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Transition évolutive vers la vie marine chez les vertébrés à respiration aérienne

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Université de La Rochelle

Rapport de travaux de recherches présenté par

François Brischoux

En vue de l'obtention de

l'Habilitation à Diriger des Recherches

Transition évolutive vers la vie marine chez les vertébrés à
respiration aérienne



Soutenue au Centre d'Etudes Biologiques de Chizé (UMR7372 CNRS-ULR) le 21
Octobre 2014 devant le jury composé de :

Yann Voituron	Université de Lyon	Rapporteur
Manuel Massot	Université Pierre et Marie Curie	Rapporteur
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I. Présentation du projet de recherche

1. Cadre général : les transitions évolutives

L'histoire évolutive des organismes est ponctuée de grandes étapes. Ce constat a notamment inspiré la théorie des équilibres ponctués, évoquée par Darwin (1859), et formalisée par Eldredge et Gould (1972). Selon cette théorie, l'émergence rapide d'un trait nouveau offrirait parfois à une lignée un avantage adaptatif déterminant dans un contexte sélectif particulier (Schluter 2000). Cet avantage, à condition d'être soumis aux processus de sélection, permettrait l'émergence très rapide de nouveaux traits associés à l'utilisation de nouvelles niches écologiques. Il serait à la base de l'apparition de nouvelles espèces adaptées à des milieux, ou à l'exploitation de ressources, particuliers. Ces transitions évolutives seraient ainsi suivies de radiations adaptatives explosives (Schluter 2000). A l'appui de cette vision par grandes étapes de l'histoire évolutive des organismes, la succession des espèces se caractérise à la fois par une apparition abrupte dans le registre fossile et par une grande stabilité suite à leur apparition.

Ces transitions concernent tous les niveaux d'intégration. Par exemple, à l'échelle cellulaire, l'hypothèse de l'endosymbiose pour expliquer l'existence des mitochondries et chloroplastes est basée sur un processus relativement brutal : la capture de bactéries par d'autres organismes au cours d'une étape majeure (Margulis 1970). A plus grande échelle, l'acquisition de la vascularisation par les végétaux a été un tournant capital dans la diversification du règne végétal, notamment pour la conquête des milieux terrestres (Raven 1993). Dans le même ordre d'idée, les transitions vers la viviparité ou l'endothermie ont façonné l'histoire évolutive des vertébrés (Shine 1995, Farmer 2000).

Un défi majeur est d'identifier à la fois les moteurs évolutifs (causes) et les mécanismes proximaux sous-jacents. Par exemple, dans le cadre de l'apparition de l'endothermie chez les vertébrés (indépendamment chez les oiseaux et les mammifères), un avantage en terme de qualité des conditions de développement embryonnaire aurait favorisé l'émergence d'un maintien de température corporelle optimale, élevée et stable (Farmer 2000). Les mécanismes physiologiques de production endogène de chaleur auraient par exemple été sélectionnés. Dans ce cadre, une approche comparative de formes ectothermes et endothermes offre la possibilité d'identifier les mécanismes mis en place pour générer et maintenir cette température corporelle élevée et stable (thermogénèse). Par ailleurs, cette hypothèse suppose que si les anatomies et physiologies respectives des oiseaux et des mammifères se ressemblent plus que ce qui pouvait être attendu par hasard, c'est l'avantage reproducteur lié à l'endothermie qui aurait canalisé tout le cortège de traits morpho-fonctionnels dans la même direction dans deux groupes zoologiques distincts (Farmer 2000).

2. Les transitions "retour"

Parmi ces grandes étapes qui jalonnent l'histoire des organismes, certaines transitions revêtent un intérêt très particulier : ce sont des transitions inverses ou retour. Il s'agit de situations où les organismes retournent vers des étapes écologiques par lesquelles leurs ancêtres sont déjà passés, par exemple retour vers un milieu abandonné au cours de l'évolution (Figure 1). Si les organismes reviennent vers une position écologique ancestrale, ils ne s'y réadaptent toutefois pas dans leurs conditions d'origine. L'histoire évolutive qui les a façonnés ne leur permet d'y revenir que de manière analogue (Figure 1).

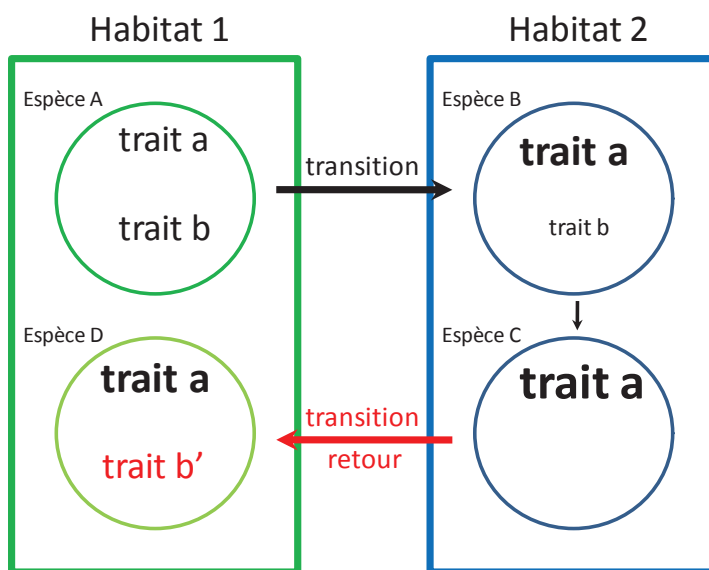


Figure 1. Exemple de transition évolutive retour. Ici, une espèce ancestrale a colonisé un nouvel habitat (habitat 2). Ce nouvel habitat à travers de nouvelles pressions de sélections a favorisé certains traits. Un retour dans le milieu ancestral (habitat 1) s'accompagne de nouvelles adaptations. Si les espèces A et D sont dans une situation homologue (même habitat), leurs adaptations sont bien différentes (analogues). La taille des caractères caractérisant chaque trait reflète la sélection (ou contre-sélection) de ce trait hypothétique dans les deux habitats.

Ces situations très particulières offrent, comme pour des transitions plus "classiques", la possibilité d'identifier les causes et les mécanismes qui accompagnent de telles étapes. Mais, l'avantage conceptuel clé de l'étude de ces situations réside dans l'homologie des pressions ancestrales et actuelles. Les mêmes causes écologiques produisent-elles les mêmes phénomènes adaptatifs ? Il est donc possible de comprendre comment des adaptations antérieures contraignent la marge de manœuvre évolutive des organismes. En conséquence, ces situations permettent de mieux comprendre la diversité de réponses évolutives à des contraintes similaires.

Par exemple, certaines études suggèrent que les ancêtres des crocodiliens actuels étaient des reptiles endothermes très actifs (voir Seymour et al. 2004). L'alternance sur une échelle de temps évolutive des modes métaboliques ectotherme-endotherme-ectotherme (transition retour) expliquerait l'existence chez cette lignée de vertébrés ectothermes de paramètres morpho-fonctionnels typiques des espèces endothermes à activité élevée (cœur cloisonné, pression sanguine élevée, architecture pulmonaire complexe, diaphragme musculaire, os fibrolamellaires, soins maternels post-nataux ; Seymour et al. 2004). Cette transition retour, et le passage par un mode métabolique différent (endothermie) a profondément façonné les crocodiliens actuels : leurs

adaptations morpho-fonctionnelles sont effectivement différentes de celles présentes chez les autres vertébrés ectothermes (Grigg & Gans 1993, Seymour et al. 2004).

3. La transition vers la vie marine chez les vertébrés à respiration aérienne

Quel que soit le type d'organisme examiné, certaines transitions offrent la possibilité d'examiner à la fois différents niveaux d'intégration. C'est typiquement le cas des grandes transitions entre habitats, qui intègrent un vaste cortège d'adaptations morpho-fonctionnelles et qui concernent tous les aspects de la physiologie, morphologie ou comportement des organismes. Cette situation offre un substrat très fertile à des investigations en écophysiologie évolutive (Mazin & de Buffrénil 1996).

J'ai choisi de proposer un projet de recherche qui repose précisément sur une grande transition entre habitats : la transition retour depuis le milieu terrestre vers le milieu marin (Mazin & de Buffrénil 1996).

Cette transition présente quatre caractéristiques cruciales. Tout d'abord, il s'agit d'une des grandes transitions-retour, cette recolonisation du milieu aquatique suit la colonisation des milieux terrestres par les formes ancestrales aquatiques des organismes terrestres actuels (Mazin & de Buffrénil 1996). Ensuite, il s'agit d'une transition évolutive très largement représentée : elle concerne un nombre important d'organismes vivants appartenant à des phylums très différents comme les plantes phanérogames, les mollusques, les arthropodes, ou les vertébrés tétrapodes (Vermeij & Dudley 2000, Figure 2). Le moteur évolutif à l'origine de cette transition pourrait être à chaque fois l'acquisition de la ressource alimentaire dans un nouveau milieu. Enfin, cette transition entre milieux est intégratrice dans le sens où elle incorpore un cortège d'adaptations morpho-fonctionnelles (morphologie, physiologie, comportement) qui permettent aux organismes de faire face aux contraintes d'un nouveau milieu radicalement différent du dernier milieu d'origine.

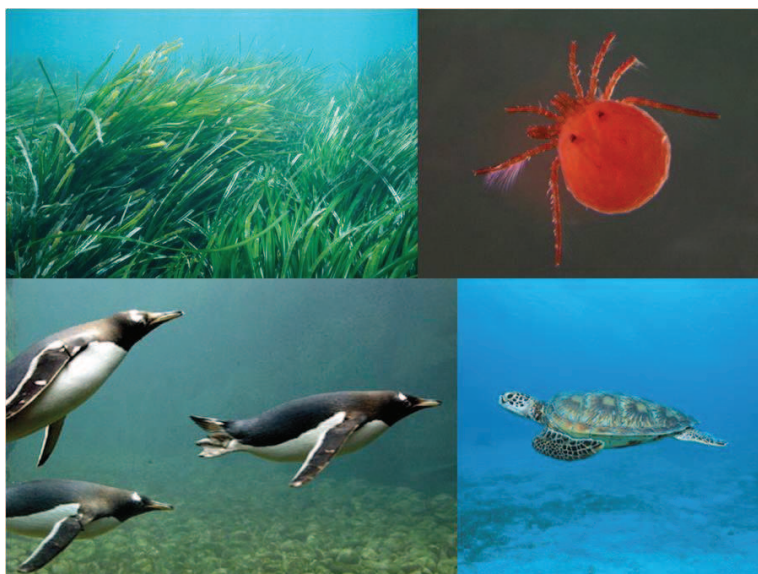
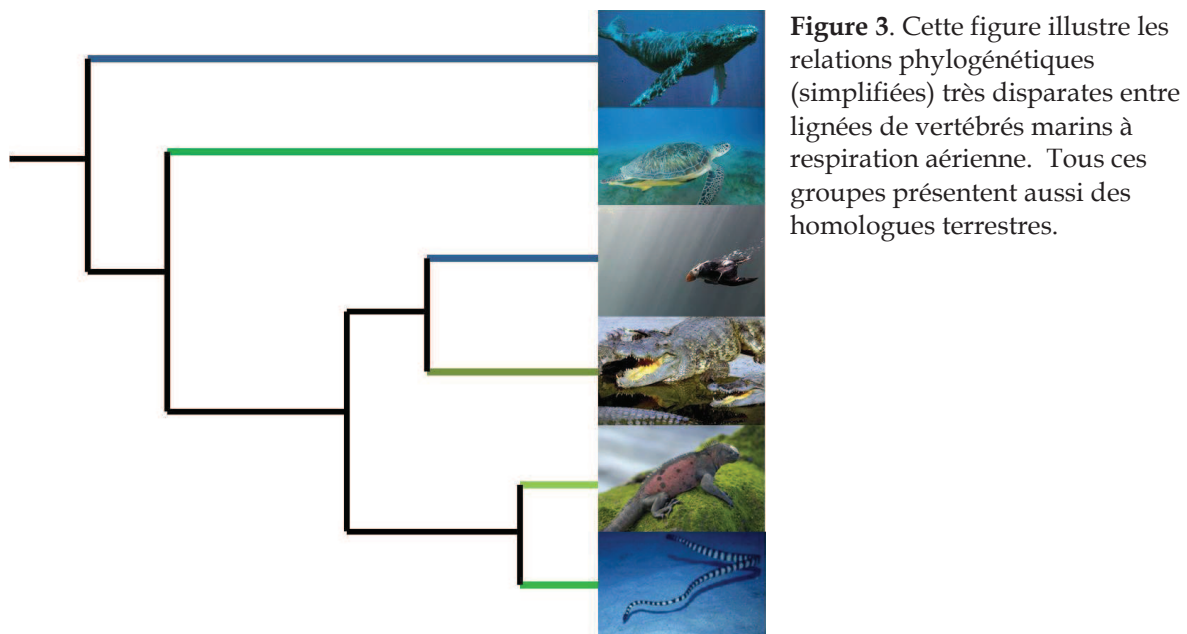


Figure 2. Quelques exemples d'espèces ayant entrepris cette transition-retour typique vers la vie marine (posidonie, acarien marin, manchots, tortue).

Parmi les grands groupes d'organismes qui ont effectué cette transition-retour, les vertébrés semblent être les mieux représentés (Vermeij & Dudley 2000, Figure 3). En comparaison aux autres groupes (plantes phanérogames et arthropodes principalement), les vertébrés présentent en effet à la fois le plus grands nombre de transitions indépendantes vers ce nouveau milieu mais aussi le plus grand nombre d'espèces utilisant le milieu marin actuellement (Vermeij & Dudley 2000). C'est sur ce groupe particulier que j'ai décidé de focaliser mon programme de recherche.

Les caractéristiques physico-chimiques du milieu marin sont très contrastées par rapport à celles du milieu terrestre. Ces différences se déclinent principalement autour de deux grands paramètres : la densité et la composition chimique. Elles imposent des pressions de sélections différentes aux organismes. En conséquence, la transition du milieu terrestre vers le milieu marin entraîne une série de modifications importantes des traits d'histoire de vie (Mazin & de Buffrénil 1996). Tout d'abord, il est remarquable de constater que les vertébrés qui sont retournés vers la vie aquatique ont conservé un mode de respiration aérienne. Ce fait illustre à quel point les transitions retours ne correspondent pas à des phénomènes d'évolution inverse (e.g., ré-acquisition d'une respiration branchiale). D'autres traits sont plus malléables, c'est le cas de nombreux comportements et de la morphologie notamment. C'est précisément sur l'équilibre entre des contraintes de paramètres physiologiques relativement rigides et les solutions éco-physiologiques plus plastiques que se concentre mon projet de recherches.



Les vertébrés marins à respiration aérienne présentent en effet des séries d'adaptations spécifiques à la vie marine (Boyd 1997, Kooyman 1989). Par exemple, le milieu marin exerce des pressions sur des attributs tels que la capacité à se mouvoir efficacement sous l'eau (et donc de poursuivre et de capturer des proies), de rester immergé pendant de longues périodes sans revenir à la surface pour respirer (et donc d'augmenter le temps passé en contact avec des proies) et de plonger à des profondeurs considérables (et donc d'augmenter la dimension de l'espace de chasse,

Kooyman 1989). Les caractéristiques morphologiques, physiologiques et comportementales qui facilitent de telles tâches sont profondément différentes de celles des organismes terrestres.

Par conséquent, les lignées de vertébrés qui ont entrepris d'exploiter le milieu marin fournissent des exemples frappants d'adaptation à la vie marine (Boyd 1997, Kooyman 1989, Butler & Jones 1997). Les informations disponibles sur certains groupes (oiseaux et mammifères principalement, Brischoux et al. 2008) montrent, que comparés à leurs homologues terrestres, ces animaux sont capables de stocker de grandes quantités d'oxygène, de réduire leur consommation d'oxygène lorsqu'ils plongent et de réduire leur susceptibilité aux pressions hydrostatiques (Butler & Jones 1997). Associées à ces adaptations à la plongée et donc à une acquisition efficace de la ressource alimentaire, ces animaux présentent aussi une morphologie hydrodynamique optimale, des membres modifiés en palettes natatoires (Fish 1998), et des structures excrétrices particulières pour maintenir leur balance hydrominérale (Schmidt-Nielsen 1998).

Les données paléontologiques ont permis de découvrir l'existence des transitions évolutives. Associées aux données génétiques, elles offrent toujours un substrat extrêmement riche pour comprendre les successions de formes qui ont permis l'invasion de nouveaux milieux. Par exemple, les vertébrés marins actuels tels que les pinnipèdes, les sphéniscidés et les cétacés partagent des caractéristiques distinctes telles qu'une morphologie hydrodynamique (fusiforme) et des membres transformés en palettes natatoires (Fish 2001). Ces formes aquatiques sont le résultat d'une série d'étapes intermédiaires partant de la morphologie ancestrale de quadrupèdes terrestres (Fish 1992). La présence dans le registre fossile de ces étapes intermédiaires permet de reconstruire les grandes étapes évolutives qui jalonnent les arbres phylogénétiques (Fish 1992).

Mais il manque de nombreux segments de l'histoire de ces transitions, en particulier ceux qui ne s'impriment pas, ou très mal dans le registre fossile. Typiquement, la physiologie, le comportement ou même l'écologie des formes intermédiaires clés restent très difficile à appréhender. Il est alors très ardu de comprendre non seulement les ajustements fins et graduels qui ont conduit aux formes actuelles ; mais surtout il est presque impossible d'identifier les pressions de sélections initiales et les traits essentiels qui ont permis aux organismes de coloniser de nouveaux milieux.

Une autre approche consiste à comparer des espèces actuelles utilisant des milieux contrastés. Par exemple, il est possible de comparer directement des mammifères ou des oiseaux marins à leurs homologues terrestres. Ce type d'investigation a permis de mettre en évidence des contrastes majeurs au niveau de la physiologie des espèces utilisant différents milieux (voir ci-dessus). Comparer des organismes différents ayant entrepris une transition similaire offre la possibilité d'identifier des convergences ou des divergences entre lignées différentes en réponse à des contraintes écologiques similaires (Figure 3). Toutefois, de telles comparaisons sont par nature limitées aux éléments actuels de continuums évolutifs généralement en grande partie constitués d'espèces éteintes. Les formes intermédiaires des

mammifères marins et des oiseaux marins ont par exemple toutes disparues, les aspects dynamiques des processus impliqués restent effacés à jamais.

De telles limites brouillent forcément l'image que l'on peut construire de ces processus évolutifs, d'autant plus que les comparaisons disponibles sont souvent limitées à quelques lignées relativement restreintes, ce qui gêne considérablement les possibilités de généralisation. Par exemple, l'étude des relations liant les capacités de plongée (duré maximum d'apnée) à la masse corporelle a permis d'identifier une relation allométrique forte entre ces deux paramètres, et ce chez des organismes aussi différents que les oiseaux, les mammifères et les tortues (Schreer and Kovacs 1997, Halsey et al. 2006a,b). En conséquence, la masse corporelle a même été proposée comme un des déterminants évolutifs clés des capacités de plongée et donc de l'évolution vers la vie marine (Halsey et al. 2006a,b). Mais en étudiant d'autres groupes zoologiques, principalement des vertébrés ectothermes aquatiques (crocodiles, iguanes, serpents, tortues), il a été montré que cette relation n'était vraie que chez les vertébrés endothermes, et que l'inclusion d'un échantillon relativement important de vertébrés ectothermes a sérieusement bouleversé un paradigme qui était trop étroitement basé sur les vertébrés endothermes (Brischoux et al. 2008).

En somme, comme souvent lorsque l'on s'intéresse à des processus ayant lieu à l'échelle des temps évolutifs, l'étude des transitions évolutives est particulièrement compliquée. Notre connaissance des processus évolutifs reste encore très limitée pour deux raisons majeures :

1. La première concerne les possibilités de généralisation des processus évolutifs connus et potentiellement impliqués dans la transition retour vers la vie aquatique. L'essentiel des connaissances acquises sur cette étape de l'évolution des vertébrés concerne un groupe très particulier de vertébrés endothermes, les oiseaux et les mammifères, et de façon plus limitée les tortues marines (Brischoux et al. 2008, Ropert-Coudert et al. 2006). Cependant les modes métaboliques endothermes et ectothermes sont extrêmement contrastés, et les contraintes qui y sont associées bien différentes (Pough 1980). Il reste tout à fait possible que la forte convergence évolutive détectée entre oiseaux et mammifères marins reste très fortement liée à l'endothermie qui impose une canalisation des traits de ces groupes d'origines différentes (Farmer 2000).

On peut s'attendre à ce que des vertébrés marins à respiration aérienne ectothermes aient suivi des chemins évolutifs différents de celui suivi par les endothermes. Notamment, par rapport aux endothermes, on s'attend à ce que cette transition-retour chez les ectothermes se soit développée à travers des processus physiologiques très économes en énergie. En outre, il est vraisemblable que les différents phylums de vertébrés marins à respiration aérienne ectothermes aient suivi des chemins évolutifs différents. Les données nécessaires pour examiner ces questions ne sont pas disponibles pour l'instant (Brischoux et al. 2008).

2. La deuxième complication concerne la difficulté à reconstruire les chemins évolutifs suivis par les différentes espèces au cours du temps. L'absence de formes intermédiaires ("chainons manquants") rend très difficile la description et la conceptualisation de la dynamique des mécanismes impliqués. Les différences observées entre homologues terrestres et marins nous montrent sans doute une partie des mécanismes par lesquels la transition a eu lieu. Mais il ne s'agit que d'une comparaison entre deux extrémités d'un continuum. Typiquement, des ajustements physiologiques, morphologiques et comportementaux fins et graduels, qui devraient être centraux et qu'il est de toutes les façons indispensable d'étudier pour comprendre les causalités mises en jeu, restent très difficiles à saisir. En l'absence de formes intermédiaires actuelles, les innovations initiales, point de départ des adaptations successives à un nouveau mode de vie, nous restent inaccessibles. L'accès à une gamme de formes intermédiaires (terrestres, semi-aquatiques, marines...) dans une lignée phylogénique donnée entre milieu terrestre et marin permettrait d'avoir accès à une image beaucoup plus dynamique des mécanismes en jeu.

L'accès à des formes intermédiaires est indispensable pour appréhender les ajustements fins et graduels qui ont accompagné les transitions évolutives (e.g., Brischox & Shine 2011). Ces formes intermédiaires sont aussi indispensables pour distinguer les différents mécanismes évolutifs sous-jacents. De telles formes intermédiaires n'ont, à ma connaissance, jamais été utilisées dans le cadre d'investigations de la transition du milieu terrestre au milieu marin.

Sur la base de ces deux constats, j'ai identifié un modèle d'étude original (squamates) qui offre la possibilité de nourrir à la fois des approches comparatives endothermes-ectothermes mais aussi ectothermes-ectothermes. Plus important, ce modèle offre une gamme de formes intermédiaires très étendue permettant d'aborder cette transition évolutive d'un point de vue dynamique.

4. Projet de recherche

4.1. Originalité du modèle

Au sein du groupe des vertébrés marins à respiration aérienne, il existe une lignée ectotherme particulièrement bien représentée : les serpents. Sur les 3000 espèces de serpents appartenant précisément au groupe des Caenophidia ("advanced snakes", ce qui exclue les espèces primitives fouisseuses), environ 225 sont totalement aquatiques et plus d'une centaine sont marines (Lillywhite et al. 2008, Figure 4). Cette diversité dans une lignée phylogénique précise pour la transition retour vers la vie aquatique est supérieure à ce qui existe chez les autres vertébrés.

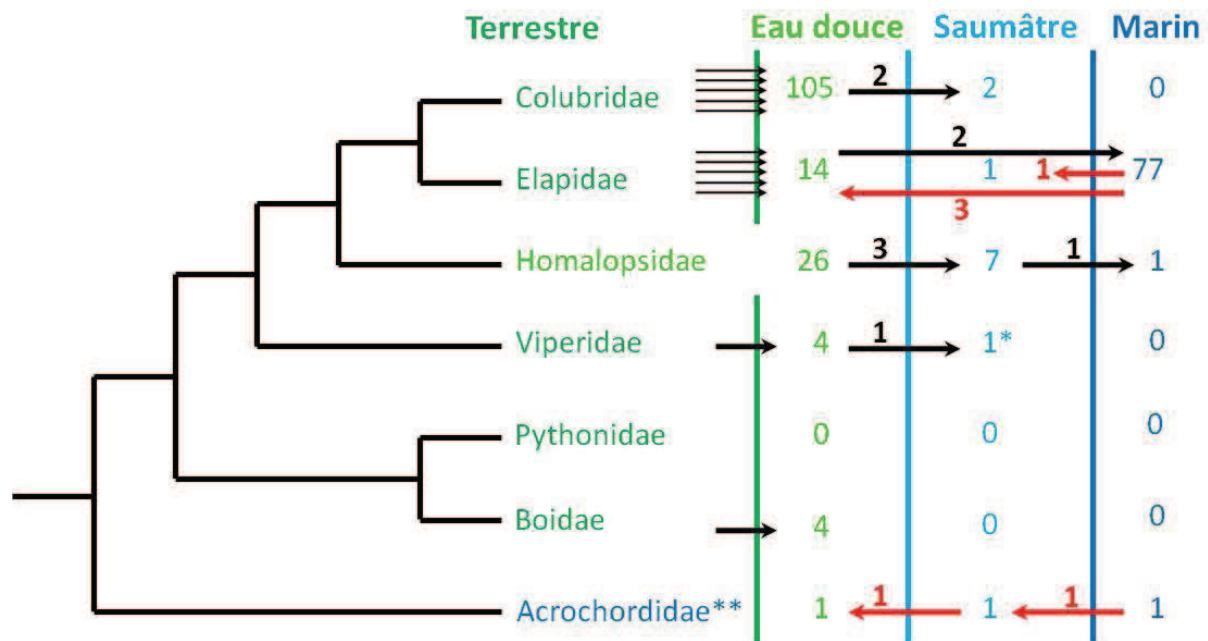


Figure 4. Cette figure illustre les transitions indépendantes multiples qui caractérisent le groupe des serpents. Elle illustre aussi la quantité de formes intermédiaires auxquelles on peut avoir accès dans le cadre de ce projet de recherche. Les nombres dans les colonnes indiquent le nombre approximatif d'espèces utilisant les différents milieux (probablement sous-estimé). Les flèches noires indiquent les transitions d'un milieu à l'autre, les nombres associés indiquent le nombre de transitions indépendantes. Les flèches rouges indiquent de nouvelles transitions inverses. * l'espèce de Viperidae saumâtre (*Agkistrodon piscivorus*) a été identifiée très récemment comme un bon candidat pour la reconstruction d'un scénario évolutif de transition vers la vie marine (Lillywhite et al. 2008). ** la famille des Acrochordidae pourrait éventuellement être un modèle atypique dans le sens où une des hypothèses actuelles évoque une forme ancestrale marine ayant donné naissance aux espèces marine, saumâtre et d'eau douce actuelles (McDowell 1979).

Le modèle serpents présente un cortège de traits particuliers, il offre des opportunités uniques d'aborder la transition entre milieu terrestre et milieu marin.

1. Les serpents ont effectué des transitions multiples et indépendantes vers tous les types de milieux aquatiques : eau douce, eau saumâtre, océan. De nombreuses espèces sont amphibies tandis que d'autres sont totalement pélagiques. Une telle diversité, unique chez les vertébrés, permet d'accéder à des formes intermédiaires sur le plan des modes de vie et de la physiologie (Figure 4).
2. Il existe une très grande diversité biogéographique de cette transition puisqu'elle concerne toutes les zones géographiques où les serpents sont présents, c'est-à-dire sur presque toutes les régions de la planète.
3. Dans des unités phylogénétiques très réduites, comme la famille ou le genre, il existe des gradients d'adaptation au milieu aquatique. Par exemple, chez les Elapidae, on trouve des espèces terrestres, des espèces dulçaquicoles, des espèces de milieux saumâtres, des espèces marines amphibies et des espèces totalement marines qui se sont totalement émancipées des liens qui les rattachaient au milieu terrestre ancestral, hormis la respiration aérienne (Figure 4).
4. Enfin, certaines espèces de serpents ont effectué de nouvelles transitions inverses. Des espèces marines ont entrepris une nouvelle transition-retour en s'éloignant des

océans pour retourner vers les milieux saumâtres et dulçaquicoles (Figure 4). Cette opportunité, unique au sein du groupe des vertébrés, offre la possibilité d'examiner une dimension supplémentaire dans le cadre de cette grande transition évolutive entre milieux.

Outre ces caractéristiques cruciales, les serpents présentent une suite de traits qui en font de très bons modèles en écophysiologie évolutive (Shine & Bonnet 2000).

5. Ces animaux sont caractérisés par une morphologie relativement simple en comparaison aux tétrapodes classiques (corps allongé, absence de membres, Gans 1975). Cette situation particulière offre un cadre simplifié pour les mesures biométriques. En conséquence, dans le cas de la transition évolutive vers la vie marine, on retrouve des espèces dont les morphologies sont extrêmement homogènes, facilitant les comparaisons entre homologues terrestres, aquatiques et marins par exemple. Des déviations subtiles par rapport à cette architecture basale étant très facilement mises en relation avec l'habitat (Aubret & Shine 2008, Brischoux et al. 2010, Brischoux & Shine 2011). Typiquement, il est possible de s'émanciper des interactions souvent très complexes qui lient les membres, leurs morphologies mais aussi l'architecture corporelle et son hydrodynamisme par exemple (Fish 2001).
6. Les serpents en général offrent une gamme étendue de tailles corporelles à l'échelle spécifique (variations interindividuelles entre adultes souvent supérieures à 50%, parfois plus de 100%). Il est donc possible d'accéder aisément aux relations allométriques qui sont à la base de nombreuses analyses morpho-fonctionnelles. Les variations allométriques intra-spécifiques sont souvent très faibles chez d'autres modèles à croissance déterminée dont la taille et la masse sont étroitement canalisées (variations presque toujours inférieures à 10%). Classiquement, ce type de contraintes impose d'approcher ces relations allométriques d'un point de vue interspécifique, en injectant dans ce type d'analyses les biais inhérents aux comparaisons d'entités phylogénétiquement disparates. Par exemple, dans le cadre de la relation qui lie la masse corporelle aux capacités d'apnée, seule l'approche interspécifique a pu être entreprise à l'heure actuelle (Schreer & Kovacs 1997, Halsey et al. 2006a,b, Brischoux et al. 2008). Cette limite pourra donc être transgressée à travers le travail proposé dans ce projet de recherche.

4.2. Hypothèse centrale

Les transitions vers la vie marine chez les vertébrés à respiration aérienne semblent liées à l'acquisition des ressources alimentaires. En fait, il s'agit même d'un des seuls points communs qui caractérisent oiseaux, mammifères, tortues, crocodiles, iguanes ou serpents. Même des formes intermédiaires amphibiennes qui maintiennent un lien fort et obligatoire avec le milieu terrestre ancestral (pour la reproduction par exemple) utilisent le milieu marin principalement pour acquérir leurs ressources alimentaires.

D'ailleurs, cette similitude suggère fortement que c'est l'acquisition de la ressource alimentaire dans un nouveau milieu (vraisemblablement en occupant de nouvelles niches, peut-être moins compétitives) qui est le principal moteur évolutif de cette transition.

Dans le cadre de mon projet de recherche, je définis cette transition comme impliquant une utilisation obligatoire et intensive du milieu marin afin d'y acquérir la ressource alimentaire. L'acquisition des ressources alimentaires doit être maximisée par la mise en place d'adaptations spécifiques permettant d'utiliser le nouveau milieu de manière efficace.

Même si ce constat n'a jamais été formalisé à ma connaissance, il est clair qu'une des caractéristiques de l'acquisition des ressources alimentaires dans le milieu marin par des vertébrés à respiration aérienne implique des niveaux élevés d'activité sur des périodes de temps longues. Ce type d'observation est corroboré par les durées de voyages en mer (dédiés à l'alimentation) mesurées chez les oiseaux et les mammifères marins par exemple (e.g., Bost et al. 2009), mais aussi par mes travaux sur le comportement de plongée chez des serpents marins amphibiens. Or les vertébrés ectothermes en général, et les serpents en particuliers sont bel et bien caractérisés par des faibles niveaux d'activité de chasse, ayant lieu sur des courtes périodes de temps, et souvent soutenus par le métabolisme anaérobie (Pough 1980).

L'hypothèse centrale de mon projet de recherche concerne donc la mise en place d'adaptations spécifiques, permettant le maintien de niveaux d'activité de recherche alimentaire intenses sur des périodes de temps longues. L'acquisition efficace des ressources alimentaires permettant en retour d'entretenir ces adaptations spécifiques coûteuses, en limitant les coûts pour d'autres activités clés telles que la croissance, la maintenance et la reproduction (Figure 5).

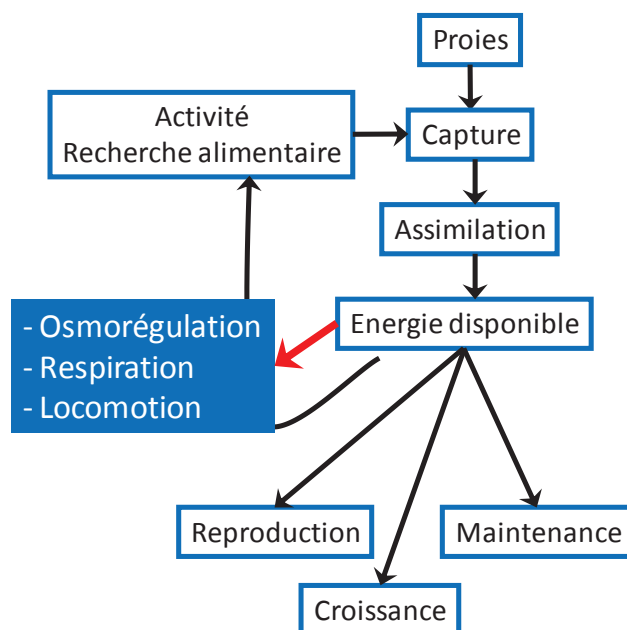


Figure 5. Illustration de l'hypothèse centrale de mon projet de recherche. La mise en place d'adaptations spécifiques concernant l'osmorégulation, la respiration et la locomotion vient supporter une activité de recherche alimentaire intense sur des périodes de temps longues au détriment d'une partie de l'énergie disponible. L'acquisition efficace des ressources alimentaires permettant en retour d'entretenir ces adaptations spécifiques coûteuses, en limitant les coûts pour d'autres activités clés telles que la croissance, la maintenance et la reproduction.

J'ai décidé d'explorer cette hypothèse en abordant les trois défis évolutifs auxquels les organismes ont dû faire face lors de la transition retour :

- a) l'osmorégulation, et donc le maintien de l'équilibre hydrominéral dans un milieu hyperosmotique ;
- b) la respiration permettant le maintien d'un métabolisme aérobie dans un milieu où l'acquisition d'oxygène nécessite des retours réguliers à la surface ;
- c) la locomotion dans l'eau en étudiant les processus de la réduction des coûts liés à une activité élevée pour progresser dans un milieu dense.

Chacun de ces volets de recherche se replace dans l'hypothèse centrale de mon projet. La diminution des coûts (recherche alimentaire) doit se faire à travers la mise en place d'adaptations spécifiques permettant le maintien d'une activité élevée en limitant les coûts pour d'autres fonctions clés telles que la reproduction, la croissance ou la maintenance. Ceci est vrai pour les trois volets de recherche envisagés : une balance hydrominérale stable doit permettre le maintien d'une activité élevée indépendamment d'un accès imprédictible à l'eau douce ; des adaptations respiratoires doivent permettre l'augmentation du temps passé en contact avec les proies (durée de plongée par exemple) ; des ajustements locomoteurs doivent permettre de diminuer les coûts liés à des déplacements extensifs dans un milieu dense.

II. Osmorégulation et transition vers la vie marine chez les tétrapodes

Dans le cadre de ce mémoire d'HDR, j'ai décidé de résumer mes travaux portant sur la gestion de l'équilibre osmotique. Les articles publiés sur ce sujet sont placés après le résumé des résultats principaux. Les autres travaux concernant mon projet de recherche (écologie alimentaire, locomotion, respiration) ou d'autres sujets sont listés au point III.

1. Résumé des travaux

L'eau de mer est hyperosmotique par rapport aux fluides corporels de la plupart des organismes. En conséquence, la plupart des espèces vont perdre de l'eau et/ou se charger en sodium à travers les surfaces perméables (Schmidt-Nielsen 1998). En addition, l'absorption d'eau de mer (inévitabile lors de la capture de proie par exemple) impose une charge en sel supplémentaire (Costa 2002, Houser et al. 2005). De fait, vivre dans l'eau de mer entraîne un risque majeur de déshydratation et d'hypernatrémie, et la plupart des vertébrés marins doivent réguler leur équilibre hydrominéral pour survivre (Schmidt-Nielsen 1998).

Les vertébrés secondairement marins présentent une diversité de structures excrétrices qui permettent d'éliminer une surcharge en sel et de maintenir l'équilibre hydrominéral dans une gamme compatible avec la vie (Schmidt-Nielsen 1998, Houser et al. 2005). Les reins des mammifères marins sont lobulés (réniculés), et les systèmes de contrecourant de leurs néphrons permettent de maintenir l'équilibre

osmotique en excréant de grande quantité d'ions dans une urine hyperosmotique (Ortiz 2001). Les reins reptiliens ne possèdent pas les anses de Henle qui caractérisent les reins des mammifères, et ils ne sont pas capables de produire une urine hyperosmotique (Peaker and Linzell 1975). Les reptiles marins au sens large (i.e., en incluant les oiseaux) possèdent des glandes à sel extrarénales capables de sécréter des solutions concentrées en sel pour maintenir leur équilibre osmotique (Peaker and Linzell 1975 ; Article I, Figure 6)

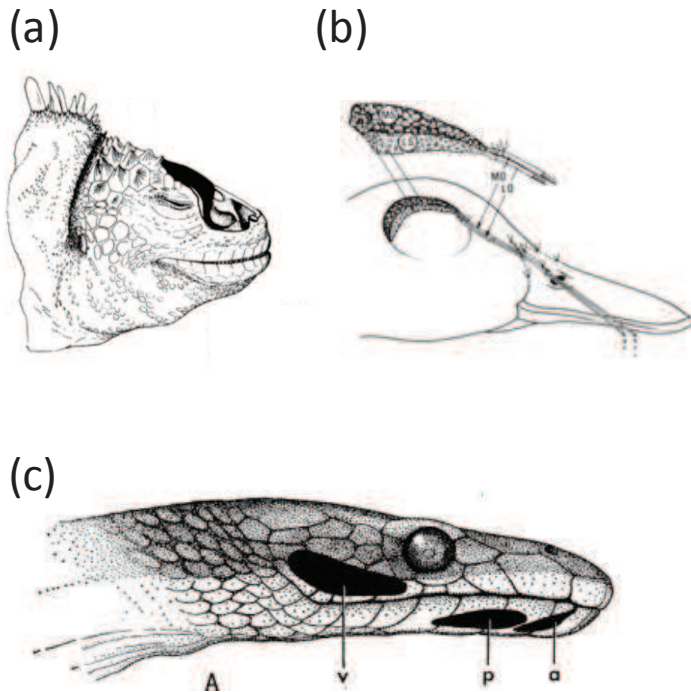


Figure 6. Glandes à sel supra-oculaires chez l'iguane marin (a) ou chez les oiseaux marins (b). Les glandes à sel semblent dérivées des glandes oculaires chez les lézards, les tortues et les oiseaux. Les serpents (glandes salivaires modifiées, c) et les crocodiles (glandes linguales modifiées) représentent des déviations par rapport à ce bauplan "classique".

Il existe très peu de restes fossiles des taxons qui ont fait la transition entre les habitats terrestres et les habitats aquatiques. Lorsqu'ils existent les fossiles ne permettent pas de clarifier des aspects cruciaux concernant la physiologie ou le comportement (Mazin et de Buffrénil 2001). Il est donc difficile de quantifier le rôle des contraintes de l'osmorégulation au cours des transitions évolutives vers la vie marine. Par exemple, la présence de glandes à sel chez les reptiles marins disparus reste un sujet très débattu (Witmer 1997, Modesto 2006, Young et al. 2010, mais voir Fernández and Gasparini 2008). En plus, les caractéristiques morphologiques seules ne permettent pas d'obtenir des réponses univoques sur les fonctions. Par exemple, les reins lobulés caractéristiques des mammifères marins sont également présents chez les ongulés terrestres (Houser et al. 2005). D'autre part, les glandes à sel existent également chez beaucoup d'oiseaux terrestres et chez des crocodiliens d'eau douce (Babonis and Brischoux 2012).

Les serpents offrent l'opportunité de clarifier le rôle des contraintes liées à l'osmorégulation durant la transition vers la vie marine. Cette lignée présente une combinaison rare de caractéristiques qui permettent de contourner la plupart des limitations expliquées ci-dessous. Tout d'abord, 4 lignées phylogénétiques de serpents ont effectué la transition vers la vie marine indépendamment, et ces 4

lignées appartiennent à 3 familles (Homalopsidae, Acrochordidae ; et au sein des Elapidae, les sous-familles Laticaudinae et Hydrophiini [Heatwole 1999]). Toutes ces transitions indépendantes montrent une évolution convergente des glandes à sel, alors qu'aucun serpent terrestre ou aquatique ne possède de telles adaptations (Babonis and Brischoux 2012). Ensuite, le grand ratio surface/volume imposé par la morphologie des serpents (Brischoux and Shine 2011) fait du maintien de l'équilibre osmotique un défi physiologique majeur pour les serpents marins. Par ailleurs, ces lignées de serpents marins se situent le long d'un continuum d'émancipation de l'environnement terrestre ancestral et couvrent une grande variété de stades écologiques entre la terre et les océans (Heatwole 1999). Certaines espèces sont parmi les tétrapodes les plus marins, complètement indépendant de l'environnement terrestre, alors que d'autres dépendent de cet environnement ancestral pour accomplir de nombreuses activités. Enfin, beaucoup d'espèces d'eau douce sont connues pour utiliser fréquemment des eaux saumâtres ou salées (Murphy 2012); et permettent d'accéder à des stades précoces le long du continuum évolutif entre la terre et les océans (i.e., des chaînons manquants qui font défaut dans les autres lignées de tétrapodes marins). Cette combinaison de traits fait des serpents un modèle particulièrement pertinent pour explorer les contraintes physiologiques liées à la salinité océanique au cours de la colonisation des environnements marins par des vertébrés terrestres.

Les serpents en tant que vertébrés ectothermes font preuve d'une très grande plasticité, mais aussi d'une très grande résistance face à des variations de leurs paramètres physiologiques (Pough 1980, Bradshaw 1997). Les serpents sont capables de faire face à des déviations de leurs paramètres physiologiques (plasmatiques par exemple) sans encourir d'effets pathologiques brutaux et/ou immédiats. Le maintien de leur balance hydrominérale est très probablement une contrainte forte limitant la capacité des serpents à conquérir le milieu marin (Dunson 1975). Par contre, leur capacité à résister à des déviations de ce paramètre sans effet pathologique brutal leur permet probablement d'utiliser le milieu marin sans mettre en place des adaptations complexes.

Pour examiner ces questions au niveau de situations écologiques qui pourraient refléter les différentes étapes évolutives entre les environnements terrestre et marin, j'ai examiné trois groupes de serpents.

1) Un espèce de serpent amphibie, d'eau douce, européenne : la couleuvre tessellée *Natrix tessellata* (Figure 7). La couleuvre tessellée a une distribution Paléartique étendue de l'Europe centrale à l'Égypte du Nord jusqu'à la Chine Occidentale. C'est un Natricinae typique qui se nourrit de poissons et d'amphibiens dans les cours d'eau, les rivières et les lacs. Bien qu'elle ne possède pas de glande à sel, certaines populations sont présentes dans des environnements saumâtres ou salés le long des côtes de la mer Adriatique, des mers Ionienne et Égée, de la mer noire et de la mer Caspienne. C'est sur une population côtière de la mer Noire en Bulgarie que j'ai travaillé.

2) Deux espèces de serpents marins amphibies : les tricots rayés *Laticauda laticaudata* et *L. saintgironsi* (Figure 7). Les tricots rayés sont des serpents marins qui ne se sont

pas totalement émancipés de l'environnement terrestre ancestral. Ils cherchent et capturent leur proie (principalement des murènes et des congres) dans les récifs coralliens mais reviennent à terre pour toutes les autres activités (digestion, mue, reproduction, etc.). Ils possèdent des glandes à sel fonctionnelles, et les deux espèces étudiées se situent le long d'un continuum d'utilisation de l'habitat : *L. saintgironsi* est plus terrestre que *L. laticaudata*. J'ai travaillé sur ces deux espèces en Nouvelle Calédonie.

3) Deux espèces de serpent totalement marin : le serpent marin à tête de tortue *Emydocephalus annulatus* et le serpent marin à ventre jaune *Pelamis platurus* (Figure 7). Ces espèces sont totalement émancipées du milieu terrestre ancestral et ne reviennent jamais à terre. *Emydocephalus annulatus* se nourrit d'œufs de poissons coralliens et j'ai travaillé sur cette espèce en Nouvelle Calédonie. *Pelamis platurus* est totalement pélagique, présent dans la totalité des Océans Indien et Pacifique tropicaux, et j'ai travaillé sur une population de la côte Pacifique du Costa Rica.



Figure 7. Illustration des espèces de serpents sélectionnées dans le cadre de ce projet. En haut à gauche, une couleuvre tessellée (*Natrix tessellata*) dans la mer Noire. En haut à droite, un tricot rayé jaune (*Laticauda saintgironsi*) en recherche alimentaire dans le lagon calédonien. En bas à gauche un serpent marin à tête de tortue (*Emydocephalus annulatus*) explorant les fonds corallien en Nouvelle Calédonie. En bas à droite un accouplement de serpents marins à ventre jaune (*Pelamis platurus*) à la surface de l'océan.

En accord avec la prédiction ci-dessus, les résultats que j'ai obtenus montrent que les individus d'une population côtière de couleuvres tessellées sont régulièrement en hypernatrémie (Article II), sans effet apparent sur plusieurs traits physiologiques ou comportementaux (e.g., hémocrite, condition corporelle ou recherche alimentaire).

Par contre, de manière contre-intuitive, même des espèces de serpents marins, possédant des glandes à sel fonctionnelles, sont également régulièrement en hypernatrémie. Les travaux que j'ai menés sur les tricots rayés de Nouvelle Calédonie, des serpents marins amphibies révèlent que ces animaux présentent également des taux de sodium circulant situés bien au-dessus des valeurs de normonatrémie (Article III).

En fait, les données de natrémie publiées dans la littérature sur de nombreuses espèces de serpents (d'eau douce, ou marines) suggèrent que l'apparition de la glande à sel ne signifie pas une régulation fine et précise des taux de sodium circulant (Article II & III). Ensemble, ces résultats suggèrent que la mise en place d'une tolérance physiologique à l'hypernatrémie a été cruciale au cours de l'évolution d'une physiologie euryhaline, et qu'elle a probablement précédé l'apparition des glandes à sel.

Grâce à cette tolérance accrue à l'hypernatrémie, la sécrétion de sodium par les glandes à sel n'interviendrait que lorsque la natrémie dépasserait des seuils élevés (e.g., entre 170 et 200 mmol.l⁻¹ chez *P. platurus*, Dunson et al. [1971]).

L'hypothèse majeure sous-jacente est que la restriction de la sécrétion active de sodium représente un moyen important pour économiser de l'énergie chez les "low-energy specialists" que sont les serpents (Pough, 1980). Si le fonctionnement coûteux des glandes à sel n'intervient que lorsque le sodium dépasse dangereusement des seuils élevés, cela permettrait de réduire substantiellement les coûts liés au fonctionnement continu des glandes à sel (Peaker and Linzell, 1975; Gutiérrez et al., 2011); ces coûts représentant probablement une dépense d'énergie qui serait excessive pour la survie de ces organismes (Pough, 1980).

En support à ces résultats, les observations concernant des espèces de serpents de milieux saumâtres ou marins montrent que pour rétablir cette balance osmotique, ces animaux sont capables de profiter d'une ressource indispensable, l'eau douce (Article III et IV). Cette déshydratation est probablement liée à la combinaison de deux processus différents : le gain de sel ou la perte en eau.

Les serpents marins amphibies (ou tricots rayés, *Laticauda* spp.) bénéficient de l'eau douce lors de leur retour à terre où celle-ci est relativement aisée à acquérir (Article III). Néanmoins, les serpents capturés sur le terrain, notamment en période estivale présentent des taux de natrémie élevée et boivent l'eau douce de manière frénétique quand celle-ci est présente (Article III).

Les serpents totalement marins (Hydrophini) ne retournent jamais à terre. L'acquisition d'eau douce est donc probablement problématique pour ces animaux. Comme les tricots rayés, ces animaux boivent abondamment l'eau douce lorsqu'elle est présente (Article IV & V). En fait, il semble même que certaines espèces passent au moins 6 à 7 mois de l'année dans un état de déshydratation (ou d'hypermatrémie) et qu'elles ne bénéficient de l'accès à l'eau douce que pendant la saison des pluies au cours de laquelle des précipitations violentes permettraient l'existence transitoire et localisée de lentilles d'eau douce à la surface de l'océan (Article V).

Que ce soit pour les espèces amphibies ou les espèces marines, le rétablissement de l'équilibre osmotique dépend très largement des conditions climatiques locales. Lors de périodes sèches, non seulement l'accès à l'eau douce est encore plus précaire mais également la salinité océanique augmente (Article VI). La condition corporelle des serpents marins répond de manière forte à ces variations que ce soit pour des espèces

amphibies ou des espèces totalement marines (Article VI). Evidemment, outre les coûts liés au fonctionnement même transitoire des glandes à sel, cette faible condition corporelle doit avoir un impact fort sur la croissance, la survie et la reproduction de ces espèces, et donc pourrait influencer la persistance des populations.

En plus de ces processus à petite échelle temporelle, cette contrainte osmotique a probablement des implications évolutives très fortes.

La capacité des serpents marins amphibies (*Laticauda* spp.) à acquérir l'eau douce à terre et à tolérer la déshydratation et l'hypernatrémie déterminent ensemble leurs tolérances environnementales et leurs distributions géographiques (Article VII).

Ce résultat montre que les patrons de spéciation au sein de ce groupe ont été influencés par les variations interspécifiques de leur sensibilité à une salinité élevée combinée au degré d'utilisation de l'environnement marin (Article VII).

Plus généralement, ces résultats suggèrent que les contraintes osmotiques ont joué un rôle dans la diversification des tétrapodes marins.

Enfin, à une échelle plus large, des analyses de la distribution des quatre lignées de serpents marins montrent que la salinité océanique contraint leur distribution actuelle. Ceci est d'autant plus fort pour des espèces qui doivent ressembler à des formes de transitions précoces (e.g., espèces amphibies, Article VIII). Au niveau spécifique, des glandes à sel plus efficaces permettent à une espèce d'exploiter des zones océaniques plus salées et donc plus grandes (Article VIII).

La salinité apparaît comme le prédicteur le plus robuste de la richesse spécifique des serpents marins. Cette richesse spécifique est négativement liée à la salinité moyenne annuelle, mais positivement liée à sa variation mensuelle (Article VIII).

Il a longtemps été admis que les tétrapodes marins (i.e., mammifères, oiseaux, tortues, serpents, lézards et crocodiles) pouvaient réguler leur natrémie grâce à des structures excrétrices spécialisées et pouvaient maintenir leur balance osmotique sans consommer d'eau douce (Randall et al. 2002, Houser et al. 2005). Ce dogme apparaît maintenant plus fragile, au moins chez les serpents pour lesquels les données récemment acquises suggèrent que même des espèces marines (avec des glandes à sel fonctionnelles) ne peuvent réguler leur balance osmotique sans accès à l'eau douce. En ouvrant ces travaux aux oiseaux, j'ai pu montrer que ces contraintes s'appliquent également avec force à d'autres lignées de tétrapodes marins (Article IX).

En fait, il semble bien que les contraintes éco-physiologiques et évolutives de la salinité océanique aient largement été négligées jusqu'à présent ; et concernent très probablement la plupart des espèces de tétrapodes marins. Ce constat ouvre des champs de recherches féconds à explorer.

2. Articles



SYMPOSIUM

Perspectives on the Convergent Evolution of Tetrapod Salt Glands

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Synopsis Since their discovery in 1958, the function of specialized salt-secreting glands in tetrapods has been studied in great detail, and such studies continue to contribute to a general understanding of transport mechanisms of epithelial water and ions. Interestingly, during that same time period, there have been only few attempts to understand the convergent evolution of this tissue, likely as a result of the paucity of taxonomic, embryological, and molecular data available. In this review, we synthesize the available data regarding the distribution of salt glands across extant and extinct tetrapod lineages and the anatomical position of the salt gland in each taxon. Further, we use these data to develop hypotheses about the various factors that have influenced the convergent evolution of salt glands across taxa with special focus on the variation in the anatomical position of the glands and on the molecular mechanisms that may have facilitated the development of a salt gland by co-option of a nonsalt-secreting ancestral gland. It is our hope that this review will stimulate renewed interest in the topic of the convergent evolution of salt glands and inspire future empirical studies aimed at evaluating the hypotheses we lay out herein.

Introduction

Discovered by Schmidt-Nielsen et al. (1958), the physiology of tetrapod salt glands has been studied in great detail. Over the past several decades, much has been learned about the basic mechanisms by which these cephalic glands facilitate the net secretion of concentrated NaCl (or KCl, in some herbivorous taxa), and there have been several thorough reviews summarizing these data (Peaker and Linzell 1975; Gerstberger and Gray 1993; Shuttleworth and Hildebrandt 1999; Hildebrandt 2001; Dantzler and Bradshaw 2009; Holmgren and Olsson 2011). Building on this foundation, recent studies of tetrapods' salt glands have taken the form of comparisons among closely related marine and freshwater species (Bennett and Hughes 2003; Babonis and Evans 2011), the role of water-regulatory proteins in modulating the secretory output of the glands (Muller et al. 2006; Babonis and Evans 2011), variation in the composition of the secretion (Butler 2002), the modulation of secretion by various endocrine and neurological agents (Reina et al. 2002; Krohn and Hildebrandt 2004; Franklin et al. 2005; Hughes

et al. 2006; Butler 2007; Cramp et al. 2007; Hughes et al. 2007; Cramp et al. 2010), phenotypic plasticity of the form and function of salt glands under various environmental conditions (Cramp et al. 2008; Babonis et al. 2009; Gutierrez et al. 2011), the combined osmoregulatory function of salt glands and other organs (Hughes 2003; Lavery and Skadhauge 2008; Babonis et al. 2011), and several recent reports of bacterial infections of salt glands (Klopfleisch et al. 2005; Brito-Echeverria et al. 2009; Suepaul et al. 2010; Oros et al. 2011). Interestingly, although the basic physiology of these glands has been quite well characterized, there have been relatively few hypotheses about the convergent evolution of this specialized tissue across taxa (but see Peaker and Linzell 1975).

The ability of salt glands to secrete concentrated salt solution and the taxonomically wide-spread association between the use of desiccating habitats and the possession of functional salt glands in tetrapods suggest that this tissue may have been critical in facilitating the invasion (or re-invasion) of desiccating environments during the evolution of tetrapods

(Brischoux et al. 2012). Despite this, the past 50 years of research have seen only few hypotheses regarding the potential mechanisms that may have led to the convergent evolution of this gland across diverse taxa (Dunson and Dunson 1973; Peaker and Linzell 1975; Taplin et al. 1982; Babonis and Evans 2011). This paucity of hypotheses regarding the convergent evolution of tetrapod salt glands undoubtedly lies in the lack of several important types of data, notably (1) a thorough catalog of the presence/absence of salt glands from extinct and extant taxa (from which to infer the number of times salt glands have originated), (2) information about the homology of salt glands alternatively named “pre-orbital,” “supraorbital,” and “nasal” (see Technau 1936), as evidenced through the embryological origin of these glands, and (3) a mechanism by which a gland with a salt-secreting function may have evolved by co-option from an ancestral gland with another function. In this review, we attempt an initial remedy to this situation by (1) providing an exhaustive list of the extinct and extant tetrapod taxa currently known to have salt glands (as well as information about the anatomical position of the salt gland in these taxa), (2) summarizing the known embryology of glands from representative taxa, and (3) synthesizing the literature regarding the molecular development of cephalic glands from model systems. We then use these combined results to propose mechanisms by which salt glands may have evolved, independently, across diverse tetrapod taxa and present a call for future empirical studies aimed at testing the hypotheses we lay out herein. Since this review is largely speculative, we believe it is important to start by clearly laying out our assumptions about tetrapod salt glands.

Assumptions

Salt glands are so-defined because they secrete a product that is more concentrated in inorganic salts (NaCl or KCl) than is the blood plasma. Although there is diversity (and in some cases, plasticity) in the type of inorganic salt secreted by salt glands (particularly among lizards), for the purposes of this review, we do not distinguish among glands of different secretory types and merely refer to all such glands as “salt glands.”

Salt glands have evolved independently, multiple times throughout the evolution of tetrapods. We, parsimoniously, assume that the minimum number of independent origins is represented by the number of unique anatomical positions occupied by salt glands across taxa (e.g., “nasal,” “lacrimal,” and

“sublingual” glands represent a minimum of three origins); however, we acknowledge that the actual number of origins may well have been much greater than this (i.e., gain of a nasal salt gland followed by loss of this gland and another independent gain would be indistinguishable from a single-gain scenario in the absence of robust fossil data).

Salt glands are not unique/novel glands, they simply have a unique/novel form/function when compared with other cephalic glands in the same species. Indeed, although salt glands are present in marine (and some desert) taxa, the homologous gland in the nonmarine sister taxon is present but not specialized for the secretion of salt. Since the homologous position in a nonmarine sister taxon is occupied by a gland with a nonsalt-secreting function, convergent evolution of salt glands has likely resulted from the repeated co-option of various existing (unspecialized) glands rather than *de novo* organogenesis.

Anatomy of salt glands in tetrapods

Across diverse tetrapod taxa (see Supplementary Table S1 for an exhaustive list of the tetrapod taxa that have been reported, thus far, to have salt glands), the anatomy of cephalic salt glands is largely consistent (Babonis et al. 2009). This tissue comprises a mass of secretory tubules that terminate blindly (i.e., without secretory acini); thus, they are called compound tubular glands. The secretory tubules are separated by vascularized connective tissue and are arranged radially around the perimeter of a central duct. Together, these structures constitute an individual lobule of the gland; multiple such lobules in association are joined by the connection of their central ducts to a main duct, the conduit whereby secreted salts exit the body (for illustrations, see Schmidt-Nielsen 1960). Unlike other types of cephalic glands, the secretory epithelium of salt glands is populated almost exclusively by salt-secreting principal cells, as exemplified by marine snakes (Dunson et al. 1971; Dunson and Dunson 1974; Babonis et al. 2009). Where variation does exist (e.g., in the salt glands of some turtles and lizards) (Abel and Ellis 1966; Cowan 1969; Van Lennep and Komnick 1970), the various cell types present in the gland are scattered throughout the secretory epithelium rather than being confined to single-function units like the mucus acini versus the serous acini of some mixed-function salivary glands. Although the size of these glands across taxa has been hypothesized to vary with the degree of marine tendency (i.e., the time spent in a marine

habitat and/or the osmolality of the food items) in birds (Technau 1936; Holmes et al. 1961; Staalnd 1967; Ernst and Ellis 1969), lizards (Hazard et al. 1998), turtles (Holmes and McBean 1964; Cowan 1969; Dunson 1970), crocodiles (Taplin 1985; Cramp et al. 2008), and snakes (Dunson and Dunson 1974, 1979), the basic tubular morphology of this tissue appears largely invariant across taxa. Interestingly, not all tetrapods inhabiting desiccating environments have a salt gland (see Supplementary Table S2 for a list of species that have been reported to lack a salt gland) suggesting much remains to be learned about the relationship between environmental constraints and salt-gland function in tetrapods.

Distribution and nomenclature of salt glands in tetrapods

Although there are many glands present in the head of the idealized tetrapod (Fig. 1A), only one (or one pair, for paired glands) is the salt gland in any given taxon (Fig. 1B). The anatomical position of the salt gland(s) in tetrapods varies quite extensively among lineages, and three main cephalic areas are currently recognized (1) nasal glands in extinct archosaurs, extant birds, and lizards, (2) orbital glands in turtles, and (3) oral glands in extant crocodiles and snakes (Supplementary Table S1). Interestingly, those glands typically described as “nasal” can vary in location from the vestibule of the nostril, (Fig. 1C, I) to small preorbital structures, midway between the nostril and the orbit (Fig. 1C, II), to the supraorbital position exemplified by the salt gland in the marine iguana, and many marine birds (Fig. 1C, III). This variation in the anatomical location of the body of the gland has resulted in variation in the nomenclature of the gland (Technau 1936) and has contributed to confusion about the homology of this gland across taxa (see later for more details on the homology of these glands). Interestingly, salt glands housed in the frontal region of the cranium are the most widespread among tetrapod lineages.

Orbital salt glands are found only in chelonians and occur in two phylogenetically divergent lineages: the sea turtles (Cheloniidae and Dermochelyidae) (Schmidt-Nielsen and Fänge 1958; Hudson and Lutz 1986) and the diamondback terrapin, *Malaclemys terrapin* (Emydidae) (Schmidt-Nielsen and Fänge 1958). Although the morphology and the function of the lachrymal glands (and their ducts) have been well characterized for turtles (Ellis and Abel 1964; Abel and Ellis 1966; Cowan 1969; Marshall 1989; Marshall and Saddler 1989), the

identity of the chelonian salt gland has been an intense subject of debate. Historically, this gland has been dubbed the nasal gland (Benson et al. 1964; Holmes and McBean 1964), the lachrymal gland (Abel and Ellis 1966) and the Harderian gland (Dunson and Taub 1967; Dunson 1969; Chieffi-Baccari et al. 1992, 1993). Although some debate still exists regarding the nomenclature of the salt-secreting glands in chelonians (Chieffi-Baccari et al. 1992, 1993), most researchers in this field still consider them to be modified lachrymal glands (Belfry and Cowan 1995; Lutz and Musick 1997; Hirayama 1998; Reina and Cooper 2000; Oros et al. 2011), and we will refer to them here as such. Although salt glands have not been reported officially in either flatback sea turtles (*Natator depressus*) or Kemp's Ridley sea turtle (*Lepidochelys kempii*), the presence of osteological characteristics consistent with large lachrymal glands in extinct chelonian sea turtles (Hirayama 1998), combined with a recent study of the phylogenetics of sea turtles (Naro-Maciel et al. 2008), suggests that salt glands are ancestral in this group.

Oral salt glands have evolved independently in at least two lineages of tetrapods: extant crocodylians and snakes. Among crocodylians, lingual salt-secreting glands were originally identified by Taplin and Grigg (1981) in the tongue epithelium from *Crocodylus porosus* and have since been identified in all species of the Crocodylidae that have been studied (Supplementary Table S1), including the freshwater species (Taplin et al. 1985). Interestingly, the other two lineages of extant crocodylians (alligatorids and gavialids) appear to have (presumably homologous) lingual glands that lack the capacity to produce a hypertonic salt secretion (Taplin et al. 1985). These observations suggest that either lingual salt glands evolved in the ancestor to all modern crocodylians, but the concentrating capacity was lost in modern alligatorid and gavialid lineages or that functional salt glands evolved by modification of unspecialized lingual glands after the crocodylids split from the alligatorid and gavialid lineages (crocodylian relationships after Man et al. 2011).

Among snakes, salt glands have evolved at least four times in lineages that have independently undergone an evolutionary transition to marine life: the file snakes (Acrochordidae) (Dunson and Dunson 1973), rear-fanged water snakes (Homalospidae) (Dunson and Dunson 1979), and, within the Elapidae, two lineages of sea snakes (Laticaudinae and Hydrophiinae) (Dunson et al. 1971). Similar to the crocodylians, all these lineages

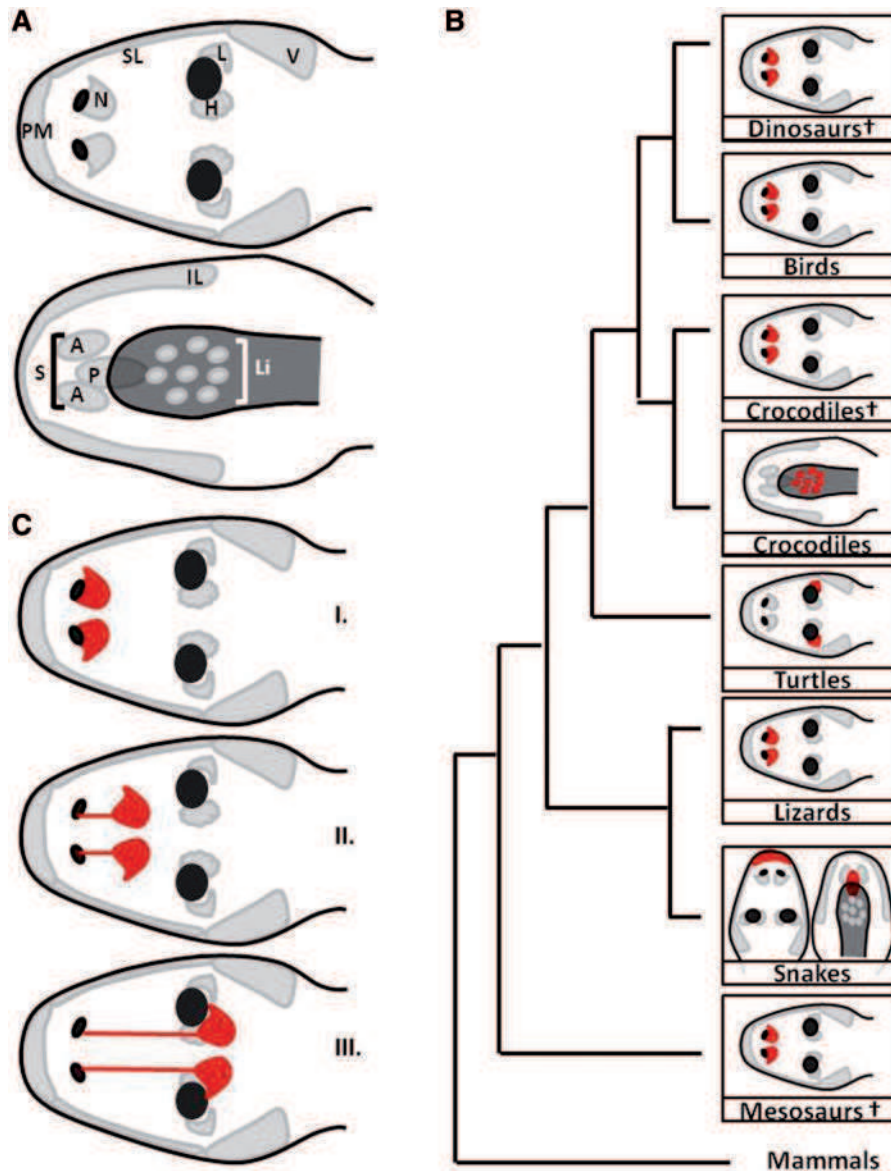


Fig. 1 Cephalic glands in the tetrapod lineages listed in Supplementary Table S1. **(A)** An idealized tetrapod exhibiting all possible cephalic glands. Top-down view with anterior to the left and posterior to the right. The cranium/maxilla is pictured on the top, and the mandible/lower jaw is pictured on the bottom. Small black ovals are nostrils, and large black ovals are eyes; glands are outlined in dark grey and filled with light grey. **(B)** Salt glands are present in representatives of each of the pictured lineages and occupy the gland in each lineage highlighted in red. Among snakes, salt glands have been identified in two different locations; however, each species of snake with a salt gland has only one of these. **(C)** An evolutionary scenario to illustrate how traditionally defined “nasal” glands (highlighted in grey) might have migrated from a position near/in the nostril (I) to either a “preorbital” (II) or “supraorbital” (III) position. The length of the duct differs in each of these scenarios, resulting in a different cranial location of the body of the gland. A, anterior (sublingual glands); H, harderian gland; IL, infralabial gland; L, lachrymal gland; Li, lingual glands; N, nasal gland; P, posterior (sublingual gland); PM, pre-maxillary gland; S, sublingual gland(s); SL, supralabial gland; T, tongue; V, venom gland. †An extinct lineage.

evolved oral salt glands: acrochordids, laticaudines, and hydrophines have a posterior sublingual salt gland located in the lower jaw beneath the tongue casing, whereas the homalopsids have a pre-maxillary salt gland. It is noteworthy that despite their relatively close ancestry with lizards, snakes followed independent evolutionary pathways leading to their

convergence on salt glands; no snakes studied thus far have a salt gland that is homologous with the nasal gland of lizards.

Embryology and homology

Glands occupying distinct cephalic positions (e.g., the lachrymal salt glands of turtles, lingual

glands in extant crocodiles, and sublingual and pre-maxillary glands in snakes) are clearly not homologous with salt glands in any other taxon. Thus, these examples represent a minimum of four independent convergent evolutionary events. The case is not as clear for the “nasal” salt glands of extinct archosaurs, modern birds, and modern lizards. Indeed, the homology of the “nasal,” “pre-orbital,” and “supraorbital” glands has been questioned (Dunson 1969), likely because of the dramatic variation in the position of the body of the gland in the cranium. From embryological studies of various bird taxa, it is known that nasal glands develop initially as an outgrowth of the nasal epithelium (Marples 1932). This primordial bud develops into the distal-most portion of the duct and grows posteriorly to the position where the body of the gland is to develop. From there, the body of the gland expands from the posterior end of the duct. If this developmental scheme is also true of nasal-gland development in extinct archosaurs (as proposed by Fernandez and Gasparini 2000; Gandola et al. 2006) and modern lizards (as yet, unstudied), it can be assumed that all glands (independent of where the mature body of the gland lies) that develop from an outgrowth of the nasal epithelium are homologous. Following these assumptions, it is reasonable to assume that the diversity of modern “nasal” glands (this term now includes those glands alternatively labeled “preorbital” and “supraorbital”) is simply a result of variation in the length of the duct of the nasal gland, resulting in a gland body that may be housed anywhere from the nostril to the supraorbital position. Although it is possible that the “nasal” salt glands of lizards are not homologous with the “supraorbital” glands of birds, we find this to be unlikely. Only detailed embryological studies of cephalic glands in lizards and birds will resolve this issue. Considering that salt glands have been positively identified in representatives of at least 8 of the 26 currently recognized families of lizards (Vidal and Hedges 2009), in at least 40 families of birds (Supplementary Table S1) representing nearly all orders of birds except the Passeriformes (Hackett et al. 2008), and several lineages of extinct crocodylians and dinosaurs, nasal salt glands may indeed be an ancestral characteristic in the diapsids.

There have been several detailed embryological studies of turtles (Ewert 1985, and references therein) including marine turtles (Miller 1985, and references therein), yet the embryology of the lachrymal gland does not appear to have been described. Despite this, all lineages (extinct and extant) of turtles have evolved salt glands in the position of the lachrymal

gland (but see Chieffi-Baccari et al. 1993). Considering that the ducts of the lachrymal glands in those turtles that have been studied all open in the same location (onto the lateral portion of the nictitating membrane) (Cowan 1973), all the glands identified as “lachrymal” among turtles are, indeed, likely homologous. The phylogenetic distance between modern lineages exhibiting salt glands (sea turtles and terrapins) makes it difficult to assess whether salt glands evolved twice among turtles (both times in the position of the lachrymal gland) or whether the lack of salt-secreting abilities of this gland among other turtles represents loss of the lachrymal salt gland subsequent to its origin in the ancestor to all turtles. Evidence of large interorbital foramina (Hirayama 1998) in the skulls of fossil emydine turtles would be suggestive of the presence of salt glands in these taxa and provide more support for a single origin of salt glands among turtles.

Embryological studies of species with oral salt glands are also lacking. The lingual salt glands of crocodylians are reported to develop from the dorsal epithelium of the tongue (Ferguson 1985), but no other data on the generation of the secretory tubules or the onset of secretory-cell identity are available. Comparative studies of lingual-gland development in alligators (or gavials) and crocodiles, with special focus on the acquisition of a salt-secreting function, would be particularly useful for understanding the molecular mechanisms that underlie convergence. Similarly, among snakes, there have been no developmental studies of either the sublingual or pre-maxillary glands. As such, we cannot distinguish between two possible scenarios among snakes that salt glands evolved multiple times (once as the sublingual gland in the file snakes, at least once [and probably twice] as the sublingual gland of laticaudine and hydrophine sea snakes, and once as the pre-maxillary gland of water snakes) or that salt glands evolved only twice, represented by the two unique anatomical positions, and that salt glands were lost in the intervening taxa. Considering, again, the phylogenetic distance between file snakes and sea snakes (or, indeed, between laticaudine and hydrophine sea snakes), we think it is more likely that salt glands evolved at least three (and potentially four) times in snakes.

Toward a coherent evolutionary hypothesis on the diversity of salt glands

The diversity in the location of modern salt glands alone suggests that this structure has evolved multiple times, independently, among modern tetrapod

taxa; however, similarities in the location of the gland and, importantly, the position of the duct, combined with the presumed embryological origin of the nasal salt glands in both extant (e.g., birds and lizards; see earlier) and extinct lineages (e.g., birds, dinosaurs, mesosaurs, and metriorhynchid crocodiles) (Supplementary Table S1), are consistent with the hypothesis that nasal salt glands were also present in the ancestor of all diapsids (Fernandez and Gasparini 2000). Interestingly, from this putative starting point, deviations are observed in turtles, one of the first groups to diverge from the ancestral diapsid form, extant crocodylians, which likely evolved from an ancestor that had lost the original nasal salt glands, and snakes, which, as a group, have likely experienced several origins of salt glands. The various origins of salt glands in nonhomologous positions may suggest that constraints specific to each of these lineages led to the development of a salt gland in these novel locations.

Gasparini et al. (2006) and Pierce et al. (2009) suggested that skull morphology among extinct crocodylians may have been influenced by a shift toward a more highly aquatic lifestyle, including changes in feeding strategy (e.g., a shift toward ambush predation) (Seymour et al. 2004) and increases in the mechanical resistance of the snout. They used these ideas to propose that the evolution of new feeding habits was likely the driving force separating the skull morphologies across species. In this light, it is possible that the shape of the snout imposed constraints in relationship to the capture of prey and that ambush predation limited the capacity of the skull to house a salt gland, leading to a second origin of salt glands among crocodylians in the soft tissue of the tongue's epithelium. Recent phylogenetic studies suggest that turtles are sister to the archosaurian lineage (Shen et al. 2011; Voronov et al. 2011) and, thus, should be placed within the Diapsida. This suggests, then, that the anapsid turtle skull is derived from a diapsid ancestor and that turtles may, therefore, have evolved from a lineage that possessed nasal salt glands (Fernandez and Gasparini 2000). Considering that the chelonian anapsid skull constitutes a major modification from the ancestral diapsid form, it is not unreasonable to hypothesize that the lachrymal position of the salt gland in turtles may have resulted from functional constraints associated with this extensive cranial remodeling. Similarly, among the four lineages of snake that evolved salt glands, it is possible that deviation from the putative ancestral nasal gland is a result of the relatively recent evolution of modern snake taxa from burrowing or aquatic ancestors

with reduced ocular structures (Walls 1940; Heise et al. 1995; Caprette et al. 2004). For example, the covering of the eye of snakes by a scale fused with the scales of the body would preclude egress of secretions to the external environment from an orbital salt gland. Functional constraints linked to ancestral ecology in this group (e.g., loss of lachrymal glands) (Taub 1966), reliance of this group on vomerolfaction, or indeed a combination thereof might well have played a significant role in the modification of oral glands.

An *evo/devo* approach to the study of convergent evolution in salt glands

To develop useful hypotheses about the mechanisms that may have supported the convergent evolution of salt glands across taxa, it is necessary to first define the features that must have appeared during the evolution of a salt-secreting gland. As aforementioned, all salt glands identified thus far have a compound tubular shape with extensive secretory epithelium that is populated in large part by principal secretory cells at the expense of the mucous cells or other cell types that typify this epithelium in unspecialized glands. To our knowledge, there have been only few studies aimed specifically at the development of cephalic glands in nonmammalian tetrapods (e.g., Marples 1932; Ellis et al. 1963; Kochva 1965; Nogawa 1978; Ovadia 1984; Chieffi Baccari et al. 1995, 1996; Rehorek et al. 2005), and all these studies are limited to morphological/histochemical surveys and lack molecular data. In contrast, the development and regeneration of salivary glands (particularly the submandibular glands, sublingual glands, and parotid glands) in mammalian models are active areas of research extending well beyond descriptive embryology to include vast details regarding the molecular regulation of gland shape and cellular identity (recently reviewed by Tucker 2007; Larsen et al. 2010; Harunaga et al. 2011; Lombaert et al. 2011). From these mammalian studies, it is possible to develop hypotheses about the molecular regulation of compound tubular shape and salt-secreting versus mucus-secreting cellular identity and, therefore, to postulate about the mechanism by which salt glands were co-opted from unspecialized glands.

Glandular organogenesis

The organogenesis of salivary glands is a well-conserved process in mammals (Tucker 2007), and Supplementary Table S3 summarizes some of the signaling molecules involved in each stage. In brief, the earliest stages of glandular development

(stage 1: pre-bud; Supplementary Table S3) involve thickening of the oral epithelium and proliferation of the gland primordium to form the initial bud (stage 2; Supplementary Table S3). Continued cell proliferation in the gland primordium leads to further outgrowth and invasion of the surrounding mesenchyme (stage 3: pseudoglandular; Supplementary Table S3), a process that relies on signaling molecules from both the epithelium of the developing gland and the surrounding mesenchyme. At the same time, the earliest rudiments of a lumen begin to form through the directed expression of apoptotic signals (in those cells destined to form the cavity of the lumen) or the expression of anti-apoptotic signals (in those cells destined to become the epithelium lining the lumen). Cells destined to become the epithelium lining the lumen begin to express polarizing signals (as apical/basal polarity is a defining feature of epithelia) by this stage, and, furthermore, some evidence suggests that cells in this stage (stage 4: cannicular; Supplementary Table S3) are already fated to become either duct cells or acinar cells (Walker et al. 2008). Extensive branching morphogenesis follows initial formation of the lumen, ultimately giving rise to the gross architecture of the gland (stage 5: terminal bud; Supplementary Table S3). This process is, again, regulated by opposing signals from the growing epithelium and the surrounding mesenchyme. Although we believe that studies of *de novo* glandular organogenesis in marine and nonmarine tetrapods will represent a new and important contribution to this field, studies of this type are unlikely to reveal the evolutionary mechanism resulting in the possession of a specialized salt-secreting gland in a marine taxon or in the possession of an unspecialized homologous gland in its nonmarine sister taxon. Thus, we use the remainder of this discussion to develop hypotheses about the co-option of an unspecialized gland that was already in place.

Co-option of an existing gland

The complete set of cephalic glands in tetrapods (Fig. 1A) includes both compound tubular and compound acinar glands of mucous, serous, and mixed function (Tucker 1958). Assuming a similar complement of shapes and functions of glands in the ancestor of modern marine taxa, two scenarios are likely for the evolution of salt glands: co-option of an existing tubular gland or co-option of an existing acinar gland. To keep these comparisons simple, this review will focus on the evolution of salt-secreting glands from ancestral glands with

a mucus-secreting or mixed (mucoserous) function. Since many cephalic glands have a mucus-secreting component (e.g., wholly mucous acini, mixed mucous, and serous acini, or mucus-secreting cells lining the ducts) (Babonis and Evans 2011), we find the hypothesis that salt glands evolved from mucous glands to be most plausible; however, the approach we apply in this section could be applied with equal validity to hypotheses invoking co-option from another ancestral type of gland.

Co-option of an existing (unspecialized or mucus-secreting) compound tubular gland likely involves a change in cellular identity without a concomitant change in glandular morphology. This process may have been gradual, whereby portions of the gland adopted a salt-secreting function simply through a gradual change in the domain of expression of signals regulating the acquisition of salt-secreting cellular identity (see Fig. 2A for an example). In contrast, co-option of an existing (unspecialized or mucus-secreting) compound acinar gland invokes a change both in the cell's identity and in the shape of the gland (Fig. 2B and C). This would involve a shift from mucus-secreting to salt-secreting cellular identity and a shift from acinar to duct/tubule cellular identity and likely resulted from either (1) loss of the acinar component of the ancestral gland by re-specification of these cells as duct/tubule cells (Fig. 2B) or (2) actual loss of the presumptive acinar epithelium and compensatory growth of the portion of the gland already specified as duct to form ductal/tubular termini (Fig. 2C). Since the acinar component of a typical mammalian salivary gland is specified early (Walker et al. 2008), evaluation of this hypothesis will require careful studies of the timing and location of expression of cell-identity markers (pre-acinar versus pre-ductal markers) (Supplementary Table S3) during early glandular development (Fig. 2D). Evidence of apoptotic signals in the pre-acinar component of salt glands and a lack of these signals in the early development of nonsalt-secreting salivary glands might suggest that the homogeneous makeup of salt glands is a result of actual loss of other cell types. In contrast, a lack of pre-acinar markers in the absence of apoptotic signals early in glandular development may support the hypothesis that these cells have undergone early re-specification as duct cells. Although Supplementary Table S3 is far from an exhaustive list of molecular components of salivary-gland development, this summary should provide a solid starting point from which to test specific hypotheses about changes in the timing or distribution/range of expression of various cell-identity markers in specialized and unspecialized glands across tetrapods.

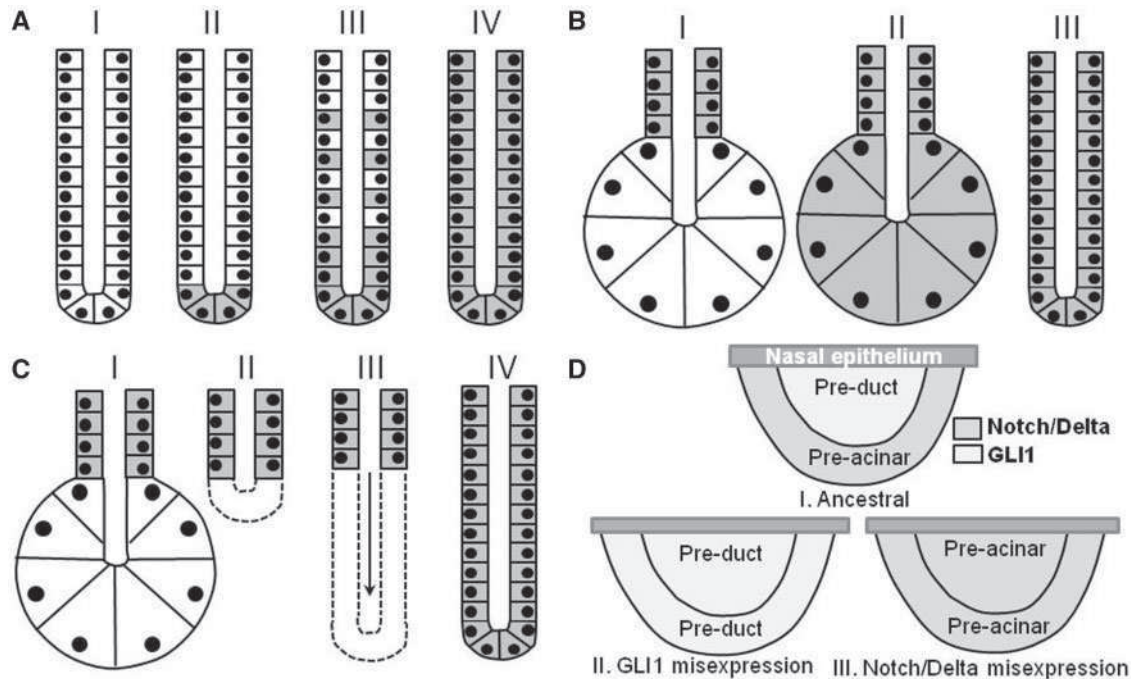


Fig. 2 Hypothetical scenarios for the co-option of an ancestral gland to form a salt gland. **(A)** The appearance of salt-secreting cells (grey) in the secretory epithelium may have occurred gradually (forms I–IV), first in only a small portion of the gland and later taking on a homogeneous distribution. **(B)** An ancestral acinar gland exhibiting nonsalt-secreting (white) cells in the acini (I) may have undergone first a transition to become populated by principal cells (grey; II) followed by a change in the shape of the gland from acinar to tubular (III). **(C)** Alternatively, loss of the acinar component of the ancestral gland (I, II), followed by elongation of the ductal/salt-secreting component (III) may have resulted in a homogeneous tubular secretory epithelium (IV). **(D)** The ancestral acinar gland may have expressed Notch/Delta (Dang et al. 2009) in the pre-acinar component and the transcription factor GLI1 (Fiaschi et al. 2011) in the pre-ductal component (I). Misexpression of GLI1 (II) in the pre-acinar component may have resulted in a shift in the identity of these cells from pre-acinar to pre-ductal. For comparison, misexpression of Notch/Delta (III) might have resulted in a gland that was homogeneously acinar in cell type.

Future directions for this research

The hypotheses we have developed in this article are speculative and clearly point out the lack of knowledge on the evolution of salt glands in tetrapods. Understanding the evolutionary history of tetrapods' salt glands is an exciting field of investigation, but it will require not only a thorough resolution of the presence and locations of salt glands throughout the evolutionary history of tetrapods (e.g., using reconstruction of ancestral states) (Witmer 1997; Fernandez and Gasparini 2000) but also a precise investigation of the functional constraints of nasal salt glands in lineages that deviate from the putative basal bauplan (nasal salt glands) and detailed molecular studies of glandular development in various taxa. Because of the number of tetrapod lineages that have independently re-invaded marine habitats, there are many examples of closely related marine and nonmarine sister taxa among tetrapods, providing abundant opportunities for comparative studies. Furthermore, there are many species that have salt

glands with mixed function (serous-secreting and mucus-secreting cells) that would also make nice developmental models (e.g., the skink *Tiliqua rugosa*) (Saint Girons et al. 1977). By examining the development of the salt gland in these species, it will be possible to identify the signals leading to the development of salt-secreting and mucus-secreting cells in the same gland at the same time. Finally, recent studies of rectal (salt) gland morphogenesis in Iago sharks (Fishelson et al. 2004) and orbital-gland morphogenesis in various nonmammalian tetrapods (Chieffi-Baccari 1996; Rehorek et al. 2005, 2007) provide a basis for assessing morphological changes occurring during the development of specialized and unspecialized cephalic glands (e.g., development of the salt-gland capsule and the associated capillaries and amplification of the basolateral membrane of principal cells) but do not provide molecular hypotheses about the signals regulating these various morphological events. These initial comparisons can then be used to (1) evaluate hypotheses about the

mechanisms leading to the acquisition of a specialized salt-secreting gland in any individual marine lineage, (2) make comparisons of developmental mechanisms of salt glands across lineages to understand the processes by which convergent evolution occurs, and (3) to compare the developmental pathways resulting in specialized and unspecialized glands to understand how existing structures may be modified through evolution. It is our hope that this review will provide a starting place for anyone interested in pursuing these ideas further.

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Supplementary Data

Supplementary Data are available at *ICB* online.

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Hypernatremia in Dice Snakes (*Natrix tessellata*) from a Coastal Population: Implications for Osmoregulation in Marine Snake Prototypes

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Abstract

The widespread relationship between salt excreting structures (e.g., salt glands) and marine life strongly suggests that the ability to regulate salt balance has been crucial during the transition to marine life in tetrapods. Elevated natremia (plasma sodium) recorded in several marine snakes species suggests that the development of a tolerance toward hypernatremia, in addition to salt gland development, has been a critical feature in the evolution of marine snakes. However, data from intermediate stage (species lacking salt glands but occasionally using salty environments) are lacking to draw a comprehensive picture of the evolution of an euryhaline physiology in these organisms. In this study, we assessed natremia of free-ranging Dice snakes (*Natrix tessellata*, a predominantly fresh water natricine lacking salt glands) from a coastal population in Bulgaria. Our results show that coastal *N. tessellata* can display hypernatremia (up to 195.5 mmol.l⁻¹) without any apparent effect on several physiological and behavioural traits (e.g., hematocrit, body condition, foraging). More generally, a review of natremia in species situated along a continuum of habitat use between fresh- and seawater shows that snake species display a concomitant tolerance toward hypernatremia, even in species lacking salt glands. Collectively, these data suggest that a physiological tolerance toward hypernatremia has been critical during the evolution of an euryhaline physiology, and may well have preceded the evolution of salt glands.

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Introduction

Living in seawater entails physiological consequences such as water loss and salt gain, and coping with these constraints represents one of the principal challenges of secondarily marine vertebrates [1]. Accordingly, marine tetrapods (i.e., mammals, birds, turtles, snakes, lizards and crocodiles) display specific adaptations related to the maintenance of osmotic balance. For instance, marine mammals have specialized nephrons which allow highly concentrated urine [2]. Although marine reptiles lack the ability to excrete excess salt in urine, they have evolved salt glands that secrete concentrated salt solution [3,4].

The widespread relationship between marine life and presence of specific salt-excretory structures, found across very different taxa, strongly suggests that the ability to excrete excess salt has been critical during the invasion of marine environments by tetrapods. However, as with most evolutionary processes, transitional steps are missing and are seldom represented by fossil remains [5]. In addition, crucial characteristics such as physiology and/or behaviour do not print well within the fossil records [5]. Yet, some research works have proposed scenarios of the evolution of an euryhaline physiology during the transition to marine life [6,7,8]. For instance, Dunson and Mazzotti [6] have proposed four successive steps that should ultimately lead to an efficient maintenance of the osmotic balance. The first step consists of a primary reliance on behavioural osmoregulation (e.g., frequent

obligate fresh water drinking [9]). The second step involves a reduction in salt gain and water loss through permeable surfaces [10,11,12]. The third and fourth steps include the evolution of rudimentary salt secreting features and their subsequent development [4], which would ultimately allow exploiting more saline, thus larger, oceanic areas [13].

Clearly, these successive stages would ultimately allow organisms to progressively become emancipated from regular access to fresh water, and thus to thrive in saline environments. Likely, this has led to the conclusion that marine tetrapods could maintain their water balance without consuming fresh water [14,15]. However, recent investigations have challenged this paradigm. Specifically, the most detailed studies performed on marine snakes have shown that species having a functional salt gland cannot equilibrate their hydromineral balance without access to fresh water [12,16]. Dehydration in seawater has been shown to occur in amphibious sea snakes (Laticaudine sea kraits) as well as in fully marine species (Hydrophine sea snakes) [12,16,17]. In addition, elevated plasmatic sodium concentrations have been measured in various marine snake species [18–25]. These studies have led to the hypothesis that the development of a physiological tolerance to hypernatremia may have been an important feature of the evolution of marine snakes [25]. However, data gathered under experimental conditions show that fresh water species lacking salt glands (including coastal presumably salt tolerant species) rapidly

accumulate large salt loads when acclimated in brackish and salt water [24,26]. In most of these cases, the resulting hypernatremia was lethal [24,26]. Taken together, these elements highlight the lack of information from groups thought to resemble transitional forms between the land and the sea (species lacking salt glands but occasionally using salty environments) to draw a comprehensive picture of the evolution of an euryhaline physiology in these organisms.

Snakes provide a suitable study system with which to clarify the steps that allowed coping with osmotic challenge [13]. Indeed, this lineage displays a unique gradient of habitat use which allows studying groups thought to resemble transitional forms between the land and the sea [13,27]. Importantly, these transitional steps can be investigated at several phylogenetic scales along a gradient of habitat use [27]. Species that are salt tolerant, but imperfectly marine (i.e., lacking salt glands) represent a powerful opportunity to investigate the early steps of the evolution of an euryhaline physiology. In the current investigation, we examine such a study system. The European Dice snake (*Natrix tessellata*) is a typical semi-aquatic fresh water natricine species that occurs over Eurasia (broadly from Italy to China [28]). Although this species relies primarily on fresh water bodies to forage for fish and amphibians, some populations are known to use, more or less extensively, brackish or saline habitats, thereby offering the possibility to investigate an intermediate step during the evolution to marine life. In this study we report natremia (plasma sodium concentration, an indicator of the osmotic challenge linked to marine life in snakes [25]) measured in free-ranging Dice snakes inhabiting a coastal ecotone between freshwater and the Black Sea in Bulgaria. In combination with a review of plasmatic sodium concentration of snakes, these results are discussed in the light of the secondary transition to marine life in tetrapods.

Materials and Methods

Ethics statement

All procedures were approved by French and Bulgarian regulations (Comité d'éthique Poitou-Charentes approval number CE2013-5 to FB; Ministry of Environment and water of Bulgaria permit to YVK: 298/09.03.2011).

Study species

Natrix tessellata is a medium-sized (up to 130 cm [29]) species with an extended Palearctic distribution: from central Europe to northern Egypt and east as far as north-western China [30,31]. It is a typical semi-aquatic natricine foraging mainly for fish, and to a lesser extent for amphibians in streams, rivers, and lakes [28].

Although the vast majority of *N. tessellata* populations rely on fresh water bodies, few do occur in saline environments along the coasts of the Adriatic Sea [28,32–35], the Ionian and Aegean Seas [36], the Black Sea ([37–40], this study) and the Caspian Sea [41,42]. In most of these cases, *N. tessellata* occurs in brackish waters of lagoons, salt marshes and river mouths.

Study site

We surveyed a population of Dice snakes on the southern Bulgarian Black Sea coast, in the “Poda” Protected Area (Fig. 1). The Poda wetland (1 km²) consists of a coastal ecotone inserted between a large predominantly freshwater reservoir (Mandra Lake) and the Black Sea (Fig. 1). Poda is mainly composed of an alternation of shallow pools of water (usually <1 m deep) intersected by embankment lands and lower, temporarily flooded areas. The proximity of the Mandra Lake and the Black Sea and seasonal climatic fluctuations create a wide variety of aquatic

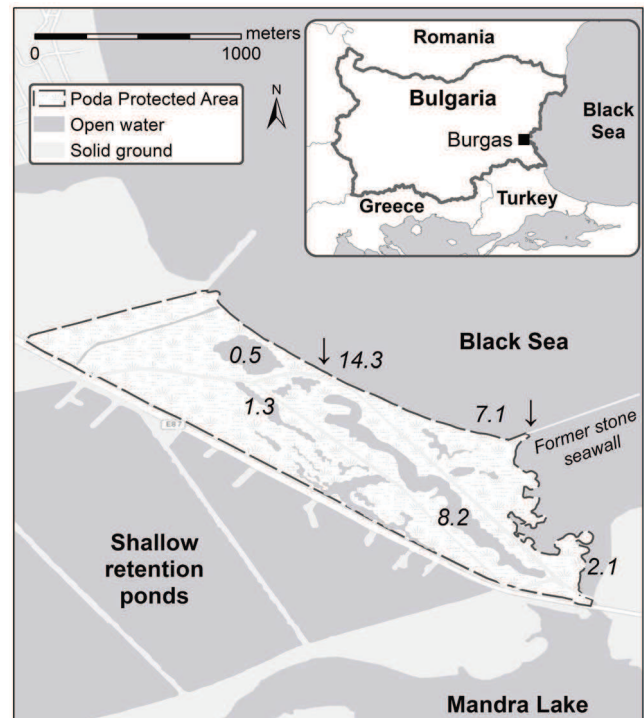


Figure 1. Map of the study area. The upper panel gives the location of the Poda Protected Areas in the vicinity of Bourgas, Bulgaria. Emergent lands are indicated in white, water is indicated in light grey. The lower panel shows the Poda Protected Area (dashed area). Emergent lands are indicated in white and water is indicated in light grey. Numbers designate salinity (‰) recorded for three ponds, two locations on the shore of the Black Sea, and one location at the mouth of the Mandra Lake. The two arrows show sites where tracks from snakes commuting between the land and the Black Sea were observed. doi:10.1371/journal.pone.0092617.g001

habitats spanning from fresh (<1‰) and brackish (1–10‰) to salt water (>10‰), including some hyperhaline waters during the summer (32–33 ‰, [43], Fig. 1).

Field procedures

In April 2012, a total number of 19 snakes were captured by hand. Snakes were typically found while basking in the sun. Individuals were measured (snout-vent length [SVL] and total length [TL], ± 0.5 cm), weighted (± 1 g), and sexed by eversion of the hemipenis. Feeding and reproductive status were assessed by gentle palpation.

Only large adult females (>140 g, N = 13) were blood-sampled to avoid putative detrimental effects of the procedure on smaller individuals, and to avoid sex effects on plasmatic parameters. Blood (~400 μ l) was sampled through cardiocentesis using 30 G-needles. A small fraction (10 μ l) of the blood was collected in a micro-capillary tube and centrifuged on site in a minihaematocrit Compur M1101 (Bayer) for 3 min to record haematocrit (packed blood cell volume, %). The remaining blood was centrifuged (3 min at 8,000 G) and the plasma was separated and stored at -25°C until assays were processed. Plasma sodium concentrations were assessed with an ISE module on a Pentra C 200 (Horiba Medical Ltd) compact chemistry analyzer.

At the end of the procedures (usually <30 min), snakes were released at the location of capture.

Water samples were collected from water bodies where *N. tessellata* were observed foraging and/or in the vicinity of which we

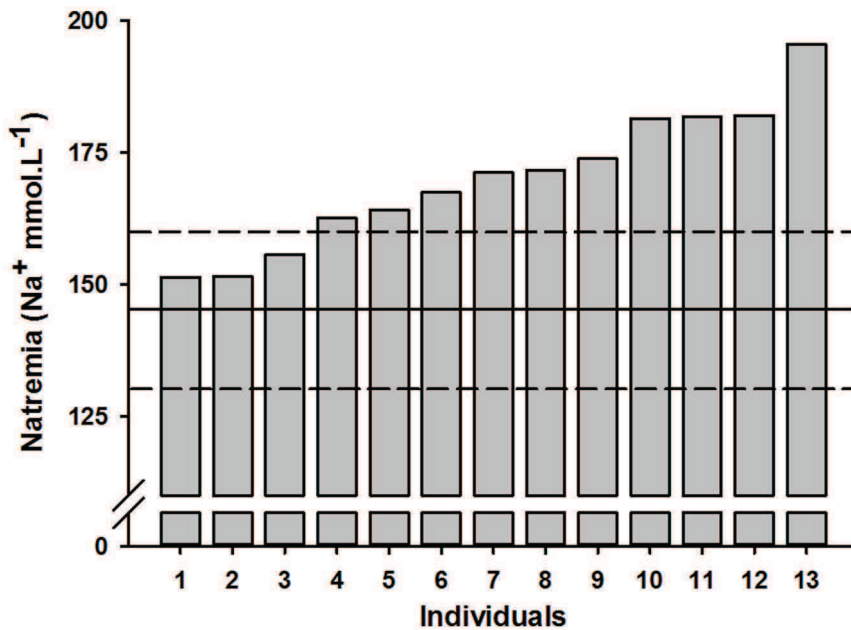


Figure 2. Natremia (plasma sodium concentration) of thirteen free-ranging individual *N. tessellata* captured at Poda Protected Area, Bulgaria. The dashed lines indicate the range of normonatremia (130–160 mmol.l⁻¹ [45]) and the horizontal black line indicates mean normonatremia (145 mmol.l⁻¹). For clarity, individuals are ranked by ascending order of natremia. doi:10.1371/journal.pone.0092617.g002

collected snakes. These sampled stations included 3 ponds within Poda, 2 sites along the coast of the Black Sea as well as 1 site situated at the mouth of the Mandra Lake (Fig. 1). Salinity (‰) was assessed with a Pocket Salt Meter (PAL-ES2, Atago).

Analyses

We quantified a body condition index (BCI) using residual scores from the linear regression between body size (SVL) and body mass (both variables were log transformed for linearity [44]). We excluded individuals with prey in the stomach from the BCI calculations.

Relationships between natremia and possible correlates (BCI, Hct) were investigated using Spearman rank correlations.

Results and Discussion

Free-ranging *N. tessellata* display highly variable plasma sodium concentrations (mean 169.9 ± 13.2 mmol.l⁻¹) ranging from normonatremia (which range from 130 to 160 mmol.l⁻¹ in non-mammalian tetrapods [45]) to hypernatremia (up to 195.5 mmol.l⁻¹, Fig. 2). Most individuals (N = 10, 77%) displayed hypernatremia, and only three snakes had values within the range of normonatremia (130–160 mmol.l⁻¹ [45]). Classically, deviations of the osmotic balance trigger several behavioural and physiological adjustments in snakes. For instance, dehydrated and/or hypernatremic individuals tend to seclude themselves in well-buffered shelters in order to reduce additional water loss [8,9]. Such behaviour is usually accompanied by a thermal depression and decreased metabolism which result in a strong reduction in activity levels [46,47]. Apparently, the high natremia we recorded did not trigger such adjustments in *N. tessellata*. Indeed, all the individuals we captured were basking in the sun, or actively moving in the open, and tried actively to evade capture. Remnants of food were palpated in three individuals that did not display particularly low plasma sodium (155.6, 167.4 and 173.8 mmol.l⁻¹ Na⁺ respectively). In addition, BCI was not related to plasma

sodium (Spearman rank correlation, $r_s = -0.47$, $p > 0.05$), suggesting an absence of long-term effect of hypernatremia on foraging.

The elevated natremia we found in some individuals could be the result of mere dehydration (i.e., the more dehydrated an individual, the more concentrated its body fluids). However, this hypothesis seems unlikely as we did not find any relationship between plasma sodium and BCI (see above) or haematocrit (Spearman rank correlation, $r_s = 0.20$, $p > 0.05$), two parameters known to correlate with hydration state [17,48]. Our results rather suggest that free-ranging *N. tessellata* gained salt during their day-to-day activities. Indeed, measurements of environmental salinity in Poda showed that most potential foraging areas were saline (up to 14.3‰, Fig. 1, see also [43]). Likely, Dice snakes foraged in water bodies that were brackish or saline, or indeed at sea (as witnessed by tracks of snakes commuting to the Black Sea, Fig. 1); and gained salt passively through permeable surfaces. Marine snakes display a significant reduction in salt gain and water loss through permeable surfaces [10–12,49], and future studies should assess skin permeability to water and sodium in *N. tessellata* and compare coastal *versus* inland populations.

Voluntary or incidental (e.g., during prey capture) salt water drinking is an additional process that leads to salt gain [15]. Accordingly, marine forms usually display an increased ability to discriminate water salt content and to avoid salt water drinking [12,16,26,50,51]. We do not know whether *N. tessellata* is able to discriminate water salt content and/or to avoid salt water drinking and such issues need to be clarified. In addition to salt water drinking avoidance, many marine taxa can rely on behavioural osmoregulation such as fresh water drinking. Indeed, dehydrated and hypernatremic marine snakes are known to drink large amounts of fresh water when available to restore osmotic balance [9,12,16,25]. Interestingly, two individuals (151.3 and 162.6 mmol.l⁻¹ Na⁺ respectively) regurgitated copious amounts of fresh water upon capture, suggesting that these individuals have drunk shortly before. The variety of aquatic habitats found in Poda (fresh, brackish and salt water, Fig 1) over a small spatial scale,

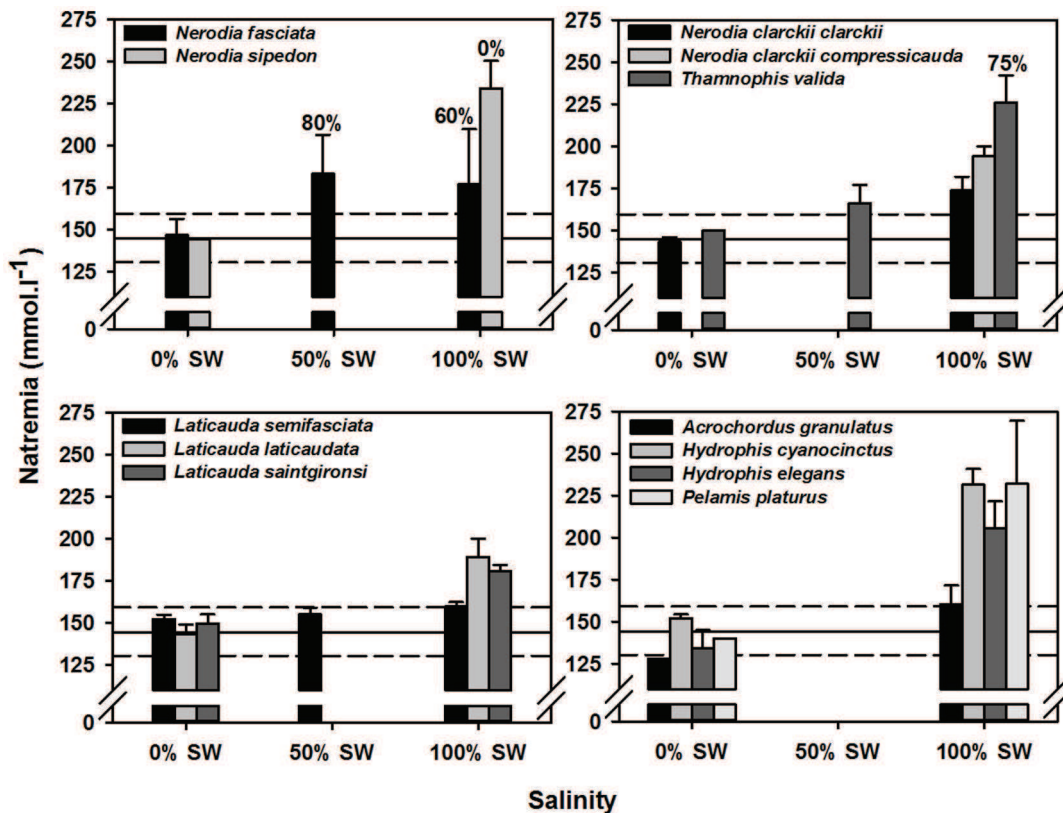


Figure 3. Published data on snake natremia. These data were available from strictly fresh water species (*Nerodia fasciata* and *N. sipedon* [24,26]), salt tolerant species lacking salt glands (*N. clarckii clarckii*, *N. clarckii compressicauda*, *Thamnophis valida* [24,26]), amphibious sea kraits with functional salt glands (*Laticauda saintgironsi*, *L. laticaudata*, *L. semifasciata* [24,25]) and fully marine sea snakes with functional salt glands (*Acrochordus granulatus*, *Hydrophis elegans*, *H. cyanocinctus*, *Pelamis platurus* [18,19,21–23]). The dashed lines indicate the range of normonatremia (130–160 mmol.l⁻¹ [45]) and the horizontal black line indicates mean normonatremia (145 mmol.l⁻¹). Numbers above the bars indicate survival rates (no number = 100%). Data are mean values per species ± SD. doi:10.1371/journal.pone.0092617.g003

may allow hypernatremic Dice snakes to easily access fresh water, and thus to periodically restore osmotic balance. Accordingly, specific environments characterized by low and/or variable salinity may have facilitated evolutionary transitions to marine life in snakes by allowing regular access to relatively fresh water over short time-scales and decreasing the cost of osmotic maintenance [13].

More generally, a review of plasmatic sodium concentration of snakes experimentally maintained in fresh water or seawater gives additional insights to our results (Fig. 3 and references therein). These data suggest that when kept in fresh water, irrespective of their primary habitat (i.e., fresh- versus seawater) all species shared similar normonatremia (~140–150 mmol.l⁻¹, Fig. 3). Similarly, in full-strength seawater, plasma sodium increased in all species regardless from their osmoregulatory attributes (i.e., presence/absence of salt glands, Fig. 3, [25]). Importantly, survival differed among species and treatments, with strictly fresh water species (*Nerodia fasciata* and *N. sipedon*) having decreased short-term (i.e., hours) survival in osmotically challenging treatments (Fig. 3). Survival decreased also for salt tolerant species, although to a lesser extent, with only one species (*Thamnophis valida*) out of three having its short-term survival decreased in full-strength seawater (Fig. 3). The other salt tolerant species survived for several days in full-strength seawater. Survival stayed high (100%) in both groups of marine adapted snakes (Fig. 3). Overall, these patterns seem to indicate that along a continuum of habitats use between fresh- and

seawater, snake species display a concomitant physiological tolerance toward high plasma sodium, even in species lacking salt glands (Figs. 2 and 3). In turn, this physiological flexibility would allow reducing detrimental effects of salt gain such as decreased activity levels and decreased short-term survival [26]. Such resistance would allow individuals to periodically access fresh water, and hence to occasionally restore their osmotic balance. Ultimately, in marine species having salt glands, such flexibility would allow excreting excess salt when natremia exceeds high thresholds [22], which would substantially decrease energetic costs linked to salt gland functioning [25].

In conclusion, the combination of these data strongly suggest that the development of a physiological tolerance toward deviations of the osmotic balance (e.g., increased plasma sodium) might have been a critical innovation in the evolution of an euryhaline physiology and may well have preceded the evolution of salt glands. Although only few populations of *N. tessellata* are found in saline environments, our results show that these populations may be salt tolerant, and use saline water bodies despite lacking salt glands. In this respect, *N. tessellata* seems a promising study model (i.e., a marine snake prototype) of the secondary transition to marine life in vertebrates.

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Author Contributions

Conceived and designed the experiments: FB YVK. Performed the experiments: FB YVK. Analyzed the data: FB YVK. Contributed reagents/materials/analysis tools: FB YVK. Wrote the paper: FB YVK.

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Variations of natremia in sea kraits (*Laticauda* spp.) kept in seawater and fresh water



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ABSTRACT

Marine tetrapods evolved specific excretory structures (e.g. salt glands) that maintain salt concentrations within a narrow range of variation. However, recent investigations showed that in some lineages (sea snakes), individuals dehydrate in seawater and cannot equilibrate their hydromineral balance without access to fresh water. How these marine species cope with salt gain is therefore puzzling. We sampled two species of amphibious sea kraits (*Laticauda saintgironsi* and *L. laticaudata*) in the field. We also experimentally investigated patterns of salt regulation, specifically variations in natremia (plasma sodium) and body mass (net water flow), in individuals transferred first to fresh water and then to seawater. Our results show that free-ranging sea kraits display hypernatremia (up to 205 mmol·l⁻¹). Experimental data showed that natremia markedly decreased in snakes exposed to fresh water and increased when they were transferred to saltwater, thereby demonstrating a marked flexibility in their relation to environmental conditions. A literature survey indicated that all free-ranging marine snake species usually display hypernatremia despite having functional salt glands. Overall, sea snakes exhibit a marked tolerance to salt load compared to other marine tetrapods and apparently trigger substantial salt excretion only once natremia exceeds a high threshold. We hypothesise that this high tolerance significantly decreases energetic costs linked to salt gland functioning.

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1. Introduction

One of the paramount challenges for marine tetrapods (i.e., mammals, birds, turtles, snakes, lizards and crocodiles) is to maintain hydromineral balance within vital boundaries (Schmidt-Nielsen, 1983). Because seawater is hyperosmotic to body fluids, marine species tend to gain salt and lose water (Schmidt-Nielsen, 1983). As a consequence, in most marine vertebrates, hydromineral balance regulation requires expenditure of energy (Schmidt-Nielsen, 1983). Marine tetrapods display a variety of structures that actively excrete excess salt. For instance, marine mammals possess a sophisticated countercurrent system with elongated nephrons that excrete large loads of ions in hypertonic urine (Ortiz, 2001). Reptiles do not produce highly concentrated urine, but they have evolved a diversity of cephalic salt glands that excrete concentrated salt solutions (Peaker and Linzell, 1975; Babonis and Brischoux, 2012).

Owing to their developed salt excreting abilities, marine tetrapods have long been thought to maintain their water balance without consuming fresh water (Randall et al., 2002; Houser et al., 2005). This long-standing dogma has been recently challenged in some lineages

(Lillywhite et al., 2012). Detailed studies performed on snakes showed that marine species cannot equilibrate their hydromineral balance without access to fresh water (Lillywhite et al., 2008, 2012). Dehydration in seawater has been documented in amphibious sea snakes (sea kraits) as well as in fully marine species (hydrophiines) (Lillywhite et al., 2008, 2012; Brischoux et al., 2012a). Interestingly, dehydration rates are dependent on the degree of emancipation from the ancestral terrestrial environment, both within and across phylogenetic lineages (Brischoux et al., 2012a; Lillywhite et al., 2012). Life in seawater can thus impose significant physiological costs that have likely influenced the secondarily evolutionary transition to marine life in tetrapods (Brischoux et al., 2012b).

Dehydration rates of marine snakes in seawater have been mainly assessed through variation in body mass, an integrative parameter that primarily informs about net water loss (Lillywhite et al., 2008, 2012; Brischoux et al., 2012a). However, underlying variations of concentrations of ions or osmolytes in body fluids that exert a crucial influence on the maintenance of osmotic balance should be investigated (Schmidt-Nielsen, 1983; Ortiz, 2001; Dantzler and Bradshaw, 2009). Although salt strongly influences osmolality, many other osmolytes, such as proteins, carbohydrate and nitrogenous wastes can also be involved (Schmidt-Nielsen, 1983). Conversely, in the marine environment, sodium is one of the primary ions that can be passively gained through permeable surfaces (Schmidt-Nielsen, 1983, but see Dunson and Robinson,

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1976; Dunson and Stokes, 1983). Therefore, natremia (plasma sodium concentration) should directly reflect the outcome of the main osmotic challenge faced by marine tetrapods (Schmidt-Nielsen, 1983), including marine snakes (Dunson, 1968, 1980; Dunson et al., 1971; Dunson and Dunson, 1973, 1974, 1975; Duggan and Lofts, 1978; Babonis et al., 2011). In support of this view, sodium excretion rates and thus presumably salt gain have been shown to influence sea snakes environmental tolerances (Brischox et al., 2012b).

We investigated salt gain and salt regulation by monitoring natremia in marine snakes transferred from fresh water to seawater. Amphibious sea snakes (sea kraits, *Laticauda* spp.) provide an excellent opportunity to investigate physiological tolerances to salinity constraints within a restricted phylogenetic framework (Brischox et al., 2012a, 2013). First, the high surface to volume ratio due to the snake body plan likely increases salt gain through permeable surfaces (Brischox and Shine, 2011). Second, sea kraits obligatorily use both marine and land environments to forage, digest, and reproduce (Heatwole, 1999). Importantly, they dehydrate in the field and drink fresh water when available in order to restore their osmotic balance (Bonnet and Brischox, 2008; Lillywhite et al., 2008; Brischox et al., 2012a). Third, species from this clade differ in their relative use of terrestrial versus marine environments and display a concomitant gradient of adaptations to marine life (Lillywhite et al., 2008, 2009; Brischox et al., 2013). Finally, life in seawater poses strong physiological challenges to sea kraits (Dunson, 1975; Brischox et al., 2012a) and likely limits their distribution (Brischox et al., 2012b, 2013). We examined natremia of free-ranging individuals and patterns of salt gain of individuals experimentally transferred from fresh water to seawater in two-closely related species of sea kraits (*Laticauda laticaudata* and *L. saintgironsi*) which vary in their degree of habitat use (marine versus terrestrial) and their susceptibility to dehydration in seawater.

Specifically, we predicted that the more marine *L. laticaudata*, which displays relatively lower dehydration rates in seawater, should display a higher resistance to salt gain in seawater and a higher salt tolerance compared to the more terrestrial *L. saintgironsi*. Conversely, we expected both species to restore normonatremia when kept in a presumably less constraining medium such as fresh water.

2. Materials and methods

2.1. Captive animals

Ten adult male *L. saintgironsi* and 10 adult male *L. laticaudata* were caught on Signal islet, New Caledonia (22°17'45 S; 166°17'34 E) between November 18th and November 20th 2011. Upon capture, the stomach of each individual was palpated in order to ensure that no recently fed individuals were included in the experiment. The snakes were weighed and subsequently kept in calico bags. On November 21th 2011, the snakes were brought back at the Aquarium des Lagons Research Facility (Nouméa, New Caledonia) where all experimental procedures were performed.

2.2. Experimental protocols

Our experimental treatment was split into two successive phases. Snakes were placed in fresh water for two days in order to allow them to drink ad libitum. This first step will be abbreviated "2-d-FW" thereafter. After the 2-d-FW treatment, snakes were handled and blood sampled through intra-cardiac punctures using 30G-needles. The blood (~300 µL representing <0.2% of a snake's body mass) was immediately centrifuged (3 min at 8000 g) and the plasma was separated and stored at -25 °C. Each snake was weighed and randomly allocated to the next experimental step.

In this second treatment, we subjected the sea kraits to different salinity levels (either fresh water or full strength seawater, thereafter

FW and SW) during 12 days; an ecologically relevant duration similar to that of a foraging trip at sea (Brischox et al., 2007). To limit cage effect, each treatment was repeated in two aquaria. Five individuals per species were subjected to each treatment (2 to 3 snakes per aquarium). Aquaria were fitted with a platform placed approximately 1 to 2 cm below the water's surface, providing to the snakes with a resting place, notably to breathe without swimming, while remaining in permanent contact with water. At the end of this treatment, snakes were recaptured, and blood sampled as described above. We did not detect any effects of the aquaria per treatment on the parameters analyzed (all $p > 0.7$) therefore pairs of aquaria were pooled for each treatment for analyses.

Two *L. laticaudata* (one for each treatment) and one *L. saintgironsi* (FW treatment) escaped during the experiment and followed the water drain which opens in the Lagoon therefore thereby reducing our final sample 4 FW and 4 SW *L. laticaudata* and 4 FW and 5 SW *L. saintgironsi*. The remaining snakes were released at the site of capture after the experiment.

2.3. Field animals

To compare natremia between experimental and free-ranging individuals, we also sampled snakes directly in the field at a near-by site (Amédée islet, 22°28'38 S, 166°28'06 E) where a tourist facility allowed us to use a similar protocol for collecting blood as described above. We collected blood from 4 male *L. saintgironsi* and 2 male *L. laticaudata* shortly after capture (<3 min).

2.4. Natremia

Plasma sodium concentrations were assessed with an ISE module on a Pentra C 200 (Horiba Medical Ltd) compact chemistry analyzer.

3. Results

3.1. General observations

When placed in fresh water (onset of the 2-d-FW period) all individuals drank abundantly, often before exploring their new environment and despite the stress of capture. During the 2-d-FW period, many individuals defecated as indicated by large amounts of nitrogenous wastes (insolubilized urates) quickly accumulating at the bottom of the aquariums.

3.2. Variations in body mass

Despite fresh water uptake, we detected a slight loss of body mass between capture and the end of the 2-d-FW period (possibly due to defecation), significant in *L. laticaudata* solely (*L. saintgironsi*: body mass 1 = 153.0 ± 21.4 g, body mass 2 = 151.3 ± 21.8, paired t-tests, $t = 1.47$, $df = 13$, $p = 0.16$; *L. laticaudata*: body mass 1 = 206.5 ± 38.2 g, body mass 2 = 201.2 ± 35.3, paired t-tests, $t = 3.15$, $df = 14$, $p = 0.007$).

In both species, we detected a significant loss of body mass during the second step of the experiment, but with no treatment effect (repeated measures ANOVA, Time effect: $F_{1, 7} = 47.67$, $p < 0.001$, Time*Treatment: $F_{1, 7} = 0.36$, $p = 0.56$, body mass 3 = 143.9 ± 6.9 g for *L. saintgironsi*; Time effect: $F_{1, 10} = 150.31$, $p < 0.0001$, Time*Treatment: $F_{1, 10} = 0.51$, $p = 0.49$, body mass 3 = 187.2 ± 10.3 g for *L. laticaudata*).

3.3. Natremia

In *L. saintgironsi*, the mean natremia of individuals sampled in the field was significantly higher compared to the mean value for individuals sampled after two days in fresh water (ANOVA, $F_{1, 20} = 36.27$,

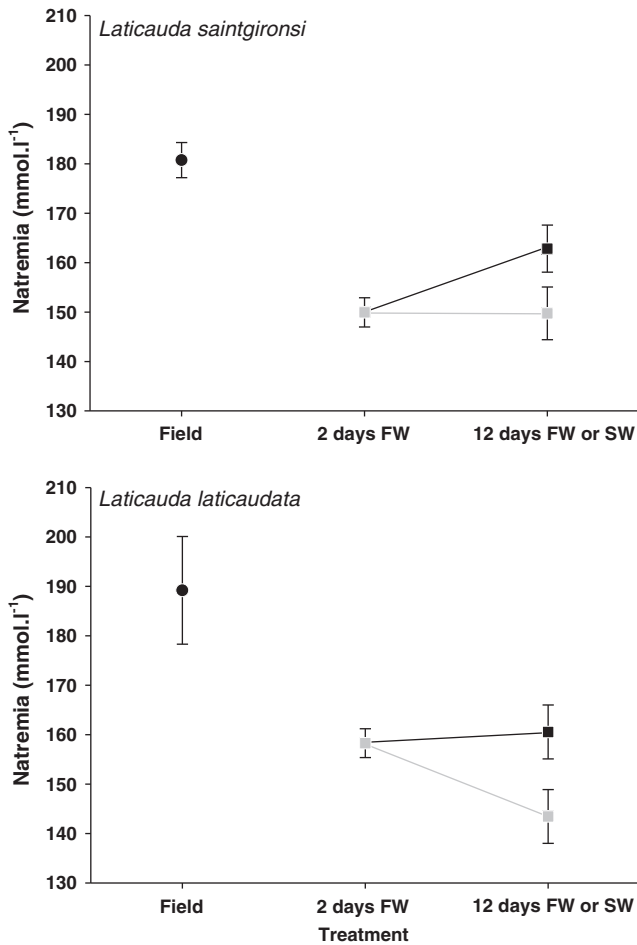


Fig. 1. Natremia (plasma sodium concentration, $\text{mmol}\cdot\text{l}^{-1}$) of sea kraits (*L. saintgironsi* and *L. laticaudata*) sampled in the field (black circles), and under experimental conditions (black and grey squares). FW and SW stand for fresh water and seawater; and are represented by grey and black symbols respectively. Connected dots indicate that the same individuals were sampled for those treatments. See text for details.

$p < 0.0001$, Fig. 1), and was higher compared to the value recorded in individuals transferred and kept in sea water for 12 additional days (ANOVA, $F_{1, 16} = 18.22$, $p < 0.001$, Fig. 1). Focusing on the experimental individuals, we found a significant treatment effect (repeated measures ANOVA, effect of treatment through time $F_{1, 6} = 6.06$, $p = 0.04$, Fig. 1). Post hoc tests revealed that the natremia of snakes kept in fresh water did not change after 12 days (Fisher's LSD, $p = 0.55$); by contrast salt gain increased in snakes transferred to saltwater ($p < 0.01$, Fig. 1).

In *L. laticaudata*, we also found that the mean natremia of individuals sampled in the field was higher compared to individuals sampled two days after transfer to fresh water (ANOVA, $F_{1, 13} = 24.64$, $p < 0.0001$, Fig. 1), and also compared to snakes 12 days after transfer to seawater (ANOVA, $F_{1, 8} = 17.79$, $p < 0.003$, Fig. 1). Focusing on the experimental individuals, we found a significant treatment effect (repeated measures ANOVA, effect of treatment through time $F_{1, 5} = 5.05$, $p = 0.05$, Fig. 1). Post hoc tests showed that the natremia of snakes kept in fresh water decreased after 12 days (Fisher's LSD, $p = 0.03$), while it stayed constant in individuals transferred to seawater ($p = 0.71$, Fig. 1).

4. Discussion

Physiological capacity for excreting salt is essential for marine vertebrates (Peaker and Linzell, 1975; Schmidt-Nielsen, 1983; Ortiz, 2001). Owing to their salt glands, sea snakes were expected to be

able to maintain their natremia within a narrow range when exposed to various salinities. Unexpectedly, our results show that 1) free-ranging sea kraits can display elevated natremia (up to $205 \text{ mmol}\cdot\text{l}^{-1}$, Fig. 1), and 2) that sea kraits can undergo important changes of natremia in response to the salt content of their aquatic environment (Fig. 1). Interestingly, such marked changes occurred relatively independently from variations in body mass, suggesting possible decoupling between natremia and net water flows between body compartments and the environment (Lillywhite et al., 2008, 2012).

The elevated natremia we recorded on free-ranging individuals could be merely a concentration of body fluid due to dehydration. Indeed, amphibious species such as sea kraits dehydrate both on land (Lillywhite et al., 2009) and at sea (Lillywhite et al., 2008; Brisichoux et al., 2012a). To cope with dehydration, and restore their water balance, sea kraits drink fresh water when available (Bonnet and Brisichoux, 2008; Lillywhite et al., 2008, this study). In our study, intake of fresh water presumably allows adjustment of natremia down to $\sim 150 \text{ mmol}\cdot\text{l}^{-1}$ (Fig. 1), a level considered as normal in snakes (Campbell, 2004). Although teasing apart the respective role of water loss (and thus concentration of body fluids) versus salt gain on natremia was not possible on the free-ranging individuals we sampled, our results suggest that despite having a functional salt gland, sea kraits display a high tolerance to hypernatremia.

Although both species are amphibious and share basic ecological traits (foraging at sea versus other activities on land: Heatwole, 1999), they also vary in their degree of emancipation from the terrestrial environment, *L. saintgironsi* being more terrestrial (Shine et al., 2003; Bonnet et al., 2005, 2009; Bonnet and Brisichoux, 2008). Each sea krait species also displays specific physiological adaptations: dehydration rate in seawater of *L. saintgironsi* (as assessed for its sister species *Laticauda colubrina*: Lillywhite et al., 2008) should be higher compared to *L. laticaudata* (Lillywhite et al., 2008). These differences are reflected by their relationship to salinity (Brisichoux et al., 2012a, 2013). Accordingly, the two species of sea kraits displayed different responses to salinity (Fig. 1). When transferred to seawater, the natremia of *L. saintgironsi* increased by 8.5% but remained stable in snakes kept in fresh water (Fig. 1). Salt gain was unlikely the result of drinking as captive sea kraits refuse to drink seawater (Lillywhite et al., 2008), our findings rather suggest that *L. saintgironsi* gain salt through permeable skin surfaces when kept in seawater for 12 days. In contrast, natremia in *L. laticaudata* rose only 1.2% following 12 days in seawater (Fig. 1). Interestingly, in *L. laticaudata* kept in fresh water, natremia continued to decrease by 9.4% (Fig. 1). These data suggest that either it took longer for *L. laticaudata* to restore osmotic balance through drinking and/or that an influx of fresh water through permeable surfaces had occurred in this species. This latter hypothesis may reveal important trade-offs with skin permeability, and deserves further study (see Dunson and Robinson, 1976; Dunson and Stokes, 1983). Overall, our experiment shows that, over an ecologically relevant time scale (duration of a foraging trip), the more terrestrial *L. saintgironsi* is more susceptible to salt gain through the skin than is the more marine *L. laticaudata*. These results dovetail remarkably well with interspecific differences in dehydration rates in seawater measured elsewhere (Lillywhite et al., 2008; Brisichoux et al., 2012a).

A review of plasma sodium concentration of marine snakes kept in fresh water or seawater provides additional insights (Table 1). Free-ranging marine snakes (including file snakes, sea kraits and hydrophines sea snakes) exhibit elevated natremia under natural conditions (Table 1). However, when transferred to fresh water, all of these species restore natremia to normal levels ($140\text{--}150 \text{ mmol}\cdot\text{l}^{-1}$: Campbell, 2004). This suggests that sea snakes share with other tetrapods (marine and terrestrial) relatively similar normonatremia as shown by the remarkable consistency of the levels they attain when hydrated (Table 1, Campbell, 2004). However, in striking contrast to other marine tetrapods (seabirds, marine mammals) sea snakes tolerate strong

Table 1

Summary of published data on plasma sodium concentration in several marine snake species (having salt glands). Most data from seawater (SW) comes from individuals captured in natural conditions; while data from fresh water acclimated (FW) snakes come from laboratory experiments. Data are mean values collected from Dunson, 1968 (a), Dunson and Dunson, 1973 (b), 1974 (c), 1975 (d), Dunson et al., 1971 (e), Duggan and Lofts, 1978 (f), Babonis et al., 2011 (g) and the present study (h).

Habits	Family	Species	Natremia (mmol·l ⁻¹)	
			FW	SW
Amphibious	Laticaudinae	<i>Laticauda saintgironsi</i> ^h	149.7	180.7
		<i>L. laticaudata</i> ^h	143.4	189.2
		<i>L. semifasciata</i> ^g	152.2	158.2
Aquatic	Acrochordidae	<i>Acrochordus granulatus</i> ^b	128.0	160.3
	Hydrophiini	<i>Hydrophis cyanocinctus</i> ^f	152.2	231.4
		<i>H. elegans</i> ^c	134.0	205.5
		<i>Pelamis platurus</i> ^{a,d,e}	140.0	232.1

hypernatremia and can sustain very high sodium concentrations in the plasma (Table 1, up to 307 mmol·l⁻¹ recorded in free-ranging *Pelamis platurus*). Although marine snakes' distributions and tolerances to salinity have been shown to correlate with the efficiency of their salt glands (Brischoux et al., 2012b), remarkably our results question the usefulness and/or efficiency of their functional salt gland. Empirical and experimental studies (Table 1) suggest that salt glands of sea snakes do not maintain normonatremia as in other marine tetrapods; instead they seem to serve to limit extreme salt loads. For instance according to Dunson et al. (1971), effective salt secretion is initiated once natremia deviate from high thresholds between 170 and 200 mmol·l⁻¹ in *P. platurus*.

We hypothesise that restricting active salt excretion to high levels of natremia represents an effective means of saving energy in these low-energy specialists (Pough, 1980). One would have expected more marine adapted species (hydrophiines) to regulate their natremia more precisely. Counter intuitively, "true" sea snakes (hydrophiines) which have relatively more highly developed and thus more efficient salt glands (Brischoux et al., 2012b) also show the highest tolerance to hypernatremia (all >200 mmol·l⁻¹, Table 1), while other species, presumably less marine-adapted (acrochordids, laticaudines: Brischoux et al., 2012b), show lower natremia under natural conditions (160–190 mmol·l⁻¹, Table 1). We suggest that, in addition to modifications of skin permeability (Dunson and Robinson, 1976; Dunson and Stokes, 1983), and the evolution of salt glands (Babonis and Brischoux, 2012) life in seawater might have substantially modified the tolerance of marine snakes to hypernatremia. A greater tolerance to hypernatremia would be beneficial since active salt excretion would occur only when plasma sodium dangerously exceeds an upper threshold. In turn, this would substantially decrease energetic costs linked to salt gland functioning (Peaker and Linzell, 1975; Gutiérrez et al., 2011) an otherwise continuous expenditure of energy that might be prohibitive in the day-to-day life of these organisms (Pough, 1980). Future studies should test this hypothesis in the context of the evolutionary transition to marine life in secondarily marine vertebrates. For instance, physiological performances (e.g. swimming) should deteriorate at a higher threshold of natremia in true sea snakes compared to other, presumably less marine-adapted species. More generally, our results support the notion that the great flexibility conferred by ectothermy is a major adaptive strategy related to the saving of energy in low-energy specialists (Pough, 1980; Shine, 2005).

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SYMPOSIUM

Dehydration and Drinking Responses in a Pelagic Sea Snake

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Synopsis Recent investigations of water balance in sea snakes demonstrated that amphibious sea kraits (*Laticauda* spp.) dehydrate in seawater and require fresh water to restore deficits in body water. Here, we report similar findings for *Pelamis platurus*, a viviparous, pelagic, entirely marine species of hydrophiine (“true”) sea snake. We sampled snakes at Golfo de Papagayo, Guanacaste, Costa Rica and demonstrated they do not drink seawater but fresh water at variable deficits of body water incurred by dehydration. The threshold dehydration at which snakes first drink fresh water is -18.3 ± 1.1 % (mean \pm SE) loss of body mass, which is roughly twice the magnitude of mass deficit at which sea kraits drink fresh water. Compared to sea kraits, *Pelamis* drink relatively larger volumes of water and make up a larger percentage of the dehydration deficit. Some dehydrated *Pelamis* also were shown to drink brackish water up to 50% seawater, but most drank at lower brackish values and 20% of the snakes tested did not drink at all. Like sea kraits, *Pelamis* dehydrate when kept in seawater in the laboratory. Moreover, some individuals drank fresh water immediately following capture, providing preliminary evidence that *Pelamis* dehydrate at sea. Thus, this widely distributed pelagic species remains subject to dehydration in marine environments where it retains a capacity to sense and to drink fresh water. In comparison with sea kraits, however, *Pelamis* represents a more advanced stage in the evolutionary transition to a fully marine life and appears to be less dependent on fresh water.

Introduction

Secondary evolutionary transitions between terrestrial and marine environments pose great difficulties for organisms and are not well understood. Such transitions involve numerous adaptations of morphology, physiology, and behavior of organisms (Mazin and de Buffrenil 2001). The maintenance of water balance in hyperosmotic environments is especially a problematic aspect of secondary marine transitions and is possibly the principal deterrent to successful marine life (Dunson 1979; Brischoux et al. 2012). Seawater (SW) is hyperosmotic to body fluids of most vertebrates, and therefore marine forms will tend to lose water and gain salts across permeable surfaces. Additional salts will be gained by drinking SW, whether incidentally or intentionally, and additional body water will be lost via pulmonary evaporation,

defecation, and excretion of waste products. Thus, living in SW incurs severe risk of dehydration.

Secondarily, marine vertebrates have evolved means of conserving water including specialized excretory structures that eliminate excess salt. Although these aspects of hydromineral balance are generally well studied, less is known regarding the means of replacing lost body water and the sources from which this water comes. Observations of drinking and behavioral responses to freshwater (FW) sources are especially scant, and recent observations suggest that some generalizations regarding drinking of SW may not be correct (Lillywhite et al. 2008).

Several independent lineages of reptiles have successfully colonized coastal waters, but comparatively few are capable of permanent residence in SW, especially in vast open seas. Sea snakes are the exception,

being widely distributed throughout much of the world's tropical marine waters (Heatwole 1999). Recent studies of amphibious sea kraits (*Laticauda* spp.) indicate that these species dehydrate in SW and require FW for water balance, in spite of possessing functional salt glands (Lillywhite et al. 2008). We do not yet know, however, whether the principal lineage of "true" sea snakes—some 60 species of Hydrophiini that are viviparous and entirely marine—also require FW. Therefore, to further understand the FW drinking requirements of sea snakes requires examination of this important marine clade.

Here, we report that yellow-bellied sea snakes *Pelamis platurus* drink FW and restore water balance when experimentally dehydrated, but, like sea kraits, do not drink SW. This finding is significant because it emphasizes some dependence on FW in what is arguably one of the more fully marine-adapted species of sea snake.

Methods

Animals and study site

We investigated dehydration and drinking behaviors in yellow-bellied sea snakes (*P. platurus*) at coastal sites in Golfo de Papagayo, Guanacaste, Costa Rica during three research trips conducted in 2010 and 2011. Snakes were collected 2–10 km offshore, during morning hours beginning after sunrise. Each snake was captured individually, either by hand or by using a handheld dip net, while the snake floated in a "float-and-wait" posture on the ocean surface (Brischoux and Lillywhite 2011). The snake was immediately transferred to a plastic container, inspected for epibionts and size, then transferred into a mesh bag for transport to shore.

Dehydration and drinking

In principle, we followed methods that were used in previous studies of sea kraits and reported by Lillywhite et al. (2008). Snakes ($n=29$) were weighed following their return to the laboratory after brief exposure to room air while lying on a dry towel until their skins were dry to the touch. Snakes were weighed to the nearest 0.1 g using a Sartorius ELT2001 electronic balance. Each snake was dehydrated by exposure to room air while being held individually inside a marked mesh bag. Mean air temperature was $25.8 \pm 0.6^\circ\text{C}$ and the mean relative humidity was $53.6 \pm 4.5\%$ during the times snakes were dehydrating.

Each animal was weighed daily (without bag) during the period of dehydration and testing, which varied from 3 to 13 days (mean 6.4 ± 1.3 days). The bags containing dehydrating snakes were kept separated on shelves and exposed to laboratory air during periods between weighings. Snakes in bags appeared remarkably calm, assumed relaxed loose coils, and moved little. After snakes had lost variable amounts of the original body mass, each was placed individually inside a plastic container half-filled with SW (approximately 2–4 l, depending on the size of snake) and observed for drinking (Fig. 1). Each snake was held overnight and re-weighed the following morning, 18–20 h later. Prior to each weighing, a snake was placed on an absorbent cloth towel, patted lightly to remove surface water, and then exposed to room air until the skin reached a dry condition as determined by touch (5–15 min). Each snake was treated similarly, and we attempted to be consistent with respect to the final condition of the skin prior to weighing.

After being weighed, each snake was then placed individually into a plastic aquarium half-filled with FW ($\sim 2\text{--}4$ l, depending on the size of the snake) and observed for drinking (Fig. 1). Each snake was held in FW overnight and re-weighed the following morning, 18–20 h later. If a snake did not drink FW, it was placed inside a mesh bag and the dehydration process was continued (in air) until the snake's mass was further reduced by variable amounts; the above protocol was then repeated. These steps were continued until drinking occurred, or until the loss of mass reached 27% of initial body mass (see below).

Drinking resulted in a gain in mass by snakes, but, to account for possible measurement error, we judged that drinking had occurred if a snake gained ≥ 1 g following 18–20 h in water. The majority of snakes that were kept in FW or SW and did not drink lost mass during similar periods.

The snakes used in these tests were collected on two different occasions. The second group of snakes ($n=11$) was returned to the laboratory and tested immediately for drinking FW using the protocol described above. These snakes were then dehydrated as above and subsequently used for further drinking tests. Also, when we tested this second group of snakes, we already knew (based on data from the first group) that the dehydration threshold for drinking FW usually exceeded a loss of 12% body mass. Therefore, we dehydrated snakes to greater deficits

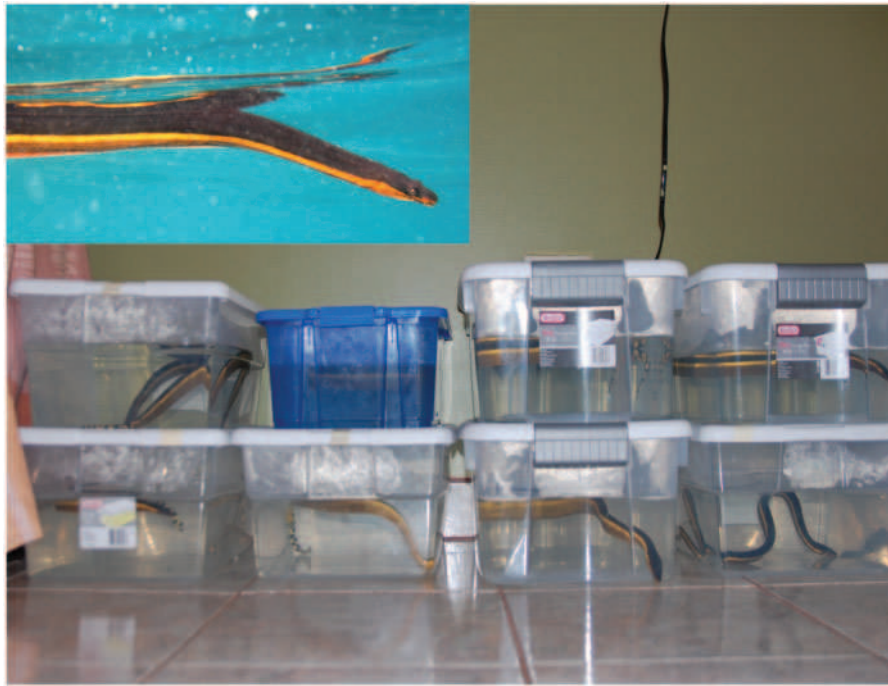


Fig. 1 Yellow-bellied sea snakes (*P. platurus*) resting in FW inside containers that were used in experiments on dehydration and drinking. The head down position of some of these snakes is typical of postures that are assumed by snakes floating at the ocean's surface (inset).

than that before we tested them for FW drinking at further intervals of dehydration (e.g., Fig. 2).

Tests of drinking in brackish water

In addition to the above experiments, 19 snakes (48.6 ± 9.1 g body mass) were tested for drinking FW immediately following capture, then dehydrated to $-19.2 \pm 1.1\%$ of their original body mass and subsequently tested for drinking in a regressive series of brackish water beginning at full SW (32 ppt), then 70% SW, 50% SW, 25% SW, 10% SW, and ending with FW (0 ppt). For each discrete step in the series, snakes were subjected to the drinking protocol described above, except that time in each salinity was 8 h. Each snake was placed in the next sequential water immediately following weighing, without any additional time in air between the salinities tested. Each snake went through the entire series of drinking tests regardless of the concentration at which drinking was first observed.

Dehydration in seawater

Five snakes were held in SW and their mass measured each day over a period of 11 days. Each snake was dried externally before weighing, as described

above. Snakes rested in SW in various positions, but usually with the head angled slightly downward, which is typical of the “float-and-wait” posture seen when snakes are floating on the ocean's surface (Fig. 1).

Data analysis

All data are expressed as mean \pm SE and were analyzed using Statview 5.0.1.0. Differences among variables were tested for significance using ANOVA and Fisher's PLSD *post hoc* tests. Percentages were log-transformed prior to analysis. Rates of loss of mass were determined using standard regression analysis.

Results

Dehydration and drinking

During two separate visits to the field site, we collected a total of 29 snakes (mass = 68.2 ± 6.3 g; range 14.6–155.8 g). Three snakes died during the initial dehydration process, with one individual refusing to drink even when dehydrated to -16.7% of original body mass. Three of the 11 snakes collected in the second sample drank FW amounting to 9.2%,

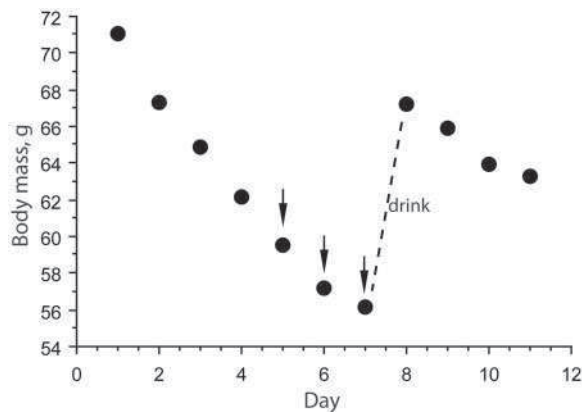


Fig. 2 Changes of body mass in an individual snake that was dehydrated by exposure to air in the laboratory. The arrows indicate points during the dehydration schedule when snakes were offered FW according to the protocol described in the text. This individual drank at the third opportunity when FW was offered at -21.4% loss of body mass. Drinking made up approximately $3/4$ of the mass deficit.

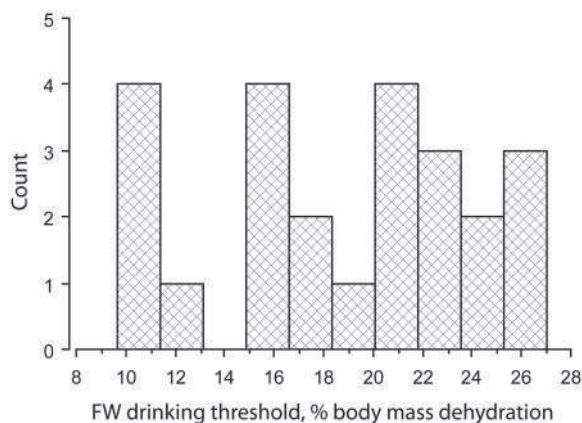


Fig. 3 Histogram illustrating variability in the threshold for drinking FW by *P. platurus*. The counts are number of snakes that drank at the indicated % loss of body mass.

10.4%, and 11.5% of the snake's body mass, respectively, immediately following capture.

Not a single snake drank SW in the laboratory. When held in FW, snakes drank measurable volumes at varying levels of dehydration (Fig. 2). The majority of snakes (85%) drank FW at dehydration deficits varying from 9.7% to 26% loss of body mass (Fig. 3). Four snakes (15% of total) refused to drink during dehydration up to 14.5%, 23%, 26%, and 27% of body mass, respectively. In comparison with sea kraits studied previously (Lillywhite et al. 2008), *Pelamis* dehydrated to greater deficits of body mass

and drank relatively greater volumes of water to replenish a greater percentage of the dehydration deficit (Table 1; Fig. 4).

Few snakes were observed drinking, but ingestion of water was evident from increases in body mass. In some cases, ingested water was also evident from a distended stomach and from water dripping from the lips. Care was taken to keep snakes level or with the head elevated during drying and weighing. In the few cases when snakes were observed to drink FW, movements of the mouth usually involved short to medium gapes with relatively rapid closure. Such drinking movements usually occurred at or near the surface of the water and were reflected in increases of mass by the snakes that we observed expressing this behavior.

Tests of drinking in brackish water

Of the 19 dehydrated snakes presented with the opportunity to drink from a regressive series of brackish water, none drank SW or 70% SW, and 5 snakes did not drink at any of the concentrations tested (Table 2). Snakes tended to drink relatively a greater volume of water at values up to 25% SW, then somewhat less at 50% SW (Fig. 5). Each of the snakes that first drank at 50% SW also drank further upon subsequent exposure to more dilute values. One of the snakes that first drank at 25% SW also drank at 10% SW, but none of the snakes that first drank at 10% SW drank again at FW. One of the 19 snakes drank 30.6% of its body mass in FW immediately following capture and prior to dehydration and subsequent testing in brackish water.

Dehydration in seawater

Regression analysis indicated that snakes kept in full SW lost mass at a rate of $0.54 \pm 0.03\%$ body mass per day. This rate was roughly an order of magnitude less than the rates of loss in air ($4.32 \pm 0.82\%$ body mass per day) at equivalent temperatures.

Discussion

We have explored the drinking behaviors of a pelagic sea snake and report that *P. platurus* does not drink SW but will drink FW when dehydrated sufficiently to induce a drinking response. This has important implications for the little-explored question of how marine vertebrates might respond to the distribution of FW sources (Lillywhite and Ellis 1994; Lillywhite et al. 2008, 2010; Lillywhite and Tu 2011;

Table 1 Dehydration parameters (mean \pm SE) for four species of sea snakes dehydrated for 2 weeks in air, then offered SW followed by FW

Species (n)	Body mass (g)	Cumulative dehydration deficit		SW ingested	FW ingested		
		g	% Mass		g	% Mass	% Deficit
<i>Laticauda colubrina</i> (14)	258.1 \pm 65.5 ^a	21.9 \pm 3.7 ^a	9.6 \pm 0.5 ^a	0	11.1 \pm 2.5 ^a	4.9 \pm 0.7	51.8 \pm 7.1 ^a
<i>L. laticaudata</i> (9)	205.1 \pm 16.2 ^a	31.5 \pm 2.3 ^a	15.5 \pm 0.5 ^b	0	15.8 \pm 2.6 ^a	8.0 \pm 2.6	51.2 \pm 7.2 ^{a,b}
<i>L. semifasciata</i> (12)	554.6 \pm 38.4 ^b	75.5 \pm 4.8 ^b	13.7 \pm 0.4 ^b	0	29.9 \pm 4.4 ^b	5.4 \pm 0.6	39.5 \pm 4.5 ^b
<i>P. platurus</i> (27)	68.2 \pm 6.3 ^c	12.29 \pm 1.6 ^c	18.3 \pm 1.1 ^c	0	8.24 \pm 0.9 ^c	13.06 \pm 1.0 ^c	77.3 \pm 7.8 ^c

Species are listed in order of decreasing terrestrial tendencies. *L. colubrina* spends considerable time on land, hiding among rocks near shoreline; *L. laticaudata* emerges onto rocks but spends most time in water; and *L. semifasciata* is nearly fully aquatic except for egg laying. *P. platurus* is pelagic and entirely marine. Data for *Laticauda* spp. are from Lillywhite et al. (2008).

Parameters with different symbols are statistically different for comparisons of species within a column (ANOVA, $P < 0.05$).

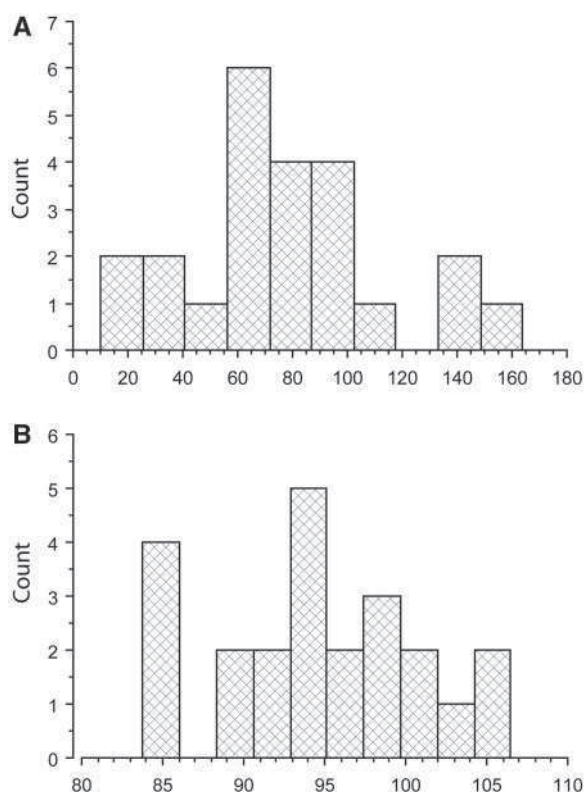


Fig. 4 Histograms illustrating variability in the amount of (A) body mass deficit that was made up by drinking FW (expressed as percentage of the mass deficit rehydrated), and the resulting (B) hydration status of the snakes (*P. platurus*) following drinking, expressed as percentage of original body mass.

Brischoux et al. 2012). In the context of water balance and responses to FW resources, *P. platurus* is especially important for two reasons: (1) It represents the only clade of marine snakes (Hydrophiini) that was previously not investigated with respect to

drinking FW. (2) It is pelagic, being the most widely distributed species of snake (southern Africa through Indo-Pacific to Central America) and the only sea snake to range into the eastern Pacific Ocean (Heatwole 1999). This species is totally marine, whereas laticaudine sea kraits we investigated previously (Lillywhite et al. 2008) are amphibious and represent separate and less advanced (transitional) stages in the adaptation to marine life by sea snakes. *Pelamis platurus* is part of the Hydrophiini clade of elapid snakes, which contains about 60+ species of viviparous, completely marine sea snakes (Slowinski and Keogh 2000; Sanders et al. 2008). Because of its pelagic habits and extensive range, *Pelamis* is arguably one of the more highly adapted species of marine snakes. Indeed, it offers a useful model for exploring distributional constraints in relation to water salinity and the evolutionary transitions from land to sea (Brischoux et al. 2012).

We found *Pelamis* to be different from sea kraits (1) in being far less inclined to drink FW (roughly two-fold higher threshold) (2) and in drinking a higher percentage of body water deficits during replenishment (Table 1; Figs. 3 and 4). Drinking a relatively larger volume of water is likely the result of the greater dehydration threshold at which these snakes first drink. Clearly, there is an evolutionary suppression of drinking response in *Pelamis* relative to sea kraits, the latter representing an earlier lineage of elapid sea snakes that evolved marine adaptations independent of the Hydrophiini (Slowinski and Keogh 2000). Because *Pelamis* exhibit a high dehydration threshold before drinking, we could not know the dehydration deficit of snakes before they were dehydrated in the laboratory. Therefore, the

Table 2 Summary of salinity thresholds at which 19 snakes (*P. platurus*) that had been dehydrated to $-19.17 \pm 4.69\%$ of their original body mass drank brackish or FW when exposed to a regressive series of salinities at the end of the dehydration period

SW	70% SW	50% SW	25% SW	10% SW	0% SW (FW)	No drinking
0	0	4 (9.3 ± 2.6)	5 (15.4 ± 3.0)	2 (13.9 ± 0.8)	3 (6.9 ± 2.0)	5

Table entries for each species indicate the number of snakes drinking from indicated water source when offered in series: 100% SW, 70% SW, 50% SW, 25% SW, 10% SW, 0% SW (FW).

Numbers in parentheses indicate the percentage of the original body mass of water ingested. Numbers are mean \pm SE.

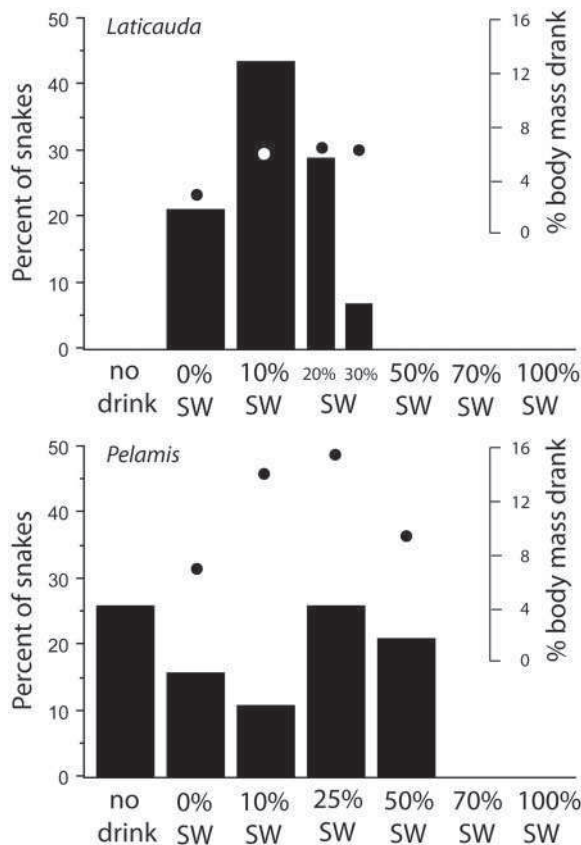


Fig. 5 Percentages of tested snakes that first drank at various salinities when tested in regressive order (100% SW to FW). The dots indicate the mean amounts of water drunk, scaled to the vertical axis on the right-hand side of the graph. The upper graph is based on data reported previously for *Laticauda* species (Lillywhite et al. 2008), and the lower graph illustrates data for *P. platurus* from the present study. All snakes were initially dehydrated to the species' mean threshold for drinking before exposing them to indicated salinities.

true body water deficit might be even greater than those we measured when snakes first drank FW. This fact renders our estimates of drinking threshold to be conservative and possibly contributes to the variation in the drinking responses measured.

Importantly, four of the snakes that were tested for drinking immediately following capture drank FW, thus indicating they were thirsty and, therefore, in a dehydrated state (Bonnet and Brischox 2008; Lillywhite et al. 2008). We are presently conducting further research to assess the extent to which *Pelamis* are naturally dehydrated at sea.

Our data also indicate that *Pelamis* drink brackish water up to 50% SW, whereas *Laticauda* spp. voluntarily drink brackish water only up to 30% SW (Fig. 5) (Lillywhite et al. 2008). Moreover, 20% of the *Pelamis* we tested did not drink water at all, and one snake refused to drink even after dehydration to a loss of 27% of its body mass. This observation reinforces the conclusion that the drinking response in this species is suppressed (or less sensitive) relative to that of *Laticauda*. Note that at lower salinities, both sea kraits and *Pelamis* drink progressively greater volumes as the salinity of the water increases (Fig. 5). However, there is a tendency in both species for the amount of water ingested to stabilize at higher salinities, and in the case of *Pelamis* to decrease at 50% SW (Fig. 5). This pattern likely reflects a trade-off between the relative amounts of water and salt that are ingested as the salinity of the water increases. None of the numerous snakes we tested ever ingested SW (Lillywhite et al. 2008; this study; unpublished observations).

The evolutionary origin of *P. platurus* is nested within the *Hydrophis* clade of sea snakes, which speciated rapidly within the past 5 Myr (Sanders et al. 2008). This and other species of sea snake conceivably represent middle to late evolutionary transition along a continuum leading to full physiological independence from FW sources. The evolution of viviparity (Sanders et al. 2008) and comparatively high rates of secretion from salt glands (Dunson 1968) bestow hydrophiine sea snakes with a high degree of adaptation to marine life compared with many other marine reptiles, including *Laticauda* spp. These conditions are reflected in a higher degree of

diversification than is characteristic of any of the other groups. Yet, *P. platurus* remains subject to dehydration in marine environments where it retains a capacity to sense and to drink FW.

The only potential sources of FW available to a pelagic species living in the open ocean are (1) fresh or brackish water lenses formed during heavy rains (Tomczak 1995) and (2) water in prey. Digestion of prey, however, incurs losses of water attributable to digestion, defecation, and excretion of salts and nitrogenous wastes via the salt glands and kidney. Increasing theoretical and empirical evidence suggests that consumption of prey might actually incur a net loss, rather than gain, of water (Peterson 1996; Henen et al. 1998; Longshore et al. 2003; Lillywhite et al. 2008; Davis and DeNardo 2010). The extent to which sea snakes of any species drink water from FW lenses in nature remains to be investigated (see also Lillywhite and Ellis 1994).

Dunson and Robinson (1976) also found that fasting *Pelamis* dehydrate in SW, and they documented drinking of FW when it was offered to dehydrated snakes. Snakes kept in SW survive for long periods if fed on FW fish (Dunson and Robinson 1976), but it remains unclear whether these snakes can survive for long periods if kept in full SW and fed marine species of fishes.

As with sea kraits, rates of net water efflux in SW are roughly an order of magnitude less than are those when snakes are in air at the same temperature. We estimated rates of net water loss using data for changes of mass in fasting snakes in SW, using the methods described by Lillywhite et al. (2008, 2009). Such rates of water loss in sea kraits vary with the aquatic tendencies of species, with more fully marine species having the lower rates (Fig. 6). We expected rates of water loss in *Pelamis* to be even lower due to its pelagic habits. Instead, net water efflux in *Pelamis* was intermediate in comparison with the three species of *Laticauda* (Fig. 6). Nonetheless, using the mean rate and assuming that 25% of the loss of mass is attributable to metabolic carbon (Lillywhite et al. 2008), we estimate that *Pelamis* can remain at sea without a source of FW for about 3 months if the lethal dehydration is about 36% of its body mass. The actual rates of net water efflux in nature are likely to be different from those measured in the laboratory, however, due to the influence of temperature, hydrostatic pressure acting on the skin when snakes are below the ocean's surface (87% of the time according to

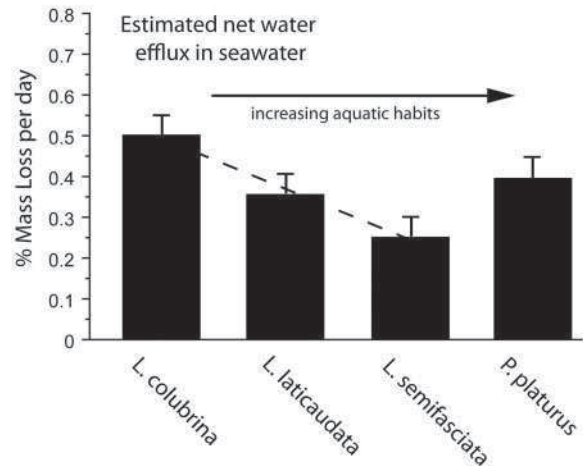


Fig. 6 Estimated rates of net water efflux for snakes kept in SW in the laboratory. Data are based on changes in mass and assume that 25% of the loss of mass is attributable to metabolic carbon (Lillywhite et al. 2008). The data for sea kraits (*Laticauda*) are from Lillywhite et al. (2009).

Rubinoff et al. 1986), and, importantly, behavior. In all likelihood, rates of water loss in freely ranging *Pelamis* are lower than those measured in the laboratory. Similarly, tolerance for dehydration might be greater than we suppose. All these factors require further investigation, which will be difficult due to the pelagic habits of this species. In spite of these limitations, knowledge of water balance in *P. platurus* and its behavioral response to water resources is important because of its position on the scale of evolutionary transition from the terrestrial to the marine habitat.

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Pelagic sea snakes dehydrate at sea

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Secondarily marine vertebrates are thought to live independently of fresh water. Here, we demonstrate a paradigm shift for the widely distributed pelagic sea snake, *Hydrophis (Pelamis) platurus*, which dehydrates at sea and spends a significant part of its life in a dehydrated state corresponding to seasonal drought. Snakes that are captured following prolonged periods without rainfall have lower body water content, lower body condition and increased tendencies to drink fresh water than do snakes that are captured following seasonal periods of high rainfall. These animals do not drink seawater and must rehydrate by drinking from a freshwater lens that forms on the ocean surface during heavy precipitation. The new data based on field studies indicate unequivocally that this marine vertebrate dehydrates at sea where individuals may live in a dehydrated state for possibly six to seven months at a time. This information provides new insights for understanding water requirements of sea snakes, reasons for recent declines and extinctions of sea snakes and more accurate prediction for how changing patterns of precipitation might affect these and other secondarily marine vertebrates living in tropical oceans.

1. Introduction

Water is essential to life, and it is a key resource especially in dehydrating environments such as deserts and ocean. The Earth's oceans teem with life, yet these salty environments are physiologically challenging because of the virtual absence of fresh water. The evolutionary transition of animals from land or fresh water to a marine habitat therefore is difficult because of the osmoregulatory challenges posed by salinity [1,2]. The successful clades of marine vertebrates that have undergone significant radiations in marine environments—bony fishes, cetaceans, pinnipeds, sea turtles, sea snakes and some birds—are thought to live independently of fresh water and to have overcome the osmoregulatory challenges by evolving anatomical and physiological specializations that maintain water balance (e.g. salt glands in birds and non-avian reptiles) [3]. However, the efficacy of such mechanisms has been questioned recently by noting dependence on fresh water that appears to limit the distribution and abundance of marine snakes [1,4–7].

The yellow-bellied sea snake, *Hydrophis (Pelamis) platurus*, is the only pelagic species of sea snake and is arguably one of the more marine-adapted species. It is the only sea snake that occurs in the eastern Pacific and, in fact, has the broadest global distribution of any species of squamate reptile. It ranges from coastal southeast Africa across the Indo-Pacific to the shores of Central America where the latitudinal distribution includes the Gulf of California to the north and Ecuador to the south [8].

We have investigated dehydration and drinking behaviour of *H. platurus* since 2009, with special focus on the population of snakes inhabiting the Golfo de Papagayo of northwestern Guanacaste, Costa Rica [4,9–11]. These marine waters and the adjoining dry forest experience drought for roughly half of the year (December through May or June) when rainfall is absent or negligible [12,13] (figure 1). Because *H. platurus* is pelagic and inhabits the open ocean, the only potential source of fresh water in its environment is a brackish

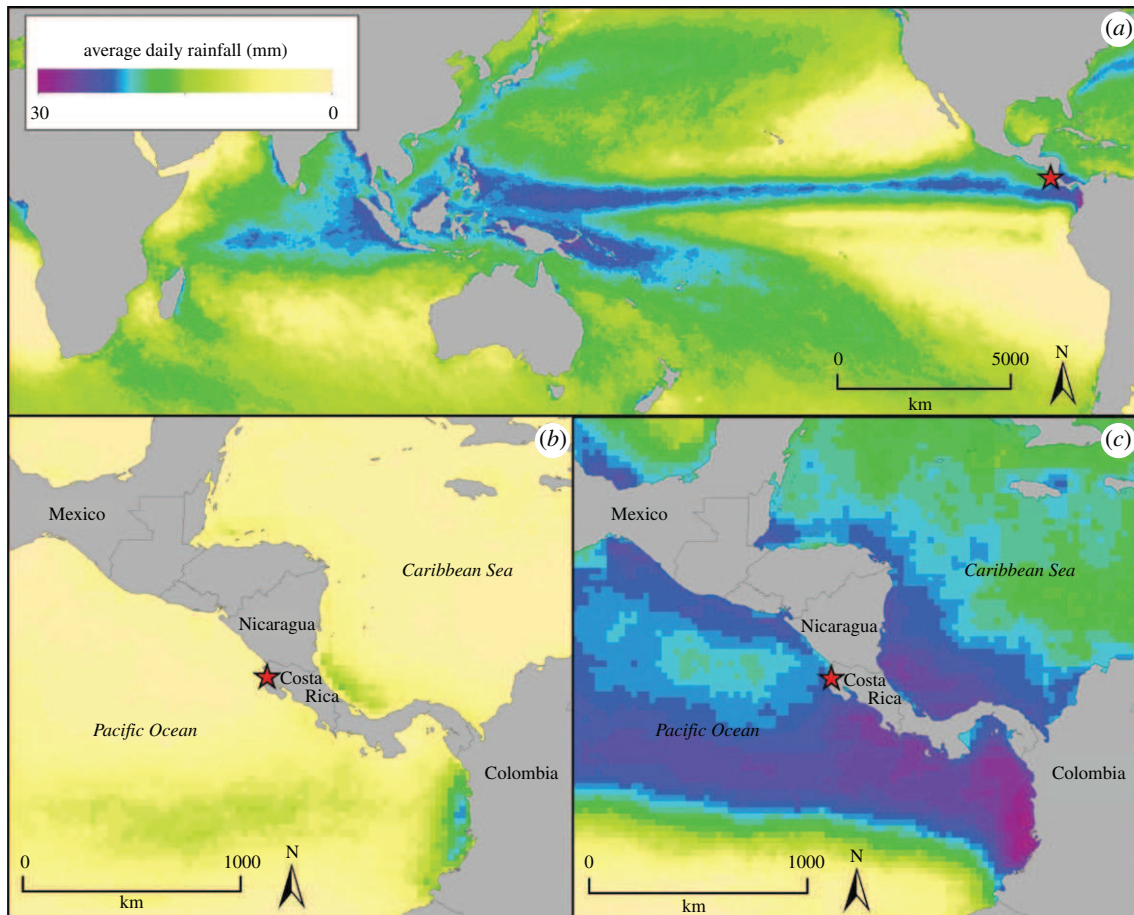


Figure 1. Spatial and temporal patterns of precipitation during the years 2010–2012. (a) Daily average rainfall for the tropical Indo-Pacific during the years 2010–2012. (b) Daily average rainfall during the dry season, December–May, 2010–2012. (c) Daily average rainfall during the wet season, June–November, 2010–2012. Data are from NASA metadata project TRMM v. 7, multisatellite precipitation. Red star represents Golfo de Papagayo, Costa Rica.

or freshwater lens that forms during heavy rainfall and, ideally, minimal mixing conditions of ocean water. Rainfall is more likely to occur over land, so the open ocean can be a virtual ‘desert’ especially during the dry season (figure 1b). Thus, we became interested to test whether this pelagic vertebrate dehydrates at sea. Here, we show that this pelagic species likely spends much of its life in a dehydrated state corresponding to cycles of prolonged seasonal drought.

2. Material and methods

We have made 10 field trips to the Guanacaste coast where, in different seasons, we collected a total of more than 500 live *H. platurus*. We sampled snakes during five to eight consecutive mornings each trip and tested whether they would drink fresh water immediately following capture. Snakes were captured using a dip net, returned to the laboratory in damp mesh bags, weighed to the nearest 0.1 g, placed in fresh water, observed for drinking and finally re-weighed the following morning *ca* 20 h later. Before each weighing to determine mass of a snake, it was gently blotted and allowed to air-dry on a towel for several minutes so the skin surface was dry to the touch and did not hold superficial water. Details and discussion of these methods may be found in previous publications [4,5,14]. Drinking is stimulated by thirst, which in turn indicates some level of dehydration prior to capture [5].

We measured the mass and length of snakes and calculated an index of body condition at capture. The body condition index (BCI) was quantified using residual scores based on linear regression of body size and body mass (log-transformed

for linearity) [15,16]. We excluded snakes from analysis if they were gravid with advanced embryos or had fish in the stomach, conditions that we determined by gentle palpation (and in some cases voluntary regurgitation of fish).

We determined the total body water (TBW) of 40 snakes we collected during three of the field sessions in Costa Rica and dried to total desiccation in a 70°C oven. Seven of these snakes appeared as healthy as the others but died of unknown causes at various times following the initial mass determination. The others were euthanized prior to drying in the oven. Two sets of measurements were made at the end of the dry season ($n = 9, 11$), while the other set was obtained at the end of the wet season ($n = 20$).

3. Results

The percentage of snakes drinking varied from 0 to 46% and exhibited a seasonal pattern, with most snakes drinking following periods of low rainfall (figure 2). The BCI was significantly lower in snakes that drank fresh water compared with those that did not drink following their capture (figure 3a). Moreover, the amount of water that snakes drank varied inversely with the BCI (figure 3b).

Using log-transformed data, mean TBW (\pm s.e.) in snakes captured at the end of the dry season ($75.2 \pm 0.3\%$ body mass) was significantly lower than that measured in snakes at the end of the wet season ($77.7 \pm 0.6\%$ body mass; *t*-test, $p = 0.0005$). In six snakes drinking fresh water, mean TBW was significantly lower before drinking ($75.8 \pm 0.9\%$ body

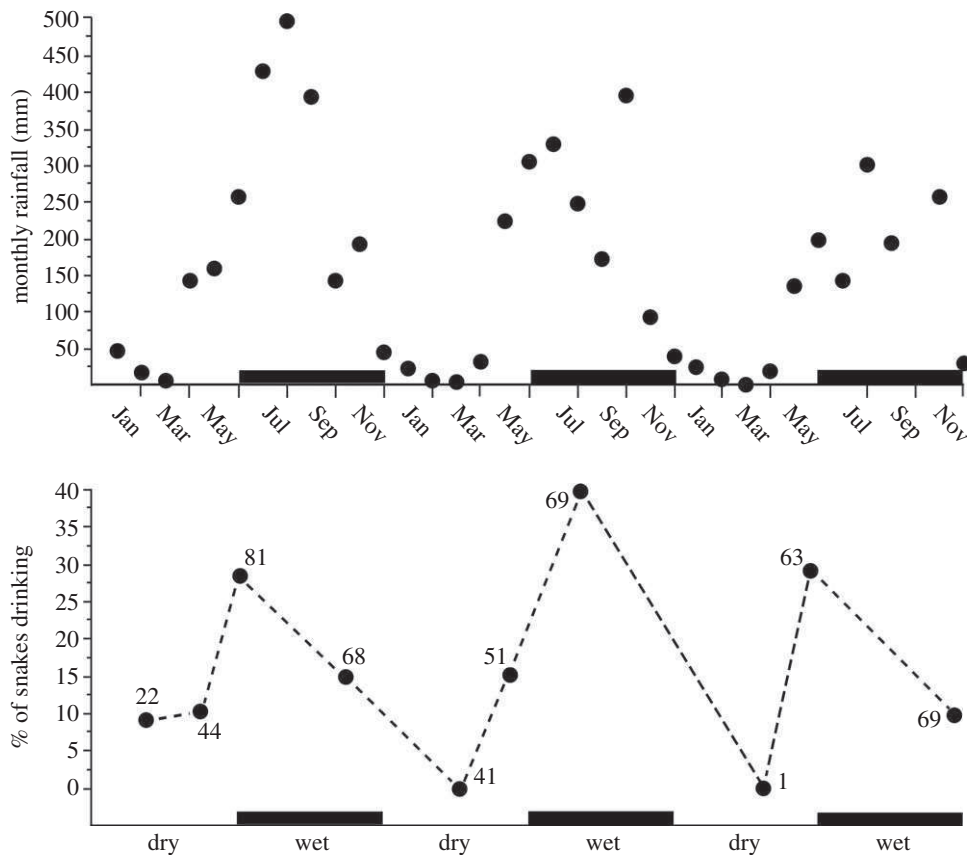


Figure 2. Patterns of monthly rainfall and the percentages of sea snakes (*H. platurus*) drinking fresh water (FW) during three drought cycles at Golfo de Papagayo, Costa Rica. Plots for rainfall are monthly totals, and the snakes drinking are percentages of the snakes sampled ($n =$ numbers next to data points) that drank FW immediately following capture from the open sea. Note that FW drinking increases following periods of several months without significant rainfall. FW drinking decreases following periods having large amounts of precipitation (see text for further explanation of the patterns). Data for rainfall are from NASA, TRMM 3B43 v. 7, and reflect monthly totals for a 25×25 km quadrat of ocean centred at the area from which snakes were collected. The data point representing a single snake (right-hand 'dry season') is included for completeness, although unusual conditions of a red tide combined with cold water, turbidity and high winds prevented a larger sampling of snakes at that time.

mass) than after drinking ($78.6 \pm 1.1\%$ body mass; paired t -test, $p = 0.0214$). The maximum TBW we measured varied from 79 to 81.6% in six individuals, and the minimum TBW we measured ranged from 73.3 to 75% in seven individuals.

4. Discussion

Previous studies have demonstrated thirst and drinking—hence dehydration—in amphibious sea kraits that spend time in terrestrial environments ([5,11] and references therein). Here, we show that pelagic sea snakes dehydrate at sea during seasonal drought. While there is an obvious seasonal pattern to drinking, both the numbers of snakes drinking and the seasonal timing of maxima and minima are variable and somewhat offset from the associated pattern of rainfall (figure 2). The observed pattern can be attributable to at least four factors. First, there is variability in the dehydration threshold at which snakes are stimulated to drink fresh water, the mean being a deficit of $-18.3 \pm 1.1\%$ s.e. loss of body mass [4]. Moreover, individuals dehydrated in the laboratory exhibit a range of such deficits spanning from 10 to 27% loss of body mass [4]. Therefore, snakes are likely to be in variable stages of dehydration and may not drink because of the variation and relative insensitivity of the response (high dehydration threshold; cf. amphibious sea kraits: [5]). Second, sea snakes dehydrate slowly in

seawater. *H. platurus* loses $0.54 \pm 0.03\%$ body mass per day in laboratory conditions, reflecting an efflux that is likely to be even smaller when snakes are in natural circumstances at sea [4]. Thus, snakes at Guanacaste are expected not to drink until well into the dry season because it requires several months to reach the dehydration threshold for drinking [4]. Third, precipitation is not necessarily tightly correlated with drinking because storms can be brief and spotty in location. Presumably, large and prolonged rain events with appropriate mixing conditions are required for the production of a freshwater lens that is suitable for drinking. Thus, a given storm might 'water' snakes at a particular location, while other individuals remain in drought perhaps only a few kilometres away. Finally, *H. platurus* are pelagic and subject to large-scale movements that involve drifting with currents [8,9,17,18]. Thus, any given collection of snakes for drinking observations might include individuals from locations having unknown histories of precipitation. Prevailing currents on the Guanacaste coast flow from south to north, so snakes drifting from more southerly and less drought-prone locations could arrive having had more recent access to fresh water than did those that might have been resident at Golfo de Papagayo for longer periods.

The BCI we measured in *H. platurus* was significantly lower in snakes that drank fresh water immediately following capture compared with those that did not drink following their capture (figure 3a), and the amount of water that

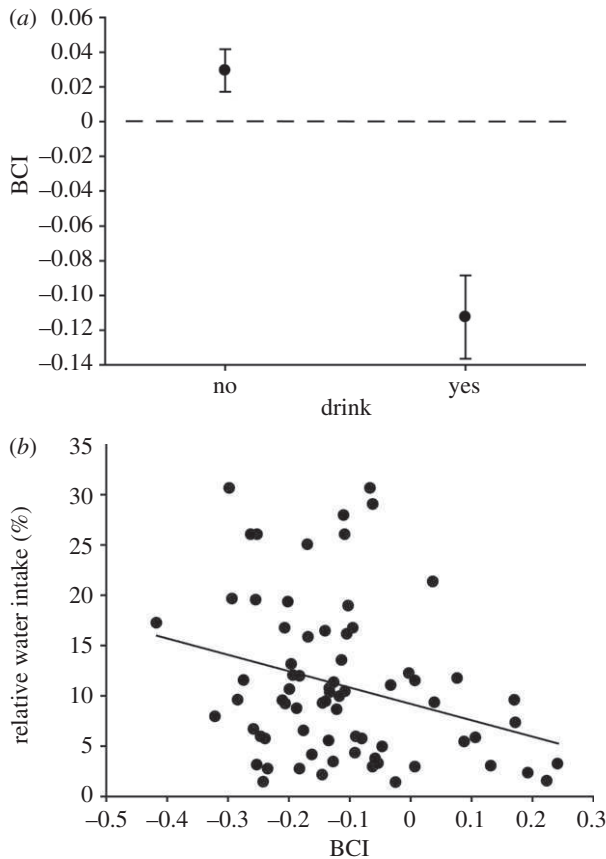


Figure 3. BCI related to consumption of fresh water by sea snakes (*H. platurus*) collected over three wet–dry cycles at Golfo de Papagayo, Costa Rica. Snakes that drink fresh water immediately following capture have significantly lower BCI than do those not drinking (a) (ANOVA, log-transformed data, $p \leq 0.0001$), and the amount of water ingested (% original body mass) varies inversely with the BCI (b) ($r^2 = 0.067$, $p = 0.0156$).

snakes drank varied inversely with the BCI (figure 3b). These results suggest that captured snakes having lower BCI reflect, at least in part, dehydration at sea. Elsewhere, it has been shown that annual increases of oceanic salinity exert a negative effect on BCI in populations of sea snakes inhabiting a lagoon at New Caledonia [6].

The body water content we measured in hydrated *H. platurus* (and some other sea snakes; H.B.L. 2012, unpublished data) is relatively high (roughly 80% body mass in hydrated individuals) compared with that of many other vertebrates, including freshwater snakes (means, 68.6–77.1% body mass [19]) and marine turtles (means, 64–66% body

mass [20]). The condition of TBW in sea snakes possibly represents a specialization that enhances dehydration tolerance, or may simply reflect a characteristically lower content of body fat compared with other species [20]. In any event, the extent of dehydration we have quantified in terms of reduced TBW (minima = 73.3–75% in seven individuals) corresponds very well with the expected reductions of TBW if snakes dehydrate to the mean threshold for drinking. A hypothetical loss of 18% body mass owing to dehydration [4] reduces TBW from 80 to 75.6% body mass. The previous measurements of TBW in this species by Dunson & Robinson (73.9% [21]) appear to represent dehydrated animals ($n = 6$).

In summary, data for spontaneous and voluntary drinking of fresh water, as well as the status of hydration and body condition, indicate unequivocally that this species of broadly distributed and exclusively marine snake dehydrates at sea and potentially remains in negative water balance for six to seven months at a time. We also conclude that such dehydration is not effectively mitigated either by functioning of salt glands or the consumption of marine fish [11,14]. Without a source of fresh water, *H. platurus* is in negative water balance in seawater and has sodium turnover rates that are too low to reflect significant ingestion of seawater [21]. Dehydration at sea by this and other sea snakes is quite possibly unique among secondarily marine vertebrates [22,23], and this phenomenon is an important consideration for determining the reasons for recent extinctions and population declines of marine snakes [24]. A more complete understanding of the water requirements in sea snakes and other secondarily marine vertebrates will potentially aid in more accurately predicting how changing precipitation patterns in the tropical oceans might impact these animals [25–27].

This research was conducted within guidelines and approval of the University of Florida IACUC.

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SYMPOSIUM

Effects of Oceanic Salinity on Body Condition in Sea Snakes

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Synopsis Since the transition from terrestrial to marine environments poses strong osmoregulatory and energetic challenges, temporal and spatial fluctuations in oceanic salinity might influence salt and water balance (and hence, body condition) in marine tetrapods. We assessed the effects of salinity on three species of sea snakes studied by mark-recapture in coral-reef habitats in the Neo-Caledonian Lagoon. These three species include one fully aquatic hydrophiine (*Emydocephalus annulatus*), one primarily aquatic laticaudine (*Laticauda laticaudata*), and one frequently terrestrial laticaudine (*Laticauda saintgironsi*). We explored how oceanic salinity affected the snakes' body condition across various temporal and spatial scales relevant to each species' ecology, using linear mixed models and multimodel inference. Mean annual salinity exerted a consistent and negative effect on the body condition of all three snake species. The most terrestrial taxon (*L. saintgironsi*) was sensitive to salinity over a short temporal scale, corresponding to the duration of a typical marine foraging trip for this species. In contrast, links between oceanic salinity and body condition in the fully aquatic *E. annulatus* and the highly aquatic *L. laticaudata* were strongest at a long-term (annual) scale. The sophisticated salt-excreting systems of sea snakes allow them to exploit marine environments, but do not completely overcome the osmoregulatory challenges posed by oceanic conditions. Future studies could usefully explore such effects in other secondarily marine taxa such as seabirds, turtles, and marine mammals.

Introduction

Secondarily marine, air-breathing vertebrates provide robust model systems with which to explore the complex effects of bio-physical parameters of the oceanic environment across a range of temporal and spatial scales. Research over the past two decades has revealed strong links between environmental parameters (e.g., sea surface temperature, primary production, sea-ice extent, El Niño or La Niña events, and fisheries offtake) on population parameters such as abundance (e.g., Baez et al. 2011), growth rates (e.g., Quillfeldt et al. 2007), survival (e.g., Rolland et al. 2010), breeding probabilities (e.g., Jenouvrier et al. 2003), breeding success (e.g., Leaper et al. 2006; Lee 2011), and aspects of individual behavior, such as spatial ecology and foraging

success (e.g., Pinaud et al. 2005; Weimerskirch et al. 2010). In several taxa, environmentally induced variation in such traits ultimately influences population dynamics (Forcada et al. 2006; Rolland et al. 2009). Understanding such links can enhance our ability to predict biotic responses to environmental perturbations (Jenouvrier et al. 2009; Wolf et al. 2010).

Although simply documenting empirical links between environmental variation and population responses is useful, an understanding of the proximate mechanisms that cause such links provides a stronger (and more general) basis for accurate prediction (Helmuth et al. 2005; Kearney and Porter 2009). In most cases, such mechanisms will include several intermediate steps between the physical properties of the marine environment and their ultimate effects

on individuals, or populations, of predators. All species of secondarily marine vertebrates use the oceanic environment to forage, so that the effects of physical oceanic parameters on apex predators likely are mediated by intermediate trophic levels (Pinaud et al. 2005). Even apparently direct effects, such as those of currents, fronts, or the extent of sea-ice on the at-sea distribution of seabirds or marine mammals, may in fact be mediated by the distribution of trophic resources (Bost et al. 2009).

Clearly, however, not all impacts of environmental variables on organismal function work through intermediate steps such as shifts in availability of food; some environmental effects act directly on the individual organism (Tomanek and Somero 2000; Helmuth et al. 2002). For example, water temperature directly affects body temperatures (and thus metabolic rates) of ectothermic vertebrates and, hence, influences the duration of their dives (Priest and Franklin 2002; Storey et al. 2008; Pratt and Franklin 2010); and substantially modifies the energy budgets of endothermic divers (de Leeuw 1996; Butler and Jones 1997; Grémillet et al. 2001). Although typically overlooked (but see Gutiérrez et al. 2011; Brischox et al. 2012), salinity poses a major physiological challenge to air-breathing marine vertebrates. Since seawater is hyperosmotic to body fluids, marine species gain salt and lose water across permeable surfaces (Schmidt-Nielsen 1983). Drinking of seawater (e.g., during prey capture) imposes a supplementary salt-load (Costa 2002; Houser et al. 2005). Thus, most marine vertebrates must regulate their osmotic balance (Schmidt-Nielsen 1983). Excreting excess salt through specific structures (salt glands in nonmammalian vertebrates [Peaker and Linzell 1975], reniculate kidneys, and elongated nephrons in marine mammals [Ortiz 2001]) can entail significant energetic costs (Schmidt-Nielsen 1983; Ortiz 2001; Gutiérrez et al. 2011). Dehydration due to osmotic loss of water to a saline medium is another risk faced by marine vertebrates (Lillywhite et al. 2008). Taken together, these elements suggest that oceanic salinity may impose significant energetic and hydric costs to air-breathing vertebrates.

Herein, we test the hypothesis that salinity may impose costs to marine tetrapods, using three species of sea snakes from the family Elapidae as our study system. Two independent phylogenetic transitions from terrestrial to marine life have occurred within this family (Hydrophiini and Laticaudinae) (Heatwole 1999). Extensive research on these taxa offers a robust ecological and physiological background to understand potential effects of salinity. Importantly,

the degree of reliance on marine versus terrestrial habitats varies extensively among species within these lineages. Hydrophiines are totally aquatic, whereas laticaudines are amphibious (Heatwole 1999). Within the laticaudines (sea kraits), some taxa use terrestrial habitats more frequently than do others (Greer 1997; Bonnet et al. 2005; Lane and Shine 2011a, 2011b), and laticaudine species vary in their ability to tolerate saline conditions (as measured by dehydration rates in seawater) (Lillywhite et al. 2008). Maintaining osmotic balance seems to pose a physiological challenge to marine snakes, and some species require access to fresh or brackish water for their survival (Bonnet and Brischox 2008; Lillywhite et al. 2008). Finally, salinity likely influenced the evolutionary transition to marine life in snakes and currently constrains the diversity and geographic distributions of sea snakes (Brischox et al. 2012).

This combination of traits renders the elapid sea snakes a powerful model system with which to explore the effects of salinity on marine vertebrates. Salinity might affect sea snakes through two pathways: (1) the energetic costs of excreting excess salt (Peaker and Linzell 1975; Gutiérrez et al. 2011) and (2) dehydration due to water loss from the body to the surrounding seawater (Lillywhite et al. 2008). Both of these processes should influence a snake's body mass (through utilization of body reserves for the former and due to water loss for the latter) and, hence, its body condition (mass relative to body length, *sensu* Bonnet and Naulleau [1995]). We thus explored the effect of salinity on the body condition of three species of sea snakes (a hydrophiine sea snake, *Emydocephalus annulatus*, and two laticaudine sea kraits, *Laticauda laticaudata* and *Laticauda saintgironsi*) from populations that we have regularly surveyed through mark-recapture studies since 2002 on the coral reefs of New Caledonia. Since these species differ in their degree of reliance on oceanic habitats (see earlier), we adopted two complementary approaches. First, we used a large time-scale analysis to compare inter-annual variation in body condition to concurrent variation in oceanic salinity. Second, we used a finer-scaled approach to explore potential effects of salinity at temporal and spatial scales relevant to each species' ecology.

Materials and Methods

Study species and study sites

Amphibious sea kraits (*Laticauda* spp.) at Signal Island
Two species of sea kraits occur in New Caledonia, *Laticauda laticaudata* and *L. saintgironsi* (Brischox

and Bonnet 2009; Lane and Shine 2011a, 2011b). Both species are amphibious: they forage at sea, mainly for anguilliform fish (moray eels, conger eels, and snake eels) (Brischoux et al. 2007, 2009, 2011) but return to small islands to digest their prey, slough their skins, mate, and lay eggs (Brischoux and Bonnet 2009). *Laticauda saintgironsi* is more terrestrial than *L. laticaudata* as measured through locomotor ability on land (Shine et al. 2003b; Bonnet et al. 2005) and habitat selection (Bonnet et al. 2009). When on land, *L. laticaudata* is mainly found under rocks that are submerged at high tide (Bonnet et al. 2009), whereas *L. saintgironsi* ventures farther inland (Bonnet et al. 2009; Lane and Shine 2011a). As a result, the primarily aquatic *L. laticaudata* spend most of the time in intimate contact with seawater, whereas the more terrestrial *L. saintgironsi* can more easily obtain freshwater during rainfall events (Bonnet and Brischoux 2008; Bonnet et al. 2009).

At sea, the two species forage in different habitats and take different prey species (Brischoux et al. 2007, 2009, 2011) and thus differ in the spatial extent and duration of their foraging trips (Brischoux et al. 2007; Fig. 1). When kept in seawater, the primarily aquatic *L. laticaudata* dehydrates less rapidly than does *L. colubrina*, a sister species of *L. saintgironsi* (Lillywhite et al. 2008; Lane and Shine 2011b).

Since 2002, we have regularly surveyed sea krait populations on Signal Island, in the South-Western Lagoon of New Caledonia (22°17'S, 166°17'E; Fig. 1). This small island is situated midway between the external barrier reef and the main island of New Caledonia (Fig. 1) (see Bonnet and Brischoux 2008; Brischoux and Bonnet 2008, 2009 for details on our field procedures). For this study, we focus on the 2002–2008 period, when we have detailed data both on sea kraits and on oceanic salinity (see later) (Fig. 2). Our mark–recapture data set included 1007 individually marked *L. laticaudata* and 1127 recaptures ($N=2134$) and 699 individually marked *L. saintgironsi* and 444 recaptures ($N=1143$, see “Analyses” later).

Turtle-headed sea snakes (*Emydocephalus annulatus*) at Nouméa

Emydocephalus annulatus is a shallow-water sea snake that spends its entire life under water (Cogger 1975; Ineich and Laboute 2002) and feeds on the eggs of damselfish, blennies, and gobies (Voriss 1966; Guinea 1996; Ineich and Laboute 2002). Since 2002, we have regularly surveyed two adjacent sites at Nouméa, New Caledonia (22°16'S, 166°26'E; Baie des Citrons and Anse Vata, separated by a few hundred meters)

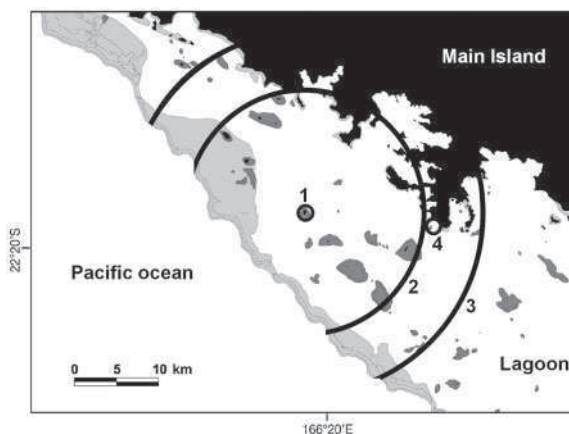


Fig. 1 Map of the southwestern lagoon in New Caledonia. Thick black circles (labeled 1, 2, 3, and 4) are centered on each study site and illustrate the spatial scales on which we focused our analyses; “1”, “2,” and “3” are centered on Signal Island (black dot within 1) and illustrate the 500 m, 14 and 21 km radii, respectively, and “4” is centered on Anse Vata and illustrates the 500 m radius (Baie des Citrons is adjacent, northwest of Anse Vata and included within that circle). See text for details. Black areas indicate emergent land (main island and small coralline islands within the lagoon), gray areas represent coral-reef flats, and light gray areas represent the barrier reef and other fringing reefs. Modified from Brischoux et al. 2007.

(Fig. 1). Details on our field procedures can be found elsewhere (Shine et al. 2003a, 2004, 2005, 2010; Shine 2005). For this study, we focus on the 2002–2008 period, as for *Laticauda* spp. Our mark–recapture data set included 443 individually marked snakes and 276 recaptures ($N=719$, see “Analyses” later).

Index of body condition

For each species, we quantified the body condition index (BCI) using residual scores from the linear regression between body size (snout-to-vent length) and body mass (both variables were log transformed for linearity) (Bonnet and Naulleau 1995). In both *Laticauda* spp., we excluded individuals with prey in the stomach and reproductive females (i.e., with vitellogenic follicles or oviductal eggs) from our calculations. For *E. annulatus*, our calculations excluded reproductive females but not recently fed individuals. Because *E. annulatus* feed only on tiny fish eggs (mean individual prey mass ~ 0.00008 g) (Shine et al. 2004), relative prey mass is trivial (e.g., 1000 eggs represent $<0.1\%$ of the snake’s mean body mass).

Salinity

Because long-term, fine-scale monitoring of salinity over contrasted spatial scales were lacking, salinity in

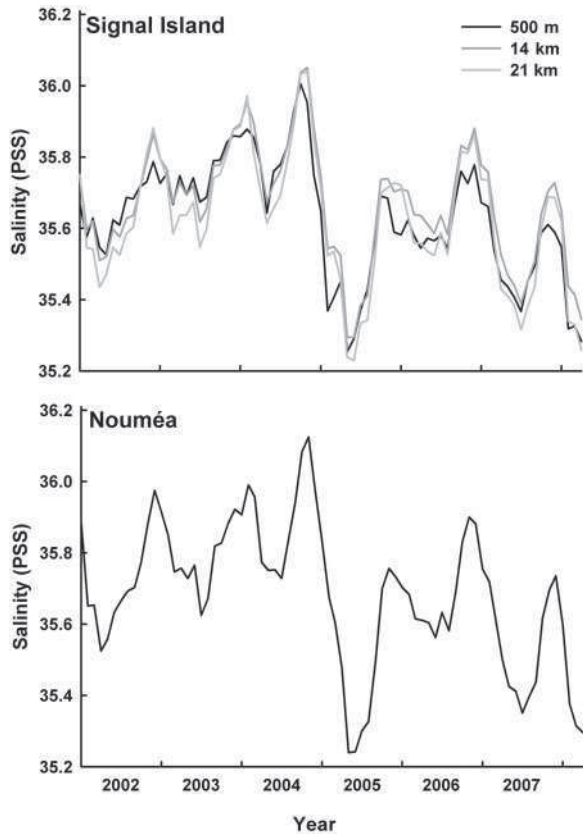


Fig. 2 Salinity around Signal Island at various spatial scales (500 m, 14 and 21 km; upper panel) and salinity around Anse Vata (Nouméa; lower panel). Curves represent monthly means between January 2002 and April 2008. PSS, Practical Salinity Scale. See text for details.

the lagoon was computed from the MARS3D (Model for Application at Regional Scales) model (further details can be found in Lazure and Dumas [2008]). The configuration is implemented on a 540 m resolution horizontal grid (i.e., fitting our smallest radii, see later) and 30 layers on a vertical grid. These layers are terrain-following and distributed to enhance resolution close to the sea's surface. This grid is oriented along the longitudinal axis of the main island of New Caledonia to optimize the number of wet cells, and the total domain encompasses both our study sites.

We used a high-resolution atmospheric model Weather Research & Forecasting (WRF) to estimate wind and heat fluxes at the sea's surface (see Lefèvre et al. 2010). The numerical solution of the BlueLink ReANalysis (BRAN) model (<http://www.marine.csiro.au/ofam1/>) was used to predict temperature, salinity, sea-surface height, and velocity of current along the lateral open boundary (e.g., open Pacific ocean outside the lagoon) (Fig. 1). High-frequency movements (tides and surges) of the sea-surface elevation were

added to the BRAN solution by harmonic composition from Advanced Circulation Model (ADCIRC) tidal components and an inverse barometer component. Finally, river flows were included, to estimate salinity near river mouths. Comparisons of predictions against data sets from coastal stations and hydrographic surveys show a good accuracy of the model (daily mean absolute error between predicted and observed values was $\sim 0.5\%$ around Signal Island and $\sim 1\%$ around Anse Vata). We used this model to predict oceanic salinity every 3 days between 2002 and 2008, but we used salinity integrated over longer durations (e.g., fortnights and months versus calculation of daily errors), thereby decreasing the overall error over the temporal scale of our analyses.

We used our information on snakes' spatial ecology to choose appropriate spatial and temporal scales for analysis of the putative links between salinity and the snakes' body condition. At our study sites, the foraging trips of *Laticauda* spp. are bimodal (Brischoux et al. 2007): either very short (<1 day, for one-third of the foraging trips) when snakes capture a prey item on the reef flats surrounding Signal Island (mean radius of ~ 500 m) (Fig. 1) or much longer (1–3 weeks, for the remaining two-thirds of the trips) in which case snakes capture their prey much further away (mean radius of ~ 14 km for *L. laticaudata* and ~ 21 km for *L. saintgironsi*) (Brischoux et al. 2007) (Fig. 1). As a consequence, we computed salinity values integrated over the vertical column within a radius of 500 m of Signal Island for both *Laticauda* spp., and within a radius of 14 km for *L. laticaudata* and 21 km for *L. saintgironsi* (Figs. 1 and 2). In contrast, *E. annulatus* is a shallow-water species, restricted to a small area around our study sites (e.g., virtually no exchange of individuals among our two study populations, despite the small distance between them [Lukoschek and Shine 2012]). We thus computed mean salinity values within a radius of 500 m, centered on Anse Vata (Figs. 1 and 2).

Analyses

To explore temporal and spatial extents of the effects of salinity on the snake's body condition, we incorporated scales relevant to each species' ecology. For each study site and/or radius, we calculated average salinity over the year, month, fortnight, and week during which a snake was captured. Because the effect of salinity will be integrated over time (i.e., a snake's body condition may reflect its history of exposure and current salinity levels), we also incorporated time-lags by calculating mean values of salinity

for the month previous to the month of capture, the fortnight previous to the fortnight of capture, and the week previous to the week of capture. Finally, we also computed mean salinity values over the month/fortnight/week 2 months/fortnights/weeks previous to the month/fortnight/week of capture. In total, we computed one time-constant model, and 10 models with different temporal scales of salinity variation for the three species, over two spatial scales for the *Laticauda* spp. This resulted in a total of 53 models: 11 for *E. annulatus* and 21 each for *L. laticaudata* and *L. saintgironsi*.

All models were linear mixed models with salinity as a fixed effect and individual identity as a random effect to account for individual heterogeneity (several individuals were captured more than once which could generate pseudoreplication). We used the lmer procedure in the lme4 package of R software (Crawley 2007). Due to temporal correlation between the various salinity variables, each variable was included in a separate model to explain variation in the BCI. To avoid multiple testing problems, we used an information theoretic approach to compare competing models and for statistical inference based on the Akaike Information Criterion (AIC) (Burnham and Anderson 2002). We began model selection with the time-constant model and models with annual salinity. Then, if the best model included annual salinity, we proceeded to examine models with salinity averaged over finer temporal scales. The best model was taken to be the one with the lowest AIC and $\Delta AIC \leq 2$ (where $\Delta AIC_i = AIC_i - \min AIC$). The AIC weights ($AICw_i$), a measure of relative likelihood of each model, were calculated as $AICw_i = \exp(-0.5 \times \Delta AIC_i) / \sum(\exp[-0.5 \times \Delta AIC])$.

Results

Annual effects

In all three species of marine snakes, models incorporating mean annual salinity were better than time-constant models (lower AICs), indicating that temporal variation in the snake's body condition was partly explained by variation in mean annual salinity (Table 1). For *Laticauda* spp., models that included mean annual salinity at the largest spatial scale relevant to snake foraging trips (within radii of 14 or 21 km around Signal Island depending on the species) (Fig. 1) were more powerful (>90% support) in explaining variation in the snake's body condition than were models that incorporated salinity variation at smaller spatial scales (Table 1).

Temporal and spatial effects of salinity

In the fully aquatic *E. annulatus*, variations in salinity over short (e.g., weekly) time scales were less successful at explaining temporal variation in the snake's body condition than was the model incorporating mean annual salinity (Table 2). A similar result was seen in the more aquatic of the two laticaudine species, *L. laticaudata*, whereby variation in the snake's body condition was most strongly linked to mean

Table 1 Selection of a model for body condition of snakes as a function of mean annual oceanic salinity

Model	Definition	AIC	ΔAIC	AICw
<i>Laticauda saintgironsi</i>				
1	21 km	-1126	0	0.94
2	500 m	-1120	5	0.06
3	<i>Constant</i>	-1115	11	0.00
<i>Laticauda laticaudata</i>				
1	14 km	-2145	0	0.98
2	500 m	-2137	8	0.02
3	<i>Constant</i>	-2119	26	0.00
<i>Emydocephalus annulatus</i>				
1	500 m	-1183	0	1.00
2	<i>Constant</i>	-1121	63	0.00

AIC is the Akaike Information Criterion. ΔAIC is the difference between the best model (lowest AIC) and the AIC of the model considered. AICw is the AIC weight representing the relative likelihood of the model considered. The best model is shown in bold face, and the italicized time-constant model is used as a reference model. See text for details and Tables 2–4 for the relative weights of these annual models when taking into account other temporal scales.

Table 2 Selection of a model for temporal fluctuations in body condition of the sea snake *Emydocephalus annulatus* as a function of variation in oceanic salinity at various temporal scales

Model	Definition	AIC	ΔAIC	AICw
1	Year	-1183.26	0.0	1.000
3	Previous 2 fortnights	-1138.76	44.5	0.000
4	Previous fortnight	-1130.17	53.1	0.000
5	Month	-1129.46	53.8	0.000
6	Previous 2 weeks	-1128.88	54.4	0.000
7	Previous month	-1124.71	59.1	0.000
8	Fortnight	-1123.71	59.6	0.000
9	Week	-1123.07	60.2	0.000
10	Previous week	-1122.33	60.9	0.000
11	Previous 2 months	-1122.19	61.1	0.000

See text for details and Table 1 for legend. Model 1 is identical to that in Table 1 but is now used as a reference model. "Previous 2 months/fortnights/weeks" stand for the salinity calculated during the month/fortnight/week 2 months/fortnights/weeks previous to the month/fortnight/week of the snake's capture.

annual salinity over a large spatial scale (Table 3). For this species, most of the top 10 models incorporated salinity values over the largest spatial scale (i.e., 14 km) (Table 3).

The more terrestrial *L. saintgironsi* showed a different pattern, with fluctuations in the body con-

Table 3 Selection of a model for body condition of the sea snake *Laticauda laticaudata* as a function of salinity at various temporal and spatial scales (500 m and 14 km from the snake's home island)

Model	Definition	AIC	Δ AIC	AICw
<i>1</i>	<i>Year—14 km</i>	-2144.92	0.0	0.998
4	Previous 2 fortnights—14 km	-2131.25	13.7	0.001
5	Previous month—14 km	-2128.98	15.9	0.000
6	Previous 2 weeks—14 km	-2128.60	16.3	0.000
7	Previous 2 months—14 km	-2128.32	16.6	0.000
8	Previous 2 weeks—500 m	-2124.87	20.1	0.000
9	Previous fortnight—14 km	-2122.73	22.2	0.000
10	Previous 2 months—500 m	-2121.94	23.0	0.000
11	Previous month—500 m	-2121.90	23.0	0.000
12	Previous 2 fortnights—500 m	-2120.90	24.0	0.000

Only the top 10 models and the time-constant model (italicized) are presented. See text for details and Table 1 for legend. Model 1 is identical to that in Table 1 but is now used as a reference model. "Previous 2 months/fortnights/weeks" represents the mean salinity calculated during the month/fortnight/week 2 months/fortnights/weeks previous to the month/fortnight/week of capture.

Table 4 Selection of a model for body condition of the sea snake *Laticauda saintgironsi* as a function of salinity at various temporal and spatial scales (500 m and 21 km from the snake's home island)

Model	Definition	AIC	Δ AIC	AICw
<i>4</i>	<i>Previous month—21 km</i>	-1149.41	0.0	0.524
5	Fortnight—21 km	-1146.05	3.4	0.098
6	Previous 2 fortnights—21 km	-1145.94	3.5	0.093
7	Previous week—21 km	-1145.79	3.6	0.086
8	Previous 2 weeks—21 km	-1145.65	3.8	0.080
9	Previous month—500 m	-1145.59	3.8	0.078
10	Previous fortnight—21 km	-1142.32	7.1	0.015
11	Previous week—500 m	-1141.77	7.6	0.012
12	Week—21 km	-1141.62	7.8	0.011
13	Fortnight—500 m	-1139.15	10.3	0.003
<i>1</i>	<i>Year—21 km</i>	-1126.05	23.4	0.000

Only the top 10 models and the time-constant model (italicized) are presented. See text for details and Table 1 for legend. Model 1 is identical to that in Table 1 but is now used as a reference model. "Previous 2 months/fortnights/weeks" represents the salinity calculated during the month/fortnight/week 2 months/fortnights/weeks previous to the month/fortnight/week of the snake's capture.

dition of this species best explained by variations in salinity over a shorter time scale (i.e., the month previous to the month of capture) (Table 4 and Fig. 3). Most other models with substantial support (i.e., Δ AIC < 4 and AICw > 0.08, Table 4) involved shorter time scales as well, bracketing a time lag spanning the week before the week of capture and the month before the month of capture (Table 4). Unlike the case with *E. annulatus* or *L. laticaudata*, the model incorporating mean annual salinity was poorly supported for the more terrestrial laticaudine species (Table 4). However, similarly to *L. laticaudata* and regardless of time scale, most of the top 10 models incorporated salinity values over the largest spatial scale (i.e., 21 km versus 500 m) (Table 4). Using model averaging (i.e., summing AIC weights of all models with salinity measured at 21 km), models that included salinity calculated at the largest spatial scale received 90.6% support among all tested models.

In all three snake species, the best models (*E. annulatus*: model 1, Table 2; *L. laticaudata*: model 1, Table 3; and *L. saintgironsi*: model 4, Table 4) indicated a negative effect of salinity on body condition (*E. annulatus*: slope = -0.221 ± 0.026 , $P_{\text{Wald}} < 0.0001$; *L. laticaudata*: slope = -0.131 ± 0.022 , $P_{\text{Wald}} = 0.0001$; and *L. saintgironsi*: slope = -0.162 ± 0.038 , $P_{\text{Wald}} = 0.002$, Fig. 3). That is, higher values of oceanic salinity consistently were associated with reduced body condition in sea snakes (all slopes were negative; values not shown).

Discussion

To our knowledge, our analysis is the first to assess the effects of variation in oceanic salinity on the body condition of free-ranging marine snakes. As expected from the physiological challenges of living in a hyperosmotic environment, sea snakes were in lower body condition during (and following) periods of high oceanic salinity, across a range of temporal and spatial scales (Tables 1–4 and Fig. 3).

There are some limits to our study, however, as our analysis did not include other environmental factors (such as water temperature or availability of food) that should also influence the body condition of free-ranging sea snakes. Incorporating such factors is difficult, for several reasons. First, the divergent life histories of the two families included in this study preclude a straightforward inclusion of these parameters. For example, including measurements of water temperature (if available) in our models would be straightforward for the totally aquatic *E. annulatus* but not for amphibious sea kraits that come back

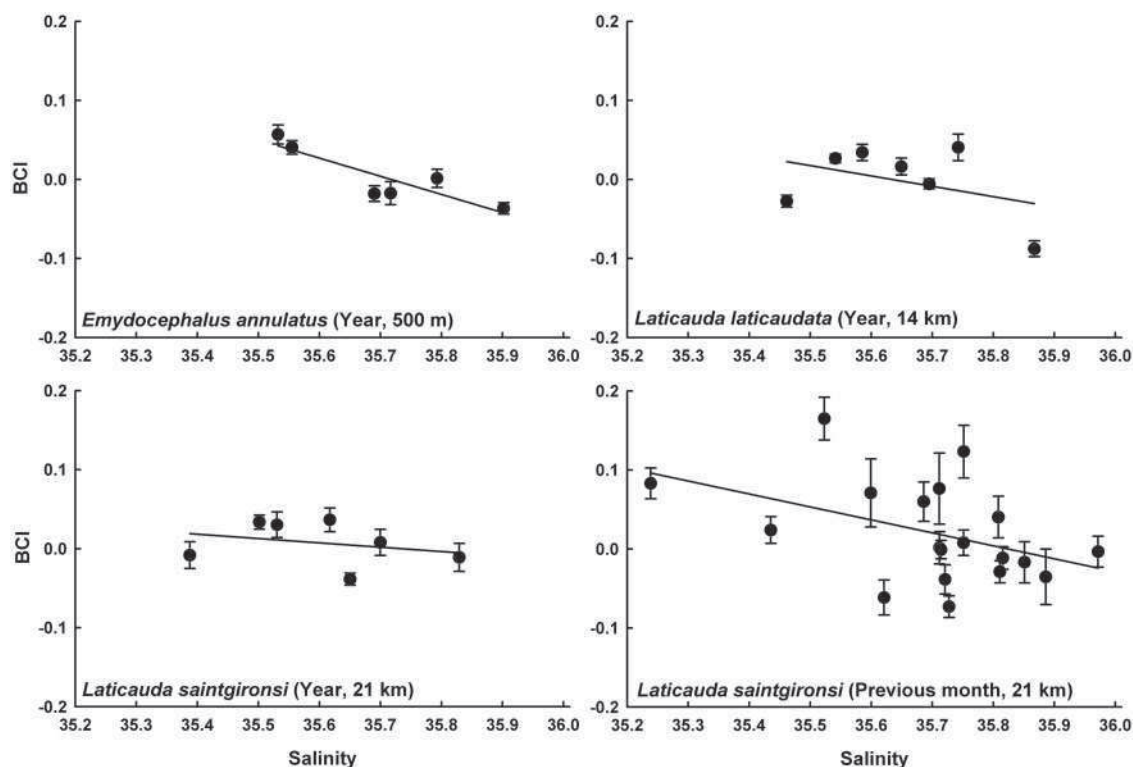


Fig. 3 Relationships between oceanic salinity (Practical Salinity Scale [PSS]) and body condition index (BCI) for three species of sea snakes. The panels show the relationship between mean annual salinity and mean body condition for *E. annulatus* (upper left panel), *L. laticaudata* (upper right panel), *L. saintgironsi* (lower left panel), and between mean salinity during the month previous to the month of capture and body condition for *L. saintgironsi* (lower right panel). Regression lines are drawn from the best models, and error bars represent standard errors. See text for details of the analyses.

on land to digest their prey. Thus, for both *L. saintgironsi* and *L. laticaudata*, thermal data would have to somehow combine at-sea and on-land thermal regimes (e.g., under beach rocks and in bird burrows) (Bonnet et al. 2009). That complexity prevents simple comparisons of similar models among species. Second, it was logistically impossible to obtain (or to model) detailed data for those parameters over the duration of our study and for the range of temporal and spatial scales we used. In addition, variation in salinity may directly affect the prey of the snakes. However, this hypothesis is not robustly supported by available data. First, the two lineages of sea snakes we examined in this study are highly divergent in their diets. Sea kraits feed on relatively large (mostly subadult and adult) anguilliform fish (Brischoux et al. 2007, 2009, 2011), whereas *E. annulatus* feeds exclusively on the eggs of damselfish, blennies, and gobies (Voris 1966; Guinea 1996; Ineich and Laboute 2002). It is unlikely that high salinity could similarly affect two contrasting life stages of two different fish lineages over similar temporal scales. Second, such putative direct effects of salinity on prey species cannot explain the different

effects of salinity we found between the most terrestrial taxon (*L. saintgironsi*—sensitive to salinity over a short temporal scale, see “Results” section) and the more aquatic species (*E. annulatus*, fully aquatic, and *L. laticaudata*, highly aquatic; both being sensitive to salinity over an annual scale). Thus, we cannot totally evaluate the impact of variation on salinity relative to other sources of variation (such as in temperature or food supply); all we can say is that our analyses suggest that oceanic salinity (a parameter largely overlooked to date) affects a sea snake’s body condition. Future studies could usefully attempt to quantify the relative contributions of various environmental parameters.

Models incorporating mean annual salinity were better predictors of the snake’s body condition than were time-constant models, as expected if (1) all three snake species were negatively affected by salinity and (2) they integrate the negative effects of salinity over a long period of time (Table 1 and Fig. 3). Incorporating variation in salinity over shorter time-scales did not improve our ability to predict variation in body condition in two of our study species: the totally aquatic *E. annulatus* and the highly aquatic

L. laticaudata; in both of these taxa, annual salinity was the best predictor of body condition among all variables tested (Tables 2 and 3). In contrast, the more terrestrial species (*L. saintgironsi*) appeared to be sensitive to fluctuations in salinity over shorter timescales (weeks to months) (Table 4). This time lag is consistent with the probable duration of a snake's most recent foraging trip at sea before capture (= 1–3 weeks) (Brischoux et al. 2007; Ineich et al. 2007). Digestion of a large meal requires 1–2 weeks (Brischoux et al. 2007; Ineich et al. 2007), so the foraging cycle (prey capture at sea and its subsequent digestion on land) is likely to last 2–5 weeks. As our analysis omitted snakes with prey items in their digestive tracts, the duration of the foraging cycle dovetails well with our conclusion that body condition in *L. saintgironsi* is affected by oceanic salinity over the preceding few weeks (Table 4).

In combination, our results suggest that *L. saintgironsi* is more sensitive to salinity over a short time than are the other taxa. There are three plausible (and complementary) reasons for this difference. First, *L. saintgironsi* is exposed to oceanic salinity only intermittently (during foraging bouts), so may be affected by conditions only at that time rather than averaged over a broader timescale. Second, the more terrestrial habits of this species (Bonnet et al. 2005, 2009; Lane and Shine 2011a) increase its access to freshwater during rare and unpredictable rainfall events (Bonnet and Brischoux 2008). Such events may allow *L. saintgironsi* to restore osmotic balance, regardless of oceanic levels of salinity (Bonnet and Brischoux 2008; see also Lillywhite et al. 2008). Third, higher dehydration rates in seawater (assessed in *L. colubrina*, a sister species of *L. saintgironsi*) (Lane and Shine 2011b) suggest that local salinity should affect body condition more rapidly in *L. saintgironsi* than in *L. laticaudata* (Lillywhite et al. 2008). All three of these processes might render body condition in *L. saintgironsi* sensitive to short-term rather than long-term levels of oceanic salinity.

Both *L. laticaudata* and *L. saintgironsi* sometimes forage close to their home island (on the reef flat within 500 m; Fig. 1) and sometimes much further away (mean radius of 14 and 21 km for *L. laticaudata* and *L. saintgironsi*, respectively) (Brischoux et al. 2007) (Fig. 1). The strongest effects of salinity on the body condition of these snakes are over the larger spatial scales (Tables 1–4), perhaps because the longer trips expose snakes to those salinity conditions for a prolonged period (Brischoux et al. 2007). In contrast, the highly sedentary *E. annulatus*

is affected by salinity levels over a small spatial scale (i.e., 500 m) (Fig. 1).

In summary, the invasion of marine habitats by terrestrial snakes has been accompanied by a wide range of morphological, behavioral, and physiological modifications that have enabled these animals to thrive in tropical oceans (Heatwole 1999; Aubret and Shine 2008; Brischoux and Shine 2011). Nonetheless, adaptations to marine life may not have completely emancipated snakes from the constraints associated with salt balance and water balance in a hyperosmotic environment (Lillywhite et al. 2008; Brischoux et al. 2012). Reflecting their ancestral dependence on freshwater, even these highly specialized marine snakes exhibited reduced body condition after periods of higher-than-average oceanic salinity. Although variation in salinity through time in the Neo-Caledonian lagoon is relatively minor (e.g., ~1 Practical Salinity Scale), sea snakes are exposed to salt overloading because for prolonged periods they remain in intimate contact with an hyperosmotic medium with very limited access to freshwater (Bonnet and Brischoux 2008; Lillywhite et al. 2008). Our analysis clearly detected negative effects despite the low range of variation in oceanic salinity. In support of these results, salinity constrains the current diversity and geographic distributions of sea snakes (Brischoux et al. 2012). Other populations of marine snakes are found in areas that fluctuate from very dilute to full-strength saltwater and may show much more dramatic effects. Also, our correlative analysis does not allow teasing apart the effects of the energetic costs of excreting excess salt (Peaker and Linzell 1975; Gutiérrez et al. 2011) and/or the dehydration due to loss of water to the surrounding sea (Lillywhite et al. 2008). Experimental approaches will be crucial for unraveling the respective contributions of these two different but complementary, physiological processes. Such experimental approaches also would improve our understanding of the mechanisms and intensity of the effect of salinity on marine snakes' body condition. In addition, future studies could usefully examine the effect of salinity on traits such as growth rates, survival, reproductive frequency, and reproductive output, as well as exploring the impacts of other environmental parameters such as temperature, rainfall, and availability of food. Fluctuations in oceanic salinity might well influence the population dynamics of this overlooked assemblage of tropical, marine, apex predators (Ineich et al. 2007; Brischoux and Bonnet 2008). Osmoregulatory constraints may be important in other secondarily marine vertebrates also, such as seabirds, turtles,

cetaceans, and pinnipeds (e.g., see Gutiérrez et al. 2011). For a comprehensive understanding of the impacts of climatic change on such animals, we cannot afford to ignore the potential role of oceanic salinity.

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Behavioral and physiological correlates of the geographic distributions of amphibious sea kraits (*Laticauda* spp.)

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ABSTRACT

The physiological costs of living in seawater likely influenced the secondary evolutionary transitions to marine life in tetrapods. However, these costs are alleviated for species that commute between the land and the sea, because terrestrial habitats can provide frequent access to fresh water. Here, we investigate how differences in the ecology and physiology of three sea krait species (*Laticauda* spp.) interact to determine their environmental tolerances and geographic distributions. These three species vary in their relative use of terrestrial versus marine environments, and they display concomitant adaptations to life on land versus at sea. A species with relatively high dehydration rates in seawater (*Laticauda colubrina*) occupied oceanic areas with low mean salinities, whereas a species with comparatively high rates of transcutaneous evaporative water loss on land (*Laticauda semifasciata*) occupied regions with low mean temperatures. A third taxon (*Laticauda laticaudata*) was intermediate in both of these traits, and yet occupied the broadest geographic range. Our results suggest that the abilities of sea kraits to acquire fresh water on land and tolerate dehydration at sea determine their environmental tolerances and geographic distributions. This finding supports the notion that speciation patterns within sea kraits have been driven by interspecific variation in the degree of reliance upon terrestrial versus marine habitats. Future studies could usefully examine the effects of osmotic challenges on diversification rates in other secondarily marine tetrapod species.

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1. Introduction

Oceanic salinity imposes an osmotic challenge to vertebrates living in seawater (Schmidt-Nielsen, 1983). This is especially true for marine tetrapods, a group that has evolved a diversity of structures that help to maintain osmotic balance (sophisticated nephrons in mammals, Ortiz, 2001; salt glands in reptiles, Peaker and Linzell, 1975). Regardless of the efficiency of these excretory structures, living in seawater inevitably entails significant physiological costs (Gutiérrez et al., 2011; Ortiz, 2001; Schmidt-Nielsen, 1983). Hyperosmotic conditions not only affect the day-to-day life of marine vertebrates, but also likely influenced their evolutionary transition to marine life (Brischoux et al., 2012a). For example, despite having salt-secreting glands, the diversity and geographic distributions of extant sea snakes are constrained by oceanic salinity (Brischoux et al., 2012a).

Although osmotic challenges apply to most or all marine tetrapods, some ecological situations alleviate these constraints. For example,

species that regularly commute between saline and terrestrial environments have increased opportunities to frequently access freshwater (i.e., shorebirds, Gutiérrez et al., 2011; sea kraits, Bonnet and Brischoux, 2008; Liu et al., 2012). In such situations, interactions between dehydration at sea and freshwater acquisition on land can influence individual movement rates (e.g., a dehydrated individual will commute more often to a source of freshwater). On a broader spatial scale, such interactions also may affect the extent of a species' geographic range. For example, species that are sensitive to dehydration at sea might be able to withstand saline conditions in the marine environment if their terrestrial habits allow regular access to freshwater sources. Such circumstances should allow species to occupy areas that are extremely saline relative to their dehydration rates, thereby increasing the extent of their geographic distributions, provided that suitable terrestrial habitats are available.

Sea kraits (*Laticauda* spp.) provide an excellent opportunity to investigate how the relative use of terrestrial versus marine environments influences environmental tolerances and geographic distributions. First, sea kraits are characterized by obligatory use of both land (to bask, digest prey, mate, and oviposit) and sea (where they forage for fish) (Heatwole, 1999). Second, this clade is divided into three major complexes (Heatwole et al., 2005), which broadly overlap in geographic range, but differ in their relative use of terrestrial versus marine

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environments. Species from the “*L. colubrina* complex” (N=4 species) are more terrestrial; species from the “*L. semifasciata* complex” (N=2 species) are more aquatic; and *L. laticaudata* (disregarding the closely related, lake-locked *Laticauda crockeri*) is intermediate (Greer, 1997; Heatwole, 1999). Third, experimental studies have shown that the three nominal species lie along a gradient of adaptations to life on land versus at sea (Lillywhite et al., 2008, 2009). Finally, life in seawater poses a major physiological challenge to sea kraits (Brischoux et al., 2012b; Dunson, 1975), and thus limits their distribution (Brischoux et al., 2012a; Lillywhite et al., 2008).

Herein, we investigate how differences in the ecology (relative use of terrestrial versus marine environments) and physiology (dehydration rates on land versus at sea) of sea kraits are related to their environmental tolerances (salinity and temperature), and ultimately, their geographic distributions.

2. Materials and methods

2.1. Study species and physiological data

Sea kraits (Laticaudinae) are amphibious snakes common throughout coral reefs of the Eastern Indian and Western Pacific Oceans (Heatwole, 1999). Sea kraits forage at sea for fish, but return to land to digest, rest, slough their skins, mate, and lay eggs (Heatwole, 1999). Importantly, these snakes rely on the frequent use of both environments (typically returning to land once every two weeks, Brischoux et al., 2007; Shetty and Shine, 2002).

Data on dehydration rates in seawater and rates of transcutaneous evaporative water loss on land in *L. colubrina*, *L. laticaudata* and *L. semifasciata* were measured experimentally using field-caught animals (see Lillywhite et al., 2008, 2009 for further details). These three species differ significantly in their relative use of terrestrial versus marine environments (Greer, 1997; Liu et al., 2012).

2.2. Geographic range and environmental data

Data on the distribution of each species of sea krait were taken from extent-of-occurrence range maps assembled by the IUCN Sea Snake Specialist Group (<http://www.iucnredlist.org/technical-documents/spatial-data>). Salinity and sea surface temperature (SST) were averaged within each species' geographic range using long-term gridded climate data available from Bio-ORACLE (Tyberghein et al., 2012). We did not use terrestrial temperature data because we lacked information on the terrestrial distributions of sea kraits. Instead, we used SST data as a proxy for air temperatures on land. The relationship between sea surface and terrestrial air temperatures should be particularly strong within the areas occupied by sea kraits because these species are largely restricted to coastlines (Bonnet et al., 2009; Lane and Shine, 2011a). Oceanic range sizes were calculated using an equal-area Behrmann projection.

3. Results

Dehydration rates in seawater were inversely related to the mean salinity within each species' oceanic range (Fig. 1a). The mean salinity within the range of *L. laticaudata* was intermediate between that of *L. colubrina* and *L. semifasciata* (ANOVA with the mean salinity in each grid cell as the dependent variable and species as the predictor, $F_{2,158,329} = 347.65, p < 0.0001$, Fig. 1a).

The mean SSTs within each species' range were inversely related to the mean rates of transcutaneous evaporative water loss (Fig. 1b). The mean SST within the range of *L. laticaudata* was again intermediate between that of *L. semifasciata* and *L. colubrina* (ANOVA with the mean SST in each grid cell as the dependent variable and species as the predictor, $F_{2,158,329} = 4039.8, p < 0.0001$, Fig. 1b).

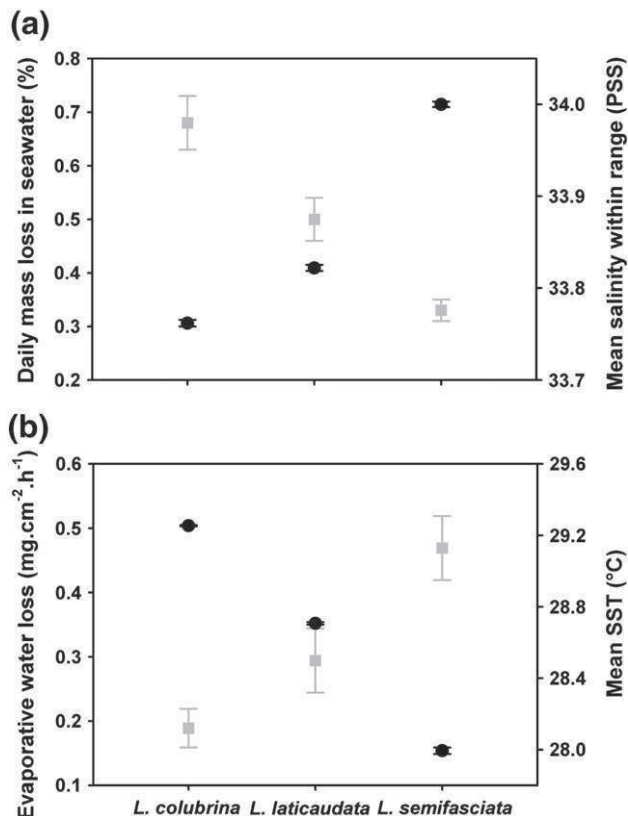


Fig. 1. Characteristics of the environmental conditions within the geographic ranges of sea kraits (*Laticauda* spp.). (a) Gray squares: daily mass loss in seawater for each species (modified from Lillywhite et al., 2008), and black circles: the mean salinity within each species' range. (b) Gray squares: transcutaneous evaporative water loss (modified from Lillywhite et al., 2009), and black circles: the mean sea surface temperature (SST) within each species range. Values shown are means ± SE.

The three species also diverged in geographic range size (Fig. 2). *L. laticaudata* occupied the broadest geographic range, whereas *L. colubrina* had a range that was intermediate in size between that of *L. semifasciata* and *L. laticaudata*.

4. Discussion

The three species of sea kraits included in our analyses differ in their reliance on aquatic versus terrestrial habitats, and exhibit a counter-gradient of physiological attributes related to water balance (Lillywhite et al., 2008, 2009). The more terrestrial *L. colubrina* is

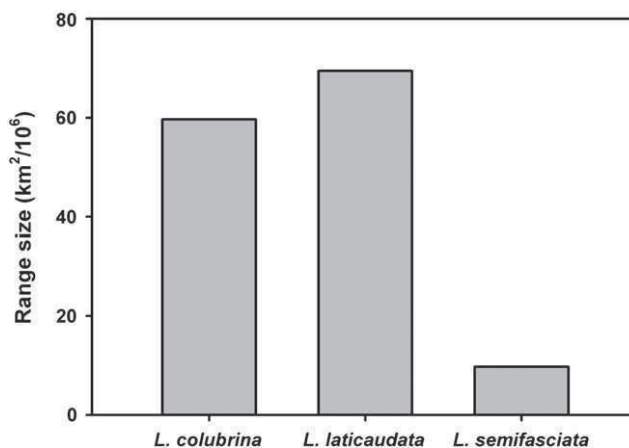


Fig. 2. Geographic range size of three species of sea kraits (*Laticauda* spp.).

resistant to desiccation on land, but sensitive to dehydration in seawater, relative to the other species. Conversely, the more aquatic *L. semifasciata* is resistant to dehydration in seawater, but relatively more sensitive to desiccation on land. *L. laticaudata* is intermediate in both traits (Fig. 1). Accordingly, the mean salinity within each species' oceanic range follows the reverse trend to that observed for dehydration rates in seawater (Fig. 1a). Sea surface temperature (a proxy for thermal conditions on land) within each species' range also follows the reverse trend to that observed for rates of transcutaneous evaporative water loss (Fig. 1b). Taken together, these results support the hypothesis that physiological constraints imposed by salinity and temperature limit the distribution and dispersal of sea kraits.

Because salinity poses a physiological challenge to sea kraits, these species have to restore their osmotic balance by acquiring fresh water during unpredictable rainfall events (Bonnet and Brischox, 2008; Guinea, 1991). However, the ease with which fresh water can be acquired depends on the degree of terrestriality of the species (Bonnet and Brischox, 2008). Precipitation is more likely to fall over tropical islands than over open ocean, and even minor rainfall events can provide fresh water for sea kraits in coastal environments (Bonnet and Brischox, 2008). In the ocean, on the other hand, heavy rainfall and appropriate environmental conditions are required to form freshwater lenses. As a consequence, the more terrestrial *L. colubrina* is more likely to acquire fresh water and restore its osmotic balance than is the more marine *L. semifasciata*, and to a lesser extent *L. laticaudata* (Brischox et al., 2012b). In addition, high rates of evaporative water loss are likely to limit the ability of *L. semifasciata* to spend significant amounts of time on land, preventing this species from accessing fresh water following rainfall events. Conversely, long periods in seawater, either during foraging trips or during dispersal over larger oceanic areas, will dehydrate *L. colubrina* more rapidly than *L. semifasciata*, and to a lesser extent *L. laticaudata* (Brischox et al., 2012b). Thus, the differential abilities of sea kraits to acquire fresh water on land and tolerate dehydration at sea are likely to influence the extent of their geographic distributions. In support of this view, the intermediate species *L. laticaudata*, moderately efficient at accessing freshwater on land (Bonnet and Brischox, 2008), but also moderately resistant to dehydration at sea, occupies the widest geographic range (Fig. 2).

Recent genetic analyses have shown that the distribution of terrestrial habitats within the oceanic range of *L. laticaudata* has little impact on gene flow (Lane and Shine, 2011b). Conversely, the more terrestrial “*L. colubrina* complex” (*L. colubrina*, *Laticauda frontalis*, *Laticauda guineai* and *Laticauda saintgironsi*) shows stronger geographic differentiation in allelic frequencies, associated with island groups (Lane and Shine, 2011b). Although we lack genetic data for the “*L. semifasciata* complex” (*L. semifasciata* and *Laticauda schistorhincha*), the existence of two species in this complex also suggests geographic differentiation. Taken together, these patterns suggest that the ability to restore osmotic balance during rainfall events might be more important for individual survival during infrequent dispersal events over large oceanic areas than is the resistance to dehydration in seawater. Accordingly, the restricted ranges of *L. semifasciata* and *L. schistorhincha* suggest that limitations on acquiring fresh water on land constrain successful dispersal in the more marine laticaudines. The intermediate ecology of *L. laticaudata* allows this species not only to colonize the widest range, but also to maintain gene flow among populations (Lane and Shine, 2011b). Collectively, these observations support the hypothesis that sea krait speciation patterns have been driven by differences in the importance of terrestrial versus marine habitats in the species' ecology (Lane and Shine, 2011b).

There are, however, several caveats to our conclusions. First, our analysis is based on correlations, and we have no direct evidence of causation. For example, it remains possible that sea snake distributions are determined by factors unrelated to osmotic challenges and that the correlations we see reflect adaptation of snake physiology to the osmotic conditions that each species experiences over its range; that is,

interspecific differences in osmoregulatory ability may be consequences rather than causes of the interspecific differences in geographic distribution. It is likely that the osmoregulatory abilities of extant snakes are evolving in relation to their present distributions. Second, our comparisons are based upon a small number of species, and the validity of our interpretations can only be tested by expanding the suite of taxa that are studied. The multiple evolutionary invasions of the marine environment throughout the tetrapod phylogeny provide abundant opportunities for such studies. For example, if it is generally true that an ability to cope with the osmotic challenges associated with marine life has influenced geographic ranges and speciation patterns of secondarily marine organisms (Brischox et al., 2012a), such processes might have contributed to the remarkably rapid radiation of hydrophiine sea snakes (Sanders et al., 2008, 2010). Future studies could usefully compare osmoregulatory capacities to geographic distributions in these and other species of secondarily marine tetrapods.

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Salinity influences the distribution of marine snakes: implications for evolutionary transitions to marine life

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Secondary transitions from terrestrial to marine life provide remarkable examples of evolutionary change. Although the maintenance of osmotic balance poses a major challenge to secondarily marine vertebrates, its potential role during evolutionary transitions has not been assessed. In the current study, we investigate the role of oceanic salinity as a proximate physiological challenge for snakes during the phylogenetic transition from the land to the sea. Large-scale biogeographical analyses using the four extant lineages of marine snakes suggest that salinity constrains their current distribution, especially in groups thought to resemble early transitional forms between the land and the sea. Analyses at the species-level suggest that a more efficient salt-secreting gland allows a species to exploit more saline, and hence larger, oceanic areas. Salinity also emerged as the strongest predictor of sea snake richness. Snake species richness was negatively correlated with mean annual salinity, but positively correlated with monthly variation in salinity. We infer that all four independent transitions from terrestrial to marine life in snakes may have occurred in the Indonesian Basin, where salinity is low and seasonally variable. More generally, osmoregulatory challenges may have influenced the evolutionary history and ecological traits of other secondarily marine vertebrates (turtles, birds and mammals) and may affect the impact of climate change on marine vertebrates.

Evolutionary transitions between habitats provide powerful opportunities to understand how selective pressures imposed by the new habitat have shaped the morphology, physiology, and behaviour of organisms. For example, secondary transitions from terrestrial to marine life provide remarkable examples of evolutionary change, driven by contrasting physical and chemical properties of the two environments (Mazin and de Buffrénil 2001). As a result, secondarily marine air-breathing vertebrates display a suite of specializations linked to marine life (Kooyman 1989). Compared to their terrestrial counterparts, these species exhibit a specialized morphology that allows efficient movement through water; their ability to store large amounts of oxygen, and to decrease rates of oxygen use, allow them to remain underwater for long periods; and their reduced susceptibility to high hydrostatic pressures allows them to dive deeply (Kooyman 1989, Boyd 1997, Butler and Jones 1997).

Adaptations of the respiratory system to marine life have attracted extensive research (Seymour and Webster 1975, Boyd 1997, Halsey et al. 2006, Brischoux et al. 2008). For example, a capacity for prolonged apnoea may well have been critical to the evolutionary success of secondarily marine air-breathing vertebrates. However, marine life poses physiological challenges other than respiration – notably, related to the chemical composition of seawater and, in particular, the high concentration of sodium chloride (Schmidt-Nielsen

1983). Because seawater is hyperosmotic relative to the internal milieu of most vertebrates, marine forms will tend to gain salt and lose water across permeable surfaces (Schmidt-Nielsen 1983). Additionally, drinking seawater (inevitable during prey capture) will impose a supplementary salt-load (Costa 2002, Houser et al. 2005). As a consequence, living in seawater entails a significant risk of dehydration, and most marine vertebrates have to regulate their hydro-mineral balance in order to survive (Schmidt-Nielsen 1983).

Secondarily marine vertebrates have evolved a diversity of excretory structures that eliminate excess salt and maintain hydro-mineral balance within a range compatible with life (Schmidt-Nielsen 1983, Houser et al. 2005). The kidneys of marine mammals are lobulated or reniculated, and the countercurrent geometry of elongated nephrons allows them to maintain osmotic balance by excreting large ion loads (Ortiz 2001). Reptilian kidneys lack the loops of Henle that are characteristic of mammals, and they are not able to excrete large ion loads in highly concentrated urine (Peaker and Linzell 1975). However, marine reptiles (*sensu lato* i.e. including birds) possess specialized extrarenal salt glands capable of excreting concentrated solutions of salt to maintain osmotic balance (Peaker and Linzell 1975).

We have very little fossil evidence of the taxa that are transitional between terrestrial and aquatic habitats – and

even when such fossils are available, they are unlikely to be preserved in enough detail to clarify critical aspects of physiology or behaviour (Mazin and de Buffrénil 2001). Hence, it is difficult to identify the role of physiological challenges (such as those linked to osmoregulation) during phylogenetic transitions to marine life. For example, the presence of salt glands in extinct marine reptiles is still a topic of active debate (Witmer 1997, Modesto 2006, Young et al. 2010, but see Fernández and Gasparini 2008). Additionally, morphological features alone may not provide unequivocal evidence as to function: for example, the specialised salt-excreting features of marine mammals (lobulated kidneys) are also seen in terrestrial ungulates (e.g. ruminants, pigs: Houser et al. 2005). Similarly, salt-excreting glands occur in many terrestrial birds and lizards and some freshwater crocodylians (Peaker and Linzell 1975).

In the current investigation, we examine a study system that facilitates exploration of the hypothesis that oceanic salinity was a major proximate challenge during evolutionary transitions from terrestrial to marine life. We use snakes as our model system because this lineage displays a combination of characteristics that circumvent some of the limitations highlighted above. First, four phylogenetic lineages of snakes independently underwent the transition to marine life; and those four lineages are spread across three Families (Homalopsidae, Acrochordidae and within Elapidae, the subfamilies Laticaudinae and Hydrophiini [Heatwole 1999]). Second, all of these independent transitions exhibit convergent evolution of salt-secreting glands (modified sub-lingual glands in Acrochordidae, Laticaudinae and Hydrophiini [Dunson 1976] and modified pre-maxillary glands in Homalopsidae [Dunson and Dunson 1979]), whereas no extant terrestrial or freshwater snakes are known to possess any such salt-secreting adaptations (Babonis et al. 2011). Third, the high ratio of surface area to volume imposed by the snake body plan (Brischoux and Shine 2011) likely makes maintaining osmotic balance a major physiological challenge for marine snakes, and some species cannot survive without access to fresh or brackish water (Lillywhite and Ellis 1994, Lillywhite et al. 2008). Finally, these lineages of marine snakes vary significantly in their degree of emancipation from the terrestrial environment, covering a continuum of intermediate ecological stages between the land and the ocean (Heatwole 1999). Some marine snakes are among the most fully marine tetrapod taxa, completely independent from land, whereas others depend upon terrestrial habitats for many of their daily activities.

This unique combination of traits within snakes provides a model system for investigating the role of oceanic salinity as a physiological challenge during the colonization of marine environments by terrestrial vertebrates, and suggests the following predictions: 1) salinity should constrain the oceanic distributions of marine snakes, and the extent of this constraint should vary concomitantly with their degree of marine life. 2) Marine snake richness should be negatively correlated with oceanic salinity, and positively correlated with variation in salinity because highly variable salinity should provide frequent access to rehydration with less saline water. 3) Snake species with more effective salt-excreting glands should extend into more saline areas, and hence be distributed over larger areas. 4) The transition from terrestrial

to marine life is most likely to have occurred in areas with low and/or variable salinity.

To test these predictions, we adopted two complementary approaches. First, we used large-scale analyses on broad phylogenetic groups (i.e. the four lineages of marine snakes) to identify abiotic factors affecting sea snake distribution, and snake richness. Second, we used a fine-scale (species-level) analysis to investigate the relationship between salt gland function and the geographic distributions of sea snake species.

Material and methods

Marine snake groups

Snakes underwent four independent transitions to marine life (ancestors of the present-day Homalopsidae, Acrochordidae and [within the Elapidae] the Laticaudinae and Hydrophiini). The potential minimum crown age for the marine adapted homalopsids is 18 My; 16 My for acrochordids; 13 My for laticaudines; and 7 My for hydrophiines (Alfaro et al. 2008, Sanders and Lee 2008, Sanders et al. 2010a). Although the minimum ages for these transitions fall in the Miocene, the transition to marine life could have occurred earlier (e.g. due to possible disparities between stem and crown ages for some of these clades). We did not include one facultatively marine species of Colubridae (the salt-marsh snake *Nerodia clarkii*) in our analyses because the osmoregulatory biology (e.g. presence or absence of a salt gland) remains unknown in this species (Babonis et al. 2011).

The species belonging to these four phylogenetic groups are conservative in their broad life-history traits (except hydrophiines, see below).

- 1) The Acrochordidae (filesnakes) consists of three extant species, placed within a single genus, *Acrochordus*. The three acrochordid species span the entire range from freshwater (*A. arafurae*) through brackish (*A. javanicus*) to saltwater (*A. granulatus*) (McDowell 1979). We considered only the two latter species in our analysis. These species are widely distributed through marine, estuarine and freshwater habitats, especially mangrove areas, and feed mainly on gobioid fishes which are located by active foraging in small crevices on the sea bottom (Voris and Glodek 1980). The posterior sublingual gland of *A. granulatus* is a salt gland (Dunson and Dunson 1973, see Biogeography and salt gland function). Freshwater drinking is important to water balance of *A. granulatus* (Lillywhite and Ellis 1994).
- 2) The Homalopsidae (Oriental-Australian rear-fanged water snakes) include 10 genera and 34 species of medium-sized snakes distributed from India across southeast Asia to northern Australia (Gyi 1970, Greer 1997, Voris et al. 2002). Eight species are coastal, living in mangrove forests, tidal mudflats, near-shore coastal waters and estuarine habitats (*Bitia hydroides*, *Cantorina violacea*, *C. annulata*, *Cerberus rynchops*, *Enhydria bennetti*, *Fordonia leucobalia*, *Gerarda prevostiana*, *Myron richardsonii*, Heatwole 1999), and we included these eight marine species in our analyses. Most research on ecophysiology linked to marine life has been conducted on the dog-faced water snake *C. rynchops*, which has the widest distribution of any homalopsid, and

is the only homalopsid species known to possess a salt gland (pre-maxillary glands, Dunson and Dunson 1979, see Biogeography and salt gland function).

- 3) The Laticaudinae (sea kraits) are present in most coral reef areas of the Indian and Western-Pacific Oceans (Heatwole 1999). These amphibious snakes forage for fish in coral reef systems but return to land to perform all other activities (digestion, mating, egg-laying, Heatwole 1999, Brischoux and Bonnet 2009). Of the eight laticaudine species (Heatwole et al. 2005, Cogger and Heatwole 2006), we did not include the brackish water, lake-locked *L. crockeri* (derived from marine ancestors) in our analysis (Cogger et al. 1987). Laticaudine sea snakes have salt glands (Dunson and Taub 1967, Dunson et al. 1971, Babonis et al. 2009, see Biogeography and salt gland function), but often drink freshwater (on land during rainfall events; Guinea 1991, Bonnet and Brischoux 2008, Lillywhite et al. 2008).
- 4) The Hydrophiini (true sea snakes) include the majority of marine snake species. They are the most truly marine of all extant reptilian taxa, never voluntarily leaving the water (Heatwole 1999). Two secondarily-derived freshwater species were excluded from our analyses (Heatwole and Cogger 1993). We thus included 54 hydrophiine species that are found mostly on coral reef areas of the Indian and Western Pacific ocean (Heatwole 1999). Although most species are benthic foragers on coral reefs, one taxon (yellow-bellied sea snake *Pelamis platurus*) is truly pelagic, and hence is the only marine snake not associated with the benthic community (Marsh et al. 1993). *Pelamis platurus* is widespread over the tropical Indo-Pacific, feeding on small fish at the sea surface (Heatwole 1999, Brischoux and Lillywhite 2011). Salt glands have been described in five different genera of hydrophiines (see Biogeography and salt gland function), and it is likely that all hydrophiines possess such glands (Dunson 1968, Dunson and Dunson 1974).

Our analyses below are largely based on these four independent examples of the transition from terrestrial to marine life (acrochordids, homalopsids, laticaudines, and hydrophiines). Due to the unique life history of *P. platurus* (see above), we also performed our analyses on hydrophiines excluding *P. platurus*, and on *P. platurus* alone.

Geographic range data

Data on the distribution of each species of marine snake were taken from extent-of-occurrence range maps assembled by the IUCN Sea Snake Specialist Group (< www.iucnredlist.org/technical-documents/spatial-data >). These range maps provided detailed information on the distribution of the four phylogenetic groups (acrochordids, n = 8083 grid cells; homalopsids, n = 779; laticaudines, n = 9085; hydrophiines, n = 26975 [excluding *P. platurus*; n = 9898; and *P. platurus* alone, n = 17077]). Range maps for each group were converted to a 0.25° grid to match the resolution of our environmental data (see below). Because we were interested in the abiotic factors associated with the transition to marine life, we excluded all snake locations from freshwater environments.

Environmental data

We investigated whether six environmental variables were correlated with the presence of each snake group: 1) mean annual salinity, 2) standard deviation of mean monthly salinity, 3) mean annual temperature, 4) standard deviation of mean monthly temperature, 5) water depth, and 6) distance to the nearest shoreline. Temperature and salinity data (0.25° resolution) were taken from the World Ocean Atlas 2009 (Antonov et al. 2010, Locarnini et al. 2010). Bathymetry data (0.017° resolution) were extracted from the ETOPO1 global relief model (Amante and Eakins 2009), and re-sampled to match the resolution of the climate data. Both climate and bathymetry data were standardized to exclude terrestrial areas using a vector shoreline dataset (National Geospatial-Intelligence Agency 1990). This shoreline dataset was also used to calculate the distance from the centre of each grid cell to the nearest shoreline (using an equidistant cylindrical projection). Pair-wise correlations between environmental variables were generally low ($r = 0.015\text{--}0.48$), with the exception of mean temperature and variation in temperature, which were significantly correlated with each other ($r = -0.77$).

Statistical analyses

To determine which environmental variables influenced sea snake presence/absence, we used classification trees as implemented in the rpart and caret libraries in R 2.12.0 (R Development Core Team). Classification trees have the advantage of flexibly incorporating variable interactions and non-linear relationships, while producing models that are easy to interpret (De'ath and Fabricius 2000). These models attempt to explain variation in a categorical response variable (in this case, sea snake presence/absence) by repeatedly splitting the data into smaller, more homogenous groups. Splits in the tree divide the response variable into two mutually exclusive subsets (nodes) using a rule based on a single explanatory variable (e.g. mean temperature $\leq 20^\circ\text{C}$). Each resulting node is then further partitioned using this splitting procedure. The end result is a decision tree consisting of numerous nodes, each of which is defined by a threshold value of an explanatory variable, a typical value of the response, and the sample size within the group (De'ath and Fabricius 2000).

Classification trees require the selection of a tree-size that represents an optimal trade-off between model parsimony and classification error. To determine the optimal-sized tree, we used a cross-validation procedure based on the area under a receiver operating characteristic curve (AUC), which is a threshold-independent measure of classification accuracy that ranges from 0.5 (random) to 1.0 (perfect). Our validation procedure involved three steps. First, we grew a nested sequence of trees of increasing size. Second, we calculated the AUC of each tree based on leave one-group out cross-validation, whereby CT models were trained on 75% of the data, and tested on the remaining 25%. To reduce sampling errors, this step was repeated 50 times for each tree. Finally, we chose the tree that had the highest AUC that was within one standard error of the maximum AUC value (Breiman et al. 1984).

Species distribution models such as classification trees require information on locations where species are absent, in addition to where they are present. Because we lacked absence data, we randomly sampled 'pseudo-absences' (Elith et al. 2006) within the latitudinal and longitudinal extents occupied by sea snakes, which roughly corresponds to the distributions of the Indian and Pacific Oceans. The number of pseudo-absence records for each group was equal to five times the number of presence records.

In a second set of analyses, we used regression trees to explore environmental constraints on sea snake species richness. IUCN range maps were intersected with a 0.25° resolution grid, and the number of species in each grid cell was summed. The procedure used to select the optimal-sized tree which adequately predicted sea snake richness was the same as that used in our presence-absence analyses, except that R² was used as a measure of model fit instead of AUC. *Pelamis platurus* was excluded from these analyses due to its unique life-history and large geographic range.

Biogeography and salt gland function

Because marine snakes have evolved specific excretory structures that eliminate excess salt, the efficiency of such salt secreting structures may limit (and thus, predict) the salinity

characteristics of the oceanic areas exploited by marine snakes. To test this hypothesis, data on maximum sodium (Na⁺) excretion rates were collected from the literature for eight species belonging to the four lineages of marine snakes (Acrochordidae: *Acrochordus granulatus*, Dunson and Dunson 1973; Homalospidae: *Cerberus rynchops*, Dunson and Dunson 1979; Laticaudinae: *Laticauda semifasciata*, Dunson and Taub 1967; Hydrophiini: *Aipysurus laevis*, *Lapemis hardwickii*, *Hydrophis elegans*, *H. major*, Dunson and Dunson 1974, and *Pelamis platurus*, Dunson 1968). We then correlated these maximum Na⁺ excretion rates with two measures of environmental tolerance: 1) the maximum salinity within each species' geographic range, and 2) geographic range size (calculated using cylindrical equal-area projections).

Results

All four groups of sea snakes were largely restricted to areas within 46 km from the nearest shoreline (Fig. 1). Within these areas, however, environmental constraints differed among the four groups.

The highest-ranked classification tree for Hydrophiini contained a secondary split on the distance to the nearest shoreline at 32.6 km (Fig. 1A), suggesting that this variable

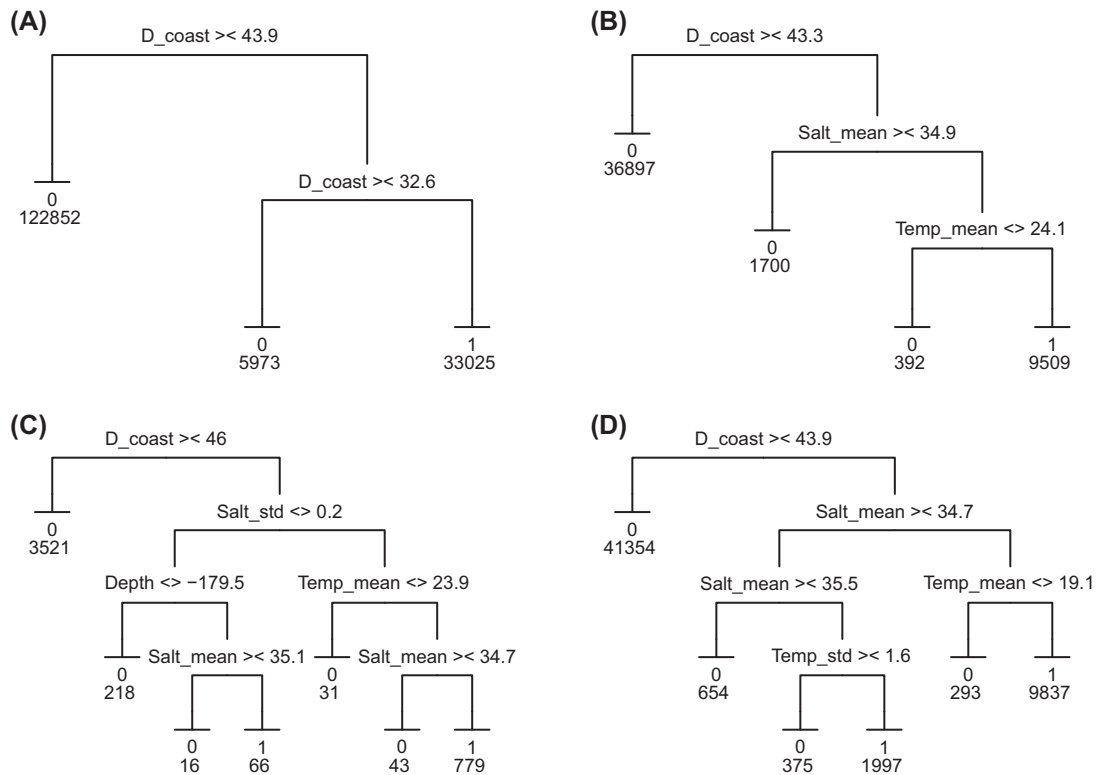


Figure 1. Highest-ranked classification trees predicting the distributions of hydrophiine (A), acrochordid (B), homalopsid (C), and laticaudine (D) sea snakes at a global scale. Trees are read from top to bottom. Each split in the tree attempts to divide the response variable (snake presence [1] or absence [0]) into homogenous groups according to a threshold value of an explanatory variable (shown above each split). A '<>' symbol indicates that cases with lower values go to the left, whereas a '>>' symbol means that cases with lower values go to the right. Sea snake presence (1) or absence (0) and sample sizes (number of 0.25° grid cells) are given below each node. D_coast = distance to the nearest shoreline (km), Depth = ocean depth (m), Temp_mean = mean annual temperature (°C), Temp_std = standard deviation of mean monthly temperature, Salt_mean = mean annual salinity (according to the practical salinity scale), Salt_std = standard deviation of mean monthly salinity.

was the sole constraint on the distribution of this group. Results were qualitatively similar when the wide-ranging *P. platurus* was considered in isolation (Supplementary material Appendix 1). However, when *P. platurus* was excluded from the Hydrophiini (Supplementary material Appendix 1), the remaining hydrophiines were also more likely to occur in warm climates (annual temperatures $> 19.9^{\circ}\text{C}$), suggesting that the broad thermal tolerance of *P. platurus* obscured the influence of temperature on the overall distribution of hydrophiines.

Environmental constraints on the distribution of Acrochordidae were more complex. Acrochordids occupied areas that were characterized by low annual salinities (< 34.9 PSS) and high annual temperatures ($> 24.1^{\circ}\text{C}$; Fig. 1B).

Homalopsid occurrence was linked to monthly variation in salinity (Fig. 1C). In regions with high salinity variation (standard deviation > 0.2), homalopsids were most likely to occur in areas with warm annual temperatures ($> 23.9^{\circ}\text{C}$) and low annual salinities (< 34.7 PSS). In areas with low variation in salinity (standard deviation < 0.2), homalopsids were more likely to be found in shallow waters (< 179.5 m) with low annual salinity (< 35.1 PSS).

Correlates of laticaudine distribution varied according to annual salinity levels (Fig. 1D). In low salinity regions (< 34.7 PSS), laticaudines were more likely to occur in areas with warm annual temperatures ($> 19.1^{\circ}\text{C}$). However, under more saline conditions (> 34.7 PSS), laticaudines occupied areas with low annual salinities (< 35.5 PSS) and more stable thermal properties (variation in monthly temperatures < 1.6). Classification trees for all groups had extremely high predictive accuracy, with all models falling within the good to excellent category of Swets (1988) (Supplementary material Appendix 2).

The best regression tree of sea snake richness also had high explanatory power ($R^2 = 63.8\% \pm 0.0137$), but was structurally complex, containing eleven variable splits

(Fig. 2). Salinity emerged as the strongest predictor of sea snake richness, with annual salinity (Fig. 3A) and variation in monthly salinity (Fig. 3B) being the most frequently selected variables in the tree. In areas with low annual salinities (< 34.2 PSS), species richness was highest in areas with warm annual temperatures ($> 22.7^{\circ}\text{C}$), shallow depths (> 117.5 m), and low annual salinities (< 33.3 PSS). Under more saline conditions (> 34.2 PSS), sea snake richness was highest in shallow areas (> 101.5 m) with low annual salinities (< 36 PSS) and high heterogeneity in monthly salinity (standard deviation > 0.2).

Maximum Na^+ excretion rates were positively correlated with maximum annual salinity within each species' oceanic range ($F_{1,6} = 9.63$, $R^2 = 0.61$, $p = 0.02$, Fig. 4A) and also with the oceanic range size of each species ($F_{1,6} = 5.93$, $R^2 = 0.49$, $p = 0.05$, Fig. 4B). That is, species with more effective salt-excreting glands penetrated into areas of higher salinity, and had broader geographic distributions.

Discussion

Collectively, our results support the hypothesis that oceanic salinity is a significant abiotic constraint on the current distributions of marine snakes. In turn, that result suggests that dealing with salinity has been a major evolutionary challenge during the transition from terrestrial to marine life in snakes. Data on oceanic salinity predicted the geographic distributions of three of our four lineages of snakes, and these effects were largely consistent with each group's degree of emancipation from the ancestral terrestrial environment. Homalopsids, acrochordids and laticaudines are restricted to estuarine habitats, mangroves, tidal mudflats, coastal waters and/or are amphibious, and thus may resemble early transitional forms along the gradient of habitat between the land and the ocean (e.g. laticaudines are amphibious and

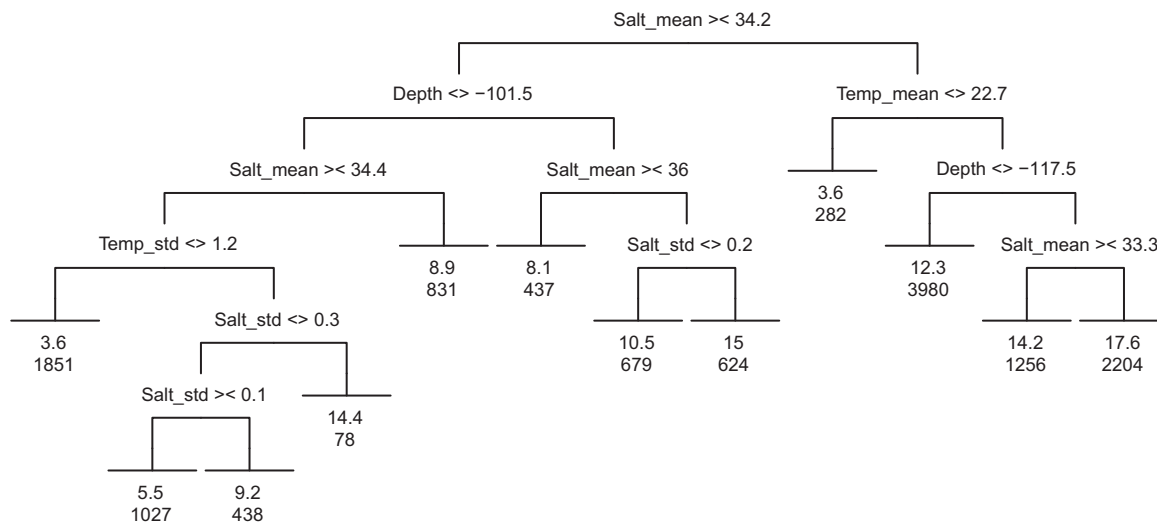


Figure 2. Highest-ranked regression tree predicting the richness of marine snakes at a global scale. The tree is read from top to bottom. Each split in the tree attempts to divide the response variable (snake richness) into homogenous groups according to a threshold value of an explanatory variable (shown above each split). A '<>' symbol indicates that cases with lower values go to the left, whereas a '><' symbol means that cases with lower values go to the right. Mean species richness and sample sizes (number of 0.25° grid cells) are given below each node. Variable abbreviations are the same as those used in Fig. 1.

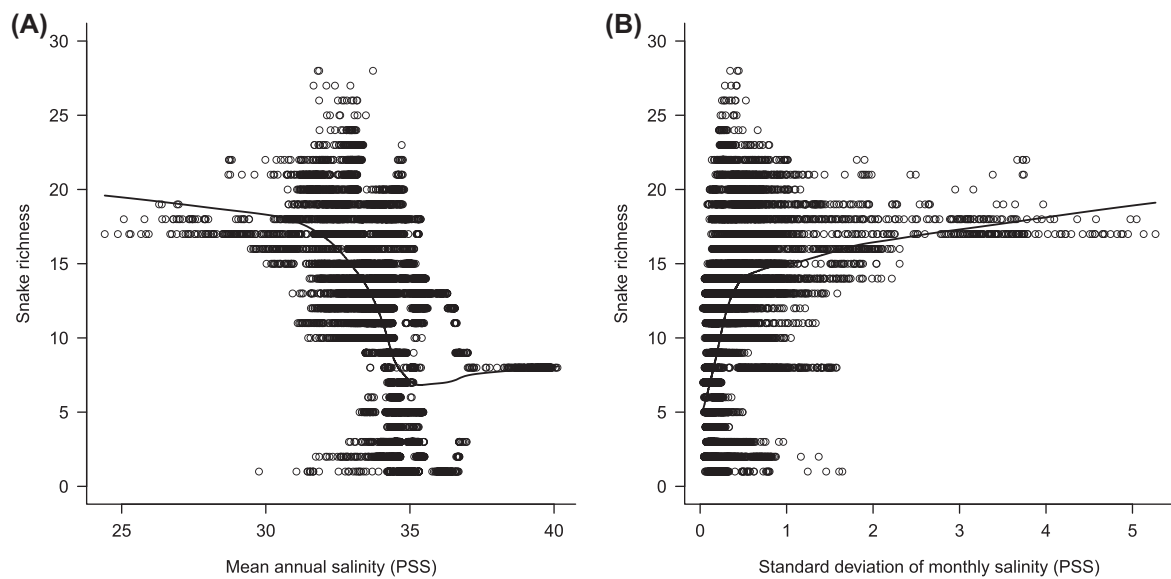


Figure 3. Relationships between sea snake richness and mean annual salinity (A), and monthly variation of salinity (B). PSS = practical salinity scale.

require extended time on land, Heatwole 1999). In contrast, salinity did not influence the distribution of hydrophiines (either with or without *P. platurus*), consistent with the fully marine habits of these species.

Geographic patterns in the species richness of marine snakes also were linked to oceanic salinity. Salinity was repeatedly included in the best regression tree of sea snake species richness, representing ~64% of the variable splits (four occurrences of mean annual salinity, and three occurrences of salinity variation, Fig. 2). Overall, species richness of marine snakes was negatively linked

to mean annual salinity, but positively related to variation in monthly salinity (Fig. 3). Although both parameters are somewhat correlated with one another, they provide complementary information. Low salinity levels should decrease the cost of osmotic maintenance through reduced salt gland functioning (Schmidt-Nielsen 1983), as well as decreased rates of water loss to the environment (Lillywhite et al. 2008, 2009). Similarly, high variation in salinity levels should allow regular access to brackish water over short time-scales (e.g. a month in our study), again decreasing the cost of osmotic maintenance, and dehydration rates. Perhaps more importantly, high variability in salinity levels is likely to reflect frequent, heavy rainfall events, during which marine species can drink fresh or slightly brackish water to restore their hydration state. Amphibious species have direct access to freshwater from precipitation on land (Bonnet and Brischox 2008, Lillywhite et al. 2008), and snakes that are at sea (e.g. 'true' sea snakes or foraging sea kraits) also have access to fresh water lenses that form at the ocean surface (Lillywhite and Ellis 1994). Importantly, low and highly variable salinity levels would be expected to alleviate the energetic costs of osmoregulation even in species having very effective salt-secreting glands (e.g. many hydrophiines). In turn, the low osmoregulatory costs associated with such environmental conditions would have presented terrestrial watersnakes with an unoccupied niche which may have promoted rapid diversification, and ultimately led to higher species richness.

At the species-level, salt gland function (maximum excretion rates of Na^+) was linked to geographic distributions in eight species of snakes belonging to the four lineages of marine snakes that we studied. Both maximum salinity within a species' range, and the size of a species' oceanic range, were positively correlated with salt gland function (Fig. 4). Although causal links remain unclear, these results strongly suggest that a more efficient salt gland (i.e. being able to excrete higher salt loads) allows a

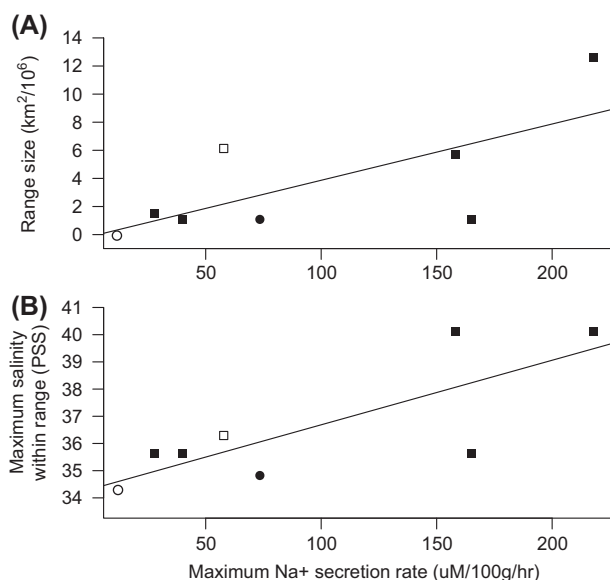


Figure 4. Maximum salinity within each species' geographic range (A) and geographic range size (B) vs the maximum Na^+ excretion rate of each species. Different symbols represent different snake lineages: open squares = Acrochordidae, open circles = Homalopsidae, filled circles = Laticaudinae, filled squares = Hydrophiini.

species to cope with more saline waters, and hence to exploit larger oceanic areas.

There are several caveats to our study, particularly relating to the resolution of the range maps used in our analyses. First, the IUCN range maps included several occurrences of sea snakes outside their core ranges (i.e. waifs). Inclusion of data points outside the range in which populations are viable might have introduced noise into our analyses. This is especially the case for extremely northern or southern locations (e.g. locations of *P. platurus* around the Cape of Good Hope). However, no obvious decision rules would have satisfactorily allowed us to remove potential waifs without biasing our results. Additionally, IUCN range maps were drawn solely over a coastal margin of 50 km, thereby potentially removing locations that shelter snake populations further at sea. However, this should produce a significant bias for three of the four lineages that we studied (Acrochordidae, Homalopsidae and Laticaudinae) because their ecologies limit their distribution to coastal waters. The situation is different for the totally marine Hydrophiini. In the case of benthic foragers (all species except *P. platurus*), geographic distributions should be constrained to waters ca 100 m deep (Heatwole 1999, Brischox et al. 2007; Fig. 2). Although this bias is likely weak because shallow waters tend to be close to shore, the actual ranges of these species might be slightly underestimated in the available maps. For the pelagic *P. platurus*, available information suggests an extensive range covering the whole Indo-Pacific (Heatwole 1999). Clearly, the coastal range from IUCN range maps will underestimate the actual range of this species. This underestimation likely influenced our finding that distance to the nearest coast-line was such a prominent explanatory factor even for *P. platurus*. Because coastal waters

are less saline than offshore waters due to extended freshwater runoff from land, limiting the range of hydrophiine sea snakes to coastal waters might explain why we detected no effect of salinity on their geographic distribution. This possibility is supported by the link between salt gland function and species distributions (Fig. 4). However, this putative bias should be conservative as it concerns the most marine adapted lineage. In spite of these limitations, the relationships we found between coarse geographic information and independently gathered physiological data suggests that the effects of salinity we detected are likely to be robust to errors in our range maps.

Globally, our results suggest that salinity plays a significant role in the current distributions and richness of marine snakes, and does so more profoundly in species which are presumably analogous to the early transitional forms between the land and the sea (i.e. amphibious and near-coastal species). This result supports the fourth prediction in our Introduction, by suggesting that specific geographic areas may have offered favourable conditions for early transitional forms to cope with salinity constraints (Dunson and Mazzotti 1989). All four independent transitions to marine life in snakes may have taken place in a single area (between Malaysia, Indonesia and northern Australia) that currently contains representatives of all marine snake lineages. The highest values of marine snake species richness occur in this area (Fig. 5), across the Sunda and Sahul shelves (hereafter 'Indonesian Basin' for simplicity). The hypothesis that this single area has played a role in all four transitions to marine life in snakes is congruent with the geographic distributions of terrestrial outgroups identified by phylogenetic analyses of the marine snake groups (Keogh 1998, Keogh et al. 1998,

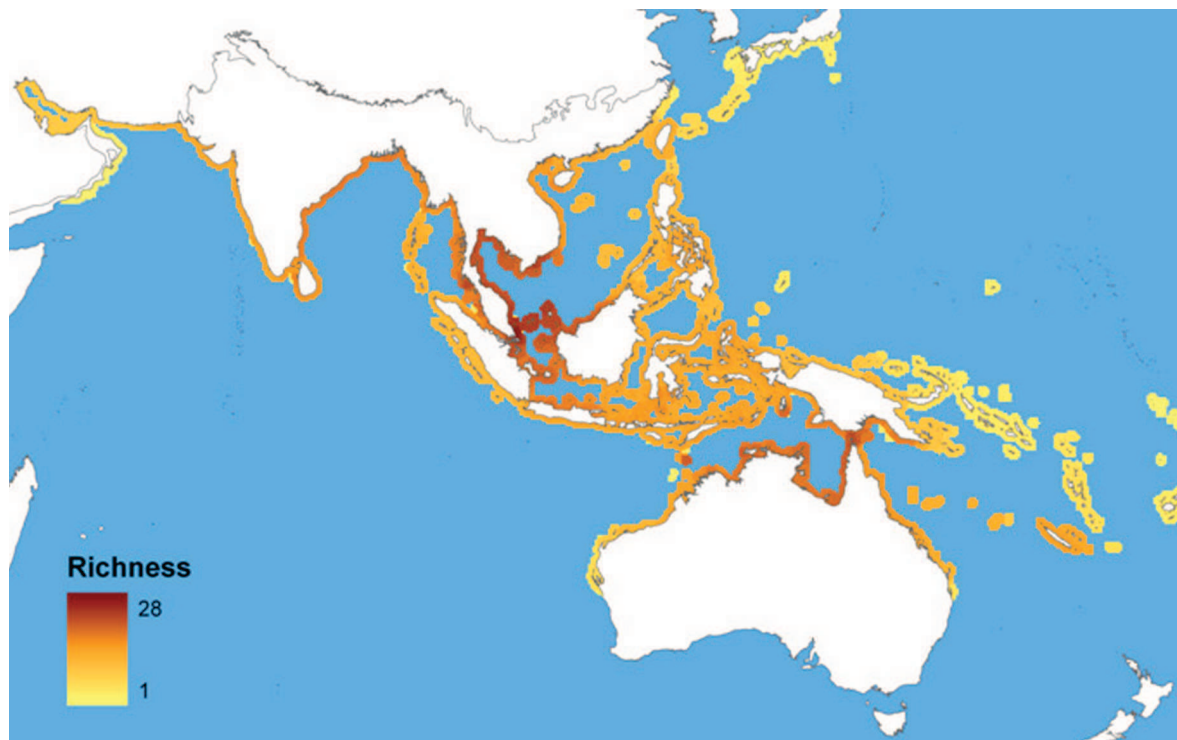


Figure 5. Map of sea snake richness at a 0.25° resolution (excluding *P. platurus*).

Alfaro et al. 2008, Sanders and Lee 2008, Sanders et al. 2008, 2010a, b). The inference that all of these transitions occurred in the same geographic area over an extended time frame (i.e. 11 My elapsed between the estimated minimum crown ages of Homalopsidae and Hydrophiinae) highlights the Indonesian Basin as offering unusually favourable environmental conditions for this major evolutionary transition. The Indonesian Basin is currently characterised by extensive interface environments between the land and the sea, such as large areas of shallow water, numerous islands and islets, as well as ragged coastlines and mangroves. In addition, the Indonesian Basin is a biodiversity hotspot, especially for coral reef ecosystems (e.g. the Coral Triangle, Green and Mous 2004). All of these biogeographic characteristics might have facilitated the transition from terrestrial to marine life in snakes. Although it is difficult to robustly infer the paleo-biogeographical history of this region, repeated marine transgressions and regressions over the Indonesian Basin during the Neogene, and a monsoonal climate, are likely to have offered somewhat similar conditions (Guo 1993, Voris 2000, Woodruff 2003, Hanebuth et al. 2011). The low salinity of the Indonesian Basin, as well as its high seasonal variation in salinity (due to the monsoonal regime) might have been critical in the evolutionary transition to marine life in snakes, providing an additional proximate cause as to why this region has served as a 'centre of origin' for biodiversity (Ekman 1953, Briggs 2000, Mora et al. 2003).

To conclude, our results suggest that salinity plays an important role in the current distributions of marine snakes in the tropical Indo-Pacific Ocean. The low and variable salinity of the Indonesian Basin is likely to have facilitated evolutionary transitions to marine life in snakes, and may indeed have been the location for all four of the transitions represented by extant marine snake species. More robust biogeographic inferences will require clarifying how salinity (among other parameters) affected rates of speciation, and extinction among marine snakes within a phylogenetic framework. Nevertheless, our findings suggest that the importance of salinity may have been underestimated in evolutionary and ecological studies of secondarily marine vertebrates (Gutiérrez et al. 2011). Future studies should examine the role of this environmental parameter in other lineages of secondarily marine vertebrates such as turtles, birds and mammals, all of which display osmoregulatory adaptations functionally similar to those of snakes. Additionally, studies on the likely impact of future climate change on marine vertebrates could usefully incorporate salinity and its forecasted changes. If oceanic salinity drives species distributions, and rainfall patterns and currents drive oceanic salinity, then changes in salinity may well mediate the impacts of climate-change on marine organisms.

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Supplementary material (Appendix E7717 at <www.oikosoffice.lu.se/appendix>). Appendix 1–2.

Article soumis à Journal of Evolutionary Biology

Marine lifestyle is associated with higher baseline corticosterone levels in birds

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Abstract

Because seawater is hyperosmotic relative to body fluids of most vertebrates, marine lifestyle is expected to strongly influence the physiology of marine tetrapods. Regulating the salt content of body fluids is energetically costly; and osmoregulatory organs may not totally overcome salt load and/or water loss. As a consequence, marine lifestyle should influence physiological systems involved in the maintenance of the physiological balance (homeostasis), in the mobilization of energetic resources (e.g., to fuel salt excretion), or in the acquisition of resources (e.g., fresh water). Corticosterone (CORT, one such “generalist” mediator) is expected to be higher in marine tetrapods as it is linked with energy expenditure and physiological stress and may activate osmoregulation. Using comparative analyses, we tested this hypothesis in birds, a lineage for which available data on baseline CORT allow comparing marine *versus* terrestrial species, and species with or without salt glands. We found that marine species (and species with salt glands) display significantly higher baseline CORT during the wintering (but not the breeding) stage. Although salt glands’ presence was tightly linked to phylogeny, our results suggest that marine life imposes strong, but overlooked, osmotic constraints on the physiology of marine birds. Such habitat-related variation is a major phenomenon to explore owing to its general implications for understanding the physiological basis of evolutionary transitions in habitat use.

Keywords. osmoregulation; birds; salinity; salt glands; corticosterone

Introduction

Evolutionary transitions in habitat use provide powerful opportunities to understand how the environment shapes the morphology, physiology or behaviour of organisms (Little 1990, Mazin and de Buffrénil 2001). For instance, tetrapods that adapted to marine life offer great examples of such evolutionary changes in organismal traits (Mazin and de Buffrénil 2001). Marine life exerts major selection on attributes such as the ability to move efficiently through water; to remain underwater for long periods without needing to surface to breathe; and to dive to considerable depths (Kooyman 1989).

Although widely studied, the respiratory physiology of marine tetrapods is not the only target of selective pressures; and other characteristics of the marine environment, notably its chemical properties, are equally expected to strongly influence the physiology of

secondarily marine vertebrates. Due to its elevated concentration of sodium chloride, seawater is hyperosmotic relative to body fluids of most vertebrates. As a consequence, these organisms tend to gain salt and/or to lose water across permeable surfaces. Accordingly, to maintain their osmotic balance, marine tetrapods have evolved specific structures that allow regulating the salt content of their body fluids (sophisticated nephrons in mammals, salt glands in non-mammalian marine tetrapods [Ortiz 2001, Babonis and Brischox 2012]).

Functional aspects of osmoregulation have attracted strong interest from the scientific community (Ortiz 2001, Peaker and Linzell 1975, Skadhauge 1981, Bentley 2002). However, some other facets of osmoregulatory physiology have been mostly overlooked to date. For instance, excreting excess salt necessitates the activation of specific physiological systems and requires energy expenditure (Schmidt-Nielsen 1983). Additionally, specific osmoregulatory organs may not totally overcome salt load and/or water loss and many marine vertebrates may experience frequent and/or prolonged periods of hypernatremia or dehydration (Brischox et al. 2012, 2013, 2014, Lillywhite et al. 2012, 2014). Clearly, all of these parameters should strongly influence the overall physiology of marine tetrapods, including systems involved in the maintenance of the physiological balance (homeostasis), in the mobilization of energetic resources (e.g., to fuel salt excretion), or in the acquisition of resources (e.g., fresh water).

Glucocorticoids (henceforth “GC”) are well-studied mediators of organismal allostasis that has attracted considerable attention from the scientific community (McEwen & Wingfield 2003; Romero et al. 2009; Angelier & Wingfield 2013). When an environmental change occurs, the activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis results in an increase of circulating GC levels; which in turn leads to specific physiological and behavioural adjustments. These adjustments are expected to allow the organisms to cope with potentially challenging situations (Wingfield et al. 1998; Sapolsky et al. 2000; Romero 2004). GC may equally respond to the aforementioned constraints of marine life for several reasons. First, excreting excess salt is energetically costly, and GC are known to be linked with energy expenditures (Astheimer et al. 1992; Landys et al. 2006; DuRant et al. 2008; Lynn et al. 2010). Second, GC, as “stress hormones”, may respond when organisms undergo some form of physiological stress, such as osmotic stress (Allen et al. 1975). Finally, GC, as mineralocorticoids, have been suggested to be key mediators of osmoregulation (notably of salt gland functioning, Allen et al. 1975, Peaker and Linzell 1975,

Skadhauge 1981, Bentley 2002). In fact, it has been demonstrated that high GC levels can be tightly linked with an activation of osmoregulation processes (Harvey & Phillips 1980; Tome et al. 1985; Arnason et al. 1986).

Taken together, these elements strongly suggest that GC levels may differ between terrestrial and marine tetrapods because of the osmotic constraints of life in seawater. In this study, we tested this hypothesis using a comparative analysis of 101 bird species for which corticosterone levels are available (CORT, the primary glucocorticoid in birds). Birds are well-suited to test such hypothesis for several reasons. First, this lineage contains both terrestrial and marine species. Second, many species of birds, including a large number of terrestrial species, are known to possess functional salt glands, while other species (terrestrial only) lack these organs (Babonis and Brischoux 2012). Finally, birds have attracted strong attention from stress physiologists over the last decades thereby producing the largest dataset of CORT levels available to date (Wingfield et al. 1995; Romero 2002; Bókony et al. 2009; Hau et al. 2010; Lendvai et al. 2013). Specifically, we tested the following predictions:

- 1) Because of the strong relationship between CORT and osmotic constraints, baseline CORT levels should be higher in marine birds as compared to terrestrial ones.
- 2) Because the functioning of the salt gland is tightly linked to CORT secretion (Allen et al. 1975), baseline CORT levels should be higher in birds possessing salt glands.

Materials and Methods

Data collection

Baseline CORT levels were extracted from the dataset published in Lendvai et al. (2013) that was completed by 6 species for which recent studies had published CORT data. In May 2013, we searched for articles on the ISI Web of Knowledge database using the keyword "corticosterone" and we kept only the studies that were conducted in free-living adult birds. We focused only on baseline CORT levels as they appear more relevant in the context of our study. Indeed, they represent the CORT levels that are unaffected by the stress of capture, and therefore, they should be a good proxy of the allostatic state of free-ranging individuals (McEwen & Wingfield 2003; Romero et al. 2009). Baseline CORT data were divided into two seasons (wintering and breeding), as these different life history stages have been shown to affect baseline CORT levels (reviewed in Romero 2002). Baseline CORT values were log-transformed for analyses.

Habitat use was collected from field guides and the authors' personal experience. We produced two straightforward habitat categories: birds were considered as following a marine lifestyle when they used extensively (e.g., to forage) marine environments (such as oceans, seas, brackish or saltwater coastal marshes) and thus were supposedly susceptible to the osmotic constraints of marine lifestyle. All other species of birds were considered as terrestrial. Three species which shifted habitats between seasons (*Calidris mauri*, *C. pusilla*, *Histrionicus histrionicus*) were categorized as terrestrial when breeding and marine when wintering as the foraging habitat used by these species strongly differed between seasons. The presence or absence of salt glands was determined based on the review published by Babonis and Brischoux (2012). Altogether we used data from 101 bird species.

To control for the effects of potentially confounding variables, we collected data on aspects of interspecific life-history variation that have been shown to be related to CORT levels. We gathered data on body mass because larger birds have lower baseline CORT levels, indicating a lower overall metabolism and/or that large mass may serve as a buffer against some stressors (Bókony et al. 2009; Hau et al. 2010). We calculated the average of male and female body mass where sex-specific body mass data were available, and used their average in the analyses. Although sex-specific analyses were not performed, the reason of collecting sex-specific body mass data was to control for potential sex-biased sampling for some species. Body mass was log-transformed before the analyses. CORT levels may vary latitudinally (Silverin et al. 1997; Breuner et al. 2003; Bókony et al. 2009). Therefore we collected data on the latitude of each study site, then we calculated the mean latitude weighted by the sample size for each species and for each life-history stage. We used the absolute values of latitude in the analyses. We also collected data for brood value (expressed as $\log_{10}[\text{clutch size} / (\text{clutch size} * \text{broods per year} * \text{average reproductive life span})]$) which may affect baseline CORT (Bókony et al. 2009). However, we did not include this variable in the final analyses for several reasons. First, brood value was available for less than half of the species under focus, thereby substantially decreasing the power of our analyses. Second, variation in brood value is partially captured by body mass in our dataset as shown by the relatively strong correlation between these two variables ($r=-0.66$, $p<0.001$, $n=41$). Finally, preliminary analyses have shown that our results remain similar when including brood value (results available from the authors upon request).

Statistical analyses

We investigated relationships between baseline CORT levels and marine/terrestrial lifestyle and the presence of salt glands with Bayesian phylogenetic mixed-effects models based on Markov chain Monte Carlo estimations, as implemented in the R-package ‘MCMCglmm’ version 2.17 (Hadfield 2010; Hadfield and Nakagawa 2010; R Core Team 2013). Shared ancestry was taken into account by specifying a phylogenetic variance-covariance matrix. In order to make our conclusions more robust, we used three different phylogenetic trees that differ in branching of the major clades: (1) Davies’ phylogenetic supertree (Davis 2008), (2) Hacket et al.’s tree (Hacket et al. 2008) and (3) the Sibley-Ahlquist tree (Sibley and Ahlquist 1990). For each tree we used the topology from the respective source with branch lengths transformed according to Nee’s method (Maddison and Maddison 2006). We also used a model where species were treated as independent samples. We compared these models using the Deviance Information Criterion (DIC) (Hadfield 2010), which is the Bayesian equivalent of the more commonly used Akaike Information Criterion. We report the parameter estimates (posterior means) and corresponding 95% credibility interval (CI) values.

First, we analysed whether marine vs. terrestrial lifestyle and the presence of salt glands were related to baseline CORT levels using different assumptions about the evolutionary history of the clades (three different phylogenies and no phylogeny, i.e. species as independent samples). In these initial models we used marine *versus* terrestrial lifestyle or the presence of salt glands, the season (wintering or breeding) and their interaction as fixed effects. Then we further investigated whether our results hold when we included body mass and latitude as potential confounding covariates in the analyses.

Table 2. Parameter estimates, corresponding 95% credibility intervals (CI) and Bayesian p-values from the MCMC linear mixed model analysing log-transformed baseline CORT levels in relation to habitat use and salt gland presence. Shared evolutionary ancestry was taken into account by the Hacket et al. (2008) phylogeny (see Table 1). Season refers to life-history stage (breeding or wintering). See text for details.

	Effect	Parameter estimate	Upper 95% CI	Lower 95% CI	p-value
Salt Glands	Intercept (terrestrial, breeding)	2.22	1.56	2.90	<0.001
	Salt Gland	-0.13	-0.92	0.64	0.734
	Season (wintering)	-0.57	-0.81	-0.27	<0.001
	Season×Salt gland	0.79	0.25	1.27	0.002
Habitat	Intercept (terrestrial, breeding)	2.05	1.45	2.63	0.001
	Marine lifestyle	0.17	-0.32	0.80	0.554
	Season (wintering)	0.52	-0.02	1.08	0.088
	Season×Marine lifestyle	-1.08	-1.72	-0.51	0.001

Results

Models using four different phylogenetic hypotheses received similar support by DIC (Table 1). As the Hacket tree performed slightly better than the rest (Table 1), we report below the models using this phylogeny. Baseline CORT during the breeding season did not differ significantly between species with or without salt glands (Table 2). However, during wintering, species possessing salt glands had significantly higher baseline CORT levels than species lacking salt glands (Table 2, Fig. 1).

Table 1. Deviance Information Criterion (DIC) for models using different phylogenetic topologies. Differences between DIC values (Δ DIC) were small, indicating that models using different topologies gave qualitatively similar results, but the Hacket et al.’s (2008) phylogeny received slightly better support for both sets of analyses. See text for details.

	Phylogenetic model	DIC	Δ DIC
Salt glands	Hacket et al. (2008)	228.2	
	Davies (2008)	230.2	2
	No phylogeny	231.8	3.6
	Sibley and Ahlquist (1990)	232.9	4.7
Habitat	Hacket et al. (2008)	228.1	
	No phylogeny	229.3	1.2
	Davies (2008)	230.2	2.1
	Sibley and Ahlquist (1990)	230.7	2.6

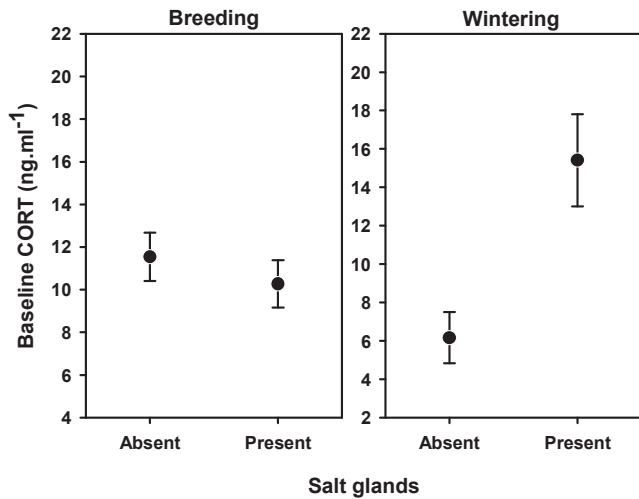


Figure 1. Baseline CORT (simple mean \pm SE) in birds with or without salt glands during the breeding and the wintering stages. Birds with salt glands display significantly higher baseline CORT during the wintering stage. See text for details.

Similarly, baseline CORT levels did not differ significantly between marine and terrestrial birds during the breeding season (Table 2), but during wintering, marine species had significantly higher baseline CORT than terrestrial species (Table 2, Fig. 2).

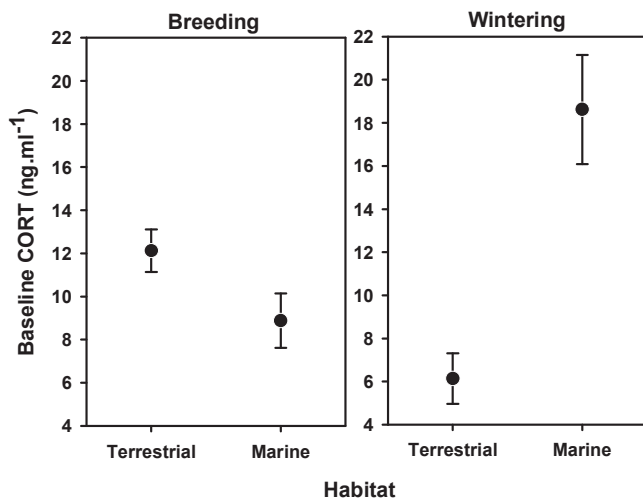


Figure 2. Baseline CORT (simple mean \pm SE) in terrestrial and marine birds during the breeding and the wintering stages. Marine birds display significantly higher baseline CORT during the wintering stage. See text for details.

The three species that shifted from terrestrial to marine habitat between breeding and wintering had higher baseline CORT in the marine habitat (Fig. 3).

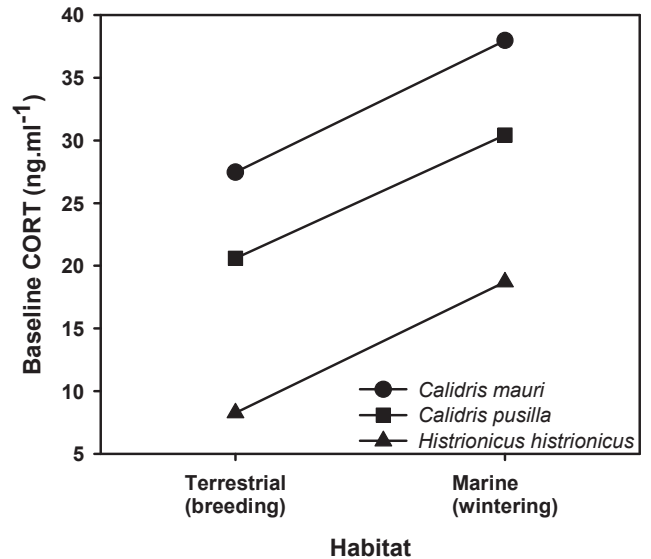


Figure 3. Baseline CORT in three species of birds (*Calidris mauri*, *C. pusilla*, *Histrionicus histrionicus*) that shift from terrestrial to marine habitat between breeding and wintering.

Body mass tended to have a negative effect on baseline CORT levels, although this effect overlapped with zero (-0.045, 95% CI [-0.176, 0.085]). Higher latitude was associated with higher baseline CORT levels, although this effect was small (0.01, 95% CI: [0.003, 0.017]). Accordingly, controlling for body size or latitude did not alter the relationships of baseline CORT levels with habitat and salt glands qualitatively. Also, although low sample sizes in some categories impeded robust analyses, we found that restricting our analyses to species for which we had data for both seasons, or comparing terrestrial and marine birds having salt glands, produced qualitatively similar patterns (results available from the authors upon request).

Discussion

Overall, our comparative analyses support the hypothesis that marine lifestyle is linked with higher baseline CORT levels in birds, presumably because of the strong osmotic challenge linked to life in seawater. Unexpectedly, we found that the relationship between baseline CORT levels and the habitat (marine *versus* terrestrial) was only apparent in winter, suggesting a strong influence of life-history stage (e.g., reproduction in summer) on the relationship between baseline CORT levels and marine life or presence of salt glands. This difference between seasons may be directly linked to the modulation of CORT secretion throughout the life cycle (Romero 2002; Romero et al. 2006). Indeed, reproduction is known to produce an important elevation of baseline

CORT levels in birds (Romero 2002). This elevation is thought to support the energetic demands of the reproductive period and this concomitant increase in energetic demands and baseline CORT levels may mask the influence of other factors. For example, because baseline CORT increases with brood value (Bókonyi et al. 2009), and because terrestrial birds often have high brood value (e.g. -0.53 ± 0.04 SE in our sample, compared to -0.98 ± 0.05 SE in marine birds), this may elevate their baseline levels above those of marine birds during reproduction; despite the possible influence of osmoregulation on baseline CORT levels in marine species. Future studies could usefully compare non-breeding and breeding individuals during the reproductive period as it would allow testing whether the positive influence of marine life/salt glands on baseline CORT levels is present during the breeding period in individuals that are not engaged into reproduction.

Among non-breeding birds, we found that marine birds display higher baseline CORT levels than terrestrial birds. Baseline CORT levels were also elevated in species with functional salt glands. Importantly, among birds with salt glands, terrestrial species (for which salt glands are smaller and less functional, Peaker and Linzell 1975) may show lower baseline CORT levels than marine birds. Taken together, these results strongly suggest that CORT secretion and osmoregulation are tightly linked. This functional link was further supported by specific examples. Indeed, species that shift from terrestrial to marine habitat between breeding and wintering had significantly higher baseline CORT when using marine habitats, against the overall trend that baseline CORT values are higher during breeding. Supporting our comparative results, intraspecific studies have found that CORT is involved in the process of osmoregulation (Peaker and Linzell 1975, Bentley 2002, Skadhauge 1981, Landys et al. 2006) but also in the functioning of the salt glands (Allen et al. 1975).

There are a few caveats to our study, notably regarding the effects of salt gland presence/absence on basal CORT levels. Indeed, salt gland seems to be an ancestral trait in most birds (Babonis and Brischoux 2012), and it may have been lost during a single evolutionary event sometime during the evolution of Passeriformes (Schoemaker 1972, Babonis and Brischoux 2012). As a consequence, for this specific analysis, we cannot robustly tease apart the respective role of phylogeny *versus* presence/absence of salt glands. Although we acknowledge that this result needs to be interpreted with caution, several elements suggest that our analysis did not generate spurious patterns. First, this result is in line with our a priori prediction. Indeed, salt gland

functioning is energetically costly, and CORT levels correlate with energy allocation and metabolic rates (Sapolsky et al. 2000; Landys et al. 2006). Detecting a significant relationship between salt gland presence and CORT levels was expected, independently of any phylogenetic considerations. Second, none of the other traits that we have included in our analysis (e.g., body mass, latitude) altered our conclusions, while, at least body mass differs extensively between passerine and non-passerine birds. This suggests that the effect of salt glands on baseline CORT levels should be relatively strong compared to other variables. If this result was a by-product of body size solely, this result would have been similar during the breeding season, because the body mass difference between groups is comparable. On the contrary, baseline CORT levels during reproduction were similar between passerine (lacking salt glands) and non-passerines (with salt glands) birds. This suggests that CORT levels may vary similarly between these two phylogenetically distinct groups, at least during reproduction. Finally, among birds with salt glands, terrestrial species (for which salt glands are smaller and less functional, Peaker and Linzell 1975) may show lower baseline CORT levels than marine birds. Importantly, there was no obvious way for us to remove such bias (e.g., to produce a passerine bird having salt glands). Despite such limitation, we believe that including this result, although putatively fragile, is critical in the context of our study.

The reasons why non-breeding baseline CORT levels are higher in marine birds (or in birds having salt glands) are probably multiple. CORT is a “generalist” physiological mediator which is involved in functions as diverse as resource acquisition, mobilisation and allocation (Landys et al. 2006; Lynn et al. 2010), metabolic rates (Astheimer et al. 1992; DuRant et al. 2008), movements and activity (Angelier et al. 2007; Crossin et al. 2012), or stress response (Wingfield et al. 1998; Angelier & Wingfield 2013). In addition, and interestingly in the context of our study, CORT as a mineralocorticoid may also be involved in the maintenance of the osmotic balance (Peaker and Linzell 1975, Skadhauge 1981, Bentley 2002). Importantly, all these functions or organismal responses are likely to be influenced by marine life. For instance, excreting excess salt is energetically costly, and CORT may be higher in marine birds (or in birds having salt glands) because they have higher energetic requirements in order to fuel salt gland functioning (Schmidt-Nielsen 1983). Similarly, marine species may experience frequent and/or prolonged period of hypernatremia or strong dehydration (i.e., osmotic stress) which may provoke the activation of the HPA axis to produce dedicated physiological and behavioural responses (e.g., to reduce

additional water loss, or to acquire fresh water when available). Finally, the higher level of CORT we detected in marine birds (or in birds having salt glands) may be a consequence of the role of CORT as a mediator of osmoregulation (Harvey & Phillips 1980; Tome et al. 1985; Arnason et al. 1986).

Although our study does not allow elucidating the physiological basis of the habitat-related variation in CORT levels we detected, our analyses strongly suggest that marine life is related to a bird's baseline CORT level; presumably because of the osmotic constraints of life in seawater, a parameter largely overlooked to date. Future experimental studies will be critical to disentangle the physiological causes of CORT variation between habitats. More generally, we believe that such habitat-related variation is a major phenomenon to explore owing to its general implications for understanding the physiological basis of evolutionary transitions in habitat use.

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III. Curriculum vitae

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IV. Liste complète des publications

2014

1. Bonnet X, Briand MJ, **Brischoux F**, Letourneur Y, Fauvel T, Bustamante P. 2014. Anguilliform fish reveal large scale contamination by mine trace elements in the coral reefs of New Caledonia. *Science of the Total Environment*, 470-471:876-882.
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8. Lillywhite HB, Sheehy III CM, **Brischoux F**, Grech A. 2014. Pelagic sea snakes dehydrate at sea. *The FASEB Journal* 28, No. 1, Supplement 860.19.
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63. Ford N B, **Brischoux F**, Lancaster D. 2004. Reproduction in the western cottonmouth, *Agkistrodon piscivorus leucostoma*, in a floodplain forest. *Southwestern Naturalist*, 49:465-471.
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Soumis

65. **Brischoux F**, Angelier F. Academia’s never-ending selection for productivity. *BioScience*.
66. **Brischoux F**, Lendvai A, Bokoni V, Angelier F. Marine lifestyle is associated with higher baseline corticosterone levels in birds. *J Evol Biol*.
67. Cook TR, **Brischoux F**. Why does the only ‘planktonic’ marine tetrapod dive? Determinants of diving in marine ectotherm. *Behav Ecol*.
68. Dupoué A, **Brischoux F**, Angelier F, DeNardo DF, Wright C, Lourdais O. Water deprivation induces a mother-offspring conflict in favour of embryos in a viviparous snake (*Vipera aspis*). *Funct Ecol*.

V. Liste des travaux présentés lors de colloques et de séminaires

Séminaires invités

1. **Brischoux F.** 2011. Women in Science and Engineering (WiSE UF) discussion on “Enhancing Your Productivity: Faculty Share Their Tips and Tricks.” Invited panellist along with Marta Wayne (Biology, UF), Susan Cameron-Devitt (Wildlife Ecology and Conservation, UF) and

Josephine Allen (Materials Science and Engineering, UF). January 24th 2011, Department of Biology, University of Florida, Gainesville, USA.

2. **Brischoux F.** 2010. Allometry of dive duration. Gillooly Lab. November 1st 2010, Department of Biology, University of Florida, Gainesville, USA.
3. **Brischoux F.** 2010. Marine ecology of sea kraits in New Caledonia. September 28th 2010, Department of Biology, University of Florida, Gainesville, USA.
4. **Brischoux F.** 2009. Sea, snakes and sun. October 8th 2009, Département Ecologie, Physiologie, Ethologie; Institut Pluridisciplinaire Hubert Curien, CNRS-ULP, Strasbourg, France.

Colloques nationaux et internationaux (* indique une présentation sous forme de poster)

1. * **Lillywhite HB**, Sheehy III C, **Brischoux F**, Grech A. Pelagic Sea Snakes Dehydrate at Sea. Experimental Biology 2014, April 26-30, 2014, San Diego.
2. * **Gherghel I**, Papeş M, **Brischoux F.** 2014. Marine and terrestrial potential distribution of sea kraits (*Laticauda*: Elapidae): implications for conservation. Oklahoma State University 25th Annual Research Symposium, February 19-21, 2014, Stillwater, Oklahoma.
3. **Lillywhite HB**, Sheehy III CM, **Brischoux F**, Heatwole H. 2013. The salt life of sea snakes. University of Florida Marine Biology Symposium, January 17-18, 2013, St Augustine, FL.
4. Calosi P, Verberk WCEP, **Brischoux F**, Spicer JL, Garland T Jr, Bilton DT. 2012. The comparative biology of diving in European diving beetles: Towards a better understanding of the allometry of diving in ectotherms and endotherms divers. SEB 2012 - Society for Experimental Biology, June 29-July 2, 2012, Salzburg, Austria.
5. Fauvel T, **Brischoux F**, Bonnet X. 2012. Indirect method to assess the distributions of cryptic top predators in coral reef habitats. SERL 2012 – 8th meeting Ecology and Behaviour, April 2-6, 2012, Chizé, France.
6. **Lillywhite HB**, **Brischoux F**, Sheehy III CM, Pfaller JB. 2012. Dehydration and freshwater drinking requirements of marine snakes. SICB Annual Meeting, January 3-7, 2012, Charleston, SC.
7. **Brischoux F**, Tingley R, Shine R, Lillywhite HB. 2012. Distributional data helps to identify evolutionary challenges: Oceanic salinity as a major constraint during the transition to marine life in snakes. SICB Annual Meeting, January 3-7, 2012, Charleston, SC.
8. **Babonis LS**, **Brischoux F.** 2012. Perspectives on salt gland evolution in marine snakes. SICB Annual Meeting, January 3-7, 2012, Charleston, SC.
9. **Pfaller JB**, Frick MG, **Brischoux F**, Sheehy III CM, Lillywhite HB. 2012. Ecology of epibiosis: What can we learn from marine reptiles? SICB Annual Meeting, January 3-7, 2012, Charleston, SC.
10. **Brischoux F**, Lillywhite HB. 2011. Light- and flotsam-dependent “float-and-wait” foraging in pelagic sea snakes (*Pelamis platurus*). University of Florida Marine Biology Symposium, January 27-28, 2011, Whitney Lab, Marineland, FL.
11. **Lillywhite HB**, Sheehy III CM, **Brischoux F**, Pfaller J. 2011. Freshwater drinking requirement in a pelagic sea snake, *Pelamis platurus*. University of Florida Marine Biology Symposium, January 27-28, 2011, Whitney Lab, Marineland, FL.
12. * Lillywhite HB, Sheehy III CM, **Pfaller J**, **Brischoux F.** 2010. Drought tolerance of pelagic sea snakes in Costa Rica. August 2010. APS Intersociety Meeting: Global Change and Global Science: Comparative Physiology in a Changing World.
13. **Brischoux F**, Bonnet X, Shine R. 2009. Foraging ecology of sea kraits in New Caledonia. Second Meeting of the Australasian Societies for Herpetology, SMASH 2009, February 20-22, 2009, Massey University, Albany, New Zealand.
14. **Brischoux F**, Bonnet X, Shine R. 2008. Foraging ecology of sea kraits in New Caledonia. French Herpetological Society Congress, October 2-4, 2008, E.C.O.L.E. de la mer, La Rochelle, France.

15. **Brischoux F**, Bonnet X, Cook TR, Shine R. 2007. Allometry of diving capacities: ectothermy versus endothermy. Petit Pois Dérivé (French congress of genetics and population dynamics), August 27-30, 2007, Université de Poitiers, Poitiers, France.
16. * **Brischoux F**, Bonnet X, Cook TR, Shine R. 2007. Snakes at sea: diving behaviour of free-ranging sea kraits. 11th Annual Meeting on Health, Science & Technology, June 14, 2007, Université François Rabelais, Tours, France.
17. **Bonnet X**, **Brischoux F**, Ineich I, Kulbicki M, Shine R, Séret B. 2005. Abundance and diet of neo-caledonian sea kraits. French Herpetological Society Congress, June 28-30 2005, Village des Tortues, Gonfaron, France.
18. **Brischoux F**. 2005. Sister species coexistence in sea kraits: resources partitioning and sympatric speciation. Neo Caledonian PhD students meeting, April 27 2005, Institut de Recherche pour le Développement de Nouméa (IRD), Nouméa, New Caledonia.
19. **Brischoux F**, Lourdaï O. 2003. How to find benefice in failure? French Herpetological Society Congress, July 2-5 2003, Laboratoire Arago, Banyuls, France.
20. **Ford N B**, **Brischoux F**, Lancaster D. 2003. Reproduction in the Western Cottonmouth, *Agkistrodon piscivorus leucostoma*, in a Stochastic Environment. 50th Anniversary Meeting, Southwestern Association of Naturalists, April 17-19 2003, University of Oklahoma.

VI. Encadrement d'étudiants

1. Thèses

- 2014-2017 : Héloïse Guillot (co-encadrement avec X. Bonnet). Importance des contaminants environnementaux sur la physiologie et l'écologie d'un vertébré aquatique, la couleuvre vipérine. Université de La Rochelle.
- 2012-2015 : Alizée Meillère (co-encadrement avec F Angelier). Eco-physiologie des oiseaux urbains. Université de La Rochelle.
- 2011-2014 : Marine Briand (comité de thèse et collaboration ; membre du jury de thèse). Place des poissons anguilliformes dans le fonctionnement des écosystèmes récifo-lagonaires de la Nouvelle-Calédonie : rôle trophique et impact des contaminations. Université de Nouvelle Calédonie.
- 2009-2012 : Thomas Fauvel (comité de thèse et collaboration). Dynamique de métapopulation chez deux prédateurs supérieurs des récifs coralliens. Université Paris VI.

2. Masters et Licences

- 2014: Alexandre Baduel, Licence Pro. Université de Besançon.
- 2014: Aurélien Bonnet, Licence Pro. Université de Besançon.
- 2014: Jules Giraud, Licence Pro. Université de Montpellier.
- 2013-2015: Iulian Gherghel, Master of Science, Oklahoma State University, co-encadrement avec le Dr. Monica Papes.
- 2012: Vhon Gracia, Master of Science, National Museum of the Philippines, co-encadrement avec les Dr. RD Papa and Dr. AC Diesmos (University of Santo Tomas).
- 2012 : Elsa Muret (co-encadrement avec X. Bonnet), Master 2 Biologie, Ecologie, Evolution. Université de Poitiers.

- 2008 : Michaël Decoux (co-encadrement avec X. Bonnet), Master 1 Biologie des populations et des Ecosystèmes. Université de Tours.
- 2005-2006 : Sophie Lorioux (co-encadrement avec X. Bonnet), DU Sciences Naturelles, Université Paris VI.
- 2005-2006 : Margot De Crignis (co-encadrement avec X. Bonnet), DU Sciences Naturelles, Université Paris VI.
- 2004 : Hervé Lelièvre (co-encadrement avec X. Bonnet), DU Sciences Naturelles, Université Paris VI.
- 2004 : Matthieu Berroneau, (co-encadrement avec X. Bonnet), Master 1 Biologie des populations et des Ecosystèmes. Université de Poitiers.

VII. Références citées

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