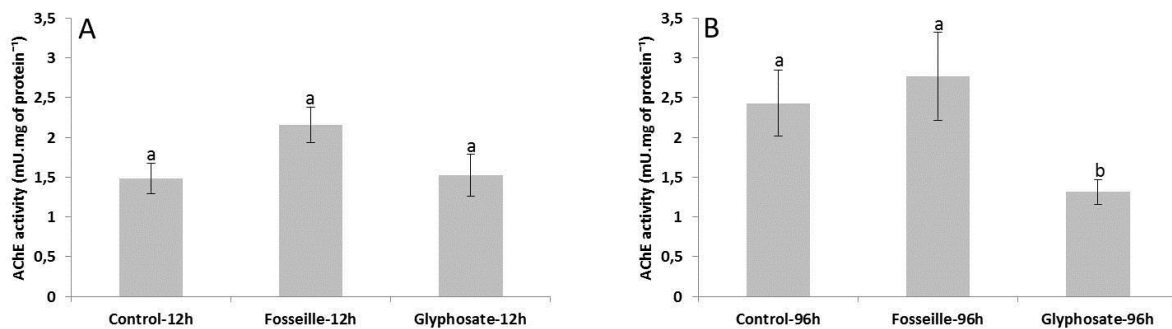


Figure 5: Comparison of AChE activity between Control, Fosseille and Glyphosate groups after 12h (A) and 96h (B) of exposure. Statistically significant differences ( $P < 0.05$ ) within experimental groups are illustrated with lowercase letters. Values are means  $\pm$  SEM.  $N = 5$  per exposure groups.

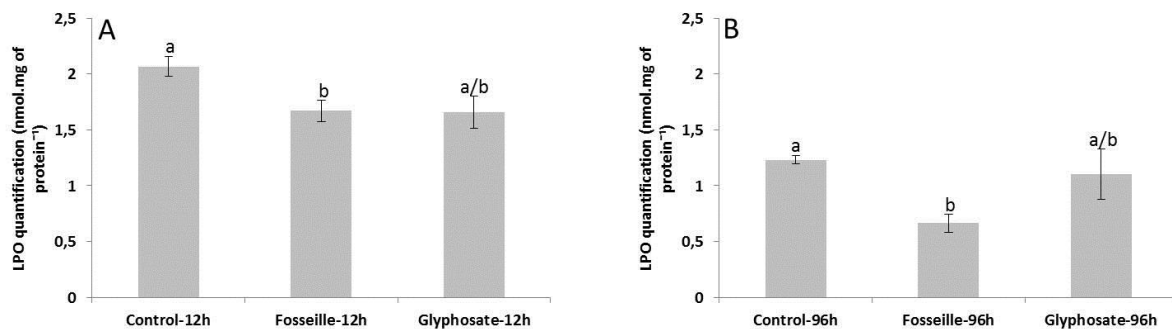


Figure 6: Comparison of LPO quantification between Control, Fosseille and Glyphosate groups after 12h (A) and 96h (B) of exposure. Statistically significant differences ( $P < 0.05$ ) within experimental groups are illustrated with lowercase letters. Values are means  $\pm$  SEM.  $N = 5$  per exposure groups.

## DISCUSSION

### **Investigation of gene expression and enzyme activities from turtles blood samples: new perspectives in the disciplinary field of environmental ecotoxicology**

Measures of gene expression and enzyme activities in the Mediterranean pond turtle *M. leprosa* were conducted exclusively from blood samples. Results showed that gene expression profiles and enzyme activities of SOD and CAT anti oxidative stress enzymes could be quantified, indicating that this approach can be performed non only from organs such as liver, heart, brain and kidneys (Gluszczak et al., 2006; Bagnyukova et al., 2006; Langiano and Martinez, 2008), but also from blood extracts. To the exception of the study of Labrada-Martagon et al. (2011) that was completed from the quantification of enzyme activities among blood samples of sea turtles, our study is the first to report gene expression profiles from blood. Because four biochemical markers were studied in *T. s. elegans* under experimental conditions along this study, i.e. SOD, CAT, AChE and LPO, and because we did not drawn sufficient blood from the very small red-eared sliders, we have not been able to perform gene expression analyses from blood extracts. Thereby, measures of gene expression for SOD and CAT, only, were performed from turtle's liver after animal euthanasia. The results obtained overall indicate that this procedure for assessing oxidative stress can be proceeded without killing animals. This technique can therefore be applied to a great diversity of animals, especially animals that are endangered of extinction. This is particularly true in the study of Labrada-Martagon et al. (2011) that focused on the Eastern Pacific green sea turtle in two contrasted coastal lagoons. Regarding the increase of threats on animal species, this approach thus offers new perspectives in the disciplinary field of environmental ecotoxicology as it will allow the study of wild populations without host sacrifice and the survey of the same individuals after CMR (capture-mark-recapture) in natural environments, as is the case for instance for *M. leprosa* (Meyer et al., 2015; Palacios et al., 2015).

### **Evolution of oxidative stress over time: what lessons can we learn about experimental design?**

Reactive oxygen species (ROS), which are produced throughout intracellular metabolism, have physiological effects at low concentrations. However, if ROS are overproduced after environmental contaminations, they may have deleterious effects on cell structures, proteins, DNA and lipids (see Gavrić et al., 2015 for a review). As a result, antioxidant enzymes, especially SOD and CAT, are activated to detoxify ROS and counteract their deleterious effects (Van der Oost et al., 2003). The first step in the detoxification process involves the

conversion of the superoxide anion ( $O_2^-$ ) in hydrogen peroxide ( $H_2O_2$ ) by the SOD enzyme and the second consists in the conversion of  $H_2O_2$  to water ( $H_2O$ ) and oxygen ( $O_2$ ) by the CAT enzyme (Halliwell, 2001). Nevertheless the sensitivity of both enzymes against ROS explains why SOD and CAT can be damaged by  $H_2O_2$  and  $O_2^-$ , respectively. Consequently, SOD and CAT can be inactivated by these two components (Hermes-Lima and Storey, 1993). Finally, the hydrogen peroxide, when not converted into water and oxygen, is metabolized in hydroxyl radicals ( $OH^-$ ) through the Fenton reaction. Hydroxyl radicals can react with lipids in the cells plasma membranes, which can cause extensive damages (Hermes-Lima, 2004) (see Figure 7 for a schematic representation of the detoxification process).

The SOD enzyme activity profile for the Control-12h group was 1.4 fold lower than that of the Control-96h group, which was unexpected. A similar result was obtained between the Control-12h and Control-96h groups for the CAT activity, the enzyme activity profile being 1.7 fold higher in the Control-12h group. These results indicate that at least one of the turtles control groups was stressed, although all turtles were maintained in tap water without any source of pollutant under experimental conditions. Because the SOD activity was not statistically different between the Control-12h, Fosseille-12h and Glyphosate-12h groups, which was not the case at 96h, it is therefore very likely that the Control-12h group could have been stressed. The dissimilar enzyme activity profiles for the SOD Control at 12 and 96 hours after starting experiments thus suggest that SOD activity could have been inactivated by  $H_2O_2$ , while the dissimilar enzyme activity profiles for the CAT Control at 12 and 96 hours suggest that CAT activity could have been activated by  $H_2O_2$ . Because gene expression for the SOD Control was similar at 12 and 96 hours after starting experiments, as it was also the case for the CAT Control, stress induced at 12 hours would imply only enzyme activity and no gene transcription.

Regarding our experimental design, two groups of 15 turtles each were acclimated two months at the lab in 20 liters water tanks. Containers were first equipped with UV lamps and basking sites. Turtles were subsequently divided in six groups of five individuals per group in tanks filled with 5cm of water without basking sites, which constituted the experimental groups that were sampled at 12 or 96 hours. Although turtles were acclimated for a period of 60 days, they might have been stressed soon after the beginning of the experiments. We identified several parameters that could indeed explain the variations in antioxidant enzyme activities: (i) turtle's handling when organizing the six experimental groups of animals; (ii) no turtle's feeding during the experimental process; (iii) impossibility of swimming due to low levels of water in tanks; (iv) absence of artificial basking sites preventing basking and/or

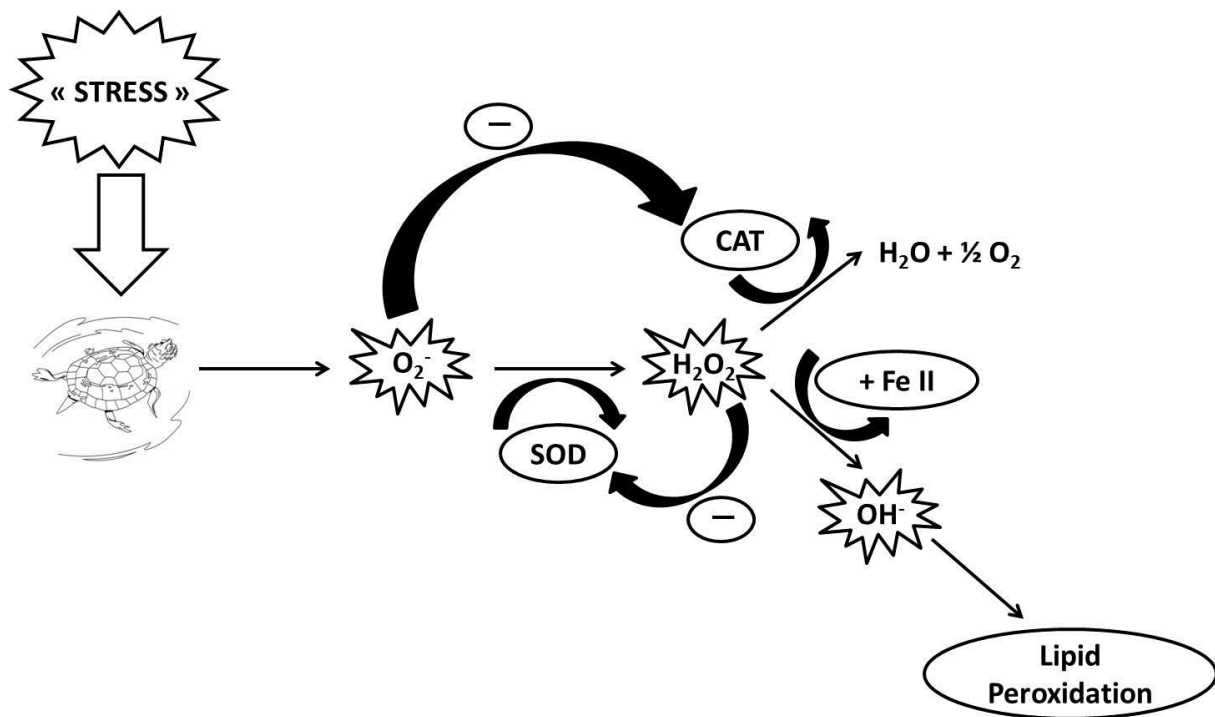


Figure 7: Schematic representation of lipid peroxidation and detoxification process involving antioxidant SOD and CAT enzymes (modified after Halliwell (2001) and Hermes-Lima (2004)).

hiding activities. The last two settings (low levels of water and absence of basking sites) were selected as such in order to force turtles to be always in contact in water, and consequently with the contaminant if any. It is therefore necessary to replicate the same settings as those applied during the acclimation phase, or to replicate settings as close as possible of those used for animal acclimation during the experimental course. Variations in enzyme activities have been already observed by others among control groups sampled at different phases but, to our knowledge, have never been discussed (see Langiano and Martinez, 2008; Modesto and Martinez, 2010). Our study thus highlights the importance to replicate control groups and to compare only control with exposed groups challenged the same interval of time. In the present study, as we showed that animals may be slightly stressed at 12h, even in the absence of contaminant, comparisons between exposed and control groups will be studied only at 96 hours after the beginning of experiments.

## **Evolution of oxidative stress within animals challenged with pesticides and polluted water**

Comparisons of SOD enzyme activities between Glyphosate-96h and Control-96h groups illustrated differences in enzyme activity profiles, which were 1.6 fold higher in the Control-96h group. However, the SOD gene expression was 3.4 fold higher in the Glyphosate-96h. If comparisons of CAT enzyme activities between the Glyphosate-96h and Control-96h groups also illustrated differences in enzyme activity profiles, these were however 1.5 fold higher in the Glyphosate-96h group. Similarly, the CAT gene expression was 2.8 fold higher in the Glyphosate-96h group. Regarding the detoxification process of ROS, which involves transformation of  $O_2^-$  in  $H_2O_2$  and transformation of  $H_2O_2$  in  $H_2O$  and  $O_2$  through SOD and CAT activities, respectively, and down regulation of both enzymes following production of  $O_2^-$  and  $H_2O_2$  (see figure 7), it is likely that the SOD enzyme was inactivated while the CAT enzyme was activated within turtles challenged with high concentrations of glyphosate-based herbicide. CAT activation also supposes that SOD enzyme already catalyzed  $O_2^-$  in  $H_2O_2$ , which means that SOD activation occurred quite early, before 96h. Assuming that the commercial formulation used during experiments induced a stress, concentrations of  $H_2O_2$  in organisms must have increased substantially. Because LPO levels were not significantly different between the Glyphosate-96h and Control-96h groups, which indicated that  $H_2O_2$  was not metabolized in  $OH^-$ , this suggests that  $H_2O_2$  was catalyzed in  $H_2O$  and  $O_2$ . The LPO measure then supports our hypothesis about an activation of the CAT enzyme. Our results therefore illustrate an oxidative stress effect of the glyphosate-based herbicide under experimental conditions, which has been already documented among many aquatic or semi aquatic vertebrate species, such as fishes, amphibians and reptiles (Relyea et al., 2005a, 2005b; Gluszczak et al., 2006; Gluszczak et al., 2007; Cavalcante et al., 2008; Costa et al., 2008; Langiano and Martinez, 2008; Poletta et al., 2009; Modesto and Martinez 2010). The AChE activity, which was lower in the Glyphostae-96h group in comparison to the Control-96h group, emphasizes a stress induced by this contaminant, as exemplified in fishes (Gluszczak et al., 2007; Modesto and Martinez 2010).

Comparisons of SOD enzyme activities between the Fosseille-96h and Control-96h groups did not illustrate statistical differences in enzyme activity profiles, though SOD enzyme activity was slightly lower in the Fosseille-96h group. However, the gene expression for the SOD enzyme was 2.2 fold higher in the Fosseille-96h group. On the opposite, comparisons of CAT enzyme activities between the Fosseille-96h and Control-96h groups illustrated differences in enzyme activity profiles, which were 1.9 fold higher in the Fosseille-

96h group. The gene expression for the CAT enzyme was 2.1 fold higher in the Fosseille-96h group. Overall, the pattern of gene expression and enzyme activity of SOD and CAT enzymes was similar for the Fosseille-96h and Glyphosate-96h groups. Regarding LPO levels, they were significantly lower in the Fosseille-96h group, which indicated that  $H_2O_2$  was not metabolized in  $OH^-$ , but was catalyzed in  $H_2O$  and  $O_2$  as stated for the Glyphosate-96h group. Therefore, if the LPO measure also supports the hypothesis about an activation of the CAT enzyme, these results illustrate an oxidative stress effect induced by the water collected in the Fosseille river. Because the AChE activity was not statistically different between the Fosseille-96h and Control-96h groups, it is likely that the glyphosate-based herbicide was probably not the main stressor involved in the physiological response of turtles (see Glusczak et al., 2007; Modesto and Martinez 2010). Other contaminants may be thereby advocated to explain the molecular and biochemical variations within turtles challenged with the Fosseille water. As wastewater treatments are used to convert water quality of waters that feed the Fosseille River, it is likely that a cocktail of chemicals may be the source of turtles stress. To conclude, surveying *M. leprosa* turtles of the Fosseille River remains a central issue to assess the impacts of the poor quality of this water course on this species specifically, and on biodiversity in general. Turtles may be indeed an excellent model to follow variations in water quality since these animals are globally less sensitive than sentinel species, such as amphibians which are lacking in this river.

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## REFERENCES

- Abell, R.** (2002). Conservation biology for the biodiversity crisis: a freshwater follow-up. *Conserv. Biol.* **16**, 1435-1437.
- Bagnyukova, T.V., Lushchak, O.V., Storey, K.B. and Lushchak, V.I.** (2007). Oxidative stress and antioxidant defense responses by goldfish tissues to acute change of temperature from 3 to 23°C. *J. Therm. Biol.* **32**, 227-234.
- Balian, E.V., Segers, H., Lévêque, C. and Martens, K.** (2008). The freshwater animal diversity assessment: an overview of the results. *Hydrobiologia* **595**, 627-637.
- Baron, J.S., LeRoy Poff N., Angermeier, P.L., Dahm, C.N., Gleick, Hairston Jr., G.N., Jackson, R.B., Johnston, C.A., Richter, B.D. and Steinman, A.D.** (2003). Sustaining healthy freshwater ecosystems. *Issues in Ecology* **10**, 1-16.
- Cavalcante, D.G.S.M., Martinez, C.B.R. and Sofia, S.H.** (2008). Genotoxic effects of Roundup® on the fish *Prochilodus lineatus*. *Mutat. Res-Gen. Tox. En.* **655**, 41-46.
- Commissariat Général au Développement Durable – Service de l'observation et des statistiques** (2015). Les pesticides dans les cours d'eau Français en 2013. Chiffres et Statistiques **697**. <http://www.developpement-durable.gouv.fr/IMG/pdf/CS697.pdf>
- Costa, M.J., Monteiro, D.A., Oliveira-Neto, A.L., Rantin, F.T. and Kalinin, A.L.** (2008). Oxidative stress biomarkers and heart function in bullfrog tadpoles exposed to Roundup Original®. *Ecotoxicology* **17**, 153-163.
- Dudgeon, D., Arthington, A.H., Gessner, M.O., Kawabata, Z.I., Knowler, D.J., Lévêque, C., Naiman, R.J., Prieur-Richard, A.H., Soto, D., Stiassny, M.L.J. and Sullivan, C.A.** (2006). Freshwater biodiversity: importance, threats, status and conservation challenges. *Biol. Rev.* **81**, 163-182.
- Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M.C. and Séralini, G.E.** (2009). Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology* **162**, 184-191.
- Gavrić, J., Prokić, M., Despotović, S., Gavrilović, B., Radovanović, T., Borković-Mitić, S., Pavlović, S. and Saičić, Z.** (2015). Biomarkers of oxidative stress and acetylcholinesterase activity in the blood of grass snake (*Natrix natrix* L.) during prehibernation and posthibernation periods. *Braz. Arch. Biol. Techn.* **58**, 443-453.
- Giesy, J.P., Dobson, S. and Solomon, K.R.** (2000). Ecotoxicological risk assessment for Roundup herbicide. *Rev. Environ. Contam. Toxicol.* **167**, 35-120.
- Gluszczak, L., dos Santos Miron, D., Crestani, M., Braga da Fonseca, M., Araújo Pedron, F. de, Duarte, M.F. and Vieira, V.L.P.** (2006). Effect of glyphosate herbicide on acetylcholinesterase activity and metabolic and hematological parameters in piava (*Leporinus obtusidens*). *Ecotoxicol. Environ. Safe.* **65**, 237-241.
- Gluszczak, L., Miron, D. dos S., Moraes, B.S., Simões, R.R., Schetinger, M.R.C., Morsch, V.M. and Loro, V.L.** (2007). Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*). *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **146**, 519-524.
- Halliwell, B.** (2001). Free radicals and other reactive species in disease. Encyclopedia of Life Sciences. Nature Publishing Group.
- Hermes-Lima, M. and Storey, K.B.** (1993). Antioxidant defenses in the tolerance of freezing and anoxia by garter snakes. *Am. Physiol. Soc.* **265**, 642-652.
- Hermes-Lima, M.** (2004). Oxygen in biology and biochemistry: role of free radicals. In Functional metabolism: regulation and adaptation. John Wiley and Sons, Inc.
- Howe, C.M., Berrill, M., Pauli, B.D., Helbing, C.C., Werry, K. and Veldhoen, N.** (2004). Toxicity of glyphosate-based pesticides to four North American frog species. *Environ. Toxicol. Chem.* **23**, 1928-1938.
- Hupfer, M. and Kleberg, A.** (2007). State and pollution of freshwater ecosystems –

- Warning signals of a changing environment. In Lozán, J.L., Grassl, H., Hupfer, P., Menzel, L. and Schönwiese, C.P. Global change: Enough water for all? Wissenschaftliche Auswertungen, Hamburg.
- Jiraungkoorskul, W., Upatham, E.S., Kruatrachue, M., Sahaphong, S., Vichasri-Grams, S. and Pokethitiyook, P.** (2002). Histopathological effects of Roundup, a glyphosate herbicide, on Nile tilapia (*Oreochromis niloticus*). *Science Asia* **28**, 121-127.
- Labrada-Martagón, V., Tenorio Rodríguez, P.A., Méndez-Rodríguez, L.C. and Zenteno-Savín, T.** (2011). Oxidative stress indicators and chemical contaminants in East Pacific green turtles (*Chelonia mydas*) inhabiting two foraging coastal lagoons in the Baja California peninsula. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **154**, 65-75.
- Langiano, V. do C. and Martínez, C.B.R.** (2008). Toxicity and effects of a glyphosate-based herbicide on the Neotropical fish *Prochilodus lineatus*. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **147**, 222-231.
- Livak, K.J. and Schmittgen, T.D.** (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the  $2^{-\Delta\Delta CT}$  Method. *Methods* **25**, 402-408.
- Lowry, O.H., Rosenbrough, N.J., Faar, A.L. and Randall, R.J.** (1951). Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
- Lushchak, O.V., Kubrak, O.I., Storey, J.M., Storey, K.B. and Lushchak, V.I.** (2009). Low toxic herbicide Roundup induces mild oxidative stress in goldfish tissues. *Chemosphere* **76**, 932-937.
- Mann, R.M. and Bidwell, J.R.** (1999). The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs. *Arch. Environ. Contam. Toxicol.* **36**, 193-199.
- Meyer, L., Du Preez, L.H., Bonneau, E., Héritier, L., Quintana, M., Valdeón, A., Sadaoui, A., Kechemir-Issad, N., Palacios, C. and Verneau, O.** (2015). Parasite host-switching from the invasive American red-eared slider, *Trachemys scripta elegans*, to the native Mediterranean pond turtle, *Mauremys leprosa*, in natural environments. *Aquat. Invasions* **10**, 79-91.
- Modesto, K.A. and Martínez, C.B.R.** (2010). Roundup® causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus*. *Chemosphere* **78**, 294-299.
- Palacios, C., Urrutia, C., Knapp, N., Quintana, M.F., Bertolero, A., Simon, G., Du Preez, L.H. and Verneau, O.** (2015). Demographic structure and genetic diversity of *Mauremys leprosa* in its northern range reveal new populations and a mixed origin. *Salamandra* **51**, 221-230.
- Peixoto, F.** (2005). Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. *Chemosphere* **61**, 1115-1122.
- Poletta, G.L., Larriera, A., Kleinsorge, E. and Mudry, M.D.** (2009). Genotoxicity of the herbicide formulation Roundup® (glyphosate) in broad-snouted caiman (*Caiman latirostris*) evidenced by the Comet assay and the Micronucleus test. *Mutat. Res-Gen. Tox. En.* **672**, 95-102.
- Polo-Cavia, N., Lopez, P. and Martin, J.** (2009). Interspecific differences in chemosensory responses of freshwater turtles: consequences for competition between native and invasive species. *Biol. Invasions* **11**, 431-440.
- Polo-Cavia, N., Lopez, P. and Martin, J.** (2010). Competitive interactions during basking between native and invasive freshwater turtle species. *Biol. Invasions* **12**, 2141-2152.
- Polo-Cavia, N., Lopez, P. and Martin, J.** (2011). Aggressive interactions during feeding between native and invasive freshwater turtle species. *Biol. Invasions* **13**, 1387-1396.
- Rapport final** – Suivi de la qualité des cours d'eau du bassin versant du Tech et des petits fleuves côtiers, 2013. Conseil Général des Pyrénées-Orientales.

- Relyea, R.A.** (2005a). The lethal impact of Roundup on aquatic and terrestrial amphibians. *Ecol. Appl.* **15**, 1118-1124.
- Relyea, R.A.** (2005b). The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecol. Appl.* **15**, 618-627.
- Reoyo-Prats, B., Aubert, D., Menniti, C., Ludwig, W., Sola, J., Pujo-Pay, M., Conan, P., Verneau, O. and Palacios, C.** (2016). Multicontamination phenomena occur more often than expected in Mediterranean coastal watercourses: study case of the Têt River (France). Submitted.
- Revenge, C. and Mock, G.** (2000). Freshwater biodiversity in crisis. Earth Trends World Resources Institute 1-4.
- Richard, S., Moslemi, S., Sipahutar, H., Benachour, N. and Seralini, G.E.** (2005). Differential effects of glyphosate and Roundup on human placental cells and aromatase. *Environ. Health Perspect.* **113**, 716-720.
- Strayer, D.L. and Dudgeon, D.** (2010). Freshwater biodiversity conservation: recent progress and future challenges. *J. N. Am. Benthol. Soc.* **29**, 344-358.
- UICN France, MNHN & SHF** (2015). La liste rouge des espèces menacées en France - Chapitre reptiles et amphibiens de France métropolitaine. Paris, France.
- Van der Oost, R., Beyer, J. and Vermeulen, N.P.** (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Phar.* **13**, 57-149.
- Vaughn, C.C.** (2010). Biodiversity losses and ecosystem function in freshwaters: Emerging conclusions and research directions. *BioScience* **60**, 25-35.
- Vazquez, M.** (2012). Dynamique de la population d'émydes lépreuses – *Mauremys leprosa*. Site Natura 2000 “Massif des Albères/Banyuls-sur-Mer”. Master dissertation, UPVD, pp. 1-27.
- Villeneuve, A., Humbert, J.F. and Larroudé, S.** (2011). Herbicide contamination of freshwater ecosystems: impact on microbial communities (INTECH Open Access Publisher).

**CHAPITRE VII. OXIDATIVE STRESS BIOMARKERS  
IN THE MEDITERRANEAN POND TURTLE  
(*MAUREMYS LEPROSA*) REVEAL STRESSFUL  
AQUATIC ECOSYSTEMS IN SOUTHERN FRANCE**

## Présentation du Chapitre VII

Suite au développement des biomarqueurs et leurs validations en mésocosme dans le chapitre précédent, le travail final de cette thèse présenté dans ce chapitre VII a consisté à appliquer ces biomarqueurs au sein de populations de tortues aquatiques sauvages. Un travail de terrain a été mené sur trois cours d'eau des Pyrénées-Orientales, contrastés au niveau des contaminants biotiques et abiotiques, afin de récolter des échantillons sanguins. Cette expérience devrait permettre d'évaluer l'état de santé des populations de tortues mais également le niveau de contamination des cours d'eau.

## **Oxidative stress biomarkers in the Mediterranean pond turtle (*Mauremys leprosa*) reveal stressful aquatic ecosystems in Southern France**

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### **Keywords**

*Mauremys leprosa*, degraded waters, Oxidative stress, Catalase, Superoxide dismutase, Gene expression, Enzyme activity

## **Abstract**

Increasing anthropogenic activities constitute the main causes of the loss of water quality and freshwater ecosystems degradation. Among all sources of pollution, some of them are regarded as a serious threat for human health and biodiversity. This is the case of chemical contaminants that are released by the agricultural practices, like for instance pesticides that are still used massively. In France, especially in the Pyrénées-Orientales (PO) department where the agricultural economy is principally based on fruit crops and vineyards, high concentrations of glyphosate and AMPA are measured in certain watercourses. Because it was shown under experimental conditions that these contaminants may impact freshwater animal species, we investigated the impact of degraded waters on the physiology of the Mediterranean pond turtle *Mauremys leprosa*, which is an endangered endemic species of the PO department. We assessed the biological response of turtles following the gene expression and the activity of two enzymes involved in the oxidative detoxification processes, namely the Catalase and the Superoxide dismutase. We showed variations in the gene expression and activity of both enzymes within turtles that were collected from contrasted environments. As expected, the most degraded water courses constituted the most stressful habitat for turtles. Surprisingly, the main source of pollution at the origin of the physiological disturbances within turtles was wastewater treatment plants releases.

## INTRODUCTION

Water degradation is a pervasive threat for watercourses worldwide, mainly due to increasing anthropogenic activities (Sala et al., 2000; Albiac et al., 2010; Bhuiyan et al., 2013). Water extraction, straightening of watercourses, dam construction, alien species introduction and chemicals pollution constitute the principal causes of the loss of water quality and freshwater ecosystems disruption (Malmqvist and Rundle, 2002; Sabater and Tockner, 2010). Regarding the chemical threat, the main pollutants that are released into natural environments are toxic gases, heavy metals, organics and biotoxics (Wang et al., 2013). If some of these pollutions can have natural origins, following for instance volcanic eruptions, anthropogenic causes are in most cases associated to municipal wastewater treatment plants, industrial and intense farming activities releases (Schwarzenbach et al., 2006; Trujillo-Reyes et al., 2014). Regarding agricultural practices, the most important sources of pollution involve fertilizers, hormones, antibiotics and pesticides (Trujillo-Reyes et al., 2014), which become a serious threat to biological communities when accumulated in freshwater bodies (Gill and Garg, 2014).

Freshwater ecosystems are represented by a total of 270.000 kilometers of watercourses throughout the French territory. Following a survey that was conducted in 2005 (Institut Français de l'Environnement, 2007), about 35% of the surface freshwaters presented a moderate to poor quality. In 2013, the presence of pesticides was revealed in more than 90% of the control points that were investigated (Commissariat général au développement durable – Service de l'observation et des statistiques, 2015: <http://www.developpement-durable.gouv.fr/IMG/pdf/CS697.pdf>), suggesting a drop in water quality. The most common compounds that were identified in the early 2000's were the glyphosate, the Aminomethylphosphonic Acid (AMPA) and the diuron (Institut Français de l'Environnement, 2007). In the early 2010's, the glyphosate and AMPA were still found in natural environments (Commissariat général au développement durable – Service de l'observation et des statistiques, 2015: <http://www.developpement-durable.gouv.fr/IMG/pdf/CS697.pdf>). The glyphosate is a weak acid that is used in combination with the polyethoxylated tallowamine (POEA), i.e., the predominant non-ionic surfactant, in herbicide commercial formulations (Giesy et al., 2000). AMPA is, on the contrary, the main degradation product of the glyphosate (Giesy et al., 2000). These two components, glyphosate and AMPA, are suspected to impact human health and biodiversity, mainly in areas with intense agricultural activities.

In the Pyrénées-Orientales (PO) department, which is situated in Southern France, the agricultural economy is principally based on fruit crops and vineyards. This may explain the



relatively high concentrations of glyphosate and AMPA that are measured in some watercourses (Unpublished report of the Conseil Général des Pyrénées-Orientales, 2013). As a result, several streams were classified in the worst of the five listed categories to account the water quality, namely the Fosseille and the Agouille de la Mar rivers. Both rivers are paradoxically home for the Mediterranean pond turtle *Mauremys leprosa* (Schweigger, 1812), which is an endemic endangered freshwater species of the PO department in France (see Palacios et al., 2015). It is indeed classified as “Vulnerable” in this country (UICN France, MNHN & SHF, 2015). With about 250 individuals that were recorded from 2012 to 2016 in the Fosseille River (Verneau, unpublished data), that population can be considered as one of the most important in the PO department. On the opposite, only about 20 individuals were identified to date along the Agouille de la Mar River after three field sessions that were conducted in 2014 and 2015 (Verneau, unpublished data). Regarding the poor water quality of the Fosseille and the Agouille de la Mar rivers, one may wonder if these two turtle’s populations were under stress in these natural environments.

It has been shown under experimental conditions, that commercial formulations of glyphosate-based herbicides induced a stress within many freshwater organisms, among others fishes (Gluszczak et al., 2006, 2007; Cavalcante et al., 2008; Langiano and Martinez, 2008; Modesto and Martinez, 2010), amphibians (Costa et al., 2008) and freshwater turtles (Héritier et al., submitted). All these studies were performed from analysis of oxidative stress biomarkers, such as gene expression and enzymatic activity of the Catalase (CAT) and Superoxide dismutase (SOD) enzymes. Because *M. leprosa* was considered as vulnerable in France, Héritier et al. (submitted) developed a procedure to measure molecular and biochemical biomarkers from blood samples collected from red-eared sliders *Trachemys scripta elegans* (Wied, 1839), as it was previously done on other reptiles such as the garter snake *Thamnophis sirtalis* (Linnaeus, 1758) (Gavrić et al., 2015) and the broad-nose caiman *Caiman latirostris* (Daudin, 1802) (Poletta et al., 2016). Our objectives in the present study were to assess CAT and SOD gene expression and enzyme activities within three Mediterranean pond turtle populations sampled in contrasted environments, i.e. degraded ecosystems, such as the Fosseille and the Agouille de la Mar rivers and non-degraded ecosystems, such as the Baillaury River. The Baillaury River is indeed a small stream in the PO department, which was classified in one of the best categories used to account water quality (Unpublished report of the Conseil Général des Pyrénées-Orientales, 2013), and which embraces the greatest turtle’s population whose size was estimated to about 315 individuals (Vazquez, 2012). We therefore hypothesized that the two most contaminated rivers in the pO

department could constitute a more stressful habitat for turtles. Indeed, the high levels of glyphosate and AMPA that were therein recorded could be a stressor for turtles as demonstrated by H eritier et al. (submitted) under experimental conditions.

## **MATERIALS AND METHODS**

### *Turtle sampling*

The Mediterranean pond turtles were sampled from three watercourses of the PO department (Figure 1A). Captures were done using catfish traps that were baited with pork liver, equipped with empty plastic bottles to avoid the risk of drowning and firmly attached to the vegetation of the shore. Traps were set three days at the Fosseille River on June-July 2015 and checked every day for turtles (Figure 1B). They were set overnight at the Agouille de la Mar and Baillaury rivers on May and July 2014, respectively (Figures 1C to 1D). Specimens were measured, weighed and marked with cuts on the marginal scales of the carapace following standard procedure for Capture-Mark-Recapture (CMR) of chelonians. Blood was then drawn from the subcarapacial sinus and preserved thoroughly in two distinct Microtainer<sup>®</sup> tubes with anticoagulant. All the samples were frozen by immersion in liquid nitrogen and transported to the lab in a cryogenic shipper (Taylor-Wharton 5LS) where they were stored at -80°C. Turtles were finally released at the exact place of capture in the field.

### *Gene expression of CAT and SOD*

Total RNA extractions were conducted from blood samples using Trizol reagent (Life Technologies) following the manufacturer's protocol. RNA quality and concentrations were determined by spectrophotometry at 280nm (Nanodrop ND-1000, Thermo Fisher Scientific Inc., MA, USA). One microgram of total RNA was used for the reverse transcription with the Maxima H Minus first strand cDNA synthesis kit (Thermo Scientific). cDNAs were finally diluted at 1/20 for the real time PCR experiments.

Primers used for the real time PCR were previously designed and tested on *M. leprosa* cDNA samples (H eritier et al., submitted). Real time PCR experiments were performed using a Light Cycler apparatus (Roche Molecular Biochemicals, Germany) in a final volume of 10 l containing 2 l of cDNA, LightCycler<sup>®</sup> 480 SYBR Green I Master Mix 1X and primers at 0.4 M final concentration. Cycling conditions were one cycle of 14' of preincubation at 95°C, followed by 45 cycles of 11s of denaturation at 95°C, 11s of annealing at 60°C and 19s of elongation at 72°C. Specificity of both primers was checked from melting curves. Relative

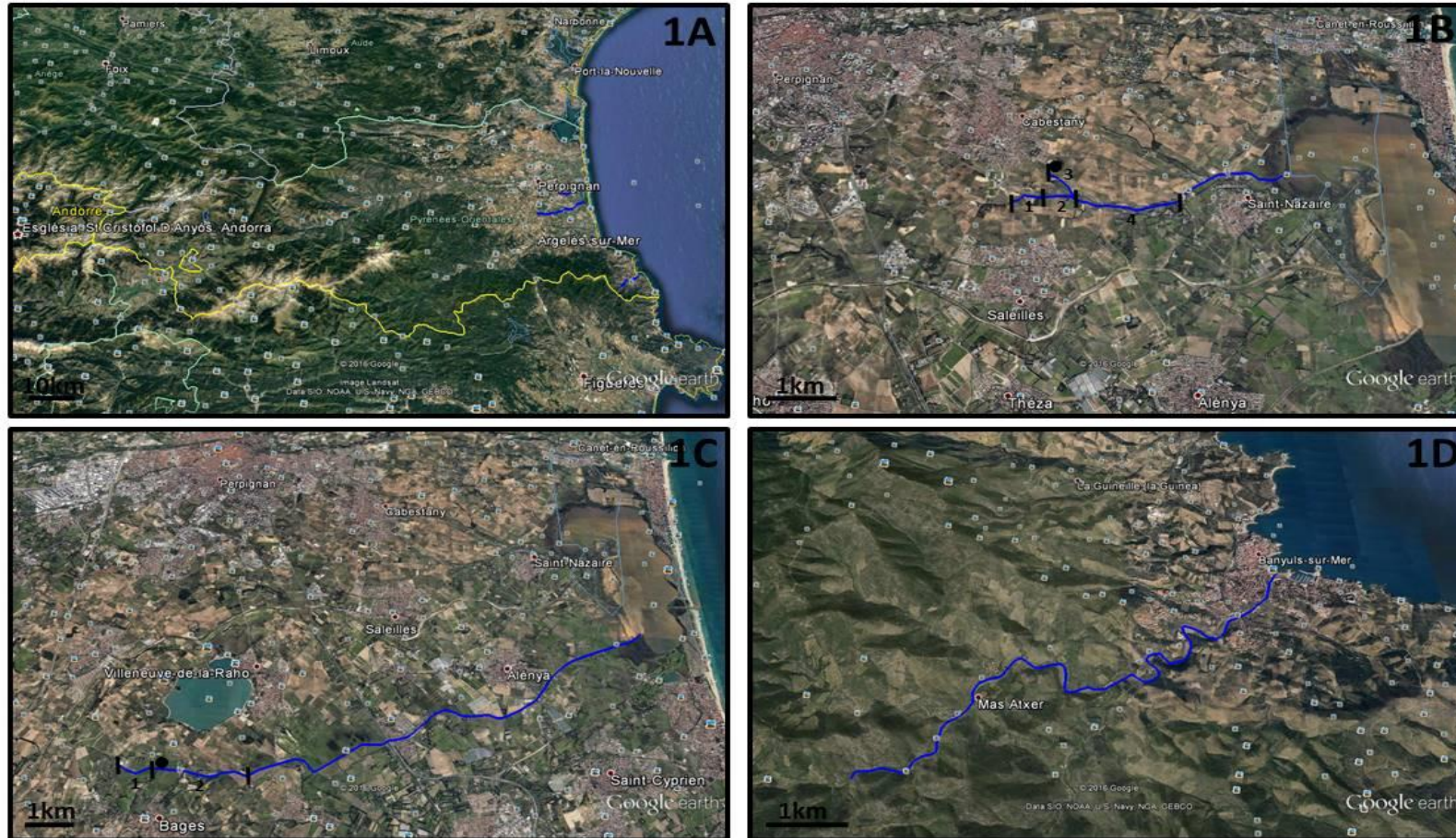


Figure 1A. Map of the Pyrénées-Orientales department. In blue, rivers of investigation: From north to south, Fosseille, Agouille de la Mar and Baillaury rivers. 1B. Representation of the Fosseille River: section 1 corresponds to the upstream of the Wasterwater Treatment Plant (WTP); section 2 corresponds to the dried part; section 3 corresponds to the outfall of the WTP; section 4 corresponds to the downstream of the WTP. The black filled circle indicates the location of the WTP. 1C. Representation of the Agouille de la Mar River: section 1 corresponds to the upstream of the WTP; section 2 corresponds to the downstream of the WTP. The black filled circle indicates the location of the WTP. 1D. Representation of the Baillaury River.

changes in gene expression were analyzed using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001).

#### *Enzyme activity of CAT and SOD*

Five  $\mu\text{l}$  of blood were diluted in 200 $\mu\text{l}$  of cold assay buffer from the Catalase Activity Colorimetric/Fluorometric Assay Kit (BioVision). After centrifugation at 4°C during 15 min at 10,000g, 40 $\mu\text{l}$  of the supernatant was then used for measuring the CAT activity at 570nm following the manufacturer recommendations. CAT activity was expressed in mU.mg of protein<sup>-1</sup>. Another blood sample of 5 $\mu\text{l}$  was also diluted in 995 $\mu\text{l}$  of distilled deionized ultra-pure water. After centrifugation at 4°C during 15min at 10,000g, 20 $\mu\text{l}$  of the supernatant was diluted in 200 $\mu\text{l}$  of WST working solution from the Superoxide Dismutase (SOD) Activity Assay Kit (BioVision) for measuring the SOD activity at 450nm following the manufacturer recommendations. SOD activity was expressed in U.mg of protein<sup>-1</sup>.

#### *Statistical analyses*

Non-parametric tests, i.e. Kruskal-Wallis test (KW) by ranks followed by Mann-Whitney U test (MW), were conducted to assess variations in mRNA gene expression and enzyme activities using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA). The statistical significance was accepted at  $P < 0.05$ . Because some wastewater treatment plants (WTP) continuously discharge processed waters in the Fosseille and Agouille de la Mar rivers, turtles were divided into several groups according to the geographical section from which they were traps along the river. On the Fosseille River, three sections were considered, i.e. upstream of the WTP, downstream of the WTP and outfall of the WTP (Figure 1B). On the Agouille de la Mar River, two sections were considered, i.e. upstream and downstream of the WTP of the village Bages (Figure 1C). On the Baillaury River, only one section was considered as no WTP discharge processed waters in the stream section where turtles were located (Figure 1D). Because it has been shown on sea turtles that antioxidant enzyme activities were not influenced by the size and the weight of investigated specimens (Labrada-Martagón et al., 2011), samples were not analyzed regarding age classes. Comparisons were thus conducted among groups of turtles sampled within distinct sections of the same rivers on one hand and within distinct sections of discrete rivers on the other.

## RESULTS

### *Field samples selected for gene expression and enzyme activities*

Field investigations allowed collecting 93 distinct turtles in the Fosseille river on June 30<sup>th</sup> and July 1<sup>st</sup> and 2<sup>nd</sup>, among which 15 were sampled upstream of the WTP, 62 downstream of the WTP and 16 on the outfall of the WTP. Fifty-two blood samples in total were used for biochemical analyses, 15 from turtles sampled upstream of the WTP, 28 from turtles sampled downstream of the WTP and 9 from turtles sampled on the outfall of the WTP. Because blood samples were not homogeneous in terms of quantity, only 12 samples from the section located upstream of the WTP, 18 from the section located downstream of the WTP and 6 from the section located on the outfall of the WTP were used for molecular analyses. On the Agouille de la Mar river, 15 turtles were collected on May 7<sup>th</sup>, among which 6 were sampled upstream of the WTP and 9 downstream of the WTP. Fourteen blood samples in total were used for molecular and biochemical analyses, 6 from turtles sampled upstream of the WTP and 8 from turtles sampled downstream of the WTP. On the Baillaury River, 55 turtles were collected on July 3<sup>th</sup>. Forty blood samples were used for biochemical analyses. For the same reasons as explained above, only 13 samples were used for molecular analyses. Regarding samples used for analyses, all age classes and sex were represented within turtle's populations of the Fosseille and Baillaury rivers. On the contrary, only adults were represented within the turtle's populations of the Agouille de la Mar River as no juveniles were trapped in the field. However the same proportion of males and females were collected.

### *CAT gene expression and enzyme activity*

Regarding comparisons across the three groups of turtles that were considered at the Fosseille river, significant variations in the CAT gene expression profiles were observed only between the groups located upstream and downstream of the WTP (MW,  $P = 0.001$ ) (Figure 2A). A similar result was observed between the same two groups for the CAT enzyme activity (MW,  $P = 0.005$ ) (Figure 2B). On the opposite, no significant variations was observed in CAT gene expression and enzyme activity profiles between the groups located upstream and downstream of the WTP at the Agouille de la Mar. Regarding comparisons across groups of turtles located in discrete rivers, significant variations in the CAT gene expression profiles were observed between the Baillaury turtles population and the group of turtles located downstream of the WTP of the Fosseille river (MW,  $P = 0.025$ ) (Figure 2A). The same result was observed between the groups of turtles located upstream of the WTP of the Agouille de la Mar and downstream of the WTP of the Fosseille river (MW,  $P = 0.009$ ) (Figure 2A). Concerning the

CAT enzyme activity, significant variations were observed between the Baillaury turtles population and all other groups of turtles (see table 1) to the exception of the group of turtles located upstream of the WTP of the Agouille de la Mar (Figure 2B). Similarly, significant variations were also observed between the groups of turtles located upstream of the WTP of the Fosseille river and downstream of the WTP of the Agouille de la Mar river (MW,  $P = 0,008$ ) (Figure 2B).

*SOD gene expression and enzyme activity*

Regarding comparisons across groups of turtles located either in distinct sections of the same river, or in sections of discrete rivers, no significant variations was observed in the SOD gene expression profiles (Figure 3A). Concerning the enzyme activity profiles, significant variations were observed only between the Baillaury population and all other groups of turtles (see table 1 and Figure 3B).

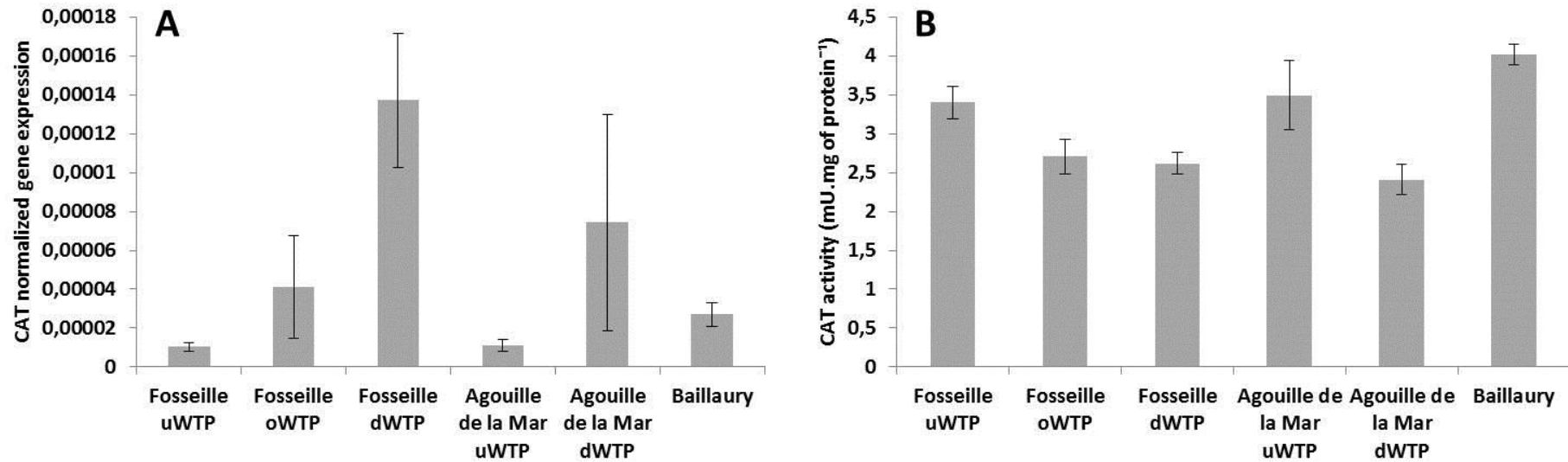


Figure 2A: CAT gene expression within groups of turtles located upstream (u), in the outfall (o) or downstream (d) of the Wasterwater Treatment Plant (WTP) of the Fosseille River, within groups of turtles located uWTP or dWTP of the Agouille de la Mar River and within the turtle's population of the Baillaury River. Data sets are normalized to the 18S rRNA gene reference. Statistically significant differences were considered at  $P < 0.05$ . Values are means  $\pm$  SEM. 2B. CAT enzyme activity within groups of turtles located uWTP, in the oWTP or dWTP of the Fosseille River, within groups of turtles located uWTP or dWTP of the Agouille de la Mar River and within the turtle's population of the Baillaury River. Statistically significant differences were considered at  $P < 0.05$ . Values are means  $\pm$  SEM.

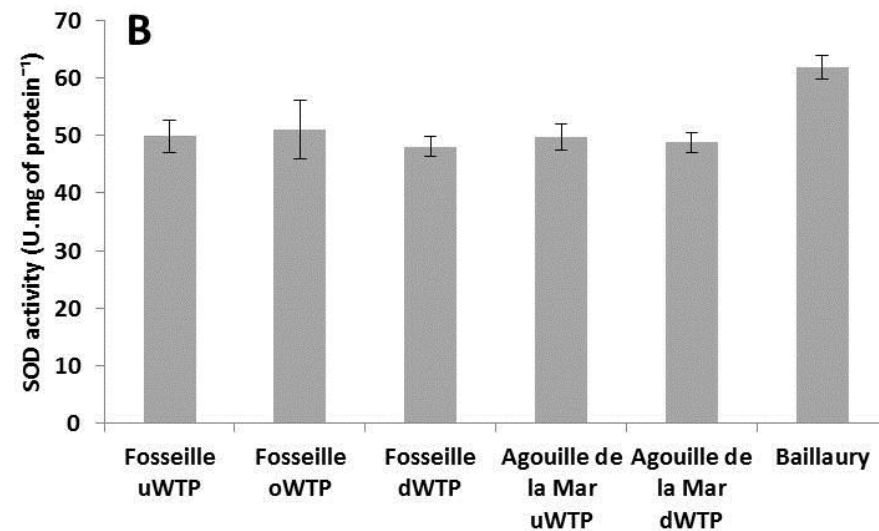
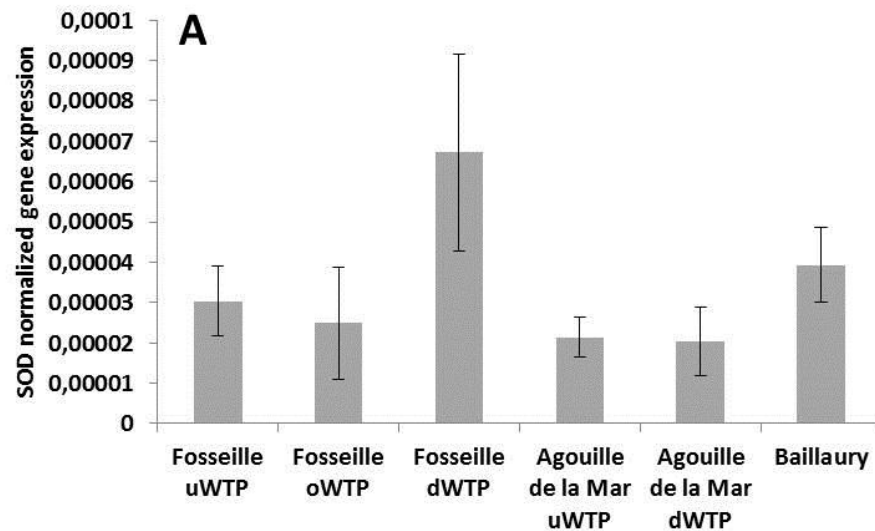


Figure 3A: SOD gene expression within groups of turtles located upstream (u), in the outfall (o) or downstream (d) of the Wasterwater Treatment Plant (WTP) of the Fosseille River, within groups of turtles located uWTP or dWTP of the Agouille de la Mar River and within the turtle's population of the Baillaury River. Data sets are normalized to the 18S rRNA gene reference. Statistically significant differences were considered at  $P < 0.05$ . Values are means  $\pm$  SEM. 3B. SOD enzyme activity within groups of turtles located uWTP, in the oWTP or dWTP of the Fosseille River, within groups of turtles located uWTP or dWTP of the Agouille de la Mar River and within the turtle's population of the Baillaury River. Statistically significant differences were considered at  $P < 0.05$ . Values are means  $\pm$  SEM.



Table 1. P values following comparisons between groups of turtles for the SOD enzyme activity (upper right) and the CAT enzyme activity (lower left).

See figure legends for abbreviations.

	Fosseille uWTP	Fosseille oWTP	Fosseille dWTP	Agouille de la Mar uWTP	Agouille de la Mar dWTP	Baillaury
Fosseille uWTP		1	0,721	0,791	0,925	0,004
Fosseille oWTP	0,084		0,986	0,607	0,606	0,002
Fosseille dWTP	0,005	0,689		0,741	0,808	0
Agouille de la Mar uWTP	0,97	0,272	0,11		0,755	0,006
Agouille de la Mar dWTP	0,008	0,423	0,614	0,081		0,001
Baillaury	0,026	0	0	0,297	0	

## DISCUSSION

### *Analysis of the biomarkers response within wild populations of turtles*

To the exception of very few studies that were conducted within animals sampled in aquatic natural environments (Labrada-Martagón et al., 2011), measures of the oxidative stress involving gene expression and enzyme activity of SOD and CAT biomarkers have, to date, only been completed within animals under experimental conditions (Gluszczak et al., 2007; Langiano et al., 2008; Costa et al., 2008; Modesto and Martinez, 2010). In the present study, while certain profiles of gene expression and enzyme activity were significantly different between groups of turtles, most of the comparisons revealed no significant differences. Nevertheless, even not significant, some of the differences observed could be viewed as trends in the anti-oxidative stress response of animals face to dissimilar environments. Indeed, the variability in the physiological response of turtles, particularly for the SOD and CAT gene expression within the outfall and downstream of the WTP Fosseille groups, as well as within the downstream WTP Agouille de la Mar group, could explain the lack of significant results.

Freshwater turtles are mostly animals with low dispersal capacities (Pearse et al. 2006; Roe et al. 2009). If this peculiarity explains the almost total absence of migration between discrete freshwater courses, turtles can however move quite easily along the same river, especially when no physical barrier prevents animal displacements. This is particularly true between the outfall and downstream of the WTP sections of the Fosseille River and between the upstream and downstream of the WTP sections of the Agouille de la Mar River (Figures 1B and 1C). Moreover, if turtles spend some of their time in water for feeding and reproduction activities, they also spend some of their time outside water for basking and nesting activities. It is thereby extremely difficult to assess their lifestyle along a circadian cycle. Our approach, which is achieved in natural environments, contrasts with the experimental procedure that was completed under controlled conditions (Héritier et al., submitted). Indeed, when juveniles of freshwater turtles were challenged to herbicide commercial formulation or degraded waters, Héritier et al. (submitted) showed between groups of five animals only, significant differences in the biochemical response of turtles. The low variability in the physiological responses would be explained by the experimental design used to investigate oxidative stress induced by water pollutions. Numerous parameters were actually fixed, such as the age of animals, the acclimation phase of turtles, the temperature of water, the turtle's number per group and the feeding activity (Héritier et al. submitted). Nevertheless, Héritier et al. (submitted) evidenced a stress within their Control group that could be the result of modifications in turtle's life traits, such as basking, feeding and

swimming activities. Because all these activities are impossible to account in natural environments, they can be responsible of large variabilities in the measurements of gene expression and enzymatic activity within animals. Consequently, some of the trends that were revealed from differences in the physiological responses within disparate groups of turtles could constitute proper signals of turtle's stress. Therefore, in order to decrease the overall background noise, our procedure for catching turtles, regardless water courses, was left unchanged. Traps were systematically checked for turtles every morning and blood samples were always taken immediately after capture.

#### *Response of CAT and SOD biomarkers within turtles face to contrasted environments*

The agricultural activities in the PO department are mostly based on fruit crops and vineyards, which is the case for the three areas of investigation. Despite the development of organic practices in farming the last 20 years, the use of pesticides and herbicides is still effective. Following a survey on the water course quality that was performed in the PO department in 2011, two rivers were classified in the worst representative category of water quality (Unpublished report of the Conseil Général des Pyrénées-Orientales, 2013). These concerned the Fosseille and the Agouille de la Mar rivers, due to high levels of glyphosate (G) and AMPA (Fosseille:  $G = 0.8\mu\text{gL}^{-1}$  in July 2011 and  $\text{AMPA} = 13\mu\text{gL}^{-1}$  in September 2011; Agouille de la Mar:  $G = 0.420\mu\text{gL}^{-1}$  and  $\text{AMPA} = 8.6\mu\text{gL}^{-1}$  in September 2011). Surprisingly, the concentration of these two pesticides among the Baillaury River was much lower than that measured in the previous rivers ( $G = 0.091\mu\text{gL}^{-1}$  and  $\text{AMPA} = 0.17\mu\text{gL}^{-1}$  in July 2011). As a result, the Baillaury River was classified in the second of the five listed categories used to the valuation of water quality (Unpublished report of the Conseil Général des Pyrénées-Orientales, 2013), which suggested that the three investigated rivers may constitute contrasted environments for endemic freshwater turtles.

Comparisons of the CAT gene expression between the groups of turtles located upstream and downstream of the WTP of the Fosseille River illustrated significant differences in gene expression profiles, which were on average 13,7 fold higher in the group located downstream of the WTP (Figure 2A). Whereas significant differences in the CAT enzyme activity were also exemplified between the same groups of turtles (Figure 2B), enzyme activity profiles were 1,3 fold lower in the group located downstream of the WTP. The transcriptional activity was thereby higher in the group showing the lowest enzyme activity. During the detoxification process, SOD and CAT enzymes are involved in the conversion of superoxide anion ( $\text{O}_2^-$ ) in hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and in the conversion of  $\text{H}_2\text{O}_2$  to water

(H<sub>2</sub>O) and oxygen (O<sub>2</sub>), respectively (Halliwell, 2001). However these two enzymes can be inactivated by the reactive oxygen species, namely H<sub>2</sub>O<sub>2</sub> for the SOD and O<sub>2</sub><sup>-</sup> for the CAT, during a negative feedback process (Hermes-Lima and Storey, 1993). Therefore, our results indicate that the CAT enzyme in the group of animals located downstream of the WTP could be inactivated, suggesting that this section of the Fosseille River may be more stressful than the upstream section. Regarding the SOD gene expression, although no significant differences were revealed between the groups of turtles located upstream and downstream of the WTP (Figure 3A), the gene expression profile for the group located downstream was however 2,21 fold higher than the group located upstream. This indicates a more intense transcriptional activity of the gene coding for the SOD within the group of turtles located in the downstream section suggesting, as discussed above, that this part of the Fosseille River is actually more stressful than the upstream section. The absence of significant differences in the SOD activity between these two groups of turtles (Figure 3B) suggests, however, that the SOD activity could be saturated by an excess of O<sub>2</sub><sup>-</sup>. This outcome would result of stressful conditions in both sections.

Comparisons in the CAT gene expression and enzyme activity profiles did not illustrated significant differences between the group of turtles located in the outfall and the groups located upstream and downstream of the WTP of the Fosseille River (Figures 2A and 2B). However, trends in variations of gene expression and enzyme activity could be observed. This was highlighted by the gene expression profiles, which were 4,0 fold higher in the outfall compared to the upstream WTP group on one hand and 3,4 fold lower in the outfall compared to the downstream WTP group on the other. Furthermore, the enzyme activity profiles were 1,25 fold lower in the outfall compared to the upstream WTP group. Regarding the SOD gene expression and enzyme activity, profiles were similar between the outfall and upstream WTP groups (Figures 3A and 3B). These result overall suggest that the outfall section of the WTP may be less stressful than the downstream section, but more stressful than the upstream section of the Fosseille River. Despite the lack of a physical barrier between the upstream and downstream WTP sections of the Fosseille River, we definitely showed that turtles located upstream of the WTP were less stressed than turtles located downstream of the WTP. This could be explained by the dried intermediate sector of about 700m (Figure 1B) between both sections (Figure 1B), which tends to restrict turtle's movements and isolate animals geographically in divergent aquatic habitats. The stressful environment in the downstream WTP section would be thereby the result of the wastewaters that are discharged continuously in the Fosseille River. Turtles located in the outfall of the WTP would be less stressed than

turtles located downstream of the WTP as the flow of discharged wastewaters at the exit of the WTP prevent sedimentation of contaminants, which is not the case in the last river section.

Regarding CAT gene expression and enzyme activity profiles within groups located upstream and downstream of the WTP of the Agouille de la Mar River (Figures 2A and 2B), although no significant difference were evidenced between groups, trends in variations of molecular and biochemical responses could be observed. Gene expression profiles were indeed 6,75 fold higher in the downstream WTP group, while enzyme activity profiles were 1,45 fold lower. Concerning SOD gene expression and enzyme activity profiles within the same two turtles groups (Figures 3A and 3B), if no significant differences were revealed between groups, no trends in variations of molecular and biochemical responses were observed either. The CAT biomarkers variations suggest that the upstream seems less stressed than the downstream WTP turtle group, as illustrated for the discrete groups located in similar sections of the Fosseille River. The absence of variations in the SOD enzyme activity between the two groups of turtles located in the Agouille de la Mar River on one hand, and the similar activity levels between these two groups and those located in the distinct sections of the Fosseille rivers on the other, suggest that the SOD activity could be saturated. As explained above for the turtle's population of the Fosseille River, our results thus suggest that both sections in the Agouille de la Mar River could be also stressfull. Finally, the absence of variations in the SOD gene expression between downstream and upstream WTP groups on one hand, and the fact that the levels in SOD gene expression for these two groups are lower than those revealed in the downstream WTP group of the Fosseille River on the other, suggest that the Agouille de la Mar River could constitute a less stressful environment than the downstream section of the Fosseille River.

In the lack of WTP and physical barriers in the Baillaury River, we considered this aquatic environment as homogeneous. Therefore, the turtle's population was not divided in distinct groups. Regarding the CAT gene expression profiles within all groups of turtles designed across the three water courses of the PO department (Figure 2A), significant differences were evidenced between the Baillaury population and the downstream WTP turtle's group of the Fosseille River. Levels of gene expression were indeed lower in the Baillaury population. Concerning the CAT activity (Figure 2B), it was significantly higher in the Baillaury population than in all other groups of turtles, to the exception of the upstream WTP group of the Agouille de la Mar River. Considering that the CAT enzyme could have been inactivated within the turtle's group located downstream of the WTP of the Fosseille River, following the production of superoxide anion  $O_2^-$ , the CAT within the Baillaury

population could be either non inactivated or less inactivated than mostly investigated turtle's groups. This suggests the existence of lower levels of  $O_2^-$  in this population, reflecting a less stressful aquatic environment. Regarding the SOD gene expression profiles (Figure 3A), although no significant differences were revealed between turtle's groups, molecular responses tended to be higher in the downstream WTP Fosseille group than in all other groups including the Baillaury population. However, the SOD activity was significantly higher for the Baillaury population than that of all other turtle's groups (Figure 3B). Considering that the activity of the SOD enzyme could be saturated following an excess of  $O_2^-$  in the populations of the Fosseille and Agouille de la Mar rivers, the SOD activity for the Baillaury population would not be saturated. This result indicates, as those inferred from the CAT biomarkers, that levels of  $O_2^-$  within turtles of the Baillaury population are likely very low compared to other turtle's populations. As a result, the Baillaury River may be probably the less stressful river.

### *Conclusions*

Experimental and environmental ecotoxicological studies were to date mainly based on the investigation of single biomarkers. Some studies, for instance, explored the expression of genes involved in detoxification processes (Meistertzheim et al., 2007; Li et al., 2012; Stecyk et al., 2012; Dominguez et al., 2015) while some others assessed the activity of enzymes involved in the same processes (Gluszczak et al., 2007; Moraes et al., 2007; Costa et al., 2008; Langiano et al., 2008; Modesto and Martinez, 2010). Nevertheless, describing the physiological processes involved in the response of organisms face to stressful conditions implies the use of both biomarkers, i.e. gene expression and enzyme activity of the same antioxidant enzyme. Our study is, to our knowledge, the first to combine both biomarkers, which allowed us to elucidate the detoxification processes in all turtle's populations and to relate physiological responses to the water courses quality.

As expected, the most degraded water courses, i.e. the Fosseille and Agouille de la Mar rivers, constituted a more stressful habitat for freshwater turtles than the Baillaury River. The absence of frogs and salamanders in the two former rivers and their presence in the latter one constitutes another evidence of the poor quality of these ecosystems. Indeed, amphibians are very sensitive to the accumulation of contaminants (Howe et al., 2004; Relyea et al., 2005a, b; Costa et al., 2008; Relyea and Jones, 2009). However, the distinct sections that were designed along a same water course, i.e. upstream and downstream of the WTP, constituted differential stressful environments, which was unexpected. Because agricultural activities are similar along the Fosseille and Agouille de la Mar rivers, they cannot be solely considered as

the main factor involved in the turtle's stress. As a consequence, the processed waters discharged by WTPs, may considerably impact the biodiversity, as illustrated within freshwater turtles.

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## REFERENCES

- Albiac J, Calvo E, Tapia J, Esteban E. 2010. Water quality and nonpoint pollution: Comparative global analysis. Re-Thinking Water and Food Security Chapter 13:251-256.
- Bhuiyan AB, Mokhtar MB, Toriman ME, Gasim MB, Ta GC, Elfithri R, Razman MR. 2013. The environmental risk and water pollution: a review from the river basins around the world. *Am-Eurasian J Sustain Agric* 7:126-136.
- Cavalcante DGSM, Martinez CBR, Sofia SH. 2008. Genotoxic effects of Roundup® on the fish *Prochilodus lineatus*. *Mutat Res-Gen Tox En* 655:41-46.
- Commissariat général au développement durable – Service de l’observation et des statistiques (2015). Les pesticides dans les cours d’eau Français en 2013. Chiffres et Statistiques 697.
- Costa MJ, Monteiro DA, Oliveira-Neto AL, Rantin FT, Kalinin AL. 2008. Oxidative stress biomarkers and heart function in bullfrog tadpoles exposed to Roundup Original®. *Ecotoxicology* 17:153-163.
- Dominguez GA, Lohse SE, Torelli MD, Murphy CJ, Hamers RJ, Orr G, Klaper R.D. 2015. Effects of charge and surface ligand properties of nanoparticles on oxidative stress and gene expression within the gut of *Daphnia magna*. *Aquat Toxicol* 162: 1-9.
- Gavrić J, Prokić M, Despotović S, Gavrilović B, Radovanović T, Borković-Mitić S, Pavlović S, Saičić Z. 2015. Biomarkers of oxidative stress and acetylcholinesterase activity in the blood of grass snake (*Natrix natrix* L.) during prehibernation and posthibernation periods. *Braz Arch Biol Techn* 58:443-453.
- Giesy JP, Dobson S, Solomon KR. 2000. Ecotoxicological risk assessment for Roundup herbicide. *Rev Environ Contam Toxicol* 167:35-120.
- Gill HK, Garg H. 2014. Pesticides: Environmental Impacts and Management Strategies. In Pesticides - Toxic Aspects. S. Soloneski ed. (InTech).
- Gluszczak L, dos Santos Miron D, Crestani M, Braga da Fonseca M, Araújo Pedron F de, Duarte MF, Vieira VLP. 2006. Effect of glyphosate herbicide on acetylcholinesterase activity and metabolic and hematological parameters in piava (*Leporinus obtusidens*). *Ecotoxicol Environ Safe* 65:237-241.
- Gluszczak L, dos Santos Miron D, Moraes BS, Simões RR, Schetinger MRC, Morsch VM, Loro VL. 2007. Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*). *Comp Biochem Physiol C Toxicol Pharmacol* 146:519-524.
- Halliwell B. 2001. Free radicals and other reactive species in disease. Encyclopedia of Life Sciences. *Nature Publishing Group*.
- Héritier L, Duval D, Galinier R, Meistertzheim AL, Verneau O. 2016. Oxidative stress induced by Glyphosate-based herbicide on freshwater turtles under experimental conditions. Submitted to Environmental Toxicology and Chemistry.
- Hermes-Lima M, Storey KB. 1993. Antioxidant defenses in the tolerance of freezing and anoxia by garter snakes. *Am Physiol Soc* 265:642-652.
- Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. *Environ Toxicol Chem* 23:1928-1938.
- Institut Français de l’Environnement 2007. Les pesticides dans les eaux données 2005. 36 pp.
- Labrada-Martagón V, Tenorio Rodríguez PA, Méndez-Rodríguez LC, Zenteno-Savín T. 2011. Oxidative stress indicators and chemical contaminants in East Pacific green turtles (*Chelonia mydas*) inhabiting two foraging coastal lagoons in the Baja California peninsula. *Comp Biochem Physiol C Toxicol Pharmacol* 154:65-75.
- Langiano V do C, Martinez CBR. 2008. Toxicity and effects of a glyphosate-based herbicide on the Neotropical fish *Prochilodus lineatus*. *Comp Biochem Physiol C Toxicol Pharmacol* 147:222-231.



- Li X, Kang Y, Zhang X, Zhu B, Fang W. 2012. Identification of a heat shock cognate protein 70 gene in Chinese soft-shell turtle (*Pelodiscus sinensis*) and its expression profiles under thermal stress. *J Zhejiang Univ Sci B* 13:465-477.
- Livak KJ, Schmittgen TD. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the  $2^{-\Delta\Delta CT}$  Method. *Methods* 25:402-408.
- Malmqvist B, Rundle S. 2002. Threats to the running water ecosystems of the world. *Environ Conserv* 29:134-153.
- Meistertzheim AL, Tanguy A, Moraga D, Thébault MT. 2007. Identification of differentially expressed genes of the Pacific oyster *Crassostrea gigas* exposed to prolonged thermal stress: Thermal stress-induced gene expression in *C. gigas*. *FEBS Journal* 274:6392-6402.
- Modesto KA, Martinez CBR. 2010. Roundup® causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus*. *Chemosphere* 78:294-299.
- Moraes BS, Loro VL, Gluszcak L, Pretto A, Menezes C, Marchezan E, de Oliveira Machado S. 2007. Effects of four rice herbicides on some metabolic and toxicology parameters of teleost fish (*Leporinus obtusidens*). *Chemosphere* 68:1597-1601.
- Palacios C, Urrutia C, Knapp N, Quintana MF, Bertolero A, Simon G, Du Preez LH, Verneau O. 2015. Demographic structure and genetic diversity of *Mauremys leprosa* in its northern range reveal new populations and a mixed origin. *Salamandra* 51:221-230.
- Pearse DE, Arndt AD, Valenzuela N, Miller BA, Cantarelli V, Sites JW. 2006. Estimating population structure under nonequilibrium conditions in a conservation context: continent-wide population genetics of the giant Amazon river turtle, *Podocnemis expansa* (Chelonia; Podocnemididae): Conservation genetics of giant turtles. *Mol Ecol* 15:985-1006.
- Poletta GL, Simoniello MF, Mudry MD. 2016. Biomarkers of oxidative damage and antioxidant defense capacity in *Caiman latirostris* blood. *Comp Biochem Physiol C Toxicol Pharmacol* 179:29-36.
- Unpublished report of the Conseil Général des Pyrénées-Orientales, 2013 - Suivi de la qualité des cours d'eau du bassin versant du Tech et des petits fleuves côtiers.
- Relyea RA. 2005a. The lethal impact of Roundup on aquatic and terrestrial amphibians. *Ecol Appl* 15:1118-1124.
- Relyea RA. 2005b. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecol Appl* 15:618-627.
- Relyea RA, Jones DK. 2009. The toxicity of Roundup Original Max® to 13 species of larval amphibians. *Environ Toxicol Chem* 28:2004-2008.
- Roe JH, Brinton AC, Georges A. 2009. Temporal and spatial variation in landscape connectivity for a freshwater turtle in a temporally dynamic wetland system. *Ecol Appl* 19:1288-1299.
- Sabater S, Tockner K. 2010. Water scarcity in the Mediterranean: Perspectives under global change. *Env Chem* 8: 15-39.
- Sala OE, Chapin III FS, Armesto JJ, Berlow E, Bloomfield J, Dirzo R, Huber-Sanwald E, Huenneke LF, Jackson RB, Kinzig A, Leemans R, Lodge DM, Mooney HA, Oesterheld M, Poff NL, Sykes MT, Walker BH, Walker M, Wall DH. 2000. Global Biodiversity Scenarios for the Year 2100. *Science* 287:1770-1774.
- Schwarzenbach RP, Escher BI, Fenner K, Hofstetter TB, Johnson CA, Gunten UV, Wehrli B. 2006. The Challenge of Micropollutants in Aquatic Systems. *Science* 313:1072-1077.
- Stecyk JAW, Couturier CS, Fagernes CE, Ellefsen S, Nilsson GE. 2012. Quantification of heat shock protein mRNA expression in warm and cold anoxic turtles (*Trachemys scripta*) using an external RNA control for normalization. *Comp Biochem Physiol Part D Genomics and Proteomics* 7:59-72.

- Trujillo-Reyes J, Peralta-Videa JR, Gardea-Torresdey JL. 2014. Supported and unsupported nanomaterials for water and soil remediation: Are they a useful solution for worldwide pollution? *J Hazard Mater* 280:487-503.
- UICN France, MNHN & SHF. 2015. La liste rouge des espèces menacées en France - Chapitre reptiles et amphibiens de France métropolitaine. Paris, France.
- Vazquez M. 2012. Dynamique de la population d'émydes lépreuses – *Mauremys leprosa*. Site Natura 2000 “Massif des Albères/Banyuls-sur-Mer”. Master dissertation, UPVD, pp. 1-27.
- Wang S, Sun H, Ang HM, Tade MO. 2013. Adsorptive remediation of environmental pollutants using novel graphene-based nanomaterials. *Chem Engin J* 226:336-347.

## Summary

Human imprint on Earth is actually so profound leading global environmental changes that affects and modifies the functioning of ecosystems. Among the natural biomes, freshwater ecosystems are remarkable habitats that comprise great species biodiversity but are also the most threatened by human activities. The main causes of the loss of water quality and disruption of freshwater ecosystems includes water pollution and the introduction of alien species.

The first part of this thesis showed invasion of parasites on native freshwater turtle populations, carried by introduced turtle species. Furthermore, it highlighted the necessary to study and describe the new invasive parasite species with more performant technics. The second part of this thesis consisted in the development of a tool to evaluate the status of the health of wild freshwater turtle populations, allowed also the level of contamination of the watercourses.

Keywords: Biological invasions; water pollution; glyphosate; *E. orbicularis*; *M. leprosa*; *T. s. elegans*

## Résumé

L'empreinte humaine sur Terre est si profonde qu'elle entraîne des changements environnementaux qui affectent et modifient le fonctionnement des écosystèmes. Parmi tous les biotopes, les écosystèmes aquatiques continentaux sont des habitats remarquables qui abritent une grande biodiversité, mais qui sont aussi les plus menacés par les activités humaines. Les principales causes de la perte de qualité de l'eau et de la perturbation des ces écosystèmes comprennent la pollution des eaux et l'introduction d'espèces exotiques.

La partie première partie de ce travail de thèse a montré des invasions parasitaires sur les populations de tortues d'eau douce indigènes, transmis par des espèces de tortues introduites. De plus, la nécessité d'étudier et de décrire les nouvelles espèces de parasites invasives avec des techniques plus performantes a été soulignée. La deuxième partie de cette thèse a consisté en l'élaboration d'un outil pour évaluer l'état de la santé des populations de tortues d'eau douce sauvages, ce qui reflète également le niveau de contamination des cours d'eau.

Mots clés : Invasions biologiques ; pollution des eaux ; glyphosate; *E. orbicularis*; *M. leprosa*; *T. s. elegans*