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Matthias Renoirt

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LA ROCHELLE UNIVERSITE

ÉCOLE DOCTORALE

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Chize (C.E.B.C.)

THÈSE

présentée par :

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pour l'obtention du grade de Docteur de La Rochelle Université

Discipline : Biologie de l'environnement, des populations, écologie

**Influence de l'habitat sur l'écologie et la physiologie du crapaud épineux
(*Bufo spinosus*)**



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PREFACE

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INTRODUCTION

I/ Anthropisation

1. Modifications environnementales et biodiversité

D'après le dernier rapport de l'IPBES (« Plateforme Intergouvernementale sur la Biodiversité et les Ecosystèmes »), les activités humaines entraînent une large modification des milieux naturels, menaçant d'extinction un grand nombre d'espèces à une vitesse alarmante (Steffen et al., 2007, 2015). En chiffres, 75% des milieux terrestres et 85 % des zones humides sont impactés jusqu'à provoquer leur disparition du fait de l'action de l'anthropisation, menant à une crise de biodiversité. En moyenne, les activités humaines menacent 25 % des espèces animales et végétales (telles que 41% des espèces d'amphibiens ou encore 25% des espèces de mammifères; Rodrigues et al., 2006), un taux bien plus élevé que la moyenne sur les 10 derniers millions d'années (Barnosky et al., 2011). Selon l'Indice Planète Vivante et depuis 1970, on assiste à une diminution, en moyenne, de 68% des populations de vertébrés (**Figure 1**). Divers facteurs sont en jeu et on dénombre ainsi cinq grandes causes globales d'origine de perte de biodiversité : les espèces envahissantes, la pollution, le changement climatique, la surexploitation des espèces et la destruction de l'habitat (dégradation et perte d'habitat, Newbold et al., 2015; Maxwell et al., 2016, **Figure 1**). Ces cinq grandes causes sont toutes d'origine anthropique et en lien direct ou indirect avec la croissance démographique humaine.

Afin de répondre aux besoins d'une démographie croissante et après la révolution industrielle de 1970, les secteurs de l'agriculture, de la pêche, des bioénergies et des industries extractives ont vu leur production augmenter considérablement (FAO, 2009, 2020; Rudel et al., 2009). Une telle augmentation exponentielle des activités humaines induit une augmentation substantielle des besoins humains dans un monde aux ressources limitées. Fatalement, cette surconsommation est probablement à l'origine de la sixième extinction majeure avec un besoin des espèces animales et végétales de s'adapter à ces nouveaux environnements (Steffen et al., 2006a, 2015). En réponse à tous ces changements, la biologie de la conservation (perte, maintien et restauration de la biodiversité) s'est considérablement développée ces trente dernières années (Mangel et al., 1996; Angermeier, 2000). La biologie de la conservation diffère de la plupart des autres sciences biologiques sur un point important : il s'agit souvent d'une discipline de crise (Soulé, 1985). Pour fournir la compréhension et les outils nécessaires au maintien de la biodiversité, de nombreuses disciplines sont utilisées (Soulé, 1985) afin de « conserver les acteurs de la pièce évolutive et le décor écologique où elle est jouée » (George Evelyn Hutchinson, 1965).

Un grand nombre d'études ont mis en avant les effets négatifs des pressions anthropiques dans le temps et dans l'espace sur la biodiversité (Venter et al., 2016; Jung et al., 2019; Kadoya et al., 2022; Li et al., 2022). Newbold et al., (2015) ont par exemple montré que la biodiversité locale pouvait être négativement influencée du fait de l'utilisation des terres à des fins anthropiques. Dans les habitats les plus touchés, la richesse spécifique, l'abondance totale et la richesse (abondance d'espèces rares) ont respectivement diminué en moyenne de 76,5%, 39,5% et 40,3% (Newbold et al., 2015). La plupart de ces études ont réalisé des modèles prédictifs qui démontrent, qu'en cas de pratiques inchangées, l'augmentation considérable de l'utilisation et de la modification des habitats se poursuivrait (Balmford et al., 2005; Alkemade et al., 2012; Visconti et al., 2016), ce qui provoquerait une accentuation de la dégradation des écosystèmes et donc de la biodiversité (Alkemade et al., 2012; Visconti et al., 2016).

Certains écosystèmes sont encore plus touchés par ces changements, et c'est le cas des zones humides, qui font parties des écosystèmes subissant le plus l'intensification de l'agriculture et où, les pertes en termes de biodiversité sont les plus conséquentes (Tilman et al., 2001; Ficetola et al., 2015). Ces écosystèmes supportent pourtant une forte biodiversité et richesse spécifique (Gibbs, 2000), et sont connus pour être particulièrement sensibles aux perturbations de l'environnement (Gibbs, 2000). S'ajoute à cela que les espèces utilisant les zones humides sont particulièrement sensibles aux conséquences de la modification de leur habitat (Erwin, 2008). Etant particulièrement dépendantes des contraintes hydriques et de la qualité de l'eau, que ce soit tout au long de leur vie ou pour des cycles reproducteurs, les populations de ces espèces subissent des déclin importants ou, pour les espèces les plus adaptables, des changements de type d'habitats (Martin et al., 2021). Mais même pour une espèce plastique, un habitat ne fournissant pas les conditions écologiques optimales pourrait impacter la qualité individuelle comme la fitness ou la valeur sélective (Donald et al., 2001), et *in fine* le maintien des populations (Wiegand et al., 2005). Le type et la qualité d'un habitat représentent ainsi des facteurs essentiels influençant la fitness des individus et des populations (Johnson, 2007; Bjørneraas et al., 2012).



Figure 1. (A) indice planète vivante (IPV) montre un déclin moyen de 68 % des populations de vertébrés suivies entre 1970 et 2016 (écart : de - 73 % à - 62 %) parmi 20 811 populations de 4 392 espèces. (B) indice habitat des espèces (IHE) mesure les pertes de surface de l'habitat propice à partir des changements d'habitats observés ou modélisés. Entre 2000 et 2018, l'indice a baissé de 2 %, ce qui indique une diminution importante et générale des habitats disponibles pour les espèces. Adaptée du rapport planète vivante (2020) WWF.

2. Artificialisation : Urbanisation et contexte agricole

L'urbanisation, de par la transformation des paysages, est le principal moteur de l'artificialisation des milieux. Les routes, bâtiments et l'ensemble des infrastructures sont favorisés au détriment des forêts, friches, prairies et cultures (Marzluff and Ewing, 2008; He et al., 2014). La perte de végétation liée à l'artificialisation du sol entraîne une cascade de changements biologiques et chimiques entraînant par exemple une augmentation du CO₂ et une diminution de l'absorption du dioxyde de carbone en lien avec l'absence de végétaux (Pickett et al., 2008; Wang et al., 2011) ; ou encore des dérèglements des cycles hydrologiques (absorption des sols) en lien avec la forte superficie bitumée des villes (50% de la surface au sol, McKinney, 2006) dont les propriétés des matériaux diffèrent des propriétés géothermiques des sols naturels (Pickett et al., 2008). Ainsi, les différents changements biotiques et abiotiques liés à cette perte d'habitats naturels (notamment la disparition de la végétation native) s'accompagnent d'une diminution de la biodiversité (McKinney, 2002). Les aires les plus urbanisées contiennent moins de la moitié des espèces de plantes, insectes, oiseaux et mammifères trouvées dans les habitats naturels (McKinney, 2002), notamment du fait de la réduction de la disponibilité en ressources naturelles pour se nourrir et se reproduire, engendrant le déclin de nombreux taxons tels que les reptiles (Germaine and Wakeling, 2001) et les amphibiens (Riley et al., 2005).

En plus de l'artificialisation des sols liés à l'urbanisation, plus d'un tiers de la surface totale des terres est utilisé pour l'agriculture. L'expansion agricole est le principal facteur de la modification des milieux, aux dépens des forêts, prairies et zones humides (Alkemade et al., 2012; Barnes et al., 2014; Jung et al., 2019; de Lima et al., 2020; Kadoya et al., 2022, **Figure 2**). En effet, les habitats fragmentés, comprenant des vestiges de parcelles d'habitat entourés de terres perturbées par l'homme, réduisent le domaine vital et altèrent les niches écologiques des organismes présents (Rudnick et al., 2012). Dans ce type de milieu, les espèces généralistes et capables de faire face à des conditions modifiées sont favorisées au détriment des espèces plus spécialisées (Rudnick et al., 2012), ce qui peut conduire à des changements de composition des communautés. Pour évaluer objectivement ces changements actuels de biodiversité, il est par ailleurs nécessaire de prendre en compte les effets biotiques retardés liés aux modifications passées des terres, qui continuent d'influencer les assemblages d'espèces actuels longtemps après les premières altérations (Jung et al., 2019).

De ce fait, il est essentiel d'étudier l'écologie de ces espèces dans des habitats contrastés afin de déterminer l'état de santé des populations, leur capacité d'adaptation, de résilience mais aussi de déterminer les différentes contraintes anthropiques sur leur persistance.

Évolution de la superficie agricole

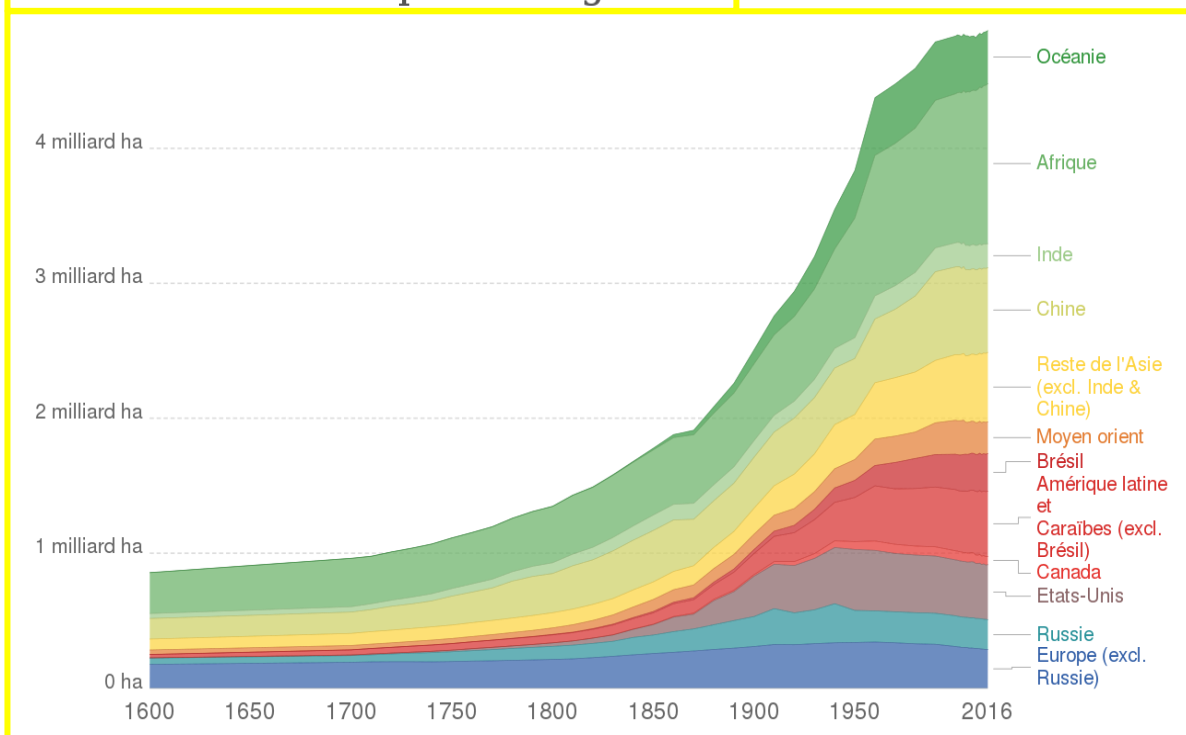


Figure 2. Utilisation totale des terres en surface pour l'agriculture (en hectares). Adaptée de History database of the Global Environment (2017).

II/ Modification des milieux : contexte agricole

Afin d'appréhender de manière exhaustive les conséquences de ces modifications, il est nécessaire de bien comprendre et d'identifier les pressions directes et indirectes que subissent les espèces sauvages, qui sont les plus exposées à ces changements. Sur l'ensemble de la surface terrestre, la Surface Agricole Utilisée (SAU) représente 4,9 milliards d'ha pour une estimation de 13 milliards d'ha de terres émergées (FAO, 2009; Feillet, 2014). Ainsi, 38% de la surface terrestre est utilisée à des fins agricoles (FAO, 2009; Feillet, 2014). En France, la SAU représente plus de la moitié (54%) du territoire national (INSEE, Agreste). Entre 1970 et 2020, la SAU par exploitation en France est passée d'environ 20 ha à environ 70 ha. Cette augmentation de l'utilisation des terres, et de la modification de l'environnement qui en découle, a des implications fortes pour la persistance des populations et pour la biodiversité, du fait de la fragmentation de l'habitat, mais aussi du fait de l'homogénéisation et de la simplification des milieux. Dans une étude de Pilotto et al., (2022) il a été montré que les périodes de croissance des populations humaines affectent et ont affecté la biodiversité des insectes à l'échelle des temps géologiques (il y a 4000 ans et il y a 10 000 ans). Ces effets peuvent être directs mais aussi indirects à l'échelle du paysage sur des sites éloignés des zones d'impacts des humains (Pilotto et al., 2022).

1. Fragmentation de l'habitat

La fragmentation est décrite comme un ensemble de processus qui transforment un habitat naturel homogène en fragments de taille variable et discontinue. Elle représente un processus complexe qui englobe deux composantes principales : (1) la perte d'habitat, qui correspond à une réduction de la surface de l'habitat considéré, et (2) la fragmentation au sens strict de cet habitat (Fahrig, 2003, **Figure 3**). Les modifications actuelles sont souvent associées à de la fragmentation et conjointement à de la perte d'habitat. Par conséquent, le terme de fragmentation d'habitat est souvent lié à une notion de perte d'habitat (contrairement à la fragmentation de l'habitat per se, voir : Fahrig, 2017) et ces deux processus conjoints sont considérés comme étant la menace principale liée à l'agriculture (Wilcove et al. 1998). A l'image de la diversité de processus qui sous-tendent la fragmentation, les conséquences sont multiples, ce qui met en exergue l'intérêt d'étudier ces conséquences sur la diversité et le fonctionnement des communautés, ainsi que sur la viabilité des populations et sur leur capacité de réponse adaptative.

La fragmentation de l'habitat peut, par exemple, se manifester par une augmentation du nombre de parcelles/fragments ainsi que leur isolement dans le paysage, par une réduction en surface d'un habitat et par la réduction de la taille de ces parcelles, mais encore, par une plus grande distance entre les fragments pouvant affecter les déplacements des individus (Fahrig, 2017, **Figure 3**).

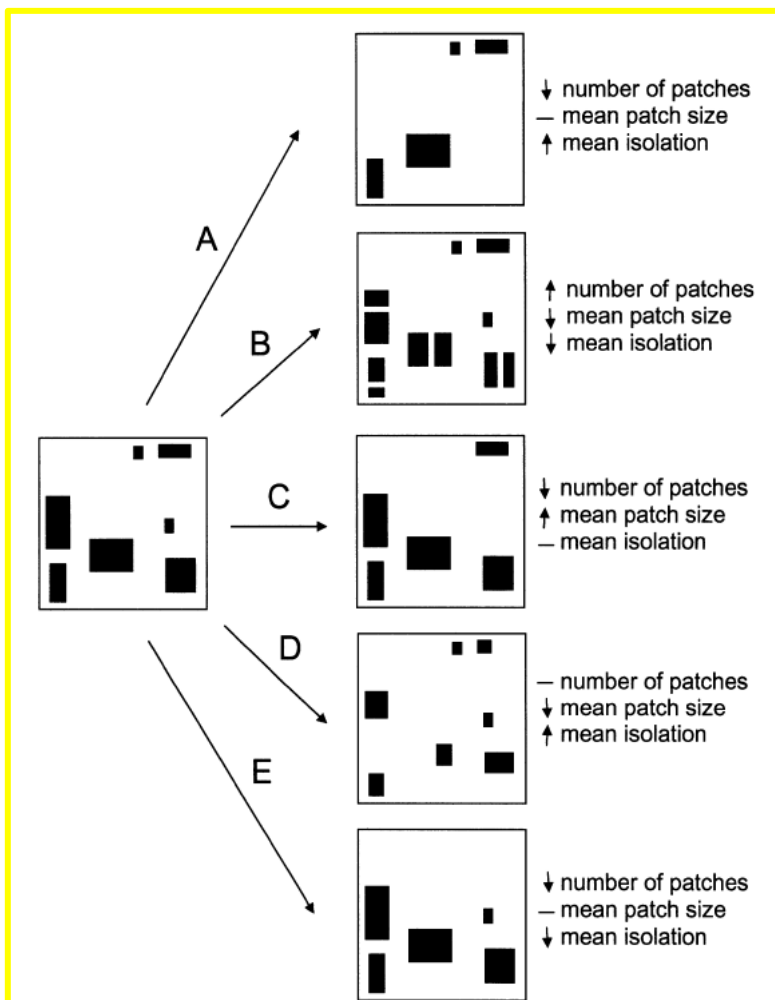


Figure 3. Illustration de la perte d'habitat entraînant certains, mais pas tous, des trois autres effets attendus de la fragmentation de l'habitat sur la configuration du paysage. Les effets attendus sont (a) une augmentation du nombre de parcelles, (b) une diminution de la taille moyenne des parcelles, et (c) une augmentation de l'isolement moyen des parcelles (distance du plus proche voisin). Les changements réels sont indiqués par des flèches. Issue de Fahrig, (2003).

Les études empiriques réalisées à ce jour suggèrent que la perte d'habitat a des effets négatifs importants et constants sur la biodiversité, avec notamment une disponibilité en habitats plus faible, limitant l'abondance totale (Debinski and Holt, 2000; Flather and Bevers, 2002; Evans et al., 2005). Cette limitation engendre une diminution de la richesse spécifique, ce qui provoque une réduction du nombre d'espèces pouvant avoir des populations viables dans ces fragments (Debinski and Holt, 2000; Gaston, 2000). De plus, la modification des mouvements des individus entre parcelles d'habitats peut entraîner des conséquences dévastatrices pour les populations. Si, l'adaptation, la diversification et l'émergence de nouvelles espèces sont facilitées par l'isolement, d'un point de vue démographique l'isolement réduit considérablement le taux de survie et de reproduction (Keller and Waller, 2002). En effet, lorsque le nombre d'individus dans une population est faible, couplé à l'isolement, le succès reproducteur diminue et le risque d'extinction augmente (Purvis et al., 2000). Dans une étude sur les mammifères terrestres, Tucker et al., (2018) ont par exemple mis en évidence le fait que la fragmentation de l'habitat limite la reproduction, la migration, la dispersion, mais aussi les zones de fourragements de ces espèces. Ainsi, la fragmentation, à travers la dégradation des écosystèmes et l'isolement des habitats, empêche les mouvements d'individus, réduit la persistance des populations, l'abondance, la richesse spécifique et la dynamique des réseaux trophiques.

La viabilité et la persistance des populations sont aussi impactées négativement d'un point de vue génétique. La baisse d'effectif, couplée à l'isolement des populations, engendrent une perte de diversité génétique, plus particulièrement au sein des petites populations (Keyghobadi, 2007; Dixo et al., 2009). Mais l'échelle temporelle joue un rôle primordial dans la fragmentation de l'habitat. En effet, certaines conséquences de la fragmentation sont progressives, et les effets sur la viabilité des populations peuvent n'être ressentis qu'après plusieurs décennies (Tilman et al., 1994; Kuussaari et al., 2009; Haddad et al., 2015). Ainsi, une espèce peut persister un temps malgré le fait que les conditions locales ne soient pas réunies pour garantir son maintien, on parle de « dette d'extinction » (Tilman et al., 1994) ; une population peut disparaître en décalage avec une fragmentation antérieure de l'habitat (Tilman et al., 1994; Kuussaari et al., 2009; Haddad et al., 2015). A l'inverse, les effets peuvent être à court terme et impacter négativement, dans un laps de temps réduit, les espèces indigènes qui utilisent cet habitat.

2. Hétérogénéité : vers une homogénéisation et une simplification des milieux

Le terme "hétérogénéité" qualifie, par définition, ce qui n'est pas homogène, c'est-à-dire ce qui est constitué d'un ensemble d'objets disparates (Baudry and Papy, 2001). A l'état naturel un milieu est considéré comme hétérogène spatialement car il est composé d'un mélange d'écosystèmes (Tschardt et al., 2012). De ce fait, les nombreux fragments homogènes composant un milieu le rendent hétérogène. Cette notion est centrale car c'est cette hétérogénéité qui confère au milieu la capacité de soutenir de nombreuses espèces (Forman and Godron, 1981; Tews et al., 2004). Cependant, l'intensification des pratiques agricoles a pour conséquence une modification très importante des paysages. Ainsi, la spécialisation des exploitations agricoles et la simplification des pratiques ont réduit l'hétérogénéité compositionnelle avec notamment une agglomération des exploitations et une augmentation de la superficie des parcelles (Benton et al., 2003). L'Agreste estime qu'en 2010, en France, les grandes exploitations assurent 80 % du potentiel de production contre 73 % en 2000. Les exploitations de petites tailles disparaissent progressivement au profit des grandes exploitations de plus de 50 ha. D'après Stoate et al., (2009), ces exploitations tendent de plus en plus à se spécialiser et cette restructuration s'accompagne d'une diminution des surfaces semi-naturelles comme les haies ou les surfaces boisées, ou encore, des bordures de champs herbacées. De ce fait, on estime aujourd'hui que 95 % de la consommation mondiale est basée sur seulement 30 espèces végétales dont 50% consacrée uniquement au blé, riz et maïs (Rahmann, 2011). En conséquence, les paysages agricoles intensifiés contiennent généralement une faible diversité de types de cultures distribués sur de grandes parcelles uniformes (Fahrig et al., 2011). Or, La composition et la structure des communautés végétales sont des composantes essentielles de l'habitat, et essentielles pour supporter les populations faunistiques (Morrison et al., 2012). La distribution spatiale d'une espèce s'explique par la notion de niche écologique (Peterson et al., 2011; Broennimann et al., 2012). Chaque espèce a un environnement spécifique qui est, normalement, favorable et indispensable à son maintien (Scott et al., 2002; Peterson et al., 2011; Broennimann et al., 2012). Ceci est dû à la pression sélective des conditions locales qui filtrent la présence des espèces (Scott et al., 2002; Tschardt et al., 2005).

Les espèces ne sont donc pas présentes partout mais uniquement dans les habitats qui correspondent à leur niche écologique (Scott et al., 2002). La réponse des espèces aux gradients environnementaux s'opère en fonction de leurs caractéristiques morphologiques et physiologiques, c'est-à-dire leurs traits fonctionnels (Díaz and Cabido, 2001). Ainsi, l'homogénéisation se réfère à la réduction de la diversité des communautés biotiques (souvent spécialisées) en faveur d'un même cortège d'espèces tolérantes aux perturbations (souvent généralistes) (McKinney and Lockwood, 1999; Olden and Rooney, 2006; Filippi-Codaccioni et al., 2010). Ces modifications peuvent entraîner une homogénéisation biotique des communautés et donc une perte de diversité (McKinney and Lockwood, 1999; Olden et al., 2004; Smart et al., 2006; Clavel et al., 2011). Cette homogénéisation biotique s'explique par des communautés présentant une diversité de réponses réduite pouvant limiter les fonctions assurées par la communauté, ainsi que leur capacité à répondre aux perturbations (McKinney and Lockwood, 1999; Olden et al., 2004; Clavel et al., 2011, **Figure 4**). Ces réponses sont liées au fait que les filtres environnementaux sélectionnent pour certains traits fonctionnels, affectant ainsi la composition de la communauté lors d'un changement de l'habitat (Mouillot et al., 2013, **Figure 4**). Par exemple, il est possible d'évaluer la réponse des communautés à l'intensification de l'agriculture en étudiant des traits tels que le régime alimentaire, la capacité de dispersion ou encore la taille corporelle (McKinney and Lockwood, 1999; Olden et al., 2004; Devictor et al., 2008). Dans les écosystèmes gérés de manière intensive, le risque d'extinction des espèces ayant une capacité de dispersion limitée, un régime alimentaire restreint et une faible fécondité est plus élevé (McKinney and Lockwood, 1999; Clavel et al., 2011; Kormann et al., 2015).

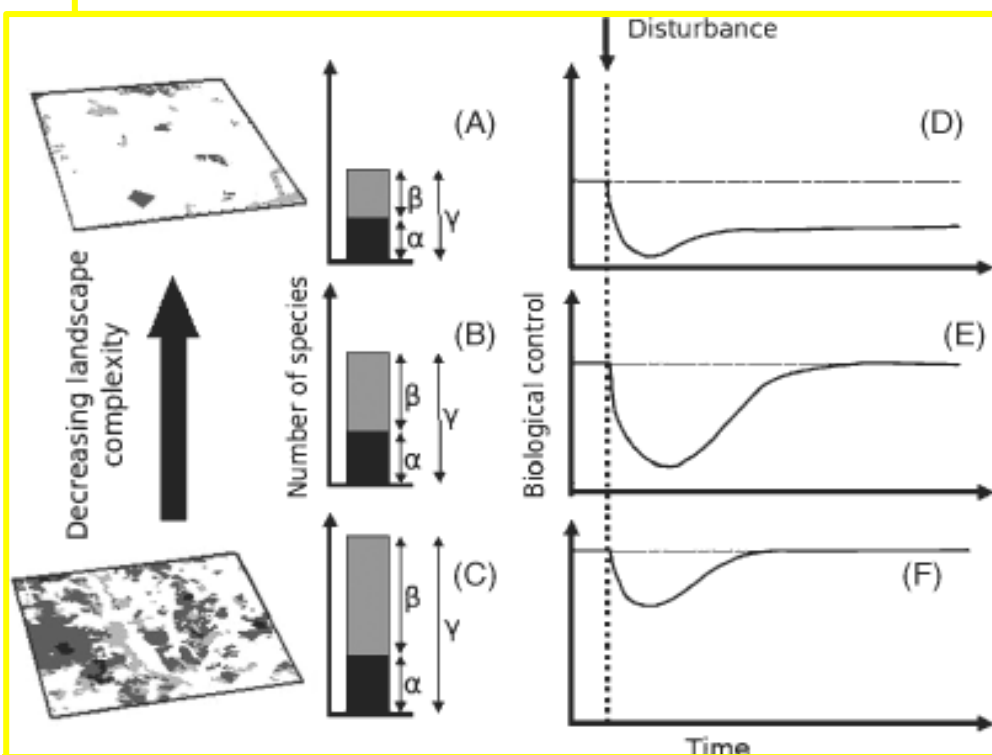


Figure 4. Complexité du paysage et résilience, la capacité à se réorganiser après une perturbation, d'un service écosystémique tel que le contrôle biologique. La complexité du paysage diminue de bas en haut, influençant les réponses aux perturbations en termes de richesse en espèces (A-C) et de contrôle biologique par les ennemis naturels (D-F). L'augmentation de la complexité du paysage améliore le pool d'espèces (A-C) et le niveau de contrôle biologique, permettant une récupération rapide du contrôle biologique après une perturbation (D-F). Les paysages complexes abritent plus d'espèces, principalement en raison de la diversité bêta plus élevée, mais souvent aussi de la diversité alpha plus élevée (non illustrée ici) et ne permettent qu'une faible baisse et un retour rapide du contrôle biologique après une perturbation (Tscharrntke et al., 2007a, modifié d'après Bengtsson et al., 2003). Issue de Tscharrntke et al., 2012

3. Modifications chimiques des milieux

En plus de la fragmentation, de l'homogénéisation et de la simplification des milieux, l'agriculture est étroitement associée à la contamination environnementale. Les pratiques agricoles modernes se basent sur l'utilisation de l'agrochimie (pesticides, fertilisants), des molécules qui altèrent et interagissent avec l'écosystème. Malgré une volonté de contrôler et réglementer les intrants dans les pratiques agricoles (date et nombre d'épandages, contrôle de toxicité etc.), les impacts négatifs directs ou indirects perdurent, engendrant des conséquences multiples dans les milieux non-cibles et sur les organismes non-cibles (Hasenbein et al., 2017; de Brito Rodrigues et al., 2019).

a. Milieux non-cibles et fertilisation

L'agrochimie est basée sur l'utilisation de produits pour protéger les récoltes mais également dans un but d'augmentation des rendements. En effet, l'azote a un rôle majeur sur la croissance des plantes et se retrouve naturellement dans l'environnement. Cependant, afin de répondre à une demande toujours plus importante, et d'accroître la production et le rendement (FAO, 2009, 2020; Rudel et al., 2009), l'utilisation de fertilisants synthétiques (engrais chimiques) s'est développée. Depuis les années 60, la vente d'engrais chimiques est en constante augmentation. Leur utilisation massive et excessive dans les cultures a engendré l'eutrophisation des eaux de surface et des nappes phréatiques (Khan and Mohammad, 2014). Par lixiviation, ruissellement et/ou dérive de pulvérisation, ces intrants chimiques peuvent se retrouver accidentellement dans les milieux aquatiques (Wood and Goulson, 2017; Borsuah et al., 2020). L'eutrophisation est l'une des principales causes de la dégradation de la qualité de l'eau (Smith and Schindler, 2009). De ce fait, les engrais chimiques en arrivant dans des milieux non-cibles (ici des milieux aquatiques) entraînent une croissance démesurée des plantes aquatiques et des algues. Ainsi, la stabilité du milieu est perturbée (Smith and Schindler, 2009; Glibert, 2017), ainsi que les espèces qui y sont indigènes (Helminen et al., 2000; Duprey et al., 2016). De plus, cette supplémentation excessive peut entraîner le développement des cyanobactéries responsables de l'anoxie d'un milieu aquatique (effets de toxicité directe également) et donc de la mort massive des espèces utilisant ce milieu (Dodds et al., 2009).

b. Pesticides

Les pesticides sont largement utilisés dans la production agricole. Ils permettent de prévenir ou contrôler les ravageurs, les maladies, les adventices et autres agents pathogènes des plantes. Parmi les pesticides, on distingue trois familles : (1) les fongicides qui luttent contre les champignons ravageurs, (2) les herbicides contre les adventices et (3) les insecticides qui luttent contre les insectes ravageurs des récoltes afin d'assurer un rendement des cultures optimal. De sérieuses inquiétudes ont été soulevées quant aux risques que représentent ces pesticides pour les espèces non-cibles et la faune sauvage (voir *Silent Spring* de Rachel Carson, 1962) et le nombre d'études concernant les effets des pesticides sur l'environnement a augmenté de 58% ces 10 dernières années (Sharma et al., 2019; Rajmohan et al., 2020, **Figure 5**).

Afin d'évaluer les effets potentiellement néfastes des pesticides sur l'environnement (contamination du sol, de l'eau et de l'air par lixiviation, ruissellement et dérive de pulvérisation) et la faune sauvage (les vertébrés et invertébrés, les plantes et d'autres organismes non-cibles) il est essentiel de prendre en compte de multiples facteurs comme la toxicité du pesticide, la dose appliquée, l'absorption sur les colloïdes du sol, les conditions météorologiques après l'application et la durée de persistance du pesticide dans l'environnement (Wood and Goulson, 2017; Borsuah et al., 2020; Gunstone et al., 2021). L'évaluation des risques des pesticides sur la faune sauvage est un processus complexe en raison de la multiplicité des types de pesticides utilisés (que ce soit la toxicité et/ou la persistance), des différences dans les périodes et les niveaux d'exposition, et finalement, des caractéristiques environnementales des zones où les pesticides sont appliqués (Gunstone et al., 2021). Dans les pays développés, les cas de toxicité aigus de la faune sauvage ont diminué au cours des dernières décennies. Le problème de la toxicité aigüe des pesticides est par contre devenu le centre d'intérêt principal des études, car plus représentatif des potentielles expositions des organismes dans les milieux naturels. Ainsi, les effets à différents niveaux sur les organismes sont multiples et les conséquences sont souvent néfastes (Gunstone et al., 2021, **Figure 5**). Il a été montré que les pesticides ont des effets délétères directs en affectant la survie des individus ou en réduisant leur capacité de reproduction (Relyea, 2004; Slaninova et al., 2009; Williams et al., 2015; Trudeau et al., 2020) ; mais également indirects en réduisant les ressources alimentaires (arthropodes ou adventices) (Hart et al., 2006; Wagner, 2020). Or, pour les méso-prédateurs (taxons supérieurs de la chaîne trophique) l'abondance et la diversité d'insectes sont essentiels à la pérennité des populations.

Mais la ressource est affectée par l'intensification de l'agriculture, à la fois par la suppression des habitats semi-naturels et l'utilisation de pesticides (Goulson, 2019; Sánchez-Bayo and Wyckhuys, 2019), ce qui risque de provoquer un déséquilibre de la chaîne trophique. Par ailleurs, plus de 120 pesticides perturbateurs endocriniens, encore utilisés, sont connus à ce jour (McKinlay et al., 2008). Certains, comme les organochlorés ou les triazoles présentent des propriétés de perturbation de la thyroïde chez les micromammifères, les oiseaux, les amphibiens et les poissons (Brucker-Davis, 1998; Boas et al., 2012; Köhler and Triebkorn, 2013, **Figure 5**). D'autres, comme l'atrazine et les organophosphorés interagissent avec le système immunitaire (Galloway and Depledge, 2001; Galloway and Handy, 2003, **Figure 5**) et altèrent les fonctions métaboliques dont découle le comportement (activité, temps de recherche de nourriture, capacité d'apprentissage), la thermorégulation et la consommation d'eau et/ou de nourriture chez les vertébrés (Story and Cox, 2001). De plus, de nombreuses études ont montré des malformations des organes, un retard de développement, une diminution de la croissance et une mortalité embryonnaire chez les organismes du biote aquatique comme les poissons, les amphibiens et les invertébrés non ciblés (Pašková et al., 2011; Cheron and Brischoux, 2020; Werner et al., 2021; Cheron et al., 2022a, 2022b, **Figure 5**).

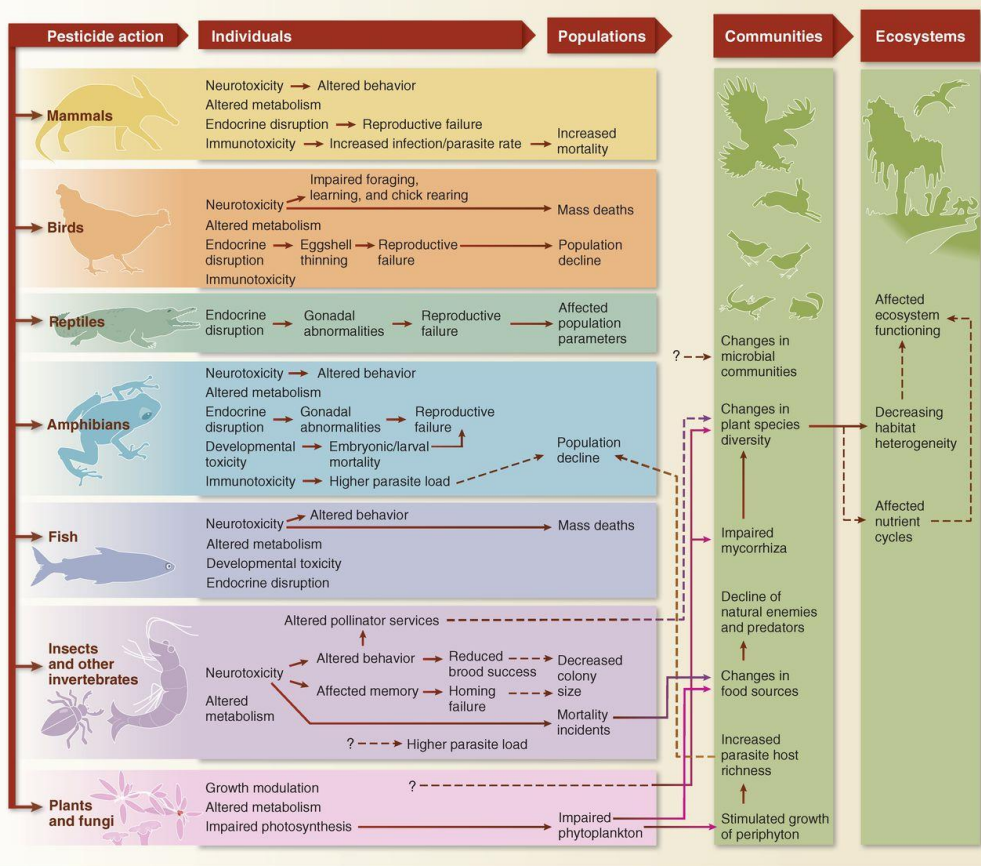


Figure 5. Effets documentés des pesticides sur la faune sauvage à différents niveaux d'organisation biologique et interrelations connues (flèches pleines) ou prouvées et anticipées (flèches en pointillés) entre ces effets. Issue de Köhler and Triebkorn, (2013).

III/ Biodiversité dans les agroécosystèmes

Lorsqu'on étudie un milieu dans son ensemble, notamment au travers des organismes qu'ils abritent, il est nécessaire de distinguer les facteurs abiotiques et les facteurs biotiques. Les facteurs abiotiques représentent les phénomènes physico-chimiques du milieu, tels que l'humidité de l'air, la lumière, la température, les structures physiques et compositions chimiques du substrat et de l'eau (pour les milieux aquatiques) ou encore la pression atmosphérique (Dunson and Travis, 1991; Hajek and Shapiro-Ilan, 2018). Ce sont les facteurs non-vivants d'un milieu pouvant influencer les organismes qui y vivent. Les facteurs biotiques, quant à eux, concernent les influences que subit un organisme dans un milieu donné du fait de la présence d'autres organismes dans ce même milieu. Ces influences peuvent être positives ou neutres (e.g. mutualisme, voir Boucher et al., 1982) mais aussi contraignantes et exercer sur un organisme donné une compétition (inter ou intraspécifique) pour la ressource et/ou pour l'accès à la reproduction (Watts and Holekamp, 2008; Schradin et al., 2010; Britton et al., 2018), par exemple. Cela peut ainsi impacter différents aspects de la vie des organismes tels que le taux de survie en réponse à la prédation, le parasitisme et/ou les maladies (Bancroft et al., 2008; Coors and De Meester, 2008; King et al., 2010) et à terme affecter la fitness. Cependant, les effets observés dépendent grandement de la physiologie de l'organisme considéré (capacité métabolique, rythme endogène d'activité, déplacement) (Olden et al., 2004; Olden and Rooney, 2006; Smart et al., 2006; Shennan, 2008; Médiène et al., 2011; Tuomainen and Candolin, 2011; Ward-Fear et al., 2021).

1. Filtres écologiques

L'assemblage des communautés dans un habitat est considérée comme une succession hiérarchique de filtres abiotiques et biotiques agissant à des échelles spatio-temporelles (Chase and Myers, 2011; Ovaskainen et al., 2017). Bien qu'on puisse distinguer les facteurs biotiques et abiotiques, les milieux naturels sont complexes et sujets à de nombreux facteurs, finalement indissociables. Par exemple, les facteurs abiotiques peuvent eux-mêmes être modifiés du fait de la présence d'autres organismes. Ainsi, presque tous les facteurs abiotiques du milieu sont filtrés et modifiés par la végétation. Mais la végétation n'a pas pour seul rôle de filtrer les facteurs abiotiques. Elle constitue en effet une source de nourriture et d'abris pour les organismes vivants (Bossenbroek et al., 1977; Gunnill, 1982; Tuomainen and Candolin, 2011; Ward-Fear et al., 2021). De fait, les notions de disponibilités et compétition pour la ressource, de régimes alimentaires, de capacité de dispersion et de fécondité deviennent essentielles pour assurer la pérennité des populations mais aussi pour la diversité des organismes qui composent l'habitat (McKinney and Lockwood, 1999; Olden et al., 2004; Devictor et al., 2008).

2. Synergie des facteurs biotiques et abiotiques

Puisque les pratiques agricoles modernes modifient à la fois directement (e.g. augmentation des apports d'engrais minéraux et de pesticides; Tschardt et al., 2005) et indirectement (e.g. simplification et homogénéisation du paysage ; Tschardt et al., 2005) les habitats, les facteurs abiotiques (climat et microclimat) et biotiques (les interactions entre espèces Schmitz et al., 2003) doivent être pris en compte pour une vue d'ensemble du maintien de la diversité dans un paysage donné. Différents exemples peuvent être sélectionnés afin de mettre en exergue les relations exigües entre biotique et abiotique, notamment en contexte paysager dégradé.

Une review de Blaustein et Kiesecker, (2002) a mis en avant les effets de nombreux facteurs abiotiques (e.g. radiation d'UV-B) et biotiques sur le déclin des espèces d'amphibiens. Par exemple, avec l'introduction d'espèces invasives qui exploitent la niche écologique d'autres espèces d'amphibiens. C'est le cas de la grenouille-taureau (*Rana catesbeiana*) et du crapaud buffle (*Rhinella marina*) introduits aux Etats-Unis et qui déciment les populations d'amphibiens (grenouilles de la famille Ranidae) (Blaustein and Kiesecker, 2002). En effet, en plus d'épuiser la ressource alimentaire des autres espèces d'amphibiens, la grenouille-taureau les prédatent, que ce soit au stade larvaire ou adulte. En réponse à ces contraintes de prédation et de compétition, la grenouille à pattes rouges (*Rana aurora*) et ses têtards, vont modifier leur utilisation de l'habitat, ce qui va rendre cette espèce plus vulnérable à la prédation par les poissons (Kiesecker and Blaustein, 1997, 1998; Blaustein and Kiesecker, 2002). Finalement, la question des effets des maladies et des pathogènes émergents, en relation avec les changements environnementaux, sur la faune sauvage se pose. Ainsi, de nombreuses recherches suggèrent que les épidémies sont le résultat d'interactions complexes multifactorielles dépendantes de l'habitat (Daszak et al., 2001; Anderson et al., 2004; Ostfeld, 2009). De ce fait, la modification des structures de l'habitat en lien avec l'ouverture du milieu (microclimat) peut engendrer des hausses de températures dans ces habitats modifiés (Oliver and Morecroft, 2014) et favoriser par exemple, la prévalence de maladie et/ou d'espèces opportunistes. De plus, ce changement de température peut influencer directement le comportement des animaux et le coût énergétique par des biais de processus physiologiques et notamment en induisant des comportements d'évitement et de thermorégulation différentiels (Huey and Tewksbury, 2009). Afin de maintenir une température corporelle optimale et donc d'éviter les coûts physiologiques, certains animaux peuvent, par exemple, limiter leur exposition au soleil (Kearney et al., 2009). C'est pourquoi, les changements des facteurs abiotiques (e.g températures, intrants chimiques) au travers de la modification de l'habitat peuvent affecter les mécanismes hormonaux et neuronaux et, influencer le comportement des organismes (Zala and Penn, 2004; Kearney et al., 2009).

3. Effets sur l'habitat

De fait, les perturbations environnementales, en dégradant les attributs physiques d'un environnement et/ou en modifiant les facteurs abiotiques qui affectent l'écologie de nombreuses espèces, peuvent diminuer la disponibilité d'un habitat approprié (Dale et al., 2000; Tuomainen and Candolin, 2011; Ward-Fear et al., 2021). Ainsi, les changements dans la structure de l'habitat modifient la disponibilité des ressources, comme les abris contre les prédateurs et les sites de nidification. Afin d'éviter une perte de fitness, les espèces vivant dans ces habitats doivent s'adapter ou rechercher des habitats offrant des conditions plus favorables (Tratalos et al., 2007). L'adaptation des organismes passe par des processus physiologiques et comportementaux influencés par les facteurs environnementaux. En lien avec ces adaptations, les interactions biotiques constituent un filtre qui va déterminer les espèces ayant la plus forte probabilité d'être dominantes (Cingolani et al., 2007). Avec l'intensification de l'agriculture qui agit comme un filtre écologique (Tschardt et al., 2005), les communautés peuvent être limitées dans leur résilience face aux perturbations et dans la diversité des réponses exprimées (McKinney and Lockwood, 1999; Olden et al., 2004; Devictor et al., 2008; Clavel et al., 2011). De telle sorte que, l'homogénéisation biotique va conduire à des communautés ayant des traits communs et une diversité de réponses réduites aux perturbations de l'environnement (McKinney and Lockwood, 1999; Olden et al., 2004; Devictor et al., 2008; Clavel et al., 2011). Par conséquent, les espèces spécialisées sont progressivement remplacées par des espèces généralistes et opportunistes (Clavel et al., 2011). Dans les milieux modifiés, les chances pour les généralistes non-indigènes de trouver les ressources nécessaires et les conditions environnementales appropriées sont plus grandes (Duncan et al., 2003). En plus de l'occupation de la niche écologique par les espèces généralistes, les espèces spécialistes sont plus sensibles à la modification du milieu parce qu'elles n'ont pas accès aux types de ressources alternatives dont disposent les espèces généralistes (Clavel et al., 2011).

IV/ Importance de considérer les facteurs combinés

Les points précédents sont en interactions et agissent en parallèle dans la nature. Ainsi, les écosystèmes modifiés sont la résultante de réponses biotiques à des perturbations pouvant être d'origine humaine (e.g. dégradation et enrichissement des sols, Hobbs et al., 2006). De ce fait, il est nécessaire d'intégrer l'ensemble des différentes pressions anthropiques (fragmentation, homogénéisation et simplification du milieu mais aussi l'utilisation d'intrants chimiques), qui modifient les interactions abiotiques et biotiques du milieu, afin de comprendre de manière globale l'effet de l'agriculture sur la persistance des populations. Pour comprendre cet effet, il est essentiel d'avoir une approche intégrée combinant un panel de marqueurs larges et agissant à différentes échelles temporelles et spatiales (écologie du paysage, génétique des populations, comportement, traits d'histoire de vie). Ainsi, ces différents marqueurs vont permettre de caractériser l'habitat agricole, son utilisation par les espèces qui y vivent, et son effet sur les populations de manière globale (en prenant en compte tous les facteurs de pression simultanément, **Figure 6**).

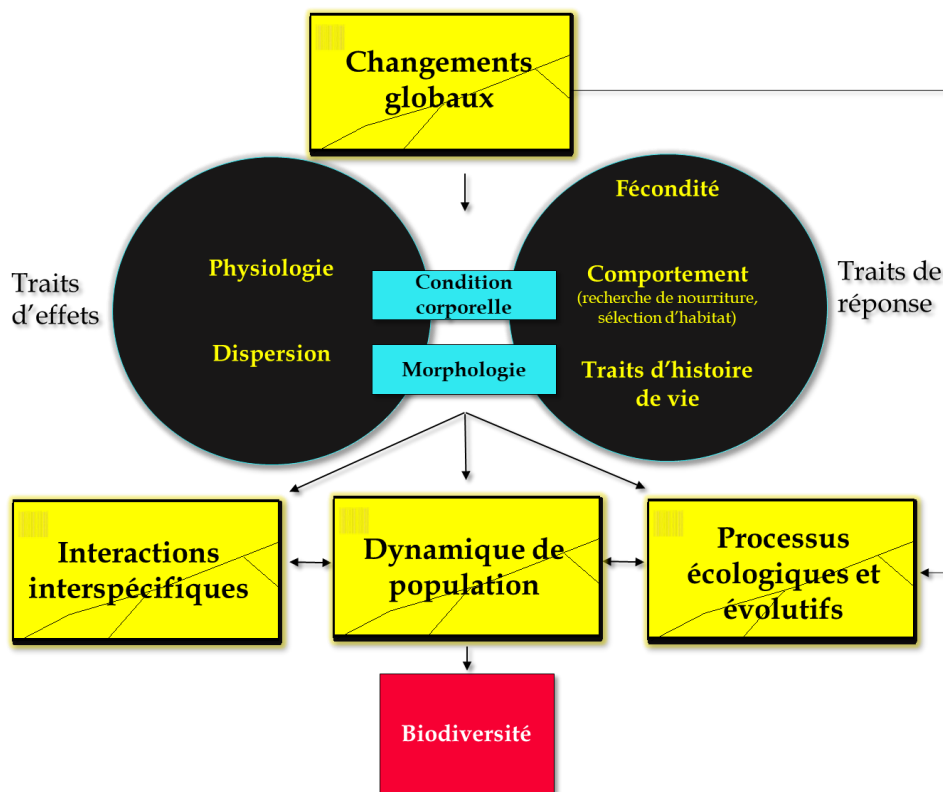


Figure 6. Voies reliant les changements environnementaux aux altérations des traits fonctionnels qui influencent finalement la biodiversité. Les modifications de l'habitat induites par l'homme s'ajoutent aux variations naturelles de l'environnement pour influencer les traits fonctionnels tels que les systèmes de sensoriels, la physiologie et la dispersion. Les changements dans les traits de réponse, tels que la fécondité, la dispersion et une variété de comportements (par exemple, la recherche de nourriture, la sélection de l'habitat), peuvent servir d'indicateurs précoces de perturbation environnementale. Les traits d'effet et de réponse sont déterminés par la condition corporelle, la taille et la morphologie d'un individu. Les changements de traits ont des effets en chaîne sur les interactions entre espèces, la dynamique des populations et les processus écologiques et évolutifs, qui influencent tous la biodiversité. Les processus écologiques et évolutifs influencent également la fonction des écosystèmes en provoquant des changements dans les taux de croissance, la productivité et les interactions trophiques. Adaptée de Kelley et al., (2018).

1. Flux génique et connectivité des populations

Pour évaluer les effets des pratiques agricoles sur la faune sauvage, il est nécessaire de déterminer si la structure de ces habitats peut influencer la structure de la population à large échelle. Avec la conversion des terres en terres arables, les habitats disparaissent ainsi que leur connectivité spatiale (Benton et al., 2003). Par conséquent, les différentes pressions anthropiques en lien avec l'agriculture moderne (fragmentation et homogénéisation du paysage) affectent la démographie et la génétique de la biodiversité (McKinney and Lockwood, 1999; Dixo et al., 2009). La diminution de la taille des populations (e.g. par la diminution de la taille de l'habitat) couplée à la baisse des déplacements (dispersion, migration) entre les populations (e.g. fragmentation, Brown and Kodric-Brown, 1977) peuvent avoir un impact sur le flux génétique responsable du maintien de la diversité génétique, des taux de consanguinité et de la dérive génétique (Lenormand, 2002; Beebee, 2005; Epps et al., 2005). Dans ce contexte la valeur adaptative des espèces est négativement impactée et le taux d'extinction est plus conséquent (Bonte and Bafort, 2019). Il est donc crucial de mieux comprendre comment les populations sauvages sont affectées et répondent aux perturbations des agroécosystèmes. Pour ce faire, la génétique du paysage est particulièrement adaptée. En combinant la génétique des populations, l'écologie du paysage et les statistiques spatiales, elle permet de décrire l'influence des structures paysagères sur la structuration spatiale de la variabilité génétique (Cosson, 2006) en reliant directement les caractéristiques du paysage à la structure des populations, à la diversité génétique et aux flux génétiques (Storfer et al., 2007; Manel and Holderegger, 2013). Finalement, il devient possible de déterminer si les populations sont récentes et adaptées à leur environnement.

2. Dynamique des populations

Les modifications de l'environnement ont lieu depuis de nombreuses années mais elles continuent de s'intensifier dans le temps et dans l'espace (Venter et al., 2016; Jung et al., 2019; Kadoya et al., 2022; Li et al., 2022). Ainsi, il est nécessaire de prendre en compte l'échelle temporelle et spatiale des conséquences anthropiques sur l'évolution du cortège d'espèces, et sur le maintien des différents organismes vivant dans ces milieux modifiés (Turner, 1989). Les effets rapides de ces modifications actuelles et les effets retardés liés aux modifications passées influencent toujours les assemblages d'espèces et la dynamique des populations d'aujourd'hui (Jung et al., 2019). Les modifications de ces assemblages et de ces dynamiques sont à la fois temporelles et spatiales, ce qui rend compliqué leur appréhension. Pourtant, elles peuvent être approchées par des suivis des populations qui combinent ces deux approches (Baillie, 1990; Witmer and Witmer, 2005; Marsh and Trenham, 2008; Mills, 2012). Ils s'articulent sur l'acquisition de données répétées et standardisées en s'appuyant sur des paramètres démographiques et géographiques comme l'occurrence, les effectifs, les taux de mortalités ou encore l'immigration au fil des générations (Baillie, 1990; Witmer and Witmer, 2005; Marsh and Trenham, 2008; Mills, 2012). Les résultats découlant de ces suivis permettent de mieux suivre l'évolution de l'état de conservation des espèces, mais aussi de possiblement améliorer la gestion de leur habitat (Baillie, 1990; Witmer and Witmer, 2005; Marsh and Trenham, 2008; Mills, 2012). En étudiant en parallèle leur écologie, il est possible de caractériser l'état de santé des populations, leur capacité d'adaptation, de résilience mais aussi de déterminer les menaces que les contraintes anthropiques font peser sur leur persistance et notamment lorsque les populations d'une même espèce se répartissent le long d'un gradient d'habitat (e.g. un gradient d'altération de l'habitat).

3. Réponses individuelles aux changements d'habitats

Pour la plupart des espèces, la biologie de la reproduction est perturbée par de nombreuses pressions anthropiques (Moore and Waring, 2001; Rhind, 2009; Seress and Liker, 2015). Or, évaluer les déterminants de la fécondité et de la qualité de la progéniture (e.g. succès reproducteur) est un aspect essentiel de l'écologie évolutive (Pianka, 2011). Le succès reproducteur est souvent dépendant de nombreux paramètres agissant en concomitance, et incluant les traits parentaux mais aussi les conditions environnementales lors d'un événement reproducteur (Kölliker et al., 2014; Ratikainen et al., 2018). De ce fait, le succès de la reproduction est dépendant de la qualité des parents (Kölliker et al., 2014; Ratikainen et al., 2018) et repose sur des interactions complexes entre physiologie, écologie et comportement, tout au long de la vie de l'individu et de l'évènement reproductif (Moczek, 1998; Bradshaw and McMahon, 2008; Cauchard et al., 2013). Les soins parentaux sont, par exemple, des comportements permettant de maximiser la survie de la descendance et donc la transmission des gènes des parents (Uller, 2008). Chez les poissons, il a été montré, que les soins parentaux et la taille des œufs sont positivement corrélés entre eux (Sargent et al., 1987). La taille de l'œuf va déterminer la taille initiale du juvénile, et les juvéniles plus gros ont un taux de mortalité plus faible (Sargent et al., 1987). Chez les oiseaux, la qualité des soins parentaux (choix du site de ponte, incubation, nourrissage des jeunes...) va aussi influencer la qualité et donc le taux de survie de la descendance (Krist, 2011). L'investissement dans la progéniture (qualité de la descendance) dépend aussi de la qualité des parents (e.g. phénotype et génotype) et va influencer la qualité de la descendance (Uller, 2008). Finalement, certains marqueurs physiologiques et/ou moléculaires peuvent nous renseigner sur la qualité parentale et sur la qualité de la progéniture. C'est par exemple le cas des télomères. Ces régions d'ADN non codantes en bout de chromosome sont utilisées comme outil moléculaire afin d'évaluer la qualité individuelle (Angelier et al., 2019; Eastwood et al., 2019). Les télomères renseignent en effet sur les réponses phénotypiques et physiologiques aux conditions environnementales et sur la fitness des individus (Heidinger et al., 2012; Herborn et al., 2014; Wilbourn et al., 2018). Les télomères longs sont associés à une meilleure survie/fitness et les stress environnementaux sont responsables du raccourcissement des télomères (Angelier et al. 2018, Chatelain et al. 2020). Les organismes vivants dans les milieux modifiés sont impactés négativement aux différents stades de leur vie (œufs/embryons, juvéniles et adultes; Crozier et al., 2008). La reproduction, et notamment le succès reproducteur, contrebalance avec les taux de mortalités et joue un rôle essentiel dans le maintien des populations, que ce soit au travers des densités d'individus ou de la diversité génétique des populations (Pulliam, 1988; Ellegren and Galtier, 2016). Etudier les déterminants du succès de la reproduction, la qualité des individus et la qualité de la descendance selon un gradient d'habitat contrasté (habitat naturel/favorable à l'espèce indigène vs habitat modifié/défavorable) permet d'obtenir de nombreux indices sur la capacité des organismes à se maintenir dans un paysage de plus en plus anthropisé et modifié, comme c'est le cas des agroécosystèmes.

V/ Les amphibiens : un taxon bio-indicateur

Avec un taux d'extinction 200 fois supérieurs aux taux historiques, les amphibiens sont le taxon le plus menacé et en déclin rapide (Roelants et al., 2007; Hoffmann, 2008; Hoffmann et al., 2010). Les causes du déclin des amphibiens sont complexes et multiples (Figure 7). Elles peuvent être globales, du fait des changements climatiques, des espèces invasives et de la propagation des maladies infectieuses émergentes (Daszak et al., 2001; Stuart et al., 2004; Alan Pounds et al., 2006; Sodhi et al., 2008; Todd et al., 2011; Egea-Serrano et al., 2012, Figure 7); ou locales avec la perte et la dégradation de l'habitat, les perturbations des micro-habitats et la pollution, telle que la contamination par les pesticides en milieu agricole (Hopkins, 2007; Alford, 2010; Blaustein et al., 2011, Figure 7). Ainsi, l'agriculture moderne est de plus en plus impliquée dans le déclin des amphibiens et ses conséquences diffèrent selon les espèces et leur sensibilité, leur mode de vie et le stade de développement considéré (Greulich and Pflugmacher, 2003; Blaustein et al., 2011; Brühl et al., 2011). Par exemple, aux stades aquatiques, les amphibiens des zones agricoles sont affectés par les mélanges de pesticides présents dans les plans d'eaux de reproduction (Mann et al., 2009). Pour la plupart des espèces d'amphibiens des régions tempérées (e.g. reproducteurs annuels itéropares), la reproduction et le développement larvaire coïncident avec l'épandage des pesticides et engrais chimiques et les impacts des facteurs combinés (intrants chimiques divers) sur ces organismes peuvent être importants (Mann et al., 2009).

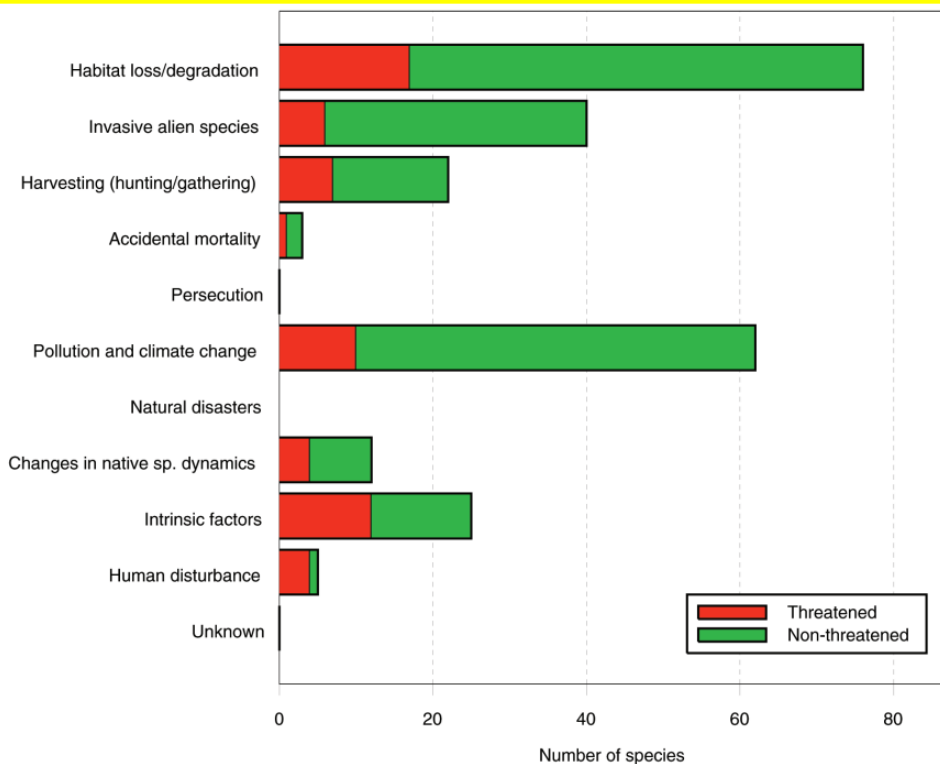


Figure 7. Principales menaces pesant sur les amphibiens en Europe. Issue de IUCN European Red list of Amphibians.

1. Cycle de vie et caractéristiques des amphibiens

Les amphibiens ont un cycle de vie biphasique (du grec *amphi*, « des deux côtés », et *bios*, « vie ») signifiant que ces animaux peuvent vivre en milieu aquatique comme en milieu terrestre. Chez ce taxon, la radiation adaptative est remarquable et, parmi les vertébrés, c'est le groupe ayant la plus grande diversité de modes de vie (Duellman and Trueb, 1994). Ces organismes sont inféodés aux milieux d'eau douce (e.g. étang, mares) où ils pondent leurs œufs (à la membrane perméable), qui se développent au cours d'une phase embryonnaire et larvaire aquatique comprenant plusieurs stades de développement (Gosner, 1960) jusqu'à la métamorphose (qui constitue en un changement de mode de vie, du milieu aquatique vers le milieu terrestre). Finalement, le stade adulte réside en milieu aquatique ou terrestre avec une dépendance aux milieux humides. Pour la grande majorité des amphibiens, le cycle de vie consiste en une alternance de déplacement entre milieux terrestre (milieux de vie) et milieux aquatiques pour la reproduction (recherche de partenaire, ponte d'œufs). La classe des amphibiens est constituée de trois ordres : les Gymnophiones (e.g. cécilies), les urodèles (e.g. salamandres, tritons) et les anoures (e.g. grenouilles et crapauds). Contrairement aux urodèles ayant une queue visible au stade adulte, les anoures en sont dépourvus. Malgré une grande diversité biologique au sein de ce taxon, certaines caractéristiques physiologiques sont communes aux amphibiens : (1) ils sont ectothermes et sont donc soumis aux contraintes environnementales (e.g. température). (2) Ils sont dépendants de l'eau pour se reproduire (e.g. milieux aquatiques) afin d'éviter la dessiccation des œufs (Hoffmann et al., 2010). (3) Finalement, l'une de leur caractéristique principale est leur peau perméable par laquelle ils réalisent des échanges gazeux et liquide avec leur environnement externe (e.g. respiration).

2. Déclin des amphibiens et répercussions

Les amphibiens constituent l'un des taxons les plus touchés par la crise de la biodiversité, dont 32% des espèces sont menacées, et 43% sont en déclin, d'après l'union internationale de pour la conservation de la nature (UICN, Rodrigues et al., 2006; Monastersky, 2014, **Figure 8**). Cinq menaces majeures pour les amphibiens ont été identifiées au cours des 20 dernières années : les changements climatiques, à l'échelle mondiale ou locale, la modification de l'habitat (e.g. perte, fragmentation, homogénéisation), les contaminants environnementaux, les espèces exotiques envahissantes, et les maladies infectieuses émergentes. C'est par exemple le cas du champignon chytride (*Batrachochytrium dendrobatidis*), qui touche principalement les populations de poisson et d'amphibiens, avec des conséquences fatales (Longcore et al., 1999; Weldon et al., 2004; Garner et al., 2005; Fisher and Garner, 2020). Il est aujourd'hui considéré comme une cause majeure du déclin et de l'extinction des espèces d'amphibiens à travers le monde (Briggs et al., 2010; O'Hanlon et al., 2018; Fisher and Garner, 2020). Ce déclin va avoir des conséquences en chaîne, les amphibiens étant un maillon essentiel de la chaîne alimentaire, à la fois proies et prédateurs. Ils régulent les maillons inférieurs de la chaîne trophique et permettent d'alimenter les maillons supérieurs de la chaîne trophique. Par ailleurs, les têtards, présents en quantité importante (Kaplan, 1987; Newman, 1987; Kaplan, 1992; Hagman and Shine, 2008), se nourrissent d'algues, et peuvent réduire l'eutrophisation des milieux aquatiques (Personius, 2017). Ainsi, leur déclin peut entraîner des répercussions secondaires sur l'ensemble des écosystèmes.

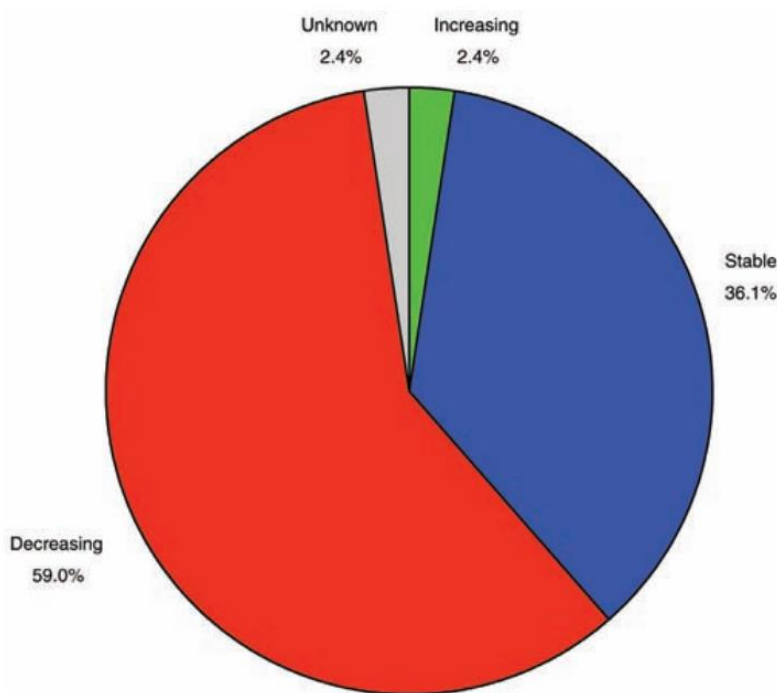


Figure 8. Tendances des populations d'amphibiens européens. Plus de la moitié (59%) des amphibiens européens voient leur population décliner, 36% sont stables, et seulement 2% sont en augmentation. Issue de IUCN European Red list of Amphibians.

3. Déclin des amphibiens en milieu agricole

Avec des besoins multiples et complexes en termes d'habitat, les amphibiens sont particulièrement sensibles à la modification de leur milieu (Gallant et al., 2007; Erwin, 2008). L'intensification de l'agriculture moderne est à l'origine de nombreux facteurs contribuant au déclin des amphibiens (e.g. perte, altération, modification et fragmentation de l'habitat), et les zones agricoles occupent actuellement 40% de la surface terrestre (FAO, 2009; Blaustein and Kiesecker, 2002; Brühl et al., 2013; Feillet, 2014). Ces différents facteurs ont contribué à une perte considérable des milieux humides, essentiels à la reproduction et au développement embryonnaire et larvaire des amphibiens (Gibbs, 2000; Tilman et al., 2001; Ficetola et al., 2015). Par conséquent, les populations d'amphibiens déclinent en raison de la diminution de la taille des populations et des pertes de flux génique et de connectivité entre les populations (e.g. dispersion, migration, colonisation) (Brown and Kodric-Brown, 1977; McKinney and Lockwood, 1999; Dixo et al., 2009; Allentoft and O'Brien, 2010). En plus de la modification structurelle de l'habitat, ce taxon est soumis à des modifications chimiques et toxiques avec des conséquences désastreuses en lien avec la particularité de leur peau (perméabilité cutanée). Dans les écosystèmes agricoles, de nombreux intrants chimiques (e.g. fertilisants, pesticides), pouvant avoir des interactions encore inconnues, sont appliqués en grande quantité chaque année (Mann et al., 2009; Relyea, 2009). En plus des effets directs sur ces organismes (e.g. milieu terrestre) (Berger et al., 2013), le ruissellement et la pulvérisation accidentelle entraînent des effets indirects dans les zones de reproduction de ce taxon (e.g. étangs et mares) (Giesy et al., 2000; Solomon and Thompson, 2003).

4. Les amphibiens comme bio-indicateurs de leur milieu

Les effets des stress environnementaux sont repérables en premier lieu au niveau populationnel, et touchent principalement les espèces les plus sensibles. Ces espèces sont ainsi indicatrices de la santé de l'environnement dans lequel elles vivent (Odum, 1996; Niemi and McDonald, 2004). Leur étude permet d'identifier les signes précurseurs des perturbations de l'environnement, et donc d'identifier un dysfonctionnement de l'écosystème. En raison de leur cycle de vie-biphasique, de leurs adaptations physiologiques spécialisées et de leurs besoins spécifiques en matière de micro-habitats, les amphibiens sont considérés comme un indicateur naturel de la santé global, à la fois pour les environnements terrestres et les environnements aquatiques (Blaustein, 1994; Blaustein et al., 1994). En outre, leur dépendance aux deux habitats (aquatique et terrestre) et leur perméabilité cutanée rendent les amphibiens sensibles aux modifications anthropiques (Wells, 2010). Les stades lors de l'embryogenèse et de l'ontogenèse (fécondation et développement externe) sont particulièrement sensibles à une exposition directe aux polluants environnementaux (milieu aquatique) (Feil and Fraga, 2012). De plus, au cours de leur stade aquatique, les larves d'amphibiens sont spécialisées dans l'utilisation du micro-habitat (recherche de nourriture, prédation) (Whiles et al., 2006). Cette spécialisation rend les larves d'amphibiens hautement sensibles aux moindres changements environnementaux (même mineurs) qui altèrent leur capacité à se protéger des prédateurs mais aussi leur comportement de recherche alimentaire (Whiles et al., 2006). Leur réaction précoce au stress et à la dégradation de l'environnement en font un taxon bio-indicateur. Finalement, la manifestation de symptômes comportementaux et physiologiques (bio-marqueurs), mais aussi la présence et l'absence de ces organismes dans les milieux permet d'obtenir des informations, qualitatives comme quantitatives, sur la qualité de l'environnement (Niemi and McDonald, 2004).

VI/ Hypothèses et objectifs de la thèse

1. Objectif global de la thèse

Les activités anthropiques sont considérées comme le principal moteur de la perte de biodiversité actuelle (Chapin III et al., 2000; Myers and Knoll, 2001; Brooks et al., 2002). De fait, les modifications des habitats peuvent influencer les traits d'histoire de vie des organismes qui les utilisent. En particulier, il a été démontré que les pratiques agricoles modernes influencent négativement la biodiversité, que ce soit de manière directe ou indirecte (Fahrig, 2003; Firbank et al., 2008; Köhler and Triebkorn, 2013). La simplification et l'homogénéisation du paysage, la fragmentation de l'habitat et la contamination par des produits phytosanitaires peuvent avoir des conséquences importantes sur la structure du paysage et donc sur l'écologie et la physiologie de la faune sauvage, et impacter négativement la persistance des populations qui exploitent ces habitats (Gibbs, 2000; Fahrig, 2003; Wiegand et al., 2005; Firbank et al., 2008; Dixo et al., 2009; Gámez-Virués et al., 2015).

Dans ce contexte, ma thèse s'intéresse à la manière dont l'environnement agricole peut influencer l'écologie et la physiologie de la faune sauvage. Les amphibiens sont particulièrement adaptés pour explorer cette thématique, ce taxon étant sensible aux contraintes environnementales et à l'ensemble des pressions citées précédemment (Niemi and McDonald, 2004; Wells, 2010). De plus, ces 40 ans dernières années ont été marquées par un déclin significatif des populations d'amphibiens (Roelants et al., 2007; Hoffmann, 2008; Hoffmann et al., 2010; Egea-Serrano et al., 2012). S'ajoute à cela que ce sont des organismes avec une mobilité réduite et donc l'état des populations d'amphibiens renseigne sur les pressions locales de l'environnement dans lequel ces espèces vivent.

2. Pertinence de *Bufo spinosus* dans l'étude

En l'occurrence, nous avons travaillé spécifiquement sur le crapaud épineux (*Bufo spinosus*). L'intérêt de cette espèce réside dans son écologie. En effet, le crapaud épineux vit et se reproduit dans une large variété de milieu, que ce soit lors de sa phase terrestre ou de sa phase aquatique. Ainsi, on retrouve l'espèce sur un gradient de milieux allant des milieux conservés -comme les milieux forestiers- jusqu'à des milieux fortement dégradés -comme les milieux agricoles. C'est une espèce répandue avec un cycle de vie biphasique, et est donc soumise à des contraintes agricoles en zones terrestres à l'âge adulte comme aquatiques lors de l'ontogenèse et de la période de reproduction. Afin de mieux comprendre la diversité des effets des habitats agricoles sur les populations d'amphibiens, les études composant cette thèse s'orientent en 3 axes et se concentrent sur un gradient de 23 sites allant de fortement forestiers à fortement dégradés.

3. Objectifs détaillés de la thèse

Tout d'abord, et afin d'évaluer les effets des pratiques agricoles sur la faune sauvage, il est nécessaire de déterminer si la structure de ces habitats peut influencer la structure de la population à large échelle (**Figure 9**). Pour ce faire, nous avons utilisé la génétique des populations en s'appuyant sur marqueurs génétiques polymorphes (e.g. marqueurs microsatellites, Trujillo et al., 2017) que nous avons relié à des caractéristiques paysagères des différents sites de reproduction (e.g. âge des mares de reproduction et nombre de mares de reproduction périphériques). Les individus peuvent également utiliser différents types d'habitat, et cette utilisation de l'habitat a été étudiée au travers de l'analyse des isotopes stables, qui nous ont permis de déterminer le type d'habitat dans lequel vivent les individus échantillonnés (Renoirt et al., 2021).

Dans un second temps, nous avons cherché à comprendre l'effet des modifications de l'habitat sur les populations de crapaud épineux (**Figure 9**). Pour cela nous avons mis en place un suivi des populations sur les différents sites de reproduction le long d'un gradient paysager (forestier à agricole). Ainsi, les présences-absences, la phénologie, les abondances d'individus, les sex-ratios ont pu être suivies tout au long de la période de reproduction sur les différentes mares, avec l'hypothèse que l'habitat pouvait contraindre la reproduction de ces animaux.

Pour finir, nous avons examiné différents indices de la qualité individuelle et des performances de reproduction d'individus issus d'habitat contrastés (**Figure 9**). Ainsi, nous avons récupéré des amplexus (couples d'individus) sur les différents sites prospectés. En conditions contrôlées, nous avons récolté différents marqueurs de la qualité individuelle et du succès reproducteur des parents, mais aussi de la descendance, que ce soit au stade embryonnaire ou aux différents stades larvaires (Amos et al., 2001; Castellano et al., 2004; Refsnider and Janzen, 2010; Wilson and Nussey, 2010; Kölliker et al., 2014; Ratikainen et al., 2018). De plus, les télomères ont été utilisés comme un proxy de la qualité individuelle, que ce soit chez les adultes comme chez la descendance (Cheron et al., 2021). L'objectif était de tester les effets de l'habitat dans lequel sont retrouvés les adultes sur la reproduction et le développement (effets parentaux pré-nataux en lien avec l'habitat et/ou les phénotypes des parents).

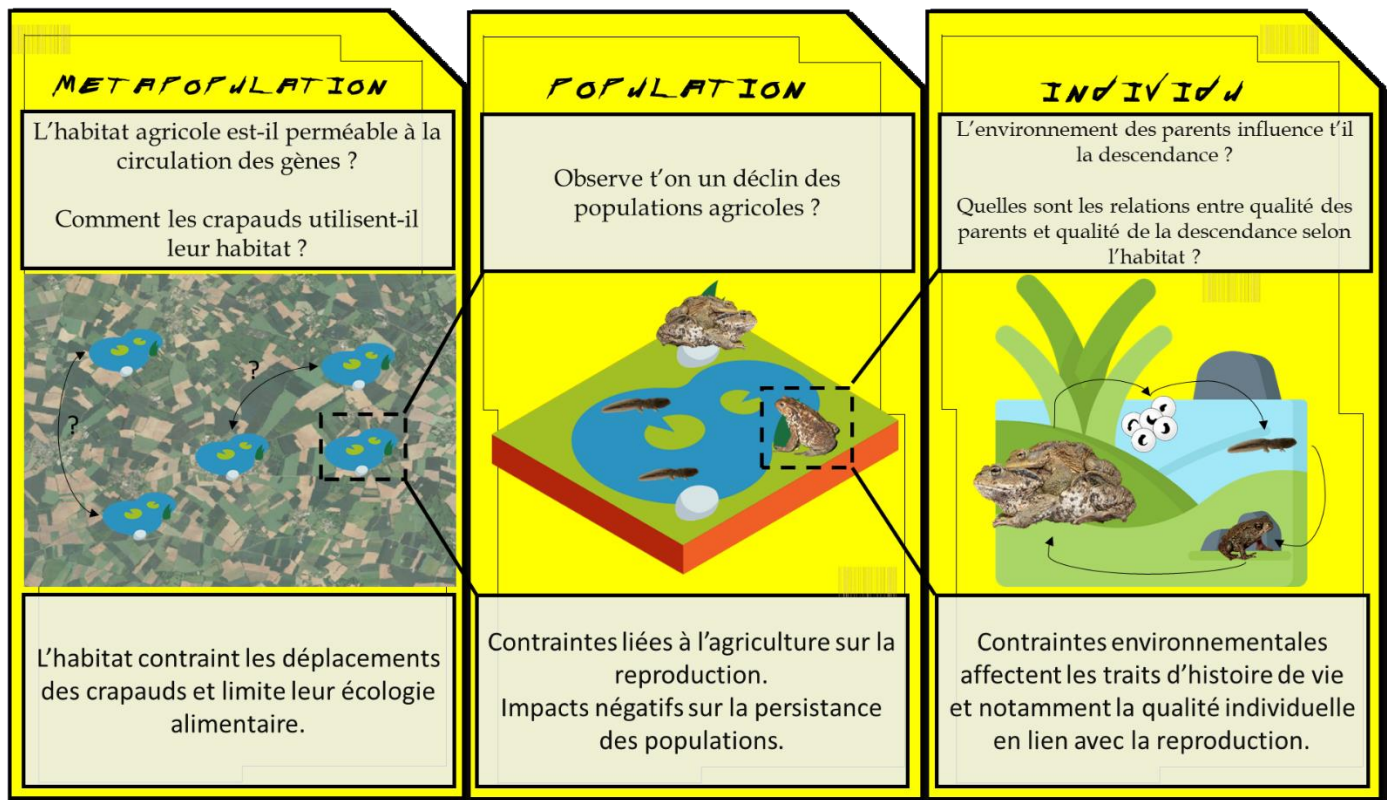


Figure 9. Echelle d'intégration prise en compte dans le cadre de cette thèse reflétant les trois chapitres. Les questions et hypothèses de travail sont illustrées à chaque niveau d'organisation. Le chapitre 1 se concentrera sur une échelle métapopulationnelle avec l'étude du possible flux génique entre mares mais également l'étude de l'écologie alimentaire en fonction de l'habitat. Le chapitre 2 se concentre sur l'étude de la structure de la population en période de reproduction en lien avec le contexte paysager. Enfin, le chapitre 3 est l'étude corrélationnelle au niveau individuel de la reproduction chez le *B.spinosus* en fonction de l'habitat d'origine.

**MATERIEL
ET
METHODES**

I/ Modèle d'étude

1. Le crapaud épineux, *Bufo spinosus*

Bufo spinosus, plus communément appelé crapaud épineux, est un amphibien de l'ordre des anoures (ce qui signifie « sans queue ») et de la famille des Bufonidae. Avant 2012, il était considéré comme une sous-espèce de *Bufo bufo* (crapaud commun actuel) et a été élevé au rang d'espèce à partir de critères morphologiques et phylogénétiques (Recuero et al., 2012, Arntzen et al., 2013 en s'appuyant sur les travaux de Schneider and Sinsch, 2004). De ce fait il se distingue du crapaud commun par une plus grande taille à l'âge adulte, des glandes parotoïdes plus volumineuses et, notamment, par la présence d'un plus grand nombre d'excroissances corporelles s'apparentant à des épines, d'où son nom de crapaud épineux. Ces deux espèces peuvent aussi être différenciées de par leur aire de répartition. Avant 2012, le crapaud commun avait une répartition Eurasiatique allant de la Grande Bretagne jusqu'au Kazakhstan. Cependant, avec l'élévation au rang d'espèce de *Bufo spinosus*, l'aire de répartition a été modifiée et le crapaud épineux est principalement présents dans différents pays du sud de l'Europe (Espagne, Italie, Portugal, Maghreb). En France, le crapaud commun est présent dans la zone Nord-est tandis que le crapaud épineux se situe dans la zone Sud-Ouest délimité par une zone de contact allant de Rouen aux Alpes-Maritimes (**Figure 10**).

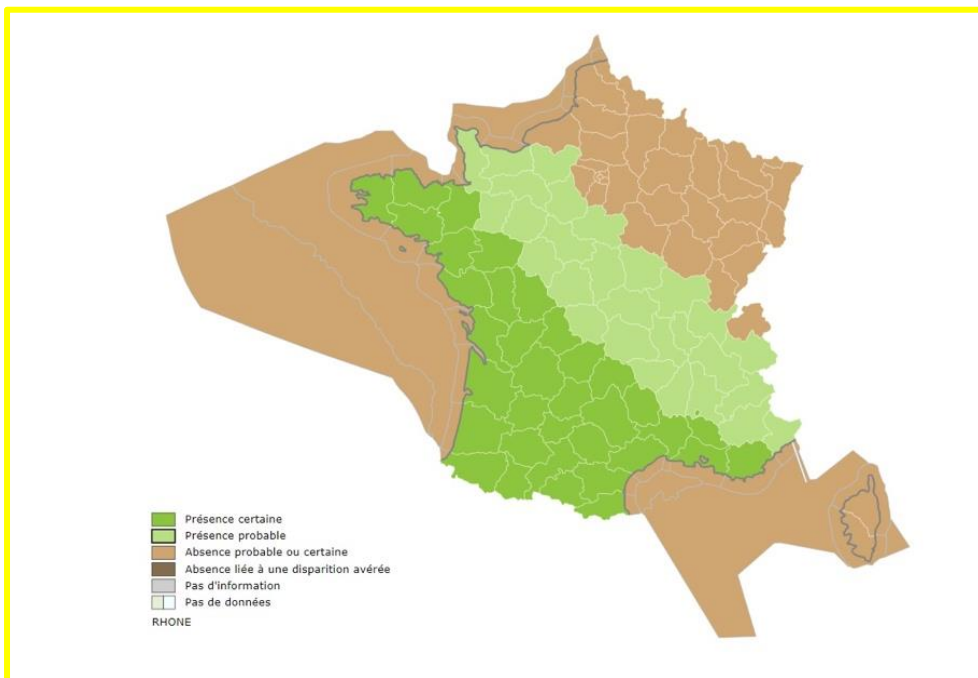


Figure 10. Aire de répartition du crapaud épineux en France. Issue de INPN (Jean-Christophe Massary).

2. Ecologie de *B. spinosus*

Le crapaud épineux est une espèce généraliste terrestre vivant dans un gradient d'habitat allant des milieux conservés comme les milieux forestiers jusqu'à des milieux fortement dégradés comme les milieux agricoles (Guillot et al., 2016). Le crapaud épineux est un méso prédateur nocturne se nourrissant exclusivement d'insectes (insectivore, Guyetan, 1967). Comme la plupart des amphibiens semi-terrestres, ces organismes ont un lien fort aux milieux aquatiques et notamment lors de la reproduction. De plus, les individus montrent une grande fidélité à leur mare de naissance (Reading et al., 1991).

3. Cycle de vie de *B. spinosus*

Ses périodes d'activités vont de mi-janvier à fin septembre, lorsque les températures chutent, il rentre en période d'hivernation (**Figure 11**). Plusieurs phases de migrations sont observées chez le crapaud épineux, une migration pré et post nuptiale et une migration post-métamorphose (Hartel & Demeter, 2015; Kovar et al., 2009). La partie terrestre du cycle annuel des crapauds se déroule dans divers environnements généralement à moins d'un kilomètre du site de reproduction (Janin et al., 2011 ; Guillot et al. 2016). La saison de reproduction a lieu à la fin de l'hiver (février - mars) durant laquelle, les crapauds mâles migrent massivement vers les sites aquatiques de reproduction où ils attendent les femelles (Reading, 1998 ; Kelleher et al., 2018 ; Brischoux & Cheron, 2019, **Figure 11**). Les mâles peuvent rester sur le site de reproduction pendant plusieurs semaines, tandis que les femelles partent peu après l'accouplement et la ponte (Davies & Halliday, 1977). Etant donné que cette espèce est qualifiée d'« explosive breeder » (grand nombre d'individus se regroupant sur une période éphémère lors de la reproduction, Ulloa et al., 2019) il n'est pas rare de la trouver en grand effectif dans ces sites de reproduction. Une fois les deux sexes réunis le mâle s'accroche au dos de la femelle et ils forment un amplexus à la suite de quoi la femelle pond progressivement un chapelet d'œufs (de 2000 à 6000 œufs) qu'elle enroule dans la végétation aquatique de la mare pendant que le mâle fertilise les œufs (reproduction externe). Les œufs se développent ensuite avec de nombreuses phases de multiplication cellulaire (voir Gosner, 1960) pour donner un têtard qui va lui aussi se développer (plusieurs stades de développement, voir Gosner, 1960) jusqu'à la métamorphose et la sortie de l'eau (**Figure 11**).

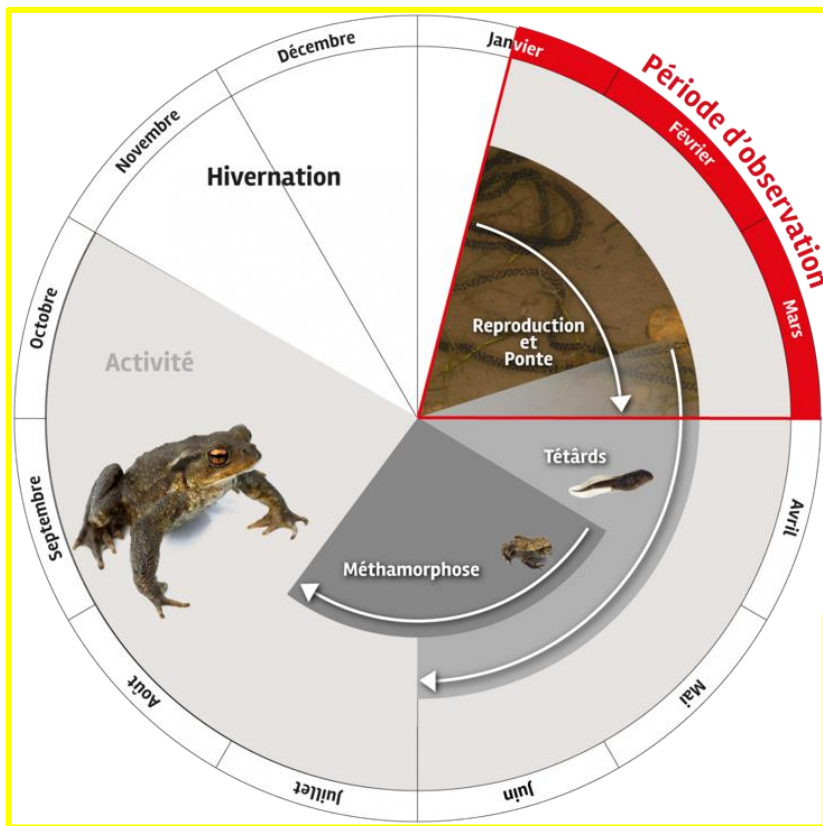


Figure 11. Cycle de vie du crapaud épineux (*B. spinosus*). Issue de Les sentinelles du climat, L'Observatoire.

II/ Echantillonnage et caractérisation de l'habitat

1. Choix des sites de suivis

Les différentes études de cette thèse se sont réalisées dans le Sud des Deux-Sèvres. Les Deux-Sèvres constituent une zone adaptée pour explorer les thématiques de dégradation de l'habitat puisque le paysage est principalement composé de monoculture en continuité avec des paysages conservés comme les forêts et des points d'eaux nécessaires à la reproduction du crapaud épineux. Ce type de paysage permet, ainsi, de sélectionner une grande diversité de sites d'échantillonnages avec des habitats variés, contrastés et entrecoupés de vestiges d'autres habitats. Les échantillonnages ont été effectués sur un gradient de 23 sites, allant de fortement forestiers à fortement dégradés, notamment par l'agriculture intensive. Certains sites ont été utilisés que dans le cadre de certaines années d'études pour des protocoles particuliers (Table 1). Tout au long de la thèse, nous avons cherché à garder un nombre suffisant de sites équitablement répartis selon le type d'habitat pour les comparer.

Sites	Catégorie	Année de prospection	Prélèvement individus	Surface agricole (ha)	Surface forestière (ha)	Chapitre I		Chapitre II		Chapitre II	
						Article I	Article II	Article III	Article IV	Article V	Article VI
Aulnay	Forêt	2019, 2020, 2021, 2022	OUI	0	301						
Labo	Forêt	2019, 2020, 2021, 2022	OUI	13	287						
Prioulet	Forêt	2019, 2020, 2021, 2022	OUI	90	190						
Melle	Agricole	2022	NON	93	5						
Javarzay	Agricole	2019, 2020, 2021, 2022	OUI	157	21						
Génard	Agricole	2022	NON	181	52						
Rimbault	Agricole	2021, 2022	OUI	184	96						
Secondigné	Agricole	2020, 2021, 2022	OUI	209	6						
Pouzou	Agricole	2022	NON	218	10						
Bernegoue 4	Agricole	2021, 2022	NON	229	7						
HL2	Agricole	2022	NON	229	14						
Les fosses	Agricole	2022	NON	234	43						
Brulain	Agricole	2021, 2022	NON	235	3						
Prérault	Agricole	2019, 2020, 2021, 2022	OUI	236	27						
Prissé	Agricole	2019, 2020, 2021, 2022	OUI	238	7						
Bernegoue 3	Agricole	2021, 2022	NON	239	10						
Bernegoue 1	Agricole	2020, 2021, 2022	OUI	243	19						
Bernardière	Agricole	2022	NON	259	1						
Asnière 2	Agricole	2021, 2022	OUI	268	18						
Bernegoue 2a	Agricole	2021, 2022	NON	270	10						
Bernegoue 2b	Agricole	2021, 2022	NON	272	10						
Ensigné	Agricole	2019, 2020, 2021, 2022	OUI	272	14						
Asnière 1	Agricole	2021, 2022	OUI	286	1						

Table 1. Tableau récapitulatif des sites prospectés et de leur utilisation dans les différentes études réalisées tout au long de la thèse.

2. Caractérisation de l'habitat

A l'aide de QGIS 2.18.2, nous avons dessiné des zones tampons autour de chaque site d'échantillonnage (mares). Ces tampons couvraient l'échelle spatiale des distances (rayon de 1km) potentielles parcourues par un individu pendant la migration de reproduction jusqu'à l'étang de reproduction (Janin et al., 2011 et Guillot et al. 2016). Pour chaque tampon, nous avons extrait la surface des champs agricoles et de la forêt. Ces surfaces ont été utilisées pour caractériser davantage le type d'habitat (forestier, mixte ou agricole) en mesurant l'aire agricole, forestière, urbaine, les linéaires de haies et les routes (**Figure 12**).

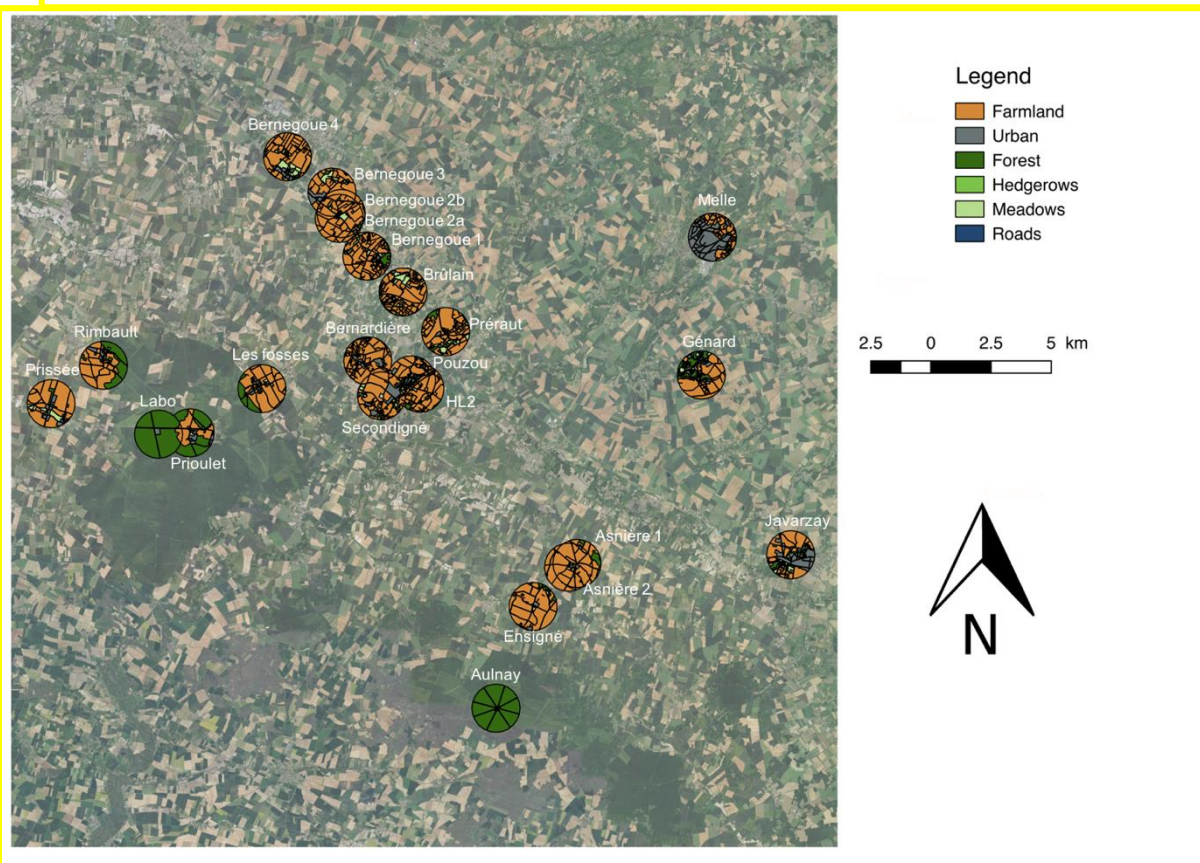


Figure 12. Sites échantillonnés durant la thèse et zone tampon représentative de la distance parcourue par un crapaud des surfaces mesurées (forestières, agricoles, de bâtis, de linéaires de haies).

Dans le cadre de l'étude sur l'abondance des populations (2022, voir **Table 1, 2**). Nous avons extrait la surface des principaux types d'habitats entourant chaque site d'étude : forêts et bois, haies, champs agricoles, prairies et bâtiments (petits villages) à l'aide de QGIS. Nous avons extrait une valeur d'axe issue d'une analyse en composantes principales (ACP) de ces cinq variables pour attribuer un score d'habitat à chaque site. Le PC1 des sites pour lesquels nous avons évalué la présence des crapauds représentait 51,9 % de la variance totale et était positivement corrélé aux champs agricoles ($r=0,82$) et négativement corrélé à la forêt ($r=-0,91$). Le PC1 des sites pour lesquels nous avons évalué l'abondance des crapauds représentait 65,0 % de la variance totale et était positivement corrélé avec les champs agricoles ($r=0,90$) et négativement corrélé avec la forêt ($r=-0,96$).

III/ Utilisation de l'habitat

Durant les premiers mois de cette thèse, j'ai pu bénéficier d'échantillons sanguins d'adultes de crapauds épineux provenant de sites contrastés issus de la thèse de Marion Cheron (**Table 1, 2**). Ces échantillons sanguins nous ont permis d'explorer les thématiques de l'utilisation de l'habitat par ces individus et de valider l'utilisation des isotopes stables comme outil. De plus, cela a permis de nombreuses collaborations externes, notamment avec Paco Bustamante (LIENSS) pour l'isotopie ainsi que Nicolas Bech (Laboratoire EBI) pour les analyses de génétiques des populations

1. Isotopie

Les analyses isotopiques ont été réalisées sur des globules rouges lyophilisés au LIENSs (La Rochelle, France). Des aliquotes de $\sim 0,3$ mg de masse sèche ont été soigneusement emballés et pliés dans des récipients en fer blanc pour permettre les analyses isotopiques avec un spectromètre de masse à flux continu (Thermo Scientific Delta V Advantage) couplé à un analyseur élémentaire (Thermo Scientific Flash EA 1112). Les résultats sont en notation δ par rapport à la bélemnite Vienna PeeDee et au N₂ atmosphérique pour $\delta^{13}\text{C}$ et $\delta^{15}\text{N}$, respectivement. Des normes de laboratoire internes (acétanilide) ont été utilisées pour vérifier la précision. Les erreurs de mesure étaient $< 0,15$ ‰ pour les deux valeurs $\delta^{13}\text{C}$ et $\delta^{15}\text{N}$ (**Table 2**).

2. Génétique des populations

A partir des échantillons sanguins, nous avons réalisé des mesures génétiques des différentes populations (**Table 2**). Pour ce faire, l'extraction d'ADN a été réalisée sur environ 4 μ L de globules rouges à l'aide d'un kit NucleoSpin® Blood (Macherey-Nagel) selon le protocole du fabricant. La qualité et la concentration de l'ADN ont été mesurées avec un Nandrop ND-1000 (Ozyme). Après une validation PCR simplex des marqueurs sur 15 individus, nous avons analysé tous les échantillons d'ADN en utilisant 12 marqueurs moléculaires microsatellites précédemment publiés : Bspi3-02 ; Bspi 3-19 ; Bspi 3-26 ; Bspi 4-14 ; Bspi 4-16 ; Bspi 4-24 ; Bspi 4-27 ; Bspi 4-28 ; Bspi 4-29 ; Bspi 4-30 , Bspi 4-25 et Bspi 3-11(Trujillo, 2017 REF). Nous avons amplifié ces loci dans 4 multiplex. Le multiplex 1 contenait les loci : Bspi 3-11 (6-FAM), Bspi 4-24 (VIC) et Bspi 4-25 (PET) ; le multiplex 2 contenait Bspi 4-16 (6-FAM), Bspi 4-30(VIC), Bspi 4-27(NED) et Bspi 4-28(PET) ; le multiplex 3 contient Bspi 4-29 (VIC), Bspi3-02(NED) et Bspi 3-19 (PET) ; le multiplex 4 contient Bspi 3-26 (6-FAM) et Bspi 4-14 (VIC). Nous avons normalisé la quantité d'ADN à 10ng/ μ L puis réalisé des réactions PCR Multiplex avec les kits Type-it Microsatellite PCR® (Qiagen) dans un volume total de 15 μ l qui comprend 7,5 μ l de Master Mix, 1,2 μ l de Primer Mix avec 0,3 μ M de chaque amorce et 5,3 μ l de RNase-free H₂O. Les réactions multiplex ont consisté en une dénaturalisation initiale (95°C ; 5 minutes), 30 cycles de dénaturalisation (95°C ; 30 secondes), recuit (pendant 90 secondes à différentes températures dans chaque réaction multiplex : les réactions multiplex 1, 2 et 4 avaient une température de recuit de 60°C et la réaction multiplex 3 avait une température de recuit de 58°C) et d'extension (72°C ; 30 secondes), et d'extension finale (60°C ; 30 minutes) Ensuite, le produit PCR a été séparé par électrophorèse sur un séquenceur automatique (ABI PRISM3730) avec le standard de taille GeneScan 500 LIZ (Applied Biosystems) à Genoscreen (Lille, France). La taille des fragments a été déterminée par inspection visuelle à l'aide de Gene-Mapper version 4.0 (Applied Biosystems). Afin de valider cette expérience, 16 individus ont été répliqués (extraits et typés séparément).

Nous avons testé la fiabilité et l'efficacité du panel de marqueurs microsatellites en vérifiant spécifiquement la présence d'allèles nuls, le déséquilibre de liaison ainsi que l'espérance de Hardy-Weinberg et le polymorphisme. Nous avons retenu tous les marqueurs microsatellites pour les analyses.

IV/ Suivi de population

Afin de comprendre l'état des populations de nos différents sites d'études, nous avons commencé un suivi des populations sur plusieurs années qui a commencé au début de cette thèse en 2020, notamment grâce à l'aide de Sabrina Tartu, Marion Cheron, Léa Lorrain-Soligon et Laure Jabaud (stagiaire de Master 2, **Table 2**). Nous avons souhaité au fur et à mesure des années rajouter le plus de sites possibles pour capturer les différences entre habitats avec le plus de précision.

Ainsi, pendant ces différentes périodes de terrain et d'échantillonnages nous avons pu mettre en place un suivi des populations pour la période de reproduction. Sur chaque site monitoré toutes les nuits, nous avons pu compter la présence et l'absence d'individus/de ponte, le nombre de mâles, de femelles et d'amplexus, s'ils sont présents dans la mare ou sur la terre, le nombre de cadavres (prédation). Tout cela en relation avec des mesures climatiques et temporelles comme l'hydrométrie, la température ambiante et la pression atmosphérique. L'heure des visites des différents sites a été randomisée afin d'éviter tout biais en lien avec l'heure de monitoring.

1. Suivi de reproduction

Nous avons évalué la présence de crapauds reproducteurs sur 23 sites (annexe 1). Ces sites ont été suivis pendant 2 à 3 nuits (séparées par 2 à 4 jours) pendant le pic d'abondance des crapauds sur leurs sites de reproduction aquatiques. Les étangs et leurs abords ont été surveillés de nuit (entre 21h et 1h du matin) à l'aide de lampes frontales afin de localiser les individus. Nous avons noté la présence (1 pour la présence et 0 pour l'absence) des individus reproducteurs et si ces individus étaient des mâles (1 pour la présence et 0 pour l'absence) ou des femelles (1 pour la présence et 0 pour l'absence) car le dimorphisme sexuel de cette espèce permet un sexage simple sans capture (Hemelaar, 1988).

2. Abondance

En 2021 et 2022, nous avons évalué l'abondance des crapauds reproducteurs dans 8 sites parmi ceux étudiés pour la présence de crapauds (**Table 1**). Ces sites ont été suivis trois fois par semaine (lundi, mercredi et vendredi) depuis la fin janvier (25 en 2021 et 31 en 2022) avant l'arrivée des premiers individus reproducteurs, jusqu'au départ des derniers individus reproducteurs (9 avril en 2021 et 11 avril en 2022). Lors de ces prospections, les étangs et leurs abords ont été surveillés de nuit (entre 21h et 1h du matin) à l'aide de lampes frontales et le nombre de mâles et de femelles aperçus a été compté. A partir de ces données de comptage nocturne, nous avons extrait les abondances (nombre total d'individus comptés [total, mâles ou femelles], nombre maximal d'individus comptés au cours d'une seule nuit [total, mâles ou femelles] et nombre moyen d'individus comptés pendant toute la saison de reproduction pour chaque site [total, mâles ou femelles]). Ce suivi n'a pas pu être réalisé sur l'ensemble des 23 sites pour des raisons logistiques. Les 8 sites ont été sélectionnés car ils représentent la variété des paysages agricoles que l'on pouvait trouver dans la région (**Figure 13**).

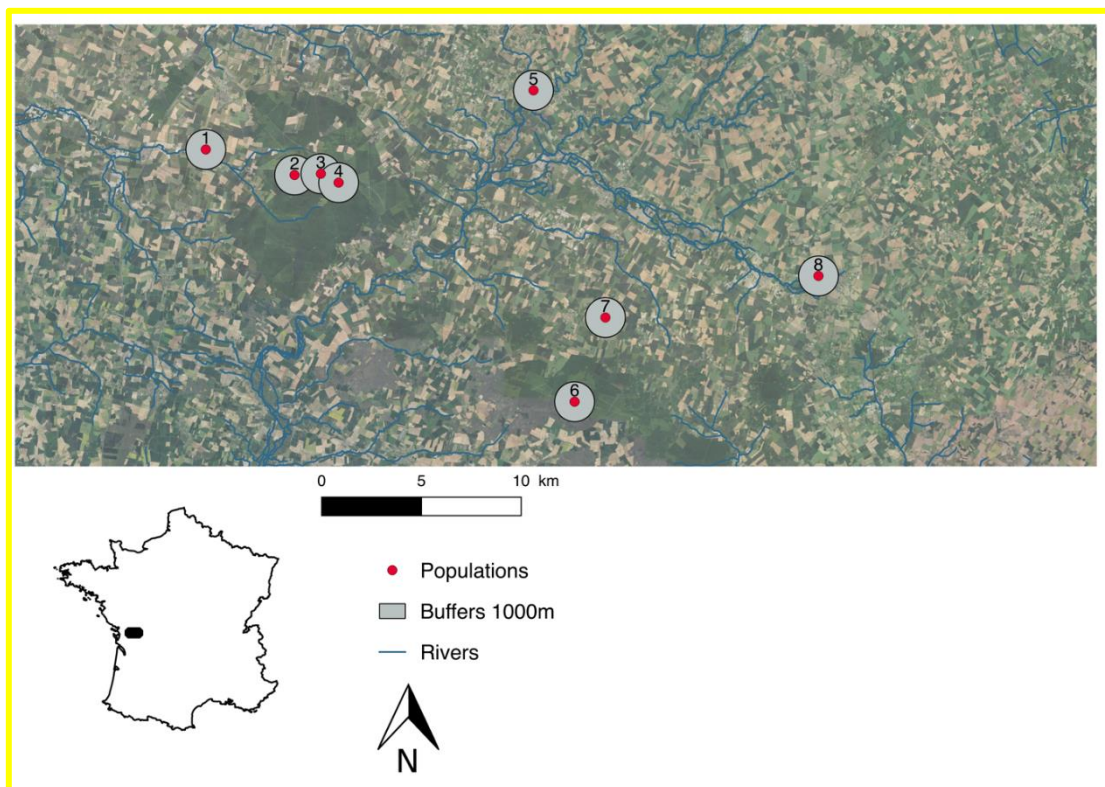


Figure 13. Localisation des sites d'étude et situation générale de la zone d'étude en France. Chaque site est représenté par un point rouge et le cercle gris représente la zone tampon (rayon de 1 km) qui a été utilisée pour évaluer le type d'habitat. Le nombre de sites d'étude est indiqué dans le tableau 1.

V/ Suivi individuel

Le design expérimental de cette thèse a été conduit dans le but de mesurer les relations entre la qualité parentale, la qualité de la descendance et l'habitat d'origine des parents. Ainsi différents marqueurs de la qualité individuelle des parents (phénotypique) ont pu être mis en relation avec leur succès reproducteur et les différents marqueurs de la qualité de la descendance, sur toute la période de reproduction et selon un gradient d'habitats contrastés (Figure 14, Table 2).

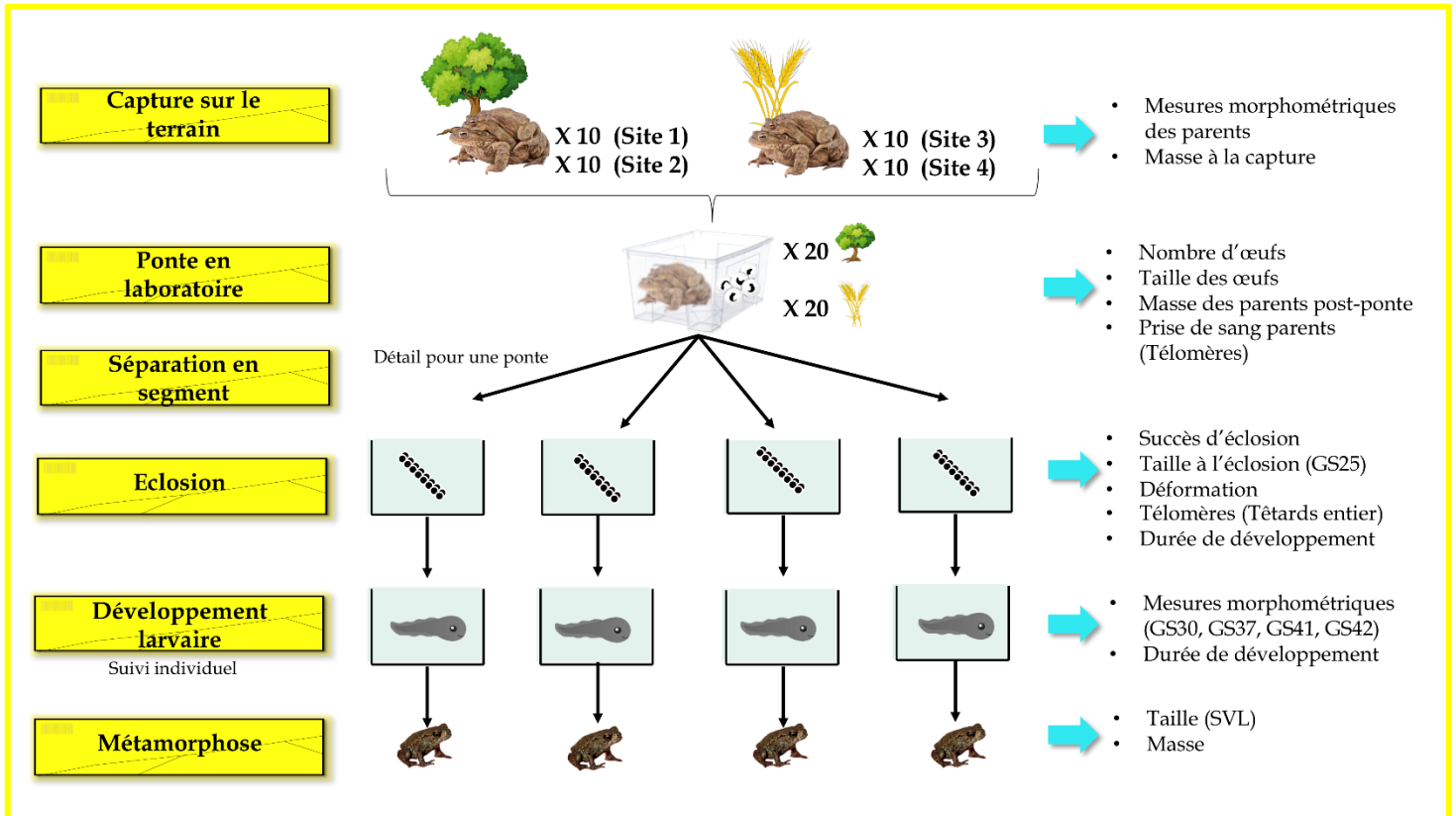


Figure 14. Design expérimental destiné à étudier l'influence de l'habitat d'origine des parents sur les traits d'histoire de vie de la descendance. Les parents sont issus de deux habitats contrastés (Forestier x2 sites et Agricoles x2 sites).

1. Capture et maintenance

L'échantillonnage a eu lieu toutes les nuits sur 3 années consécutives à la même période (mi-janvier à début avril 2020, 2021 et 2022) sur le panel de 23 sites de reproduction situés à proximité du laboratoire (CEBC, 46° 8' 48,64 "N ; 0°25'30,86 "W). Tous les sites ont été suivis avant le début de la saison de reproduction (mi-janvier) jusqu'à après la fin de la saison de reproduction afin de pouvoir déterminer les dates exactes du début et de la fin de la reproduction de cette espèce. Concernant les captures d'individus, elles ont été réalisées de nuit à la lampe frontale et les paires de crapauds (amplexus) ont été capturées à l'aide d'un filet puis ramenés en laboratoire. Cet échantillonnage nous a permis de récupérer les premiers amplexus sur sites. Ceci permet aussi d'éviter la compétition pour l'accès à la reproduction des mâles et d'obtenir le premier mâle sur la femelle.

Une fois les individus récoltés sur les différents sites de reproduction, les amplexus sont par la suite ramenés en laboratoire (boîtes de transport) afin de les faire pondre en milieu contrôlé (**Figure 15**) et aussi de s'assurer de la paternité et d'éviter la multi-paternité (Myers and Zamudio, 2004; Knopp and Merilä, 2009). Des tests préliminaires nous ont permis de constater qu'un amplexus séparé de force, se remet rapidement en amplexus. Ils sont ensuite placés dans des bacs en plastique de 59x36x28 cm dans 30L d'eau (**Figure 15**). Une roche ainsi qu'une branche préalablement stérilisée (eau en ébullition 100°C / 30 minutes) sont placées dans le bac afin de permettre à la femelle de pondre plus confortablement (enrouler la ponte, **Figure 15**). La journée, les bacs sont observés toutes les heures afin de récupérer la ponte le plus tôt possible. Une fois les différentes données récoltées les individus et la descendance (œufs, têtards) sont tous libérés sur leur site de capture ou le site d'origine des parents (pour la descendance).



Figure 15. Couples de crapauds épineux dans les bacs de pontes. La ponte est le chapelet d'œufs sur l'image de droite.

2. Traits et marqueurs parentaux

a. Mesures morphométriques

A la capture, l'amplexus est donc séparé afin de mesurer la masse pré-ponte des deux individus (mâle et femelle). Une fois que la femelle a pondu, différentes mesures morphométriques sont réalisées sur les deux parents à l'aide d'un pied à coulisse (SVL, Taille des pattes, des glandes, largeur des bras et masse post-ponte).

b. Fécondité

Une fois la ponte récupérée, elle est placée dans un aquarium lui-même placé sur une feuille de papier millimétré. De plus, 250ml d'eau sont rajoutés dans l'aquarium afin d'éviter la dessiccation de la ponte. La manipulation de la ponte s'est faite avec soin pour limiter la dégradation des œufs. Cette ponte est par la suite photographiée (CANON EOS 80D – 50 mm) de dessus (**Figure 16**). A l'aide du logiciel image J (Schneider et al., 2012) toutes les photos sont analysées. Ceci nous permet de mesurer la fécondité (taille de la ponte/nombre d'œufs, taille des œufs) en sélectionnant au hasard 5 segments de 10 cm de long pour chaque ponte. Nous avons compté le nombre d'œufs par segment. Le nombre moyen d'œufs par segment de 10 cm a été utilisé pour évaluer la fécondité en l'extrapolant à la taille totale de la ponte.

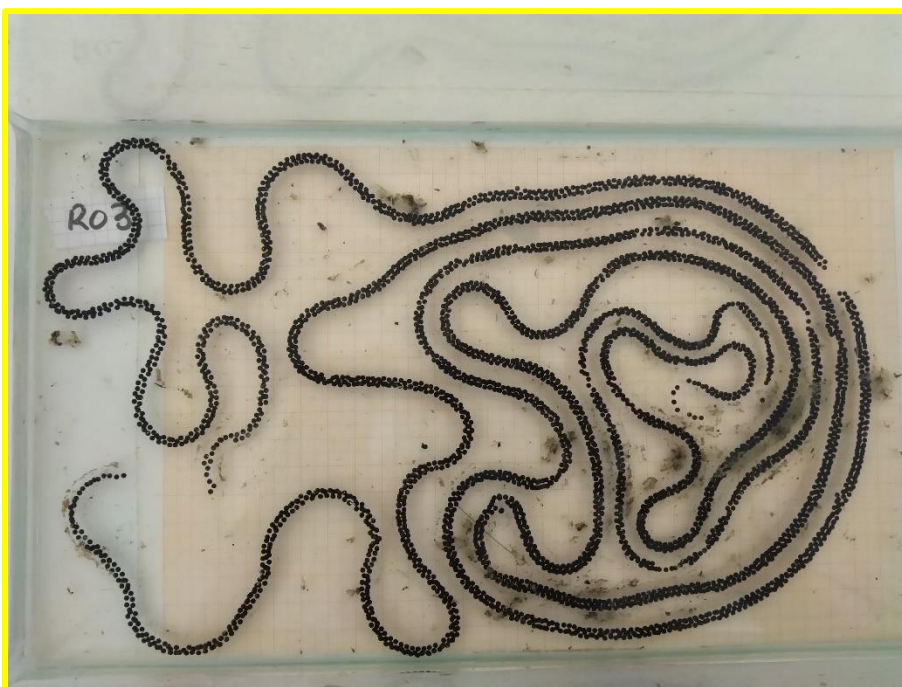


Figure 16. Ponte numéro 3 provenant du site de reproduction « Rimbaut » (Voir Table 1).

c. Télomères

Un échantillon sanguin n'excédant pas 1% de la masse de l'individu est aussi prélevé pour les deux parents post-ponte. La prise de sang est réalisée par cardiocentèse à l'aide d'une seringue de 1 ml et d'une aiguille héparinée de 30 G (Brischoux et al., 2018). Le sang total a été centrifugé pendant 3 minutes et les globules rouges (sang d'origine, séparation du plasma) ont ensuite été conservés à -20°C jusqu'à leur analyse.

Les analyses des télomères adultes ont été mesurées à l'aide d'une technique de PCR quantitative en temps réel (voir : Cawthon, 2002). Ces analyses ont été effectuées au CEBC à l'aide de Cécile Ribout. L'ADN génomique a été extrait de globules rouges préalablement congelés en utilisant le kit DNeasy Blood and Tissue (Qiagen) et en suivant le protocole du fabricant. Pour les échantillons de sang, des amorces télomères universelles ont été utilisées, et le gène témoin à copie unique recombination activating gene 1 (RAG1) a été sélectionné et amplifié à l'aide d'amorces spécifiques conçues pour le crapaud épineux en utilisant la méthodologie d'alignement des séquences : RAG1-F 5'-GGGTCCTGATAGCCGAAA-3' et RAG1-R 5'-CATCATAACCTGTACCCCGGA-3'. Ce gène à copie unique a déjà été utilisé avec succès chez de multiples espèces (oiseaux : Sebastiano et al., 2020 ; poissons : Petitjean et al., 2020 ; reptiles : McLennan et al., 2019), y compris chez les amphibiens (Canestrelli et al., 2021).

La qPCR a été réalisée sur trois plaques pour chaque gène (RAG1 et télomère) en utilisant 7,5 ng d'ADN par réaction. Nous avons utilisé des amorces pour le télomère et le gène de la copie unique à des concentrations de 800 et 300 nM, respectivement. Pour les éclosions, des dilutions sérielles d'ADN provenant d'un échantillon groupé de dix têtards ont été incluses sur la plaque (en triplicata). Cela a permis de contrôler l'efficacité de l'amplification des réactions en générant une courbe standard à six points (50,0 à 1,5 ng). Pour l'amplification des télomères et de RAG1, la spécificité d'amplification de ces amorces a pu être validée en utilisant les courbes de fusion qui ont montré des résultats simples et propres. Pour tenir compte de la variation inter-plaques, un échantillon de crapaud de référence a été analysé en triplicat dans toutes les plaques. Les échantillons ont été assignés au hasard aux plaques PCR et analysés en double. Les valeurs de seuil de cycle (Ct) déterminées pour les duplicatas ont été moyennées, et les échantillons présentant un écart standard de Ct > 0,2 entre les duplicatas ont été répétés.

3. Traits et marqueurs de la descendance

a. Suivi du développement chez les amphibiens

Nous avons utilisé Gosner, (1960) afin de déterminer les stades de développement larvaire. Nous avons utilisé les clefs de détermination morphologique pour sélectionner les stades suivants: stade GS25 (Stade à l'éclosion, rétractation des branchies), stade GS30 (Taille des bourgeons caudaux), stade 37 (Nombre de doigts des pattes postérieures) , stade GS41 (Rétractation du tube ventral), stade 42 (Présence des deux pattes antérieures) et stade GS46 (métamorphose, sortie de l'eau) (voir Gosner, 1960) + stade GS46, 5 jours après la métamorphose afin de palier au poids possiblement biaisé de par le fait que les métamorphes puissent être gorgés d'eau.

b. Développement embryonnaire

Pour chaque ponte, nous avons sous-échantillonné de façon aléatoire 4 segments de 34 œufs (**Figure 17**). Nous avons relâché le reste de la ponte sur le site de capture de leurs parents.

Ensuite, nous les avons transférés et placés dans des aquariums de 2L remplis d'eau de robinet déchlorée. Les aquariums sont placés sur des étagères, dans une pièce à température constante de 17°C. 4 néons reproduisant la lumière du soleil ont été placés au milieu de ces étagères. Les volets de la pièce ont été fermés tout le long de l'expérience. La lumière était allumée de 8h à 18h. Les changements d'eau s'effectuaient 1 fois par semaine.

Nous avons mesuré différents marqueurs pendant le développement embryonnaire/ Tout d'abord, nous avons placé le segment dans une boîte de Pétri avec une échelle millimétrique. Nous avons pris des photos (**Figure 17**) et les avons analysées avec le logiciel image-J (Schneider et al., 2012) pour obtenir la taille des 34 œufs par segment (diamètre).

Lors de l'éclosion, qui se situe entre le stade 22 et 25, nous avons décidé de sélectionner le stade 25 (rétractation des branchies larvaires) comme stade à l'éclosion puisque le caractère morphologique de ce stade est plus facile à discerner. Pour chaque segment, nous avons noté la durée du développement embryonnaire, de la ponte à l'éclosion (stade 25 de Gosner, Gosner, 1960). Le succès d'éclosion (nombre de têtards ayant atteint le stade 25 par rapport au nombre d'embryons non développés) a également été enregistré.



Figure 17. Boîte de Pétri avec 4 segments aléatoires de 34 œufs, de la ponte numéro 9 du site de Aulnay.

c. Développement Larvaire

Sur le nombre de têtards après éclosion, nous avons conservé de manière aléatoire 1 têtard par segment mis en développement individuellement, dans des aquariums (18x13x18cm) et dans 2L d'eau non chlorée. Les têtards sont en développement jusqu'à la métamorphose (développement larvaire) et sont pris en photos aux 6 stades choisis (voir plus haut). En suivant Cheron et al, 2021, nous avons mesuré, pour chaque stade sélectionné, la longueur totale des têtards, la longueur du corps du têtard et la longueur de la queue du têtard. Pour ce faire, nous avons placé les têtards dans des boîtes de Pétri (échelle millimétrique) avec de l'eau provenant de leur propre aquarium. Nous avons pris des photos et analysé les images en prenant des mesures morphométriques à l'aide du logiciel Image-J (Schneider et al., 2012, **Figure 18**).

Lors de la métamorphose (stade 46 de Gosner, Gosner, 1960), nous avons mesuré les crapauds nouvellement métamorphosés (SVL) et pesé et transféré individuellement dans une boîte en plastique (17x15x9cm) avec une serviette en papier humide comme substrat et un abri. Nous avons répété les mesures 5 jours après la métamorphose et relâché les individus sur leur site d'origine.

Nous avons également mesuré le nombre de jours entre les stades de développement ainsi que le développement total. Et enfin, la mortalité des têtards tout au long du développement a été mesurée.

Les têtards sont nourris ad-libitum avec des épinards issus de l'agriculture biologique hachés.

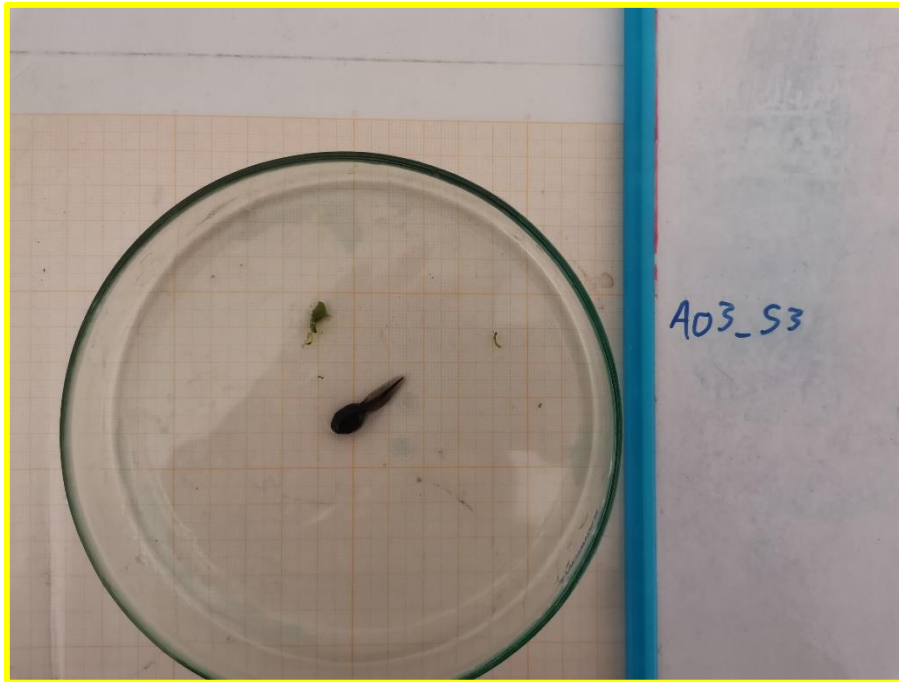


Figure 18. Têtard au GS37 originaire de Aulnay et du segment 3.

d. Analyse des télomères

A GS25 (Gosner Stage 25), nous avons sélectionné au hasard un têtard à l'éclosion par segment et par ponte qui a été euthanasié pour l'analyse des télomères. Nous avons utilisé entièrement les individus sélectionnés et nous avons relâché les individus restants sur le site de capture de leurs parents. Nous avons suivi un protocole précédemment établi (McLennan et al., 2019) pour l'analyse des télomères et adapté aux crapauds épineux (Cheron et al., 2021).

La longueur des télomères des têtards à l'éclosion a pu être déterminée par analyse quantitative (qPCR ; BioRad CFX 96 ; Bio-Rad, USA). Conformément aux instructions du fabricant, les échantillons ont d'abord été digérés avec de la protéinase K et l'ADN a été extrait à l'aide du kit tissulaire Nucleospin (MachereyNagel). Un spectrophotomètre Nanodrop ND1000 (Thermo Scientific) a été utilisé pour évaluer la pureté et la concentration de l'ADN (**Table 2, Figure 19**).

Récapitulatif

Chapitre	Question	Indice	Mesures et prélèvements	Article
I	L'habitat agricole est-il perméable à la circulation des gènes ?	Marqueurs génétiques microsatellites	Sang (cellules sanguines)	Article I : Soumis à <i>Conservation Genetics</i>
	Comment les crapauds utilisent-il leur habitat ?	Isotopes stables ($\delta^{15}N$ & $\delta^{13}C$)		Article II : Renoirt et al., 2020. <i>AGEE</i>
II	Observe t'on un déclin des populations agricoles ?	Présence/ Absence	Suivi de populations/Monitoring	Article III : Renoirt et al., 2021; <i>Herpetological Journal</i>
		Abondance et présence/absence		Article IV : Soumis à <i>AGEE</i>
III	Quelles sont les relations entre qualité des parents et qualité de la descendance selon l'habitat ?	Qualité des parents	Mesures morphométriques, condition corporelle, fécondité	Article IV, Renoirt et al., 2022; Under review in <i>Current Zoology</i> Article V, en préparation
		Longueur des télomères	Sang (cellules sanguines) pour les parents	Article V, en préparation
		Qualité de la descendance	Mesures morphométriques, suivi du développement, survie	Article IV, Renoirt et al., 2022; Under review in <i>Current Zoology</i> Article V, en préparation
		Longueur des télomères	Têtards entier pour la descendance	Article V, en préparation

Table 2. Tableau récapitulatif des mesures utilisées pour chaque étude tout au long de la thèse.

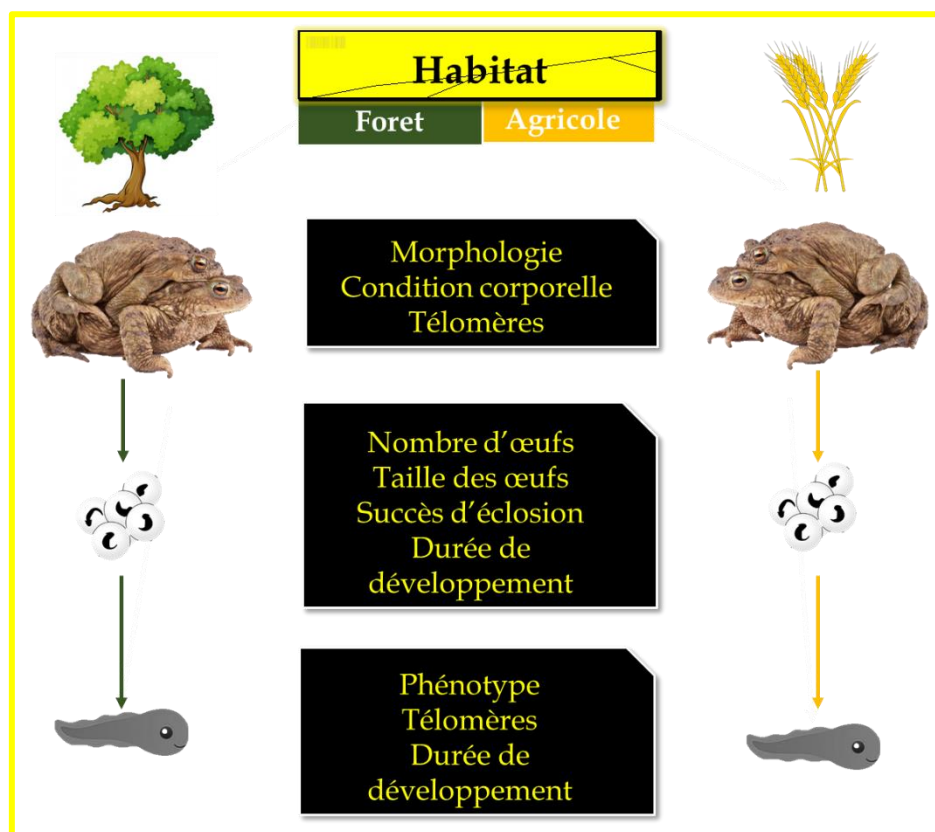


Figure 19. Schéma récapitulatif des mesures récoltées à chaque niveau de vie de l'individu (parents, embryonnaire, larvaire).

CHAPITRE 1

Connectivité et utilisation des habitats
forestiers et agricoles

Ce chapitre s'appuie sur l'article 1 et 2 et se base sur un échantillonnage qui a eu lieu en 2019 sur lequel seuls les mâles ont été échantillonnés.

En lien avec une intensification des pratiques agricoles et une démographie croissante, l'agriculture a plus que doublé entre 1970 et aujourd'hui (FAO, 2009, 2020; Rudel et al., 2009). Cette intensification des pratiques agricoles moderne est le premier facteur de la perte de biodiversité (Chapin et al., 2000; Myers & Knoll, 2001; Brooks et al., 2002) et modifie structurellement le paysage avec par exemple la simplification et la fragmentation de l'environnement et/ou par la contamination environnementale (e.g. intrants chimiques, Myers & Knoll, 2001; Brooks et al., 2002; Fahrig, 2003; Relyea, 2009). De ce fait, les organismes vivant dans ces habitats modifiés et dégradés sont soumis à un vaste panel de facteurs pouvant contraindre leur persistance. Les pratiques de conservation et de gestion efficaces des espèces en déclin et de leurs habitats nécessitent une compréhension des processus naturels et anthropiques à l'origine de la disparition ou de la persistance d'une espèce donnée. Il est donc important d'étudier les interactions entre les espèces, telles que l'écologie alimentaire et l'utilisation de l'habitat, afin d'évaluer leur potentiel en tant que moteurs du déclin des espèces.

Les études d'écologie des populations offrent des exemples marquants des contraintes d'échantillonnage lorsqu'un ensemble d'individus, représentant une petite fraction de la population totale, est échantillonné à partir d'emplacements sélectionnés pour reconstituer des dynamiques écologiques complexes (Moore et McCarthy, 2016 ; Jin et Yang, 2020). Or, les difficultés à suivre les populations d'amphibiens, en particulier *B.spinosus* qui ne présentent pas de motifs spécifiques, rendent difficile la mise en place de suivi démographique. Les difficultés d'application de ces techniques aux amphibiens nous a conduit à l'utilisation de marqueurs isotopiques et génétiques pour comprendre les caractéristiques des populations.

Dans un premier temps, afin d'évaluer de manière exhaustive les impacts des pratiques agricoles sur la biodiversité dans des paysages complexes, c'est-à-dire des paysages entremêlant des habitats agricoles et des vestiges d'autres types d'habitats (micro-habitats : vraisemblablement plus favorables), il est nécessaire d'évaluer dans quelle mesure les habitats agricoles sont effectivement utilisés par une espèce donnée et dans quel type d'habitat vivent les individus étudiés (**Article 1**). Pour cela, nous avons utilisé la signature en isotopes stables de l'azote et du carbone afin de distinguer l'utilisation de l'habitat de *B. spinosus* (**Article 1**). Les signatures isotopiques des tissus ou échantillons sanguins se sont avérées être un outil puissant pour déduire comment les animaux utilisaient différents paysages (par exemple, Marra et al. 1998 ; Hobson 1999 ; Chamberlain et al. 2000 ; Hobson et al. 2001 ; Meehan et al. 2001 ; Rubenstein et al. 2002). En effet, les valeurs $\delta^{13}\text{C}$ des tissus animaux indiquent les sources de carbone alimentaire, ce qui permet de distinguer l'ingestion de production primaire marine ou terrestre, de composants végétaux ou animaux, et de plantes C3 ou C4, entre autres, très présentes dans notre région d'étude. Les valeurs $\delta^{15}\text{N}$ des tissus animaux reflètent en grande partie la position trophique de l'animal, car les valeurs des organismes augmentent de façon prévisible avec l'accroissement des niveaux trophiques et elles peuvent également indiquer les processus azotés régissant la base d'un réseau alimentaire.

Des études impliquant l'étude de signatures isotopiques des proies et de contenus stomacaux ont montré sur la base d'un échantillon de 1158 éléments de proie provenant de 91 crapauds faisant du crapaud épineux un prédateur opportuniste. Ces résultats montrent que le régime alimentaire de *B. spinosus* comprend au moins 42 familles d'invertébrés différentes. Les fourmis (Formicidae) et les coléoptères semblent être les proies les plus importantes dans le régime alimentaire de *B. spinosus* (Vallvé & Sanchez-Iglesias, 2018). Ces proies se nourrissent directement dans le milieu et donc avoir un impact direct sur la signature isotopique des crapauds renseignant sur le régime alimentaire et l'enrichissement potentiel en azote dans le milieu.

Dans un système aussi dynamique, les informations sur les niveaux de connectivité des populations entre des lieux de reproduction et d'hivernage particuliers et la connaissance des schémas de migration sont cruciaux pour comprendre pleinement les limites et les menaces qui pèsent sur les populations d'amphibiens. L'application de marqueurs moléculaires avec des taux de mutation plus élevés, tels que les microsatellites, pourrait améliorer le niveau de compréhension de la population. On sait que les modifications structurelles du paysage peuvent impacter le flux génétique entre les populations, la diversité génétique locale, la consanguinité et la dérive génétique menant à une mise en péril de la persistance des espèces. De ce fait, nous avons cherché à déterminer si la structure de ces habitats peut influencer la structure de la population à large échelle. Pour ce faire, nous avons utilisé la génétique des populations en s'appuyant sur marqueurs génétiques polymorphes (e.g. marqueurs microsatellites, Trujillo et al., 2017). De plus, nous avons relié des caractéristiques paysagères des différents sites de reproduction (e.g. âge des mares de reproduction et nombre de mares de reproduction périphériques) à la structure génétique des populations de crapauds épineux.

II/ Article 1

Stable isotopes of a terrestrial amphibian illustrate fertilizer-related nitrogen enrichment of food webs in agricultural habitats

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Short communication

Stable isotopes of a terrestrial amphibian illustrate fertilizer-related nitrogen enrichment of food webs in agricultural habitats

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To comprehensively assess the impacts of agricultural practices on biodiversity in complex landscapes mixing both agricultural habitats and remnants of other (presumably more favorable) types of habitats, a prerequisite is to evaluate to which extent agricultural habitats are actually used by a given species. Here, we tested whether the stable isotope method can help to discriminate habitat use of a wild vertebrate, the spined toad (*Bufo spinosus*). We expected habitat to influence their $\delta^{13}\text{C}$ values and the use of fertilizers to increase $\delta^{15}\text{N}$ values of individuals from agricultural landscapes. Based on 114 toads from seven sites characterized by contrasted habitats (agricultural, forest or mixed habitats), we found that toad blood $\delta^{15}\text{N}$ values were positively related to agricultural surface area, a result that was corroborated by diverging blood $\delta^{15}\text{N}$ values between habitat categories. Conversely, toad $\delta^{13}\text{C}$ values did not vary according to the habitat. Our results suggest that isotopic values (especially $\delta^{15}\text{N}$) could be a powerful tool to assess agricultural habitat use in terrestrial taxa. Further studies should usefully investigate whether individual $\delta^{15}\text{N}$ values can be used as a fingerprint of other constraints of agricultural habitats (e.g., contaminants) in agricultural landscapes.

Key-words: *Bufo spinosus*, agriculture, amphibians, habitat use, forest, $\delta^{15}\text{N}$, $\delta^{13}\text{C}$.

Anthropogenic activities are considered as the main factors responsible for the current loss of biodiversity (Chapin et al., 2000; Myers & Knoll, 2001; Brooks et al., 2002). Among these anthropogenic changes, modern agricultural practices have been shown to negatively influence biodiversity both directly and indirectly. The direct negative impacts of agriculture on flora and fauna are mainly linked to the destruction and simplification of habitat structures (Fahrig, 2003). Indirect effects are mainly mediated by the increasing reliance on chemical inputs that aim at improving crop productivity (Köhler & Triebkorn, 2013). For instance, the use of large amounts of fertilizers can eventually lead to disruptions of ecosystem functioning (Huang et al., 2017). In addition, the toxic effects of pesticides on non-target components have attracted considerable interest (Köhler & Triebkorn, 2013). Clearly, both direct and indirect effects are expected to affect the species inhabiting agricultural landscapes (McLaughlin & Minneau, 1995; Köhler & Triebkorn, 2013).

In some cases, it is relatively straightforward to assess the consequences of these habitat modifications on the ecology of animal species (e.g., when nesting trees or shrubs are lacking for birds, Mohring et al. 2021). Yet, in most cases, agricultural landscapes will be intersected with remnants of other types of habitats (e.g., small woods, hedgerows) that should allow the persistence of populations. In these cases, assessing the actual use of agricultural habitats *versus* remnant of native habitats is logistically complicated, especially when the species under focus is relatively mobile (i.e., most animal species). Nonetheless, in order to comprehensively assess the consequences of agricultural practices on biodiversity, it is necessary to evaluate to which extent such habitat is actually used by a given species (Street, 2016).

Stable isotopes can provide insights in this respect (Robinson 2001, Rubenstein and Hobson 2004, Perkins et al. 2014, Newton 2016). The concept of the isotopic niche is based on the fact that an animal's chemical composition is influenced by what it consumes (Fry, 2006). Stable nitrogen isotope values ($\delta^{15}\text{N}$) are mostly used as a proxy of trophic position, but can be also a relevant proxy of consumers' foraging habitat (Kelly, 2000). In our context, because fertilizers widely used in agriculture show relatively high $\delta^{15}\text{N}$ (e.g., manure and compost [$\delta^{15}\text{N}$ up to 16.2 ‰], ammonium sulphate [$\delta^{15}\text{N}$ up to 6.6 ‰] and ammonium nitrate [$\delta^{15}\text{N}$ up to 2.2 ‰], Bateman & Kelly, 2007), it is expected that trophic chains influenced by fertilization will be enriched in $\delta^{15}\text{N}$ (Anderson & Cabana, 2005; Bateman & Kelly, 2007). As a consequence, individuals relying on agricultural areas to forage should display higher $\delta^{15}\text{N}$ values than individuals using other types of habitats. In contrast, stable carbon values ($\delta^{13}\text{C}$) vary little along the food chain, and often depend on foraging habitats ($\delta^{13}\text{C}$ source). Typically, $\delta^{13}\text{C}$ varies among specific primary producers (Farquhar et al. 1989), and we can use this parameter to examine differences in trophic support and thus presumably habitats.

In this study, we tested whether blood $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values can help to discriminate the foraging habitats of a wild vertebrate, the spined toad (*Bufo spinosus*). This species is particularly well suited to test these hypotheses for several reasons. First, this widespread amphibian can live in a variety of habitats and persist even in highly modified agricultural areas (Guillot et al., 2016, see also Salazar et al. 2016, Leeb et al. 2020). Second, spined toads forage for invertebrates and its prey spectrum has been shown to be highly conserved between habitats (Zamora-Camacho & Comas, 2017). Third, the terrestrial part of the life cycle occurs within 1 km from the breeding (sampling) sites, which allow a straightforward classification of the surrounding landscapes potentially used in the day-to-day life of individuals (Janin et al., 2011, Guillot et al., 2016). Finally, the remarkably long lifetime of erythrocytes of amphibians (Altland & Brace 1962) indicates that stable isotopes from red blood cells actually reflect habitat use during the terrestrial life of our study species prior to breeding (see also Cloyed et al. 2015). In order to test whether isotopic values of spined toads can discriminate their foraging habitats, we assessed $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ of red blood cells (herein blood) from 114 individuals from seven sites ranging from forested areas to highly agricultural sites.

Material and methods

Study species

In Western Europe, Spined toad (*Bufo spinosus*) is one of the most common species of amphibians. As most anuran species, spined toads have a biphasic life-cycle with an extensive use of terrestrial habitats during most of the year, and a short breeding season (~1 month) in ponds. During breeding, male toads massively migrate towards ponds where they wait for females (Brischoux & Cheron, 2019) and a large number of males can be easily sampled at each pond.

Study sites and sampling

Sampling took place in February 2020 on seven breeding ponds situated in the south of the “Département des Deux-Sèvres” nearby the laboratory (46° 8'48.64"N; 0°25'30.86"W). Two sites were located in highly forested areas, three sites in agricultural landscapes, and the remaining two sites at the interface between forested areas and agricultural areas (Appendix 1). Distances between different sites within a habitat type (e.g., ~18 km between the two forested sites; at least ~12 km between agricultural sites) were large enough to minimize spatial autocorrelation (Janin et al., 2011; Guillot et al. 2016). Such site selection allowed making simple habitat classifications (Table 1). Using QGIS 2.18.2 and satellite images (Google Earth), we drew a buffer around each pond (1000 m radius spanning the spatial scale travelled by toads during the breeding migration, Janin et al., 2011; Guillot et al. 2016) and we extracted the surface area of agricultural fields (Table 1).

We focused our sampling on the first breeding males arriving at each breeding sites. Captures occurred at night using a headlamp to locate individuals. Upon sighting, each toad (total N=114, 3–21 different individuals per sampling sites, Table 1) was captured with a net, and a blood sample was collected (approx. 100 µl) via cardiocentesis using a 1 ml syringe and a 30-G heparinized needle (Brischoux et al., 2018). All individuals were released at their location of capture after blood collection.

Stable isotope analyses

Whole blood was centrifuged, and red blood cells subsequently stored at -20°C until analysis. Isotopic analyses were carried out on freeze-dried red blood cells at the LIENSs (La Rochelle, France). Aliquots of ~0.3 mg dry mass were analysed with a continuous flow mass spectrometer (Thermo Scientific Delta V Advantage) coupled to an elemental analyser (Thermo Scientific Flash EA 1112). Results are in δ notation relative to Vienna PeeDee Belemnite and atmospheric N₂ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively. Internal laboratory standards (acetanilide) were used to check accuracy. Measurement errors were < 0.15 ‰ for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values.

Statistical analyses

All data were tested for homogeneity of variance, residues independence and normality with the Bartlett test, Dubin-Watson test and Shapiro-Wilks test, respectively. We also checked the residues normality using diagnostic plots. All statistical analyses were carried out with R.Studio v 1.2.1335 (R Core Team, 2019). We fitted linear mixed models (LMER, package lmerTest, Kuznetsova et al., 2015) to assess differences in blood $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values across agricultural surface area, with “sites” as a random factor. We analyzed these models with variances analysis (ANOVA). We also analyzed differences in blood $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values between categories (see habitat classification). We followed these analyses by post-hoc tests to performed pair-wise comparisons between categories using Tukey-Kramer tests for unbalanced sample sizes (implemented in the TukeyHSD function).

A site was characterized by very low sample size (N=3, site #6, Table 1, Appendix 1). Excluding this site from our analyses yielded similar results and we present results with the whole dataset below.

Table 1. Summary of the sampling design (habitat categories, number of individuals) and of the corresponding toad blood mean (\pm standard deviation) of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values.

Sites	Categories	n	Agricultural surface area (ha)	$\delta^{15}\text{N}$ (mean \pm sd)	$\delta^{13}\text{C}$ (mean \pm sd)
1	Forest	16	0	0.49 \pm 0.54	-24.79 \pm 0.26
2	Forest	21	15	1.33 \pm 1.61	-23.93 \pm 0.53
3	Mixed	16	54	2.52 \pm 1.83	-23.62 \pm 0.38
4	Mixed	20	104	2.72 \pm 1.70	-24.41 \pm 0.59
5	Agricultural	19	260	3.43 \pm 1.64	-24.35 \pm 0.47
6	Agricultural	3	261	7.28 \pm 0.20	-23.10 \pm 0.76
7	Agricultural	19	286	4.77 \pm 1.86	-24.45 \pm 0.63

Results

Blood $\delta^{15}\text{N}$ values were positively related to agricultural surface area ($F_{1,107} = 16.022$, $p < 0.001$). Blood $\delta^{15}\text{N}$ values were significantly different between habitat types ($F_{2,107} = 6.967$, $p < 0.001$, Fig. 1) with post-hoc tests showing that $\delta^{15}\text{N}$ of toads from forest habitats were significantly lower than $\delta^{15}\text{N}$ of toads from agricultural habitats ($p < 0.001$, Fig. 1), while individuals from mixed habitats were not different from the two other categories (both $p > 0.089$, Fig. 1).

Blood $\delta^{13}\text{C}$ values did not vary according to the agricultural surface area ($F_{1,107} = 0.120$, $p = 0.730$). Accordingly, $\delta^{13}\text{C}$ values were similar between habitat types ($F_{2,107} = 0.201$, $p = 0.81$).

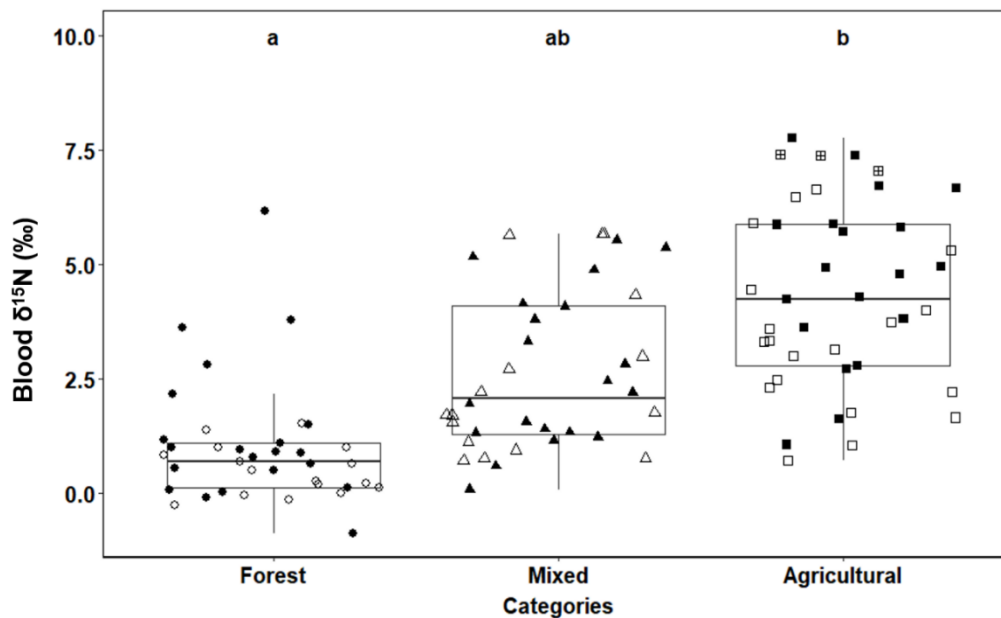


Figure 1. Blood $\delta^{15}\text{N}$ values of toads from forest, mixed and agricultural habitats. The bottom and top of the boxes represent the first and third quartile, the line across the box represents the median, the whiskers represent the minimum and maximum values. The circles, triangles and squares represent individual data points: \circ Site 1, \bullet Site 2, \blacktriangle Site 3, \triangle Site 4, \blacksquare Site 5, \boxtimes Site 6, \square Site 7. Different letters indicate significant differences.

Discussion

Overall, we found that habitat type influences isotopic values of toads. Blood $\delta^{15}\text{N}$ values were positively related to agricultural surface area. These results were corroborated by diverging toad $\delta^{15}\text{N}$ values between habitat categories with lower $\delta^{15}\text{N}$ values found in individuals from forest habitats and higher $\delta^{15}\text{N}$ values found in toads from agricultural areas. Conversely, blood $\delta^{13}\text{C}$ values of toads did not vary according to the habitat.

Toads that breed in ponds surrounded by agricultural environments, and thus likely living in such environments (Guillot et al., 2016, Salazar et al. 2016, Leeb et al. 2020), were characterized by higher blood $\delta^{15}\text{N}$ values than individuals breeding in ponds surrounded by forest. Two different hypotheses could explain such results. First, toads from agricultural landscapes may forage on different food items (higher in the trophic web) than individuals living in forest. This hypothesis seems unlikely as toad diet remains similar amongst habitats (Zamora-Camacho & Comas, 2017). In addition, the remarkably large variation of $\delta^{15}\text{N}$ values between habitat types (-0.9 to 7.9 ‰, Fig. 1), spans approximately 2.1 – 2.7 theoretical trophic levels (DeNiro & Epstein, 1981). Although proportional abundances of invertebrates of different trophic levels may differ between habitat types (but see Zamora-Camacho & Comas, 2017), it can hardly explain the large variation of $\delta^{15}\text{N}$ - hence “trophic levels” - we found. Detailed analyses of the diet of toads and the $\delta^{15}\text{N}$ values of prey items in each habitat (see Perkins et al. 2014 for an example of food web stable isotopic table) are required to test this hypothesis.

More likely, agricultural fertilization leads to very high amounts of ^{15}N -enriched fertilizers (e.g., manure, compost, ammonium sulphate and to a lesser extent ammonium nitrate, Bateman & Kelly, 2007) deposited on arable land (Tamm, 2012). Relatively high $\delta^{15}\text{N}$ values of these fertilizers increases $\delta^{15}\text{N}$ baselines that propagate through the trophic webs in agricultural landscapes (Anderson & Cabana, 2005). Such process has already been highlighted in tadpoles developing in waters with high nitrate concentrations (Trakimas et al., 2011).

It is important to emphasize the large variations in $\delta^{15}\text{N}$ values between individuals from the same habitat type or study site (Table 1, Fig. 1). Such result seems to suggest different individual strategies of (micro-) habitat use to forage within a similar landscape (Miaud & Sanuy, 2005; Indermaur et al., 2009). This indicates that the influence of nitrogen fertilization on toad $\delta^{15}\text{N}$ values is spatially restricted. Such result suggests that, at least in our setting, $\delta^{15}\text{N}$ values can help to understand the use of micro-habitats, movement and/or dispersal and individual strategies in agricultural landscapes (Rickers et al., 2006; Dammhahn & Goodman, 2014). Future studies using both individual tracking (i.e, radio-tracking) and isotope analyses will be critical to test for this hypothesis.

In contrast to $\delta^{15}\text{N}$ values, our results showed that blood $\delta^{13}\text{C}$ values did not differ between toads from agricultural habitats and individuals living in forest. It is likely that the surface area occupied by corn fields (the main C4 plant expected to influence $\delta^{13}\text{C}$ values in agricultural habitats, Schwertl et al., 2005) is too low to significantly influence $\delta^{13}\text{C}$ values of individual toads using agricultural habitats: In our study area, corn crop represents only ~4.5% of agricultural surface area (Agreste, 2016). Future studies are required to investigate both the presence of toads and their potential prey in different crop types. Additionally the prey of toads could be sampled to assess their trophic level in each habitat to provide a better understanding of the habitat's nutrient flow and its possible influence on toads' use of various habitats (Perkins et al., 2014). Finally, future studies could incorporate other isotope markers such as sulphur or oxygen to further determine the nutrients in each habitat's food web (Penna et al. 2020). Complementary approaches such as compound-specific isotopic analyses of amino acids may well prove useful to assess both fertilizer-related (e.g., phenylalanine) and trophic-related (e.g., glutamic acid) effects on $\delta^{15}\text{N}$ (McMahon & McCarthy 2016).

To conclude, we suggest that $\delta^{15}\text{N}$ values could be a powerful tool to assess habitat use in a terrestrial meso-predator such as the spined toad. Whether such approach could be used on other taxa living in agricultural landscape deserve further investigations. Interestingly, $\delta^{15}\text{N}$ values, as an index of agricultural habitat use, may also help to reveal individual susceptibility to disturbances linked to modern agricultural practices (i.e., pesticides). Futures studies should usefully investigate whether individual $\delta^{15}\text{N}$ values can be used as a fingerprint of concentrations of contaminants in agricultural landscapes.

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III/ Article 2

Does agricultural landscape influence population genetics in a widespread terrestrial amphibian?

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Abstract

Structural modifications of the landscape by modern agricultural practices (simplification, homogenisation and fragmentation) are known to influence gene flow between populations, local genetic diversity, inbreeding and genetic drift which can lead populations into extinction vortices. Amphibians are particularly susceptible to habitat fragmentation and represent a component of biodiversity well suited to investigate the effects of agricultural practices on vertebrate population genetics. We examined the influence of the agricultural habitat in addition to pond density and age of the pond on the genetic diversity and genetic structure (10 microsatellites markers) of 8 populations (N=146 individuals) of Spined toads (*Bufo spinosus*), a philopatric terrestrial amphibian that persist in agricultural landscapes. Overall, we found low genetic structure and no significant relationships between habitats characteristics (habitat type, pond density or breeding site age) and markers of genetic diversity. These results suggest that local habitat structure within an extended rural area allows gene flow and homogenized genetic structure across spined toad populations despite intensive agricultural practices. Future studies should extend to several amphibian species to identify if specific life-history traits (e.g., dispersal ability) can mediate responses to intensive agriculture.

Keywords: genetic diversity, genetic structure, geographic distance, habitat, microsatellites

Modern agricultural practices are recognized as major sources of perturbation for biodiversity (Firbank et al. 2008). Habitat modifications linked to agriculture (e.g., land consolidation) reduce the presence, occurrence and quality of natural habitats required for the persistence of populations (Fahrig 2003). In Europe, land consolidation that occurred after World War II has homogenized rural landscapes, from a complex matrix of small plots, to very large monoculture fields (Benton et al., 2003). Such agricultural revolution has also led to a decrease in the spatial connectivity between remnants of native habitats in which wild populations persist (Firbank et al. 2008). Both processes (i.e., habitat loss and habitat fragmentation) can negatively impact biodiversity (McKinney and Lockwood 1999; Dixo et al. 2009). For instance, reduced surface area of native habitat can decrease population size by directly reducing carrying capacity, resources quantity and quality, or by increasing predation pressure (Soulé 1987; Murdoch et al. 1996). Complementarily, habitat fragmentation may negatively influence dispersal between vestiges of native habitats, which can ultimately lead to population isolation (Brown and Kodric-Brown, 1977). Such process can influence gene flow between populations and consequently local genetic diversity (Lenormand 2002; Epps et al. 2005), inbreeding and genetic drift (Beebee 2005; Bani et al. 2018) which can lead populations into extinction vortices (Bonte and Bafort 2019).

Amphibians are well suited to assess the effects of agricultural practices on population genetics (Beebee 2005; Cushman 2006). Indeed, amphibians are highly impacted by land conversion (Stuart et al. 2008; Hof et al. 2011) because of a suite of traits such as a complex biphasic life cycle (Becker et al. 2007;) and comparatively low dispersal capacity (Hillman et al. 2014) that make them susceptible to habitat fragmentation. Consequently, it is expected that habitat modification linked to intensive agriculture should influence spatial connectivity between populations and thus gene flow and associated genetic diversity (Gauffre et al. 2022).

Herein, we investigated the influence of habitat type on the genetic diversity and genetic structure across 8 populations (N=146 individuals) of Spined toads (*Bufo spinosus*) a philopatric terrestrial amphibian that persist in agricultural landscapes (Guillot et al. 2016). Based on 10 microsatellites molecular markers, we analyzed the genetic differentiation between habitat types (agricultural, forest or mixed habitats, Renoirt et al. 2021a), and we included in our analyses other relevant habitat characteristics (density of breeding ponds, age of sampled ponds) that may influence genetic structure.

Material and methods

Study species and sampling

Spined toad (*Bufo spinosus*) is a common amphibian species occurring in Western Europe. Terrestrial adults reach aquatic sites during a relatively short breeding season (~1 month). Males migrate massively at the beginning of the breeding season and remain at the breeding sites during several weeks while females occur transitorily to mate and lay eggs (Brischoux and Cheron 2019). Because numerous males can be captured at a given site, we focused on this sex and captured and drew blood from 146 males (Renoirt et al. 2021a).

Study sites

We monitored eight breeding ponds (Appendix 1). These breeding sites display different proportions of habitat type such as forests, hedgerow networks and agricultural areas. To assess these proportions, we drew a buffer around each pond (1000 m radius spanning the spatial scale travelled by toads during the breeding migration, Guillot et al. 2016) and we extracted the surface area of agricultural fields, and forest cover using QGIS.org 3.22 (QGIS, 2022) and aerial orthophotos available from Geoportail and GoogleEarth (Appendix 2). This allowed us to categorize the breeding sites as agricultural, forest or mixed. We also reported the number of neighbouring potential breeding sites (i.e., pond density). Archives of municipalities allow us also to access the age of each pond. Two sites could not be accurately aged because they already existed since a presumably very long time and we therefore set their age at 200 years for our analyses.

Genetic analyses

Details on DNA extraction and analyses are given in Appendix 3.

We tested the reliability and effectiveness of the 10 microsatellite markers (Trujillo et al. 2017) testing the presence of null alleles, linkage disequilibrium as well as Hardy-Weinberg expectation and polymorphism (Appendix 4). All microsatellite loci were polymorphic (AR ranged from 2 to 8.121 and HE ranged from 0.2 to 0.917) and retained for analyses (Appendix 5).

We estimated genetic differentiation computing F_{st} values between populations (Weir and Cockerham 1984) with FSTAT v. 2.9.3.2 (Goudet 2001). The significance was calculated using global tests implemented in FSTAT v. 2.9.3.2 with a level of significance adjusted for multiple tests using the standard Bonferroni correction. At the individual scale, we combined Euclidian and genetic distances into simple Mantel test (with 9999 permutations), using GENALEX software v 6.2 (Peakall and Smouse, 2006). We used this method to test “Isolation By Distance” (*i.e.*, IBD) between individuals. Then, we investigated also the global genetic structure using the individual-based approach implemented in STRUCTURE (Pritchard et al. 2000) (with the admixture model and the option of correlated allele frequencies between populations). This approach used Bayesian clustering analyses to estimate both the number (K) of genetic cluster(s) and the associated admixture coefficient of individuals. As recommended by Evanno et al. (2005), we replicated 20 independent runs of STRUCTURE for each value of K (with K varying from 1 to 10). Each run had a burn-in of 10,000 and a total number of 1 million iterations. To determine the most likely number of genetic clusters (K), we used the method implemented in STRUCTURE HARVESTER version 0.6.9 (Earl and vonHoldt 2012).

Statistical analyses

All statistical analyses were carried out with R v4.2.1 (R Core Team 2019). We checked residual normality using diagnostic plots after tested all data for homogeneity of variance, residues independence and normality with the Bartlett test, Dubin-Watson test and Shapiro-Wilks test, respectively. We fitted linear models to assess relationship between genetic diversity parameters and habitat type, pond density and pond age.

Results and Discussion

We found relatively low F_{ST} values (mean=0.018±0.013, Table 1) suggesting a low genetic structure. Although few pairwise comparisons between sites were significant (Table 1), these values are convergent with the Mantel test that revealed a weak but significant IBD ($F_{1,10583} = 205.6$, $r^2 = 0.019$, $P < 0.001$, Appendix 6). Such pattern was also corroborated by the results from Bayesian clustering analyses (Appendix 7). Indeed, although these approaches inferred a highest ΔK value for $K=4$ (Appendix 7), they also yielded a low ΔK value suggesting a poor convergence between the 20 independent runs (Appendix 7). This echoes the fact that IBD can disturb the approach implemented in STRUCTURE, which can in turn discern multiple clusters where there is only a single large area with isolation by distance (Meirmans 2012).

Table 1. Pairwise F_{ST} values for each population comparison (below diagonal) and their significance level (above diagonal). P-value threshold is adjusted with the Bonferroni correction, $P = 0.0018$. Number of the study sites refers to Appendix 1 and Appendix 2.

	1	2	3	4	5	6	7	8
1	-	*	*	*	*	*	*	*
2	0.0261	-	NS	NS	NS	NS	NS	NS
3	0.0497	0.0206	-	NS	NS	NS	*	*
4	0.026	0.0145	0.0018	-	NS	NS	*	NS
5	0.0399	0.0168	-0.0145	0.0038	-	NS	NS	NS
6	0.0451	0.0175	0.0173	0.0139	0.0111	-	NS	NS
7	0.0361	0.0192	0.0189	0.0216	0.0157	0.0051	-	NS
8	0.0344	0.0165	0.0149	0.0088	0.0032	0.0096	0.006	-

Interestingly, this pattern dovetails well with the lack of relationships between habitat characteristics and markers of genetic diversity. For instance, the surface area of agriculture or forest around each breeding site was not linked to AR, He or Fis (agricultural surface area: all $P > 0.38$; forest surface area: all $P > 0.49$, Figure 1). Similarly, the number of neighbouring breeding ponds around each breeding site was not linked to AR, He or Fis (all $P > 0.24$, Figure 1). Finally, AR, He and Fis were not related to the age of the breeding sites (all $P > 0.38$, Figure 1).

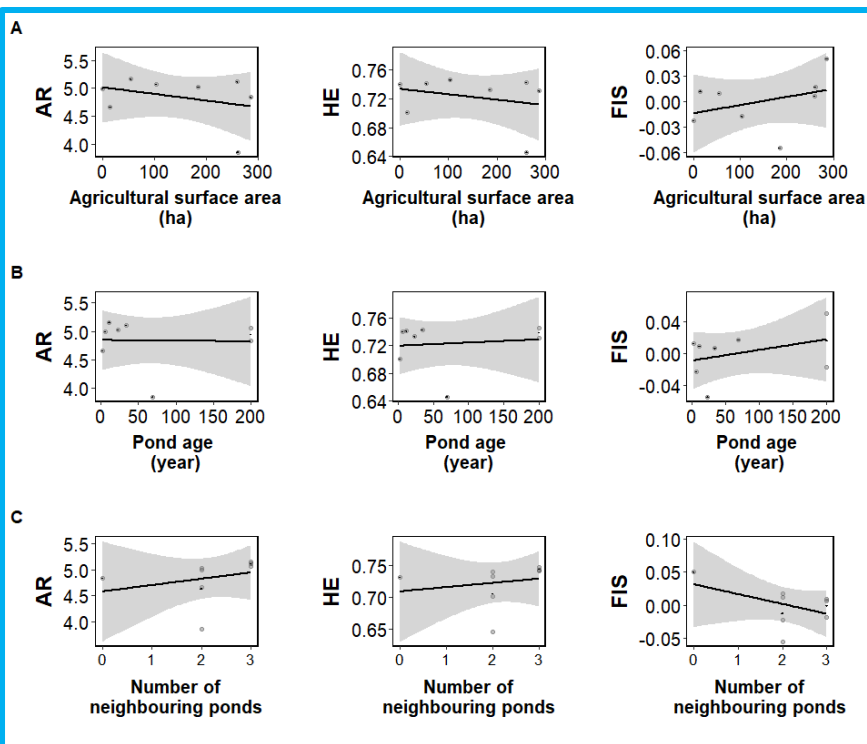


Figure 1. Genetic diversity parameters (AR: allelic richness, HE: expected heterozygosity, FIS: Fixation index) according to (A) agricultural surface area (ha), (B) the age of each study site and (C) the number of neighbouring breeding ponds around each study site. Grey shading indicates 95% confidence intervals.

Collectively, these results suggest that local habitat structure within an extended rural area remains permeable enough to maintain gene flow and to homogenize genetic structure across spined toad populations despite intensive agricultural practices. However, it is noteworthy that the population collected in the site #1 is significantly and genetically different from all other (Table 1). Interestingly, despite its proximity with other sites (Appendix 1), this site displayed lowest AR and He and highest Fis (Appendix 2) suggesting a genetic impoverishment. Future studies should usefully investigate the processes underlying this specificity.

Our study is based on a polymorphic panel of 10 microsatellite markers and our methodological approach appears reliable. Yet, we cannot rule out potential caveats affecting genetic diversity and fine genetic structure detections. For instance, our sampling was focused on males solely for logistical reasons (see above). Future studies should include females to explore potential sex-specific bias in dispersal ability and genetic structure (Li and Kokko 2019). This may reveal important at a time when female toads have been highlighted to be more susceptible to agricultural habitats than males (Renoirt et al. 2021b).

The overall genetic similarity between our study sites contrasts with the results from a similar study performed on marbled newts in the same area (Gauffre et al. 2022). This study highlighted a positive influence of pond density on genetic diversity and a negative influence of agriculture on gene flow and connectivity (Gauffre et al. 2022). Such contrast suggests that species-specific ecological traits (e.g., locomotor performance) can mediate the negative influence of intensive agriculture on dispersal due to habitat loss and reduced connectivity. Future studies should usefully include several amphibian species to identify which life-history traits (e.g., dispersal ability, microhabitat selectivity, susceptibility to desiccation or to predation) can induce divergent responses to intensive agriculture.

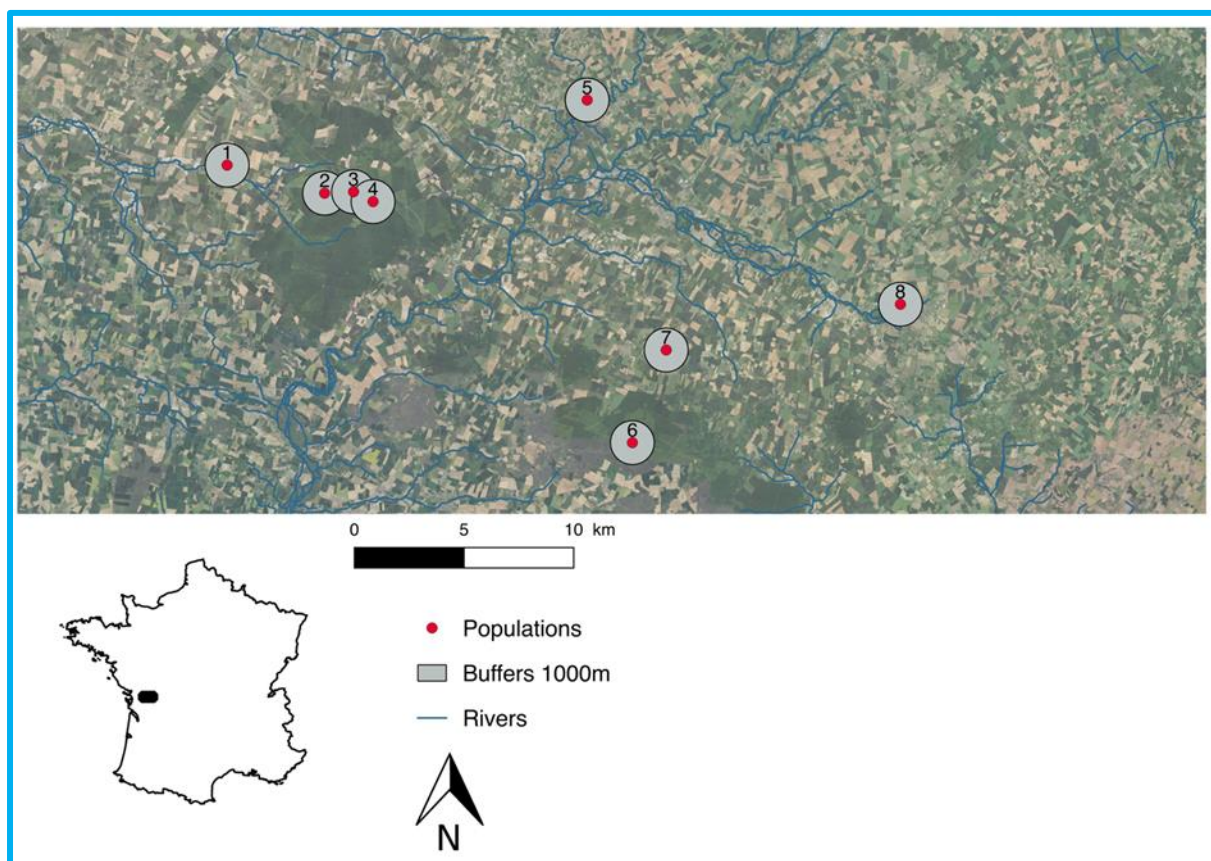
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Appendix 1. Localization of the study sites and general situation of the study area in France. Each site is depicted by a red dot and the grey circle represents the buffer (1 km radius) that was used to assess habitat type. Number of the study sites refers to Table 1 and Appendix 2.

Sites	Categories	n	Age	Ponds number around sites	Agricultural surface area (ha)	Forest surface area (ha)	Latitude	Longitude
1	Agricultural	20	69	2	261	14	46.15568	-0.48309
2	Forest	22	2	2	15	289	46.14708	-0.4259
3	Mixed	20	>200	3	104	188	46.14694	-0.40787
4	Mixed	18	11	3	55	226	46.14446	-0.39561
5	Agricultural	7	34	3	260	40	46.18868	-0.27222
6	Forest	19	6	2	0	209	46.04904	-0.23728
7	Agricultural	20	>200	0	286	18	46.08755	-0.21963
8	Agricultural	20	23	2	185	17	46.11075	-0.08225

Appendix 2. Summary of the sampling design (habitat categories, number of individuals, age of ponds in year, ponds number around breeding sites (1km radius), agricultural and forest surface area (ha)).

DNA extraction was performed on approximately 4µL of red blood cells using a NucleoSpin® Blood kit (Macherey-Nagel) according to manufacturer's protocol. DNA quality and concentration were measured with a Nandrop ND-1000 (Ozyme). After a simplex PCR validation of the markers on 15 individuals, we analysed all DNA samples using 10 previously published microsatellite molecular markers: Bspi3-02; Bspi 3-19; Bspi 3-26; Bspi 4-14; Bspi 4-16; Bspi 4-24; Bspi 4-27; Bspi 4-28; Bspi 4-29 and Bspi 4-30 (Trujillo et al., 2017). These loci were amplified in 4 multiplexes and fluorescently labelled. Multiplex 1 contained only the locus: Bspi 4-24 (VIC) ; multiplex 2 contained Bspi 4-16 (6-FAM), Bspi 4-30(VIC), Bspi 4-27(NED) and Bspi 4-28(PET); multiplex 3 contained Bspi 4-29 (VIC), Bspi3-02(NED) and Bspi 3-19 (PET); multiplex 4 contained Bspi 3-26 (6-FAM) and Bspi 4-14 (VIC). We normalised DNA quantity at 10ng/µL then performed PCR Multiplex reactions with Type-it Microsatellite PCR® (Qiagen) kits in a total volume of 15 µl that includes 7.5 µl of Master Mix, 1.2 µl of Primer Mix with 0.3 µM of each primer and 5.3 µl of RNase-free H₂O. Multiplex reactions consisted of initial denaturalization (95°C; 5 minutes), 30 cycles of denaturalization (95°C; 30 seconds), annealing (during 90 seconds at different temperatures in each multiplex reaction: multiplex reactions 1, 2 and 4 had an annealing temperature of 60°C and multiplex reaction 3 had a annealing temperature of 58°C) and extension (72°C; 30 seconds), and final extension (60°C; 30 minutes) Then, PCR product have been separated by electrophoresis on an automated sequencer (ABI PRISM3730) with the GeneScan 500 LIZ size standard (Applied Biosystems) at Genoscreen (Lille, France). Fragment size was determined using Gene-Mapper version 4.0 (Applied Biosystems) by visual inspection. In order to validate this experiment, 16 individuals were replicated (extracted and typed separately).

Appendix 3. DNA extraction and analyses.

First, we tested the presence of null alleles or scoring errors due to stuttering according to the method implemented in Microchecker v.2.2.3 (Van Oosterhout et al., 2004). We tested the linkage disequilibrium using exact tests (1200 permutations), as implemented in FSTAT v.2.9.3.2 (Goudet, 2001). Then, we assessed the departure from Hardy-Weinberg expectations using GENALEX software v 6.2 (Peakall and Smouse, 2006). We adjusted the threshold of significance with the standard Bonferroni corrections involving for multiple tests (Rice, 1989). Finally, we investigated genetic diversity : allelic richness (AR), expected heterozygosity (*He*), and fixation index (*Fis*) using FSTAT v.2.9.3.2 (Goudet, 2001). Among the 80 tests, 2 exhibited null alleles. Only 1 of the 80 populations-locus combinations deviated significantly from Hardy-Weinberg expectations (P-value threshold = 0.0006 after Bonferroni correction) (Appendix 5). Moreover, no evidence of linkage disequilibrium was detected (P-value threshold= 0.001 after Bonferroni correction). As these null alleles as well as Hardy Weinberg deviance did not affect global Hardy-Weinberg expectance, we retained all microsatellite markers for analyses. All microsatellite loci were polymorphic within all populations (see Appendix 5 for details).

Appendix 4. Test of the reliability and effectiveness of the microsatellite markers panel

Microsatellite markers								
	*03-02				*3-19			
Site	AR	HE	FIS	NA	AR	HE	FIS	NA
1	2,316	0,541	0,124	no	2,562	0,562	0,11	no
2	2,911	0,632	-0,079	no	2,918	0,581	0,262	no
3	2,774	0,578	-0,125	no	2,981	0,671	0,18	no
4	2,968	0,644	0,137	no	2,957	0,655	0,152	no
5	2,989	0,643	0,556	no	3	0,667	0	no
6	2,962	0,627	-0,007	no	2,981	0,68	0,395	no
7	2,665	0,545	0,174	no	2,985	0,676	0,335	<i>yes</i>
8	2,869	0,621	-0,016	no	2,983	0,676	0,187	no
Mean	2,83	0,604	0,096	no	2,957	0,646	0,203	no

Microsatellite markers								
	*3-26				*4-14			
Site	AR	HE	FIS	NA	AR	HE	FIS	NA
1	2	0,512	0,28	no	5,668	0,818	-0,039	no
2	2,272	0,529	0,141	no	5,831	0,838	-0,08	no
3	2,295	0,463	-0,188	no	6,094	0,851	-0,109	no
4	2,353	0,537	0,233	no	7,37	0,877	0,002	no
5	2	0,429	-0,333	no	6,681	0,905	0,053	no
6	2,333	0,525	0,153	no	7,052	0,892	-0,121	no
7	2	0,513	0,026	no	6,415	0,851	0,152	no
8	2	0,508	-0,28	no	6,79	0,862	-0,044	no
Mean	2,158	0,502	0,004	no	6,758	0,862	-0,023	no

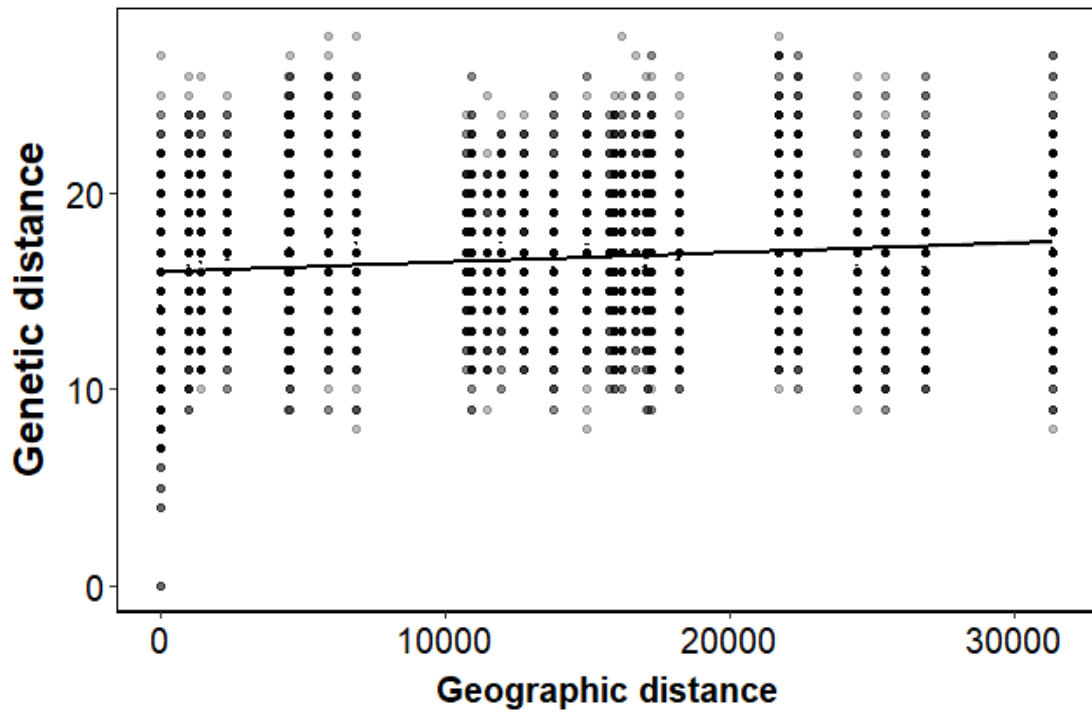
Microsatellite markers								
	*4-16				*4-24			
Site	AR	HE	FIS	NA	AR	HE	FIS	NA
1	3,835	0,723	0,011	no	4,117	0,755	-0,059	no
2	3,656	0,681	0,065	no	6,395	0,848	-0,018	no
3	4,899	0,787	-0,017	no	7,081	0,882	0,036	no
4	4,67	0,742	-0,048	no	6,089	0,842	-0,056	no
5	4,967	0,81	-0,235	no	6,681	0,893	-0,12	no
6	4,766	0,759	-0,04	no	7,107	0,896	-0,116	no
7	3,288	0,695	-0,08	no	6,172	0,863	0,131	no
8	4,619	0,726	-0,101	no	6,905	0,889	-0,068	no
Mean	4,359	0,74	-0,056	no	6,882	0,859	-0,034	no

Microsatellite markers								
*4-27					*4-28			
Site	AR	HE	FIS	NA	AR	HE	FIS	NA
1	5,252	0,741	0,006	no	4,284	0,746	-0,2	no
2	6,665	0,863	-0,001	no	5,875	0,834	-0,144	no
3	7,136	0,9	-0,056	no	6,749	0,886	0,097	no
4	7,612	0,907	-0,041	no	6,796	0,877	-0,076	no
5	7,407	0,917	0,065	no	6,429	0,869	0,014	no
6	6,33	0,849	-0,053	no	6,349	0,864	-0,036	no
7	6,908	0,895	0,059	no	6,654	0,874	-0,087	no
8	7,202	0,897	-0,003	no	6,124	0,849	-0,177	no
Mean	7,165	0,871	-0,003	no	6,512	0,85	-0,076	no

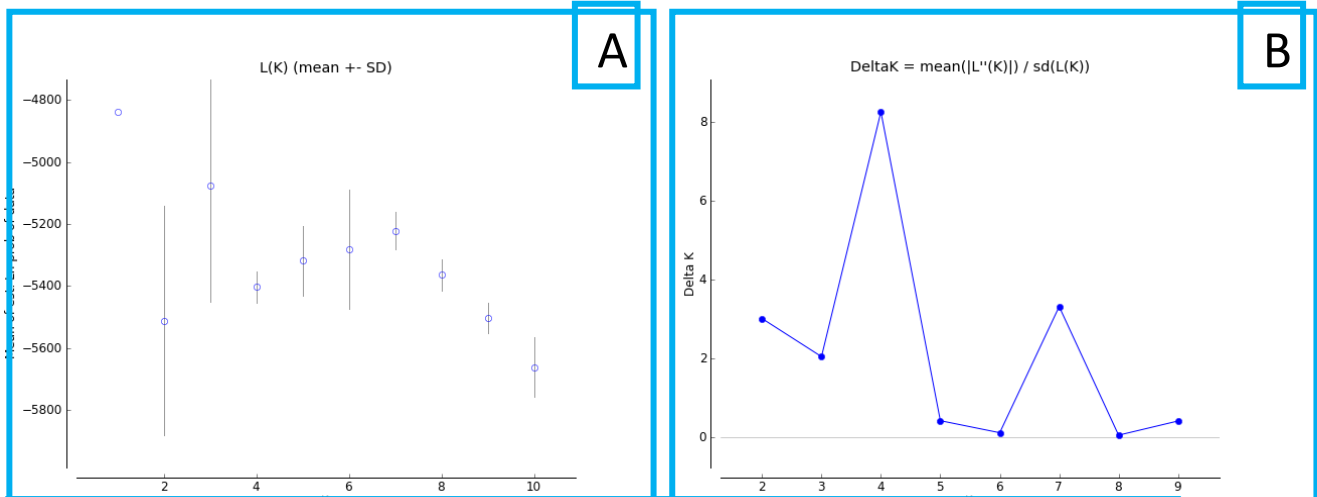
Microsatellite markers								
*4-29					*4-30			
Site	AR	HE	FIS	NA	AR	HE	FIS	NA
1	6,336	0,863	0,073	no	2,169	0,2	-0,051	no
2	7,742	0,916	0,106	no	2,36	0,285	-0,118	no
3	7,047	0,875	0,086	no	3,596	0,568	-0,231	no
4	7,648	0,895	-0,117	no	3,15	0,43	0,095	no
5	8,121	0,917	0,065	no	2,846	0,381	-0,125	no
6	7,211	0,892	-0,121	no	2,826	0,415	-0,205	no
7	7,676	0,914	-0,037	no	3,565	0,482	-0,2	no
8	7,776	0,909	0,01	no	2,96	0,389	-0,155	no
Mean	7,687	0,898	0,008	no	2,96	0,394	-0,124	no

Mean			
Site	AR	HE	FIS
1	3,854	0,646	0,017
2	4,663	0,701	0,012
3	5,065	0,746	-0,018
4	5,161	0,741	0,009
5	5,112	0,743	0,006
6	4,992	0,74	-0,023
7	4,833	0,731	0,05
8	5,023	0,733	-0,055
Mean	5,027	0,722	-0,001

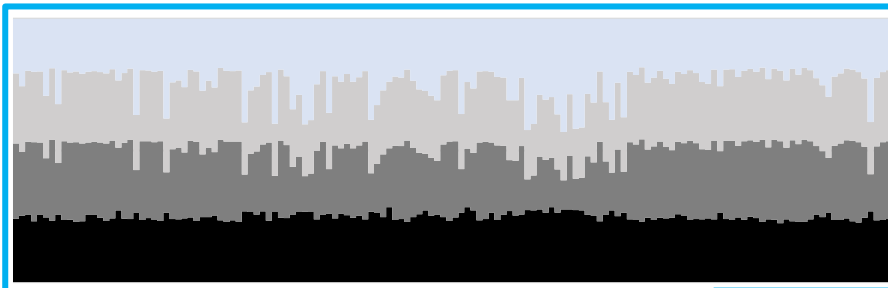
Appendix 5. Genetic diversity parameters for the microsatellite markers calculated for each population. N: number of samples analysed; AR: allelic richness and He: expected heterozygosity. Fis (i.e. Fixation index) is represented in italics and bold when significantly deviating from Hardy-Weinberg expectations (i.e. significantly different from 0; $P < 0.0006$ after Bonferroni adjustment). NA: presence of Null Allele.



Appendix 6. Relationship between genetic distance (i.e. Log 10 transform genetic distances) and geographic distance (computed from the geographic coordinates of our sampling points).



Appendix 7. Results from STRUCTURE and STRUCTURE HARVESTER for Spined toad (*Bufo spinosus*) individuals (n= 146). A-The mean log likelihood of the data, and associated variance across the 20 replicates (white circles), and B- Evanno's Delta K statistic (blue circles) per number of simulated genetic clusters (K, from 1 to 10). As attested by the general form of the curve as well as by the low Delta K values, The MCMC chains did not converge toward a precise number of genetic clusters K suggesting a weak genetic structure. These figures have been realized using STRUCTURE HARVESTER software version 0.6.1 (Earl & von Holdt 2012).



Plot of STRUCTURE (Pritchard et al., 2000) results concerning the genetic population structure of the *B. spinosus* (n= 146). Mean of proportions of ancestry for investigated individuals, inferred at K = 4: the highest value of the mean likelihood. Each vertical bar represents an individual; each color represents a different genetic cluster inferred by STRUCTURE analyses.

IV/ Conclusion

Dans ce chapitre nous avons montré qu'il était possible d'évaluer l'utilisation de l'habitat à partir des valeurs isotopiques (notamment le $\delta^{15}\text{N}$) et que l'habitat agricole par la supplémentation en engrais pouvait influencer la susceptibilité individuelle aux perturbations liées à l'agriculture intensive moderne (**Article 1**). De par les grandes variations des signatures isotopiques sanguines en $\delta^{15}\text{N}$ au sein d'un même type d'habitat (Forestier, mixte et agricole), nous avons pu mettre en évidence une utilisation individuelle différentielle de l'habitat, sûrement en lien avec une utilisation de micro-habitat. C'est par exemple le cas des haies dans les paysages agricoles, qui peuvent servir de refuge et/ou de corridor écologique pour les populations d'animaux vivant dans ces milieux. En raison de cette possible utilisation différentielle de l'habitat par ces organismes, il devient intéressant de se demander si la structure du paysage peut influencer la structure génétique des populations et si du coup, l'environnement agricole par rapport à un environnement forestier montre une plus faible diversité génétique. Nous avons mis en évidence à partir de l'**article 2** des résultats qui concorderaient avec une utilisation de micro-habitat et possiblement de corridor écologique dans les paysages dégradé. Ainsi, la diversité génétique globale des populations de crapauds épineux reste homogène peu importe le milieu. On s'attendait à ce que les paysages agricoles contraignent les déplacements d'individus et en conséquence impacte la diversité génétique des populations. Cependant, on peut conclure que ce type de milieu reste perméable aux déplacements d'individus et n'impacte pas la diversité génétique. Ces résultats tendent à être explorés plus en profondeur étant donné qu'ils contrastent avec les résultats d'une étude similaire réalisée sur les tritons marbrés dans la même région (Gauffre et al. 2022). Les auteurs de cette étude ont, par exemple, mis en évidence une influence négative de l'agriculture sur le flux génétique et la connectivité (Gauffre et al. 2022). De ce fait, nous avons suggéré que les traits écologiques spécifiques aux espèces et au sexe peuvent médier l'influence négative de l'agriculture intensive sur la dispersion.

Finalement, dans ce chapitre nous suggérons des voies de réflexion et d'investigation sur la contamination environnementale (e.g. pesticides), qui au même titre que les engrais azotés, pourrait se retrouver dans les organismes des animaux qui vivent en milieu agricole et avoir des répercussions néfastes sur leur pérennité. Aussi, nous suggérons des études plus approfondies sur l'utilisation des micro-habitats pouvant servir de rempart et/ou de tampon aux contraintes agricoles qui sévissent dans ces milieux. Pour finir, nous recommandons d'intégrer les femelles dans les études de l'utilisation de l'habitat et de la diversité génétique puisqu'elles peuvent contraindre la reproduction par leur abondance, leur période de reproduction limitée dans le temps et par le coût de la reproduction qui est souvent plus important chez ce sexe (Glutton-Brock and Vincent, 1991; Cussac, 1999; Gowaty and Hubbell, 2009).

CHAPITRE 2

Etat des populations des habitats forestiers et agricoles

Dans ce chapitre (**Article 3 et 4**), nous nous sommes concentrés à comprendre l'effet des modifications de l'habitat sur les populations de crapauds épineux. Ce chapitre s'axe autour d'une approche terrain avec un suivi de populations d'une durée de 3 ans le long d'un gradient d'habitat et sur un total de 23 sites de reproductions. A l'aide des PCA nous avons établi un gradient scoré allant de milieu fortement boisé jusqu'à des milieux fortement agricoles. On a pu recenser tout au long des périodes de reproduction de nombreux paramètres en lien avec l'état de santé des populations comme les présences-absences, la phénologie, les abondances d'individus et les sex-ratios en suivant l'hypothèse que l'habitat pouvait contraindre la reproduction.

Le suivi des tendances démographiques des espèces est important pour aider à déterminer le statut des listes, évaluer les besoins de gestion de l'habitat et suivre les réponses des espèces aux actions de conservation et de gestion (Lovett et al. 2007).

Au cours des dernières décennies, les biologistes ont documenté des changements ou des déclin de population soudains ou rapides chez les amphibiens (par exemple, Blaustein et al., 1994 ; Blaustein et al., 2011 ; Meyer et al., 1998 ; Houlahan et al., 2000 ; Green, 2003) avec une série de facteurs de changement (par exemple, Collins et Storfer, 2003). Il s'agit notamment des réponses dépendantes de la densité (par exemple Wilbur, 1990), des effets climatiques et des altérations de l'habitat par les humains (par exemple Hanski, 1998 ; Alford et Richards, 1999 ; Carlson et Edenhamn, 2000 ; Hartel, 2005 ; Gollmann et al., 2002) et des maladies (Ariel et al., 2005). Les amphibiens sont maintenant considérés comme des bio-indicateurs des écosystèmes où des changements majeurs de population peuvent avoir un impact sérieux sur d'autres espèces, soulignant l'importance de surveiller les tendances à long terme. Ceci est particulièrement pertinent dans les paysages fragmentés, où les espèces qui fonctionnent selon une dynamique de métapopulation peuvent être sérieusement affectées. Les séries de données à long terme générées par les suivis de population facilitent les prédictions sur la façon dont elles évoluent dans le temps, ce qui constitue une information essentielle pour établir une stratégie de conservation valable (Ferri et al., 2017).

Au cours de cette thèse, nous avons cherché à démarrer une série de données à long terme sur la démographie du crapaud épineux sur plusieurs sites. La présente étude a été réalisée dans un paysage fragmenté des Deux-Sèvres, dans l'ouest de la France, où les modifications importantes de l'environnement résultant des activités agricoles, y compris l'utilisation d'agrochimie. Les activités agricoles dans les paysages fragmentés sont deux facteurs majeurs identifiés dans le déclin des amphibiens (Blaustein et al., 1994 ; Battisti et al., 2016). Dans la zone d'étude, la migration des amphibiens vers les étangs de reproduction a lieu principalement de décembre à mars chez ces anoues. Les échantillons représentent donc principalement des adultes reproducteurs.

Les questions clés abordées dans cette étude sont 1) les crapauds épineux montrent-ils des tendances de population sur plusieurs années ? 2) Existe-t-il des preuves de déclin de la population en lien avec l'agriculture ? 3) Dans quelle mesure les effectifs fluctuent-ils ?

II/ Article 3

Unusual lack of reproduction in toad populations from agricultural

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Unusual lack of reproduction in toad populations from agricultural habitats

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Anthropogenic alterations of habitats can have detrimental consequences for biodiversity. Documenting these effects require monitoring in multiple sites that vary in the degree of alterations over long temporal scales, a task that is challenging. Yet, simple naturalist observations can reveal major ongoing events affecting wild populations, and serve as a basis for further investigations. We quantified breeding parameters of toad (*Bufo spinosus*) populations from forested (preserved) and agricultural (altered) habitats. We found that reproduction did not occur at the sites surrounded by agriculture, while it occurred successfully in ponds from forests. Males were present at all sites, but females, amplexus, egg strings and tadpoles remained absent from agricultural sites. Observations made at the same sites indicated that breeding occurred during previous years. Our observations of habitat- and sex-specific lack of reproduction may have critical consequences for the persistence of populations of a widespread amphibian species in agricultural areas.

Key-words: Amphibian, *Bufo spinosus*, breeding, conservation, reproductive success

Biodiversity is dramatically affected by human activities leading to an alteration of ecosystems (Chapin et al., 2000; Myers & Knoll, 2001; Brooks et al., 2002). Human activities, such as intensive farming, generate habitat alteration, fragmentation and simplification (e.g. Maron & Fitzsimons, 2007). In addition, agricultural landscapes often suffer from the massive use of pesticides, which contaminate the environment and the wildlife (Schäffer et al., 2007).

As a consequence, these modern agricultural practices can have detrimental impacts on fauna and flora (Myers & Knoll, 2001; Brooks et al., 2002; Fahrig, 2003; Relyea, 2009). In order to persist in these altered habitats, wildlife must adjust to these ongoing changes. However, the ability of a species to persist in agriculture landscapes can be jeopardized when critical elements necessary to perform its life-cycle are missing in the environment. For example, the lack of trees or shrubs can impair the ability of some bird species to breed in simplified landscapes (Newton, 1994; Verhulst et al., 2004). Similarly, amphibian populations will disappear if suitable breeding ponds are missing following habitat simplification (Alex Smith & Green, 2005). In addition to habitat alteration, other effects can be linked to the increasing use of chemical inputs that aim to improve crop productivity in agricultural habitats (McLaughlin & Minneau, 1995; Köhler & Triebkorn, 2013). For instance, pesticides are used to control pests (e.g., weeds, insect, fungi) that negatively impact crop productivity. These pesticides can have toxic effects on non-target components. For example, they have been shown to negatively impact reproduction in wildlife species, through various mechanisms that spans from direct toxic or sublethal effects (Mnif et al., 2011; Cheron and Brischoux, 2020) to alterations of ecosystem functioning (e.g., disruption of the food web, Relyea & Hoverman, 2008).

The direct effects of habitat alteration on population persistence are relatively easy to assess (see above). Yet, assessments of indirect effects of agricultural practices on population persistence are more challenging and require population monitoring in multiple sites that vary in their habitat structure (i.e., degree of alteration and fragmentation). To document these effects, simple naturalist observations can be important because they often help to reveal major ongoing and detrimental events that affect wild populations (Sagarin & Pauchard, 2010; Sagarin & Pauchard, 2012; Mauz & Granjou, 2013).

During the course of a study that aimed to compare toad (*Bufo spinosus*) populations between forested (preserved) areas and agricultural (simplified) habitats, we opportunistically quantified breeding parameters (number of males, presence of amplexus, egg strings and tadpoles) in both types of habitat in Western France (Fig. 1). The spined toad (*Bufo spinosus*) is a widespread species that can live in a variety of habitats and has been previously shown to persist even in highly modified agricultural areas (Arntzen et al., 2014, Guillot et al., 2016). As in most anuran species, spined toads have a biphasic life-cycle with an extensive use of terrestrial habitats during most of the year, and a short breeding season (~1 month) in aquatic sites (ponds) where mating occurs and eggs and tadpoles develop (Reading, 1998; Kelleher et al., 2018; Brischoux & Cheron, 2019). The breeding season occurs at the end of winter (February - March). During this period, male toads migrate towards aquatic breeding sites where they wait for females (Reading, 1998). Males can remain at the breeding site for several weeks, while females leave shortly after mating and egg-laying (Davies & Halliday, 1977). Eggs and tadpoles develop over three to four months before metamorphosis and subsequent dispersal in nearby terrestrial habitats. Reproductive events can be easily assessed later in the season (when breeders have left the breeding site) by monitoring the presence of egg strings and tadpoles.

The terrestrial part of the annual cycle of toads occurs in various environments usually within one km from the breeding pond (Janin et al., 2011; Guillot et al., 2016). Two of our study sites were located in forested areas where forest cover represented > 95% within a circle of a one km radius centered on the breeding pond; while three sites were located in agricultural areas (composed mainly of large fields) where forest cover was always < 35% within the same surface area (Fig. 1). Forest and agricultural sites were situated in close proximity (max distance 12 km) in order to avoid diverging climatic conditions that may affect timing of reproduction.

Observations were made from early January (week one) to late June (week 26) 2020. At the onset of the reproductive period (from week one to week 11) all study sites were monitored every night. Observations were stopped from week 12 to week 16 because of the lockdown linked to the COVID-19 pandemic. Observations resumed on week 17 on a monthly basis until late June (week 26) in order to assess the presence of developing tadpoles.

Due to of the diverging reproductive behavior of males and females (see above), we made the following observations. Males were individually counted when abundances were < 10 individuals and number of individuals was approximated by increment of 10 individuals when abundances were > 10 individuals. Females remain only briefly at the breeding pond, and amplexus occurs in areas where precise quantification is precluded (in highly vegetated areas or deeper water). As a consequence, we assessed female presence through the observation of amplexus and qualified for each site whether amplexus was observed or not (present/absent). When reproduction occurred, large numbers of egg strings and tadpoles precluded direct enumeration and successful reproduction was assessed with the presence/absence of egg strings and tadpoles.

We emphasize that our opportunistic observations are qualitative rather than quantitative for most parameters recorded as they were not directly linked to the primary goal of the surveys we performed (assessment of reproductive success across habitats).

Observations are summarized in Table 1. Overall, we found that reproduction did not occur at the three sites from agricultural habitats, while it occurred successfully in breeding ponds from forested areas (presence of egg strings and tadpoles, Table 1).

At all of our study sites, breeding males were present, yet with variable abundances (Table 1). Mean number of adult males was 19.0 ± 28.4 (range 0-100) for agricultural sites and 15.6 ± 8.3 (range 0-30) for forest sites (Table 1). These numbers suggest that abundances of reproductive males did not seem to be related to the surrounding habitat structures. Indeed, some sites from agricultural areas displayed numbers of males that equaled or even exceeded those from forested habitats (Table 1). Importantly, the onset of the reproductive period (first observations of males occurring at the study sites) was similar between habitat types (occurring on week 5, Table 1), suggesting that climatic (micro-) conditions did not significantly influence reproduction between sites. These observations tend to further indicate that the lack of reproduction we recorded (see below) may not be linked to a lack of breeding males (although one agricultural site was characterized by lower abundances, Table 1), but rather to a lack of reproductive females.

Indeed, the most clear-cut difference between our study sites was linked to the presence of females (assessed through the presence of visible amplexus, Table 1) and their reproductive success (assessed through the presence of egg strings and developing tadpoles, Table 1). Amplexus was observed on very few nights (one or two nights) at two of the agricultural sites, and was not observed at the other agricultural site. Conversely, amplexant pairs were observed steadily almost every night over six weeks at the sites surrounded by forest. No egg-strings or developing tadpole were observed at all three sites from agricultural habitats, while egg strings and developing tadpoles were present at the two forest sites. Importantly, these observations suggest that females did not migrate to breed in sites surrounded by agricultural areas and, thus, that habitat-specific and sex-specific responses to habitat perturbations occurred in adult females.

Table 1. Summary of the data collected during our surveys. Male abundances show min-max number of individuals observed for each week. Female presence or absence was assessed through observations of amplexus. The presence of egg strings and developing tadpoles was also documented. "ND" stands for "no data". "NO" refers to absence of individuals at periods during which presence was expected, while "-" refers to absence of individuals at periods when absence was expected.

Observations	Sites	Habitat	Week number												
			1-4	5	6	7	8	9	10	11	12-16	17	21	26	
Number of males	A	Agriculture	0	1-3	1	0	0	0	0	0	0	ND	-	-	-
	B	Agriculture	0	40	10-40	3-10	1-3	1	1	0	0	ND	-	-	-
	C	Agriculture	ND	ND	ND	100	70	50	50	50	50	ND	-	-	-
	D	Forest	0	10-20	20	20	10-20	10-20	10	10	10	ND	-	-	-
	E	Forest	0	30	30	20	10-20	20	20	10	10	ND	-	-	-
Presence of amplexus	A	Agriculture	NO	NO	NO	NO	NO	NO	NO	NO	NO	ND	-	-	-
	B	Agriculture	NO	NO	YES	YES	NO	NO	NO	NO	NO	ND	-	-	-
	C	Agriculture	ND	ND	ND	YES	NO	NO	NO	NO	NO	ND	-	-	-
	D	Forest	NO	YES	YES	YES	YES	YES	YES	YES	YES	ND	-	-	-
	E	Forest	NO	YES	YES	YES	YES	YES	YES	YES	YES	ND	-	-	-
Presence of egg strings	A	Agriculture	NO	NO	NO	NO	NO	NO	NO	NO	NO	ND	-	-	-
	B	Agriculture	NO	NO	NO	NO	NO	NO	NO	NO	NO	ND	-	-	-
	C	Agriculture	ND	ND	ND	NO	NO	NO	NO	NO	NO	ND	-	-	-

	D	Forest	N O	NO	YE S	YE S	YE S	YE S	YE S	YE S	N D	-	-	-
	E	Forest	N O	NO	YE S	YE S	YE S	YE S	YE S	YE S	N D	-	-	-
Presence of tadpoles	A	Agriculture	-	-	-	-	-	-	-	-	N D	NO	NO	N O
	B	Agriculture	-	-	-	-	-	-	-	-	N D	NO	NO	N O
	C	Agriculture	-	-	-	-	-	-	-	-	N D	NO	NO	N O
	D	Forest	-	-	-	-	-	-	-	-	N D	YE S	YE S	N O
	E	Forest	-	-	-	-	-	-	-	-	N D	YE S	YE S	N O

It is important to stress that our observations are unreplicated and preliminary and that we have not observed this phenomenon in previous years. Therefore, these observations do not give any strong clue regarding the mechanisms through which habitat-specific and probably sex-specific lack of reproduction has occurred. Yet, previous observations made at the same study sites (Guillot et al., 2016; MC and FB unpublished data) indicate that breeding successfully occurred at some of these agricultural sites at least in 2015 and in 2019; 2 years during which we monitored reproduction at some of those sites and for which egg strings and developing tadpoles were observed. Although we acknowledge the limitations of our observational study, we believe it is important to document, at least in a qualitative way, a potential problem for the persistence of the populations of a widespread amphibian species in agricultural areas (Guerry & Hunter, 2002, Boissinot et al., 2019); and we urge other researchers to share similar observations.

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Author contributions

F.B. and F.A. proposed the initial idea and together with M.R. and M.C. contributed to its development. M.R., M.C. and F.B. performed field work. M.R. and M.C. tabulated the resulting data. All authors discussed the results, and substantially contributed to the writing.

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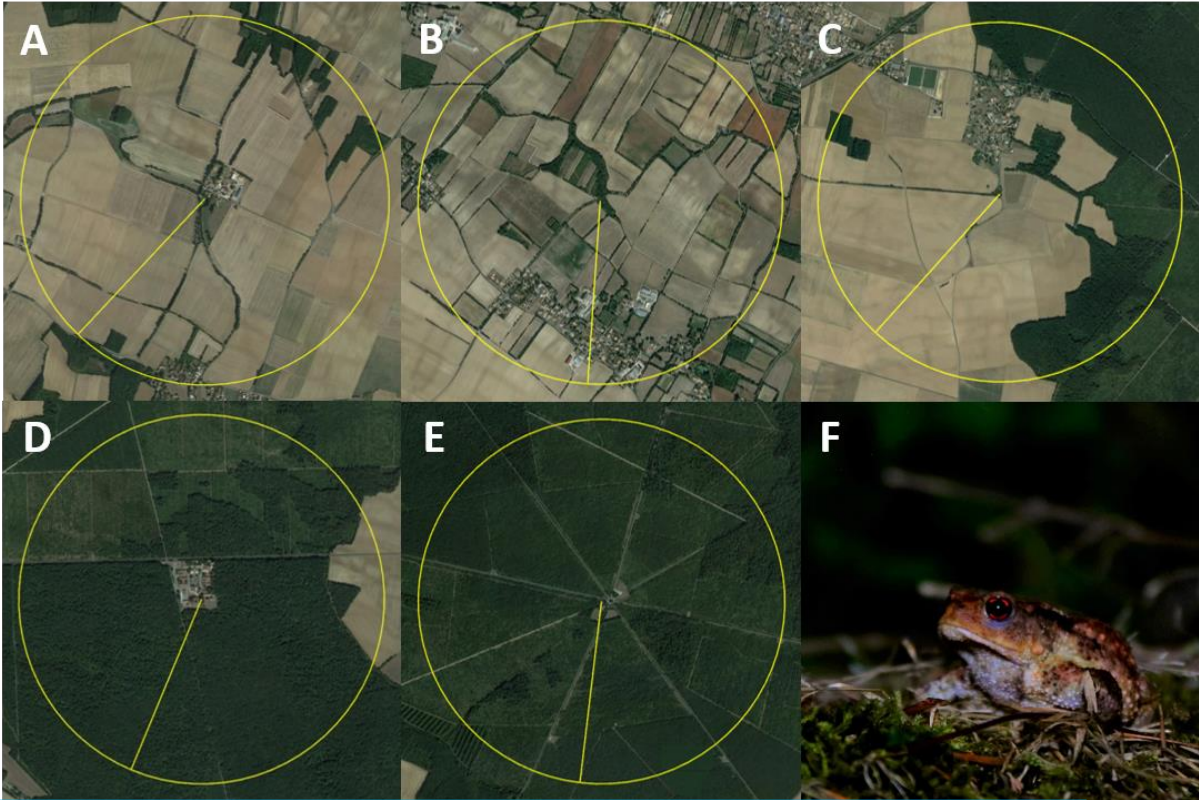


Figure 1. A-D: Aerial pictures (Google Earth) of the five study sites with the one km radius surrounding breeding ponds used to illustrate the contrast between three agricultural sites (A, B and C) and two forested sites (D and E). Letters in the pictures relate to site numbers in Table 1. F: Picture of an individual *Bufo spinosus* in the field in South Deux-Sèvres, France (© Matthias Renoirt).

III/ Article 4

Evidence for ongoing population declines of a widespread amphibian in agricultural landscapes

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Modern agricultural practices are suspected to play a major role in the ongoing erosion of biodiversity. In order to assess whether this biodiversity loss is linked to past habitat modifications (e.g., land consolidation) or to current consequences of modern agriculture (e.g., use of agrochemicals), it remains essential to monitor species that have persisted in agricultural landscapes to date. In this study, we assessed the presence, abundance and recent population trends of one such species, the spined toad (*Bufo spinosus*) along a gradient of habitats from preserved (forests) to highly agricultural sites in rural Western France. Our results showed that both presence and abundance of spined toads were markedly lower in reproductive ponds surrounded by intensive agriculture. The most salient result of our study is the ongoing decline of this species in farmland habitats. Indeed, this result suggests that unknown factors are currently affecting a widespread terrestrial amphibian previously thought to persist in agricultural landscapes. These factors have recently induced strong population declines over the course of a few years. Future investigations are required to identify these factors at a time when anthropogenic activities are currently leading to unprecedented rates of biodiversity loss.

Key-words: abundance; agroecosystems; *Bufo spinosus*; biodiversity; presence

Anthropogenic activities are currently leading to unprecedented rates of biodiversity loss (Chapin III et al., 2000; Myers and Knoll, 2001; Brooks et al., 2002). Indeed, human activities are now recognized to be responsible of climate change (Vitousek, 1994; Steffen et al., 2007), major shifts in land use (Klein Goldewijk and Ramankutty, 2004; Young et al., 2005) and environmental contamination (Rudel et al., 2009; Saleh and Aglan, 2018); all of which can individually and/or interactively affect wildlife (de Brito Rodrigues et al., 2019; Trudeau et al., 2020; Wagner, 2020; Gunstone et al., 2021).

Among the various sources of anthropogenic disturbances to natural ecosystems, modern agricultural practices are suspected to play a major role in the ongoing erosion of biodiversity for several reasons (Altieri, 1999; Dudley and Alexander, 2017). First, agriculture is responsible for the alteration and the reduction of natural habitats and landscape homogenization (Fahrig, 2003). For instance, in Europe, changes in land-use politics that occurred post World War II (WWII) have induced a large scale land consolidation (Benton et al., 2003; Tschardtke et al., 2005). The ancestral rural matrix of small plots and meadows bordered by a dense network of hedges has been homogenized to extended fields hosting monocultures (Benton et al., 2003; Tschardtke et al., 2005). Concomitantly, this revolution has provoked a reduction in the spatial connectivity between patches of favourable habitats among the agricultural landscape, limiting therefore the persistence of wildlife (Benton et al., 2003). Second, detrimental effects of modern agriculture are linked to the massive use of chemical substances that are used to increase agricultural yields (Geiger et al., 2010). Many pesticides are used to control pests in crops (e.g., weeds, insect, fungi) but they have been suspected or shown to detrimentally impact non-target species (Hasenbein et al., 2017; de Brito Rodrigues et al., 2019), either directly through their toxic sublethal or lethal effects (Relyea, 2004; Slaninova et al., 2009; Williams et al., 2015) or indirectly through alterations of ecosystem functioning (e.g., reduced food availability, Hart et al., 2006; Wagner, 2020).

As a result, agricultural practices are expected to detrimentally affect wildlife through different temporal scales. Indeed, at least in Western Europe, most of the landscape changes linked to land consolidation have occurred after WWII (Antrop, 2000) and the subsequent habitat homogenisation and fragmentation have been mostly achieved by the mid 70's or early 80's (Griffin, 1979; Skole and Tucker, 1993; Harper et al., 2007; Rudel et al., 2009). As a consequence, it is expected that the effects of such processes on the persistence of wild populations have already been acting for several decades (Debinski and Holt, 2000; Fuller et al., 2015). In contrast, the temporal scale of the consequences of agrochemical use on wildlife is much more complex to assess. Indeed, although the reliance on chemical inputs has progressively increased with the development of modern agriculture, the type (fertilizers *versus* pesticides), the quantity and the chemical composition (active compounds) of agrochemicals have constantly changed over time; most notably to circumvent issues linked to the adaptive resistance of pests and, more recently, in response to growing societal concerns (Howden et al., 2007; Bhandari, 2014; Prashar and Shah, 2016; Hawkins et al., 2019; Sharma et al., 2019). Taken together, these ideas suggest that the consequences of agriculture on wildlife linked to the reduction of natural habitats should have already occurred and that impoverished biodiversity in agricultural areas should be a ghost of past landscape changes (Harding et al., 1998; Cousins, 2009; Surasinghe and Baldwin, 2014). In contrast, current negative trends of wildlife population should be related to the consequences of agrochemicals either directly or due to their interactions with the constraints of habitat structure described above (Potts et al., 2010; Oliver and Morecroft, 2014) or due to their interactions with current climatic modifications ((De Frenne et al., 2019; De Lombaerde et al., 2022). As a consequence, it seems critical to continue to monitor the populations of species that have persisted in agricultural landscapes.

In this study, we assessed the presence, abundance and recent population trends of one such species, the spined toad (*Bufo spinosus*) in rural Western France. The spined toad is a terrestrial amphibian that has been shown to persist in agricultural habitats (Guillot et al., 2016) when critical landscape elements necessary to perform its life-cycle are still present in the environment (i.e., reproductive ponds). Indeed, as most terrestrial amphibians, this species has a biphasic life cycle with an extensive use of terrestrial habitats during most of the year and a short breeding season in ponds where mating occurs and eggs and tadpoles develop (Reading, 1998; Semlitsch, 2008; Kelleher et al., 2018). In order to describe the effects of agriculture on the persistence of this species, we used three complementary approaches. First, in 2021 and 2022, we assessed the presence of reproductive individuals in ponds (N=23 sites) located along a gradient of habitats from preserved (forests) to highly agricultural sites. Second, on a representative subsample of the same sites (N=8 sites) and during the same years, we quantified abundances of reproductive individuals (males and females) during the whole reproductive season (~2.5 months). Finally, on a few sites that have been monitored for other purposes since 2015 (N=5 sites), we used capture data of reproductive males as an index of abundances to describe temporal trends.

Material and methods

Study species

Spined toad (*Bufo spinosus*) is one of the most common terrestrial amphibians in Western Europe. This species can live in a wide variety of habitats and has been shown to persist in agricultural areas (Guillot et al., 2016). Juveniles and adults are terrestrial most of the year, but reproduce in aquatic sites (ponds) where eggs and larvae develop during 2 to 3 months (Reading and Clarke, 1983; Reading, 1998; Kelleher et al., 2018). At the beginning of the reproductive season, toads migrate to breeding sites where males can remain for several weeks, while females occur shortly for mating and egg-laying (Reading and Clarke, 1983; Reading, 1991, 1998).

Presence, abundance and recent population trends

First, in 2021 and 2022, we assessed the presence of reproductive toads in 23 sites (Appendix 1). These sites were monitored during 2 to 3 nights (separated by 2 to 4 days) during the peak of toad abundance at their aquatic breeding sites (from our abundance surveys, see below). During these surveys, ponds and their surroundings were monitored at night (between 9 pm and 1 am) with headlamps to locate individuals. We recorded the presence (1 for presence and 0 for absence) of breeding individuals and whether these individuals were males (1 for presence and 0 for absence) or females (1 for presence and 0 for absence) as the sexual dimorphism in this species allows straightforward sexing without capture (Hemelaar, 1988).

Second, in 2021 and 2022, we assessed the abundance of reproductive toads in 8 sites from the ones surveyed for toad presence (Appendix 1). These sites were monitored three times a week (Monday, Wednesday and Friday) from late January (25th in 2021 and 31st in 2022) before the arrival of the first reproductive individuals, until the departure of the last reproductive individuals (April 9 in 2021 and April 11 in 2022). During these surveys, the ponds and their surroundings were monitored at night (between 9pm and 1 am) with headlamps and the number of males and females sighted were counted. From these nightly count data, we extracted abundances (total number of individuals counted [total, males or females], maximum number of individuals counted during a single night [total, males or females] and mean number of individuals counted during the whole breeding season for each site [total, males or females]). Such monitoring could not be carried out on all the 23 sites for logistical reasons. The 8 sites were selected because they represent the variety of agricultural landscapes that could be found in the area (Appendix 1).

Finally, we used data collected for other studies to assess recent population trends in 3 sites situated in agricultural settings and 2 sites situated in preserved habitats (Appendix 1). On these sites, only males were monitored (Guillot et al., 2016; Brischoux et al., 2018; Brischoux and Cheron, 2019; Brischoux et al., 2021; Renoirt et al., 2021a; unpublished data).

Since 2015, we aimed at capturing 20 to 40 per study sites. Although such sample sizes were readily obtained during a single night during the reproductive peak at the beginning of our projects, it became increasingly difficult to obtain these numbers during the subsequent years in those given sites (see results). We used these capture data (number of captured males during a single night situated around the peak of reproduction) in order to monitor broad proxy of abundances across years. Although we acknowledge that this dataset has not been designed to thoroughly monitor toad abundances, we emphasize that the trends in the number of captured individuals across years should describe, at least in a qualitative way, the recent population trends in specific areas.

Habitat classification

The terrestrial part of the life cycle of toads occurs usually within 1 km from the breeding ponds (Kovar et al., 2009; Janin et al., 2011; Guillot et al., 2016). As a consequence, from aerial pictures of each study site (GoogleEarth), we drew buffers with a radius of 1 km, corresponding to the potential distance travelled by an individual to reach a breeding site (Kovar et al., 2009; Janin et al., 2011; Guillot et al., 2016). We extracted surface area of the main habitat types surrounding each study site: forests and woods, hedges, agricultural fields, meadows and buildings (small villages) using QGIS. We used the PC1 value from a principal component analysis (PCA) of these five variables to attribute a habitat score to each site. The PC1 of the sites for which we assessed toad presence accounted for 51.9% of the total variance and was positively correlated with agricultural fields ($r=0.82$) and negatively correlated with forest ($r=-0.91$). The PC1 of the sites for which we assessed toad abundances accounted for 65.0% of the total variance and was positively correlated with agricultural fields ($r=0.90$) and negatively correlated with forest ($r=-0.96$). For the sites used for assessing recent population trends, we used habitat categories (agriculture *versus* forest, see Renoirt et al. 2021a for details).

Statistical analyses

We used Generalized Linear Mixed Models (GLMM) with a binomial distribution to assess the influence of the habitat score on the presence (1) or absence (0) of toads (overall, males or females). Some sites were monitored 2 years of our study (N=10), while others were monitored once (N=13). Year was set as a fixed factor and site identity as a random factor in our models.

We used Generalized Linear Mixed Models (GLMM) to assess the influence of habitat score on toad abundance (total, maximum and mean number) for all individuals and males or females, separately. Most sites were monitored during 2 years (N=7), while one site was included during a single year (N=1), as previously we added year as a fixed factor and site identity as a random factor in our models.

Finally, we used GLMM to analyse our proxy of recent population trends with the number of captured males as a response variable, and year and habitat category (agriculture *versus* forest) as predictors, we used site identity as a random factor in our models (the number of sites varied according to year).

Results

Toad presence

Models including overall toad presence were identical as those including the presence of males solely, with only 4 sites (all agricultural with positive PC1 scores) for which males were present but females absent.

We found a significant negative effect of the habitat score on the presence of both males ($X^2=9.525$, $df=1$, $p=0.002$) and females ($X^2=11.77$, $df=1$, $p<0.001$), with presence decreasing with increasing agriculture (Fig. 1).

Toad abundance

In males, reproductive phenology was not affected by the habitat score either for arrival dates ($X^2=0.052$, $df=1$, $p=0.820$) or departure dates ($X^2=2.233$, $df=1$, $p=0.135$), but we found a difference between years for both parameters (respectively $X^2=5.774$, $df=1$, $p=0.016$ and $X^2=16.532$, $df=1$, $p<0.001$). In females, dates of arrival at reproductive sites were not affected by habitat score ($X^2=0.160$, $df=1$, $p=0.689$) while departure date were earlier in agricultural sites ($X^2=8.346$, $df=1$, $p=0.004$) and both dates varied between years (respectively $X^2=10.951$, $df=1$, $p<0.001$, $X^2=18.346$, $df=1$, $p<0.001$).

We found a significant negative effect of the habitat score on the total number of individuals ($X^2=1573.68$, $df=1$, $p<0.001$), the total number of males ($X^2=1456.19$, $df=1$, $p<0.001$) and the total number of females ($X^2=87.45$, $df=1$, $p<0.001$). These numbers varied between years in females ($X^2=75.46$, $df=1$, $p<0.001$) but not in males ($X^2=0.79$, $df=1$, $p=0.375$).

We found a significant negative effect of the habitat score on the maximum number of individuals ($X^2=279.15$, $df=1$, $p<0.001$), the maximum number of males ($X^2=230.29$, $df=1$, $p<0.001$, Fig 1) and the maximum number of females ($X^2=38.40$, $df=1$, $p<0.001$, Fig 1). These numbers varied between years in both males ($X^2=22.89$, $df=1$, $p<0.001$) and females ($X^2=8.65$, $df=1$, $p=0.003$).

Finally, we found a significant negative effect of the habitat score on the mean number of individuals ($F_{1,12}=9.057$, $df=1$, $p=0.011$), the mean number of males ($F_{1,12}=8.80$, $df=1$, $p=0.012$) and a marginally negative significant effect for the mean number of females ($F_{1,12}=4.044$, $df=1$, $p=0.064$). These numbers did not vary between years for both sexes (respectively $F_{1,12}=0.356$, $df=1$, $p=0.562$, $F_{1,12}=1.034$, $df=1$, $p=0.328$).

Population trends

We found a significant interaction between the habitat type and the number of males captured between years ($F_{2,16}=6.59$, $p=0.02$). Number of captured males in agricultural sites decreased strongly between years ($F_{1,11}=63.34$, $p<0.0001$, Fig. 2), while the number of captured individuals from forest sites remained steady ($F_{1,5}=0.08$, $p=0.78$, Fig. 2).

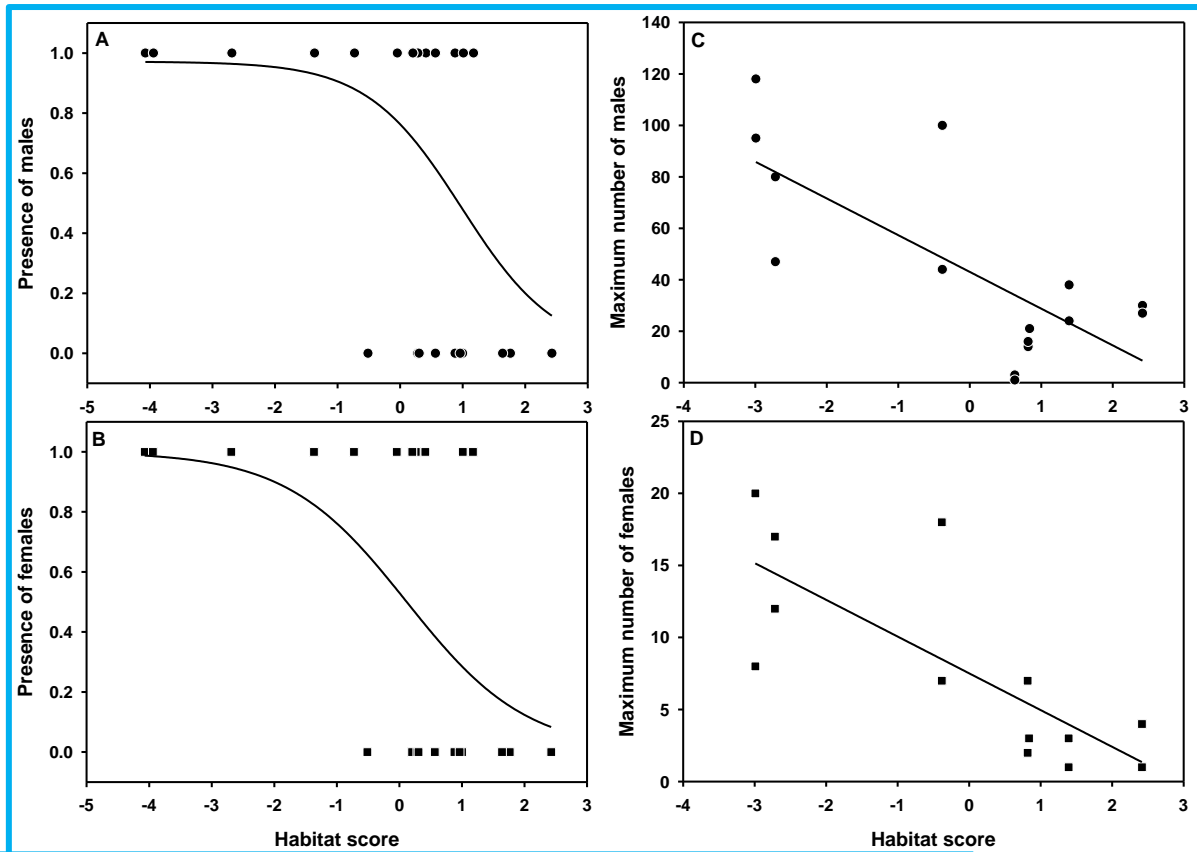


Figure 1. Right panels: presence (1) and absence (0) of males (A) and females (B) spined toads during two years (2021 and 2022) in 23 breeding sites situated along a gradient of habitats from preserved sites (negative scores) to intensive agriculture (positive scores). Left panels: abundances (maximum number of individuals counted during a single night during the breeding peak) of males (C) and females (D) spined toads during two years (2021 and 2022) in 8 sites situated along a gradient of habitats from preserved sites (negative scores) to intensive agriculture (positive scores). All sites were not monitored during all years and details can be found in Appendix 1.

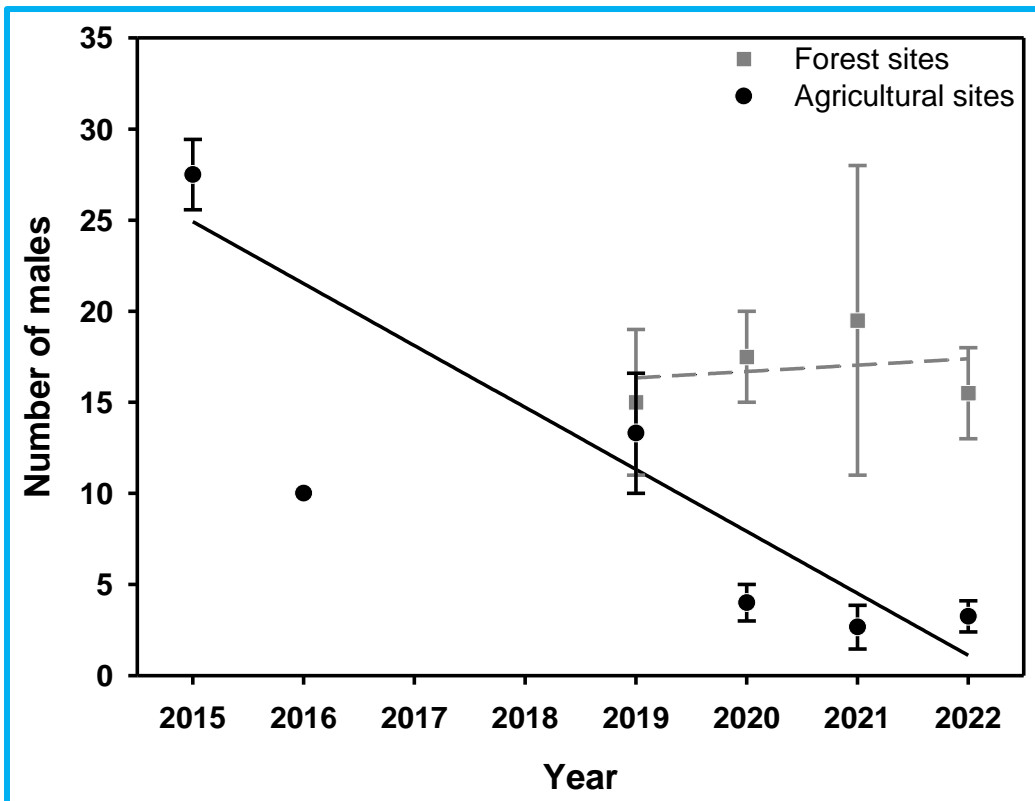


Figure 2. Number of males captured during the course of other studies on spined toads (see methods for details). We used these capture data (number of captured males during a single night situated around the peak of reproduction) in order to monitor broad proxy of abundances across years. All sites were not monitored during all years and details can be found in Appendix 1.

Overall, we found that both presence and abundances of a widespread terrestrial amphibian were markedly lower in reproductive ponds surrounded by intensive agriculture. The most salient, yet worrisome, result of our study is the ongoing decline of this species in such farmland habitats. Indeed, this result suggests that unknown factors are currently affecting amphibian populations and have recently induced strong population decreases over the course of a few years.

Although spined toads have been earlier shown to persist in agricultural habitats (Guillot et al., 2016), our results show that, as for many other farmland species, agriculture negatively influenced the presence of reproductive individuals at breeding sites (Keller and Waller, 2002; Williams et al., 2015; Tucker et al., 2018). Importantly, although such effect was found in both males and females, in 4 of the study sites (25% of the sites with a positive habitat score and thus characterized by intensive agriculture) we did not observe any reproductive female. Such result dovetails relatively well with previous observations made on the same species and which have highlighted the lack of reproductive females at some sites situated within agricultural landscapes (Renoirt et al., 2021b). Although the putative sex-specific mechanisms presumably affecting females more than males in agricultural habitats remain unknown, the lack of reproductive females at some breeding ponds is likely to jeopardize population persistence in agricultural habitats.

In sites where spined toads were present for reproduction, abundances of both males and females were strongly reduced in agricultural habitats. Several hypotheses can explain this result. First, for growing juveniles and adult individuals, agricultural habitats may be characterized by lower carrying capacity, both in term of terrestrial microhabitats availability (buffered and concealed retreat sites to evade predation and decrease thermal and hydric constraints, Tuomainen and Candolin, 2011; Oliver and Morecroft, 2014) during the terrestrial part of the life cycle and/or in term of trophic resources availability (decreased abundances of prey, Hart et al., 2006; Wagner, 2020). Such constraints would inevitably increase intraspecific competition for these limiting resources and thus decrease abundances of toads in agricultural habitats. Second, for developing eggs and larvae, the quality of aquatic breeding sites may be lower in agricultural landscapes. For instance, the presence of environmental contaminants in such sites (Bókony et al., 2018; Leeb et al., 2020) may well negatively influence the survival of embryos and larvae (Bókony et al., 2018; Cheron et al., 2022a, 2022b) and/or the quality of metamorphic individuals (Boone et al., 2005). We emphasize that these hypotheses are not mutually exclusive and it is likely that reduced abundances of spined toads in agricultural landscapes may result from complex interactions between various habitat-specific constraints. Deciphering the relative role of these different constraints will require future investigations.

Importantly, our results on recent population trends can give further insights into these processes. Indeed, although we emphasize that these data were not designed to thoroughly assess population abundances over time and thus need to be handled with caution; the trends we highlighted seem to indicate very recent population decreases in agricultural habitats as compared with preserved forest habitats. In line with the ideas developed above (see Introduction), such result may indicate that the structural constraints of agricultural habitats (linked to previous landscape homogenization and fragmentation) may not be the primary driver of the current decreased presence and abundances of spined toads. Indeed, based on examination of aerial photographs (GoogleEarth) and on our knowledge of the study area, no structural changes have occurred since the beginning of our surveys (e.g., land consolidation). In combination with recent population trend, such information suggests that the decline we are currently witnessing may be linked either indirectly to a concomitant decline in prey abundances (Hart et al., 2006; Wagner, 2020) and/or to other factors affecting directly spined toads. In this respect, the potential role of environmental contamination seems a likely candidate knowing the detrimental effects of agrochemicals on wildlife (Kendall and Akerman, 1992) and more specifically on amphibians (Baker et al., 2013; Trudeau et al., 2020). Alternatively, but not exclusively, it is also plausible that recent changes in climatic conditions, which apply more strongly in open habitats than under forest canopies (De Frenne et al., 2019; De Lombaerde et al., 2022), affected amphibian populations in agricultural areas (e.g., due to increased temperature and reduced precipitation, Lawler et al., 2010). Whatever the underlying mechanisms, the putative sex-specific mechanisms presumably affecting females more than males (see above, Renoirt et al., 2021b) are required to be deciphered.

Overall, our study potentially highlights a worrying recent decline in the populations of a widespread terrestrial amphibian previously thought to persist in agricultural landscape. We emphasize that unknown factors are currently affecting these populations very rapidly. Future investigations are required to identify these factors at a time when anthropogenic activities are currently leading to unprecedented rates of biodiversity loss.

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Supplementary file

Site#	Habitat score	Presence	Abundance	Captures
1	-4.07	2021-2022	2021-2022	2019-2022
2	-3.94	2021-2022	2021-2022	2019-2022
3	-2.69	2022		
4	-1.37	2021-2022	2021-2022	
5	-0.73	2022		
6	-0.51	2022		
7	-0.04	2022		
8	0.20	2021-2022	2021-2022	2015, 2019-2022
9	0.21	2021-2022	2021-2022	
10	0.26	2021-2022	2021-2022	
11	0.28	2021-2022		
12	0.28	2022		
13	0.30	2021		
14	0.41	2021	2021	
15	0.56	2021-2022		
16	0.88	2021-2022		
17	0.96	2021		
18	1.00	2022		
19	1.01	2022		2015, 2016, 2019, 2021, 2022
20	1.17	2021-2022	2021-2022	2015, 2020-2022
21	1.64	2022		
22	1.76	2022		
23	2.42	2021		

Appendix 1. Description of the sampling details for our three complementary approaches (presence, abundance and index of population trends using capture data). Sites are numbered (Site#) according to ascending habitat score (Habitat score) from the PC1 values of a principal component analysis (see methods for details). Years monitored are indicated for each parameter (Presence, Abundance, Captures) used in our analyses.

IV/ Conclusion

Dans ce chapitre nous avons mis en évidence un déclin récent et inquiétant des populations de crapaud épineux en milieu agricole. Ce résultat tend à remettre en doute la persistance de cette espèce dans les paysages agricoles. Etant donné que des études précédentes, sur des sites d'études communs (voir Guillot et al., 2016), ont montré la présence d'individus reproducteurs, et malgré le fait que nous ne connaissons pas les causes, nous soulignons des effets rapides du contexte paysager sur la reproduction des populations de crapauds épineux. De plus, comme supposé dans le chapitre précédent les femelles ont l'air plus contraintes par ce type d'habitat, et notamment en période de reproduction, que les mâles. Ces résultats appuient les suggestions précédentes indiquant d'intégrer les femelles dans des études de génétiques des populations afin de déterminer leur rôle relatif dans la reproduction, dans le maintien de la diversité génétique et dans la persistance des populations en milieu agricole. Nous suggérons aussi que l'agriculture intensive peut impacter directement et/ou indirectement le succès reproducteur et les populations futures de crapaud épineux à travers la qualité individuelle, la qualité de la descendance et à travers de possibles interférences de la contamination environnementale.

CHAPITRE 3

**Etude de la qualité de la descendance en lien
avec la qualité parentale et selon un contexte
paysager**

Dans ce chapitre, et étant donné que l'environnement agricole a l'air de contraindre la reproduction du crapaud épineux, nous nous sommes concentrés sur les relations entre la qualité individuelle des parents, le succès reproducteur et la qualité de la descendance en fonction de l'habitat d'origine. Il faut prendre en compte que cette thèse a eu lieu pendant la période de pandémie de la « COVID 19 » et que, en plus du confinement, nous avons été confrontés à une absence de reproduction en lien avec une absence de femelles, pour les sites agricoles (**Article 3**). De ce fait, nous avons quand même décidé de valoriser les résultats obtenus en écartant la notion d'habitat et en nous concentrant sur les relations entre qualité parentale, succès reproducteur et qualité de la descendance à partir d'une étude corrélative (**Article 5**). De ce fait, nous avons réalisé l'année suivante la même expérience tout en prenant en compte la variable d'habitat d'origine des parents afin de comparer les mêmes relations mais selon un contexte paysager dégradé-conservé (**Article 6**). A noter que l'article 6 n'est pas totalement finalisé et des modifications supplémentaires seront apportées par la suite.

Pour les écologistes et les biologistes, les effets parentaux sur la descendance sont actuellement un centre d'intérêt particulier (Badyaev and Uller, 2009) puisque la question de savoir comment la variation de l'environnement fourni par les parents affecte le phénotype de la progéniture est centrale (Groothuis et al., 2005; Green, 2008; Marshall and Keough, 2007). Dans de nombreuses études il a été mis en avant des relations significatives entre les traits parentaux et les traits de la progéniture telle que l'immunité (Soler et al., 2003; Kilpimaa et al., 2005; Pitala et al., 2007), le comportement (Forstmeier et al., 2004), l'histoire de vie (Hunt and Simmons, 2002; Fox et al., 2004; Charmantier and Garant, 2005) mais surtout la morphologie (Kruuk et al., 2001; McAdam et al., 2002; DiBattista et al., 2009) et le rythme de développement (Fox, 1994; Rauter and Moore, 2002; Winn, 2004). De ce fait, bien que leur ampleur diffère selon les traits de la progéniture, les environnements et les étapes de l'histoire de la vie, les effets parentaux sont omniprésents. Les effets parentaux dépendent souvent de l'environnement et peuvent être plus prononcés dans les environnements de mauvaise qualité (McAdam et al., 2002; Charmantier et al., 2004). Ceci est bien soutenu par les observations selon lesquelles la variance environnementale, à laquelle les effets parentaux contribuent, augmente alors que l'héritabilité diminue dans les environnements de mauvaise qualité (Merilä and Sheldon, 2001; Charmantier and Garant, 2005).

Par exemple, la qualité du régime alimentaire est un déterminant environnemental clé de la taille corporelle individuelle, qui reflète souvent la condition (Blanckenhorn, 2000). Ainsi, des effets maternels ou paternels dépendants de l'environnement peuvent être sélectionnés par la variation environnementale du régime alimentaire (par opposition à des effets parents d'origine potentiellement indépendants de l'environnement, tels que l'empreinte génomique, voir par exemple Fitch et al., 1998 ; Lloyd, 2000). Alors, les individus qui acquièrent une condition élevée dans un environnement riche en ressources peuvent bénéficier du transfert de leur condition à leur progéniture, améliorant ainsi la condition physique de cette dernière (Mousseau and Fox, 1998; Pál and Miklós, 1999; Qvarnström and Price, 2001) et si l'environnement (par exemple, le régime alimentaire) que les parents connaissent prédit l'environnement que leur progéniture rencontrera, les parents peuvent être sélectionnés pour optimiser le phénotype de la progéniture pour cet environnement (Gilchrist and Huey, 2001; Rotem et al., 2003; Holbrook and Schal, 2004). Cela permet à la progéniture soit d'avoir une condition plus élevée et de mieux se porter dans un environnement de mauvaise qualité (e.g. milieux agricoles), soit, que la progéniture sera plus résistante aux contraintes environnementales d'un milieu dégradé.

II/ Article 5

What are the contributions of maternal and paternal traits to fecundity and offspring development? A case study in an amphibian species, the spined toad (*Bufo spinosus*)

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Assessing the determinants of reproductive success is critical but often complicated because of complex interactions between parental traits and environmental conditions occurring during several stages of a reproductive event. Here, we used a simplified ecological situation – an amphibian species lacking post-oviposition parental care and for which breeding site selection can be excluded as a parental effect – and a common garden approach to investigate the relationships between parental (both maternal and paternal) phenotypes (body size and condition) and reproductive success (fecundity, egg size, embryonic and larval duration, larval and metamorphic morphology). We found significant effects of maternal phenotype on fecundity, hatching success and hatchling size, as well as on the duration of larval development. Interestingly, and more surprisingly, we also found significant contributions of the paternal phenotype occurring both during early (embryonic development duration) and relatively late (duration of larval stages close to the metamorphosis) offspring development. Our findings indicated very few additive effects of both parental traits but rather separate maternal and paternal influences on specific stages of offspring development. Although our study focused on morphological traits solely, we suggest several hypotheses involving physiological costs of development. Future studies are required to decipher the mechanisms underlying our findings in order to clarify the mechanistic basis of the links between parental phenotypes and offspring development.

Keywords: Phenotype - Reproductive success - Clutch quality - Embryonic development - Larval development.

Assessing the determinants of fecundity and offspring quality; and thus reproductive success remains an essential question in evolutionary ecology (Pianka, 2011). This is especially the case at a time when natural (ancestral) environmental constraints on the reproductive biology of most species are disrupted by novel, additional anthropogenic sources of perturbations (Moore and Waring, 2001; Rhind, 2009; Seress and Liker, 2015). Indeed, reproductive success often depends on a variety of parameters which include both parental traits and environmental conditions. Importantly, parental traits and environmental conditions can interact during multiple stages of a reproductive event to affect reproductive success (Kölliker et al., 2014; Ratikainen et al., 2018). It is thus critical to thoroughly investigate determinants of reproductive success across a variety of taxa if we are to understand the consequences of anthropogenic global change on the persistence of populations (Dahlhoff et al., 2008; Massot et al., 2008; Loarie et al., 2009; Auer and Martin, 2013).

Reproductive success is known to be particularly dependent on the quality of parental organisms (Kölliker et al., 2014; Ratikainen et al., 2018). Such parental quality is expressed through complex interactions between ecology, physiology and behaviour during reproduction (Moczek, 1998; Bradshaw and McMahon, 2008; Cauchard et al., 2013). For instance, parental quality can influence reproductive success through processes that include the selection of suitable reproductive sites, the selection of suitable mates, the production of gametes and the energetic investment during embryonic development (Amos et al., 2001; Refsnider and Janzen, 2010; Cauchard et al., 2013; Kölliker et al., 2014; Ratikainen et al., 2018). In addition to these traits which occur relatively early during reproduction, other determinants of reproductive success can occur later and are often expressed through the parental care to the progeny and the quality of the environment where reproduction takes place (Clutton-Brock, 2019). In many cases, both environmental characteristics and parental traits interact to determine reproductive success, sometimes during several stages of a reproductive event (Hoy et al., 2016). As a consequence, it is often difficult to tease apart the relative contributions of parental (including both maternal and paternal organisms) and environmental factors on reproductive success (Ridley, 2007; Hoy et al., 2016).

Some ecological situations offer relevant opportunities to simplify such complex interactions between environmental and parental characteristics. This is typically the case for species lacking post-oviposition parental care and for which breeding site selection can be excluded as a parental effect (Heisswolf et al., 2005; Refsnider and Janzen, 2010). Indeed, such situation allows to reduce the influence of parental organisms to few simple factors of the reproductive investment, such as the quality of the gametes and the energetic investment in the eggs (Ratikainen et al., 2018). In such context, it is thus possible to directly assess how maternal and paternal traits (proxies of individual quality, Wilson and Nussey, 2010) can affect fecundity and offspring quality; and thus reproductive success (Ratikainen et al., 2018).

Amphibians are one such taxa allowing to simplify complex interactions between environmental and parental traits. Indeed, many amphibian species lack parental care (Wells, 2010) and lay their eggs communally (i.e., in the same breeding pond, Doody et al., 2009). In these taxa, maternal phenotype (e.g., body size, mass and condition) has been shown to positively affect fecundity and egg size (Castellano et al., 2004) which, in turn, positively affect subsequent larval quality (Laugen et al., 2002; Loman, 2002). Interestingly, as in most vertebrate species, the influence of paternal phenotype on reproductive success has been overlooked to date (Moiron et al., 2020 but see Lange et al., 2021), presumably because of the predominant role of females for reproduction in most systems (Parker and Begon, 1986; Caro et al., 2008). Yet, it is widely recognized that reproductive success depends on both maternal and paternal traits, even in species in which the contribution of paternal organisms to reproduction is reduced to the fertilization of the eggs (Brommer and Rattiste, 2008; Germain et al., 2016). It is thus essential to include paternal phenotypes in addition to maternal traits to thoroughly assess the effects of parental traits on fecundity and offspring quality, and thus reproductive success.

In this study, we used a common garden experiment to investigate the relationships between parental (both maternal and paternal) phenotypes (body size and condition) and reproductive success in an amphibian species that breeds communally and lacks post-oviposition parental care, the spined toad (*Bufo spinosus*). Pairs of breeding toads (amplexus) were captured in the field after that mate selection occurred and brought back to the laboratory before egg laying, which allowed us to monitor the whole reproductive event (from egg laying to metamorphosis) under controlled conditions. With such a design, we were able to assess whether maternal and paternal phenotypes relate to each other (i.e., indicating assortative mating, Chajma and Vojar, 2016 but see Green, 2019), and to assess how maternal and paternal phenotypes influenced fecundity (number and size of the eggs), embryonic development traits (duration, mortality and hatchling size) and larval development traits (duration, mortality, morphology across key developmental stages) up to metamorphosis (body size, mass and condition).

Material and methods

Study species and sampling

Spined toad (*Bufo spinosus*) is one of the most common amphibian species in western Europe. Breeding occurs between late winter and early spring (mid-January to late March) depending on the climatic conditions. During breeding, adults converge to reproductive ponds where they pair and lay their eggs. Both embryonic and larval developments occur in reproductive ponds.

Sampling took place in February 2020 on 3 breeding sites situated nearby the laboratory (CEBC, 46° 8' 48.64"N; 0°25'30.86"W). All sites were monitored from the onset of the breeding season (mid-January). Captures were conducted at night using headlamp and toad pairs (hereafter amplexus) were caught using a net. We collected a total of 23 amplexus which were brought back to the laboratory until laying.

Parental traits and fecundity

At the laboratory, each amplexus was separated and males and females were individually weighted (± 0.01 g). Each pair was reunited in plastic containers (59x36x28 cm) containing 30L of dechlorinated tap water, a rock, and a branch (for egg attachment). Each amplexus was monitored several times a day until completion of egg-laying, which occurred after 0-7 days (2.54 ± 0.38).

When egg-laying was completed, males and females were individually weighted and body size (snout to vent length, SVL) was measured using a calliper (± 0.01 mm). All individuals were released at the site of capture within one day after laying.

The clutch of *Bufo spinosus* is formed by elongated egg strings containing 3000-5000 eggs (Cheron et al., 2021a). In order to assess the fecundity of each amplexus, each egg string was placed in a container (35x20x25cm) containing 2 cm of dechlorinated tap water and a scale (graph paper). A picture was taken in order to measure the total length of the egg string using ImageJ software (Schneider et al., 2012). For each clutch, we randomly selected 5 segments of 10 cm long and individually counted the number of eggs within each segment. Mean number of eggs per 10cm segment was calculated and used to assess fecundity (number of eggs) for each clutch based on the length of the egg strings.

Embryonic and larval development

For each clutch, we randomly subsampled 4 pieces containing 34 eggs that were kept for our experiment. The remaining eggs were released at their site of origin. Each piece was placed in a Petri dish above graph paper and a picture was taken in order to measure egg size (diameter) using the ImageJ software (Schneider et al., 2012). We collected a total of 136 values of egg diameter per clutch. Each segment was then individually transferred in glass tanks (18x13x18cm) containing 2L of dechlorinated tap water (changed every week) until hatching. Hatching occurred at Gosner stage 25 (Gosner, 1960) and for each segment we recorded the duration of embryonic development and the number of undeveloped embryos.

Upon hatching, we randomly selected 6 tadpoles per segments to monitor the larval development until metamorphosis (N= 138 tadpoles). The remaining individuals were released at their site of origin. Tadpoles were raised individually in glass tanks (18x13x18cm) containing 2L of dechlorinated tap water. During the larval development, tadpoles were fed *ad libitum* with organic chopped spinach and water was changed every week. We used morphological features to classify developmental stages according to Gosner (1960). We selected Gosner stages 25, 30, 37, 41 and 42 (hereafter, GS 25, GS 30, GS 37, GS 41 and GS 42, respectively) in order to monitor larval development (Cheron et al., 2021b). For each stage, we measured total length and tail length following Cheron et al. (2021a). Each tadpole was put into a Petri dish with the water from its own tank, and photographs were taken from above the Petri dish. Morphological measurements were performed with the software ImageJ (Schneider et al., 2012).

At metamorphosis (Gosner stage 46, Gosner, 1960), toadlets were measured (SVL) and weighted and individually transferred to a plastic box (17x15x9cm) with damp paper towel as substrate and a shelter. Measurements were reiterated 5 days after metamorphosis and individuals were then released at their site of origin.

All the experiments took place in a thermally controlled room with the temperature set at 17 °C (both air and water). The photoperiod was controlled (12 h dark–12 h light).

Statistical analyses

All data were tested for homogeneity of variance, residues independence and normality with the Bartlett test, Dubin-Watson test and Shapiro-Wilks test, respectively. We also checked the residues normality using diagnostic plots. All statistical analyses were carried out with R.Studio v 1.2.1335 (R Core Team., 2019).

For adult individuals, we quantified a body condition index (BCI) using residual scores from the linear regression between body size and body mass independently in males ($F_{1,21} = 14.78$, $r^2 = 0.385$, $P < 0.001$) and females ($F_{1,21} = 48.38$, $r^2 = 0.682$, $P < 0.001$).

We fitted linear mixed models (LMER, package lmerTest. Kuznetsova et al., 2015) to assess relationships between parental traits and fecundity, embryonic traits, and larval traits across.

First, to study the relation between fecundity and parental morphology (SVL, mass and BCI), we fitted two models with clutch size or egg size as dependent variables and parental morphometrics measurements as explanatory variables.

Second, for regarding embryonic development, we fitted models with hatching success, development duration or hatchling length (GS25) as dependant variable and parental morphometrics measurements as explanatory variables (SVL and BCI).

Finally, to test whether there was a relation between parental traits and larval development, we fitted several models with larval development duration, the development duration between different stages (for example: GS30 to GS37), tadpoles length at each stages and morphology of metamorphic individuals (SVL and BCI) as dependent variables and parental traits as explanatory variables (SVL and BCI).

Then, we performed model selection using Akaike criterion corrected for small sample size (AICc) to obtain the most parsimonious model with maximum likelihood. We selected random factors for each model with Akaike criterion. After random effects comparison, the models chosen were compared with each other and with the null model. We selected the model with the smallest AICc and we based model selection on $\Delta AICc < 2$ (Posada and Buckley, 2004). With this model selection, we determined the models with variables that had a significant effect. These models are further detailed (Table 1). The models with variables that do not have a significant effect are shown in Appendix 1).

Results

Relationships between parental traits

We did not find any relationship between male and female body size ($F_{1,21} = 0.016$, $P = 0.969$), body mass ($F_{1,21} = 0.122$, $P = 0.730$) or body condition ($F_{1,21} = 0.018$, $P = 0.966$).

Parental determinants of fecundity and egg size

Snout-vent length (SVL) and body condition index (BCI) were positively related to clutch size in females (SVL $F_{1,21} = 9.782$, $r^2 = 0.285$, $P = 0.005$ and BCI $F_{1,21} = 10.46$, $r^2 = 0.332$, $P = 0.004$, Figure 1, Table 1), but not in males (all $P > 0.666$, Table 1).

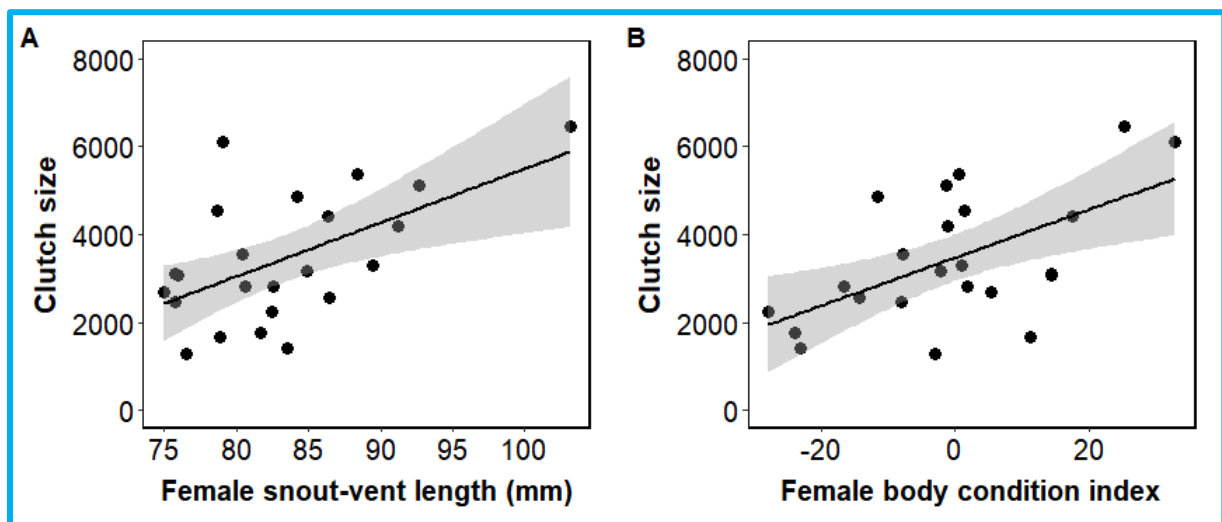


Figure 1. A: Relationship between female body size (SVL, mm) and fecundity (clutch size). B: relationship between female body condition and fecundity (clutch size). Grey shading indicate 95% confidence intervals.

		Df	AICc	Δ AICc	AICcWt	LogLik
<i>Parental fecundity</i>						
Clutch size	SVL_F	4	371.29	0.00	0.98	-178.88
	SVL_F + SVL_M	5	378.91	7.62	0.02	-184.35
	null	3	385.32	14.03	0.00	-187.55
	SVL_M	4	383.50	22.21	0.00	-193.12
Clutch size	BCI_F + BCI_M	5	373.26	0.00	0.94	-179.87
	BCI_F	4	378.99	5.73	0.05	-184.38
	BCI_M	4	386.97	13.71	0.00	-188.38
	null	3	393.50	20.24	0.00	-193.12
<i>Embryonic development</i>						
Hatching success	BCI_F	3	2827.09	0.00	0.56	-1410.54
	BCI_F + BCI_M	4	2827.60	0.5	0.44	-1409.79
	null	2	2838.10	11.0	0.00	-1417.05
	BCI_M	3	2838.79	11.7	0.00	-1416.39
Embryonic development duration	SVL_M	4	-1779.10	0.00	0.57	893.56
	SVL_F + SVL_M	5	-1777.06	2.04	0.21	891.53
	null	3	-1776.81	2.28	0.18	893.41
	SVL_F	4	-1774.02	5.08	0.04	891.02
<i>Larval development</i>						
Hatchling length GS25	BCI_F	4	-1174.80	0.00	0.82	591.45
	null	3	-1171.70	3.10	0.18	588.88
	BCI_F + BCI_M	5	-1161.54	13.27	0.00	585.84
	BCI_M	4	-1158.24	16.56	0.00	583.17
Larval development duration	BCI_F	3	1009.35	0.00	0.93	-501.58
	null	2	1015.60	6.25	0.04	-505.75
	BCI_M	3	1017.38	8.03	0.02	-505.60
	BCI_F + BCI_M	4	1017.38	8.03	0.02	-505.60
Days between GS37 and GS41	SVL_M	3	714.90	0.00	0.55	-354.36
	SVL_F + SVL_M	4	717.02	2.12	0.19	-354.36
	null	2	717.06	2.16	0.19	-356.49
	SVL_F	3	719.15	4.25	0.07	-356.48
Hatchling length GS30	BCI_F + BCI_M	4	-91.89	0.00	0.58	50.10
	BCI_F	3	-91.05	0.85	0.38	48.61
	null	2	-84.89	7.00	0.02	44.49
	BCI_M	3	-84.67	7.23	0.02	45.42

Table 1. Models selection of the effects of parental phenotype (body size: SVL, body condition index: BCI, males: M and females: F) on reproductive success. AIC models with dependent variables and explanatory variables. Df stands for the degree of freedom of the models. AICc represent the Akaike criterion corrected value in ascending order. Δ AICc represent the interval between the AICc value of the most parsimonious model and the other models. AICcWT is the weight of the explanatory part of each model. LogLik is a model fitted by maximum likelihood. Only models for which we found significant influences of parental traits on offspring development are shown in this Table. All other (non-significant) models tested are detailed in Appendix 1.

The size of the eggs was not related to female or male traits (all $P > 0.307$).

Clutch size was negatively related to eggs size ($F_{1,3126} = 32.38$, $r^2 = 0.102$, $P < 0.001$, Table 1, Figure 2).

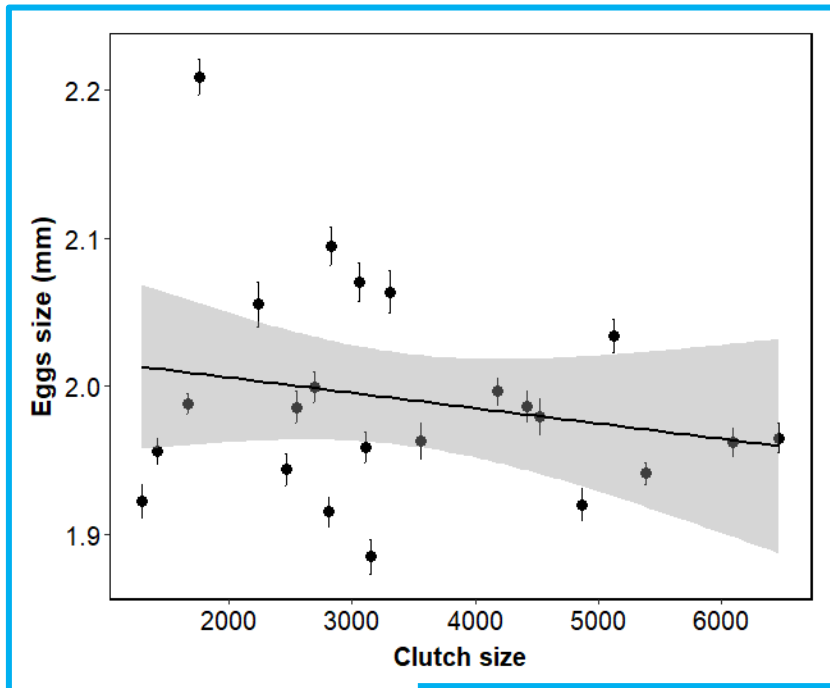


Figure 2. Relationship between fecundity (clutch size) and eggs size (mm). Grey shading indicate 95% confidence intervals.

Parental influences during embryonic development

Female BCI was positively related to hatching success and hatchling total length (respectively, $F_{1,3126} = 13.82$, $r^2 = 0.382$, $P < 0.001$ and $F_{1,89} = 15.90$, $r^2 = 0.689$, $P < 0.001$, Table 1, Figure 3) and male SVL was negatively related to embryonic development duration ($F_{1,90} = 10.564$, $r^2 = 0.096$, $P < 0.001$, Table 1, Figure 4). Other parental and embryonic traits were not related to each other (Appendix 1).

Eggs size and embryonic development duration were not related to hatching success (respectively $F_{1,3126} = 2.158$, $P = 0.141$ and $F_{1,3126} = 1.035$, $P = 0.307$), and eggs size was not related to hatchling size ($F_{1,424} = 0.3382$, $P = 0.561$).

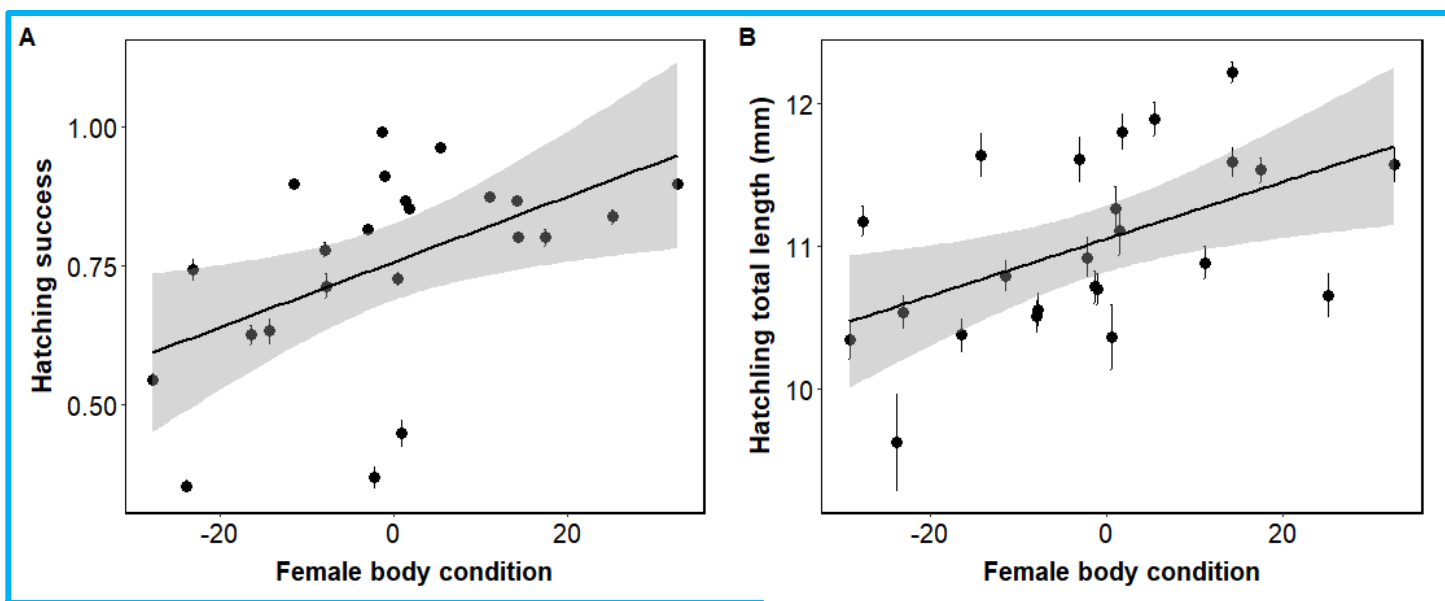


Figure 3. Relationships between female body condition and A: Hatching success (%) and B: hatchling size (total length, mm). Grey shading indicate 95% confidence intervals.

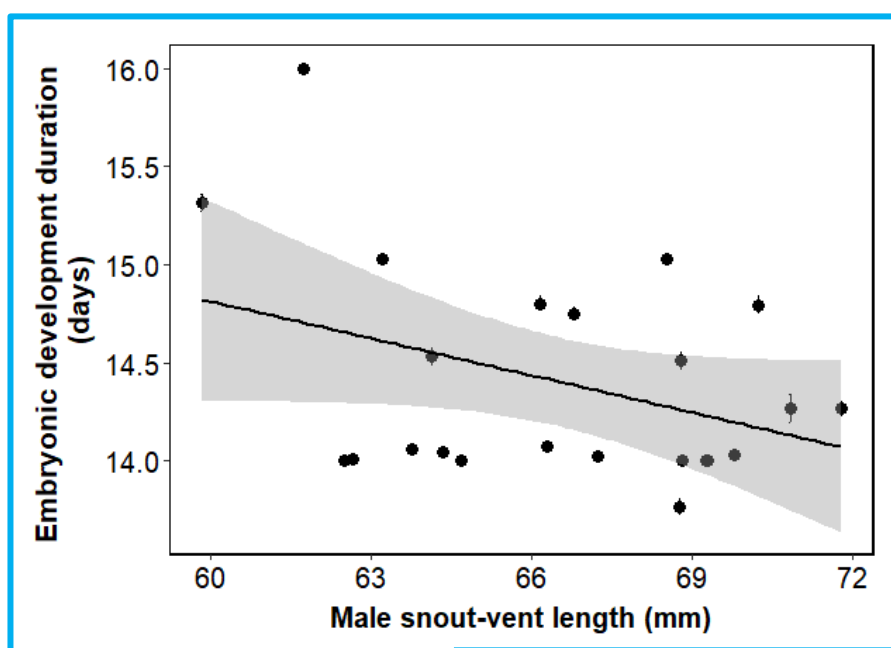


Figure 4. Relationship between embryonic development duration (days) and male body size (SVL, mm). Grey shading indicate 95% confidence intervals.

Larval development

Female BCI was positively related to the total duration of larval development and negatively related to the total length of tadpoles at GS30 solely (respectively, $F_{1,132}=8.484$, $r^2 = 0.599$, $P = 0.004$ and $F_{1,136}=8.373$, $r^2 = 0.510$, $P = 0.004$, Table 1, Figure 5).

Male SVL was negatively related to larval development duration between GS37 and GS41 ($F_{1,133}=4.259$, $r^2 = 0.237$, $P = 0.041$, Table 1, Figure 6).

Other parental and larval traits were not related to each other (Appendix 1).

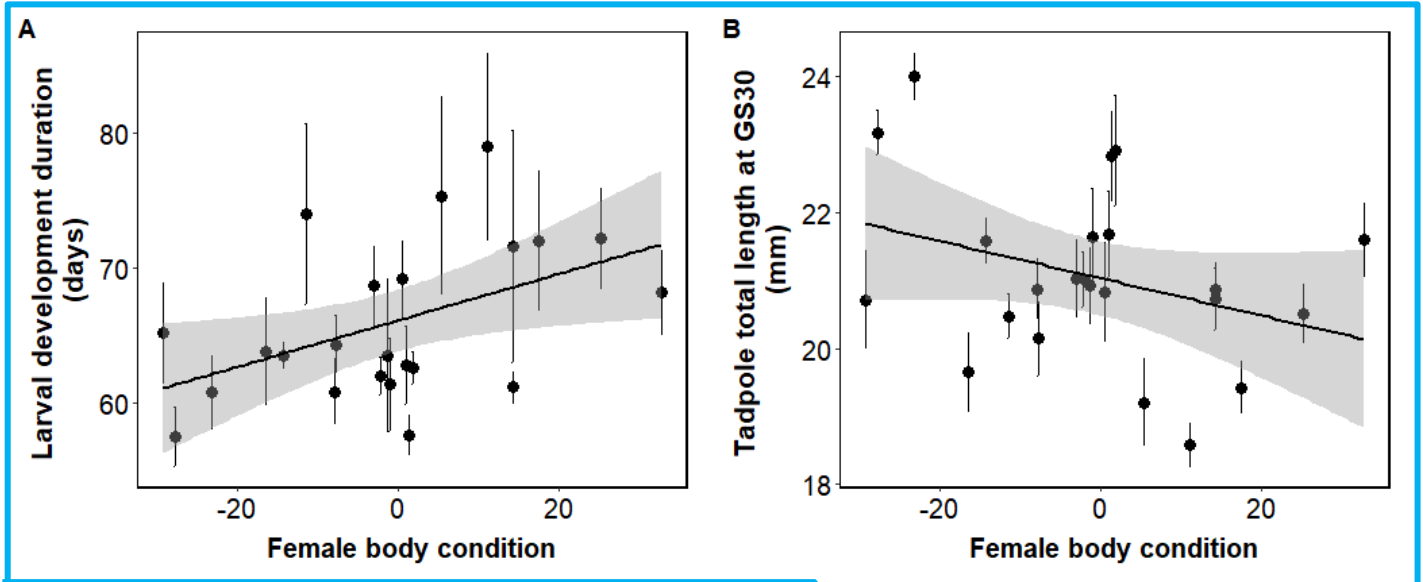


Figure 5. Relationships between female body condition and A: larval development duration (days) and B: tadpole size at GS30 (total length, mm). Grey shading indicate 95% confidence intervals.

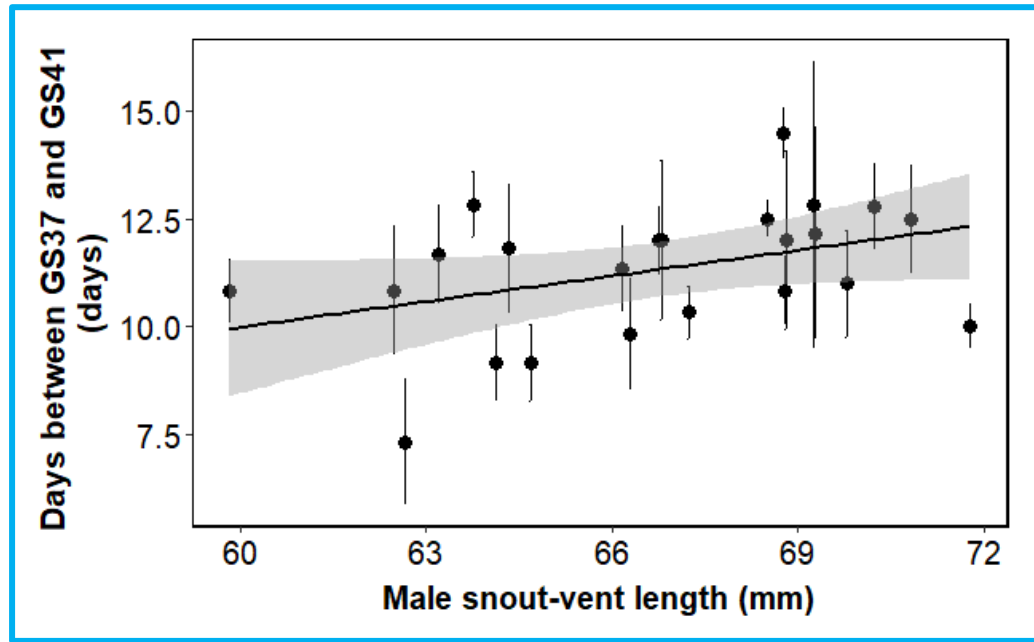


Figure 6. Relationship between male body size (SVL, mm) and the duration of the first steps of metamorphosis (elapsed days between GS 37 and GS41). Grey shading indicate 95% confidence intervals.

In this study, we investigated the effects of both maternal and paternal phenotypes on embryonic and larval development in an amphibian species. As expected, we found significant effects of maternal phenotype on fecundity, hatching success and hatchling size, as well as on the duration of larval development. Interestingly, and more surprisingly, we also found significant contributions of the paternal phenotype occurring both during early (embryonic development duration) and relatively late (duration of larval stages close to the metamorphosis) offspring development. Importantly, our results indicated very few additive effects of both parental traits but rather separate maternal and paternal influences on offspring development. Such lack of additive effect dovetails remarkably well with the lack of morphology-related assortative mating in our study species (see also Marco and Lizana, 2002).

Parental determinants of fecundity and egg size

We found that fecundity was strongly linked with maternal morphology. As expected, clutch size was positively related with female body size and body condition, indicating that larger and bigger females laid higher number of eggs. Such positive effect of female morphology on fecundity is a widespread relationship that has already been highlighted in amphibians (Gibbons and McCarthy, 1986; Castellano et al., 2004) as well as in other taxa (Hines, 1988; Blackmore and Lord, 2000). Interestingly, fecundity was also the sole trait for which our analyses highlighted an additive effect of both female and male morphology. Indeed, the model including body condition of both male and female performed better at explaining fecundity than models including the same parameter separately for both sexes (Table 1). This was an unexpected result as, in our study species, vitellogenesis occurs during the months prior to the reproduction (Feder and Burggren, 1992; Aranzábal, 2011; Rastogi et al., 2011). In our study model, it was also expected that females lay the entirety of their eggs during a single mating event (Kouba et al., 2009; Rastogi et al., 2011; Guy et al., 2020). Conversely, such result indicates that females may be able to modulate the number of eggs laid according to the body condition of their mate, and thus could assess the quality of the male with which they reproduce in order to adjust their reproductive effort (Reyer et al., 1999).

Future studies are required to investigate the mechanistic basis of such result in order to understand how female adjust their clutch size to the quality of their mate. Overall, parental condition appears as a major determinant of reproductive success through its influence on fecundity (Castellano et al., 2004).

Egg size was not related to parental phenotypes, but was negatively related to clutch size. Such result indicates that the classical trade-off between egg number and egg size apply in a species that produce remarkably high number of tiny eggs, as already highlighted in amphibians (Gould et al., 2022) as well as in other taxa (Jørgensen, 1984; Berven, 1988; Elgar, 1990). The lack of a direct relationship between parental phenotypes and egg size can plausibly be related to the relatively weak relationship between fecundity and egg size as well as the relatively large variance in egg size data (Figure 2).

Parental influence during embryonic development

We found strong relationships between maternal phenotype and embryonic development. First, females in better condition produced clutch with greater hatching success. In this species and its close relative *B. bufo* (Trujillo et al., 2017), hatching success has been either related to fertilization success (Touzot et al., 2020) or to embryonic mortality (Cheron and Brischoux, 2020; Cheron et al., 2021a). Our result demonstrates that the quality (body condition) of females positively influenced embryonic survival while our analyses revealed that male phenotype does not significantly affect hatching success. This result also reinforces the fact that, in this species, hatching success can be used as an index of clutch quality (Cheron et al., 2021a). Second, females in better condition produced longer hatchlings. This result suggests that the quality (body condition) of females can positively affect the quality of hatchlings (size). Such effect is likely to be mediated through condition-specific investment in egg composition (energetic reserves to sustain embryonic development, Bernardo, 1996; Kaplan and King, 1997; Mousseau and Fox, 1998). Such result further suggests potential long-term influence as larger hatching size has been shown to positively influence the survival of larvae facing relatively high levels of predation and competition during larval development (Kaplan and Phillips, 2006; Martin and Pfennig, 2010 see also : Benard, 2004; Burraco et al., 2017).

Interestingly, although our analysis does not reveal an influence of male traits on fertilization success (see above), we found that male traits were linked to the duration of embryonic development. The eggs fertilized by larger males produced embryos that developed quicker. The mechanisms underlying such result remain unknown and deserve specific investigations. Yet, it is likely that this effect is mediated through the quality of the sperm produced by larger – presumably older - males (Gasparini et al., 2010; Roth et al., 2010). Although, age-dependent sperm quality in amphibian species is expected to decrease through senescence (Hettyey et al., 2012), study found that older males displayed same fertilization capacity (e.g motility and concentration) as younger males (Watt et al., 2021).

In addition, it has also been shown that younger males produced more atypical spermatozoa than older males (Watt et al., 2021). Other, complementary mechanisms could potentially involve size-specific macro-molecular composition of spermatozoa affecting the duration of the first steps of fertilization (e.g., acrosome composition, chromatin unpacking) and/or the embryonic development (Gusseck and Hedrick, 1971; Lohka and Masui, 1983; Carroll Jr et al., 1991). Future studies are required to precisely identify the mechanisms that link paternal size and embryonic development duration. Such paternal influence on embryonic development duration may be critical to the survival of minute and immobile embryos that are susceptible to predation (Zamudio et al., 2016). Furthermore, longer embryonic development may also induce carry-over effects on the following larval stage duration, a potentially deleterious consequence if spawning takes place in ephemeral water bodies.

Parental influence during larval development

Parental phenotypes continued to influence offspring development during larval stages. Specifically, female body condition was positively related to the total duration of larval development. This suggests that larvae originating from larger clutch - but smaller eggs (see above) - took more time to successfully develop up to metamorphosis. This result further indicates that the trade-off between clutch size and egg size we detected can eventually bear long term consequences across the larval stage (Ficetola et al., 2011). Interestingly, this effect was supported by the negative relationship we found between female body condition and tadpole size during a key stage of larval somatic growth (GS30, Cheron et al., 2021b). Again, tadpoles that originated from larger clutch - but smaller eggs (see above) - were smaller during somatic growth. Surprisingly, we failed to detect such effects during later phases of larval development, and two different but non-mutually exclusive hypotheses could explain this lack of longer-term effects. First, later larval phases correspond to critical phases dedicated to the onset of metamorphosis. The remarkable modifications of tadpole morphology, behaviour and physiology (Cheron et al., 2021b) during such stages may have obscured such effect. Second, it is plausible that mechanisms of compensatory growth may have allowed smaller tadpoles to reach similar body size than their counterpart originating from smaller clutch (Hector et al., 2012). Such compensatory growth is likely knowing that tadpoles need to reach a minimal size to successfully complete metamorphosis (Wilbur and Collins, 1973). Although we lack behavioural data (e.g., feeding rates, activity level, Cheron et al., 2021b) to test for this hypothesis, future studies should usefully investigate whether tadpole behaviour could compensate for lower growth rates during early phases of larval development and whether such putative effects are mediated by parental phenotypes. Finally, another puzzling result involves the discrepancy between the larger size of hatchlings (GS 25) originating from larger clutch (see above) and the reverse trend we detected during later larval developmental stages (GS 30). Such effect may be mediated by the costs of quicker embryonic development, as it has been shown that embryos that develop more rapidly produced tadpoles with shorter telomeres and unbalanced oxidative status (Burraco et al., 2017; Cheron et al., 2021b, 2022a, 2022b).

If such hypothesis holds true, this could indicate that embryos that developed rapidly accumulated damages (i.e., unbalanced oxidative status, increased rates of telomeres attrition, Burraco et al., 2017) that require slowing down early phases of larval growth to be compensated for (Burraco et al., 2020). Acquiring detailed data on telomere size and oxidative status during the whole larval development is a logistically complicated endeavour and future studies are required to test for such hypothesis.

Interestingly, although our analysis does not reveal an influence of male phenotype on early larval phases, we found that male body size was linked to the duration of late stages of larval development. Specifically, larger males produced tadpole that developed quicker during the first steps of metamorphosis (i.e., GS37 to GS 41, Cheron et al., 2021b). Such delayed effect of paternal phenotype on larval development remains puzzling. The mechanistic bases of such phenomenon clearly represent potentially fruitful avenue of investigations. Importantly, this larval stage is situated at a pivotal stage with a marked reduction in locomotion performances which can affect the ability of tadpoles to evade predation (Johnson et al., 2015; Lindgren et al., 2018). Additionally, during this specific stage, decreasing the costs of locomotion (lower activity levels and swimming speeds) may allow tadpoles to dedicate more energy to metamorphosis (Beck and Congdon, 2003; Wright et al., 2011; Kirschman et al., 2017; Ruthsatz et al., 2020a). Overall, a decrease in the duration of this larval stage may allow tadpoles both to reduce the duration of a stage during which they are more susceptible to predation (i.e., decreased locomotion, Cheron et al., 2021b) and to reduce to duration of a stage during which feeding is precluded thereby limiting energy expenditure linked to organismal maintenance and to favour energy allocation to metamorphosis (Cheron et al., 2021b).

Parental influences on metamorphic individuals

Finally, we failed to detect any links between parental traits and the morphology of toadlets during their first days of terrestrial life. As stated above, the remarkable modifications of tadpole morphology, behaviour and physiology (Cheron et al., 2021b) during and following metamorphosis may have negatively affected our ability to detect such effects. It is plausible that the characteristics of embryonic and larval development may carry-over later in life (Bouchard et al., 2016; DiGiacopo and Hua, 2020; Garcia et al., 2017; Ruthsatz et al., 2020b; Zeitler et al., 2021), and future studies investigating longer-term effects of parental phenotypes on young toadlets up to adult life may usefully reveal functional links between parental quality and offspring development (Ensminger et al., 2018; Parker and Begon, 1986).

Conclusion

Overall, our study allowed to highlight significant contributions of both maternal and paternal phenotypes to fecundity and offspring development in a study species lacking parental care. As expected, we found significant effects of maternal phenotype on fecundity, hatching success and hatchling size, as well as on the duration of larval development. Interestingly, we also found significant contributions of the paternal phenotype occurring both during early (embryonic development duration) and relatively late (duration of larval stages close to the metamorphosis) offspring development. This is especially interesting as, in amphibians, the role of fathers has often been reduced to egg fertilization (Byrne and Silla, 2020; Kouba et al., 2009). Our findings indicated very few additive effects of both parental traits but rather separate maternal and paternal influences on specific stages of offspring development. Although our study focused on morphological traits solely, we suggest several hypotheses involving physiological costs of development (e.g., telomere attrition, oxidative status, Burraco et al., 2017; Saino et al., 2005). These physiological markers may well mediate, at least in part, the relationships between parental phenotypes and offspring development (Van Leeuwen et al., 2015; Wells, 2014). The potential underlying costs of development may bear strong consequences later in life (Burraco et al., 2020). Clearly, future studies should investigate the mechanisms underlying our findings in order to clarify the mechanistic basis of the links between parental phenotypes and offspring development we highlighted.

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Appendix

		Df	AICc	Δ AICc	AICcWt	LogLik
Eggs size	null	3	-18398.86	0.00	1	9202.43
	SVL_M	4	-18382.30	16.56	0	9195.16
	SVL_F	4	-18380.87	17.99	0	9194.44
	SVL_F + SVL_M	5	-18364.32	34.54	0	9187.17
Hatching success	null	3	2838.10	0.00	0.53	-1417.05
	SVL_M	4	2840.03	1.94	0.20	-1417.01
	SVL_F	4	2840.07	1.98	0.20	-1417.03
	SVL_F + SVL_M	5	2842.01	3.92	0.07	-1417.00
Hatchling length GS25	null	3	-1119.21	0.00	0.96	562.64
	SVL_F	4	-1112.74	6.48	0.04	560.42
	SVL_M	4	-1109.44	9.78	0.01	558.77
	SVL_F + SVL_M	5	-1103.10	16.11	0.00	556.63
Larval development duration	null	2	1015.60	0.00	0.53	-505.75
	SVL_M	3	1017.46	1.86	0.21	-505.64
	SVL_F	3	1017.66	2.06	0.19	-505.74
	SVL_F + SVL_M	4	1019.55	3.95	0.07	-505.62
Days between GS25 and GS30	null	2	550.10	0.00	0.47	-273.00
	SVL_M	3	551.23	1.13	0.27	-272.53
	SVL_F	3	552.16	2.06	0.17	-272.99
	SVL_F + SVL_M	4	553.32	3.22	0.09	-272.51
Days between GS30 and GS37	null	2	911.69	0.00	0.55	-453.80
	SVL_F	3	913.76	2.08	0.19	-453.79
	SVL_M	3	913.77	2.08	0.19	-453.79
	SVL_F + SVL_M	4	915.87	4.19	1.00	-453.79
Days between GS41 and GS42	null	2	878.58	0.00	0.43	-437.25
	SVL_M	3	879.52	0.94	0.27	-436.67
	SVL_F	3	880.29	1.70	0.18	-437.05
	SVL_F + SVL_M	4	881.26	2.67	0.11	-436.47
Days between GS42 and GS46	null	2	540.06	0.00	0.43	-266.94
	SVL_F	3	540.33	0.27	0.38	-266.01
	SVL_F + SVL_M	4	543.02	2.95	0.10	-269.46
	SVL_M	3	543.32	3.25	0.09	-268.57
Tadpoles length GS30	null	2	-86.18	0.00	0.45	46.18
	SVL_M	3	-84.89	1.29	0.24	44.49

	SVL_F + SVL_M	4	-84.59	1.59	0.20	46.44
	SVL_F	3	-83.34	2.85	0.11	44.76
Tadpoles length GS37	null	2	722.72	0.00	0.49	-359.31
	SVL_F	3	724.05	1.33	0.25	-358.93
	SVL_M	3	724.76	2.04	0.18	-359.29
	SVL_F + SVL_M	4	726.12	3.41	0.09	-358.91
Metamorph length GS46	null	2	-307.83	0.00	0.37	155.96
	SVL_M	3	-306.97	0.24	0.24	156.58
	SVL_F	3	-306.91	0.23	0.23	156.55
	SVL_F + SVL_M	4	-306.02	0.15	0.15	157.17
Metamorph weight GS46	null	2	-590.38	0.00	0.38	297.24
	SVL_M	3	-589.64	0.74	0.26	297.92
	SVL_F	3	-589.23	1.16	0.21	297.71
	SVL_F + SVL_M	4	-588.46	1.92	0.14	298.39
Metamorph BC GS46	null	2	-830.72	0.00	0.54	417.40
	SVL_M	3	-828.75	1.96	0.20	417.47
	SVL_F	3	-828.62	2.09	0.19	417.41
	SVL_F + SVL_M	4	-826.63	4.09	0.07	417.47
Larval mortality	null	2	634.54	0.00	0.62	-472.71
	SVL_F	3	637.57	3.03	0.14	-474.46
	SVL_M	3	638.96	4.42	0.13	-473.53
	SVL_F + SVL_M	4	640.72	6.18	0.11	-475.77
Eggs size	null	3	-18398.86	0.00	1	9202.43
	BCI_M	4	-18381.84	17.02	0	9194.93
	BCI_F	4	-18380.83	18.03	0	9194.42
	BCI_F + BCI_M	5	-18363.84	35.02	0	9186.93
Embryonic development duration	null	3	-1777.06	0.00	0.97	891.53
	BCI_M	4	-1769.62	7.44	0.03	888.82
	BCI_F	4	-1756.63	10.44	0.00	887.32
	BCI_F + BCI_M	5	-1759.20	17.	0.00	884.61
Days between GS25 and GS30	null	2	550.10	0.00	0.68	-273.00
	BCI_M	3	551.94	1.84	0.19	-272.88
	BCI_F	3	552.15	2.05	0.13	-272.99
	BCI_F + BCI_M	4	554.00	3.90	0.00	-272.85
Days between GS30 and GS37	null	2	911.69	0.00	0.44	-453.80
	BCI_F	3	912.77	1.08	0.26	-452.21
	BCI_M	3	913.47	1.78	0.18	-452.04
	BCI_F + BCI_M	4	914.27	2.58	0.12	-453.50

Days between GS37 and GS41	null	2	717.06	0.00	0.38	-355.11
	BCI_M	3	717.72	0.66	0.28	-356.49
	BCI_F + BCI_M	4	718.28	1.22	0.21	-354.66
	BCI_F	3	719.23	2.17	0.13	-356.19
Days between GS41 and GS42	null	2	878.58	0.00	0.67	-437.25
	BCI_F	3	880.30	1.72	0.28	-432.76
	BCI_F + BCI_M	4	885.06	6.48	0.03	-432.96
	BCI_M	3	886.34	7.76	0.01	-436.84
Days between GS42 and GS46	null	2	540.06	0.00	0.47	-266.94
	BCI_F	3	541.16	1.10	0.27	-268.97
	BCI_M	3	542.15	2.09	0.17	-269.46
	BCI_F + BCI_M	4	543.28	3.22	0.09	-268.97
Tadpoles length GS37	null	2	722.72	0.00	0.29	-359.31
	BCI_M	3	722.89	0.17	0.27	-358.13
	BCI_F + BCI_M	4	723.00	0.28	0.25	-357.15
	BCI_F	3	723.61	0.89	0.19	-358.58
Metamorph length GS46	null	2	-307.83	0.00	0.30	155.96
	BCI_F + BCI_M	4	-307.59	0.24	0.26	158.30
	BCI_F	3	-307.36	0.46	0.23	157.12
	BCI_M	3	-307.11	0.72	0.21	156.88
Metamorph weight GS46	null	2	-590.38	0.00	0.29	297.24
	BCI_F	3	-590.34	0.04	0.28	298.46
	BCI_F + BCI_M	4	-590.02	0.36	0.24	299.51
	BCI_M	3	-589.51	0.87	0.19	298.03
Metamorph BC GS46	null	2	-830.72	0.00	0.54	417.40
	BCI_F	3	-828.80	1.91	0.21	417.50
	BCI_M	3	-828.62	2.09	0.19	417.41
	BCI_F + BCI_M	4	-826.68	4.03	0.07	417.50
Larval mortality	null	2	634.54	0.00	0.62	-472.71
	BCI_F	3	637.42	2.88	0.18	-473.68
	BCI_M	3	637.68	3.14	0.12	-474.06
	BCI_F + BCI_M	4	639.52	4.98	0.08	-474.98

Appendix 1. Details on non selected models to explore effects of parental phenotype (body size: SVL, body condition index: BCI, males: M and females: F) on reproductive success. The models which we found significant influences of parental traits on offspring development are shown in Table 1. AIC models with dependent variables and explanatory variables. Df stands for the degree of freedom of the models. AICc represent the Akaike criterion corrected value in ascending order. Δ AICc represent the interval between the AICc value of the most parsimonious model and the other models. AICcWT is the weight of the explanatory part of each model. LogLik is a model fitted by maximum likelihood.

III/ Article 6

Parental habitats affect reproduction and offspring development in a widespread amphibian

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Abstract

Anthropogenic activities and particularly environmental change related to agriculture have been shown to individually and interactively affect natural ecosystem functioning and wildlife persistence. Agricultural activities affect the structure of rural landscapes through homogenization and simplification of habitats but also their connectivity and modern agricultural practices rely heavily on chemical inputs. Environmental contamination interactively affect individual survival, population persistence, and thus biodiversity in agricultural landscapes. Indeed, small and fragmented remnants of optimal habitats within agricultural landscapes can allow survival of adult individuals but may decrease reproduction and/or negatively affect offspring quality and thus survival. Yet, several species have been shown to persist in agricultural landscapes. Whether reproductive performances are affected by the constraints of agricultural environments in species that persist in such habitats remains to be tested while taking into consideration that early life-stages are known to be more susceptible to environmental constraints. Spined toad (*Bufo spinosus*) is a widespread terrestrial amphibian occurring throughout a wide range of habitat types ranging from intensive agriculture to preserved (forests) habitats. We collected pairs of Spined toads originating from agricultural habitats and preserved areas. In experimental conditions we monitored reproductive performances, with a comprehensive set of markers, throughout the whole reproductive event from oviposition to offspring metamorphosis in order to compare whether individuals originating from intensive agriculture would display altered reproductive output. We failed to detect significant effects of the habitat type on the phenotype of reproductive individuals or direct effects of parental habitat on reproductive performances. However, we found several traits linked to reproductive success (clutch size, hatching success, larval development duration and larval morphology) that were influenced by parental habitat through interactions with parental phenotype suggesting that parental habitat can alter the relationships between parental phenotype and reproductive performances. We conclude that agricultural environment showed to be restrictive for the offspring and the question of the persistence of amphibians in these environments arises. We lack an understanding of the underlying mechanism and future studies need to focus on more specific aspects of parental to offspring transfer and identify environmental variables that may play into its relationships.

Keywords: Phenotype - Reproductive success - Clutch quality - Embryonic development - Larval development.

Anthropogenic activities are now recognized as major causes of the current loss of biodiversity (Chapin III et al., 2000; Myers and Knoll, 2001; Brooks et al., 2002). These activities induce critical changes in climatic conditions (Salinger, 2005; Diffenbaugh and Field, 2013), environmental contamination (Relyea, 2004; Hart et al., 2006; Slaninova et al., 2009; Williams et al., 2015; Trudeau et al., 2020; Wagner, 2020) and land-use changes (Alkemade et al., 2012; Barnes et al., 2014; Jung et al., 2019; de Lima et al., 2020; Kadoya et al., 2022), all of which have been shown to individually and interactively affect natural ecosystems functioning and persistence of wildlife (Rudnick et al., 2012; Newbold et al., 2015).

Among these various Human-induced environmental perturbations, modern agricultural practices have been highlighted as a prominent factor affecting biodiversity (Schmitzberger et al., 2005; Rudnick et al., 2012; Newbold et al., 2015). The detrimental effects of agricultural practices on biodiversity are linked to two main processes. First, agricultural activities affect the structure of rural landscapes through habitat homogenization and simplification (e.g., linked to land consolidation, Rudnick et al., 2012), thereby reducing both the surface area of optimal habitats for wildlife (Rudnick et al., 2012) but also their connectivity (i.e., fragmentation, (Rudnick et al., 2012)). These changes in habitat structure have been shown to influence wildlife both directly (e.g., reduced surface area of suitable habitats, refs) and indirectly (e.g., reduced resources availability, (Hart et al., 2006; Williams et al., 2015; Wagner, 2020)). Second, modern agricultural practices heavily rely on chemical inputs to improve crop productivity (i.e., fertilizers and pesticides, Sandrini et al., 2022)). Although these substances are specifically used to target pests, growing evidence now suggests that agrochemicals can affect non-target species, either because these species occur in agricultural habitats or because these substances can be transported to non-target environments (Hasenbein et al., 2017; de Brito Rodrigues et al., 2019; Gunstone et al., 2021). The resulting environmental contamination can affect wildlife both directly (i.e., through toxic effects inducing mortality; or through sublethal effects disrupting normal organismal functions) and indirectly (i.e., because pesticides reduce resources availability) (Relyea, 2004; Hart et al., 2006; Slaninova et al., 2009; Williams et al., 2015; Trudeau et al., 2020; Wagner, 2020). These two processes are known to interactively affect individual survival, population persistence and thus biodiversity in agricultural landscapes.

Yet, several species have been shown to persist in agricultural landscapes, albeit usually at lower abundances than in preserved areas and assessing the temporal stability of these relictual populations is a critical endeavour (Daily et al., 2003; Harvey et al., 2006; Olden and Rooney, 2006; Prugh et al., 2008; Filippi-Codaccioni et al., 2010). Such population persistence should occur through two main processes. First, individual survival may occur within small and fragmented remnants of optimal habitats (Scott et al., 2002; Tschamntke et al., 2005; Peterson et al., 2011; Broennimann et al., 2012). Second, actual reproduction should occur to allow recruitment and individual renewal through time (Baker et al., 2015; Foster et al., 2015).

This later process is of prime importance because life in suboptimal environments is known to affect reproductive output and fitness in a wide array of organisms (Moore and Waring, 2001; Rhind, 2009; Seress and Liker, 2015). Indeed, small and fragmented remnants of optimal habitats within agricultural landscapes can allow survival of adult individuals but may decrease reproduction (e.g., through reduced resources availability and thus energetic reserves needed to fuel reproduction) and/or negatively affect offspring quality and thus survival (Luck, 2003; Newton, 2004; Vander Haegen, 2007). In addition, early life-stages are known to be more susceptible to environmental constraints (including those linked to agricultural practices, (Leet et al., 2011; Luo et al., 2016)) than adults, and development under suboptimal environmental conditions can bear strong consequences later in life (Gicquel et al., 2008; Moe et al., 2013). In turn, decreased reproduction and lower offspring quality will ultimately influence recruitment thereby producing extinction vortices (Gaggiotti and Hanski, 2004). Whether reproductive performances are affected by the constraints of agricultural environments in species that persist in such habitats remains to be tested.

Yet, testing this hypothesis is logistically complicated for several reasons. First, it requires to identify a study species that occur in both agricultural environments and in habitats that are not affected by these activities (Kleijn et al., 2006, 2011). Many species are specialized in one or another habitat type thereby precluding such comparisons (Clavel et al., 2011; Ramiadantsoa et al., 2018). Second, discrete populations of such species should be segregated between habitat types in order to make sure that movements between habitat types do not occur within the day-to-day life of such organisms. Most species are relatively mobile and can commute between different habitats thereby precluding a straightforward assessment of the exclusive use of agricultural environments. Finally, such species should be logistically easy to maintain in captivity in order to monitor reproductive performances under controlled conditions and thus to avoid direct influence of the habitat type during a reproductive event (i.e., common garden, de Villemereuil et al., 2016, 2022)).

In this study, we used a study model that fulfills all of these conditions. Spined toad (*Bufo spinosus*) is a widespread terrestrial amphibian distributed across Western Europe and occurring throughout a wide range of habitat types ranging from intensive agriculture to preserved (forests) habitats (Guillot et al., 2016). Because toads are characterized by comparatively low mobility, samplings of individuals within contrasted habitat types have been shown to reflect their overall habitat use (Renoirt et al., 2021a). Their biphasic life style (terrestrial adults reproducing seasonally in small water bodies where eggs and larvae develop, Doody et al., 2009; Wells, 2010)) allow to sample reproductive individuals within discrete habitat types. Finally, reproduction can be obtained in captivity (Renoirt et al., 2022), thereby allowing to monitor reproductive performances (from egg laying through embryonic and larval development to metamorphosis, Renoirt et al., 2022) of individuals originating from agricultural or preserved sites.

We collected pairs of Spined toads originating from agricultural habitats (2 sites, N=10 pairs per site) and preserved areas (2 sites, N=10 pairs per site) and brought them back to the laboratory before oviposition. We monitored reproductive performances throughout the whole reproductive event from oviposition to offspring metamorphosis on order to compare whether individuals originating from intensive agriculture would display altered reproductive output. Specifically, we used a comprehensive set of markers in order to test whether the quality of reproductive individuals differed between habitat types (morphology, condition, telomere length) and whether the origin of parents (agricultural *versus* forest areas) influence their reproductive performances throughout offspring development (fecundity, duration of embryonic development, hatching success, telomere length of hatchlings, larval development duration, larval morphology and survival). We predicted that

We predicted that markers of parental quality will be negatively impacted by the agricultural environment but positively by the forest environment. Thus, we suggest that embryonic and larval quality markers will be influenced by parental quality through the environment of origin of the parents with indirect negative effects of the agricultural environment and positive effects of the forest environment.

Material and methods

Study species and sampling

In Western Europe, the Spined toad (*Bufo spinosus*) is a widespread amphibian. As most terrestrial amphibians, Spined toads have a biphasic life-cycle with terrestrial adults reproducing in aquatic environments where eggs and larvae develop (Griffiths, 1997; Tiegs et al., 2016). Reproduction occurs between late winter and early spring (mid-January to late March) during which reproductive individuals gather in small water bodies (ponds, (Griffiths, 1997).

In 2021, we monitored 4 breeding ponds situated in contrasted landscapes (see below) nightly using headlamp throughout the breeding season. Toad pairs (amplexus) were captured with a net upon their arrival at each breeding site before oviposition occurred and brought back to the laboratory until spawning. Adult individuals were released once oviposition occurred.

Sampling sites and habitat classification

Sampling took at 4 breeding sites near the laboratory (CEBC, 46° 8' 48.64 "N; 0°25'30.86 "W, Deux-Sèvres, France,) in a typical rural landscape mixing intensive agricultural activities and preserved areas (forests). During their terrestrial phase, adult toads are generally located within 1km of their breeding site (Janin et al., 2011; Guillot et al. 2016). Based on this geographic scale (buffer of 1 km radius around reproductive ponds), we selected 2 study sites surrounded by preserved areas (forest surface area representing 100% and 93% within buffers) and surrounded by intensive agricultural activities (crop field surface area representing 66% and 92% within buffers), thereby allowing simple habitat classifications (i.e., agriculture *versus* forest, Guillot et al., 2016; Renoirt et al., 2021a). Importantly, a previous investigation validated such classification (Renoirt et al., 2021a).

Parental traits

Once at the laboratory, captured pairs were transitorily separated and males and females were individually weighted with an electronic scale (pre-laying mass, ± 0.01 g). Each pair was then placed in a plastic containers (59x36x28 cm) filled with 30L of dechlorinated tap water and containing a branch and a stone. All pairs reformed an amplexus in these containers. We monitored the pairs several times a day until the end of oviposition which was achieved between 0 and to 17 days (6.23 ± 0.86) after captures. Once oviposition was achieved, males and females were individually weighted with an electronic scale (post-laying mass, ± 0.01 g), measured (snout to vent length, SVL) with a caliper (± 0.01 mm) and a blood sample was collected with a 1ml syringe and a 30G heparinised needle through cardiocentesis for telomere analyses (see below). All individuals were then released at their site of capture.

Monitoring of reproductive performances

Fecundity

Spined toad clutches consist of an elongated egg string (refs). After oviposition, each string was carefully collected and placed in a container (35x20x25cm) containing 2 cm of dechlorinated water with graph paper. . Each string was photographed from above in order to quantify fecundity (number of eggs). Pictures were analyzed with Image-J software (Schneider et al., 2012). We measured the length of the whole egg string. We randomly selected 5 segments of 10 cm long within each clutch. For each segments, we counted the number of eggs. The average number of eggs per 10 cm segments was used to assess fecundity by extrapolating it to the egg string total length.

Embryonic development

We randomly sampled 3 segments of 34 eggs for each clutch to monitor embryonic development (the remaining eggs were released at the site of capture). Each segment was placed in a petri dish with graph paper and photographed from above. These pictures were used to measure the size (diameter) of each egg using image-J software (Schneider et al., 2012).

Each segment was transferred in 2L glass tanks filled with dechlorinated tap-water (water was changed every week). For each segment we monitored embryonic development using Gosner (1960) until hatching in order to assess embryonic development duration. Hatching occurred at Gosner stage 25 (Gosner 1960). Upon hatching, for each segment, we individually counted the number of live hatchlings and the number of eggs that did not successfully develop in order to quantify hatching success.

We randomly selected 1 live hatchling per segment for telomere analyses (see below).

Larval development

Each hatchling was placed individually in 2 L of dechlorinated water in glass containers (18x13x18cm). Throughout larval development, tadpoles were fed ad-libitum with chopped organic spinach. We monitored larval development using Gosner (1960) and assessed larval morphology across key developmental stages following Cheron et al (2021). These stages corresponded to Gosner (1960) stages 25, 30, 37, 41 and 42 (hereafter, GS 25, GS 30, GS 37, GS 41 and GS 42, respectively). For each selected stage, each individual was transitorily placed in petri dishes (with graph paper) with water from their own aquarium and photographed from above. Using Image-J software (Schneider et al., 2012), we measured total length, body length and tail length.

Metamorphic individuals

Upon metamorphosis (Gosner stage 46, Gosner, 1960), toadlets were measured (Snout to Vent length, SVL), weighed and individually transferred to a plastic box (17x15x9cm) with damp paper towel as substrate and a shelter. We repeated measurements 5 days after metamorphosis and released individuals at their site of origin.

All the experiments took place in a thermally and photoperiod controlled room with the temperature set at 17 °C (both air and water) and 12 h dark-12 h light.

Telomere length

Both blood of parents and randomly selected hatchlings (see above) were used for telomere analyses.

We adapted a previously established protocol (McLennan et al., 2019) for telomere analysis.

Adult telomeres were measured on red blood cells using a quantitative real-time PCR technique (see: Cawthon, 2002). Genomic DNA was extracted from previously frozen red blood cells using the DNeasy Blood and Tissue kit (Qiagen) and following the manufacturer's protocol.

Hatchling telomeres were measured on whole individuals because of their small size. Hatchling telomere length could be determined from quantitative analysis (qPCR; BioRad CFX 96; Bio-Rad, USA). In accordance with the manufacturer's instructions, the samples were first digested with proteinase K and DNA was extracted using the Nucleospin tissue kit (MachereyNagel). A Nanodrop ND1000 spectrophotometer (Thermo Scientific) was used to evaluate the purity and concentration of the DNA.

For blood samples and hatchling, universal telomere primers were used, and the control single-copy gene recombination activating gene 1 (RAG1) was selected and amplified using specific primers designed for the spined toad using the sequence alignment methodology: RAG1-F 5'-GGGTCCTCTGATAGCCGAAA-3' and RAG1-R 5'-CATCATAACCTGTACCCCGGA-3'. This singlecopy gene has previously been used successfully in multiple species (birds: Sebastiano et al., 2020; fish: Petitjean et al., 2020; reptiles: McLennan et al., 2019), including amphibians (Canestrelli et al., 2021).

qPCR was performed on three plates for each gene (RAG1 and telomere) using 7.5 ng of DNA per reaction. We used primers for the telomere and single copy gene at concentrations of 800 and 300 nM, respectively. For hatchling, serial dilutions of DNA from a pooled sample of ten tadpoles were included on the plate (in triplicate). This allowed for monitoring the amplification efficiency of the reactions by generating a six-point standard curve (50.0 to 1.5 ng). For telomere and RAG1 amplification, the amplification specificity of these primers could be validated using the melting curves which showed simple and clean curve results. To account for inter-plate variation, a reference toad sample was analyzed in triplicate in all plates. Samples were randomly assigned to PCR plates and analyzed in duplicate. Cycle threshold (Ct) values determined for duplicates were averaged, and samples with a standard deviation of Ct > 0.2 between duplicates were repeated.

Amplification efficiencies reached 89.50 ± 1.85 (mean \pm SE, %) for the adults' telomere and 91.80 ± 0.99 for adults' RAG1. For hatchling, amplification efficiencies reached 95.30 ± 2.80 (mean \pm SE, %) for the telomere and 88.20 ± 4.60 for RAG1. The relative telomere length (expressed as the T/S ratio) was calculated as the number of telomere copies (T) relative to the singlecopy gene (S; RAG1) (see: Cawthon, 2002). Inter-plate variation for the T/S ratio was 6.18% and the ICC-inter-plate was 0.85.

Statistical analyses

All statistical analyses were conducted with with R v 4.2.1 (R Core Team., 2019). All data were tested for homogeneity of variance, residues independence and normality with the Bartlett test, Dubin-Watson test and Shapiro-Wilks test, respectively. We also checked the residues normality using diagnostic plots.

For adult toads, we quantified a body condition index (BCI) using residual scores from the linear regression between body size and body mass independently in males ($F_{1,38} = 61.03$, $r^2 = 0.606$, $P < 0.001$) and females ($F_{1,38} = 90.78$, $r^2 = 0.697$, $P < 0.001$).

We fitted linear mixed models (LMER, package lmerTest. Kuznetsova et al., 2015) to assess relationships between isotopes and habitat type, parental traits (also telomeres), habitat type and fecundity, embryonic traits, and larval traits (also telomeres). For each models, we included parents capture sites and clutch as random variable. Based on Renoirt et al., (2021) it was determined that blood nitrogen of male and female toads could discriminate habitat type ($F_{1,74} = 32.393$, $r^2 = 0.542$ $P < 0.001$) while there was no relationship with blood carbon and habitat ($F_{1,74} = 2.569$, $P = 0.250$).

First, we investigate whether parents' phenotype were different according to habitat type. We fitted models with parental morphometrics measurements (SVL and BCI) as dependent variables and habitat type as explanatory variables.

To study the relation between fecundity, parental morphology (SVL and BCI) and habitat (Agricultural and Forest), we fitted two models with clutch size or egg size as dependent variables and parental morphometrics measurements as explanatory variables in interaction with the habitat.

Regarding embryonic development, we fitted models including hatching success, development duration or hatchling length (GS25) as dependant variable and parental morphometrics measurements as explanatory variables (SVL and BCI) in interaction with the habitat.

To test whether there was a relation between parental traits (SVL, BCI or telomere length), habitat type and larval development (total length, body length, tail length or days between each stage) we performed repeated measures anova (stats package). For each model (lmer for total length, body length, tail length and glmer for days, lme4 package), parental traits, gosner stages and habitat type and their interaction were included as fixed factor. Individual identity was nested inside their own clutch and included as random effect. We dropped all non-significant interactions from these models (anova, package stats). We performed post hoc analysis using emmeans package and investigated statistical differences of the slope of the interaction at each level of stage.

We investigate a potential relationship between parental telomeres, parental morphometrics measurements and habitat types. We fitted models with parental telomeres length as dependent variables and parental morphometrics measurements as explanatory variables in interaction with the habitat types. To study the relation between parents and descendants, we fitted different models between parental telomeres length in interaction with the habitat types (explanatory variables) and fecundity (clutch size, eggs size) and embryonic (embryonic development duration, hatching success and hatchling length at GS25) and larval traits (larval development duration, morphology measurements at each stage and larval telomere length) as dependent variables.

Finally, we used linear model to determine whether the inter-group variation is greater than the intra-group variation. This allows us to use the average telomere length of a single clutch as a proxy for the telomere length of their siblings. We looked at a potential relationship between embryonic traits (explanatory variable) in interaction with the habitat type and tadpole telomere length (dependent variable). Next, we looked at the relationship between hatchling telomere length as an explanatory variable in interaction with the habitat type, and the larval traits after S25 (larval development time, total tadpole length at different stages) as dependent variable.

Finally, we performed survival analysis using log-rank method ("survival" packages) to assess mortality rate until metamorphosis. This method allowed us to estimate the rate at which death occurs over time and whether mortality is more susceptible to occur at a specific stage. We examined differences across habitat with a Cox model. The test for assumption of proportional hazards for Cox regression which show that our model was appropriate.

Results

Does parental habitat influence parental phenotype?

None of the parental traits we investigated (body size, body condition or telomere length) differed between habitat types (Table 1).

Table 1. Relation between parental traits and reproductive performance according to habitat. M= males and F= Females

Parameter	Factor	Df	F value	P value
SVL_F	Habitat	1,38	3.528	0.201
BCI_F	Habitat	1,38	0.461	0.567
SVL_M	Habitat	1,38	2.218	0.145
BCI_M	Habitat	1,38	0.234	0.676
Female telomeres size	SVL_F	3,36	0.113	0.738
	Habitat	3,36	0.059	0.809
	SVL_F * Habitat	3,36	0.109	0.742
	BCI_F	3,36	1.351	0.253
	Habitat	3,36	2.241	0.143
	BCI_F * Habitat	3,36	0.068	0.794
Males telomeres size	SVL_M	3,36	1.028	0.317
	Habitat	3,36	0.667	0.419
	SVL_M * Habitat	3,36	0.595	0.445
	BCI_M	3,36	0.871	0.356
	Habitat	3,36	0.333	0.567
	BCI_M * Habitat	3,36	0.659	0.659

Does parental habitat influence reproductive performances?

All of the parameters we investigated to assess reproductive performances were similar between parental habitat types (Table 2).

In addition, tadpole survival was similar between parental habitat types (log-rank test, $X_1=0.4$, $p=0.5$).

Table 2. Reproductive performance relationship according to habitat. EDD= Embryonic Development Duration.

Parameter	Factor	Df	F value	P value
Clutch size	Habitat	1.38	0.001	0.983
Eggs size	Habitat	1.4078	0.530	0.542
Hatching success	Habitat	1.4078	0.270	0.604
EDD	Habitat	1.2495	0.091	0.933
Hatchling length	Habitat	1.437	2.622	0.113
Hatchling telomeres size	Habitat	1.109	0.123	0.727
Total Larval development duration	Habitat	1,29	0.065	0.800
Stage specific larval development duration	Habitat	1,31	0.029	0.8667
	Stage	4,419	402.33	<0.001
	Habitat*stage	4,419	0.702	0.591
Tadpole Total length	Habitat	1,39	0.323	0.573
	Stage	4,402	1545.13	<0.001
	Habitat*stage	4.406	1.80	0.13
Toadlets SVL	Habitat	1.67	0.413	0.523

Does parental habitat mediate the relationships between parental phenotype and reproductive performances?

Taking into account parental phenotype, most of the traits related to reproductive performances were similar between parental habitat types (Table 3).

However, we found several metrics of reproductive performances for which parental habitat types had a significant influence through parental phenotype (Table 3).

Clutch size was affected by the interaction between paternal body size and parental habitat type (Table 3). Clutch size was negatively correlated with males SVL from forest habitats (-141.05 ± 57.92 , $P = 0.020$, Figure 1) but was not correlated with those from agricultural habitats (50.67 ± 57.46 , $P = 0.380$, Figure 1).

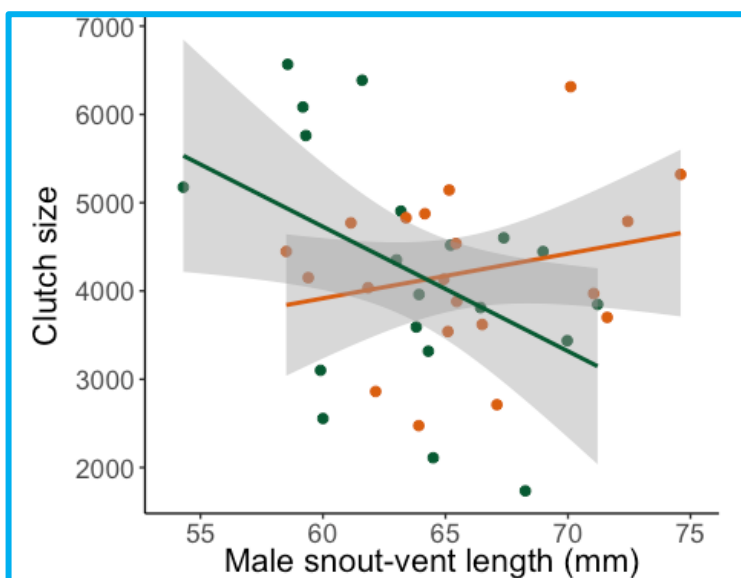


Figure 1. Relationship between male body size and fecundity (clutch size). Grey shading indicate 95% confidence intervals. The orange was the individuals coming from the agricultural environment. The green was the individuals coming from the forest environments.

Hatching success was affected by the interaction between maternal body size and parental habitat type (Table 3). Hatching success was positively correlated with females SVL from forested habitats (0.10 ± 0.04 , $P = 0.030$, Figure 2), while negatively correlated with females SVL from agricultural habitats (-0.11 ± 0.04 , $P = 0.020$, Figure 2).

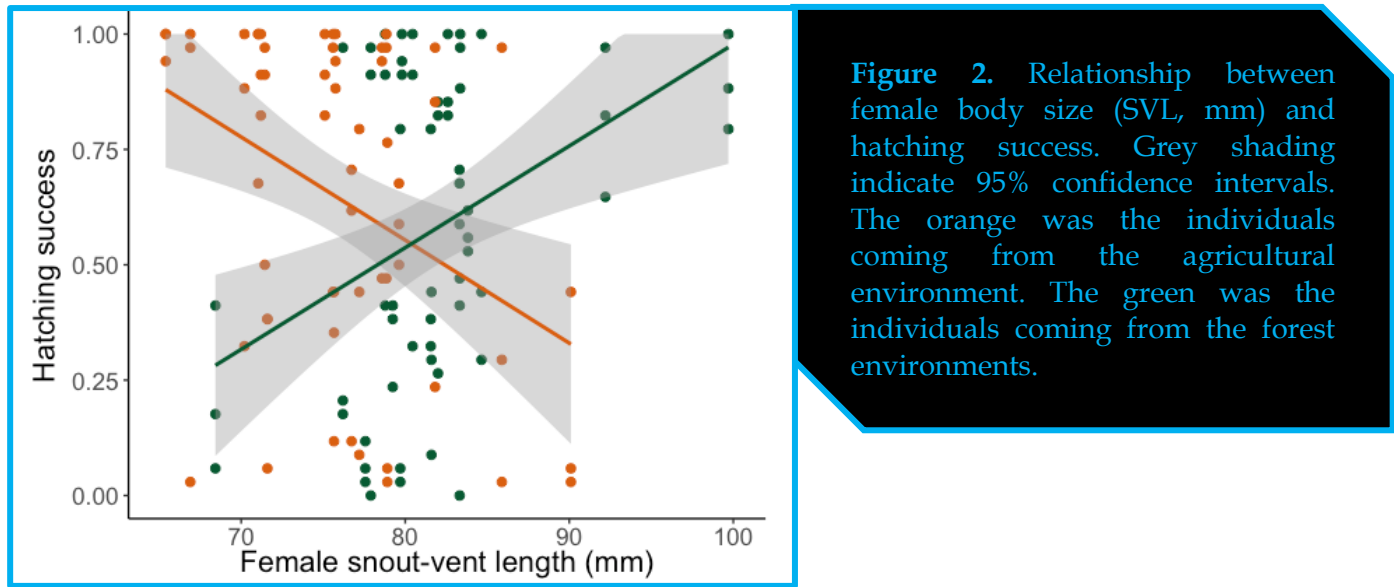


Figure 2. Relationship between female body size (SVL, mm) and hatching success. Grey shading indicate 95% confidence intervals. The orange was the individuals coming from the agricultural environment. The green was the individuals coming from the forest environments.

Total larval development duration was affected by the interaction between both maternal and paternal body condition and parental habitat type (Table 3). Total larval development duration was not correlated with the body condition of forest males (0.69 ± 0.55 , $P = 0.23$, Figure 3) but negatively correlated with that of males from agricultural habitats (-0.74 ± 0.41 , $P = 0.038$, Figure 3). Larval development duration was negatively correlated with the body condition of forest females (-0.38 ± 0.18 , $P = 0.041$, Figure 3) but not correlated with that of females from agricultural habitat (0.32 ± 0.28 , $P = 0.270$, Figure 3). These results were corroborated with stage-specific durations (Table 3).

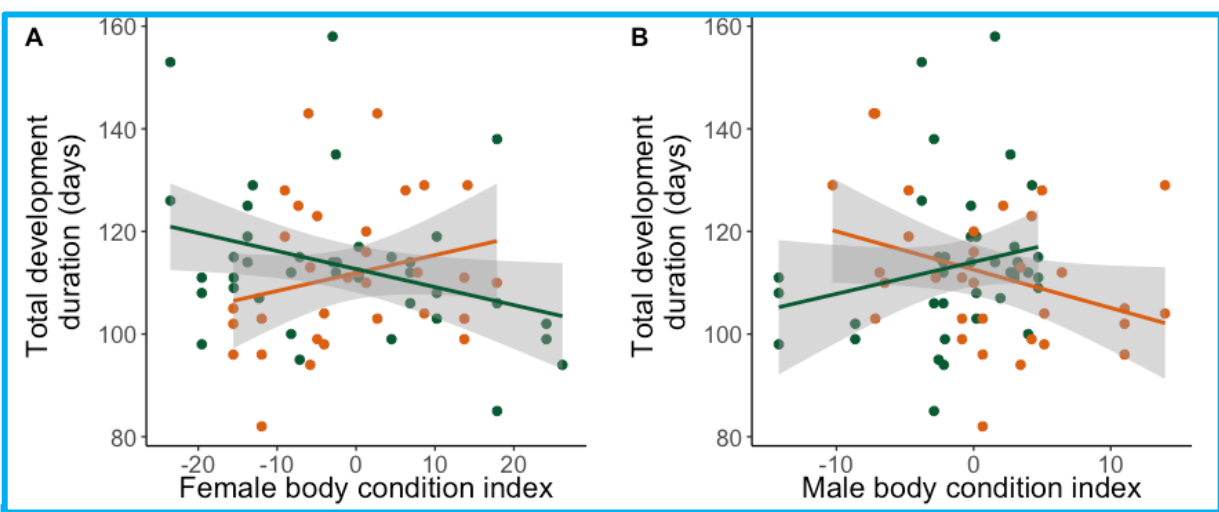


Figure 3. Relationships between female body condition index (BCI) and embryonic development duration (days). Grey shading indicate 95% confidence intervals. The orange was the individuals coming from the agricultural environment. The green was the individuals coming from the forest environments.

According to habitat type, female body condition affected differentially the days between GS 30 and GS 37 (Positive relation in agricultural toads : 0.487 ± 0.137 , $P < 0.001$, no relation in forest toads : 0.001 ± 0.087 , $P = 0.921$) and between GS37 and GS 41 (Negative relation in agricultural toads : -0.377 ± 0.140 , $P = 0.008$, no relation in forest toads : -0.014 ± 0.087 , $P = 0.869$). According to habitat type, male body condition affected differentially the days between GS 30 and GS 37 (Negative relation in agricultural toads : -0.666 ± 0.189 , $P = 0.005$ and a positive relation in forest toads : 0.637 ± 0.257 , $P = 0.014$). In addition, stage-specific durations were also differentially affected between habitat types by paternal telomere length (Table 3). In agricultural toads, male telomere length was positively correlated to the days between GS30 and GS37 (32.69 ± 8.72 , $P < 0.001$) while we found a negative relation for forested toad (-33.82 ± 10.28 , $P = 0.011$, Figure 4).

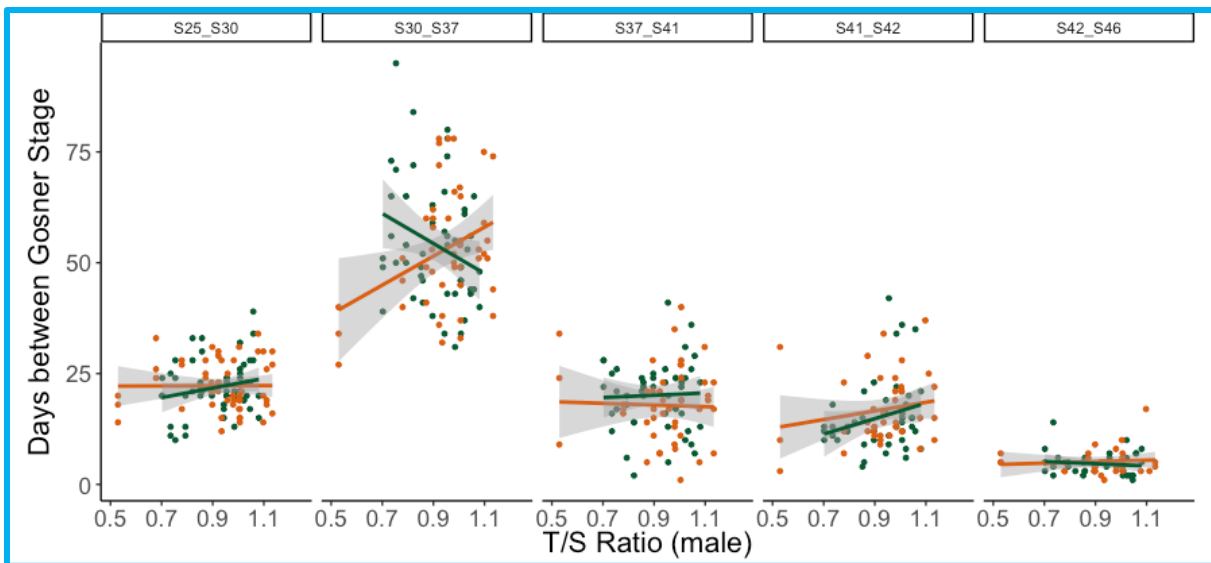


Figure 4. Relationships between male telomere length (T/S ratio) and larval development between Gosner stage. Grey shading indicate 95% confidence intervals. The orange was the individuals coming from the agricultural environment. The green was the individuals coming from the forest environments.

Finally, larval development duration was affected by the interaction between hatchling telomere size and parental habitat type (Table 3). For tadpoles coming from agricultural parents the relationship was positive (80.82 ± 32.30 , $P = 0.020$, Figure 5) while the relation was not significant for those from forest parents (-8.68 ± 30.83 , $P = 0.78$, Figure 5).

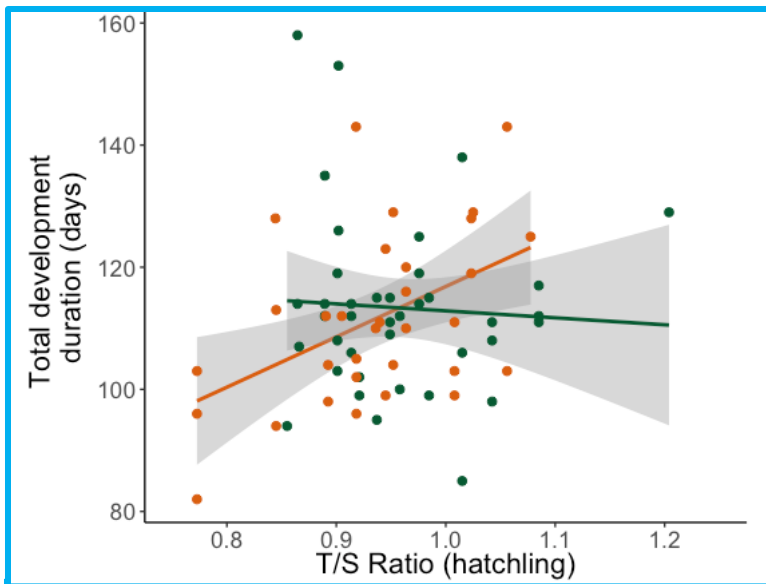


Figure 5. Relationships between larval development duration (days) and hatchling telomere length (see material and methods, T/S ratio). Grey shading indicate 95% confidence intervals. The orange was the individuals coming from the agricultural environment. The green was the individuals coming from the forest environments.

Larval morphology (tadpole total length) was affected by the interaction between maternal body size, body condition and telomere length (Table 3); and paternal body condition and telomere length differentially according to parental habitat type (Table 3). For maternal body size, the differences were located during somatic growth phase GS30. At GS30, both habitats displayed opposite trends, with a negative relationship in individuals originating from agricultural parents and a positive relationship in tadpoles originating from forest parents (Respectively -0.02 ± 0.001 , $P = 0.003$; 0.01 ± 0.004 , $P=0.012$, Figure 6). For maternal body condition, at GS30, agricultural toads displayed no relationship (-0.004 ± 0.003 , $P = 0.184$) and a positive slope for forest (0.005 ± 0.002 , $P=0.03$, Figure 6). For maternal telomere length, post hoc analyses showed that this relation was located at GS30 with a non significant relation for forest female (0.169 ± 0.238 , $P = 0.481$, Figure 6) and a negative relation for agricultural toad (-0.760 ± 0.233 , $P = 0.008$, Figure 6). For paternal body condition, post hoc analyses showed that this relation was located at GS30 (non significant relation for agricultural toads : 0.007 ± 0.005 , $P = 0.148$ and negative slope for forest toads : -0.016 ± 0.006 , $P = 0.012$). For paternal telomere length, post hoc analyses showed that this relation was located at GS30. For tadpoles coming from agricultural parents the relationship was negative (-0.538 ± 0.200 , $P = 0.007$) while the relation was marginally positive for those from forest parents (0.454 ± 0.252 , $P = 0.074$, Figure 6).

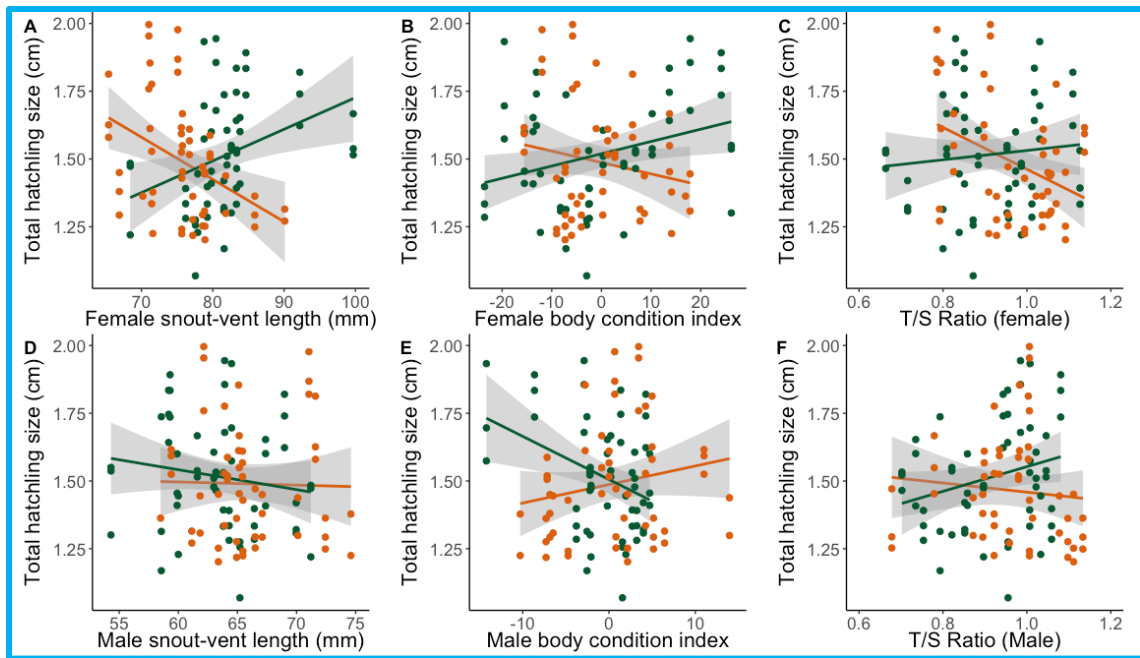


Figure 6. Relationships between total hatchling length and parental traits for males and females (SVL, BCI and T/S ratio). Grey shading indicate 95% confidence intervals. The orange was the individuals coming from the agricultural environment. The green was the individuals coming from the forest environments.

Table 3. Relation between parental traits and reproductive performance according to habitat. M= males, F= Females, EDD= Embryonic Development Duration and LDD = Larval Development Duration.

		Df	F value	P value	
Clutch size	SVL_F	3.36	13.307	< 0.001	
	Habitat	3.36	0.375	0.544	
	SVL_F * Habitat	3.36	0.229	0.634	
	BCI_F	3.36	12.020	0.096	
	Habitat	3.36	0.229	0.634	
	BCI_F * Habitat	3.36	0.390	0.536	
	TELO_F	3.36	1.448	0.237	
	Habitat	3.36	1.059	0.311	
	TELO_F * Habitat	3.36	1.125	0.296	
	SVL_M	3.36	1.227	0.275	
	Habitat	3.36	0.055	0.816	
	SVL_M * Habitat	3.36	5.522	0.024	
	BCI_M	3.36	0.467	0.499	
	Habitat	3.36	0.073	0.941	
	BCI_M * Habitat	3.36	0.021	0.963	
	TELO_M	3.36	0.025	0.875	
	Habitat	3.36	0.092	0.763	
	TELO_M * Habitat	3.36	0.093	0.763	
	Eggs size	SVL_F	3.4076	2.769	0.104
		Habitat	3.4076	0.067	0.797
SVL_F * Habitat		3.4076	0.084	0.773	
BCI_F		3.4076	0.570	0.455	
Habitat		3.4076	0.379	0.602	
BCI_F * Habitat		3.4076	0.022	0.963	
TELO_F		3.4076	0.339	0.563	
Habitat		3.4076	0.615	0.438	
TELO_F * Habitat		3.4076	0.449	0.507	
SVL_M		3.4076	2.507	0.122	
Habitat		3.4076	0.279	0.601	
SVL_M * Habitat		3.4076	0.179	0.675	
BCI_M		3.4076	0.661	0.422	
Habitat		3.4076	0.760	0.515	
BCI_M * Habitat		3.4076	1.839	0.185	
TELO_M		3.4076	0.114	0.737	
Habitat		3.4076	0.152	0.698	
TELO_M * Habitat		3.4076	0.319	0.575	
Hatching success		SVL_F	3.4076	0.132	0.012
		Habitat	3.4076	0.375	<0.001
	SVL_F * Habitat	3.4076	10.989	<0.001	
	BCI_F	3.4076	0.789	0.759	
	Habitat	3.4076	0.234	0.629	
	BCI_F * Habitat	3.4076	0.046	0.831	
	TELO_F	3.4076	1.946	0.395	
	Habitat	3.4076	0.525	0.995	
	TELO_F * Habitat	3.4076	0.038	0.841	
	SVL_M	3.4076	0.754	0.545	
	Habitat	3.4076	0.393	0.084	
	SVL_M * Habitat	3.4076	3.164	0.067	
	BCI_M	3.4076	1.733	0.399	
Habitat	3.4076	0.176	0.689		

	BCI_M * Habitat	3.4076	0.210	0.655
	TELO_M	3.4076	3.682	0.141
	Habitat	3.4076	0.350	0.971
	TELO_M * Habitat	3.4076	0.016	0.899
EDD	SVL_F	3.2493	2.519	0.121
	Habitat	3.2493	0.045	0.947
	SVL_F * Habitat	3.2493	0.007	0.978
	BCI_F	3.2493	0.089	0.767
	Habitat	3.2493	0.011	0.929
	BCI_F * Habitat	3.2493	1.960	0.171
	TELO_F	3.2493	0.161	0.691
	Habitat	3.2493	1.821	0.190
	TELO_F * Habitat	3.2493	1.885	0.179
	SVL_M	3.2493	0.496	0.486
	Habitat	3.2493	0.046	0.946
	SVL_M * Habitat	3.2493	0.040	0.949
	BCI_M	3.2493	10.033	0.003
	Habitat	3.2493	0.077	0.808
	BCI_M * Habitat	3.2493	0.144	0.706
	TELO_M	3.2493	0.039	0.950
	Habitat	3.2493	0.021	0.988
	TELO_M * Habitat	3.2493	0.024	0.961
LDD	SVL_F	3.67	0.311	0.580
	Habitat	3.67	0.385	0.539
	SVL_F * Habitat	3.67	0.412	0.525
	BCI_F	3.67	0.035	0.852
	Habitat	3.67	0.032	0.961
	BCI_F * Habitat	3.67	4.352	0.047
	TELO_F	3.67	1.542	0.227
	Habitat	3.67	0.289	0.595
	TELO_F * Habitat	3.67	0.401	0.533
	SVL_M	3.67	1.439	0.239
	Habitat	3.67	1.463	0.235
	SVL_M * Habitat	3.67	1.546	0.222
	BCI_M	3.67	0.062	0.937
	Habitat	3.67	0.094	0.932
	BCI_M * Habitat	3.67	4.351	0.042
	TELO_M	3.67	3.696	0.038
	Habitat	3.67	1.042	0.319
	TELO_M * Habitat	3.67	0.964	0.338
			0.136	0.713
Days between gosner stages	SVL_F	1,33		
	Habitat	1,33	1.571	0.211
	Stage	4,411	2.328	0.055
	SVL_F * Habitat	1,32	1.490	0.223
	SVL_F * Stage	4,411	0.023	0.999
	Habitat * Stage	4,411	0.965	0.426
	SVL_F * Habitat * Stage	4,411	0.978	0.419
	BCI_F	1,31	0.025	0.874
	Habitat	1,29	0.006	0.937
	Stage	4,41	416.965	0.000

	BCI_F * Habitat	1,31	0.315	0.578
	BCI_F * Stage	4,410	4.205	0.002
	Habitat * Stage	4,410	0.728	0.573
	BCI_F * Habitat * Stage	4,410	4.337	0.002
	Telo_F	1,27	0.503	0.484
	Habitat	1,27	0.279	0.602
	Stage	4,395	6.431	0.000
	Telo_F * Habitat	1,267	0.359	0.554
	Telo_F * Stage	4,396	0.538	0.708
	Habitat * Stage	4,395	0.019	0.999
	Telo_F * Habitat * Stage	4,396	0.031	0.998
	SVL_M	1,33	0.195	0.661
	Habitat	1,33	0.101	0.753
	Stage	4,411	1.269	0.281
	SVL_M * Habitat	1,32	0.111	0.741
	SVL_M * Stage	4,411	0.121	0.975
	Habitat * Stage	4,411	1.718	0.145
	SVL_M * Habitat * Stage	4,411	1.777	0.132
	BCI_M	1,31	0.018	0.893
	Habitat	1,29	0.017	0.896
	Stage	4,41	409.634	0.000
	BCI_M * Habitat	1,31	3.270	0.071
	BCI_M * Stage	4,410	0.067	0.992
	Habitat * Stage	4,410	0.750	0.559
	BCI_M * Habitat * Stage	4,410	3.327	0.011
	Telo_M	1,27	1.448	0.230
	Habitat	1,27	2.511	0.114
	Stage	4,395	9.817	0.000
	Telo_M * Habitat	1,267	2.428	0.120
	Telo_M * Stage	4,396	0.741	0.565
	Habitat * Stage	4,395	5.879	0.000
	Telo_M * Habitat * Stage	4,396	5.788	0.000
Total length tadpoles	SVL_F	1,37	0.016	0.899
	Habitat	1,36	3.532	0.068
	Stage	4,393	5.998	0.000
	SVL_F * Habitat	1,37	3.360	0.075
	SVL_F * Stage	4,393	1.116	0.349
	Habitat * Stage	4,393	3.567	0.007
	SVL_F * Habitat * Stage	4,393	3.751	0.005
	BCI_F	1,39	0.004	0.948
	Habitat	1,37	0.301	0.586
	Stage	4,393	1563.974	0.000
	BCI_F * Habitat	1,39	0.045	0.833
	BCI_F * Stage	4,397	0.108	0.980
	Habitat * Stage	4,393	2.110	0.079

	BCI_F * Habitat * Stage	4,397	2.798	0.026
	Telo_F	1,35	0.484	0.491
	Habitat	1,35	0.688	0.413
	Stage	4,378	17.444	0.000
	Telo_F * Habitat	1,35	0.555	0.461
	Telo_F * Stage	1,378	4.235	0.002
	Habitat * Stage	4,378	3.630	0.006
	Telo_F * Habitat * Stage	4,378	3.538	0.008
	SVL_M	1,37	0.577	0.452
	Habitat	1,37	1.885	0.178
	Stage	4,394	8.559	0.000
	SVL_M * Habitat	1,37	1.958	0.170
	SVL_M * Stage	4,393	3.081	0.016
	Habitat * Stage	4,393	0.631	0.640
	SVL_M * Habitat * Stage	4,393	0.739	0.566
	BCI_M	1,35	0.034	0.855
	Habitat	1,37	0.374	0.545
	Stage	4,394	1537.469	0.000
	BCI_M * Habitat	1,35	2.703	0.109
	BCI_M * Stage	4,389	0.651	0.626
	Habitat * Stage	4,394	1.745	0.139
	BCI_M * Habitat * Stage	4,389	2.912	0.021
	Telo_M	1,35	0.000	0.988
	Habitat	1,35	1.320	0.258
	Stage	4,389	22.127	0.000
	Telo_M * Habitat	1,35	1.189	0.283
	Telo_M * Stage	4,389	1.590	0.176
	Habitat * Stage	4,389	3.218	0.013
	Telo_M * Habitat * Stage	4,389	3.349	0.010
Hatchling telomeres size	SVL_F * Habitat	3.107	1.350	0.252
	BCI_F * Habitat	3.107	0.015	0.902
	TELO_F * Habitat	3.107	3.131	0.095
	SVL_M * Habitat	3.107	1.141	0.293
	BCI_M * Habitat	3.107	0.471	0.497
	TELO_M * Habitat	3.107	0.717	0.403

		Df	F value	P value
Hatchling telomeres size	Eggs size	3.109	1.894	0.173
	Habitat	3.109	0.295	0.588
	Eggs size * Habitat	3.109	0.305	0.582
Hatchling telomeres size	Hatching success	3.107	0.072	0.783
	Habitat	3.107	3.025	0.085
	Hatching success * Habitat	3.107	5.175	0.025
Hatchling telomeres size	EDD	3.107	12.998	< 0.001
	Habitat	3.107	2.752	0.103
	EDD * Habitat	3.107	2.703	0.105
LDD	Hatchling telomeres size	3.67	2.610	0.120
	Habitat	3.67	4.043	0.056
	Hatchling telomeres size * Habitat	3.67	4.017	0.045
Metamorphs morphometrics traits	SVL_F	3.67	2.556	0.114
	Habitat	3.67	5.114	0.271
	SVL_F * Habitat	3.67	5.091	0.274
	BCI_F	3.67	0.016	0.899
	Habitat	3.67	0.423	0.517
	BCI_F * Habitat	3.67	0.044	0.834
	TELO_F	3.67	0.740	0.392
	Habitat	3.67	3.268	0.076
	TELO_F * Habitat	3.67	2.837	0.097
	SVL_M	3.67	0.037	0.848
	Habitat	3.67	3.718	0.582
	SVL_M * Habitat	3.67	3.889	0.528
	BCI_M	3.67	0.173	0.679
	Habitat	3.67	0.288	0.593
	BCI_M * Habitat	3.67	0.012	0.976
	TELO_M	3.67	0.404	0.527
	Habitat	3.67	0.932	0.337
	TELO_M * Habitat	3.67	0.775	0.382
	Hatchling telomeres size	3.67	0.641	0.426
	Habitat	3.67	1.457	0.232
	Hatchling telomeres size * Habitat	3.67	1.357	0.248

In this study, we examined the influence of contrasted habitat types (intensive agriculture *versus* preserved forest) on the phenotype of reproductive individuals as well as on their reproductive performance. Interestingly, we failed to detect significant effects of the habitat type on the phenotype of reproductive individuals or direct effects of parental habitat on reproductive performances. Importantly, we found several traits linked to reproductive success (clutch size, hatching success, larval development duration and larval morphology) that were influenced by parental habitat through interactions with parental phenotype. Taken together, these results suggest that parental habitat can alter the relationships between parental phenotype and reproductive performances.

Parental habitat and parental phenotype

We did not find significant effects of habitat type on the phenotype of reproductive individuals. Indeed, reproductive toads from preserved forest habitat were similar that individuals living in intensive agricultural landscape in terms of body size, body condition and telomere length. These findings contrast with the results from other studies, which found that toads were larger and in better condition in agricultural habitats as compared to forests (Guillot et al., 2016; Zamora-Camacho and Comas, 2017). This appears surprising as one of these studies (Guillot et al., 2016) focused on the same study species in the same geographic area. Importantly, our study design differs from that of Guillot et al. (2016) for several aspects. First, the number of sites included by Guillot et al. (2016) was larger (N=12 sites) while the number of study sites included in the current study was lower (N=4 sites) due to the logistical complexity linked to the monitoring of reproductive performances. Second, Guillot et al. (2016) focused solely on single males (not involved in amplexus) found in reproductive ponds while our study focused on paired (amplexed) individuals from both sexes. Finally, in order to avoid any confounding effects of reproductive phenology on parental phenotype and reproductive performances (Saino et al., 2005; Clark et al., 2014; Jonsson and Jonsson, 2014), we focused our study on the first individuals that arrived and mated at our study sites, while Guillot et al. (2016) opportunistically sampled individuals independently of the temporal patterns of arrival at reproductive ponds. All of these differences may have played a role in the contrasts between the findings of Guillot et al. (2016) and those from the current study. Future studies should usefully focus on a larger number of study sites and sample during the whole reproductive period in order to test whether these contrasts can be reconciled.

Direct effects of parental habitat on reproductive performances

Similarly to the absence of effect of parental habitat on parental phenotype discussed above, we failed to detect significant direct effect of parental habitat on reproductive performance. Fecundity, hatching success, development durations (embryonic and larval) or tadpole phenotype (morphology and telomere length) appeared similar irrespective of the parental habitat. Such result was surprising owing to the well-known influence of parental habitat on reproduction in general or specifically in agricultural context (Hinsley and Bellamy, 2000; Benton et al., 2003; Britschgi et al., 2006). Importantly, these results could lead to the idea that altered environments (intensive agriculture) do not affect reproductive performance in our study species. Yet, taking into account the (complex) interactions between parental habitat and parental phenotype on reproductive performance challenged this simplistic hypothesis (see below). It is also plausible that offspring development is optimized for specific habitats through parental effects (Mcginley et al., 1987; Uller, 2008; Jørgensen et al., 2011). That is, individuals living and reproducing in a specific set of environmental conditions may produce offspring that develop better in these specific environmental context (Stearns, 1976; Ricklefs and Wikelski, 2002). Because our study design involved a 'common garden' approach to allow straightforward comparisons of reproductive performances between habitat types, we may have failed to detect such phenotypic matching between the conditions experienced by reproductive parents and those that will be experienced by their developing offspring (refs). Future studies should usefully adopt a 'cross-fostering' approach between agricultural and forest reproductive sites in order to test for this hypothesis.

Parental habitat alters the relationships between parental phenotype and reproductive performances

In strong contrast with the elements discussed above, we found that several key traits linked to reproductive performances were affected by parental habitat types through (complex) interactions with parental phenotype. Clearly, taking into account the influences of parental phenotype on reproductive outputs (Hendry et al., 2001; Donelson et al., 2008) challenges the results yielded from simple direct comparisons of reproductive performance between habitat types. These results further emphasize that deciphering the effects of altered habitats on biodiversity deserves to adopt a comprehensive approach in order to thoroughly investigate both habitat-specific influences in interaction with parental-dependent traits.

More specifically, we found that clutch size was differentially influenced by paternal body size between agricultural or forest habitats. In individuals from forest habitat, clutch size decreased with male body size while this relationship did not occur in individuals from agricultural sites. Such result indicates that reproductive females from forest habitats may be able to modulate the number of eggs laid according to the body size of their mate as already demonstrated in a frog species (Reyer et al., 1999).

The contrasted responses between parental habitat type could plausibly be linked to the fact that larger males are presumably older individuals in forest habitats while this may not be the case in agricultural habitats (Zamora-Camacho and Comas, 2017). Whether larger and older males originating from forest habitats are perceived as of poorer quality by females remains puzzling and should deserve specific investigations. Whatever the underlying mechanisms, this result suggests that the ability of a reproductive female to estimate the quality of her mate and thus to adjust her reproductive effort may be altered in agricultural habitats.

Importantly, we found that the relationship between female phenotype (body size) and hatching success was strongly affected by habitat type. In our study species, previous studies have found that hatching success was linked to embryonic mortality (Cheron et al., 2021a, Renoirt et al., 2022) and not to (a deficit of) fertilization as in the closely related common toad (Touzot et al., 2020). As expected, hatching success was positively correlated to female body size in forest habitat, suggesting that larger females produce not only larger clutches, but eggs of better quality. In contrast, we found that hatching success of the clutch produced by females originating from agricultural habitats decreased with increasing female body size, while the positive relationship between female size and clutch size was similar between habitat types. This suggests that, for individuals originating from agricultural habitats, larger females produce larger clutches of poor quality eggs which fail to successfully develop into hatching tadpoles. As above, the mechanisms underlying such size-specific responses need to be deciphered, but may plausibly be linked to aging patterns (refs), or altered trade-offs between clutch size and egg quality (refs) linked to altered resources in agricultural habitats (refs). Whatever the underlying mechanisms, this result suggests that the production of live and healthy tadpoles is altered in agricultural habitats, a process that may affect population persistence (Bridges, 2000; Babini et al., 2016).

Key larval developmental traits were also differentially affected by habitat types through interactions with parental traits. Larval development duration was affected by paternal condition in individuals produced by parents originating from agricultural habitats, suggesting that agricultural fathers in better condition produced faster developing offspring. Conversely, larval development duration was affected by maternal condition in individuals produced by parents originating from forest habitats, suggesting that forest mothers in better condition produced faster developing offspring. Interestingly, most of these effects were found during key developmental stages, notably those linked to the peak of larval somatic growth (i.e., GS30 and GS37, Gosner 1960, Cheron et al., 2021b). Importantly, larval development duration is a critical trait that will affect reproductive success, notably if reproduction occurs in ephemeral water bodies. In temporal ponds, faster developing tadpoles will have higher probability to achieve metamorphosis and thus to survive (Travis, 1983; Altwegg, 2002). Yet, accelerated development can bear long-term consequences through processes linked to physiological cost of development (e.g., oxidative stress, telomere length, Cheron et al., 2022).

In support of this hypothesis, we found that in agricultural individuals, males with longer telomere produced tadpoles that will grow more slowly during somatic growth phases, and that tadpoles with longer telomere grow more slowly, while both relationships were non-significant in forest habitats. We emphasize that the opposite sex-specific (maternal *versus* paternal) influences on larval development duration depending on habitat needs to be investigated to understand the relative contributions of both paternal and maternal phenotype on determining the duration of larval stage. Whatever the underlying mechanisms, these results suggest that the determinants of larval development duration are altered in agricultural habitats as compared to forest habitats and investigating the ultimate effects of larval development duration on habitat-specific reproductive success is required in order to understand the impact of intensive agriculture on spined toads.

Finally, tadpole morphology was also differentially affected by habitat types through interactions with parental traits. Again, all of these effects occurred during the somatic growth of larvae (GS30), a key developmental stage (Cheron et al., 2021b). In individuals originating from forest parents, female traits (size and condition) positively influenced tadpole size while in individuals originating from agricultural parents, females traits (size and telomere length) negatively affected tadpole size. Paternal contributions to tadpole size were negative in both habitats (paternal body condition in forest individuals and paternal telomere length in agricultural individuals). Tadpole size is a critical parameter that will affect mobility, survival and thus metamorphic success (Semlitsch, 1990), and the fact that maternal traits negatively affect this parameter in agricultural sites may affect overall reproductive success in these habitats. As above, opposite sex-specific (maternal *versus* paternal) influences on tadpole size needs to be investigated to understand the relative contributions of both paternal and maternal phenotype on determining larval size.

Conclusion

Overall, our study highlight strong contrasts between the absence of direct effects of habitat type on parental traits or on reproductive performance and the altered relationships between parental traits and reproductive performances between contrasted habitats. Most of the mechanisms underlying the patterns we found needs to be deciphered and discussing these putative mechanisms is both highly speculative and out of the scope of the current study. Yet, investigating these mechanisms will be critical to improve our understanding of the effects of habitat quality on reproduction and population persistence. Our results emphasize variable, often opposite, effects of parental traits on key metrics of offspring development according to habitat types. We believe that taking into account the (complex) interactions between parental habitat and parental phenotype that determine reproductive performance is essential in order to understand the ultimate effects of altered habitats (i.e., intensive agriculture) on reproductive success and thus population persistence. Finally, we also believe that our results may open new fruitful avenues of research which should aim at understanding the variable, often opposite, effects of paternal *versus* maternal phenotypes on the parameters we investigated.

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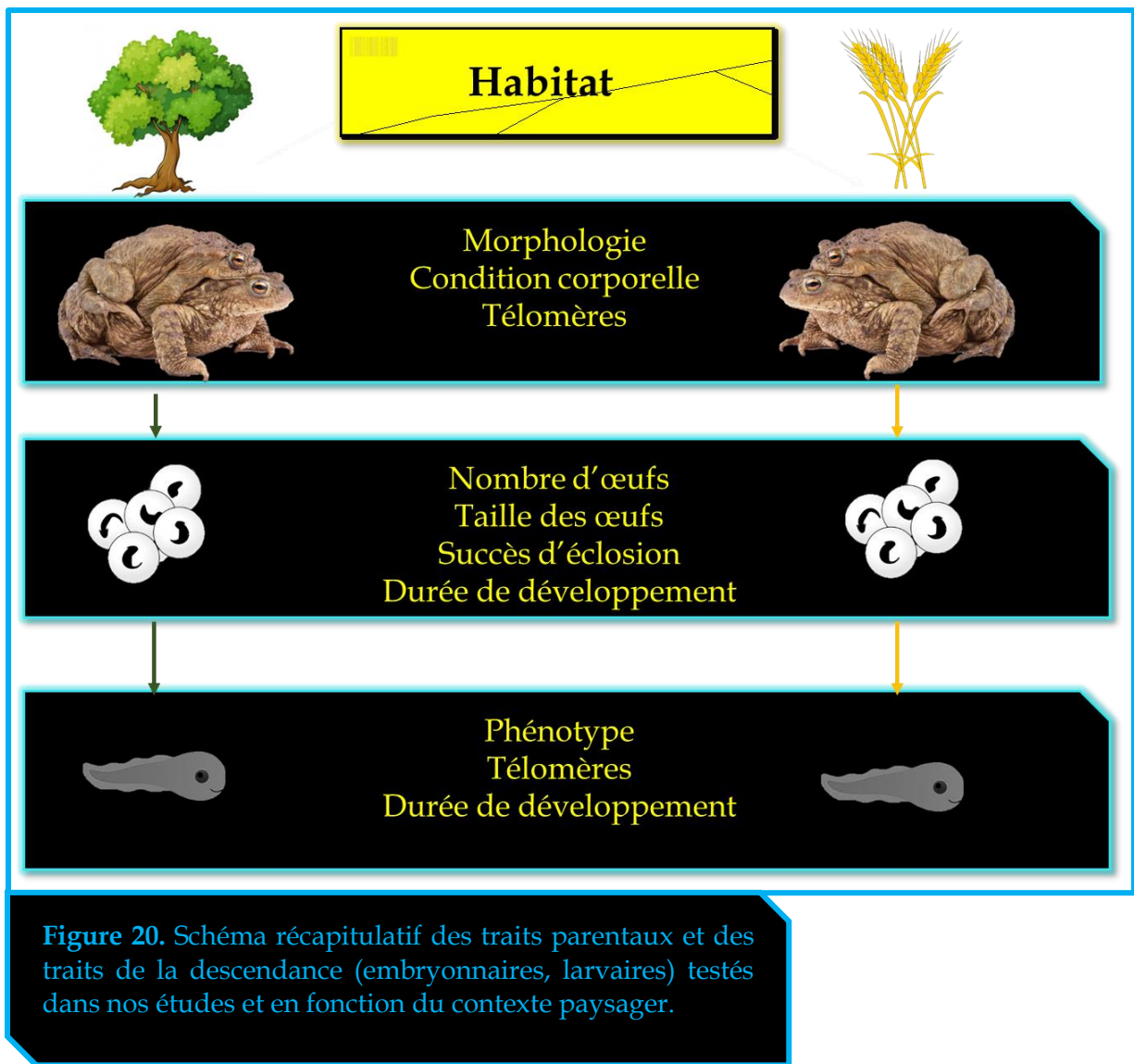
IV/ Conclusion

Dans ce chapitre (**Article 5 et 6**) nous avons pu mettre en évidence des contributions des traits, de la qualité individuelle, maternels et paternels à la fécondité et au développement de la progéniture que ce soit en relation ou pas avec un contexte paysager allant de milieux conservés à dégradés (**Figure 20**).

Dans l'**article 5** nous avons pu voir que les traits de qualité de la femelle (SVL, BCI) influencent la fécondité, le succès d'éclosion, la taille des têtards et la durée de développement larvaire (**Figure 21**). Contre toute attente, nous avons trouvé des contributions significatives du phénotype paternel sur la durée de développement embryonnaire (**Figure 21**). Etant donné que nous ne connaissons pas les mécanismes sous-jacents à ces résultats, nous suggérons d'étudier les coûts physiologiques du développement de la progéniture (par exemple, l'attrition des télomères, le statut oxydatif, Burraco et al., 2017 ; Saino et al., 2005) afin de mieux comprendre les résultats obtenus dans cette étude, nous avons réalisé l'année suivante une étude similaire en prenant en compte la longueur des télomères pour les parents comme pour la descendance (**Article 6**).

Dans l'**article 6** nous avons trouvé des relations entre les traits de qualité parentale (SVL, BCI, Télomères) et les traits de la qualité de la progéniture aux différents stades du développement (embryonnaire, comme larvaire, **Figure 22**). Les résultats obtenus dans l'**article 5** sont pour la plupart confirmés dans l'**article 6** et notamment l'influence du père sur la descendance. Cependant, nous avons pu observer des résultats divergents en fonction du contexte paysager, avec comme observation que le milieu agricole a l'air de contraindre et/ou de perturber la reproduction des individus, contrairement aux parents issus des milieux forestiers (**Figure 22**). Le plus étonnant étant l'absence de relations directes du type d'habitat sur les traits parentaux, la fécondité et les traits de la descendance. Ceci suggère que l'habitat influence la descendance à travers les traits parentaux. De ce fait, les mécanismes qui sous-tendent les modèles que nous avons trouvés sont complexes et difficiles à expliquer à partir de notre étude. Cependant, nous suggérons des voies d'investigations supplémentaires sur ces mécanismes puisque leur explication permettrait une meilleure compréhension des effets de la qualité de l'habitat sur la reproduction et notamment sur la persistance de ce type d'espèces dans ce genre de milieu.

Finalement, et étant donné les effets négatifs des intrants chimiques sur les populations d'amphibiens, nous pouvons suggérer une possible influence des pesticides (contamination) contraignant la reproduction de cette espèce et la réponse de la descendance à travers les traits parentaux.



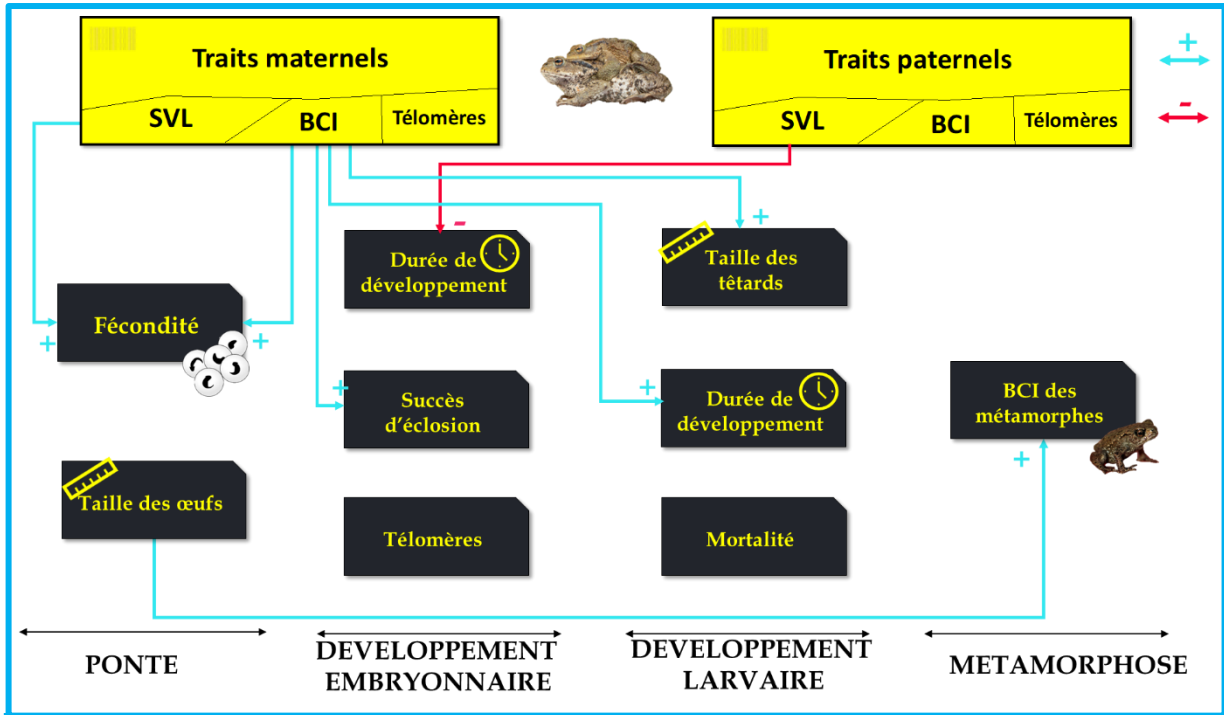


Figure 21. Schéma récapitulatif des résultats obtenues dans l'article 5. Relation entre qualité parentale et qualité de la progéniture.

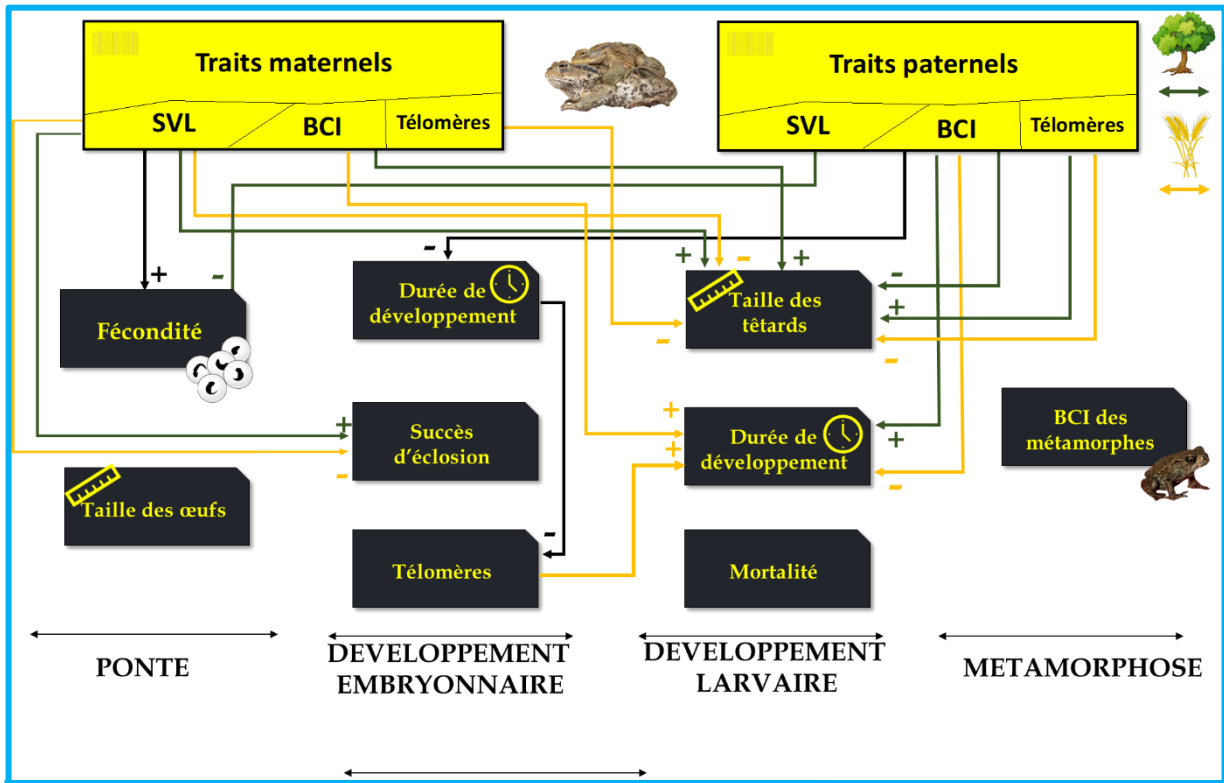


Figure 22. Schéma récapitulatif des résultats obtenues dans l'article 6. Relation entre qualité parentale et qualité de la progéniture selon le type d'habitat.

PERSPECTIVES

Cette thèse avait pour objectif d'évaluer les effets des contraintes environnementales d'un milieu modifié par l'agriculture moderne intensive, sur la persistance des populations d'amphibiens. Pour ce faire, nous avons utilisé une multitude d'approches complémentaires permettant une vision large du maintien des populations dans ce type de paysage. La base de ces travaux a été une approche comparative entre des populations issues de milieux conservés (naturels ; forestiers) et plus ou moins dégradés (modifiés ; agricoles). Dans un premier temps, afin de comparer les populations forestières et les populations agricoles, nous nous sommes appuyés sur un panel d'indicateurs pour examiner la structure des populations (marqueurs micro-satellites) et l'écologie alimentaire (isotopes stables). Nous avons ensuite déterminé, à travers une approche terrain, l'état des populations de crapauds épineux le long d'un gradient d'habitat, en réalisant un suivi des populations sur le terrain. Enfin, expérimentalement, nous avons évalué la qualité individuelle (télomères, phénotypes) ainsi que son impact sur la reproduction et la descendance (fécondité, qualité embryonnaire et larvaire) à travers un contexte forestier - agricole. Chaque étude a déjà été discutée dans les différents articles présentés plus haut (**Article 1, 2, 3, 5, 6, Annexe 1, 2**). Dans cette partie « perspective » on se concentre sur la mise en relation des différentes parties de la thèse et sur l'identification des faiblesses des études réalisées, permettant d'avancer des pistes d'explorations futures afin de mieux comprendre les mécanismes pouvant expliquer nos résultats.

I/ Utilisation effective de l'habitat par *B. spinosus*

L'étude de l'écologie alimentaire du crapaud épineux à partir des isotopes stables (**Article 1**) a permis de mettre en avant l'utilité de cette méthode dans la détermination du milieu de vie des individus selon un contexte forestier-agricole. Par la même occasion, nous avons pu montrer que la supplémentation en azote (engrais) dans les milieux agricoles conduit à des modifications des maillons de la chaîne trophique avec des augmentations des ratios isotopiques de l'azote à la base des réseaux trophiques pouvant avoir des répercussions sur l'ensemble du réseau alimentaire. Nous montrons dans l'**article 2** que la structure de l'habitat en milieu agricole intensif reste suffisamment perméable au maintien de la diversité génétique et que la structure génétique des populations de crapauds épineux est homogène, sauf pour un site d'étude particulier.

1. Les marqueurs isotopiques de tous les maillons de la chaîne trophique

Notre étude réalisée dans l'**article 1** a permis de mettre en évidence des effets de l'environnement agricole sur l'écologie alimentaire des populations de crapauds épineux en lien avec la fertilisation de l'environnement. Notre étude s'est exclusivement concentrée sur le crapaud épineux, et la question de l'augmentation de la signature isotopique en azote sanguin ($\delta^{15}\text{N}$) sur d'autres organismes vivant dans ce type de paysage reste ouverte. De plus, nous manquons de précisions sur les conséquences physiologiques et structurelles (populationnelles) d'une telle augmentation par rapport à des milieux conservés. Malgré une absence de résultats directs entre physiologie des organismes et supplémentation, Shehab and Guo, 2021 ont montré dans une étude que la fertilisation en azote pour augmenter le rendement et la qualité du sorgho (plante originaire d'Afrique) a mis en avant une accumulation d'acide cyanhydrique (HCN) dans les plantes, ce qui augmenterait le risque de toxicité pour les animaux, en particulier dans des conditions de sécheresse. Ces résultats sont particulièrement intéressants dans un contexte de réchauffement climatique et d'ouverture du milieu (modification des températures, Lawler et al., 2010; De Frenne et al., 2019; De Lombaerde et al., 2022) en paysage agricole. Les isotopes stables sont particulièrement étudiés dans le domaine marin, mais adaptés aux milieux terrestres (Kelly, 2000; Sherwood and Rose, 2005; Hyodo, 2015; McMahon et al., 2015). Notre étude ne semble pas montrer de contraste sur les signatures isotopiques en carbone sanguin ($\delta^{13}\text{C}$) en fonction des habitats. Avec la culture du maïs présente dans la région (Agreste, 2016), une plante C_4 , on se serait cependant attendu à des effets de ce type de culture sur les signatures isotopiques en carbone ($\delta^{13}\text{C}$) des populations de crapaud épineux.

Dans un premier temps, et en considérant le fait que le crapaud épineux est un méso-prédateur généraliste, il pourrait être intéressant de recenser les différentes cultures présentes dans le domaine vital du crapaud épineux (autour des mares de reproduction) afin de déterminer la signature isotopique des producteurs primaires (plantes C₃ et C₄) à la base de la chaîne alimentaire. Le fractionnement différentiel des isotopes stables de carbone durant la photosynthèse fait que les plantes C₄ et les plantes C₃ ont des signatures d'isotopes de carbone différentes (Kelly, 2000). Plus précisément, le carbone fixé par les plantes terrestres C₃ ($\delta^{13}\text{C} = -27\text{‰}$, plage = -35 à -21‰) peut être distingué de celui fixé par les plantes C₄ ($\delta^{13}\text{C} = -13\text{‰}$, gamme = -14 à -10‰), car il contient relativement peu d'isotopes ¹³C (Boutton, 1991; Ehleringer, 1991). Cette différence est due à la discrimination contre les isotopes ¹³C par l'enzyme primaire de fixation de CO₂ (RuBP) des plantes C₃. Chez les plantes C₄, l'enzyme primaire de fixation du CO₂ (PEP carboxylase) ne discrimine pas le ¹³C aussi fortement que celle des plantes C₃ (O'Leary, 1981; Farquhar et al., 1989). Ensuite, parce que les maillons trophiques inférieurs sont les premiers à subir les changements de leur environnement et que ces changements se répercutent sur les niveaux trophiques supérieurs, ces changements pourraient être mieux appréhendés en évaluant la signature isotopique des différents maillons du réseau trophique jusqu'au crapaud épineux (Hyodo, 2015). Plus précisément, déterminer la signature isotopique des consommateurs primaires et de toutes les espèces d'insectes plus hautes dans la chaîne trophique (consommateur secondaire, tertiaire etc, Hyodo, 2015). Ceci nous permettrait, tout d'abord, d'apporter des connaissances précises et fondamentales sur le régime alimentaire de cette espèce et de comparer l'écologie alimentaire des individus issus de milieux dégradés par rapport à celle des individus issus de milieux conservés ; mais aussi de déterminer les conséquences de cette supplémentation en engrais azoté sur les organismes de niveaux trophiques supérieurs et notamment ceux en bout de chaîne (« super prédateurs »). Les résultats épars des valeurs sanguines azotées ($\delta^{15}\text{N}$) pour un même type d'habitat suggèrent une utilisation différentielle des micro-habitats et de possibles déplacements individuels entre ces micro-habitats.

2. Micro-habitat et continuité écologique

Dans l'**article 2**, nous avons montré que les déplacements des populations de crapauds épineux ne semble pas contraint par l'environnement. On s'attendait cependant à ce que l'environnement agricole ait un effet négatif sur la diversité génétique des populations, en lien avec les contraintes d'un habitat dégradé. L'unique site pour lequel la structure génétique de la population était différente des autres populations est un site présentant une structure particulière, étant difficilement accessible (voir **article 2**). La population présente sur ce site est génétiquement différente des autres populations même proches, avec des taux d'hétérozygotie et de richesse allélique plus faibles et des taux de consanguinité plus élevés. Ces résultats suggèrent un appauvrissement génétique de cette population, suggérant des échanges réduits de cette population avec les populations voisines. Nous recommandons donc d'étudier avec plus de précision la structure générale de ce site, et plus généralement des sites isolés, pour y déterminer les différents processus poussant à cette baisse de diversité génétique. Étant donné que les micro-habitats possèdent des caractéristiques physiques et écologiques différentes de son environnement direct, il est nécessaire de prendre en considération le fait que les contraintes environnementales ne s'appliquent pas de la même manière dans un environnement donné et que les organismes utilisent différenciellement l'habitat (Cody, 1985; Martin, 1998; Smith and Ballinger, 2001; Jorgensen, 2004). Les linéaires de haies en milieu agricole vont avoir des caractéristiques différentes de l'habitat agricole lui-même et vont permettre de connecter les populations entre elles, notamment dans le cas du crapaud épineux (Fischer et al., 2013; Guillot et al., 2016; Boissinot et al., 2019). Dans le cas de notre étude, l'étude des micro-habitats et de la connectivité de ce site de reproduction pourrait expliquer en partie la perte de diversité génétique de cette population. Dans plusieurs études il a été montré que les contraintes de l'environnement agricole pouvaient diminuer la disponibilité de micro-habitats favorables; mais aussi la disponibilité des ressources trophiques engendrant de la compétition pour l'accès à la ressource (Walther et al., 2009; Bàrberi et al., 2010; Abdala-Roberts et al., 2019). Par exemple, Reverter et al., (2021), ont montré que les cultures affectent négativement la structure de la végétation et la biomasse des arthropodes au-delà des limites de la parcelle cultivée.

Aussi, Van Wilgenburg et al., 2001 ont montré une différence de microclimat (température et humidité) entre les habitats forestiers et agricole. Le rôle des forêts est fondamental dans les réponses des communautés au changement climatique (Zellweger et al., 2020). Les canopées fermées protègent des effets du changement microclimatique par leur effet de refroidissement, ralentissant les changements dans la composition de la communauté, tandis que les zones ouvertes ont tendance à accélérer le changement de la communauté par des effets de chauffage locaux (Zellweger et al., 2020). Ainsi, les forêts forment des habitats avec des microclimats favorables aux amphibiens.

Les amphibiens sont en effet particulièrement sensibles au réchauffement climatique car de nombreuses espèces ont des besoins en humidité et des marges de sécurité thermique étroites, résultant des températures de l'air et du corps proches de leurs maximums thermiques critiques (CTmax, Nowakowski et al., 2017). De ce fait, les changements des conditions climatiques qui s'appliquent plus fortement dans les habitats ouverts (augmentation des température et réduction des précipitations, Lawler et al., 2010; De Frenne et al., 2019; De Lombaerde et al., 2022) peuvent affecter les populations d'amphibiens. Une étude de De Lombaerde et al., (2022), a mis en évidence que les microclimats forestiers se réchaufferont, dans le temps, à un rythme plus lent que les zones non forestières, ce qui suggère que les espèces adaptées à des conditions de croissance plus froides pourraient trouver un abri et survivre plus longtemps que prévu sur un site forestier donné (De Lombaerde et al., 2022). Dans le cadre du changement climatique futur, ces résultats soulignent le rôle potentiel des forêts en tant que micro refuges pour la biodiversité (De Lombaerde et al., 2022). Malgré les preuves croissantes de leur importance pour la dynamique et les processus écosystémiques, comme la réponse des organismes au changement climatique, les microclimats sont souvent négligés en écologie et en évolution. Les microclimats affectent le fonctionnement physiologique des organismes qui, à leur tour, influencent la structure, la composition et le fonctionnement des écosystèmes (Zellweger et al., 2019, 2020). Ainsi, l'analyse des microclimats à l'échelle du paysage permet de comprendre les interactions climat-espèces et la dynamique de la distribution des espèces qui ont des implications dans les réponses des espèces et des écosystèmes au changement global (Zellweger et al., 2019, 2020). Il devient donc nécessaire d'étudier les différences de microclimats entre les habitats et l'étude par radio-tracking des populations de crapauds épineux est un outil à prendre en considération.

Malgré des résultats surprenants sur la structure génétique des populations de crapauds épineux en contexte agricole, nos résultats contrastent avec une étude similaire sur la génétique des populations des tritons (*Triturus marmoratus*) en Deux-Sèvres. Gauffres et al. (2022) ont mis en évidence une influence positive de la densité des étangs sur la diversité génétique locale et une influence négative de la couverture des terres arables sur le flux génétique et la connectivité des populations. La différenciation génétique joue un rôle essentiel dans la persistance des espèces et il existe deux types d'isolement qui sont pertinents pour la persistance des populations de crapauds en paysage fragmenté. Il s'agit de : 1) l'isolement par la distance (IBD), où les populations qui sont plus éloignées les unes des autres deviennent plus isolées avec le temps ; et 2) l'isolement par la barrière (IBB), où la présence d'obstacles aux mouvements peut isoler les populations (Macdonald et al., 2020).

Dans une étude, similaire à la nôtre, au Royaume-Unis, Macdonald et al., (2020) ont pu mettre en évidence le fait que les populations reproductrices de crapaud communs étaient plus différentes génétiquement avec l'augmentation de la contrainte liée à une barrière géographique (IBB). Aussi, ils ont trouvé une absence de différenciation génétique selon l'IBD pouvant s'expliquer par le fait que les crapauds communs sont moins philopatrics qu'on le pense et que la disponibilité réduite de bassins de reproduction adéquats peut pousser davantage de migrants à se disperser sur de plus grandes distances, ce qui pourrait améliorer le mélange génétique de la métapopulation. Ces résultats peuvent être mis en exergue avec les résultats que nous avons obtenus dans l'**article 2**.

La génétique des populations est intimement en lien avec les déplacements d'individus entre population (migration, taux de recrutement), et on ne peut pas exclure le fait que les capacités locomotrices du crapaud épineux puissent avoir un rôle majeur dans le maintien de la diversité génétique en milieu dégradé. Zamora-Camacho, (2018) ont étudié la performance de locomotion de crapauds calamites (*Epidalea calamita*) entre habitat dégradé et conservé. Il a montré que les crapauds de l'habitat agrosystème avaient une vitesse de course plus élevée et des arrêts plus fréquents, ce qui pourrait contribuer à augmenter la vigilance ou se camoufler des prédateurs, probablement en relation avec l'ouverture de l'habitat (Trouilloud, Delisle & Kramer, 2004 ; Zamora-Camacho, 2018). De ce fait, les femelles pourraient être d'autant plus impactées et contraintes par le milieu agricole en raison de l'ouverture du milieu et de pression de prédation plus forte en l'absence d'abris. Ce phénomène pourrait expliquer l'abondance relativement plus faible des femelles dans le milieu agricole (**Articles 3 et 4**). Pourtant, cette absence de femelles pourrait constituer un biais dans notre échantillonnage de la diversité génétique des populations.

3. Le radio-tracking et le Capture-Marquage-Recapture (CMR) comme outil

Les différentes études des **articles 1, 2, 3 et 4** soulèvent beaucoup de questions et de nombreuses hypothèses, qui ne suffisent en l'état pas à expliquer les mécanismes sous-jacents, ces questions pourraient être mieux approchées en réalisant un suivi des populations non pas seulement pendant la période de reproduction mais sur une année complète et sur les sites d'hivernage. Le radio-monitoring (**Figure 23**) pourrait être une solution afin de mieux comprendre l'écologie de ces animaux, l'utilisation de l'habitat et du micro-habitat, les mouvements populationnels et notamment des femelles, mais également la dispersion post-natale (métamorphose). Sinsch et al., (2012), ont réalisé un suivi de populations de *E. calamita* par radio-tracking. Dans cette étude, le rayon de migration n'était pas différent en fonction du sexe, mais était trois fois plus faible pour les populations provenant de zones sablonneuses par rapport à celles vivant sur des sols argileux, probablement en raison de la rareté des abris humides entraînant des déplacements plus fréquents et plus éloignés (Sinsch et al., 2012). Ce type d'étude pourrait être adapté à des suivis de populations de crapauds épineux en contexte agricole et forestier et permettrait de mettre en évidence de possibles contraintes structurelles de l'habitat en étudiant son utilisation par les populations.

Dans la littérature les amphibiens sont caractérisés comme ayant des capacités de dispersion limitées, une forte fidélité au site et un habitat de reproduction spatialement disjoint formant, de ce fait, des métapopulations (Sinsch, 1990; Blaustein et al., 1994; Duellman and Trueb, 1994; Beebee, 1996; Berry, 2001; Beebee, 2005). Cependant, dans une revue de la littérature par Alex Smith and M. Green, (2005) il est mis en avant des preuves solides que la dispersion des amphibiens n'est pas aussi uniformément limitée qu'on le pense souvent et que certaines populations d'amphibiens sont structurées comme des métapopulations, mais pas toutes. De ce fait, le CMR est une méthode communément utilisée pour estimer les tailles de populations, leur évolution dans le temps, les taux de recrutement et la survie. Elle s'applique parfaitement bien aux amphibiens et aux thématiques étudiées dans les **articles 1, 2, 3 et 4** selon un contexte paysager. Honeycutt et al., (2019) mettent en avant le manque d'information des taux démographiques de base tels que la survie et les mouvements. De plus, ils mettent en avant l'intérêt de considérer les stades de vie et les sexes puisqu'ils contribuent souvent de manière différente à la dynamique des populations. Dans cette étude (Honeycutt et al., 2019) les résultats suivants concordent avec l'intérêt d'intégrer les femelles (et possiblement l'âge des individus) puisqu'ils ont mis en évidence, chez deux espèces d'amphibiens, que les jeunes individus se déplacent plus loin que les individus plus âgés, et que les femelles se déplacent plus loin que les mâles chez les deux espèces. Ainsi, la CMR et le radio-monitoring pourrait être des outils utiles afin d'estimer les applications nécessaires à la conservation des espèces d'amphibiens vivant dans des milieux dégradés et de mieux comprendre les contraintes de cet environnement sur les populations.



Figure 23. Grenouille commune portant un émetteur « tag » radiogoniométrique autour de la taille. Crédit photo par Betsy Roznik

II/ Dynamique des populations et influence de l'habitat

Nous avons montré dans les **articles 3 et 4** un effectif moindre global pour les deux sexes, en défaveur des sites agricoles par rapport aux sites forestiers. De plus, cet effet paraît d'autant plus fort pour les femelles, avec une abondance plus faible voire nulle de femelles en milieu agricole pendant la reproduction. Après avoir montré l'importance de connaître l'utilisation effective de l'habitat par *B. spinosus*, nous avons commencé à mettre en place un suivi à long terme (4 ans) qu'il serait important de continuer puisque nous avons pu montrer une baisse des populations en milieux agricoles.

1. Abondance et suivi des populations

Dans les **articles 3 et 4**, nous avons pu mettre en évidence le fait que l'agriculture influence négativement la présence d'individus reproducteurs sur les sites de reproduction (Keller and Waller, 2002; Williams et al., 2015; Tucker et al., 2018). Bien que les deux sexes soient soumis à cet effet, les contraintes agricoles semblent affecter les femelles plus que les mâles, pouvant ainsi compromettre la persistance des populations dans les habitats agricoles. Les hypothèses pouvant expliquer ces résultats sont multiples et la réduction des abondances de *B. spinosus* en milieu agricole résulte sûrement d'interactions complexes entre diverses contraintes spécifiques à l'habitat et au sexe. C'est pourquoi l'intérêt de réaliser une étude de structure des populations en prenant en compte les femelles pourrait apporter des résultats plus contrastés entre les différents habitats. Étant donné leur faible abondance par rapport aux mâles mais aussi leurs tailles corporelles plus conséquentes qui peuvent avoir un rôle majeur dans les déplacements elles peuvent contraindre le maintien de la diversité génétique des populations en cas d'absence. La longueur du corps est souvent associée à des jambes plus longues, et un corps plus lourd détermine généralement une masse musculaire plus élevée (Wassersug & Sperry, 1977 ; Ficetola & De Bernardi 2006). Cependant, des différences de vitesse de course selon le sexe et en fonction de la longueur des membres ont pu être démontrées chez une espèce d'amphibien (*Epidalea calamita*, Zamora-Camacho, 2018). La longueur des membres et la vitesse de déplacement peut possiblement s'expliquer par la capacité d'amples qui semblent façonner des membres antérieurs plus longs et une pression de prédation plus importante chez les mâles (Zamora-Camacho, 2018). Pour une vitesse donnée, les mâles ont utilisé des taux de course plus élevés que les femelles et seraient mieux adaptés à l'évitement des prédateurs (Zamora-Camacho, 2018).

Les modifications de l'environnement agricole couplées aux changements des conditions climatiques accentuent et exacerbent les contraintes sur les organismes (comme les amphibiens) qui ont une relation étroite avec l'environnement (température, précipitation etc). Nous supposons que les traits d'histoire de vie (capacité de dispersion, sensibilité à la dessiccation, à la température ou encore à la prédation) différentiels peuvent induire une sensibilité divergente des espèces d'amphibiens à l'agriculture intensive. Ainsi, nous recommandons de mettre en place des analyses comparatives de structure génétique des populations avec différentes espèces d'amphibiens, portant sur les deux sexes, afin de déterminer les différents mécanismes qui contraignent et/ou favorisent les différents processus de la diversité génétique. Ces études pourraient aussi être couplées à une étude précise de la structure du paysage en agriculture intensive. A l'aide d'études futures, il est possible d'établir le rôle relatif de ces différentes contraintes.

Chez les amphibiens le sexe n'est pas déterminé par la température ambiante (Détermination Thermodépendante du Sexe, TSD) cependant plusieurs études ont montré des exceptions quant à la détermination du sexe dans des cas de très fortes températures pendant l'incubation (Shine et al., 2002; Eggert, 2004; Bull, 2008; Nakamura, 2009; Bickford et al., 2010) qui ne concerne pas l'air de répartition de notre modèle d'étude. L'absence de femelles en milieu agricole ([Article 3 et 4](#)) pourrait s'expliquer par un potentiel sexe ratio biaisé dès la naissance selon le milieu et le sexage des têtards serait une voie de recherche à prendre en considération. Pour cela, il faudrait dans un premier temps évaluer chez *B. spinosus* si le sexe est déterminé à la naissance ou s'il est neutre (Eggert, 2004). Ensuite, nous suggérons d'établir les mécanismes qui déterminent le sexe chez cette espèce (e.g. Navarro-Martín et al., 2012; Lambert et al., 2016, 2018; Khan, 2021) . Et finalement, sexer les têtards selon un contexte paysager et/ou selon des caractéristiques intrinsèques audit habitat (e.g. gradient de température, Lambert et al., 2018). Dans une étude de Ujhegyi and Bókony, 2020 sur *Bufo bufo* les deux auteures ont mis en avant l'utilité de la coloration de la peau comme marqueur sexuel non invasif chez les crapauds communs juvéniles. De ce fait, elles ont trouvé un dichromatisme sexuel significatif, les mâles étant plus jaunes-gris (moins rouges) et plus brillants que les femelles (Ujhegyi and Bókony, 2020). Cependant, elles mettent bien en avant les limites de leur étude et précisent bien, que la mesure de la couleur de la peau est insuffisante pour identifier le sexe d'un individu ou le sex-ratio d'un seul groupe mais peut être utile pour des comparaisons qualitatives des sex-ratios entre groupes lorsque aucun autre moyen de sexage phénotypique n'est possible (Ujhegyi and Bókony, 2020).

2. Squelettochronologie

L'une des faiblesses de nos différentes études ([Article 1](#), [2](#), [3](#), [4](#), [5 et 6](#)) est l'absence de connaissance sur l'âge des individus. Zamora-Camacho and Comas, 2017 ont comparé des populations de crapauds calamite entre milieux conservés et dégradés et ont pu constater que l'âge moyen de *E. calamita* était plus bas dans les agrosystèmes que dans les milieux conservés. Ces résultats peuvent s'expliquer par la mortalité accrue due aux facteurs de stress environnementaux des agrosystèmes (Zamora-Camacho and Comas, 2017). Aussi, les crapauds des agrosystèmes étaient plus grands malgré leur jeune âge, suggérant un taux de croissance accéléré. Ainsi, les crapauds des agrosystèmes pourraient compenser les événements reproductifs moins nombreux - en raison de leur vie plus courte - par un investissement reproductif plus élevé à chaque tentative puisque les crapauds des agrosystèmes présentaient des indicateurs accrus d'investissement reproductif. De ce fait, l'âge des populations de crapaud épineux dans nos études pourrait apporter de nombreuses réponses quant aux questions que l'on se pose. La squelettochronologie est une méthode permettant de déterminer avec précision l'âge des individus mais est aussi invasive pour les organismes, nécessitant un échantillon osseux (Frétey and Garff, 1992; Padian and Lamm, 2013; Brum et al., 2019). Cependant, dans le cas de nos études, la plupart des sites de reproductions sont à proximité de routes. Lors de la migration pré-reproduction du crapaud épineux, de nombreux individus traversent les routes afin d'accéder aux sites de reproduction, résultant en la mort de nombreux individus sur des échelles de temps courtes (Ashley and Robinson, 1996; Gibbs and Shriver, 2005; Glista et al., 2008). Il pourrait être intéressant d'utiliser ces cadavres afin de prélever des échantillons osseux et de les faire analyser pour déterminer l'âge moyen des individus composant les populations autour des sites de reproduction. De plus, ce type d'étude peut être mis en place, hors période d'hivernage et de reproduction, puisque, la nuit, les individus sont souvent présents sur les routes et subissent le même sort. Il serait aussi possible de récupérer le contenu stomacal ou les fèces afin d'étudier avec plus de précision le régime alimentaire de *B. spinosus* ([Article 1](#), Dodd, 2010). Ainsi, cette étude permettrait d'établir des liens majeurs entre écologie alimentaire ([Article 1](#)), génétique des populations ([Article 2](#)), suivis des populations ([Article 3 et 4](#)) et reproduction selon un contexte paysager ([Article 5 et 6](#)).

III/ Traits d'histoire de vie en lien avec l'agriculture

Dans les **articles 5 et 6**, nous avons pu mettre en évidence des relations entre marqueurs de la qualité parentale et marqueurs de la qualité de la descendance, que ce soit en relation avec l'habitat ou non. Nous avons discuté, plus haut, des contraintes populationnelles tandis que dans cette partie, nous allons nous intéresser aux traits d'histoire de vie. En effet, l'état de santé des individus peut se répercuter sur la santé des populations.

1. Liens étroits entre traits parentaux et descendance

Les résultats obtenus dans les **articles 1, 2, 3 et 4** suggèrent que la reproduction de cette espèce est potentiellement influencée par l'habitat. Ce processus biologique est essentiel aux maintiens des populations et à la continuité des espèces. La reproduction peut exacerber les contraintes de l'environnement, étudier ce processus en lien avec l'habitat pourrait permettre d'apporter des éléments de réponses quant aux hypothèses et aux perspectives précédentes (**Article 1, 2, 3 et 4**).

De fait, la fécondité est le potentiel reproductif d'un individu (généralement chez les femelles) sur une période donnée (Ramirez Llodra, 2002; Bradshaw and McMahon, 2008) et est sujette à des variations inter-individuelles (Hedgecock and Pudovkin, 2011; Wootton, 2012). Comme détaillé dans les **articles 5 et 6**, la variabilité environnementale, l'écologie, la physiologie et le comportement au cours des différentes phases d'un événement de reproduction vont déterminer le succès reproducteur (Moczek, 1998; Bradshaw and McMahon, 2008; Cauchard et al., 2013; Kölliker et al., 2014; Ratikainen et al., 2018) à partir d'interactions complexes de caractéristiques extra- et/ou intrinsèques de l'individu tout au long d'un événement reproductif (Hoy et al., 2016). Ainsi, dans l'**article 5**, nous avons étudié les relations entre les différents marqueurs de la qualité parentale sur le succès reproductif, et plus précisément, sur la qualité de la descendance tout au long du développement. Dans l'**article 6** en complément, nous avons testé les effets de l'habitat sur la reproduction. L'événement reproductif a ainsi été suivi de la ponte à la métamorphose, et différentes relations entre les phénotypes parentaux (pouvant être affectés par leur milieu de vie), la fécondité et les traits de développement des larves ont pu être évaluées en fonction d'un contexte paysager (conservé *versus* dégradé).

2. Accouplement assortatif et habitat

Dans les **articles 5 et 6**, nous avons pu mettre en évidence l'absence de relation de phénotype (SVL, BCI) entre les mâles et femelles des différents habitats. La reproduction des amphibiens est particulièrement bien documentée et l'accouplement assortatif reste un sujet controversé au sein de ce taxon. En effet, de nombreuses études mettent en évidence un accouplement assortatif selon la taille (Gramapurohit and Radder, 2012; Chajma and Vojar, 2016) alors que d'autres démontrent une absence d'un tel type de choix de la part des individus (Marco and Lizana, 2002; Lee and Park, 2009). Sur la base de données existantes, et en particulier chez un autre reproduction explosif, *Bufo bufo* (Chajma and Vojar, 2016), nous avons fait l'hypothèse d'une relation entre les phénotypes parentaux pour indiquer un éventuel accouplement assortatif. Une étude de Green, (2019) explique que le biais de publication qui empêche la publication de résultats non significatifs et le paradoxe de Simpson auraient tendance à augmenter significativement la prévalence et l'occurrence des résultats en accord avec l'accouplement assortatif. Les résultats obtenus dans nos études montrent qu'il n'y a pas d'accouplement assortatif entre les mâles et les femelles. En outre, dans une étude de Marco and Lizana, (2002), il a été démontré que les crapauds mâles (*B. bufo*) ne font pas de distinction entre les différentes espèces et les congénères, le sexe et/ou la taille des femelles (gravides ou non) pendant la saison de reproduction. Nos résultats suggèrent des effets maternels et paternels sur la reproduction (**Article 5 et 6**) mais aucune influence de l'habitat sur le phénotype des adultes (**Article 6**). Ce résultat est étonnant puisqu'il a été montré à de nombreuses reprises les variations et la plasticité phénotypiques des organismes en lien avec l'habitat (Scheiner, 1993; Galloway, 2005; Miner et al., 2005; Chevin and Lande, 2011).

3. L'influence du phénotype paternel sur la descendance

Dans les **articles 5 et 6**, nous avons pu mettre en évidence des relations entre marqueurs de la qualité parentale et marqueurs de la qualité de la descendance, que ce soit en relation avec l'habitat ou non. Nous avons trouvé des effets forts du phénotype maternel sur le succès reproducteur (e.g. fécondité, succès d'éclosion etc, Gibbons and McCarthy, 1986; Blackmore and Lord, 2000; Castellano et al., 2004; Green, 2015) et, de manière plus surprenante, des effets du phénotype paternel en lien avec le développement embryonnaire ou larvaire. Résultats intéressants puisque chez les amphibiens, le rôle du mâle dans la reproduction est souvent réduit à la fertilisation des œufs (Kouba et al., 2009; Byrne and Silla, 2020). De plus, dans l'**article 6** nous avons pu confirmer des marqueurs parentaux en lien avec l'habitat sur les marqueurs du succès reproducteur et de la qualité de la descendance. Ces relations avec l'habitat sont d'autant plus intéressantes puisque les relations sont inverses selon l'habitat d'origine des parents. Malgré un manque de compréhension des mécanismes sous-jacent, l'environnement agricole s'est révélé restrictif pour la progéniture et la question de la persistance des amphibiens dans ces environnements se pose une nouvelle fois. Nous suggérons donc de futures approches et hypothèses (voir ci-dessous) afin de mieux comprendre les mécanismes en jeux.

D'autres études pourraient expliquer l'investissement reproductif et les relations entre les métriques parentales et la descendance en fonction de l'habitat. C'est le cas de quelques facteurs tels que l'investissement énergétique dans la progéniture, la qualité des gamètes et la sélection du site de reproduction (Heisswolf et al., 2005; Refsnider and Janzen, 2010; Ratikainen et al., 2018). Etant donné que dans nos études le mâle influence la durée de développement larvaire et que la survie du têtard en lien avec son développement pourrait être dépendante de la qualité du père nous avons cherché à mesurer la qualité spermatique. Une étude de Hettyey and Török, (2005) a mis en avant la possibilité de récolter des échantillons spermatiques lors de la reproduction. Cependant, nos tentatives pour récolter les spermatozoïdes du mâle lors de la reproduction en suivant le protocole de Hettyey and Török, (2005) se sont révélées infructueuses. Etudier la motilité, le nombre de spermatozoïdes anormaux/déformés (teratospermie, Humann-Guillemot et al., 2018), le taux de fécondation des ovules et le taux de multi paternité (Sztatecsny et al., 2006) pourrait éclairer nos résultats. Nous suggérons donc une voie de recherche sur la qualité spermatique des mâles reproducteurs en fonction des différents marqueurs de la qualité individuelle, des marqueurs de la qualité de la descendance et de l'habitat, possiblement couplé à de la contamination. Dans les **articles 5 et 6**, les effets du mâle sur la descendance sont probablement médié par la qualité des spermatozoïdes positivement corrélés avec la taille et l'âge du mâle (Gasparini et al., 2010; Roth et al., 2010).

Bien que l'on s'attende à ce que la senescence impacte négativement la qualité du sperme (Hettyey et al., 2012), Watt et al., (2021), ont récemment mis en évidence le fait que les mâles âgés et jeunes avaient la même capacité de fertilisation. De plus, les nombreux facteurs de l'habitat peuvent influencer la qualité spermatique à travers l'influence du père et il se pourrait que les résultats que nous observons soient en lien avec ces processus. Par exemple, Rick et al., 2014 ont étudié les effets d'une exposition à long terme à des niveaux écologiquement pertinents de rayonnement UVA sur la qualité du sperme chez les épinoches à trois épines (*Gasterosteus aculeatus*). Les auteurs ont mis en avant que l'exposition à des niveaux élevés d'UVA ambiants a eu des effets néfastes à la fois sur la vitesse des spermatozoïdes, ce qui prouve que le rayonnement UV affecte les traits ciblés par la sélection sexuelle pré- et post-copulatoire (Rick et al., 2014). Aussi, dans une étude de (Perobelli et al., 2010) visant à mettre en évidence les effets de différents pesticides (dieldrine, endosulfan, dichlorvos, perméthrine + effets cocktail, voir Perobelli et al., 2010) par le régime alimentaire sur les paramètres de reproduction des rats mâles (morphologie et motilité des spermatozoïdes, la production quotidienne de spermatozoïdes (DSP) etc), ils ont pu mettre en évidence que la motilité des spermatozoïdes était significativement réduite chez la plupart des groupes. Il est aussi possible d'aller plus loin dans l'étude en déterminant les mécanismes complémentaires de la fécondation de l'œuf par le spermatozoïde en tenant compte de la durée de fertilisation selon la taille du spermatozoïde ainsi qu'en prenant en compte la composition macromoléculaire de ce dernier (composition de l'acrosome, décondensation de la chromatine, Gusseck and Hedrick, 1971; Lohka and Masui, 1983; Carroll Jr et al., 1991) en possible lien avec un contexte paysager.

IV/ Le paysage agricole, un environnement aux contraintes multiples

Aux contraintes agricoles en lien avec la structure du paysage s'ajoutent les contraintes physico-chimiques du milieu influencées par les rejets d'intrants chimiques par l'agriculture intensive.

1. Environnement chimique

En observant certaines conséquences des fertilisants sur ces organismes ([Article 1](#)), la question de l'exposition aux pesticides se pose. Étant donné le fait que dans notre étude on détecte une signature isotopique modifiée liée aux engrais azotés et que les pesticides sont également très utilisés dans l'agriculture moderne, on peut s'attendre à une exposition plus forte chez les individus qui montrent une signature isotopique de l'azote sanguin ($\delta^{15}\text{N}$) modifiée par l'agriculture. De nombreuses études ont démontré les effets néfastes de ces intrants sur les amphibiens (Baker et al., 2013; Trudeau et al., 2020). Dans une étude de Brühl et al., (2011), sur les effets de 7 produits pesticides sur *Rana temporaria*, il a été montré que la mortalité variait de 100 % après une heure à 40 % après sept jours à la dose recommandée sur l'étiquette des produits homologués. Chez notre espèce, des altérations du développement larvaire et embryonnaire ont été mises en évidence, en réponse à différents composés très répandus, comme l'AMPA et le Nicosulfuron, à des doses subléthales (Cheron and Brischoux, 2020; Cheron et al., 2022a, 2022b). Plus particulièrement, il a été montré qu'un métabolite du glyphosate à des concentrations reflétant celles de l'environnement entraînait une mortalité sélective des embryons de crapaud épineux et donc la sélection de phénotype résistant (Cheron and Brischoux, 2020; Cheron et al., 2022a).

Cet exemple démontre le fait que les pesticides terrestres sont toxiques et ont un effet négatif à grande échelle sur les populations d'amphibiens. Du fait du mode de vie particulier de nombreux amphibiens (phase de reproduction aquatique et phase de dispersion terrestre), il pourrait être intéressant de déterminer et de comparer les effets d'une exposition aux pesticides pendant et hors période de reproduction. Pour les amphibiens terrestres, l'accumulation de pesticides par contact cutané est une des principales voies d'exposition dans les paysages agricoles et peut contribuer au déclin généralisé des amphibiens. Van Meter et al., 2015 ont cherché à mettre en évidence le transfert des pesticides à travers le derme des amphibiens par (1) expositions directes (pulvérisation) ou (2) expositions indirectes (contamination par le sol après pulvérisation).

De ce fait, Van Meter et al., 2015 ont montré une absorption cutanée pour de multiples pesticides provenant à la fois de la pulvérisation directe et de l'exposition indirecte du sol. Dans une étude de Brischoux et al., (2021, **Annexe 1**), nous avons pu mettre en évidence un différentiel de perméabilité cutanée entre les individus en période de reproduction et hors reproduction. Ces résultats suggèrent un échange entre l'organisme et l'environnement plus important pendant la saison de reproduction, pouvant possiblement exacerber les effets d'une contamination en milieu non-cible (Glinski et al., 2018; Purucker et al., 2021).

Les amphibiens sont des espèces dont une partie du cycle de vie est aquatique, la question de la contamination lors de la reproduction et lors du développement larvaire et embryonnaire se pose une nouvelle fois. Ainsi, les abondances des populations de crapauds épineux en milieu agricole pourraient être influencées négativement par une survie des embryons et des larves réduite (Bókony et al., 2018; Cheron et al., 2022a, 2022b) et une qualité des individus métamorphiques (Boone et al., 2005) plus faible. Etant donné les effets néfastes des produits agrochimiques sur la faune (Kendall and Akerman, 1992) et plus spécifiquement sur les amphibiens (Baker et al., 2013; Trudeau et al., 2020), la contamination environnementale est un candidat sérieux quant à la toxicité par 1/ contamination indirecte (e.g milieux et espèces non-cibles, aquatique) avec par exemple une baisse de l'abondance de proies et donc de la disponibilité alimentaire (Hart et al., 2006; Wagner, 2020), et par 2/ contamination directe (e.g épandage, milieu terrestre) sur les populations d'amphibiens. Ainsi, les intrants chimiques peuvent avoir des conséquences négatives sur le taux de recrutement et donc sur les abondances au sein des populations (**Article 3 et 4**).

2. Adaptation locale

A la suite des résultats de Cheron and Brischoux, (2020) et Cheron et al., (2022a) qui mettent en évidence une mortalité sélective chez les embryons de têtards face à un contaminant répandue (AMPA) et dans une étude complémentaire de Tartu et al., (2022, [Annexe 2](#)), nous avons justement étudié les effets du métabolite primaire du glyphosate (l'acide aminométhylphosphonique : AMPA) sur une possible mortalité sélective, suggérant que l'exposition au glyphosate pourrait avoir sélectionnée des individus résistants à l'AMPA. Ainsi, on s'attend à ce que les effets de l'AMPA soient moins importants sur la descendance provenant de parents originaires de milieux agricole que pour ceux issus de parents forestiers. Malgré des effets négatifs de l'AMPA sur les paramètres de fitness, et notamment à des stades précoces du développement, nous n'avons pas trouvé de différence entre les individus provenant d'habitats forestiers (sans AMPA) et ceux provenant d'habitat agricole (soumis à l'AMPA depuis plusieurs générations). De ce fait, nous suggérons que le flux génétique entre les populations exposées et préservées ainsi que la dynamique temporelle et/ou spatiale de la contamination, peuvent affecter la probabilité d'émergence de ces adaptations. Finalement, les effets de la contamination à l'AMPA restent néfastes et l'AMPA participe au déclin des populations d'amphibiens sauvages. On aurait pu s'attendre à ce que la contamination environnementale influence aussi la diversité génétique des populations. Cependant, nos résultats indiquent le contraire ([Article 2](#)) et les résultats de Tartu et al., (2022) tendent à soutenir l'absence de signaux de résistance aux pesticides accrus en paysage agricole ([Annexe 2](#)). Ce qui suggère et concorde avec l'hypothèse de la mobilité des crapauds épineux et de l'homogénéisation des populations ([Article 2](#)).

Dans un premier temps, et étant donné le contraste entre les environnements conservés et dégradés, nous suggérons de prendre en considération les possibles effets des produits phytosanitaires sur le succès reproducteur et sur la qualité de la descendance au stade embryonnaire, larvaire voir à des stades tardifs, après la métamorphose. Au stade larvaire, les tailles de têtard et la durée de développement sont souvent impactés par la qualité parentale et l'habitat. Ces résultats peuvent s'expliquer par des processus en lien avec l'activité, la locomotion et le comportement aux différents stades de développement (Cheron et al., 2021).

Par exemple, la locomotion et le comportement subissent des changements significatifs au cours du développement du têtard, avec une phase d'augmentation de l'activité et de la locomotion suivie d'une phase de stase et/ou de réduction de la locomotion et de la complexité comportementale, à un stade pivot où la croissance somatique diminue et où des changements morphologiques significatifs (métamorphose) se produisent.

Cependant, des analyses de survie n'ont permis de constater aucune différence de mortalité larvaire en fonction de l'habitat, et ont montré des relations non différentes pour des stades de développement larvaire tardifs. Ceci pourrait s'expliquer par des systèmes de mécanismes compensatoires qui permettent au têtard d'atteindre la métamorphose. Cependant, ces systèmes compensatoires peuvent avoir des coûts et des répercussions sur la suite de la vie des individus (Burraco et al., 2020). Ils peuvent servir de médiateurs entre les marqueurs parentaux et de la descendance (Van Leeuwen et al., 2016; Wells, 2014). Par exemple, nous suggérons d'étudier les coûts physiologiques du développement à l'aide de marqueurs physiologiques comme le statut oxydatif et l'attrition des télomères (Saino et al., 2005; Burraco et al., 2017). Par ailleurs, il serait intéressant de suivre les métamorphes jusqu'à l'âge adulte après les avoir étudiés tout au long du développement tout en considérant un possible transfert de contaminant par la mère. On sait, par exemple, que les herbicides sulfonylurés sont des perturbateurs de l'activité normale de l'ACHé ayant des répercussions sur l'activité et le comportement (dos Santos Miron et al., 2005; Modesto and Martinez, 2010). Etant donné le fait que la mère a un fort effet sur la qualité de la descendance et médie de nombreux paramètres du succès reproducteur, des études plus poussées sur les transferts maternels à la descendance via par exemple la gangue de la ponte, en relation avec la contamination environnementale (e.g. pesticides) sont nécessaires afin de déterminer si ces composés sont transmissibles à la descendance. Le transfert maternel est un sujet d'étude prometteur chez ces organismes et certaines études traitent déjà de la bioaccumulation en lien avec le transfert maternel et ses répercussion sur le développement larvaire des amphibiens (Hopkins et al., 2006; Bergeron et al., 2010). Finalement, la question des stressseurs additifs contraignants de l'environnement agricole antagonistes et/ou synergiques, qui peuvent avoir un effet « multi stress » sur les individus, se pose (Breitburg et al., 1998; Folt et al., 1999; Storfer, 2003; Ormerod et al., 2010; Lupi et al., 2021). Ainsi, les différents facteurs de stress qui interagissent avec l'environnement (e.g. disponibilité de la ressource alimentaire, changements climatiques de l'habitat, prédation et compétition etc) peuvent être combinés à des effets cocktails en lien avec la contamination environnementale comme par exemple, des effets joints et en interaction du glyphosate (Tartu et al., 2022) et des fertilisants ([Article 1](#)) dans les eaux de surface. L'approche multi-stress est une voix de recherche nécessitant des investigations supplémentaires notamment aux différents stades de vie des amphibiens (Embryonnaire, larvaire, métamorphe et adulte).

3. Nouvelles contraintes

Dans les habitats agricoles nous avons pu voir que les contraintes de ces milieux sont exacerbées par les autres facteurs environnementaux qui menacent l'équilibre et la dynamique des populations de crapauds épineux. En plus des contraintes paysagères liées à la dégradation de l'habitat, les organismes vivant dans ces milieux peuvent être soumis à la présence d'espèces introduites opportunistes. Ces espèces engendrent généralement des conséquences négatives pour les espèces indigènes, notamment en lien avec la prédation et/ou la compétition pour l'accès à la ressource (Hobbs, 2000). Lors de nos études de terrain nous avons pu mettre en avant la présence d'une espèce introduite (*Rattus norvegicus*) qui prédate les populations de crapauds épineux et qui peut avoir un effet conséquent sur leur persistance en milieu agricole (**Annexe 3**). *Rattus norvegicus* est une des espèces de mammifère les plus envahissantes, originaire du nord-est de la Chine (Puckett et al., 2016). Falaschi et al., (2020), ont décrit les conséquences multiformes des invasions biologiques sur les amphibiens indigènes et identifié les mécanismes et stratégies potentiels qui pourraient mieux permettre la persistance à long terme des espèces indigènes. Cependant, malgré la capacité des amphibiens indigènes à répondre aux espèces invasives (dans notre cas : introduite), en modulant certains aspects de leur comportement, de leur morphologie ou de leur cycle de vie (Hobbs, 2000; Falaschi et al., 2020; Melotto et al., 2021), on ne sait toujours pas dans quelle mesure la plasticité phénotypique et l'évolution rapide peuvent aider les espèces indigènes à résister à ces impacts. De plus, les milieux dégradés (notamment les milieux agricoles) sont favorables aux espèces non-indigènes capables de faire face à des conditions modifiées (Rudnick et al., 2012). Ces espèces sont favorisées au détriment des espèces avec des exigences de milieu plus contraignantes (Rudnick et al., 2012). Ainsi, en plus d'être soumises à de la contamination environnementale (**Article 1**), à une modification structurelle de leur environnement pouvant (Gauffre et al., 2022), ou non, impacter la diversité génétique (**Article 2**) et à des modifications environnementales pouvant engendrer une diminution de l'abondance d'individus en période de reproduction (principalement de femelles, **Article 3 et 4**), les populations de crapauds épineux peuvent être soumises à des contraintes par les espèces invasives (**Annexe 3**). Malgré l'aspect anecdotique de notre étude (**Annexe 1**), ce type d'observation permet de mettre en avant de possibles effets supplémentaires et additifs aux contraintes environnementales d'un milieu dégradé (multi-stress).

CONCLUSION

Dans cette thèse, nous avons cherché à déterminer les impacts de l'agriculture intensive, à une échelle populationnelle et individuelle, sur une espèce d'amphibien sensible aux contraintes environnementales : le crapaud épineux (*Bufo spinosus*). Nous avons choisi une approche comparative, lors de la période de reproduction de cette espèce, le long d'un gradient d'habitat allant d'habitats conservés à des habitats fortement dégradés (Figure 24). De plus, ces études se sont appuyées sur un panel de marqueurs permettant d'approfondir les connaissances portant sur l'écologie alimentaire, l'utilisation de l'habitat, la structure génétique des populations, l'état de santé des populations et la reproduction chez cette espèce (Figure 24).

A l'aide d'une approche par les isotopes stables, nous avons pu discriminer le milieu de vie des individus selon le type d'habitat (Figure 24). Afin de mieux appréhender cette utilisation de l'habitat, nous avons couplé cette étude à une approche génétique à partir de marqueurs microsatellites (Figure 24). De ce fait, nous avons pu mettre en avant que la structure génétique des populations de crapauds épineux est homogène et que la structure de l'habitat en milieu agricole intensif reste suffisamment perméable au maintien de la diversité génétique (Figure 24). Ces deux études ont servi à mettre en évidence l'intérêt d'intégrer l'étude des micro-habitats et du radio-tracking afin de mieux appréhender l'écologie et les déplacements de cette espèce à travers l'habitat (Figure 24).

Dans un second temps, nous avons cherché à comprendre l'effet des modifications de l'habitat sur les populations de crapaud épineux (Figure 24). Pour ce faire, nous avons mis en place un suivi des populations (sur 3 ans) de cette espèce et nous avons pu constater que le milieu agricole pouvait contraindre la reproduction de cette espèce et notamment pour les femelles (Figure 24). Ainsi, nous mettons en avant l'intérêt d'intégrer les femelles dans les études de structure génétique des populations, et de manière opportuniste (cadavres d'individus), intégrer l'âge des crapauds composant les différentes populations à l'aide de la squelettochronologie (Figure 24).

Suite à ces résultats montrant que les environnements agricoles contraignent la reproduction des crapauds épineux, nous avons finalement examiné différents indices de la qualité individuelle et des performances de reproduction selon un contexte paysager (Figure 24). A partir d'une approche expérimentale nous avons suivi la reproduction de ces organismes de la ponte à la métamorphose et avons récolté différents marqueurs du succès reproducteur et de la qualité individuelle (phénotype et génotype) chez les parents et la descendance (Figure 24). De ce fait, nous avons pu mettre en évidence des relations intéressantes entre les marqueurs de la qualité parentale et de la descendance, notamment du père mais aussi selon le contexte paysager suggérant de possibles contraintes agricoles (site d'origine des parents) sur la descendance (Figure 24). Plus précisément, nous avons trouvé plusieurs traits liés au succès de la reproduction (taille de la ponte, succès de l'éclosion, durée du développement larvaire et morphologie larvaire) qui étaient influencés par l'habitat parental par le biais d'interactions avec le phénotype parental suggérant que l'habitat parental peut modifier les relations entre le phénotype parental et les performances reproductives (Figure 24).

Nous suggérons donc de prendre en considération la qualité spermatique et l'âge du père afin d'expliquer les mécanismes sous-jacents aux effets paternels sur la descendance. Pour finir, les résultats de cette thèse soulignent l'importance de prendre en compte les possibles effets des produits phytosanitaires (et les effets cocktails) et les effets stresseurs additifs (multi-stress) sur le succès reproducteur, sur la qualité de la descendance aux différents stades de vie de l'individu (Figure 24).

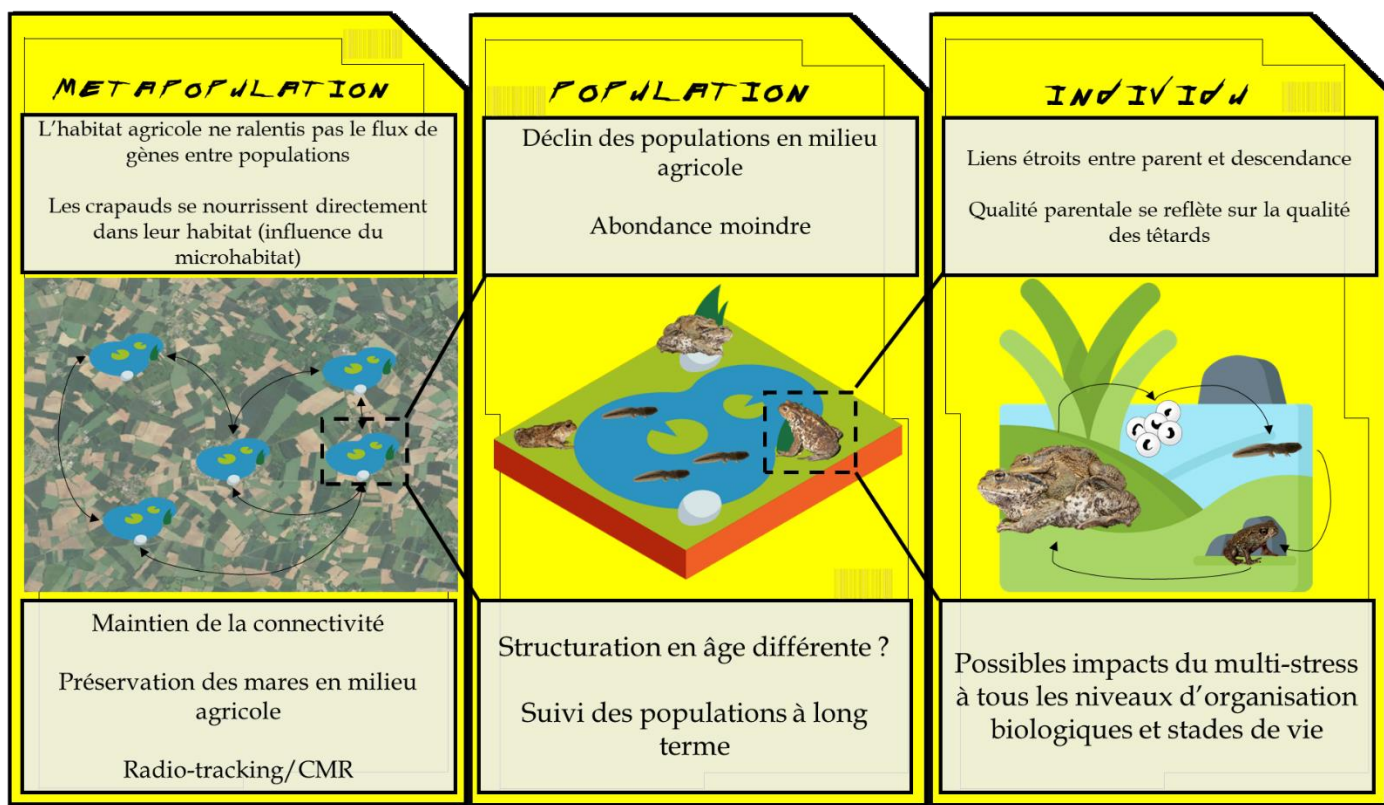


Figure 24. Food for thought : Récapitulatif des trois chapitres de la thèse indiquant les résultats et perspectives.

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ANNEXES

Annexe 1 : Brischoux, F., Cheron, M., Renoirt, M., & Lourdais, O. (2021). Getting ready for a long bath: skin permeability decreases prior to aquatic breeding in male toads. *The Science of Nature*, 108(6), 1-6.

Annexe 2 : Sabrina, T., Matthias, R., Marion, C., Léa-Lise, G., Solenn, C., & François, B. (2022). Did decades of glyphosate use have selected for resistant amphibians in agricultural habitats?. *Environmental Pollution*, 119823.

Annexe 3 : Renoirt, M., Cheron, M., Tartu, S., Angelier, F., & Brischoux, F. (2022). *Bufo spinosus* (Spined Toad). Predation. *Herpetological Review*.



Getting ready for a long bath: skin permeability decreases prior to aquatic breeding in male toads

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Abstract

Vertebrate skin regulates exchanges between the organism and its environment and notably plays a fundamental role in regulating water fluxes. Dynamic changes of skin resistance to water fluxes are expected to occur in species that regularly shift between habitat types especially if these habitats differ in their hydric properties (e.g., terrestrial versus aquatic). We investigated changes of skin permeability using a study system (terrestrial toads) where reproduction induces a transition from terrestrial to freshwater habitats and a prolonged immersion that can last several weeks in males. In this system, the simultaneity between skin shedding and the onset of breeding suggests that the production of new integument layers prior to immersion for reproduction may regulate water influxes. We found that the skin permeability of male toads decreases significantly prior to breeding, suggesting that skin shedding at the onset of breeding regulates water fluxes to alleviate osmotic costs of immersion during reproduction. The continued decrease of skin permeability detected during breeding suggests that additional mechanisms interact with skin structure to further decrease permeability to water during a prolonged immersion. Future studies are required to assess whether changes in skin permeability to water tradeoffs with other skin characteristics (gas exchanges) relevant to aquatic breeding amphibians.

Keywords *Bufo spinosus* · Water relations · Cutaneous evaporative water loss · Habitat · Reproduction

Introduction

Species that shift between habitats during their lifetime must face environmental constraints that can be highly divergent between habitat types. Typical examples of the consequences of such habitat shifts have been thoroughly described in anadromous or catadromous fish. In these species, reproductive migrations between fresh- and seawater are characterized by highly different chemical compositions (e.g., high salt concentration in seawater) and involve significant changes to the osmoregulatory apparatus that allow osmotic balance either in hyperosmotic marine or hyposmotic freshwater habitats

(Edeline 2007; Tseng and Hwang 2008; Bowerman et al. 2017). Other examples have been documented in amphibious species that commute between aquatic and terrestrial habitats (Mazin and de Buffrénil 2001) which diverging physico-chemical characteristics have been shown to influence several traits such as locomotor performance (Bonnet et al. 2005) or environmental tolerance (Brischoux et al. 2013).

Vertebrate skin provides physical protection and regulates exchanges between the organism and its environment, including the regulation of water fluxes (Lillywhite 2006). Accordingly, the resistance of the skin to water passage has been shown to be highly variable and range from very low in aquatic vertebrates to high values in species adapted to xeric environments (Lillywhite 2006). Dynamic changes in skin resistance to water fluxes are expected to occur in species that regularly shift between habitat types, especially if these habitats differ in their hydric properties (e.g., terrestrial versus aquatic). For instance, many amphibians shift between habitats during reproduction with a transition from terrestrial to aquatic sites.

The integument of amphibians is characterized by very little keratin and a thin stratum corneum (Lillywhite 2006). As a

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consequence, transcutaneous water loss is high, and amphibians tend to dehydrate rapidly under dry conditions (e.g., on land, Burggren and Vitalis 2005). Terrestrial species display specific adaptations to reduce dehydration such as an elevated ability to absorb water cutaneously (Bentley 1971; Hillyard et al. 1998) or increased skin lipids (Withers et al. 1984). Yet, many terrestrial amphibian species reproduce in aquatic habitats where mating occurs and eggs and larvae develop (Dodd 2010). In this context, the ability of terrestrial amphibians to absorb water through the skin can become a constraint when they shift to an aquatic lifestyle for reproduction.

During aquatic reproduction, male toads typically experience an osmotic challenge during the prolonged period (several weeks) of immersion in freshwater, presumably due to water influx (Brischoux and Cheron 2019). Despite a significant decrease in plasma osmolality following immersion, breeding toads can maintain a relatively elevated plasma osmolality even after prolonged periods of immersion suggesting that water influx remains limited (Brischoux and Cheron 2019). In this respect, the integument may well play a significant role in regulating water influxes (Bentley 1971; Shoemaker et al. 1992, Boutilier et al., 1992). Because male bufonid toads shed their skin (i.e., production of new integument layers) just prior to the shift to aquatic reproduction (Jørgensen & Larsen 1961), it is likely that the new skin has specific properties that allow for reduction in water fluxes (Wu et al. 2017).

In this study, we assessed the skin permeability (cutaneous evaporative water loss, CEWL) of reproductive and non-reproductive male spined toads (*Bufo spinosus*) in order to test whether a shift from terrestrial to aquatic lifestyle influences skin permeability in this terrestrial species. First, we hypothesized that skin-shedding prior to reproduction is associated with lower skin permeability during aquatic breeding in order to limit water influxes and thus osmotic costs of reproduction (Brischoux and Cheron 2019). Second, irrespectively of reproduction, we expected that there would be regional variation in CEWL depending on the body area of the toads. We predicted that the dorsal area — presumably exposed to more desiccant conditions than the ventral area, which remains in close contact with relatively humid substrate — should display lower CEWL values than the ventral area or the pelvic area that is involved in transcutaneous water intake (Jørgensen 1994).

Material and methods

Study species and sampling

The spined toad (*Bufo spinosus*) is one of the most common anuran species in Western France. As with most toad species, it is characterized by a biphasic lifestyle with breeding occurring in aquatic habitats (ponds) where eggs and

tadpoles develop, while the remaining cycle occurs in terrestrial habitats (Reading and Clarke, 1983, Brischoux et al. 2018). In late winter (February–March), male toads migrate to breeding ponds where they wait for females during several weeks (Brischoux et al. 2018). Females remain only transitorily at breeding sites, and typically return to land within a few hours once mating and egg-laying has occurred (Brischoux et al. 2018). Because aquatic life (immersion at breeding sites) is disproportionately longer in males, we focused our investigations on this sex only.

In order to comprehensively assess skin permeability of toads both during terrestrial and aquatic (reproductive) phases, toads were sampled during three distinct time periods. First, we captured individuals during the non-reproductive period in early October when climatic conditions allow toads to resume activity after aestivation during drier months (summer). During this period, we opportunistically captured individuals that were foraging on roads situated nearby the pond monitored during the breeding season (see below). Within a few days (4–18 October 2019), we were able to collect 19 adult male toads. Second, at the onset of the reproductive period, during male migration to breeding sites, we captured individuals upon their arrival at the breeding pond, but before they actually entered water. To do so, sampling was conducted at night using headlamps to detect male toads located on land and moving in the direction of the pond. These individuals were located within 10 m of the pond edges. Importantly, male toads shed their skin just before reaching breeding ponds (Jørgensen and Larsen 1961), and the individuals that we captured during this time period were all showing remnants of loose old skin suggesting that skin shedding had occurred. Due to the massive migration of male toads to aquatic breeding sites, all individuals ($N=19$) were captured during a single night (29 January 2020). Finally, we captured individuals that were immersed at the breeding pond for a significant time period (i.e., ~3 weeks after the arrival of the first individuals). Sampling was conducted at night using headlamps, and toads immersed in water were captured with a net. Similarly to the previous capture session, all individuals ($N=20$) were captured during a single night (21 February 2020). For clarity, we will refer to these three time periods as “non reproductive”, “arrival at breeding site”, and “aquatic breeding” hereafter.

After capture, individuals were brought back to the laboratory and maintained in plastic containers with a shelter and either a damp substrate (paper towel) for “non reproductive” and “arrival at breeding site” individuals, or water allowing full immersion for “aquatic breeding” individuals. The snout–vent length (SVL) of each individual was measured with electronic calipers (± 0.01 mm), and CEWL measurements (see below) were performed the day following capture.

All individuals were released at their location of capture after measurements.

CEWL measurements

Measurements were carried out at room temperature (20 °C), and toads were acclimated to this temperature for at least 2 h before measurement. We used an AquaFlux AF200 (Biox, London) and the Bioxsoftware AquaFlux 6.2 to calibrate and compute CEWL rate ($\text{g m}^{-2} \text{h}^{-1}$). We used an in vivo nail cap with rubber O-ring (diameter 2.6 mm) to insure a complete seal between the device and the toad's skin. Contact was maintained by gently restraining the toad and applying steady but slight pressure to the probe against the skin. Trials continued until the CEWL reading stabilized ($\pm 0.02 \text{ g m}^{-2} \text{ h}^{-1}$) for 180 s. If any movement (by the toad or the operator) caused a leak in the seal between the probe and the toad (detected as a sudden change in water flux), the trial was repeated. The AquaFlux unit was calibrated at the beginning of each trial. Each measurement was performed in triplicates at each body area (see below), and we used mean values for analyses.

Because we expected regional variation of CEWL depending on the body area of toads (see above), measurements were performed at three different body regions, namely the dorsal area, the ventral area, and the pelvic patch dedicated to cutaneous drinking (Jørgensen 1994). For dorsal measurements of CEWL, we targeted an area situated between the parotid glands because this area contained fewer warts than the remaining dorsal area, thereby allowing a correct seal between the Aquaflux's probe and the toad's skin. For ventral measurements of CEWL, we targeted an area situated between the forelegs. Finally, the CEWL measurements of the pelvic patch were straightforward to perform because this specific area is easily recognizable by its location (pelvis) and color (pinkish, due to high vascularization, in contrast to the creamy white belly of the toads).

Statistical analyses

Relationships among CEWL values measured at different body regions (dorsal, ventral, or pelvic patch) were assessed using linear models. Differences in CEWL among body regions and time periods were assessed using general linear models, as were relationships between toad size and CEWL within body regions and time periods. All analyses were performed with Statistica 12.

Results

Overall, the values of CEWL measured at different body regions (dorsal, ventral, or pelvic patch) were strongly correlated (dorsal–ventral: $F_{1,75} = 579.74$, $r^2 = 0.88$, $p < 0.0001$,

dorsal–pelvic patch: $F_{1,75} = 322.94$, $r^2 = 0.81$, $p < 0.0001$, ventral–pelvic patch: $F_{1,75} = 1176.60$, $r^2 = 0.94$, $p < 0.0001$, Fig. 1), and similar results were found when restricting analyses to the different time periods (all $r^2 > 0.30$, all $p < 0.008$).

CEWL differed significantly across body regions ($F_{2,165} = 15.05$, $p < 0.0001$, Fig. 2) and time periods ($F_{2,165} = 119.40$, $p < 0.0001$, Fig. 2), with no significant interactions ($F_{4,165} = 0.32$, $p = 0.86$). Dorsal CEWL was lower than both ventral CEWL and CEWL measured from the pelvic patch ($p < 0.008$), and ventral CEWL was higher than CEWL measured from the pelvic patch ($p = 0.006$). For all body areas, the CEWL was the highest during the non-reproductive period and was the lowest during aquatic breeding (all $p < 0.0001$).

Toad size did not influence CEWL within body regions and periods (all $p > 0.11$), but we found a significant interaction between the time period and SVL for dorsal CEWL

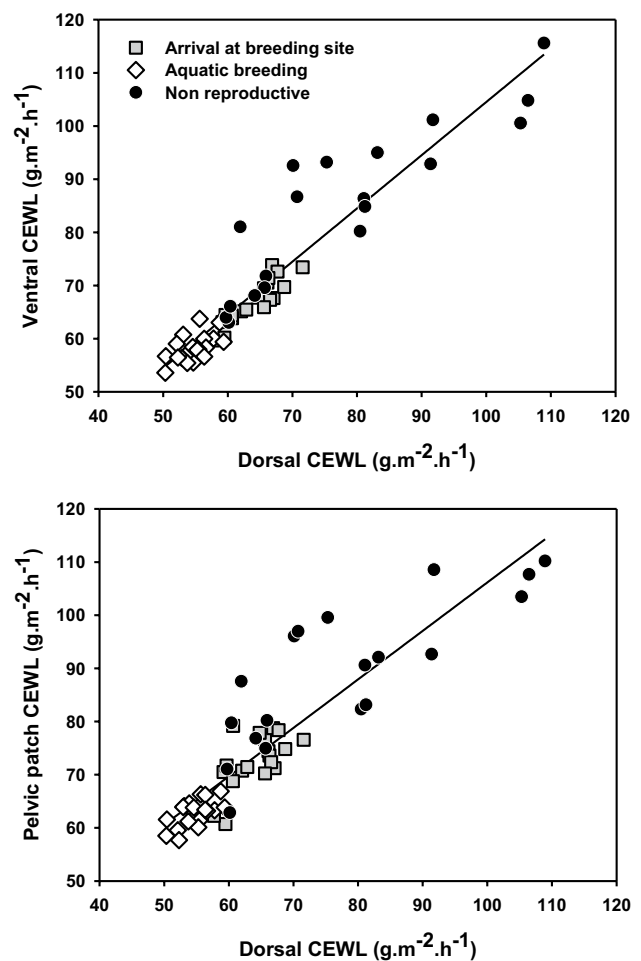


Fig. 1 Relationships between dorsal cutaneous evaporative water loss (CEWL) and ventral CEWL (upper panel) or pelvic patch CEWL (lower panel) during the “non-reproductive” (black circles), “arrival at breeding site” (grey squares) and “aquatic breeding” (white diamonds) sampling periods

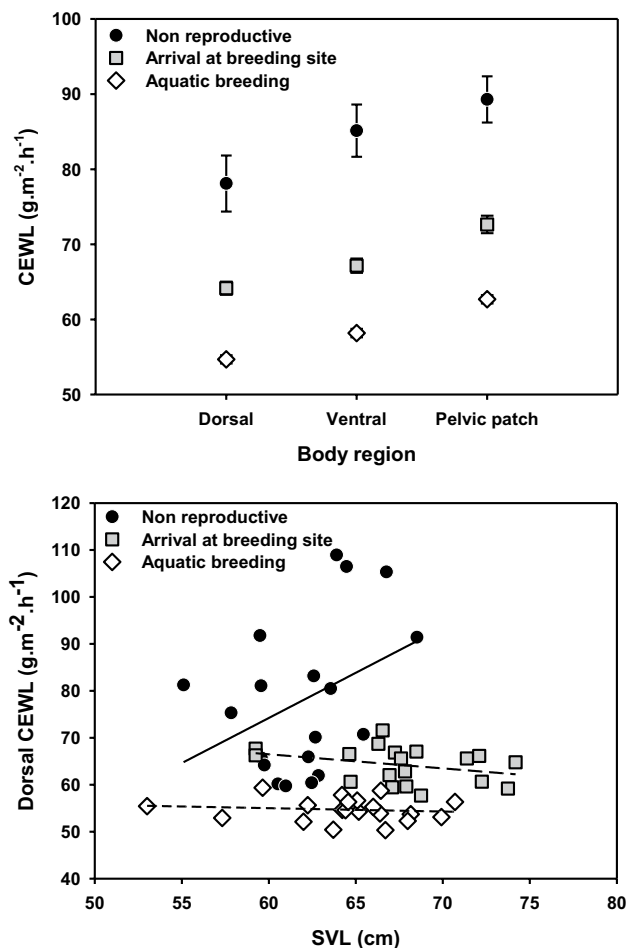


Fig. 2 Cutaneous evaporative water loss (CEWL) measured at three body areas (dorsal, ventral and pelvic patch) during the “non-reproductive” (black circles), “arrival at breeding site” (grey squares) and “aquatic breeding” (white diamonds) sampling periods (upper panel) and relationships between toad size (snout-vent length, SVL) and dorsal cutaneous evaporative water loss (CEWL) during the “non-reproductive” (black circles), “arrival at breeding site” (grey squares) and “aquatic breeding” (white diamonds) sampling periods (lower panel)

($F_{2,52} = 3.71$, $p = 0.03$, Fig. 2) and a similar, but marginal, interaction for ventral CEWL ($F_{2,52} = 2.66$, $p = 0.08$). Interestingly, inter-individual variation of CEWL was almost three times greater during the non-reproductive period (CV ranging from 15.0 to 20.8 depending on body region) than just prior to the arrival at the breeding pond (CV: 5.9–6.9) or during aquatic breeding (CV: 4.0–4.5).

Discussion

We found that the skin permeability of male spined toads decreases significantly prior to breeding. This suggests that the skin shedding that occurs at the onset of breeding (i.e., production of new integument layers with specific properties) may regulate water fluxes in order to alleviate osmotic costs linked to the protracted period of immersion during aquatic breeding (Brischoux and Cheron 2019).

Our results highlighted the regional differences in CEWL, where the dorsal area, which is exposed to more desiccant air, had lower CEWL relative to ventral areas. In addition, the pelvic patch (involved in transcutaneous water intake) had higher CEWL than the two other areas. Yet, the values of CEWL measured at different body areas were highly correlated, suggesting strong regional covariations of this parameter. Interestingly, these regional differences were strongly marked during the non-reproductive period but were also maintained prior to or during aquatic breeding, suggesting that the specific regional properties of skin structure (i.e., keratin, stratum corneum, or lipid layer composition or thickness) were conserved during breeding.

Despite these regional variations, we found that the CEWL of all body areas decreased significantly prior to aquatic breeding and continued to decrease following immersion. Reduced water influxes after shedding may allow individuals to dampen strong deviations in osmotic balance following prolonged immersion in freshwater (Brischoux and Cheron 2019). Importantly, shedding induces a temporary disruption of cutaneous integrity (increased water gain and sodium loss) that lasts a few hours post-sloughing (Jørgensen 1949; Wu et al. 2017). This process lines up particularly well with our field observations, which suggest that skin-sloughing is achieved during terrestrial migration prior to immersion in water. Interestingly, our results highlight that there is a continued decrease in CEWL following immersion in water, suggesting that the affinity for water of the new skin is further reduced during a protracted period of immersion. In addition to possible structural modifications of the skin, a shift to aquatic breeding is likely to involve strong modifications to endocrine regulations (Bentley 1971). In this respect, prolactin is known to influence skin permeability, and notably to induce a reduction in permeability to water (Bentley 1971). Clearly, further studies are required in order to assess how structural skin characteristics (e.g., keratin, stratum corneum, or lipid layer composition or thickness) and endocrine regulation (e.g., prolactin) interact to influence permeability to water during aquatic breeding.

In addition, skin shedding and the subsequent shift to an aquatic lifestyle seem to disrupt the relationship between body size and CEWL. Indeed, during the non-reproductive

period, toad size was positively related to dorsal CEWL and to a lesser extent to ventral CEWL. This result indicates that, during the terrestrial part of their life cycle, smaller individuals — which are presumably more sensitive to dehydration because of a higher surface area to volume ratio — have a lower CEWL which is likely to reduce susceptibility to dehydration. This relationship disappeared during breeding, suggesting that the structural properties of the new skin and/or endocrine regulations interacting to reduce skin permeability are not linked to toad size.

There are some caveats to our investigations notably linked to the fact that we measured CEWL (i.e., water effluxes) rather than water influxes, while aquatic breeding is expected to modify skin permeability to water influxes rather than water effluxes. Yet, both directions of water fluxes are tightly linked and our results are in line with predicted changes. Accordingly, the data on the CEWL of the pelvic patch relative to other body areas suggest that this area, which is dedicated to promote water influxes (cutaneous drinking), also has higher CEWL values and, hence, that the mechanisms responsible for water absorption (e.g., aquaporins, Suzuki et al. 2007) also play a significant role in water loss.

Finally, the breeding strategy of terrestrial toads, and especially the prolonged immersion in water during reproduction, is likely to modify not only water relations but also gas exchanges (Shoemaker et al. 1992, Boutilier et al., 1992, Burggren and Vitalis 2005). For instance, it is likely that submerged breeding toads rely more strongly on cutaneous gas exchange than terrestrial non-reproductive individuals. Future studies should test whether the shift in skin properties that we detected have consequences for the cutaneous gas exchanges of reproductive and non-reproductive toads. In addition, because of the remarkable difference of residence time at breeding ponds between males and females (Brischoux et al. 2018), comparative studies are required to test whether the skin properties of reproductive females undergo similar changes to that of males, in order to assess how divergent reproductive strategies (immersion duration) influence sexual dimorphism in the responses of skin permeability to habitat shifts.

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Author contribution François Brischoux and Olivier Lourdais conceived and designed the study. Marion Cheron and Matthias Renoit performed data collection. François Brischoux performed data analysis and writing of the first draft of the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Data will be made available upon reasonable request.

Code availability Not applicable.

Declarations

Ethics approval All applicable institutional and/or national guidelines for the care and use of animals were followed. This work was approved by the French authorities under authorizations number 2015–11-20x-01192, 16–392, and R-45GRETA-F1-10.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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1 **Did decades of glyphosate use have selected for resistant**
2 **amphibians in agricultural habitats?**

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10

11 **Abstract**

12 Glyphosate-based herbicides are used worldwide, and glyphosate's primary metabolite
13 (aminomethylphosphonic acid: AMPA), is globally retrieved in surface waters. AMPA induces
14 various adverse effects on aquatic wildlife, including selective mortality, which suggests that
15 glyphosate exposure may have selected for AMPA-resistant individuals. We tested this
16 hypothesis using spined toads (*Bufo spinosus*), an amphibian found in a variety of habitats, from
17 AMPA-exposed agricultural lands to AMPA-free forested areas. We predicted that the
18 offspring of individuals originating from agricultural habitats would develop AMPA-resistance
19 - and be less prone to develop adverse effects from- AMPA exposure. To investigate this
20 question, we performed a common garden brood-rearing experiment. The embryos and larvae
21 of 40 spined toad pairs captured in agricultural and forest ponds were exposed either to an
22 environmental relevant concentration of AMPA (0.4 $\mu\text{g L}^{-1}$) or to control conditions (n=8160
23 embryos, n=240 tadpoles). We monitored development durations, developmental abnormalities
24 and morphology, measured across key developmental stages. Although we observed significant
25 effects of AMPA on fitness parameters in each group, these effects were not exacerbated in
26 individuals from AMPA-free habitats. We suggest that temporal and/or spatial dynamics of
27 contamination, as well as gene flow between exposed and preserved populations, may hinder
28 adaptive divergence between populations. Yet, we show strong adverse effects of AMPA
29 exposure at early developmental stages. AMPA could therefore be one of the numerous causes
30 of declining wild amphibian populations.

31 **Key-words:** AMPA; selective pressure; habitat; spined toad; *bufo spinosus*

32

33 INTRODUCTION

34 Habitat heterogeneity across space and time is a strong driver of phenotypic variability (Stearns,
35 1989), and such heterogeneity is expected to favour genotypes able to produce different
36 phenotypes in response to environmental variations (Stearns, 1992). Originally thought to
37 unfold over long periods of time, it has now been demonstrated that adaptive evolution, which
38 is influenced by phenotypic plasticity (Wund, 2012), can occur during very short timescales
39 (Boag and Grant, 1981; Hairston Jr et al., 2005; Whitehead et al., 2017). Human activities are
40 nowadays considered as the dominant pressures affecting the fitness of many species and have
41 been shown to act as potent selective pressures (Palumbi, 2001; Stearns, 1992; Whitehead et
42 al., 2017). Among anthropogenic activities affecting biodiversity, land-use change and
43 especially intensive agriculture is a significant global pressure, converting natural habitats to
44 intensely managed systems (Dou et al., 2021; Dudley and Alexander, 2017).

45 The responses of wildlife to agricultural land-use are numerous. For instance, agricultural lands
46 have been associated with decreased hatching success in American kestrels *Falco sparverius*
47 (Touihri et al., 2019), modified dispersion and exploratory movements in Iberian lynx *Lynx*
48 *pardinus* (Gastón et al., 2016) or increased chemical defence in adult common toads *Bufo bufo*
49 (Bókony et al., 2019). With regard to the specific effects of pest control agrochemicals, they
50 can act at various levels from genetics to phenotypic plasticity, resulting in physiological,
51 behavioural and fitness alterations (Saaristo et al., 2018). Considering wild population can
52 develop resistance to agrochemicals (Almeida et al., 2021; Cothran et al., 2013), those
53 substances could thus impact the capacity of populations to persist into the future by altering
54 the strength and targets of evolutionary selection.

55 Among the numerous agrochemicals used in agricultural landscapes, glyphosate [N-
56 (phosphonomethyl)glycine] has been considered as a virtually ideal herbicide (Duke and

57 Powles, 2008). Glyphosate is a low-cost highly effective broad-spectrum herbicide, and is the
58 only molecule that is highly effective at inhibiting the enzyme 5-enolpyruvyl-shikimate-3-
59 phosphate synthase of the shikimate pathway. The shikimate pathway is found specifically in
60 microorganisms and plants, therefore glyphosate was considered to be one of the least toxic
61 pesticides to animals (Herrmann and Weaver, 1999; Williams et al., 2000). Yet, its widespread
62 use in agricultural lands has contaminated soils and waters. In the wild, glyphosate is rapidly
63 and primarily metabolized to 2-Amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
64 (AMPA). AMPA has a longer half-life than its parent compound, 76-240 days and 2-142 days,
65 respectively (Annett et al., 2014; Giesy et al., 2000; Grunewald et al., 2001) and is more
66 frequently detected in surface waters worldwide (Silva et al., 2018). In addition, it is now
67 recognized that glyphosate and AMPA can cause severe acute and chronic toxicological effects
68 such as cytotoxicity and genotoxicity, increased oxidative stress, disrupted estrogen pathway,
69 impaired cerebral functions, increased mortality and embryonic developmental failure (Cheron
70 et al., 2022; Cheron and Brischoux, 2020; Gill et al., 2018; Howe et al., 2004; Lanctôt et al.,
71 2014, 2013; Mann and Bidwell, 1999; Matozzo et al., 2020, 2018; Navarro-Martín et al., 2014;
72 Peillex and Pelletier, 2020; Suppa et al., 2020; Trudeau et al., 2020; Tsui and Chu, 2003) ; with
73 higher teratogenic effects of AMPA in comparison to glyphosate (Zhang et al., 2021).

74 Importantly, it has recently been shown that AMPA, at concentrations similar to those measured
75 in the wild, could alter embryonic development, induce selective mortality and affect hatchling
76 phenotype in an amphibian species, the spined toad *Bufo spinosus* (Cheron et al., 2022; Cheron
77 and Brischoux, 2020). Individuals that survived being more resistant to both AMPA and
78 AMPA-mediated effects, these findings suggest that long-term use of glyphosate could act as
79 a selective pressure (Cheron et al., 2022). Spined toads should be particularly well-suited to
80 investigate the selective effects of AMPA, as they are widespread in Western Europe, they
81 occur in a variety of habitats and persist in agricultural areas (Guillot et al., 2016). Finally, the

82 aquatic embryonic and larval phases of the spined toad, coincide with the timing of glyphosate
83 application in agroecosystems (Berger et al., 2013; Lenhardt et al., 2015). Individuals could
84 therefore be exposed to agrochemicals across their entire life. Earlier studies found no
85 glyphosate resistance in agricultural habitats in different anuran species (Bókony et al., 2017;
86 Cothran et al., 2013), but those tadpoles were exposed to high concentrations of glyphosate and
87 not to AMPA, which has a longer half-life and higher teratogenic effects (Zhang et al., 2021).

88 The aim of the present study was to investigate whether offspring of individuals originating
89 from glyphosate-exposed habitats, such as agricultural areas, would be resistant to AMPA, in
90 comparison to offspring originating from glyphosate-free habitats. To do so we captured
91 breeding spined toads from forested (preserved) and agricultural (altered) habitats, and reared
92 their offspring under common-garden conditions. To test whether individuals from agricultural
93 habitats were resistant to AMPA, we exposed half of their offspring to an environmentally
94 relevant concentration of AMPA, and the other half to control (without AMPA) conditions,
95 during the embryonic and larval development until metamorphosis. We quantified fitness
96 parameters in both groups and predicted that historically exposed populations (agricultural
97 origin) would be less impacted by AMPA exposure than preserved populations (forest origin).
98 More specifically, because selective mortality has been shown to occur during embryonic
99 development, we predicted that embryos from forest sites should be more sensitive to AMPA
100 than their agricultural counterparts. Because of this AMPA-mediated selective mortality of
101 susceptible embryos, we predicted that surviving larvae from forest sites should respond
102 similarly than those from agricultural areas.

103 **MATERIALS AND METHODS**

104 *Study sites and capture*

105 We selected four breeding ponds in contrasted habitats (**Figure S1**), two of which were located
106 in highly forested areas and the two others in agricultural landscapes; all situated in the southern
107 part of the Deux-Sèvres department (France). Such site selection allowed making simple habitat
108 classifications (“Forest” vs “Agricultural”). Pond distances were considered large enough to
109 avoid sampling individuals from a same population (i.e., ~18 km between the forest sites; ~20
110 km between the agricultural sites, shortest distance between ponds: 4 km) (Kovar et al., 2009;
111 Sinsch, 1988). To ascertain the classification of our habitats, in 2020, AMPA analyses were
112 conducted monthly (February to June) in water from the two monitored forest ponds. AMPA
113 concentrations were below limits of detection ($< 0.030 \mu\text{g L}^{-1}$). In contrast, in one of the
114 monitored agricultural ponds and in another one not included in the present study, AMPA
115 concentrations in June ranged between 0.17 and $2.8 \mu\text{g L}^{-1}$. Between 28/01/21 and 22/02/21,
116 we captured 10 spined toad amplexed pairs per sites. Pairs were spotted at night by patrolling
117 along the breeding sites with head lights. In each pond, the 10 first couples were collected to
118 guarantee comparable individual quality between the different sites.

119 *Housing, treatment and measurements*

120 Each pair was housed in a 20L aquarium containing a branch for the female to wrap her egg
121 strings around, after laying (2 -17 days after capture) males and females were released in their
122 habitat. Within 6h post laying, the egg strings (hence “clutch”) from both forest and agricultural
123 origin were removed from the aquarium, and six segments of 34 eggs per clutch (n=240
124 aquariums) were placed in individual aquariums with 2L of dechlorinated tap water in common-
125 garden conditions. Among the six segments of a same clutch, three were exposed to $0.4 \mu\text{g L}^{-1}$
126 ($\pm 0.01 \mu\text{g L}^{-1}$) of AMPA (AMPA group) and the remaining three segments, non-exposed to
127 AMPA, constituted the control group. The AMPA solution was obtained by dissolving
128 commercial crystalline powder (Aminomethylphosphonic acid, 99% purity, ACROS
129 ORGANICS™) with dechlorinated tap water. The selected AMPA concentration ($0.4 \mu\text{g L}^{-1}$)

130 was representative of actual concentrations found in surface water in our study area and based
131 on a previous study showing sublethal effects at environmental concentrations (Cheron and
132 Brischoux, 2020). We monitored the duration of embryonic development (between egg-laying
133 and hatching), once approximately 90% of the 34 eggs of a segment reached Gosner stage 25
134 (Gosner, 1960), we calculated three metrics: the hatching success (percentage of hatched
135 tadpoles), the rate of deformed tadpoles (percentage of individuals with oedema, crooked
136 spines, or other deformities, see **Figure S2**) and the rate of late tadpoles (percentage of
137 individuals which Gosner stage was at least 5 stages later than Gosner stage 25, see **Figure S2**).
138 Those metrics were calculated for n=8160 individuals (240 segments of 34 eggs). From the 34
139 hatchlings originating from each segment, one healthy looking tadpole was selected, this
140 resulted in 240 experimental individuals (n=120 in each experimental group), the remaining
141 tadpoles were released in the breeding pond of their parents. Each tadpole was individually kept
142 in a 2L aquarium either with dechlorinated tap water or AMPA according to the treatment
143 experienced during embryonic development. As a consequence, each individual was exposed
144 during both embryonic and larval development to the same treatment. The larval development
145 was monitored through six key Gosner stages (25, 30, 37, 41, 42 and 46). Tadpoles'
146 development was checked daily and when a tadpole reached one of those stages it was
147 photographed on graph paper for body, tail, and total length (snout to tail tip) measurements
148 using the free software ImageJ (<https://imagej.nih.gov/ij/>), as these traits are known to exhibit
149 plasticity in response to AMPA contamination (Cheron and Brischoux, 2020). Upon
150 metamorphosis we calculated the scaled mass index (SMI) developed by Peig and Green (2009)
151 with the following formula: $SMI = \text{body mass} \times (0.996/SVL)^{0.398}$ and mortality was monitored
152 from hatching to metamorphosis.

153 From the egg stage until metamorphosis, the 240 tadpoles were kept under simulated 12:12 h
154 day and night period, in a room at 17°C to avoid any variation of the basal metabolism and

155 therefore development. Water was changed weekly, with an addition of AMPA at 0.4 $\mu\text{g L}^{-1}$
156 for the contaminated tanks. Upon hatching, the tadpoles were fed with organic ground spinach
157 *ad libitum*.

158 *Statistical analysis*

159 Our data was tested for homogeneity of variance and normality, we also checked normality of
160 the residuals using diagnostics plots. Some variables slightly diverged from normality (see
161 violin plots **Figure S3**). Yet, because the F-statistic is extremely robust to violation of the
162 normality assumption when sample sizes are equivalent among groups and degrees of freedom
163 are large (both conditions were met in our analyses), we also used parametric test to analyse
164 those variables. We used linear and generalized mixed-effect models (LMMs and GLMMs) to
165 test the effects of AMPA treatment according to habitat on fitness parameters. It has to be
166 noticed that in the forest group, there was one clutch where the deformity rate was very high
167 (ranging from 50 to 100% for all segments), this clutch was therefore discarded from further
168 analyses, resulting in n=234.

169 The response variables were: embryonic phase duration, hatching success, deformity rate and
170 late rate calculated for n=7956 eggs, then during the larval phase, for the 234 tadpoles followed
171 until metamorphosis the response variables were: duration to reach the next monitored stage,
172 body length, tail length and snout to tail tip length (STL) and SMI (Peig and Green, 2009) at
173 stage 46. Mortality was analysed with a binomial model (GLMM) and all other response
174 variables were analysed with Gaussian models (LMMs). In each models, fixed variables were:
175 treatment (AMPA versus Control), habitat (agricultural vs forest), their interaction and the
176 clutch identity was set as a random variable. Mixed-effects models were fitted using the ‘lmer’
177 or ‘glmer’ functions of the ‘lme4’ package (Bates et al., 2015) in R v.3.6.3 (R Core Team,
178 2019). Kenward-Roger approximation was used to calculate degrees of freedom. For

179 calculating linear contrasts, we used the ‘lsmeans’ package (Lenth, 2016). From each model,
180 we report the treatment effect (difference between control and AMPA-exposed animals) for
181 each habitat type as least-squares means with standard errors (SEs) and with 84% confidence
182 intervals (CIs). Comparing two 84% CIs will give an approximate $\alpha = 0.05$ test for the
183 difference (Payton et al., 2003); thus, in our case, lack of overlap between the two 84% CIs
184 indicates a significant difference in the AMPA effect between forest and agricultural habitats.
185 Finally, time-dependent survival according to treatment and habitat was tested using a Kaplan-
186 Meier analysis with the ‘survival’ package (Therneau, 2022).

187 RESULTS

188 Embryonic development duration (i.e. from egg-laying to hatching) was not related to AMPA
189 exposure, in neither habitat, nor were hatching success, late rate or morphological measures
190 (n=7956, **Figure 1, Table 1**). However, the proportion of deformed hatchlings (n=7956) was
191 higher in eggs exposed to AMPA in forest individuals only (**Figure 1, Table 1**). During the
192 embryonic phase, we did not observe differential responses to AMPA exposure according to
193 the habitat (**Figure 1**).

194 During the larval phase (n=234), in the agricultural group only, the overall duration to reach
195 metamorphosis was longer in AMPA exposed individuals in comparison to controls (**Table 1**).
196 This result was the consequence of a ~2-day delay to reach Gosner stage 30, added to a ~4-day
197 delay to reach Gosner stage 42 (**Figure 2, Table 1**). In the Forest group, AMPA-exposed
198 individuals reached Gosner stage 30 approximately 2-days after control individuals (**Figure 2,**
199 **Table 1**). Yet in the forest group, this delay was cleared after stage 30 and until metamorphosis
200 (**Figure 2, Table 1**).

201 The strongest effects of AMPA on morphological measures were observed at Gosner stage 30,
202 where AMPA-exposed tadpoles from both habitats had shorter tails than controls, resulting in

203 shorter total length (STL, **Table 1**). In contrast, at Gosner stage 37, AMPA-exposed tadpoles
204 had longer bodies than controls in the Agricultural group (**Table 1**). At metamorphosis (Gosner
205 stage 46), SVL and body condition (SMI) were not related to AMPA exposure in either habitat
206 (**Figure 2, Table 1**), nor was overall mortality (**Table 1**). Overall, during the larval phase and
207 until metamorphosis, the effects of AMPA on morphology; body condition and time-dependent
208 survival were not habitat-dependent (**Figure 2, S4, Table 1, S1**). For each Gosner stages,
209 survival was not different in exposed or control tadpoles in either habitat ($p > 0.160$ for all tests,
210 **Figure S4**).

211 **DISCUSSION**

212 Contrarily to our prediction, several decades of glyphosate exposure in agricultural lands did
213 not confer an adaptive response to spined toad tadpoles. Although AMPA exposure did increase
214 the proportion of deformed hatchlings, which are known to be non-viable (Beattie et al., 1992;
215 Chinathamby et al., 2006; F. Brischoux personal observation), the effects of AMPA were only
216 significant in the forest populations but not stronger than in the agricultural group. In addition,
217 the effects of AMPA exposure on morphology were not habitat-dependent, although in the
218 forest group, AMPA-induced morphological differences persisted over a longer time
219 (Agricultural: stage 30 and 37; Forest: stages 30). Yet, they were cleared at metamorphosis and
220 among the toadlets that survived until metamorphosis (47.5% and 59.2% in the agricultural and
221 forest groups, respectively), AMPA exposure was not related to SVL neither to body condition
222 or mortality. AMPA exposure increased the total developmental length in tadpoles from the
223 agricultural group, yet the development duration was not significantly different from that of
224 forest individuals. Thus, individuals from both habitats responded similarly to AMPA exposure,
225 we have therefore no evidence of AMPA-resistance in spined toads originating from
226 agricultural habitats, and we could explain this result by the several complementary and non-
227 mutually exclusive hypotheses that follow.

228 A previous study conducted by Cheron and Brischoux (2020), reported non-monotonic effects
229 of AMPA on embryonic mortality. Embryonic mortality was higher than for controls at
230 concentrations which were around $0.07 \mu\text{g L}^{-1}$ and $0.32 \mu\text{g L}^{-1}$, whereas AMPA did not
231 influence embryonic mortality at higher concentrations ($3.6 \mu\text{g L}^{-1}$). In the present study we
232 monitored a higher proportion of non-viable hatchlings in AMPA-exposed individuals ($0.4 \mu\text{g}$
233 L^{-1}) in forest individuals only. In France, environmental concentrations of AMPA in aquatic
234 environments range from $0.1 \mu\text{g L}^{-1}$ to $6.6 \mu\text{g L}^{-1}$ (data from Water Agencies “Agence de l’eau
235 Loire Bretagne”). In the area where we conducted our study, maybe AMPA concentrations in
236 water bodies were too high to lead to high enough selective embryonic mortality in spined toads
237 (e.g. $>3.6 \mu\text{g L}^{-1}$ as observed in Cheron and Brischoux (2020)), to represent a strong selection
238 pressure for this species. In addition, these concentrations in water bodies are likely to vary
239 within a year, according to temperature, precipitation, and between years, according to the
240 nature of the crop, i.e. cultivated or fallow land (Edwards, 1975; Grandcoin et al., 2017; Medalie
241 et al., 2020). Therefore, within a same pond, some years could select towards AMPA-resistant
242 individuals, whereas in other years there would be no selection. Given the longevity of spined
243 toads (ca. 10 years in the wild) it is likely that a homogenization of genotypes would occur
244 between generations and mask AMPA-resistant individuals.

245 Although exposure to non-persistent chemicals may last for only a short period of time, it is
246 important to examine their long-term effects and the existence of any sensitive life stage. Any
247 delay in metamorphosis or morphological changes could impact demographic processes of the
248 population, potentially leading to declines or local extinction. Another explanation could
249 therefore be that AMPA-exposed tadpoles, that present an upregulated oxidative stress response
250 (Cheron et al., 2022), will be selected against later in their life as observed in other taxa. For
251 example, in three-spined sticklebacks *Gasterosteus aculeatus*, a temperature-related growth
252 rate increase induced oxidative damage during adulthood (Kim et al., 2019). In European staling

253 *Sturnus vulgaris*, oxidative damage in early-life affected inflammatory response in adults
254 (Nettle et al., 2017). Male Soay sheep *Ovis aries* lambs with lower protein carbonyls, a marker
255 of oxidative damage, were less likely to survive their first-winter (Christensen et al., 2016), and
256 this relationship was not observed in female lambs. AMPA-driven selection could thus occur
257 in adult spined toads, it would therefore be interesting to conduct transgenerational studies to
258 verify this hypothesis. Interestingly, during the field season of 2020 conducted in our study
259 area, females did not migrate to breed in sites surrounded by agricultural areas (Renoirt et al.,
260 2021), this could result from a sex-specific AMPA-related selection towards males rather than
261 females on sexually mature individuals (Renoirt et al., 2021). Yet we cannot exclude that some
262 biotic or abiotic factors, other than AMPA exposure, would render the monitored ponds
263 unattractive to female spined toads, as, at least during the following year, breeding females
264 were observed in other agricultural sites.

265 Finally, we could explain this result by the fact that spined toad migration distance could be
266 larger than expected, and instead of having habitat-related populations, we could be studying
267 one unique population on our entire study area covering 76 km². Although we followed
268 individuals from distant ponds (>4 km), smaller unmonitored ponds comprised between our
269 study sites could act as breeding zones, where AMPA-resistant individuals would potentially
270 reproduce with forest non-resistant individuals, resulting in a homogenised spined toad
271 population with mixed genotypes. Adult spined toads' foraging area is not delimited by habitat
272 structure (Indermaur et al., 2009) and from one pond to the next, there could be genetic mixture
273 leading to a metapopulation inhabiting a large territory. In other *Bufo* species such as *Bufo*
274 *calamita*, the connectivity between neighbouring breeding ponds can be maintained up to a
275 distance of 12 km according to the nature of the soil (Sinsch et al., 2012). The absence of
276 adaptive response to AMPA exposure in agricultural toads in comparison to forest toads could
277 therefore result from the absence of habitat-dependent genetic pools. Our results contrast with

278 that of Almeida et al. (2021), which report differential genetic adaptation in *Daphnia magna*
279 populations according to pesticides most commonly used in a 200m radius around the studied
280 pond. Considering the classic pattern of isolation by distance, which is determined by a positive
281 correlation between genetic differentiation among populations and geographic distances
282 (Wright, 1943), an organism's dispersal capacities, but also longevity is likely to affect gene
283 flow between populations and therefore local adaptations. Further studies using genetics to
284 identify segregated populations in areas more or less sprayed by glyphosate would be needed,
285 in order to better address the question of AMPA as a selection pressure.

286 Still, we show strong effects of AMPA on deformity occurrence at hatching, morphology and
287 developmental duration regardless the parents' habitat. These results suggest teratogenic
288 effects of AMPA and a potential disruption of hormones involved in developmental plasticity.
289 AMPA and glyphosate exposure have been reported to induce deformities in several aquatic
290 vertebrates such as amphibians and fish (Babalola et al., 2019; Bach et al., 2018; Smith et al.,
291 2019; Zhang et al., 2021), and as previously mentioned, deformed hatchlings are not viable
292 (Beattie et al., 1992; Chinathamby et al., 2006). Metamorphosis in amphibians is principally
293 orchestrated by thyroid hormones (THs) and glucocorticoids (GCs), which in turn act on a
294 number of other hormones (Lorenz et al., 2009; Navarro-Martín et al., 2012; Sachs and
295 Buchholz, 2019). Exposure to glyphosate and metabolites are likely to disrupt these hormonal
296 axes and the expression of targeted genes. For instance, exposure to Roundup WeatherMax®
297 in natural wetlands altered the mRNA levels of thyroid- and stress-related genes of wood frogs
298 (*Lithobates sylvaticus*) tadpoles (Lanctôt et al., 2013). The effects of AMPA on tadpole
299 morphology and development may threaten wild populations. Shorter tails and larger bodies
300 are maladaptive traits to cope with predators. For example, tadpoles reared in predator-exposed
301 ponds had shorter bodies, deeper tail fins and longer tails (Buskirk and Relyea, 1998). A shorter
302 body and a longer tail shall confer increased swimming speed, acceleration, and

303 manoeuvrability, which are adaptive traits to predation avoidance. A longer time to develop
304 may also be non-adaptive in the wild, increasing the risk of pond desiccation before emergence.
305 AMPA exposed tadpoles may therefore be easier prey to catch and may have a higher mortality
306 rate in the wild.

307 **CONCLUSION**

308 Despite strong evidences obtained in laboratory conditions from previous studies (i.e. increased
309 embryonic mortality in the AMPA-exposed group), the results of this study give little evidence
310 that glyphosate's primary degradation product, AMPA, induces a selective pressure in the wild.
311 Still, we have to remain cautious on our conclusions, since housing conditions were optimal
312 and do not reflect reality. Moreover, it has to be noticed that our monitoring of larval stages
313 was conducted on healthy looking individuals, which may be better able to cope with AMPA
314 exposure. Within and between years temporal and spatial dynamics of contamination, as well
315 as gene flow between exposed and preserved populations may hinder adaptive divergence
316 between populations. In addition, wild organisms are exposed to a multitude of biotic (e.g.
317 density, food scarcity, predation) and abiotic (e.g. temperature, desiccation probability, other
318 contaminants) stressors, which interact and may mask the effects of AMPA alone. Further
319 studies taking into account the presence of multiple stressors could help to better pinpoint the
320 habitat-related effects of AMPA on wildlife. Yet, we show strong evidence of adverse effects
321 of AMPA exposure at early developmental stages. AMPA exposure over a very short period of
322 time (embryonic development: ~17 days) leads to deformities, which are lethal. This result is
323 alarming, although glyphosate's primary metabolite increases teratogenicity, exposed spined
324 toad populations have not developed resistance to AMPA. Glyphosate application in
325 agricultural lands could therefore be one of the numerous causes of declining wild amphibian
326 populations.

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333 **ETHICS STATEMENT**

334 All applicable institutional and/or national guidelines for the care and use of animals were
335 followed. This work was approved by the French authorities (COMETHEA ethic committee
336 and Ministère de L'Enseignement Supérieur, de la Recherche et de L'innovation) under permits
337 APAFIS#13477-2018032614077834 v7, APAFIS#23728-2020011613221913 v4 and
338 DREAL/2020D/8041.

339

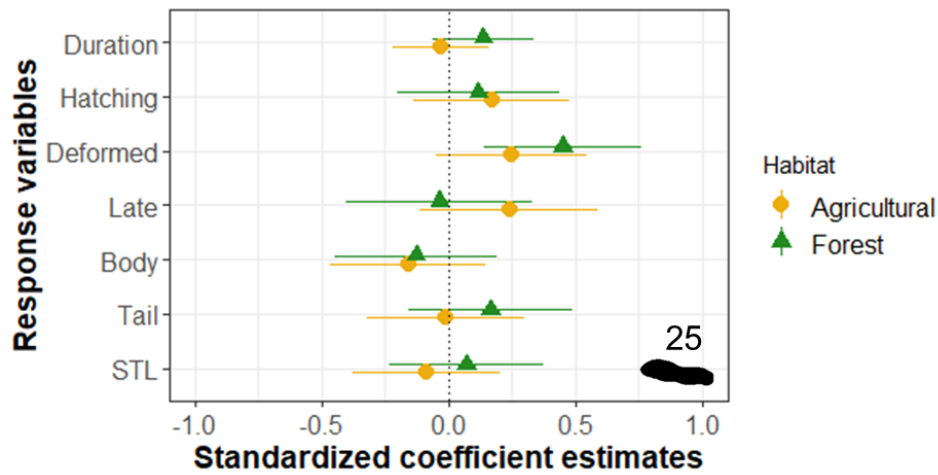
340

341

342 **Table 1. Effect of AMPA on fitness parameters according to habitat in developing spined toads.** Values are estimates, standard errors (SE),
343 degrees of freedom (df), upper and lower limits (CL) of the 84% confidence intervals obtained from the contrasts of the treatment (control vs
344 AMPA) × habitat (agricultural vs forest) interaction of LMMs and GLMMs. Embryonic stage duration, hatching success, deformity rate and late
345 rate were calculated out of 234 segments of 34 eggs (n=7956), from stage 25 metrics are obtained for n=234 individuals (sub-sample sizes are
346 given for each stages). Total development duration spans from egg-laying to metamorphosis (Gosner stage 46), toadlet's body condition was
347 calculated using the scaled mass index of Peig and Green (2009). Mortality was categorized as 0= alive, 1=dead. STL = snout to tail length, SVL
348 = snout to vent length. Values in bold are significant at the 0.05 level. Reference level is "control".

349

Response variables		Agricultural					Forest				
		Estimate	SE	df	lower CL	upper CL	estimate	SE	df	lower CL	upper CL
Embryonic stages											
	n	<i>Control Agricultural (n=2040) - AMPA Agricultural (n=2040)</i>					<i>Control Forest (n=1938) - AMPA Forest (n=1938)</i>				
Laying → 25	Duration	-0.064	0.18	188	-0.44	0.32	0.27	0.189	188.2	-0.12	0.67
	Hatching success	5.60	4.82	188.2	-4.480	15.7	3.91	5.03	188.7	-6.6	14.4
	Deformity rate	2.92	1.69	188.1	-0.61	6.45	5.34	1.76	188.6	1.66	9.01
	Late rate	3.13	2.20	188.4	-1.47	7.73	-0.46	2.29	189.5	-5.25	4.33
25	n	<i>Control Agricultural (n=60) - AMPA Agricultural (n=59)</i>					<i>Control Forest (n=54) - AMPA Forest (n=56)</i>				
	STL	-0.007	0.012	188.2	-0.032	0.017	0.006	0.0123	188.6	-0.020	0.032
	Body length	-0.006	0.006	188.2	-0.018	0.006	-0.005	0.006	188.8	-0.018	0.008
	Tail length	-0.001	0.008	188.2	-0.018	0.017	0.009	0.009	188.7	-0.009	0.028
Larval stages											
	n	<i>Control Agricultural (n=59) - AMPA Agricultural (n=53)</i>					<i>Control Forest (n=53) - AMPA Forest (n=55)</i>				
25 → 30	Duration	2.11	0.91	181.9	4.02	0.20	2.22	0.93	180.3	0.28	4.16
	STL	-0.061	0.023	179.5	-0.013	-0.108	-0.055	0.023	179.2	-0.103	-0.007
30	Body length	-0.019	0.010	179.8	0.002	-0.040	-0.014	0.010	179.3	-0.035	0.008
	Tail length	-0.041	0.014	179.5	-0.011	-0.071	-0.041	0.015	179.2	-0.071	-0.010
	n	<i>Control Agricultural (n=49) - AMPA Agricultural (n=46)</i>					<i>Control Forest (n=47) - AMPA Forest (n=52)</i>				
30 → 37	Duration	0.52	2.60	159.5	-4.92	5.96	1.84	2.54	157.3	-3.48	7.15
	STL	0.048	0.031	159.8	-0.017	0.113	0.047	0.030	157.5	-0.017	0.110
37	Body length	0.037	0.014	160.1	0.008	0.067	0.023	0.014	157.8	-0.006	0.052
	Tail length	0.006	0.022	158.8	-0.040	0.052	0.027	0.022	156.7	-0.018	0.072
	n	<i>Control Agricultural (n=43) - AMPA Agricultural (n=43)</i>					<i>Control Forest (n=42) - AMPA Forest (n=50)</i>				
37 → 41	Duration	-3.22	1.75	147.7	-6.89	0.45	-2.70	1.69	142.4	-6.23	0.84
	STL	0.002	0.037	146	-0.074	0.078	-0.052	0.035	141.3	-0.125	0.022
41	Body length	0.007	0.019	150	-0.032	0.046	-0.007	0.018	144.2	-0.044	0.031
	Tail length	-0.003	0.026	145.1	-0.058	0.052	-0.048	0.025	140.7	-0.101	0.005
	n	<i>Control Agricultural (n=23) - AMPA Agricultural (n=31)</i>					<i>Control Forest (n=32) - AMPA Forest (n=39)</i>				
41 → 42	Duration	4.27	2.03	115	0.003	8.530	-0.053	1.72	99	-3.66	3.56
	STL	-0.076	0.080	114.4	-0.243	0.091	0.020	0.067	98.6	-0.122	0.161
42	Body length	0.015	0.020	107.8	-0.028	0.057	0.009	0.017	94.1	-0.026	0.045
	Tail length	-0.096	0.079	116.9	-0.262	0.069	-0.003	0.067	100.9	-0.144	0.139
	n	<i>Control Agricultural (n=23) - AMPA Agricultural (n=31)</i>					<i>Control Forest (n=32) - AMPA Forest (n=39)</i>				
42 → 46	Duration	0.80	0.85	119.1	-0.98	2.58	0.04	0.72	103.3	-1.48	1.56
	SVL	0.005	0.024	117.8	-0.046	0.055	0.006	0.020	101.7	-0.036	0.049
46	Toadlet SMI	-0.011	0.009	116.2	-0.030	0.008	0.004	0.008	99.9	-0.012	0.020
	n	<i>Control Agricultural (n=23) - AMPA Agricultural (n=31)</i>					<i>Control Forest (n=32) - AMPA Forest (n=39)</i>				
Laying → 46	Total duration	7.52	3.58	107.2	0.01	15.03	1.70	2.95	95	-4.50	7.90
	n	<i>Control Agricultural (n=60) - AMPA Agricultural (n=60)</i>					<i>Control Forest (n=57) - AMPA Forest (n=57)</i>				
25 → 46	Mortality	-0.62	0.37	∞	-1.39	17 0.16	-0.36	0.38	∞	-1.16	0.43



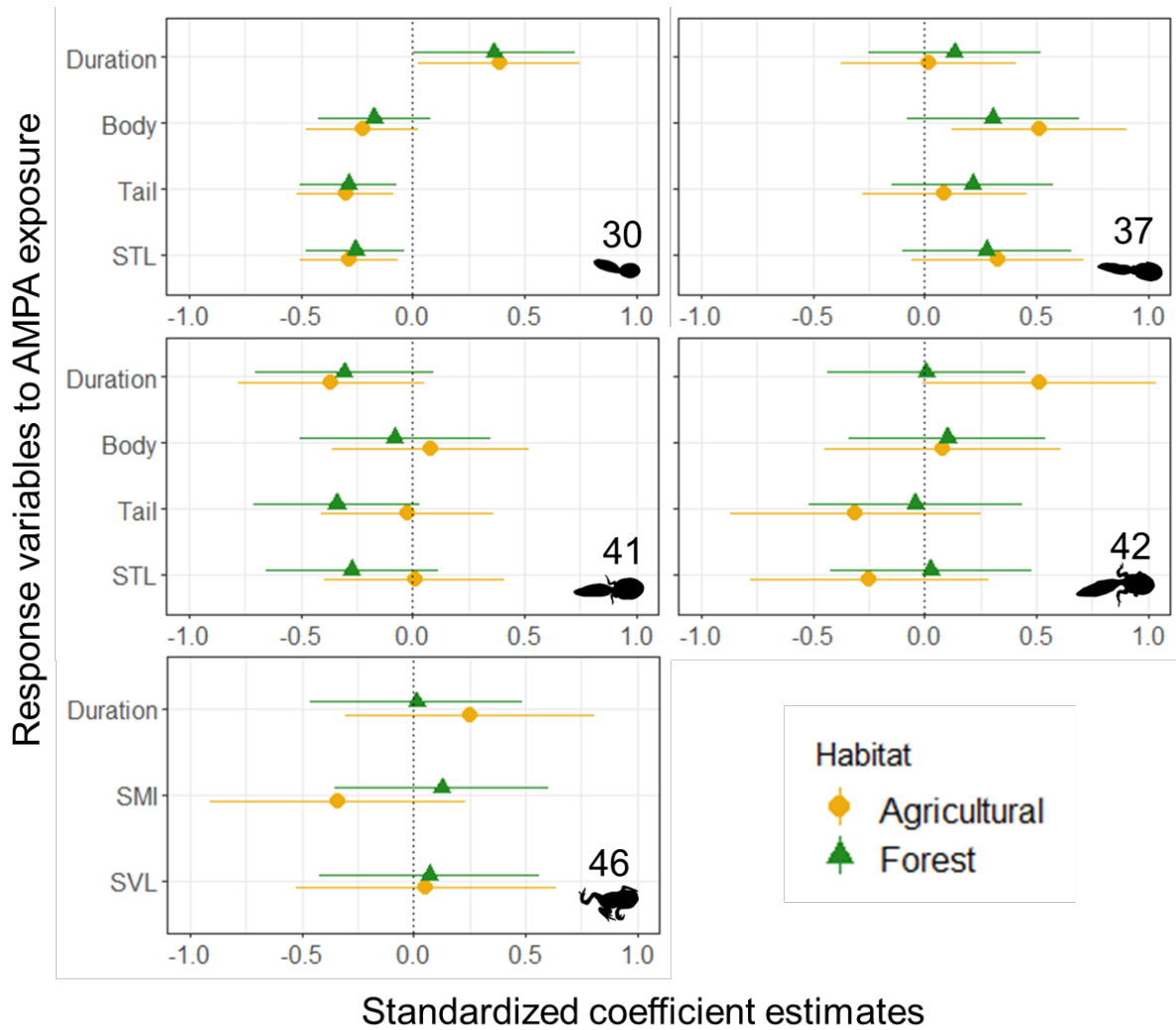
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352 **Figure 1. Effects size of fitness parameters according to habitat in response to AMPA**
 353 **exposure during the embryonic stage of spined toads.** The variables are the duration of the
 354 embryonic stage (egg-laying to hatching), hatching success, deformity rate, late rate and
 355 morphological measures (body length, tail length and snout to tail tip length = STL). Embryonic
 356 stage duration was obtained from n=234 egg segments. Hatching success, deformity rate and
 357 late rate were calculated from n=7956 eggs, and morphological measures on n=234 hatchlings.
 358 The figure illustrates scaled estimates of model outputs and 84% confidence interval from
 359 mixed effect models. An effect is considered as significant when its confidence interval does
 360 not cross zero, the effects between habitats are considered significant when their confidence
 361 intervals do not overlap. Yellow circles represent agricultural originating individuals and green
 362 triangles forest individuals.

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367 **Figure 2. Effects size of fitness parameters according to habitat in response to AMPA**
 368 **exposure during the larval stage of spined toads.** The variables represent the duration of the
 369 larval stage and morphological measures (body length, tail length, snout to tail tip length =
 370 STL). For Gosner stage 46, we used the measure snout to vent length (SVL) as tail resorption
 371 was complete in most individuals, and calculated their scaled mass index as a measure of body
 372 condition. Sample sizes for each stage are given in **Table 1**. The figure illustrates scaled
 373 estimates of model outputs and 84% confidence interval from mixed effect models. An effect
 374 is considered as significant when its confidence interval does not cross zero, the effects between
 375 habitats are considered significant when their confidence intervals do not overlap. Yellow
 376 circles represent agricultural originating individuals and green triangles forest individuals.

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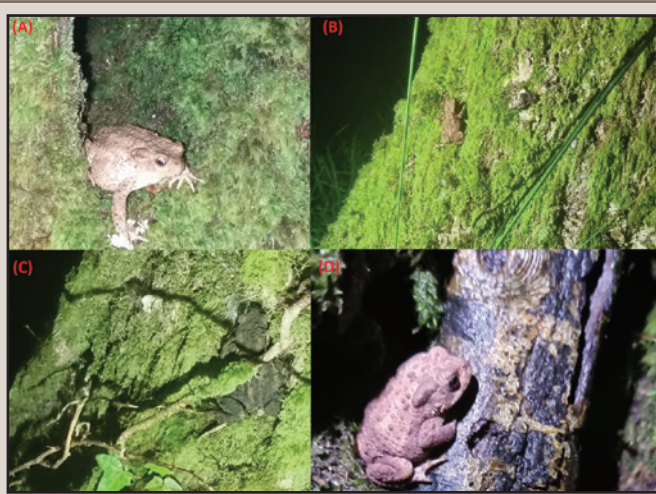


FIG. 1. Four individuals of *Bufo bufo* found climbing up to ca. 120 cm in trees at Fairwood Common on the Gower Peninsula, Swansea, Wales, U.K.

cm SVL) resting ca. 120 cm high in a tree fork at 2208 h on 2 June 2021, 30 min after sunset (Fig. 1A). Since then, we have observed an additional six separate instances of *B. bufo* climbing into trees at the same site. On the nights of 23 and 24 June 2021, there were a total of 13 *B. bufo* found, five of which were located at heights between 30–120 cm in trees. Figure 1B and 1C show a juvenile and adult respectively, exhibiting this arboreal behavior. On 6 July 2021 at 2257 h, another juvenile (Fig. 1D) was found climbing a tree at a steep angle (ca. 70°) ca. 90 cm off the ground.

The conditions have varied when this behavior has been observed, with high humidity (ca. 80%) and temperatures ranging from 14–18°C. The sightings all occurred within an hour of sunset, with damp ground conditions. The available routes by which the *B. bufo* could have reached the locations where we observed them involved climbs ranging from 45 to nearly 90°, and the behavior was not limited to adult *B. bufo*.

Bufo bufo is an opportunistic and generalist predator of small invertebrates (Gittins 1987. *Amphibia-Reptilia* 8:13–17; Vignoli et al. 2009. *Life Environ.* 59:47–57; Mallov and Stojanova 2010. *Biotechnol. Biotechnol. Eq.* 24(Suppl):263–269; Crnobrnja-Isailovi et al. 2012. *J. Herpetol.* 46:562–567) and these prey items were commonly found on tree trunks in the area, including those which toads had climbed. However, as they were also common at ground level, it is difficult to conclude that arboreal behavior in *B. bufo* provides specific advantages for feeding compared to foraging at ground level. We suggest that general exploratory activity, perhaps largely consisting of active foraging, leads *B. bufo* to move around any part of the environment it can feasibly access, including tree trunks.

The paucity of previous published records of arboreal activity in *B. bufo*, and the consideration of it as a firmly terrestrial species (Arnold and Ovenden 2002, *op. cit.*), are perhaps more to do with human search patterns than toad activity. Herpetologists searching for *B. bufo* (particularly in the U.K. where no arboreal reptiles or amphibians are native) typically search on the ground. Our observations suggest that researchers may benefit from expanding active searches for *B. bufo* to include tree trunks in suitable habitats, particularly where forks and cavities provide opportunities to securely rest. Although arboreal behavior has been documented in *B. bufo* previously (Bringsøe 2016, *op. cit.*), published observations remain rare and the species is

still considered firmly terrestrial. Our observations add, to our knowledge, the first records of arboreality for *B. bufo* in the U.K. and suggest that it may be an under-reported but common behavioral strategy in this well-studied species.

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BUFO SPINOSUS (Spined Toad). PREDATION. Bufonids are well known for their ability to produce steroid toxins (bufadienolides) mainly from their parotoid glands, but also from their dorsal tegument (Chen et al. 2017. *Ecol. Evol.* 7:8950–8957). These toxins are associated with a bitter taste and inhibit Na⁺/K⁺-ATPase activity with effects ranging from nausea to heart failure. They are mainly used as an antipredator defense to repel or kill potential predators. Despite their potent skin toxins, many bufonids are preyed upon by reptilian (Costa and Trevelin 2020. *Herpetol. Notes* 13:649–660), avian (Bordignon et al. 2018. *PLoS ONE* 13:e0193551; Blancas-Calva and Castro-Torreblanca 2021. *Reptil. Amphib.* 28:227–228) and mammalian species (Cabrera-Guzmán et al. 2014. *J. Pest Sci.* 88:143–153). In Europe, mammalian predators of *Bufo* spp. (mainly mustelids: Smiraldo et al. 2019. *Mammal Rev.* 49:240–255) have evolved specialized behaviors to avoid intoxication by bufadienolides: most species aim at the ventral surface (which contains fewer toxins (Bringsøe and Holden 2021. *Herpetozoa* 34:57–59) and peel the skin of individuals to have access to toxin-free viscera and muscles (Henry 1984. *Rev. Ecol.* 39:291–296).

While monitoring the reproduction of *Bufo spinosus* in western France, we noticed remains of male and female individuals at one of our study sites (46.17313°N, 0.46059°W; WGS 84). Most carcasses were characterized by open abdomens and evisceration as well as skinned legs and missing muscles (Fig. 1A, B). When predation involved reproductive females,



FIG. 1. A male (A) and a female (B) *Bufo spinosus* preyed upon by a *Rattus norvegicus* in western France: A) the male has been eviscerated (1), another wound is visible in the pelvic area (2) and muscles of the right hind limb are missing (3); B) The female has been wounded in the ventral area (4) with non-consumed eggs clearly visible, the gular area (5), and eviscerated at the pelvic area (6), muscles of the left hind limb are missing (7) and both forelimbs (which bones are visible – 8) are missing.



FIG. 2. Predation of *Bufo spinosus* by *Rattus norvegicus* in western France. Screenshot taken from the video footage referenced in the text.

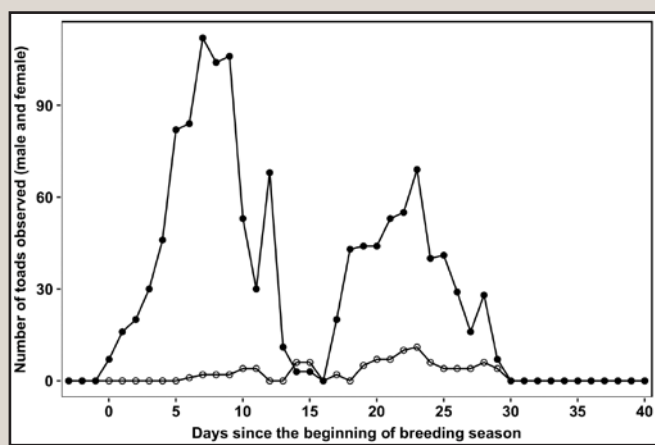


FIG. 3. Number of toads observed on the site in western France since the beginning of the breeding season (28 January 2021). Black dots represent live individuals and white dots represent carcasses of toads preyed upon by *Rattus norvegicus*.

eggs were not consumed (Fig. 1B), presumably because toad eggs also contain bufadienolides (Zhou et al. 2021. *J. Nat. Prod.* 84:1425–1433). Concomitantly, we observed a *Rattus norvegicus* (Brown Rat) swimming across the breeding pond with a live *B. spinosus* in its mouth. We set up camera traps (WiMius invisible trail camera) where toad remains have been found in order to validate our visual observation. One of our camera traps recorded a predation event of a male *B. spinosus* (Fig. 2): a brown rat actively tried to turn a *B. spinosus* on its back in order to access its ventral surface (video available at: <http://dx.doi.org/10.26153/tsw/41042>). During the course of our monitoring, the maximum number of carcasses observed in one night was 11, but these predation events likely occurred during the whole reproductive period (Fig. 3). *Rattus norvegicus* density appeared to be low, as most footage recorded only one individual, except for a single occasion when two rats could be observed simultaneously (video available at: <http://dx.doi.org/10.26153/tsw/41042>). Although we have monitored several ($N = 8$) breeding sites of *B. spinosus* since 2015, we interestingly only recorded 5 carcasses of toads on other sites and these remnants were characteristic of mustelid predation (i.e., entirely peeled skin, Henry 1984. *Rev. Ecol.* 39:291–296).

Rats are opportunist omnivores known to prey on amphibians (Watts 1981. *The Rodents of Australia*. Angus and Robertson, Sydney, New South Wales, Australia. 321 pp.; Breed and Ford 2007.

Native Mice and Rats. Clayton: CSIRO Publishing, Collingwood, Victoria, Australia. 185 pp.; Velo-Antón and Cordero-Rivera 2011. *Herpetol. Notes* 4:299–301) including toxic bufonids (Shine 2010. *Q. Rev. Biol.* 85:253–291; Cabrera-Guzmán et al. 2014. *J. Pest Sci.* 88:143–153). Yet, to our knowledge, rodent predation on toxic toads has not been described previously in Europe. Given the number of dead *B. spinosus* observed during the course of our observations, the potential impact on the breeding population might be substantial (Lodé 1996. *Ethol. Ecol. Evol.* 8:115–124).

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ELACHISTOCLEIS CESARII. DERMATOPHAGY. *Elachistocleis cesarii* is a microhylid with a wide geographic distribution occurring in the Caatinga, Cerrado, Pantanal, and Atlantic Forest domains in Brazil (Toledo et al. 2010. *Zootaxa* 2418:50–60). Dermatophagy is observed in several species of amphibians and reptiles, which either consume their own shed epidermis or that of conspecifics (Weldon et al. 1993. *J. Herpetol.* 27:219–228). In amphibians, shedding or molting of the skin of adults can last from a few days to several weeks, and the shed skin is frequently consumed (Vitt and Caldwell 2013. *Herpetology: An Introductory Biology of Amphibians and Reptiles*. Fourth edition. Academic Press, San Diego, California. 776 pp.). Dermatophagy may be associated with reclaiming proteins lost in the molting process (Bustard and Maderson 1965. *Herpetologica* 21:306–308). It may also help regulate cutaneous microorganisms, like fungi and bacteria, and in some species, it may even reduce *Batrachochytrium*



FIG. 1. A) *Elachistocleis cesarii* from the Municipality of Trairí, Ceará, Brazil; B) pulling the shed skin from its left forelimb (red arrow); C) the shed skin stretched between the forelimbs and the mouth (yellow arrows).

PHOTO BY LUCAS L. BEZERRA

RESUME

Un grand nombre d'études ont mis en avant les effets négatifs des pressions anthropiques dans le temps et dans l'espace sur la biodiversité. Parmi ces pressions anthropiques, les activités et l'expansion agricole jouent un rôle principal dans la modification des milieux et dans la perte de biodiversité. De fait, la question de la persistance des espèces animales dans ce type de milieux se pose. C'est dans ce contexte que ma thèse s'axe. Afin d'étudier les réponses des organismes à un milieu dégradé et les contraintes du paysage sur différents traits d'histoire de vie et l'écologie, je travaille spécifiquement sur une espèce d'amphibien occupant des milieux allant de fortement conservés à fortement dégradés. Afin de comparer les populations de crapauds épineux (*Bufo spinosus*) forestières et les populations agricoles, j'utilise un vaste panel de marqueurs pour examiner (1) la structure génétique des populations (marqueurs micro-satellites), (2) l'écologie alimentaire (isotopes stables), (3) la qualité individuelle (télomères, morphologie, traits de développement) et son impact sur la reproduction. De ce fait et au cours de cette thèse, j'ai pu mettre en relation de nombreux facteurs associés aux paysages agricoles qui soulèvent de nombreuses questions quant au maintien des populations de crapauds épineux. Ainsi, nous avons pu montrer un effet significatif des fertilisants sur la signature isotopique en $\delta^{15}\text{N}$ des populations de *B.spiniosus*. Aussi, nous avons pu souligner que l'environnement agricole reste suffisamment perméable au maintien de la diversité génétique. Cependant, nous avons mis en évidence de nombreuses contraintes de ce milieu sur la reproduction des populations d'amphibiens, que ce soit par la faible (voir l'absence) abondance de femelles sur les sites de reproduction, et/ou directement sur le succès reproducteur et la qualité de la progéniture. Ces résultats suggèrent de possibles effets à long terme sur les populations d'amphibiens et nous suggérons d'approfondir les différentes voies de recherche que nous suggérons tout au long de cette thèse afin de mieux comprendre les mécanismes sous-jacents à ces résultats et de trouver des solutions quant à la pérennité des espèces sauvages qui n'ont d'autres choix que de s'adapter.

A large number of studies have highlighted the negative effects of anthropogenic pressures in time and space on biodiversity. Among these anthropogenic pressures, agricultural activities and expansion play a major role in the modification of environments and in the loss of biodiversity. Questions whether animal species persist in this type of environment arises. My thesis is based on this context. We aimed at study the responses of organism to a degraded environment and the landscape constraints on life history traits and ecology. My work is focused specifically on an amphibian species persisting in habitat ranging from highly conserved to highly degraded by agricultural activities. In order to compare forest and agricultural populations of model species (Spined toad, *Bufo spinosus*), I relied on a wide variety of markers to examine (1) population genetic structure (micro-satellite markers), (2) feeding ecology (stable isotope), (3) individual quality (telomeres, morphology, developmental traits) and the impact on reproduction. As a result, I was able to connect many factors associated with agricultural landscapes that raised many questions about the persistence of spined toad populations. We were able to show a significant effect of fertilizers on the $\delta^{15}\text{N}$ isotopic signature of *B.spiniosus* populations. Moreover, we highlighted that agricultural environment allows genetic diversity between populations. However, using correlative approaches, we pointed out various constraints of this environment on the reproduction of amphibians populations, either through low (or no) abundance of females on breeding sites, and/or directly on reproductive success and offspring quality. These results suggest possible long-term effects on amphibian populations, and we suggest that the various avenues of research we suggested throughout this thesis should be pursued in order to better understand the mechanisms underlying these results and to find solutions for the sustainability of wild species that have no choice but to adapt.