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Léa Lorrain-Soligon

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ÉCOLE DOCTORALE
Euclide

Centre d'Études Biologiques de Chizé UMR 7372

THÈSE

présentée par :

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Conséquences de l'augmentation de la salinité sur la faune des marais littoraux :
le cas des amphibiens



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Sommaire

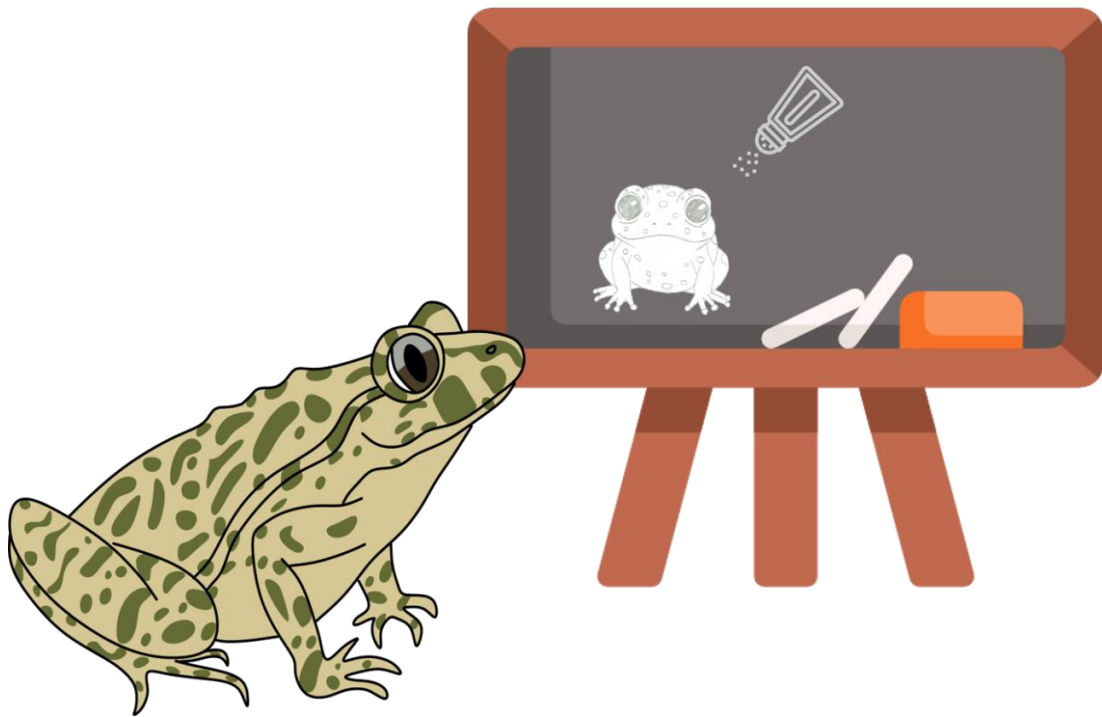
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Introduction



I) Changements globaux : des phénomènes liés à l'action de l'Homme

I.1. Anthropocène et crise d'extinction de la biodiversité

L'Homme influence fortement la biosphère. Ce fait est maintenant reconnu et indiscutable, tant et si bien que pour désigner l'ère géologique actuelle on parle d'Anthropocène. Les conséquences des activités humaines sont de fait devenues des forces évolutives majeures (Vitousek et al. 1997; Pievani 2014), et génèrent des changements significatifs sur des laps de temps très courts. Du fait de leur rapidité, ces changements ont des conséquences désastreuses sur la biodiversité (Walther et al. 2002; Parmesan and Yohe 2003; Parmesan 2006; Fey et al. 2015; Arneeth et al. 2020), que ce soit au niveau individuel, au niveau des populations, ou au niveau de l'ensemble des écosystèmes (Bellard et al. 2012). La biodiversité connaît ainsi la 6^{ème} grande crise d'extinction de son histoire (Barnosky et al. 2011; Pievani 2014; Pimm et al. 2014; Ceballos et al. 2017; Tickner et al. 2020), avec une rapidité d'extinction sans précédent et de nombreux dommages sur les écosystèmes et les biomes (Hoekstra et al. 2005). Etant donné l'ampleur globale des changements qui ont lieu actuellement, ils sont qualifiés de changements globaux.

I.2. Principaux changements globaux étudiés

Les écosystèmes sont tous naturellement soumis à des variations. Mais les activités humaines ont conduit à des variations très marquées en terme de transformation de l'habitat et d'altération des cycles biogéochimiques (Vitousek et al. 1997).

L'un des principaux changements globaux étudié (et médiatisé) est lié aux changements climatiques. Notre planète est actuellement confrontée à un changement rapide des températures globales, appelé « réchauffement climatique », qui est principalement, sinon exclusivement, dû aux activités humaines (Kellogg 1991; Vitousek 1994; Houghton et al. 2001), et à l'altération du cycle biogéochimique du carbone (Vitousek et al. 1997). Depuis 1950, l'analyse des valeurs extrêmes de température montre que les températures maximales et minimales quotidiennes extrêmes ont augmentées dans la plupart des régions, l'augmentation la plus importante ayant été reportée au Canada et en Eurasie où les températures maximum journalières ont augmentée de 1 à 3°C (Brown et al. 2008). Selon le dernier rapport de l'IPCC

(Intergovernmental Panel on Climate Change), la température moyenne sur terre va continuer d'augmenter très rapidement, de +2°C à +7°C d'ici à 2100.

Ces variations de températures pourraient mener à des changements d'habitats (Mohseni et al. 2003; Mantyka-pringle et al. 2012), mais les dégradations d'habitat en elles même sont également la cause de disparition d'espèces (Nuissl and Siedentop 2021). Et les activités humaines liées à ces modifications de l'habitat conduisent aussi à de la pollution. Par exemple, l'augmentation de la présence des terres agricoles a conduit à une augmentation accrue de l'utilisation des pesticides (Köhler and Triebkorn 2013; Gatto et al. 2016), et des fertilisants (Foley et al. 2005) qui ont des conséquences désastreuses pour la biodiversité, et conduisent à l'eutrophisation des eaux de surface et les nappes phréatiques à travers le monde (Ansari et al. 2010). Mais malgré les nombreuses études des contaminations environnementales, certains aspects de ces contaminations demeurent peu étudiés, comme c'est le cas de la salinisation des milieux (Cunillera-Montcusí et al. 2022). A titre d'exemple, sur la période 1950-2022, 160722 articles scientifiques ont été publiés concernant l'étude des pesticides (parmi lesquels 10951 en 2022), 80908 articles ont été publiés concernant l'étude de la pollution métallique (parmi lesquels 9490 en 2022), 35305 articles ont été publiés concernant l'étude de l'eutrophication (parmi lesquels 2810 en 2022), et 7540 articles ont été publiés concernant l'étude de la salinisation (parmi lesquels 921 en 2022) (Figure 1).

II) Un changement global récemment mis sur le devant de la scène : la salinisation des milieux

II. 1. La salinisation des milieux

La salinité désigne la masse de solides inorganiques dissous trouvée dans l'eau et peut être considérée comme la somme de la fraction massique des principaux cations (Na^+ , Mg^{2+} , Ca^{2+} , K^+), anions (Cl^- , SO_4^{2-}), et espèces carbonatées (HCO_3^- , CO_3^{2-}) (Dugan and Arnott 2022). Parce que la salinité est dominée par des constituants ioniques chargés, elle peut être approximée par la conductivité ; une mesure de la capacité de l'eau à conduire le flux électrique, mesurée à 25°C. Un milieu est généralement considéré comme étant doux si la salinité est comprise entre 0 et 1 g.l⁻¹ (correspondant à 1 PSU : Practical Salinity Units, ou 1 ‰), saumâtre si la salinité est comprise entre 1 et 30 g.l⁻¹, et salé si la salinité est supérieure à 30 g.l⁻¹ (Bal et al. 2022). La salinisation, quant à elle, se définit comme étant l'augmentation de la concentration en ions

Na^+ , K^+ , Cl^- , CO_3^{2-} , SO_4^{2-} dans l'environnement (Feistel et al. 2015). La salinisation peut être d'origine anthropique, et on parle alors de pollution saline. Elle se présente souvent sous la forme de sels de chlorure (e.g. NaCl , CaCl_2 , MgCl_2), et cette pollution anthropogénique est en constante augmentation (Dugan and Arnott 2022).

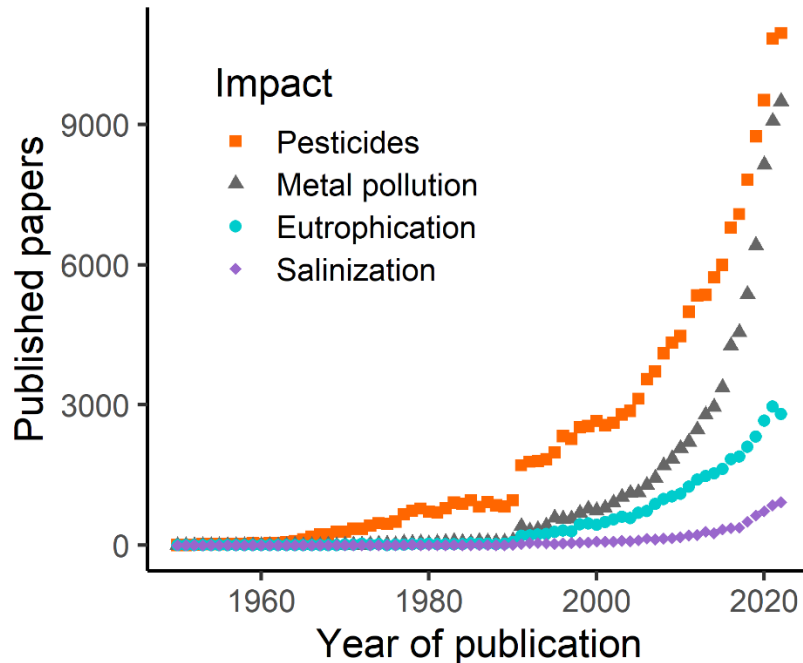


Figure 1 : Nombre d'articles publiés par année, entre 1950 et 2022, concernant l'étude des pesticides, de la pollution métallique, de l'eutrophication, et de la salinisation. Les données ont été récupéré depuis Web of Science, le 14 Juillet 2023, en recherchant respectivement les termes "Pesticide", "Metal pollution", "Eutrophication" et "Salinization".

Aujourd'hui, de plus en plus de masses d'eau continentales se salinisent (Williams 1999, 2001), ce phénomène est d'une importance majeure et en constante augmentation (Williams 1999). Bien qu'encore peu étudiée, la salinisation se produit actuellement à des taux sans précédent (Herbert et al. 2015; Cunillera-Montcusí et al. 2022), que ce soit en France (Kloppmann et al. 2011) ou à travers le monde (Wicke et al. 2011; Singh 2021; Cunillera-Montcusí et al. 2022). Elle trouve son origine dans au moins deux processus : la salinisation primaire et la salinisation secondaire (Kloppmann et al. 2011; Herbert et al. 2015; Hien 2017). Alors que la salinisation primaire désigne l'accumulation naturelle de sel dans les écosystèmes, la salinisation secondaire représente l'augmentation de la concentration en sel provoquée par l'action directe de l'Homme ou par ses activités (Herbert et al. 2015; Izam et al. 2021), la différence entre les deux étant majoritairement une différence d'échelle temporelle. En effet, la salinisation primaire représente des processus longs tels que les transitions entre

les aires glaciaires et interglaciaires (Herbert et al. 2015). La salinisation secondaire quant à elle désigne des modifications de la salinité sur des échelles de temps beaucoup plus courtes (Herbert et al. 2015). Les mécanismes impliqués dans la salinisation secondaire des milieux sont nombreux et divers (Figure 2), et sont souvent en lien avec une dérégulation des mécanismes naturels.

II.2. La salinisation : les mécanismes en jeu

II.2.1. *Augmentation du niveau de la mer*

Le niveau de la mer a augmenté entre 1900 et 2009 à un taux moyen de 1.7 ± 0.2 mm par an (Church and White 2011; Planton et al. 2015). Mais loin de s'atténuer, nombre de modèles ont prédit que l'augmentation du niveau de la mer allait encore s'accroître (Gornitz 1995; Domingues et al. 2008; Herbert et al. 2015). Cette augmentation est estimée en moyenne à 1 m d'ici à 2100 (Vermeer and Rahmstorf 2009; Bakker et al. 2017), mais elle pourrait atteindre 1.80 m selon certains modèles (Nicholls and Cazenave 2010). Cette augmentation est principalement due à la fonte des glaces (Rignot et al. 2011; Planton et al. 2015), et à l'expansion thermique des océans (Planton et al. 2015), tous deux liés au réchauffement climatique. Nicholls et al. (1999) ont suggéré que d'ici à 2080, 22% des zones humides côtières pourraient disparaître du fait de l'élévation du niveau de la mer. Cette tendance à l'élévation du niveau de la mer se vérifie sur la grande majorité des océans du globe (Planton et al. 2015). Au-delà de l'augmentation du niveau de la mer, la proximité avec le milieu marin entraîne également des intrusions souterraines d'eau salée dans les nappes continentales (Knighton et al. 1991; Visschers et al. 2022).

II.2.2. *Tempêtes marines*

Les changements climatiques vont aussi conduire à une augmentation de la fréquence et de l'intensité des événements météorologiques extrêmes, tels que les tempêtes marines et les submersions qui y sont associées (Nicholls et al. 1999; McLean et al. 2001; Knutson et al. 2010; Dettinger 2011; Trenberth et al. 2015; IPCC 2022). Dans les milieux tropicaux par exemple, il a été montré que d'ici à 2100, l'intensité des tempêtes va augmenter de 2 à 11% (Knutson et al. 2010). Les tempêtes côtières, dont les événements sont stochastiques, peuvent entraîner des entrées périodiques et brutales d'eau de mer dans des habitats autrement principalement d'eau douce (Gunzburger et al. 2010), changeant l'habitat de manière imprévisible.

II.2.3. Evaporation qui dépasse les précipitations et les apports d'eau

Avec le réchauffement climatique on assiste également à des changements dans les régimes des précipitations et de l'évaporation qui vont conduire à une augmentation de la salinité. En l'occurrence, les changements climatiques conduisent à des changements dans la magnitude et le calendrier des précipitations (Gornitz 1995; Domingues et al. 2008; Herbert et al. 2015), mais aussi à une augmentation de l'évaporation, et la salinité peut alors augmenter si l'évaporation excède les précipitations et/ou les apports d'eau (Al-Shammiri 2002; Mills et al. 2013; Obianyo 2019; Çolak et al. 2022). Cela est particulièrement vrai pour les climats secs et arides (Mills et al. 2013; Jeppesen et al. 2020; Hassani et al. 2021), mais touche également le milieu marin (Schroeder et al. 2017).

II.2.4. Causes humaines directes

La salinisation peut également être une conséquence directe de l'action humaine, du fait de créations de retenues qui provoquent des détournements des masses d'eau ou des réductions du flux d'eau (Williams 2001; Zarfl et al. 2015; Reid et al. 2019), une extraction accrue des masses d'eau souterraines et de surfaces (Pannell and Ewing 2006; Daliakopoulos et al. 2016; Zaman et al. 2018; Reid et al. 2019; Peters et al. 2021; Çolak et al. 2022), mais également des apports anthropogéniques de sel (Herbert et al. 2015; Dugan et al. 2017), tels que le salage des routes en hiver (Findlay and Kelly 2011; Van Meter and Swan 2014; Coldsnow et al. 2017), l'irrigation avec de l'eau salée ou saumâtre ou encore la surutilisation de fertilisants (Williams 2001; Pannell and Ewing 2006; Daliakopoulos et al. 2016; Zaman et al. 2018), l'extension de l'agriculture (Singh 2021), et le remplacement de la végétation par des plantes à racines moins profondes (Williams 2001). Les activités humaines telles que l'agriculture, les activités minières, le déchargement des eaux usées, et le salage des routes en hiver, pourraient aussi augmenter la variabilité temporelle de la salinité (Moyano Salcedo et al. 2022).

L'augmentation de la salinité résulte ainsi de plusieurs causes (voir Figure 2), dont certaines interdépendantes et qui sont parfois synergiques (Williams 2001). Ces variations de la salinité ont de multiples conséquences, et les interrelations complexes entre les variations des ions de sel et les conséquences chimiques, biologiques et géologiques sur l'environnement naturel, social et le patrimoine bâti ont été appelées syndrome de salinisation de l'eau douce (Freshwater Salinization Syndrome ; FSS ; Kaushal et al. 2021). Si de multiples zones humides telles que des rivières, des lacs et des mares sont touchées par la salinisation des milieux (Hart

et al. 2003; Herbert et al. 2015; Cunillera-Montcusí et al. 2022), c'est particulièrement le cas des zones humides côtières.

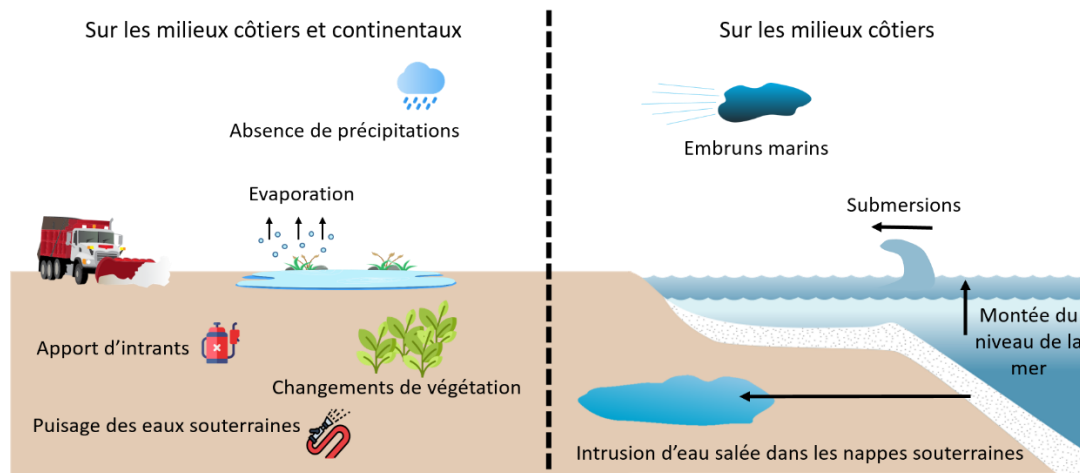


Figure 2 : Diverses causes, directes ou indirectes, pouvant conduire à la salinisation des milieux naturels.

II.4. Vulnérabilité des zones côtières

II.4.1. La biodiversité des milieux côtiers

A l'interface entre le milieu terrestre et le milieu océanique, les milieux côtiers sont de grands réservoirs de biodiversité. Ils abritent une grande diversité d'habitats et d'espèces et sont souvent représentés par une mosaïque de biotopes aux conditions environnementales spécifiques (Janas et al. 2019; Hobohm et al. 2021). Ils remplissent de nombreux services, non seulement pour l'Homme mais pour l'ensemble de l'écosystème (Maynard and Wilcox 1997; Garbutt et al. 2017; Janas et al. 2019; Pétilion et al. 2023). Ils servent par exemple un rôle de séquestration du carbone, de protection du milieu terrestre (Barbier et al. 2008; Nelson and Zavaleta 2012; De Battisti 2021; Dupuy et al. 2022), mais également un rôle dans les flux d'oxygènes, de nutriments et de soluté, et dans la production primaire (Janas et al. 2019).

II.4.2. Les milieux côtiers et les changements globaux

Mais se situer à l'interface entre deux milieux présente aussi des risques, et les milieux côtiers sont impactés par une grande variété de changements globaux en interaction (Maynard and Wilcox 1997; McLean et al. 2001; Barua et al. 2021; Martínez-Megías and Rico 2021). Il en résulte que près de 36% des espèces habitant ces écosystèmes sont aujourd'hui menacées d'extinction (Perennou et al. 2020). Ces écosystèmes sont en effet de plus en plus touchés par le retrait des

zones littorales et leur artificialisation (Bird 1985), l'expansion des zones urbaines et de l'agriculture (McLean et al. 2001; Newton et al. 2018; Martínez-Megías and Rico 2021), par une utilisation accrue des pesticides (Ccanccapa et al. 2016), la prolifération des espèces envahissantes (Taylor et al. 2021; Martínez-Megías and Rico 2021), l'augmentation des températures (Martínez-Megías and Rico 2021), mais également par l'accumulation de barrages et de réservoirs et la pollution plastique (Taylor et al. 2021). Par ailleurs, si les milieux côtiers peuvent subir des variations des niveaux de sel dû à des processus naturels, tels que le dépôt de sel via les embruns marins (Benassai et al. 2005), ils sont également sévèrement touchés par la salinisation du fait des activités anthropiques et des changements globaux (Nicholls et al. 1999; McLean et al. 2001; Herbert et al. 2015).

Contrairement aux organismes marins (ou amphihalins et euryhalins) qui vivent dans des environnements à salinité fluctuantes et ont développé des mécanismes particuliers pour tolérer ces fluctuations (Towle et al. 2001; Rivera-Ingraham and Lignot 2017; Theuerkauff et al. 2018) tels que la régulation de la perméabilité des membranes à l'eau, le remodelage des cellules, la production d'une urine hyperosmotique et l'augmentation de l'expression et de l'activité des transporteurs d'ions (Evans 2009; Kültz 2015; Rivera-Ingraham and Lignot 2017; Grosell and Oehlert 2023), les organismes d'eau douce qui vivent sur la partie terrestre des milieux côtiers sont également soumis à ces contraintes mais possèdent des capacités de tolérance au sel nettement moindres. Les organismes marins et les organismes d'eau douce sont ainsi séparés par une limite biogéographique (de ~ 150 mOsm/kg), à travers laquelle la plupart des espèces sont physiologiquement incapables de pénétrer (Remane and Schlieper 1972). Et on retrouve ainsi, chez les organismes d'eau douce, de nombreuses conséquences à habiter un milieu trop salé.

III) Conséquences de la salinisation des milieux pour la biodiversité des milieux d'eau douce

III.1. Au niveau individuel

Tous les organismes ont besoin de maintenir un équilibre osmotique entre la concentration en sel dans l'eau et la concentration en sel dans leurs fluides internes et leurs cellules (Hellebusi 1976; Bradley 2009). Dans un milieu trop salé, les individus vont ainsi osmoréguler (Lillywhite and Evans 2021), et réguler les flux hydriques et ioniques afin de maintenir l'homéostasie en

fonction des gradients osmotiques présents dans l'environnement (Schultz and McCormick 2012; Evans and Kültz 2020). Les organismes peuvent par exemple perdre ou gagner de l'eau et des ions salins via différentes structures telles que la membrane des cellules, les capillaires sanguins, la peau, le système respiratoire, les poumons, l'intestin, la vessie, les glandes à sel ou encore les reins (Bentley 2002). Certaines espèces ont même développé des glandes à sel, c'est par exemple le cas des oiseaux (Gutiérrez 2014), [même si tous les oiseaux n'ont pas de glandes à sel, tels que les passereaux (Peña-Villalobos et al. 2013)] et certains reptiles (Shoemaker and Nagy 1984), ces structures permettant aux organismes de contrôler le taux de sodium et de l'éliminer via une solution concentrée.

Que les espèces d'eau douce possèdent ou ne possèdent pas des structures capables d'excréter les ions salins en excès, elles sont généralement intolérantes à une dose élevée de sel, et la salinité du milieu devient toxique passée un niveau et une durée significative (Kumar and Afaq 2022), et peut par exemple provoquer du stress oxydant (Bal et al. 2021, 2022) et des dommages cellulaires (Koleva et al. 2017). Vivre dans un milieu trop salé induit aussi des coûts métaboliques et énergétiques liés à l'osmorégulation (Peña-Villalobos et al. 2013; Rivera-Ingraham and Lignot 2017), entraînant une remobilisation des protéines et des lipides (Wang et al. 2022), ce qui limite l'énergie allouée à de nombreuses fonctions essentielles (Herbert et al. 2015). C'est notamment le cas de la respiration (Kidder III et al. 2006), de la croissance, la fécondité, ou encore l'acquisition d'énergie (Pinder et al. 2005; Cañedo-Argüelles et al. 2013; Herbert et al. 2015; Alkhamis et al. 2022), affectant particulièrement les stades précoces de développement (e.g. les larves et les juvéniles), qui sont les plus sensibles (Sokolova 2013; Walker et al. 2023). Une exposition prolongée au sel peut ainsi conduire, à terme, à l'apparition de malformations (Hieu et al. 2021), mais aussi à de la mortalité (Cañedo-Argüelles et al. 2013).

Certaines caractéristiques, notamment morphologiques, pourraient permettre aux individus de s'ajuster à ces contraintes. Par exemple les individus les plus grands pourraient avoir une tolérance plus élevée grâce à un ratio surface/volume plus petit comparé aux individus plus petits (Bentley 2002), limitant les échanges avec le milieu extérieur. Les individus en meilleure condition ont également un avantage face à l'augmentation de la salinité parce que la lipolyse (dégradation des lipides) leur permet de fournir plus d'énergie (Luo et al. 2022). La tolérance des individus à la salinité passe aussi par nombre d'adaptations comportementales et physiologiques. Dans un milieu salé, les individus peuvent boire et uriner à des taux différents (et ce afin d'absorber moins et/ou d'excréter plus de sel), mais également retenir leur urée afin de conserver un milieu interne dont la pression osmotique est

plus élevée par rapport à leur environnement extérieur (Hazon and Flik 2002). L'osmorégulation fait également intervenir la synthèse d'osmolytes (Yancey et al. 1982; Willett and Burton 2002), pour maintenir une pression osmotique constante à travers la membrane cellulaire, ce qui permet aux individus de compenser les changements de salinité intervenant à l'extérieur (Seale and Breves 2022). Chez les vertébrés, le contrôle de l'osmorégulation passe aussi par des mineralocorticoïdes, par la prolactine, et par des hormones de croissance analogues à l'insuline I, qui contrôlent la synthèse de nouvelles protéines de transport, la prolifération et de la différenciation cellulaire (McCormick and Bradshaw 2006).

Au-delà de ces adaptations, un des mécanismes le plus communs pour échapper à la salinité est de s'y soustraire par l'évitement (Cañedo-Argüelles et al. 2013). Les changements de salinité vont ainsi affecter la structure des communautés.

III.2. Au niveau des communautés et du réseau trophique

La salinité est parmi les facteurs les plus importants définissant les limites biogéographiques dans les habitats aquatiques (Lee et al. 2022). Premièrement, la tolérance à la salinité varie en fonction des espèces (Herbert et al. 2015; Tadić et al. 2021; Arnott et al. 2023), et la salinisation peut ainsi conduire à un remplacement continu des taxons intolérants au sel par ceux qui peuvent supporter des concentrations en sel élevées (Radke et al. 2003). Les communautés des zones humides pourraient ainsi subir un remplacement vers des communautés uniquement composées des espèces les plus tolérantes à la salinité (Herbert et al. 2015), et ce soit en provoquant la mortalité directe des individus et des espèces les plus sensibles (Cañedo-Argüelles et al. 2013) soit en provoquant un changement dans la distribution de ces espèces (Gunter 1956). A terme, la salinisation peut donc altérer les interactions interspécifiques (Herbert et al. 2015) et la structure des communautés (Hart et al. 2003).

L'augmentation de la salinité va ainsi considérablement diminuer l'abondance, la richesse spécifique et la diversité (Pinder et al. 2005; Finlayson et al. 2013; Anufrieva and Shadrin 2018; Cunillera-Montcusí et al. 2022; Hébert et al. 2022; González-Sansón et al. 2022; Kendall et al. 2022; Ersoy et al. 2022), ainsi que la richesse génétique (Johannesson and Andre 2006), et ce à tous les niveaux trophiques (Hintz and Relyea 2019). Mais les effets de la salinité peuvent aussi être indirects, affectant en impactant par exemple les ressources disponibles pour les consommateurs (Finlayson et al. 2013). Tous ces impacts devraient particulièrement affecter les milieux côtiers, qui sont parmi les plus touchés par la salinisation.

III.3. Impact particulier pour les organismes sur les milieux côtiers

Les environnements proches des rivages, tels que les étangs et mares côtières, les estuaires et les zones intertidales, sont parmi les milieux aquatiques les plus exigeants sur le plan physiologique (Blewett et al. 2022). Les animaux habitant ces environnements connaissent des conditions qui fluctuent fortement sur des échelles temporelles et spatiales relativement courtes, et notamment en terme de salinité (Castillo and León 2021; Ghalambor et al. 2021). Vivre dans ces habitats nécessite de pouvoir tolérer les perturbations physiologiques induites par ces fluctuations environnementales. Les espèces vivant sur les milieux côtiers devraient être relativement tolérantes à la salinisation parce que, étant constamment exposées à une certaine concentration en sel, elles pourraient être localement adaptées à la salinité (Long et al. 2021). Mais les espèces côtières sont tout de même fortement impactées par l'augmentation de la salinité (McLean et al. 2001; Amores et al. 2013), et les intrusions d'eau marine peuvent fortement transformer ces écosystèmes (Visschers et al. 2022). De nombreuses espèces endémiques de ces écosystèmes sont euryhalines (et peuvent donc supporter de larges variations de salinité), cependant la littérature disponible montre que l'augmentation brutale de la salinité entraîne une perte de biodiversité et modifie la structure des communautés (Herbert et al. 2015). Par ailleurs, les effets de la salinisation montrent souvent leurs plus grands impacts aux stades juvéniles des individus (Herbert et al. 2015), affectant ainsi le recrutement des populations et réduisant leur avantage concurrentiel vis-à-vis des taxons halophiles (Martínez-Megías and Rico 2021). Et au sein de ces écosystèmes côtiers particulièrement vulnérables, il existe des espèces qui elles même sont elles aussi particulièrement sensibles : c'est le cas des amphibiens.

IV) Les amphibiens, un groupe particulièrement sensible à l'augmentation de la salinité

IV.1. Intérêt des amphibiens, vulnérabilité et menaces qui pèsent sur les amphibiens

Les amphibiens, appelés ainsi du fait de leur cycle de vie biphasique (du grec *amphi* « double » et *bios* « vie » : « qui vit dans deux éléments », Figure 3), jouent un rôle primordial au sein de leurs deux écosystèmes de vie. Les têtards premièrement représentent une biomasse considérable, et ils jouent de plus un rôle primordial en transformant la matière organique,

mais également en régulant le périphyton et le phytoplancton (Rowland et al. 2017). Chez de nombreux amphibiens, l'habitat de croissance des embryons et des larves est nettement différent des habitats des juvéniles et des adultes (Wells, 2007, Figure 3) et, de par ce changement d'habitat, les amphibiens participent à un transfert de nutriments pendant la métamorphose, du milieu aquatique vers le milieu terrestre (Bashinskiy et al. 2023).

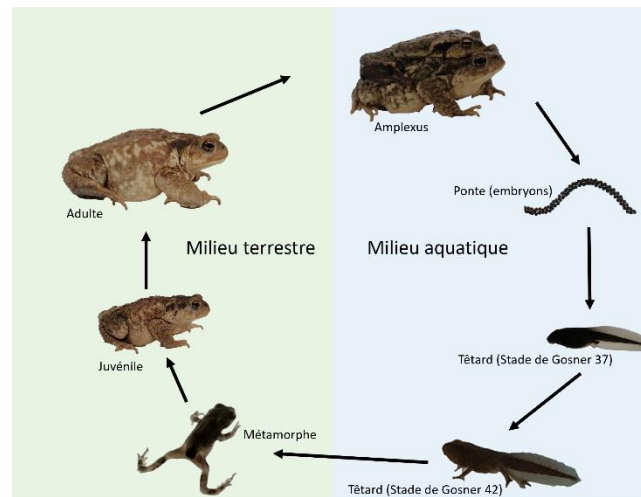


Figure 3 : Illustration du cycle de vie des anoures (photos représentant le crapaud épineux, *Bufo spinosus*), et leur mode de vie à la fois terrestre et aquatique. Stades de vie selon Gosner (1960)

Pourtant, de nombreuses menaces pèsent sur les amphibiens (Beebee and Griffiths 2005; McCallum 2007; Blaustein et al. 2011), ce groupe étant considéré comme le plus menacé parmi les vertébrés (Stuart et al. 2004; Barnosky et al. 2011; Ceballos et al. 2017). De fait, que ce soit au niveau Européen ou au niveau mondial, plus d'un quart des amphibiens sont menacés, et plus de la moitié est en déclin (Houlahan et al. 2000; Stuart et al. 2004; Temple et al. 2009). Ce déclin est estimé à 211 fois plus élevé que les taux d'extinction historiques (McCallum 2007). Il est particulièrement inquiétant du fait que les populations d'amphibiens déclinent même dans les lieux en apparence bonne santé (Denoël et al. 2019). Déjà menacés par de nombreux facteurs, tels que la contamination environnementale (Blaustein et al. 2011), l'augmentation de la température (Zhu et al. 2022), l'invasion par les espèces envahissantes (Bucciarelli et al. 2014), la fragmentation des milieux (Cushman 2006), ou encore la présence de parasites ou de maladies (Blaustein et al. 2011), ils sont particulièrement sensibles à l'augmentation de la salinité.

IV.2. La sensibilité des amphibiens

Cette sensibilité accrue s'explique par le fait que le cycle de vie complexe des amphibiens (à la fois terrestre mais aussi aquatique), ainsi que leurs faibles capacités de dispersion (Wells 2007) les exposent aux contraintes de ces deux milieux (López-Alcaide and Macip-Ríos 2011). Par ailleurs, les œufs et les larves d'amphibiens sont constamment inféodés aux milieux aquatiques et ce jusqu'à leur métamorphose (Wake and Koo 2018). Les amphibiens, à tous leurs stades de vie, possèdent également une peau très vascularisée et hautement perméable, servant de surface pour la respiration et l'osmorégulation (Shoemaker and Nagy 1984; Hillyard 1999; Bentley 2002; López-Alcaide and Macip-Ríos 2011; Wake and Koo 2018), dont les capacités chemosensorielles sont similaires à celles des cellules gustatives sur la langue des mammifères (Hillyard et al. 2007). Cette surface perméable les rend hautement sensibles à la perte d'eau par évaporation et à l'absorption de sel (Martin and Nagy 1997; Hillyard 1999; Hillyard et al. 2007), et leur confère de faibles capacités d'osmorégulation (Katz 1989). Ainsi, un environnement dont la salinité est supérieure à 25‰ la concentration en sel de l'eau de mer (entre 8 et 9 g.l⁻¹ de sel) représente une contrainte majeure pour un amphibien (Balinsky 1981). Pour lutter contre ces contraintes, de nombreux mécanismes sont mis en place.

IV.3. Mécanismes mis en place

IV.3.1. Mécanismes morphologiques

Si certaines espèces de vertébrés ont développé des glandes à sel (Shoemaker and Nagy 1984; Gutiérrez 2014), ce n'est pas le cas des amphibiens (Shoemaker and Nagy 1977). Les amphibiens sont toutefois les premiers organismes à avoir développé un néphron distal pour faire face aux conditions négatives d'un milieu terrestre dépourvu d'eau (Uchiyama and Yoshizawa 1992; Hazon and Flik 2002) qui pourrait aussi leur permettre de faire face à un environnement trop salé et donc déshydratant.

D'autres mécanismes existent (voir Figure 4) telles que des aquaporines présentes dans la vessie et les reins des amphibiens et dont le rôle est de faciliter les échanges d'eau (Ogushi et al. 2007), mais également, chez les têtards, des éléments liés à la structure des branchies internes (Uchiyama and Yoshizawa 1992). L'épaisseur de la peau des amphibiens peut également varier afin de limiter la perméabilité et donc les échanges avec le milieu extérieur (Gordon et al. 1961; Balinsky 1981). Par ailleurs, les individus plus grands sont moins sensibles, car ils ont un rapport surface/volume plus petit, ce qui diminue la perte relative d'eau

(Balinsky 1981). Mais au-delà des mécanismes morphologiques, les réponses physiologiques sont les plus courantes. Plusieurs mécanismes sont ainsi mis en place.

IV.3.2. Mécanismes physiologiques

On dénote premièrement des mécanismes visant à maintenir l'osmolalité interne élevée (plus élevée que celle de l'environnement extérieur) afin de limiter la perte d'eau. Cela met en jeu une augmentation de l'activité du cycle de l'ornithine, permettant une synthèse accrue de l'urée dans le sang et donc l'augmentation de sa concentration dans l'organisme, ainsi qu'une augmentation de la concentration en sodium et chlorure, permettant de maintenir l'équilibre osmotique, et donc de réduire le stress osmotique ainsi que la déshydratation (Balinsky et al. 1961; Gordon 1965; McBean and Goldstein 1967; Balinsky 1981; Liggins and Grigg 1985; Katz 1989; Hillyard 1999; Hidalgo et al. 2020; Álvarez-Vergara et al. 2022). Ce phénomène est notamment retrouvé chez des espèces particulièrement tolérantes telles que le Xénope lisse (*Xenopus laevis*). En effet, après exposition à une solution hypersaline pendant plusieurs semaines, les Xénopes multiplient par plus de 15 leur taux d'urée sanguine et la concentration plasmatique du sodium, les individus multipliant par 2 l'activité du cycle de l'ornithine (McBean and Goldstein 1967, 1970).

Ces mécanismes sont associés au besoin de conserver de l'eau dans l'organisme. En fait, une certaine tolérance à la déshydratation, associée à la capacité de stocker de grands volumes d'eau dans la vessie, permet vraisemblablement aux amphibiens d'exploiter les environnements terrestres où l'efflux d'eau par évaporation dépasse l'afflux provenant de l'alimentation et de la boisson (Shoemaker and Nagy 1977; Hazon and Flik 2002). Chez les têtards, ce processus est encore plus développé, et passe par une diminution du débit urinaire, accompagnée d'une baisse du transport de sodium cutané, et la libération d'hormones hypophysaires postérieures (Balinsky 1981). Cela va notamment permettre la rétention de l'urine (Balinsky et al. 1961). Ce mécanisme est très développé chez les têtards (Wu et al. 2014) et, pour les espèces terrestres, durant leur phase de vie adulte (Hillyard 1999). Mais ce mécanisme va dépendre de la salinité du milieu à laquelle sont acclimatés les individus. Par exemple, plus la préférence d'habitat d'un amphibien est vers l'eau douce, plus la concentration de sodium nécessaire pour saturer son système d'absorption de sodium est faible (Greenwald 1972).

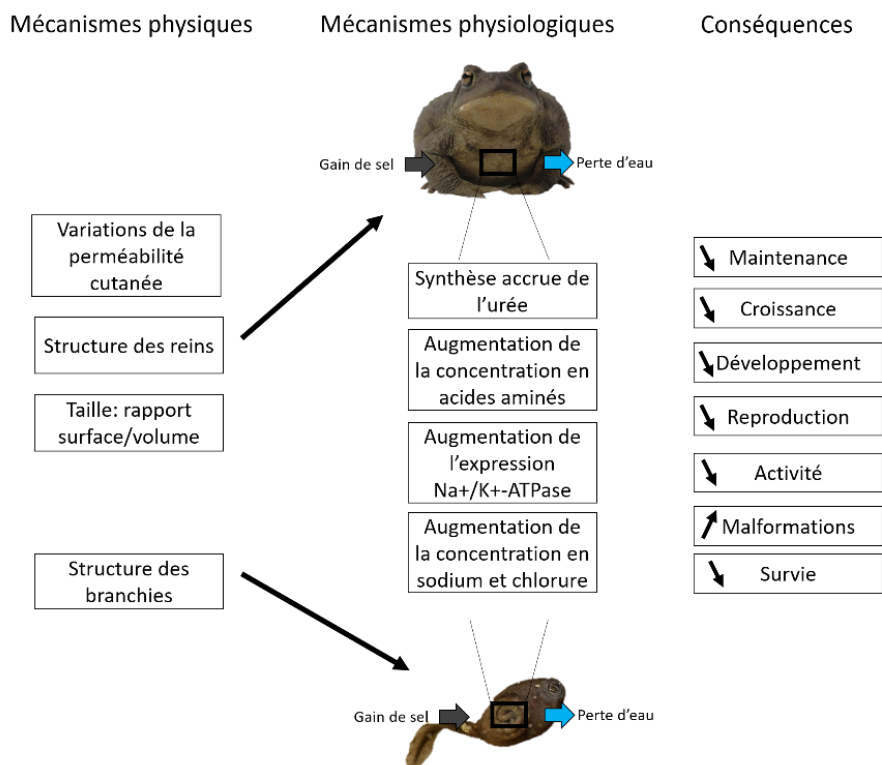


Figure 4 : Schéma illustrant les mécanismes physiques et physiologiques mis en place pour faire face aux milieux salés, et les conséquences de l'exposition à la salinité chez les amphibiens aux stades adultes et larvaires, illustré par des individus de *Bufo spinosus*.

Les amphibiens, confrontés à un milieu trop salé, peuvent aussi avoir recourt à une augmentation de la concentration en acides aminés (Gordon 1965), ou à une augmentation de l'expression Na⁺/K⁺-ATPase (Hillyard 1999; Bernabò et al. 2013; Wu et al. 2014) qui permet elle aussi aux individus de maintenir la concentration de leurs fluides internes supérieur à celle du milieu extérieur (Evans 2009; Henry et al. 2012).

L'ensemble de ces mécanismes physiologiques sont régis par l'expression d'hormones osmorégulatrices telles que la corticostérone et l'aldostérone (Tornabene et al. 2022b), mais également l'angiotensine II (responsable de la rétention en eau, de l'augmentation de l'absorption dans les reins, et de l'augmentation de la sécrétion d'aldostérone ; McCormick & Bradshaw, 2006), la vasotocine (favorisant la tonicité des vaisseaux) et l'hydrine (qui a des fonctions utérotoniques et antidiurétiques ; Hazon & Flik, 2002). Les différents mécanismes physiologiques cités précédemment peuvent s'exprimer simultanément ou non, et peuvent également évoluer dans le temps, notamment en fonction des stades de développement (Balinsky 1981; Hopkins and Brodie 2015). Chez les adultes, la synthèse et le stockage accru de l'urée vont être favorisés, alors que chez les larves, la diminution du transport de sodium

va être favorisée (Balinsky 1981), ce qui explique probablement les différences de tolérances retrouvées au sein du groupe des amphibiens (Figure 5).

IV.4. Différences de tolérance au sein du groupe des amphibiens

IV.4.1. Entre espèces

Premièrement, les différences de tolérance à la salinité s'expriment entre espèces (Brown and Walls 2013; Hua and Pierce 2013; Hopkins and Brodie 2015) (Figure 5). Certaines espèces tolérantes sont bien connues et décrites, telles que *Fejervarya cancrivora* (Darwin 1859; Gordon et al. 1961; Dunson 1977) et *Lithobates berlandieri* (McCoid 2005) qui tolèrent des taux de salinité atteignant jusqu'à 39 g.l⁻¹, mais aussi *Rhinella marina* (Liggins and Grigg 1985), *Bufo viridis* (Gordon 1962) et *Xenopus laevis* (Hewitt and Power 1913; Munsey 1972), qui peuvent tous tolérer des concentrations en sel avoisinant 15 g.l⁻¹. Si cette tolérance n'a été décrite que chez certaines espèces, elle n'est pourtant pas aussi rare que ce qui a été longtemps été admis, et elle existe en réalité chez de nombreuses autres espèces. En effet 144 espèces (provenant de 65 genres et 28 familles) peuvent utiliser des environnement salés (Hopkins and Brodie 2015). On remarque même des tolérances différentes au sein d'espèces du même groupe, par exemple le groupe des Bufonidés où toutes les espèces présentent des tolérances au sel différentes (Bernabò et al. 2013). Et malgré le fait que les amphibiens soient osmotiquement sensibles, beaucoup sont certainement capables de persister dans des habitats salins (Hopkins and Brodie 2015), la tolérance au sel pouvant ainsi varier également en fonction des adaptations locales, et notamment au sein des populations d'une même espèce.

IV.4.2. Entre les populations d'une même espèce

Il existe en effet des réponses différentes au sein d'une même espèce en fonction des populations, suggérant des mécanismes d'adaptation locale (Figure 5). Ces adaptations existent notamment en fonction de la proximité des populations à l'océan, les individus vivant à proximité de l'océan ou étant habitué à des milieux plus salés étant moins perturbés par le stress provoqué par une augmentation de la salinité, que les populations des milieux d'eau douce (Licht et al. 1975; Gomez-Mestre and Tejedo 2003; Hopkins et al. 2016, 2017). Les espèces vivant près de la côte pourraient ainsi être plus résilientes à une augmentation de la salinité (Gunzburger et al. 2010), avec une moindre mortalité et moins d'anomalies de développement (Gomez-Mestre and Tejedo 2003), mais aussi une communauté bactérienne de leur peau

adaptée à la salinité (Gabriel et al. 2021). Ces différences de tolérance entre les populations pourraient notamment s'exprimer du fait d'une expression génique différente (Albecker et al. 2021).

Au-delà de l'adaptation locale, Wu et al. (2014) ont mis en évidence le rôle très important de l'acclimatation, parce que les individus exposés directement à des milieux trop salés ne survivent pas, au contraire de ceux qui sont au préalable exposés à des salinités intermédiaires. L'acclimatation rendrait ainsi les individus plus tolérants (Liggins and Grigg 1985; Bernabò et al. 2013; Wu et al. 2014), certaines espèces telles que *Rhinella marina*, ayant des capacités d'acclimatation remarquables (Kosmala et al. 2020). Mais malgré les formidables capacités d'acclimatation et d'adaptation des amphibiens, les larves et œufs des amphibiens, quelle que soit la population, demeurent très sensibles à l'augmentation de la salinité.

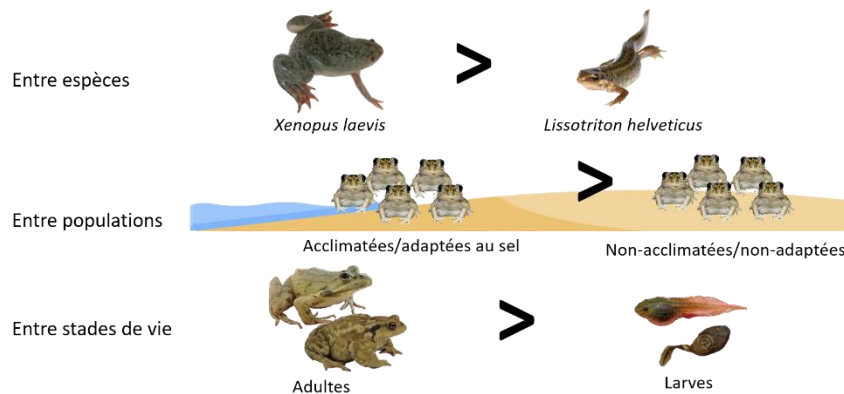


Figure 5 : Illustration des différences de tolérances à la salinité attendues au sein du groupe des amphibiens.

IV.4.3. Entre les stades de vie

En effet, au sein d'une même espèce, certains stades sont plus sensibles. C'est le cas des œufs dont la membrane est très perméable et qui peuvent donc absorber de l'eau et des solutés depuis leur environnement (Venturino et al. 2003), mais aussi des larves, car les œufs et les larves sont tous deux très dépendants du milieu aquatique (Wells 2007; Wake and Koo 2018). Ainsi, les adultes seraient moins sensibles que les stades juvéniles, le développement larvaire étant excessivement difficile dans les milieux salés. Chez les anoures, il a par exemple été montré qu'à 9 g.l⁻¹, on peut s'attendre à ce que 21% des œufs, 27% des larves et 50% des adultes survivent (Albecker and McCoy 2017). Une concentration de sel de 4.15 g.l⁻¹ va provoquer 50% de mortalité chez les œufs d'anoures, alors que 5.5 g.l⁻¹ sont nécessaires pour provoquer la

mortalité de 50 % des larves, et 9.0 g.l⁻¹ pour provoquer la mortalité de 50 % des adultes (Albecker and McCoy 2017). Les effets de la salinité changent au cours du développement, et pourraient par exemple n'apparaître que pendant le stade juvénile (Dahrouge and Rittenhouse 2022), ou disparaître au cours du développement, parce que les individus deviennent de moins en moins sensibles à mesure que les branchies s'internalisent (Uchiyama and Yoshizawa 1992). Quel que soit le stade de vie, les amphibiens vont en tout cas faire face à de nombreuses contraintes dans les milieux salés (Figure 4).

V) Conséquences de l'exposition à la salinité chez les amphibiens

V.1. Impacts au niveau de la reproduction et du développement larvaire

Premièrement, une osmolalité élevée dans le milieu diminue la motilité et la vitesse du sperme (Wilder and Welch 2014; Byrne et al. 2015). Les populations des milieux côtiers et des milieux continentaux présentent également des différences de succès reproducteur. Par exemple, les individus sur les milieux côtiers ont une fécondité réduite et pondent des œufs plus petits par rapport aux individus issus de milieux continentaux, un pattern qui n'a pas été attribué à l'augmentation en sel mais au substrat déshydratant des milieux côtiers (Marangoni et al. 2008). Par ailleurs, il a été démontré que la densité des masses d'œufs était plus élevée en eau douce qu'en eau saumâtre (Karraker et al. 2008).

Au-delà de la reproduction, les œufs d'amphibiens exposés à la salinité éclosent plus tôt et présentent une réduction du succès d'éclosion (Haramura 2016; Tornabene et al. 2021a). Les larves d'amphibiens sont également connues pour être sensibles même à des niveaux de salinité modérés (Chinathamby et al. 2006). C'est d'autant plus important que les œufs et les larves sont complètement inféodés au milieu aquatique (Wake and Koo 2018). C'est pourquoi les femelles évitent généralement de pondre dans les milieux trop salés (Haramura 2008; Albecker and McCoy 2017), même si les femelles côtières conservent une probabilité élevée de pondre dans les milieux saumâtres (Albecker and McCoy 2017). Et pour les femelles qui pondent leurs œufs dans ces milieux saumâtres, les effets de la salinité continuent pendant l'ensemble du développement larvaire.

V.2. Impacts particuliers chez les larves

V.2.1. Effets sur la survie et la croissance

Premièrement, chez les têtards, la salinité peut provoquer des diminution de la synthèse des hormones thyroïdiennes (Gomez-Mestre et al. 2004), et des changements dans les hormones osmorégulatrices telles que la corticostérone et l'aldostérone (Tornabene et al. 2021b, 2022b). Ces modifications peuvent provoquer une réduction de la croissance qui apparait pendant le développement larvaire (Christy and Dickman 2002; Wu and Kam 2009; Lukens and Wilcoxon 2020), mais également à la métamorphose (Bernabò et al. 2013). L'augmentation de la salinité impacte négativement la survie en provoquant la mortalité directe des individus (Christy and Dickman 2002; Chinathamby et al. 2006; Sanzo and Hecnar 2006; Karraker et al. 2008; Rios-López 2008; Bernabò et al. 2013; Jones et al. 2015; Albecker and McCoy 2017).

Mais les effets sur la survie peuvent également être indirects, notamment du fait de malformations (Gosner and Black 1957; Karraker 2007; Hopkins et al. 2013a; Brady 2013; Meiler 2016). Ces malformations peuvent constituer en la présence d'œdèmes abdominaux et/ou de malformations axiales, tels que des défauts de flexion dorsale et latérale de la queue (Karraker 2007), ainsi qu'une réduction ou une absence de croissance de certains tissus tels que les poumons, les yeux, les bourgeons des membres, voir même la tête, avec parfois des organes manquants (Hopkins et al. 2013a). Ces effets peuvent être liés soit à la réduction des flux entrant d'eau (Gosner and Black 1957), soit au fait d'une quantité d'énergie insuffisante dans les milieux trop salés, car une forte proportion d'énergie est allouée à l'osmorégulation (Gomez-Mestre et al. 2004). Une étude a également révélé un effet négatif de l'augmentation de la concentration en sel sur l'intégrité des branchies (Bernabò et al. 2013). Les principales modifications des branchies observées sont l'augmentation de la sécrétion muqueuse, le décollement de la couche externe, l'altération de la surface épithéliale, des phénomènes de dégénérescence, l'apparition de corps résiduels et l'immigration de macrophages (Bernabò et al. 2013). Ces altérations morphologiques de l'épithélium branchial peuvent interférer avec la fonction respiratoire et la régulation osmotique (Uchiyama and Yoshizawa 1992; Gomez-Mestre et al. 2004).

V.2.2. Avancement de la métamorphose

Les larves d'amphibiens présentent une grande plasticité dans le timing de la métamorphose (Denver et al. 1998). En fait, si les conditions de croissance sont favorables, la métamorphose devrait être repoussée pour garder l'avantage d'un environnement de croissance optimal

(Relyea 2007). A l'inverse, si les conditions et l'environnement de croissance sont mauvais, la métamorphose devrait être accélérée pour minimiser le temps passé dans ces conditions de croissance défavorables (Wassersug and Sperry 1977; Relyea 2007). Ainsi, dans un milieu trop salé, on peut s'attendre à une métamorphose avancée. Ce type de réponse a été montrée par Denver et al. (1998) en réponse à une diminution du niveau d'eau, et par Sanzo and Hecnar (2006) et Wijethunga et al. (2016) en réponse à une augmentation de la salinité.

V.2.3. Retard de la métamorphose

Mais la métamorphose peut aussi être plus tardive, avec un retard de développement pour les individus exposés à des milieux trop salés (Christy and Dickman 2002; Gomez-Mestre et al. 2004; Chinathamby et al. 2006; Lukens and Wilcoxon 2020). Par exemple, chez *Epidalea calamita*, les larves qui se développent dans des eaux salées atteignent la métamorphose jusqu'à 9 jours plus tard que leurs congénères qui se développent en eau douce (Gomez-Mestre et al. 2004). La métamorphose peut aussi ne pas avoir lieu si la salinité est trop élevée, les têtards ne franchissant pas les périodes clés du développement (Christy and Dickman 2002; Meiler 2016).

Christy and Dickman (2002) ont suggéré que l'allongement de la période larvaire pourrait être liée à une diminution du taux de différenciation des cellules avec la salinité. Ces retards de développement peuvent également être expliqués par une production diminuée d'aldostérone (Lukens and Wilcoxon 2020). Mais ces taux de différenciation des cellules plus faibles vont exposer les individus plus longtemps à cette salinité élevée (Christy and Dickman 2002), mais également au risque de prédation (Lukens and Wilcoxon 2020), et au risque de dessiccation. Cependant, une métamorphose retardée pourrait également être bénéfique, et favoriser une taille corporelle plus élevée, afin de compenser une période de stress environnemental (Chinathamby et al. 2006; Squires et al. 2010; Kearney et al. 2014).

V.2.4. Effets sur le comportement

Les effets que l'on retrouve sur la croissance et le développement pourraient être expliqués par des changements au niveau du comportement des individus. En effet, les larves exposées à des niveaux de salinité élevés expriment une activité et un comportement de recherche alimentaire réduits (Hall et al. 2017; Tornabene et al. 2021a), peu de réponses aux stimuli (Sanzo and Hecnar 2006; Karraker 2007), des performances de nage diminuées (Denoël et al.

2010; Haramura 2016), et des comportements erratiques (Tornabene et al. 2021a). Ces effets sur le comportement peuvent notamment être liés aux malformations des individus, puisque par exemple des malformations de la queue vont provoquer des difficultés de nage, peu de réponses face aux stimuli et une réduction de l'activité (Sanzo and Hecnar 2006; Karraker 2007). Cela pourrait rendre les têtards vulnérables par rapport aux prédateurs (Squires et al. 2008; Kearney et al. 2016) mais également vulnérables à la compétition inter spécifique et limiter leur capacité de recherche alimentaire (Denoël et al. 2010). Si ces effets sont retrouvés pour des expositions longues à la salinité (sur l'ensemble du développement larvaire), une exposition de courte durée (quelques minutes ou quelques heures) va induire un comportement exploratoire plus développé (LoPiccolo 2022), probablement lié à une augmentation du stress liée à une augmentation de la salinité, et parce que les individus cherchent à échapper à ce milieu contraignant.

V.2.5. Susceptibilité aux maladies

Des effets indirects peuvent aussi être observés. Notamment il a été observé sur différentes espèces que l'exposition à une salinité élevée augmentait la susceptibilité des têtards aux infections, aux parasites et aux moisissures et donc augmentait le risque de contracter des maladies (Milotic et al. 2017; Hall et al. 2020; Coughlan et al. 2020). Mais des effets contraires sont également observés. En effet il a été montré chez *Litoria aurea* et *Litoria fallax* (White 2006; Stockwell et al. 2015; Clulow et al. 2018; Callen et al. 2023), mais aussi chez *Epidalea calamita* (Bramwell 2011), que l'augmentation de la salinité provoquait une diminution de la prévalence du champignon chytride (*Batrachochytrium*), une diminution de la transmission des maladies aux congénères, et une diminution de la sensibilité à ce champignon, pour des salinités qui restent dans les limites physiologiques de ce que peut tolérer l'animal (entre 0.5 à 4.5 g.l⁻¹ en moyenne). Le champignon chytride préférerait en effet les environnements au potentiel osmotique faible (Gleason et al. 2006).

De nombreuses études suggèrent ainsi que les salinités intermédiaires pourraient être bénéfiques alors que les salinités les plus basses pourraient être préjudiciables (Robles Mendoza et al. 2009; Meiler 2016; Ahumada-García et al. 2018). Chez d'autres organismes aquatiques, des augmentations modérées de la salinité ont été utilisées pour contrôler et prévenir les infections à *Saprolegnia* (Martinez-Palacios et al. 2004), et il a été démontré que ces augmentations permettaient d'améliorer la croissance et d'éviter les parasites (Barany et al. 2021).

V.3. Impacts particuliers chez les adultes

A ce jour, la majorité des études concernant l'impact de la salinisation sur les amphibiens ont été conduites sur les larves, mais peu d'études ont été conduites sur les stades adultes (Walker et al. 2023). Par exemple, dans leur revue de la littérature publiée en 2015, Hopkins et Brodie font référence à 32 études concernant les œufs et les larves, 16 concernant les adultes, et seulement 1 concernant les juvéniles, pour décrire la tolérance expérimentale des amphibiens à la salinité. La plupart de ces études sont publiées avant les années 2000 et sont celles qui ont conduit à l'identification des mécanismes d'osmorégulation chez les amphibiens. Quelques études plus récentes, comme celle de Daneri et al. (2007), ont montré que les individus adultes exposés à des milieux plus salés pouvaient augmenter leurs battements cardiaques, probablement en réponse à une augmentation de l'énergie nécessaire pour compenser les coûts liés à l'osmorégulation. Chez *Pelophylax nigromaculatus*, une étude conduite sur 40 jours a aussi montré que, dans un traitement salé, les électrolytes augmentaient, la concentration en protéines augmentait, ainsi que la créatinine (qui est un indicateur des fonctions rénales) (Park and Do 2020). Les adultes semblent donc être, tout comme les larves, capables de réagir rapidement à une augmentation de la salinité. Toutefois, les études conduites sur les adultes demeurent rares, et tout particulièrement concernant une exposition chronique à la salinité (Walker et al. 2023).

Les adultes étant plus mobiles que les stades larvaires, les amphibiens adultes pourraient surtout être capables de se soustraire à la salinité par l'évitement. On s'attend donc à ce que l'exposition à la salinité affecte la distribution des amphibiens. La présence des adultes étant déjà limitée par la présence d'eau salée, et les larves et les œufs étant plus sensibles que les adultes (Albecker and McCoy 2017), on s'attend donc à retrouver non seulement moins d'individus dans les milieux les plus salés, mais surtout moins de femelles, et beaucoup moins de stades juvéniles.

On retrouve pourtant la présence d'adultes et de larves sur les milieux côtiers (Hopkins and Brodie 2015). Mais, au vu des variations de salinité sur ces milieux (en conditions naturelles mais aussi liées aux événements climatique extrêmes), et malgré une possible adaptation locale, cette présence ne devrait pas se faire sans impacts sur la reproduction et le développement larvaire, mais également sur les individus adultes eux-mêmes.

VII) Problématique

Si la sensibilité des amphibiens à la salinité est bien connue, elle a majoritairement été déterminée dans le cadre du salage des routes en hiver, sur des communautés qui sont naïves à l'exposition au sel. Cette thèse vise à comprendre le cas particulier des milieux côtiers, avec des communautés qui devraient être acclimatées ou adaptées au sel et qui sont confrontées à la salinisation via différents mécanismes propres à ces milieux. Il est attendu que l'ensemble des processus en jeu impactent à la fois l'écologie, la physiologie, le comportement et le développement des amphibiens, et nous allons aborder ces problématiques au travers de trois axes d'étude, regroupant les effets sur les communautés, les individus adultes, et la reproduction ainsi que le développement embryonnaire et larvaire (Figure 6), et ce en fonction des adaptations locales liées à la population d'origine.

Axe I : Variations de salinité sur les milieux côtiers, et structuration des communautés en fonction de ces variations

- 1) Tendances long terme de la salinité sur les mares des milieux côtiers : Effets du climat, des événements climatiques extrêmes, et du niveau de la mer
- 2) Impact d'un événement climatique extrême (la tempête Xynthia) sur les communautés d'amphibiens
- 3) Distribution des différentes espèces d'amphibiens côtiers en fonction du gradient de salinité (en conditions naturelles)

Axe II : Influence de la salinité sur les adultes

- 1) Influence de la salinité sur la physiologie (osmolalité et immunologie) et le comportement (performance et activité)
- 2) Conséquences d'une exposition chronique à la salinité sur la croissance, le comportement et le métabolisme
- 3) Influence de l'état hydrique sur la sélection de la salinité du milieu

Axe III : Influence de la salinité sur la reproduction, le développement embryonnaire et larvaire

- 1) Influence de la salinité de l'environnement sur la taille et la reproduction : comparaison entre populations côtières et continentales
- 2) Influence d'une exposition chronique à la salinité sur le développement embryonnaire et larvaire
- 3) Influence de la salinité sur la reproduction, et des variations de salinité au cours du développement embryonnaire et larvaire

Les études prédictives concernant les réponses physiologiques des organismes et des populations à la menace que constitue les changements de salinité font sensiblement défaut (Lee et al. 2022), et cette thèse va permettre de mieux comprendre l'effet de la salinité sur les amphibiens au travers de différents niveaux d'intégration (Figure 6) mais également au travers de différentes approches (écologie, physiologie, comportement, développement), ainsi que de comprendre la résilience de ces différents niveaux d'intégration. L'ensemble de ces études pourraient également permettre d'apporter des pistes pour la gestion de ces espèces à fort enjeu patrimonial, en évaluant leur réponse à des niveaux de salinité élevés, mais également les manières d'améliorer leur résilience, et les habitats qui leur seraient favorables.

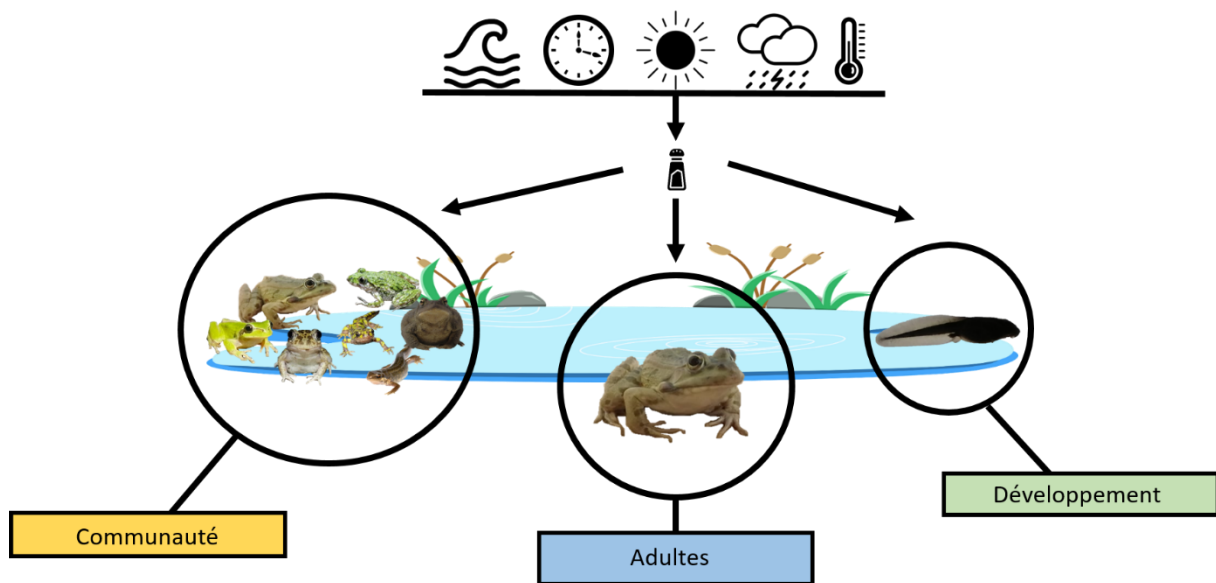


Figure 6 : Schéma illustrant les différents types de variations de la salinité et les différents niveaux d'intégration abordés au cours de cette thèse.

Méthodes



I) Sites d'étude

Au cours de cette thèse, 11 sites ont été prospectés (Figure 7) pour 1) relever les niveaux de salinité et/ou 2) relever la présence, ou capturer différentes espèces d'amphibiens. Ces sites correspondent à des sites côtiers ou continentaux. Les sites côtiers sont caractérisés par des niveaux de sel plus élevés que ceux retrouvés sur les milieux continentaux (voir Chapitre III). Sur ces sites, différents éléments sont prospectés tels que des mares (Figure 8AB) mais aussi des dunes sableuses (Figure 8CD), ou encore des habitats terrestres et des routes. Une partie de ces approches ont été possibles grâce aux suivis sur le long terme mis en place au sein des espaces protégés (réserves naturelles gérées par la LPO).

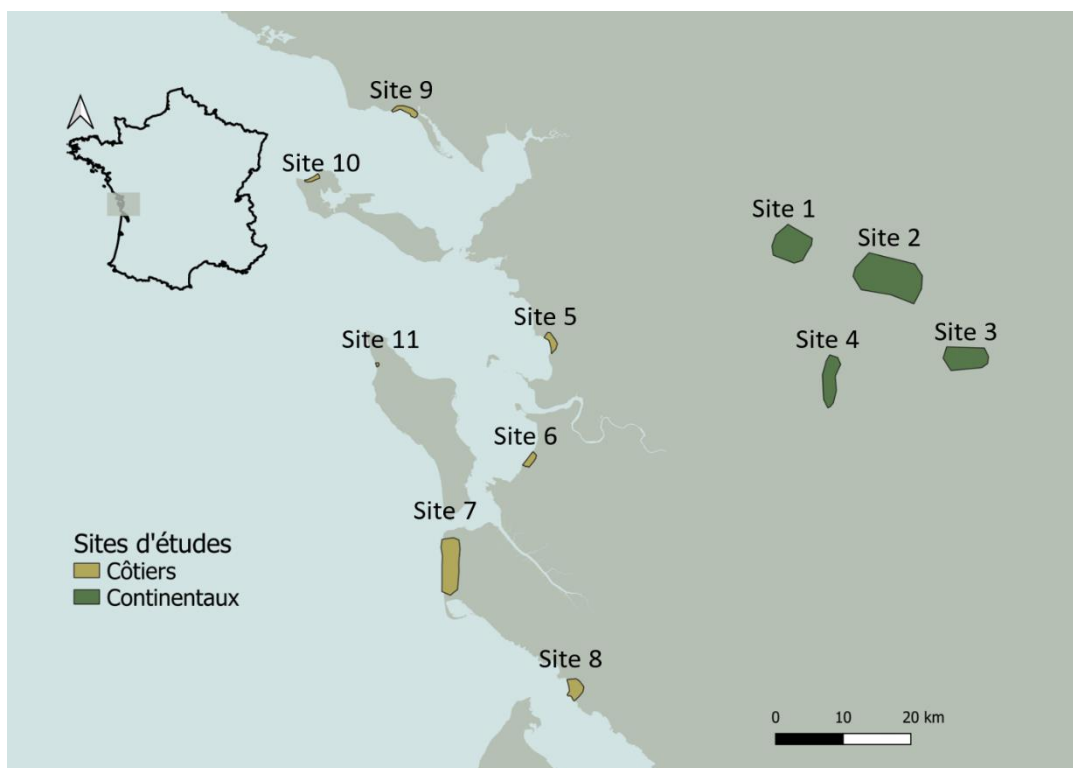


Figure 7 : Sites d'études prospectés durant la thèse. Les sites correspondent à des positions continentales (4 sites : 1 à 4) ou côtières (7 sites : 5 à 11). Les sites 9, 10 et 11 ont été prospectés pendant le travail de thèse, et les résultats publiés (Lorrain-Soligon et al. 2022c, d), mais ces travaux n'ont pas été inclus dans le manuscrit final. Sites 1, 2, 3 et 4 : Forêt de Chizé et alentours, Site 5 : Réserve Naturelle Nationale du Marais d'Yves, Site 6 : Réserve Naturelle Nationale de Moëze-Oléron, Site 7 : Forêt de la Coubre, Site 8 : Forêt de Suzac, Site 9 : Réserve Naturelle Nationale de la casse de la Belle Henriette, Site 10 : Forêt du Lizay (site insulaire), Site 11 : Dune des huttes (site insulaire).

II) Les espèces étudiées

Pour mieux comprendre l'impact de ce processus sur les amphibiens en général, nous avons travaillé non pas sur une espèce, mais sur la diversité d'amphibiens retrouvés sur les milieux côtiers dans l'Ouest de la France (7 espèces). La durée moyenne de vie de ces espèces varie entre 5 à 10 ans.

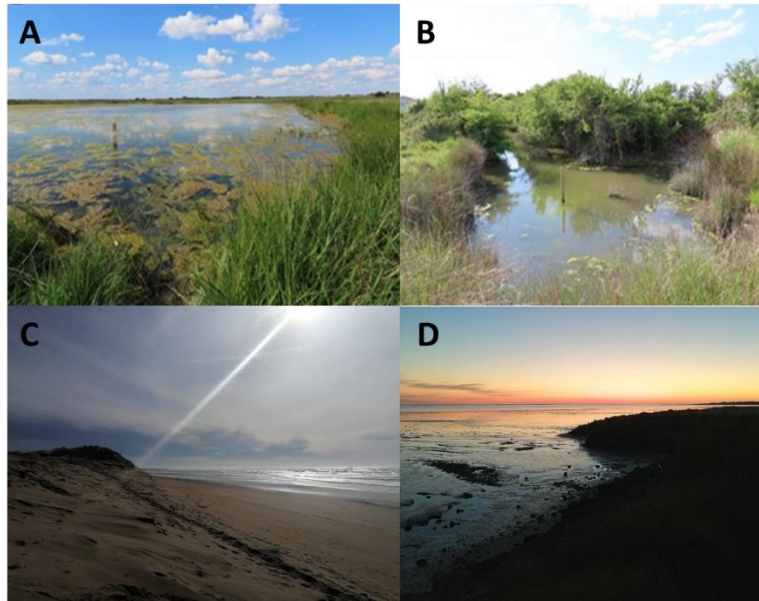


Figure 8 : Illustration de différents types de sites visités. AB : mares (crédit photo : Céline Dufour), CD : milieux côtiers.

II.1. La grenouille verte : *Pelophylax* sp.

Au sein de nos zones d'étude, on retrouve trois espèces d'anoures du genre *Pelophylax* (Figure 9A): la grenouille rieuse (*P. ridibundus* - Pallas, 1771), la grenouille de Perez (*P. perezi* - Seoane, 1885) et la grenouille de Graf (*P. kl. grafi* - Crochet, Dubois, Ohler & Tunner, 1995) (Speybroeck et al. 2018). Ces trois espèces sont regroupées sous la dénomination de *Pelophylax* sp, et appartiennent à la famille des Ranidae. Il s'agit en fait d'un complexe d'espèce différenciables uniquement par analyses génétiques (Dufresnes et al. 2019). L'accouplement a lieu au printemps via un amplexus axillaire, et la fécondation est externe. Ces grenouilles pondent dans la végétation des amas de 4000 à 12000 œufs.

Les grenouilles du genre *Pelophylax* sont communes et largement répandues (Duguet et al. 2003). Elles sont principalement aquatiques et actives autant de jour que de nuit, fréquentant une large gamme d'habitat, où la végétation est abondante et où les plans d'eau

sont ensoleillés. Ces espèces sont connues pour être relativement tolérantes au sel (Sillero and Ribeiro 2010; Natchev et al. 2011; Mollov 2020), des individus adultes de cette espèce ainsi que des têtards et des pontes ayant été retrouvés sur le terrain jusque 10 g.l⁻¹ (Natchev et al. 2011), et dans des conditions extrêmes des adultes et juvéniles ont aussi été observé jusque 28 g.l⁻¹ (Sillero and Ribeiro 2010). La tolérance des adultes a été testée en condition expérimentale jusque 8.8 g.l⁻¹ (Katz 1975), et la tolérance des œufs jusque 1 g.l⁻¹ (Ortiz-Santaliestra et al. 2010). Dans le cadre de cette thèse, cette espèce a été suivie sur le terrain en terme d'abondance, de répartition, et de paramètres populationnels, mais également expérimentalement au laboratoire.

II.2. Le pélobate cultripède : *Pelobates cultripes*

Le pélobate cultripède (Cuvier, 1829, Figure 9B) est un anoure faisant partie de la famille des Pelobatidae. Chez cette espèce la reproduction a lieu à la fin de l'hiver, l'amplexus est lombaire, et la fécondation est externe. Les femelles pondent jusqu'à 3 400 œufs en un long ruban pouvant faire jusqu'à 1 m. Ce ruban s'enroule autour de la végétation. Les juvéniles émergent en été, mais les larves peuvent hiberner et terminer leur métamorphose l'année suivante.

Le pélobate vit dans les pinèdes, les dunes côtières et les terres agricoles. Il est inféodé aux milieux côtiers. Ce crapaud fouisseur se rencontre le plus souvent la nuit, se retirant en journée dans des terriers d'environ 10 cm de profondeur. La reproduction a lieu généralement dans les eaux claires assez profondes à végétation abondante. La reproduction de cette espèce est contrainte par la salinité, et elle a majoritairement lieu dans des habitats où la salinité varie entre 1 g.l⁻¹ et 10 g.l⁻¹ (Thirion 2014). Expérimentalement, la tolérance des têtards a été testée jusque 9 g.l⁻¹ (Burraco and Gomez-Mestre 2016). Dans le cadre de cette thèse, cette espèce a été suivie sur le terrain en terme d'abondance et de répartition.

II.3. La rainette méridionale : *Hyla meridionalis*

La rainette méridionale (Böttger, 1874, Figure 9C) est un anoure de la famille des Hylidae. La reproduction a lieu au printemps. L'amplexus est axillaire et la fécondation est externe. La ponte, pouvant contenir de 800 à 1500 œufs, est déposée en de nombreux petits amas agglutinés, fixés à la végétation aquatique, contenant chacun une trentaine d'œufs.

Cette espèce est commune, et utilise une grande variété de plans d'eau pour se reproduire (prés inondés, mares, fossés ; Duguet et al. 2003; Speybroeck et al. 2018). La tolérance à la salinité de cette espèce n'est pas connue, mais elle est connue chez *Hyla cinerea*. Chez cette espèce, les adultes et les larves ont été retrouvées sur le terrain jusque 23.4 g.l⁻¹ (Albecker and McCoy 2017), et la tolérance en condition expérimentale s'élève chez les œufs à 4 g.l⁻¹ (Albecker and McCoy 2017), et chez les larves à 10 g.l⁻¹ (Brown and Walls 2013). Dans le cadre de cette thèse, cette espèce a été suivie sur le terrain en terme d'abondance et de répartition.

II.4. Le pélodyte ponctué : *Pelodytes punctatus*

Le pélodyte ponctué (Daudin, 1803, Figure 9D) est un anoure appartenant à la famille des Pelodytidae. L'amplexus est lombaire et la fécondation est interne. A la fin de l'hiver, les femelles pondent jusqu'à 1 600 œufs en plusieurs cordons qu'elles enroulent autour d'une tige ou d'un brin d'herbe.

L'espèce se retrouve souvent près de plans d'eau ensoleillés peu profonds (mares à végétation abondante, fossés, ruisseaux à faible courant) mais affectionne également les milieux arborés. Il s'agit d'une espèce nocturne assez discrète mais agile. L'espèce a été retrouvée dans des mares jusqu'à 9 g.l⁻¹ (Thirion 2014). Dans le cadre de cette thèse, cette espèce a été suivie sur le terrain en terme d'abondance et de répartition.

II.5. Le crapaud épineux : *Bufo spinosus*

Le crapaud épineux (Daudain, 1803, Figure 9E) est le plus grand crapaud retrouvé en Europe de l'Est (Speybroeck et al. 2018), appartenant à la famille des Bufonidae. Récemment élevé au rang d'espèce au même titre que le crapaud commun (*Bufo bufo*) (Recuero et al. 2012), il est retrouvé à l'Ouest de la France. Les adultes se reproduisent à la fin de l'hiver par amplexus axillaire (Speybroeck et al. 2018), et la fécondation est externe. Leurs pontes sont constituées de deux cordons pouvant contenir jusqu'à 10000 œufs (Miaud and Muratet 2018).

Le crapaud commun est très ubiquiste et il n'est pas rare de le retrouver près des habitations et dans les zones urbanisées. Il privilégie toutefois les vastes plans d'eau profonds très ensoleillés. La tolérance au sel du crapaud épineux n'est pas connue mais chez le crapaud

commun, la tolérance expérimentale de ses larves est estimée à 4.8 g.l⁻¹ (Bernabò et al. 2013). Dans le cadre de cette thèse, cette espèce a été suivie sur le terrain en terme d'abondance, de répartition, et de paramètres populationnels, mais également expérimentalement au laboratoire.

II.6. Le triton palmé : *Lissotriton helveticus*

Le triton palmé (Razoumowsky, 1789, Figure 9F) est un urodèle appartenant à la famille des Salamandridae. Pendant la reproduction qui a lieu au début du printemps, les mâles courtisent la femelle via une parade nuptiale, en agitant la pointe de leur queue pour diriger vers la femelle les phéromones de leur cloaque. La fécondation est interne. Les œufs (jusqu'à 500 œufs pour une femelle) sont pondus un à un et enroulés individuellement dans une feuille par les femelles.

C'est une espèce opportuniste qui habite une grande variété de plans d'eau. Les larves de cette espèce ont été retrouvées sur le terrain jusque 21.95 g.l⁻¹ (Spurway 1943). Dans le cadre de cette thèse, cette espèce a été suivie sur le terrain en terme d'abondance et de répartition.

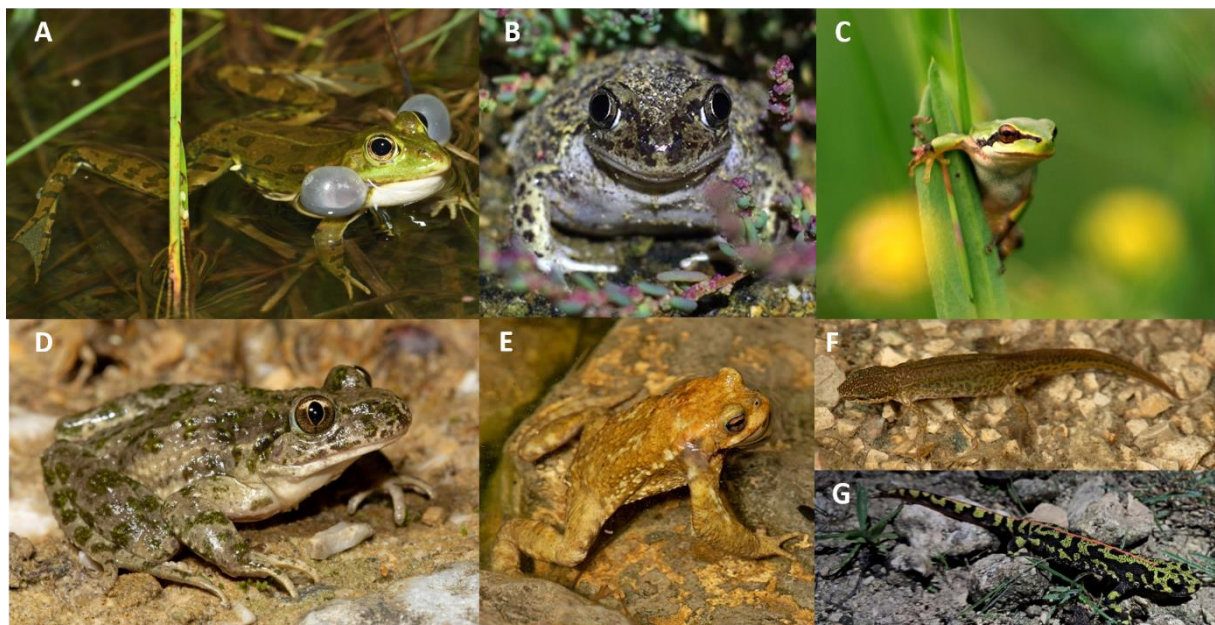


Figure 9 : Photos des différentes espèces étudiées. (A) *Pelophylax* sp. (B) *Pelobates cultripes*, (C) *Hyla meridionalis*, (D) *Pelodytes punctatus*, (E) *Bufo spinosus*, (F) *Lissotriton helveticus*, (G) *Triturus marmoratus*.
Crédits photos : ABCG : Marko Jankovic, DEF : Jean-Pierre Vacher.

II.7. Le triton marbré : *Triturus marmoratus*

Le triton marbré (Latreille, 1800, Figure 9G) est un urodèle appartenant à la famille des Salamandridae. Au début du printemps, les mâles de cette espèce réalisent une parade nuptiale pour séduire les femelles, en arquant le dos tout en se dressant sur leurs pattes et en se penchant vers elles. La fécondation est interne. Les femelles pondent jusqu'à 400 œufs, et les déposent un par un dans des feuilles repliées.

Le triton marbré est opportuniste, mais son habitat de prédilection se constitue généralement de terrains vallonnés montagneux, de prairies et de terres agricoles non loin d'un point d'eau. La période aquatique dure généralement de février à juin. La tolérance au sel du triton marbré n'est pas connue, mais chez des espèces proches, telles que *Triturus dobrogicus*, les juvéniles ont été retrouvés sur le terrain jusque 1.72 g.l^{-1} (Mester et al. 2013). Dans le cadre de cette thèse, cette espèce a été suivie sur le terrain en terme d'abondance et de répartition.

III) Approches utilisées

Pour comprendre les effets complexes de la salinité sur les amphibiens, à différents niveaux d'intégration, différentes approches ont été utilisées, à la fois sur le terrain mais aussi au laboratoire. Dans cette section, chaque paramètre étudié est brièvement introduit, mais les méthodes complètes sont indiquées dans le matériel et méthodes de chaque article.

III.1. Mesures de la salinité

Premièrement, comprendre les effets de la salinité implique de pouvoir connaître les niveaux de salinité présents à la fois dans l'environnement, mais aussi dans nos traitements expérimentaux (dont les niveaux de salinité ont été calqués sur ceux mesurée *in natura*). Tout au long de cette thèse, il a donc été réalisé des mesures de salinité, le plus souvent au conductimètre (YSI Professional Plus). Cette salinité est toujours donnée en g.l^{-1} .

III.2. Ecologie des espèces : distribution sur des milieux à salinité contrasté

Les différentes espèces d'amphibiens sont connues pour présenter des différences de tolérance à la salinité (Hopkins and Brodie 2015), mais la tolérance à la salinité varie aussi entre les stades de vie au sein d'une même espèce (Albecker and McCoy 2017). Ces différences de tolérance devraient influencer la distribution des espèces et des stades de vie, car les individus devraient choisir des milieux correspondant à leur gamme de tolérance, et les femelles devraient choisir des milieux favorables au développement de leurs larves. Pour comprendre si ces tolérances différentielles au sel s'expriment effectivement sur les milieux côtiers, nous avons échantillonné différentes mares (lieux privilégiés de la présence des amphibiens au printemps), ayant une salinité contrastée, afin d'estimer si les espèces se répartissent en fonction de cette contrainte, en conditions naturelles, ou suite à un évènement climatique extrême, sur toutes les espèces décrites précédemment. Voir Chapitre I.

III.3. Physiologie

III.3.1. L'osmolalité : une mesure de base pour comprendre les effets de la salinité

Les variations de salinité ont des impacts considérables sur les individus, en particulier parce que les individus régulent la concentration de leur milieu interne. Dans un milieu hyperosmotique (plus concentré en sel que le milieu intérieur de la cellule), par osmose, des flux d'eau ont lieu depuis l'intérieur vers l'extérieur de l'organisme, et des flux de sel ont lieu depuis l'extérieur vers l'intérieur de la cellule, ce qui peut provoquer la plasmolyse. A l'inverse, dans un milieu hypoosmotique (moins concentré en sel que le milieu intérieur de la cellule), par osmose, des flux d'eau ont lieu depuis l'extérieur vers l'intérieur de la cellule, et des flux de sel ont lieu depuis l'intérieur vers l'extérieur de la cellule, ce qui va provoquer la turgescence. Enfin, dans un milieu isotonique (où la salinité du milieu extérieur est égale à celle du milieu intérieur), les flux d'eau et de sel ont lieu dans les deux sens, on parle d'équilibre osmotique. Ces flux hydriques et ioniques ont été estimés à travers des mesures de l'osmolalité plasmatique, chez *Pelophylax* sp. (Figure 10, Chapitre II), et *Bufo spinosus* (Figure 11, Chapitres II et III).

III.3.2. Perméabilité cutanée

La peau des vertébrés assure une protection physique et régule les échanges entre l'organisme et son environnement, y compris la régulation des flux d'eau et de sel (Lillywhite 2006). Chez les amphibiens, la perte d'eau transcutanée peut être élevée et ils ont tendance à se déshydrater rapidement dans des milieux secs (Burggren and Vitalis 2005; Brischoux et al. 2021a). Pour comprendre si la salinité peut impacter cette perméabilité, selon de protocole de Brischoux et al. (2021), nous avons utilisé un Aquaflux (BIOX), constitué d'une chambre de mesure close sans mouvement d'air, équipée d'un condenseur (à un température maintenue à -7.5 °C environ) permettant de convertir l'eau évaporée en glace, et ainsi de mesurer les pertes d'eau cutanée, chez *Bufo spinosus* (Figure 11, Voir Chapitre II).

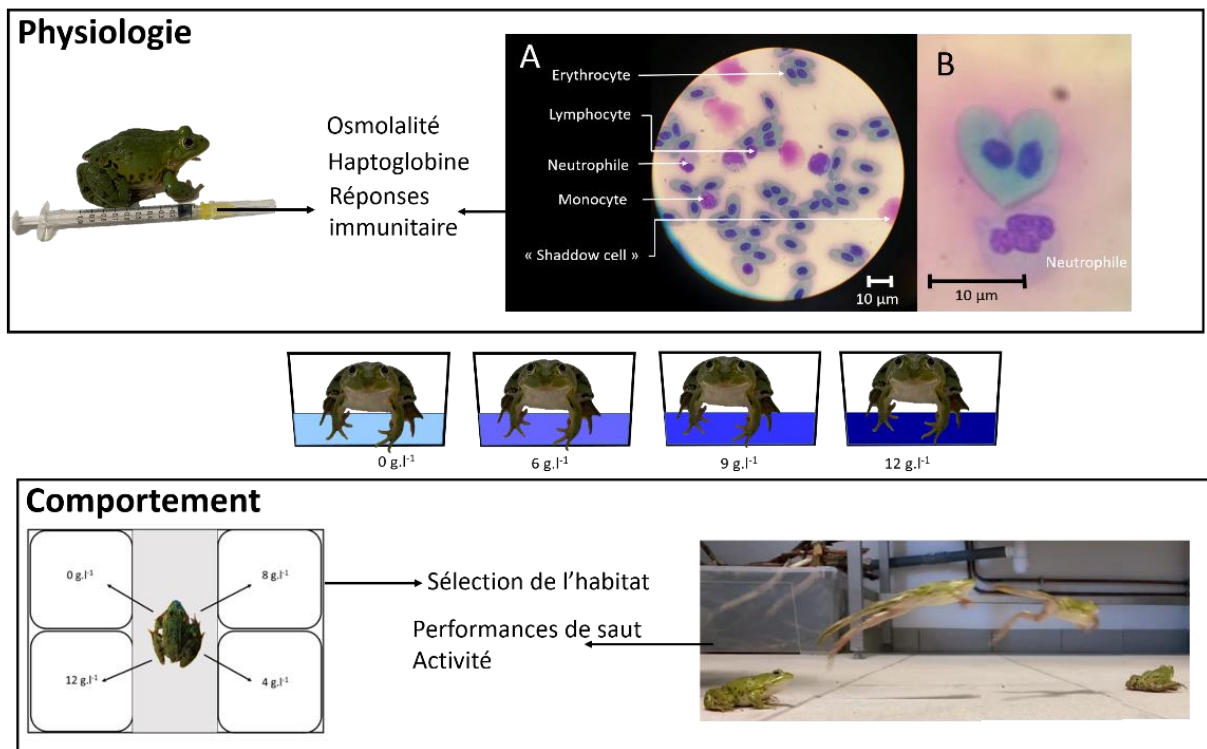


Figure 10 : Schéma illustrant différents paramètres mesurés chez des individus adultes de grenouilles vertes exposés à différents niveaux de salinité. Pour les réponses immunitaires : (A) Observation au microscope d'une lame colorée au May-Grünwald-Giemsa (Gx1000), et types cellulaires observés (les « shadow cells » correspondent à des cellules qui ont éclaté), et (B) neutrophile ainsi qu'un érythrocyte à la forme particulière (Gx1000). Voir Chapitre II.

III.3.3. Haptoglobine

Ces flux hydriques et ioniques importants ayant lieu entre l'intérieur et l'extérieur des cellules pourraient potentiellement endommager la membrane cellulaire. Pour mesurer la présence de

ces possibles dommages cellulaires chez les amphibiens, chez *Pelophylax* sp., nous nous sommes basés sur la mesure de la concentration en haptoglobine, qui est une protéine qui se lie à l'hémoglobine libérée lors de la plasmolyse des hématies, pour atténuer les dommages causés par les composants réactifs de l'oxygène pendant la réaction inflammatoire (Andersen et al. 2012). Voir Figure 10 et Chapitre II.

III.3.4. Comptage des leucocytes

Parmi les effets physiologiques notables, l'exposition à salinité élevée pourrait augmenter la sensibilité des têtards aux infections (Milotic et al. 2017; Hall et al. 2020). En effet, les amphibiens exposés au sel présentent des réponses leucocytaires induites par le stress (Davis et al. 2008). Pour évaluer l'effet de l'exposition au sel sur le statut immunologique (Burraco and Gomez-Mestre 2016; Hall et al. 2020), nous avons compté des leucocytes d'adultes de *Pelophylax* sp. par observation directe des cellules sur des frottis sanguins, colorés au May-Grünwald-Giemsa. Nous avons identifié et compté la proportion de neutrophiles, de lymphocytes, de monocytes, d'éosinophiles et de basophiles, sur 100 leucocytes. Voir Figure 10 et Chapitre II.

III.3.5. Télomères

Les télomères sont des structures nucléoprotéiques, situées aux extrémités des chromosomes et sujettes à un raccourcissement à chaque cycle de division cellulaire (Harley et al. 1990; Olovnikov 1996). Ils empêchent les extrémités chromosomiques d'être reconnues comme des cassures double brin et les protègent de la fusion et de la dégradation (Blackburn 1991). Ils sont ainsi des témoins de la qualité individuelle (Le Vaillant et al. 2015). Les télomères sont constitués de segments d'ADN répétitifs à forte teneur en G-C et sont considérés comme étant très sensibles aux dommages induits par le stress oxydant (Houben et al. 2008). Nous avons analysé les différences de tailles de télomères chez des individus larvaires de *Bufo spinosus* exposés à un stress salin. Voir Figure 12 et Chapitre III.

III.3.6. Métabolisme

Un environnement extérieur stressant pourrait modifier l'allocation d'énergie vers la maintenance (Heino and Kaitala 1999). On s'attend ainsi à ce qu'une exposition prolongée à la

salinité impacte le taux métabolique basal et la respiration (Kidder III et al. 2006). Pour mesurer les impacts sur le métabolisme, chez *Bufo spinosus*, en suivant la méthodologie de Dezetter et al. (2022), nous avons mesuré le taux de consommation de l'oxygène (un proxy du métabolisme standard, SMR; $\text{ml}\cdot\text{h}^{-1}$) et la production de dioxyde de carbone ($\text{ml}\cdot\text{h}^{-1}$) sur des individus au repos à 22°C , grâce à un système de respirométrie à flux ouvert, et à l'analyse des flux sortant sur le logiciel Expedata (Sable Systems). Voir Figure 11 et Chapitre II.

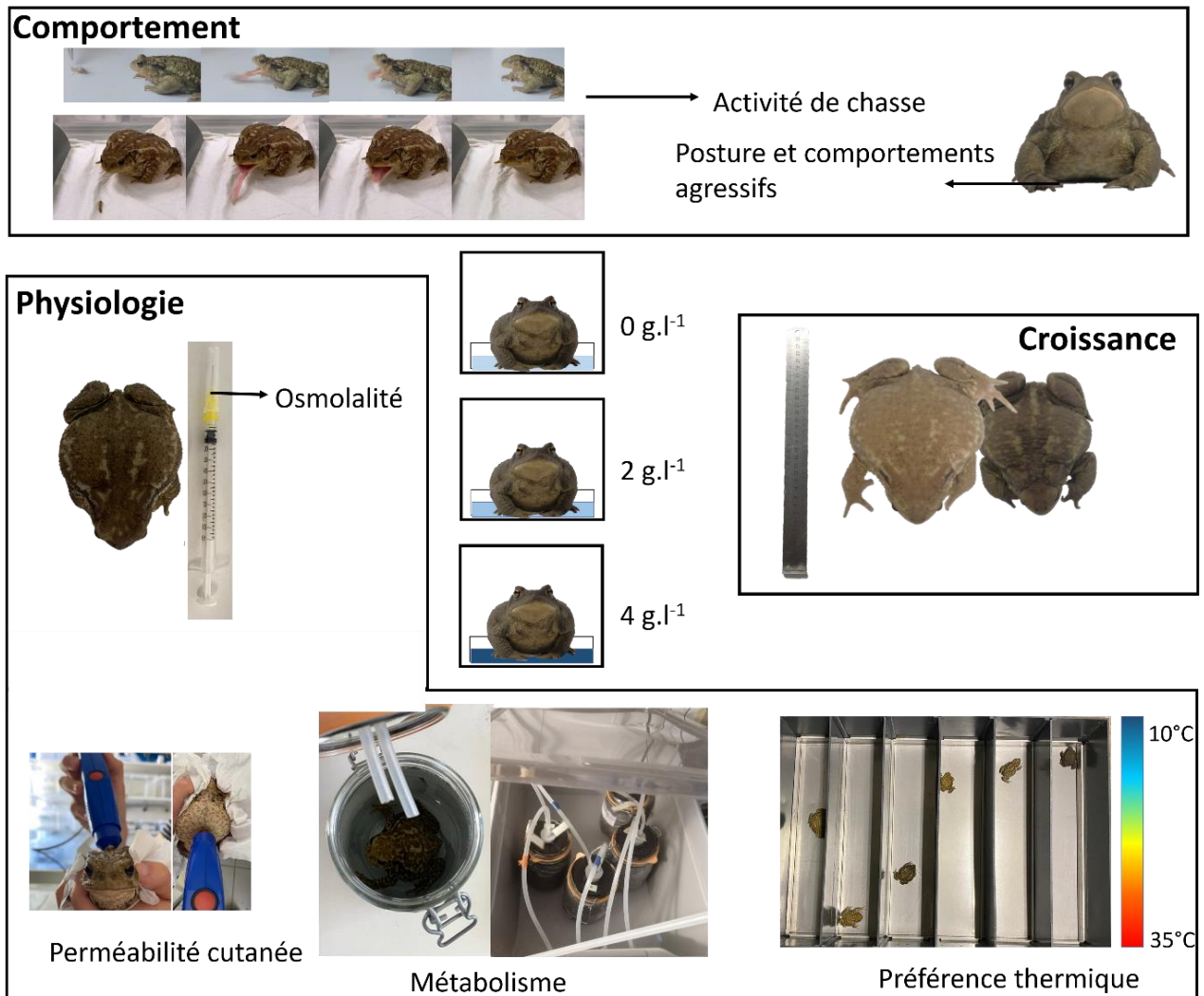


Figure 11 : Schéma illustrant différents paramètres mesurés chez des individus adultes de crapauds épineux exposés à différentes concentrations en sel. Voir Chapitre III.

III.3.7. Préférences thermiques

La salinité pourrait aussi diminuer la tolérance à d'autres facteurs environnementaux. En l'occurrence, il a été montré que la salinité influençait la tolérance thermique. De fait, la

tolérance thermique maximale des têtards peut décliner dans des salinités élevées (Chuang et al. 2022b). Par ailleurs, certaines études ont été réalisées concernant l'interaction entre salinité et température chez les larves (Rogell et al. 2009; Heard et al. 2014; Hopkins et al. 2017; Traversari 2021; Dahrouge and Rittenhouse 2022), mais ces effets n'ont jamais été testé chez les adultes, qui pourraient pourtant être assez mobiles pour réaliser une sélection de leur habitat thermique. En suivant la méthodologie de Lange et al. (2022), nous avons utilisé un gradient thermique (6 tubes PVC de 102 × 72 × 25.5 cm) allant de 10 à 35 °C pour évaluer la température corporelle individuelle préférée (T_{pref}) d'individus adultes de *Bufo spinosus* exposés ou non à la salinité, grâce à des mesures de la température corporelle toutes les heures pendant 10h (thermomètre infra-rouge Fluke 572 Infrared Thermometer, resolution 0.1 °C, Accuracy ± 0.75 °C). Voir Figure 11 et Chapitre II.

III.4. Comportement

III.4.1. Comportement des larves

Les larves exposées à une salinité plus élevée expriment une réduction de leur activité et des comportements de recherche de nourriture (Hall et al. 2017; Tornabene et al. 2021a), des réponses aux stimuli (Sanzo and Hecnar 2006; Karraker 2007) et des performances de nage (Denoël et al. 2010; Haramura 2016), mais réalisent aussi des mouvements erratiques (Tornabene et al. 2021a). Nous avons approfondi ces aspects en exposant des larves de *Bufo spinosus* à différentes concentrations en sel, et en étudiant notamment leur activité, leur vitesse de nage, leur utilisation de l'espace, et la complexité de leur mouvement, grâce au logiciel ToxTrac (Rodriguez et al. 2018). Voir Figure 12 et Chapitre III.

III.4.2. Performance de saut et activité

Si les effets de la salinité sur le comportement ont été largement étudiés chez les larves, ces effets ont rarement été déterminés chez les individus métamorphiques, et encore moins sur les adultes. Pour pallier à ces manques, nous avons quantifié à la fois l'activité d'adultes (*Pelophylax* sp., Figure 10, Chapitre II) et de métamorphes (*Bufo spinosus*, Figure 12, Chapitre III) soumis à différents traitements de salinité (à l'aide d'une échelle de score allant de 1 à 5), mais également leur performance de saut, en faisant sauter les individus 6 fois, et en retenant la distance moyenne parcourue au cours de ces 6 sauts.

III.4.3. Comportement de prédation

Des effets de l'augmentation du sel sur l'efficacité de la recherche alimentaire ont déjà été suggérés chez les larves d'amphibiens, en relation avec une diminution de l'activité (Chambers 2011; Van Meter et al. 2012; Hall et al. 2017). Après avoir testé les effets sur l'activité des adultes, nous avons donc également testé directement leur efficacité à la prédation, en analysant le comportement de chasse d'individus adultes de *Bufo spinosus* face à des proies mobiles. Voir Figure 11, Chapitre II.

III.4.4. Sélection de la salinité

La capacité des individus à évaluer la qualité de leur habitat influence fortement leur survie et leur performance. Les individus peuvent ainsi réagir de manière comportementale à un changement soudain des conditions environnementales. Un tel changement est attendu avec l'augmentation prévue de la salinité. Les amphibiens semblent être capables de détecter la disponibilité d'eau douce dans leur environnement (Hillyard 1999), et les femelles de certaines espèces semblent pouvoir sélectionner des sites de ponte afin d'éviter les sites à forte salinité (Viertel 1999; Haramura 2008; Albecker and McCoy 2017). Mais aucune preuve n'existe concernant le choix que font les individus en dehors des choix liés à la reproduction. Pour mieux comprendre cette sélection, nous avons confronté des individus adultes de *Pelophylax* sp., déshydratés ou non, face à un choix entre 4 salinités : 0, 4, 8 et 12 g.l⁻¹, et avons estimé différents paramètres parmi lesquels l'orientation des individus, leur nombre de trajets, et le temps que les individus passent dans chacun des traitements. Voir Figure 10, Chapitre II.

III.5. Développement/Croissance

III.5.1. Reproduction et développement embryonnaire

Le sel a de nombreux effets sur la reproduction des amphibiens. Premièrement, une osmolalité élevée dans le milieu diminue la survie, la motilité et la vitesse du sperme (Wilder and Welch 2014; Byrne et al. 2015). Mais l'effet de la salinité sur les paramètres du succès reproducteur demeurent largement méconnus. Pour mieux comprendre ces effets, nous avons testé, chez des crapauds épineux (*Bufo spinosus*) côtiers et continentaux, mais également des individus côtiers exposés à de l'eau douce ou de l'eau saumâtre, des paramètres liés au succès reproducteur (fécondité [nombre d'œufs], taille des œufs, masse de la ponte, densité des œufs,

perte de poids des parents, taux d'éclosion, taux de malformation). Voir Figure 12, Chapitre III.

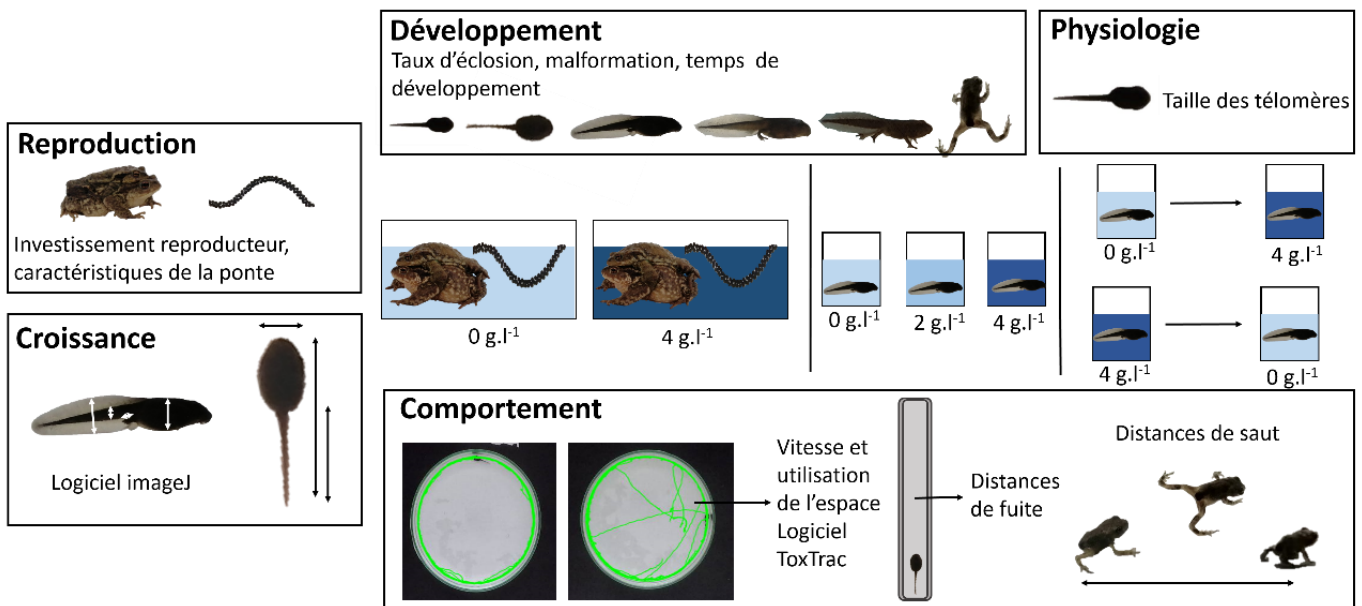


Figure 12 : Schéma illustrant différents paramètres chez des individus aux stades adultes, embryonnaires, larvaires et métamorphiques de crapauds épineux exposés à différents niveaux de salinité. Voir Chapitre III.

III.5.2. Développement larvaire

Les effets du sel sur les amphibiens ont, à ce jour, surtout été étudiés chez les larves, et particulièrement sur leur développement et leur croissance (Christy and Dickman 2002; Gomez- Mestre and Tejado 2003; Gomez- Mestre et al. 2004; Sanzo and Hecnar 2006; Wu and Kam 2009; Bernabò et al. 2013; Wood and Welch 2015; Wijethunga et al. 2016; Hopkins et al. 2016; Lukens and Wilcoxon 2020; Tornabene et al. 2021a). Mais les effets de l'acclimatation locale sur la réponse développementale et comportementale des larves d'amphibiens restent mal compris. Particulièrement, la manière dont salinité du milieu dans laquelle les parents pondent impacte l'ensemble du développement larvaire n'a pas été étudié. Nous avons étudié les effets du sel sur le développement larvaire de *Bufo spinosus*, au travers de différents stades de développement [stades de Gosner (Gosner 1960)], pour une exposition chronique, et en réaction à des changements de salinité au cours de leur développement (Figure 12, Chapitre III).

III.5.3. Croissance des adultes

Si les effets de la salinité sur le développement larvaire sont bien connus, aucune étude à ce jour n'a testé les effets de la salinité sur la croissance des adultes. Pour comprendre ces effets, nous avons exposé, de manière chronique (>6 mois), des adultes de *Bufo spinosus* à des niveaux de salinité faibles, et évalué la croissance des individus suite à cette exposition, grâce à des mesures morphologiques avant et après exposition (Figure 11, Chapitre II).

Chapitre I : Variations de salinité sur les milieux côtiers, et structuration des communautés en fonction de ces variations

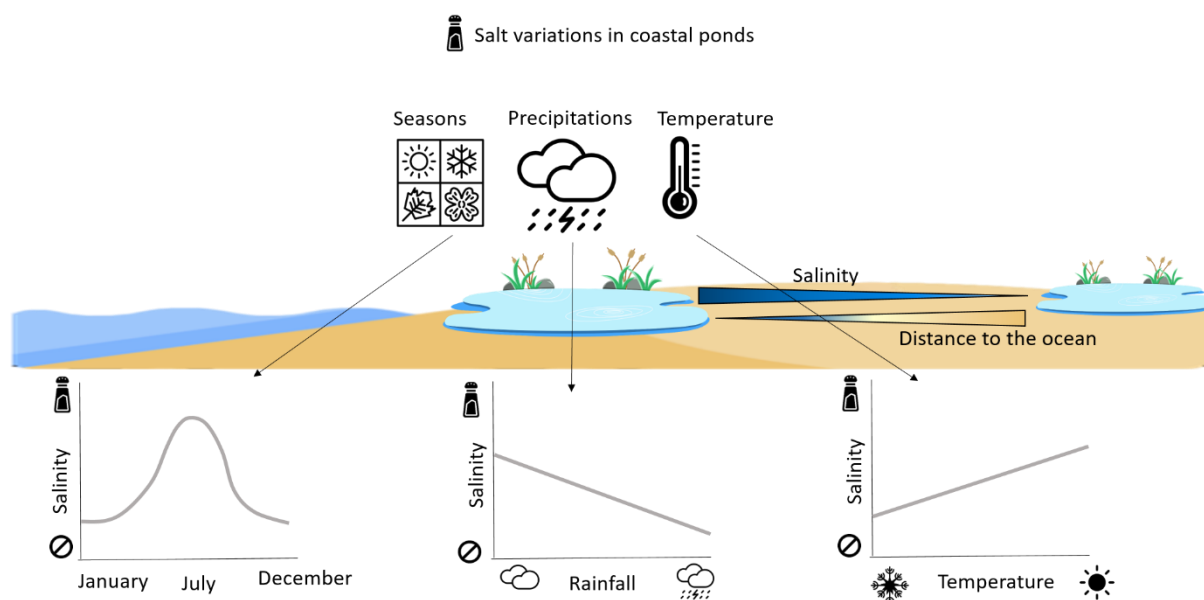


Photo par Marko Jankovic

Article I

Long-term trends of salinity in coastal wetlands: Effects of climate, extreme weather events, and sea-water level

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Abstract

Coastal wetlands play major roles as reservoirs of biodiversity and provide many ecosystem services and protection from extreme weather events. While they play major roles worldwide, they are affected by a large variety of anthropogenic threats, among which salinization has been less studied, despite the major role of salinity on biodiversity and ecosystem functioning. In this study, we investigated the variations of salinity across long-term (1996-2020) and seasonal (monthly records) temporal scales and spatial (varying distance to the coastline) scales in water bodies of two typical temperate coastal wetlands situated on the Atlantic coast of France. We complemented our analyses with models of seawater levels computed at both sites across 2000-2020. Our detailed data set allowed for highlighting that salinity in ponds varied seasonally (higher during summer), but also spatially (higher closer to the seashore). Both decreased precipitation and increased temperature induced an increased strength of this spatial gradient of salinity through time. We also highlighted contrasted long-term patterns of salinity changes on these two coastal wetlands, linked to the responses to marine flood, allowing to document the temporal dynamics of salinity following a massive intrusion of seawater. Complementarily, at both sites, water levels at high tides increased through time, a pattern which can induce additional salinization. To our knowledge, our study is the first to investigate long-term changes in salinity in coastal wetlands through natural processes (e.g. seaspray, seasonal variations) and ongoing climate perturbations (e.g. marine surges linked to extreme weather events, increased temperature and decreased precipitations).

Key-words: Freshwater, Marine submersion, Precipitation, Salinization, Seasonal variations, Temperature

1. Introduction

Coastal wetlands are situated at the boundary between the land and the sea (Janas et al., 2019). Despite a restricted extent of the interface between oceanic and terrestrial biomes, coastal wetlands represent a significant surface area worldwide (Hopkinson et al. 2019). While they play significant ecological roles (Davis et al. 2014; Hopkinson et al. 2019; Hobohm et al. 2021), coastal wetlands are currently affected by a large variety of anthropogenic threats such as eutrophication, chemical pollution, land use, invasive species, and ongoing modification of climatic conditions (Barua et al. 2021; Martínez-Megías and Rico 2021). Among these threats, changes in salinity regimes (environmental salinization) across spatial and temporal scales relevant to coastal wetlands have been less studied to date, despite the major role of this environmental parameter in the functioning of these ecosystems (Herbert et al. 2015).

Coastal ecosystems are naturally exposed to spatial and temporal variations of salinity (Barua et al. 2021). Such natural variations of salinity (i.e. primary salinization, Herbert et al., 2015) are linked to the specific geographic position of coastal ecosystems, and notably the exchanges between terrestrial and oceanic environments (Xue et al. 2013). The natural processes which affect variations of salinity are primarily linked to landward aerial transport of dry or wet oceanic salts (Zaman et al. 2018), such as sea-sprays (Benassai et al. 2005). As a consequence, coastal ecosystems are characterized by spatial and temporal heterogeneity of salinity (Estévez et al. 2019; Ranjbar and Ehteshami 2019; Fu et al. 2021; Ghaleb et al. 2021). The spatial determinants of

salinity are mainly linked to the proximity of salt sources (i.e. distance to the coastline; Wu, Liu, and Huang, 2017) while salinity can also change temporally from short-(hours) to longer temporal scales (years) (Gutierrez and Johnson 2010; Ghosh et al. 2013). Complementarily, climatic conditions (e.g. temperature, rainfall, wind regimes) interact with salt deposition and can influence variations of salinity (Morcillo et al. 2000; Perigaud et al. 2003). For instance, salinity increases when evaporation exceeds precipitation (Hassani et al. 2021), which might naturally occur seasonally (De Luis et al. 2009).

Coastal ecosystems are also susceptible to secondary salinization, the increase in salt concentration caused by the direct actions of humans or their activities (Herbert et al. 2015; Izam et al. 2021), because of several processes operating at different temporal scales. First, ongoing climatic changes are expected to induce a significant increase in sea level (Gornitz 1995; Domingues et al. 2008; Church and White 2011; Herbert et al. 2015). Second, current climatic changes are expected to increase the intensity and frequency of extreme events, such as marine storms and associated surges (Dettinger 2011; Trenberth et al. 2015; IPCC 2022), even if this increase is more uncertain for temperate areas. Third, alterations of hydric cycles due to changes in the magnitude and timing of precipitation (Neubauer and Craft 2009; Martínez-Megías and Rico 2021) should affect freshwater inputs and change evaporation rates (Mills et al. 2013; Herbert et al. 2015; Jeppesen et al. 2020). Finally, other anthropogenic activities can directly lead to an increase in salinity, such as groundwater pumping or water abstraction which have been shown to increase seawater

inundation and saline intrusion (Reid et al. 2019; Peters et al. 2021). All of these processes and the associated increased salinity, may severely transform coastal areas (Knighton et al. 1991; Visschers et al. 2022).

However, despite the major ecological impacts of salinization, the spatio-temporal variations of salinity in coastal areas, and more importantly, their recent changes in response to climatic modifications remain poorly known. In order to better understand these variations in salinity, it is essential to investigate the effects of environmental factors that might influence salinization with high temporal resolution time series of salinity measurements (Lee et al. 2022). Such investigations are currently of crucial importance because they will allow assessment of the extent of salinity variations in coastal environments (either natural or induced by climate change), and thus to understand the consequences of these changes on the resilience of these ecosystems.

In this study, we investigated the spatio-temporal variations of salinity across long-term (decades) and fine temporal (monthly records) and spatial (varying distance to the coastline) resolutions in water bodies of two typical temperate coastal wetlands situated on the Atlantic coast of France. On both sites, water salinity was recorded each month (for 24 years on 24 ponds in one site, and for 10 years on 31 ponds in the other site). Based on these detailed data sets, our objectives were multiple. First, we analysed the seasonal and spatial variations of salinity. We hypothesized that salinity should be greater during summer because of reduced precipitation and higher temperatures, and that salinity

should be higher closer to the seashore because of landward deposition of seaspray. Second, we analysed the long-term changes in salinity and related these changes to concomitant variations in precipitation and temperature in order to highlight potential effects of ongoing climate change on this parameter. We hypothesized that increased temperatures and decreased precipitation over time (Schär et al. 2004; Briffa et al. 2009) should increase salinity levels across our time series. We also hypothesized that these long-term changes should interact with the spatial variations of salinity, with proportionally greater salinity increase closer to the seashore. Finally, we complemented our analyses with models of seawater levels computed in front of both sites across 2000-2020 in order to investigate whether fluctuation in this parameter can contribute to further salinization through local inundations. We hypothesized that seawater levels which occasionally provoke landward inundations should increase in frequency and intensity over time.

2. Methods

2.1. Study sites and field procedures

Salinity of water bodies were measured on two coastal wetlands from the western coast of France: the « Réserve Naturelle Nationale de Moëze-Oléron » (45°53'33.36"N, 1°04'59.16"W, hereafter MO) and the « Réserve Naturelle Nationale du marais d'Yves » (46°2'40.735"N, 1°3'16.906"W, hereafter MY) (See figure I1). These two study sites, separated by ~15 km, are situated on the Atlantic coast of France (Département de la Charente-Maritime), an area characterized by a temperate climate. The two sites are natural reserves characterized by diverse habitat types which cover a continuum between intertidal sandy area, through salt and freshwater marshes; up to sand dunes and grasslands along with increasing distance from the seashore. On both sites, the presence of small water bodies (ponds) were favoured in order to improve biodiversity. Both sites span a similar surface area (218 ha for MO and 204 ha for MY, Figure I1) and are relatively low (mean elevation: MY= 2.64 m ± 0.02 SE NGF; MO=2.90 m ± 0.01 SE NGF) coastal wetlands displaying a coastal sand dune (3.30 m high NGF both at MY and MO) situated near the high sea limit (see Lorrain-Soligon et al., 2021 for topographic profiles). Both sites were differentially hit by storm Xynthia (that occurred in 2010, Bertin et al., 2014): one site was completely submerged by seawater (MY), while many ponds at the other site (MO) were spared from the storm because they were protected by a second additional dune (see Lorrain-Soligon et al., 2021).

At MO, salinity of 24 ponds was measured (distance to the ocean: mean=564.52 m ± 59.99, min=57.69 m, max=1079.24 m, Figure I1) from January 1996 to December 2019. At MY, the salinity of 31 ponds was measured (distance to the ocean: mean=564.29 m ± 24.95, min=186.83 m, max=836.76 m, Figure I1) from January 2010 (corresponding to the seawater intrusion by storm Xynthia) to December 2019. These represent small body of still water (<500m²), presenting similar characteristic of habitat types, surrounding and aquatic vegetation, and depth (see Lorrain-Soligon et al., 2023, for detailed description). On each pond, the salinity was recorded once per month during 24 years (at MO) and 10 years (at MY). The salinity was recorded using a portable refractometer until 2000 (Cond 330i), and then using a conductimeter (YSI Professional Plus, ProQuatro Multiparameter Meter, and Multi 340i with TetraCon 325 probe), and was repeatedly measured at the same location for each pond (20 cm from the pond edge, 10 cm below the water surface).

2.2. Meteorological parameters

We included yearly and monthly data of temperature and precipitation using the *easyclimate* package (Moreno and Hasenauer 2016) on R 3.6.3 (R Core Team 2020). This package allows extraction of maximum temperature, which is expected to influence evaporation, and total precipitation on a daily scale. We extracted these data from La Rochelle meteorological station (46°9'0.684"N, 1°8'43.08"W) situated at 17.4 km and 26.6 km from MY and MO respectively, between 1996 and 2020.

2.3. *Estimates of the water levels across 20 years*

To investigate the possible impact of changes in sea level on coastal wetland salinization, we simulated the hydrodynamic circulation in the study area using the numerical model SCHISM (Zhang et al. 2016), which solves the primitive equations on unstructured grids using advanced numerical methods. We used a hydrodynamic model because sea water levels strongly vary spatially while the only tide gauge available over the studied period (La Pallice, see Figure I1) is located about 20 and 30 km to the North of both study sites. This spatial variability of water levels is due to spatial variations of tides and storm surges. Thus, Dodet et al. (2019) showed that, over the modelled domain and over the period 1998-2018, the mean tidal range varied from 3.28 and 3.83 m and the maximum storm surge varied from 1.15 and 1.57 m. To a lesser extent, the tide gauge data has some gaps while the model is continuous over the studied period. SCHISM was implemented in 2DH (two dimensional horizontal, which means that the model uses one single layer on the vertical) over the 'Pertuis Charentais' area with a spatial resolution ranging from 2000 m along the open boundary to 100 m near the shoreline, as described in Savelli et al. (2019). The model starts at mean sea-level and uses a ramp of 1 day. A sensitivity analysis reveals that the model converges when using longer ramps, which is due to the small dimensions of the modelled domain. The model is forced by amplitudes and phases of the 18 main tidal constituents, linearly interpolated from the regional tidal model of Bertin et al. (2012) and fields of sea-level pressure and 10 m winds originating from the CFSR reanalysis (Saha et al. 2010). The

hydrodynamic model used in this study is well-established and was previously developed to compute sediment transport and morphological changes (Guerin et al. 2016) and bed shear stress and biofilm resuspension in front of MO (Savelli et al. 2019). The model was run over the period 2000-2020 and hourly time series of water levels were extracted in front of each studied wetland. Due to the 2DH (two dimensional horizontal) barotropic configuration, the model cannot capture long-term changes in sea levels due to large-scale circulation patterns and global warming. In order to account for these processes which are relevant at the scale of the studied period (2000-2020), yearly mean sea levels observed at the nearby tide gauge of La Rochelle (data available at <https://data.shom.fr/>) were computed and linearly added to model outputs. This approach was validated against the available water level data at La Rochelle tide gauge, which are reproduced with a root mean squared discrepancy of 0.13 m. Extreme water levels are only substantially underestimated during Xynthia storm, mostly because the effects of short waves were not accounted for in the simulation, as discussed in Bertin et al. (2014).

2.4. *Statistical analyses*

All statistical analysis were performed using R 3.6.3 (R Core Team 2020) and Rstudio v1.1.419. We computed Linear Models (LMs) or Linear Mixed Models (LMMs). For all test computed, models accuracy was tested using the `check_model` function from the *performance* package (Lüdtke et al. 2020). When models did not fit the data, the response variable was log transformed or log+1 transformed.

2.4.1. Seasonal variations of salinity

We investigated seasonal variations of salinity (all years combined), by computing LMMs with each value of salinity recorded as dependent variables (log+1 transformed), month and its quadratic form as covariates, and pond as a random effect. We also investigated the climatic drivers of these variations by computing LMMs with mean salinity (measured for each month and each pond, all years combined) as the dependent variable (log transformed), mean temperature or precipitation as covariates, and pond as a random effect. As temperature and precipitation were highly correlated (cor=-0.145, p-value<0.001) these variables were tested in two separate models.

For these analyses, we calculated a mean temperature for each month (across the study period) and a mean precipitation (mean of the sum of precipitations recorded during each month across the study period).

2.4.2. Spatial variations of salinity

We analysed salinity records according to distance to the ocean of the ponds where salinity was recorded, by computing LMMs with each value of salinity recorded as dependent variables, distance to the ocean as the covariate (log+1 transformed), and pond as a random effect.

2.4.3. Long term changes in salinity

Across the whole study period (January 1996 to December 2019 at MO, and January 2010 to December 2019 at MY), we evaluated long-term temporal trends of salinity by computing LMMs with each value of salinity recorded as the dependent variable (log+1 transformed), year as the

covariate, and pond as a random effect. Additionally, we investigated trends of maximum and minimum salinity (for all ponds and all years: one value of maximum and minimum salinity for each pond and each year), using either minimum or maximum salinity as dependent variables, year as the covariate, and pond as a random effect. We also investigated the climatic drivers of salinity trends, by computing LMMs with mean salinity (one value of mean salinity for each pond and each year) as the dependent variable, mean temperature across the year or total precipitation as covariates, and pond as a random effect. Because previous analyses demonstrated that salinity recorded in 2010 at MY was exceptionally high due to the marine flooding driven by storm Xynthia (Lorrain-Soligon et al. 2021), we removed this year from our analysis at MY, in order to investigate only variations of salinity according to environmental parameters under normal conditions.

Finally, to investigate whether the spatial gradient of salinity (distance to coastline) changes across the study period, we extracted, for each year, the slope of the relationships between salinity and distance to the ocean, and computed LMs with these slope as dependent variables, and year, temperature (mean temperature of the year) or precipitation (sum of the precipitations of the year) as a covariate.

2.4.4. Estimates of the high tides' water level

To investigate the variations of estimated water levels across the study period (2000-2020 for both sites), we computed yearly-mean values of water levels exceeding 2.4 m/MSL (Mean Sea Level, the water level is thus given with respect to mean sea level),

which corresponds to spring tides. Indeed, under neap tides, the sea level is too low to induce any flooding and salinization of the studied ponds. Although this value is somehow arbitrary, it has no impact on inter-annual variability and upward trend of sea levels.

We then computed LMs with these estimated water levels at high tides as dependent variables, and year as a covariate.

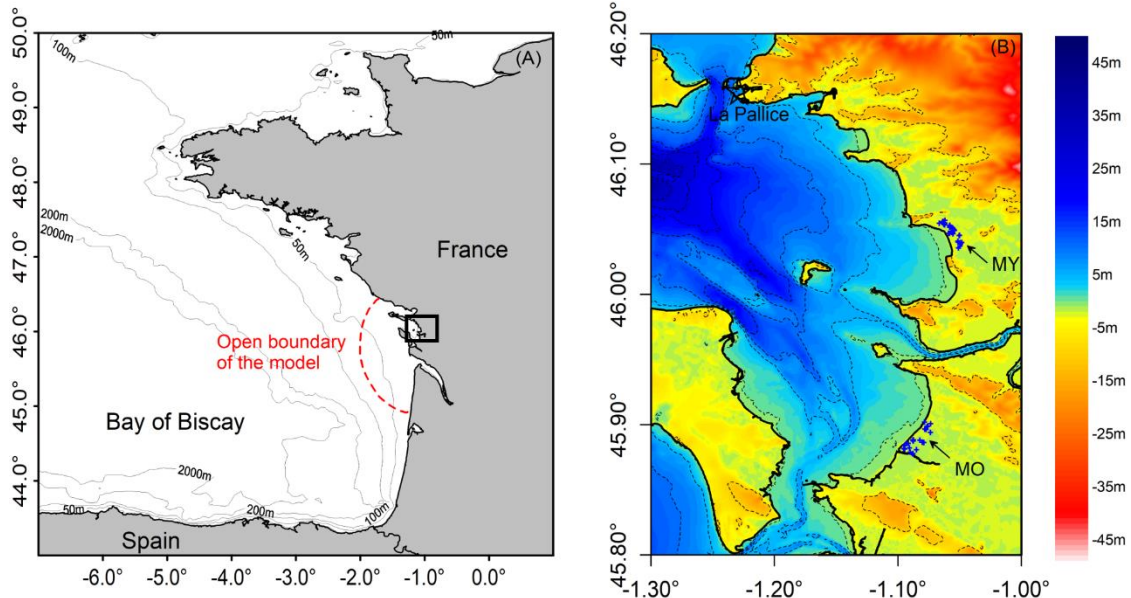


Figure I1: (A) Map of the study area relative to Western France, including open boundary of the model run for estimated sea water levels, and (B) map representing the two study sites (MO and MY) and the study ponds (blue points), as well as the tidal gauge and point use for climatic data (La Pallice). Bathymetry is indicated with the colour scale. At MO, position of the study ponds ranged from 58 m to 1080 m from the seashore. At MY, position of the study ponds ranged from 187 m to 840 m from the seashore. MO (« Réserve Naturelle Nationale de Moëze-Oléron ») and MY (« Réserve Naturelle Nationale du marais d'Yves »)

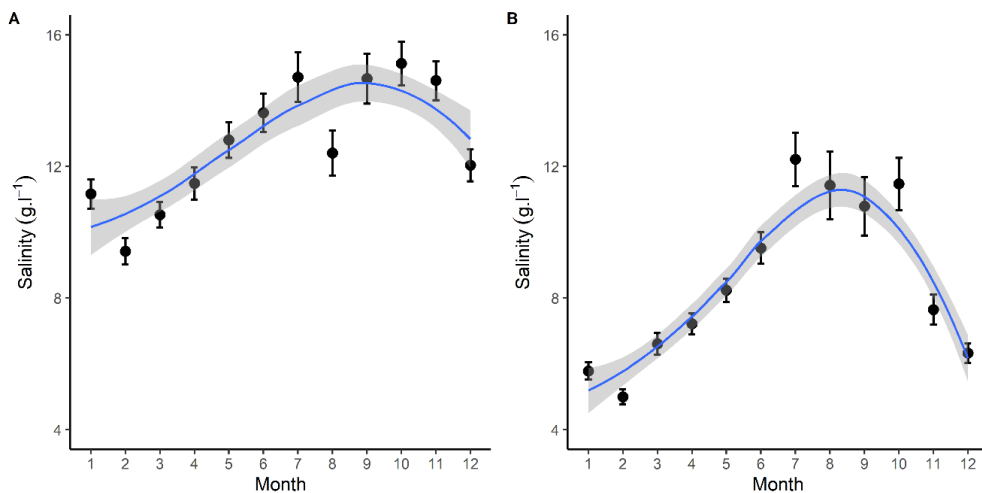


Figure I2: Variations of salinity by month at MO (A) and MY (B). Mean \pm SE, and linear trend line \pm SE. MO : « Réserve Naturelle Nationale de Moëze-Oléron » and MY : « Réserve Naturelle Nationale du marais d'Yves »

3. Results

3.1. *Seasonal variations of salinity*

Across the whole study period, salinity varied seasonally (monthly variation), according to a quadratic form, with maximum salinity attained in summer on both sites (Figure I2, Table I1). In both sites, these variations were significantly explained by temperature, with salinity increasing with increasing temperature (MO: Estimate=0.002, SE<0.001, t-value=14.780, p-value<0.001; and MY: Estimate=0.002, SE<0.001, t-value=8.96, p-value<0.001), and by precipitation, with salinity decreasing with increasing precipitation (MO: Estimate<-0.001, SE<0.001, t-value=-2.368, p-value=0.019; MY: Estimate<-0.001, SE<0.001, t-value=-3.645, p-value<0.001).

3.2. *Spatial variations of salinity*

At MO and MY, salinity decreased with increasing distance to the ocean (MO: Estimate<-0.001, SE<0.001, t-value=-3.41, p-value=0.003; MY: Estimate=-0.001, SE<0.001, t-value=-2.048, p-value=0.050).

3.3. *Long term changes in salinity*

Across the whole study period, salinity decreased at MY, considering either mean, minimum, or maximum values (Table I1). At MO, results were more complex with a significant increase in minimal values of salinity across time (Table I1), and no significant trends for maximal and mean values (Table I1). The long-term variations of salinity were linked to precipitation, with salinity decreasing with increasing precipitation at both sites (MO: Estimate<-0.001, SE<0.001, t-value=-3.562, p-value<0.001; and MY: Estimate<-0.001, SE<0.001, t-value=-3.282, p-value=0.001),

but not to temperature (at MO: Estimate=-0.011, SE=0.016, t-value=-0.695, p-value=0.487; and at MY: Estimate=0.066, SE=0.044, t-value=1.524, p-value=0.130).

Across years, the spatial gradient of salinity (slope of the relationships between salinity and distance to the coastline) increased at MO (Estimate<-0.001, SE<0.001, t-value=-7.449, p-value<0.001, Figure I3A), an effect that was marginally related to decreased precipitation (Estimate<0.001, SE<0.001, t-value=1.881, p-value=0.073), but not to temperature (p-value=0.238). At MY, there was no difference in the strength of the spatial gradient of salinity (Estimate<0.001, SE<0.001, t-value=0.327, p-value=0.752, Figure I3B), and no relationships with temperature or precipitation (both p-values>0.269).

3.4. *Estimates of the high tides' water level*

In both sites, water levels at high tides increased over time (at MO: Estimate=0.002, SE<0.001, t-value=4.735, p-value<0.001; at MY: Estimate=0.002, SE<0.001, t-value=4.330, p-value<0.001; Figure I4). These data show that open water surface can exceed the height of the protective coastal sand dunes during significant amount of time (9 days at MO and 7 days at MY).

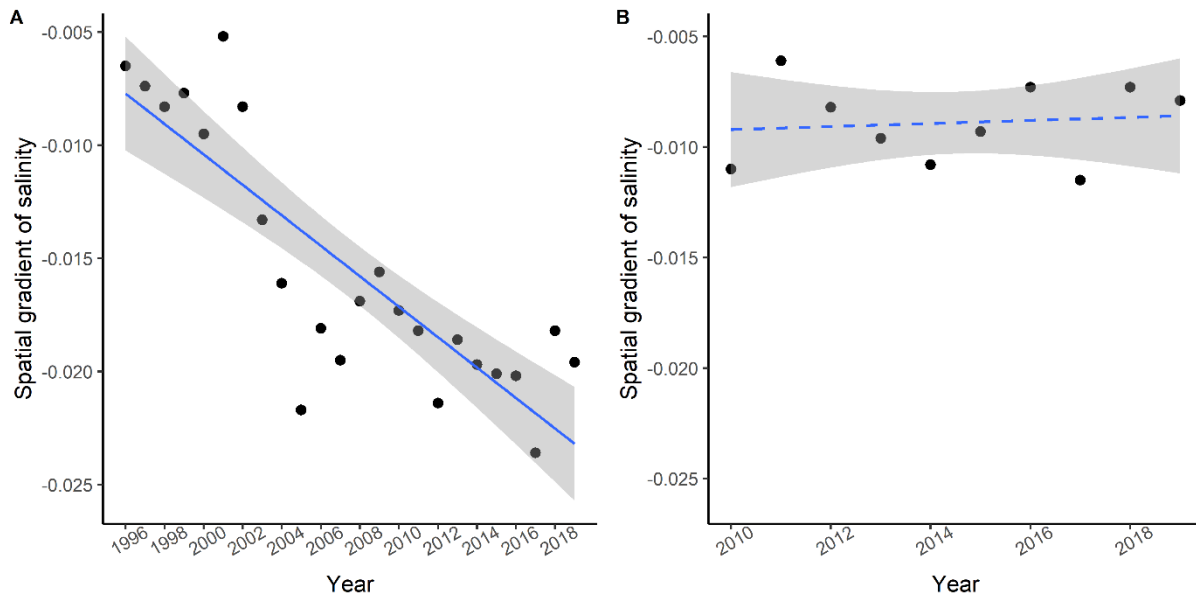


Figure I3: Variations of the spatial gradient of salinity (slope of the regression between salinity and distance to the ocean) through time, at MO (A) and MY (B). MO : « Réserve Naturelle Nationale de Moëze-Oléron » and MY : « Réserve Naturelle Nationale du marais d'Yves ». Dashed line represent a non-significant relation

4. Discussion

Several studies have investigated contemporary long-term salinity changes in marine environments and highlighted contrasted patterns linked to variable freshwater inputs and evaporation patterns (Samuelsson 1996; Lin et al. 2001; Omstedt and Axell 2003; Meredith and King 2005; Li et al. 2017; Tukenmez and Altioek 2022). Very few studies have investigated such variations for freshwater or continental water bodies (Collins and Russell 2009; Benjankar et al. 2021; Dugan and Arnott 2022). To our knowledge, our study is the first to investigate long-term changes in salinity in coastal wetlands susceptible to salinization through natural processes (e.g. seaspray, Benassai et al., 2005) and ongoing climate perturbations (e.g. marine surges linked to extreme weather events, Nordio et al., 2023). Our detailed data set allowed for highlighting that salinity in ponds varied seasonally, being higher during summer months, but also spatially, as salinity increase closer to the seashore. Importantly, this spatial gradient of salinity

linked to landward salt deposition strengthened through time. These variations of salinity are linked to both increased temperature and reduced precipitations. Complementarily, in both sites, water levels at high tides increased through time, a pattern which can induce an additional source of salinization.

At both sites, salinity varied seasonally, with lowest salinity in winter and highest salinity in summer. Such seasonal variation was expected given the concomitant variations in climatic conditions (temperature and precipitation) in temperate areas. Indeed, we showed that the seasonal variation of salinity is driven both by temperature and precipitation, as salinity increased with increasing temperature and decreasing precipitation. Both variables are likely to affect evaporation patterns interactively (Hassani et al. 2021), which naturally occur seasonally (De Luis et al. 2009). Few other studies have investigated seasonal

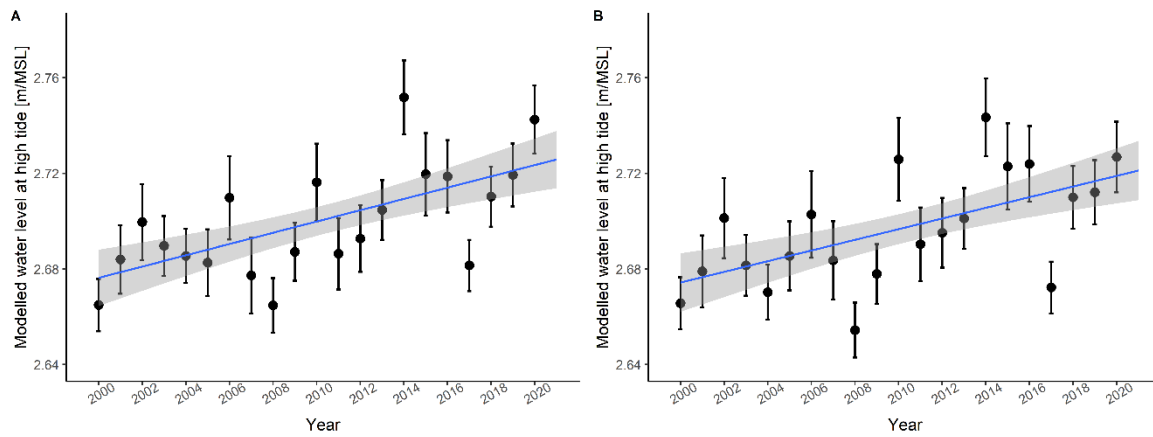


Figure I4: Variations of water level at high tides by year at MO (A) and MY (B), given in m/MSL (Mean Sea Level, the water level is thus given with respect to mean sea level). Mean \pm SE. MO : « Réserve Naturelle Nationale de Moëze-Oléron » and MY : « Réserve Naturelle Nationale du marais d'Yves »

variations of salinity in closed waterbodies (ponds) such as the ones monitored in this study (Collins and Russell 2009; Benjankar et al. 2021). Conversely to our findings, such studies highlighted winter salinity peaks attributed to the use of road de-icing salts (Collins and Russell 2009). These contrasted patterns highlight the importance of direct anthropogenic activities on the disruption of seasonal variation of salinity in (fresh-) water systems.

As expected from landward deposition of seaspray, we found that, overall, salinity was higher in coastal ponds situated closer to the coastline, despite the small spatial extent of our study sites (situated from ~50m to 1000m from the coastline). Importantly, we found that, over time, the strength of this spatial gradient of salinity increased significantly on one of our study sites (MO). That is, the slope of the relationship between pond salinity and distance to the coastline increased through time. This effect was mainly driven by increased salinity closer to the seashore rather than by decreased salinity farther from the seashore. Such a result highlights a remarkable spatial heterogeneity in the susceptibility of these coastal ponds to

salinization over a relatively small spatial scale. Complementarily, this result reinforces the notion that ongoing climate change is currently inducing significant salinization, and that this process strongly alters the spatial gradient of salinity. Despite the strengthening of the spatial gradient of salinity, we did not find a consistent trend of salinity over time, but found contrasted responses in our two study sites. Indeed, in one of our study sites (MO), salinity (maximum and mean) remains relatively stable across time, but minimum values of salinity increased through time. This site was spared from marine submersion linked to storm Xynthia due a protection dune (Lorrain-Soligon et al. 2021). The second site however (MY), was completely submerged by storm Xynthia (Lorrain-Soligon et al. 2021). Our salinity recordings started at the onset of this marine submersion and documented the temporal dynamics of this parameter following a massive intrusion of seawater. We showed that salinity was maximum following storm Xynthia and progressively decreased through time to reach lower and temporally stable values at ~3 years post-storm (see Lorrain-Soligon et al. [2021] for some salinity values recorded before

Xynthia). These lower values remained stable through time, although we emphasize that the following 7 years are likely too short of a time scale to investigate potential long-term changes in salinity. Importantly, other studies have shown that effects of this marine submersion were long-lasting both for fluxes of marine elements (salt, sediments, and organic matter) through the food web (Lorrain-Soligon et al. 2022d) and for the direct negative impact of salinization on biodiversity (Lorrain-Soligon et al. 2021). Our data suggest that the frequency of future marine submersions may well jeopardize the resilience capacity of these coastal wetlands, which may affect the recolonization dynamics of wildlife.

Long-term variations in salinity are hypothesized to be mainly due to low precipitation and excess evaporation linked to increased temperature (Al-Shammiri 2002; Mills et al. 2013; Obianyo 2019). Our results suggest that salinity increase may be related to precipitation, while we failed to detect any influence of thermal conditions, despite the strong effect of both parameters on seasonal variations of salinity (see above). These results suggest that long-term salinization is primarily driven by changes in the magnitude and the timing (seasonality) of precipitation, the effects of which are strengthened by climate change and, notably, the increased frequency of droughts (Mills et al. 2013; Herbert et al. 2015; Jeppesen et al. 2020).

Finally, the numerical modelling of sea levels and the computation of yearly-mean values at high tides also shows a significant increase over time at both our study sites. This pattern is explained firstly by the

nodal tidal cycle (18.6 year period, which can influence sea water levels, Baart et al., 2012; Royer, 1993; Yndestad et al., 2008), which reached a minimum in 2006 and maximum in 2015. The minimum values observed in 2003-2005 also correspond to a period characterized by a weaker storminess in the region (Chaumillon et al. 2019). Over the studied period, water levels increased by about 0.10 m. This increase is explained mostly by long term sea-level rise and, to a lesser extent, by an increase in storminess from 2003-2005 to 2014-2017. More specifically, these data show that open water surface can exceed the height of the protective coastal sand dunes during significant amount of time. Such data highlight additional risks of salinization through seawater inundations, which frequency has been shown to increase with global changes in several areas of the globe (Nicholls et al. 1999; McLean et al. 2001; Knutson et al. 2010; Dettinger 2011; Trenberth et al. 2015), even though they have not been formally tested in temperate areas. A previous study have emphasized the importance of coastal sand dunes as a protection of coastal wetlands against seawater intrusions (Lorrain-Soligon et al. 2021), but these natural barriers may well prove inefficient in the long-term, given that by the 2080s, sea-level rise is expected to cause the loss of as much as 22% of the world's coastal wetlands (Nicholls et al. 1999).

Whatever the underlying causes, salinization is known to negatively affect the rich biodiversity of coastal ecosystems (Hellebusi 1976; Bradley 2009; Schultz and McCormick 2012; Evans and Kültz 2020). Variations in salinity can thus affect species geographic distribution (Gunter 1956; Brischoux et al. 2021b), and in turn

community structure (Hart et al. 2003; Anufrieva and Shadrin 2018; Cunillera-Montcusí et al. 2022; González-Sansón et al. 2022; Kendall et al. 2022; Ersoy et al. 2022), throughout trophic levels (Hintz and Relyea 2019). In our study area, a previous study have shown that storm Xynthia has led to a decrease in amphibians species richness (Lorrain-Soligon et al. 2021). Other components of local biodiversity (birds, reptiles, invertebrates, plants) might be affected as well, which need to be further investigated. In addition, these changes of salinity levels can also influence habitat structure and provoke landward habitat shifts (Visschers et al. 2022). Finally, salinization is known to affect hydrology by destabilizing the water column (i.e. salt water is denser than fresh water), thus leading to water mixing issues (Koretsky et al. 2012; Dugan and Arnott 2022), changes in biogeochemistry (e.g. lack of oxygenation, Ladwig, Rock, and Dugan, 2021; Dugan and Arnott, 2022), and affect soils characteristics (e.g. decreased carbon content and increased organic matter recalcitrance, Neubauer, Franklin, and Berrier, 2013). Taken together, these various consequences of coastal wetland salinization suggest that salinity fluctuations are likely to dramatically transform - and impact the functioning of - coastal ecosystems (Denny 1994; Cañedo-Argüelles et al. 2013; Herbert et al. 2015; Cunillera-Montcusí et al. 2022; Visschers et al. 2022). Despite growing interest on the outcome of human-induced salinization, we emphasize that the consequences of these processes at large spatial and temporal scales are still to be investigated (Cunillera-Montcusí et al. 2022). Further research should focus on the impacts on biodiversity in relation to the spatio-temporal salinity gradients, particularly in

summer when water levels are expected to decrease, leading to high salinity pulses (Martínez-Megías and Rico 2021). In addition, forecasting models should incorporate more information on critical factors that might influence salinity changes, across large spatial and temporal scales, not only for water bodies but also for soil, as terrestrial salinization is a critical but overlooked process (Greenway and Munns 1980; Rath and Rousk 2015; Singh 2016; Ayub et al. 2020).

5. Conclusion

We highlighted the consequences of both a marine submersion and of ongoing climate change, the latter inducing a long-term increase in salinity and disrupting the spatial gradient of salinity in coastal ecosystems. Models of water levels at high tides further highlight a temporal increase, suggesting a higher susceptibility to marine intrusion. Taken together, these elements strongly suggest that coastal wetlands are threatened by salinization. We emphasize the importance of recording long-term series for a large variety of locations, as the salinity trends can be highly variable and site-specific.

Table I1: Effects of temporal scales (year, month and month²) on salinity at MO and MY (absolute values of salinity are log+1 transformed, not minimum and maximum values). MO : « Réserve Naturelle Nationale de Moëze-Oléron » and MY : « Réserve Naturelle Nationale du marais d'Yves »

Time scale	Site	Variable	Covariate	Estimate	SE	t-value	p-value
Seasonal	MO	Salinity	Month	0,006	<0,001	13,14	<0,001
	MO	Salinity	Month ²	-0,0003	<0,001	-10,41	<0,001
	MY	Salinity	Month	0,008	<0,001	13,47	<0,001
	MY	Salinity	Month ²	-0,0006	<0,001	-12,31	<0,001
Inter-annual	MO	Minimum salinity	Year	0,09	0,031	2,935	0,003
	MO	Mean salinity	Year	<0,001	<0,001	0,991	0,322
	MO	Maximum salinity	Year	-0,033	0,055	-0,606	0,545
	MY	Minimum salinity	Year	-0,481	0,042	-11,49	<0,001
	MY	Mean salinity	Year	<-0,001	<0,001	-30,48	<0,001
	MY	Maximum salinity	Year	-1,6161	0,106	-15,18	<0,001

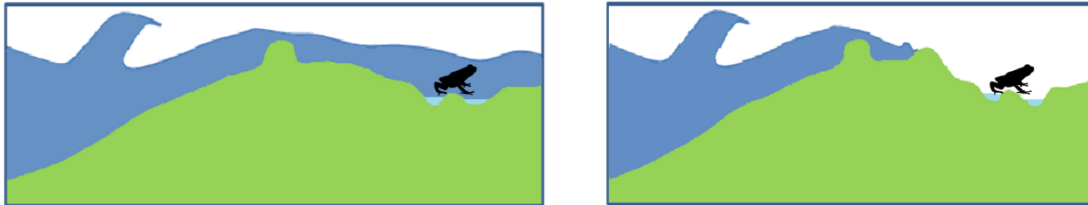
Article II

Slight variations in coastal topography mitigate the consequence of storm-induced marine submersion on amphibian communities

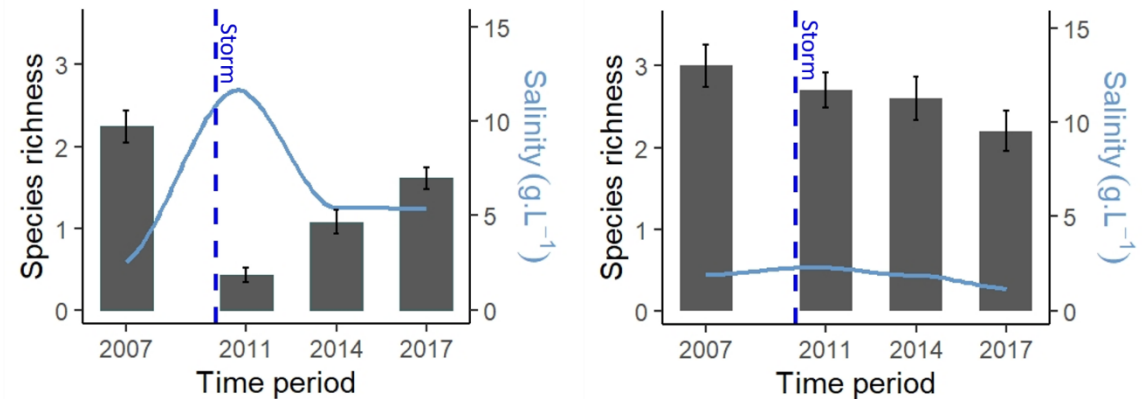
Léa Lorrain-Soligon¹, Frédéric Robin^{2,3,4}, Pierre Rousseau³, Marko Jankovic⁴, François

Brischoux¹

Small topography differences inducing different submersion patterns



Inducing site specific responses in salinity and species richness following storm Xynthia



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Slight variations in coastal topography mitigate the consequence of storm-induced marine submersion on amphibian communities

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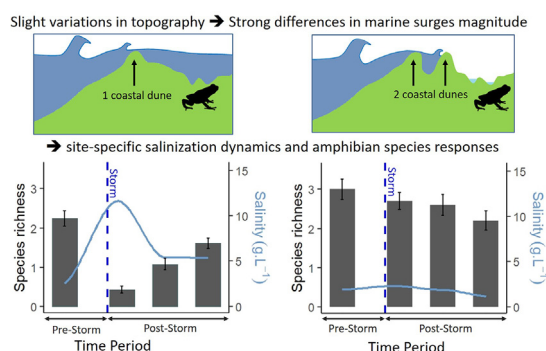
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HIGHLIGHTS

- Coastal marine submersions are expected to increase in frequency and intensity.
- Such extreme weather events threaten coastal wetlands.
- Long-term surveys pre/post marine submersion revealed site-specific responses.
- Variations in topography influenced salinization and associated wildlife responses.
- Topographical features can buffer consequences of such extreme weather events.

GRAPHICAL ABSTRACT



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ABSTRACT

The rise in sea-level and the increase in frequency and intensity of extreme weather events (i.e., storms and associated surges) are expected to strongly impact coastal areas. The gradual impacts of sea-level rise may allow species to display adaptive responses to overcome environmental changes. In contrast, the abruptness of marine submersions during extreme weather events can induce changes that may exceed the ability of species to respond to brutally changing environments. Yet, site-specific topographical features may buffer the expected detrimental effects of marine submersions on wildlife. In order to test such topographical effects, we examined the long-term consequences of a major marine submersion (storm Xynthia) on the amphibian communities of two French Atlantic coastal wetlands that slightly differ in their topography and, thus, their susceptibility to marine submersion. Amphibians were monitored on 64 ponds for up to 13 years, using acoustic and visual methods, in conjunction with environmental parameters (e.g., pond topology, vegetation, salinity). We found that the amphibian communities at the two neighboring sites displayed different responses to the marine submersion linked to storm Xynthia. As predicted, slight differences in local topography induced strong differences in local magnitude of the landward marine surge, influencing salinization dynamics and associated consequences on wildlife (amphibians). The different species responses show that amphibian richness can recover to that of pre-storm conditions, but with significant changes in the composition of the community. Our results suggest that amphibian presence post-submersion in coastal wetlands results from an interaction between species traits (e.g., tolerance

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to elevated salinity), site-specific topography, and environmental parameters. Finally, our study emphasizes that relatively modest landscaping management may be critical to allow wildlife to successfully recover after a marine submersion.

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1. Introduction

Anthropogenic activities have led to unprecedented rates of global climate change (Vitousek et al., 1997). Among the multiple consequences of climate change, the progressive rise in sea-level, as well as the increase in frequency and intensity of extreme weather events (i.e., storms and associated surge), are expected to strongly impact biodiversity and particularly in coastal areas (McLean et al., 2001; Herbert et al., 2015; Wang et al., 2016; Maxwell et al., 2019; Chen et al., 2020). Although coastal wetlands are recognized as some of the most diverse and productive habitats on earth (Denny, 1994; McLean et al., 2001), they are currently particularly vulnerable because of several interacting threats (i.e., agriculture, land use, urbanization, sea level rise) (McLean et al., 2001).

Gradual changes of habitats linked to sea-level rise are expected to strongly affect coastal wetlands (Neubauer and Craft, 2009; Traill et al., 2011; Geselbracht et al., 2015). Yet, the relatively long temporal scale over which these changes are expected to occur may allow species to display adaptive responses. For instance, relatively mobile species could progressively adjust their coastal distribution, and thus gradually disperse to more suitable habitats (Convertino et al., 2012). Coastal wetland salinization is one of the main environmental changes linked to sea-level rise (Herbert et al., 2015), and associated osmoregulatory challenges are known to negatively affect wildlife (Greenberg et al., 2006; Pedersen et al., 2014; Gutiérrez, 2014; Rivera-Ingraham and Lignot, 2017). Nonetheless, progressive increases in salinity, such as those due to a gradual rise in sea levels, can allow species to display adaptive responses. Indeed, such responses have already been shown in various organisms such as fishes (Purcell et al., 2008), amphibians (Gomez-Mestre and Tejedo, 2003), aquatic snakes (Brischoux et al., 2017), waterbirds (Gutiérrez, 2014) and rodents (Getz, 1966).

In contrast, the sudden and unpredictable nature of marine submersions during extreme weather events can induce changes that may exceed the ability of species to disperse toward more suitable habitats and/or adapt to a new set of environmental conditions, especially when marine submersion events induce direct and brutal salinization of inland soil and water (Hopkins and Brodie, 2015; Wang et al., 2016). These short pulses of high salt concentrations may lead to extreme mortality events (Gunzburger et al., 2010; Herbert et al., 2015), in contrast to exposure to moderate salinity levels during a longer time period (Marshall and Bailey, 2004). Yet, to our knowledge, very few studies have examined the effects of extreme events, such as storm-induced marine submersions on wildlife from coastal areas (but see Greenberg et al. (2006); Ameica y Juárez et al. (2013)), especially in the context of the salinization induced by these marine submersions (but see Kokelj et al. (2012)).

Importantly, specific geographical features of coastal wetlands may dampen the expected effects of marine submersions on these ecosystems (Loder et al., 2009; Wamsley et al., 2010). For instance, low-lying coastal wetlands without relief should be particularly exposed to marine submersions (Eliot et al., 1999; Wang et al., 2012), while the presence of barriers may dampen the effects of extreme weather events (McLean et al., 2001). As a consequence, site-specific topographical features may buffer the expected detrimental effects of marine submersions on wildlife. Given the forecasted increase in the frequency and intensity of such extreme weather events (Dettinger, 2011), identifying such topographical features may prove useful for proposing landscaping solutions that aim to preserve these essential coastal ecosystems.

We examined the consequences of a major marine submersion (Xynthia) on Atlantic coastal wetlands in France. Xynthia was an exceptionally violent windstorm which crossed the Western European coast between 27 February and 1 March 2010, leading to the submersion of a large number of coastal sites for a few days to a few weeks (Durant et al., 2018). In order to identify the consequences of marine submersions linked to storm Xynthia, we investigated coastal amphibian communities, because these taxa display several traits that should increase their susceptibility to marine surges. For instance, amphibians are known to be highly sensitive to salinity levels because of their permeable skin and their comparatively low ability to osmoregulate in hyperosmotic environments (Shoemaker et al., 1992; Wells, 2007). Increasing salinity is known to decrease survival for amphibians across all life stages (Albecker and McCoy, 2017), but this is especially true for eggs and tadpoles that develop in aquatic environments (Hopkins and Brodie, 2015; Albecker and McCoy, 2017; Welch et al., 2019). In addition, they are characterized by low dispersal capacities (Wells, 2007), thereby affecting their ability to evade sudden detrimental conditions. Although several studies have assessed the effects of salinization on amphibians, particularly on tadpoles (Christy and Dickman, 2002; Smith et al., 2007; Hopkins and Brodie, 2015), less work has been performed on their long-term responses to marine submersions (but see Gunzburger et al., 2010), presumably because such an approach requires logistically complex surveys encompassing pre- and post-storm monitoring (Walls et al., 2019).

In this study, we monitored the amphibian community during a time period (13 years) that surrounded storm Xynthia, in the ponds of two neighboring natural reserves on the Western Atlantic coast of France. Both sites are relatively low coastal wetlands displaying a coastal sand dune close to the sea limit. Importantly, these two neighboring coastal sites display slightly different topographies with one of them having an additional sand barrier. We posited that these small differences in topography should influence the magnitude of landward marine surge and determine the responses of amphibians. We specifically predicted that:

- ponds from the more protected site should be less affected by marine surge and, thus, should display relatively low and constant salinity levels across time, while ponds from the less protected site should be characterized by elevated salinity levels post-storm.
- amphibian richness should be negatively affected by salinity levels, and should decline post-storm in the less protected site.
- species more tolerant to salinity should recover rapidly following a marine surge while species more sensitive to osmotic stress may be extirpated.

2. Material and methods

2.1. Study sites

The study was carried out on two reserves in Western France: one in the "Réserve Naturelle Nationale de Moëze-Oléron" (45°53'33.36"N, 1°04'59.16"W, hereafter MO, Fig. 1), and one other in the "Réserve Naturelle Nationale du marais d'Yves" (46°2'40.735"N, 1°3'16.906"W, hereafter MY, Fig. 1). These two study sites, separated by ~15 km straight line, are situated on the Atlantic coast of France (Département de la Charente-Maritime), and cover a similar surface area (218 ha for

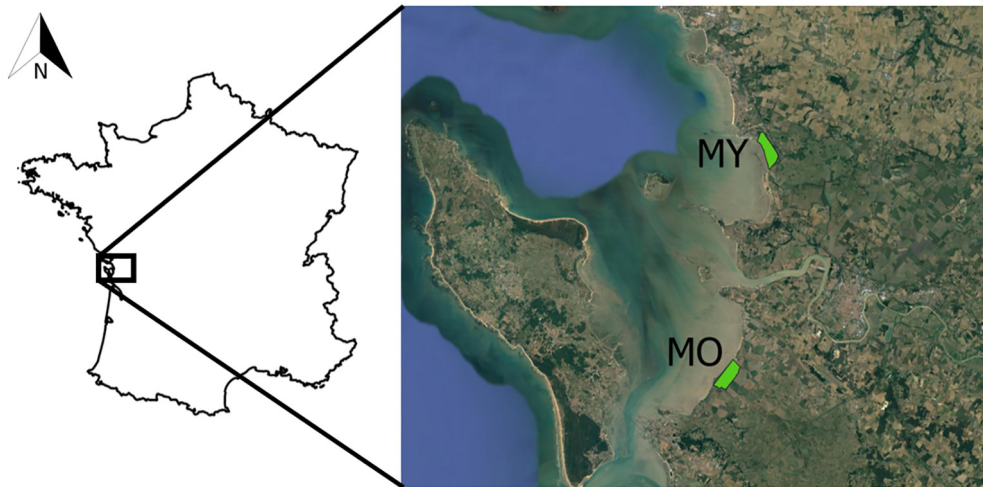


Fig. 1. Map of the study area relative to Western France illustrating the two study sites, using an OpenStreetMap basemap. Green areas represent the two study sites (MY and MO). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

MO and 204 ha for MY, Fig. 1). The two reserves were hit by Xynthia on 27 February 2010, with the same intensity (maximum wind intensity of 130–140 km/h, see Météo France (<http://tempetes.meteo.fr/spip.php?article132>) and Appendix A).

Both sites are relatively low (mean elevation: MY = 2.64 m \pm 0.02 SE; MO = 2.90 m \pm 0.01 SE) coastal wetlands displaying a coastal sand dune (3.30 m high both in MY and MO) situated nearby the high sea limit (Fig. 2), which is followed by typical coastal wetlands composed of a matrix of salt marsh, meadows, and freshwater ponds. Importantly, these two neighboring coastal sites display slightly different topographies with MO having an additional barrier (a fossil sand dune) running parallel to the shore and situated ~500 m inland (Fig. 2). The two sites are exposed to an oceanic climate (Cfb according to Köppen classification).

2.2. Sampling

2.2.1. Periods of sampling

For each site, amphibian monitoring in breeding ponds was performed both before and after storm Xynthia. Specifically, sampling occurred a few years before Xynthia (in 2007 and 2008 for MY and MO, respectively; hereafter “pre-storm”), the year after Xynthia (in 2011 for both sites, hereafter “1 year post-storm”), 4 years after Xynthia (in 2014 for both sites, hereafter “4 years post-storm”) and again a few

years later (in 2017 and 2019 for MY and MO respectively; hereafter “more than 4 years post-storm”).

2.2.2. Sampling ponds

For each site, amphibian presence was monitored in natural breeding ponds during the spring (see Section 2.2.3). At MY, 54 ponds were monitored during the 4 time periods mentioned above. These breeding sites vary in their size, ranging from 36 m^2 to 156,824 $\text{m}^2</math>. These ponds are situated at an average distance of 556 m (± 24 m SE) from the ocean, ranging from 27 to 832 m. At MO, 10 ponds were monitored during the 4 time periods mentioned above. The size of these sites ranges from 20 $\text{m}^2</math> to 70 $\text{m}^2</math>. Their position relative to the ocean is 429 m (± 36 m SE), ranging from 262 m to 637 m. The positions of the ponds relative to site-specific topography are represented in Fig. 2.$$$

2.2.3. Monitoring of amphibian presence

In order to take differences in phenology and detectability of the amphibian species occurring at each breeding pond into account, we performed three successive monitoring sessions for each pond, according to Petitot et al. (2014). These surveys occurred in the spring during the breeding period of amphibians in Western France (Miaud and Muratet, 2018). In March and April, surveys were conducted at night (2 h after sunset) during the activity peak of breeding amphibians; while for the last session (May), surveys occurred during daytime in

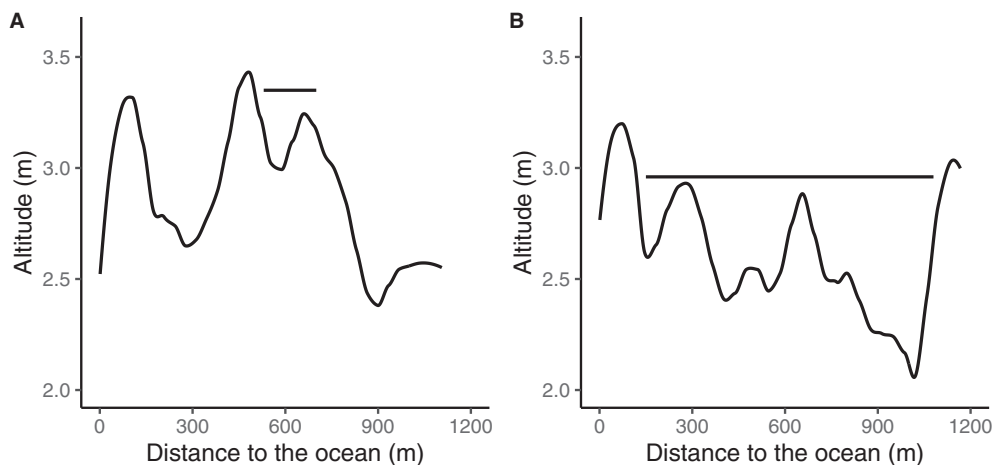


Fig. 2. Coastline topographic profile of MO (A) and MY (B) highlighting the presence of the additional small dune in MO. The relative position of the pond surveyed is indicated by a horizontal line and shows that ponds in MO were all situated behind the additional sand dune.

order to visually assess indices of effective reproduction (i.e., egg and tadpole presence).

At each site, two complementary survey methods were performed successively at each session. First, an acoustic survey was performed for 5 min at a distance of four meters from the pond edges. This method allowed us to assess species presence based on mating or territorial calls. Second, a visual survey was performed during another 5 min by slowly walking around the pond edge with a headlamp to detect individuals present nearby the pond edge (on land or in water). These two methods were occasionally (in order to limit the destruction of aquatic vegetation) complemented by the use of a net in order to assess the presence of the most secretive species, but it did not yield additional species that were not detected by the two methods described above.

2.2.4. Environmental parameters

For each pond, we recorded 9 environmental parameters: Water salinity, type of habitats, habitat permanence, area of the pond, shortest distance to the ocean, surrounding vegetation, aquatic vegetation, number of other species found, and water depth (see below). These parameters were chosen because of their possible impact on amphibian presence and diversity (Başkale and Kaya, 2009; Shulze et al., 2012; Hopkins and Brodie, 2015). Each parameter was recorded each year of survey, allowing to determine differences between years, and differences pre- and post-submersion. Water salinity (g.l^{-1}) of the breeding ponds was recorded in May using a Multi 340i multimeter (WTW, Xylem analytics). At MY, salinity was measured at 53 out of the 54 ponds monitored, while at MO, salinity was measured at 5 out of the 10 ponds surveyed. For each pond, we also characterized the type of habitat (pond or ditch), the habitat permanence (temporary or permanent water body), the area of the pond and the shortest distance to the ocean (both evaluated using QGIS 3.4.7), the surrounding vegetation (expressed in percentage cover), the aquatic vegetation (with a score ranging from 0 to 3, adapted from Lachavanne et al. (1995) and corresponding to diversity, i.e. number of species which can be differentiated, and water surface occupied by aquatic vegetation, evaluated visually, in April), the number of other species found in the pond (fishes, insect larvae, and crayfish), and the water depth (measured with a ruler in April).

2.3. Data analysis

For species presence, we used presence/absence data (coded as 1 and 0 respectively), and considered a species to be present for a given year in a given pond if we had detected the presence of adults (either visually or acoustically), eggs, or larvae during at least one of the surveys.

To determine whether there were differences in the number of species between sites and time period, and variations of salinity between sites and time periods, we ran GLMs (General Linear Models) with site, time period, and their interaction as predictors. We computed GLMs on the total number of species present at each site and on salinity, using a Poisson and a Gaussian distribution, respectively. These distributions were chosen based on the fit to our data, and by checking overdispersion of residuals, and effects were assessed with a backward model selection using chi-squared tests. We computed Dunnett post-hoc tests on our GLMs using the “multcomp” package in order to assess within and across site and time period differences. These models could not be computed for each species separately, and species responses were investigated using Kruskal Wallis and Dunn post hoc test (package “PMCMR”). Multiplicity of responses (as the analyses included only 0 and 1) were corrected for multiplicity with the Holm correction, to account for ex aequo.

In addition, to determine whether environmental parameters affected amphibian presence at each site, we also tested the effects of our 9 environmental variables (see Section 2.2.2) on species presence (except for type of pond and permanence in MO, because ponds are all temporary and pond stricto sensu), using GLMs with a Poisson

regression. In these models, we first implemented the whole set of environmental parameters, for which correlations were tested with a Pearson correlation test. Variables were standardized before analyses. Beginning from a model with 9 parameters, we then carried out a backward model selection using Chi-square tests (Bolker et al., 2009).

All data analyses were performed using R 3.6.3 (R Core Team, 2020) and Rstudio v1.1.419.

3. Results

3.1. Salinity

We found an effect of the site, time period, and their interaction on salinity (Table 1, Fig. 3). More specifically, pre-storm salinity was similar between MO and MY (Fig. 3). Salinity was significantly higher at MY 1 year post-storm than either pre-storm values at MY or 1 year post-storm at MO (Fig. 3). Salinity at MY significantly decreased 4 years post-storm and more than 4 years post-storm but remained higher than pre-storm values and salinity measured at MO for the same periods (Fig. 3). In contrast, salinity at MO remained steady across time periods (Fig. 3).

3.2. Species richness

At MY, these surveys led to the detection of 6 different species composed of 5 anurans (*Pelophylax* sp. [Green frogs], *Hyla meridionalis* [Mediterranean tree frog], *Pelodytes punctatus* [Common parsley frog], *Bufo spinosus* [Spined toad] and *Pelobates cultripes* [Western spadefoot toad]) and 1 urodele (*Triturus marmoratus* [Marbled newt]).

At MO, surveys allowed to detect 6 species composed of 4 anurans (*Pelophylax* sp. [Green frogs], *Hyla meridionalis* [Mediterranean tree frog], *Pelodytes punctatus* [Common parsley frog] and *Pelobates cultripes* [Western spadefoot toad]) and 2 urodeles (*Lissotriton helveticus* [Pal-mate newt] and *Triturus marmoratus* [Marbled newt]).

Interestingly, 5 of these species were found at both of our study sites.

We found an effect of the site, time period, and their interaction on species richness (Table 2, Fig. 4). More specifically, pre-storm species richness was similar between MO and MY (Fig. 4). Species richness was significantly lower at MY 1 year post-storm than either pre-storm values at MY or 1 year post-storm at MO (Fig. 4). Species richness at MY increased post-storm progressively attaining similar values than MO more than 4 years post-storm, and regaining pre-submersion levels (Fig. 4). At MO, we observed a slight but not significant decrease of species richness across time periods (Fig. 4).

3.3. Species responses

In MY, the presence of all species strongly decreased post-storm (Fig. 5). Some species (*Pelophylax* sp., *Hyla meridionalis*, *Pelodytes punctatus*) recovered pre-storm level 4 years post-storm or more than 4 years post-storm (Fig. 5ABC). Two species (*Pelobates cultripes*, *Triturus marmoratus*) disappeared (Fig. 5DE), and one species (*Bufo spinosus*) appeared for the first time more than 4 years post-storm (Fig. 5F).

In MO, most species did not seem to be directly impacted by storm Xynthia (Fig. 5). More specifically, *Pelophylax* sp., *Hyla meridionalis*, *Pelodytes punctatus*, *Pelobates cultripes* and *Triturus marmoratus* showed stable trends over time (Fig. 5GHJ). Interestingly, the two urodele

Table 1
Effect of the site, time period, and their interaction on salinity.

Tested variable	Parameters	Df	Deviance	AIC	Scaled deviance/LRT	p-Value
Salinity	Time period	3	4265.6	1180.7	99.748	<0.001
	Site	1	2928.8	1109.7	24.925	<0.001
	Time period:site	7	4565.4	1192.2	122.54	<0.001

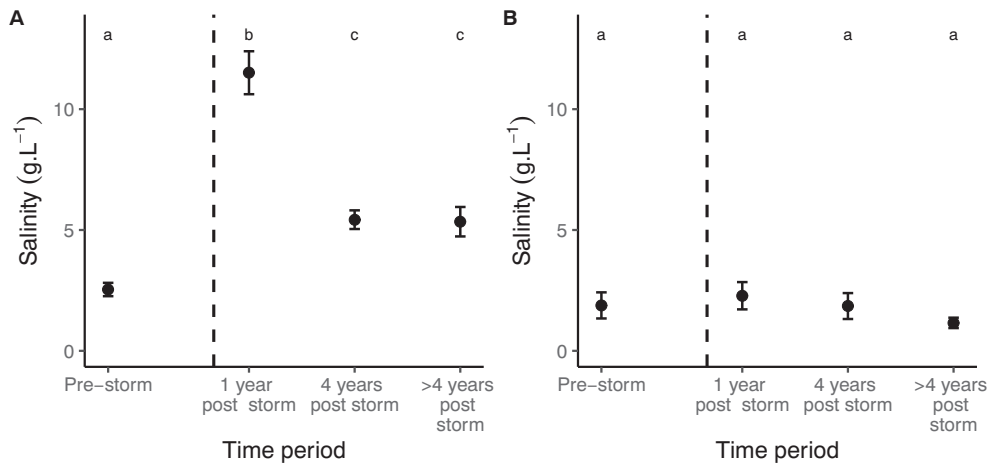


Fig. 3. Mean (\pm SE) salinity measured for the four time periods of our surveys at MY (A) and MO (B). The vertical dashed line represents the year of storm Xynthia (2010). Different letters represent a significant difference at $\alpha = 0.05$.

Table 2
Effect of the site, time period, and their interaction on species richness.

Tested variable	Parameters	Df	Deviance	AIC	Scaled deviance/LRT	p-Value
Species richness	Time period	3	295.36	784.92	56.547	<0.001
	Site	1	270	763.55	31.186	<0.001
	Time period: site	7	326.55	814.1	111.43	<0.001

species displayed change over time, but these changes did not seem to be related to storm Xynthia (Fig. 5KL).

Detailed statistical analyses can be found in appendices B and C.

3.4. Environmental parameters influencing diversity

In MY, 4 parameters out of the 9 tested were found to be significant, with higher amphibian diversity found in ponds (rather than ditches) with low salinity levels, low levels of surrounding vegetation, and high aquatic vegetation (Table 3). All other tested parameters (pond permanence, pond area, distance to the ocean, depth, or presence of other species) were not retained during the selection procedure.

In MO, we did not observe an effect of any of the 9 tested parameters (Table 3, only the results for the same parameters that were significant for MY are presented for the sake of comparison).

4. Discussion

Overall, we found that two neighboring coastal wetlands displayed different responses to the marine submersion linked to storm Xynthia. As predicted, slight differences in local topography (i.e., an additional sand dune at MO, Fig. 2) induced strong differences in the local magnitude of landward marine surge, influencing salinization dynamics and associated consequences for wildlife (amphibians).

The protective aspect of the additional sand dune at MO (Fig. 2) is exemplified by the salinity levels measured before and after storm Xynthia. Indeed, at this site, mean salinity remained steady across a relatively long time period (11 years), with no increase in salinity one year post-storm, suggesting that the marine surge did not reach the studied ponds. The constant salinity measured at this site further suggests that salinization of nearby land has not induced progressive salt percolation across soil layers (i.e. salt infiltration in groundwater, Gunzburger et al., 2010). In contrast, at MY (the less protected site displaying a single coastal sand dune), salinity measured at the studied ponds drastically changed following storm Xynthia. Although pre-storm salinity was

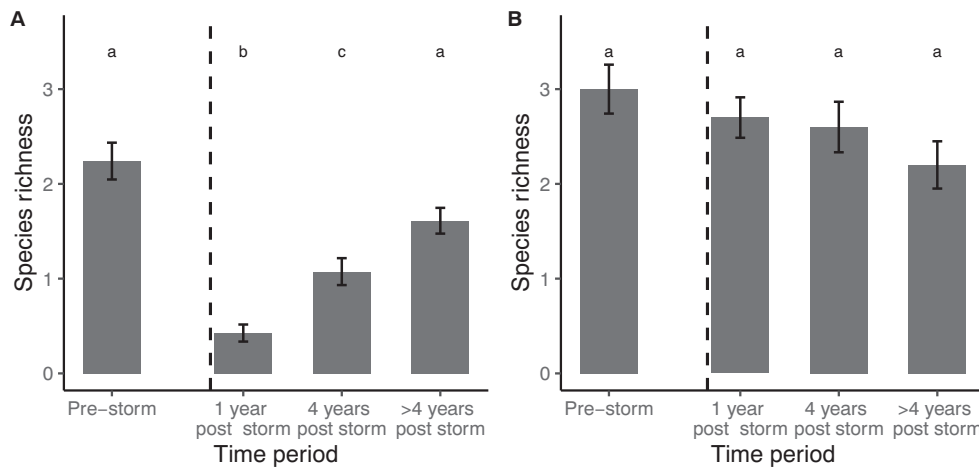


Fig. 4. Mean (\pm SE) amphibian diversity measured for the four time periods of our surveys at MY (A) and MO (B). The vertical dashed line represents the year of storm Xynthia (2010). Different letters represent a significant difference at $\alpha = 0.05$.

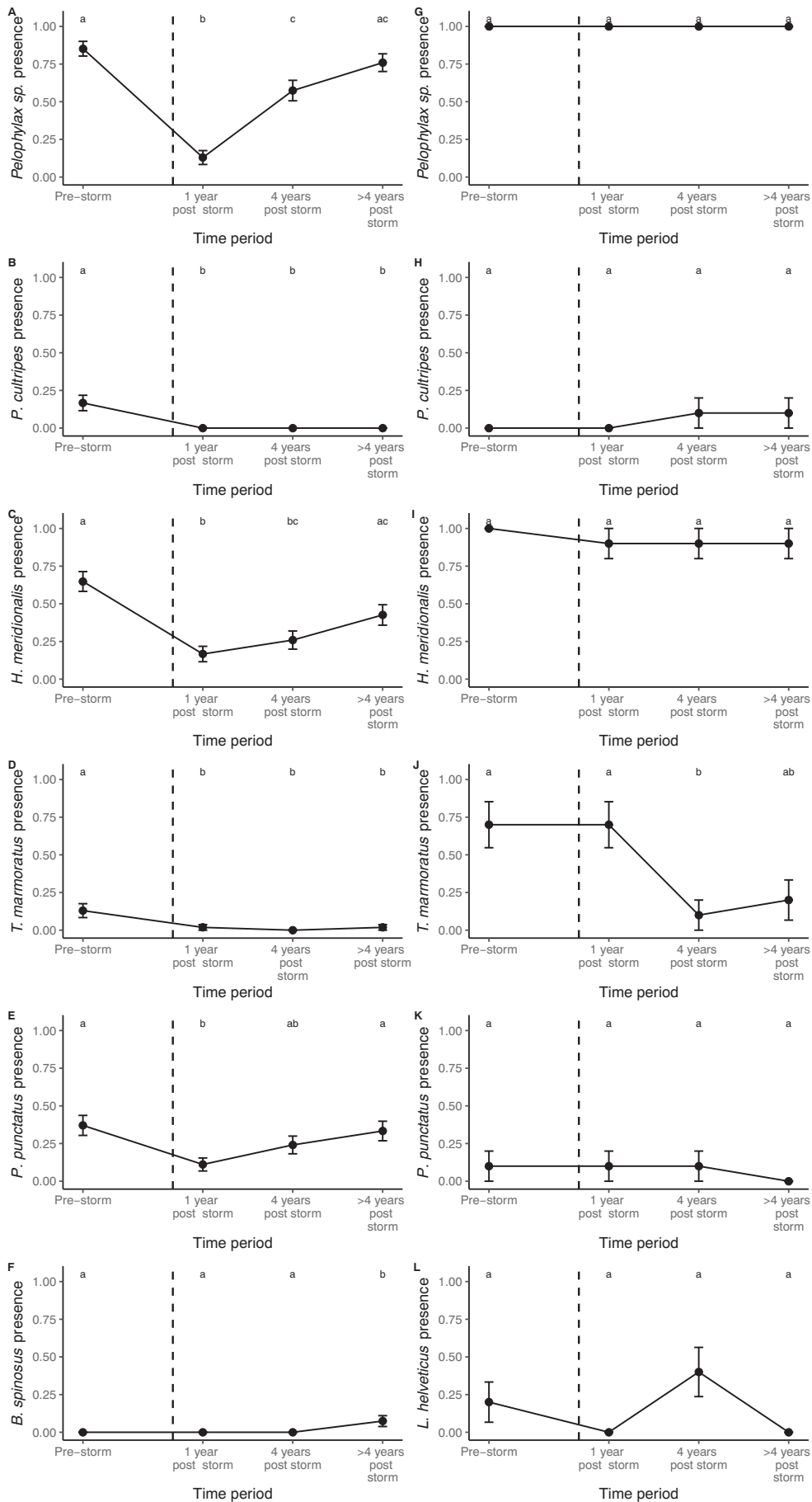


Table 3

Influence of habitat characteristics on species presence. Only significant factors are shown for MY. The same factors (although not significant) are shown for MO for comparison.

Site	Parameters	Estimate	SE	z	p
Marais d'Yves	Typology (pond)	0.68	0.159	4.269	<0.001
	Surrounding vegetation	-0.226	0.099	-2.269	0.023
	Aquatic vegetation	0.273	0.082	3.311	<0.001
	Salinity	-0.147	0.073	-2.025	0.042
Moëze-Oléron	Typology (pond)	-	-	-	-
	Surrounding vegetation	0.057	0.17	0.337	0.736
	Aquatic vegetation	0.118	0.303	0.388	0.698
	Salinity	0.072	0.178	0.405	0.686

low and similar to MO, the landward marine surge induced a massive increase in salinity, reaching a value of $\sim 11 \text{ g.l}^{-1}$ one year post-storm. These elevated salinity levels decreased post-storm (Fig. 3) but remained relatively high (5 g.l^{-1}) and did not decrease back to pre-storm conditions even 7 years post-storm. This result suggests potentially long-lasting influences of marine surges on coastal wetland salinity and contrasts with other studies showing rapid salinity decrease after storms (Frazer et al., 2006). Further studies are required in order to investigate the site-specific salinization dynamics linked to landward marine surges.

Similar to salinity, amphibian species richness responded differently at the two study sites. At MO, where the salinity of ponds remained low and constant, amphibian richness remained relatively constant across the study period for most species. Nevertheless, at this site, two urodele species displayed strong fluctuations over time (*T. marmoratus*, *L. helveticus*), but these fluctuations were not attributable to Xynthia, and may be linked to the increasing presence of non-native Louisiana crawfish, *Procambarus clarkii*, known to affect urodeles (Cruz et al., 2006). In addition, none of the pond characteristics we included in our analyses significantly explained the number of species observed, probably because most ponds are characterized by very similar features. In strong contrast, at MY, amphibian richness seems to have been affected by the change in salinity linked to Xynthia. Indeed, the mean number of species per pond showed a massive decrease following storm Xynthia, reaching the lowest values (~ 0.43 species per ponds) one year post-storm. Amphibian richness then progressively increased, attaining values similar to pre-storm conditions, 7 years after the storm. Importantly, salinity was a significant predictor of the number of species observed in ponds, suggesting that the increase in salinity due to storm Xynthia was responsible for the decrease in species richness found at MY. There are three different, but not mutually exclusive hypotheses to explain this result. First, the high salinities linked to storm Xynthia may have directly affected amphibians by inducing mortality of juvenile and adult individuals (Albecker and McCoy, 2017). Second, relatively high salinity values in breeding ponds may have inhibited the ability of surviving adults to reproduce (Albecker and McCoy, 2017). Third, reproduction may have occurred, but high salinity of breeding ponds may have induced direct mortality of developing embryos and/or larvae (Christy and Dickman, 2002; Kearney et al., 2014; Hopkins and Brodie, 2015). Indeed, some amphibian species are relatively tolerant to salinity and may be able to reproduce in brackish conditions (Hopkins et al., 2020). Yet, even in relatively salt-tolerant species, eggs and larvae have decreased survival linked to salinity (Natchev et al., 2011). These three hypotheses involve different processes with different consequences for population dynamics (e.g., affecting adult survival versus reproduction). Future studies should investigate the relative contributions of these different possible responses on the influence of marine submersions on coastal amphibian communities.

Despite the strong influence of storm Xynthia on amphibians at MY, some studies have highlighted that recovery can occur (Schriever et al., 2009; Gunzburger et al., 2010; Wang et al., 2016; Maxwell et al., 2019; Chen et al., 2020), and it is noteworthy in our study that the amphibian community did recover to pre-storm conditions 7 years after the submersion. Yet, different species diverged strongly in their ability to recover from the negative impact of storm Xynthia (Fig. 5). Most notably, three species were clear drivers of the increase in amphibian richness back to pre-storm conditions. These species were *Pelophylax* spp., *H. meridionalis* and *P. punctatus*, and have all been described as salt tolerant (Sillero and Ribeiro, 2010; Natchev et al., 2011; Thirion, 2014; Hopkins and Brodie, 2015). In our study sites, these species can be found in water bodies with markedly elevated salinities (i.e., maximum salinity of 11.8 g.l^{-1} for *P. punctatus*, 13.7 g.l^{-1} for *H. meridionalis* and 17.1 g.l^{-1} for *Pelophylax* sp.). In contrast, two species were lost, or almost lost, after storm Xynthia (i.e., *P. cultripes*, *T. marmoratus*). *T. marmoratus* is a newt, that is characterized by comparatively lower mobility (Wells, 2007). Such a trait may have prevented adult individuals from evading detrimental conditions during storm Xynthia, or may have impeded other individuals from less impacted nearby areas to recolonize our study sites in MY. The reasons for the disappearance of *P. cultripes* are less clear, because this species is known to be relatively tolerant to salinity (Stănescu et al., 2013; Thirion, 2014). Because *P. cultripes* is a burying species which shelters in sandy soils, it is possible that sheltered individuals may have died during the submersion. Finally, it is noteworthy that a new species, the Spined toad (*Bufo spinosus*), appeared for the first time more than 4 years after submersion, highlighting that the colonization of some individuals from new species can occur following environmental perturbations (pattern also shown in Gunzburger et al. (2010), were some species are only present in overwashed wetlands post-storm).

These different species-specific responses highlight that, although amphibian richness could recover to that of pre-storm conditions, the composition of the community can change (Marroquín-Páramo et al., 2020; Stockwell et al., 2020), with one third of the species being lost at MY after Xynthia. Interestingly, because several pond characteristics were shown to be positively related to amphibian diversity (e.g., pond type, aquatic vegetation), it is likely that such features may positively influence either species resilience to environmental perturbations (Bernhardt-Römermann et al., 2011; Suazo-Ortuño et al., 2018; Marroquín-Páramo et al., 2020) or to favor recolonization from nearby and less impacted sites [i.e. shelters (Walsh, 1983)]. This may be the case for *P. cultripes*, which appeared in MO post-storm, while it was the first to disappear in MY, suggesting that MO could be one of these shelters for surrounding sites. Future studies should investigate the interactions between salt-tolerance and ecological characteristics (e.g., micro-habitat use, mobility) in order to understand the drivers of the species-specific responses that we have highlighted.

5. Conclusions

Our study is, to our knowledge, the first multi-species long term survey of the consequences of marine submersion induced salinization on coastal amphibians (but see Gunzburger et al. (2010)). Most notably, we highlight that the presence of amphibians in coastal wetlands post-submersion results from an interaction between species-specific traits (tolerance to elevated salinity, ecology) and site-specific characteristics (topography). This latter category is especially interesting to take into account at a time when the frequency and intensity of extreme weather events, such as marine submersion, are expected to increase (Dettinger, 2011). Habitats management can promote adaptation to

Fig. 5. Mean (\pm SE) species presence measured for the four time periods of our surveys at MY (left panels) and MO (right panels). A, G: *Pelophylax* sp., B, H: *Hyla meridionalis*, C, I: *Pelodytes punctatus*, D, J: *Pelobates cultripes*, E, K: *Triturus marmoratus*, F: *Bufo spinosus* and L: *Lissotriton helveticus*. The vertical dashed line represents the year of storm Xynthia (2010). Different letters represent a significative difference at $\alpha = 0.05$.

extreme climatic events (Maxwell et al., 2019) and, in this respect, our study shows that relatively modest landscaping management (i.e., the creation of additional barriers similar to the small sand dune found at MO) may be critical to allow amphibians to successfully recover after a marine submersion, either by providing a barrier to marine surge and/or by increasing the surface area of land that remains unsubmerged, providing an elevated refuge. Clearly, it is plausible that such a refuge may also provide protection for other wildlife susceptible to marine submersion and may, thus, offer a cost-effective solution to buffer the detrimental consequences of marine submersion on coastal wetlands.

CRedit authorship contribution statement

FB & FR: Conceptualization; PR & MJ: Data collection; FR: Data curation; LLS, FR, FB: Formal analysis; FR & FB: Funding acquisition; LLS, FB, FR: Writing - original draft; LLS, FB, FR, PR & MJ: Writing - review & editing. All authors have reviewed and edited the manuscript and approved the final version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability statement

Data will be made available upon reasonable request.

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2021.145382>.

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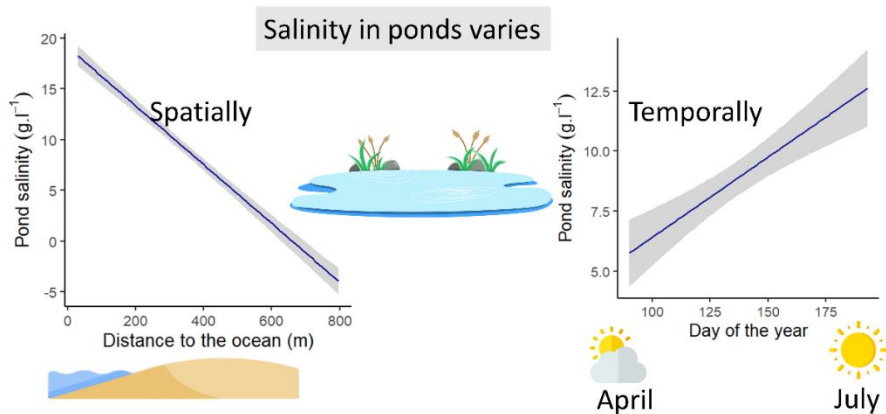
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Article III

Some like it salty: Spatio-temporal dynamics of salinity differentially affect anurans and caudates in coastal wetlands

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A process which will differentially impact amphibian species

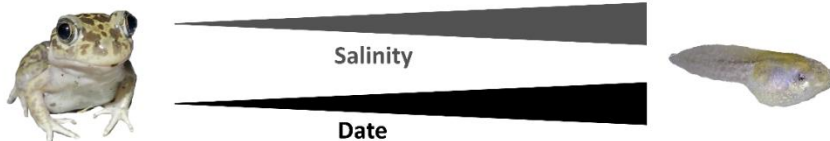


Found up to 16 g.l⁻¹ salinity



Found up to 3.5 g.l⁻¹ salinity

And disrupts the match between salinity selected by adults and those experienced by their offspring




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Some like it salty: Spatio-temporal dynamics of salinity differentially affect anurans and caudates in coastal wetlands

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Abstract

1. Coastal wetlands are naturally subjected to salinity, which is expected to increase through global climate changes. Most species will be affected by these changes, leading to major consequences for community structure and ecosystem functioning.
2. We investigated variation of salinity of temporary wetlands across spatial (1,000m from the ocean) and temporal (across three breeding season) scales relevant to coastal biodiversity and used amphibians (six species, sampled across one breeding season) as a model to investigate the consequences of the spatio-temporal variation of salinity in 24 ponds situated on the Atlantic coast of France.
3. We show that salinity is a highly dynamic environmental variable that varies widely both across spatial and temporal scales. The spatio-temporal dynamics of salinity are a critical factor structuring amphibian communities that affect the main amphibian phylogenetic groups (caudates vs. anurans) differently. Temporal variation in salinity disrupts the match between salinity selected by reproductive adults and those experienced later by their developing offspring, which negatively affect reproductive success.
4. Future changes in coastal salinity are likely to affect the structure and functioning of these ecosystems, excluding salt-intolerant species and eventually leading to less diverse communities of salt-tolerant species.

KEYWORDS

abundances, amphibians, life stages, reproductive success, species richness

1 | INTRODUCTION

Coastal wetlands are among the most diverse and productive ecosystems on earth (Hobohm et al., 2021). Situated at the interface between land and open sea (Janas et al., 2019), coastal areas are composed of a complex mosaic of biotopes concentrated across

restricted spatial scales, and shelter a large diversity of species and habitats (Janas et al., 2019; Maynard & Wilcox, 1997). In addition to their rich biodiversity (Maynard & Wilcox, 1997), coastal wetlands also provide ecosystem services with key role in oxygen and nutrient fluxes and primary production (Janas et al., 2019). However, coastal wetlands are particularly vulnerable to ongoing global

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changes (Barua et al., 2021; Hobohm et al., 2021; Martínez-Megías & Rico, 2021).

Among the various factors that affect these rich habitats, salinisation is an important – yet understudied – threat (Cunillera-Montcusí et al., 2022). Coastal wetlands are naturally subjected to spatially and temporally variable salinity mainly as a result of landward sea spray deposition, which creates a spatial and temporal salinity gradient (Benassai et al., 2005; Estévez et al., 2019; Fu et al., 2021; Ranjbar & Ehteshami, 2019). However, salinity is expected to increase locally as a result of several processes all linked to anthropogenic activities and ongoing climate changes (Le et al., 2019; Reid et al., 2019). In addition to the impacts linked to sea-level rise (Church & White, 2011; Vermeer & Rahmstorf, 2009), extreme storm events (Maxwell et al., 2019) and specific anthropogenic activities (i.e., groundwater pumping; Peters et al., 2021; Reid et al., 2019), salinity also can increase in temperate areas as a consequence of current modifications of climatic conditions (i.e., increased temperature and decreased precipitation; Çolak et al., 2022; Schroeder et al., 2017). Freshwater inputs to coastal wetlands are reduced because of decreasing riverine flows (Schroeder et al., 2017). Complementarily, evaporation can seasonally exceed precipitations – especially in spring and summer – and thus increase the salinity of coastal wetlands subjected to moderate salt input by sea sprays (Benassai et al., 2005). Taken together, all of these processes are expected to significantly affect the heterogeneity and associated spatio-temporal dynamics of salinity (Ghalambor et al., 2021).

The importance of considering salinisation and its consequences for wildlife is linked to the fact that most species have to regulate the osmolality of their body fluids in order to survive (Hoffmann et al., 2009; Potts, 1954). Most species display physiological mechanisms that aim at regulating water and ion fluxes to maintain homeostasis (i.e., osmoregulation; Evans & Kültz, 2020; Schultz & McCormick, 2012). These mechanisms are metabolically expensive and can trade-off with other functions (Herbert et al., 2015), and, ultimately, may jeopardise survival (Cañedo-Argüelles et al., 2013; Hall & Burns, 2002; Moorman et al., 1991). Although all trophic levels can be affected by increased environmental salinity (Hintz & Relyea, 2019), there is a wide variation in susceptibility among species (Castillo et al., 2018; Halse et al., 2003; Venâncio et al., 2022; Venâncio, Castro, Ribeiro, Antunes, Abrantes, et al., 2019), and among life stages (Paiva et al., 2020). In freshwater environments, early life stages of amphibians, mussels and zooplankton have been identified as the most sensitive taxa to salinity exposure, whereas some fish species, water moulds and macrophytes can be more tolerant (Venâncio et al., 2022). This species-specific susceptibility to salinity can have major consequences for ecosystem functioning (Cunillera-Montcusí et al., 2022). Indeed, susceptible species can avoid elevated salinity through habitat selection (Venâncio et al., 2020), thereby inducing shifts in home ranges size or location of intolerant species (O'Dell et al., 2021). Ultimately, these processes can reduce biodiversity (Hébert et al., 2022; Hintz & Relyea, 2019) and change community structure and composition (Findlay & Kelly, 2011; Hart et al., 2003; Hintz & Relyea, 2019; Venâncio, Castro, Ribeiro, Antunes, & Lopes, 2019).

Amphibian taxa are particularly susceptible to salinity (Venâncio et al., 2022). Indeed, amphibians are characterised by a relatively permeable skin that allows not only gas exchanges, but also ion and water fluxes (Hillyard et al., 2007; Wake & Koo, 2018), and relatively low dispersal abilities constraining movements when environmental conditions are harsh (Wells, 2007). As a consequence, amphibian species richness and distribution decrease with increasing salinity (Başkale & Kaya, 2009; Karraker et al., 2008; Smith et al., 2007), suggesting that salinity is a limiting factor for amphibian diversity (Hall et al., 2017). Conversely, some amphibian species have been shown to live and reproduce in habitats ranging from brackish to nearly marine (Greenwald, 1972; Hopkins & Brodie, 2015), which suggest that salt tolerance may not be as rare as it is been assumed previously, especially for species occurring in coastal habitats (Hopkins et al., 2016; Hopkins & Brodie, 2015), and that tolerances to salinity vary between species (Babbitt et al., 2006; Brown & Walls, 2013; Hopkins & Brodie, 2015; Hua & Pierce, 2013; Lorrain-Soligon et al., 2021).

In addition to these species-specific tolerances to salinity, amphibians also can display variable levels of tolerance to salinity within species. Indeed, most amphibians are characterised by a complex biphasic life cycle, with eggs and tadpoles developing in aquatic habitats and adults displaying various levels of terrestriality (Wells, 2007). Accordingly, eggs and larvae are less tolerant to elevated salinity than adults (Albecker & McCoy, 2017), and some evidence suggest that reproductive individuals can express habitat selection and avoid saline water for egg-laying (Albecker & McCoy, 2017; Haramura, 2008; Lorrain-Soligon, Robin, et al., 2022; Viertel, 1999) to improve their reproductive success (Lukens & Wilcoxon, 2020; Tornabene et al., 2021). Taken together, these elements suggest that variability in both within- and between-species susceptibility to salinity could be a major factor determining the structure of amphibian coastal communities (Lorrain-Soligon et al., 2021) and may significantly affect within- and between-species distribution across the spatial and temporal salinity gradient. However, the current literature lacks data on the effect of salinity across amphibian community (but see Lorrain-Soligon et al., 2021; Smith et al., 2007), across multiple sites and a long time scale, which is key to understanding how they will behave to the predicted increase in salinity.

In order to better understand how salinity changes naturally in coastal environments and how amphibians can respond to such spatial and temporal variation, we studied the amphibian community in a typical coastal wetland situated on the Atlantic coast of France, where amphibians are subjected to natural salinity variations. Firstly, we assessed the spatial (distance to the coastline) and temporal (seasonal across spring and summer) dynamics of salinity during 3 years in 24 coastal ponds. Secondly, on the same sites during one breeding season, we assessed amphibian presence and abundances across key life stages (adults, larvae and metamorphs) to investigate how community and diversity vary with salinity. Thirdly, we also investigated how the spatio-temporal dynamics of salinity can affect the fitness of individuals by quantifying malformations of larvae and metamorphs, as elevated salinity can alter embryonic and larval

development and induce malformations (Hopkins et al., 2013). We hypothesised that amphibian diversity should decrease with increasing salinity (Smith et al., 2007) as most amphibian species seem to be highly susceptible to salinity, that larvae should be present only in low-salinity ponds, as they are less tolerant to elevated salinity compared to adults (Albecker & McCoy, 2017), and that localised seasonal increase of salinity throughout the embryonic and larval development should negatively affect offspring quality because of the detrimental effects of salinity on development (Lukens & Wilcoxon, 2020; Tornabene et al., 2021).

2 | METHODS

2.1 | Study site and sampling

The study was carried out on a coastal wetland from the Eastern Atlantic coast (Réserve Naturelle Nationale de Moëze-Oléron, France, 45°53'33.36"N, 1°04'59.16"W). This site is composed of a continuum of habitat types (brackish and freshwater marshes and ponds) with increasing distance from the seashore (Figure 1).

We surveyed 24 ponds, during 32 sessions that occurred between 31/03/2021 and 12/07/2021 (Figure 1). Twenty ponds were prospected during 32 sessions, three ponds were prospected during three sessions, and one pond was prospected during 18 sessions. Each pond was prospected at an even number of night and day sessions. Successive sessions at each site were separated by a minimum of 24h and a maximum of 5 days. These ponds were chosen to express a large salinity gradient, which vary during the season. Characteristic of all ponds and number of visits are given in Table S1.

For each session, we measured water salinity (g/L) and temperature (°C) using a conductimeter (YSI Professional Plus) at 15 cm

deep. We also estimated the percentage of surface area covered by aquatic vegetation. Additionally, to complement our measures of salinity performed in 2021 and better assess the temporal variations of salinity, we aggregated data of pond salinity recorded once a month in April, May, June and July in 2018 and 2019 for 20 of the 24 ponds studied in 2021, using the same methodology as that used in 2021.

The surface area of each pond as well as its distance to the ocean at high tide were measured using QGIS 3.4.7. Both variables varied widely across our study sites (surface area 36–3,250 m² [mean 490.47 m² ± 159.21 SE]; distance to the ocean 30–796 m [356.10 m ± 47.64 m SE]) but were not related to each other (Pearson correlation test: $r = -0.088$, $p\text{-value} = 0.712$).

For each prospection, we used three complementary survey methods to assess abundance of amphibian species. First, during diurnal surveys, we used binoculars at a distance of 10m from the pond edges to count individuals present on the whole water surface and on the edges of the ponds. Second, during diurnal and nocturnal sessions, we completed this survey by counting all individuals visible (≤ 2 m on land or in water) by slowly walking around the pond (with a headlamp at night), and searching under cover objects situated up to 2m from its edge on land to search for both hidden anurans and caudates. Finally, these visual surveys were complemented by direct sampling (using a net with fine mesh [1-mm]) to assess the presence of secretive larvae. To standardise this survey method, we sampled a pond according to its perimeter. We performed five dip netting manoeuvres every 10m. Such direct sampling also allowed us to ascertain whether individuals display malformations or abnormalities (Hopkins et al., 2013). We checked for the presence of malformation in all species and life stage captured, and these malformations consisted of oedema, axial malformations, reduced tail size, and skin infections. These complementary methods allowed us to assess the abundance of larvae, metamorphic or adult individuals for each species.

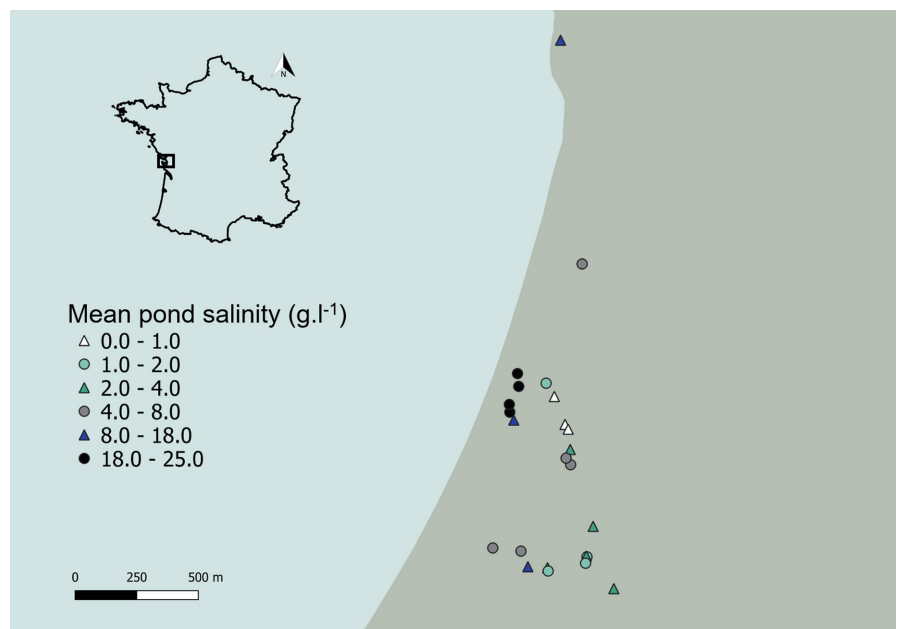


FIGURE 1 Location of the 24 study ponds in western France, with mean salinity during the whole study period (from 31/03/2021 to 12/07/2021).

This protocol led to the identification of six amphibian species (four anurans, two caudates): the green frog *Pelophylax* sp. (composed of a hybridogenic complex indistinguishable by sight; Dufresnes et al., 2017), the Mediterranean tree frog (*Hyla meridionalis*), the common parsley frog (*Pelodytes punctatus*), the Western spadefoot toad (*Pelobates cultripes*), the palmate newt (*Lissotriton helveticus*) and the marbled newt (*Triturus marmoratus*).

2.2 | Review of salt tolerance

We collected published data on salt tolerance in caudates and anurans obtained both during field studies and under laboratory conditions. We complemented the seminal review of Hopkins and Brodie (2015) with additional data published since then (Table S2). For consistency, we used the same methodology as Hopkins and Brodie (2015) to extract salinity data: "For every species where environmental salinity was measured at the time of field observation, we determined the maximum salinity concentration in which the animal was found. There are many measurement units used in the salinity literature, with very little standardisation or consistency (e.g., conductivity, specific conductance, mOsm/L, g/L, mg/ml, mequiv/L, specific gravity, ppt [parts per thousand], ppm [parts per million], psu [practical salinity unit]). To facilitate accurate comparison among species and studies, we converted all values into ppt (g/L Cl⁻). For those species whose salt tolerance had been experimentally examined in the laboratory, we determined the maximum upper limit of tolerance by arbitrarily defining this as the concentration of salt in which >50% of individuals survived. For non-lethal measures, we recorded the upper limit as that concentration which first caused a statistically significant negative effect." In addition, as in Hopkins and Brodie (2015), when a species had been found to occur in multiple salinities, we retained in our collection (see Table S2) the highest value.

These data were not used to perform quantitative statistical analyses, but we provide qualitative comparisons in Section 4 below.

2.3 | Data analysis

2.3.1 | Spatio-temporal variation in salinity

The spatio-temporal variation in salinity at our study sites was investigated with a linear mixed model (lmer) with salinity as a dependent variable, distance to the ocean, day of the year (the number of days since 01/01/2021), and their interaction as covariates, and pond as random effect, using all salinities measured during the 32 sessions. For clarity, only three dates are represented in Figure 2 corresponding to the beginning (31/03/2021), middle (20/05/2021) and end (12/07/2021) of the survey. To complement our measures of salinity performed in 2021 and to better assess the temporal variations

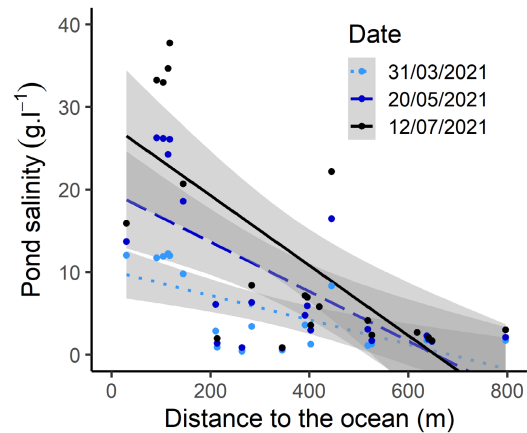


FIGURE 2 Spatial (according to distance to the ocean) and temporal (across surveys) variation of salinity on 24 coastal ponds. For clarity, only data corresponding to the beginning (31/03/2021), middle (20/05/2021) and end (12/07/2021) of the survey are represented. Points are observed values, lines indicate relationships with increasing distance from the seashore and grey shadings indicate 95% confidence intervals.

of salinity, we aggregated pond salinity data recorded in April, May, June and July in 2018 and 2019 for 20 of the 24 ponds studied in 2021 with all of the data recorded in 2021. The temporal variations of salinity were then investigated using lmer with distance to the ocean, day of the year, and their interaction as covariates, and pond and year as random effects. We previously tested for the effect of year to see if there was a difference in salinity between years, by setting a lmer with year as a dependent variable and pond as a random effect, but we found no variations between years (Sum Sq=32.356, $F_{1,771}=2.732$, $p=0.099$).

2.3.2 | Species presence and abundances

In order to model each species abundance, we used the N-mixture method of Royle (2004). This method allows estimating abundance of species using multiple counts of unmarked individuals, taking into account imperfect detection probability, and for variables influencing species detection and occupancy (Royle, 2004). For anuran species, we modelled for each species the estimated abundance of adults, metamorphs and larvae. Low sample sizes (three observations for each group) precluded these analyses for adult *P.punctatus* and adults and metamorphic individuals of *P.cultripes*. For caudate species, we modelled for each species the estimated abundance of adults and larvae. For the two groups (anurans and caudates), we also computed the same statistics using the abundance of all species considering each life stage separately (*Pelophylax* sp., *H.meridionalis*, *P.punctatus* and *P.cultripes* in anurans, and *L.helveticus* and *T.marmoratus* in caudates).

For each species and each developmental stage, we thus ran N-mixture models with the matrix of the 32 observations of the given

species and stage on the 24 study ponds, and tested a combination of models including, as variables explaining occupancy, maximum salinity recorded during the whole study period, its quadratic form, aquatic vegetation, the interaction between aquatic vegetation and either maximum salinity or the quadratic form of maximum salinity, and the additive effect of the surface area of the pond. Aquatic vegetation was always excluded during the model selection procedure. Maximum salinity was selected, instead of any other values of salinity, because maximum salinity represents the most constraining level of salinity that may influence habitat selection throughout the breeding season. It allows us to test whether adults could potentially predict temporal change in salinity that their larvae will experience later. For all models, we also tested if detection probability was temperature-dependent (using water temperature data recording at each pond during each prospection), as well as dependent on the day of the year (and its quadratic form, which was always excluded during the model selection procedure) or if it was constant over time. We used Poisson regression which fitted well our count data, except for the two caudate species, where abundance was lower and for which we used a zero inflated Poisson distribution. The best model was retained with selection by Akaike information criterion (AIC). The estimated abundances were represented using the package *ggeffects*.

In order to model the richness of amphibians' species, we used generalised linear mixed models (glmers) with Poisson or Zero Inflated Poisson distribution. We evaluated species richness of adults, larvae, metamorphs, and total (adults, larvae and metamorphs together) separately for anurans and caudates, and finally estimated total species richness regrouping all anuran species and all caudate species for all life stages. In our models, we included as covariates salinity measured at each prospection, its quadratic form, the interaction between aquatic vegetation and either salinity or the quadratic form of salinity, and the additive effect of the surface area of the study ponds. Pond was set as a random effect. We retained the best model with selection by AIC. The estimated specific richness were represented using the package *ggeffects*.

2.3.3 | Salinity and life stages

Differences in susceptibility (salinity tolerated by individuals) between life stages (analysed on all species grouped together) were investigated with lmer with salinity as a dependent variable (i.e., all pond salinities for prospection in which individuals were found – when no individuals were found, the pond and its associated salinity were not used in this analysis), day of the year (the number of days since 01/01/2021), life stages, and their interaction as dependent variables, with pond as random effect. This allowed us to highlight the mean salinity in which each life stage was found, and to compare with other life stages. We also provided empirical data on the minimum, maximum and mean pond salinity in which each life stage of each species were found, as well as the date of first and last observation (Table 1).

2.3.4 | Malformations occurrence

In order to assess the influence of pond salinity on occurrence of malformed individuals (analysed on all species grouped together), we used Poisson glmers with number of malformed individuals as a dependent variable, pond salinity or day of the year as a covariate, and pond as a random effect.

All statistical analysis were performed using R 3.6.3 (R Core Team, 2020) and Rstudio version 1.1.419.

3 | RESULTS

3.1 | Spatio-temporal variation of salinity

Considering only 2021, salinity varied with time (Sum Sq=3,353.9, $F_{1,24}=15.981$, $p<0.001$), distance to the ocean (Sum Sq=74.5, $F_{49,535}=17.406$, $p<0.001$), and their interaction (Sum Sq=1,307.3, $F_{49,535}=6.229$, $p<0.001$). Indeed, salinity decreased when distance to the ocean increased, but increased with date (Figure 2). During the whole survey, mean salinity among the five ponds closest from the ocean was of 22.19 g/L (± 2.18 SE) and mean salinity among the five ponds the farthest from the ocean was of 1.96 g/L (± 0.11 SE). Across our survey period, salinity ranged from 0.44 to 12.26 g/L at the beginning of the breeding season to 0.89 to 37.73 g/L at the end of the breeding season.

Data of salinity recorded in 2018, 2019 and 2021 in 20 study ponds yielded similar results, with salinity seasonally increasing in ponds (Estimate=0.072, SE=0.004, $t=20.346$, $p<0.001$), and increasing closer to the ocean (Estimate=-0.030, SE=0.006, $t=-4.631$, $p<0.001$), but no interaction between distance to the ocean and day of the year.

3.2 | Species presence and abundances

Overall, amphibians were present in 20 (83.33%) of the 24 surveyed ponds. Ponds that did not shelter any species during the whole breeding season were characterised by higher salinity both at the beginning (9.80 g/L ± 0.99 SE) and the end of the survey (30.45 g/L ± 9.56 SE). Detailed data on the minimum and maximum salinity for each species and life stages are given in Table 1.

Green frogs were found in high-salinity ponds (16.19 g/L for one individual and 15 g/L for two individuals). Except for these anecdotal observations, all anuran species were found in ponds where salinity was below 9 g/L (Table 1). In caudates, no individual was found in ponds where salinity exceeded 4 g/L (Table 1).

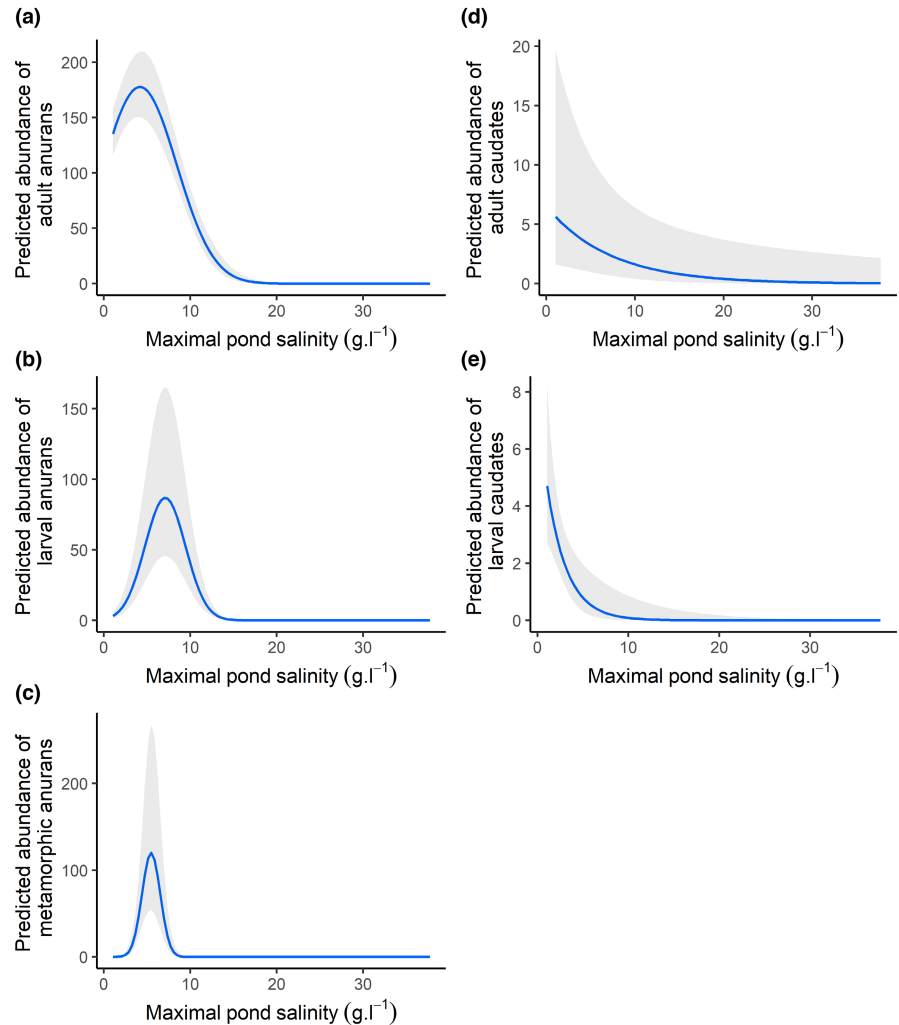
For all anurans species and all life stages, we found a significant quadratic response of abundance to maximum salinity, with abundance being higher at intermediate salinities (Figure 3A–C; Tables 2, S3). For some, but not all, anuran species, we also found that increasing pond surface area increased abundance of individuals (Tables 2, S3). Lastly, there was no effect of aquatic vegetation (alone or in

TABLE 1 Presence of each developmental stage of each species according to pond salinity (min, max and mean \pm SE).

Group	Species	Life stage	Salinity (g/L)			Average	SE	Number of ponds	Number of observations	Total number of individuals	Mean presence	First observation	Last observation
			Min	Max	SE								
Anurans	<i>Pelophylax</i> sp.	Adults	0.14	16.19	2.90	2.16	20	386	6,487	16.76	FD	LD	
		Larvae	1.23	8.96	4.23	2.04	6	66	794	12.03	03/05/2021	LD	
		Metamorphs	1.97	8.41	5.03	2.2	4	15	321	21.40	26/06/2021	LD	
<i>Hyla meridionalis</i>		Adults	0.4	7.85	2.71	1.77	16	77	256	3.21	FD	LD	
		Larvae	1.2	8.96	3.79	2.08	9	96	1,024	10.67	03/05/2021	25/06/2021	
		Metamorphs	1.66	8.96	5.1	2.14	6	20	260	13	07/06/2021	01/07/2021	
<i>Pelodytes punctatus</i>		Adults	3.66	4.22	3.87	0.3	1	3	3	1	11/04/2021	22/04/2021	
		Larvae	1.11	8.96	4.58	1.86	5	80	2,946	36.83	FD	26/06/2021	
		Metamorphs	3.88	8.96	6.6	1.29	5	25	339	13.56	09/05/2021	06/07/2021	
<i>Pelobates cultripes</i>		Adults	1.41	4.66	3.08	1.77	3	4	4	1	14/04/2021	03/05/2021	
		Larvae	5.11	8.96	6.94	1.34	2	13	105	8.08	07/06/2021	LD	
		Metamorphs	5.32	6.47	5.9	0.81	1	2	2	1	01/07/2021	06/07/2021	
Caudates	<i>Lissotriton helveticus</i>	Adults	0.44	2.4	1.12	0.63	3	10	10	1	FD	30/04/2021	
		Larvae	1.2	2.17	1.55	0.31	1	12	18	1.5	03/05/2021	25/06/2021	
		Adults	0.44	3.78	1.35	1.13	4	9	20	2.22	FD	03/05/2021	
	<i>Triturus marmoratus</i>	Larvae	1.2	2.25	1.67	0.31	2	28	103	3.68	03/05/2021	01/07/2021	

Note: We reported number of observations (i.e., the number of occasions on which species were encountered across the study period on all study sites), and the total number of individuals found, and the average number of individuals for the periods when observations were not zero (mean presence). FD stands for first day and indicates that a species (or a life stage) was already present when the surveys began (31/03/2021). LD stands for last day and indicates that a species (or a life stage) was still present when the surveys ended (12/07/2021).

FIGURE 3 Predicted abundance relative to salinity using N-mixture models for anurans (left panels) and caudates (right panels), in adults (a, d), larvae (b, e) and metamorphic individuals (c). Salinity was the main predictor in all cases, but for some species we also found a significant effect of pond surface (see Section 3 for details). Grey shadings indicate 95% confidence intervals. Note that as a result of variable richness and abundance, the scales of the y-axes are different. See Table S3 for details on analyses performed on each species separately.



interaction with salinity). Anuran detection probability also varied with temperature and day of the year (Table S3). In species in which day of the year had an effect, detection probability decreased when day of the year increased in adults, but increased with day of the year in larval and metamorphic individuals.

In caudates, abundance decreased strongly with increasing maximum salinity (Table 2; Figure 3D,E), but this effect was not significant in *Lissotriton helveticus* (Table S3). For *Triturus marmoratus*, we also found that increasing pond area decreased abundance of individuals (Table S3). Caudate detection probability also varied with temperature and day of the year (Table S3). In species in which day of the year had an effect, detection probability decreased when day of the year increased in adults, but increased with day of the year in larvae and metamorphic individuals. Lastly, there was no effect of aquatic vegetation (alone or in interaction with salinity).

Total species richness (for all stages of all species) followed a quadratic form, and was maximal for intermediate – temporally matched – salinities (Table 3). For all life stages, we observed that richness of anurans species was higher at intermediate salinities (quadratic effect), whereas richness of adult caudates and total

caudates decreased with increasing salinity (Table 3; Figure S1). We detected no significant relation between larval caudates and salinity. In adult anurans, we found that richness increased with the surface area of the pond (Table 3), but this variable was not retained in any other model. Lastly, there was no effect of aquatic vegetation (alone or in interaction with other variables) on species richness.

3.3 | Salinity and life stages

The salinity in which we found each species varied according to life stages (Sum Sq=13.827, $F_{4,598}=9.524$, $p<0.001$), date (Sum Sq=16.874, $F_{1,598}=46.491$, $p<0.001$), and their interaction (Sum Sq=13.630, $F_{4,598}=9.389$, $p<0.001$). In anurans, the salinity of the ponds where larvae or metamorphs were found was higher than that of adults because these life stages occurred later than adults at those sites (Figure 4). This pattern was corroborated by the highest mean salinity in which we found tadpoles and metamorphs compared to adults (Table 1). Comparatively, for caudates, larvae occurred later than adults, but the salinity in which adults and larvae were found was similar (Figure 4, Table 1).

TABLE 2 Environmental variables affecting abundance of anurans and caudates (all life stages analysed separately).

Group	Life stage	Environmental parameter	Estimated abundance			
			Estimate	SE	z	p
Anurans	Adults	Maximal salinity	0.19	0.038	4.95	<0.001
		Maximal salinity ²	-0.027	0.003	-8.05	<0.001
		Surface	0.001	<0.001	8.52	<0.001
		Temperature	0.042	0.005	8.69	<0.001
		Day of the year	-0.016	<0.001	-23.69	<0.001
	Larvae	Maximal salinity	1.323	0.162	8.15	<0.001
		Maximal salinity ²	-0.088	0.011	-7.96	<0.001
		Surface	0.002	<0.001	5.65	<0.001
		Temperature	0.109	0.012	8.8	<0.001
	Metamorphs	Maximal salinity	1.406	0.244	5.77	<0.001
		Maximal salinity ²	-0.096	0.021	-4.62	<0.001
		Surface	0.004	<0.001	9.39	<0.001
Temperature		0.245	0.018	14	<0.001	
Caudates	Adults	Maximal salinity	-0.139	0.06	-2.3	0.021
		Temperature	-0.104	0.058	-1.78	<0.001
		Day of the year	-0.058	0.014	-4.07	<0.001
	Larvae	Maximal salinity	-0.452	0.152	-2.97	0.002
		Temperature	0.092	0.034	2.72	0.006
		Day of the year	0.011	0.004	2.5	0.012

Note: Temperature and day of the year were used as covariates to model detection probability. Only variables retained in the best models are represented. See Table S3 for details on analyses performed on each species separately. Maximal salinity² indicate the quadratic form of maximal salinity.

TABLE 3 Environmental variables affecting species richness in anurans and caudates (all life stages combined or analysed separately).

Life stage	Species	Environmental parameter	Species richness			
			Estimate	SE	z	p
Total	Total (anuran and caudate)	Salinity	0.181	0.08	2.249	0.025
		Salinity ²	-0.025	0.006	-3.847	<0.001
	Anuran	Salinity	0.25	0.083	2.997	0.003
		Salinity ²	-0.029	0.007	-4.343	<0.001
	Caudate	Salinity	-0.635	0.279	-2.278	0.023
Adults	Anuran	Salinity	0.011	0.006	1.83	0.067
		Salinity ²	-0.002	<0.001	-4.318	<0.001
		Surface	<0.001	<0.001	2.815	0.005
	Caudate	Salinity	-2.239	0.691	-3.241	0.001
Larvae	Anuran	Salinity	1.334	0.25	5.346	<0.001
		Salinity ²	-0.095	0.022	-4.389	<0.001
	Caudate	Salinity	<-0.001	0.002	-0.235	0.814
Metamorphs	Anuran	Salinity	4.085	0.582	7.022	<0.001
		Salinity ²	-0.266	0.047	-5.647	<0.001

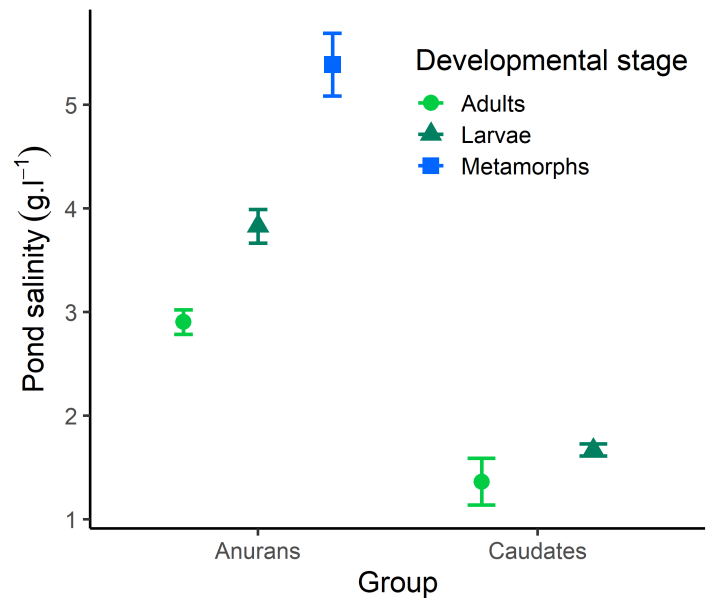
Note: Only variables retained in the best models are represented. Salinity² indicate the quadratic form of salinity.

3.4 | Malformations occurrence

We did not find any malformed individuals in caudates. Conversely, we found several malformed larvae and metamorphic individuals in

anurans ($N=17$, 0.35%). Malformations were diverse and included oedema, axial malformations, reduced tail size, and skin infections. In anurans, the number of malformed individuals increased with time (Estimate=0.035, SE=0.010, $t_{1,417}=3.415$, $p<0.001$) and increasing

FIGURE 4 Salinities (mean \pm SE) in ponds where different life stages of anurans (adults, larvae and metamorphs) and caudates (adults and larvae) occurred.



pond salinity (Estimate = 1.051, SE = 0.242, $t_{1,434} = 4.340$, $p < 0.001$), malformed individuals occurring solely in ponds wherein salinity was above 5 g/L.

4 | DISCUSSION

Overall, we show that salinity is a highly dynamic environmental variable that varies widely in coastal wetlands across spatial (up to 1 km) and temporal (spring and summer, across 3 years) scales relevant to local biodiversity. Using amphibians as a study model, we show that the spatio-temporal dynamic of salinity is a critical structuring factor of the community of these taxa. The most salient result from our study is the strong contrast between caudates and anurans, across all life stages. Indeed, caudates avoided brackish waters resulting in a strong decrease in richness and abundances with increasing salinity. Conversely, in anurans, richness and abundances were maximal for intermediate salinity, and decreased at around 8 g/L. In anurans, we found that larvae and metamorphic individuals developed in waters wherein salinity was higher than those selected by adults to reproduce, which can negatively affect reproductive success as exemplified by the decreased offspring quality (increased malformations) with increasing salinity.

4.1 | Spatio-temporal dynamics of salinity

Continental water bodies (i.e., rivers and lakes) have attracted considerable research effort to understand the consequences of freshwater salinisation (Cunillera-Montcusí et al., 2022), while marshes and ponds have been largely ignored to date despite their crucial role for biodiversity (Céréghino et al., 2014; Teixeira et al., 2014). In our study focused on such small water bodies, we found that salt concentration can vary widely, both spatially and temporally (Benjankar

et al., 2021; Collins & Russell, 2009). Spatial variations in salt concentration have already been shown in ponds (Benjankar et al., 2021), but never on such a small spatial scale (~1 km). In our study sites, salinity increased with decreasing distance to the ocean. This spatial variation is likely to be a result of exchanges between terrestrial and oceanic systems (Xue et al., 2013) and to the landward transport of sea spray (Benassai et al., 2005). Interestingly, we found a strong seasonal increase in pond salinity, presumably linked to increasing temperature and decreasing precipitations – and thus increasing evaporation – across spring and summer (Collins & Russell, 2009). We based our analyses across spring and summer during 3 years, and our results suggest that seasonal variation of salinity follows a predictable seasonal pattern. However, we emphasise that future studies are required to thoroughly quantify the seasonal variations of salinity (including autumn and winter) over longer-term (multiple years) in order to document whether current shifts in climatic conditions (i.e., temperature, precipitations, but also wind regimes that all interact to influence evaporative patterns; Richter & Xie, 2008) can affect the temporal variation of salinity and the hydroperiod in natural systems (Estévez et al., 2019; Ghalambor et al., 2021), and how such shifts will ultimately influence biodiversity.

4.2 | Variation in tolerance to salinity in coastal amphibians

Our results highlighted variable susceptibility between species suggesting different tolerance to salinity (Bray et al., 2019). We found very few effects of pond size and no effect of aquatic vegetation on amphibians' richness and abundance across all life stages (even if anuran richness and abundance was found to increase with increasing pond size, and caudate richness and abundance was found to decrease with increasing pond size), which emphasises the critical role of salinity as a structuring factor of the amphibian community.

Importantly, no species was found above 16 g/L which seems to constitute a threshold to amphibians' presence, and corroborate the global susceptibility to salinity in amphibians (Hopkins & Brodie, 2015). Taken together, our results point to a strong contrast between caudates and anurans across all life stages. In caudates, richness and abundance decreased strongly with small increments in salinity strengthening the fact that although few species can be salt-tolerant (Izzo et al., 2021; Licht et al., 1975), these taxa may be more susceptible to salt than anurans (Hopkins et al., 2013). Indeed, we found that anuran richness and abundance (across all life stages) were maximal for intermediate salinity (around 5–6 g/L) but also that some species occurred at remarkably high salinity. Such a contrast may be related to a weaker efficiency of the physiological mechanisms involved in osmoregulation in caudates as compared to anurans (i.e., reliance on urea, free amino acids and/or ionic concentrations in body fluids to reduce salt and water fluxes; Licht et al., 1975). To date, to the best of our knowledge, no study has formally compared mechanisms of osmoregulation and their associated efficiency between anurans and caudates. Complementarily, the seminal review of Hopkins and Brodie (2015), conducted for all life stages, suggested a slightly higher (but not significant) tolerance to salinity in anurans ($8.9 \text{ g/L} \pm 8.9 \text{ SD}$) than in caudates ($6.6 \text{ g/L} \pm 6.6 \text{ SD}$) in field studies; and a similar tolerance (respectively $9.0 \text{ g/L} \pm 6.6 \text{ SD}$ and $10.1 \text{ g/L} \pm 7.2 \text{ SD}$) under laboratory conditions. We have updated the review of Hopkins and Brodie (2015) with recent references and these new data point to similar patterns both in the field (mean tolerance in anurans $8.2 \text{ g/L} \pm 8.1 \text{ SD}$ and in caudates $6.3 \text{ g/L} \pm 6.0 \text{ SD}$) and under laboratory conditions (mean tolerance in anurans $8.1 \text{ g/L} \pm 6.0 \text{ SD}$ and in caudates $7.7 \text{ g/L} \pm 5.3 \text{ SD}$). As already highlighted in Hopkins and Brodie (2015), we believe this apparent discrepancy may be linked to bias in research effort between caudates and anurans. Indeed, the salt-tolerance of caudates seems less studied than it is in anurans (16 vs. 90 species studied in the field, 9 vs. 48 species studied under laboratory conditions; see Hopkins & Brodie, 2015). In addition, laboratory studies of salt-tolerance have used highly variable salinity conditions which preclude direct and straightforward comparisons (see also Hopkins & Brodie, 2015). In order to assess whether caudates and anurans universally differ in their salt-tolerance, we emphasise that future field studies should focus on both taxonomic groups where they co-occur, in a similar way to the current study. This also should be tested in sites where amphibian-specific richness is higher, because in our study site only six amphibian species are found, which limits computation of statistical analyses whenever specific richness is low. In addition, laboratory tests should be performed on similar test salinities in order to support large-scale comparisons. Finally, assessing whether caudates and anurans differ in osmoregulation mechanisms and associated efficiency will allow further crucial insights in order to understand putative differences in salt-tolerance and, thus, differential responses of these amphibian phylogenetic groups to environmental salinisation.

Interestingly, the higher tolerance of anurans to salinity in comparison to caudates fails to satisfactorily explain the quadratic

patterns we found across all life stages. Indeed, higher tolerance to salinity should have influenced the strength of the relationship between richness or abundances and salinity (i.e., the slope should be weaker in anurans) but not its shape (i.e., the quadratic effect of salinity and thus the lower richness and abundances of caudates at low salinity were unexpected). The fact that anurans seem to avoid fresh water may indicate weaker predation pressure (Velasco et al., 2019) or weaker pathogen transmission (such as chytridiomycosis; Clulow et al., 2018) in brackish water as compared to fresh water, which may induce habitat selection toward sites characterised by lower predation (i.e., lower abundances of crawfish) or pathogens in species that can tolerate moderate salinity (i.e., anurans) but not in salt-intolerant species (i.e., caudates). Finally, these patterns may reveal trade-offs linked to adaptive processes. Indeed, coastal anurans may have evolved specific physiological adaptations to promote life in hyperosmotic environments (Albecker & McCoy, 2019; Gomez-Mestre & Tejedo, 2003; Hopkins et al., 2016), notably through mechanisms that allow them to reduce salt intake (or increase excess salt excretion) and/or to increase water influxes (or reduce water effluxes, Lorrain-Soligon, Bichet, et al., 2022; Lorrain-Soligon, Robin, et al., 2022). In turn, these mechanisms may become maladaptive in hyposmotic water bodies because of elevated salt loss and excessive water influxes (Lorrain-Soligon, Bichet, et al., 2022), leading to massive oedema and mortality (L. Lorrain-Soligon, pers. obs). To date, we lack evidence to suggest that physiological mechanisms needed in hyperosmotic environments can affect performance in fresh water (Gomez-Mestre & Tejedo, 2003). Future studies should usefully investigate the mechanistic bases of the non-monotonic response of coastal anurans to environmental salinity. Whatever the underlying mechanisms, this non-monotonicity (somewhat similar to the "intermediate disturbance" hypothesis; Sheil & Burslem, 2013) appears to be a common pattern of response to salinity across a wide array of organisms [e.g., fish (Hieu et al., 2021), amphibians (Ahumada-García et al., 2018), invertebrates (Kefford et al., 2007), and plants (Sauer et al., 2016)].

4.3 | Seasonal increase in salinity and life-stages

Amphibian reproduction is negatively influenced by salinity (Karraker, 2007; Tornabene et al., 2021). For instance, salinity can directly affect sperm viability and fertilisation processes in these taxa characterised by external fertilisation (Green et al., 2021), and both field and experimental studies have shown that frogs avoid saline water for spawning (Albecker & McCoy, 2017; Viertel, 1999). In caudates, the salinities of ponds where larvae were found were similar to those of ponds where adults reproduced. Although larvae appeared later than adults, this emphasises the fact that adults selected low-salinity sites to reproduce characterised by a relatively moderate seasonal increase in salinity. Conversely, in anurans, tadpoles occurred when salinity was significantly higher than that experienced by reproductive adults. In this case, the seasonal increase in salinity induced a temporal disconnection between the salinity selected by

adults to reproduce and the salinity experienced by their developing offspring. In turn, the elevated salinity in which anuran larvae developed increased occurrence of malformations and abnormalities in both larvae and metamorphic individuals. This result dovetails well with previous laboratory studies which highlighted that increased salinity during embryonic and larval development induced malformations (Brady, 2013; Gosner & Black, 1957; Hopkins et al., 2013; Karraker, 2007), which ultimately decrease fitness and survival (Michel & Burke, 2011). Interestingly, the occurrence of malformed individuals (only found in anuran larvae and metamorphs) increased with increasing salinity and solely in sites wherein salinity exceeded 5 g/L. It is noteworthy that the proportion of malformed individuals we found remained very low, suggesting either that malformations increased mortality and/or susceptibility to predation (i.e., selective disappearance; Blaustein & Johnson, 2003) or that most larvae of coastal anurans were tolerant to salinity (Albecker & McCoy, 2019). Future studies are required to test for these hypotheses.

5 | CONCLUSION

Coastal aquatic ecosystems are naturally subject to salinity variation. In response to these variations, we show that species express different responses, affecting their distribution and their reproduction across the salinity gradient. Future changes in coastal salinity will be likely to affect the structure and functioning of freshwater ecosystems (Castillo et al., 2018). Indeed, even a small increase of salinity may be sufficient to induce changes in community structure and interspecific interactions (Venâncio, Castro, Ribeiro, Antunes, Abrantes, et al., 2019). Amphibian species seem to persist in saline habitats and can probably subsist in environments when gradual increase in salinity can promote adaptive responses (Delaune et al., 2021). Yet, brutal shifts in salinity (i.e., linked to extreme weather events; Lorrain-Soligon et al., 2021) may overcome population persistence (Venâncio et al., 2022). Colonisation by tolerant taxa and interactions between species will be key drivers of community persistence in salinised ecosystems (Kefford et al., 2016).

AUTHOR CONTRIBUTIONS

Conceptualisation: LLS, FR, FB. Developing methods: LLS, FB. Conducting the research: LLS. Data analysis: LLS, CB, FB. Data interpretation: LLS, FR, CB, FB. Preparation of figures & tables: LLS, CB, FB. Writing: LLS, FR, CB, FB.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data will be made available upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Chapitre II : Influence de la salinité sur les adultes

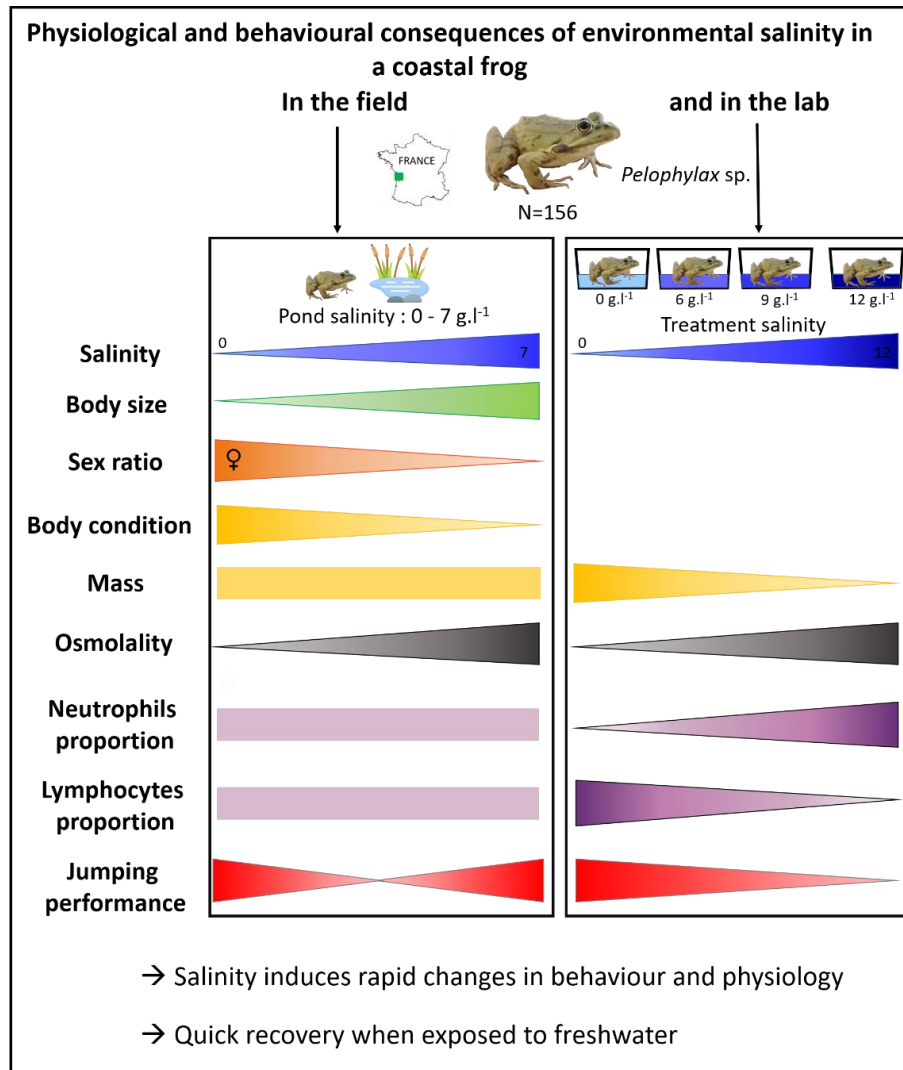


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Article IV

From the field to the lab: physiological and behavioural consequences of environmental salinity in a coastal frog

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From the Field to the Lab: Physiological and Behavioural Consequences of Environmental Salinity in a Coastal Frog

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Environmental salinization is recognized as a global threat affecting biodiversity, particularly in coastal ecosystems (affected by sea level rise and increased frequency and severity of storms), and the consequent osmoregulatory challenges can negatively affect wildlife. In order to assess whether coastal species can respond to changes in environmental salinity, it remains essential to investigate the consequences of exposure to salinity in an environmentally-relevant context. In this study, we assessed the consequences of exposure to environmental salinity in coastal frogs (*Pelophylax* sp., N = 156) both in the field and experimentally, using a comprehensive combination of markers of physiology, behaviour and ecology. Exposure to salinity in the field negatively affected physiological parameters (osmolality, monocytes and eosinophils counts), as well as body condition and locomotor performance, and influenced size- and sex-specific habitat selection. Further, we demonstrated in a controlled experiment that short-term exposure to salinity strongly affected physiological parameters (salt influxes, water effluxes, immunity-related stress markers) and locomotor performance. Most of these effects were transient (water and salt fluxes, locomotor performance) once optimal conditions resumed (i.e., freshwater). Taken together, our results highlight the need to investigate whether exposure to environmental salinity can ultimately affect individual fitness and population persistence across taxa.

Keywords: coastal ecosystems, immunological changes, locomotor performances, osmolality, *Pelophylax* sp., salinization

1 INTRODUCTION

Current global changes are causing numerous modifications to both abiotic and biotic conditions, which threatens biodiversity (Vitousek et al., 1997; Pievani, 2014). Although research has highlighted the detrimental consequences of some relatively conspicuous and mediatized components of global changes (e.g., global warming, Walther et al., 2002; land-use changes and habitat loss, Jantz et al., 2015; invasive species, Early et al., 2016), other—presumably less obvious—environmental parameters have been proportionally less studied. A typical example of such a scarcely studied impact of global change is environmental salinization (Cunillera-Montcusí et al., 2022). Yet, environmental salinization is recognized as a global threat currently affecting oceanic, freshwater and terrestrial ecosystems worldwide (Herbert et al., 2015). For instance, oceanic salinity has increased in areas where evaporation exceeds precipitation (Helm et al., 2010), but also in colder

areas due to global climate changes (e.g., Antarctic, Meredith and King, 2005), potentially inducing major shifts in species distributions (Brischoux et al., 2021). A large proportion of Earth's freshwater wetlands have already been affected by salinization (Cañedo-Argüelles, 2020; Cunillera-Montcusí et al., 2022; Hébert et al., 2022), inducing large shifts in wetland communities and their associated ecosystem functions (Herbert et al., 2015). Even terrestrial environments can be affected by this process (Daliakopoulos et al., 2016), which can deteriorate soil fertility in irrigated arable lands (Singh, 2019; Ondrasek and Rengel, 2021), constrain soil-plant-water transfers (Munns et al., 2020), and affect microbial activity and carbon balance (Gavrichkova et al., 2020).

Coastal habitats—recognized as some of the most diverse and productive habitats on Earth (Hobohm et al., 2021)—are particularly at risk of increasing salinization because of their position at the boundary between land and sea (McLean et al., 2001). Indeed, although coastal wetlands are frequently subjected to moderate salinity levels (i.e., sea spray deposition linked to landward winds, Benassai et al., 2005), they have become particularly exposed to salinization because of two processes that function at different temporal scales. First, predicted sea level rise should induce a progressive increase in environmental salinity of coastal wetlands (Morris et al., 2002), that will ultimately produce landward shifts of these habitats where it is possible (Di Nitto et al., 2014; Field et al., 2016). Second, predicted increases in the frequency and intensity of extreme weather events (i.e., storms and associated marine floods, Dettinger 2011) are expected to induce sudden, unpredictable and abrupt salinization (Delaune et al., 2021).

Salinization-induced osmoregulatory challenges are expected to alter ecological assemblages and exert severe constraints on wildlife (Cañedo-Argüelles et al., 2013; Herbert et al., 2015). Indeed, physiological adaptations are required to live in a specific range of salinity (Beadle, 1957; Kirschner, 1991; Hallows and Knauf, 1994), and coping with high salinity (exceeding the species tolerance range) can be challenging (Rivera-Ingraham and Lignot, 2017). Mechanisms involved in osmoregulation are metabolically costly and thus should be traded off against allocation to other functions, such as growth (Munns and Tester, 2008), reproduction (Morris et al., 2002; Herbert et al., 2015), and activity (Santos et al., 2007; Van Meter and Swan, 2014). Exposure to relatively high salinity can also induce more direct effects, such as malformations (Morris et al., 2002; Santos et al., 2007) or cell damage (Koleva et al., 2017). Most coastal species are subjected to relatively moderate levels of salinity in their day-to-day life (Greenberg et al., 2006), and living in such environments may provide a powerful selective context allowing these species to display adaptive responses to tolerate salinity (Purcell et al., 2008; Hopkins et al., 2016). In order to assess whether coastal species can adapt to the expected changes in environmental salinity, it remains essential to investigate the consequences of exposure to salinity in an environmentally-relevant context.

Amphibians are well-suited study models to investigate such a research question (Hopkins and Brodie, 2015). Although previously thought as mostly intolerant to elevated salinities,

amphibians are relatively diverse and abundant in coastal wetlands (Hopkins and Brodie, 2015). In addition, they are strongly dependent on the availability of fresh water, and are particularly sensitive to salt because of their highly permeable skin (comparatively to other species), which is involved in water, gas and ion exchanges (Hillyard, 1999; Quaranta et al., 2009). In larvae, it has been observed that increased salinity affects whole body osmolality (Gomez-Mestre et al., 2004; Lukens and Wilcoxon, 2020; Tornabene et al., 2022), and negatively influences immune responses (Burraco and Gomez-Mestre 2016) and, in turn, increases the susceptibility of tadpoles to infections and diseases (Milotic et al., 2017; Hall et al., 2020). In addition, increased costs of osmoregulation have been shown to reduce physical performance (Gomez-Mestre et al., 2004; Kearney et al., 2016) and salinity has been shown to reduce activity, speed or movement (Squires et al., 2008; Denoël et al., 2010; Wood and Welch, 2015; Hall et al., 2017), affecting foraging efficiency (Chambers, 2011; Hall et al., 2017) and antipredator responses (Watkins, 1996; Squires et al., 2008; Johnson et al., 2015). Although adult life-stages should be affected by saline conditions (Hopkins and Brodie, 2015; Park and Do, 2020; Lorrain-Soligon et al., 2021), the physiological and behavioural consequences have been less studied than those in larvae. Given the influence of adult life-stages in population dynamics—and thus population persistence—in amphibians (Wells, 2007), such caveats can hamper our ability to thoroughly assess whether coastal amphibians can sustain the expected change in environmental salinity.

In this study, we adopted a comprehensive approach to assess the behavioural and physiological consequences of exposure to environmental salinity in adults of a coastal frog (*Pelophylax* sp., $N = 156$) originating from freshwater and brackish ponds in wetlands from the Atlantic coast of France. First, we used a field-based approach to assess the consequences of pond salinity on 1) physiology [osmolality (Gomez-Mestre et al., 2004), hemoglobin-binding protein (Wang et al., 2020), blood cell composition (Burraco and Gomez-Mestre 2016)], 2) locomotor performance [jumping distance, (Alexander et al., 2012; Kearney et al., 2016; Sanabria et al., 2018)] and 3) ecological responses [morphology and sex-ratio (Lorrain-Soligon et al., 2022b)]. On the same individuals, and for the same measures, we then used an experimental approach to precisely measure the consequences of short-term (48 h) exposure to different levels of salinity (0, 6, 9 or 12 g L⁻¹) similar to those recorded in the field (min: 0 g L⁻¹, max: 16 g L⁻¹). This experiment was preceded and followed by acclimation in freshwater (0 g L⁻¹) to assess whether individuals were able to recover from the effects of exposure to salinity.

First, we hypothesized that pond salinity should influence the distribution of individuals according to their body size (because water and ion fluxes should be linked to surface area to volume ratio, Gordon et al., 1961) and to their sex (because of the energetic cost of osmoregulation, Rivera-Ingraham and Lignot, 2017). Accordingly, we predicted that we should capture larger individuals and more males (as compared to females) in ponds with relatively high salinity. Second, we hypothesized that salinity should influence both physiological markers and performance

(Gutiérrez, 2014). Accordingly, we predicted that, both in the field and under experimental conditions, high levels of salinity should increase plasmatic osmolality (Gomez-Mestre et al., 2004), increase hemoglobin-binding proteins concentration (as salinity induces damages to cells, Koleva et al., 2017), and increase the proportion of neutrophils compared to lymphocytes (Burraco and Gomez-Mestre 2016), but decrease jumping performance and activity (Alexander et al., 2012; Kearney et al., 2016; Sanabria et al., 2018). We predicted that these effects should dissipate when normal conditions (access to freshwater) are restored (Park and Do, 2020). Finally, we further hypothesized that individuals from brackish sites should be locally adapted (Hua and Pierce, 2013), and we predicted that individuals originating from high salinity ponds to be less impacted.

2 METHODS

2.1 Study Site, Species and Field

Procedures

2.1.1 Study Site, Studied Species and Ethics Statement

The study was carried out on the “Réserve Naturelle Nationale de Moëze-Oléron” (45°53'33.36"N, 1°04'59.16"W), located in the Atlantic coast of France (Charente-Maritime). In the study area, *Pelophylax* sp. are composed of viable and fertile hybrids (Graf's hybrid frog, *P. kl. grafi*) of the Marsh frog (*P. ridibundus*) and the Perez's frog (*P. perezi*, Speybroek et al., 2018). These frogs are distinctly aquatic, and active both during the day and at night. In our study site, *Pelophylax* sp. can be found in ponds in which salinity ranges from 0.14 to 16.19 g L⁻¹ (mean: 2.86 ± 2.17 g L⁻¹).

This work was approved by the French authorities under permits R- 45GRETA-F1-10, 135-2020 DBEC and APAFIS#30169-2021022515546003 v3.

2.1.2 Field Procedures

Individuals were captured at night, between 10 and 12 p.m. at 13 different ponds (each separated by less than 1.3 km from one another, see **Supplementary Appendix A**) from 29/03/2021 to 29/05/2021. Salinity (measured with a conductimeter YSI Professional Plus) at the capture sites ranged from 0.10 to 7.16 g L⁻¹ (mean = 3.25 g L⁻¹ ± 2.16 g L⁻¹). On these sites, we captured a total of 156 individuals, 36 in freshwater ponds (<1 g L⁻¹: range 0.10–0.76 g L⁻¹, mean = 0.50 g L⁻¹ ± 0.24 g L⁻¹), and 120 in brackish ponds (>1 g L⁻¹: range 1.46–7.16 g L⁻¹, mean = 4.07 g L⁻¹ ± 1.76 g L⁻¹), as freshwater ponds were less common than brackish ones (see **Supplementary Appendix B** for details on capture dates, salinity of the pond, and sex of captured individuals). All captured individuals weighed more than 15 g, a threshold that allow sexing in our study population. Upon capture, individuals were brought to the laboratory, where they were weighed (with a portable electronic balance ±0.1 g) and measured for body size (snout-vent length: SVL) and hindlimb length (left leg) using a caliper (±0.1 mm). Sex was assessed based on the presence of secondary characteristics (Speybroek et al., 2018). We performed tests of jumping performance and evaluated activity scores (see below).

Finally, we collected blood samples (~1% body mass, Diehl et al., 2001; Soulsbury et al., 2020) through cardiocentesis, using 1 ml syringes and heparinized 30G needles. These blood samples were performed within 2 h after capture (between 2 and 4 a.m.). Collected blood was placed in a 0.675 ml microcentrifuge tube and centrifuged (7 min at 2000 G), and plasma fractions were collected and stored at -18 °C in sealed microtubes until analyses.

2.2 Housing Conditions

Frogs were housed individually in transparent plastic boxes (14 × 16 × 9 cm) with a perforated cover to minimize evaporation while providing sufficient ventilation. Each box was filled with water (see details below) to allow permanent contact with water while allowing aerial respiration. Frogs were not fed during the experiment. They were kept in a room with natural photoperiod.

2.3 Experimental Design

Our experiment consisted of three successive stages:

- 1) Because frogs were captured in ponds with varying salinity (see above), boxes were first filled with freshwater (0.34 g L⁻¹ salinity ± 0.007 SE) for 24 h to allow frogs to restore osmotic balance. This preparatory stage, hereafter termed “acclimation”, was intended to establish plasma normosmolality in all individuals. At the end of this stage, individuals were weighed, tests of jumping distance and activity scores were performed, and a blood sample was collected. Individuals were then randomly allocated to the experimental groups. Experimental groups were identical in term of mass, size (SVL) and sex distribution (all *p*-values > 0.751; see **Supplementary Appendix C**).
- 2) In the second stage (hereafter termed “Exposure”), we subjected for 48 h the frogs to one of four salinity levels: freshwater (0.35 g L⁻¹ salinity ± 0.003 SE, N = 47 individuals, hereafter 0 g L⁻¹ for simplicity) and three different brackish water treatments, namely 6 g L⁻¹ (6.11 g L⁻¹ salinity ± 0.01 SE, N = 46 individuals), 9 g L⁻¹ (9.14 g L⁻¹ ± 0.02 SE, N = 30 individuals) and 12 g L⁻¹ (12.08 g L⁻¹ salinity ± 0.040 SE, N = 33 individuals). Water was prepared by dissolving sea salt in tap water. Individuals were weighed every 24 h, while tests of jumping distance and activity scores were performed and a blood sample was collected at the end of the exposure to salinity treatments.
- 3) In the last stage (hereafter termed “Recovery”), each treatment water was replaced by freshwater (0.35 g L⁻¹ salinity ± 0.009 SE) for 24 h in order to allow individuals to restore their osmotic balance. Jumping performance and activity scores were assessed at 12 and 24 h and a final body mass measure was collected at the end of this stage. Individuals were then released at the site of capture. We did not collect blood at the end of the recovery stage for ethical reasons (i.e., to limit both the number of punctures and the amount of blood withdrawn). We relied on an indirect assessment of osmotic balance at the end of the recovery stage by measuring salt effluxes (salinity of the water, see below). Relationships between salt fluxes and osmolality were assessed during the treatment stage of the experiment and

verified that both parameters were linked (see below). Unfortunately, eight individuals that had been exposed to the 12 g L^{-1} treatment died, either during exposure or during recovery, and determinants of mortality (e.g., morphology) were analysed (see below).

2.4 Measurements

2.4.1 Behavioural Parameters

2.4.1.1 Activity Scores

We evaluated the condition of each individual using an activity score during daily manipulation (i.e., during weighing). These scores were set on a scale ranging from 1 to 5. To score activity, we referred to the following quantifications: 1) Weak individuals with low muscular strength (which is scored by the experimenter holding the frog and evaluating its posture and its reaction to manipulation) and no tendency to engage in escape behaviour, no or very few movements (individuals were almost motionless). These individuals did not jump if not stimulated. 2) Weak individuals with low muscular strength, and no tendency to engage in escape behaviour, few movements. These individuals responded weakly to manipulation but did not jump if not stimulated. 3) Individuals moved, had low muscular strength, tried to evade manipulation, jumped even if not stimulated. These individuals did not move energetically and stopped jumping after one or two jumps if not stimulated. 4) Individuals moved energetically, tried to evade manipulation, had perceptible muscular strength, jumped even if not stimulated, but did stop jumping after five or six jump if not stimulated. 5) Individuals moved energetically, tried to evade manipulation, had perceptible muscular strength, jumped even if not stimulated, and did not stop jumping and moving during whole duration of the experiment. For consistency, all activity score assessments were performed by the same person (LLS).

2.4.1.2 Jumping Distances

Jumping performance was assessed by measuring the distance travelled by a frog for each jump during six consecutive jumps (Greenberg and Palen, 2021). Individuals were gently taken from their boxes and placed on the floor. In most cases, jumps occurred naturally, but on a few instances (i.e., for frogs from the 12 g L^{-1} group) jumping was elicited by gently probing the tip of the urostyle with a finger. Distance of each jump was measured with a ruler ($\pm 0.5 \text{ cm}$) and divided by the SVL of frogs to provide a more meaningful measure of locomotor performance (Van Damme and Van Dooren, 1999). The frogs were allowed to jump on an area of 9 m^2 ($3 \times 3 \text{ m}$). The six consecutive jumps were measured by following the frog in its displacements. These assays were always performed at night, between 2 and 4 a.m., except for the measures made during recovery (after 12 h), which were performed between 2 and 4 p.m. Maximum jumping distances were also analysed and produced similar results than mean jumping distance (results not shown).

2.4.2 Physiological Parameters

2.4.2.1 Salt Fluxes and Osmolality

During all stages, the salinity of water was recorded daily (and each 12 h during the recovery period) to approximate the

direction of diffusion of salt based on the change in water salinity, using a calibrated real-time conductivity meter (Testo 240, Testo AG, Germany). Plasma osmolality (mOsmol kg^{-1}) was measured from $10 \mu\text{l}$ aliquots on a Vapro2 osmometer (Elitech group).

2.4.2.2 Hemoglobin-Binding Protein

Individuals exposed to relatively high salt concentrations may present cells with ruptured membranes (Koleva et al., 2017), releasing free hemoglobin. Hemoglobin-binding proteins can be found in some amphibians species (in a newt species, *Taricha granulosa*, see Francis et al., 1985), but not in all (for example in *Xenopus tropicalis*, see Wicher and Fries, 2006) and are known to bind free hemoglobin in order to mitigate damages caused by reactive oxygen components during inflammation (Quaye, 2008; Andersen et al., 2017). We attempt to quantify these hemoglobin-binding proteins in $7.5 \mu\text{L}$ plasma samples using a commercially-available assay (TP801, Tri-Delta Diagnostics, United States). This colorimetric test measures the preservation of the peroxidase activity of bounded hemoglobin, which should be proportional to the concentration of the hemoglobin-binding proteins present in the plasma sample. We followed the instructions provided by the manufacturer with slight modifications following Matson et al. (2012). The standards, which were included in duplicate in each plate ($n = 2$), ranged from 2.5 to 0.039 mg mL^{-1} . For 15 samples, values were below the standard curve and were therefore transformed to 0. The measured concentration of the hemoglobin-binding proteins varied from 0 to 2.45 mg mL^{-1} . Using the standards, we calculated the intra- and inter-plate coefficients of variation to 2 and 4%, respectively. A negative ($7.5 \mu\text{l}$ of diluent) control was also included in duplicate in each plate. We measured plasma absorbance (spectrophotometer Anthos 2010; Biochrom) at 600 nm , on $7.5 \mu\text{l}$ plasma, before adding the final reagent that induced the colorimetric reaction. Final absorbance values at 600 nm were corrected by the pre-scan absorbance values at 600 nm , which allowed us to control for sample differences in colour and cloudiness (Matson et al., 2012). We have to emphasize that this assay is still at its infancy in amphibians. The role of such proteins in these taxa is not well understood and further studies are needed in order to thoroughly describe hemoglobin-binding proteins in amphibians, and/or to develop a specific assay. Moreover, we used a kit aimed to measure binding activity to human hemoglobin. The affinity of frog's proteins to human hemoglobin, used in this assay, is still unknown. As such, our assay could also have measured the peroxidase activity, instead of hemoglobin-binding proteins concentration.

2.4.2.3 Leukocyte Counts

Neutrophils are potent effector cells of the innate immune response, which are relatively rapid and perform non-specific defences (Demas et al., 2011). In comparison, lymphocytes are implicated in the acquired immune response, and are generally slower and induce pathogen-specific responses that are an additional line of defence (Demas et al., 2011), even if they have innate-like properties (Flajnik, 2018). They play a key role in adaptive immunity, and can differentiate into memory cells and are involved in the production of antibodies

(immunoglobulin) (Du Pasquier et al., 1972; Sprent and Tough, 1994). A stress (i.e., a factor experienced by individuals beyond their tolerance range, that can decrease individuals fitness and/or increase mortality), such as salinity in our context, should modify the proportion of these leucocytes. Indeed, neutrophils and lymphocytes are affected by stress in opposite directions (Davis et al., 2008), as a stress may induce redistribution of lymphocytes from the blood to other body compartments (Dhabhar et al., 1995; Dhabhar, 2002; Davis et al., 2008), causing a significant reduction in their circulating numbers, while neutrophils are redirected from body compartments to the blood (Bishop et al., 1968; Davis et al., 2008). Thus, an increase in N:L ratio is considered to be a measure of an amphibian's response to stress agents (Davis et al., 2008; Silva et al., 2020). In fact, high N:L ratios may indicate high levels of glucocorticoids (Davis et al., 2008), which alters leukocyte components in response to stress, even if not all organisms express a correlation between circulating glucocorticoid levels and N:L ratios, as these two variables play different roles in the stress response (Müller et al., 2011; Davis and Maney, 2018).

Monocytes are the precursors of tissue macrophages and act as cell phagocytes associated with innate defences against bacterial infections (van Furth and Cohn, 1968). They serve as phagocytic and antigen-presenting cells (Claver and Quaglia, 2009). Little is known about eosinophil function in amphibians, but there is some evidence that they respond to trematode infestations (Claver and Quaglia, 2009). Their increase is thought to be a common response to contaminants, as eosinophils respond to stimuli such as parasitic infections or pollutants (Kiesecker, 2002; Claver and Quaglia, 2009). The exact functions of basophils in amphibians have not yet been well-established (Claver and Quaglia, 2009), but they can participate in innate immune responses (by secreting proteins such as toxins) and serve as antigen-presenting cells (Demas et al., 2011). They also play an important role in early inflammation reactions (Claver and Quaglia, 2009).

To assess the effect of exposure to salt on leukocyte profile (see Burraco and Gomez-Mestre, 2016; Hall et al., 2020), a drop of blood was smeared on a slide. Slides were air-dried and fixed in absolute methanol for 5 min, and then coloured using May-Grünwald-Giemsa staining (3 min bath in May-Grünwald; 1 min bath in tampon solution, and 10 min bath in Giemsa 1/20). Neutrophils, lymphocytes (and the corresponding ratio of neutrophils to lymphocytes, hereafter N:L ratio), monocytes, eosinophils and basophils were counted by a single observer (LLS). Up to 100 leucocytes were counted, using a X100 ocular (Microscope Primo Star 8) microscope (total magnification $\times 1,000$) and immersion oil.

Hemoglobin-binding proteins and leukocytes analyses were performed on 40 individuals (N = 120 samples: three blood samples per individuals, on 10 individuals per treatment). On these individuals, the four experimental groups had similar mass, size (SVL) and sex distribution (all p -values > 0.771; see **Supplementary Appendix C**).

2.5 Statistical Analysis

2.5.1 Effects of Exposure to Salinity in the Field

To assess how salinity of the ponds influences ecological responses (sex ratio: proportion of females), morphology [SVL,

mass, body condition index (BCI, calculated as the residuals from the regression of log body mass on log SVL)], physiology [osmolality, blood properties (hemoglobin-binding protein, N:L ratio, leukocyte profiles)], and behaviour [jumping performance, and activity (as activity is rated from 1 to 5, we evaluated it as a proportion)], we performed LMs (linear models) or quasibinomial GLMs (generalized linear models; for leukocytes profiles and activity) with salinity as continuous explanatory variable, or the quadratic form when models better fitted with a quadratic variation of salinity (using a backward selection procedure; likelihood ratio tests). As amphibians are sexually dimorphic (Wells, 2007), sex was also included as covariate in all models, as well as the interaction between salinity and sex. However, the effect of sex was never retained in the final models. Moreover, for sex ratio, we included Julian date as a covariate, but it was excluded during the model selection procedure. Finally, to understand if variations in jumping distance were influenced by individual morphology more than by pond salinity, we also computed LMs on jumping performance with SVL or hindlimb length as covariate. Mean jumping distance was not linked to body size (Estimate = 0.059, SE = 0.039, t -value = 1.513, p -value = 0.132) or hindlimb length (Estimate = 0.048, SE = 0.048, t -value = 1.018, p -value = 0.31).

2.5.2 Effects of Experimental Exposure to Salinity

2.5.2.1 Variations During Acclimation

Variations in all the studied parameters (body mass, osmolality, N:L ratio, leukocyte proportions, hemoglobin-binding protein concentration, jumping performance and activity) during acclimation were assessed using LMMs (linear mixed models) or quasibinomial GLMMs (generalized linear mixed models), and Tukey post-hoc tests. Time was set as an explanatory variable, and individual as a random effect. Differences between experimental groups after acclimation were assessed using LMs and quasibinomial GLMs with treatment as a covariate, with Tukey post-hoc tests.

2.5.2.2 Variations During Exposure and Recovery

The effect of salinity was assessed using four salinity treatments (0, 6, 9 and 12 g L⁻¹).

The effect of each treatment was assessed on mass (using absolute body mass changes, values at 48 h exposure compared to the beginning of exposure for variations during exposure, values at 24 h recovery compared to the end of exposure for variations during recovery). These effects were also assessed on changes in the concentration of the treatment (using variation in water concentration, values at 48 h exposure compared to the beginning of the exposure), and on changes in concentration of the recovery treatment (using variation in water concentration, values at 24 h recovery compared to the beginning of the recovery). Finally, the effect of each treatment was assessed on osmolality, hemoglobin-binding protein and the N:L ratio of individuals (at 48 h exposure compared to the beginning of the exposure), and on jumping performance (corrected by individuals size, at 48 h exposure compared to the beginning of exposure for variations during exposure, and at 24 h recovery

compared to the end of exposure for variations during recovery). All these effects were assessed using LMMs.

The effect of each treatment on the leukocyte profiles at 48 h treatment, and on activity (as activity is rated from 1 to 5, we evaluated it as a proportion; at 48 h exposure compared to the beginning of exposure for variations during exposure, and at 24 h recovery compared to the end of exposure for variations during recovery), was assessed using quasibinomial GLMMs.

In all of these models, we included treatment, time, pond salinity, sex and SVL as covariates, as well as the interactions between treatment and time, treatment and pond salinity, treatment and SVL and treatment and sex as covariates. As we performed multiple measures on a single individual, individual was set as a random effect. The best model was selected using a backward selection procedure (0.1 cut-off *p*-value) using likelihood-ratio tests, and only the retained parameters were represented. The effects of pond salinity and sex were never retained in the selection procedure. Each model performed were checked for accuracy by analyzing residuals distribution and over/under dispersion.

In order to facilitate comprehension, the intermediate measures (at 24 h exposure and 12 h recovery) that were assessed for some variables (i.e., body mass and changes in treatment salinity at 24 h exposure, and changes in salinity and jumping distance at 12 h recovery), are not presented in results, but are represented in **Supplementary Appendix D** for treatment and **Supplementary Appendix E** for recovery. Statistics were computed and gave the same results as when we compared only the end of the treatment and the end of the recovery. Detailed statistics are also presented in **Supplementary Appendix F** for treatment, and **Supplementary Appendix G** for recovery.

2.5.3 Mortality

Finally, to understand determinants of mortality during our treatments, we computed GLMs with quasibinomial distribution and mass, loss in mass during the treatment, size (SVL), BCI, sex, salinity of the pond and osmolality as covariates. We did not include salinity of treatments, as only eight individuals from the 12 g L⁻¹ treatment died. Because of this low sample size, we did not compute a complete model with all these variables together, and all analyses for these different covariates were conducted separately.

All data analysis were performed using R 3.6.3 (R Core Team, 2020) and Rstudio v1.1.419.

3 RESULTS

3.1 Effects of Exposure to Salinity in the Field

3.1.1 Sex-Ratio and Morphology

Sex ratio (proportion of females compared to total number of individuals) decreased with pond salinity (**Figure 1A**; **Table 1**). Individuals were larger in ponds with higher salinity (**Figure 1B**; **Table 1**), and heavier, as body mass increased with increasing salinity (**Table 1**). BCI was lower in ponds with higher salinity (**Table 1**).

3.1.2 Physiological Parameters

Osmolality increased with pond salinity (**Figure 1C**; **Table 1**). Neither the proportion of neutrophils, lymphocytes and basophils, nor the N:L ratio were correlated with pond salinity (**Table 1**). The proportion of monocytes decreased, while the proportion of eosinophils increased, with increasing pond salinity (**Table 1**). Details on cell counts from blood smears are given in **Supplementary Appendix H**. Hemoglobin-binding protein concentration was not correlated with pond salinity (**Table 1**).

3.1.3 Jumping Performance and Activity

Mean jumping distance was correlated with pond salinity with a curvilinear relationship (**Table 1**). Jumping distance decreased with pond salinity <~4 g L⁻¹, and increased with pond salinity >~4 g L⁻¹. Activity scores were not correlated with pond salinity (**Table 1**).

3.2 Effects of Experimental Exposure to Salinity

The effects of acclimation in freshwater are summarized in **Supplementary Appendix I**. At the onset of the experimental exposure to salinity, all groups were similar for all the parameters measured (**Supplementary Appendix I**), except for osmolality (**Supplementary Appendix I**), where values for individuals exposed to 12 g L⁻¹ were slightly higher compared to those exposed to 6 g L⁻¹ (Estimate = 9.684, SE = 3.356, *z*-value = 2.885, *p*-value = 0.023).

3.2.1 Body Mass

The changes in body mass during exposure were different between treatments (**Figure 2A**; **Supplementary Appendix J**).

Individuals from the freshwater group showed a slight mass loss during exposure (Estimate = -1.151, SE = 0.430, *z*-value = -2.677, *p*-value = 0.021) (**Figure 2A**; **Supplementary Appendix F**). Individuals exposed to 6 g L⁻¹ showed a relatively stable pattern with no mass changes (Estimate = -0.470, SE = 0.435, *z*-value = -1.080, *p*-value = 0.527) (**Figure 2A**; **Supplementary Appendix F**). Individuals exposed to 9 g L⁻¹ lost mass during exposure (Estimate = -1.490, SE = 0.538, *z*-value = -2.768, *p*-value = 0.016) (**Figure 2A**; **Supplementary Appendix F**), as well as individuals exposed to 12 g L⁻¹ (Estimate = -6.270, SE = 0.537, *z*-value = -11.666, *p*-value < 0.001) (**Figure 2A**; **Supplementary Appendix F**). At the end of the exposure, body mass was similar between individuals exposed to 0, 6 and 9 g L⁻¹ (all *p*-values > 0.277), but significantly lower for individuals at 12 g L⁻¹ (all *p*-values < 0.004) (**Figure 2A**). The body size of individuals did not influence the mass changes across treatments (**Supplementary Appendix J**).

3.2.2 Salt Influx and Osmolality

The changes in salt concentration of water during exposure were different between treatments (**Figure 2B**; **Supplementary Appendix J**). In the freshwater group, treatment concentration remained constant during exposure (*p*-value = 0.973) (**Figure 2B**; **Supplementary Appendix F**). In the 6 g L⁻¹ treatment (Estimate = -0.338, SE = 0.079, *z*-value = -4.274, *p*-value < 0.001), the 9 g L⁻¹

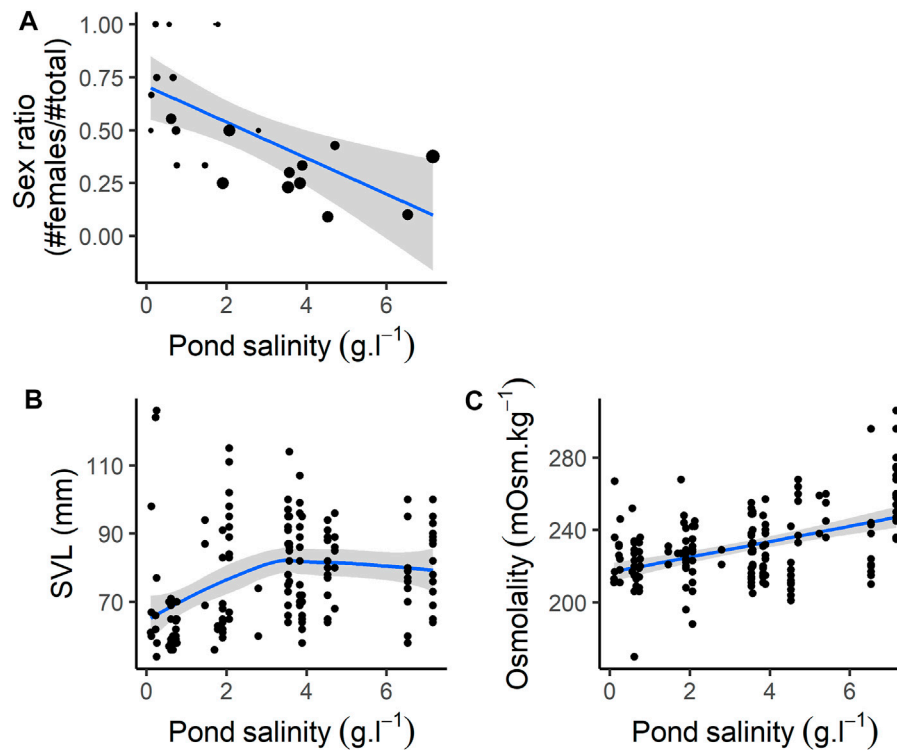
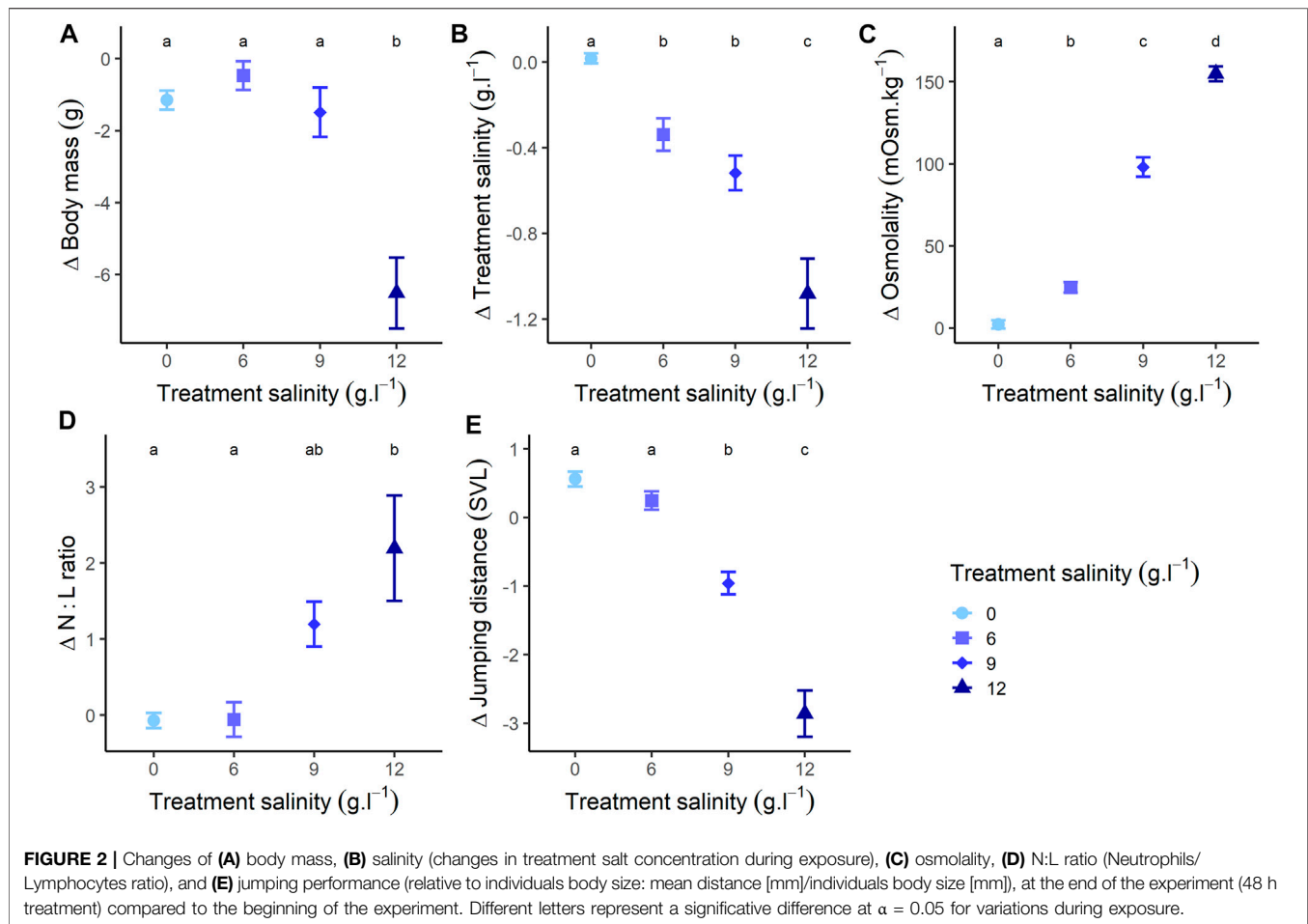


FIGURE 1 | Individual characteristics according to pond salinity. **(A)** Sex ratio (note that size of the dots are proportional to the sample size, ranging from 1 to 16, see **Supplementary Appendix B**), **(B)** SVL (size, mm), **(C)** Osmolality.

TABLE 1 | Effect of pond salinity on osmolality, SVL (size, mm), mass (g), body condition (BCI), Sex ratio, hemoglobin-binding protein, Neutrophils/Lymphocytes ratio (N:L ratio), leucocytes counts, jumping distance (as the jumping distance corrected by individuals SVL), and activity. Computed on N = 156 individuals (see also **Supplementary Appendix B** for number of individuals captured on each site), except for hemoglobin-binding proteins and leucocytes counts, where it was computed on N = 40 individuals.

Comparison	Covariable	R ²	F	Estimate	SE	t/z	p-value
Sex ratio (#Females/#Total)	Salinity	-	-	-0.276	0.085	-3.236	0.001
SVL	Salinity	0.11	10.61	7.087	1.859	3.811	<0.001
	Salinity ²	0.11	10.61	-0.714	0.246	-2.897	0.004
Mass	Salinity	0.008	2.194	1.653	1.116	1.481	0.141
	Salinity ²	0.218	22.62	-0.003	0.001	-5.062	<0.001
BCI	Salinity	0.218	22.62	0.016	0.004	3.559	<0.001
	Salinity ²	0.218	22.62	-0.003	0.001	-5.062	<0.001
Osmolality	Salinity	0.194	40.81	4.269	0.668	6.388	<0.001
Neutrophil proportion	Salinity	-	-	-0.019	0.036	-0.544	0.59
Lymphocyte proportion	Salinity	-	-	0.002	0.015	0.145	0.886
N:L ratio	Salinity	-0.025	0.053	-0.003	0.012	-0.23	0.819
Monocyte proportion	Salinity	-	-	-0.088	0.04	-2.178	0.036
Eosinophil proportion	Salinity	-	-	0.136	0.063	2.144	0.039
Basophil proportion	Salinity	-	-	0.066	0.126	0.527	0.601
Hemoglobin-binding protein concentration	Salinity	0.007	1.281	-0.011	0.01	-1.132	0.265
Jumping performance	Salinity	0.157	15.48	-0.871	0.16	-5.446	<0.001
	Salinity ²	0.157	15.48	0.103	0.021	4.881	<0.001
Activity	Salinity	-	-	<0.001	<0.001	-0.018	0.985



treatment (Estimate = -0.518 , SE = 0.097 , z -value = -5.317 , p -value < 0.001) and the 12 g L^{-1} treatment (Estimate = -1.069 , SE = 0.098 , z -value = 10.936 , p -value < 0.001), concentration decreased during exposure (Figure 2B; Supplementary Appendix F). At the end of the exposure, changes in concentration were similar for the 6 g L^{-1} and 9 g L^{-1} treatments (p -value = 0.448), but significantly lower for the 0 g L^{-1} treatment and higher for the 12 g L^{-1} treatment (all p -values < 0.005) (Figure 2B). The body size of individuals did not influence the changes of treatment concentration across treatments (Supplementary Appendix J).

Accordingly with the observed diverging salt influxes, the changes in plasma osmolality values during exposure were different between treatments (Figure 2C; Supplementary Appendix J). Osmolality of individuals in the freshwater group remained stable during the whole experiment (Estimate = 2.722 , SE = 3.457 , z -value = 0.739 , p -value = 0.711) (Figure 2C; Supplementary Appendix F). Conversely, osmolality increased in individuals exposed to 6 g L^{-1} (Estimate = 24.781 , SE = 3.158 , z -value = 7.114 , p -value < 0.001), 9 g L^{-1} (Estimate = 98.067 , SE = 5.512 , z -value = 23.350 , p -value < 0.001) and 12 g L^{-1} (Estimate = 153.839 , SE = 3.659 , z -value = 35.372 , p -value < 0.001) (Figure 2C; Supplementary Appendix F). As a consequence, osmolality was different across all groups at the end of the exposure (all p -value < 0.001). Additionally, variations of body

mass were negatively correlated with osmolality (Estimate = -0.027 , SE = 0.005 , t -value = -5.480 , p -value < 0.001), and change in treatment concentration (i.e., the salt concentration of their housing water) were negatively correlated with osmolality (Estimate = -0.003 , SE < 0.001 , t -value = -5.783 , p -value < 0.001). The body size of individuals did not influence the osmolality changes across treatments (Supplementary Appendix J).

3.2.3 Leukocytes Counts

The changes in lymphocyte proportion during exposure were different between salinity treatments (Supplementary Appendix J). More specifically, lymphocyte proportion remained stable for individuals in the freshwater group (p -value = 0.842), but increased for individuals exposed to 6 g L^{-1} (p -value = 0.032), and decreased for individuals exposed to 9 g L^{-1} (Estimate = -0.488 , SE = 0.079 , z -value = -6.166 , p -value < 0.001) and 12 g L^{-1} (Estimate = -0.775 , SE = 0.087 , z -value = -8.895 , p -value < 0.001). At the end of the exposure, lymphocyte proportions were similar between individuals exposed to 0 and 6 g L^{-1} (all p -values > 0.102), but significantly lower for individuals exposed to 9 g L^{-1} (all p -values < 0.026) and 12 g L^{-1} (all p -values < 0.016).

The changes in neutrophil proportion during exposure were different between salinity treatments (Supplementary Appendix

J). Neutrophil proportions remained constant in the 0 g L^{-1} (p -value = 0.662) and 6 g L^{-1} (p -value = 0.218) treatments but increased in the 9 g L^{-1} (Estimate = 0.838, SE = 0.095, z -value = 8.825, p -value < 0.001) and 12 g L^{-1} (Estimate = 0.969, SE = 0.094, z -value = 10.312, p -value < 0.001) treatments. At the end of the exposure, neutrophil proportions were similar for individuals exposed to 12 g L^{-1} and 9 g L^{-1} (p -value = 0.813), and for individuals exposed to 0 g L^{-1} and 6 g L^{-1} (p -value = 0.710). All other comparisons were significant (all p -values < 0.001).

As a consequence, during exposure, N:L ratios varied differently across treatments (Figure 2D; Supplementary Appendix J). This ratio remained stable for individuals in the freshwater group (p -value = 0.859) or exposed to 6 g L^{-1} (p -value = 0.886), but increased for individuals exposed to 9 g L^{-1} (Estimate = 1.197, SE = 0.397, z -value = 3.012, p -value = 0.005) and 12 g L^{-1} (Estimate = 2.195, SE = 0.397, z -value = 5.522, p -value < 0.001) (Figure 2D; Supplementary Appendix F). At the end of the exposure, N:L ratios were similar for individuals exposed to 9 g L^{-1} and 12 g L^{-1} (p -value = 0.054) and for individuals exposed to 0 g L^{-1} and 6 g L^{-1} (p -value = 0.997), but the other comparisons were significant (all p -values < 0.037).

The changes in eosinophil proportion during exposure were different between salinity treatments (Supplementary Appendix J). Eosinophil proportions remained constant for individuals exposed to 0 g L^{-1} (p -value = 0.224) and 6 g L^{-1} (p -value = 0.567), but decreased for individuals exposed to 9 g L^{-1} (Estimate = -0.858, SE = 0.247, z -value = -3.470, p -value < 0.001) and 12 g L^{-1} (Estimate = -1.101, SE = 0.273, z -value = -4.035, p -value < 0.001). Overall, at the end of exposure, eosinophil proportions did not differ between treatments (for all p -value > 0.101), except for individuals exposed to 0 g L^{-1} which had a higher eosinophil proportions compared to those exposed to 12 g L^{-1} (p -value = 0.016).

Conversely, monocyte proportions and basophil proportions did not vary through time and treatment during the experiment (both p -value > 0.314) (Supplementary Appendix J). Additionally, the concentration of hemoglobin-binding proteins did not vary through time and treatment during the experiment (p -value = 0.520) (Supplementary Appendix J). For all blood cell counts and hemoglobin-binding protein, the body size of individuals did not influence their response to exposure to salinity across treatments (Supplementary Appendix J).

3.2.4 Behaviour

The changes in jumping distance during exposure varied among treatments (Figure 2E; Supplementary Appendix J). Individuals from the freshwater group showed an increase in jumping distance during exposure (Estimate = 0.561, SE = 0.158, z -value = 3.547, p -value < 0.001), and individuals exposed to 6 g L^{-1} showed no variation in jumping distance (Estimate = 0.246, SE = 0.160, z -value = 1.539, p -value = 0.126) (Figure 2E; Supplementary Appendix F). Conversely, jumping distance decreased for individuals exposed to 9 g L^{-1} (Estimate = -0.958, SE = 0.198, z -value = -4.838, p -value < 0.001), and 12 g L^{-1} (Estimate = -2.901, SE = 0.203, z -value = -14.313, p -value < 0.001) (Figure 2E; Supplementary Appendix F). At

the end of the exposure, jumping distance was similar between individuals exposed to 0 and 6 g L^{-1} (p -value = 0.957), but significantly lower for individuals in other treatments (for all, p -value < 0.031) (Figure 2E). There was an effect of size as, for all treatments, jumping performance decreased more for larger individuals (Estimate = -0.050, SE = 0.005, t -value = -9.317, p -value < 0.001) (Supplementary Appendix J).

The changes in activity during exposure were also different among treatments (Supplementary Appendix J). Individuals exposed to 0 g L^{-1} (p -value = 0.956), 6 g L^{-1} (p -value = 0.998) and 9 g L^{-1} (p -value = 0.836) showed stable activity scores during exposure. Conversely, activity scores of individuals exposed to 12 g L^{-1} decreased (Estimate = -0.860, SE = 0.181, z -value = -4.750, p -value < 0.001). At the end of the exposure, activity scores were similar between individuals exposed to 0, 6 and 9 g L^{-1} (all p -values > 0.999), but significantly lower for individuals at 12 g L^{-1} (all p -values < 0.001). The body size of individuals did not influence the activity changes across treatments (Supplementary Appendix J).

3.3 Effects of Recovery in Freshwater

3.3.1 Body Mass

The changes in body mass during recovery were different across treatments (Figure 3A; Supplementary Appendix J). Individuals previously exposed to 0 and 9 g L^{-1} treatments showed a stable pattern during recovery (for both p -value > 0.240) (Figure 3A; Supplementary Appendix G). On the contrary, body mass of individuals previously exposed to 6 g L^{-1} significantly decreased (Estimate = -0.947, SE = 0.432, z -value = -2.191, p -value = 0.030) (Figure 3A; Supplementary Appendix G). Conversely, the body mass of individuals previously exposed to 12 g L^{-1} significantly increased (Estimate = 6.661, SE = 0.564, z -value = 11.812, p -value < 0.001) (Figure 3A; Supplementary Appendix J) and attained body mass similar to that of the beginning of the exposure (Estimate = 0.202, SE = 0.589, z -value = 0.344, p -value = 0.732) (Figure 3A). At the end of the recovery, body mass was similar among treatments (for all p -value > 0.059) (Figure 3A). We found an effect of body size on the pattern of body mass gain only in individuals exposed to 12 g L^{-1} (Supplementary Appendix J). In this group, larger individuals gained more mass (Estimate = 0.143, SE = 0.067, t -value = 2.150, p -value = 0.042) (Supplementary Appendix J).

3.3.2 Salt Efflux

The changes in salt concentration of water during recovery were different among treatments (Figure 3B; Supplementary Appendix J). Salt concentration remained stable for individuals previously exposed to 0 g L^{-1} (p -value = 0.390) (Figure 3B; Supplementary Appendix J). Conversely, salt concentration increased for individuals previously exposed to 6, 9 and 12 g L^{-1} (for all p -values < 0.001) (Figure 3B; Supplementary Appendix G). After 24 h recovery, changes in salt concentration were different among all treatments (all p -values < 0.001) (Figure 3B). We found an effect of body size on the pattern of variation in salt concentration in individuals previously exposed to 6 g L^{-1} (Estimate = 0.002, SE < 0.001, t -value = 3.486, p -value = 0.001), 9 g L^{-1} (Estimate =

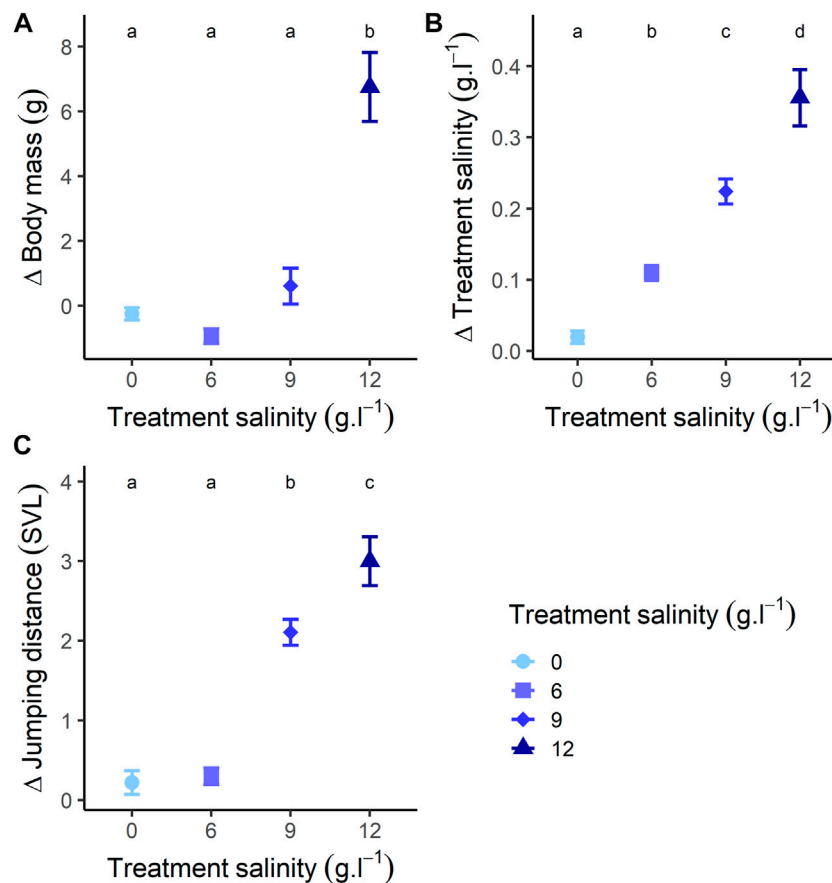


FIGURE 3 | Changes of **(A)** body mass, **(B)** salinity (changes in salt concentration during recovery), and **(C)** jumping performance (relative to individuals body size: mean distance [mm]/individuals body size [mm]), at the end of the recovery (24 h recovery) compared to the beginning of the recovery. Different letters represent a significant difference at $\alpha = 0.05$ for variations during recovery.

0.004, SE < 0.001, t-value = 5.181, p -value < 0.001) and 12 g L⁻¹ (Estimate = 0.010, SE = 0.001, t-value = 6.200, p -value < 0.001) (**Supplementary Appendix J**). Increase in salt concentration was higher for larger individuals.

3.3.3 Behaviour

The changes in jumping distance during recovery were different across treatments (**Figure 3C**; **Supplementary Appendix J**). Jumping distance of individuals previously exposed to 0 g L⁻¹ remained stable (Estimate = -0.251, SE = 0.138, z-value = -1.822, p -value = 0.164) (**Figure 3C**; **Supplementary Appendix G**). Jumping distance increased for individuals previously exposed to 6 g L⁻¹ (Estimate = 0.331, SE = 0.135, z-value = 2.453, p -value = 0.039) (**Figure 3C**; **Supplementary Appendix G**). Jumping distance of individuals previously exposed to and 9 g L⁻¹ increased (Estimate = 2.108, SE = 0.162, z-value = 13.015, p -value < 0.001), and reached values greater than those recorded at the beginning of the exposure (Estimate = 1.150, SE = 0.178, z-value = 6.446, p -value < 0.001) (**Figure 3C**; **Supplementary Appendix G**). Similarly, jumping distance of individuals previously exposed to 12 g L⁻¹ increased (Estimate = -2.993, SE = 0.183, z-value = -16.339, p -value < 0.001), and

became similar to jumping performance at the beginning of exposure (p -value = 0.999) (**Figure 3C**; **Supplementary Appendix G**). After 24 h recovery, jumping distance was similar among individuals previously exposed to 0, 6 and 12 g L⁻¹ (all p -values > 0.545), but significantly higher for individuals at 9 g L⁻¹ (all p -values < 0.012) (**Figure 3C**). There was an effect of size where, for all treatments, the increase in jumping performance was lower for larger individuals (Estimate = -0.061, SE = 0.009, z-value = -6.905, p -value < 0.001) (**Supplementary Appendix J**).

The changes in activity during recovery were different among treatments (**Supplementary Appendix J**). Individuals previously exposed to 0 g L⁻¹ (p -value = 0.992), 6 g L⁻¹ (p -value = 0.991) and 9 g L⁻¹ (p -values = 0.977) did not vary in activity scores during recovery. Conversely, activity scores of individuals previously exposed to 12 g L⁻¹ increased (Estimate = 0.827, SE = 0.196, z-value = 4.226, p -value < 0.001). Overall, activity scores between the beginning of the treatment and the end of the recovery were similar (p -value = 0.981). At 24 h recovery, activity was similar among all groups (all p -values > 0.993). The body size of individuals did not influence the activity changes across treatments (**Supplementary Appendix J**).

TABLE 2 | Summary of the effects of increasing salinity on morphological, physiological and behavioural parameters (both in the field and experimentally). NS, Non-significant, - indicates variables that have not been tested, computed on N = 156 individuals (0 g L⁻¹: N = 47; 6 g L⁻¹: N = 46; 9 g L⁻¹: N = 30; 12 g L⁻¹: N = 33), except for hemoglobin-binding proteins and leucocytes counts, where it was computed on N = 40 individuals (10 per treatments).

Explanatory variables	Effects of increasing salinity		
	Field (0.10–7.16 g L ⁻¹)	Experimental	
		Exposure (0–12 g L ⁻¹)	Recovery
Sex ratio (#Females/#Total)	Decrease	-	-
SVL	Increase	-	-
Mass	NS	Decrease	Increase
BCI	Decrease	-	-
Treatment concentration	-	Decrease	Increase
Osmolality	Increase	Increase	-
Lymphocyte proportion	NS	Decrease	-
Neutrophil proportion	NS	Increase	-
N:L ratio	NS	Increase	-
Eosinophil proportion	Increase	Decrease	-
Monocyte proportion	Decrease	NS	-
Basophil proportion	NS	NS	-
Hemoglobin-binding protein concentration	NS	NS	-
Jumping performance	Increase for intermediate salinities (4 g L ⁻¹)	Decrease	Increase
Activity	NS	Decrease	Increase

TABLE 3 | Effects of mass, loss of mass, SVL (size), body condition, sex, pond salinity and osmolality on individual mortality in the 12 g L⁻¹ salinity treatment (computed only on individuals exposed to the 12 g L⁻¹ salinity treatment, N = 33 individuals).

Covariate	Estimate	SE	t-value	p-value
Mass	-0.145	0.06	-2.4	0.023
Loss in mass	0.124	0.092	1.353	0.186
SVL	-0.141	0.064	-2.199	0.028
BCI	-10.432	11.94	-0.874	0.382
Sex	-0.065	0.841	-0.077	0.939
Pond salinity	0.371	0.192	1.934	0.053
Osmolality	-0.045	0.047	-0.962	0.336

The overall effects of salinity (both in the field and at the lab) on morphological, physiological and behavioural parameters are summarized in **Table 2**.

3.4 Mortality

During our experiments, eight individuals died after being exposed to the 12 g L⁻¹ treatment: five during treatment, three during recovery. Mortality was linked to morphology and occurred in smaller and lighter individuals (**Table 3**).

4 DISCUSSION

Our study showed that the physiology and behaviour of a coastal frog are influenced by environmental levels of salinity, both in the field and experimentally. Our study species can be found in ponds in which the salinity (ranging from 0 to 16 g L⁻¹) is similar to those experienced by other species found in coastal marshes (Hopkins and Brodie, 2015). The salinity experienced in the field (0–7 g L⁻¹) was correlated with physiological parameters (osmolality, monocytes and eosinophils counts), negatively

correlated with body condition and locomotor performance and seems to influence size- and sex-specific habitat selection (see also Lorrain-Soligon et al., 2022b). Experimentally, we demonstrated that short-term exposure to environmental salinity significantly affects physiological parameters [salt influxes (osmolality), water effluxes (body mass), immunity (leukocyte counts)] and locomotor performance (jumping distances and activity). Recovery in freshwater indicates that most of these effects were transient (water and salt effluxes, locomotor performance). Taken together, these results suggest that salinity can have important, but transient, effects on behaviour and physiology even in a species thought to be salt-tolerant (Natchev et al., 2011; Mollov, 2020).

4.1 Insights From Field Investigations

In the field, individuals from brackish ponds were larger but lighter, had higher osmolality and had lower jumping performance than their counterparts from freshwater sites. Such results suggest that brackish ponds are colonized by larger individuals, presumably because smaller individuals have a larger skin surface area to volume ratio, which could increase transcutaneous rates of water loss and salt gain (Gordon et al., 1961) thereby limiting their ability to remain in brackish sites. Yet, individuals from brackish ponds were lighter and had lower locomotor performance and several complementary and non-mutually exclusive hypotheses can explain such results. First, it is possible that the effects of elevated salinity on locomotor performance (Squires et al., 2008; Denoël et al., 2010; Wood and Welch, 2015; Hall et al., 2017) influence foraging efficiency, thereby decreasing energy gain and thus decreasing body condition. Second, lower body condition in individuals from brackish sites may be a mere consequence of dehydration (water effluxes) linked to contact with brackish water (Shoemaker and Nagy, 1977; Gonzalez, 2012; Brischoux et al., 2017; Lorrain-Soligon et al., 2022a). Third, osmoregulation can be

energetically costly (Kidder et al., 2006; Rivera-Ingraham and Lignot, 2017) and individuals living in brackish water may experience higher energy expenditure, thereby reducing their body condition (De Boeck et al., 2000). Teasing apart the relative contribution of these complementary processes deserves further investigation.

Interestingly, more males were located in brackish sites than females. These results are relatively consistent with the processes highlighted above. Indeed, adult females mobilize large amount of energy to reproduce (Wells, 2007; Hayward and Gillooly, 2011) and energetic constraints of life in brackish water (either directly linked to osmoregulation, or indirectly linked to foraging efficiency, see above) may negatively interact with the energetic requirements of reproduction. Complementarily, such a biased sex-ratio may also be linked to habitat selection processes for which amphibian females have been shown to select less brackish sites to mate and lay their eggs in order to decrease the negative consequences of elevated salinity on embryonic and larval development (Haramura, 2008; Albecker and McCoy, 2017; Lorrain-Soligon et al., 2022b). It is noteworthy that we focused our investigation on pond salinity and we did not assess other parameters. Future studies should usefully investigate the effects of such environmental characteristics in relation with salinity.

Neither the proportions of neutrophils, lymphocytes nor basophils were influenced by pond salinity. However, the proportion of monocytes decreased, while the proportion of eosinophils increased, with increasing pond salinity. Monocytes act as cell phagocytes associated with innate defences against bacterial infections (Davis et al., 2008), and eosinophils respond to stimuli such as parasitic infections or pollutants (Kiesecker, 2002; Claver and Quaglia, 2009). As such, it seems that salinity could influence leukocyte profiles in the sampled ponds. One hypothesis could be that salinity differentially affected the different leukocytes (Burraco and Gomez-Mestre, 2016). Indeed, increased salinity is thought to exert immunosuppressive effects (Gutiérrez et al., 2013; Gutiérrez, 2014). Alternatively, salinity could influence the type and/or the frequency of the encountered pathogens. For instance, in one amphibian genus (two species; *Litoria aurea* and *Litoria fallax*), it has been shown that *Batrachochytrium* infection and transmission is reduced with increasing salinity (White, 2006; Stockwell et al., 2015; Clulow et al., 2018). In birds, it has been shown that a relatively lower diversity of blood parasites is observed in saline habitats because of a reduced abundance in invertebrate vectors (Yohannes et al., 2009; Gutiérrez, 2014). Thus, the observed differences in leukocyte profiles could reflect changes in the prevalence and virulence of pathogens and parasites with salinity. To tease apart these two hypotheses, parasites and pathogens need to be sampled in individuals to understand if the change in leukocyte proportion is a consequence of a shift in parasites and pathogens presence and/or virulence with increasing salinity. Hemoglobin-binding protein did not covary with salinity. This suggests that damage to blood cells (and thus the presence of free hemoglobin) due to salinity, as highlighted in some species (Koleva et al., 2017), does not occur in coastal frogs. Nevertheless, it is important to note

that the maximum salinity in which we captured individuals remains moderate (7 g L^{-1}) and it is possible that exposure to higher salinity may have induced greater effects (see below).

4.2 Insights From Experimental Investigations

4.2.1 Water and Salt Influx and Osmolality

The main physiological consequence linked to exposure to salinity that we observed was an increase in the osmotic concentration of body fluids (Brischoux and Kornilev, 2014; Lukens and Wilcoxon, 2020; Park and Do, 2020; Nagy et al., 2021; Tornabene et al., 2022) and a concomitant increase in water loss (Gonzalez, 2012). The increase in osmolality for individuals in saline treatments is linearly related to water salinity, and reveals salt influxes (Licht et al., 1975; Brischoux and Kornilev, 2014; Nagy et al., 2021). This linearity suggests that salt intake occurs through passive mechanisms, a process that resemble osmoconformity (i.e., passive diffusion of ions according to the osmolality of the environment, see also Weick and Brattstrom, 2021). Such a result is strengthened by the fact that water salinity—and not body size, sex or salinity of the pond of origin—affected osmolality. Importantly, we also measured a significant decrease in the salinity of the water during the experimental treatment. Such a result indicates that individuals have gained salt from the water (as corroborated by an increase in osmolality) and/or have diluted the treatment water due to water loss (as corroborated by a decrease in body mass in some treatments, Brischoux et al., 2017). Interestingly, body mass loss was observed for the highest salinity tested (12 g L^{-1}), suggesting that contrarily to salt gain, water loss occurred only in the high salinity treatment. Such a result further indicates that mechanisms that aim at limiting water loss are at play (e.g., electrolyte production, Gordon 1965; Park and Do 2020), and that these mechanisms are efficient at limiting water loss in amphibians, at least up to 9 g L^{-1} . Finally, during recovery in freshwater, we found the opposite responses. Although we did not collect blood at the end of this stage, salt concentration in the water during the recovery stage of our experiment increased linearly with the salinity of the preceding treatment, suggesting that individuals flushed excess salt passively according to the osmotic gradient. In addition, water absorption (body mass gain) occurred in individuals previously exposed to the 12 g L^{-1} treatment (Hillyard et al., 1998). Collectively, these results suggest that ion and water balance equilibration may occur rapidly when freshwater is available (Shoemaker and Nagy, 1977).

4.2.2 Immunological Changes

It has been observed in different amphibian species that exposure to high salinity increases the susceptibility of tadpoles to infection (Milotic et al., 2017; Hall et al., 2020). In addition, salt-exposed amphibians exhibit stress-induced leukocyte responses (Davis et al., 2008). We show that individuals exposed to a high salinity treatment express a greater N:L ratio, driven by a decrease in the proportion of lymphocytes and an increase in the proportion of neutrophils. This process has already been highlighted as a response to high salinity, in larval amphibians (Burraco and Gomez-Mestre, 2016) and terrapin

(Ashley et al., 2021). Although we did not assay glucocorticoids in the current study, it is noteworthy that such response may be a corticosterone-driven stress response, even if corticosterone and N:L ratio are not always correlated and cannot be used interchangeably as indicators of stress (Müller et al., 2011; Davis and Maney, 2018). This could have cascading effects on multiple physiological pathways (metabolic rate, reactive oxygen species production, and immune system, Burraco and Gomez-Mestre, 2016). Consistent with this reasoning, we observed a decrease in the proportion of eosinophils with increasing salinity, which, in association with increased neutrophil and decreased lymphocyte proportions, strengthens a stress response which may be linked to increasing levels of glucocorticoid hormones (Davis and Maerz, 2010). In our study, it remains to be tested if N:L ratios are correlated with glucocorticoid hormones, as this is not always the case (Müller et al., 2011; Davis and Maney, 2018). These changes in leukocytes profiles can be the result of an increased susceptibility to infections or parasites, in correlation with other immune responses (Al-Murrani et al., 2002; Davis et al., 2008), even if the prevalence of parasites and pathogens can be reduced in saltwater (Yohannes et al., 2009; Gutiérrez, 2014; Clulow et al., 2018). However, it has to be emphasized that the increase in N:L ratios in response to infections is not consistent among amphibians species (Gervasi et al., 2014), and it remains to be tested whether it is the case in our study species. Moreover, knowledge of baseline data (not known in our study species) is critical for a thorough interpretation of leucocyte profiles (Davis and Maney, 2018). Interestingly, we showed that this response occurs rapidly (48 h), mainly in water salinity that exceeds those of sites in which we captured the study individuals. Such relatively high salinity may well explain the variable responses we observed between the field and the lab in leucocytes proportion (Table 2). It may be insightful to examine these parameters under the same range of salinity both in the field and in the lab. Because previous work has shown that salinity induces cell damage (Koleva et al., 2017), we expected (but did not find) an increase in the concentration of hemoglobin-binding proteins for saline treatments, as these proteins are expected to bind free hemoglobin and thereby reduce their detrimental consequences (Andersen et al., 2012). It is plausible that the duration of exposure to salinity in our experimental setting was short enough to spare erythrocytes (48 h compared to 84 h in Koleva et al., 2017), a hypothesis supported by the lack of damaged cells in our blood smears (pers. obs.). Hemoglobin-binding proteins have been found in some amphibians (*Taricha granulosa*, see Francis et al., 1985), but not in all (for example in *Xenopus tropicalis*, see Wicher and Fries, 2006). The role of such proteins in amphibians is still not well understood and further studies are needed in order to thoroughly describe hemoglobin-binding proteins in amphibians, and/or to develop a specific assay. Moreover, we used a kit aimed to measure binding activity to human hemoglobin, and whether frogs' proteins are binding to the human hemoglobin remains unknown. As it is, our assay could also have measured the peroxidase activity, instead of hemoglobin-binding proteins concentration.

4.2.3 Behavioural Consequences

At the end of exposure, we observed that individuals exposed to a high salt concentration expressed a decrease in activity and in jumping

performance, which can have direct effects on survival, by decreasing foraging ability and anti-predator responses (Wassersug and Sperry, 1977; Squires et al., 2008; Denoël et al., 2010). Three hypotheses can explain such results. First, hyperosmolality linked to salt gain may have disrupted the processing of sensory information (Dole et al., 1994). Second, energetic costs of osmoregulation may trade-off with physical activity even if this trade-off is expected to occur over longer time-scales (i.e., several weeks, see Gomez-Mestre et al., 2004; Peña-Villalobos et al., 2016). More likely, decreased performance may be a mere consequence of dehydration (both salt gain and water loss) as this process can strongly limit locomotor abilities in amphibians (Kearney et al., 2018; Greenberg and Palen, 2021). Indeed, our results show that salt gain and performance loss are both linearly correlated with salinity. In contrast, the decreased activity scores seem to be related to water loss because both occur solely in the highest salinity tested (12 g L^{-1}). Importantly, and similarly to the other parameters investigated, locomotor performance returned to initial levels when individuals are returned in freshwater, which highlights the transient effect of salinity in amphibians (Park and Do, 2020), and the high lability of their performance (Gatten and Clark, 1989).

4.3 Overall Salinity Impact

Pelophylax sp. have been described to be relatively tolerant to salinity (Natchev et al., 2011), but our data show that *Pelophylax* sp. adults can be affected by exposure to saline conditions. Eight individuals from the highest salinity treatment died either during treatment or shortly following their return to freshwater. This mortality was observed for smaller individuals, which suggests that a smaller body size—and thus relatively higher surface area to volume ratio—may increase susceptibility to salinity. However, we found an opposite effect of size on mass and performance changes. Indeed, at 12 g L^{-1} , larger individuals lost more water and exhibited a greater decrease in their performance. During recovery, these individuals gained more mass. As smaller individuals have been shown to be more susceptible to water loss with high salinity (Gordon et al., 1961), this seems to be a counterintuitive result: due to their comparatively lower surface area to volume ratio, we would have expected larger individuals to be less susceptible to salinity. It is plausible that, for relatively high levels of salinity, the absolute surface of exchange (skin surface) may be more important to take into consideration than the surface area to volume ratio. Interestingly, although it has been shown that saline populations showed a higher osmotolerance (Licht et al., 1975; Gomez-Mestre and Tejedo, 2003), we found no effect of the salinity of the pond of origin. The lack of effect of the salinity of the pond of origin could be explained by the fact that all ponds were located on a relatively small spatial scale, and because *Pelophylax* sp. is a relatively mobile species (Wells, 2007). Such a hypothesis could be tested using continental individuals (never exposed to salinity).

5 CONCLUSION

Our study highlights the importance of considering the impact of sublethal salinity concentrations, since it could affect organismal behaviour (jumping performance and activity) and physiology

(body mass, osmolality, immunology) at a very short time scale. Adult amphibians are clearly susceptible to environmental salinity, but these effects are remarkably transient once returned to freshwater. Despite the transient character of the effects that we found, we cannot rule out potential long-term effects of exposure to salinity, as it is well-known that stressful conditions, even during short time frames, can affect organismal physiology at longer time-scales (Masero et al., 2017). Further studies are required to investigate whether individuals that never experienced saline conditions (e.g., continental) could display similar short-term responses to salinity, in order to test for local adaptations to low but variable salinity. It is also critical to investigate whether exposure to environmental salinity can ultimately affect individual fitness and population persistence.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by French authorities under permits R- 45GRETA-F1-10, 135-2020 DBEC and APAFIS#30169-2021022515546003 v3.

AUTHOR CONTRIBUTIONS

LLS, FR and FB conceived the ideas and designed methodology; LLS realized field sessions and the experiments; CB realized hemoglobin-binding proteins measurements; LLS realized

blood smears observations and osmolality measurements, all authors analyzed the data; LLS and FB led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2022.919165/full#supplementary-material>

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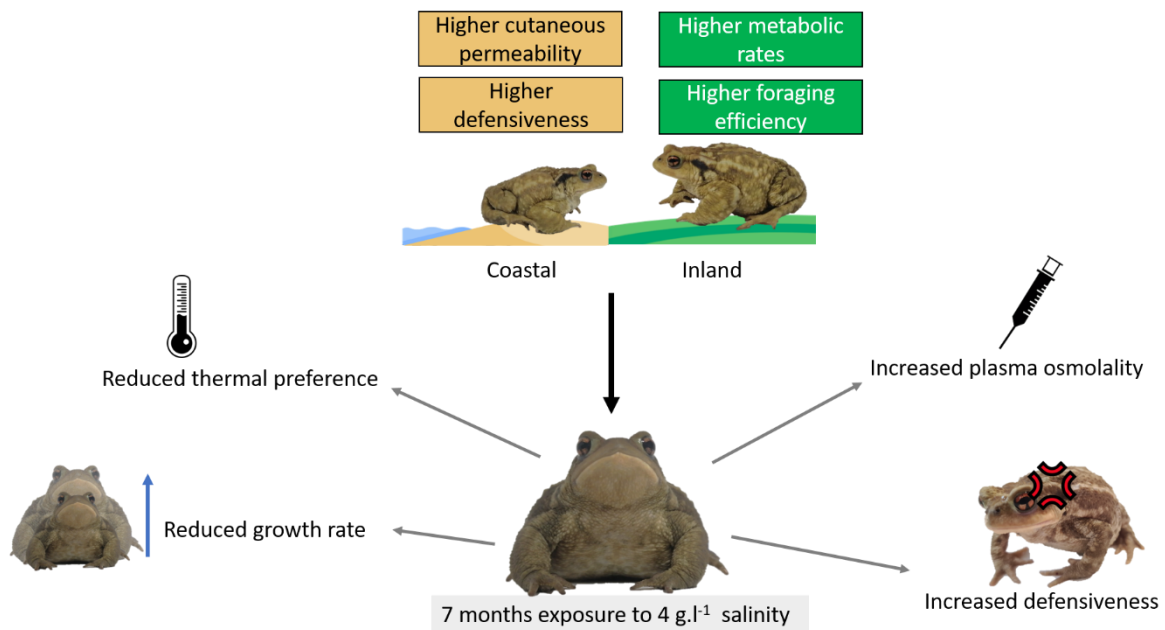
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Article V

Long-term exposure to salinity reveals site-specific physiological and behavioural responses in coastal and continental adult toads

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Abstract

Salinization is a global change currently affecting many coastal and inland water bodies at unprecedented rates. Such salinization can have multiple consequences, most of which are linked to mechanisms involving osmoregulation, which can occur at moderate salinity exposure. These mechanisms are metabolically costly and can affect many functions. However, most studies on the effect of salinity have mainly been focused on juvenile life stages and for short period of times. In this study, we evaluated the impact of sublethal environmental salinity (7 months-long exposure to 0, 2 or 4 g.l⁻¹ salinity) in adult individuals of a widespread amphibian, the spined toad (*Bufo spinosus*), originating from coastal (salt-exposed) or inland (naïve to salinity) environments. Overall, we found strong effects of moderate exposure to salinity on both coastal and inland toads leading to increased osmolality, reduced thermal preference and growth, and increased expression of defensive behaviours. Coastal and inland individuals, irrespective of exposure to salinity, differed in cutaneous permeability, metabolic rates, defensiveness and foraging. Interestingly, defensiveness and thermal preference were more affected by exposure to salinity in coastal individuals, which might overall allow them to cope with constant exposure to salinity impacts. Our study demonstrates that salinization can have negative effects on critical traits (hydro-mineral balance, metabolic rates, growth and behaviour) of adult anurans, and emphasize the importance to increase research effort on the impact of salinity on adult life-stages including both experimental and long-term field studies.

Key-words: Cutaneous permeability, Defensiveness, Growth, Foraging, Metabolism, Thermal preference

1. Introduction

Salinization is a global change currently affecting many coastal and inland water bodies at unprecedented rates (Williams 1999, 2001; Wicke et al. 2011; Singh 2021; Cunillera-Montcusí et al. 2022). While salinity can vary naturally in coastal ecosystems (primary salinization; Herbert et al. 2015), it has been shown to change in response to human activities (secondary salinization; Herbert et al. 2015, Izam et al. 2021). Secondary salinization can be due to direct human actions such as anthropogenic salt inputs (Herbert et al. 2015; Dugan et al. 2017), including the application of road de-icing salt (Findlay and Kelly 2011; Van Meter and Swan 2014), creation of water reservoirs and/or modified water flows (Williams 2001; Zarfl et al. 2015; Reid et al. 2019), or increased extraction from ground and surface water bodies (Pannell and Ewing 2006; Daliakopoulos et al. 2016; Zaman et al. 2018; Reid et al. 2019; Peters et al. 2021; Çolak et al. 2022). In addition, salinization is also affected by climatic modifications, with salinity increasing when evaporation exceeds precipitations (Al-Shammiri 2002; Mills et al. 2013; Obianyo 2019; Çolak et al. 2022). In coastal environments, salinization can also occur because of sea-level rise (Nicholls and Cazenave 2010; Church and White 2011; Planton et al. 2015; Bakker et al. 2017), and increasing frequency and intensity of sea storms and associated marine floods (Nicholls et al. 1999; McLean et al. 2001; Knutson et al. 2010; Dettinger 2011; Trenberth et al. 2015; IPCC 2022).

Such salinization can have multiple consequences, which have been coined as Freshwater Salinization Syndrome (FSS, Kaushal et al., 2021). For instance,

freshwater organisms are generally intolerant to salinity which can induce salt toxicosis following exposure to high levels during significant durations (Kumar and Afaq 2022). Mechanisms underlying such toxicity can involve oxidative stress (Bal et al. 2021, 2022) and cellular damages (Koleva et al. 2017; Bal et al. 2022). Yet, the prominent consequences of exposure to salinity are linked to mechanisms involving osmoregulation (Hellebusi 1976; Bradley 2009; Lillywhite and Evans 2021) which can occur at moderate salinity exposure (Hazon and Flik 2002). Indeed, living in a hyperosmotic environment affect water and ionic fluxes and can induce dehydration due to osmotic loss of water (Bentley 2002; Lillywhite et al. 2014; Brischoux et al. 2017). In order to maintain their internal homeostasis, most organisms rely on physiological and/or behavioural compensatory mechanisms aiming at regulating water and ionic fluxes depending on the salinity of their environment (Schultz and McCormick 2012; Evans and Kültz 2020). The mechanisms involved in excreting excess ions and maintaining hydration are metabolically costly (Pistole et al. 2008; Peña-Villalobos et al. 2013; Herbert et al. 2015; Álvarez-Vergara et al. 2022). In turn, these energetic costs can affect many functions such as respiration (Kidder III et al. 2006), fecundity (Pinder et al. 2005; Woodley et al. 2023), growth and energy acquisition (Pinder et al. 2005; Cañedo-Argüelles et al. 2013; Herbert et al. 2015; Ayub et al. 2020; Alkhamis et al. 2022), immunity (Bal et al. 2022; Lorrain-Soligon et al. 2022a), activity and food intake (Bal et al. 2022; Lorrain-Soligon et al. 2022a; Zhou et al. 2022), which might overall impact growth rates (Templeman 1936; Davis 1958; Britz and Hecht 1989; Tandler et al. 1995;

Kumlu et al. 2000; Fielder et al. 2005), and can lead to malformations (Hieu et al. 2021) and mortality (Cañedo-Argüelles et al. 2013). However, most parameters regarding the effect of salinity have mainly been tested on juveniles life stages (Walker et al. 2023).

Importantly, different mechanisms can be involved to alleviate the consequences of exposure to salinity. For instance, ectothermic organisms may behaviourally adjust their body temperature by selecting thermal micro-habitats in order to regulate their energetic expenditure in response to both increased metabolic rates and reduced food intake (Huey and Kingsolver 2019). In addition, the skin of vertebrate can regulates exchanges between the organism and its environment, including the regulation of water fluxes (Lillywhite 2006). Dynamic changes of skin resistance to water fluxes are expected to occur in response to salinity in order to reduce susceptibility to dehydration (Brischoux et al. 2021a). Finally, and ultimately, it is expected that the mechanisms involved in response to salinity can vary between populations, because individuals may exhibit local adaptation to increased salinity in areas where exposure to salinity is more frequent (DeFaveri and Merilä 2014; Long et al. 2021). More importantly, tolerance to salinity can also vary according to duration of exposure with, counterintuitively, more detrimental consequences following acute rather than chronic exposure to salinity (McIver 2022). Under natural conditions individuals can be exposed for long period of times to relatively moderate levels of salinity (Pétillon et al. 2008; Martin et al. 2009; Lorrain-Soligon et al. 2022a; Lubińska-Mielińska et al. 2023). However, most

studies conducted to date have mostly tested acute exposure to salinity, or chronic exposure but for less than 40 days (Walker et al. 2023).

It is expected that tolerance to salinity reflects occurrence of certain biological traits, including gill-breathing, semi-permeable skin, multiple life stages, and limited mobility (Walker et al. 2023). These characteristics emphasized the sensitivity of amphibians to increasing salinity (Venâncio et al. 2022), due to their permeable skin (Venturino et al. 2003; Hillyard et al. 2007; Wake and Koo 2018), low dispersal abilities (Wells 2007), and eggs and tadpoles that are restricted to the aquatic environment (Karraker and Gibbs 2011; Wake and Koo 2018). This comparatively elevated sensitivity to salinity of amphibians has been extensively studied in early life stages (Albecker and McCoy 2017; Venâncio et al. 2022). During embryonic and larval stages, salinity has been shown to affect survival (Christy and Dickman 2002; Chinathamby et al. 2006; Sanzo and Hecnar 2006; Karraker et al. 2008; Rios-López 2008; Bernabò et al. 2013; Jones et al. 2015; Albecker and McCoy 2017), growth (Christy and Dickman 2002; Wu and Kam 2009; Lukens and Wilcoxon 2020), development (Christy and Dickman 2002; Gomez-Mestre et al. 2004; Chinathamby et al. 2006; Lukens and Wilcoxon 2020), physiology (Gomez-Mestre et al. 2004; Tornabene et al. 2021b, 2022b), and behaviour (Sanzo and Hecnar 2006; Karraker 2007; Denoël et al. 2010). To date, very few studies have investigated the effect of salinity on adults (reviewed in Walker et al. 2023), while this life stage can also be susceptible to environmental salinity both in the water and on land, for

extended duration (Lorrain-Soligon et al. 2022a, c).

In this study, we evaluated the impact of sublethal environmental salinity in adult individuals of a widespread amphibian, the spined toad (*Bufo spinosus*), originating from coastal (salt-exposed) or inland (naïve to salinity) environments. After 7 months' exposure to low environmentally relevant levels of salinity (0, 2 or 4 g.l⁻¹), we measured osmolality, growth, metabolic rates, cutaneous evaporative water loss, preferred temperature, and behaviour (foraging, activity, and defensiveness). We hypothesized that individuals exposed to low levels of salinity should express higher osmolality (Lorrain-Soligon et al. 2022c), which should increase metabolic costs (Peña-Villalobos et al. 2016), resulting in lower thermal tolerance (Sanabria et al. 2018; Chuang et al. 2022b), and altered energetic allocation to other competing functions such as growth (Christy and Dickman 2002; Wu and Kam 2009; Lukens and Wilcoxon 2020). Additionally, as amphibians can adjust skin resistance to water fluxes (Balinsky 1981; Brischoux et al. 2021a), we hypothesized that such response may occur following exposure to salinity. Finally, exposure to salinity should impact behavioural responses, reduce activity (Lorrain-Soligon et al. 2022a) and increase defensiveness (Zhou et al. 2022).

2. Methods

2.1. *Study species*

The spined toad (*Bufo spinosus*) is a relatively large species of toad found in Western Europe (Speybroeck et al. 2018). Adults reproduce in aquatic environments at the end of the winter (most often in February, Speybroeck et al. 2018), but spend most of their time on land. The species tolerance to salt is not known, but in a close species (*Bufo bufo*), larvae are known to tolerate up to 4.8 g.l⁻¹ (Bernabò et al. 2013). Tolerance of adults has never been assessed.

2.2. *Study sites and field procedures*

From 08/09/2022 to 01/11/2022 we prospected various coastal (distance to coastline 0.82±0.44 SE km, Article VII) and inland areas (distance to coastline 43.84±7.18 SE km, Article VII). These individuals were opportunistically captured on roads. We captured 45 individuals in coastal locations (22 females [68.36mm ± 1.48 ES] and 23 males [61.26mm ± 0.98 ES]) and 45 individuals in inland locations (23 females [79.65mm ± 2.32 ES] and 22 males [67.36mm ± 0.80 ES]). Individuals were captured by hand, immediately placed in a transport box (14*16*9cm), and brought to the laboratory (thermally controlled room with air temperature set at 22 ° and photoperiod set at 12 h dark-12 h light) immediately after field sessions for further measurements (see below). A previous study has shown that individuals captured on the coast are smaller than individuals captured inland (Article VII). Similarly, plasma osmolality in coastal individuals was significantly higher than plasma osmolality in inland individuals (Article VII), indicating that

coastal individuals were exposed to salinity.

2.3. *Laboratory maintenance*

At the laboratory, individuals were weighed (with a portable electronic balance ±0.1 g) and measured for body size (snout-vent length: SVL) using a caliper (±0.1 mm). Sex was assessed based on the presence of secondary sexual characteristics. They were then housed individually in a plastic container (L 70.5 x 139.8 x H 16 cm) with a substrate (absorbent paper), a shelter (PVC pipe cut in length) and a Petri dish (diameter 10cm) filled with tap water for drinking. There were fed with 3 crickets and 2 mealworms twice a week, and drinking water was offered twice a week. Until 14/11/2022, all individuals were kept in acclimation and were thus all given freshwater. From 8/11/2022 to 10/11/2022, we collected blood (via cardiocentesis) on all individuals in order to obtain pre-exposition samples. All individuals weighted more than 15 g and we collected 100 µL of blood (representing no more than 10% of blood volume). The blood was centrifuged for 7 min at 2000 g, plasma was separated and stored at -20 °C for further physiological analysis (pre-exposure osmolality values). Individuals were once again measured (SVL) and weighted.

On 14/11/2022, all individuals were randomly assigned to a salinity treatment: either 0 g.l⁻¹ salinity (control), 2 g.l⁻¹ salinity (low salinity) or 4 g.l⁻¹ salinity (moderate salinity). These treatments were chosen to represent the salinity to which individuals are exposed naturally in coastal environments (individuals found up to 6 g.l⁻¹ during reproductive events). Exposure to salinity occurred through drinking water

(Petri dish). 30 individuals were assigned to each treatment (15 coastal individuals and 15 inland individuals). 7 coastal females ($68.29\text{mm} \pm 1.71$ ES), 8 coastal males ($61.00\text{mm} \pm 2.13$ ES), 8 inland females ($79.50\text{mm} \pm 4.01$ ES) and 7 inland males ($67.86\text{mm} \pm 1.45$ ES) were assigned to the 0 g.l^{-1} salinity treatment. 7 coastal females ($68.57\text{mm} \pm 1.46$ ES), 8 coastal males ($60.88\text{mm} \pm 1.87$ ES), 8 inland females ($79.50\text{mm} \pm 4.31$ ES) and 7 inland males ($67.71\text{mm} \pm 1.11$ ES) were assigned to the 2 g.l^{-1} salinity treatment. 8 coastal females ($68.25\text{mm} \pm 3.76$ ES), 7 coastal males ($62.00\text{mm} \pm 0.87$ ES), 7 inland females ($80.00\text{mm} \pm 4.31$ ES) and 8 inland males ($66.63\text{mm} \pm 1.65$ ES) were assigned to the 4 g.l^{-1} salinity treatment. Osmolality before exposure (but after acclimation) was similar between treatments (Linear Model: SumSq= 2956, F-value=1.998, p-value=0.142) and sites (Linear Model: SumSq=1699, F-value=2.296, p-value=0.133). The exposure started on 14/11/2022 and lasted until 28/06/2023 to 12/07/2023 (7 months). Individuals were once again measured (SVL) and weighted. From 28/06/2023 to 12/07/2023 we collected blood (via cardiocentesis) on all individuals in order to obtain post-exposure samples. We collected 100 μL of blood (representing no more than 10% of blood volume). The blood was centrifuged for 7 min at 2000 g, plasma was separated and stored at -20 °C for further physiological analysis (post-exposure osmolality values).

During the whole experimental procedure, individuals were checked once a day for their condition. At the end of the experiment, all individuals were released at their site of capture.

2.4. Behavioural tests

From 09/01/2023 to 13/02/2023, during 26 occasions, we recorded anti-predator responses of individuals. During the day, we approached the terrarium of individuals and identified 1) the position of the toad (under its shelter [1] or outside its shelter [0]), and 2) we assessed the behaviour of the toad when simulated (neutral or defensive), and 3) we recorded their activity on a scale of 1 to 5 following (Lorrain-Soligon et al. 2022a).

Further, from 06/03/2023 to 08/03/2023, we evaluated their foraging activity and feeding success. Toads expressed an active hunting behaviour, with individuals orienting to their prey, before capture. Individuals were unfed for 3 days before this experiment, and were placed in a plastic box ($35*24*12$ cm), above which we placed a GoPro HERO [GoPro, San Mateo, CA, USA]. Individuals were placed at an extremity of the box, and 3 crickets were placed at the other extremity. The behaviour was recorded for 2 minutes. During this 2 minutes we extracted 1) reaction time of individuals (time before turning toward and staring at the prey), 2) number of capture attempts and 3) number of successful captures, allowing to compute a success rate.

2.5. Physiological measurements (pre and post exposure)

2.5.1. Osmolality (pre and post exposure)

Plasma osmolality (mOsmol.kg^{-1}) was measured from 10 μL aliquots on a Vapro2 osmometer (Elitech group, France).

2.5.2. Metabolism (post exposure)

Following (Dezetter et al. 2022), we measured the rate of oxygen consumption (O_2 proxy for SMR) and the rate of carbon

dioxide excretion (CO_2) (both $\text{ml}\cdot\text{h}^{-1}$) on resting individuals at 22°C (temperature to which individuals were acclimated). We used an open-flow respirometry system. We measured gas exchanges during the day during 9 hours. Individuals were placed in a 500 mL test chamber. 7 individuals were tested simultaneously. To prevent any feces or urine during the test, individuals were placed in different 500 mL test chamber during the night, which allow them to be acclimated to the chambers and to excrete feces and urine during this phase. Nonetheless, 7 individuals defecated during measurements, and were rerun later. We used an eight-channel flow controller and meter ($\pm 1\text{ml}\cdot\text{min}^{-1}$, Flowbar-8 Sable Systems, Las Vegas, USA) to provide a constant influx of air ($92.004 \pm 2.137 \text{ml}\cdot\text{min}^{-1}$) to eight test chambers. Seven chambers were used for measurements on the toads and one was left empty for baseline measurements. We saturated the entering air with water vapor, using a double bubbling system in a climatic chamber set at a dew point temperature of 10°C . Individuals were put in acclimation for 30 minutes with oxygen fluxes at 8:00 each morning from 28/06/2023 to 12/07/2023. Individuals remained in the chamber for 9 hours (in order to compute 3 trials, 20 minutes per individual), with a continuous air-flow being analyzed. The efflux of each chamber was sequentially selected with a gas switcher (RM-8 Multiplexer, Sable Systems, Las Vegas, USA) in order to collect the final air samples. Air passed through a column of desiccant (Drierite) before entering an O_2 analyzer (FC10A, Sable Systems, Las Vegas, USA) and a FoxBox (Sable Systems, Las Vegas, USA) to measure CO_2 . Output signals of different metrics were continuously recorded with an UI-2

interface (Sable Systems, Las Vegas, USA). As in Dezetter et al. (2022), we used respirometric equations derived from Withers (Withers 1977), which corrected for CO_2 and O_2 entering and exiting the chamber and the flow rate of air to calculate O_2 and CO_2 concentrations.

2.5.3. Cutaneous permeability (post exposure)

Following Brischoux et al. (2021a), we measured Cutaneous Evaporative Water Loss (CEWL, $\text{g}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$) rates at 22°C (temperature to which individuals were acclimated), using an AquaFlux AF200 (Biox, London) and the Bioxsoftware AquaFlux 6.2. We used an in vivo nail cap (diameter 2.6 mm, cap calibration 0.45), and measured CEWL on the toads' skin in three areas: the dorsal area, the ventral area, and the pelvic patch. Measures were taken by ensuring that the cap was sealed with the skin. We waited that the values of CEWL stabilized for more than 100 s ($\pm 0.02 \text{g}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$) before stopping the measurements. Each measurement was performed in triplicates at each body area, from 28/06/2023 to 12/07/2023.

2.5.4. Thermal preference (post exposure)

Following Lange et al. (2022), we used a thermal gradient (PVC box, $102 \times 72 \times 25.5 \text{cm}$) to assess individual preferred body temperature (T_{pref}). The thermal gradient ranged from 10 to 35°C , and was composed of six compartments ($68 \times 16 \text{cm}$) in 2-mm-thick aluminium. Each compartment was provided with a shelter (half PVC tubes of $66 \times 15 \times 7.5 \text{cm}$) that covered the entire length of the compartment. The thermal gradient was heated in one end by a heating cord set on

35 °C, heated in the middle by a heating cord set on 27 °C, and cooled at the other end by copper pipes, cooled by a stream of antifreeze solution in a cold unit, set on 5 °C. The gradient was placed in a temperature-controlled room (mean air temperature 16 °C) to maintain thermal gradient stability over time. The tests were performed from 08/06/2023 to 23/06/2023, for 10 hours each day from 8:00 to 18:00. Skin surface temperature was taken every hour using an infrared thermometer (Fluke 572 Infrared Thermometer, resolution 0.1 °C, Accuracy ± 0.75 °C). The skin surface temperature was collected in the ventral area, due to its direct contact with the thermal gradient.

2.6. *Statistical analyses*

All statistical analyses were performed using R 3.6.3 (R Core Team 2020) and Rstudio v1.1.419. We computed Linear Models (LMs), Linear Mixed Models (LMMs), or Generalized Linear Mixed Models (GLMMs) with binomial distribution, from *lme4* package (Bates et al. 2015). For all test computed, models accuracy was tested using the *check_model* function from the *performance* package (Lüdecke et al. 2020). In all models, variables were selected by top-down selection, and only the retained variables are presented in the final models.

2.6.1. Growth

To assess the effect of acclimation on growth, for both sites, we computed LMs with difference in size between capture and the end of acclimation as a dependant variable, and the interaction between site (coastal or inland) and SVL before acclimation as explanatory variables.

To assess the effect of treatment on growth, for both sites, we computed LMs with difference in size (SVL) between end of the acclimation and end of the treatment as a dependant variable, and site (coastal or inland), treatment (0, 2 and 4 g.l⁻¹), SVL before treatment and their interaction as explanatory variables.

2.6.2. Osmolality, cutaneous permeability and thermal tolerance

We computed the effects of treatment and sites on osmolality, and mean cutaneous permeability (either dorsal, ventral, or pelvic area), by setting LMs with site (coastal or inland), treatment (0, 2 and 4 g.l⁻¹) and their interaction as explanatory variables.

To analyse ventral thermic preference, we performed a LMM by site, given the difference between coastal and inland individuals, and set treatment (0, 2 and 4 g.l⁻¹) as an explanatory variable, and individual as a random effect (each individual having 10 measures of its ventral temperature).

For cutaneous permeability and ventral thermic preference, we also added the interaction with SVL as a covariable, but it was always excluded during the model selection procedure.

2.6.3. Metabolism

Log transformed VO₂, a proxy for SMR (standard metabolic rate), and log transformed VCO₂ were both analysed using LMMs, with site (coastal or inland), treatment (0, 2 and 4 g.l⁻¹) and their interaction as explanatory variables, as well as mass at the moment of the measure, given its effect on metabolism (see also (Dorcas et al. 2004; Dezetter et al. 2022)) and

sex, and individual as a random effect (each individual performing 3 runs).

2.6.4. Behavior

We assessed the effect of treatment and site on hiding frequency, defensiveness, activity (rated on a scale of 1 to 5) and capture efficiency by setting binomial

GLMMs with site (coastal or inland), treatment (0, 2 and 4 g.l⁻¹) and their interaction as explanatory variables, and individuals as a random effect. Lastly, we tested these effects on reaction time to moving preys by setting a LMM with site (coastal or inland), treatment (0, 2 and 4 g.l⁻¹) and their interaction as explanatory variables, and individuals as a random effect.

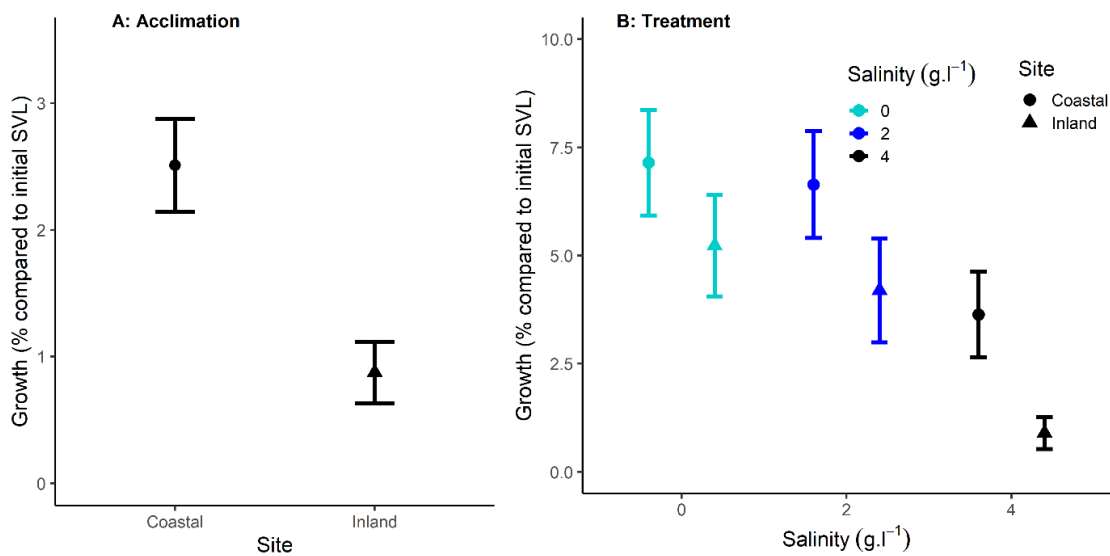


Figure V1- Growth in SVL (Snout-Vent-Length) during (A) acclimation (according to site solely) or (B) treatment (according to site and salinity treatment), expressed as a percentage compared to SVL (A) at capture or (B) at the end of acclimation.

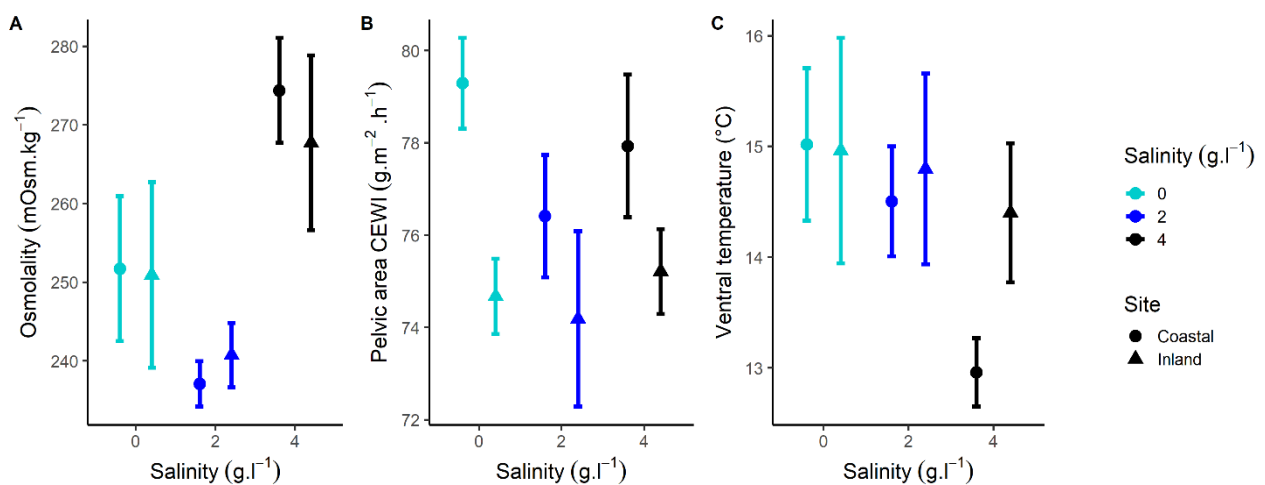


Figure V2- Osmolality (A), cutaneous evaporative water loss measured at the pelvic area (B), and ventral temperature in the thermal gradient (C), according to site and treatment.

3. Results

3.1. Growth

During acclimation, when individuals were all exposed to freshwater, growth rates were influenced by site, SVL (Snout-Vent-Length) and their interaction. Growth rates were higher in coastal individuals compared to inland ones (Estimate= 8.309, SE= 3.511, t-value= 2.367, p-value=0.020, Figure V1A), and decreased with increasing body size (Estimate= -0.151, SE= 0.043, t-value= -3.515, p-value<0.001, Figure V1A), this relation being steeper in coastal individuals as compared to inland ones (Estimate= 0.109, SE= 0.052, t-value= 2.111, p-value=0.038, Figure V1A).

After treatment, growth was influenced both by treatment and SVL, but not their interaction. Indeed, growth was lower in the 4 g.l⁻¹ treatment compared to the 2 g.l⁻¹ (Estimate= -3.085, SE= 0.998, t-value= -3.090, p-value=0.008) or 0 g.l⁻¹ (Estimate= -3.926, SE= 0.998, t-value= -3.933, p-value<0.001) salinity treatments (Figure V1B). Growth in the 0 g.l⁻¹ and 2 g.l⁻¹

treatment was equivalent (Estimate= -0.841, SE= 0.998, t-value= -0.842, p-value=0.678, Figure V1B). Growth rates also decreased with increasing body size (Estimate= -0.197, SE= 0.044, t-value= -4.497, p-value<0.001).

3.2. Osmolality

After treatment, osmolality was influenced by treatment salinity but not the site of origin (Figure V2A). Indeed, osmolality was higher in the 4 g.l⁻¹ treatment compared to the 2 g.l⁻¹ (Estimate= 32.167, SE= 8.065, t-value= 3.989, p-value<0.001) or 0 g.l⁻¹ (Estimate= 19.722, SE= 8.134, t-value= 2.425, p-value=0.045) salinity treatments. Osmolality was equivalent between the 0 g.l⁻¹ and 2 g.l⁻¹ salinity treatments (Estimate= 12.445, SE= 8.134, t-value= 1.530, p-value=0.282).

3.3. Cutaneous permeability and thermal preference

Cutaneous permeability (CEWL), either from the dorsal (Estimate= 1.815, SE= 0.914, t-value= 1.987, p-value=0.050), ventral (Estimate= 1.347, SE= 0.728, t-value= 1.850, p-value=0.068), or pelvic (Estimate= 3.188,

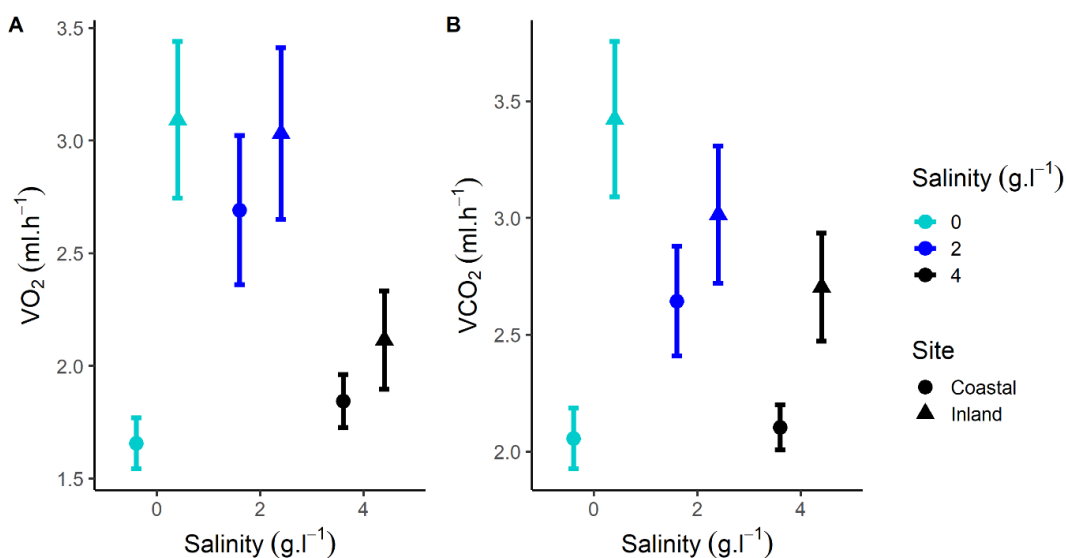


Figure V3- Oxygen consumption (A) and CO₂ production (B), according to site and treatment.

SE= 1.066, t-value= 2.991, p-value=0.004, Figure V2A) area, was influenced by the site of origin and was higher in coastal individuals compared to inland ones.

Thermal preference varied between coastal and inland individuals. In coastal individuals, ventral preferred temperature was significantly lower for individuals exposed to 4 g.l⁻¹ than for individuals exposed to 0 g.l⁻¹ (Estimate= -0.137, SE= 0.048, t-value= -2.840, p-value=0.019), and marginally lower for individuals exposed to 4 g.l⁻¹ compared to individuals exposed to 2 g.l⁻¹ (Estimate= -0.109, SE= 0.048, t-value= -2.248, p-value=0.075), while it was equivalent between individuals exposed to 0 or 2 g.l⁻¹ (Estimate= 0.029, SE= 0.048, t-value= 0.591, p-value=0.826) salinity (Figure V2C). In inland individuals, ventral preferred temperature was equivalent between treatments (SumSq= 0.006, F-value=0.074, p-value=0.929, Figure V2C).

3.4. Metabolism

VO₂, a proxy for SMR, was influenced by the site of origin (Figure V3A), the sex, but not the treatment. Indeed, VO₂ was higher in inland individuals compared to coastal ones (Estimate= 0.104, SE= 0.033, t-value= 3.158, p-value=0.002), and was higher in females as compared to males (Estimate= 0.206, SE= 0.033, t-value= 6.273, p-value<0.001).

Similarly, VCO₂ was influenced by the site of origin (Figure V3B), the sex, but not the treatment. Indeed, VCO₂ was higher in inland individuals compared to coastal ones (Estimate= 0.093, SE= 0.029, t-value= 3.155, p-value=0.002), and was higher in females as compared to males (Estimate= 0.187, SE= 0.029, t-value= 6.379, p-value<0.001).

3.5. Behavior

Hiding frequency was only influenced by the site of origin, coastal individuals being more often hidden than inland ones (Estimate= 0.125, SE= 0.063, z-value= 1.984, p-value=0.047, Figure V4A). Defensiveness was influenced by treatment, site of origin, and their interaction. Indeed, defensiveness was higher in coastal individuals compared to inland ones, in which this behaviour was mainly expressed at 2 and 4 g.l⁻¹ salinity (Figure V4B, Table V1). In coastal individuals only, defensiveness increased with increasing treatment salinity (Figure V4B, Table V1). Similarly, activity was influenced by treatment, site of origin, and their interaction (Table V1). Indeed, activity was overall higher in coastal individuals compared to inland one, but mostly at 0 and 4 g.l⁻¹ salinity (Figure V4C, Table V1). Additionally, if activity was constant across treatments in coastal individuals, it was lower in the 0 and 4 g.l⁻¹ treatments compared to the 2 g.l⁻¹ treatment in inland individuals (Figure V4C, Table V1).

Lastly, reaction time to moving prey was slightly but not significantly longer in coastal individuals compared to inland ones (Estimate= 5.556, SE= 3.257, t-value= 1.706, p-value=0.092, Figure V5A), and capture efficiency was lower in coastal individuals compared to inland ones (Estimate= 0.687, SE= 0.312, t-value= 2.202, p-value=0.031, Figure V5B).

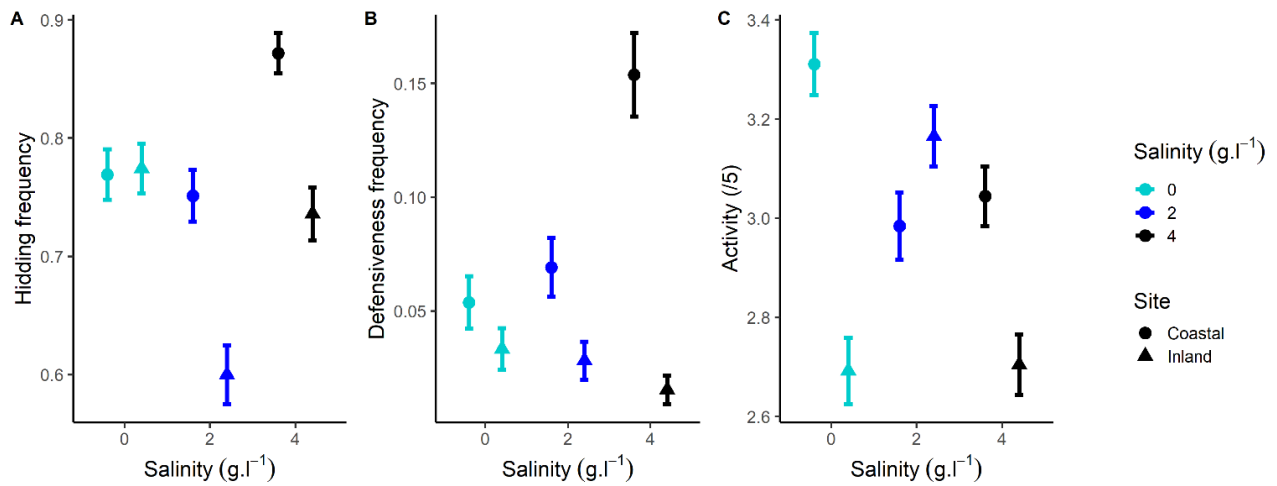


Figure V4- Hiding frequency (A), defensiveness frequency (B) and activity rated on a scale from 1 to 5 (C), according to site and treatment.

4. Discussion

Overall, we found strong effects of chronic exposure to moderate salinity on both coastal and inland toads leading to increased osmolality, reduced thermal preference and growth, and increased expression of defensive behaviour. Coastal and inland individuals, irrespective of exposure to salinity, differed in cutaneous permeability, metabolic rates, defensiveness and foraging. Interestingly, defensiveness and thermal preference were more affected by exposure to salinity in coastal individuals.

Exposure to salinity (4 g.l⁻¹) increased plasma osmolality, suggesting that our experimental treatment affected the hydro-mineral balance of adult toads. Although this result was expected, it is noteworthy that this response has been previously highlighted for significantly higher levels of salinity (e.g., 10 and 12 g.l⁻¹, Hopkins et al. 2016, Lorrain-Soligon et al. 2022a, c). Our study allows to demonstrate that even relatively moderate salinity can significantly alter the hydro-mineral balance of toads. This result

coastal toads reproducing in ponds up to 3.9 g.l⁻¹ salinity displayed higher osmolality than their inland counterpart from salt-free environments (Article VII). We hypothesized that adjustments of cutaneous permeability could be a possible proximate mechanism involved in the regulation of hydric and ionic fluxes (Dicker and Elliott 1970; Hazon and Flik 2002; Evans 2009). Contrary to this hypothesis, we did not find any influence of exposure to salinity on CEWL. However, interestingly, we found that coastal individuals have a higher cutaneous permeability than inland individuals, a difference that was detectable after 7 months of captivity in similar conditions. The higher CEWL of coastal individuals should increase their susceptibility to dehydration, a counterintuitive result given their exposure to salinity and desiccant substrates (Marangoni et al. 2008; Lorrain-Soligon et al. 2022a, b). Alternatively, higher cutaneous permeability could allow coastal individuals to absorb water more efficiently, should it be brackish, a process that may allow coastal individuals to regulate their hydro-mineral status. Levels of hormonal mediators, such as

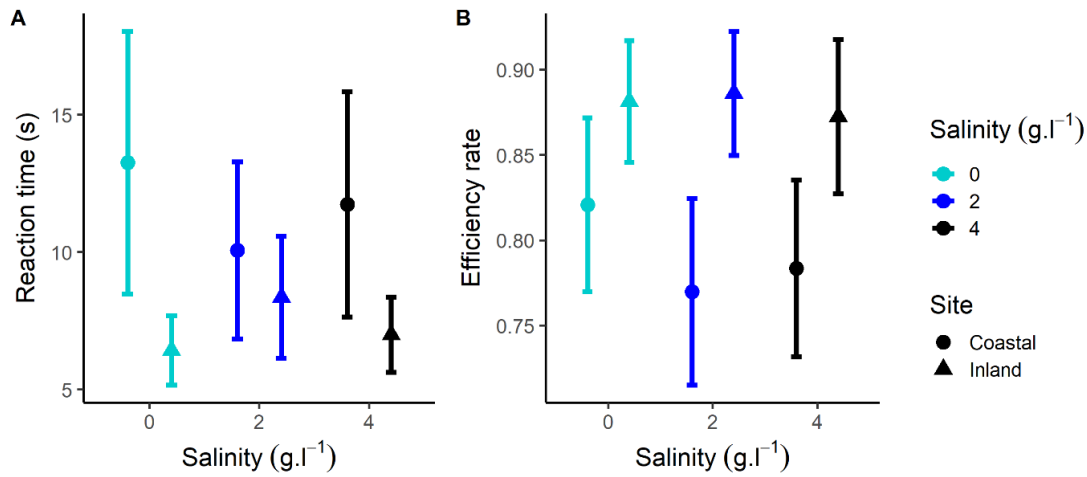


Figure V5- Reaction time to moving prey (A) and capture rate efficiency (number of actual captures divided by number of tentative captures, B), according to site and treatment.

corticosteroids, known to impact drinking behavior (Fuentes et al. 1996; Bentley 2002) or prolactin, known to reduce skin permeability to water (Bentley 2002), should be investigated together with cutaneous permeability in order to validate this hypothesis.

Several studies, including studies on amphibians, have demonstrated that SMR (standard metabolic rate) increased when individuals are exposed to increased salinity (Kammerer et al. 2010; Peña-Villalobos et al. 2016; Álvarez-Vergara et al. 2022). Indeed, the mechanisms involved in the hydro-mineral regulation are metabolically costly (Pistole et al. 2008; Peña-Villalobos et al. 2013; Herbert et al. 2015). Contrarily to these previous findings, we did not find any influence of exposure to salinity on metabolic rates (O_2 consumption and CO_2 production), suggesting that our experimental treatment did not trigger a metabolic increase. As above, it is noteworthy that these previous studies involved markedly higher levels of salinity (~10, 12 and 25 g.l⁻¹, Kammerer et al. 2010, Peña-Villalobos et al. 2013, 2016, Álvarez-Vergara et al. 2022) while our experiment was designed to reproduce salinity experienced by these individuals in

the wild. Similarly, these studies involved shorter exposure to salinity (5 to 45 days) while we exposed toads to salinity during an extended duration (7 months). It is plausible that shorter-term metabolic response also occurred in our study individuals, a hypothesis that remained to be tested. Nonetheless, and similarly to our results on CEWL, we found a strong effect of the site of origin, with coastal toads having higher O_2 consumption and CO_2 production than their inland counterparts. Such result could be related to acclimation to longer-term (life-long) exposure to salinity or, indeed, early life conditions (e.g., embryos and larvae developing in brackish ponds) which are known to influence life-long metabolic trajectories in ectothermic organisms (Dezetter et al. 2022). Future studies should usefully test whether salinity during developmental conditions can influence metabolic trajectories later in life.

Importantly, although we failed to highlight any effect of exposure to salinity on metabolic rates, our results on growth rates give further perspectives regarding energetic allocation in toads exposed to salinity. That is, despite similar metabolic rates, toads exposed to moderate salinity (4

g.l⁻¹) displayed reduced growth rates. Such result suggests that energetic allocation to growth was altered in these individuals. This further suggest that energetic costs of osmoregulation may not be translated by overall higher metabolic rates, but may induce trade-offs between competing organismal functions. In support of this hypothesis, we found that during acclimation when all individuals were exposed to freshwater, coastal individuals expressed higher growth rates. Because coastal individuals are smaller than inland ones, a pattern that has been attributed to exposure to salinity (Article VII), this result suggest that once osmotic constraints are relaxed (access to freshwater), coastal toads are able to display compensatory growth mechanisms, as shown in larvae (Squires et al. 2010; Wu et al. 2012). Importantly, to our knowledge, our study is the first to assess the impact of salinity on growth rates in post-metamorphic amphibians, and whether limited growth in salt-exposed toads is linked, as in larvae, to reduced thyroid hormones levels (Gomez-Mestre et al. 2004) and to changes in osmoregulatory hormones such as corticosterone and aldosterone (Tornabene et al. 2021b, 2022b) should be tested.

Salinity can constrain thermal tolerance in ectotherms (Sardella et al. 2008; Shaughnessy and McCormick 2018; Chuang et al. 2022b). Few studies assessed the effects of both temperature and salinity in amphibians, but not simultaneously, either in the lab (Peabody and Brodie 1975; Dunson 1977; Komaki et al. 2016; Burraco and Gomez-Mestre 2016), or in the field (Dunson 1977), in larvae or post-metamorphic individuals. The maximal thermal tolerance of tadpoles has been shown to decline with increasing salinity

(Chuang et al. 2022b). In our study, we demonstrated that, in adult individuals, coastal toads exposed to moderate salinity (4 g.l⁻¹) select lower temperatures. Such lower T_{pref} may be related to increased costs of osmoregulation in saline environments, because lower body temperatures should decrease metabolic rates and energetic expenditures (Huey and Kingsolver 2019). Such result further suggest that interactive effects of temperature and salinity might be detrimental to adults, as shown in amphibian larvae (Rogell et al. 2009; Heard et al. 2014; Dahrouge and Rittenhouse 2022). Whether the tolerance of adult individuals to salinity is altered as higher temperatures remains to be tested.

In addition to physiological traits (e.g., osmolality, metabolic rates), we found that exposure to moderate salinity (4 g.l⁻¹) altered behavioural responses (increased defensiveness and reduced activity). In fish, exposure to salinity increased the frequency of aggressive interaction and cannibalism (de Oliveira et al. 2020), and lead to higher timidity, reduced sociability and decreased reproductive occasions (Zhou et al. 2022). In these taxa, increased aggressiveness has been related to changes in hormones levels such as serotonin and dopamine (Liang et al. 2023) or testosterone (Higby et al. 1991), known to mediate aggressive behaviour. Hormonal mediators involved in behavioural responses in amphibians exposed to salinity should be investigated. Decreased activity levels linked to exposure to salinity are similar to what has been found in other amphibian species whether in larvae (Sanzo and Hecnar 2006; Karraker 2007; Denoël et al. 2010; Haramura 2016; Hall et al. 2017; Tornabene et al. 2021a) or in adults (Lorrain-Soligon et al. 2022a, b), suggesting

that altered activity may be a repeatable response of amphibians to salt stress. In inland individuals, activity was higher at 2 g.l⁻¹ salinity. This result is in line with the lower (but not significantly) osmolality in the 2 g.l⁻¹ treatment, and may suggest beneficial effects of intermediate salinities, as previously suggested (Meiler 2016; Lorrain-Soligon et al. 2023). We also demonstrated that coastal individuals expressed higher hiding frequency and defensive behaviours, which may be related to local conditions in coastal environments. For instance, weaker predation pressure (Velasco et al. 2019) and lower competition (most amphibians being intolerant to salt, Hopkins & Brodie, 2015) experienced in coastal environments may orient the behavioural responses of these individuals when confronted with stimuli. Importantly, some of these effects were more marked in individuals exposed to salinity, suggesting a role of salinity in behavioural expression. In addition to these effects on temperament, we found that coastal individuals' express longer reaction time to prey, as well as lower capture rates. These results dovetail relatively well with previous studies on larval stages, in which larvae exposed to salinity expressed reduced foraging activity and behaviour (Hall et al. 2017; Tornabene et al. 2021a). In other taxa, salinity has also been shown to reduce predation rates (Garton and Stickle 1980). These patterns could be linked to lower energetic investment in foraging activity linked to trade-offs between energetic allocation to osmoregulation and other competing functions. Alternatively, increasing salinity has also been found to interfere with the reception, transmission and processing of information as well as in the control and accuracy of tongue

protraction in other bufonid amphibians (Dole et al. 1994), which could explain the effect on capture efficiency we found. Whether reduced foraging abilities in response to salinity are linked to energetic trade-offs, decreased cognitive ability or a combination thereof remain to be tested.

For some studied variables (thermal preference and aggressiveness), coastal individuals seem to be more affected by salinity than their inland counterparts. These patterns were unexpected, as coastal populations are thought to be acclimated or locally adapted to saline conditions (Licht et al. 1975; Gomez-Mestre and Tejedo 2003; Hopkins et al. 2016, 2017). Yet, coastal individuals expressed a marked lability in some of their responses. For instance, we highlighted compensatory growth during acclimation (access to freshwater) linked to their overall smaller body size attributed to field exposure to salinity (Article VII). In addition, coastal individuals exposed to moderate salinity were able to adjust their thermal preference, presumably to cope with altered salinity-related energetic allocation. Whether such responses can alleviate the consequences of chronic exposure in the wild remains to be assessed. Importantly, if larval amphibians exposed to high salinity have been shown to express higher mortality (Christy and Dickman 2002; Chinathamby et al. 2006; Sanzo and Hecnar 2006; Karraker et al. 2008; Rios-López 2008; Bernabò et al. 2013; Jones et al. 2015; Albecker and McCoy 2017), we were unable to show such effects on adult anurans exposed to moderate but environmentally realistic salinity, indicating that the effects of environmental salinity on adults are mainly sublethal.

5. Conclusion

Our study demonstrates that salinization can have negative effects on critical traits (hydro-mineral balance, metabolic rates, growth and behaviour) of adult anurans. Although the effects of salinity in amphibians have been mainly shown on early-life stages, our study strongly suggest that post-metamorphic stages - neglected to date (Walker et al. 2023) - are highly susceptible to this environmental parameter. Given the importance of adult individuals in population dynamics (Wells 2007; Muths et al. 2011), and the forecasted salinization of freshwater environments worldwide (Singh 2021; Cunillera-Montcusí et al. 2022), it seems critical to increase research effort on the impact of salinity on adult anurans including both experimental and long-term field studies.

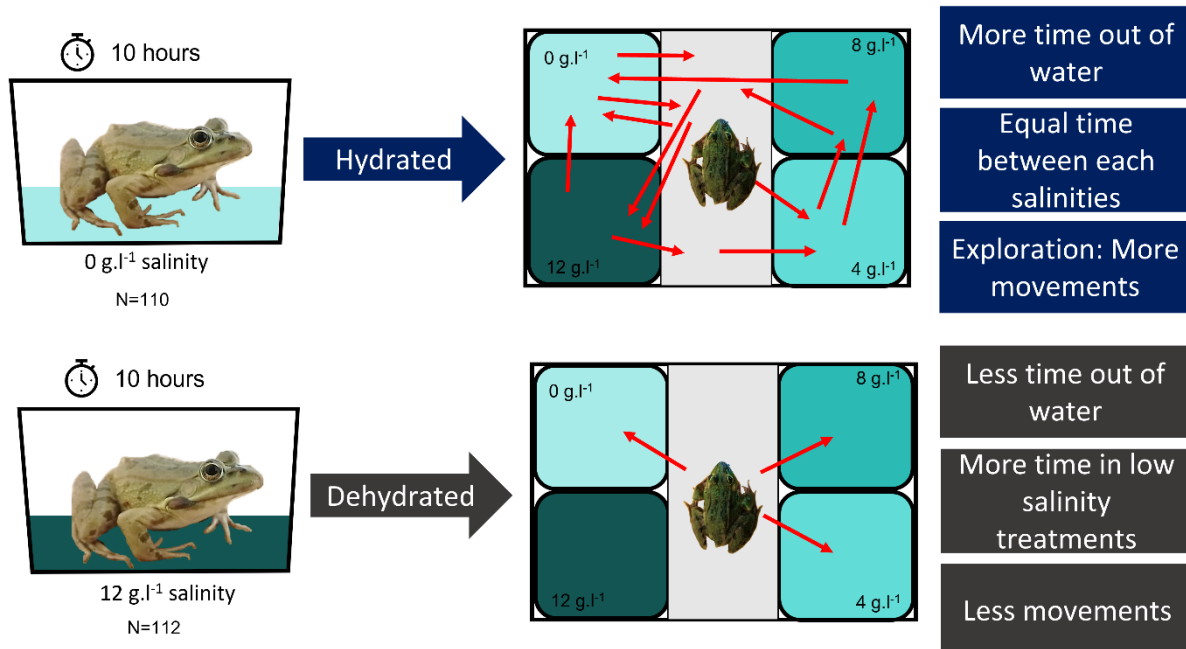
Table V1 - Post-Hoc analyses for the effect of site and treatment on aggressiveness and activity.

Variable	Group	Comparison	Estimate	SE	z-ratio	p-value
Defensiveness	Treatment 0	Coastal-Inland	0.480	0.360	1.332	0.183
	Treatment 2	Coastal-Inland	0.898	0.365	2.462	0.014
	Treatment 4	Coastal-Inland	2.303	0.434	5.304	<0.001
	Coastal	Treatment 0- Treatment 2	-0.251	0.300	-0.839	0.679
	Coastal	Treatment 0- Treatment 4	-1.050	0.263	-3.985	<0.001
	Coastal	Treatment 2- Treatment 4	-0.799	0.243	-3.292	0.003
	Inland	Treatment 0- Treatment 2	0.167	0.416	0.402	0.915
	Inland	Treatment 0- Treatment 4	0.773	0.499	1.550	0.268
	Inland	Treatment 2- Treatment 4	0.606	0.513	1.183	0.464
Activity	Treatment 0	Coastal-Inland	0.207	0.056	3.680	<0.001
	Treatment 2	Coastal-Inland	-0.059	0.056	-1.059	0.290
	Treatment 4	Coastal-Inland	0.118	0.057	2.078	0.038
	Coastal	Treatment 0- Treatment 2	0.104	0.055	1.883	0.144
	Coastal	Treatment 0- Treatment 4	0.084	0.055	1.526	0.279
	Coastal	Treatment 2- Treatment 4	-0.020	0.056	-0.358	0.932
	Inland	Treatment 0- Treatment 2	-0.162	0.057	-2.858	0.012
	Inland	Treatment 0- Treatment 4	-0.005	0.058	-0.081	0.996
	Inland	Treatment 2- Treatment 4	0.157	0.057	2.777	0.015

Article VI

Hydric status influences salinity-dependent water selection in frogs from coastal wetlands

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Hydric status influences salinity-dependent water selection in frogs from coastal wetlands

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ABSTRACT

The environment is heterogeneous across spatial and temporal scales, and the behavioural responses required to adjust individuals' needs to resource availability across such variable environments should be under selective pressure. Coastal wetlands are characterized by a diversity of habitats ranging from fresh- to salt water; and individuals occurring in such complex habitats need to adjust their habitat use based on their osmotic status. In this study, we experimentally tested whether an amphibian species (*Pelophylax* sp.) occurring in coastal wetlands was able to discriminate and select between different salinity concentrations (0, 4, 8 and 12 g.l⁻¹) and whether hydric status (hydrated versus dehydrated) influenced salinity-dependent water selection. We found that frogs selected water based on salinity differentially between hydrated and dehydrated individuals, with the later favoring lower salinities likely to improve their osmotic status. Interestingly, we highlighted the ability of frogs to select lower salinity before having access to water, suggesting that frogs can assess water salinity without actual contact. In coastal wetlands where salinity of water bodies can dynamically vary through space and time, such behavioural osmoregulation process is potentially a key factor affecting individual movements, habitat choice and thus species distribution. Our study further highlights the importance of salinity-dependent habitat heterogeneity and especially the presence of freshwater environments as structuring factors for the amphibian community.

1. Introduction

The environment is heterogeneous across spatial and temporal scales. Such variation affects species distribution according to the availability of resources through processes of habitat selection [1]. Habitat selection by a species can also vary according to environmental characteristics (e.g., climate, predation pressure) [2, 3, 4], life-history stages (e.g., age, growth, reproduction) [1, 5, 6, 7], physiological status (e.g., energy budget, immune status, water balance) [8, 9], or a combination thereof [1]. As a consequence, the ability of individuals to assess habitat quality should strongly influence their performance and survival, and the behavioural responses required to adjust individual needs to resource availability are expected to be under strong selective pressure [1].

Freshwater is a vital resource that influences the ecology and behavior of animal species, especially in environments where water is restricted or unavailable [10, 11]. Indeed, free (drinking) water can be

limited across temporal (e.g., season) and spatial (e.g., rivers, ponds) scales in the environment [12, 13]. Restricted freshwater availability (or water salinity, see below) causes physiological dehydration [14], which can affect a wide array of physiological and behavioural individual characteristics [15]. For instance, dehydration is related to an increased plasma osmolality, decreased plasma volume, increased protein content [16, 17, 18] and has been shown to induce increased stress levels [19], increased oxidative damages [20] all of which can lead to impaired fitness or mortality [15, 21].

In addition to these physiological mechanisms, dehydrated individuals often develop specific responses that aim to decrease additional water loss and to promote water acquisition, such as drinking behavior in freshwater [22, 23]. Typical examples of these responses involve major changes in activity [14, 24, 25], reproduction [26, 27], or locomotor performances [28]. In order to evade the detrimental effects of water restriction, individuals can actively move in the environment to seek for and acquire water to hydroregulate [15] and such process can

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affect habitat selection. Indeed, drinking water availability has been shown to influence population distribution [12], and trades-off with predation risks or food quality [29, 30, 31]. Ultimately, access to freshwater and/or osmoregulation has been suggested to influence evolutionary mechanisms at a global scale [32, 33].

These hydroregulatory mechanisms are particularly important in coastal environments where organisms are subject to complex interactions between marine and terrestrial influences [34, 35]. In brackish or salt water, dehydration occurs through salt gain and water loss, and most organisms have to regulate the osmolality of their body fluids in order to survive [36]. Coastal wetlands are characterized by a high diversity of habitats ranging from fresh to salt water [34, 37]. As a consequence, individuals occurring in such complex environments can dynamically adjust their habitat use according to their osmotic status [23, 38]. Indeed, dehydrated individuals are expected to select - and to move to - habitat that will allow to restore their osmotic balance [39, 40]. Yet, behavioural responses to limit exposure to dehydration and/or osmotic disequilibrium of species occurring in the complex habitat matrix of coastal wetlands have been overlooked to date (but see [23, 40]).

Amphibians are particularly well-suited to investigate this question for several reasons. First, amphibian species are abundant in coastal ecosystems [41]. Second, these taxa are particularly dependent on fresh water availability because of their complex life cycle which requires both aquatic (i.e., eggs and larvae) and terrestrial environments [42]. Third, their highly vascularized and permeable skin makes them particularly susceptible to water salinity [43–45]. Indeed, detrimental effects of relatively high salinity have been highlighted across life-history stages (e.g., eggs, larvae and adult individuals, [46]). Fourth, their comparatively low dispersal ability prevents large scale movements to evade detrimental conditions, and amphibians display strong habitat selection at a short spatial scale [47]. Finally, although some species have been shown to be locally adapted to relatively high salinity [39, 48, 49], their habitat preference remain markedly oriented toward lower salinity when available [50]; presumably because of the fitness costs associated to dehydration [25]. In addition, there are evidences that reproductive females may select oviposition sites based on salinity [46, 51, 52] because low salinity is necessary for successful embryonic and larval development [46, 53]. Yet, whether such habitat selection occurs independently from the constraints associated to embryonic and larval development remains an open question. Similarly, whether individual's osmotic status influences habitat selection in amphibians has not been investigated to date.

In this study, we experimentally tested whether individuals from an amphibian species (*Pelophylax* sp.) occurring in both fresh- and brackish water in coastal wetlands were able to discriminate and select between different salinity concentrations (0, 4, 8 and 12 g.l⁻¹). We further assessed whether hydric status (hydrated versus dehydrated) influenced salinity-dependent water discrimination and selection by experimentally manipulating individual's hydration state after an acclimation to freshwater (0 g.l⁻¹) or brackish water (12 g.l⁻¹). We evaluated behavioral preference for and avoidance of salinity through quantification of the time spent in the four salinity treatments, and made the following predictions:

- Because of the physiological costs associated to elevated salinity, individuals should avoid elevated salinity.
- Dehydrated individuals should select for salinities that should improve their hydric status.

2. Material and methods

2.1. Study site, species and sampling

The study was carried out on the « Réserve Naturelle Nationale de Moëze-Oléron » (45°53'33.36"N, 1°04'59.16"W), situated in the

Atlantic coast of France (Département de la Charente-Maritime). In our study area, *Pelophylax* sp. are mainly composed of viable and fertile hybrids (Graf's hybrid frog, *P. kl. grafi*) of the Marsh frog (*P. ridibundus*) and the Perez's frog (*P. perezi*) [54].

Individuals were captured at night ca. 11pm during 34 sampling events, which occurred, from 03/06/2021 to 13/07/2021, on fresh (<1 g.l⁻¹) and brackish (>1 g.l⁻¹) ponds, with salinity ranging from 0.11 g.l⁻¹ to 8.41 g.l⁻¹ (mean = 3.50 g.l⁻¹ ± 2.41 SE, measured with a conductimeter YSI Professional Plus). A total of 222 individuals were captured and tested (40 from freshwater ponds, and 182 from brackish ponds).

2.2. Manipulation of the hydric state

Individuals were weighted (with a portable electronic balance ±0.1 g), measured (Snout-vent length [SVL] ±0.1 cm), and sexed (131 females and 88 males). To manipulate the osmotic status of individuals, we subjected frogs to an acclimation period (10 h) either in freshwater (0.36 g.l⁻¹ ± 0.01, N = 120) or in brackish water (12.13 g.l⁻¹ ± 0.10, N = 122). Frogs were individually placed in small containers (14×16×9 cm) containing one of the treatments and the water level was adjusted to individual size to allow continuous contact with water.

Body size and body mass between these two groups were similar at the onset of the acclimation period (body mass: 32.03 g ± 1.60 at 0 g.l⁻¹ and 32.67 g ± 1.65 at 12 g.l⁻¹, lm test: Estimate = 0.646, SE = 2.297, t-value = 0.281, p-value = 0.779; body size: 70.53 mm ± 1.00 at 0 g.l⁻¹ and 70.89 mm ± 1.01 at 12 g.l⁻¹, lm test: Estimate = 0.371, SE = 1.423, t-value = 0.257, p-value = 0.797).

After 10 h of acclimation, individuals were weighted (±0.1 g) to verify that water loss (and thus dehydration) occurred in the 12 g.l⁻¹ group. To further assess that our procedure actually affected osmotic status, we further assessed plasma osmolality in a subsample of individuals (N = 5 in each treatment). Blood samples (50 µl) were obtained through cardiocentesis, centrifuged for 7 min at 3000 G and plasma was separated from blood and stored at -20 °C. Plasma osmolality (mOsmol.kg⁻¹) was measured from 10 µl aliquots on a Vapro2 osmometer (Elitech group).

2.3. Activity and selection of salinity

After the 10 h acclimation period, individuals were placed out of water for 2 h before the actual behavioural tests.

To test for water selection, we constructed an arena in a large container (50 * 33 * 27 cm) containing 4 water compartments (14×16×9 cm) at each corner and a central area (leveled to upper part of the compartments) providing a substrate without water. Each compartment contained water with different salinities: 0 g.l⁻¹ (0.36 g.l⁻¹ ± 0.01 SE), 4 g.l⁻¹ (4.12 g.l⁻¹ ± 0.10 SE), 8 g.l⁻¹ (8.07 g.l⁻¹ ± 0.12 SE) and 12 g.l⁻¹ (12.13 g.l⁻¹ ± 0.10 SE) (Fig. 1). These values were selected to mimic the salinity of ponds where frogs were present at our study site (mean: 2.86 ± 2.17 g.l⁻¹, min: 0.14 g.l⁻¹, max: 16.19 g.l⁻¹).

The location of each treatment in the experimental arena was randomly set for each trial.

The test begins with 30 min of acclimation, during which individuals were placed in the central area (out of water) in a mesh cage that prevented the frog to actually reach the water compartments (Fig 1a). After 30 min, the cage was removed (Fig 1b), and the behavior of each individual was recorded for 2 h with a GoPro Hero 8.

After the experiments, individuals were shortly released at their site of capture.

At the end of the 30 min acclimation period (before actual access to water compartments), we assessed the orientation of the individuals toward one of the water compartments using the orientation of the antero-posterior axis of the individuals toward one of the water treatments. This variable can reflect whether individuals can discern water salinity without actual contact (prevented by the mesh cage).

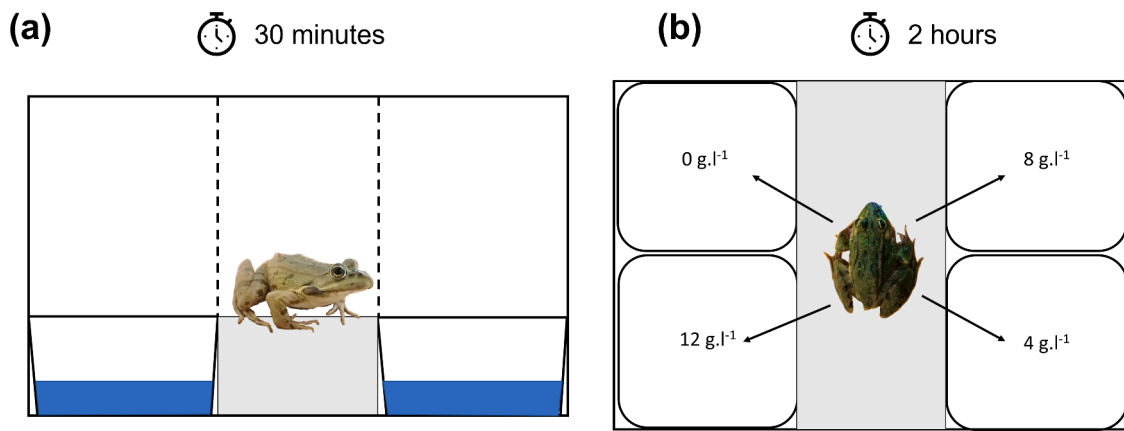


Fig. 1. Experimental set up (a) with the mesh cage and (b) after the mesh cage has been removed.

Once the mesh cage was removed, from the 2 h footages for each individual we extracted the following variables:

- Latency before moving in one of the water compartments.
- Time spent out of the water and time spent in each water compartment.
- Total number of movements (between compartments including the central area out of water).

2.4. Statistical analyses

2.4.1. Manipulation of the hydric state

We compared osmolality and body mass after acclimation (at 0 g.l^{-1} or 12 g.l^{-1}) with lmer tests (Linear Mixed-Effects models) with individual identity as a random effect, and Tukey post-hoc tests.

2.4.2. Orientation without access to water

We analyzed orientation toward water compartments with Fisher's chi square tests, and Fisher's post-hoc tests.

2.4.3. Activity and selection of salinity

2.4.3.1. Activity. We used lms (linear models) with acclimation to salinity (0 g.l^{-1} or 12 g.l^{-1}) as a covariate to investigate the influence of hydric status on the latency before reaching a water compartment and on the time spent out of water; while the total number of movements was analyzed using poisson glm with acclimation to salinity as a covariate.

2.4.3.2. Water selection. We used Fisher's chi square tests, and Fisher's post-hoc tests to test whether hydric status influenced the salinity of the first visited compartment.

For total time spent in each salinity, we used binomial glmer with the salinity treatment as a covariate, and individual identity as a random effect. Because of the large difference of time spent out of versus in water between hydric status (see results), this analysis was performed with time spent in each treatment transformed as a proportion of the total time spent in water.

The influence of individual morphology (body size) on the proportion of time spent in each treatment between hydric status was investigated using glmer (interaction between treatment and body size [SVL]) with individual identity set as a random effect.

For all analyses, we included interaction with salinity of the pond of capture as a covariate, and interaction with sex as a covariate. We never found any significant effect of these two variables (salinity of the pond of capture, all $p > 0.062$; sex, all $p > 0.094$), and these variables were excluded from our final models.

All data analysis were performed using R 3.6.3 [55] and Rstudio

v1.1.419.

3. Results

3.1. Manipulation of the hydric state

Acclimation at 12 g.l^{-1} significantly affected hydration state of individuals as shown by the higher loss of body mass (i.e., water effluxes) in this group. Frogs acclimated to 12 g.l^{-1} lost on average $4.98 \pm 0.27 \text{ g}$ ($\sim 15.6\%$ of their initial body mass, Estimate = -4.98 , SE = 0.272 , t-value = -18.280 , p-value < 0.001) while individuals from the 0 g.l^{-1} group lost less mass, with an average of $0.53 \pm 0.19 \text{ g}$ ($\sim 1.2\%$ of their initial body mass, Estimate = -0.54 , SE = 0.189 , t-value = -2.817 , p-value = 0.006). Body mass loss differed significantly between groups (lm test: estimate = -14.435 , SE = 0.73 , $t = -19.83$, $p < 0.001$).

In addition to water loss illustrated by change in body mass, acclimation at 12 g.l^{-1} significantly affected plasma osmolality (which reflects both water loss and salt gain) of individuals that increased from $245.4 \text{ mOsm.kg}^{-1} \pm 4.26 \text{ SE}$ to $364.4 \text{ mOsm.kg}^{-1} \pm 7.33 \text{ SE}$ (Estimate = 119.00 , SE = 5.848 , t-value = 20.35 , p-value < 0.001), while it slightly, but not significantly decreased in individuals maintained at 0 g.l^{-1} (from $249.0 \text{ mOsm.kg}^{-1} \pm 3.36 \text{ SE}$ to $242.6 \text{ mOsm.kg}^{-1} \pm 5.09 \text{ SE}$, Estimate = -6.40 , SE = 5.492 , t-value = -1.165 , p-value = 0.244). Final osmolality differed significantly between groups (lm test: estimate = -121.80 , SE = 7.38 , $t = 16.50$, $p < 0.001$).

3.2. Orientation without access to water

During the 30 min period in the mesh cage, hydrated individuals did not preferentially orient to specific compartments (water compartments with different salinities or the dry area, $p = 0.08$, Fig. 2). In strong contrast, dehydrated individuals significantly avoided to orient toward the compartment containing water with elevated salinity (12 g.l^{-1} , all $p < 0.012$, Fig. 2), significantly oriented more often toward the dry area (all $p < 0.037$, Fig. 2) but were equally oriented toward other compartments (all $p > 0.768$, Fig. 2). Such diverging orientation pattern is exemplified by the significant difference of the number of individuals that oriented toward the elevated salinity compartment (12 g.l^{-1}) between hydrated and dehydrated individuals ($p = 0.025$, all other $p > 0.195$, Fig. 2).

3.3. Activity and selection of salinity

Once the mesh cage was removed, patterns of activity and compartment use were strongly different between hydrated and dehydrated individuals.

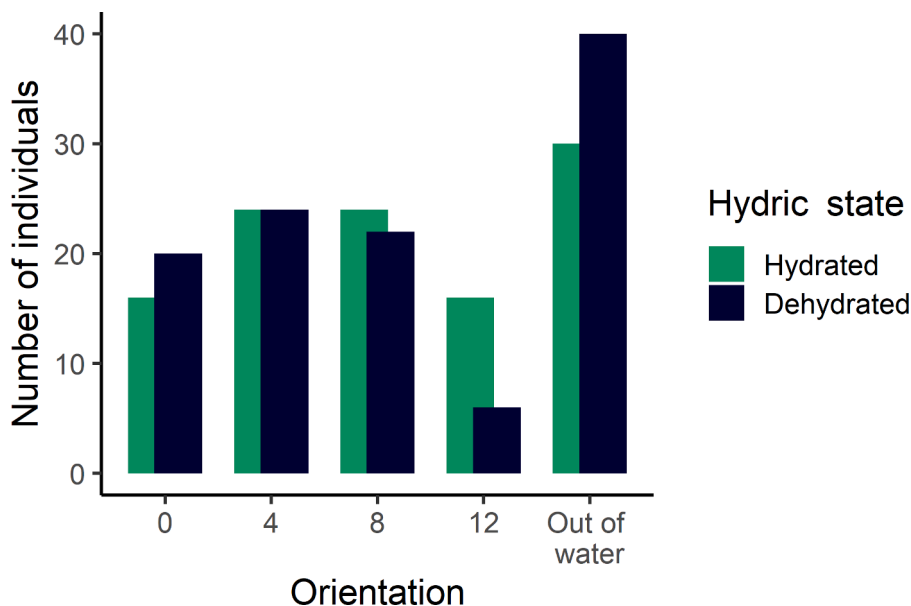


Fig. 2. Number of individuals that oriented toward each compartment while in the mesh cage (no access to water) according to their hydric state.

3.3.1. Activity

The latency before reaching a water compartment was longer in dehydrated individuals than in hydrated ones (15.04 min ± 2.072 SE and 2.04 min ± 0.642 SE respectively, lm test: Estimate = -13.001, SE = 2.169, t-value = -5.994, p-value < 0.001). Similarly, hydrated individuals moved more than dehydrated ones (18.68 ± 1.531 SE versus 2.23 ± 0.240 SE movements respectively, glm test: Estimate = 2.124, SE = 0.192, t-value = 11.05, p-value < 0.001).

During the whole duration of the experiment, hydrated individuals spent more time out of water than dehydrated ones (66.82% ± 3.25 SE and 4.32% ± 1.25 SE, lm test: Estimate = -62.494, SE = 3.482, t-value = -17.950, p-value < 0.001, Fig. 3).

3.3.2. Water selection

Focusing on the first visited water compartment, hydrated individuals selected equally between salinities (p = 0.125). In contrast, dehydrated individuals significantly avoided the water compartment containing water with elevated salinity as the number of individuals

choosing the water at 12 g.l⁻¹ for their first visit was significantly lower than those choosing either 0 g.l⁻¹ or 4 g.l⁻¹ (all p < 0.024) but not different from those choosing 8 g.l⁻¹ (p = 0.116). This diverging choice in first visited water compartment is exemplified by the significant difference of the number of individuals that first visited the elevated salinity (12 g.l⁻¹) compartment between hydrated and dehydrated individuals (p = 0.040, all other p > 0.323).

A similar result was found across the whole duration of the experiment. Overall, if both hydrated and dehydrated individuals spent a different amount of time in each compartment (hydrated individuals: Sum sq = 16,404, F-value = 5.9673, p-value < 0.001; dehydrated individuals: Sum sq = 66,833, F-value = 13.73, p-value < 0.001), the proportion of time spent in each treatment was different for the two groups (Fig. 4, Table 1). Hydrated individuals spent more time in 0 g.l⁻¹ than in other compartments (all p < 0.033) but used equally these other compartments (all p > 0.555) (Fig. 4, Table 1). In contrast, dehydrated individuals spent significantly less time in 12 g.l⁻¹ than in other compartments (all p < 0.001) but used equally these other compartments (all

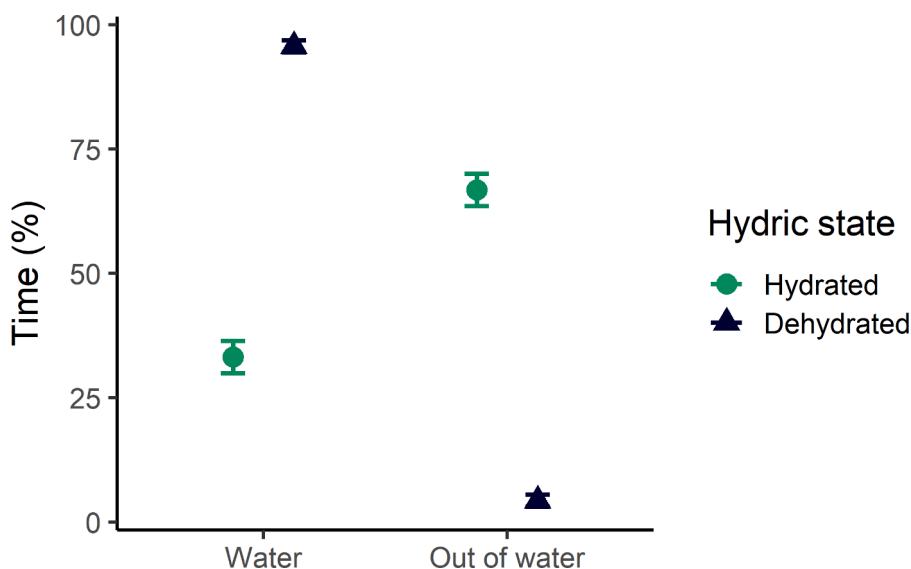


Fig. 3. Proportion of time in water (irrespective of salinity) and out of water for hydrated and dehydrated individuals.

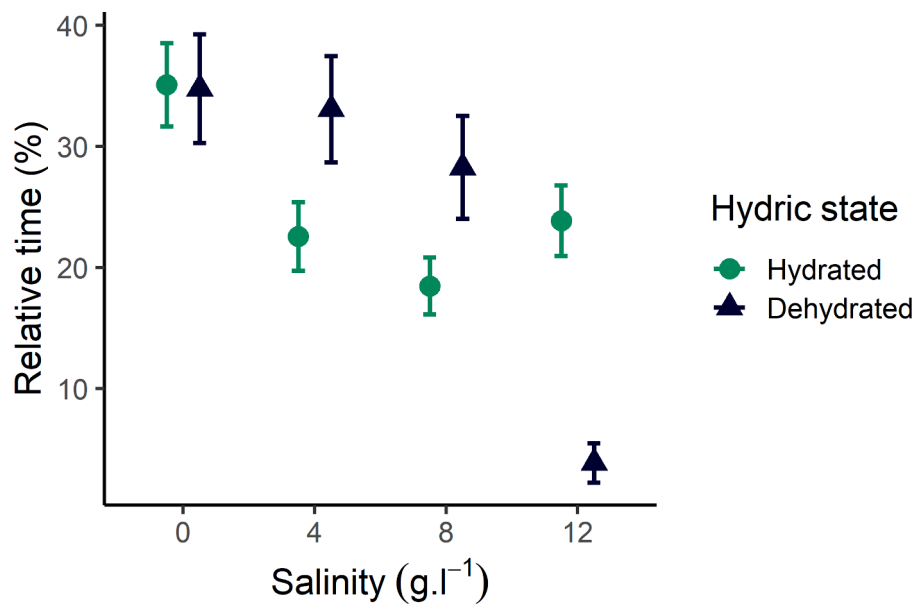


Fig. 4. Proportion of time spent in each treatment (as a proportion of the total time in water) according to the hydric state of individuals.

Table 1

Pair-wise comparisons of the proportion of time spent in each treatment (as a proportion of the total time in water) in hydrated and dehydrated individuals.

		Estimate	SE	t-value	p-value	
Hydrated individuals N = 110	0 g/l-4 g/l-1	12.543	4.119	3.045	0.013	
	0 g/l-8 g/l-1	16.63	4.119	4.037	<0.001	
	0 g/l-12 g/l-1	11.225	4.119	2.725	0.033	
	4 g/l-8 g/l-1	4.087	4.119	0.992	0.754	
	4 g/l-12 g/l-1	-1.318	4.119	-0.32	0.989	
	8 g/l-12 g/l-1	-5.405	4.119	-1.312	0.555	
	Dehydrated individuals N = 112	0 g/l-4 g/l-1	1.69	5.482	0.308	0.99
		0 g/l-8 g/l-1	6.496	5.482	1.185	0.636
0 g/l-12 g/l-1		30.921	5.482	5.641	<0.001	
4 g/l-8 g/l-1		4.806	5.482	0.877	0.817	
4 g/l-12 g/l-1		29.231	5.482	5.333	<0.001	
8 g/l-12 g/l-1		24.425	5.482	4.456	<0.001	

$p > 0.636$).

The amount of time spent in each compartment differed between hydrated and dehydrated individuals (all p -value < 0.04), except for the freshwater compartment (0 g.l^{-1}) in which hydrated and dehydrated individuals spent a similar amount of time (lm: Estimate = 0.323, SE = 5.654, t-value = 0.057, p -value = 0.955).

Interestingly, we found an effect of the size of the tested individuals on the water selection in dehydrated individuals solely (Fig. 5). Indeed, in this group, larger individuals tended to select less often the freshwater treatment (Estimate = -0.783, SE = 0.412, t-value = -1.900, p -value = 0.06, Fig. 5) but significantly selected more often the 8 g.l^{-1} salinity treatment (Estimate = 1.017, SE = 0.385, t-value = 2.639, p -value = 0.009, Fig. 5). For hydrated individuals, there was no differences according to size in the observed responses (all p -value > 0.09).

4. Discussion

Our experiment allows to demonstrate that frogs select water bodies based on their salinity and that such selection differ between hydrated and dehydrated individuals. Experimental manipulation of the hydric status (acclimation to 0 g.l^{-1} or 12 g.l^{-1}) prior to behavioural tests influenced the orientation of individuals toward specific water compartments, but also activity patterns and the amount of time spent in each salinity.

One of the most salient results from our study is the ability of frogs to orient to (and thus select) lower salinity before actually having a direct access to water. Indeed, detection of water (or soil) salinity has been usually shown to require contact between water and chemosensory organs [56, 57, 58]. In our context, the mesh cage prevented individuals to have direct access to water, suggesting that frogs can assess water salinity without contact. Such result further suggests that indirect environmental cues can be used by frogs to assess water salinity. Two different but not mutually exclusive mechanisms can be hypothesized in our context. First, olfaction may be used to assess water salinity (based on both sodium and chloride ions) as it has been recently shown in fish [59, 60]. Yet, as stated above, such example involved actual contact between water and olfactory organs. Volatile compounds have been shown to be used by shore insects to select for the salinity of their habitat [61]. However, these volatile compounds seems to be produced by specific bacteria [62, 63] and such salinity-dependent bacterial activity seems unlikely in our experimental context where treatment water was freshly prepared before each behavioural trial. Alternatively, it is also plausible that frogs can visually assess water salinity based on differing reflection of light between salt- and fresh water [64]. Moreover, fresh and saltwater differ in their depolarization ratio [65]. Amphibians have been shown to detect polarized light [66, 67, 68], and are able to use polarization patterns for orientation [66]. Although our experimental setup do not allow to tease apart these different but not mutually exclusive mechanisms, our result highlight that frogs can assess water salinity without direct contact. Such ability may reveal critical for individuals occurring in coastal wetlands where the salinity of water bodies can dynamically vary through space and time and where selection of adequate water salinity can ultimately influence reproductive success and individual survival. Future experimental studies are required to assess whether frogs rely on olfactory or visual cues (or a combination thereof) to orient toward low salinity water bodies.

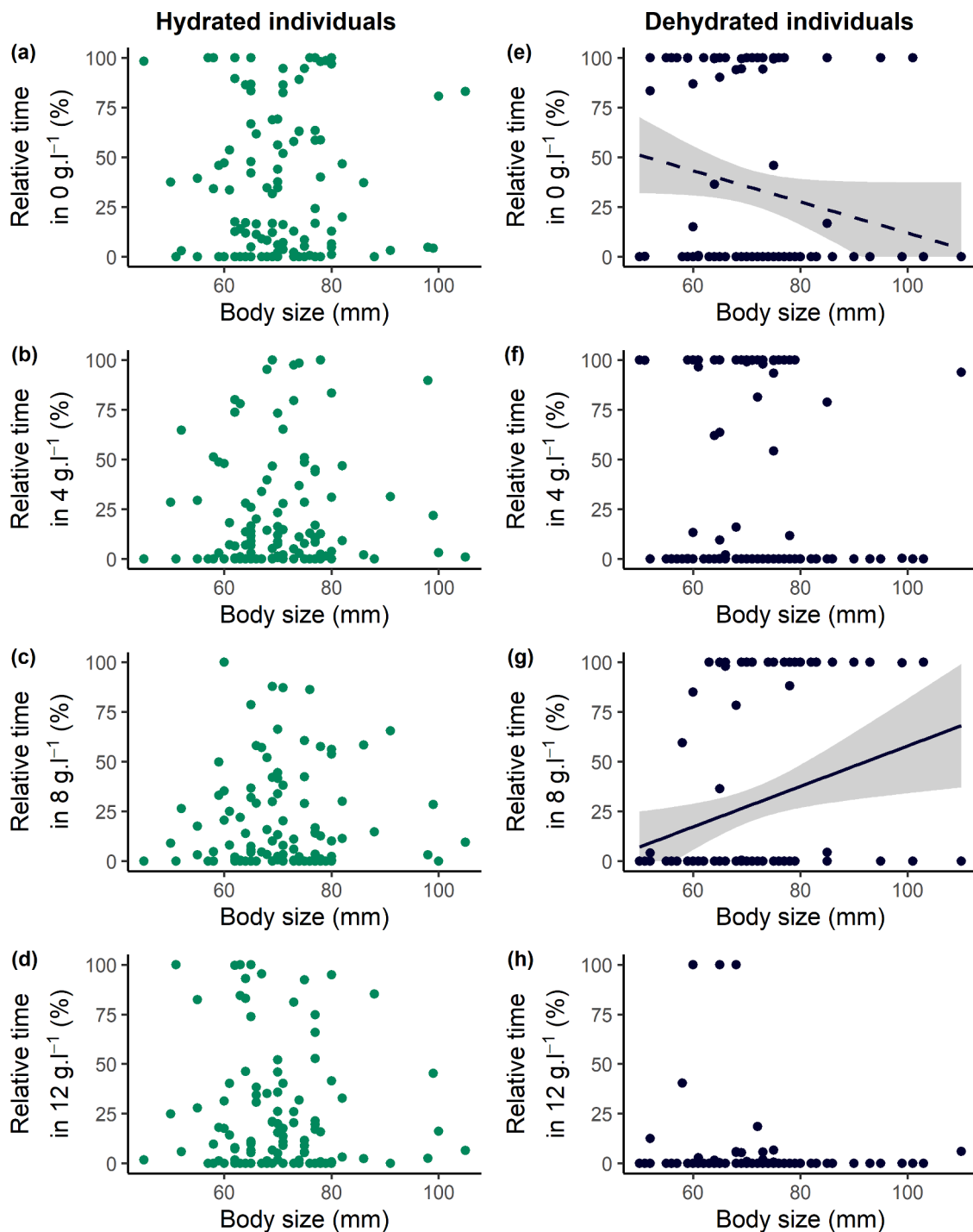


Fig. 5. Effect of individual morphology (body size) on the proportion of time spent in each treatment (as a proportion of the total time in water), for hydrated individuals (left column, abcd) or dehydrated individuals (right column, efgh).

Activity patterns were highly divergent between hydrated and dehydrated individuals. Overall, hydrated individuals took less time to explore water compartments and moved more during the behavioral trials. Such differences dovetail relatively well with the expected consequences of dehydration on activity levels and locomotor performances [24, 25]. Interestingly, hydrated individuals spent more time emerged than their dehydrated counterparts, indicating that dehydrated individuals actively seek contact with water in order to allow cutaneous water absorption (“cutaneous drinking”, [69]) to equilibrate their hydromineral balance [50]. Conversely and as expected, such need to osmoregulate did not occur in hydrated individuals which thus

remained out of water for longer period of time. Future studies should usefully explore whether similar contrasts can occur following dehydration in air rather than in brackish water.

Importantly, water selection differed between hydrated and dehydrated individuals. Overall, hydrated individuals spent most of their immersed time in freshwater. Yet, when using the other water compartments, they spent similar amount of time in each of the salinity treatment irrespective of salt concentration. Such result indicates that although hydrated individuals preferred to be in contact with fresh water, they did not actively avoid the highest salinity compartment as compared to the two lower salinities. In strong contrast, dehydrated

individuals actively selected for water salinities below the one to which they were acclimated. They spent similar amount of time in freshwater and in the two intermediate salinities, all of which would allow individuals to correct, at least in part, for both dehydration and elevated osmolality. As shown in other species [40, 70], dehydrated individuals actively avoided the highest salinity presumably to avoid additional water loss and salt gain, both of which are likely to jeopardize activity, locomotion [71, 72, 73] and ultimately survival [74]. Interestingly, we found a small but significant effect of body size on water selection, as expected from the larger surface area to volume ratio of smaller individuals making them more susceptible to water loss due to high salinity [75]. In dehydrated individuals, smaller frogs spent more time in freshwater and less time in brackish water (8 g.l^{-1}), while no effect of body size was apparent in hydrated individuals.

Finally, we did not find any influence of the salinity of the pond on which individuals were captured. Such result is likely to be linked to the spatial and temporal dynamics of salinity in our environmental context [34, 35]. Indeed, our study species is relatively mobile [47] and it is plausible that individuals move between ponds depending on environmental characteristics (including salinity) and may have experienced a relatively large panel of salinity across this coastal habitat [34, 35], which may lessen putative local acclimation to the salinity condition of each pond.

5. Conclusions

Our study shows that coastal frogs can assess water salinity and select water bodies based on both their salinity and the individuals' hydric and/or osmotic status, which indicate behavioural omoregulation. In coastal wetlands where salinity of water bodies can dynamically vary through space and time [34], such ability is potentially a key factor affecting individual movements, habitat choice and thus species distribution. We emphasize that knowledge of factors affecting habitat choice can have direct implications for the management of natural habitats and may greatly influence conservation actions [1]. In this respect, our study highlights the importance of salinity-dependent habitat heterogeneity and especially the presence of freshwater environments as structuring factors for the amphibian community.

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Data availability statement

Data will be made available by the corresponding author upon acceptance.

Ethics statement

This work was approved by the French authorities under permits R-45GRETA-F1-10, 135-2020 DBEC and APAFIS#30,169-2,021,02 2,515,546,003 v3.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data Availability

Data will be made available on request.

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Chapitre III : Influence de la salinité sur la reproduction, le développement embryonnaire et larvaire

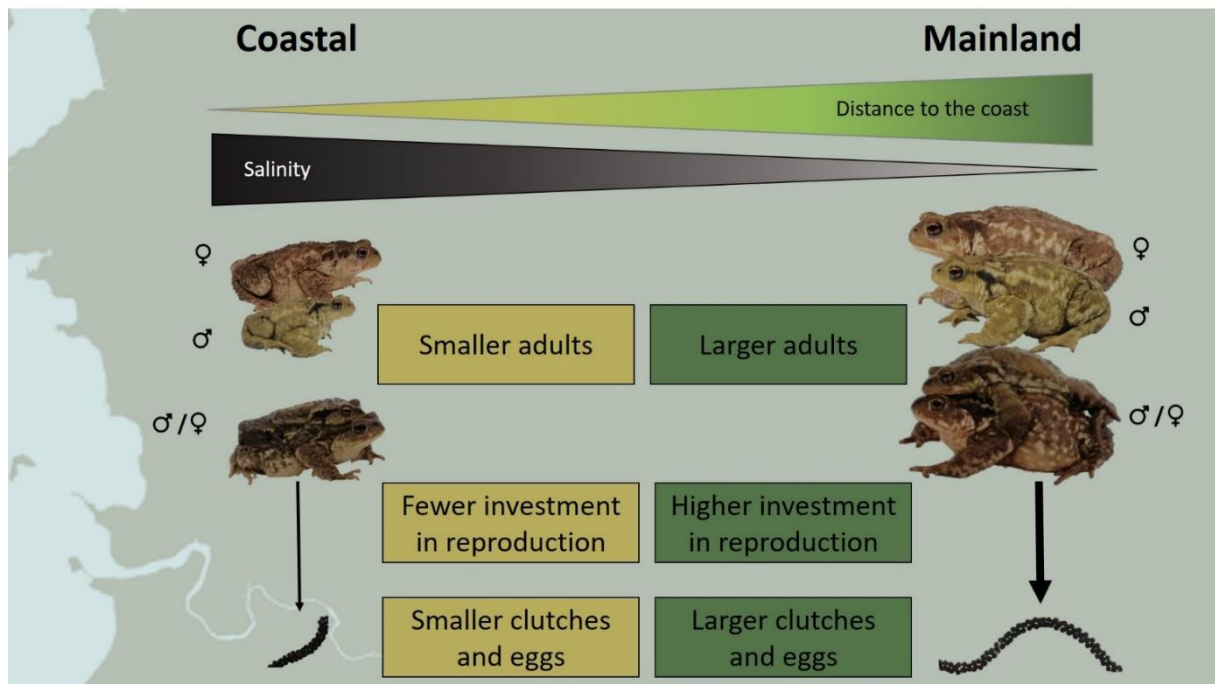


Photo par Jean-Pierre Vacher

Article VII

The costs of living on the coast: reduction in body size and size-specific reproductive output in coastal populations of a widespread amphibian

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Abstract

Body size is a critical component of organismal biology. Body size is known to be influenced by a plethora of environmental conditions, among which exposure to large scale variations of salinity has been comparatively overlooked. Yet, exposure to salinity is known to affect energetic allocation toward growth and reproduction. In this study, we investigated the morphological differences between inland and coastal individuals of spined toads (*Bufo spinosus*) in Western France. We measured adult morphology both outside and during the reproductive season on 190 individuals, and assessed reproduction in pairs originating from inland (N=20) and coastal (N=30) environments. Overall, we found that adult coastal toads were smaller and lighter than inland individuals. Reproductive correlates of these differences included lower fecundity and smaller egg size (but higher egg density) in coastal females. Interestingly, these differences were not allometric correlates of body size, as coastal females invested proportionally less in all components of reproduction (fecundity, egg size and egg protection). These results suggest altered resource allocation to growth and reproduction in coastal amphibians, which may be related to the marked spatial gradient of salinity (measured in reproductive ponds) and the associated costs of osmoregulation (higher osmolality in coastal individuals), for which local adaptation and higher tolerance to salinity remains to be tested.

Key-words: Amphibians, Fecundity, Inland, Morphology, Reproduction, Salinity

1. Introduction

Variation in body size among individuals in a population and among different populations is associated with many other biological traits. For instance, body size can influence large scale interactions such as trophic interactions, population dynamics and can affect both the structure and functioning of communities (Elton 1927; Cohen et al. 1993; Jobling 1997; Brown et al. 2004; Hildrew et al. 2007). At the individual scale, body size can influence most (if not all) life history traits such as metabolic rates (Kleiber 1947; Brown et al. 2004; Nagy 2005), growth rates (Davidowitz and Nijhout 2004), susceptibility to predation (Margulies 1989; Hart and Bychek 2011), foraging and resource acquisition (Mittelbach 1981; Brown et al. 1993; Greenleaf et al. 2007), age at maturity (Blueweiss et al. 1978; Roff 2001), survival and longevity (Smith 2002; Badwan and Harper 2021).

Body size is also a critical determinant of fitness through its influences on reproduction. Indeed, a larger body size can increase reproductive success (Berglund and Rosenqvist 1990; Bosch and Vicens 2006) through effects on mate selection (assortative mating; Crespi, 1989; Han & Fu, 2013; Shine et al., 2001), fecundity (Honěk 1993; Pincheira-Donoso and Hunt 2017), and egg and/or offspring size (Ito 1997; Marshall et al. 2000; Moran and McAlister 2009). Body size often governs the amount of resources that individuals can invest during a reproductive event (George 1994). This amount of resources will affect fecundity (Briegel 1990; Honěk 1993; Calvo and Molina 2005) and egg and/or offspring size (Ito, 1997; D. J. Marshall et al., 2000; Moran & McAlister, 2009), two reproductive

parameters that are known to trade-off against each other (Lasne et al., 2018; Smith & Fretwell, 1974). In turn, larger eggs have higher hatching success (Metz et al. 2010; Xu et al. 2019), produce larger offspring (Pepin et al. 1997), and overall result in better offspring fitness (Xu et al. 2019). Importantly, egg and/or offspring size can also be adjusted by the parents based on the expected offspring performances under a given set of environmental conditions (Fox et al. 1997; Marshall et al. 2008), emphasizing the importance of environmental characteristics in shaping individuals' body size.

Although body size has been shown to influence tolerance to fluctuating environmental conditions (Preziosi et al. 1996; Brown and Sibly 2006), body size itself can also be influenced by environmental conditions (Moran and McAlister 2009). Differences in size can be observed between populations of a same species (Mousseau and Roff 1989; Vitasse et al. 2009) because, throughout the range of a species, individuals are likely to experience different sets of environmental conditions (Frederiksen et al. 2005). Accordingly, geographic variation in body size has often been related to latitude or altitude (Loeschcke et al. 2000; Vitasse et al. 2009; Vinarski 2014) because of the resulting clines in temperature. Temperature is a strong determinant of individual body size, with body size and egg/offspring size decreasing with increasing average temperature (Skadsheim 1989; Moran and McAlister 2009; Gardner et al. 2011; Sheridan and Bickford 2011), a process which has been linked to the temperature size rule (TSR; Atkinson, 1994; Sentis et al., 2017).

Among the various environmental parameters which may influence body size, large scale variations of salinity have been comparatively overlooked. Coastal habitats are constantly exposed to salinity (McLean et al. 2001; Hobohm et al. 2021), a pattern which can be attributed to landward transport of sea-spray (Benassai et al. 2005) or seawater infiltration (Gopinath et al. 2015), both in aquatic (Hoque et al. 2016; Little et al. 2022) and terrestrial (Szabo et al. 2016; Su et al. 2020) habitats, thereby inducing a strong contrast with inland environments. Salinity is an important factor, as most organisms (e.g., numerous invertebrates, vertebrates but also plants) have to osmoregulate in order to survive (i.e., osmoregulators) (Bradley, 2009; Munns & Tester, 2008). Facing fluctuating salinity osmoregulators rely on physiological and behavioural mechanisms to regulate hydric and ionic fluxes in order to maintain homeostasis (Schultz and McCormick 2012; Evans and Kültz 2020). These mechanisms are metabolically costly, and energetic allocation to fuel these expensive mechanisms trade-off with resources available to growth and reproduction in a large variety of organisms including fishes, freshwater invertebrates, plants, and microbes (Stearns 1989; Pinder et al. 2005; Munns and Tester 2008; Cañedo-Argüelles et al. 2013; Herbert et al. 2015; Alkhamis et al. 2022). As a consequence, salinity has been shown to influence development rates, growth and ultimately body size in a wide variety of freshwater taxa (Pinder et al. 2005; Hopkins et al. 2013b, 2014; Lambret et al. 2021).

Amphibians are one such taxa known to be susceptible to environmental variations due to their low dispersal abilities (Wells

2007), permeable skin, eggs without shell, and complex life cycle (López-Alcaide and Macip-Ríos 2011). They have limited abilities to maintain their homeostasis relative to the environment (Katz 1989), and they have been shown to be particularly susceptible to salinity (see for example Lorrain-Soligon, Bichet, et al., 2022; Lorrain-Soligon et al., 2021), even in terrestrial habitats (Traversari 2021; Lorrain-Soligon et al. 2022c; Vegso et al. 2022). However, some amphibian species can persist in brackish habitats (Greenwald 1972; Hopkins and Brodie 2015), highlighting variable tolerances between species (Hopkins and Brodie 2015), and/or populations (e.g. populations originating from brackish water being more tolerant than populations originating from freshwater; Gomez-Mestre & Tejedo, 2003; Hopkins et al., 2016, 2017; Licht et al., 1975). These differences among populations have been useful to demonstrate phenotypic selection (Gomez-Mestre and Tejedo 2003), and variable gene expression associated with local salinity in amphibians (Albecker et al. 2021). In terms of morphology, the size of individuals has been shown to increase when distance to the ocean increases (decreasing exposure to salinity) at a very short spatial scale (<1 km; Lorrain-Soligon, Robin, et al., 2022), but neither variations of adult morphology on a larger spatial scale, nor their consequences for reproductive effort have been tested to date (but see Marangoni et al., 2008).

In this study, we investigated the morphological differences between inland and coastal individuals of spined toads (*Bufo spinosus*). We verified salt-exposure by measuring salinity in coastal and inland sites and osmolality in coastal and inland toads. We quantified adult morphology

(body size, body mass, body condition, sexual size dimorphism) both outside and during the reproductive season on 190 individuals. We further quantified reproduction in pairs originating from inland (N=20 pairs) and coastal environments (N=30 pairs) and assessed investment in reproduction as well as fecundity and egg size. We expected differences between coastal and inland populations, as exposure to salinity should alter resource allocation, and, in individuals originating from coastal environments, we predicted (1) reduced body size of adults in response to exposure to salinity (Lorrain-Soligon et al. 2022c), (2) reduced fecundity and egg size in response to the smaller size of adults (Ito 1997; Marshall et al. 2000; Moran and McAlister 2009), and (3) a comparatively smaller investment in reproduction linked to increased costs of osmoregulation (Herbert et al. 2015).

2. Methods

2.1. Study species

The spined toad (*Bufo spinosus*) is the largest toad species in western Europe, females being larger than males (Speybroeck et al. 2018). This species is terrestrial during most of its annual cycle, except during late winter - early spring when adults converge to water bodies to reproduce (Brischoux et al. 2018). During this aquatic reproductive period, males clasp females in an axillary amplexus until egg laying. The species is largely distributed across Europe and found both in coastal and inland habitats (Speybroeck et al. 2018).

2.2. Study sites and field procedures

We captured by hand coastal and inland individuals during two different time periods, in Western France (Figure VII1). Three coastal ponds (distance to coastline 0.54 ± 0.24 SE [Standard Error] km) and four inland ponds (distance to coastline 52.94 ± 6.13 SE km) were prospected (see Figure VII1) to collect amplexant pairs during the reproductive period (16/02/2022 to 02/03/2022) when individuals are readily available at reproductive ponds. Various sites were chosen in order to capture the first arriving amplexant pairs at each site, and to avoid any site effect. Salinity measured (Pocket Salt Meter PAL-ES2, Atago) at these reproductive sites confirmed that coastal sites were salt-exposed (mean salinity: 2.67 ± 0.69 g.l⁻¹, range 1.8-3.9 g.l⁻¹) while inland sites were not exposed to salt (salinity: 0.0 g.l⁻¹).

Outside the reproductive period (08/09/2022 to 01/11/2022) when individuals resumed activity after

aestivation we prospected various coastal (distance to coastline 0.82 ± 0.44 SE km, Figure VII1) and inland areas (distance to coastline 43.84 ± 7.18 SE km, Figure VII1). These non-reproductive individuals were opportunistically captured on roads, which induced relatively dispersed captures across wide coastal and inland areas. As a consequence, for these individuals, we could not define specific capture "sites" (see Figure VII1 and statistical analyses below).

During the reproductive period, we captured 30 coastal amplexant pairs (60 individuals in amplexus) and 20 inland amplexant pairs (40 individuals in amplexus). Amplexant pairs were captured by hand, placed in a transport box (14*16*9cm), and brought to the laboratory (thermally controlled room with air temperature set at 17 ° and photoperiod set at 12 h dark-12 h light) immediately after field sessions for further measurements (see below). During the non-reproductive period, we captured 45 individuals (23 males and 22 females) from various coastal areas, and 45 individuals (22 males and 23 females) from inland areas. Individuals were captured by hand, placed in a transport box (14*16*9cm), and brought to the laboratory (thermally controlled room with air temperature set at 17°C and photoperiod set at 12 h dark-12 h light) immediately after field sessions for further measurements (see below).

To confirm that salt exposure in coastal toads induced osmotic consequences, we collected blood (via cardiocentesis) on 10 coastal males (originating from one coastal pond: salinity = 2.80 g.l⁻¹, distance to the ocean= 811.65m), and 10 inland males (originating from one inland pond: salinity

= 0.00 g.l⁻¹; distance to the ocean= 50371.30 m) on 08/03/2023 (different animals as than those described above). Osmolality was computed only in males because during the reproductive period males are more easily captured than females. Only individuals weighing more than 15 g were sampled and we collected 100 μ L of blood (representing no more than 10% of blood volume). The blood was centrifuged for 7 min at 2000 g, plasma was separated and stored at -20 °C. Plasma osmolality (mOsmol.kg⁻¹) was measured from 10 μ L aliquots on a Vapro2 osmometer (Elitech group, France). Plasma osmolality in coastal individuals was significantly higher than plasma osmolality in inland individuals (Linear Model: Estimate=8.500, SE=3.855, $t_{1.5}$ =2.205, p-value=0.041; 231.3 \pm 2.906 mOsmol.kg⁻¹ in coastal individuals, and 222.8 \pm 2.533 mOsmol.kg⁻¹ in inland individuals).

2.3. Measurements

At the laboratory, all individuals were measured (snout-vent length, SVL) using a caliper (\pm 1 mm) and weighed using an electronic balance (\pm 0.1g). Amplexing individuals collected during the reproductive season were transitorily separated for these measurements and shortly reunited to further quantify reproduction (see below). A body condition index (BCI) was computed as the residuals of the linear regression between log(SVL) and log(body mass).

2.4. Reproductive effort

Separated amplexant pairs (for measurements, see above) re-formed systematically as soon as the partners were brought back into contact. Once the amplexant pairs were reunited, each pair was placed in a plastic container (35*55*26

cm) containing freshwater (~20L, salinity: 0.3 g.l⁻¹) as well as branches for laying support. Amplexant pairs were left in these tanks until egg laying (mean duration before egg laying: 66 \pm 0.75h, range 10-175h). Once egg laying was completed, individuals were again weighed to calculate loss in body mass.

The clutch of bufonid toads is formed by egg strings containing ~3,000–10,000 eggs (Miaud and Muratet 2018). In order to assess fecundity, each egg string was placed in a container (35 \times 20 \times 25 cm) containing 2 cm of dechlorinated tap water and a scale (graph paper). A picture was taken from above in order to measure the total length of the egg string using ImageJ software (Schneider et al. 2012). For each clutch, we randomly selected 6 segments of 10 cm long and individually counted the number of eggs within each segment. The mean number of eggs per 10-cm segment was calculated and used to assess fecundity (number of eggs) for each clutch based on the length of the egg strings. We also calculated egg density as the number of eggs divided by the length of the egg strings. Finally, on a subsample of 100 randomly selected eggs in each egg strings, we measured egg diameter.

Adult individuals as well as eggs strings were then released at their site of capture.

2.5. Statistical analyses

For all statistical analyses, locations represent the difference between coastal and inland environments, while sites represent the different sites or ponds prospected within each location.

All data analyses were performed using R 3.6.3 (R Core Team 2020) and Rstudio v1.1.419.

2.5.1. Adults' morphology

As individuals captured during the breeding season had a larger body size (SVL) than those captured after the breeding season, both in coastal (during the breeding season: $70.28 \text{ mm} \pm 1.38 \text{ SE}$; after the breeding season: $64.53 \pm 1.04 \text{ SE}$; Linear Model: Estimate=5.750, SE=1.826, $t_{1,103}=3.149$, p-value=0.002) and in inland (during the breeding season: $83.95 \text{ mm} \pm 1.54 \text{ SE}$; after the breeding season: $73.64 \pm 1.54 \text{ SE}$; Linear Models: Estimate=10.306, SE=2.186, $t_{1,83}=4.715$, p-value<0.001) locations, we investigated differences in SVL between coastal and inland locations separately for the two periods (during the breeding season or outside the breeding season).

For individuals captured during the breeding season we tested for the existence of assortative mating, either in coastal or inland populations, by running Pearson correlation tests between male and female body size (SVL). We tested for the effect of location (coastal or inland) on individuals' body size (SVL), body mass and body condition (BCI), separately for males and females. These effects were assessed by computing LMMs (Linear Mixed Models, packages *lme4* [Bates et al., 2015] and *lmerTest* [Kunzetsova et al., 2017]) with location as an explanatory covariate, and the site as a random effect.

For individuals captured outside the breeding season, we tested for the effect of location (coastal or inland) on individuals' body size (SVL), body mass and body condition (BCI), separately for males and females. These effects were assessed by computing LMs (linear models) with location as an explanatory covariate.

2.5.2. Reproduction

- Fecundity and egg size

We tested for the differences in egg string length (mm), clutch size (number of eggs), egg density (number of eggs/mm), and egg diameter (mm), between locations by computing LMMs with location as an explanatory variable and site as a random effect. We also tested for the correlation between egg density or egg diameter and egg number by using a LMM with egg density or egg diameter as a dependent variable, clutch size, location and their interaction as covariates, and site as a random effect. These variables were selected by top-down selection, and only the retained variables are presented in the final models.

- Adults' investment in reproduction

We tested for the effect of location (coastal or inland) on individuals' variations in mass (Δ mass, computed as the difference in mass between time at capture and after laying, as a proportion of mass at capture [%]), separately for males and females. These effects were assessed by computing LMMs (Linear Mixed Models) with location as an explanatory covariate, and the site as a random effect.

We tested for the differences in laying time (time elapsed between capture and laying) between locations by computing LMMs with location as an explanatory variable and site as a random effect.

We tested for the effects of females' body size (SVL), females' body mass, and females' variations in mass (Δ mass, computed as the absolute difference in mass between time at capture and after laying [g]) on clutch size, by computing LMMs with clutch size as a dependant variable, and females SVL, mass or Δ mass as well as their interaction with location

(coastal or inland) as explanatory variable, and site as a random effect. In order to understand if these effects (location and females' SVL, mass or Δ mass) were

additive or interactive, we performed a top-down selection procedure to retain significant effects.

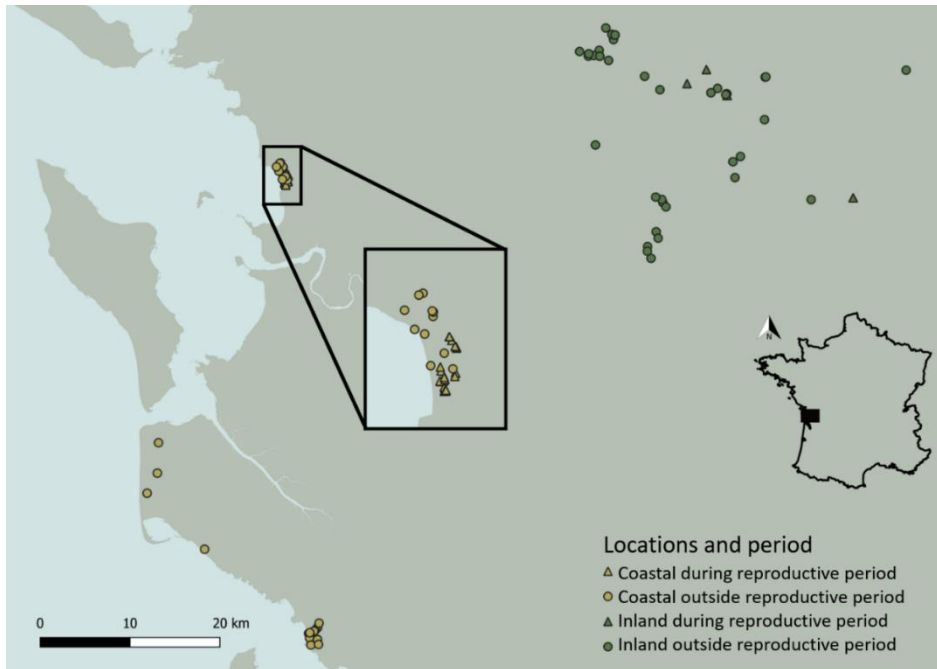
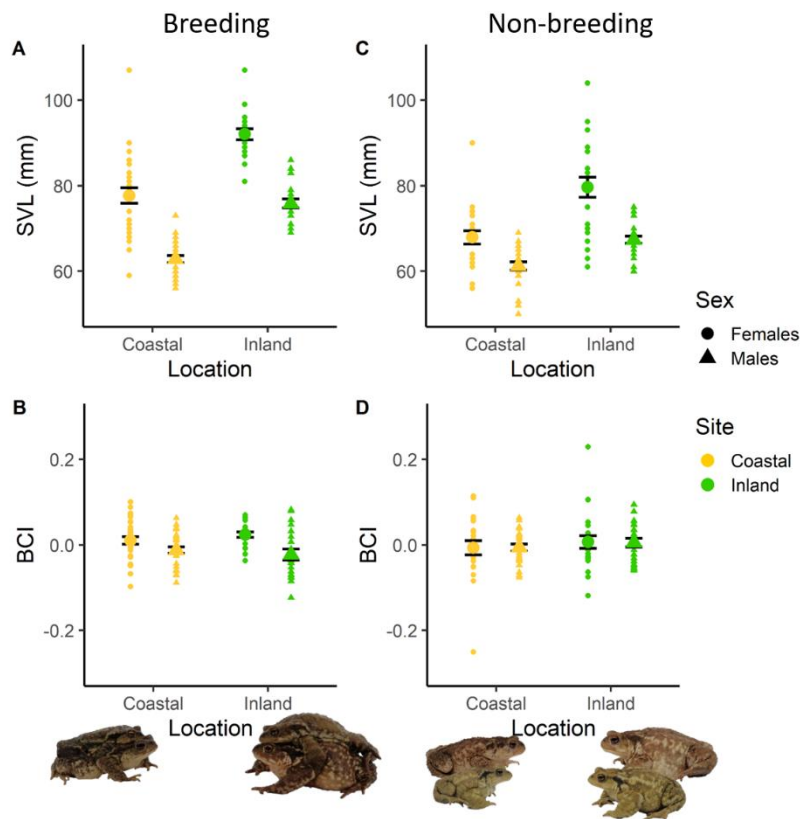


Figure VII1 -Locations of the coastal and inland sites where reproductive and non-reproductive individuals were captured in Western France.



FigureVII2 - Body size (SVL, A; C) and body condition (B; D) according to location and sexes, for individuals captured during the breeding season (left column) or outside the breeding season (right column). Means \pm SE and raw data points.

3. Results

3.1. Body size between coastal and inland populations

For individuals captured during the reproductive season, the body size (SVL) and mass of females and males were higher in inland population compared to coastal ones (Table VIII1, Figure VII2A). BCI of females was higher in inland locations compared to coastal ones, but did not vary in males (Figure VII2B, Table VIII1).

Across amplexant pairs, male and female SVL were not correlated in coastal populations ($r=0.115$, 95% CI=[-0.256,0.456], $t=0.611$, $df=28$, $p\text{-value}=0.546$), but we found a marginally non-significant correlation in inland amplexant pairs ($r=0.442$, 95% CI=[-0.001,0.740], $t=2.093$, $df=18$, $p\text{-value}=0.051$).

For individuals captured during the non-reproductive period, SVL and mass were higher in inland populations compared to coastal ones, both in males and females (Table VIII1, Figure VII2C). BCI was higher in males in inland compared to mainland populations, but did not vary in females according to location (Table VIII1, Figure VII2D).

3.2. Reproductive effort between coastal and inland populations

Egg strings were longer in inland individuals (Estimate=2011.003, SE=605.112, $t_{1.6}=3.323$, $p\text{-value}=0.017$). Accordingly, clutch size (number of eggs) was higher in inland locations (Estimate=2749.442, SE=613.069, $t_{1.5}=4.485$, $p\text{-value}=0.007$, Figure VII3A). However, egg density (number of eggs divided by egg string length) was higher in coastal populations (Estimate=0.206, SE=0.088,

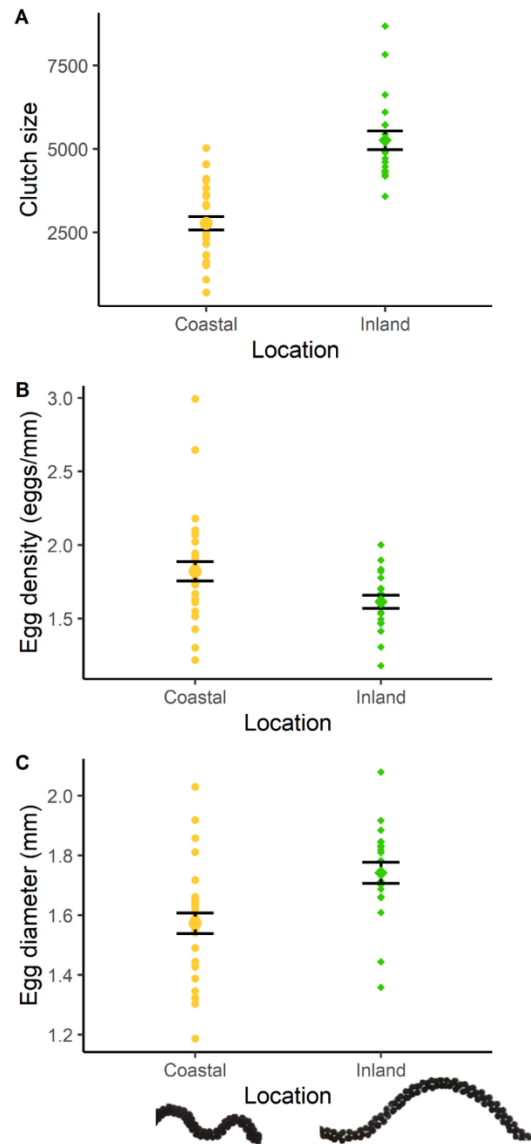


Figure VII3 – Clutch size (number of eggs, A), egg density (B) and egg diameter (C), according to location (coastal [N=30] and inland [N=20]), for individuals captured during the breeding season. Means \pm SE and raw data points.

$t_{1.48}=-2.348$, $p\text{-value}=0.023$, Figure VII3B). Indeed, egg density was negatively related to clutch size across locations (Estimate<0.001, SE<0.001, $t_{1.48}=-3.420$, $p\text{-value}<0.001$). Egg diameters varied according to clutch size (Estimate<0.001, SE<0.001, $t_{1.46}=2.786$, $p\text{-value}=0.007$), location (Estimate=-0.522, SE=0.183, $t_{1.46}=2.849$, $p\text{-value}=0.006$), and their

interaction (Estimate<0.001, SE<0.001, $t_{1.46} = -2.527$, p-value=0.015; Figure VII4). Eggs were larger in inland populations, but egg diameter increased with clutch size in coastal clutches (Estimate<0.001, SE<0.001, $t_{1.25} = 2.375$, p-value=0.025; Figure VII4) while it remained constant across clutch size in inland clutches (Estimate<0.001, SE<0.001, $t_{1.18} = -0.881$, p-value=0.390; Figure VII4). Body mass change during reproduction differed according to location both in males and females (Table VII1). Within sexes, inland males have gained mass, while coastal males have lost mass (Table VII1). Coastal females have lost less mass compared to inland ones (Table VII1).

Latency between capture and egg-laying was slightly shorter in coastal individuals, but this difference was not significant (respectively $57.81h \pm 8.83$ SE and $78.49h \pm 9.25$ SE; Estimate=22.499, SE= 14.540, $t_{1.5} = 1.547$, p-value= 0.180).

We showed that clutch size was highly dependent on female morphological traits (namely body size [SVL], body mass, and loss in mass), location, and their interaction (Figure VII5A, Table VII1). Indeed, clutch size increased with female SVL (Figure VII5A, Table VII1), but this relationship was steeper in inland individuals (Estimate= 177.41, SE= 29.68, $t_{1.18} = 5.978$, p-value<0.001) compared to coastal ones (Estimate=93.81, SE=11.36, $t_{1.28} = 8.255$, p-value<0.001). Clutch size was also positively related to female body mass, but the interaction between location and body mass was not significant (Table VII1). In addition, clutch size was negatively related to female loss in mass, but the interaction between location and body mass loss was not significant (Figure VII5B, Table VII1).

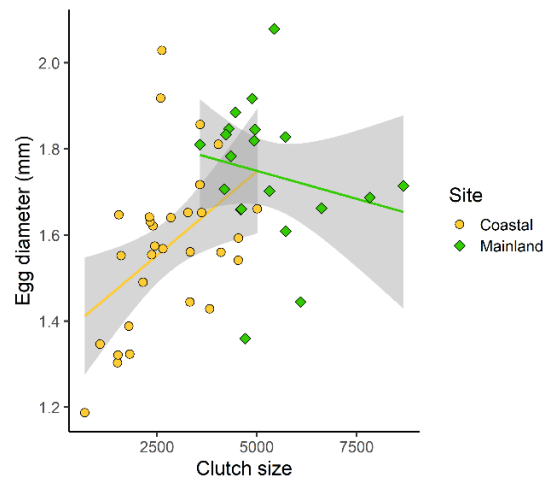


Figure VII4 - Relationships between eggs diameter (mm) and clutch size (number of eggs) according to location (coastal [N=30] and inland [N=20]). Means \pm SE and raw data points.

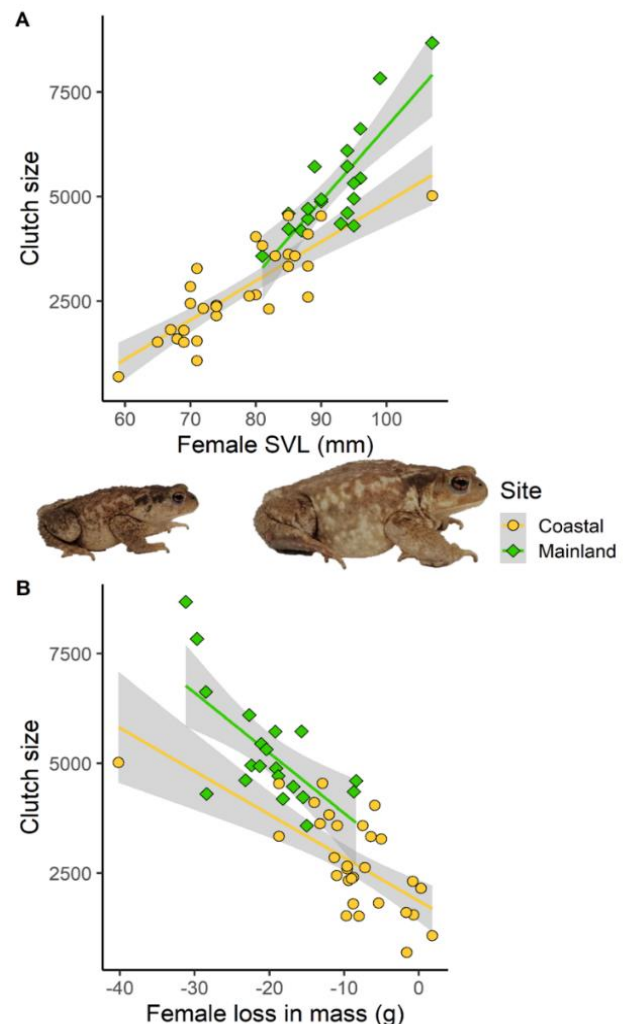


Figure VII5 - Relationships between (A) Females body size and (B) females Δ body mass and clutch size (number of eggs) according to location (coastal [N=30] and inland [N=20]), for individuals captured during the breeding season. Means \pm SE and raw data points.

4. Discussion

Overall, we found that coastal toads were smaller and lighter than inland individuals, both during and outside the reproductive season. Reproductive correlates of these morphological differences included lower fecundity and smaller egg size, but higher egg density, in coastal females as compared to inland individuals. Interestingly, these differences were not mere allometric correlates of smaller body size in coastal individuals, as coastal females produced proportionally smaller clutches than their inland counterparts. In combination with fecundity-dependent body mass loss and egg density, these results suggest altered resource allocation to growth and reproduction in coastal amphibians.

4.1. Smaller body size in coastal toads

Individuals from coastal populations were smaller and lighter than their inland counterparts, suggesting that coastal habitats altered resource allocation to growth in these individuals. Coastal environments are constantly exposed to salt, notably due to the effect of landward sea-spray (Benassai et al. 2005) inducing a spatial gradient of salinity from the seashore both in aquatic (Lorrain-Soligon et al., 2022; Santoro et al., 2006, see also our salinity recordings) and terrestrial environments (Angiolini et al. 2013; Canfora et al. 2014). As a result, direct salt exposure or salt exposure resulting from individual's diet (Nagy et al. 2021) affected osmotic balance in coastal individuals (higher plasma osmolality, see also Lorrain-Soligon et al., 2022 for an other species of coastal amphibian), further suggesting that these individuals experienced higher osmotic costs which can trade-off with energetic allocation

toward growth (Munns and Tester 2008; Herbert et al. 2015). These costs could involve increased energetic expenditures linked to osmoregulation, or alternatively, other costs linked to an increased tolerance to higher osmolality, and future studies should investigate the mechanisms underlying these osmotic costs. Importantly, such osmoregulatory constraints may affect coastal individuals throughout their life. During larval development, tadpoles exposed to salinity express a stunted growth (Gomez-Mestre et al. 2004; Hopkins et al. 2013b, 2014; Lukens and Wilcoxon 2020), which can carry-over after metamorphosis (Lewis et al. 2021). During juvenile and adult (terrestrial) stages, exposure to salted substrates may also influence energetic allocation between osmoregulation and growth (Herbert et al. 2015). Interestingly, Marangoni et al. (2008) highlighted the same pattern of body size reduction near the coast in two anurans species (*Pelobates cultripes* and *Epidalea calamita*) and attributed this pattern to osmotic consequences of highly desiccating sandy substrates, and Hyeun-Ji et al. (2020) highlighted dwarfism in *E. calamita* populations exposed to drier and warmer climatic conditions. These hypotheses indicated that other component of coastal environments, such as desiccating substrate or possibly desiccating winds, also linked to hydric stress, can lead to decreased body size similarly to our study. These studies dovetail relatively well with our hypothesis related to osmotic stress. Indeed, dehydration and salt exposure trigger similar mechanisms of water and ionic regulations (Shoemaker and Nagy 1977; Bentley 2002; Hazon and Flik 2002) and, in addition to energetic costs of osmoregulation, both affect activity levels (Feder and Londos 1984; Titon Jr et al. 2010;

Lorrain-Soligon et al. 2022a) and thus resource acquisition (Feder and Londos 1984; Yuqing et al. 2021).

Alternatively, but not exclusively, other ecological characteristics of coastal habitats could also explain the results we found. For instance, weaker predation pressure (Velasco et al. 2019) and lower competition (most amphibians being intolerant to salt, Hopkins and Brodie 2015) in coastal habitats could affect growth rates and individual body size (Van Buskirk and Yurewicz 1998; Relyea 2001). Similarly, growth rates and individual body size are highly dependent on resource types and availability (Dunham 1978; Festa-Bianchet et al. 2004; Dmitriew 2011). For instance, species diversity and especially invertebrates diversity and abundance are known to decrease with increasing salinity (Pinder et al. 2005; Finlayson et al. 2013; Cunillera-Montcusí et al. 2022; González-Sansón et al. 2022; Kendall et al. 2022; Ersoy et al. 2022). However, coastal habitats are also known to be highly productive interface environments (Hobohm et al. 2021) and arthropod (one of the main food source of toads, Wells, 2007) diversity and abundances have been shown to be relatively high in coastal habitats (Polis and Hurd 1996; Barrett et al. 2005; Brunbjerg et al. 2015), notably because of the positive effects of both terrigenous and marine sources of nutrients on trophic webs (Polis and Hurd 1996). Thus, the size reduction in coastal population is unlikely to be related only to a reduction in resources abundance. This hypothesis seems supported by the fact that coastal toads are smaller and lighter than their inland counterpart; but have overall (except for females during reproductive period)

similar body condition (an index of body reserves, Schulte-Hostedde et al., 2005).

Complementarily, although we did not investigate the physiological contrasts between coastal and inland toads, it is likely that specific physiological mechanisms mediate the link between environmental constraints and body size. In this respect, corticosterone, a pleiotropic mediator involved in energetic allocation (Crespi & Warne, 2013), osmoregulation (McCormick and Bradshaw 2006) and stress response (including osmotic stress, Hopkins et al., 2016; Tornabene, Hossack, et al., 2021), as well as aldosterone (involved in ionic and water regulations in amphibians, Hillyard et al., 2009; Tornabene et al., 2022), may be key parameters to consider in order to investigate the mechanistic bases of the differences we found. Finally, although some of the data we collected may have included juvenile individuals (i.e., sampling outside the reproductive season), our data gathered on reproductive individuals clearly show that the pattern we found is attributable to smaller adult body size in coastal individuals. Interestingly, these data show that sexual maturity is attained at smaller body size in coastal individuals (i.e., 59 and 56 mm SVL respectively for the smallest female and male involved in an amplexus) as compared to inland individuals (i.e., 81 and 69 mm SVL respectively for the smallest female and male involved in an amplexus). Such result may indicate that the age-size relationship in toads (characterized by indeterminate growth, Duellman & Trueb, 1994) may be altered in coastal environments. This may further suggest that coastal individuals may reproduce at younger age than inland ones, a potentially

major shift in life-history strategy linked to lower survival in sites exposed to salt (Hall et al. 2020; Lorrain-Soligon et al. 2022a), a hypothesis which would need to be tested (e.g., using skeletochronology, Bastien & Leclair, 1992; Sinsch, 2015).

4.2. Reproductive correlates of smaller size in coastal toads

In coastal amplexant pairs, we failed to find any relationships between male and female body size. In contrast, we found a marginally non-significant positive relationship between male and female body size in inland amplexant pairs. Such result may suggest that smaller body size in coastal individual may alter processes linked to size-related mate selection and/or assortative mating (Crespi 1989; Shine et al. 2001). Yet, we suggest this result to be taken with caution as assortative mating remains a topic of debates in amphibians (Green 2019) and because such pattern was not previously found in inland populations of the same study species (Renoirt et al. 2022).

We found that coastal females produced both smaller clutches and smaller eggs. This result could be attributed to allometric-dependent reproductive investment (Ito, 1997; D. J. Marshall et al., 2000; Moran & McAlister, 2009). That is, because coastal females are smaller, they produce fewer and smaller eggs, a result found by Marangoni et al. (2008) in two coastal anurans. Yet, we found that the slope of the relationship between female size and fecundity differed between coastal and inland individuals. Such result demonstrates that coastal females produced proportionally smaller clutch than their inland counterparts, and further suggest that size-relative energetic

investment in reproduction was lower in coastal females. This result is further supported by complementary indices of energetic investment in reproduction. For instance, egg density was higher in coastal females, suggesting that the amount of vitelline membrane deposited by the oviduct around the eggs (i.e., egg jelly composed of glycoproteins, Bonnell & Chandler, 1996; Yurewicz et al., 1975) was lower. Such lower investment in egg jellies (further suggested by the lower body condition for coastal females) may bear strong consequences for embryonic development, as vitelline membrane has been shown to enhance egg fertilization and to protect developing embryos from pathogens (Altig and McDiarmid 2007). Taken together, these results suggest that energetic investment during vitellogenesis and vitelline membrane production by the oviduct were proportionally lower in coastal females.

Importantly, the lower reproductive energetic investment of coastal females not only affected fecundity and egg protection, but also egg size. That is, coastal females produced smaller eggs. Yet, perhaps more importantly, we found a positive correlation between egg size and clutch size in coastal females, but not in inland females. Such result is interesting insofar as, usually, fecundity negatively trades-off with egg size (Lasne et al., 2018; Smith & Fretwell, 1974). This pattern also contrasts with what would have been expected from the fractional egg hypothesis (Ricklefs 1968; Nussbaum 1981; Ford and Seigel 2010), which posits that species with large clutch sizes and small eggs (typical of Bufonid toads, Wells, 2007) would add additional offspring and not change offspring size should extra energy become available

(Ricklefs 1968; Nussbaum 1981). We found that increased clutch size is concomitant with increased egg size in coastal females solely; suggesting that higher energy available to reproduction positively influenced both parameters simultaneously. In strong contrast, inland females followed the prediction of the fractional egg hypothesis, with egg size being independent from increasing clutch size.

Similarly to the effect on body size discussed above, we believe that the underlying proximate mechanisms involve the spatial gradient of salinity between coastal and inland sites (Santoro et al. 2006; Angiolini et al. 2013; Canfora et al. 2014; Lorrain-Soligon et al. 2022c); and the associated energetic costs linked to osmoregulation (Goolish and Burton 1989; Gutiérrez et al. 2011). The deviation from an allometric-dependent reproductive investment strongly suggests the existence of additional energetic costs in reproductive females from coastal sites. Other alternative hypotheses (e.g., involving predation pressure, competition, resource availability or dehydration) are unlikely to explain this pattern, and would have induced similar size-dependent reproductive investment between locations (see Marangoni et al. 2008). Importantly, these additional energetic costs affected all components of reproduction (fecundity, egg size and egg protection) suggesting strong potential consequences for future embryonic and larval development and survival (Giménez and Anger 2001; Moran and McAlister 2009; Olson 2019; Xu et al. 2019).

Interestingly, if coastal brackish water bodies are suboptimal for embryonic and

larval development (Hopkins et al. 2013b, 2014; Albecker and McCoy 2017; Lukens and Wilcoxon 2020; Tornabene et al. 2021a), one would have expected coastal females to produce larger and more protected eggs in order to improve survival of their offspring under such detrimental environmental conditions (a similar pattern as what has been found in halophile species exposed to low salinity environments e.g., Giménez & Anger, 2001). We suggest that the additional energetic costs linked to osmoregulation in coastal sites may induce a selfish maternal effect (Marshall et al., 2008; Schwarzkopf & Andrews, 2012), with females allocating proportionally less energy to their offspring in order to favor their own homeostasis. This hypothesis needs to be tested by formally measuring osmotic costs (and osmolality) in coastal and inland females. Alternatively, females may be able to adjust their reproductive effort (fecundity and egg size) according to the environment in which their offspring will develop, to improve their offspring performance and survival in order to maximize their own lifetime reproductive success (Fox et al., 1997; Kudo & Nakahira, 2005; D. Marshall et al., 2008; Marshall & Uller, 2007; Schwarzkopf & Andrews, 2012). In our context, this hypothesis can found three different explanations. First, coastal females may produce fewer and smaller eggs because they reproduce in brackish ponds where competition, predation and pathogens pressures are lower (Yohannes et al. 2009; Gutiérrez 2014; Clulow et al. 2018; Hintz and Relyea 2019). Second, coastal females may produce fewer and smaller eggs because they reproduce in brackish water, which they may consider as a poor-quality environment for their offspring given the detrimental effects of salinity on embryonic and larval

development (Albecker & McCoy, 2017; Lukens & Wilcoxon, 2020; Tornabene et al., 2021). Finally, it is also possible that because of our experimental design for which we maintained amplexant pairs in freshwater, coastal females may have produced fewer and smaller eggs because they may consider freshwater as a poor-quality environment (see Fox et al., 1997; D. Marshall et al., 2008) if they are locally adapted to live and reproduce in saltier environments. Whether females can modify reproductive allocation so rapidly (e.g., during short captivity) deserves to be investigated. Future studies are required to test whether the patterns we found are a result of a selfish strategy improving future survival of females or a manipulative strategy adjusting reproductive effort to local (brackish) developmental conditions of their offspring.

5. Conclusion

Coastal toads are characterized by smaller

body size and size-specific reproductive output compared to inland individuals. These patterns may be related to the marked spatial gradient of salinity found in coastal areas and the subsequent additional costs of osmoregulation in saltier habitats. Assessing whether females adjust their reproductive effort to favor their own fitness or that of their offspring in such suboptimal environments remains to be tested. Whatever the underlying mechanisms, the differences we found between inland and coastal populations remain intriguing as growth and reproduction of coastal individuals appears limited in these habitats. To better understand the fitness consequences of living along the coast, it remains essential to decipher whether salt exposure, as well as other environmental contrasts between coastal and inland habitats, can induce local adaptation and to test if coastal adults, embryos and larvae display an increased tolerance to salt exposure than their inland counterparts.

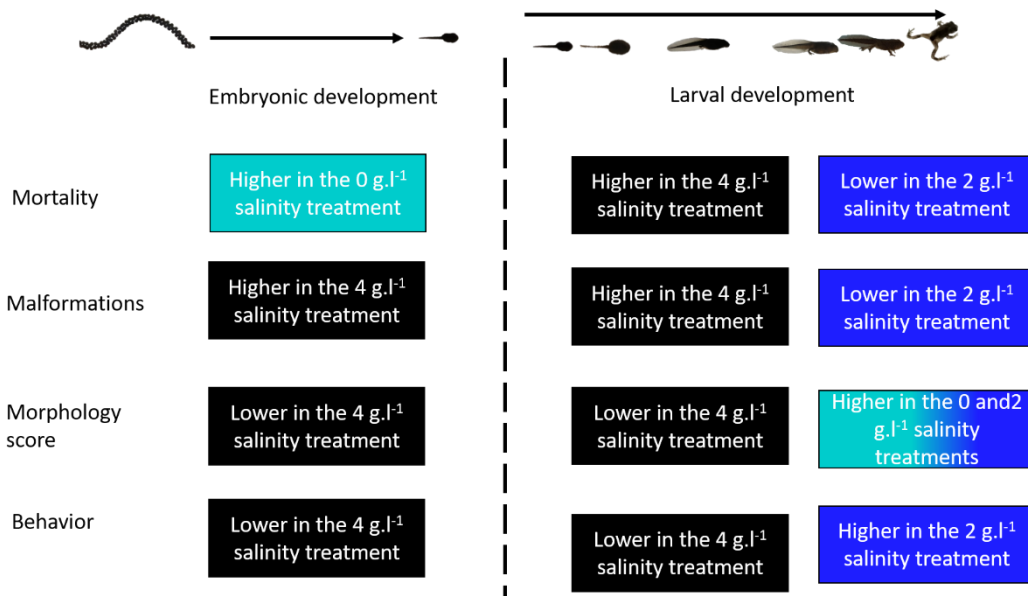
Table VIII - Models outputs for individuals captured during breeding season [concerning body size (SVL), body mass, body condition (BCI), Δ body mass and clutch size] or outside breeding season [concerning body size (SVL), body mass and body condition (BCI)]. Only variables retained during the selection procedures are shown.

Period	Dependent variable	Selected covariates	Estimate	SE	t-value	df	p-value	
During breeding season	Females SVL (mm)	Location (Inland-Coastal)	15.095	3.928	3.843	1.6	0.008	
	Males SVL (mm)	Location (Inland-Coastal)	13.940	2.271	6.139	1.4	0.004	
	Females mass (g)	Location (Inland-Coastal)	47.303	12.235	3.866	1.4	0.007	
	Males mass (g)	Location (Inland-Coastal)	22.235	2.768	8.033	1.4	0.001	
	Females BCI	Location (Inland-Coastal)	0.026	0.011	2.254	1.2	0.029	
	Males BCI	Location (Inland-Coastal)	0.019	0.013	1.455	1.5	0.152	
	Females delta mass (%)	Location (Inland-Coastal)	-5.339	2.036	-2.623	1.7	0.012	
	Males delta mass (%)	Location (Inland-Coastal)	3.973	1.485	2.676	1.5	0.010	
	Clutch size	Female SVL (mm)		93.81	12.51	7.497	1.46	<0.001
		Location (Inland-Coastal)		-6548.4	2616.5	-2.503	1.46	0.016
		Female SVL: Location		83.6	29.13	2.87	1.46	0.006
		Females body mass (g)		42.476	4.267	9.955	1.47	<0.001
		Location (Inland-Coastal)		699.628	264.04	2.65	1.47	0.011
		Females loss in mass (g)		-112.061	17.882	-6.267	1.47	<0.001
	Clutch size	Location (Inland-Coastal)		1370.648	477.4	2.871	1.7	0.023
Outside breeding season	Females SVL (mm)	Location (Inland-Coastal)	11.698	2.828	4.136	1.43	<0.001	
	Males SVL (mm)	Location (Inland-Coastal)	6.103	1.274	4.788	1.43	<0.001	
	Females mass (g)	Location (Inland-Coastal)	28.669	7.169	3.999	1.43	0.002	
	Males mass (g)	Location (Inland-Coastal)	10.343	1.824	5.672	1.43	<0.001	
	Females BCI	Location (Inland-Coastal)	0.008	0.022	0.374	1.43	0.711	
	Males BCI	Location (Inland-Coastal)	0.022	0.013	1.765	1.43	0.085	

Article VIII

Site-specific developmental and behavioural responses in coastal and continental tadpoles exposed to environmental salinity

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More pronounced effects in coastal individuals compared to inland ones

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Abstract

Salinization is predicted to intensify due to the impacts of climate change, with expected consequences for biodiversity and ecosystem functioning, due to osmoregulation's costs, and particularly in early-life development. Amphibians are particularly susceptible to environmental salinity due to their highly permeable skin, this sensitivity being especially elevated in eggs and larvae. In this study, we investigated tolerance to salinity in eggs and larvae of a widespread amphibian species (spined toad, *Bufo spinosus*) originating from salt-exposed (coastal) populations and salt-free (inland) populations. We experimentally tested the effect of different salinities (0, 2 and 4 g.l⁻¹) mimicking those measured in salt-exposed (coastal) and salt-free (inland) environments. We showed that exposure to moderate environmentally-relevant salinity (4 g.l⁻¹) altered both embryonic and larval development in individuals originating from both inland and coastal populations. Specifically, increased salinity induced increased malformations, decreased body size and survival and altered behavior. Interestingly, irrespective of origin, individuals exposed to the intermediate salinity treatment (2 g.l⁻¹) performed better for most traits investigated. The effects of salinity were even higher in coastal individuals originating from salt-exposed populations compared to inland ones originating from salt-free populations, suggesting maladaptive performance. The consequences of such effects for coastal populations may be difficult to anticipate, but increasing salinity will certainly affect populations at demographic levels. As response to salinity is often species-specific in amphibians, the response in other coastal species remains to be investigated.

Key-Words: Coastal, Eggs, Inland, Larvae, Maladaptive, Salinization

1. Introduction

Salinization - the increase in environmental salinity - is predicted to intensify due to the impacts of climate change (Le et al. 2019; Reid et al. 2019) and increased water demand (Cañedo-Argüelles et al. 2013). Many inland freshwater bodies are becoming more saline due to human activities (Williams 2001), which include groundwater pumping (Reid et al. 2019; Peters et al. 2021), disposal or accidental spillage of saline waste water (Vengosh et al. 2014), use of salt to de-ice impervious surfaces such as roads (Findlay and Kelly 2011; McGuire and Judd 2020), and reduction in freshwater flow and droughts (Reid et al. 2019). In addition to these causes, coastal ecosystems are expected to experience further salinization because of the forecasted sea level rise (Vermeer and Rahmstorf 2009; Bakker et al. 2017), and the increase in frequency and intensity of marine storms and associated marine submersions (Knighton et al. 1991; Dettinger 2011; Visschers et al. 2022). Although salinization remains relatively overlooked, it is a major phenomenon to investigate due to its expected consequences for biodiversity and ecosystem functioning (Cañedo-Argüelles et al. 2013; Herbert et al. 2015).

Indeed, most organisms have to osmoregulate (Bradley 2009). When salinity fluctuate, organisms display physiological compensatory mechanisms in order to regulate water and ion fluxes in order to maintain internal homeostasis (Schultz and McCormick 2012; Evans and Kültz 2020). Yet, the mechanisms involved in the regulation of the hydro-mineral balance are metabolically expensive (Herbert et al. 2015). Salinity, through its

effect on energy budget and/or disrupted homeostasis (i.e., hypernatremia and/or dehydration) has been shown to negatively influence critical organismal functions such as foraging (Chuang et al. 2022a), activity (Leite et al. 2022; Moniruzzaman et al. 2022), anti-predator responses (Hoover et al. 2013), growth (Liu et al. 2022), but also to induce oxidative stress (Lassoued et al. 2023) and to induce altered development leading to malformations (Hieu et al. 2021), potentially affecting survival (Cañedo-Argüelles et al. 2013; Moniruzzaman et al. 2022).

In many organisms, early life stages do not tolerate as elevated salinity as adults (Lee et al. 2003; Paiva et al. 2020; Liu et al. 2022; Walker et al. 2023). As a consequence, elevated salinity can affect both reproduction and reproductive success (Hart et al. 2003). For instance, salinity has been shown to decrease sperm performance (Green et al. 2021) and fecundity (Pinder et al. 2005). In addition, salinity has been shown to negatively influence early-life development in several freshwater taxa, notably because the high energy expenditure allocated to osmoregulation (Sokolova 2013) induces a trade-off with the energetic demands of growth and development (Pinder et al. 2005; Lambret et al. 2021). In turn, these early influences of salinity can impact many aspects of a living organism later in life because consequences of early-life development are known to carry-over (Masero et al. 2017; Urzúa et al. 2018; Traversari 2021).

Amphibians are particularly susceptible to environmental salinity due to their highly permeable skin used for gas exchange, and for ions and water transport (Shoemaker

and Nagy 1984; Martin and Nagy 1997), and because of their comparatively limited ability to osmoregulate (Katz 1989). Early-life stages (i.e. eggs and larvae) are more susceptible to salinity than adults (Albecker and McCoy 2017). Amphibian eggs have very low osmoregulatory abilities (Karraker and Gibbs 2011), and eggs and larvae are often entirely dependent on - and thus restricted to - the aquatic environment compared to adults (Wells 2007). Embryos and larvae are thus highly sensitive to moderate salinity levels (Chinathamby et al. 2006), even if the responses to increasing salinity is often species-specific (Tornabene et al. 2021a). Increasing salinity has been shown to lead to decrease embryonic and larval survival (Christy and Dickman 2002; Chinathamby et al. 2006; Dougherty and Smith 2006; Sanzo and Hecnar 2006; Karraker et al. 2008; Bernabò et al. 2013; Jones et al. 2015), and to increase deformities (Sanzo and Hecnar 2006; Karraker 2007; Hopkins et al. 2013a; Brady 2013; Meiler 2016; Tornabene et al. 2021a) such as abdominal edema and axial malformations.

In addition to these ultimate effects affecting survival, salinity can also strongly influence other parameters of embryonic and larval development of amphibians. For instance, salinity can affect the duration of embryonic development (Haramura 2016; Tornabene et al. 2021a) and the developmental stage at hatching (Haramura 2007; Karraker and Gibbs 2011; Tornabene et al. 2021a). In larvae, salinity may depress thyroid hormones (Gomez-Mestre et al. 2004), and induce changes in hormones involved in osmoregulation such as corticosterone and aldosterone (Tornabene et al. 2021b, 2022b). These processes can lead to prolonged larval

development (Christy and Dickman 2002; Gomez-Mestre and Tejedo 2003; Lukens and Wilcoxon 2020) and reduced growth (Gomez-Mestre and Tejedo 2003; Gomez-Mestre et al. 2004; Wu and Kam 2009; Bernabò et al. 2013; Wood and Welch 2015; Haramura 2016; Lukens and Wilcoxon 2020; Tornabene et al. 2021a). In addition to these physiological mechanisms, such effects are likely to be mediated by behavioural alterations. Indeed, larvae exposed to higher salinity express reduced activity and foraging behavior (Hall et al. 2017; Tornabene et al. 2021a), low responses to stimuli (Sanzo and Hecnar 2006; Karraker 2007), low swimming performance (Haramura 2016), erratic movements (Tornabene et al. 2021a), and reduced speed (Denoël et al. 2010), all of which can, in turn, affect their susceptibility to predation and competition.

Taken together, all these elements point to detrimental impacts of salinization on amphibian populations (Karraker et al. 2008). Yet, it is noteworthy that exposure to moderate levels of salinity can induce adaptive selection and improve tolerance to salinity in coastal amphibians (Gomez-Mestre and Tejedo 2003; Hopkins et al. 2016; Tornabene et al. 2021a). As a consequence, individuals originating from brackish coastal habitats should be more tolerant to increased salinity compared to naïve individuals originating from strictly freshwater habitats. Yet, the effects of local adaptation on the developmental and behavioral responses of amphibian larvae to salinity remains poorly investigated.

In this study, we investigated tolerance to salinity in eggs and larvae of a widespread amphibian species (spined toad, *Bufo*

spinus) originating from salt-exposed (coastal) populations and salt-free (inland) populations. We experimentally tested the effect of different salinities (0, 2 and 4 g.l⁻¹) mimicking those measured in salt-exposed (coastal) and salt-free (inland) environments. During the embryonic development, we recorded survival (hatching rate) and duration of development (time to hatching). Upon hatching, we assessed telomere length and oxidative status of larvae. During the larval development, we assessed survival, duration of development, malformation, morphology and growth. We also investigated behavioural characteristics (activity, swimming speed, use of space, behavioral complexity). Finally, once metamorphosis was completed, we investigated the consequences of the salinity experienced during embryonic and larval development on metamorphic individuals. We predicted that the highest salinity treatment should negatively affect individuals, and that these effects should be more pronounced in naïve individuals originating from salt-free environments (Gomez-Mestre and Tejedo 2003). We also predicted that individuals originating from salt-free (inland) and salt-exposed (coastal) populations should perform better in freshwater (Gomez-Mestre and Tejedo 2003).

2. Methods

2.1. Study species and egg collection

The spined toad (*Bufo spinosus*) is a widespread amphibian species found in western Europe (Speybroeck et al. 2018; Meek 2022). Breeding occurs in late winter, during which adults converge to reproductive ponds where they mate and lay their eggs constituted of elongated egg strings (Miaud and Muratet 2018). Egg laying, embryonic and larval developments occur in ponds.

Already composed pairs (amplexus) were captured in 3 coastal and 4 inland locations (see Article VII). As coastal and inland populations were geographically separated by more than 50 km, amplexus collected within these locations should be sufficiently distant to provide an accurate assessment of population-level differences in their susceptibility to salinity. Captures occurred at night (between 20 pm and 4 am) from 16/02/2022 to 02/03/2022. For inland populations, four ponds were prospected (coordinates: 46,1707, -0,4561; 46,1558, -0,4831; 46,0489, -0,2373; 46,1458, -0,4248) where salinity was 0.0 g.l⁻¹. For coastal populations, three ponds were prospected (coordinates: 46,0469, -1,0516; 46,0359, -1,05307; 46,039504, -1,049723) where salinity ranged from 1.8 to 3.9 g.l⁻¹ (mean=2.67 ± 0.69 g.l⁻¹).

On each location (coastal/inland), 20 amplexus were captured (20 coastal amplexus and 20 inland amplexus). Individuals were captured by hand, placed in a transport box (14*16*9cm), and brought to the laboratory immediately after field sessions.

At the laboratory, each pair was placed in containers (35*55*26 cm) containing freshwater (~20L, 0.3 g.l⁻¹) and branches for laying support in a thermally controlled room with temperature set at 17 °C and controlled photoperiod (12 h dark-12 h light). Amplexus were left in these tanks until the egg strings were laid (min time=10h, max=175h; mean=66h ± 45). Upon laying, 6 segments of each egg string, containing 30 eggs each (i.e. a total of 180 eggs for each egg string) were selected randomly and kept for our experiment. Adults and the remaining eggs (i.e. 1500-8500) were released at their site of origin.

2.2. Experimental design

2.2.1. Treatment salinity

Our goal was to mimic the range of salinity concentrations found in nature, and we produced 3 treatments: control (0 g.l⁻¹ salinity: 0.3 ± 0.0 g.l⁻¹), low (2 g.l⁻¹ salinity 2.0 ± 0.05 g.l⁻¹) and moderate (4 g.l⁻¹ salinity 4.0 ± 0.06 g.l⁻¹), in 2 L tanks (13*18*18cm, n=240) which correspond to salinity levels found in coastal environments (see above).

2.2.2. Egg segments (n=240)

Each clutch was represented by 2 segments of 30 eggs in each of the experimental treatments (i.e. for each clutch: 2 segments in the control treatment, 2 segments in the low treatment, and 2 segments in the moderate treatment). Each segment was placed in a glass tank containing 2 l of water and water was changed once a week. To prevent osmotic shock, and because natural salinity fluctuations do not exceed ± 2 g.l⁻¹ a day, segments exposed to the low or moderate treatments were gradually exposed to the salinity of the treatment, with an increase of 1 g.l⁻¹ a day (Hsu et al. 2018). Following this procedure, segments in the low treatment reached 2 g.l⁻¹ in 2

days, and segments exposed to moderate treatment reached 4 g.l⁻¹ in 4 days. Each segment was monitored twice a day until tadpoles hatched and reached a free feeding stage (Gosner stage 25 [Gosner 1960], hereafter GS25) (Gomez-Mestre and Tejado 2003).

2.2.3. Tadpoles (n=226)

Upon hatching, one undeformed tadpoles of each segment (e.g. 6 tadpoles per clutch, 2 from the control, 2 from the low treatment, and 2 from the moderate treatment) was selected randomly, placed individually in a glass tank containing 2 l of water and kept for the remaining of the experiment, and their development was monitored until the end of the metamorphosis. Additional 4 tadpoles per segment were kept at -80°C for physiological measurements (oxidative stress and telomeres length, see below). All other tadpoles were released at the site where their parents were captured. In 14 segments (7 control, 2 low and 5 moderate) representing 9 clutches, no individuals survived.

During the whole larval development, water was changed once a week, and tadpoles were fed ad libitum with thawed organic spinach. Individuals were checked twice a day for their developmental stage. We selected Gosner stages 25, 30, 37, 41 and 42 (Gosner 1960, hereafter, GS 25, GS 30, GS 37, GS 41 and GS 42, respectively), according to (Cheron and Brischoux 2020). Individuals were kept until metamorphosis, which occurred at Gosner stage 45 (hereafter GS45). At this stage, individuals were transferred in a plastic box (14*16*9cm) containing a humid paper towel and a shelter.

Three coastal individuals in the control treatment did never reached metamorphosis (two remained at GS39 and one remained at GS41) and were removed from the experiment. At the end of this experimental procedure, individuals were released at the site where their parents were captured.

2.3. Morphological and survival measurements

At GS 25, we assessed hatching success (proportion of eggs that have hatched) and deformation rates (number of larvae that hatched but were malformed divided by the total number of hatched individuals). For each larval stages (GS 25, GS 30, GS 37, GS 41 and GS 42), we measured total length, body length, tail length, and head width. Additionally, from GS 37 to GS 42, we also measured tail height, tail muscle height, body height and hindlimb length. Upon metamorphosis (GS45), we measured the snout-to-vent length (SVL) of toadlets as well as their body width and body height. Five days after the onset of metamorphosis, toadlets were weighted (OHAUS scale, precision ± 0.001g).

For all measurements, each tadpole was put into a Petri dish with the water from its own tank (except for GS45 where individuals were placed in the Petri dish without water), and photographed from above and laterally. Morphological measurements were performed with the software ImageJ (Schneider et al. 2012). All individuals were checked daily and abnormalities or mortality were recorded.

2.4. Physiological measurements

120 tadpoles (20 tadpoles per treatment and per site) were used for telomere analysis. Telomeres are nucleoprotein structures,

located at the ends of chromosomes and subject to shortening with each cycle of cell division (Harley et al. 1990; Olovnikov 1996). They prevent chromosome ends from being recognized as double-strand breaks and protect them from fusion and degradation (Blackburn 1991). Telomeres consist of repetitive DNA segments high in G-C and are considered to be highly susceptible to damage induced by oxidative stress (Houben et al. 2008), and are known to shorten in response to a stress (Breuner et al. 2013). For telomere analysis, whole individuals were digested with proteinase K, and DNA was extracted using the Nucleospin Tissue Kit (MachereyNagel). The DNA concentration and purity were assessed with a Nanodrop ND1000 spectrophotometer (Thermo Scientific). Telomere length was determined by quantitative PCR (qPCR; BioRad CFX 96; Bio-Rad, USA).

2.5. *Activity, locomotion, and behavioral complexity*

Upon hatching (GS25) but before tadpoles were transferred to their individual aquaria, we assessed general activity of individuals belonging to each segment. We used a scale of 1 to 5 to assess the proportion of live individuals that were active (moving) with 1: 0 to 20% of hatchlings being active, 2: 20-40%, 3: 40-60%, 4: 60-80% and 5: 80-100%.

Just after hatching (GS25), for individuals that were kept in individual aquaria, we assessed their position in the water column (at the bottom of their aquarium, or within the water column). We also performed a test of escape from predation: we gently stimulated (touched) tadpoles with a pipette, and their response (escape or no escape) was recorded.

At GS37, a pivotal stage characterized by the peak of activity and associated behaviour (Cheron et al. 2021), we performed a comprehensive set of behavioural measures. Following Cheron et al. (2021), tadpoles were removed from their tank and placed in an individual arena filled with the water from their tank (Petri dish, diameter 13.5 cm, water level 1.5 cm). Tadpoles were left acclimated for 20 min, and then video recorded for 35 min, using a camera placed above the arena (GoPro HERO; GoPro, San Mateo, CA, USA). Videos were analyzed with the software ToxTrac (Rodriguez et al. 2018). We used a white background in order to create a contrast that allowed the software to follow the tadpoles. The successive positions of a tadpole (defined from the x- and y- calibrated coordinates within the Petri dish) were then extracted per frame, and the distance moved between two consecutive frames was calculated. Using 'Tracking RealSpace' data, we determined the total duration of activity; the total distance travelled (both absolute and relative to body size), mean swimming speed (both absolute and relative to body size), and frequency of positioning at less than 50mm to the center of the arena as tadpoles can be more visible to predators in open areas (see Denoël et al., 2012 and references therein). We thus expect that swimming at the center of the aquarium could be considered as a "risky" behavior.

Additionally, as in (Cheron et al. 2021) we evaluated DFA (Detrended fluctuation analysis) and DFAC (corrected detrended fluctuation analysis) using the *fractal* package (version 2.0-4, (Constantine and Percival 2017)). DFA is a robust method used to estimate the degree to which time series are long-range dependent and self-

affine (Cannon et al. 1997), which is now currently used in the study of animals behavior (Alados and Huffman 2000; MacIntosh et al. 2011). Following (MacIntosh et al. 2013), to compute DFA, we coded our behavioral sequences as binary time series $[z(i)]$: an immobile individual is coded as -1, while a mobile individual (an individual is considered moving if it moves more than 0.25 mm/S) is coded as 1, at 1 s intervals to length N (for 35 minutes). Description of how DFA and DFAc were computed was presented in (Peng et al. 1995; MacIntosh et al. 2013). DFA is inversely related to the fractal dimension, a classical index of structural complexity (Mandelbrot 1982), and thus smaller values reflect greater complexity.

At GS45, toadlets were positioned on a 2 m² plane surface, and were stimulated (touched) with a pipette. Activity was recorded with a scale ranging from 1 to 5 (from 1 if the individual is amorphous, with little or no response to stimuli, to 5 if the individual moves energetically, following (Lorrain-Soligon et al. 2022a)). Additionally, we recorded their posture (0: prostrate, 1: stand straight), and their ability to jump (0: no jump, 1: at least one jump). Then, we computed jumping performance of metamorphic individuals which have performed jumps, according to (Lorrain-Soligon et al. 2022a). Indeed, jumping performance was assessed by measuring the distance travelled by a metamorph for each jump during 6 consecutive jumps (Greenberg and Palen 2021). Mean distance was then extracted.

2.6. Statistical analysis

We investigated the differences within sites (coastal and inland) and within treatments (0, 2 or 4 g.l⁻¹ salinity), as well as the

interaction between treatment and sites. We always added clutch as a random effect as 6 individuals of each clutch were randomly assigned to the three treatments.

2.6.1. Embryonic development

To test for differences in hatching success, malformation rate and general activity between sites and treatment, we performed binomial Generalized Linear Mixed Models (GLMMs) with sites, treatment and their interaction as covariates, and with clutch as a random effect. To investigate the effect on time to hatching, we performed Linear Mixed Models (LMMs) with sites, treatment and their interaction as covariates, and with clutch as a random effect.

We also tested if telomeres length at the end of embryonic development was influenced by treatment, site, tadpoles' size, embryonic development duration and hatching success using a LMM with the interactions between all these variables and clutch as a random effect. Best variables were retained using a top-down selection, and only the retained variables are presented.

2.6.2. Larval development

To test for the effect of treatment and site on larval mortality, we computed Kaplan-Meier curves of survival using the *survival* package (Therneau 2022).

As morphological variables were all highly correlated, we performed PCA on all the morphological metrics taken at each developmental stages, and extracted the coordinates of the first axis (explaining most part of the variation), hereafter termed as morphology score, to allow analyze the variation of all variables

together. At GS25 and GS30, PCA were realized on total length, tail length, body length, and body width. At GS37, GS41 and GS42, PCA were realized on total length, tail length, body length, body width, tail muscle height, tail height, body height and hindlimbs length. At GS45, PCA were realized on SVL, body width, and body height. An increase of the score indicates an increase of all these parameters. To test for the effect on the morphology score at each stage, we performed LMMs with sites, treatment and their interaction as covariates, and with clutch as a random effect. Results for body length solely are also presented for clarity.

To investigate the effect on development time, we retained the time (in day) between GS25 and the specific developmental stage, and between the previous Gosner stage and the specific developmental stage, and we performed Linear Mixed Models (LMMs) with sites, treatment and their interaction as covariates, and with clutch as a random effect.

At GS25, to test for the effect on position within the water column and escape from predation (both binomial variables with 0/1 responses) we performed binomial GLMMs with sites, treatment and their interaction as covariates, and with clutch as a random effect.

At GS37, as behaviour variables were all highly correlated, we performed PCA on all the behaviour metrics (DFA, DFAC, total distance, total distance corrected by individual body size, mean speed, mean speed corrected by individuals body size, total duration of activity, frequency of positioning at less than 50 mm to the center), and extracted the coordinates on

the first axis (explaining most part of the variation), hereafter termed as behaviour score, to allow analyze the variation of all variables together. An increase of the score indicates an increase of total distance, mean speed and total duration of activity, but a decrease in DFA and DFAC and of the frequency of positioning at less than 50 mm to the center.

To test for the effect on the behavior score, we performed LMM with sites, treatment and their interaction as covariates, and with clutch as a random effect. For clarity, we also detailed the result found for each behaviour variable. The effect on DFA, DFAC, total distance traveled and average speed were assessed using LMMs with sites, treatment and their interaction as covariates, and with clutch as a random effect. In order to test the effect on frequency of positioning at less than 50 mm to the center we performed binomial GLMMs with sites, treatment and their interaction as covariates, and with clutch as a random effect.

2.6.3. Metamorphosis

As morphological variables were all highly correlated, we performed PCA on all the morphological metrics taken and extracted the coordinates on the first axis (explaining most part of the variation), hereafter termed as morphology score, to allow analyze the variation of all variables together. At GS45, PCA were realized on body length, body width, and body height. An increase of the score indicates an increase of all these parameters.

To test for the effect on the morphology score, as well as body mass and mean jump distance we performed LMMs with sites, treatment and their interaction as

covariates, and with clutch as a random effect.

To test for differences in activity (as a proportion compared to 5 being the maximum value for activity) between sites and treatment, we performed binomial GLMMs with sites, treatment and their

interaction as covariates, and with clutch as a random effect. We also looked for the differences in individuals' attitude (stand straight, performed jumps) but without realizing statistical analysis.

All data analysis were performed using R 3.6.3 (R Core Team 2020) and Rstudio v1.1.419. Statistical results are given in tables VIII1, VIII2 and VIII3.

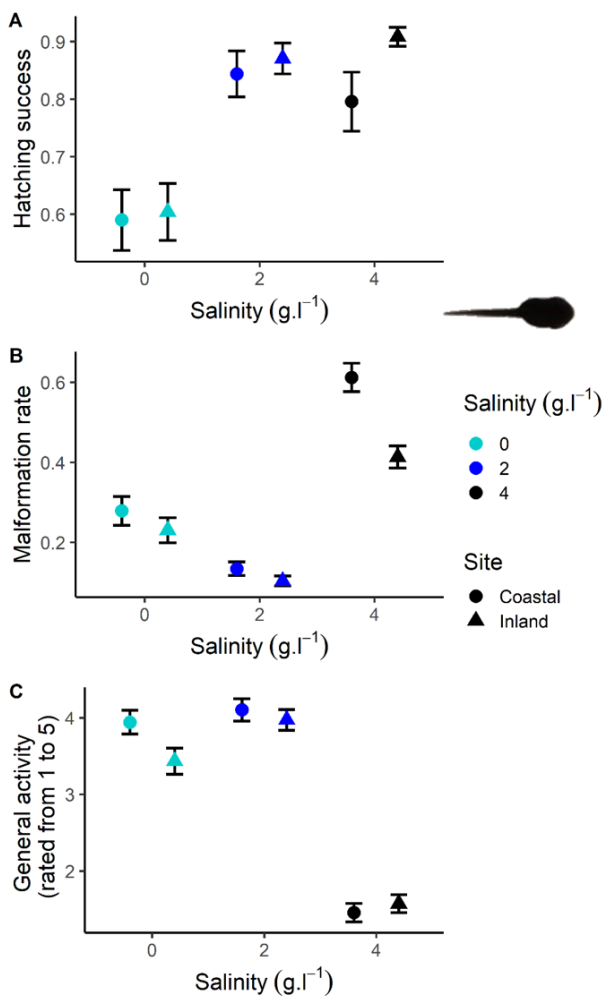


Figure VIII1 - (A) Hatching success (proportion of eggs that have hatched), (B) Malformation rate (proportion of malformed larvae that hatched but are, on the total number of hatched individuals) and (C) General activity (on a scale of 1 to 5, describing the proportion of individuals that were active; 1=0-20%, 5=80-100%), for the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Mean \pm SE.

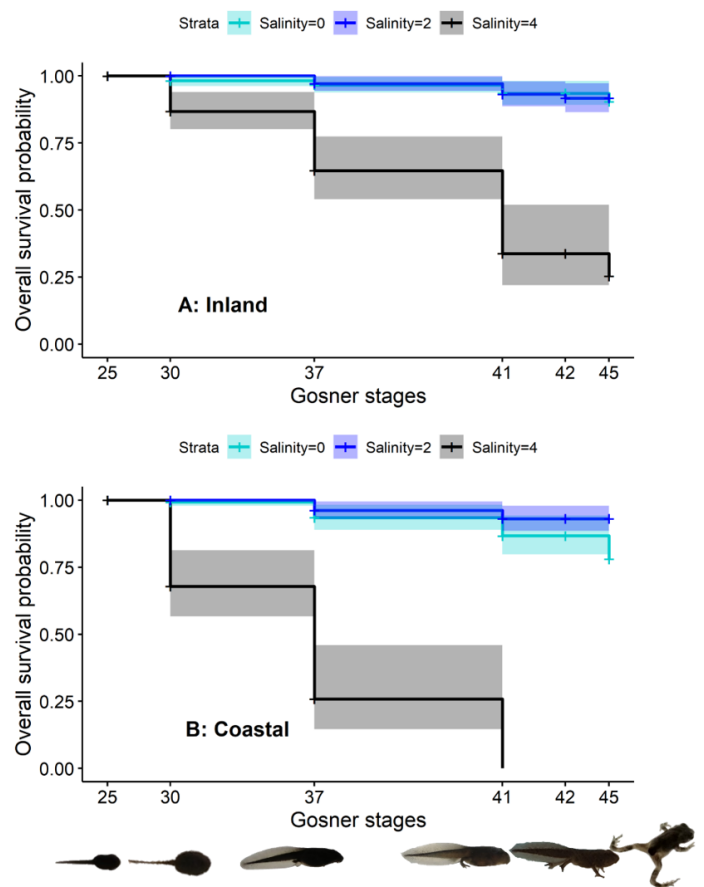


Figure VIII2 -Curves of Kaplan-Meier for estimated survival of (A) inland and (B) coastal individuals in the three treatments (0, 2 and 4 g.l⁻¹).

3. Results

3.1. Embryonic development

Hatching success was higher in the 2 g.l⁻¹ and 4 g.l⁻¹ salinity treatments compared to the 0 g.l⁻¹ treatment, with no differences between coastal or inland individuals (Figure VIII1A). However, malformation rate was higher in the 4 g.l⁻¹ salinity treatments compared to the 0 g.l⁻¹ and 2 g.l⁻¹ salinity treatments, and higher in coastal individuals compared to inland ones in the 4 g.l⁻¹ salinity treatment (Figure VIII1B).

General activity was also higher in the 0 g.l⁻¹ and 2 g.l⁻¹ salinity treatments compared to the 4 g.l⁻¹ treatment, with no differences between coastal or inland individuals (Figure VIII1C). For inland individuals, time to hatching decreased with increasing salinity, but not in coastal individuals.

Upon hatching, we found that telomere length was positively influenced by tadpole size (Estimate=0.051, SE=0.014, t-value=3.548, p-value=0.001) and

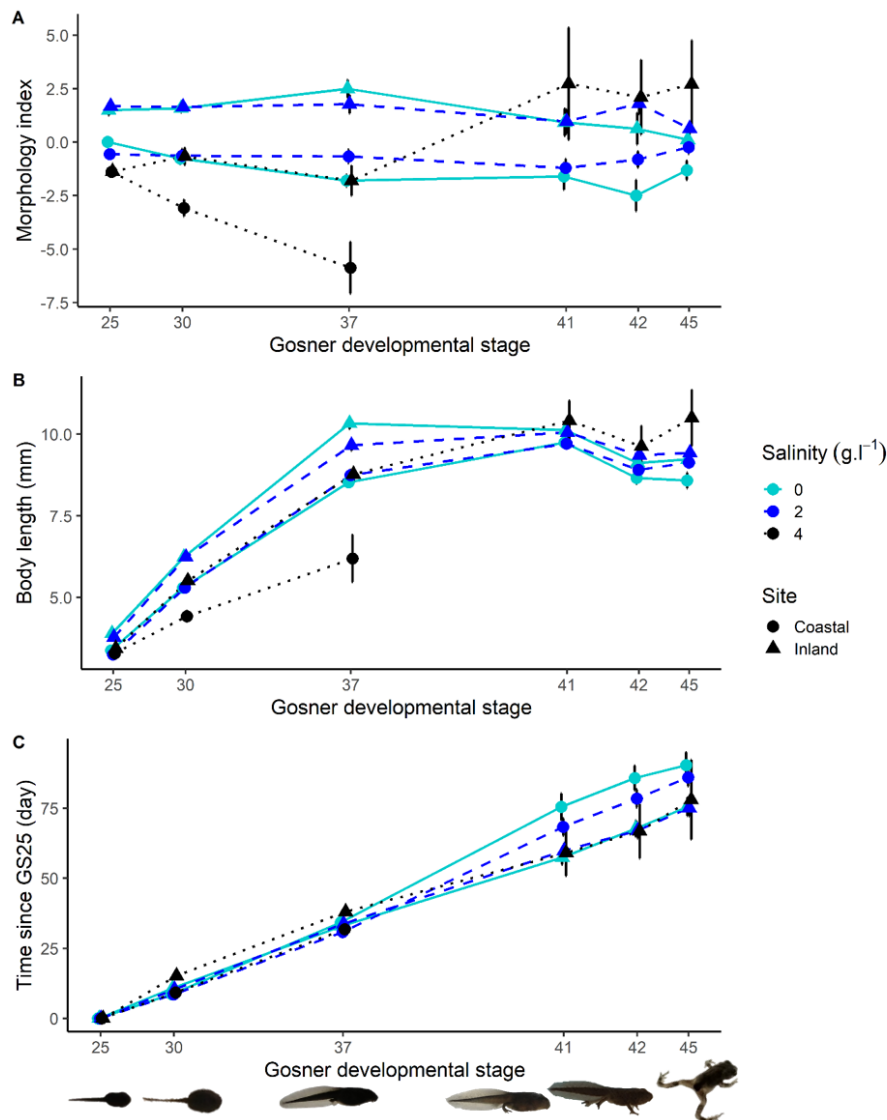


Figure VIII3 – (A) Morphology score, extracted for the first axis of the PCA between all the different metrics variables for all developmental stages (see text for details), (B) body length, and (C) time between Gosner stage 25 (hatching) and each studied stage, for each stage and the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Mean ± SE.

embryonic development duration (Estimate=0.056, SE=0.014, t-value=3.990, p-value<0.001). We found no effect of treatment, site, or their interaction.

3.2. Larval development

3.2.1. Survival

Mortality during the whole development was higher in the 4 g.l⁻¹ salinity treatment (Figure VIII2). In the 4 g.l⁻¹ salinity treatment, only 4 inland individuals survived to GS45 (Figure VIII2A), while no coastal individuals survived to GS41 (Figure VIII2B). The survival was relatively low in coastal individuals in the 0 g.l⁻¹ salinity treatment (Figure VIII2B). Malformation rate was higher in the 4 g.l⁻¹ salinity treatment, but the number of malformed individuals decreased with increasing developmental stage.

3.2.2. Morphology

At the beginning of the larval development, individuals in the 0 and 2 g.l⁻¹ treatments had a similar morphology score and body length, both being larger compared to individuals in the 4 g.l⁻¹ salinity treatment (Figure VIII3AB). At the end of the larval development, individuals in the 2 g.l⁻¹ treatment had a larger morphology score and body length compared to individuals in the 0 g.l⁻¹ treatment (Figure VIII3AB), and individuals in the 4 g.l⁻¹ treatment seemed to have a larger morphology score and body length compared to individuals in the other treatments (Figure VIII3AB), although this was not significant. Overall, across the larval development, inland individuals had a larger morphology score and body length compared to coastal ones (Figure VIII3AB).

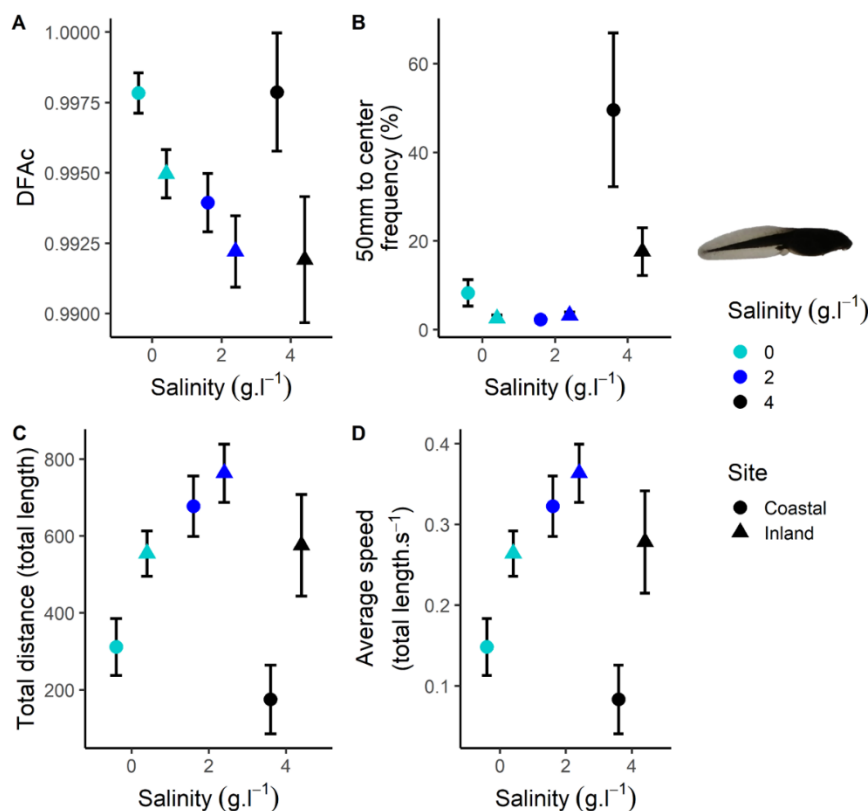


Figure VIII4 - Behavioural metrics studied at Gosner stage 37. (A) Corrected DFA (DFAC: Detrended fluctuation analysis, indicating the complexity of the movements, the higher it is, the less the movement is complex), (B) Frequency of individuals positioning at less than 50 mm to the center of the arena, (C) total travelled distance corrected by individuals' total length, and (D) Average speed, corrected by individuals' total length, for the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Mean \pm SE.

3.2.3. Development duration

During early stages of development (GS30-GS37), inland individuals were slower to develop (Figure VIII3C). Conversely, at later developmental stages (GS41-GS45), coastal individuals were slower to develop (Figure VIII3C). Salinity (moderate treatment) affected development rate in inland individuals during early developmental stages solely (GS30-GS37), but this effect was transient and at later developmental stages, we found no significant differences between treatments (Figure VIII3C).

3.2.4. Behaviour

At GS25, individuals in the 4 g.l⁻¹ treatment were positioned at the bottom of their aquarium more often while individuals in the 0 g.l⁻¹ and 2 g.l⁻¹ salinity treatments moved within the water column. Similarly, individuals in the 4 g.l⁻¹ treatment expressed fewer escape response to predation compared to individuals in the 0 g.l⁻¹ and 2 g.l⁻¹ salinity treatments. Coast and inland individuals expressed no differences in these behavior.

At GS37, the behavior score was always higher in the 2 g.l⁻¹ salinity treatment compared to the 0 g.l⁻¹ and 4 g.l⁻¹ salinity treatments, and always higher in inland individuals compared to coastal ones. In particular, DFA and DFAc were lower in the 2 g.l⁻¹ treatment and in inland individuals (Figure VIII4A). Position to the center point of the arena was higher in the 4 g.l⁻¹ salinity treatment compared to the 0 and 2 g.l⁻¹ salinity treatments, and higher in coastal individuals compared to inland ones (Figure VIII4B). Total distance traveled and average speeds were higher in the 2 g.l⁻¹ salinity treatment and for inland individuals (Figure VIII4CD).

3.3. *Metamorphic individuals*

3.3.1. Survival

No coastal individuals in the 4 g.l⁻¹ salinity treatment survived to metamorphosis, and only 4 inland individuals in the 4 g.l⁻¹ salinity treatment survived to metamorphosis (Figure VIII2). The survival at GS45 was also very low in coastal individuals in the 0 g.l⁻¹ salinity treatment (Figure VIII2B). At metamorphosis, no individuals presented evidence of malformation.

3.3.2. Morphology

Morphology score was always higher in inland individuals, and particularly higher for inland individuals in the 4 g.l⁻¹ salinity treatment (Figure VIII3A). The morphology score between individuals in the 0 and 2 g.l⁻¹ salinity treatment was similar (Figure VIII3A). The same pattern was found for body mass (Figure VIII5A).

3.3.3. Behaviour

At GS45, activity between individuals in the 0 and 2 g.l⁻¹ salinity treatment was similar (Figure VIII5A). Inland individuals in the 4 g.l⁻¹ salinity treatment all stood straight (Figure VIII5C) and all actively jumped (Figure VIII5D). Mean jumping distance was similar between all treatments and between coastal and inland individuals, but with a non-significant trend to be higher in inland individuals (Figure VIII5E).

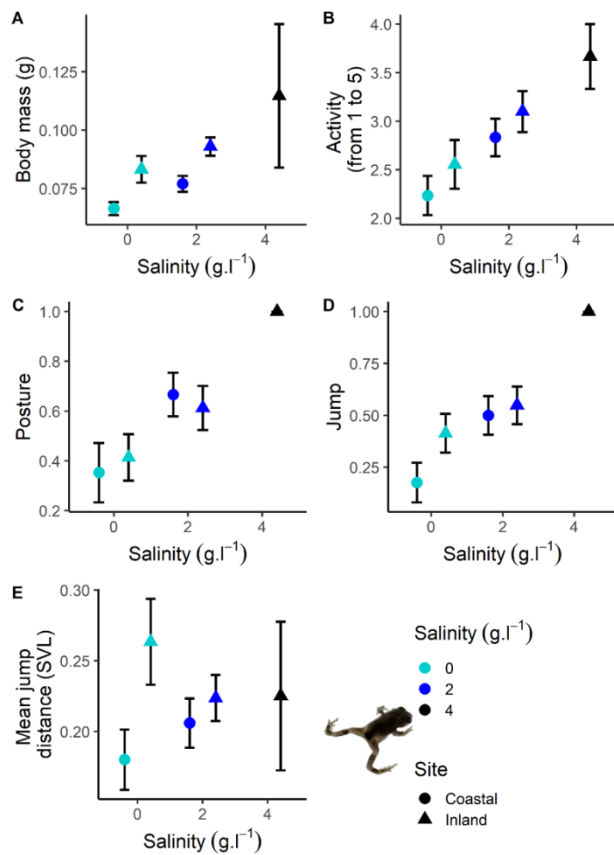


Figure VIII5 – Metrics studied at J+5 after metamorphosis (Gosner stage 45). (A) Individuals body mass, (B) Activity (measured from 1 to 5 according to (Lorrain-Soligon et al. 2022): 1 individuals did not react to stimuli, 5: individuals are very active and realized multiple jumps), (C) Individuals posture (0: prostrate, 1: stand straight), (D) Individuals realizing jumps (0: no jump, 1: at least one jump), and (E) mean jump distance, for individuals that have realized jumps, for the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Mean ± SE.

4. Discussion

In this study, we show that exposure to moderate environmentally-relevant salinity (4 g.l⁻¹) altered both embryonic and larval development in both individuals originating from naïve (salt-free) and coastal (salt-exposed) populations. Specifically, increased salinity induced increased malformations, decreased body size and survival and altered behavior. Contrary to our predictions, these effects

were higher in coastal individuals originating from salt-exposed populations compared to inland ones originating from salt-free populations. Interestingly, irrespective of origin, individuals exposed to the intermediate salinity treatment (2 g.l⁻¹) performed better for most traits investigated.

4.1. Embryonic development

Embryonic development duration decreased with increasing treatment salinity in inland individuals. Conversely, in coastal individuals, embryonic development duration increased in individuals exposed to moderate salinity solely. As a consequence, embryonic development duration was longer in inland individuals at the lower salinities (0 and 2 g.l⁻¹), but shorter at 4 g.l⁻¹ compared to coastal individuals. Differences in development time according to salinity have already been shown in amphibians (see Christy and Dickman 2002; Sanzo and Hecnar 2006; Wu and Kam 2009; Wijethunga et al. 2016; Lukens and Wilcoxon 2020). Faster developmental rates in a stressful environment (such as our moderate salinity treatment) should allow individuals to evade such detrimental conditions more rapidly (Wassersug and Sperry 1977; Relyea 2007), a hypothesis that is supported in individuals originating from inland (salt-free) populations. Contrarily, we found that individuals originating from coastal (salt-exposed) populations were longer to develop when exposed to our highest salinity level. Although such result could suggest local adaptation to higher salinity in these individuals, complementary metrics of fitness (malformation, morphology and activity) do not support this hypothesis. Although it is plausible that osmoregulation mechanisms triggered

to cope with high salinity altered developmental rates (Gomez-Mestre et al. 2004), the counterintuitive contrast we highlight between coastal and inland individuals remains puzzling.

Interestingly, lower hatching success in both coastal and inland individuals were found for freshwater (0 g.l⁻¹ salinity). Some evidence suggests, indeed, that intermediates salinities could be beneficial while lower salinities may be detrimental (Martnez-Palacios et al. 2004; Robles Mendoza et al. 2009; Ahumada-García et al. 2018). This could be due to lower parasites and pathogens prevalence at intermediate salinity levels (Robles Mendoza et al. 2009; Ahumada-García et al. 2018; Clulow et al. 2018; Callen et al. 2023). In any case, hatching success may be poorer *in natura* as 4 g.l⁻¹ salinity has been shown to reduce ability of sperm to survive and swim (Wilder and Welch 2014), an effect which we were unable to demonstrate as our clutches were all obtained in freshwater.

In addition, we show no variation in telomere's size according to treatment or site, while telomere's size is known to be a reliable measure of cellular aging (Harley et al. 1990; Olovnikov 1996) and of exposure to stress (Houben et al. 2008; Breuner et al. 2013). We thus found that an exposure to environmental salinity during embryonic development (~ 15 days) is not enough to induce telomere shortening. Yet, as expected, we found increasing telomeres length with increasing tadpoles' size and embryonic development duration (Angelier et al. 2019), both of which varied with exposure to salt. Environmental salinity can thus impact telomeres length through these indirect effects. Finally, telomeres length should usefully be

measured at later stages of larval development in order to test whether a longer exposure to salinity triggers telomere attrition.

4.2. Larval development

During larval development, we found that survival and growth are reduced, and malformation rate increased in brackish water (Christy and Dickman 2002; Wu and Kam 2009; Hopkins et al. 2013a; Lukens and Wilcoxon 2020). In the 4 g.l⁻¹ salinity treatment, individuals were the smallest. These individuals had a higher frequency of positioning at less than 50 mm of the center of the arena, where tadpoles can be more visible to predators (see Denoël et al., [2012] and references therein), which can be considered as a "risky" behavior, but also less complex behavior (smaller DFA, Mandelbrot 1983), leading, in natural environments, to a reduced probability of resource encounters (Bartumeus 2007; Viswanathan et al. 2008), and a tendency to be less active, which corroborate the results of previous studies (Sanzo and Hecnar 2006; Chuang et al. 2022a).

Interestingly, in the control treatment, individuals also expressed a smaller DFA and thus a less complex behavior (Mandelbrot 1982). Control individuals also displayed a smaller speed and lower distance travelled compared to the 2 g.l⁻¹ salinity treatment. As in other studies, we also found that survival and body length increased at intermediate salinities compared to freshwater (Meiler 2016; Nakkrasae et al. 2016; Ahumada-García et al. 2018; Barany et al. 2021; Lambret et al. 2021). The reasons why intermediate salinities may be beneficial during embryonic development have been investigated (see above). The underlying

mechanisms are likely to be similar for developing tadpoles, but pattern during larval development remains to be explored.

4.3. *Metamorphic individuals*

An intriguing pattern is that we did not find differences between treatments in the time to reach metamorphosis while such result has been often described (Christy and Dickman 2002; Sanzo and Hecnar 2006; Wu and Kam 2009; Wijethunga et al. 2016; Lukens and Wilcoxon 2020). We found a difference in the time to metamorphosis between coastal and inland individuals, with inland individuals reaching metamorphosis sooner than coastal individuals. This is an important results as time and size at metamorphosis influence adults fitness (Semlitsch et al. 1988). The optimal timing to metamorphosis involves trade-offs between growth, development, and survival of both the larvae and the future adult (Stearns and Koella 1986; Semlitsch et al. 1988). Tadpoles may adopt two different developmental trajectories: They can metamorphose later at a larger body size but remain in contact with suboptimal environmental conditions longer or they can metamorphose sooner at a smaller size in order to escape adverse aquatic conditions (Semlitsch et al. 1988; Day and Rowe 2002). However, we found that inland individuals metamorphose sooner at a larger body size compared to coastal individuals. Interestingly, complete development was not achieved in 3 coastal individuals exposed to the control treatment, which may be attributed to maladaptation of these individuals to freshwater environments ((Brady 2013), but see (Gomez-Mestre and Tejedo 2003)). Individuals in coastal and inland populations also differed in size and mass

at metamorphosis. Indeed, inland individuals were larger and heavier compared to coastal individuals, which, in turn, may affect juvenile survival, adult size, size at first reproduction, mating success, and reproductive output (Werner 1986; Semlitsch et al. 1988; Relyea 2007).

Importantly, at metamorphosis, individuals in the 4 g.l⁻¹ salinity treatment had a tendency to perform better, with a seemingly higher activity, size and mass (see also Dahrouge and Rittenhouse [2022]). This could be linked to environmental selection of the few individuals that were able to survive in this harsh environment. Although this hypothesis is in line with our predictions, it fails to successfully explain the high mortality that occurred in coastal individuals exposed to our moderate salinity treatment (see below). Indeed, these effects were found only in inland individuals, the only populations in which few individuals survived at 4g.l⁻¹ to metamorphosis. As suggested by Dahrouge and Rittenhouse (2022), the effects of high-salinity during larval development can carry-over later during the juvenile life stage, so that these surviving inland individuals can experiment detrimental effects later in development, and longer-term monitoring of these individuals during their juvenile and adult life-stages would be valuable .

4.4. *Differences in sensitivity between coastal and inland populations*

Higher salinities are known to be harmful to both eggs and larvae (Nakkrasae et al. 2016; Albecker and McCoy 2017), in both coastal and inland populations (Albecker and McCoy 2017). Individuals native to

brackish environments are expected to have a stronger tolerance to salinity (Gomez-Mestre and Tejedo 2003), because of local adaptation (Hopkins et al. 2016). However, in our study, survival and all metrics of development were severely reduced at 4 g.l⁻¹ in coastal individuals compared to inland ones. In contrast, we found that inland individuals performed better under all treatments. Different hypotheses can explain this counterintuitive lower tolerance to salinity in coastal individuals. First, it is possible that, in coastal populations, salinity gradients within ponds occur (e.g., linked to differential density of salt- versus fresh water, (Millero and Poisson 1981)). As a consequence, such salinity gradients may allow reproductive females to select areas characterized by lower salinities to lay their eggs, and/or larvae to select such areas to develop. Future studies should usefully investigate whether such processes occur *in natura*. Second, we performed our study under laboratory conditions, with which we were not able to test for complementary parameters that may enhance survival in natural saline conditions such as weaker predation pressure (Velasco et al. 2019) or weaker pathogen transmission (Clulow et al. 2018). Lastly, the reduced development and survival we highlighted in coastal individuals may be the result of the smaller size of the eggs in these individuals (Article VII), because enlarged egg size increase hatching success, as well as offspring and larval fitness (Xu et al. 2019). In addition, it is also plausible that this reduced performance of coastal individuals is linked to the fact that adults lay their eggs in freshwater, a salinity that did not correspond to the salinity of their environment. Future studies, based on more natural settings (i.e., mesocosms), are

required to disentangle these different hypotheses.

Importantly, the increased mortality in the highest salinity treatment can reveal a selective process: as the environment is unfavorable, this could induce high mortality in susceptible individuals and select for the few individuals that are able to survive in this harsh environment. Although this hypothesis is supported by our results for inland individuals (see above), it fails to successfully explain the high mortality that occurred in coastal individuals exposed to our moderate salinity treatment. Further studies are required to decipher the underlying mechanisms, including investigation of the physiological mechanisms used by individuals from coastal and inland populations to cope with elevated salinity.

5. Conclusion

In this study, we showed very high sensitivity to relatively elevated salinity (albeit at lower levels than what have been reported in literature, see Albecker and McCoy 2017). Our results on individuals originating from coastal populations seem to suggest maladaptive performance (Brady 2013) as coastal individuals performed poorly both in fresh- and brackish water. The consequences of such effects for coastal populations may be difficult to anticipate, but the predicted increase in salinity is likely to have severe demographic consequences (Karraker et al. 2008). However, the responses of larval anurans to salinity are often species-specific (Tornabene et al. 2021a), which strengthens the importance to test these responses on different species to better

understand the effects of environmental salinization on the biodiversity of coastal wetlands.

Table VIII1 – Analyses on the different variables studied, at the different Gosner developmental stages (GS25 and GS30), the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹).

Developmental stage	Metrics	Covariates	Df	SumSq	F-value	Chisq	p-value
GSS25	Hatching success	Treatment	2	-	-	37.19	<0.001
		Site	1	-	-	0.053	0.819
		Treatment:Site	2	-	-	1.831	0.4
	Malformation rate	Treatment	2	-	-	244.237	<0.001
		Site	1	-	-	1.898	0.168
		Treatment:Site	2	-	-	2.702	0.259
	General activity	Treatment	2	-	-	34.425	<0.001
		Site	1	-	-	0.756	0.384
		Treatment:Site	2	-	-	0.677	0.713
	Time to hatching	Treatment	2	30.712	25.949	-	<0.001
		Site	1	0.141	0.239	-	0.628
		Treatment:Site	2	101.755	85.972	-	<0.001
	Morphology index	Treatment	2	204.369	116.912	-	<0.001
		Site	1	12.183	13.939	-	<0.001
		Treatment:Site	2	48.054	27.49	-	<0.001
	Position within the water column	Treatment	2	-	-	19.148	<0.001
		Site	1	-	-	1.005	0.316
		Treatment:Site	2	-	-	0.852	0.653
Predation test	Treatment	2	-	-	18.602	<0.001	
	Site	1	-	-	2.306	0.129	
	Treatment:Site	2	-	-	0.824	0.662	
GS30	Time between GS25 and GS30	Treatment	2	194.878	41.236	-	<0.001
		Site	1	73.797	31.23	-	<0.001
		Treatment:Site	2	136.325	28.846	-	<0.001
	Morphology index	Treatment	2	201.998	87.705	-	<0.001
		Site	1	39.077	33.933	-	<0.001
		Treatment:Site	2	0.976	0.424	-	0.655

Table VIII2 – Analyses on the different variables studied, at GS37, for the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹).

Developmental stage	Metrics	Covariates	Df	SumSq	F-value	Chisq	p-value
GS37	Time between GS25 and GS37	Treatment	2	98.93	3.068	-	0.05
		Site	1	86.277	5.351	-	0.024
		Treatment:Site	2	240.323	7.453	-	<0.001
	Time between GS30 and GS37	Treatment	2	26.393	0.97	-	0.382
		Site	1	0.34	0.025	-	0.875
		Treatment:Site	2	255.868	9.406	-	<0.001
	Morphology index	Treatment	2	104.549	13.869	-	<0.001
		Site	1	83.346	22.113	-	<0.001
		Treatment:Site	2	31.972	4.241	-	0.017
	Behaviour index	Treatment	2	147.87	8.895	-	<0.001
		Site	1	83.379	10.031	-	0.002
		Treatment:Site	2	17.59	1.058	-	0.349
	DFA	Treatment	2	0.008	6.992	-	0.001
		Site	1	0.005	7.133	-	0.008
		Treatment:Site	2	0.001	0.707	-	0.495
	DFAc	Treatment	2	<0.001	4.607	-	0.012
		Site	1	<0.001	6.613	-	0.011
		Treatment:Site	2	<0.001	0.65	-	0.523
	50 mm to center frequency	Treatment	2	-	-	248.21	<0.001
		Site	1	-	-	6.357	0.012
		Treatment:Site	2	-	-	75.35	<0.001
	Total distance (size)	Treatment	2	3138449	8.923	-	<0.001
		Site	1	1201962	6.835	-	0.009
		Treatment:Site	2	364830	1.037	-	0.357
Mean speed (size/mm)	Treatment	2	0.707	8.838	-	<0.001	
	Site	1	0.279	6.979	-	0.009	
	Treatment:Site	2	0.086	1.069	-	0.346	
Total duration of activity	Treatment	2	681113	5.471	-	0.005	
	Site	1	355377	5.709	-	0.018	
	Treatment:Site	2	57447	0.462	-	0.631	

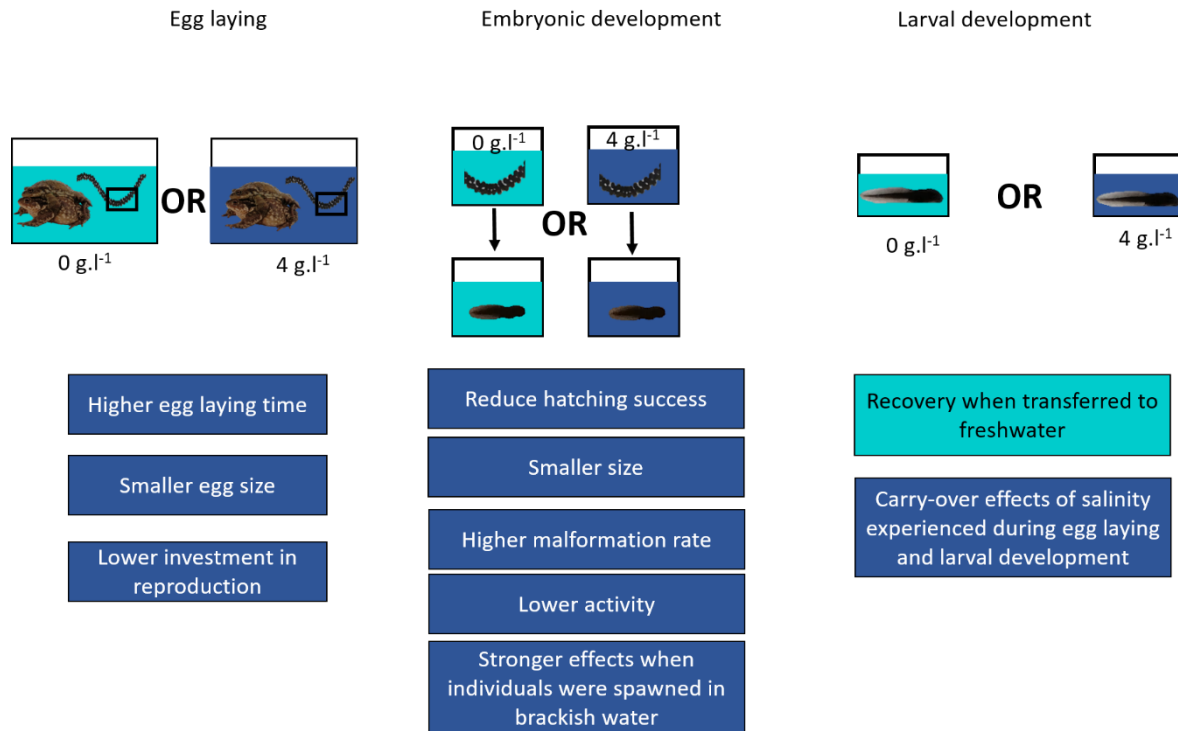
Table VIII3 - Analyses on the different variables studied, at the different Gosner developmental stages (GS41, 42 and GS45), for the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹).

Developmental stage	Metrics	Covariates	Df	SumSq	F-value	Chisq	p-value
GS41	Time between GS25 and GS41	Treatment	2	143.05	0.305	-	0.738
		Site	1	2900.1	12.378	-	0.001
		Treatment:Site	2	564.11	2.408	-	0.125
	Time between GS37 and GS41	Treatment	2	8.8	0.024	-	0.976
		Site	1	3243	17.594	-	<0.001
		Treatment:Site	2	221.5	1.202	-	0.276
	Morphology index	Treatment	2	7.334	0.453	-	0.637
		Site	1	95.32	11.792	-	0.002
		Treatment:Site	2	1.748	0.216	-	0.643
GS42	Time between GS25 and GS42	Treatment	2	389.8	0.791	-	0.457
		Site	1	3276.8	13.301	-	0.001
		Treatment:Site	2	322.4	1.309	-	0.256
	Time between GS41 and GS42	Treatment	2	173.632	3.163	-	0.047
		Site	1	75.207	2.739	-	0.108
		Treatment:Site	2	0.328	0.012	-	0.913
	Morphology index	Treatment	2	37.987	3.361	-	0.04
		Site	1	83.45	14.766	-	<0.001
		Treatment:Site	2	4.621	0.818	-	0.369
GS45	Time between GS25 and GS45	Treatment	2	225.15	0.462	-	0.632
		Site	1	2397.33	9.839	-	0.003
		Treatment:Site	2	201.87	0.829	-	0.366
	Time between GS42 and GS45	Treatment	2	31.541	2.043	-	0.136
		Site	1	2.061	0.267	-	0.608
		Treatment:Site	2	2.119	0.275	-	0.602
	Morphology index	Treatment	2	19.715	3.087	-	0.051
		Site	1	21.305	6.672	-	0.013
		Treatment:Site	2	5.204	1.63	-	0.205
	Mass at 5 days	Treatment	2	0.003	3.893	-	0.024
		Site	1	0.003	7.735	-	0.008
		Treatment:Site	2	<0.001	0.236	-	0.628
	Activity	Treatment	2	-	-	1.721	0.423
		Site	1	-	-	0.299	0.585
		Treatment:Site	2	-	-	0.02	0.887
Mean jump distance	Treatment	2	0.948	1.268	-	0.294	
	Site	1	1.343	3.592	-	0.067	
	Treatment:Site	2	0.391	1.047	-	0.312	

Article IX

Variations of salinity during reproduction and development affect ontogenetic trajectories in a coastal amphibian

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Abstract

Salinization of coastal freshwater bodies is increasing over time and threatens coastal biodiversity. Coastal ecosystems are submitted to temporal variations of salinity. Species that exploit such habitats can thus be exposed to brackish water at different life stages. However, the impacts of short term variations of salinity on wildlife remain poorly understood. This is particularly true for coastal amphibians, due to the strong dependency of early life stages (embryos and larvae) on aquatic environments. In order to investigate the effect of salinity during egg laying and embryonic and larval development of coastal amphibians, we used a full-factorial design to expose reproductive adults, eggs and larvae of coastal spined toads (*Bufo spinosus*) to fresh (0 g.l^{-1}) or brackish water (4 g.l^{-1}). At egg laying, we evaluated parental investment in reproduction. During embryonic and larval development, we assessed effects on survival, development, and growth. We highlighted strong effects of environmental salinity on reproduction (egg laying time, egg size, and investment in reproduction). Responses to salinity were highly dependent on the developmental stages of exposure. These effects carried over when exposure occurred at egg laying or during embryonic development, highlighting the importance of the environmental conditions during early life on ontogenetic trajectories. We also highlighted partial compensation when individuals were transferred to freshwater. Whether the magnitude of these responses can allow coastal biodiversity to overcome salinization remain to be assessed.

Key-words: Carry-over effects, Embryonic development, Freshwater, Recovery, Reproductive investment, Tadpoles

1. Introduction

Salinization of freshwater bodies is strongly increasing over time (Williams 2001). Coastal wetlands are particularly subjected to salinization because of salt water intrusions (Knighton et al. 1991; Visschers et al. 2022), reductions in freshwater flow resulting from infrastructures, droughts and increased abstraction (Reid et al. 2019), rising sea levels (Vermeer and Rahmstorf 2009; Church and White 2011; Bakker et al. 2017), and the predicted increase in frequency and intensity of marine surges (Nicholls et al. 1999; McLean et al. 2001; Knutson et al. 2010; Dettinger 2011; Trenberth et al. 2015; IPCC 2022). Overall, estimates suggest that 150 million ha of forest and wetlands are affected by salinization worldwide (Wicke et al. 2011).

Salinization is expected to strongly affect coastal biodiversity (Amores et al. 2013; Debue et al. 2022). Indeed, most organisms need to maintain their internal homeostasis and rely on physiological compensatory mechanisms to regulate water and ion fluxes (Schultz and McCormick 2012; Evans and Kültz 2020). Most species have specific ranges of tolerance, and crossing salinity boundaries activates mechanisms that aim at regulating ionic and hydric fluxes (Evans 2009). These mechanisms include modulation of drinking and urination rates, regulation of the permeability of membranes to water, remodeling of cells and tissues, and increased expression and activity of ion transporters (Evans 2009; Kültz 2015; Rivera-Ingraham and Lignot 2017). However, these mechanisms are often metabolically costly and the associated energetic expenditures can affect energetic

allocation to other functions (Herbert et al. 2015; Rivera-Ingraham and Lignot 2017). As such, salinity can influence critical functions such as foraging (Chuang et al. 2022a), activity (Leite et al. 2022; Moniruzzaman et al. 2022), anti-predator responses (Hoover et al. 2013) and growth (Liu et al. 2022). In addition, elevated salinity can also increase the occurrence of malformations (Hieu et al. 2021), potentially leading to increase mortality (Cañedo-Argüelles et al. 2013; Moniruzzaman et al. 2022; Woodley et al. 2023). Salinity in which adult individuals live can also influence their reproductive investment, such as egg production rate and fecundity (Pinder et al. 2005; Froneman 2023; Woodley et al. 2023), egg size (Article VII), or the age at first reproduction (Woodley et al. 2023). In addition, increasing salinity is known to reduce sperm performance, motility and velocity (Wilder and Welch 2014; Byrne et al. 2015, 2022; Green et al. 2021). Ultimately, salinization can alter community structure (Hart et al. 2003; Anufrieva and Shadrin 2018), across all trophic levels (Hintz and Relyea 2019).

Importantly, due to their particular position between terrestrial and oceanic systems, coastal ecosystems are naturally submitted to variations of salinity (Xue et al. 2013). The natural processes which affect variations of salinity are primarily linked to landward aerial transport of dry or wet oceanic salts (Zaman et al. 2018), and coastal ecosystems are thus known to experiment temporal and spatial heterogeneity of salinity (Estévez et al. 2019; Ranjbar and Ehteshami 2019; Fu et al. 2021). Importantly, these natural variations are expected to change in response to climatic changes (altered thermal and

rainfall patterns, Morcillo et al., 2000; Perigaud et al., 2003), salt water intrusions (Knighton et al. 1991; Visschers et al. 2022) and marine surges (Nicholls et al. 1999; McLean et al. 2001; Knutson et al. 2010; Dettinger 2011; Trenberth et al. 2015; IPCC 2022), which can lead to a brutal increase in salinity. However, the impacts of these short term variations of salinity on wildlife remain poorly understood.

This is especially true for coastal amphibians. Indeed, amphibians are particularly sensitive to salinity due to their highly permeable skin used for gas exchanges, and for ions and water transport (Shoemaker and Nagy 1984; Martin and Nagy 1997), and to their relatively low osmoregulatory abilities (Katz 1989). Early life stages (i.e. eggs and larvae) are particularly susceptible to environmental salinity (Albecker and McCoy 2017) because of their strong dependency on aquatic environment compared to adults (Wells 2007) and to their comparatively lower osmoregulatory abilities (Karraker and Gibbs 2011). Increasing salinity has been shown to lead to decrease embryonic and larval survival (Christy and Dickman 2002; Chinathamby et al. 2006; Dougherty and Smith 2006; Sanzo and Hecnar 2006; Karraker et al. 2008; Bernabò et al. 2013; Jones et al. 2015), and to increase deformities (Sanzo and Hecnar 2006; Karraker 2007; Hopkins et al. 2013a; Brady 2013; Tornabene et al. 2021a), time to hatching (Haramura 2016; Tornabene et al. 2021a), hatching success (Tornabene et al. 2021a), larval duration (Christy and Dickman 2002; Gomez-Mestre and Tejedo 2003; Lukens and Wilcoxon 2020; Tornabene et al. 2021a), and to reduce growth and thus to induce smaller body size (Wu and Kam 2009; Bernabò et al. 2013;

Wood and Welch 2015; Haramura 2016; Lukens and Wilcoxon 2020; Tornabene et al. 2021a, b).

Still, amphibians are known to be very plastic in behavioral and morphological traits (Hoverman and Relyea 2008), and their developmental traits can vary in response to environmental cues (Chivers et al. 2001; Warkentin 2011). For instance, tadpoles can modulate their growth rates according to environmental conditions, with longer development in optimal conditions but faster development and early metamorphosis in harsh conditions (Werner 1986; Vonesh and Warkentin 2006). Additionally, it has been suggested that the influence of a given environmental factor on development and growth of tadpoles is highly dependent on the stage and condition during which they experience such factor (Denver et al. 2002). This can be highly relevant for changes in salinity. For example, *Fejervarya limnocharis* and *Litoria ewingii* tadpoles released from salinity stress at early larval stages reached a size at metamorphosis similar to that of tadpoles maintained in low salinity throughout development, indicating compensatory growth (Squires et al. 2010; Wu et al. 2012). Yet, the efficiency of such compensatory mechanisms may vary if individuals are exposed earlier or later during larval development (Hsu et al. 2018). Additionally, whether parents can influence the tolerance of their offspring according to the salinity in which they are laying eggs is unknown.

In order to investigate the effect of salinity during egg-laying and embryonic and larval development of coastal amphibians, we used a full-factorial experimental design to expose reproductive adults, eggs

and larvae of coastal spined toads (*Bufo spinosus*) to fresh (0 g.l⁻¹) or brackish water (4 g.l⁻¹, Figure IX1). More specifically, reproductive pairs (amplexus) were exposed to fresh or brackish water prior to egg-laying (Figure IX1). Following egg laying, we exposed eggs to either fresh or brackish water until hatching (Figure IX1). Following hatching, we exposed larvae to either fresh or brackish water (Figure IX1). At egg laying, we evaluated parental investment in reproduction. During the complete embryonic and larval development, we assessed effects of each experimental treatment on survival (hatching rate, larval survival), development (duration of embryonic and larval development, malformation), and growth (body length, tail length, tail

height). We predicted that:

- Brackish water should impact reproductive investment and egg size due to the costs associated with osmoregulation (Rivera-Ingraham and Lignot 2017), and alter fecundity because salinity can impact sperm motility (Byrne et al. 2015, 2022).
- Salt exposure should decrease embryonic survival, larval survival and growth (Albecker and McCoy 2017; Lukens and Wilcoxon 2020; Tornabene et al. 2021a), regardless of the stage of exposure. These effects should be alleviated in individuals transferred to freshwater, because of the resilience capacities of amphibians to salt exposure (Squires et al. 2010; Wu et al. 2012; Hsu et al. 2018; Lorrain-Soligon et al. 2022a).

Table IX1 – Number of surviving individuals in each treatment at each life stage

Embryonic treatment	Number of individuals in the treatment	Surviving individuals at GS25	Larval treatment	Number of individuals in the treatment	Surviving individuals at GS25	Surviving individuals at GS30
FF	66	66	FFF	36	36	30
			FFS	30	30	15
FS	102	70	FSF	53	37	14
			FSS	49	33	2
SF	66	65	SFF	33	33	22
			SFS	33	32	18
SS	83	31	SSF	46	20	7
			SSS	37	11	2

2. Methods

2.1. Study species

Spined toad (*Bufo spinosus*) is a common amphibian species found in western Europe (Speybroeck et al. 2018; Meek 2022). Breeding occurs in late winter, during which adults converge to reproductive ponds where they mate (amplexus) and lay their eggs constituted of 2 eggs strings which can be constituted of up to 10000 eggs (Miaud and Muratet 2018). Egg laying, embryonic and larval developments all occur in ponds.

2.2. Captures and measurements

Already composed amplexant pairs were captured on one coastal pond situated in the Réserve Naturelle Nationale du Marais d'Yves (location: 46.04013, -1.05363). Captures occurred at night (between 20 pm and 4 am) on 21/02/2023 and 22/02/2023. 21 amplexant pairs were captured by hand, placed in a transport box (14*16*9cm), and brought to the laboratory immediately after field sessions. At the laboratory, individuals were kept in a thermally controlled room with temperature set at 17 °C and controlled photoperiod (12 h dark–12 h light). Pairs were transitorily separated and all individuals were measured (snout-vent length, SVL) using a caliper (± 1 mm) and weighed using an electronic balance (± 0.1 g). Once measurements were completed, separated amplexant individuals were shortly reunited and pairs re-formed systematically as soon as the partners were brought back into contact.

2.3. Experimental procedures

Amplexant pairs, embryos and larvae were exposed to either freshwater or

brackish water, following a full factorial design (Figure IX1).

At the end of the experiment, individuals were thus exposed to one of the eight following treatments:

- FFF ; clutch obtained in freshwater, embryonic development in freshwater, larval development in freshwater
- FSF ; clutch obtained in freshwater, embryonic development in brackish water, larval development in freshwater
- FFS ; clutch obtained in freshwater, embryonic development in freshwater, larval development in brackish water
- FSS ; clutch obtained in freshwater, embryonic development in brackish water, larval development in brackish water
- SFF ; clutch obtained in brackish water, embryonic development in freshwater, larval development in freshwater
- SSF ; clutch obtained in brackish water, embryonic development in brackish water, larval development in freshwater
- SFS ; clutch obtained in brackish water, embryonic development in freshwater, larval development in brackish water
- SSS ; clutch obtained in brackish water, embryonic development in brackish water, larval development in brackish water

During the whole experimental procedure, water was changed once a week, and tadpoles were fed ad libitum with organic spinach. Individuals were checked twice a

day for their specific developmental stage and for mortality.

2.3.1. Egg laying (n=21)

Once the amplexant pairs were reunited, they were placed in a plastic container (35*55*26 cm) containing either freshwater (~20l, salinity: 0.3 g.l⁻¹, 10 amplexant pairs) or brackish water (~20l, salinity: 4.0 g.l⁻¹, 11 amplexant pairs) as well as branches for laying support. The two groups did not differ in size (freshwater: females= 74.7 mm ± 1.61 SE, males= 64.1 mm ± 1.00 SE; brackish water: females= 75.9 mm ± 2.08 SE, males= 62.7 mm ± 1.22 SE; differences between females in the two groups: Estimate=1.209, SE=2.604, t_{1,19}=0.464, p-value=0.648; differences between males in the two groups: Estimate=1.373, SE=1.601, t_{1,19}=0.857, p-value=0.402). Amplexant pairs were left in these tanks until egg laying. Adult individuals were then released at their site of capture.

12 pieces of each egg strings, containing 30 eggs each (i.e. a total of 360 eggs for each egg string) were selected randomly and kept for our experiment, and the remaining eggs (i.e. 100- 4500) were released at the site of origin of their parents.

2.3.2. Embryonic development (n=232)

The 2 treatments were applied in 2 L individual tanks (13*18*18cm, n=232, table IX1) containing one of the two treatment (freshwater: 0 g.l⁻¹ salinity: 0.3 ± 0.0 g.l⁻¹; brackish water : 4 g.l⁻¹ salinity 4.0 ± 0.06 g.l⁻¹). Water was changed once a week. Each clutch was represented by 6 segments of 30 eggs in each of the experimental treatments. To prevent osmotic shock, segments for which the clutch was obtained in another salinity than which their embryonic development occur (i.e. clutch

that were obtained at 0 g.l⁻¹ but for which embryonic development will occur at 4 g.l⁻¹ and clutch that were obtained at 4 g.l⁻¹ but for which embryonic development will occur at 0 g.l⁻¹) were gradually exposed to the salinity of the treatment, with an increase or a decrease of 1 g.l⁻¹ a day (Hsu et al. 2018). This way, segments were exposed to their final treatment over 4 days. Each segment were kept in the laboratory until tadpoles hatched and reached a free feeding stage (Gosner stage 25, hereafter GS25) (Gomez-Mestre and Tejedo 2003). Embryos were checked twice a day.

2.3.3. Larval development (n=232)

Upon hatching (GS25), we selected 1 individual per segment, and these individuals were once again distributed in two treatments: freshwater (0 g.l⁻¹ salinity: 0.3 ± 0.0 g.l⁻¹), and brackish water (4 g.l⁻¹ salinity 4.0 ± 0.06 g.l⁻¹). For each clutch, three segments and thus three tadpoles were thus exposed to each treatment (FFF, FFS, FSF, FSS, SFF, SFS, SSF, SSS). In some segments, no individuals survived until GS25. In these cases, individuals were replaced by other individuals from the same treatments but originating from different clutches in order to keep a total of 232 tadpoles that were followed during larval development (see table IX1 for the count of tadpoles in each treatment). All other tadpoles were released at the site where their parents were captured.

Similarly to embryos, when salinity changed (i.e. tadpoles for which embryonic development occur at 0 g.l⁻¹ but larval development will occur at 4 g.l⁻¹ and tadpoles for which embryonic development occur at 4 g.l⁻¹ but larval development will occur at 0 g.l⁻¹), tadpoles

were gradually exposed to the salinity of the treatment, with an increase or a decrease of 1 g.l⁻¹ a day to prevent osmotic shock (Hsu et al. 2018). Larval development was monitored until G30 (Gosner 1960), a pivotal stage when somatic growth decreases and significant morphological changes occur according to (Cheron et al. 2021). At the end of this experimental procedure, individuals were released at the site where their parents were captured.

2.4. Measurements

2.4.1. Reproductive effort (n=21)

Once egg laying was completed, individuals were again weighed to calculate variations in body mass. Additionally, each clutch was weighed using an electronic balance ($\pm 0.1\text{g}$).

In order to assess fecundity, each egg string was placed in a container (35 × 20 × 25 cm) containing 2 cm of dechlorinated tap water and a scale (graph paper). A picture was taken from above in order to measure the total length of the egg string using ImageJ software (Schneider et al. 2012). For each clutch, we randomly selected 6 segments of 10 cm long and individually counted the number of eggs within each segment. The mean number of eggs per 10-cm segment was calculated and used to assess fecundity (number of eggs) for each clutch based on the length of the egg strings. We also calculated egg density as the number of eggs divided by the length of the egg strings. Finally, on a subsample of 100 randomly selected eggs in each egg strings, we measured egg diameter.

2.4.2. Embryonic development (n=232)

At GS 25, hatching success (proportion of eggs that hatched, on the segment of 30 eggs) and deformation rates (proportion of larvae that hatched but were malformed,

on the total number of hatched individuals) were computed. Additionally, for each segments, we described general activity of all hatchlings as being, on a scale of 1 to 5, the proportion of live individuals that were active (1=0-20%, 2=20-40%, 3=40-60%, 4=60-80%, 5=80-100%).

2.4.3. Larval development (n=232)

At GS25 and GS30, we measured total length, body length, tail length, head width, tail height, tail muscle height, body height. Each tadpole was put into a Petri dish with the water from its own tank, and photographed.

Morphological measurements were performed with the software ImageJ (Schneider et al. 2012).

Additionally, at GS25 and GS30, three behavioural parameters were tested, during a 2 minutes-long period:

- occurrence of abnormal rotations, which are a sign of neurotoxic stress (see Denoël et al. (2012) and references therein)
- position within the water column (surface or bottom of their aquarium)
- activity following a scale ranging from 1 to 5 (from 1 if the individual is amorphous, with little or no response to stimuli, to 5 if the individual move energetically).

2.5. Statistical analyses

2.5.1. Reproductive effort

We evaluated the effect of salinity (clutches laid in freshwater [0 g.l⁻¹] or in brackish water [4 g.l⁻¹]) on clutch length, clutch size (number of eggs), clutch mass, egg density (clutch size divided by clutch length), egg diameter, laying time, variation of body mass (for females and males) by setting linear models (LMs), with salinity as an

explanatory variable. We also tested for the relationships between egg diameter and clutch size in each salinity by setting a LM with egg diameter as a dependent variable, and clutch size, salinity and their interaction as explanatory variables. Finally, we tested for the effect of the interaction between female SVL and salinity, and between variation of body mass of females during egg laying and salinity on clutch size and clutch mass, using LMs. For these models, best variables were retained using a top-down selection, and only the retained variables are presented.

2.5.2. Embryonic development

We evaluated the effect of salinity of spawning (clutch laid in freshwater [0 g.l⁻¹] or in brackish water [4 g.l⁻¹]), and of embryonic development (individuals developing in freshwater [0 g.l⁻¹] or brackish water [4 g.l⁻¹]).

As morphological variables were all highly correlated, we performed PCA on all the morphological metrics taken at each life stages, and extracted the coordinates on the first axis (explaining most part of the variation), hereafter termed as morphology index, to allow analyses of the variation of all variables together. At GS25 and GS30, PCA were realized on total length, tail length, body width, tail muscle height, tail height and body height. An increase of the index indicates an increase of all these parameters. We computed these effects on time to hatching and morphology index at GS25 with Linear Mixed models (LMMs), and on hatching success, malformation rate, general activity, tadpole activity (both rated on a scale from 1 to 5), abnormal rotations, and breathing at surface using binomial Generalized Linear Mixed

Models (GLMMS). For all these models, we have set salinity of spawning, salinity of embryonic development and their interaction as explanatory variable, and the clutch as a random effect (as multiple individuals originated from a single clutch). Best variables were retained using a top-down selection, and only the retained variables are presented.

2.5.3. Larval development

We evaluated the effect of salinity of spawning (clutches laid in freshwater [0 g.l⁻¹] or in brackish water [4 g.l⁻¹]), salinity of embryonic development (individuals developing in freshwater [0 g.l⁻¹] or brackish water [4 g.l⁻¹]), and salinity of larval development (individuals developing in freshwater [0 g.l⁻¹] or brackish water [4 g.l⁻¹]).

We computed these effects on duration between S25 and S30, growth rate between GS25 and GS30, and morphological index at GS30 with LMMs, and on mortality, abnormal rotations, surface breathing and activity (rated on a scale from 1 to 5) using GLMMS. For all these models, we have set salinity of spawning, salinity of embryonic development, salinity of larval development and their interaction as explanatory variable, and the clutch as a random effect (as multiple individuals originated from a single clutch). Best variables were retained using a top-down selection, and only the retained variables are presented. At GS30, only 2 individuals were malformed (in FFS and FSF) and thus statistics were not computed.

All data analysis were performed using R 3.6.3 (R Core Team 2020) and Rstudio v1.1.419.

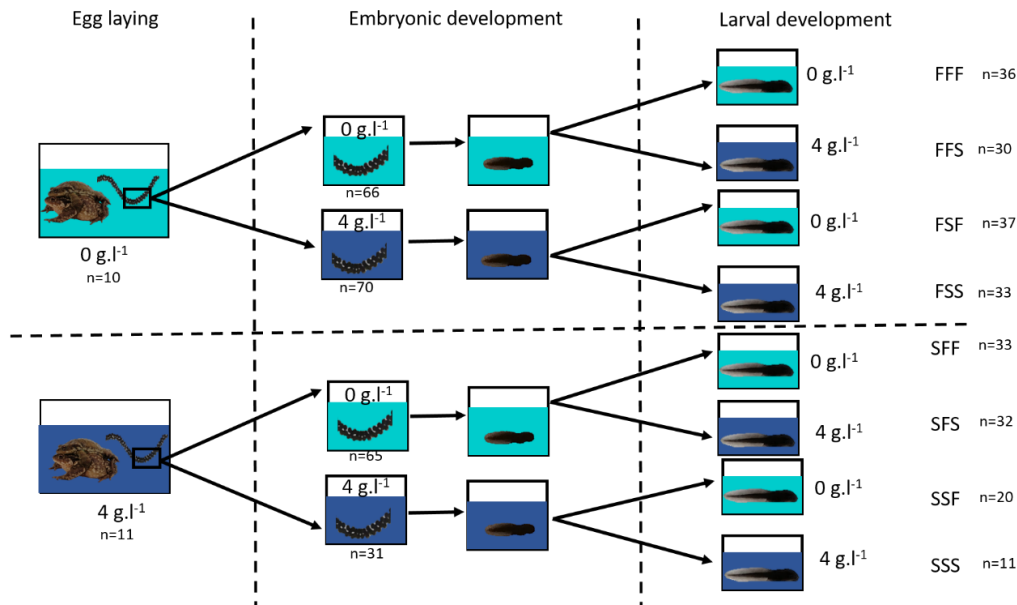


Figure IX1 – Experimental design used to assess the effects of salinity during different stages of development in *Bufo spinosus*. Colours represent salinity treatments (light blue = fresh water [0 g.l⁻¹], dark blue = brackish water [4 g.l⁻¹]).

3. Results

3.1. Reproductive effort

Clutches produced in freshwater or in brackish water were similar for length (Estimate=70.05, SE= 263.58, $t_{1.19}=0.27$, p-value=0.793), number of eggs (Estimate=182.60, SE= 405.40, $t_{1.19}=0.45$, p-value=0.658), and thus egg density (Estimate=0.14, SE= 0.16, $t_{1.19}=0.87$, p-value=0.395). However, clutches produced in freshwater were heavier (Estimate=28.21, SE= 13.07, $t_{1.19}=2.16$, p-value=0.044), and their eggs were slightly (but not significantly) larger (Estimate=0.11, SE= 0.06, $t_{1.19}=1.80$, p-value=0.088, Figure IX2A). Laying time was shorter for individuals that laid eggs in brackish water compared to those that laid eggs in freshwater (Estimate=-31.77, SE= 14.84, $t_{1.19}=-2.14$, p-value=0.046, Figure IX2B).

Egg diameter was related to the interaction between clutch size and salinity (Estimate<0.001, SE<0.001, $t_{3.17}=2.65$, p-value=0.017, Figure IX2C). Egg diameter was not influenced by clutch size in clutches

laid in freshwater (Estimate<0.001, SE<0.001, $t_{1.8}=1.21$, p-value=0.260, Figure IX2C), but increased with increasing clutch size in clutches laid in brackish water (Estimate<0.001, SE<0.001, $t_{1.9}=2.40$, p-value=0.040, Figure IX2C).

Interestingly, females spawning at 0 g.l⁻¹ (-13.56 % ± 2.23 SE) and 4 g.l⁻¹ (-12.21 % ± 1.36 SE) have lost similar amount of mass during egg laying (Estimate=1.35, SE=2.56, $t_{1.19}=0.53$, p-value=0.604). Males reproducing at 0 g.l⁻¹ have lost mass (-1.63 % ± 2.19 SE) while males reproducing at 4 g.l⁻¹ have gained mass (3.79% ± 1.27 SE) during egg laying (Estimate=5.41, SE=2.47, $t_{1.19}=2.19$, p-value=0.041).

Clutch size was positively related to female SVL (Estimate=85.63, SE=29.81, $t_{1.19}=2.87$, p-value=0.010) and female loss of mass during egg laying (Estimate=141.51, SE=35.46, $t_{1.19}=3.99$, p-value=0.008). Clutch mass was positively related to female body mass (Estimate=5.29, SE=1.22, $t_{1.19}=4.33$, p-value<0.001) and to clutch size (Estimate=0.04, SE=0.01, $t_{1.19}=4.38$, p-value<0.001).

3.2. Embryonic development

Hatching success was higher for clutch produced in freshwater (Estimate=0.480, SE=0.194, $z=2.649$, $p\text{-value}=0.014$), and higher in embryos that developed in freshwater (Estimate=0.972, SE=0.064, $z=15.103$, $p\text{-value}<0.001$). The interaction between the salinity of spawning and embryonic development was significant (Estimate=-1.493, SE=0.141, $z=-10.580$, $p\text{-value}<0.001$, Figure IX3A).

Malformation rate was higher in embryos which developed in brackish water (Estimate=0.893, SE=0.114, $z=7.840$, $p\text{-value}<0.001$), but not related to salinity during spawning (Estimate=0.086, SE=0.242, $z=0.357$, $p\text{-value}=0.721$). The interaction between the salinity of spawning and embryonic development was significant (Estimate=-1.059, SE=0.301, $z=-3.524$, $p\text{-value}<0.001$, Figure IX3B). Time to hatching was higher in embryos which developed in brackish water (Estimate=2.013, SE=0.458, $t_{1,221}=4.399$, $p\text{-value}<0.001$), but not related to salinity during spawning (Estimate=0.097, SE=0.631, $t_{1,27}=0.153$, $p\text{-value}=0.880$). The interaction between the salinity of spawning and embryonic development was significant (longer time to hatching was only found in individuals that were laid in freshwater but developed in brackish water, Estimate=-2.429, SE=0.734, $t_{1,218}=-3.309$, $p\text{-value}=0.001$).

General activity (Estimate=1.164, SE=0.173, $z=6.735$, $p\text{-value}<0.001$), tadpoles' activity (Estimate=1.141, SE=0.117, $z=9.706$, $p\text{-value}<0.001$) and morphology index (Estimate=2.916, SE=0.227, $t_{1,228}=12.826$, $p\text{-value}<0.001$, Figure IX3C) were all influenced by salinity experienced during embryonic development solely, and were

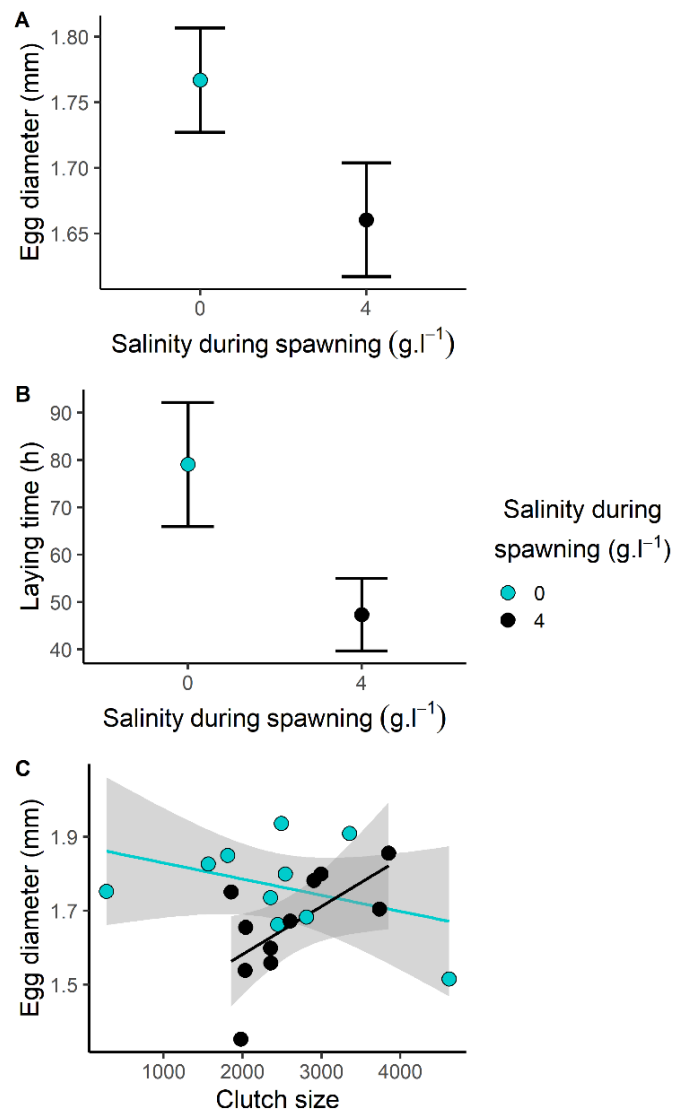


Figure IX2 - Effects of salinity during spawning on reproductive characteristics. (A) Egg diameter, (B) clutch size (number of eggs), (C) relationships between clutch size and egg diameter, for clutches produced in freshwater (0 g.l⁻¹) or brackish water (4 g.l⁻¹). Data are presented as mean ± SE.

higher for embryos that developed in freshwater. Abnormal rotations were influenced neither by salinity during spawning, salinity during embryonic development, or their interaction (all $p\text{-values}>0.322$). Breathing at surface varied with salinity during embryonic development (being higher for individuals that develop in freshwater, Estimate=0.925, SE=0.054, $z=17.254$, $p\text{-value}<0.001$), the interaction between salinity during spawning and salinity during embryonic development (Estimate=0.231, SE=0.087,

$z=2.666$, $p\text{-value}=0.008$, Figure IX3D), but not with salinity during spawning alone (Estimate=0.150, SE=0.081, $z=1.848$, $p\text{-value}=0.080$).

3.3. Larval development

Mortality at GS30 was influenced by salinity to which individuals were exposed during embryonic development (higher for individuals which embryonic development occurred in brackish water, Estimate=3.405, SE=0.005, $z=676.3$, $p\text{-value}<0.001$, Figure IX4A) and during larval development (higher for larvae developing in brackish water, Estimate=1.421, SE=0.005, $z=282.5$, $p\text{-value}<0.001$, Figure IX4A), but not their interaction.

Duration between S25 and S30 varied with salinity of embryonic development (longer for individuals which embryonic development occurred in brackish water, Estimate=1.409, SE=0.635, $t_{1.101}=2.218$, $p\text{-value}=0.029$, Figure IX4B), and the interaction between salinity of embryonic development and of larval development (Estimate=3.307, SE=1.095, $t_{1.91}=3.020$, $p\text{-value}=0.003$, Figure IX4B), as well as the interaction between salinity of spawning and of larval development (Estimate=-2.914, SE=0.768, $t_{1.94}=-3.793$, $p\text{-value}<0.001$, Figure IX4B).

Growth rate between GS25 and GS30 was only influenced by salinity of embryonic development, and was higher for

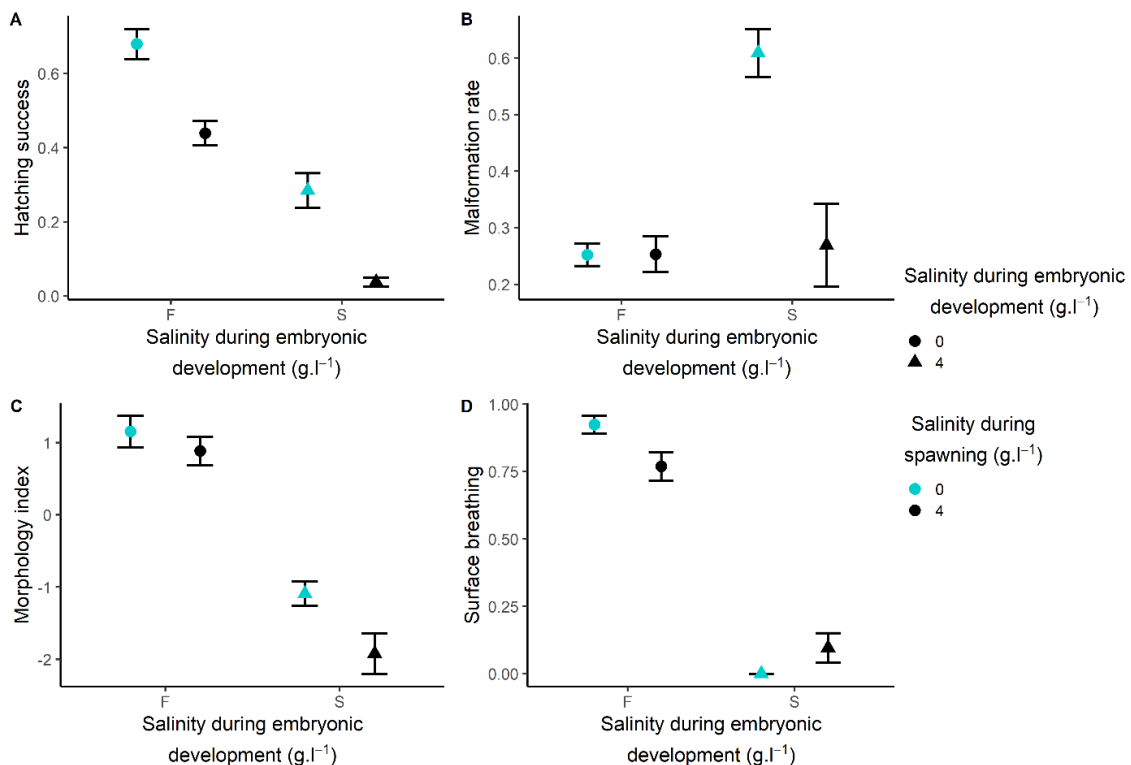


Figure IX3 - Effects of salinity during spawning (clutches laid in freshwater [0 g.l⁻¹] or brackish water [4 g.l⁻¹]) and embryonic development (individuals developing in freshwater [0 g.l⁻¹] or brackish water [4 g.l⁻¹]) on different aspects of embryonic development assessed upon hatching (Gosner stage 25). (A) Hatching success, (B) malformation rate, (C) Morphology index (extracted from the PCA performed on the different morphologic measurement), and (D) surface breathing. Data are presented as mean ± SE.

individuals which embryonic development occurred in brackish water (Estimate=0.081, SE=0.036, $t_{1.110}=2.240$, p-value=0.027, Figure IX4C).

Morphological index at GS30 was influenced by salinity to which individuals were exposed during embryonic development (higher for individuals which embryonic development occurred in freshwater, Estimate=1.976, SE=0.827, $t_{1.109}=2.389$, p-value=0.019, Figure IX4D)

and larval development (higher for larvae developing in freshwater, Estimate=1.476, SE=0.612, $t_{1.94}=2.413$, p-value=0.018, Figure IX4D), but not their interaction.

Abnormal rotation marginally increased (Estimate=1.583, SE=0.860, $z=1.840$, p-value=0.066, Figure IX4E), but surface breathing (Estimate=-0.618, SE=0.333, $z=-1.855$, p-value=0.064, Figure IX4F) and activity (Estimate=-0.350, SE=0.147, $z=-2.387$, p-value=0.017) decreased for larvae that developed in brackish water.

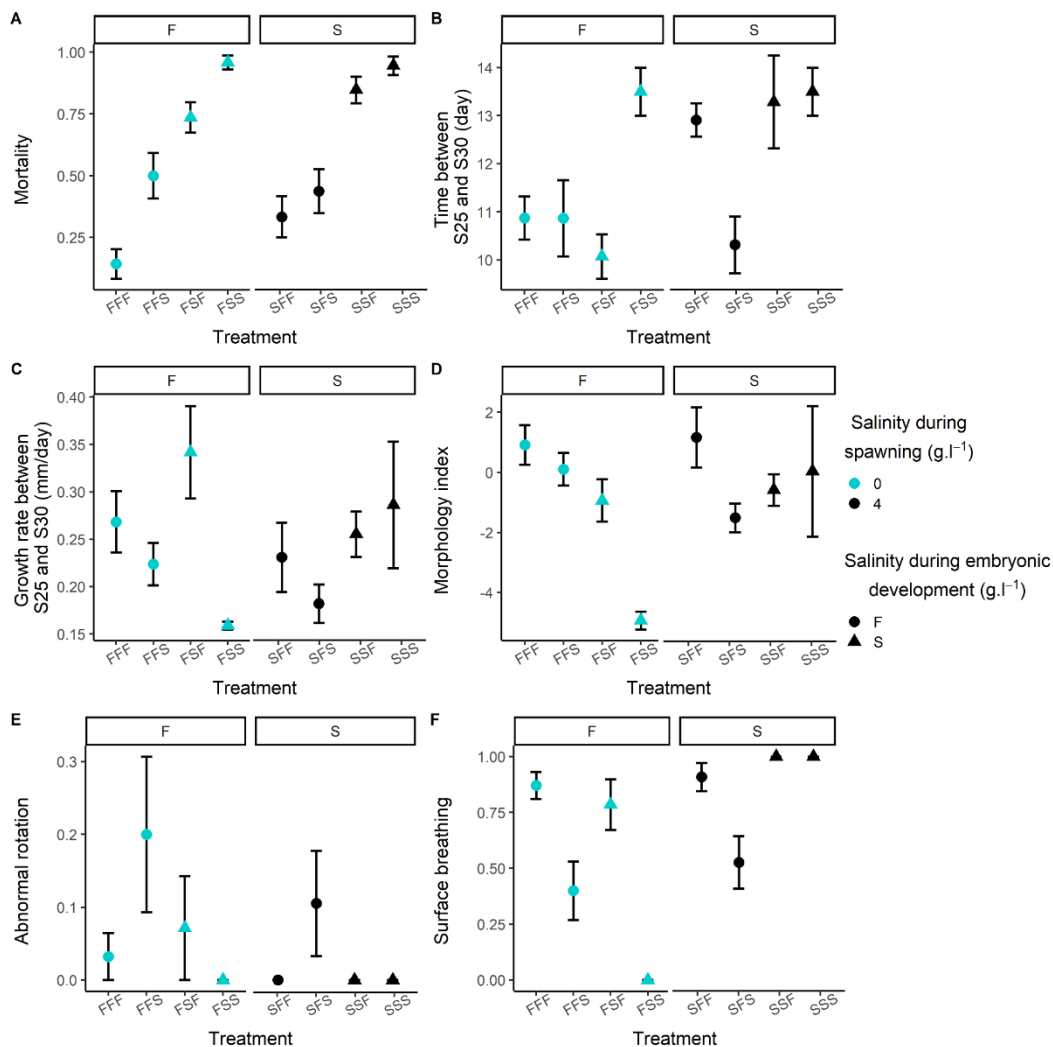


Figure IX4 – Effects of salinity during spawning (clutches laid in freshwater [0 g.l⁻¹] or brackish water [4 g.l⁻¹]), embryonic development (freshwater [0 g.l⁻¹] or brackish water [4 g.l⁻¹]) and larval development (freshwater [0 g.l⁻¹] or brackish water [4 g.l⁻¹]) on different characteristics of larval development measured at Gosner stage 30. (A) Mortality rate, (B) duration between GS25 and GS30, (C) growth between GS25 and GS30, (D) Morphology index (extracted from the PCA performed on the different morphologic measurement), and (E) abnormal rotations and (F) surface breathing. Data are presented as mean ± SE. Treatments are illustrated in Figure IX1.

4. Discussion

In this study, we demonstrated that environmental salinity can negatively affect reproduction, as well as embryonic and larval development in a coastal anuran. Importantly, our full-factorial design allowed to highlight that early exposure to brackish water did not induce increased tolerance during later exposure (i.e., tadpoles spawned in brackish water did not perform better in brackish water). However, individuals returned to freshwater after being exposed to brackish water expressed partial recovery, indicating resilience to salinity stress.

4.1. Reproduction

Clutch size did not differ between freshwater and brackish water treatment, and clutch size increases with female size and female mass loss for both treatments, with no difference between the two treatments, which indicate no effects of the treatments on these relationships. Such results further suggest that clutch size might depend on energetic investment that occurred prior to egg laying (Article VII). Interestingly, we found that salinity influenced some reproductive parameters in adults, suggesting a rapid modulation of reproductive effort based on the salinity to which amplexus were exposed. Indeed, amplexus exposed to brackish water produced their eggs more rapidly than those kept in fresh water. Two opposite hypotheses can explain such result. First, females may lay their eggs more rapidly in brackish water in order to evade an osmotically constraining environment. Conversely, because adult individuals originated from salt-exposed coastal environment, they may consider brackish water as a favourable environment if they

are locally adapted to such conditions; but delay laying in freshwater because they consider this environment as suboptimal. This later hypothesis seems relatively unlikely as our first hypothesis is further supported by the fact that, in brackish water, eggs were smaller but not fewer. This result suggests that females produced eggs of comparatively lower quality in brackish water, as larger eggs are known to improve hatching success and offspring fitness (Xu et al. 2019; Renoirt et al. 2022). The number of undeveloped eggs (presumably unfertilized given the negative effect of salinity on sperm, (Wilder and Welch 2014; Green et al. 2021)) was also higher in brackish water.

Interestingly, despite the strong effect of salinity on egg size, females from both treatments have lost a similar amount of mass. In combination with smaller eggs, this result could be linked to a relatively larger investment in egg jellies (e.g., that protect the egg strings) in females exposed to brackish water, a hypothesis that is not supported by clutch mass which was higher for females exposed to freshwater. More likely, the fact that females have lost a similar amount of mass but laid smaller eggs might be linked to direct costs of osmoregulation in adult individuals exposed to brackish water, which needs to be further investigated.

The fact that clutch mass was higher in the freshwater treatment is likely linked to water absorption due to osmotic exchanges with the environment (Venturino et al. 2003; Karraker and Gibbs 2011; López-Alcaide and Macip-Ríos 2011), or due to higher investment in glycoproteins surrounding the eggs (egg jellies which are critical to egg development, Bonnell &

Chandler, 1996; Yurewicz et al., 1975). More importantly, we found a positive correlation between egg size and clutch size for clutches laid in brackish water but not for clutches laid in freshwater, while fecundity was expected to negatively trade off with egg size (Lasne et al., 2018; Smith & Fretwell, 1974), because species with large clutch sizes and small eggs (typical of Bufonid toads, Wells, 2007) should add additional offspring and not change offspring size should extra energy become available (Ricklefs 1968; Nussbaum 1981). In our context, we found that increased clutch size is concomitant with increased egg size in clutches laid in brackish water suggesting that complex interactions between prior energetic investment in reproduction and current spawning conditions govern reproductive effort.

Lastly, reproducing males have lost mass in freshwater, but gained mass in brackish water. Such result could be linked to ejaculate volume, with males in freshwater producing more sperm than their counterparts exposed to brackish water. This hypothesis seems is corroborated by the reduced proportion of fertilized eggs in the brackish treatment. Yet, we cannot rule-out the fact that this result may also be linked to reduced sperm survival and/or swimming abilities in brackish water (Wilder and Welch 2014; Byrne et al. 2015, 2022).

4.2. Embryonic development

We show that embryonic development duration increased for individuals that were exposed to 4 g.l⁻¹ salinity during embryonic development, but only when they were spawned in freshwater. Increased development time in response to salinity have already been described in

amphibians (see (Christy and Dickman 2002; Sanzo and Hecnar 2006; Wu and Kam 2009; Wijethunga et al. 2016; Lukens and Wilcoxon 2020)), an effect that has been attributed to a response to evade development in an osmotically stressful environment (Gomez-Mestre et al. 2004). At hatching, individuals in the 4 g.l⁻¹ treatment were smaller (irrespective of the salinity in which they were spawned), which have already been described (Wu and Kam 2009; Wood and Welch 2015; Haramura 2016; Lukens and Wilcoxon 2020; Tornabene et al. 2021a) and which is probably linked to higher costs of osmoregulation (Gomez-Mestre et al. 2004). Individuals in the 4 g.l⁻¹ treatment also presented a higher malformation rate (Gosner and Black 1957), and lower activity as well as lower expression of breathing at the water surface. Interestingly, hatching success was always lower for individuals originating from a clutch that was spawned in brackish water irrespective of the salinity in which the remaining embryonic development occurred, indicating long lasting effects of the salinity in which the clutch was produced (and thus long-lasting effect of very early exposure to salinity during embryonic development).

4.3. Larval development

Overall, development duration was longer for individuals that were spawned in brackish water, indicating long lasting and carry-over effect of the salinity in which adults lay eggs, and accumulating effects as in (Tornabene et al. 2021a). Individuals that developed in brackish water during embryonic and larval development (FSS and SSS) were smaller (Squires et al. 2010), and individuals that experimented salinity during either embryonic or larval development (FSF, FFS, SSF and SFS) or

during both stages (FSS and SSS) expressed higher mortality (Squires et al. 2010; Wu et al. 2012; Albecker and McCoy 2017; Hsu et al. 2018). Interestingly, individuals that were exposed to brackish water during larval development (FFS or SFS) had similar size than those exposed to freshwater (FFF or SFF), while those exposed only during embryonic development (FSF or SSF) were smaller. These responses remain consistent regardless of the salinity in which clutches were laid. This further suggest that salinity experienced during embryonic development is more detrimental than salinity experienced during larval development, which is in line with the idea that early developmental stages (embryos) are more sensitive to salinity than later developmental stages (larvae) (Albecker and McCoy 2017). This is likely due to the development of internal gills (the main organs responsible for ion and water balance in tadpoles, (Uchiyama and Yoshizawa 1992) which improve the osmoregulation ability at this developmental stage.

The detrimental effects of salinity on growth could be linked to changes in activity. Indeed, larvae exposed to higher salinity express reduced activity and foraging behaviors (Hall et al. 2017; Tornabene et al. 2021a), lower responses to stimuli (Sanzo and Hecnar 2006; Karraker 2007), lower swimming performance (Haramura 2016), erratic movements (Tornabene et al. 2021a), and reduced speed and distance (Denoël et al. 2010). Interestingly, in our study we show higher frequency of abnormal rotation in individuals that were exposed to brackish water during larval development solely (FSF or SSF), which is likely to be linked to

the change of salinity between embryonic and larval development. These individuals also tend to swim less at the surface to breathe.

Interestingly, at GS30, the few surviving individuals that were exposed to brackish water during the entire development (SSS) tend to perform better. Because this treatment induced the highest mortality, this result suggests a selective process toward the few individuals displaying a suite of traits (yet to be deciphered) that allowed their survival in such harsh environment. It is noteworthy that the detrimental effects of salinity during larval conditions can also carry-over during later life stages (Dahrouge and Rittenhouse 2022) and the long-term costs of development in brackish water remain to be investigated.

Importantly, we highlighted higher survival, larger size, and more optimal behaviour in individuals exposed to freshwater during larval development after being exposed to brackish water during their embryonic development (FSF and SSF), compared to individuals that were exposed to brackish water during their whole development (FSS and SSS) (Wu, Gomez-Mestre, and Kam, 2012). However, these individuals still expressed a smaller size compared to individuals that were exposed to freshwater during most of their development (FFF and SFF). Taken together, these results suggest partial compensatory responses, rather than full compensation or over-compensation (Ali et al. 2003). Indeed, individuals can increase their food intake in response to a period of depressed growth (Xie et al. 2001), and future studies should aim at investigating differences in foraging activity, efficiency,

and/or food conversion efficiency in tadpoles.

Compensatory growth may act as an adaptive mechanism because it increases the chance of an organism attaining a critical size at a given developmental stage and at maturity, which can ultimately influence fitness (Dmitriew 2011). It is a likely mechanism in response to osmotic stress in coastal organisms (Hsu et al. 2018), allowing to overcome the negative effects of salinity by expressing catch-up growth when the salt stress ceases. Importantly, such process has been shown to occur in coastal amphibian species such as *Fejervarya limnocharis*, *Fejervarya cancrivora* and *Litoria ewingii* (Squires et al. 2010; Wu et al. 2012; Hsu et al. 2018). However, recent research suggests that, although compensatory growth can produce rapid benefits, it is also associated with delayed costs during later stages of development and/or adult life. Such costs include increased risk of predation, decreased resistance to starvation, decreased protein maintenance and increased muscle lesions, reduction in locomotor performance, reduced age at sexual maturation and higher telomere attrition rate (Metcalf and Monaghan 2001). Indeed, accelerated growth is known to induce accumulated cellular damages which ultimately may impair fitness later in life (Mangel and Munch 2005). In organisms with complex life cycles, the consequences of altered growth and developmental trajectories can persist after metamorphosis (Pechenik 2006), which effects need to be further investigated.

5. Conclusion

In this study, we highlighted strong effects of environmental salinity in reproduction (egg laying time, egg size, and investment in reproduction) in a coastal amphibian. Embryonic and larval responses to salinity were highly dependent on the developmental stages at which exposure occurred. We also highlighted carry-over effects of the salinity in which individuals were spawned, and of the salinity experienced during embryonic development, highlighting the importance of the environmental conditions during early development on ontogenetic trajectories. Importantly, we also highlighted partial compensation when individuals were transferred to – presumably less stressful – freshwater, which can be a useful response in coastal environments in which salinity is known to fluctuate spatially and temporally. Whether the magnitude of these responses can allow coastal biodiversity to overcome the salinization linked to global changes remain to be assessed.

Discussion Générale



I) Résultats de la thèse

Les zones humides côtières sont caractérisées par des variations de salinité qui s'appliquent spatialement [salinité plus élevée près de la côte (Chapitre I)], et au cours de la saison, car la salinité est plus élevée en été, lorsque les températures augmentent et les précipitations diminuent (Chapitre I). Cet effet de la distance à la côte sur la salinité est aussi renforcé avec le temps, la pente de cette relation s'accroissant au fil des années (Chapitre I). Ces changements sont liés aux variations de température (la salinité augmente quand la température augmente) et de pluviométrie (la salinité augmente quand la pluviométrie diminue ; Chapitre I). Mais au-delà de ces variations graduelles, la salinité peut aussi augmenter de manière brutale sur les milieux côtiers, notamment lorsqu'ils sont frappés par des submersions marines, comme cela a été le cas lors de la tempête Xynthia (Chapitre I).

Les amphibiens sont fortement impactés par ces changements. Au niveau des communautés, la richesse spécifique en amphibien peut diminuer massivement à la suite d'une tempête marine, ce phénomène impactant toutes les espèces (Chapitre I). Mais si certaines espèces peuvent récupérer leur abondance initiale après la tempête, ce n'est pas le cas de toutes, montrant ainsi une différence de sensibilité et de résilience (Chapitre I). Cette différence de sensibilité à l'augmentation de la salinité s'applique de même à l'échelle des sites de reproduction, les espèces se répartissant sur les mares en fonction de leur tolérance au sel (Chapitre I). La richesse spécifique demeure cependant maximale pour des salinités intermédiaires, aux alentours de 9 g.l⁻¹ (Chapitre I). Cet effet de la salinité sur la distribution des espèces s'observe également sur les individus au sein des populations, la distance à l'océan, et avec elle le gradient associé de salinité (Chapitre I), se traduisant, à une échelle plus large (entre les populations côtières et continentales), par des modifications de la morphologie ainsi que de l'investissement dans la reproduction (Chapitre III).

Ces effets s'observent par ailleurs au niveau individuel, où les individus adultes exposés au sel, en condition naturelle ou expérimentalement, expriment un comportement altéré, mais aussi des impacts au niveau cellulaire et immunitaires (Chapitre II). De manière chronique, même pour des salinités faibles (4 g.l⁻¹, salinité inférieure à ce qui est retrouvé sur les milieux côtiers), les adultes expriment une réduction de leur croissance, de leur préférence thermique, et des impacts sur leur comportement ainsi que leurs performances de capture de proies (Chapitre II), et les têtards et embryons expriment une mortalité et des malformations accrues, ainsi que des effets importants sur la croissance, le développement, et le

comportement (Chapitre III), qui se retrouvent déjà au moment de la ponte, car les femelles investissent moins dans la reproduction lorsque le milieu de ponte est salé (Chapitre III). De manière intrigante, alors que la diversité en amphibiens est maximale pour des salinités intermédiaires (Chapitre I), les larves et adultes subissent déjà des effets du sel à des niveaux très faibles (Chapitres II et III). Les larves côtières semblent également exprimer une maladaptation locale (Chapitre III), tandis que les adultes semblent exprimer une tolérance plus élevée à la salinité lorsqu'ils viennent de milieux plus salés (Chapitre II). Cependant à la fois les communautés (Chapitre I), les adultes (Chapitre II), et dans une certaine mesure les larves (Chapitre II) sont capables de résilience. Cette résilience est encore favorisée chez les adultes qui sont capables de se diriger vers des milieux présentant une salinité optimale (Chapitre II).

Si nos travaux ont permis une meilleure vision des variations de la salinité sur les milieux côtiers mais également de l'impact de la salinité sur les amphibiens dans son ensemble, de nombreuses questions ont également été soulevées, et nos travaux ont permis de faire des liens entre différentes composantes.

II) Salinisation

II.1. Variations spatiales et temporelles de la salinité sur les mares côtières, changements globaux, et paramètres à considérer

Les zones humides côtières sont des zones de transition critiques qui relient les habitats terrestres, d'eau douce et marins (Hobohm et al. 2021; Venâncio et al. 2022). Si ces habitats sont essentiels, et si la présence d'eau douce est une caractéristique primordiale de ces habitats (McLean et al. 2001; Hobohm et al. 2021; Venâncio et al. 2022), nous avons montré que la salinité sur ces milieux augmentait au cours de la saison, mais également en fonction de la distance à l'océan. De manière importante, ces effets sont également renforcés dans le temps et notamment du fait de l'augmentation de la température et de la diminution des précipitations, mais aussi par les tempêtes marines, et le niveau des marées (Chapitre I).

Si nous avons étudié les effets de la salinisation sur les eaux de surface, et plus particulièrement sur les mares, qui étaient jusque-là négligées dans les études sur les effets de la salinisation (Cunillera-Montcusí et al. 2022), d'autres composantes des milieux côtiers

restent peu étudiées, et c'est le cas à la fois des eaux souterraines (Castaño-Sánchez et al. 2020) mais aussi des sols (Hassani et al. 2021). Ces informations sont importantes car beaucoup d'organismes sont strictement dépendants de ces interfaces, comme c'est le cas des arthropodes (Renault et al. 2016) et des plantes (Ayub et al. 2020). Une accumulation excessive de sels solubles dans la zone racinaire des plantes peut affecter négativement leur taux de croissance (Ayub et al. 2020), leurs capacités d'évapotranspiration (Greenway and Munns 1980), ou réduire directement l'absorption d'eau (Munns and Tester 2008; Parihar et al. 2015), et impacter l'hydratation et la survie des arthropodes (Renault et al. 2016). Une salinité excessive dans le sol diminue également le fonctionnement biologique des micro-organismes, perturbant le cycle de l'azote et l'apport de matière organique (Rath and Rousk 2015; Singh 2016). Si le consensus général est que les sols affectés par la salinité se retrouvent principalement dans les zones arides, il est maintenant reconnu que la salinisation des sols est susceptible de se produire dans tous types de milieux (Hassani et al. 2021), ce qui place en exergue l'intérêt d'étudier ce type de données dans une grande diversité d'écosystèmes, et sur des longues séries temporelles. Ce type de suivis est souvent difficile et, à ce titre, cette thèse met également en avant l'intérêt des suivis à long terme réalisés par les réserves naturelles ou espaces protégés par les gestionnaires, qui permettent de disposer de telles données. Des données précises et fiables sur la répartition spatiale des sols affectés par le sel sont importantes pour élaborer des plans d'action pour la gestion des sols, de l'eau et de la végétation (Hassani et al. 2021), et pourraient être récoltées grâce à des collaborations entre différents acteurs.

Par ailleurs, si dans nos études nous nous sommes principalement penché sur les effets du sel marin (NaCl), les effets du sel ne sont pas les mêmes en fonction du type de sel considéré (Walker et al. 2023). Les effets de la salinisation dépendent de la composition et des concentrations des ions, et notamment des « cocktails chimiques » d'ions, dont les effets sont peu connus, créés par les activités anthropiques. La combinaison de différents ions (par exemple Na^+ , K^+ , Cl^- , CO_3^{2-} , SO_4^{2-}) et la mobilisation d'autres éléments ou ions (par exemple Cu, Mn, Zn, Sr, NH_4^+ , PO_4^{3-}) peut entraîner des conséquences extrêmement différentes et complexes propres à l'habitat (Cunillera-Montcusí et al. 2022).

II.2. Reconquête des milieux d'eau douce

Si la salinité peut augmenter de manière brutale sur les milieux côtiers, nous avons montré qu'elle pouvait aussi diminuer, même si cette diminution se fait sur des échelles spatiales plus longues (Chapitre I). Nous avons ainsi montré une résilience au moins partielle des écosystèmes côtiers à la salinisation. A l'issue d'une tempête marine, la récupération jusqu'à un retour aux conditions de salinité initiales pouvait ainsi prendre 7 ans (Chapitre I), une durée jusqu'ici suffisante mais qui pourrait s'avérer bien trop longue si, comme c'est attendu, la fréquence des tempêtes marines venait à augmenter (Nicholls et al. 1999; McLean et al. 2001; Knutson et al. 2010; Dettinger 2011; Trenberth et al. 2015; IPCC 2022).

Les capacités de récupération dépendent aussi de l'écosystème considéré. Par exemple, Oliveira et al., (2021) ont montré que les capacités de récupération dépendaient de la qualité du substrat, les effets étant plus délétères dans les cours d'eau exclusivement bordés d'arbres riverains de haute qualité (les aulnes en comparaison des chênes) en raison d'une activité microbienne réduite. Mais la montée du niveau de la mer par exemple peut également avoir des répercussions différentes en fonction de l'habitat humain, les conséquences dépendant des taux d'accrétion verticale et de l'espace pour la migration horizontale, qui peuvent être limités par la présence d'infrastructures (McLean et al. 2001). Pour bien comprendre les milieux les plus à risque, les propriétés aggravant ou diminuant les effets de la salinisation doivent être étudiées sur un grand nombre d'écosystèmes. Mais la première étape de la préservation des zones humides d'eau douce consistera surtout à identifier les zones humides actuellement en cours de salinisation et celles à risque de salinisation à l'avenir (Herbert et al. 2015). Cela implique de suivre la salinité sur différentes composantes de l'écosystèmes (eaux de surface, eaux souterraines, mais aussi sols), et ce dans de nombreux types de milieux, afin de posséder des suivis à long terme de la salinité sur plusieurs parties du globe [les études sur la salinisation souffrant de biais géographiques (Cunillera-Montcusí et al. 2022)]. Quoi qu'il en soit, la salinisation, qu'elle impacte les sols où les masses d'eau, va avoir des impacts directs sur la biodiversité.

III) Impacts de la salinisation sur les communautés et les populations

III.1. Répartition des espèces en fonction de la salinité

Que ce soit des suites d'une tempête marine (Chapitre I) ou sur des mares présentant des niveaux de salinité différents, nous avons montré que les espèces et les populations étaient impactées par les niveaux de salinité de leur milieu, et se répartissaient en fonction de cette contrainte (Figure 13). Si on s'attend à des différences d'effet entre les variations liées à des mécanismes naturels ou liés à des changements globaux, Delaune et al. (2021) qui ont étudié le changement de réponse entre des variations graduelles ou brutales de la salinité chez des organismes zoo- et phytoplanctoniques n'ont que très rarement détecté une différence entre les variations abruptes et graduelles de la salinité. Dans nos études, nous avons montré que les submersions marines entraînaient une augmentation brutale de la salinité, provoquant la disparition des espèces d'amphibiens les plus sensibles, tandis qu'en condition naturelle les individus se répartissent en fonction de leur tolérance (Chapitre I, Figure 13). Nos résultats suggèrent que, face à une augmentation graduelle de la salinité, les espèces pourraient ajuster leur répartition, ce qui n'est pas le cas à la suite d'une submersion marine. Mais les effets des tempêtes marines pourraient aussi être indirects, à travers des variations de la composition de la communauté (Davis et al. 2023), qui demeurent à être élucidés dans le cadre des variations naturelles de la salinité.

Les études récentes sur les effets de la salinisation se sont principalement concentrées sur les invertébrés aquatiques (Cunillera-Montcusí et al. 2022; Walker et al. 2023). Malgré leur rôle prépondérant dans le fonctionnement des écosystèmes (par exemple, le cycle des nutriments), les micro-organismes d'eau douce ont reçu moins d'attention. Il en va de même pour les niveaux trophiques supérieurs tels que les oiseaux, les poissons, les amphibiens et les reptiles (Cunillera-Montcusí et al. 2022; Walker et al. 2023), ainsi que certaines espèces terrestres (Desender and Maelfait 1999; Renault et al. 2016). La focalisation étroite sur certains groupes taxonomiques empêche une évaluation correcte des risques que la salinisation fait peser sur la biodiversité mondiale (Cunillera-Montcusí et al. 2022).

III.2. Changement de la composition de la communauté

La salinité est un facteur structurant majeur de la biodiversité (Hart et al. 2003; Findlay and Kelly 2011; Hintz and Relyea 2019; Venâncio et al. 2019a). Lorsque la salinité est trop élevée, elle provoque ainsi un déclin brutal de la richesse spécifique, que ce soit pour les anoues ou les urodèles (Chapitre I, Figure 13). Chez d'autres taxons tels que les crustacés certains auteurs se sont intéressés non seulement à la richesse spécifique (diversité taxonomique) mais aussi à la richesse fonctionnelle, et ont montré que la perte de diversité fonctionnelle était plus faible que la perte de diversité taxonomique (Hébert et al. 2022): autrement dit, même si le nombre d'espèces diminue, ces espèces présentaient toutes à peu près le même rôle fonctionnel, diminuant l'impact sur les écosystèmes. Les amphibiens, en France et particulièrement sur notre zone d'étude, présentent un nombre d'espèce beaucoup moins important, ce qui complique l'estimation de la perte de diversité fonctionnelle. Mais ce type d'étude pourrait être conduit dans des régions du monde où la diversité en amphibiens est beaucoup plus élevée, comme c'est le cas en Asie (Das and van Dijk 2013). Ces études pourraient permettre de comprendre si l'exposition au sel chez les amphibiens va réduire les services écosystémiques rendus par ce groupe taxonomique, comme c'est le cas chez d'autres organismes (Zhang et al. 2022).

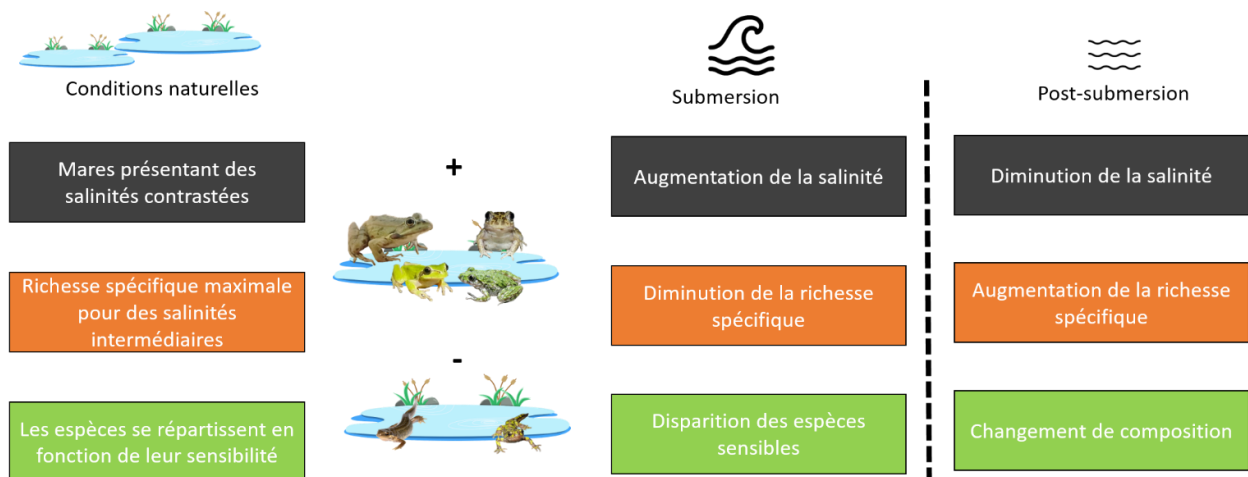


Figure 13 : Effets de la salinité sur les communautés d'amphibiens, en conditions naturelles ou suite à un évènement climatique extrême.

Il est également important de considérer le fait que les effets observés de la salinité sur une espèce sont souvent liés aux espèces avec lesquelles elles coexistent, les interactions entre les espèces pouvant ainsi moduler la tolérance au sel, ce qui rend difficile la prédiction de la tolérance des individus dans leur milieu naturel, qui est en fait régie par des interactions

complexes (Arnott et al. 2023). Les taxons ne répondent pas seuls aux variations de salinité dans leur milieu, mais réagissent en interaction avec les autres composantes de leur environnement (Lee et al. 2022), les interactions interspécifiques pouvant modifier les effets du stress lié à la salinité, et les changements dans la composition de la communauté qui en résultent (Bray et al. 2019). Pour mieux comprendre ces interactions complexes, des études en mésocosme pourraient être réalisées, pour intégrer les liens existant au sein d'un écosystème complexe, et les mécanismes indirects en jeu.

III.3. Différence de tolérance entre les anoures et les urodèles

Nos résultats ont permis de mettre en évidence la tolérance plus élevée des anoures par rapport aux urodèles (Chapitre I). Un tel contraste peut être lié à une plus faible efficacité des mécanismes physiologiques impliqués dans l'osmorégulation chez les urodèles par rapport aux anoures (par exemple le recours à l'urée, aux acides aminés libres et/ou aux concentrations ioniques dans les fluides corporels pour réduire les flux de sel et d'eau, Licht et al. 1975). A ce jour, à notre connaissance, aucune étude n'a formellement comparé les mécanismes d'osmorégulation entre les anoures et les urodèles. Deux études différentes ont déjà montré que, si les mécanismes physiologiques responsables de la tolérance à la salinité ne sont toujours pas pleinement compris, les modifications des hormones osmorégulatrices telles que la corticostérone et l'aldostérone sont des candidats privilégiés (Tornabene et al. 2021b, 2022b). Dans ces deux études séparées, les auteurs ont de fait montré, sur une espèce d'anoure puis d'urodèle, que l'expression de ces deux hormones été différentes entre les deux groupes. Chez les urodèles, les réponses en terme d'aldostérone sont plus faibles, ce qui laisse à suggérer que, chez ce groupe taxonomique, d'autres hormones peuvent jouer un rôle plus important dans l'acclimatation et la tolérance à la salinité (Tornabene et al. 2022b). Pour d'autres taxons, il a été montré qu'il existait une base génétique à la tolérance. De fait, les gènes codant pour les pompes à calcium (Ca^{2+} ATPases) et à Sodium-Potassium ($\text{Na}^{+}/\text{K}^{+}$ ATPases), sont des cibles de sélection particulièrement courantes (Velotta et al. 2022). Chez les amphibiens, une étude récente a permis de montrer que certains gènes sont associés à l'adaptation locale à la salinité chez les amphibiens (Albecker et al. 2021). Il serait donc intéressant d'identifier si des différences de gènes codants pour des réponses à la salinité existent entre anoures et urodèles (et au sein de ces groupes, entre espèces), et si ces différences permettent d'expliquer les différences de tolérance à la salinité observées.

III.4. Les espèces envahissantes

Il est connu que la salinisation pourrait, à terme, conduire à une accélération de l'invasion par les espèces envahissantes (Cañedo-Argüelles et al. 2013). De fait, l'augmentation de la salinité peut ouvrir la voie aux espèces invasives qui seront plus tolérantes aux conditions de salinité en place. Cela a par exemple été démontré pour *Rhinella marina* (Wijethunga et al. 2016), dans sa zone d'invasion dans l'Est de l'Australie, où la salinité des mares dans lesquelles on retrouve l'espèce envahissante facilite plutôt que contraint l'expansion continue du front d'invasion de ces crapauds vers le sud. *Rhinella marina* et *Xenopus laevis*, deux espèces d'amphibien envahissantes bien connues (Measey et al. 2016), peuvent toutes deux survivre dans une eau contenant 40% d'eau de mer, soit 14 g.l⁻¹ de sel (Munsey 1972; Liggins and Grigg 1985). D'autres espèces envahissantes moins connues, comme *Pelophylax ridibundus*, sont relativement tolérantes à des seuils de salinité moyennes (Karraker 2007; Sillero and Ribeiro 2010; Natchev et al. 2011), ce pattern ayant également été retrouvé dans notre zone d'étude, avec une tolérance expérimentale testée jusqu'à 12 g.l⁻¹ (Chapitre II), et des individus retrouvés sur le terrain jusqu'à 16 g.l⁻¹ (Chapitre I), l'espèce étant, parmi les espèces présentes sur notre zone d'étude, la plus tolérante à la salinité. Elle pourrait donc surpasser les autres espèces d'amphibien dans sa capacité à tolérer la salinité et notamment dans le cadre d'une submersion marine (Chapitre I). De manière intéressante, les espèces invasives, de par leur tolérance, pourraient aussi permettre de préserver la fonctionnalité des milieux côtiers en cas de salinisation (Pétillon et al. 2023). Les différences entre la tolérance des espèces invasives et des espèces natives devraient être testées expérimentalement, et prises en compte pour mettre en place des mesures de conservation adaptées (Liu et al. 2022).

III.5. Tolérance et résilience

Si les populations et communautés côtières sont soumises à la salinisation, il est important de noter qu'elles peuvent être résilientes (Figure 13). Les capacités de résilience des amphibiens étant bien connues (Beranek et al. 2021), nous avons montré que, après une tempête marine, les communautés d'amphibiens peuvent se reconstituer (Pereira et al. 2019; Lorrain-Soligon et al. 2021), au moins en terme de richesse spécifique. Nous nous sommes penché uniquement sur les réponses en terme présence/absence des espèces, mais les espèces pourraient également être impactées en terme d'abondance. Une étude conduite sur 524 espèces (considérant tous types d'espèces, et les événements climatiques extrêmes, parmi lesquels les

tempêtes marines) a permis de montrer que la majorité des populations (80.4%) montre des mécanismes de compensation lors d'événements météorologiques extrêmes pour réduire leurs effets délétères (Neilson et al. 2020). Cependant, pour les populations qui ont été impactées négativement, les conséquences démographiques ont été sévères. Près de 20 % des populations suivies ont connu des déclinés de plus de 50 % après un évènement climatique extrême, et 6,8 % des populations ont disparu. Par ailleurs, la résilience des populations n'est pas courante, car 80,0 % des populations qui ont diminué en abondance ne se sont pas rétablies aux niveaux pré-perturbation (au moins pendant la période d'étude considérée) (Neilson et al. 2020). Enfin, bien que la salinité soit connue comme un moteur majeur de la structure des communautés et de la diversité, les conséquences que la salinisation peut avoir à l'échelle des écosystème restent à comprendre (Cunillera-Montcusí et al. 2022).

IV) Au niveau individuel : effet chez les adultes et chez les larves

IV.1. Reproduction

Les adultes côtiers sont plus petits que ne le sont leurs homologues continentaux (Chapitre III, Figure 14), mais investissent également moins dans la reproduction, résultant en des œufs plus petits qui donnent naissance à des têtards plus petits et moins performants (Chapitre III, Figure 14). Puisque les femelles côtières sont soumises naturellement à des milieux plus salés, nous avons émis l'hypothèse que ces limitations pourraient être liées à un « selfish maternal effect » (Marshall et al. 2008), du fait des coût liés à l'osmorégulation (Herbert et al. 2015). De fait, les individus côtiers, mâles et femelles, présentent également un métabolisme plus faible (Chapitre II, Figure 14), ce qui pourrait limiter l'énergie disponible. De manière importante, cette limitation au niveau de la reproduction est retrouvée lorsque des couples en amplexus sont exposés de manière très brève à des niveaux de salinité faibles (4 g.l^{-1}), illustrant des différences importantes au niveau du temps de ponte et de la taille des œufs (Figure 15), indiquant que les individus peuvent ajuster leur investissement très rapidement, même si les mécanismes sous-jacents demeurent inconnus. Cette hypothèse pourrait être testée plus avant en capturant les adultes (issus de milieux côtiers et de milieux continentaux) en dehors de la période de reproduction, et en les élevant pendant une année jusqu'à la reproduction suivante dans les même conditions expérimentales (présence d'eau douce pour les individus issus des

deux types de milieux, et même accès aux ressources), afin de voir si ces limitations sur la reproduction se poursuivent.

Ces effets négatifs de la salinité que nous avons retrouvés sont maintenus pendant l'intégralité du développement embryonnaire (Figure 15), larvaire (Figure 16), mais également post-métamorphique (Figure 17).

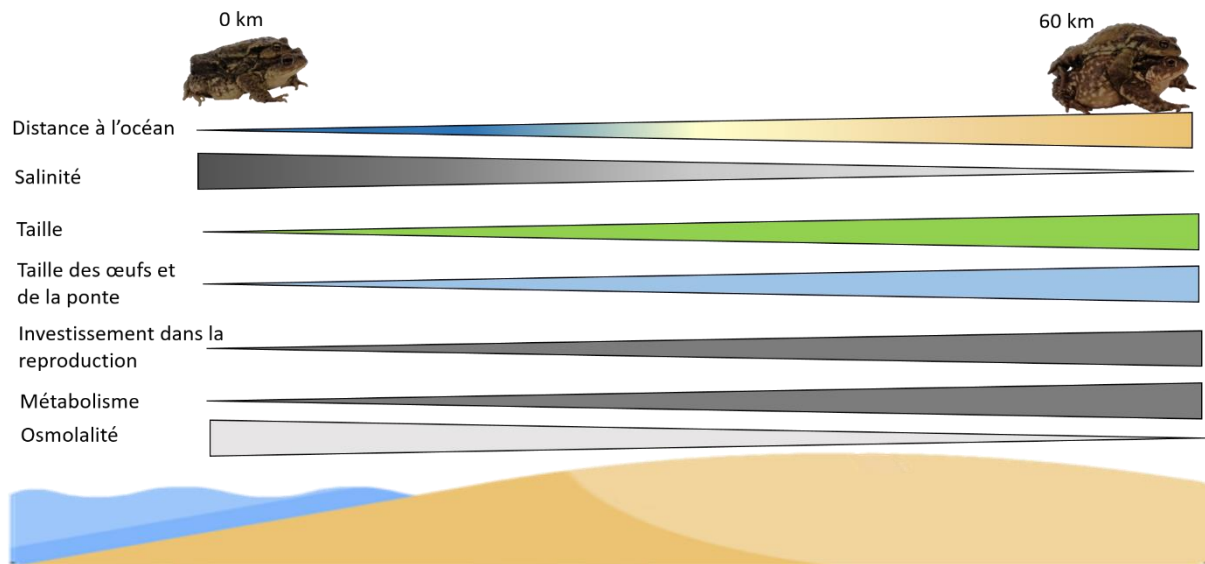


Figure 14 : Effets de la salinité sur les d'amphibiens (concentrés sur *Bufo spinosus*). Ces effets ont été observés en fonction du gradient de distance à l'océan, et du gradient de salinité associé.

IV.2. Effets physiologiques

Dans nos études, nous avons montré, chez les larves, une sensibilité marquée au sel, à des salinités plus faibles que celles attendues dans la littérature (voir par exemple Albecker and McCoy 2017, qui décrivent une LC_{50} de 5.5 g.l^{-1} chez les larves d'anoures, alors que dans nos études la LC_{50} se situe en deçà de 4 g.l^{-1} pour les larves de crapauds épineux, Figure 16). Si ces effets se retrouvent déjà à la fin de la période embryonnaire (Figure 15), à ce stade, on ne retrouve pas d'effet du traitement sur la taille des télomères (Chapitre III). Pourtant, la taille des télomères est connue être une mesure fiable du vieillissement cellulaire (Harley et al. 1990; Olovnikov 1996) et de l'exposition à un stress (Houben et al. 2008; Breuner et al. 2013). La taille des télomères n'a été évaluée qu'au stade 25 du développement larvaire, correspondant à l'éclosion, qui intervient environ 2 semaines seulement après la ponte. Si une exposition de moins de 2 semaines ne semble donc pas affecter la taille des télomères, on peut tout de même émettre l'hypothèse qu'une exposition prolongée le ferait. Ce paramètre reste donc à tester sur

des individus à un stade plus avancé, et sur des individus capturés sur le terrain, d'autant que ce paramètre n'a jamais été testé dans le cadre d'une exposition à la salinité.

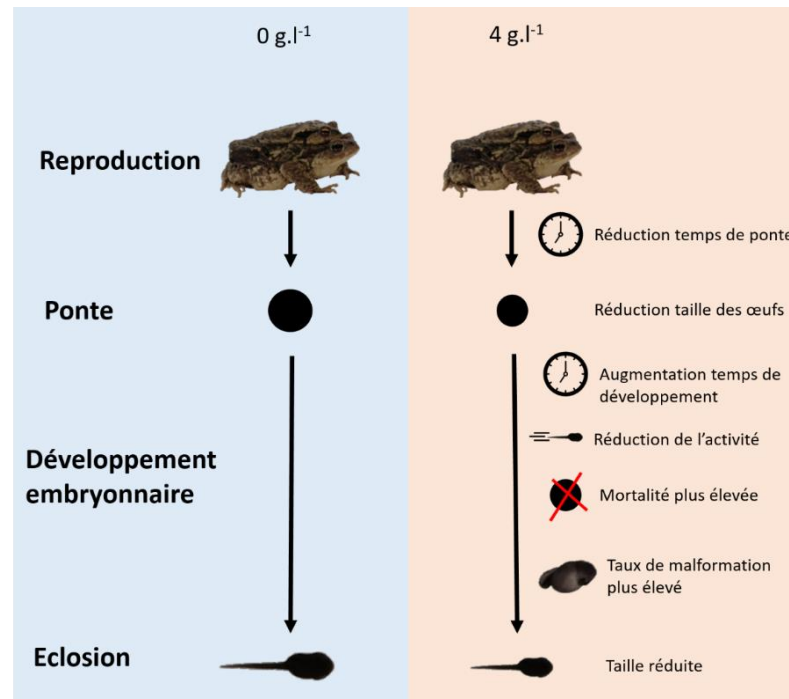


Figure 15 : Effets de la salinité sur la reproduction et le développement embryonnaire, observés sur *Bufo spinosus*.

Chez les adultes, la sensibilité au sel est moins marquée, même si bien présente. Nous avons premièrement montré qu'une salinité relativement élevée (12 g.l⁻¹), mais également des salinités plus faibles (4 g.l⁻¹) provoquaient de la déshydratation et une augmentation de l'osmolalité plasmatique (Chapitre II, Figure 17). Mais ces mécanismes de régulation ont un coût, et l'augmentation de la salinité a notamment été montré augmenter le métabolisme de nombreuses espèces (Kammerer et al. 2010; Peña-Villalobos et al. 2016; Álvarez-Vergara et al. 2022). A l'inverse, nous n'avons pas montré, après 6 mois d'exposition au sel, de différence au niveau du métabolisme (Chapitre II, Figure 17). Cette différence pourrait être due à la durée d'exposition relativement longue (>6 mois) appliquée dans notre étude, ce qui implique que le taux métabolique pourrait varier en fonction de la phase d'exposition. Les coûts de l'osmorégulation pourraient être médiés par des mécanismes hormonaux qui impliquent la corticostérone, la prolactine, les hormones de croissance, l'angiotensine II, et l'aldostérone connues pour leur rôle dans la régulation hydro-minérale chez les amphibiens (McCormick and Bradshaw 2006; Tornabene et al. 2022a). On s'attend également, pour les individus exposés en condition chronique à la salinité, à des effets sur le stress oxydant (Bal et al. 2021, 2022) et, en conséquence, sur la taille des télomères (Houben et al. 2008), qui restent à mesurer.

En condition chronique notamment, on s'attend également à des effets au niveau du développement.

IV.3. Effets sur la croissance et le développement

Chez les larves premièrement, nous avons observé des effets sur le développement et la croissance. De manière étonnante, durant l'intégralité du développement embryonnaire et larvaire, nous avons observé que les individus performaient mieux sous des salinités intermédiaires (c'est-à-dire 2 g.l⁻¹, plutôt que 0 g.l⁻¹ ou 4 g.l⁻¹) (Chapitre III). Ce résultat pourrait être la conséquence d'une osmolalité plasmatique similaire à l'osmolalité d'un milieu à 2 g.l⁻¹, ce qui pourrait limiter la perte d'eau dans ce type de milieu. Nous n'avons pas mesuré l'osmolalité interne des têtards de crapauds épineux, utilisés pendant cette étude, mais des études futures pourraient permettre d'éclaircir cette hypothèse. Alternativement, ce résultat pourrait être lié au fait qu'une augmentation très légère de salinité est bénéfique, parce que cette salinité pourrait réduire la fréquence des parasites et des infections chez les amphibiens (Robles Mendoza et al. 2009; Ahumada-García et al. 2018; Clulow et al. 2018). Au-delà d'un certain seuil, la salinité provoque tout de même des problèmes de développement, de croissance, et de mortalité (Figure 16).

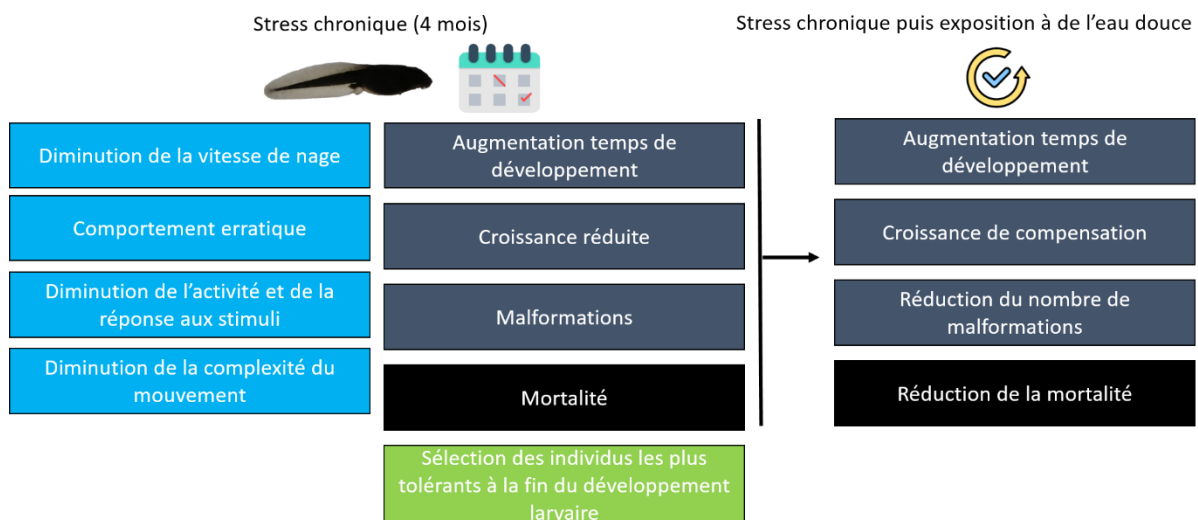


Figure 16 : Effets d'une exposition chronique à la salinité, mais également d'une exposition à de l'eau douce après exposition à la salinité, sur le développement larvaire, observé chez *Bufo spinosus*.

Au-delà des effets du traitement expérimental, notre étude montre aussi des effets du site d'origine des parents, avec des effets accrus de la salinité chez les individus côtiers (Chapitre III). Une mortalité plus élevée notamment pourrait être un processus évolutif

permettant de réduire la densité des larves, et ainsi d'affecter positivement d'autres traits dans le milieu naturel (Karraker et al. 2008).

Chez les adultes, les effets du sel sur la croissance et le développement n'avaient jamais été testés. Dans nos études, nous avons montré premièrement que, si les adultes côtiers sont plus petits que ne le sont les adultes continentaux (Chapitre III, Figure 14), lorsque les individus issus des deux milieux sont exposés à de l'eau douce, les individus côtiers expriment une croissance plus rapide (compensatoire) par rapport aux individus continentaux (Chapitre II), traduisant ainsi un relâchement du stress lié à un milieu plus salé sur la côte. Cette observation est corroborée par la croissance plus faible des adultes exposés expérimentalement à de l'eau salée (Chapitre II, Figure 17), que ce soit pour les individus côtiers ou continentaux, ce qui vient confirmer l'hypothèse que la taille plus petite des individus observés sur les milieux côtiers (Chapitre II) est principalement le fait des limitations liées aux milieux salés.

IV.4. Effets sur le comportement

Chez les têtards, nous avons montré que les individus exposés à de l'eau douce exprimaient un comportement moins complexe, mais aussi une vitesse et une distance parcourue moindres par rapport aux individus en traitement à 2 g.l⁻¹ (Chapitre III), conduisant, dans les milieux naturels, à une réduction de la probabilité de rencontre avec des ressources (Bartumeus 2007; Viswanathan et al. 2008). Comme précédemment, cela peut être expliqué par l'effet bénéfique de salinités intermédiaires sur l'osmorégulation et la réduction du parasitisme. Chez les amphibiens, il a été montré que les perturbations anthropiques (augmentation de température et de salinité) subies à l'état larvaire pourraient parfois n'affecter les individus qu'à des stades ultérieurs, post-métamorphiques (Dahrouge and Rittenhouse 2022). Ainsi, les effets bénéfiques que nous avons observé à 2 g.l⁻¹ de sel chez les têtards d'amphibien (Chapitre III) pourraient provoquer des effets négatifs n'apparaissant qu'après la métamorphose.

Si, chez les larves, les effets sur le comportement sont importants dans les milieux salés (Chapitre III, Figure 16), à la fin du développement, les individus issus de ce traitement ont tendance à être plus performants, avec une activité, une taille et une masse plus élevées (voir aussi Dahrouge and Rittenhouse, 2022). Comme suggéré ci-dessus, cela pourrait être lié à un processus sélectif où seuls les individus les plus aptes survivent dans cet environnement difficile. Mais, la salinité subie à l'état larvaire pourrait également avoir des effets « carry

over » et affecter les réponses comportementales et physiologiques des individus survivant jusqu'au stades juvénile et adultes (Vegso et al. 2022).

Chez les adultes, nous avons montré des effets sur l'activité, les performances de saut, la sélection de l'habitat, la capture de proies, mais aussi des comportements défensifs ou de cachette (Chapitre II, Figure 17). Ces résultats traduisent ainsi des effets du sel sur le comportement qui avaient initialement été principalement démontrés sur les larves. Cependant, nous n'avons pas mesuré le même type de comportements que ceux observés chez les larves (vitesse, complexité du mouvement, mouvements erratiques), et ce type de comportement pourrait être testé, notamment à des niveaux de salinité plus élevés chez les adultes.

IV.5. Tolérance et résilience

Si la salinité a un impact important à la fois sur les larves (Chapitre III) mais aussi sur les adultes (Chapitre II), nous avons aussi montré une tolérance et des capacités de résilience remarquables chez ces deux stades de vie. Les amphibiens adultes sont très résistants à l'exposition au sel au moins jusque 9 g.l^{-1} , et peuvent récupérer leur condition initiale lorsqu'ils ont accès à de l'eau douce après avoir été exposés à un traitement à une salinité de 12 g.l^{-1} , malgré la mortalité observée chez certains individus (Chapitre II). Chez les larves, nous avons montré une résilience au moins partielle avec une croissance compensatoire (Squires et al. 2010; Kearney et al. 2014) et un comportement optimal restauré (même si tous les individus n'en sont pas capables) après un transfert d'un traitement à salinité 4 g.l^{-1} vers de l'eau douce (Chapitre III), mais également une tolérance variable qui induit la sélection des individus les plus performants (Chapitre III). Ainsi, pour les deux stades, il existe au moins une résilience au niveau individuel, et une résistance qui peut se faire au niveau des populations parce que certains individus sont plus tolérants, ce qui permet à la population de persister. Cependant, comme nous avons montré que l'eau douce n'était pas forcément optimale pour le développement larvaire (Chapitre III), et qu'un transfert direct en eau douce pouvait provoquer des œdèmes et de la mortalité chez les adultes (Chapitre II), la résilience pourrait être favorisée en privilégiant un transfert vers des salinités intermédiaires plutôt que vers de l'eau douce.

La résilience des individus peut également être comportementale (Chapitre II). De fait, les individus peuvent éviter certains types d'habitat, en utilisant comme information leur

propre expérience passée (Doligez et al. 2008), et moduler leurs comportements pour limiter l'exposition à la salinité (Ashley et al. 2021). Ainsi, nous avons montré que les individus adultes sont capables de repérer la présence d'habitats d'eau douce, ces habitats d'eau douce leur permettant de se rétablir (Chapitre II). Cependant, les mécanismes de la sélection de l'habitat en fonction la salinité (sans contact direct) demeurent inconnus, mais pourraient se faire de manière visuelle (degrés de polarisation de l'eau douce par rapport à l'eau saumâtre (Zhang et al. 2019) ou olfactive (Evans 1988), et ces mécanismes doivent également être étudiés.

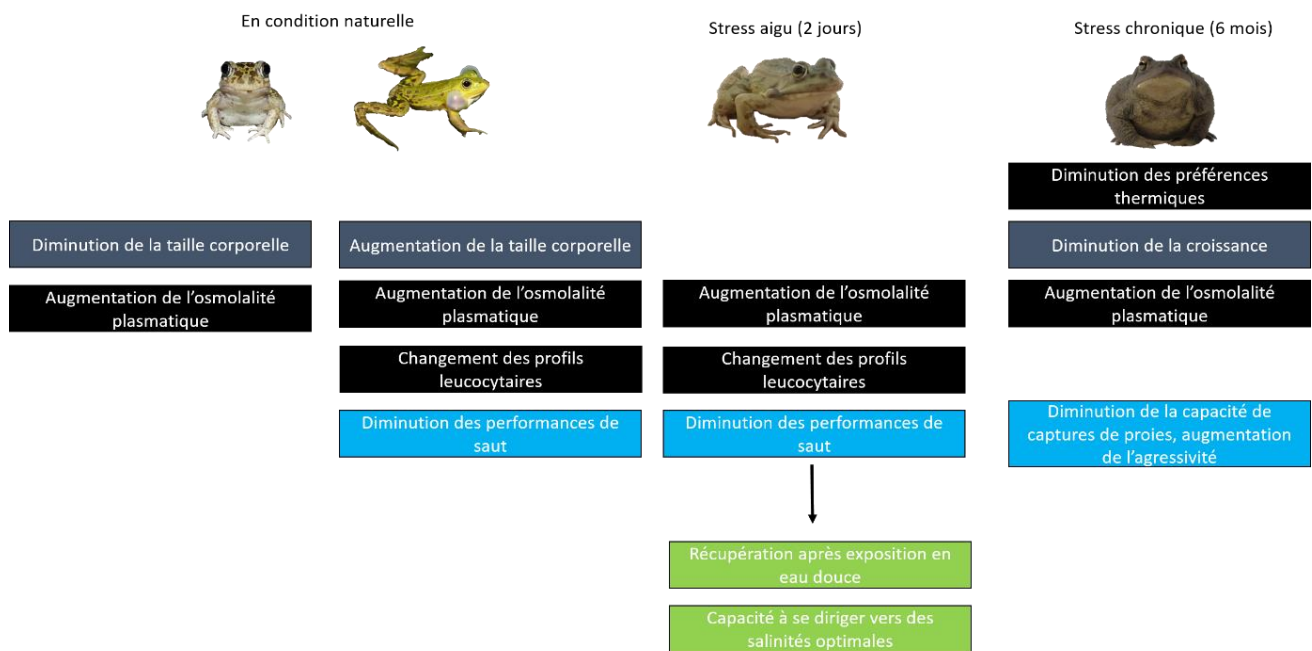


Figure 17 : Effets de la salinité à différentes échelles de temps, mais également d'une exposition à de l'eau douce après exposition à la salinité, sur les amphibiens adultes, observé chez *Pelobates cultripes*, *Pelophylax sp*, et *Bufo spinosus*.

IV.6. Absence d'adaptation locale ?

Les individus vivant à proximité de l'océan ou dans des milieux salés démontrent généralement une tolérance accrue à une augmentation de la salinité, et une adaptation locale (Licht et al. 1975; Gomez-Mestre and Tejedo 2003; Hopkins et al. 2016, 2017; Albecker et al. 2021; Gabriel et al. 2021). Dans nos études, nous avons montré que, pour des adultes capturés sur la côte, les individus issus de mares d'eau saumâtres n'étaient pas plus résistants à la salinité que ceux issus de mares d'eau douce, et n'étaient pas non plus capables d'une meilleure discrimination des milieux d'eau saumâtre (Chapitre II). Ces résultats pourraient être expliqués par le fait que, sur les milieux côtiers, les mares saumâtres et d'eau douce sont présentes sur une échelle spatiale restreinte, et les individus peuvent ainsi circuler d'une mare

à une autre, réduisant ainsi les effets d'une possible adaptation locale. Chez les individus adultes de *Bufo spinosus*, nous avons montré que les individus issus de populations côtières présentaient certains effets plus marqués que leurs homologues continentaux, notamment au niveau du choix thermique (Chapitre II). Ce résultat pourrait être un mécanisme d'adaptation locale, permettant aux individus de réduire leurs dépenses énergétiques, et les effets sur le plus long terme demeurent à étudier, afin de déterminer s'ils favorisent la tolérance des populations côtières. De manière contre-intuitive, nous avons aussi montré que les larves issues de parents côtiers (issus de mares allant jusque 6 g.l⁻¹ de sel) n'étaient non seulement pas tolérantes à la salinité (pour une salinité pourtant inférieure à ce qui est retrouvé sur les milieux côtiers, Chapitre III), mais étaient en plus moins tolérantes que ne le sont les larves issues de parents continentaux (qui n'ont jamais été exposés au sel, Chapitre III). Comme évoqué précédemment, d'autres paramètres pourraient augmenter la survie dans les milieux naturels, tels qu'une pression de prédation (Velasco et al. 2019) ou une transmission de pathogènes (Clulow et al. 2018) moindres, qui peuvent améliorer la survie des individus côtiers dans leur habitat naturel. De plus, le fait que la mortalité soit élevée dans le traitement le plus salé est néfaste pour les individus, mais pourrait être positif pour les populations, en réduisant la compétition intra-spécifique. L'ensemble de ces paramètres pourraient être pris en considération avec une étude en mésocosme.

Par ailleurs, puisque les femelles sélectionnent des sites de ponte moins salés (Albecker and McCoy 2017), et si des gradients de salinité existent au sein d'une même mare (ce paramètre pouvant être testé sur le terrain), nous pouvons émettre l'hypothèse que les femelles choisissent de pondre dans des zones au sein des mares où la salinité est inférieure à 4 g.l⁻¹, les têtards évitant ou n'ayant ainsi jamais à connaître une salinité aussi élevée à ce stade de leur vie. De fait, au-delà de l'adaptation locale, l'acclimatation est également très importante (Liggins and Grigg 1985; Bernabò et al. 2013; Wu et al. 2014), d'autant plus que nous avons mis en évidence le fait que la salinité variait temporellement sur les milieux côtiers, sur une échelle de temps très courte (Chapitre I). Puisque les têtards côtiers pourraient, dans leur milieu naturel, être confrontés à une augmentation très graduelle de la salinité, il serait important de tester l'hypothèse d'une acclimatation progressive à partir d'une salinité faible, pour déterminer si celle-ci pourrait augmenter la tolérance à des salinités plus élevées.

Enfin, l'expression de certains gènes peut expliquer la tolérance à la salinité (Albecker et al. 2021; Lee et al. 2022), mais le succès de la reproduction pourrait aussi dépendre de l'âge des individus (Kara 1994; Saraux and Chiaradia 2022) ou de leur âge cellulaire (taille des

téломères, Eastwood et al. 2019)). Tous ces paramètres pourraient varier entre les adultes côtiers et continentaux, expliquant les différences de fitness observées.

IV.7. Différence de tolérance entre espèce

La tolérance à la salinité varie entre espèces (Chapitre I), montrant des réponses espèces dépendantes à la salinité (Hopkins and Brodie 2015; Tornabene et al. 2021a), qui placent en exergue l'importance de tester ces effets sur différentes espèces pour mieux comprendre les répercussions sur l'ensemble de la communauté. Ces effets espèces dépendants pourraient par exemple expliquer les résultats que nous avons observé sur le développement larvaire chez le crapaud épineux (Chapitre III). Cette espèce n'étant pas connue pour être particulièrement tolérante au sel, les résultats pourraient varier en considérant une espèce tolérante retrouvée dans les mêmes milieux, telle que la grenouille verte (Chapitres I et II).

Comme la tolérance à la salinité varie entre les espèces, certaines espèces sont aussi avantagées par une salinité élevée, ou désavantagées par une salinité faible. Par exemple, comme évoqué précédemment, la tolérance à la salinité peut ouvrir la voie à des espèces envahissantes, parce que celles-ci sont plus tolérantes à la salinité (Rios-López 2008; Cañedo-Argüelles et al. 2013; Wijethunga et al. 2016). Certaines espèces peuvent également être impactées par la diminution de la salinité, comme certains poissons sténohalins (Xin et al. 2022). Il serait ainsi intéressant d'étudier non pas l'effet de l'augmentation de la salinité, mais l'effet de la diminution de la salinité sur les milieux côtiers, sachant que certains effets délétères ont été observés en eau douce chez les larves de crapauds épineux (Chapitre III) et que l'abondance des espèces d'anoures sur la côte est maximale pour des salinités intermédiaires (Chapitre I). Dans les régions côtières de haute latitude, l'augmentation des précipitations et la fonte des glaces entraînent une baisse de la salinité à des échelles de temps rapides (Rabe et al. 2011; McCrystall et al. 2021). Par exemple, plusieurs études ont prévu qu'une grande partie de la mer Baltique pourrait devenir presque complètement douce d'ici 2100, avec des impacts néfastes sur ses communautés actuellement adaptées aux conditions d'eau saumâtre (Janssen et al. 1999; Meier et al. 2006; Kniebusch et al. 2019). De fait, une baisse de la salinité de 2 g.l⁻¹ devait entraîner une réduction par 3 de la biomasse de la pêcherie de cabillaud de la mer Baltique (Thøgersen et al. 2015). Les effets à plus large échelle de cette diminution de la salinité devraient donc potentiellement être testés, sur les amphibiens côtiers et sur l'ensemble des

espèces adaptées aux conditions salines. Par ailleurs, d'autres paramètres pourraient modifier les effets de la salinité.

V) Effets interactifs

Les effets de la salinité sur les organismes vivants sont souvent médiés par l'effet d'autres stressseurs environnementaux (Velasco et al. 2019). L'interaction avec ces stressseurs environnementaux sont d'autant plus importants à considérer dans le cadre des zones humides côtières, qui subissent de multiples pressions anthropogéniques (Martínez-Megías and Rico 2021). Parmi ces facteurs, la température et la salinité sont particulièrement importants à considérer, parce que la survie et la répartition des individus sont fortement dépendantes de leurs tolérances physiologiques, et notamment vis-à-vis de la température et de la salinité. Une augmentation de ces paramètres, de manière indépendante, va provoquer de la mortalité directe, mais aussi des effets sublétaux sur la croissance, l'acquisition de ressources, les fonctions immunitaires, la reproduction le comportement, et les performances (Herbert et al. 2015; Huey and Kingsolver 2019; Thorall et al. 2023).

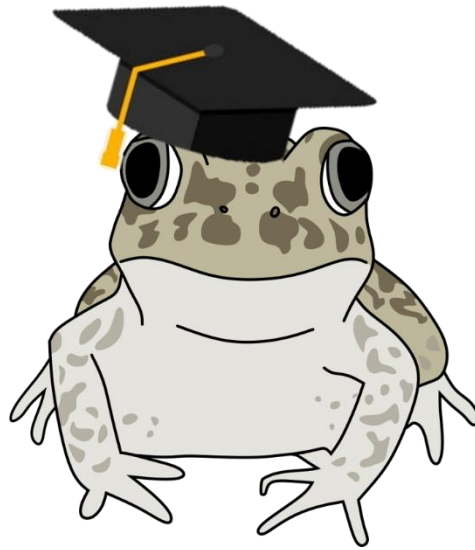
Par exemple, chez les invertébrés, les lamproies et les poissons, il a été montré que la température pouvait influencer les effets directes et indirectes de la salinité (Kelly et al. 2016; Souissi et al. 2016; Trancart et al. 2016; Walker et al. 2020; Vilas-Boas et al. 2020; Kendall et al. 2022; Shaughnessy and McCormick 2022; Ma et al. 2022). De fait, pour de nombreux organismes, des températures plus élevées se traduisent par une capacité réduite à s'acclimater ou à s'adapter aux changements de salinité.

Les amphibiens sont très sensibles à l'augmentation de la température (López-Alcaide and Macip-Ríos 2011) et de la salinité (Lorrain-Soligon et al. 2022a), ce qui est particulièrement marqué chez les larves (Albecker and McCoy 2017; Sinai et al. 2022). Pourtant, très peu d'études ont été conduites sur les interactions entre ces deux facteurs chez les amphibiens, si ce n'est des études portant sur la tolérance thermique des larves et des individus métamorphiques en réponse à une augmentation de la salinité (Sanabria et al. 2018; Chuang et al. 2022a), et sur l'interaction entre température et salinité sur les probabilités d'infection (Heard et al. 2014) et leur effet sur le développement embryonnaire et larvaire (Rogell et al. 2009; Hopkins et al. 2017; Dahrouge and Rittenhouse 2022).

Si certaines études ont été conduites chez les larves, très peu d'études ont été conduites sur les effets seuls de la salinité ou de la température chez les amphibiens adultes. Par ailleurs, à ce jour, aucune étude sur l'interaction entre les deux facteurs n'a été conduite chez ces derniers. Pourtant, ces effets interactifs pourraient être important à tester car, à des températures élevées, les individus pourraient subir un "effondrement métabolique", en raison de la diminution de leur apport énergétique et de l'accélération des coûts métaboliques (Huey and Kingsolver 2019), qui pourrait réduire leur tolérance à la salinité, puisque moins d'énergie est disponible pour l'osmorégulation au même titre que les autres fonctions essentielles. Par ailleurs, le stress thermique peut augmenter les besoins en ressources tout en altérant la capacité d'acquérir des nutriments (Litchman and Thomas 2023). Les études futures devraient donc se pencher sur ces interactions, mais d'autres interactions sont également à considérer, telles que les relations entre salinité et pesticides (Wood and Welch 2015; Lewis et al. 2020; Álvarez-Vergara et al. 2022), ou entre salinité et modification de l'habitat (Berger et al. 2019). La connaissance de l'ensemble de ces effets pourra permettre de mettre en place des mesures de conservation adaptées.

Quoi qu'il en soit, il est important de reconnaître que les zones humides côtières ont évolué et ont persisté face à des conditions environnementales changeantes pendant des milliers d'années, et ont donc une capacité inhérente à réagir aux changements environnementaux futurs (Neubauer and Craft 2009). Mais de nombreuses études ont mis en lumière l'intérêt d'atténuer les effets du changement climatique pour éviter une interférence dangereuse avec la capacité des écosystèmes naturels à s'y adapter (Salazar et al. 2007; Pétilion et al. 2023).

Conclusion



Pour conclure, nos résultats suggèrent une augmentation de la salinité sur les milieux côtiers (Figure 18), en réponse aux changements globaux mais aussi de manière saisonnière (mais dont l'effet est renforcé par les changements globaux). Les salinités élevées qui en résultent induisent des effets négatifs sur les amphibiens, aussi bien au niveau individuel, qu'à celui des populations ou des communautés (Figure 18). Malgré cela, les amphibiens démontrent des capacités de tolérance et surtout de résilience remarquables. Au niveau des communautés, si les espèces les plus sensibles disparaissent quand la salinité augmente, la résilience peut avoir lieu grâce à la présence d'espèces tolérantes, et à la recolonisation par des individus non impactés sur des zones adjacentes. Au niveau individuel, si les individus subissent des effets négatifs, la résilience peut avoir lieu parce que les individus sont capables de retrouver leur condition initiale lorsque les conditions de salinité retournent à la normale et d'exprimer des mécanismes de compensation, mais également parce qu'ils peuvent choisir de se diriger vers des milieux à salinité optimale.

Nos données, dont certaines sont issues de suivis sur le long terme initiés par des gestionnaires de réserves ou d'espaces naturels, placent en exergue l'intérêt d'instaurer des suivis à long terme pour comprendre les dynamiques régissant les milieux naturels et les changements globaux qui les affectent. Nos études soulignent par ailleurs le fait que la présence d'amphibiens dans les zones humides côtières résulte d'une interaction entre des traits spécifiques à l'espèce (tolérance à une salinité élevée, écologie) et des caractéristiques spécifiques au site (topographie et caractéristique des mares). Cette dernière catégorie est particulièrement intéressante à considérer dans le cadre de la salinisation des milieux, puisqu'une gestion paysagère relativement modeste peut être essentielle pour permettre aux amphibiens, mais aussi potentiellement à d'autres espèces, de faire face à ce changement global.

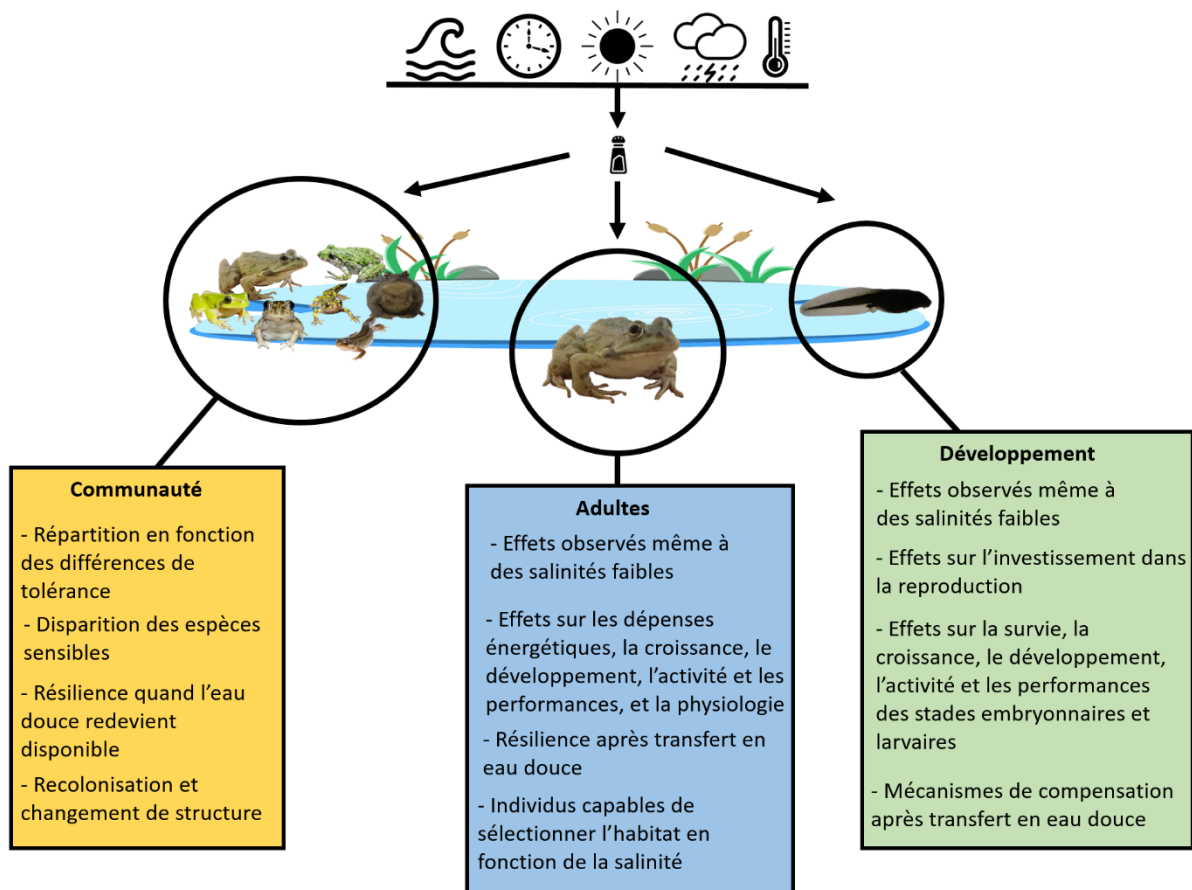


Figure 18 : Conclusion générale de la thèse, reprenant les différentes raisons des variations de salinité sur les milieux côtiers, et les effets observés sur les communautés, les populations et les individus.

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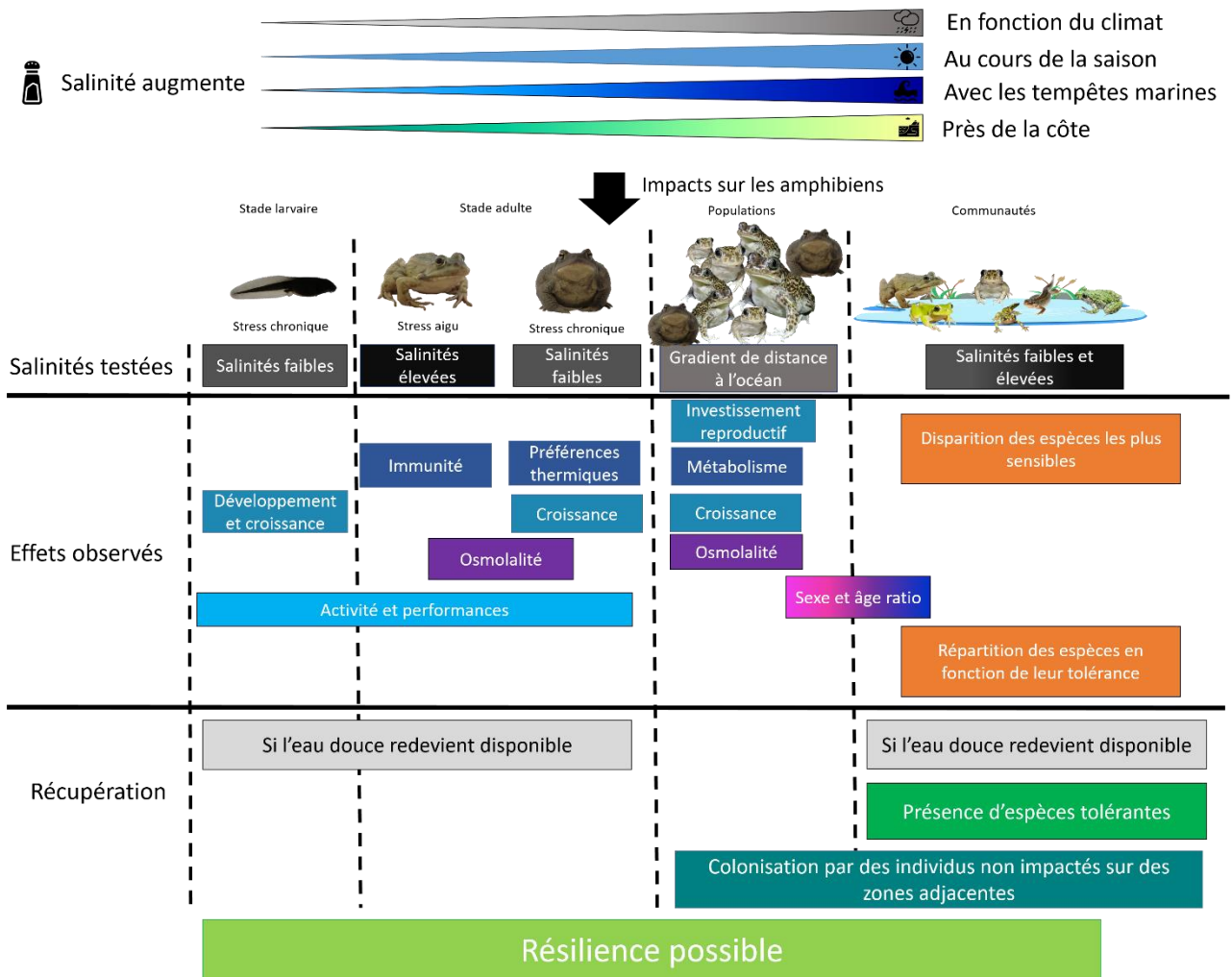
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Graphical abstract général



Conséquences de l'augmentation de la salinité sur la faune des marais littoraux : le cas des amphibiens

Résumé :

Les zones humides côtières sont caractérisées par des variations spatiales, temporelles, et liées aux changements globaux, de la salinité. Ces variations de salinité peuvent influencer négativement la biodiversité très riche des zones humides côtières. C'est particulièrement le cas pour les amphibiens, caractérisés par un tégument perméable et de faibles capacités de dispersion. Si la sensibilité des amphibiens à la salinité est bien connue, elle a majoritairement été déterminée dans le cadre du salage des routes, sur des communautés qui sont naïves à l'exposition au sel. Néanmoins, les populations côtières d'amphibiens, exposées régulièrement à ces variations de salinité, devraient présenter une tolérance à ce paramètre environnemental en réponse à des processus d'adaptation locale. Nous avons examiné comment la salinisation des milieux côtiers influence l'écologie, la physiologie, le comportement et le développement des amphibiens côtiers. A tous les niveaux d'intégration (communautés, populations, individus), nous avons montré des impacts négatifs de l'exposition au sel, affectant la composition de la communauté, la distribution des individus, leur croissance, leur développement, leur physiologie et leur comportement. Cependant, les communautés, les individus adultes, et les larves, sont tous capables de résilience physiologique et comportementale au travers de mécanismes de compensation. Nos travaux ont permis une meilleure compréhension des effets des variations de la salinité sur la biodiversité des zones humides côtières, et d'apporter des pistes de gestion pour compenser les effets de ce changement global.

Mots clés : Comportement, Développement, Milieux côtiers, Physiologie, Reproduction, Salinisation.

Consequences of increased salinity on coastal marshes' fauna : the case of amphibians

Summary :

Coastal wetlands are characterized by spatial, temporal, and global change-related variations in salinity. These variations in salinity can negatively impact the biodiversity of coastal wetlands. This is particularly the case for amphibians, characterized by a permeable integument and low dispersal abilities. Although amphibians' sensitivity to salinity is well known, it has mainly been studied in the context of road de-icing salt, in communities that are naïve to salt exposure. Nevertheless, coastal populations of amphibians, regularly exposed to these salinity variations, should express a higher tolerance to this environmental parameter in response to local adaptation processes. We investigated how salinization of coastal environments influences the ecology, physiology, behaviour and development of coastal amphibians. At all levels of integration (community, population, individual), we have shown negative impacts of salt exposure, affecting the composition of the community, the distribution of individuals, their growth, their development, their physiology and their behaviour. However, communities, adult individuals, and larvae all expressed physiological and behavioural resilience through compensatory mechanisms. Our work has led to a better understanding of the effects of salinity variations on coastal wetlands' biodiversity, and to provide management options to compensate for the effects of this global change.

Keywords : Behaviour, Coastal ecosystems, Development, Physiology, Reproduction, Salinization.



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