Université Fédérale
Toulouse Midi-Pyrénées

THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

L'Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)

Présentée et soutenue par :

Jérémie SOUCHET

Le 9 Décembre 2020

Effets de l'hypoxie d'altitude sur le développement embryonnaire et les performances juvéniles chez la Couleuvre vipérine, *Natrix maura*, dans le contexte actuel du changement climatique

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École doctorale et spécialité :

ED SEVAB : Écologie, biodiversité et évolution

Unité de recherche :

Station d'Écologie Théorique et Expérimentale, CNRS – Université Paul Sabatier,
UMR 5321, 2 route du CNRS, 09200 Moulis, France

Directeurs de Thèse :

Hervé PHILIPPE & Fabien AUBRET

Composition du Jury :

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Fabien AUBRET	Chargé de recherches	Co-directeur
Eric J. GANGLOFF	Assistant Professor	Encadrant / Invité

Finesse, subtilité, herpétologie…

TABLE DES MATIÈRES

Notes

Les crédits ne sont appliqués qu'aux documents n'appartenant pas à l'auteur.

L'ensemble des dessins d'illustrations des chapitres ont été réalisés par Hugo Le Chevalier

PRÉFACE

Associer les serpents et les montagnes dans ce manuscrit de thèse n'a rien de nouveau car cette histoire et ce projet de recherche prennent leurs origines, il y a environ 2000 ans, dans la mythologie grecque.

"Cependant le Carthaginois, troublant la paix du monde, se dirige vers les sommets chevelus des monts Pyrénéens. Des plateaux escarpés de leur cime orageuse, les Pyrénées contemplent de loin l'Ibère séparé du Celte, et conservent entre deux grandes contrées un divorce éternel. Ces montagnes ont reçu le nom de la fille de Bébryx, par le crime d'Alcide, son hôte. Dans le cours de ses travaux, il s'acheminait vers les royaumes lointains du triple Géryon. Captivé par Bacchus à la cour du Cruel Bébryx, il y laissa Pyréné séduite et bien à plaindre d'avoir été si belle. L'infortunée ! le dieu qui causa son malheur, ce dieu, s'il est permis de le croire, fut aussi cause de sa mort. Elle mit au monde un serpent : redoutant le courroux de son père, égarée, elle abandonna sur l'heure ses pénates chéris. Seule alors, au fond des antres, elle pleura la nuit passée aux bras d'Alcide, elle raconta aux sombres forêts les promesses du héros, elle accusa son ravisseur et ses ingrates amours : déchirée enfin par les bêtes, vainement elle tendit les bras à son hôte et invoqua le secours de ses armes. De retour et vainqueur, le Tirynthien arrosa de larmes ces membres mutilés ; il pâlit éperdu en retrouvant les traits de sa vierge bien-aimée. Aux éclats des douleurs d'Hercule, les sommets de la montagne tremblèrent ébranlés ; ses gémissements plaintifs appelaient Pyréné, et partout les rochers et les repaires des bêtes féroces redirent Pyréné. Il déposa enfin ses restes dans un tombeau et leur dit en pleurant un dernier adieu. Le temps n'a point détruit la mémoire de cet hommage, et ces montagnes conserveront dans tous les siècles ce nom tant déploré."

Extrait de : Les Puniques de Silius Italicus. Traduction M.E.-F. Corpet et M.N.-A. Dubois (1836-1838)

Cette histoire dans laquelle Pyréné donne naissance à un serpent dans les montagnes garde encore aujourd'hui tout son sens. Ainsi, le Pic du Midi de Bigorre, situé très en amont de la chaîne de montagnes et offrant une vue panoramique sur près de 300km, pourrait être le tombeau de Pyréné. Cette idée est confortée par le fait que sur la face Est de ce pic est visible un serpent, figé dans les plis mêmes de la roche. Et c'est exactement ici, au Pic du Midi de Bigorre que mes expériences de thèse et en partie le développement et la naissance de nombreux serpents en condition d'hypoxie ont lieu. *Coïncidence ou destinée ?*

AVANT-PROPOS

Les travaux de recherche présentés dans cette thèse ont été réalisés dans le cadre d'un contrat doctoral à la Station d'Écologie Théorique et Expérimentale du CNRS de Moulis, UMR 5321, 2 route du CNRS 09200 Moulis, France. Ce projet n'aurait pas pu être mené à bien sans les soutiens financiers et/ou techniques :

- du Laboratoire d'Excellence TULIP (LabEx TULIP, France)
- du Programme Opérationnel de Coopération Territoriale Espagne-France-Andorre sur les Ectothermes des Pyrénées (POCTEFA EctoPyr, Espagne-France-Andorre)
- de l'Observatoire Midi-Pyrénées (OMP, France)
- de la société Nouvelles Pyrénées (N'Py, France)
- du Centre de Récupération des Amphibiens et des Reptiles de Catalogne (CRARC, Espagne)
- de l'association BOMOSA (Andorre)
- de l'association Nature En Occitanie (NEO, France)
- de l'Université Toulouse III - Paul Sabatier (UPS, France)
- de l'Ecole Doctorale Sciences Ecologiques, Vétérinaires, Agronomiques et Bioingéniérie (SEVAB, France)

BILAN DES ACTIONS SCIENTIFIQUES

Articles

- **Souchet J.**, Gangloff E. J., Micheli G., Bossu C., Trochet A., Bertrand R., Clobert J., Calvez O., Martinez-Silvestre A., Darnet E., Le Chevalier H., Guillaume O., Mossoll-Torres M., Barthe L., Pottier G., Philippe H. & Aubret F. **2020**. High-altitude hypoxia impacts perinatal physiology and performance in a potential montane colonizer. Physiological plasticity in lizard embryos exposed to high-altitude hypoxia. *Integrative Zoology*. DOI: 10.1111/1749-4877.12468 ***(Chapitre 3)***

- Lucati F., Poignet M., Miro A., Trochet A, Aubret F., Barthe L.? Bertrand R., Buchaca T., Caner J., Darnet E., Denoël M., Guillaume O., Le Chevalier H., Martinez-Silvestre A., Mossoll-Torres M., O'Brien D., Calvez O., Osorio V., Pottier G., Richard M., Saas I., **Souchet J.,** Tomas J. & Ventura M. **2020**. Multiple glacial refugia and restricted but effective present-day gene flow shaped the genetic structure of an endemic newt from the Pyrenees. Molecular Ecology. DOI: 10.1111/mec.15521 ***(Hors cadre de la thèse)***

- Le Chevalier H., Mari-Mena N., Carro B., Prunier J.G., Bossu C., Darnet E., **Souchet J**., Guillaume O., Calvez O., Bertrand R., Barthe L., Pottier G., Martinez-Sylvestre A., Verdaguer-Foz I., Mossol-Torres M., Trochet A. & Aubret F. **2019**. Isolation and characterization of fourteen polymorphic microsatellite markers in the viperine snake, *Natrix maura*. *Ecology and Evolution* **9**:11227-11231. ***(Annexe 2)***

- Kouyoumdjian L., Gangloff E. J., **Souchet J**., Cordero G.A., Dupoué A. & Aubret. F. **2019**. Transplanting gravid lizards to high elevation alters maternal and embryonic oxygen physiology, but not reproductive success or hatchling phenotype. *Journal of Experimental Biology* **222**. ***(Annexe 3)***

- Grassini S., Valli K., **Souchet J**., Aubret F., Segurini G.V., Revonsuo A. & Koivisto M. **2019**. Pattern matters: Snakes exhibiting triangular and diamond-shaped skin patterns modulate electrophysiological activity in human visual cortex. *Neuropsychologia* **131**:62-72. ***(Hors cadre de la thèse)***

- Gangloff E. J., Sorlin M., Cordero G. A., **Souchet J**. & Aubret F. **2019**. Lizards at the peak: Physiological plasticity does not maintain performance in lizards transplanted to high altitude. *Physiological and Biochemical Zoology* **92**(2):189-200. ***(Annexe 4)***

- Trochet A., Deluen M., Bertrand, R., Calvez O., Martinez-Silvestre A., Verdaguer-Foz I., Mossol-Torres M., **Souchet J**., Darnet E., Le Chevalier H., Guillaume O. & Aubret F. **2019**. Body Size Increases with Altitude Elevation in Pyrenean Brook Salamanders (*Calotriton asper*). *Herpetologica* **75**(1):30-37. ***(Hors cadre de la thèse)***

- Trochet A., Dupoué A., **Souchet J**., Bertrand R., Deluen M., Murarasu S., Calvez O., Martinez-Silvestre A., Verdaguer-Foz I., Darnet E., Le Chevalier H., Mossoll-Torres M., Guillaume O. & Aubret F. **2018**. Variation of preferred body temperatures along an altitudinal gradient: a multi-species study. *Journal of Thermal Biology* **77**:38-44. ***(Hors cadre de la thèse)***

- Cordero G. A., Andersson B. A., **Souchet J**., Micheli G., Noble D. W. A., Gangloff E. J., Uller T. & Aubret F. **2017**. Physiological plasticity in lizard embryos exposed to high-altitude hypoxia. *Journal of Experimental Zoology part A: Ecological and Integrative Physiology: 1-10.* ***(Annexe 5)***

- Aubret F., Bignon F., Bouffet-Halle A., Blavillain G., Kok P. J. R. & **Souchet J**. **2017**. Yolk removal generates hatching asynchrony in snake eggs. *Scientific reports* **7**: 3041. ***(Hors cadre de la thèse)***

- **Souchet J**. & Aubret F. **2016**. Revisiting the fear of snake in human: the role of aposematic signals. *Scientific Reports* **6**: 37619. ***(Hors cadre de la thèse)***

- Bonnet X., Lecq, S., Lassay JL., Ballouard JM., Barbraud C., **Souchet J**., Mullin S.J. & Provost G. **2016**. Forest management bolsters native snake populations in urban park. *Biological Conservation* **193**:1-8. ***(Hors cadre de la thèse)***

- ***(Accepté dans Biological Journal of the Linnean Society)*** **Souchet J.**, Gangloff E. J., Bossu C., Darnet E., Le Chevalier H., Poignet M., Trochet A., Bertrand R., Calvez O., Martinez-Silvestre A., Mossoll-Torres M., Guillaume O., Dupoué A., Perrin C., Clobert J., Barthe L., Pottier G., Philippe H. & Aubret F. High temperatures limit developmental resilience to high-elevation hypoxia in the snake *Natrix maura* (Squamata: Colubridae). ***(Chapitre 4)***

- ***(Accepté dans Herpetological Review)*** Martínez-Silvestre A., Trochet A., Calvez O., Poignet M., Le Chevalier H., Souchet J., Guillaume O., Bertrand R., Mossoll-Torres M., Aubret F., Soler J?, Miró A., Lucati F., Ventura M., Barthe L., Pottier G., Marschang R. & Bosch J. Presence of the pathogenic fungus Batrachochytrium *dendrobatidis* in wild populations of the Pyrenean brook salamander (*Calotriton asper*, Dugès, 1982). ***(Hors cadre de la thèse)***

- ***(Soumis dans Diversity and Distributions)*** Dupoué A., Trochet A., Richard M., Sorlin M., Teulière Quillet J., Vallé C., Rault C., Berroneau M., Berrneau M., Lourdais O., Blaimont P., Bertrand R., Guillon M., Pottier G., Calvez O., Guillaume O., Le Chevalier H., **Souchet J.,** Le Gaillard J. F., Clobert J., Aubret F. Genetic and demographic trends shaped by climate change on both range margins of a cold-adapted lizard. ***(Hors cadre de la thèse)***
- ***(In prep.)*** Sinervo B., Bertrand R. Darnet E., Le Chevalier H., Trochet A., Dupoué A., **Souchet J.,** Calvez O., Perrin C., Martin-Garcia R., Barthe L ., Pottier G., Martinez-Silvestre A., Verdaguer-Foz I., Mossoll-Torres M., Guillaume O., Gangloff E. J., Blaimont P., Heulin B., Miles D. B., D'Amico F., Clobert J. & Aubret F. Ecophysiological Species Distribution Models for Pyrenean Ectotherms under Climate Change. ***(Hors cadre de la thèse)***
- ***(In prep.)*** Boissinot A., Grillet P., **Souchet J.,** Morin-Pinaud S. & Lourdais O. Selection of post-breeding habitat in the Common Frog (*Rana temporaria*) in a hedgerows landscape in western France. ***(Hors cadre de la thèse)***

Conférences

- **Souchet J**., Grassini S. & Aubret F. **2019**. Role of aposematic signals in fear of snakes at human. *14th Ecology and Behaviour meeting*, Toulouse (France)
- **Souchet J**., Gangloff E. J., Aubret F. & Philippe H. **2019**. Effects of high altitude hypoxia in snake development and plasticity. *14th Ecology and Behaviour meeting*, Toulouse (France)
- **Souchet J**., Grassini S. & Aubret F. **2018**. Les rôles des signaux aposématiques dans la peur des serpents. *46ème congrès de la Société Herpétologique de France,* Carnoules (France)
- **Souchet J**. **2017**. Altitudinal colonization and adaptability to hypoxia at reptiles. *13th Ecology and Behaviour meeting*, Chizé (France)
- **Souchet J**. **2016**. Colonisation altitudinale et adaptabilité à l'hypoxie chez les reptiles. *Journée DiPEE Midi-Pyrénées. Rencontre transdisciplinaire de sept laboratoires sur le thème : « Comportement et Environnement »*, Toulouse (France)
- **Souchet J**., Chevalier T., Lecq S., Provost G. & Bonnet X. **2013**. Suivi de populations de serpents en forêt de Chizé - Extension à un site du Mans. *29ème festival de Ménigoute : 8ème rencontres nationales sur la conservation des Amphibiens et Reptiles*, Ménigoute (France)

Posters

- Trochet A., Guillaume O., Calvez O., Clobert J., Perrin C., Bousquet M., Bertrand R., **Souchet J**., Barthe L., Pottier G., Altimir A., Mossoll-Torres M., Madrenys E., Martinez- Silvestre A. & Aubret F. **2016**. Projet Interreg POCTEFA ECTOPYR. *44ème congrès de la Société Herpétologique de France*, Namur (Belgique)
- **Souchet J**., Micheli G., Bossu C. & Aubret F. **2016**. Colonisation altitudinale et adaptabilité à l'hypoxie : une contrainte ignorée du réchauffement climatique sur la biodiversité. *44ème congrès de la Société Herpétologique de France*, Namur (Belgique)
- **Souchet J**., Sarraude T. & Aubret F. **2015**. Perception de la signalétique de danger chez les enfants : exemple des signaux aposématiques animaux. *43ème congrès de la Société Herpétologique de France*, Toulouse (France)

Médiations

- Conférence (**2019**). Des serpents dans les nuages. *Festival Pint of Science*, Toulouse
- Radio (**2019**). L'intelligence des reptiles. *Emission « le Nid de Pie » sur campus FM*, Toulouse
- Conférence (**2018**). Des serpents dans les nuages. *La nuit européenne des chercheur.e.*s, Toulouse
- Court métrage (**2018**). Les gardiens de la montagne. *Produit par le projet Poctefa Ectopyr*
- Clip vidéo (**2017**). Partage Ta Science, l'écophysiologie avec Jérémie Souchet. *13ème congrès Ecology and Behaviour,* Chizé
- Radio (**2017**). Changement climatique et biodiversité. *Emission « la puce à l'oreille » de la radio de l'Association des Naturalistes d'Ariège*
- Conférence (**2017**). Impacts du réchauffement climatique sur la faune des Pyrénées. *Les mercredis de l'ANA*, Foix
- Article de Presse (**2016**). Moulis teste l'adaptabilité des reptiles au Pic du Midi de Bigorre. *La Dépêche du Midi.*
- Article de presse (**2015**). Jérémie, L'étudiant qui veut savoir d'où vient la peur des serpents. *La Gazette Ariègeoise*
- Reportage (**2014**). Couleuvres et vipères, les serpents de nos régions - Des serpents suivis à la trace*. Emission « Les Animaux de la 8 - Les Deux-Sèvres, une terre animale méconnue »*

FORMATIONS EFFECTUÉES

- Enseignement dans le secondaire et encadrement de groupes d'étudiants de Master 1 lors de stage de formation d'une semaine au Centre d'Etudes Biologiques de Chizé – CNRS de Chizé
  ***Année** : 2016 / **Nombre d'heure de formation** : 25 heures*

- Formation à l'utilisation d'animaux à des fins scientifiques sur faune sauvage non hébergée, Niveau concepteur délivrée par le Muséum National d'Histoire Naturelle (MNHN), l'Office National de la Chasse et de la Faune Sauvage (ONCFS) et le Centre National de la Recherche Scientifique (CNRS)
  ***Année** : 2017 / **Nombre d'heure de formation** : 57 heures*

- Formation de tutorat à la prise de sang intracardiaque chez les ophidiens délivrée par Albert Martinez Silvestre, Docteur vétérinaire diplômé du European College of Zoo Medicine (spécialité en herpétologie) et responsable du Centre de Récupération des Reptiles et des Amphibiens de Catalogne (CRARC ; Masquefa, Espagne)
  ***Année** : 2018 / **Nombre d'heure de formation** : 3,5 heures*

- Formation maintien de capacité « Les points limites » délivrée par la délégation Occitanie Ouest du Centre National de la Recherche Scientifique (CNRS)
  ***Année** : 2019 / **Nombre d'heure de formation** : 3,5 heures*

- Participation à la Nuit européenne des chercheur.e.s à Toulouse pour « ma thèse en 5 min chrono »
  ***Année** : 2018 / **Nombre d'heure de formation** : 18 heures*

- Membre actif (pigiste et conférencier) de l'association Ad Naturam spécialisée dans la vulgarisation de l'écologie scientifique (https://adnaturam.org)
  ***Année** : 2017 à 2019 / **Nombre d'heure de formation** : 25 heures*

Total des heures de formations effectuées : **132 heures**

REMERCIEMENTS

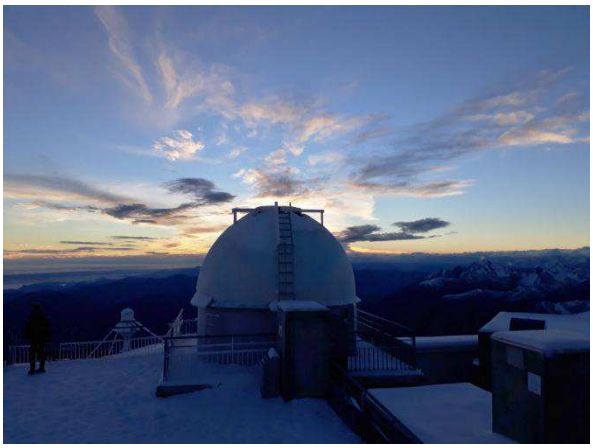
Les remerciements, ce moment attendu et redouté. D'un côté cela signifie que le projet et l'épreuve se terminent mais d'un autre côté ils nous mettent souvent face à un avenir incertain. Néanmoins, c'est toujours un plaisir de prendre quelques instants pour repenser à tous ces moments partagés.

La Station d'Ecologie Théorique et Expérimentale ne portait pas encore ce nom quand j'y suis venu pour la première fois en 2015. A cette époque le laboratoire se nommait "Station d'Ecologie Expérimentale" et je venais y réaliser mon stage de Master 2**.** Le stage traitait de la peur des serpents à travers la perception des signaux aposématiques animaux chez l'enfant. Un sujet à la frontière de la sociologie et de l'écologie qui m'a permis de découvrir l'Ariège et surtout la richesse de ses paysages. Une fois tombé sous le charme du Couserans, difficile de le quitter, c'est pourquoi j'ai été ravi de commencer cette thèse, un an après avoir quitté la station.

Avant toute chose, j'aimerais remercier **Fabien Aubret** et **Hervé Philippe** de m'avoir fait confiance et de m'avoir financé ce projet de recherche en me donnant l'opportunité de travailler sur les effets de l'hypoxie d'altitude chez une espèce de serpent, la Couleuvre vipérine. Une espèce qu'en temps que naturaliste amateur et herpétologue j'apprécie énormément.

Travailler sur l'hypoxie d'altitude nécessite de pouvoir exposer son modèle d'étude à cette condition. Pour cela, rien de mieux que de travailler sur un pic mythique des Pyrénées : le Pic du Midi de Bigorre. Avec son observatoire culminant à 2877 m et sa position géographique très en avant de la chaine des Pyrénées, le Pic du Midi de Bigorre offre, en plus des conditions de travail incroyables, une vue imprenable sur le massif. C'est un site touristique depuis les années 2000 mais c'est avant tout un site historiquement fort depuis 1878 pour la recherche en astronomie et en météorologie. C'est l'ensemble de ce que représente le Pic du Midi de Bigorre ainsi que **toutes les personnes qui y travaillent** et que j'ai eu le plaisir de rencontrer durant 3 campagnes de récolte de données que je souhaite remercier. Merci pour l'ensemble des connaissances transmises sur le système solaire et sur l'histoire du Pic. Merci pour l'aide apportée pour me faciliter au maximum les conditions de travail, pour votre intérêt et votre curiosité vis-à-vis de mon projet de recherche et merci pour tous les moments partagés de jour comme de nuit et surtout pour ce concert de Christophe Willem. Merci aussi et surtout d'avoir mis en place un téléphérique car les ascensions quotidiennes auraient été

longues et fastidieuses. Et pardon à tous ceux qui avec moi dans la cabine, sachant que je transportais des serpents, ont passé quelques longues minutes entre stress et fascination.

(A gauche) Une des cabines du téléphérique permettant d'atteindre sans effort, et en 15 min seulement, le sommet du Pic du Midi de Bigorre. (A droite) Levé de soleil par dessus les coupoles enneigées au Pic du Midi de Bigorre depuis la salle de travail.

Bien entendu tout ce travail, toutes ces expériences n'auraient pu être possibles sans l'aide technique de nombreuses personnes. Un merci aussi grand que possible à **Eric J. Gangloff**, pour ton aide sur l'ensemble de mon travail. De la conception des protocoles aux analyses stats en passant par l'aide à la rédaction et les discussions, tu as été d'une aide précieuse, ta sympathie et ta gentillesse n'enlevant rien au reste. J'espère avoir le plaisir de continuer à travailler avec toi. Ces remerciements vont aussi à **Elodie Darnet** et **Hugo Le Chevalier** pour toutes leurs aides sur les expériences. Etant incapable de me dédoubler, vous m'avez permis de survivre en prenant à votre charge une grosse partie du travail. Merci pour votre aide et votre soutien, sans vous les soirées post manips ne seraient surement pas les mêmes. Un grand merci également à **Audrey Trochet** et **Olivier Calvez** pour leur soutien, leur dynamisme et leur aide dans toutes les parties administratives et éthiques du projet. Sans vous il aurait été difficile de réaliser tout ce projet dans de bonnes conditions. Un merci également à tous les stagiaires que j'ai eu le plaisir d'encadrer et de collaborer **Gaëlle Micheli, Coralie Bossu, Sophie Murarasu, Amandine Hibert, Noémie Hennes, Léa Brun, Manon Poignet, Alicia Josserand, Lény Kerekdjian** et **Lauriane Begué**. Vous avez passé de quelques jours à quelques mois avec moi au Pic du Midi de Bigorre mais j'espère que vous avez apprécié votre passage à Moulis. Merci spécial à **Marine Deluen, Ayala Loisel, Laura Kouyoumdjian, Laurane Winandy, Jérôme G. Prunier, Audrey Trochet, Elodie Darnet, Isabel Cantera** et **Kévin Liautaud**, mes correcteurs des première et dernières minutes, qui ont pris le temps de lire, de corriger et de remanier ce manuscrit mais surtout qui m'ont sorti des impasses dans lesquelles je me suis souvent trouvé.

Un merci va également à l'ensemble des membres du projet interreg Poctefa Ectopyr, projet dans lequel mes travaux de thèse s'inscrivent. Merci à vous pour tous les échanges autour des reptiles et des Amphibiens des Pyrénnées. Pour ne citer que ceux qui ne l'ont pas été précédemment, merci à **Jean Clobert, Olivier Guillaume, Christine Perrin, Romain Bertrand, Marion Bousquet, Andréaz Dupoué, Rebbecca Martin Garcia, Laurent Barthe, Gilles Pottier, Albert Martinez-Silvestre, Isabel Verdaguer, Marc Mossol-Torres** et **Edgar Madrenys**. Merci à l'ensemble de l'équipe administrative de la SETE, **Marion Bousquet**, **Séverine Bonzom**, **Jade Manaud** et **Myriam Istoczak** pour leur soutien et leur aide dans toutes les longues procédures de missions, d'achats, de conventions. Merci également à **Dominique Pantalacci**, sans qui les procédures administratives de l'Ecole Doctorale et de l'Université seraient un veritable calvaire. Merci aussi à **Thomas Deruelles** pour son aide logistique durant la création des élevages et des dispositifs expérimentaux. Enfin, j'aimerais envoyer mes remerciements certes plus généraux mais néanmoins sincères à l'ensemble du personnel de la Station de Moulis. Merci pour votre aide au quotidien et tous les échanges que nous avons pu avoir durant ces nombreuses années.

Un bref mais néanmoins grand merci à **Stéphane Lecq, Xavier Bonnet, Alexandre Boissinot** et **Olivier Lourdais** qui m'ont ouvert les premiers les portes de la recherche et des reptiles. Je n'en serais pas là sans votre confiance et les enseignements que vous m'avez transmis dans mes premières années de stage. Un merci spécial à **Stéphane** qui n'a pas écouté les critiques de mes enseignants à mon égard et sans qui rien n'aurait commencé.

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Finalement, un doctorat à Moulis, perdu dans les vallées du Couserans ne pourrait pas se faire sans la présence d'une vie sociale forte (#looseranslife). Vous êtes nombreux à enrichir mon quotidien et je vais essayer de n'oublier personnes : **Marine, Vincent, Audrey, Olivier, Elodie, Nico, Hugo, Laurane, Rik, Léa, Mathieu, Julien, Elvire, Lisa, Alice, Etienne, Louis, Nico, Anne-Sophie, Jérôme, Claire-Lise, Kévin, Robin, Alice, Floriane, Théo, Pauline, Pierre, Diego, Jeff, Nuria, Vinicius, Mathew, Matthieu, Jarad, Fanny, Arnaud, Yuval, Eloise, Orlane, Laura, Alicia, Eric, Maya, Allan, Kéoni, Aisha, Jonathan, Sarah, Azenor**. Pour ces soirées à la boussole ou ailleurs dans les diverses colocs, ces concerts au

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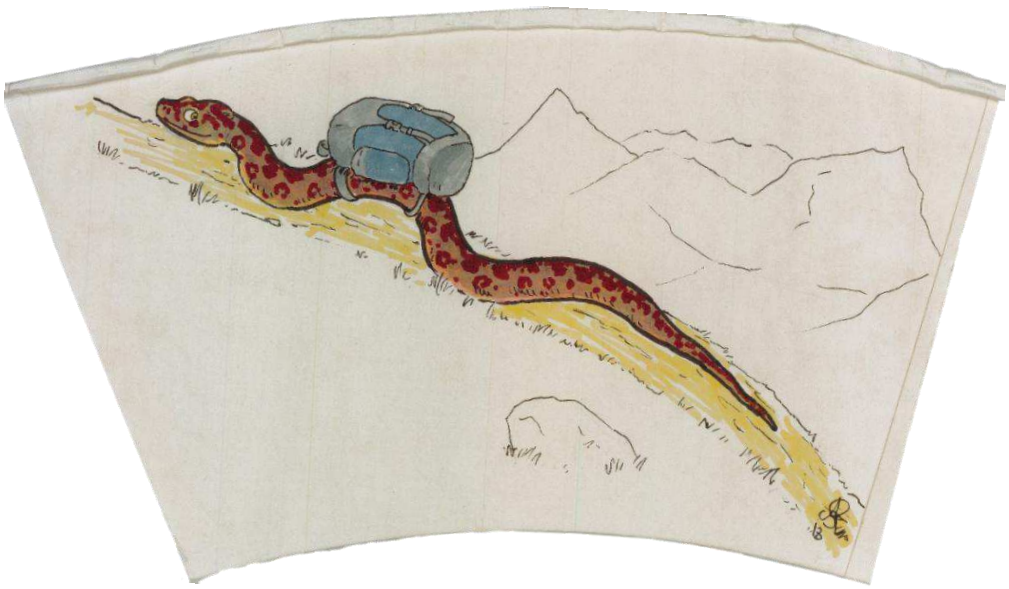
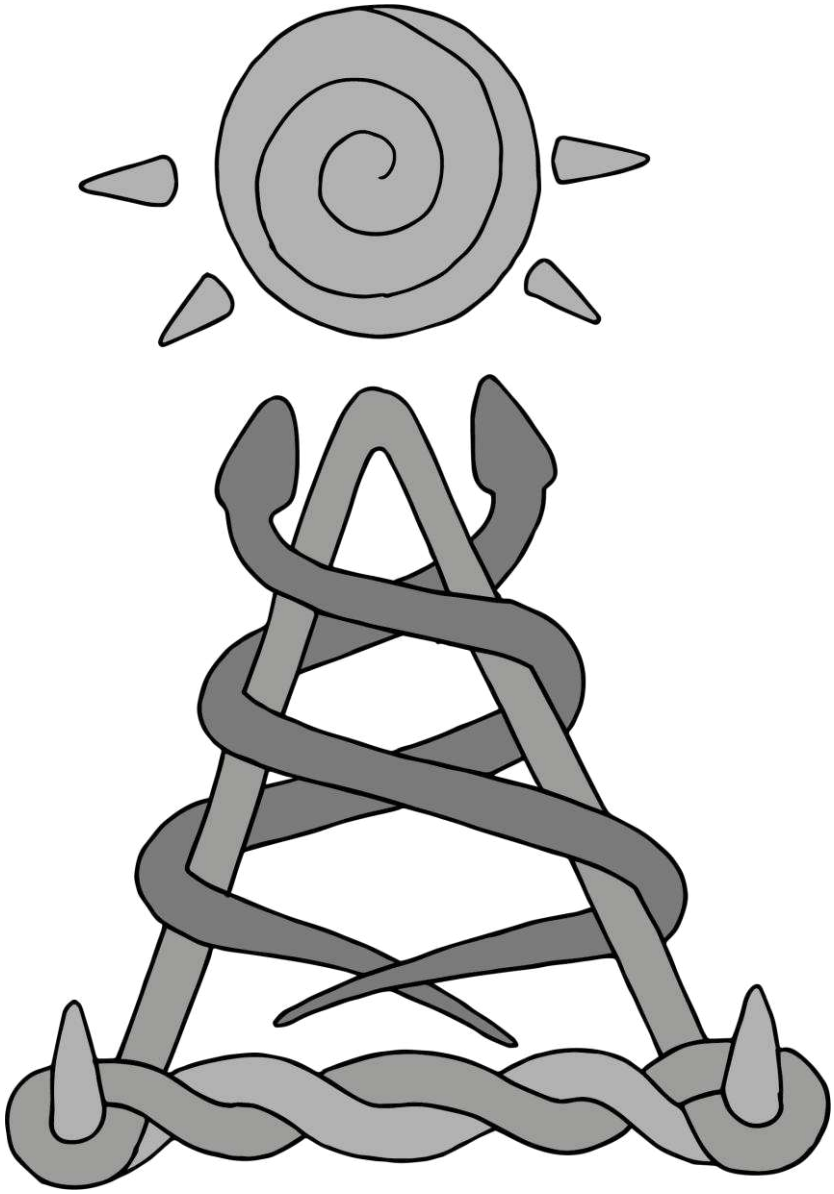


Représentation cartoonesque de la montée en altitude de la Couleuvre vipérine. Dessin réalisé en quelques minutes sur un goblet, le samedi matin du 46ème congrès de la Société herpétologique de France en 2018 (©Marion Jouffroy).

Chapitre 1.

INTRODUCTION GÉNÉRALE



1.1. Modifications environnementales et biodiversité

1.1.1. Extinctions de masse et perte de biodiversité

Au cours des 3,5 milliards d'années écoulées, 99% des 4 milliards d'espèces estimées ont disparu (Barnosky et al., 2011). La disparition des espèces est un processus continu dans le temps. Bien que certains événements de grande ampleur, appelés extinctions de masses, peuvent venir rythmer ce processus. Au cours de l'évolution des espèces, cinq extinctions de masse ont été identifiées (Raup and Sepkoski, 1982). Elles sont datées de la fin de l'Ordovicien (environ -445 millions d'années), de la fin du Dévonien (environ -360 millions d'années), de la fin du Permien (environ -245 millions d'années), de la fin du Trias (environ -200 millions d'années) et enfin de la fin du Crétacé (environ -66 millions d'années ; Figure 1). Chacune de ces crises a entrainé la disparition de 35% à 57% des familles d'animaux et de plantes, englobant 75% à 96% des espèces présentes au moment de la crise (Jablonski, 1994; Barnosky et al., 2011). Par conséquent, ces extinctions massives sont considérées comme des hécatombes mondiales entrainant une chute brutale de la biodiversité et menant parfois à la disparition de familles entières (Bambach, 2006).

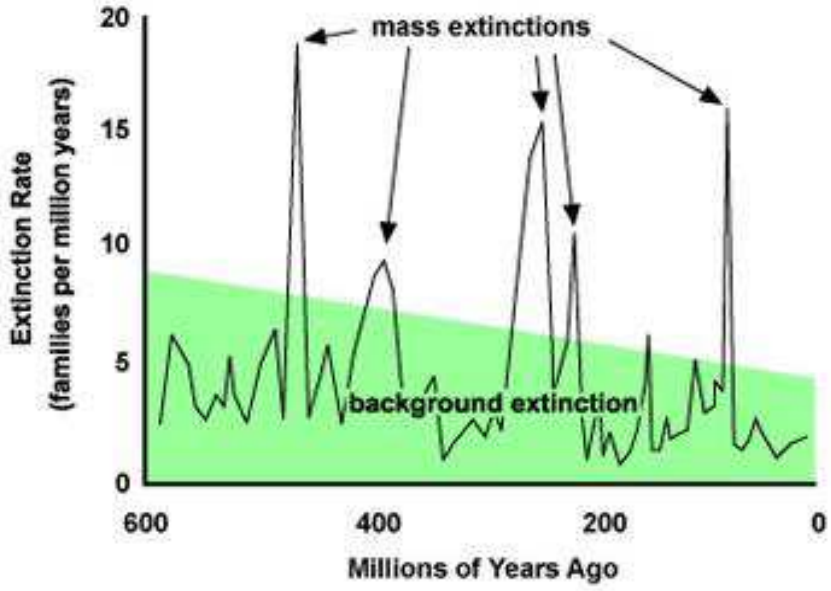


Figure 1 : *Évolution du taux d'extinction des familles d'espèces par million d'années. Les cinq pics représentent les cinq grandes extinctions de masses (à la fin de l'Ordovicien, du Dévonien, du Permien, du Trias et du Crétacé). La courbe du taux d'extinction de fond standard (dans l'aplat vert noté "background extinction") représente l'ensemble des niveaux d'extinction d'espèces considérés normaux à l'échelle temporelle présentée. (The University of California Museum of Paleontology's Understanding Evolution).*

En parallèle des extinctions massives, il existe aussi le phénomène continu d'extinction des espèces (noté "background extinction" dans la Figure 1). Le taux d'extinction des espèces, lié à ce phénomène continu d'extinction, varie en fonction de causes internes et externes qui compromettent la capacité de survie et de reproduction de ces espèces (Pievani, 2014). La perte de diversité génétique, les interactions interspécifiques ou les modifications environnementales font partie de ces facteurs pouvant conduire à l'extinction d'une espèce. Plus généralement, la détérioration des habitats et les modifications brutales des conditions environnementales sont des facteurs pouvant causer une perte de biodiversité importante. Dans le cas des extinctions de masse, celles-ci sont brutales et résultent d'événements rarissimes de forte intensité comme l'explosion d'une étoile, de fortes éruptions volcaniques ou encore des collisions répétées avec des astéroïdes. Ces événements ont eu pour conséquences une modification abrupte du climat de la planète, des fluctuations du niveau de la mer et des changements des proportions des gaz de l'atmosphère. Actuellement, de nombreux travaux scientifiques suggèrent que le taux d'extinction actuel des espèces est très supérieur au taux de base du phénomène continu d'extinction. Ces éléments démontrent que la perte de biodiversité est exceptionnellement rapide au cours des derniers siècles, indiquant que nous entrons dans une 6ème extinction de masse (Figure 2; Raup, 1991; Pimm et al., 1995; Alroy, 1996; MacPhee and Sues, 1999; Cardillo et al., 2008; May, 2010; Stork, 2010; Dirzo et al., 2014; Ceballos et al., 2015).

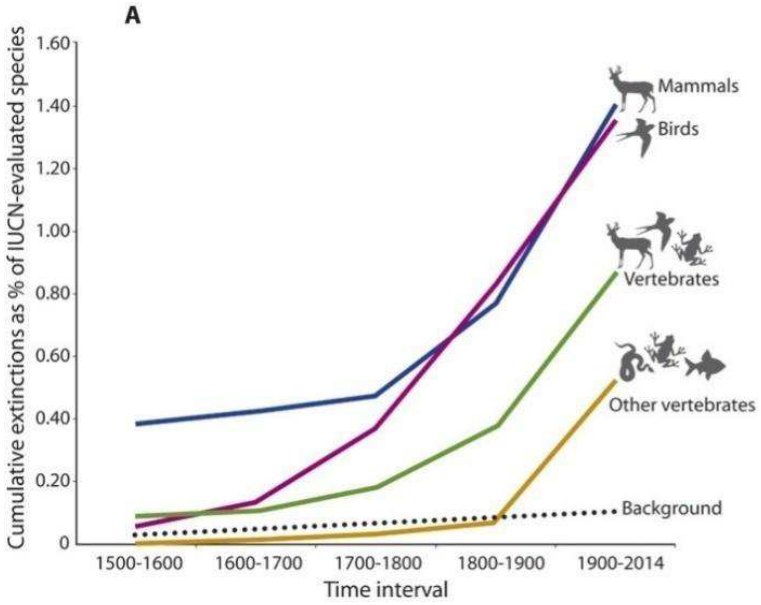


***Figure 2** : Le graphique montre le pourcentage cumulatif du nombre d'espèces éteintes par rapport aux nombres d'espèces évaluées par l'UICN chez les mammifères (5513; 100% de celles décrites), les oiseaux (10 425; 100%), les reptiles (4414; 44%), les amphibiens (6414; 88%), les poissons. (12 457; 38%), et tous les vertébrés réunis (39 22; 59%). La courbe noire en pointillé représente le processus d'extinction continu (i.e. nombre d'extinctions attendues avec une vitesse constante de 2 extinctions d'espèces par million d'espèces par an. (Ceballos et al., 2015).*

Dans la plupart des groupes de Vertébrés qui font l'objet d'études approfondies depuis plusieurs années, des déclins importants du nombre d'espèces et de l'abondance d'individus dans leurs populations ont été décrits (Murphy and Romanuk, 2014). Ces effondrements ont pour conséquences des effets en cascade au sein des communautés entrainant des perturbations dans le fonctionnement des écosystèmes et provoquant une accélération de leur détérioration (Lecq, 2013). La perte globale de la biodiversité actuelle a des origines multifactorielles principalement liées aux activités anthropiques. L'augmentation du déclin des espèces et des populations a débuté aux prémices de l'agriculture et s'est fortement accélérée depuis le début de l'industrialisation (Kareiva et al., 2007). Les modifications récentes et importantes dans les pratiques agricoles associées à la mondialisation et à l'augmentation des échanges entre pays ont fortement impacté la qualité des environnements. Parmi les causes majeures entrainant une perte de biodiversité à l'échelle globale, peuvent être citer : la perte d'habitat et la fragmentation des milieux (Griffith et al., 1989; Lefeuvre, 1992; Boissinot et al., 2014; Haddad et al., 2015), les pollutions sonores (Marzluff, 2001; Habib et al., 2007; Lengagne, 2008; Barber et al., 2010; Slabbekoorn, 2013; Meillère et al., 2015), les pollutions lumineuses (Witherington and Martin, 2000; Hölker et al., 2010; Rich and Longcore, 2013; Bashiri, 2014; Bliss-Ketchum et al., 2016; Brei et al., 2016), les pollutions chimiques (Newman, 1979; Winner and Atkinson, 1986; Treshow and Anderson, 1989; Cooper, 1993; Nahmani et al., 2005; Taylor et al., 2005; Smith et al., 2007), les introductions d'espèces invasives (Didham et al., 2005; Grice, 2006; Molnar et al., 2008; Clavero et al., 2009; Doherty et al., 2016), la surexploitation (*e.g.* surpêche, chasse et braconnage; Bodmer et al., 1997; Keane et al., 2005; Dutton and Squires, 2008; Halpern et al., 2008; Casas et al., 2009; Costello et al., 2012a; Ripple et al., 2016; Romero-Muñoz et al., 2020) et le changement climatique (Parmesan et al., 1999; Wilson et al., 2005; Parmesan, 2006; Pounds et al., 2006; Thomas et al., 2006; Jeppesen et al., 2010; Ohlberger, 2013; Pacifici et al., 2015; Urban, 2018).

Face aux modifications environnementales, les organismes peuvent mettre en place deux stratégies : la migration ou la modification phénotypique. Dans le premier cas de figure, les individus se déplacent pour rester dans des conditions favorables, entrainant un changement dans la répartition spatiale des populations (Parmesan and Yohe, 2003; Sinervo et al., 2010; Lenoir et al., 2020). Dans le second cas, les populations vont voir leur composition phénotypique modifiée afin de pouvoir s'acclimater aux nouvelles conditions. Ces modifications phénotypiques peuvent s'opérer aux niveaux physiologique, morphologique, comportemental ainsi qu'au niveau des traits d'histoire de vie. La modification phénotypique des populations peut s'obtenir par la plasticité phénotypique qui est une réponse possible à court terme mais aussi par la sélection naturelle, une réponse à long terme (Stearns, 1989). La plasticité phénotypique correspond à la capacité d'un

génotype à produire un phénotype différents dans ce nouvel environnement (Pigliucci, 2001). La sélection naturelle quand à elle favorise le maintien des génotypes qui produisent les phénotypes les plus adaptés aux nouvelles conditions environnementales (Hoffmann and Sgrò, 2011).

1.1.2. La plasticité phénotypique comme réponse aux modifications environnementales

La plasticité phénotypique est définie comme étant la capacité d'un génotype à produire différents phénotypes quand il est exprimé dans des environnements distincts (Pigliucci, 2001). Les modifications du phénotype d'un individu sont considérées comme des réactions de l'organisme face aux différents facteurs environnementaux (Pigliucci, 2001; Clobert et al., 2010). La norme de réaction décrit quant à elle la gamme des phénotypes produits par un même génotype dans des conditions environnementales différentes. Cette notion a été abordée la première fois par Woltereck en 1909. Il a démontré que des espèces de daphnies, des petits crustacés d'eau douce mesurant quelques millimètres, adoptaient des morphologies différentes selon la présence ou non de prédateurs dans leur milieu (Woltereck, 1909). Bien que les normes de réaction s'expriment en fonction de la relation qui existe entre un trait donné du phénotype et les différentes conditions environnementales, elle est souvent et arbitrairement, dans un souci de représentation, réduite à deux dimensions (*e.g.* un trait phénotypique comme la taille d'un individu à la naissance selon un facteur environnemental comme la température d'incubation de l'œuf, Figure 3; Pigliucci, 1998, 2001; Clobert et al., 2010). Il sera alors considéré qu'un individu présente de la plasticité phénotypique si son phénotype change en réponse aux modifications des conditions environnementales. Dans un cadre simple, où un seul trait phénotypique et un seul facteur environnemental sont considérés, la norme de réaction devra dans ce cas avoir une pente différente de zéro (Figure 3A et 3B). De plus, les conditions environnementales peuvent fortement affecter l'intensité d'expression des gènes (Zhou et al., 2012). Alors, dans un même environnement, un même gène peut ainsi être exprimé de manière différente entre deux individus d'une même espèce modifiant alors la réponse phénotypique et entrainant des normes de réaction différentes (Figure 3C; Pigliucci, 2001; Clobert et al., 2010).

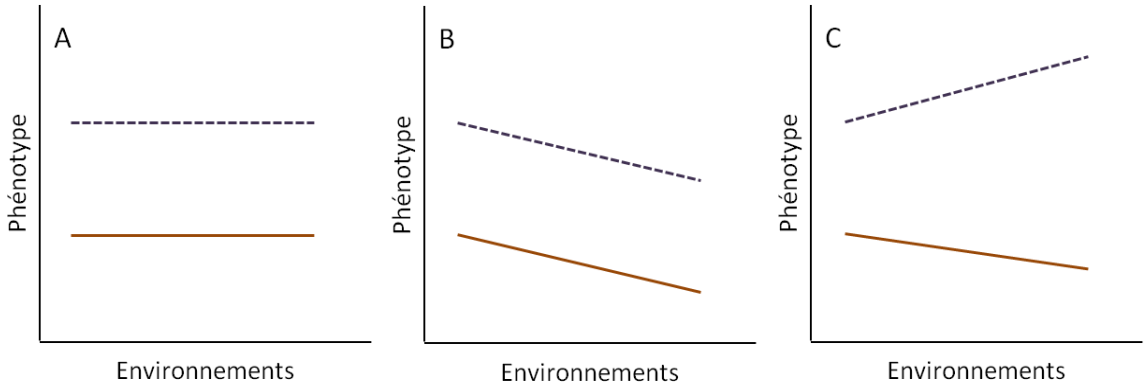


Figure 3 *: Normes de réaction d'un trait phénotypique (e.g. la taille à la naissance d'un individu) selon un trait environnemental (e.g. la température d'incubation de l'œuf) exprimés par deux génotypes différents (ligne continue et pointillée). (A) Les deux normes de réaction sont identiques et planes. Aucune plasticité n'est exprimée par l'un ou l'autre des génotypes. (B) Les deux normes de réaction ont une pente de même direction et de même amplitude. Les deux génotypes expriment une plasticité identique. (C) Les normes de réaction sont différentes entre les deux génotypes. Ils expriment une plasticité différente induite par une variabilité génotypique (d'après Pigliucci, 2001; Clobert et al., 2010).*

La plasticité phénotypique d'un individu peut être caractérisée par différents critères. Premièrement, la plasticité peut être réversible ou irréversible (Clobert et al., 2010; Beaman et al., 2016). Souvent, quand elle est exprimée durant le développement embryonnaire, la plasticité phénotypique sera irréversible (Pigliucci et al., 2006). C'est le cas par exemple de la détermination du sexe chez certains reptiles, qui est dépendante de la température d'incubation (Janzen and Paukstis, 1991; Sarre et al., 2004). A l'inverse, certaines formes de plasticité sont réversibles et correspondent à des modifications du phénotype qui peuvent s'opérer tout au long de la vie d'un individu (Clobert et al., 2010). C'est notamment le cas de la plasticité comportementale. En cas de stress de prédation par exemple, un organisme peut mettre en place des défenses couteuses, en énergie notamment, pendant un temps limité et ainsi répondre rapidement à la menace perçue (Gabriel, 2005). Deuxièmement, la plasticité phénotypique peut être adaptative ou non adaptative (Ghalambor et al., 2007; Clobert et al., 2010; Forsman, 2015). Dans le cas où le phénotype produit dans un nouvel environnement favorise la fitness de l'individu, la plasticité phénotypique sera alors considérée comme adaptative (Clobert et al., 2010). A l'inverse, la plasticité non adaptative inclut toutes les réponses phénotypiques à une condition environnementale donnée qui ne permettent pas d'améliorer la fitness (Clobert et al., 2010). Il est important de noter que le phénotype produit en réponse à une variation environnementale peut entrainer, chez les individus, des coûts de maintenance physiologique ayant des impacts à long terme. En effet, la plasticité phénotypique qui a lieu durant le développement de l'individu peut déclencher des réponses plastiques qui ne seront avantageuses que durant le développement ou les premiers stades de vie (Gluckman et al., 2005)

mais qui pourront avoir des effets délétères plus tard dans la vie (Gluckman et al., 2008; Lee et al., 2016; Walczyńska et al., 2016; Le Roy et al., 2017; Mitchell et al., 2018b).

Enfin, il est important de noter que le rôle de la plasticité phénotypique comme accélérateur ou frein à l'évolution des populations (et à plus long terme des espèces) est encore débattu (Piersma and Drent, 2003; Pigliucci et al., 2006; Whitman and Agrawal, 2009; Forsman, 2015). La plasticité phénotypique peut être vue comme "empêchant" l'adaptation évolutive en réduisant l'efficacité des pressions sélectives qui permettent le maintien des génotypes produisant les phénotypes les plus adaptés (Gibert, 2020). Cependant, la plasticité phénotypique peut permettre, dans un premier temps (*i.e.* à court terme), aux organismes de s'acclimater aux modifications abruptes des conditions environnementales et donc de survivre (Munday et al., 2017). Dans un second temps (*i.e.* à long terme), la sélection naturelle, peut sélectionner les génotypes produisant les phénotypes les plus adaptés (Aubret and Shine, 2009). Enfin, le maintien de la plasticité est principalement déterminé par les coûts qu'elle engendre et par la variabilité spatio-temporelle des conditions environnementales.

1.1.3. Les impacts du changement climatique sur la biodiversité

L'une des causes majeures de l'accélération du processus d'extinction des espèces est le changement climatique, induit par les activités anthropiques. Actuellement, une augmentation globale des températures de surface de la Terre de 2°C à 2,5°C d'ici 2100 est considérée comme très probable (Pachauri et al., 2014; IPPC, 2018). Cependant, les rapports récents du Groupe d'experts Intergouvernemental sur l'Évolution du Climat (GIEC, *IPCC* en anglais) estiment que les températures à la surface de la Terre devraient en moyenne augmenter de 1°C à 6,5°C d'ici 2100, par rapport à la période 1986-2005, en fonction des différents scenarii (Figure 4A et 4B ; Pachauri et al., 2014; IPPC, 2018). Cependant, si on ne tient compte que de la moyenne des températures à la surface des terres émergées, l'augmentation moyenne de la température est actuellement 1,5 fois plus importante que la moyenne à l'échelle du globe (Figure 4C). Le GIEC estime alors que l'augmentation des températures pour les terres emergées devrait être de l'ordre de 1,5°C à 10°C en fonction des différents scenarii (Arneth et al., 2019). À l'échelle mondiale, une hausse significative du nombre de jours anormalement chauds a été enregistrée depuis 1950. Ces augmentations de température vont être associées à des vagues de chaleur plus intenses et plus fréquentes entrainant un accroissement des risques de sécheresses et de leurs intensités (Trenberth et al., 2014; Diffenbaugh et al., 2017; Cattiaux et al., 2018), modifiant le cycle de l'eau et augmentant le niveau d'eau des océans. Dans des régions non limitées en eau, l'augmentation de la température peut favoriser l'évaporation, entrainant par la suite une augmentation des

précipitations et donc des inondations (Palmer and Räisänen, 2002; Cattiaux et al., 2018). Enfin, avec l'augmentation des températures, les événements extrêmes, les cyclones ou encore les incendies, devraient voir leur intensité augmenter (Cattiaux et al., 2018).

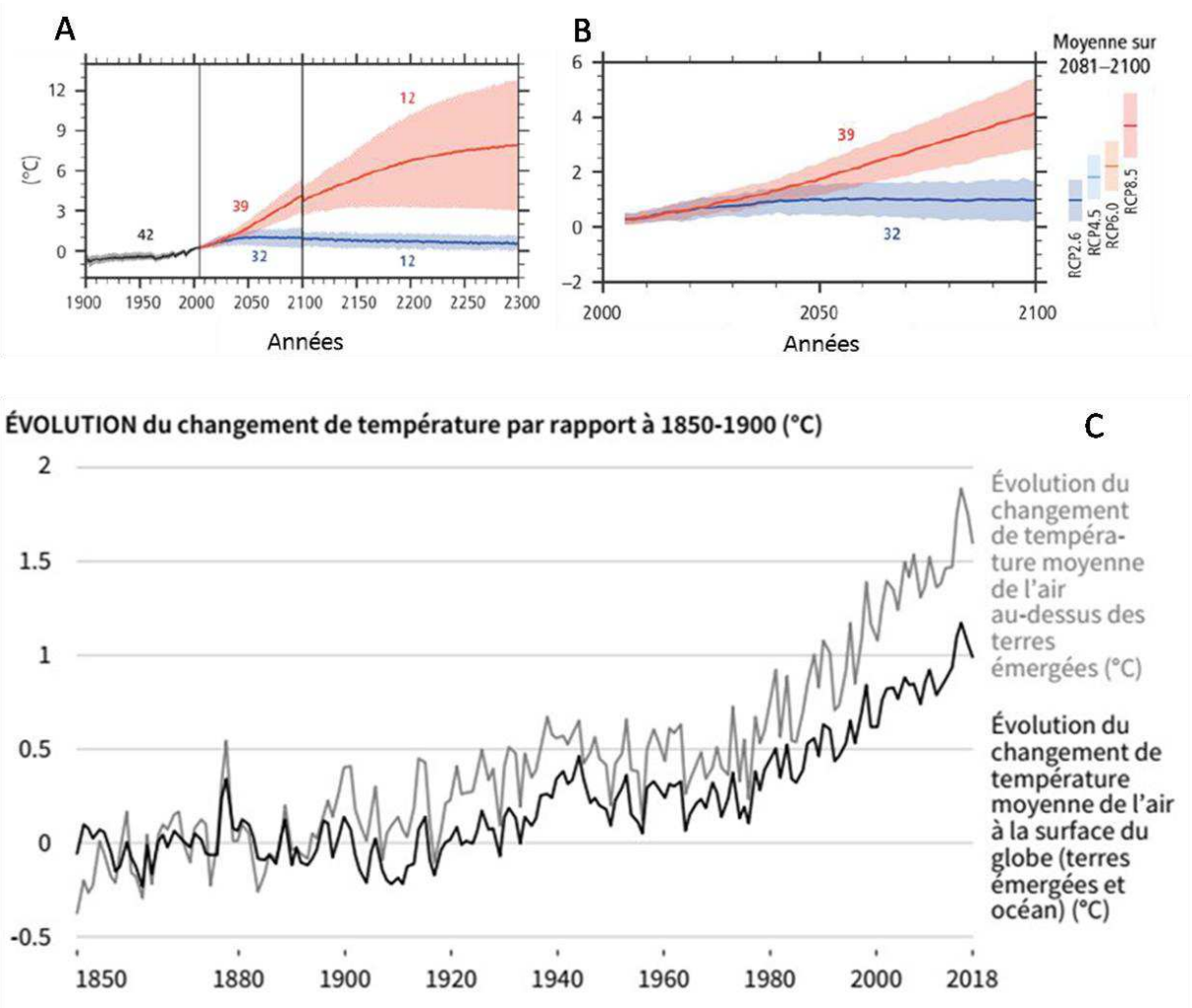


Figure 4 : *Évolution de la température moyenne de la surface de la Terre observée et prévue. (A) Séries chronologiques des changements annuels globaux de la température moyenne de surface pour la période 1900-2300 pour les scenarii RCP2.6 (en bleu) et RCP8.5 (en rouge). (B) Zoom sur la température moyenne de surface entre 2006 et 2100, déterminée par des simulations multi-modèles. (C) Données observées des températures moyennes à la surface du globe et des températures moyennes au dessus des terres émergées. (A et B : Pachauri et al., 2014 ; C : Arneth et al., 2019).*

Les modifications environnementales résultant des changements climatiques peuvent avoir des effets en cascade sur les écosystèmes et les communautés (Jeppesen et al., 2010; Ohlberger, 2013). La hausse des températures, par des effets directs ou indirects, peut mener à l'extinction de populations et au déclin de la biodiversité (Parmesan et al., 1999; Wilson et al., 2005; Pounds et al., 2006; Pacifici et al., 2018; Urban, 2018). De manière directe, le réchauffement climatique peut augmenter les températures environnementales jusqu'à ce qu'elles excèdent le seuil de tolérance

thermique de nombreux organismes (Sinervo et al., 2010). Cette augmentation des températures seraient ainsi susceptible de causer la mort des individus par surchauffe ou de modifier leurs périodes d'activité. Par exemple, dans les milieux désertiques chauds, des vagues de chaleur ont entrainé une forte mortalité aviaire (McKechnie and Wolf, 2010). En restant cachés dans des abris plus froids, les individus limiteraient leur risque de surchauffe mais cela diminuerai le temps et l'énergie alloués à des activités essentielles comme l'alimentation, menaçant leurs fonctions physiologiques de base. Ces effets de la température peuvent être particulièrement importants chez les ectothermes dont le métabolisme est accéléré sous des températures élevées (*cf. section 1.2.2*). En effet, un métabolisme accéléré augmente les besoins en nourriture et donc la vulnérabilité à la famine (Dillon et al., 2010). Lorsque chez des individus d'une espèce les fonctions physiologiques de base sont compromises, cela peut impacter l'ensemble de la population (Sinervo et al., 2010). En milieu océanique, par exemple, une vague de chaleur a entrainé une mortalité importante de l'algue *Scytothalia dorycarpa* à sa limite géographique de répartition chaude. La disparition de ces populations marginales d'algues a réduit l'aire de répartition de l'espèce d'environ 100 km^2, soit une perte de 5% de sa répartition mondiale (Smale and Wernberg, 2013). De manière plus indirecte, le changement climatique peut entrainer le déclin des populations par des modifications dans la phénologie de reproduction ou de migration des organismes (McCarty, 2001; Crick, 2004). Chez les oiseaux par exemple, un décalage entre la période de reproduction et l'abondance des proies nécessaires au nourrissage des poussins, dû au réchauffement climatique, à été observé (Buse et al., 1999). Cela peut entrainer une mortalité importante et un échec de la reproduction dans certaines populations. De nombreuses autres espèces quant à elles modifient par déplacement leur aire de répartition, entraînant une modification des communautés d'espèces à l'échelle mondiale (Crick, 2004; Perry et al., 2005; Chen et al., 2011). L'augmentation des températures peut également altérer le développement des individus avec par exemple, chez des ectothermes, une croissance et une maturité plus rapide des individus juvéniles mais avec une taille corporelle adulte qui sera réduite (Invertébrés: Frazier et al., 2006; Brans and Meester, 2018; Poissons: Loisel et al., 2019). De plus, le taux de mortalité lié à une hausse des températures ne permet pas toujours le maintien des populations à long terme, même quand la reproduction reste possible. (Frazier et al., 2006; Santos, 2007; Bestion et al., 2015b).

Pour faire face au changement climatique, les espèces – comme évoqué plus haut – peuvent répondre de deux manières différentes. En raison des multiples impacts que le réchauffement climatique va induire dans leurs environnements, certaines espèces se déplaceront, notamment vers les pôles (Parmesan and Yohe, 2003; Lenoir et al., 2020), mais elles vont aussi, dans le but de

retrouver des conditions thermiques plus favorables, remonter en altitude (Bässler et al., 2013; Pauchard et al., 2016; Freeman et al., 2018; Sinervo et al., 2018). D'autres espèces pourraient quant à elles, rester et persister dans leur environnement, en modifiant leur composition phénotypique permettant aux populations de ce maintenir malgré les variations climatiques qu'elles subissent.

1.1.4. La plasticité phénotypique dans le cadre du changement climatique

La nécessité de comprendre les réponses des espèces face au changement climatique devient une priorité de plus en plus urgente (Du et al., 2013). En effet, les impacts du réchauffement climatique sur la biodiversité sont importants (*cf. section 1.1.3*) et la survie des espèces, animales ou végétales, terrestres ou aquatiques, dépend, notamment à travers la plasticité phénotypique, de réagir rapidement à ces augmentations de température. Actuellement plusieurs études ont montré que certaines populations réagissaient à cette contrainte thermique en modifiant leur physiologie (*e.g.* Canto et al., 2009; Bradshaw and Holzapfel, 2010; Chown et al., 2010; Seebacher et al., 2015; Mitchell et al., 2018a; Bennett et al., 2019; Figure 5). Par exemple, chez les coccinelles à deux points, *Adalia bipunctata*, l'augmentation des températures printanières a fait diminuer le pourcentage d'individus mélaniques dans les populations (de Jong and Brakefield, 1998). Cette coloration sombre est un avantage chez les ectothermes (Clusella Trullas et al., 2007), leur permettant de mieux capter les radiations solaires et ainsi de faire augmenter plus rapidement leur température corporelle dans un contexte de température froide. Cela permet aux individus de pouvoir allouer efficacement leur énergie à d'autres besoins physiologiques comme la reproduction. Dans le cas des coccinelles à deux points, l'augmentation des températures réduit donc l'intérêt de cette coloration mélanique. D'autres espèces vont quant à elles modifier leur phénologie (*e.g.* Roy and Sparks, 2000; Chmielewski and Rötzer, 2001; Cotton, 2003; Badeck et al., 2004; Crick, 2004; Edwards and Richardson, 2004; Charmantier et al., 2008; Richardson et al., 2013; Figure 5). Les études menées sur la reproduction des oiseaux en sont de bons exemples. Chez les oiseaux insectivores, l'abondance des proies au moment de l'éclosion est nécessaire à la survie de la couvée. Avec une augmentation des températures, le pic d'abondance des insectes a lieu plus tôt dans la saison. Les oiseaux qui dépendent de cette ressource alimentaire voient aussi leur reproduction se réaliser plus tôt (Crick et al., 1997; Visser et al., 1998, 2004). Néanmoins, les modifications dans la phénologie des espèces ne s'opéreront pas aux mêmes vitesses, augmentant le risque de décalage temporel dans les interactions trophiques et menaçant la survie des espèces (Thackeray et al., 2010). Enfin, les espèces peuvent aussi répondre au changement climatique et à l'augmentation des températures en modifiant leurs traits d'histoire de vie notamment à travers la dynamique des populations (*e.g.* Deutsch et al., 2008; Le Galliard et al., 2010; Ozgul et al., 2010; Jenouvrier et al., 2018; Figure 5).

Avec l'augmentation des températures, le nombre d'individus dans la population ainsi que dans les différentes classes d'âge peut être modifié (*e.g.* Whitfield et al., 2007; Daufresne et al., 2009; Vindenes et al., 2014; Cunningham et al., 2017; McClelland et al., 2018). Ces modifications de la dynamique de population peuvent être dues, chez les espèces ectothermes par exemple, à des taux de croissances plus rapides mais qui donneront néanmoins des individus plus petit à l'âge adulte avec un taux de survie réduit (Vindenes et al., 2014; Bestion et al., 2015a; Loisel et al., 2019).

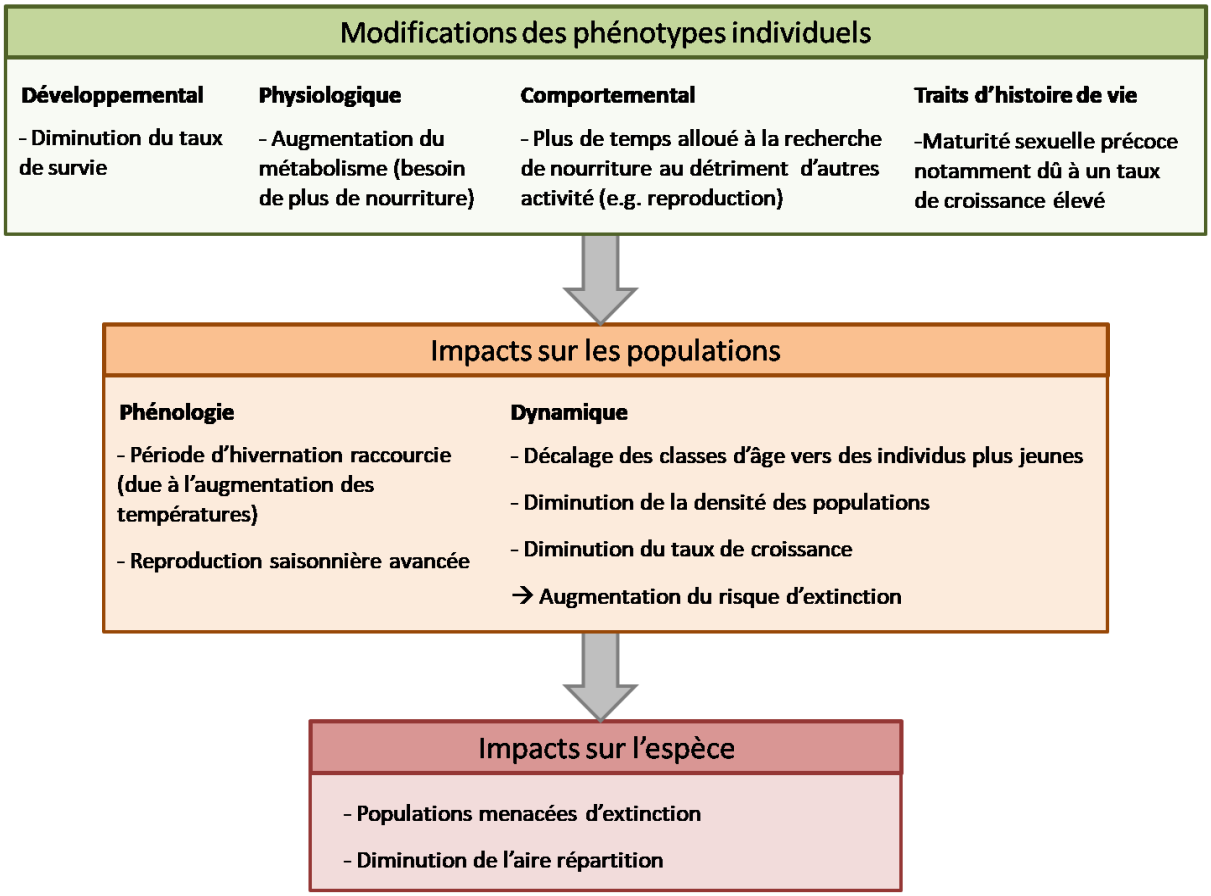


***Figure 5** : Exemples de modifications phénotypiques des individus en réponse au changement climatique entrainant des changements à l'échelle populationnelle et à l'échelle des espèces* (Parmesan, 2006; Charmantier et al., 2008; Bestion et al., 2015a; Sinervo et al., 2018).

En conclusion, la plasticité phénotypique pourrait être particulièrement importante pour permettre aux organismes de faire face à l'évolution rapide des conditions environnementales (Ghalambor et al., 2007; Gienapp et al., 2008; Nicotra et al., 2010). Dans un premier temps la plasticité phénotypique pourrait conférer une plus grande tolérance aux changements des conditions thermiques (Ghalambor et al., 2007; Donelson et al., 2018). Dans un second temps, la sélection naturelle pourrait favoriser le maintien des génotypes qui produiront les phénotypes les plus adaptés aux nouvelles conditions climatiques (Hoffmann and Sgrò, 2011). Bien qu'il soit largement accepté

qu'une forte diversité génétique au sein d'une population augmente les chances de s'adapter à un nouvel environnement (Jump et al., 2009), certains auteurs suggèrent néanmoins que le potentiel d'adaptation génétique, à travers la sélection naturelle, pourrait être limité par le changement climatique à venir (Gienapp et al., 2008; Merilä, 2012). Dans le contexte du changement climatique, les espèces ectothermes, capables de réagir efficacement aux modifications spatiales et temporelles de l'environnement (Angilletta et al., 2002), pourraient être, dû à la forte sensibilité thermique qu'inclut la stratégie ectothermique, plus vulnérables à l'augmentation des températures (Brans and Meester, 2018).

1.2. Les dépendances thermiques chez les ectothermes

1.2.1. Quelques généralités sur l'ectothermie

Sur l'ensemble des espèces animales connues, les ectothermes - c'est-à-dire toutes les espèces animales à l'exception des oiseaux et des mammifères - représentent environ 99% de la diversité (Wilson, 1992; Atkinson and Sibly, 1997). Les ectothermes ont un métabolisme qui, contrairement à celui des endothermes, ne produit qu'une quantité négligeable de chaleur, ce qui ne leur permet pas de s'affranchir de la température de l'environnement dans lequel ils se trouvent. Certaines espèces ectothermes font office d'exception et sont capables de produire de manière significative de la chaleur métabolique. C'est le cas, par exemple, de certains insectes volants (Roberts and Harrison, 1999), de quelques pythons (Bartholomew, 1982) ou encore de certains grands « poissons » comme le Thon rouge (Kitagawa et al., 2001). L'ectothermie permet de diminuer les coûts de maintenance de l'organisme en réduisant drastiquement la régulation physiologique, une part importante de la dépense énergétique des individus (Pough, 1980; Nagy, 2005). En effet, pour une même masse corporelle, le taux métabolique de base d'une espèce ectotherme sera 10 à 20 fois inférieur à celui d'une espèce endotherme (Figure 6; Nagy, 2005). Cette stratégie leur permet d'allouer une grande partie de leur budget énergétique à d'autres fonctions comme la croissance et la reproduction.

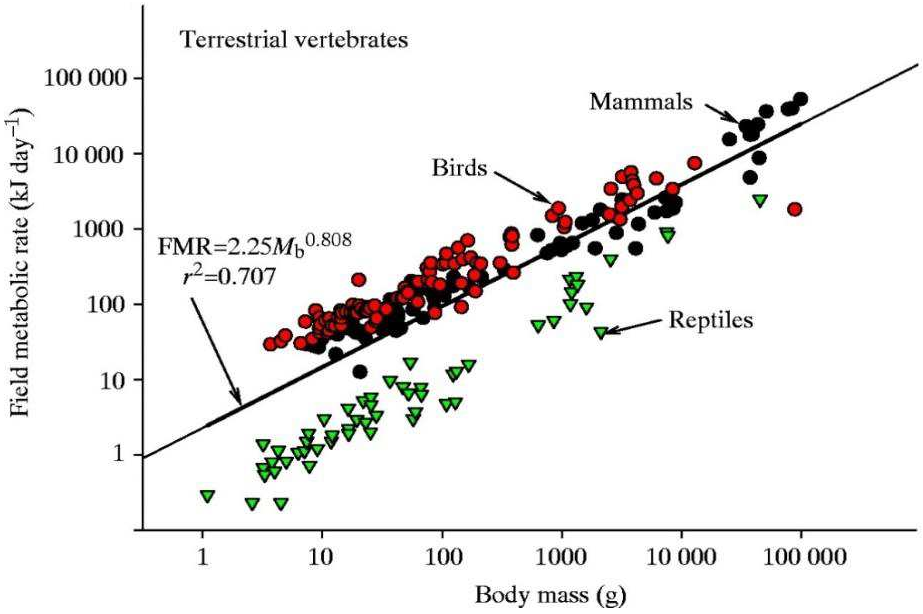


***Figure 6** : Dépense énergétique journalière chez 229 espèces de Vertébrés terrestres en fonction de leur masse corporelle. Les cercles représentent les espèces endothermes, en rouge les Oiseaux et en noir les Mammifères. Les triangles représentent les espèces ectothermes, en vert les reptiles (Nagy, 2005).*

Les organismes ectothermes peuvent être classés en deux groupes, les homéothermes et les poïkilothermes. Les ectothermes homéothermes, qui vivent principalement dans des milieux où la température est constante dans le temps, auront une température corporelle constante proche de celle de leurs milieux. A l'inverse, les ectothermes poïkilothermes, vivant dans des milieux dont la température varie, subissent des variations importantes de leur température corporelle. Cependant, la plupart des ectothermes poïkilothermes peuvent, par des ajustements comportementaux dit de thermorégulation, maintenir des températures corporelles stables malgré des variations thermiques de leur environnement (Heinrich, 1974; Beitinger and Fitzpatrick, 1979; Brattstrom, 1979; Huey, 1982; Danks, 2004; Lagerspetz and Vainio, 2006). Par exemple, les organismes vont, en fonction de la température du milieu et de leurs besoins physiologiques, ajuster le temps passé à se chauffer ou le temps passé sous abri pour atteindre leur température corporelle cible (Hertz et al., 1993; Christian et al., 2006; Kearney et al., 2009). Cette introduction n'abordera que les dépendances thermiques chez les reptiles non-aviens.

1.2.2. Les dépendances thermiques chez les reptiles non-aviens

La température corporelle chez les espèces ectothermes est un facteur clé de leur écologie qui va influencer à la fois leur physiologie et leur comportement (Figure 7; Huey and Stevenson, 1979; Angilletta et al., 2002). La vitesse des réactions chimiques, c'est-à-dire la catalyse enzymatique, et par conséquent l'ensemble du métabolisme des individus, augmente avec la température (Licht,

1966; Somero, 2004). La température optimale pour un individu correspond alors à l'optimum thermique de fonctionnement de l'organisme pour une activité donnée (Angilletta et al., 2002). Ainsi, les espèces ectothermes présentent des plages de températures corporelles pour lesquelles leur physiologie et donc leur performance sont optimales. Ces plages de températures sont bornées par une température corporelle critique minimum et une température corporelle critique maximum (Figure 7), au-delà de ces températures, l'organisme ne peut plus fonctionner (Angilletta et al., 2002).

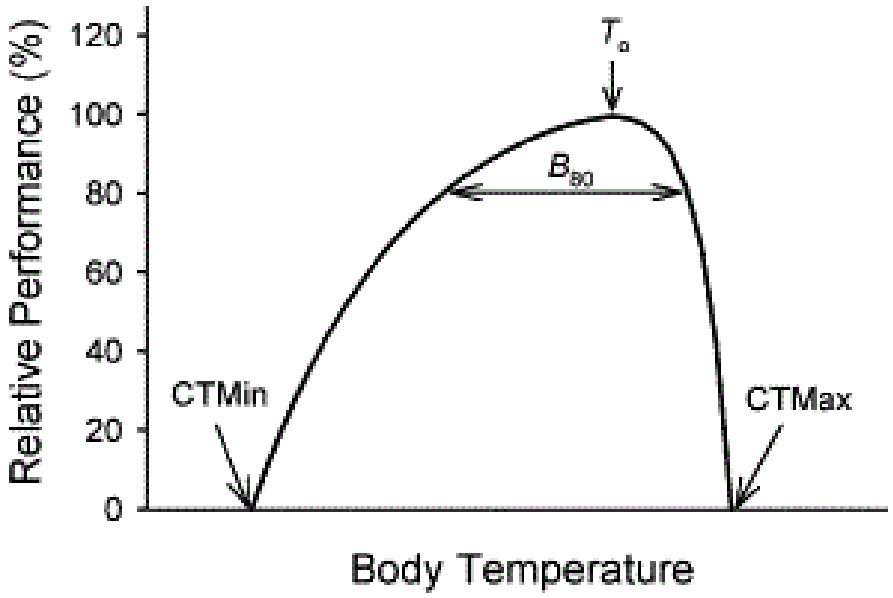


***Figure 7** : Relation schématique entre la température corporelle d'un individu ectotherme et ses performances. La température optimale (T_O) est la température pour laquelle la performance est maximale. La gamme de performance à 80% (B80) est l'intervalle de températures pour lesquelles la performance est supérieure à 80% du maximum. Le seuil critique minimum (CT_{Min}) et le seuil critique maximum (CT_{Max}) correspondent aux températures corporelles minimale et maximale entre lesquelles la performance est possible (Angilletta et al. 2002).*

La variation de réponse face à des modifications de températures a été mesurée chez de nombreuses espèces et sur un grand nombre de traits individuels tels que les réactions enzymatiques (Seebacher et al., 2003; Somero, 2004), le taux métabolique (Beyer and Spotila, 1994; Dorcas et al., 2004; Gangloff et al., 2016), la locomotion (Bennett, 1980; Braña and Ji, 2000; Lailvaux, 2007), la digestion (Hopkins et al., 2004; Bestion et al., 2017), la reproduction (Schwarzkopf and Shine, 1991), ou encore la croissance (Dutton et al., 1975). Chez les ectothermes, la taille des individus, sous une contrainte thermique, est décrite par la règle taille-température (TSR, *Temperature Size Rule* en anglais; Atkinson, 1994; Atkinson and Sibly, 1997; Angilletta and Dunham, 2003; Hessen et al., 2013; Horne et al., 2015; Walczyńska et al., 2016, 2017; Osmond et al., 2017). Cette règle, qui ne tient pas compte de l'association de la température à d'autres paramètres abiotiques, stipule que la taille corporelle des adultes est réduite avec l'augmentation de la température (Atkinson, 1994). En effet, les

individus d'une population soumise à des températures plus chaudes que les conditions optimales de l'espèce grandiront plus rapidement mais atteindront une taille adulte plus petite (Atkinson, 1994; Angilletta and Dunham, 2003). Ces individus, ayant atteint leur maturité plus rapidement, auront néanmoins un taux de survie plus faible, entrainant à une échelle populationnelle, une diminution du nombre d'individus au sein de la population (Huey and Kingsolver, 1989; Bestion et al., 2015a).

1.2.3. Les dépendances thermiques chez les embryons de Reptiles non-aviens

Chez les espèces ectothermes, les embryons présentent une sensibilité thermique marquée (Bull, 1980; Deeming and Ferguson, 1991; Shine and Harlow, 1993; Andrews et al., 2000; Brichard, 2004; Lourdais et al., 2004). La température d'incubation chez les espèces ovipares ou de gestation chez les espèces vivipares va influencer la durée du développement embryonnaire. En effet, chez les reptiles, une baisse de la température d'incubation de 10°C multiplie par deux ou trois la durée du développement (Shine, 2004; Noble et al., 2018). La température va aussi influencer le succès de l'éclosion. Par exemple, chez les serpents ratiers, où les températures optimales de développement sont comprises entre de 24°C à 30°C, un écart de 2°C de cette gamme de température entraine une diminution du succès d'éclosion de 30% (Du and Ji, 2008). Chez certaines espèces de crocodiles, chez la plupart des espèces de tortues et chez quelques espèces de lézards, la température est un facteur déterminant le sexe des nouveaux nés (Janzen and Paukstis, 1991; Sarre et al., 2004; Shine, 2004). Chez les Alligators et les Caïmans, une température d'incubation inférieure à 31°C produit uniquement des individus femelles tandis qu'une incubation à des températures supérieures à 33°C ne produit que des individus mâles (Ferguson and Joanen, 1982; Iungman and Piña, 2013). Néanmoins, à partir d'un certain stade embryonnaire, le sexe de l'individu sera fixé malgré des variations possibles de la température (*i.e.* plasticité phénotypique irreversible; Janzen and Paukstis, 1991; Sarre et al., 2004). Les conditions de température durant le développement peuvent avoir d'autre effets sur le phénotype des juvéniles, comme sur la taille corporelle, les taux de croissance, les performances locomotrices ou les préférences thermiques (Deeming and Ferguson, 1991; Shine et al., 1997; Wapstra, 2000; Blouin-Demers et al., 2004; Booth, 2006; Watkins and Vraspir, 2006; Warner, 2014). Néanmoins, des températures élevées peuvent imposer des contraintes de croissance qui ne seront visibles que tardivement dans la vie de l'individu (Angilletta and Dunham, 2003), comme par exemple, selon la TSR (*cf. section 1.2.2*), une taille adulte réduite.

1.2.4. Les reptiles non-aviens face au changement climatique

L'exposition des espèces ectothermes à de fortes températures contraint le comportement et la performance des individus (Huey, 1982; Angilletta et al., 2010; Huey et al., 2012). Chez les embryons, cette contrainte est d'autant plus importante qu'ils ne peuvent pas ou peu se déplacer pour maintenir des températures optimales par des comportements de thermorégulation (Li et al., 2014; Telemeco et al., 2016; Cordero et al., 2018; Shine and Du, 2018). Bien que la physiologie embryonnaire puisse tolérer une large gamme thermique (Du and Ji, 2008; Du et al., 2010a; Andrews and Schwarzkopf, 2012), l'augmentation de la température, induite par le réchauffement climatique, aura potentiellement un impact négatif sur le développement embryonnaire (Andrews and Schwarzkopf, 2012; Mitchell et al., 2018b). Certaines études prédisent qu'avec ces augmentations de températures les individus seront de plus en plus petits (Daufresne et al., 2009; Gardner et al., 2011; Sheridan and Bickford, 2011) et que le sexe ratio des nouveaux nés sera modifié (Cunningham et al., 2017). Ces changements (*i.e.* taille des individus et sexe ratio) auront pour conséquences des changements dans la structure et la dynamique des populations d'espèces ectothermes (Daufresne et al., 2009; Le Galliard et al., 2010; Cunningham et al., 2017). Cela va également, par l'augmentation de la croissance juvéniles et une diminution du taux de survie à l'âge à l'adulte, affecter la démographie des populations, quand elles n'entraineront pas leur extinction (Whitfield et al., 2007; Sinervo et al., 2010; Bestion et al., 2015a). Chez les individus adultes d'espèces ectothermes, les augmentations de température à long terme pourront avoir des répercutions majeures sur la composition du microbiote intestinal en diminuant jusqu'à 30% sa diversité spécifique modifiant probablement le régime alimentaire des individus (Bestion et al., 2017). L'augmentation de la température va également modifier les comportements de dispersion des individus (Deutsch et al., 2008; Le Galliard et al., 2010; Bestion et al., 2015b; Pellerin et al., 2019). Certains individus vont développer des phénotypes permettant de s'acclimater aux températures plus élevées, alors que d'autres vont devoir migrer pour trouver des températures plus favorables. Cela pourrait entrainer, au sein d'une espèce, une ségrégation spatiale des phénotypes thermiques, facilitant l'adaptation locale au réchauffement climatique pour les individus non migrateurs (Bestion et al., 2015b). Par conséquent, en réaction au réchauffement climatique, certaines espèces déplacent progressivement leur étendue géographique vers des environnements aux conditions climatiques pour lesquelles elles sont adaptées (Sorte et al., 2010; Wernberg et al., 2011). Ces modifications pourraient entrainer une augmentation de la compétition interspécifique. C'est le cas par exemple du Lézard des murailles, *Podarcis muralis*, qui en remontant en altitude pourrait entrer en compétition pour la ressource alimentaire ou les zones de reproduction avec le Lézard de Bonnal, *Iberolacerta bonnali*, déjà présent en altitude (Pottier, 2012).

1.3. Le concept de la remontée altitudinale

La redistribution des espèces vers les pôles est en train de devenir une réponse biologique importante à la hausse des températures globales dans les écosystèmes marins et terrestres (Parmesan and Yohe, 2003; Chen et al., 2011; Sunday et al., 2012). Dans les régions montagneuses, la distance physique nécessaire pour suivre la remontée des enveloppes climatiques est considérablement réduite par rapport à celle nécessaire au suivi de la remontée vers les pôles (Loarie et al., 2009; Chen et al., 2011). Cela peut réduire les effets du changement climatique dans ces régions montagneuses (Loarie et al., 2009) et favoriser une migration rapide des organismes le long du gradient d'altitude lorsque le climat se réchauffe (Lenoir et al., 2008; Bertrand et al., 2011; Freeman et al., 2018). Actuellement, de nombreuses espèces migrent le long du gradient altitudinal de zones montagneuses (Walther et al., 2002; Parmesan and Yohe, 2003; Hickling et al., 2006; Lenoir et al., 2008; Chen et al., 2011; Pottier, 2012; Bässler et al., 2013; Pauchard et al., 2016; Freeman et al., 2018; Bani et al., 2019). En Europe, il a été constaté au sommet de plusieurs massifs montagneux que le réchauffement climatique a permis une augmentation de la richesse en espèces végétales, avec un enrichissement cinq fois plus important entre 2007 et 2016 qu'entre 1957 et 1966 (Steinbauer et al., 2018). Tout comme les vallées de basse altitude ont pu servir de refuge à de nombreux organismes au cours des dernières glaciations (Hewitt, 1999; Tzedakis, 2004), les zones d'altitude pourraient jouer un rôle similaire permettant aux espèces de faire face au réchauffement climatique (Sinervo et al., 2018). Ceci serait d'autant plus vrai chez les espèces ectothermes qui subissent davantage les fluctuations climatiques, modifiant leur température corporelle (Angilletta et al., 2002; Brans and Meester, 2018; Trochet et al., 2018) et affectant leurs performances physiologiques (Angilletta et al., 2010; Huey et al., 2012). Cependant, en remontant en altitude, les organismes seront soumis à de nouvelles contraintes, comme l'augmentation des radiations UV, la diminution des températures environnementales ou de la quantité de dioxygène, pouvant limiter leur chance de colonisation. Par exemple, l'intensité des radiations UV peut entrainer des changements dans la structure de l'ADN des espèces (Li et al., 2018). Ou encore, en altitude la diminution de la pression atmosphérique de l'air entraine une réduction de la pression partielle de chacun de ses gaz, notamment du dioxygène (O_2; représentant 21% du volume de l'air au niveau de la mer), sans modifier leur proportion respective (Millet and Debevec, 2020; Richalet, 2020). Ainsi, à 3000 m d'altitude, pour un volume donné, la quantité de dioxygène est réduite de 30% (*i.e.* équivalent à 15% d'O_2 au niveau de la mer; Figure 8; Bouverot, 2012; Cordero et al., 2017a). Cette diminution de la disponibilité en dioxygène, appelée hypoxie d'altitude, peut avoir des effets aigus et chroniques sur les organismes (Powell and Hopkins, 2010; Storz et al., 2010).

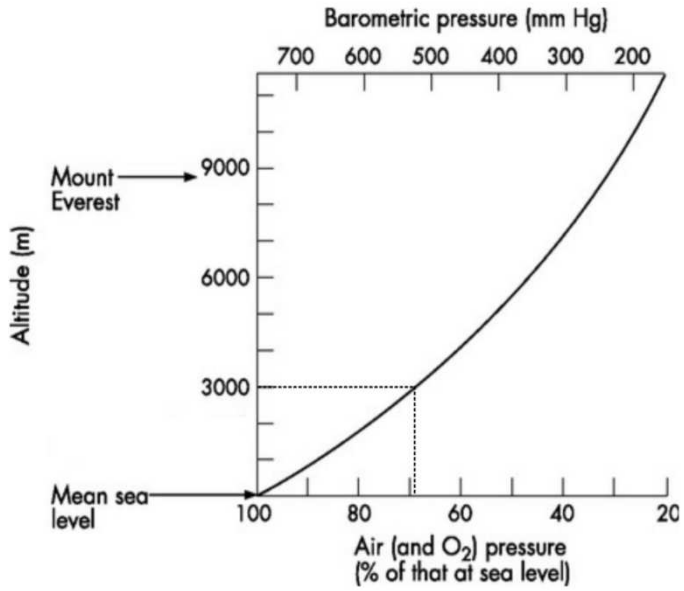


***Figure 8** : Représentation de la disponibilité en dioxygène de l'air en fonction de l'altitude (par rapport au niveau de la mer) et de la pression atmosphérique (d'après Bouverot, 2012).*

1.4. Effets de l'hypoxie chez les Vertébrés

L'hypoxie est une contrainte environnementale forte qui aura des effets chez tous les organismes ayant une respiration aérobie. Le fonctionnement cellulaire d'un organisme aérobie utilise le dioxygène comme fixateur d'électrons pour sa production d'énergie par la mitochondrie (Kelly et al., 2001). Quand un organisme est soumis temporairement à une diminution de la disponibilité du dioxygène, l'hypoxie est considérée comme aiguë. Dans le cas où cette contrainte se prolonge, l'hypoxie est alors considérée comme chronique. Les effets aigus et chroniques, ainsi que leurs coûts associés sur le long terme, varient beaucoup selon les espèces (Monge and Leon-Velarde, 1991; Golan and Huleihel, 2006).

La réponse des organismes endothermes adultes à l'hypoxie d'altitude est largement documentée chez les Oiseaux (Faraci, 1991; Monge and Leon-Velarde, 1991; Scott and Milsom, 2006; Ramirez et al., 2007; Scott, 2011; Lague et al., 2016) et les Mammifères (Monge and Leon-Velarde, 1991; Storz et al., 2004; Ramirez et al., 2007) incluant bien entendu les Humains (Beall et al., 2002; Beall, 2006; Erzurum et al., 2007). Chez l'ensemble de ces espèces, les réponses immédiates liées à l'hypoxie d'altitude comprennent généralement une augmentation du rythme cardiaque (McManus et al., 1974; Faraci, 1991; Peacock, 1998; Scott and Milsom, 2006) et une hyperventilation, qui se traduit par une augmentation du taux de dioxygène dans le sang et, en parallèle, une baisse du taux de

dioxyde de carbone (Faraci, 1991; Monge and Leon-Velarde, 1991; Peacock, 1998; Scott and Milsom, 2006). L'hypoxie chronique entraine chez les endothermes adultes une réduction du rythme cardiaque (Monge and Leon-Velarde, 1991; Peacock, 1998) et des taux métaboliques (Ramirez et al., 2007; Lague et al., 2016). Les organismes présentent également une augmentation de la taille du cœur (Faraci, 1991; Monge and Leon-Velarde, 1991) et de la concentration de l'hémoglobine dans le sang (Faraci, 1991; Peacock, 1998; Scott and Milsom, 2006; Scott, 2011).

La réponse des organismes ectothermes à l'hypoxie d'altitude est, à l'inverse des endothermes, moins documentée. Il est néanmoins notable que l'on peut trouver chez les Arthropodes un éventail remarquable d'adaptations à l'hypoxie en fonction de la variabilité des habitats où ils se retrouvent. Cela concerne par exemple la capacité de passer des voies métaboliques aérobies aux voies anaérobies, la capacité à réduire leurs taux métaboliques ou encore à élargir le volume de leur système trachéal (Hoback and Stanley, 2001). Du fait de l'absence de système respiratoire actif chez ces espèces, la réduction de la disponibilité en dioxygène entraine une réduction de la taille des individus (Peck and Chapelle, 2003) et limite les performances et la fitness (Dahlhoff et al., 2019). Dans le cadre de cette introduction, il ne sera abordé en détail que les effets de l'hypoxie chez les reptiles non-aviens. Chez ces espèces, les études se sont principalement penchées sur le développement embryonnaire liés à l'enfouissement des œufs dans les sites de pontes (Kam, 1993; Crossley and Altimiras, 2005; Iungman and Piña, 2013; Cordero et al., 2017b; Wearing et al., 2017; Williamson et al., 2017) ou encore aux effets sur le métabolisme liés à l'immersion dans l'eau, notamment chez les tortues aquatiques adultes (Altland and Parker, 1955; Boyer, 1963; Jackson, 1973; Gatten, 1987; Stone et al., 1992; Herman and Smatresk, 1999; Baker et al., 2007; Krivoruchko and Storey, 2015). A l'inverse, les effets de l'hypoxie de haute altitude chez les reptiles non-aviens sont bien moins connus et n'ont reçu qu'une attention récente (González-Morales et al., 2015; Lu et al., 2015; Cordero et al., 2017a; Li et al., 2018, 2020; Gangloff et al., 2019; Kouyoumdjian et al., 2019; Parker and Dimkovikj, 2019). Cependant, il est envisageable que les effets de cette hypoxie de haute altitude soient similaires à ceux de l'hypoxie liés à l'enfouissement dans le sol ou à l'immersion dans l'eau.

1.4.1. Effets de la condition hypoxique chez les embryons de reptiles non-aviens

Tout d'abord, il est important de noter que les reptiles non-aviens ovipares ne présentent pas ou peu de soins parentaux post-ponte (While et al., 2015) alors que l'essentiel du développement embryonnaire se produit après la ponte des œufs dans des nids souterrains sans surveillance (Packard et al., 1988; Ackerman and Lott, 2004). Par conséquent, les embryons subissent dans le nid

des fluctuations de température, d'humidité et de concentration en dioxygène (Packard et al., 1988; Deeming and Thompson, 1991; Ackerman and Lott, 2004). Chez ces espèces, même de courtes expositions des embryons à l'hypoxie (*i.e*. hypoxie aiguë) peuvent avoir des effets durables sur la croissance et le développement ultérieurs (Crossley and Altimiras, 2005; Williamson et al., 2017). Chez des embryons d'Alligator d'Amérique, *Alligator mississippiensis*, une exposition à une hypoxie aiguë de 50% (*i.e.* équivalent à 10% d'O_2 au niveau de la mer) engendre une baisse du rythme cardiaque chez les embryons qui s'enchaine une tachycardie une fois les embryons replacés en normoxie (*i.e.* 21% d'O_2 au niveau de la mer; Crossley and Altimiras, 2005). A l'inverse, des embryons du lézard, *Bassiana duperreyi*, placés temporairement en hypoxie à 30% ont accéléré leur rythme cardiaque (Du et al., 2010a). Cette réponse à l'hypoxie aiguë – accélération du rythme cardiaque – a permis aux individus de maintenir des taux métaboliques similaires à ceux des embryons incubés en normoxie, ne modifiant pas la durée d'incubation ainsi que le phénotype à l'éclosion (*e.g.*la taille des individus, la masse cardiaque relative, la vitesse locomotrice, le taux de survie et de croissance juvénile).

Quand les embryons de reptiles non-aviens sont placés en condition d'hypoxie chronique de 30%, 40% ou 50% (respectivement équivalent à environ 15%, 12% et 10% d'O_2 au niveau de la mer), leurs réponses varient en fonction des différents ordres (*i.e.* Crocodiliens, Chéloniens et Squamates; Porteus et al., 2011) ainsi qu'en fonction des différents niveaux d'hypoxie chronique (Annexe 1, Tableau 1). Ces résultats suggèrent la présence de seuils dans la réponse à l'hypoxie chez les embryons de reptiles non-aviens.

Dans le cas d'une incubation dans une condition hypoxique chronique de 30% (*i.e.* équivalent à 15% d'O_2 au niveau de la mer), une des réponses phénotypiques généralement observée est une diminution des taux métaboliques embryonnaires (Figure 9), notamment à travers une réduction de la fréquence cardiaque (Cordero et al., 2017a; Kouyoumdjian et al., 2019) et de la consommation de dioxygène (Cordero et al., 2017a). Cependant, dans certains cas, comme chez les alligators, une hypoxie chronique à 30%, n'affecte pas la fréquence cardiaque des embryons (Crossley and Altimiras, 2005) alors qu'elle l'accélère chez certains lézards (Du et al., 2010a). Chez les alligators, cette condition hypoxique entraine également une diminution de la pression artérielle (Crossley and Altimiras, 2005). Au niveau du développement embryonnaire (Figure 9), des études ont montré que ni la masse embryonnaire, ni la durée d'incubation, ni le succès d'éclosion n'étaient affecté par une hypoxie chronique de 30% (Crossley and Altimiras, 2005; Du et al., 2010a; Iungman and Piña, 2013; Kouyoumdjian et al., 2019; Li et al., 2020). Cependant, une étude à montré une légère augmentation

de la durée d'incubation chez le Lézard des murailles, *Podarcis muralis* (Kouyoumdjian et al., 2019). Enfin, à l'éclosion (Figure 9), la taille corporelle des individus est réduite chez les lézards (Du et al., 2010a; Cordero et al., 2017a; Kouyoumdjian et al., 2019; Li et al., 2020) mais pas chez les alligators (Owerkowicz et al., 2009; Iungman and Piña, 2013). Dans le cas de certains lézards, une augmentation du volume du système cardiovasculaire a été mesurée (Du et al., 2010a; Cordero et al., 2017a). Des études ont également montré, chez des lézards, que la condition hypoxique chronique de 30% subit durant l'incubation n'affectait pas les taux de croissance et de survie juvénile (Du et al., 2010a) et n'affectait pas non plus les performances locomotrices (Du et al., 2010a; Li et al., 2020).

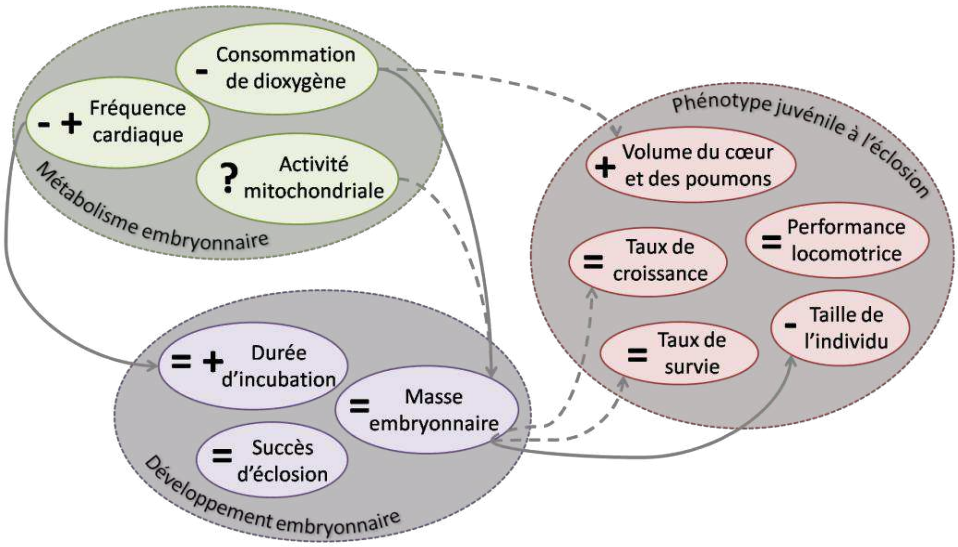


Figure 9 *: Représentation simplifiée des effets d'une hypoxie chronique de 30% durant l'incubation chez des Squamates (i.e. équivalent à 15% d'O_2 au niveau de la mer) sur le métabolisme embryonnaire et sur le phénotype juvénile à l'éclosion. Les flèches en trait plein représentent des causalités certaines et les flèches en pointillés représentent des causalités possibles mais non mesurées.*

1.4.2. Effets de la condition hypoxique chez les adultes de reptiles non-aviens

Chez les adultes des reptiles non-aviens, comme pour les embryons, les réponses à l'hypoxie sont diverses (Porteus et al., 2011; Annexe 1, Tableau 2). Dans le cas des Squamates, placés en condition d'hypoxie aiguë de 30%, les réponses connues du métabolisme sont l'augmentation de la fréquence cardiaque (Pough, 1973; Gratz, 1979) ainsi qu'une diminution de la fréquence respiratoire (*i.e.* ventilation pulmonaire; Pough, 1973; Gratz, 1979) qui s'accompagnent d'une augmentation du dioxygène consommé (Gratz, 1979). Par exemple, chez le serpent aquatique *Nerodia rhombifer*, la consommation de dioxygène et la ventilation augmente également de manière significative après de longues phases d'apnée (*i.e.* 10 à 15 min; Gratz, 1979). A l'inverse, chez un autre serpent aquatique, *Acrochordus javanicus*, dont les phases de repos diurnes se font en apnée au fond de l'eau, la

fréquence de respiration est plus basse comparée à celle mesurée durant les phases de nage et le rythme cardiaque plus important (Pough, 1973). Cette condition hypoxique aiguë entraine également, chez des lézards, une augmentation de la concentration d'hémoglobine et une augmentation du nombre d'hématocrites dans le sang (Kouyoumdjian et al., 2019).

Dans le cas d'une condition hypoxique chronique de 30%, les Squamates répondent par différents changements métaboliques (Figure 10). Comme durant une hypoxie aiguë de 30%, la fréquence cardiaque des individus augmentent (Boyer, 1966). Cependant, la fréquence respiratoire augmente mais pas la consommation de dioxygène (Boyer, 1966; Bartlett and Birchard, 1983). Chez certaines espèces, la composition sanguine est aussi modifiée par cette condition hypoxique avec une augmentation du nombre d'hématocrites (Vinegar and Hillyard, 1972; Weathers and White, 1972), de la concentration en hémoglobine (Megía-Palma et al., 2020) ainsi que de l'affinité des globules rouges (Vinegar and Hillyard, 1972) l'ensemble favorisant le transport du dioxygène dans l'organisme. Néanmoins, chez d'autres espèces, cette condition hypoxique n'influence pas ces paramètres (Weathers and White, 1972; Gangloff et al., 2019). L'ensemble des modifications métaboliques en réponse à une hypoxie chronique de 30% chez les Squamates modifie également de manière négative la condition corporelle des individus (Gangloff et al., 2019; Megía-Palma et al., 2020) ainsi que leurs performances locomotrices (Gangloff et al., 2019).

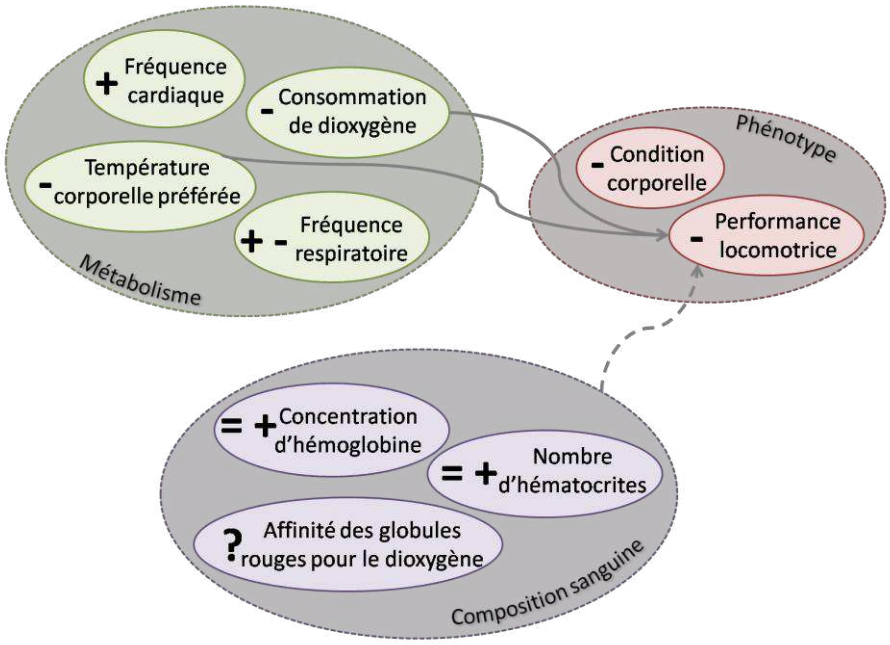


***Figure 10** : Représentation simplifiée des effets d'une hypoxie chronique de 30% (i.e. équivalent à 15% d'O_2 au niveau de la mer) durant la vie adulte de Squamates sur le métabolisme, la composition sanguine et le phénotype. Les flèches en trait plein représentent des causalités certaines et les flèches en pointillés représentent des causalités possibles mais non mesurées.*

1.4.3. Effets de la température en condition d'hypoxie chez les reptiles non-aviens

Plusieurs espèces de reptiles sont déjà adaptées à la vie à des altitudes extrêmement élevées (*i.e. les Lézards du genre Liolaemus*: Marquet et al., 1989; le Lézards des palissades, *Sceloporus occidentalis* et le Lézard de Sagebrush, *Sceloporus graciosus*: Adolph, 1990; le Lézard de Bonnal, *Iberolacerta bonnali;* Pottier, 2012; *Quedenfeldtia trachyblepharus:* Bouazza et al., 2016; les serpents du genre *Thermophis* : Li et al., 2018; *Phrynocephalus vlangalii:* Wu et al., 2018). Ces observations témoignent que la colonisation et la vie à très haute altitude sont possibles pour les espèces ectothermes. Néanmoins, si la température venait à augmenter dans les zones de haute altitude (*cf*. *section 1.1.3*), le mécanisme de production énergétique aérobie des tétrapodes ectothermes pourrait être limité par la diminution de la disponibilité en dioxygène (Gangloff and Telemeco, 2018a). Chez les reptiles adultes, par exemple, une exposition à des températures élevées augmente la consommation de dioxygène et affecte la régulation du métabolisme énergétique (Gangloff et al., 2016). Même si dans un premier temps l'augmentation des températures peut augmenter les performances des reptiles (Angilletta et al., 2002), elles peuvent dans un second temps être réduite en hypoxie (Li et al., 2016). Avec l'augmentation de la température et donc du métabolisme (*cf. section 1.2.2*), les systèmes respiratoires et cardiovasculaires des espèces pourraient être dans l'incapacité de fournir les apports en dioxygène suffisants aux tissus pour le maintien du métabolisme (Pörtner, 2002; Jackson, 2007; Pörtner and Knust, 2007; Verberk et al., 2016; Pörtner et al., 2017). Cette théorie de l'Oxygène et des Capacités de Tolérance Thermique Limitée (OCLTT pour *Oxygen and Capacity Limited Thermal Tolerance* en anglais; Figure 11) a été exposée par Pörtner en 2002. Elle stipule qu'à haute température, les faibles niveaux de dioxygène disponibles limitent les taux métaboliques maximums en activité, dont les valeurs convergent alors vers les taux métaboliques au repos (Pörtner et al., 2017). Cette réduction du métabolisme maximum réduit à son tour la tolérance des organismes à l'augmentation des températures. Dans un premier temps, chez les organismes ectothermes, cette inadéquation entre la demande en dioxygène de l'organisme et sa capacité à la fournir réduira les performances des individus mais permettra de maintenir les taux métaboliques basaux. Cependant, à des températures proches des températures critiques maximales des organismes, la réduction des capacités de transport du dioxygène et sa disponibilité pourraient ne pas suffire à maintenir les taux métaboliques basaux (Gangloff and Telemeco, 2018a). Pour réduire ce risque, de nombreuses espèces ectothermes aquatiques et terrestres abaissent par thermorégulation leur température corporelle (Dupré and Wood, 1988; Dupré et al., 1988; Jackson, 2007) ou leur préférence thermique (Megía-Palma et al., 2020) en condition d'hypoxie afin de réduire la demande en dioxygène de l'organisme.

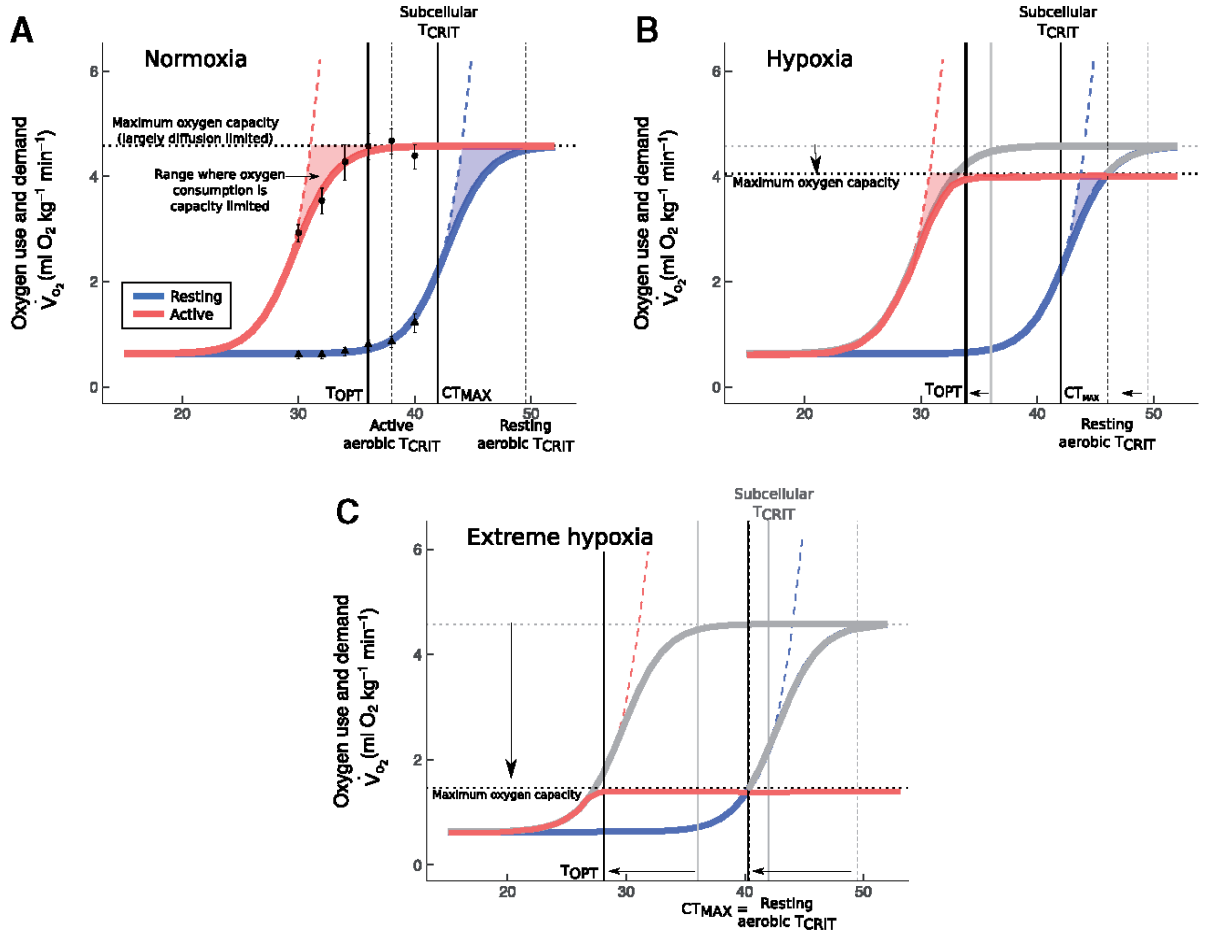


Figure 11 *: Schéma illustrant les relations proposées entre les taux métaboliques au repos et en activité chez les tétrapodes ectothermes en fonction de la température et des niveaux d'oxygène.* T_{OPT}*; Température optimale,* T_{CRIT}*; Température critique aux repos et en activité,* CT_{MAX}*; Température critique maximal. Le graphique (A) affiche ces relations dans une condition normoxique. Le graphique (B) représente ces relations en condition d'hypoxie modérée. Ce niveau d'hypoxie ne devrait avoir aucun effet sur la* CT_{MAX} *ou le métabolisme au repos mais la* T_{CRIT} *en activité sera réduite, ce qui réduira le métabolisme maximal et la* T_{OPT}*. Le graphique (C) représente les relations avec une exposition à une hypoxie extrême. Ce niveau d'hypoxie réduira la* CT_{MAX} *parce que la* T_{CRIT} *au repos est inférieur à la* T_{CRIT} *subcellulaire. La* T_{CRIT} *en activité sera également fortement affectée avec de fortes réductions du métabolisme et de la* T_{OPT}*.*

Chez les embryons de reptiles, le développement embryonnaire est dépendant d'une disponibilité adéquate de dioxygène (Vleck and Hoyt, 1991). L'augmentation des températures accélère la fréquence cardiaque des embryons et augmente leur besoin en dioxygène (Du et al., 2010a; b; Hall and Warner, 2020). Elle augmente aussi la respiration mitochondriale (Sun et al., 2015). De plus, quand les températures d'incubation s'approchent des températures létales, le rythme cardiaque et la production de dioxyde de carbone continuent d'augmenter tandis que la consommation de dioxygène plafonne (Hall and Warner, 2020). Ces informations, en accord avec l'OCLTT, démontrent que les besoins en dioxygène de l'embryon ne peuvent pas être assurés à des températures d'incubation trop élevées. C'est pourquoi, en condition d'hypoxie et de températures élevées, l'

incapacité des embryons à effectuer des comportements de thermorégulation pour diminuer leur température corporelle (Telemeco et al., 2016; Cordero et al., 2018) diminue leur succès d'éclosion (Iungman and Piña, 2013; Flewelling and Parker, 2015; Smith et al., 2015; Hall and Warner, 2020). Ces conditions vont aussi réduire la taille du corps ainsi que les performances physiques des juvéniles à l'éclosion (Iungman and Piña, 2013; Liang et al., 2015). Il est donc concevable que des niveaux de dioxygène faibles limitent les niches thermiques des espèces en limitant le succès du développement embryonnaire (Smith et al., 2015).

Étant donné l'interdépendance des température et la disponibilité en dioxygène sur les systèmes physiologiques, une compréhension des mécanismes physiologiques par lesquels les ectothermes répondent à l'hypoxie à haute altitude est nécessaire pour caractériser la capacité des individus, et par extension des populations, à monter en altitude (Storz et al., 2010).

1.5. Problématique et hypothèses générales

1.5.1. Contexte général

Les travaux de recherche présentés dans ce manuscrit s'inscrivent dans le cadre du réchauffement climatique. En effet, avec l'augmentation des températures prévue par le GIEC *(cf. section 1.1.3*), nous pouvons imaginer, à l'instar de nombreuses autres espèces, que les reptiles non-aviens, vont monter en altitude en suivant l'évolution des enveloppes thermiques (*cf. section 1.3*). Certains individus pourraient présenter une plasticité phénotypique adaptative leur permettant de s'acclimater aux conditions d'hypoxie d'altitude, notamment à travers la capacité à se maintenir puis de se reproduire en haute altitude. En effet, de nombreuses espèces ont déjà rencontré ces conditions à travers les migrations passées dues aux alternances des cycles de glaciations et de réchauffements planétaires qui ont lieu depuis plusieurs milliers d'années. Dans ce cas, les individus ayant une plus grande plasticité phénotypique dirigeront l'extension de l'espèce (Yeh and Price, 2004; Lavergne and Molofsky, 2007; Storz et al., 2010; Molina-Montenegro et al., 2012). En effet, les individus présentant un génotype capable de produire des phénotypes adaptés à cette nouvelle contrainte seront sélectionnés. Actuellement, les modèles prédictifs des futures répartitions des espèces ne tiennent pas compte des contraintes que l'hypoxie pourrait imposer à l'établissement des populations de reptiles non-aviens à haute altitude. En effet, si beaucoup d'études ont exploré les effets de l'hypoxie de haute altitude chez les oiseaux (Black and Snyder, 1980; Faraci, 1991; Monge and Leon-Velarde, 1991; Scott and Milsom, 2006; Ramirez et al., 2007; Scott, 2011; Lague et al.,

2016) ou les mammifères (Monge and Leon-Velarde, 1991; Peacock, 1998; Beall et al., 2002; Storz et al., 2004; Beall, 2006; Erzurum et al., 2007; Ramirez et al., 2007; Storz, 2016), celles concernant les reptiles non-aviens sont récentes et moins nombreuses (Weathers and McGrath, 1972; Lu et al., 2015; Cordero et al., 2017a; Li et al., 2018, 2020; Gangloff et al., 2019; Kouyoumdjian et al., 2019; Plasman et al., 2020). Actuellement, il n'existe que peu de connaissance sur les impacts de l'augmentation des températures à ces altitudes sur le maintien des populations de Vertébrés ectothermes déjà soumis à une réduction de la disponibilité en dioxygène (*cf*. *section 1.4*). Cependant, l'augmentation globale des températures prévue par l'*IPPC* (*cf*. *section 1.1.3*), prévoit également augmenter la hausse des températures dans les milieux de haute altitude. Cela pourrait éventuellement exposer les populations nouvellement établies à haute altitude à la double contrainte d'une faible disponibilité en oxygène associée à des températures environnementale non optimales.

Selon ce contexte actuel des connaissances, plusieurs scénarios sont utilisés pour mesurer l'impact de l'hypoxie d'altitude, en association ou non à une augmentation des températures, sur la Couleuvre vipérine, *Natrix maura* :

- Scénario 1 : Un environnement normoxique (*i.e.* équivalent à 21% d'O_2 au niveau de la mer) avec des températures favorables (*i.e.* 24°C et 28°C), qui correspond, pour des populations de basse altitude, aux conditions actuelles, sans augmentation des températures.
- Scénario 2 : Un environnement normoxique (*i.e.* équivalent à 21% d'O_2 au niveau de la mer) avec une température élevée (*i.e.* 32°C), qui correspond au scénario du réchauffement climatique pour des populations de basse altitude qui ne migrent pas.
- Scénario 3 : Un environnement hypoxique (*i.e.* équivalent à 15% d'O_2 au niveau de la mer) avec des températures favorables (*i.e.* 24°C et 28°C), qui correspond, pour des populations de basse altitude, aux conditions environnementales qu'elles pourront rencontrer en migrant en haute altitude.
- Scénario 4 : Un environnement hypoxique (*i.e.* équivalent à 15% d'O_2 au niveau de la mer) avec une température élevée (*i.e.* 32°C), qui correspond aux conditions environnementales futures en haute altitude, si le changement climatique perdure, et qui pourront être rencontrées par des populations ayant migré et qui se seraient maintenues en haute altitude.

1.5.2. Pourquoi étudier la Couleuvre vipérine

Afin d'essayer d'apporter des éléments de réponse, cette thèse s'intéresse aux effets de l'hypoxie d'altitude aiguë et chronique, associés à l'augmentation des températures sur la physiologie

embryonnaire et les performances physiques juvéniles, chez une espèce de Vertébré ectotherme, la Couleuvre vipérine (*Natrix maura*, Linnaeus, 1758; *cf. section 2.1*). La Couleuvre vipérine est une espèce originaire du Maghreb, où il est possible de la rencontrer jusqu'à 2600 m d'altitude. L'espèce a colonisé tout le bassin Méditerranéen (Pottier, 2016). Toujours en expansion vers le Nord, elle colonise progressivement les Pyrénées (Guicking et al., 2008; *cf*. *section 2.1.1*). La Couleuvre vipérine est une espèce ectotherme poïkilotherme (*cf*. *section 1.2*) avec une mobilité restreinte, ce qui fait d'elle une espèce représentative d'un milieu et de ses conditions environnementales à une échelle précise. En effet, comme beaucoup de reptiles, la Couleuvre vipérine peut être considérée comme un bio indicateur du milieu dans lequel elle se trouve (Hall, 1980; Manolis et al., 2002; Thompson et al., 2008; Brischoux et al., 2009; Marsili et al., 2009; Mullin and Seigel, 2011). De par son écologie (*cf. section 2.1*), la Couleuvre vipérine est, comme de nombreux Vertébrés ectothermes, vulnérable aux variations de température qui vont impacter son cycle biologique (Angilletta et al., 2002; *cf. section 1.2*). D'un autre côté, c'est une espèce largement inféodée aux milieux aquatiques (Vacher and Santos, 2010; Pottier, 2016) où elle y consomme un large éventail d'espèces de poissons (Santos et al., 2000). Son écologie, qui l'oblige à réaliser des apnées de longue durée pourrait probablement permettre à la Couleuvre vipérine de mieux faire face à des conditions hypoxiques que d'autres espèces de Squamates (*cf. section 2.1.2*). Enfin, la Couleuvre vipérine est un serpent, largement répandu en basse altitude dans les Pyrénées. Facile à capturer, cette espèce se maintient aisément en captivité et pond un nombre d'œufs suffisant (Vacher and Santos, 2010) pour réaliser des plans expérimentaux solides (*cf. section 2.3.1*). Grâce à ses caractéristiques écologiques, à son historique de colonisation et à la facilité de travail qu'elle offre, la Couleuvre vipérine est un modèle d'étude adéquat pour comprendre les impacts de l'augmentation des températures en relation avec l'hypoxie d'altitude sur un organisme ectotherme, dont la remontée altitudinale devrait accélérer à la faveur du changement climatique. Notamment dans les montagnes méditerranéennes où les effets de l'augmentation des températures sont particulièrement marqués (Lebourgeois et al., 2012).

1.5.3. Hypothèses générales

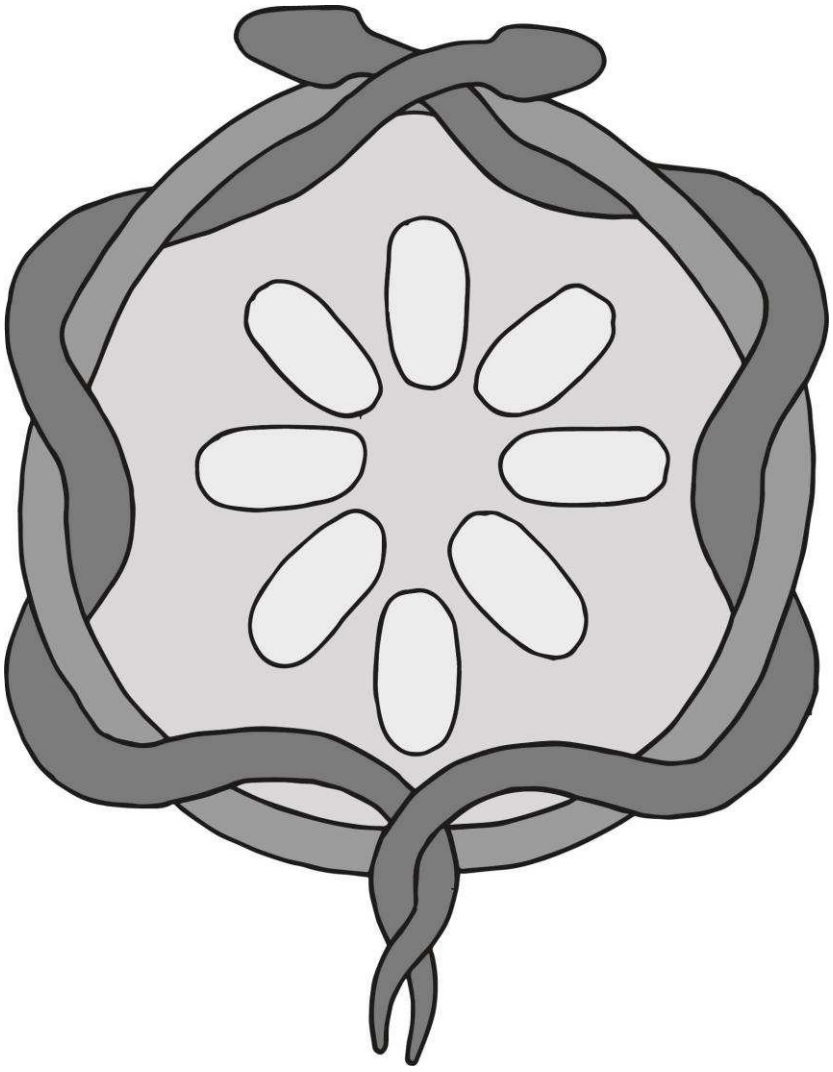
Dans un premier temps, les effets négatifs liés à l'hypoxie d'altitude pourraient être atténués par la plasticité phénotypique des individus, permettant le maintien des populations à haute altitude. A partir des études menées chez les reptiles non-aviens (*cf*. *sections 1.4.1*), il est possible de penser que les embryons de Couleuvre vipérine exposés à une hypoxie chronique de 30% auront un métabolisme réduit, notamment à travers une diminution de la fréquence cardiaque et de la consommation d'oxygène. Ces modifications entraineront probablement des variations développementales des embryons comme une durée d'incubation plus longue mais sans modifier le

succès d'éclosion. Il est également possible de penser que le phénotype à l'éclosion soit modifié avec notamment des juvéniles plus petits. De plus, tant que les températures environnementales resteront optimales, la demande en oxygène du métabolisme ne sera pas augmentée. Dans cette condition et selon le modèle mécaniste de l'*OCLTT* (*cf. section 1.4.3*), les individus devraient maintenir leurs performances locomotrices.

Dans un deuxième temps, l'augmentation des températures devrait accélérer l'ensemble du métabolisme embryonnaire. En condition de normoxie, Cela devrait entrainer, compte tenu des connaissances actuelles (*cf. section 1.2.4*), une augmentation du rythme cardiaque et une diminution de la durée d'incubation. A l'éclosion, les juvéniles devraient être plus petits et leurs performances locomotrices améliorées. Néanmoins, l'augmentation des températures durant l'hypoxie chronique de 30% devrait négativement impacter l'ensemble du développement embryonnaire ainsi que le phénotype juvénile (*cf. section 1.4.3*). Ce contexte devrait réduire fortement la capacité de la Couleuvre vipérine à se maintenir en condition d'hypoxie d'altitude entrainant potentiellement l'extinction des populations.

Chapitre 2.

MODÈLE D'ÉTUDE ET PROCÉDURES EXPÉRIMENTALES



2.1. Modèle d'étude : La Couleuvre vipérine

2.1.1. Répartition et variations géographiques

La Couleuvre vipérine, *Natrix maura* (Linnaeus, 1758), est une espèce circum-méditerranéenne présente en Afrique du Nord où elle peut se retrouver jusqu'à 2600 m d'altitude (Maroc, Algérie, Tunisie), sur toute la péninsule ibérique (Espagne, Portugal), sur les trois quarts méridionaux de la France mais aussi à l'extrême Ouest de la Suisse et l'extrême Nord-Ouest de l'Italie (Pottier, 2016). Cette espèce a colonisé les environnements montagneux des Pyrénées à travers les cycles historiques de réchauffement et de refroidissement (Gómez and Lunt, 2007). La péninsule ibérique ayant servi de refuge glaciaire pendant le Pléistocène lui a permis de recoloniser les Pyrénées et l'Europe occidentale depuis 12 000 ans, pendant l'Holocène (Guicking et al., 2008). Dans les Pyrénées, la Couleuvre vipérine est largement présente sur l'ensemble de la chaine. Néanmoins, comme pour de nombreuses autres espèces de reptiles du massif, sa répartition altitudinale varie fortement en fonction des zones biogéographiques (Pottier, 2016). Dans l'Ouest des Pyrénées, la Couleuvre vipérine a été observée jusqu'à 1000 m d'altitude sur le versant Sud (Gosá and Bergerandi, 1994) et seulement jusqu'à 700 m sur le versant Nord (Bea, 1985). A l'Est de la chaîne, l'espèce est présente jusqu'à 1500 m en Catalogne (Llorente et al., 1995; Santos, 2015) et jusqu'à 1200 m dans les Pyrénées-Orientales (Geniez and Cheylan, 2012). Sur le versant Nord des Pyrénées Centrales, en Ariège par exemple, la Couleuvre vipérine atteint seulement les 1000 m d'altitude (Pottier et al., 2008; Aubret et al., 2015). Néanmoins, de nouveaux modèles écophysiologiques prédictifs suggèrent qu'à l'horizon 2100, l'espèce présenterait un risque modéré de disparition en milieu de plaine, mais pourrait profiter de nouveaux refuges climatiques parmi les plus hauts sommets des Pyrénées. Une région dont elle est actuellement exclue en raison de l'environnement climatique frais et de la courte saison d'activité (Sinervo et al., in prep.). La phylogéographie de l'espèce, basée sur des analyses moléculaires (ADN mitochondrial), est complexe avec quatre lignées évolutives connues (Guicking et al., 2008; Pottier, 2016). Des travaux génétiques récents ont permis le développement de microsatellites spécifiques à la Couleuvre vipérine (ADN nucléaire; Le Chevalier et al., 2019; Annexe 1) afin de travailler plus particulièrement sur la structuration génétique de l'espèce à petite échelle sur des individus de la vallée du Lez et de la vallée de la Bouigane (Ariège, France). Ce sont des individus de ces mêmes populations - qui semblent montrer une structuration génétique forte - qui sont étudiés dans cette thèse. En effet, le long de ces deux rivières, les analyses montrent trois populations génétiques différentes, deux en amont et une en aval avec un mélange génétique de ces trois populations à l'endroit où les deux rivières se rejoignent (Figure 1 ; données non publiées).

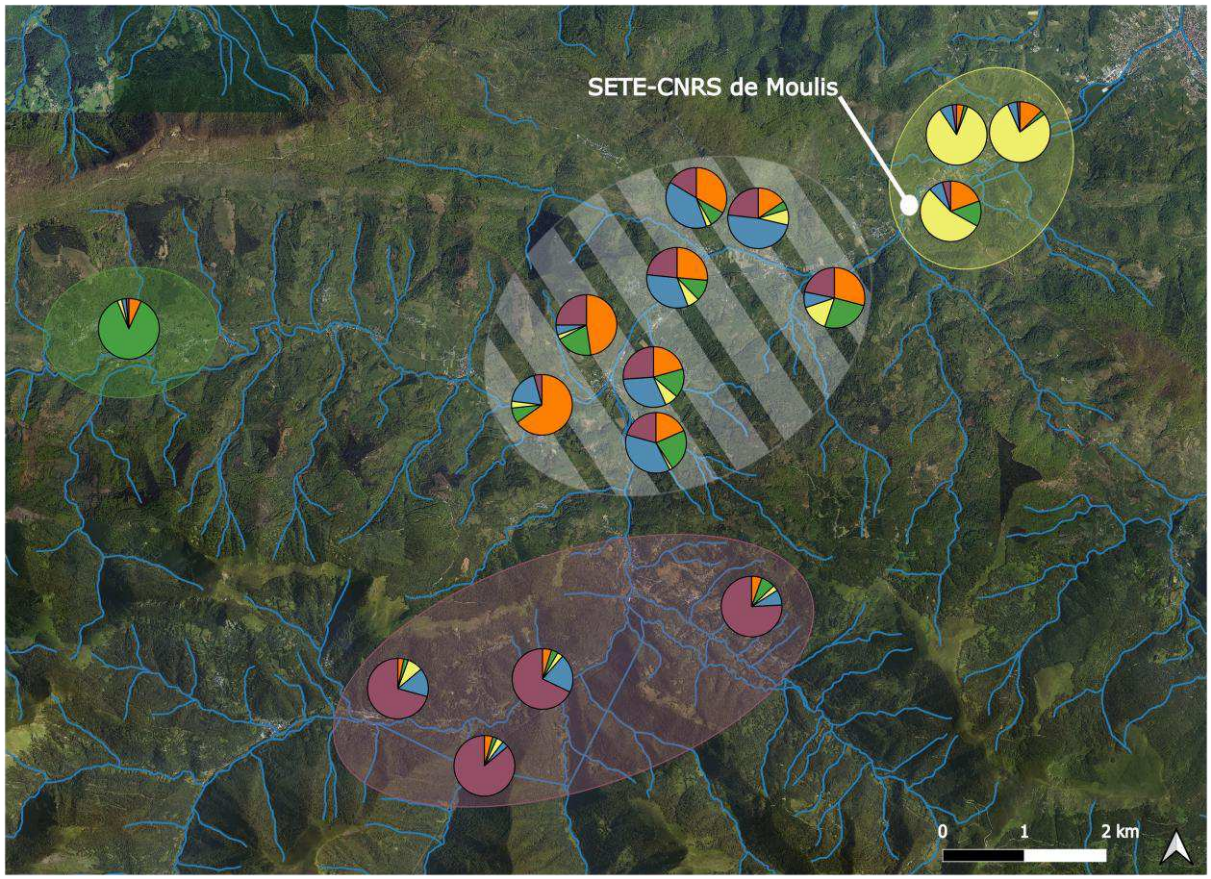


Figure 1 *: Représentations des différentes populations génétiques potentielles de Couleuvre vipérine dans la vallée du Lez et la vallée de la Bouigane.*

2.1.2. Ecologie et biologie générale

La Couleuvre vipérine (Figure 2) est une espèce de l'Ordre des *Squamates*, Sous-Ordre *Serpentes*, et de la Famille des *Natricidés* (De Massary et al., 2019). L'espèce est active autour de mars, dès que la température du gîte d'hivernage atteint les 10°C, et ce jusqu'au mois d'octobre (Vacher and Santos, 2010). Ce serpent ovipare se reproduit du mois d'avril au mois de juin. Les femelles peuvent stocker dans leurs oviductes le sperme de plusieurs mâles (Pottier, 2016). Une fois fécondées, elles se regroupent et pondent une quinzaine d'œufs en moyenne (Hailey and Davies, 1987; Vacher and Santos, 2010) dans des sites de pontes dits communautaires où règnent des conditions environnementales favorables au développement des embryons dont l'incubation dure d'environ 46 à 90 jours (Vacher and Santos, 2010). Néanmoins, la taille de la ponte augmente avec la taille des femelles, échelonnant le nombre d'œufs de 2 à 16 par ponte, voire plus dans de rares cas connus (Vacher and Santos, 2010). C'est une espèce largement inféodée aux milieux aquatiques (Vacher and Santos, 2010; Pottier, 2016) où elle y consomme un large éventail d'espèces de poissons (Santos et al., 2000) et d'amphibiens en fonction des localités et de la disponibilité des habitats (Pottier, 2016). La dispersion aquatique des adultes ou des juvéniles est assez faible avec une capacité de

déplacement estimée entre 500 m et 1000 m par jour (Hailey and Davies, 1987; Boissinot et al., 2013). Les performances de nage et d'apnée de l'espèce sont dépendantes de la température de l'eau (Aubret et al., 2015). La vitesse de nage chez la Couleuvre vipérine augmente avec les températures et est maximum entre 25°C et 30°C (Hailey and Davies, 1986a; Aubret et al., 2015) et diminue au delà de ces températures. De plus il est important de noter que, chez cette espèce, la vitesse de nage est supérieure à la vitesse de déplacement sur terre pour une température donnée (Isaac and Gregory, 2007). Concernant, les temps d'apnée, ils sont maximum à des températures plus basses (*i.e.* 10°C ; Aubret et al., 2015). En effet, comme tous les ectothermes, les besoins en oxygène de la Couleuvre vipérine sont dépendants des taux métaboliques, eux-mêmes dépendant de la température corporelle (Pough, 1980). La capacité d'apnée de l'espèce pourrait donc être un indicateur de ses besoins en oxygène (Stevenson et al., 1985). De plus, des études ont démontré que des serpents semi-aquatiques, comme l'est la Couleuvre vipérine, abaissaient leur rythme cardiaque pendant les phases de plongée (Johansen, 1959; Jacob and McDonald, 1976), diminuant probablement dans un même temps les besoins en oxygène de l'organisme. Ces capacités d'apnée importantes chez la Couleuvre vipérine pourraient être une stratégie de défense anti prédateur (Scribner and Weatherhead, 1995). En effet, cette espèce, ne s'éloigne que peu des milieux aquatiques (Isaac and Gregory, 2007) où elle y prend la fuite lorsqu'elle est menacée (Scribner and Weatherhead, 1995). Sa capacité à maintenir des apnées longues serait alors un avantage considérable pour se cacher le plus longtemps possible d'un prédateur par exemple.

Figure 2 : *Photographies de Couleuvres vipérines. (A gauche) Femelle adulte "morphe bilineata", Moulis (09, France), le 25 juillet 2016. (A droite) Nouveau né en comportement d'intimidation, Moulis (09, France), le 18 septembre 2018.*

2.2. Législation et autorisations

La Couleuvre vipérine est représentée à tous les niveaux de la liste rouge de l'Union Internationale pour la Conservation de la Nature (UICN ou *IUCN* en anglais). Elle dispose du statut LC (Least Concern : Préoccupation mineure) sur la liste rouge mondiale et européenne mais sur la liste rouge française, elle dispose du statut NT (Near Threatened : Quasi menacé). Cependant, sur la liste rouge régionale Occitanie, ce statut se restreint à LC (Least Concern : Préoccupation mineure). La Couleuvre vipérine est une espèce protégée, listée dans l'Annexe 3 de la Convention de Berne, ainsi que dans la Directive 2010/63/EU du parlement européen et du conseil du 22 septembre 2010 relative à la protection des animaux utilisés à des fins scientifiques. Elle est également listée dans l'Article 3 de l'arrêté du 19 novembre 2007 fixant les listes des amphibiens et des reptiles protégés sur l'ensemble du territoire français ainsi que les modalités de leur protection. De ce fait, toute manipulation, capture, transport, maintien en captivité ou destruction de cette espèce sont interdits. Afin de réaliser cette thèse entrainant l'utilisation de cette espèce à des fins scientifiques, les dérogations et formations nécessaires ont été obtenues. Dans un premier temps, un arrêté préfectoral portant autorisation de captures, enlèvements et prélèvements sur des reptiles et amphibiens protégés (n° 2017-s-02 du 30 mars 2017) a été délivré par la Direction Régionale de l'Environnement de l'Aménagement et du Logement (DREAL) de la préfecture d'Ariège, de l'Aude, de la Haute-Garonne, des Hautes-Pyrénées et des Pyrénées Orientales. Dans un deuxième temps, j'ai réalisé une formation réglementaire pour l'utilisation d'animaux de la faune sauvage non-hébergée à fins scientifiques – niveau concepteur (décret n° 2013-118 du 01 Février 2013 et agrément du Ministère chargé de l'Agriculture n° I-75-MNHN-F1-15 du 17 juin 2015) délivrée par le Muséum national d'Histoire naturelle (MNHN), le Centre National de la Recherche Scientifique (CNRS) et l'Office National de la Chasse et la Faune Sauvage (ONCFS). Enfin, j'ai rédigé une demande d'autorisation de projet afin d'obtenir la validation éthique du projet, qui à été obtenue auprès du Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation (n° APAFIS#16359-201808011445465 v4).

2.3. Méthodes utilisées

2.3.1. Maintien en captivité et incubation

Les femelles gestantes de Couleuvres vipérines ont été capturées à la main dans leur milieu naturel lors de de prospection à vue. Cette période de capture s'est étalée de mi juin à début juillet lorqu'il était possible de detecter, par la palpation des organes génitaux femelles, la présence d'œufs en

developpement. Au total, 51 femelles de Couleuvre vipérine réparties sur 3 années (12 en 2016, 17 en 2017 et 22 en 2018) ont été capturées (Figure 3). Les individus ont ensuite été ramenés à la Station d'Ecologie Théorique et Expérimentale de Moulis (42.958394 N, 1.086440 E; 436 m ASL).

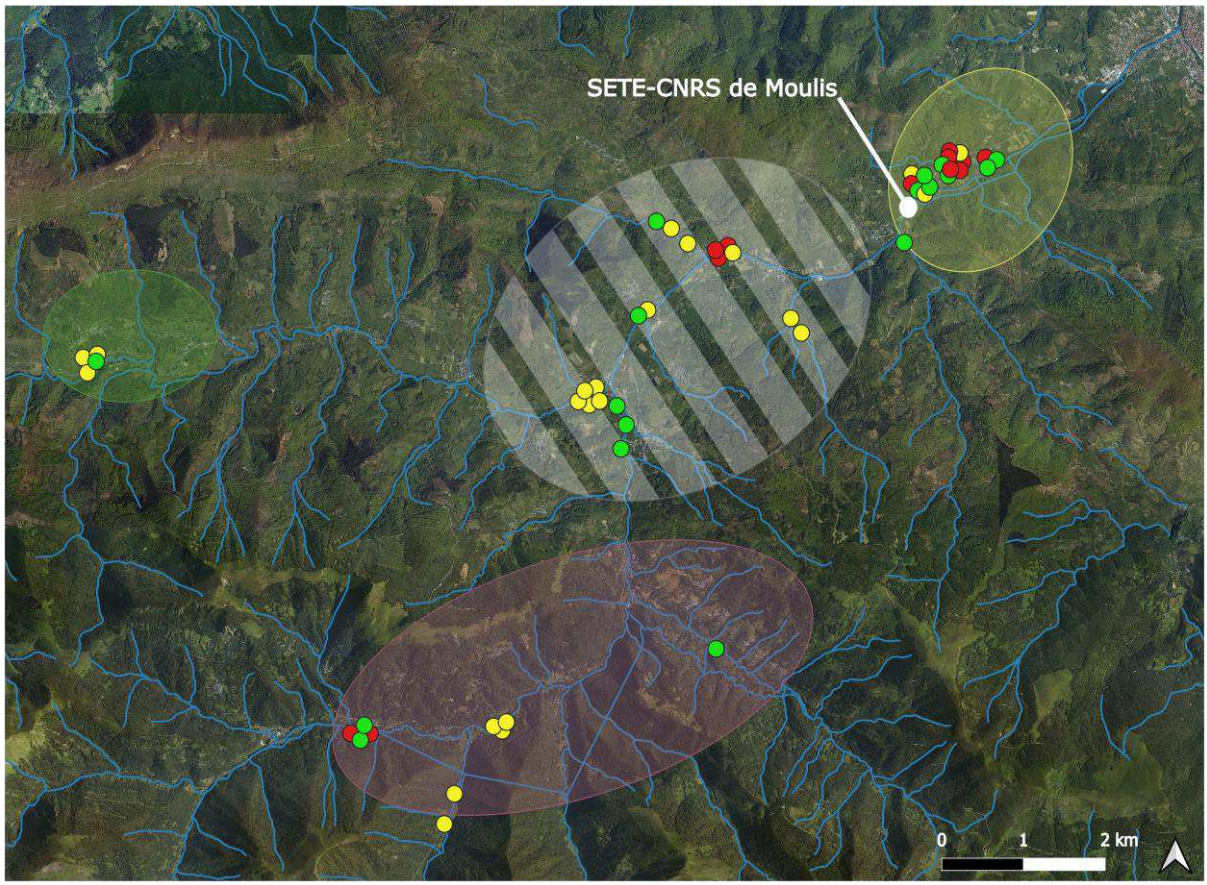


***Figure 3** : Localisation des captures des femelles gestantes de Couleuvre vipérine dans la vallée du Lez et dans la vallée de la Bouigane. 12 individus ont été capturé en 2016 (cercles rouges), 17 individus ont été capturé en 2017 (cercles verts) et 22 individus ont été capturé en 2018 (cercles jaunes). Les ovoides représentent, à titre indicatif, les différentes populations génétiques potentielles (cf. section 2.1.1, Figure 1).*

A l'entrée dans l'élevage, les Couleuvres vipérines capturées ont suivi un traitement sanitaire, où les individus sont essuyés avec un tissu enduit de Frontline© pour éliminer les parasites externes comme les acariens. Le tissu n'est pas passé sur la tête des individus pour éviter toute inhalation, ce qui pourrait entrainer des problèmes neurologiques. Les femelles ont ensuite été placées pendant 72 heures dans des terrariums temporaires individuels (40 x 30 x 8 cm) contenant chacun un substrat en papier absorbant et une cachette en tuile. Les femelles ont été privées d'eau pendant 12 heures afin qu'elles n'évacuent pas le produit appliqué en se baignant. Suite à cette période de restriction d'eau, une coupelle d'eau, suffisamment grande pour qu'elles puissent s'immerger, a été placée dans chaque terrarium temporaire et l'eau était changée 3 à 4 fois par jour. Après ces 72 heures de

traitement, les femelles ont été placées individuellement dans leur terrarium final contenant un substrat en paillage de chanvre sec, une gamelle d'eau *ad libitum*, une cachette en tuile et une boite de ponte (une boite plastique opaque de 10 x 8 x 6 cm avec un couvercle et une entree de 3 x 3 cm sur la façade) contenant un de la vermiculite humide (1 volume d'eau pour 5 volumes de vermiculite, un substrat minéral qui à la capacité de se maintenir humide sur plusieurs jours). La Couleuvre vipérine est une espèce diurne avec une tolérance thermique optimale allant de 22°C à 30°C (Vacher and Santos, 2010). Afin de fournir des conditions proches de celles du milieu naturel, les femelles ont été éclairées de 7 heures à 19 heures par des néons U.V. Le gradient thermique dans le terrarium était maintenu par une lampe chauffante de 42 Watts, fournissant une source de chaleur aux animaux avec un point chaud maximal à 32°C, et une salle climatisée à 20°C.

Les pontes se sont étalées de fin juin à fin juillet et les femelles de Couleuvre vipérine ont en moyenne pondu 11 œufs chacune (± 4,7 œufs). A la ponte, les œufs de chaque femelle ont été mirés, c'est-à-dire que la présence d'un embryon est observée par transparence de la coquille à l'aide d'une source lumineuse. Au total, 574 œufs ont été pondus et seulement 521 étaient viables. Ils ont ensuite été marqués individuellement à l'aide d'un crayon sans encre nocive, pesés (± 0.01 g) et distribués dans les différents traitements dans les 12 heures qui suivent la ponte (Figure 4). Les œufs ont été déposés dans une boite plastique (20 cm x 15 cm x 5 cm) avec un fond de 2 cm de vermiculite humide (1 volume d'eau pour 5 volumes de vermiculite) puis placés dans un incubateur (ExoTerra Model PT-2445, Rolf C. Hagen Inc., USA) aux températures de traitement choisies pour les expériences, c'est-à-dire à 24°C, 28°C ou 32°C ou dans les conditions d'oxygène choisies pour les expériences, c'est-à-dire en normoxie à 436 m ASL : PO_2 ~20.1 kPa ou en hypoxie à 2877 m ASL : PO_2 ~15.3 kPa (Bouverot, 2012; Cordero et al., 2017a). Des bols d'eau placés dans chaque incubateur ont garanti une humidité ambiante de 100% tout au long de l'incubation.

Figure 4 : *(A gauche) Œufs de Couleuvre vipérine fraichement pondus, Moulis (09, France), le 28 juillet 2016. (A droite) Œufs de Couleuvre vipérine individuellement marqués et déposés sur un fond de vermiculite avant d'être placés dans un incubateur, Moulis (09, France), le 19 juillet 2018.*

Chez les reptiles, la durée d'incubation est principalement dépendante de la température (Shine, 2004; Du and Ji, 2008; Noble et al., 2018). Durant cette thèse, l'ensemble des conditions d'incubation à entrainé des durées d'incubation allant de 30 à 72 jours, avec des éclosions s'étalant de début aout à fin septembre (sur les années 2016, 2017 et 2018). Au total, 468 juvéniles sont nés avec un succès d'éclosion moyen de 87,9%, avec un minimum de 68,2% et un maximum de 100%. A l'éclosion (Figure 5), les juvéniles ont été marqués individuellement (*cf. section 2.3.3*) et placés par date d'éclosion (par 6 individus maximum) dans des terrariums (15 cm x 10 cm x 5 cm) contenant un substrat en paillage de chanvre sec, une gamelle d'eau *ad libitum* et une cachette (Figure 4). Les terrariums ont ensuite été placés dans des incubateurs à 20°C. Cette température a été choisie car les juvéniles de Couleuvre vipérine maintenus à cette température ont montré des niveaux élevés de survie et de croissance (données non publiées).

Toutes les femelles ont été ramenées à leur site exact de capture dans les deux semaines qui ont suivi la ponte. Une fois les expériences terminées, les juvéniles de Couleuvre vipérine ont été nourris et relâchés au site de capture maternel (Figure 3).

Figure 5 : *(A gauche) Eclosion en cours du juvénile de Couleuvre vipérine identifié E4, Pic du Midi de Bigorre (65, France), le 10 août 2017. (A droite) Juvéniles de Couleuvre vipérine dans leur terrarium, Pic du Midi de Bigorre (65, France), le 15 août 2017.*

2.3.2. Mesure de la fréquence cardiaque des embryons

La fréquence cardiaque est un bon indicateur du taux métabolique et de la fonction cardiovasculaire chez les amniotes ectothermes (Crossley and Burggren, 2009). La fréquence cardiaque est fortement corrélée au taux de consommation d'oxygène des serpents et des lézards (Greenwald, 1971; Bennett, 1972; Butler et al., 2004; Du et al., 2010a; Kouyoumdjian et al., 2019). Pour mesurer la fréquence cardiaque embryonnaire des Couleuvres vipérines, nous utilisons un moniteur numérique d'œufs Buddy® (MK2, Avitronics, Cornwall, UK ; Figure 6) initialement développé pour mesurer la fréquence cardiaque des embryons d'oiseaux dans l'industrie avicole et ses applications de recherche (Lierz et al., 2006). Cependant, cet appareil s'est avéré tout à fait pertinent et fonctionnel pour mesurer les battements cardiaques des embryons dans les œufs des reptiles non aviens comme les lézards (Du et al., 2009, 2010a; c; Sartori et al., 2015; Cordero et al., 2017a; Kouyoumdjian et al., 2019), les tortues (Du et al., 2009, 2010b; c; McGlashan et al., 2012, 2015; Sartori et al., 2015) ou encore les serpents (Aubret, 2013a; Aubret et al., 2016a; b, 2017). Le système Buddy® projette un faisceau infrarouge sur la surface de l'œuf et détecte les minuscules distorsions de la coquille causées par les battements cardiaques embryonnaires (Du et al., 2009). Néanmoins, en fin d'incubation, il devient plus difficile de mesurer le rythme cardiaque des embryons. En effet, les autres impulsions comme les impulsions musculaires, également sous forme de vibrations externes, génèrent des signaux plus forts que les battements cardiaques de l'embryon ce qui ne permet pas au moniteur Buddy® de mesurer les battements cardiaques simultanés (Lierz et al., 2006).

Au moment des mesures des battements cardiaques, le moniteur Buddy® est placé dans un incubateur réglé à la température du traitement d'incubation des œufs. Puis chaque œuf est délicatement placé sur le coussin du capteur infrarouge pour la lecture de la fréquence cardiaque (une lecture stable est obtenue au bout d'environ 30 secondes ; Figure 6). Les œufs ne sont placés que brièvement (≤ 1 min) dans le moniteur Buddy® pour éviter une augmentation de la température de l'œufs due à l'exposition aux capteurs infrarouges (Sartori et al., 2015; Hulbert et al., 2017).

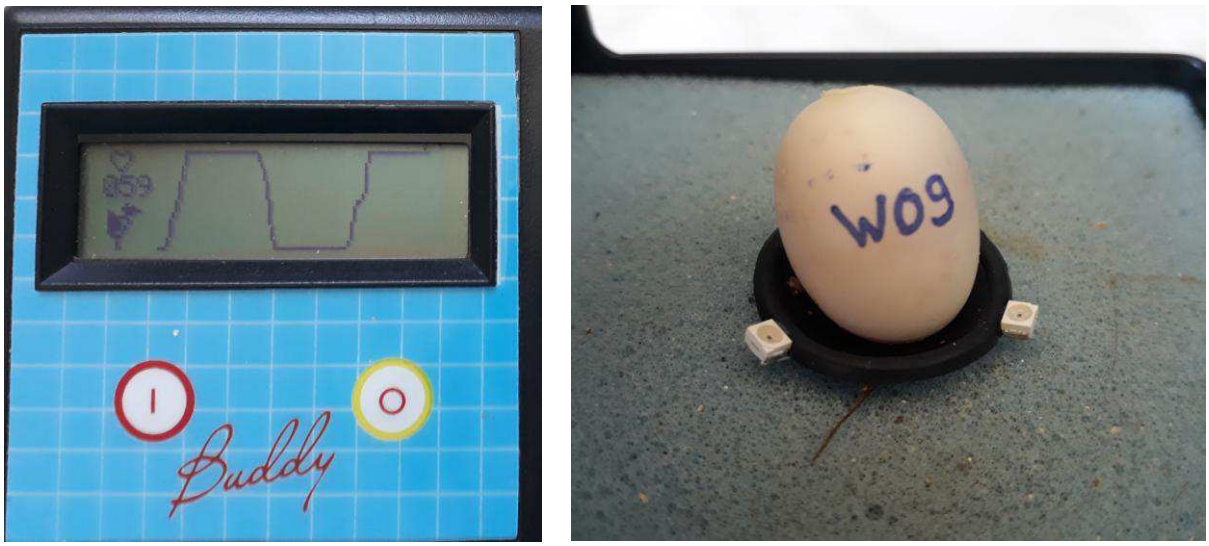


Figure 6 : *(A gauche) Mesure du rythme cardiaque d'un embryon de Couleuvre vipérine sur l'écran du Buddy®, Pic du Midi de Bigorre (65, France), le 28 juillet 2017. (A droite) Œufs de Couleuvre vipérine, identifié W09, posé sur le coussin du capteur infrarouge du Buddy®, Pic du Midi de Bigorre (65, France), le 28 juillet 2017.*

2.3.3. Mesures morphologiques et marquage des juvéniles

Dans les 24 heures suivant l'émergence, plusieurs mesures morphologiques ont été réalisées sur chaque juvénile. Ils ont tout d'abord été pesés à l'aide d'une balance numérique (±0,01 g) puis la longueur du corps (du museau au cloaque), ainsi que la longueur totale (du museau à la pointe de la queue) ont été mesurées à l'aide d'un mètre ruban (±0,1 mm). L'ensemble de ces mesures a été répété après chaque expérimentation. Cela nous permet, en plus de calculer la condition corporelle des individus, de mesurer leur taux de croissance. Toujours à l'éclosion, le reste des réserves lipidiques non utilisées pendant le développement embryonnaire a été pesé à l'aide d'une balance numérique (±0,01 g). Les juvéniles ont également été sexés par éversion des hémipénis. Ils ont ensuite été individuellement marqués à l'aide d'un cautère basse température (Bovie®) selon la technique de marquage à chaud (Figure 7). Cette technique consiste à brûler de façon superficielle l'épiderme de certaines écailles ventrales et latérales du flan gauche de l'individu selon un code

préalablement défini (Figure 7). C'est une méthode non invasive, efficace et semi permanente chez les reptiles même après plusieurs mues (Winne et al., 2006).

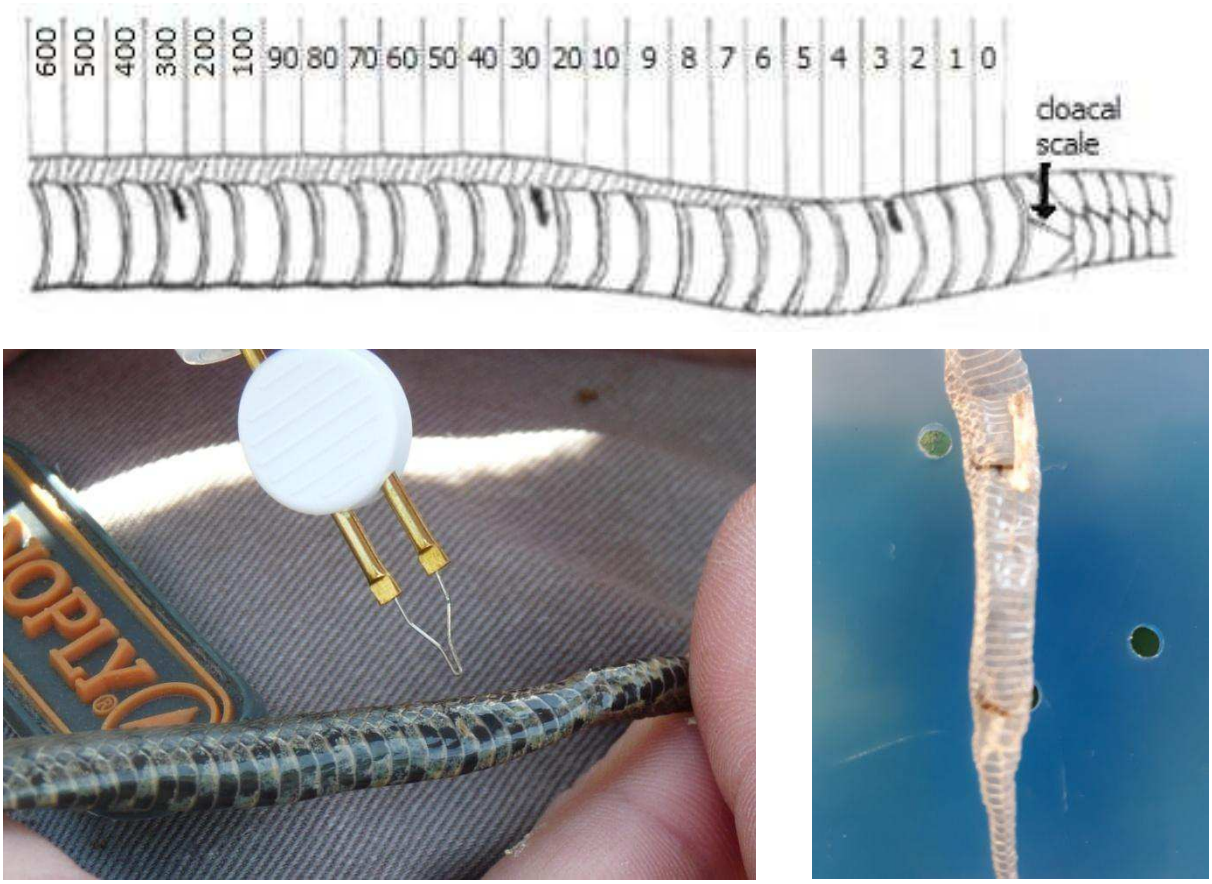


Figure 7 : *(En haut) Représentation schématique du système de codage utilisé pour le marquage des juvéniles de Couleuvres vipérines. Ici selon le code, l'individu porte le numéro 333 (© anonyme). (En bas à gauche) Marquage d'un juvénile de Couleuvre vipérine à l'aide d'un cautère basse température, Alas, (09, France), le 30 mars 2017. (En bas à droite) Visualisation de la persistance du marquage sur la mue d'un juvénile de Couleuvre vipérine, Pic du Midi de Bigorre (65, France), le 24 aout 2017.*

2.3.4. Mesures des performances physiques des juvéniles

Les performances maximales de vitesse de nage et d'apnée permettent d'évaluer la limitation potentielle due à l'hypoxie de l'organisme à atteindre ses capacités maximales. Les deux tests de performances ont été effectués à une température d'eau de 25°C, soit dans la gamme de températures optimales des performances de la Couleuvre vipérine, qui a un optimum thermique corporel d'environ 25°C à 30°C (Hailey and Davies, 1986a). A titre de comparaison, chez d'autres espèces comme la Couleuvre rayée, des vitesses maximales ont été enregistrées avec une température corporelle de 28°C (Stevenson et al., 1985).

La procédure utilisée pour estimer la performance de vitesse de nage a été validée pour les serpents dans d'autres études (Shine and Shetty, 2001; Aubret, 2004; Aubret et al., 2005) et adaptée pour les juvéniles de Couleuvre vipérine. Une caméra numérique grand angle à haute définition (25 *fps*, modèle Sony HDR-XR160E, Sony Corporation) a été installée au-dessus d'un bassin de natation linéaire de 100 cm x 20 cm x 20 cm et a enregistré les essais de chaque juvénile de Couleuvre vipérine (Figure 8). Le bassin de natation a été rempli avec 5 cm de hauteur d'eau maintenue à 25°C. A différent pas de temps en fonction des expériences, chaque juvénile de Couleuvre vipérine a nagé 10 longueurs consécutives. Si l'individu ne repartait pas, une simple touche de la queue avec le doigt de l'expérimentateur le faisait repartir. Les données brutes ont été extraites des fichiers vidéo et la vitesse de nage ($cm.s^{-1}$) a été mesurée pour chaque longueur avec le logiciel Tracker (Brown, 2019).

Figure 8 : *Bassin de natation pour les mesures des performances de nage des juvéniles de Couleuvre vipérine, Moulis (09, France), le 17 aout 2017.*

La procédure utilisée pour estimer la performance d'apnée a été validée pour les Couleuvres vipérines dans une étude antérieure (Aubret et al., 2015). Un aquarium en verre (20 cm x 15 cm x 25 cm) a été rempli avec 20 cm de hauteur d'eau maintenue à 25°C. Chaque juvénile de Couleuvre vipérine a été placé dans un tube de PVC opaque de 10 cm de longueur et de 2 cm de diamètre, fermé à une extrémité et lesté pour assurer sa stabilité sous l'eau. L'ensemble a été totalement immergé dans l'eau et placé le long du bord en verre de l'aquarium. A chaque tentative de remontée du serpent, celui-ci était légèrement apeuré par le tapotement de la vitre par l'expérimentateur. Ce stimulus est répété jusqu'à ce que, malgré la pression exercée, le besoin de respirer l'emporte. Le test s'arrête à l'instant où le juvénile de Couleuvre vipérine arrive à la surface. Le temps écoulé entre l'immersion et le retour à la surface a été enregistré à l'aide de chronomètres numériques (± 1 s).

2.3.5. Mesures des taux métaboliques au repos des embryons et des juvéniles

Les taux métaboliques au repos des embryons et des juvéniles correspondent à leur consommation d'oxygène et à leur production de dioxyde de carbone. Un système de circuit fermé a été utilisé pour mesurer les échanges gazeux entre le milieu et les individus (Foxbox-C Field O_2 and CO_2 Analysis

System, Sable Systems, Inc., Las Vegas, NV, USA ; Figure 9). Les œufs et les juvéniles de Couleuvre vipérine ont été placés individuellement à l'intérieur d'une chambre métabolique de 250 ml, elle-même placée dans un incubateur (Figure 9). La température de l'incubateur correspond à la température du traitement d'incubation des œufs ou de la température de maintien des juvéniles. Une fois les équipements en place, de l'air extérieur était injecté dans la chambre métabolique pendant 10 min à un débit de 400 $ml.min^{-1}$. Cette étape est indispensable et permet d'homogénéiser les niveaux de concentration en oxygène et dioxyde de carbone de l'air de la chambre métabolique avec celles du milieu extérieur qui sert de référentiel. Ensuite, les vannes ont été fermées pendant 60 min, scellant la chambre métabolique hermétiquement. Après cette heure, les vannes ont été ouvertes pour rétablir le flux d'air. L'air entrant à un débit de 400 $ml.min^{-1}$ provient du milieu extérieur et chasse l'air présent dans la chambre métabolique. Cet air chassé traverse un tube de Drierite afin d'enlever toutes traces d'humidité puis des capteurs mesurent les pourcentages de dioxygène et de dioxyde de carbone présent dans l'air. Les données ont ensuite été analysées à l'aide du logiciel ExpeData (v.1.7.30, Sable Systems, Inc.) permettant de calculer le volume de dioxygène consommé et le volume de dioxyde de carbone produit par l'individu durant la période de 60 min où la chambre était scellée (Lighton, 2018).

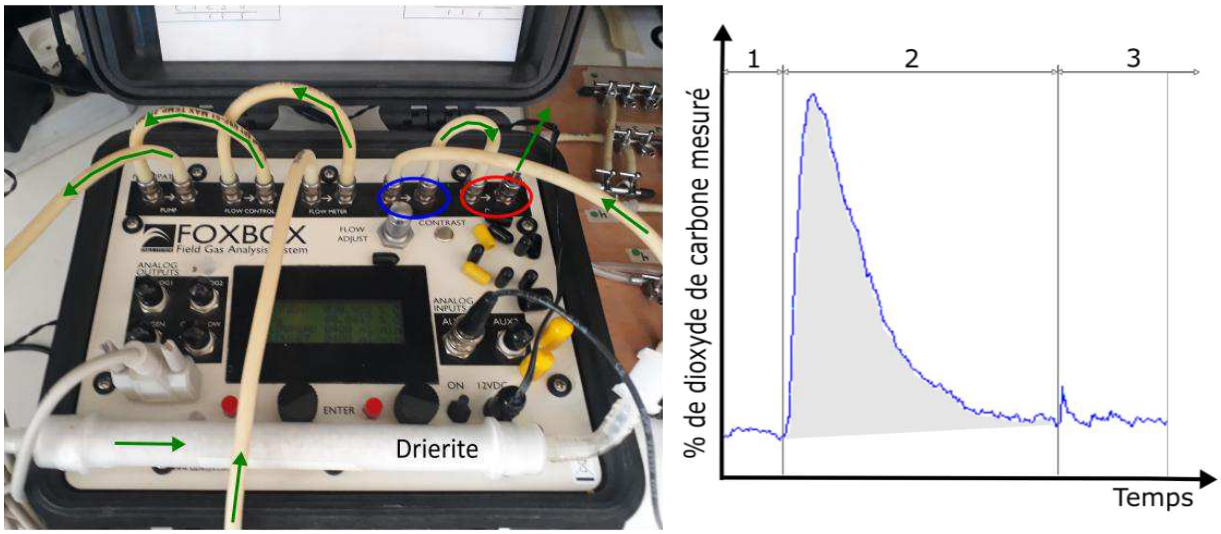
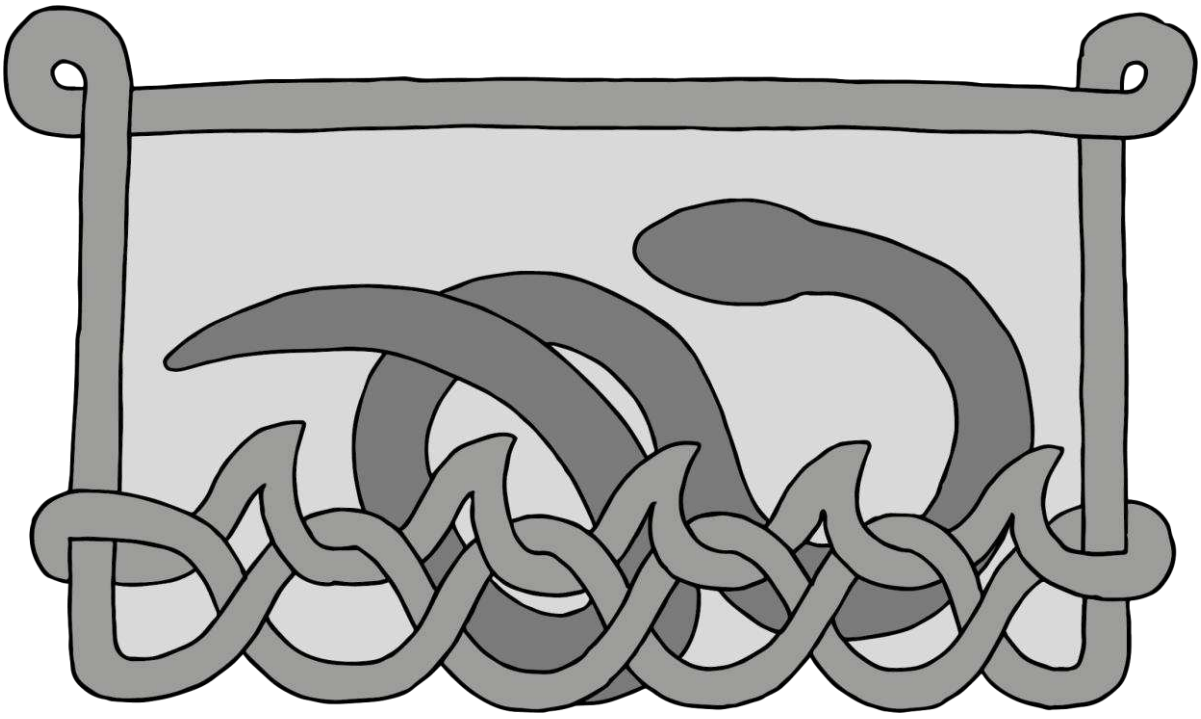


Figure 9 *: (A gauche) Système de mesures des taux de concentration du dioxygène et du dioxyde de carbone (Foxbox-C Field O_2 and CO_2 Analysis System), Moulis (09, France), le 12 septembre 2018. Les flèches vertes indiquent le sens de circulation de l'air en provenance de la chambre métabolique, le cercle bleu représente l'emplacement du capteur de mesure du pourcentage de dioxyde de carbone et le cercle rouge représente l'emplacement du capteur de mesure du pourcentage de dioxygène. (A droite) Mesure du pourcentage de dioxyde de carbone (CO_2) présent dans l'air en fonction du temps. La partie 1 représente le pourcentage en CO_2 de l'air extérieur (ligne de base), la partie 2 représente le pourcentage en CO_2 de l'air extrait de la chambre métabolique après 60 minutes, la partie 3 représente le pourcentage en CO_2 de l'air passant par la chambre métabolique en circuit ouvert. L'aire en gris représente, après calcul sur le logiciel ExpeData, le volume de CO_2 produit par le juvénile de Couleuvre vipérine.*

Chapitre 3.

EFFETS DE L'HYPOXIE D'ALTITUDE SUR LE DÉVELOPPEMENT EMBRYONNAIRE ET LES PERFORMANCES JUVÉNILES



3.1. Présentation et hypothèses du chapitre

Ce chapitre tente de mettre en évidence, dans un premier temps, les effets de l'hypoxie d'altitude sur le développement embryonnaire chez la Couleuvre vipérine. Pour s'affranchir des effets croisés de la température et de l'hypoxie, tout le developpement s'effectue à la température optimale d'incubation (*i.e.* 28°C). L'objectif est de déterminer si les embryons de Couleuvre vipérine sont capables, à travers la plasticité phénotypique, de s'adapter aux nouvelles conditions environnementales. Dans un second temps, ce chapitre s'interresse aux performances physiques des juvéniles à leur température optimale de performance (*i.e.* 25°C). Pour cela, la première étape consiste à mesurer la vitesse de nage et la durée d'apnée des individus en condition hypoxique puis de les comparer au groupe témoin, incubé et testé en condition de normoxie. L'objectif est de savoir si les juvéniles incubés et testés en hypoxie peuvent maintenir des performances équivalentes à celles du groupe témoin. Puis pour la seconde étape de test les groupes sont transférés d'une condition à l'autre. Le but est de savoir si les performances des juvéniles sont modifiées avec un retour à un niveau d'oxygène normal, mais aussi de connaitre les effets d'une hypoxie aigue sur les performances physiques des individus incubés et nés en basse altitude. Dans ce contexte, nous prédisons qu'en condition hypoxique et à une température optimale de developpement ou de performance :

- La fréquence cardiaque des embryons (*i.e*. un proxy du métabolisme *cf*. *section 2.3.2*) sera diminuée entrainant une réduction du métabolisme.
- La durée de développement ne sera pas impactée.
- La réduction du métabolisme durant le développement va modifier le phénotype à l'éclosion avec des juvéniles plus petits qui auront des taux de croissance plus faible.
- Les performances des juvéniles seront réduites.
- Après avoir été transférés en basse altitude, les performances des juvéniles seront améliorées alors que pour les juvéniles incubés en normoxie puis tranférés en haute altitude les performances seront réduites.

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3.2. Résumé

Les changements climatiques entraînent des modifications dans l'aire de répartition de nombreux organismes, notamment le long de gradients altitudinaux dans les écosystèmes montagnards. Cependant, le fait de monter en altitude expose les organismes à une plus faible disponibilité De l'oxygène, ce qui peut réduire le succès de reproduction et le développement des organismes ovipares. Pour tester cette possibilité chez une espèce colonisatrice du milieu montagnard, nous avons incubé artificiellement des embryons de Couleuvre vipérine, *Natrix maura*, en utilisant un plan expérimental où chaque ponte est séparée en deux traitements. Un groupe est incubé dans des conditions d'hypoxie (extrême haute altitude; 2877 m au-dessus du niveau de la mer ; disponibilité d'O_2 équivalente à 72 % de celle du niveau de la mer). L'autre groupe est incubé en normoxie (groupe témoin ; basse altitude ; 436 m au-dessus du niveau de la mer). Le succès de l'éclosion n'a pas varié entre les deux traitements. Les embryons qui se développaient en extrême haute altitude avaient un rythme cardiaque plus élevé en moyenne et les juvéniles ont éclos plus tôt, étaient plus petits à l'éclosion et nageaient plus lentement que ceux incubés en basse altitude. La transplantation réciproque des juvéniles dix jours après l'éclosion a en outre montré que les serpents qui se sont développés en altitude, une fois transférés de nouveau en en basse altitude, n'ont pas retrouvé la pleine performance de nage montrée par les serpents incubés et testés à basse altitude. Ces résultats suggèrent que l'hypoxie de haute altitude n'empêchera pas les organismes ectothermes ovipares de produire des petits viables, mais elle peut poser des défis physiologiques importants pour le développement de la progéniture. Ces limitations de performance au début de la vie imposées par l'hypoxie pourraient avoir des conséquences négatives sur les phénotypes adultes, y compris sur les traits liés à la condition physique.

Mots clés *: Métabolisme embryonnaire; Plasticité développementale; Natrix maura; Hypoxie d'altitude; Performances locomotrices*

High-elevation hypoxia impacts perinatal physiology and performance in a potential montane colonizer

Short running title: Snake development in hypoxia

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Jérémie Souchet[1,†], Eric J. Gangloff[1,2], Gaëlle Micheli[1], Coralie Bossu[1], Audrey Trochet[1], Romain Bertrand[1], Jean Clobert[1], Olivier Calvez[1], Albert Martinez-Silvestre[3], Elodie Darnet[1], Hugo Le Chevalier[1], Olivier Guillaume[1], Marc Mossoll-Torres[4,5], Laurent Barthe[6], Gilles Pottier[6], Hervé Philippe[1,7] & Fabien Aubret[1]

1- Station d'Ecologie Théorique et Expérimentale du Centre National de la Recherche Scientifique, UMR 5321 CNRS – Université Paul Sabatier, 09200 Moulis, France.
2- Department of Zoology, Ohio Wesleyan University, Delaware, Ohio, USA
3- Catalonia Reptile and Amphibian Rescue Center (CRARC), 08783 Masquefa, Barcelona, Spain
4- Bomosa, Pl. Parc de la Mola, 10 Torre Caldea 7º, AD700 Les Escaldes, Andorra
5- Pirenalia, c/ de la rectoria, 2 Casa Cintet, AD200 Encamp, Andorra
6- Nature En Occitanie, 14 rue de Tivoli, 31 000 Toulouse, France
7- Département de Biochimie, Centre Robert-Cedergren, Université de Montréal, Montréal, Canada

† Corresponding author. E-mail: *jeremie.souchet@gmail.com*

Author Contributions

JS and FA contributed to experimental design and logistics. JS, GM, CB and FA conducted experiments. JS, EJG and FA conducted statistical analyses. JS, EJG, FA, AT, RB, JC, OC, OG, AMS, ED, HLC, MMT, LB, GP and HP drafted the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

3.3. Introduction

Climate envelopes are typically much narrower across altitudinal than latitudinal gradients (Loarie et al., 2009; Chen et al., 2011), fostering rapid migration along the elevational gradient as the climate warms (*e.g.* Bässler et al., 2013; Freeman et al., 2018; Parmesan and Yohe, 2003; Pauchard et al., 2016; Walther et al., 2002). While lower-elevation valleys may have provided refuge to many organisms during past glaciation events (Hewitt, 1999; Tzedakis, 2004), elevated areas may play a similar role for escaping global warming (Sinervo et al., 2018). As high-elevation environments may represent climate refugia, it is important to identify constraints on upslope colonization. While it is well established that warming may promote range expansion towards higher altitudes, organismal function may be affected by the decrease in the oxygen partial pressure (for instance, oxygen availability is 25% lower at 2500 m above sea level [ASL] compared to sea level; Powell and Hopkins, 2010; Storz et al., 2010). Yet, there are many unanswered questions regarding the effects of high elevation hypoxia on the ability of ectothermic vertebrates to colonize and adapt to these elevations.

The acute and chronic effects of high-altitude hypoxia on organismal function seem to vary widely among taxa. Well documented in birds and mammals (Beall et al., 2002; Lague et al., 2016; Monge and Leon-Velarde, 1991; Storz et al., 2004), the acute effects of hypoxia commonly include hyperventilation, tachycardia, altitude sickness, and the down-regulation of non-essential physiological functions (such as digestion). These studies also demonstrate that chronic effects range from an alteration of cardiorespiratory pathways (increased lung and heart size, increased blood pressure), blood composition (increased haematocrit, increased haemoglobin concentration), and muscle performance (increased vascularization, increased amount of myoglobin and mitochondria) to effects on embryonic development, birth size, and early growth rates (Beall et al., 2002; Lague et al., 2016; Monge and Leon-Velarde, 1991; Storz et al., 2004). The consequences of high-altitude hypoxia, induced by elevation, are less well known in reptiles. However, we can surmise that they might be similar to the consequences of high-altitude hypoxia in birds and mammals or to underwater or underground hypoxia in reptiles. For instance, even short exposures to hypoxia can have lasting effects on subsequent growth and development of turtle embryos, including reduced mass at hatching, decreased oxygen consumption, and depressed metabolism, despite a comparable incubation period (Cordero et al., 2017a; Kam, 1993). Incubation in hypoxic conditions is known to reduce embryo heart rates (in lizards: Cordero et al., 2017a; Kouyoumdjian et al., 2019), produce smaller juveniles with decreased growth rate during the first months of life (in alligators: Owerkowicz et al., 2009; in turtles: (Wearing et al., 2015), and increase heart and lung size at birth (in alligators: Owerkowicz et al., 2009, and lizards: Cordero et al., 2017a). Chronic hypoxia specifically elicits

changes in the cardio-respiratory pathways (increases lung and heart size, higher blood pressure; Cordero et al., 2017a; Crossley and Altimiras, 2005; Iungman and Piña, 2013; Wearing et al., 2015); increases haematocrit and haemoglobin concentration (Gangloff et al., 2019; González-Morales et al., 2015; Lu et al., 2015; Newlin and Ballinger, 1976; Vinegar and Hillyard, 1972; Weathers and White, 1972); and alters muscle physiology (increases vascularisation and myoglobin concentration; Jochmans-Lemoine and Joseph, 2018). Although many of the physiological and anatomical changes that accumulate under chronic hypoxia improve function under low O_2 partial pressure (PO_2; *i.e.*, individuals show acclimation), these changes may only partially compensate for reduced oxygen availability. For example, low weights at birth and reduced growth in juveniles have been reported in a variety of vertebrate taxa, from humans (Monge and Leon-Velarde, 1991) to turtles (Wearing et al., 2015). Rats and mice showed delayed brain growth due to long-term exposure to hypoxia (Golan and Huleihel, 2006), cognitive effects which may be true in reptiles as well (Sun et al., 2014).

Predicting if and how animals will adapt to high altitude under global warming requires a detailed study of physiological, morphological and behavioural responses to hypoxia across an altitudinal gradient in a species that undergoes upward range expansion. This knowledge is incomplete, particularly in snakes. The successful colonisation of higher elevations in animals escaping warming temperatures depends on their ability to cope with lower partial pressure in oxygen so that they can (1) move, acquire food, mate and escape predators and (2) produce eggs (embryos) able to develop, hatch, and survive. Here we focused on the latter as effective colonization (*i.e.*, all former steps) depends on successful recruitment of offspring (Warner et al., 2012; Aubret, 2013a; While et al., 2015). To identify physiological, morphological, and behavioral alterations associated with altitude-induced hypoxia, we performed an elevation transplant experiment utilizing a generally low-elevation ectothermic species. In our experiment, we exposed eggs of the viperine snake, (*Natrix maura,* Linnaeus 1758; Colubridae)*,* to two alternative incubation treatments: extreme high elevation (EHE, above current range limits, *i.e.* hypoxia) and low elevation (LE, native elevation, *i.e.* normoxia).

The viperine snake is a circum-mediterranean species that has been colonizing mountainous and lowland environments alternately in conjunction with historical warming and cooling cycles (Gómez and Lunt, 2007). The Iberian Peninsula provided a glacial refuge during the Pleistocene and allowed the viperine snake to re-colonize the Pyrenees and Western Europe from 12,000 years ago onwards during the Holocene (Guicking et al., 2008). This aquatic species (Vacher and Geniez, 2010) has been recorded up to 1000 m ASL in France (Aubret et al., 2015) and 1500 m ASL in Spain (Martinez-Rica and Reiné-Viñales, 1988; Santos, 2015). We collected gravid females from low elevation (475 m ± 43 m ASL) and, using a split-clutch design, incubated the eggs at low elevation (normoxia, 95% O_2

availability compared to sea level equivalent) or at extreme high elevation (hypoxia, 72% O_2 availability compared to sea level equivalent). We monitored embryo physiology (heart rates; an indicator of cardiovascular output and a proxy for metabolism in ectothermic amniotes; Crossley and Burggren, 2009) and egg mass throughout the incubation. We then measured important aspects of hatchling phenotype (body mass and body size) and two aspects of fitness-related performance (sprint swimming speed and apnea duration) of the juveniles (Aubret et al., 2015). Finally, we tested whether expected deleterious effects of incubation at extreme high elevation would persist after hatchlings are returned to low elevation, which would indicate that changes in physiology and performance are due to remodeling of related pathways beyond the immediate restrictions of oxygen reduction.

3.4. Materials and methods

3.4.1. Experimental design

We captured 12 gravid female viperine snakes along the banks of the Lez River (Department of Ariège, France) between June and July 2016. Capture sites spanned from 432 m to 518 m ASL. A total of 113 eggs were obtained between 8 July 2016 and 28 July 2016 (mean clutch size ± SD = 9.4 ± 4.3 eggs). All females were returned to their exact site of capture within two weeks of egg-laying. 23 eggs were infertile or died within the first 7 days post-oviposition, leaving 90 eggs from 11 females allocated to two treatments for experiments (Figure 1): low elevation (LE) and extreme high elevation (EHE). The LE treatment was located at the Theoretical and Experimental Ecology Station of Moulis, National Center for Scientific Research (SETE-CNRS; 42.958394 N, 1.086440 E; 436 m ASL; PO_2 ~20.1 kPa) and the EHE treatment was located at the Observatory Midi-Pyrénées of the Pic du Midi de Bigorre (42.936389 N, 0.142472 E; 2877 m ASL; PO_2 ~15.3 kPa). This difference in elevation results in a decrease in atmospheric pressure, with associated reduction in the partial pressure of gases, including oxygen, carbon dioxide, and water vapor (Millet and Debevec, 2020; Richalet, 2020). Most relevant to our hypotheses is the 25% reduction in oxygen availability at the Pic du Midi de Bigorre lab in comparison to sea level (Bouverot, 2012; Cordero et al., 2017a).

Eggs were weighed using a digital scale to the nearest 0.01 g within 12 hours of oviposition, individually marked for identification with a pencil, and allocated to LE and EHE treatments using a split-clutch design within 24 hours of oviposition. Because egg mass influences both embryo metabolism and hatching phenotype (Nelson et al., 2004; Aubret, 2013a), and egg mass varied

among clutches (Kruskal-Wallis test: H = 61.97, Df = 10, P < 0.001), eggs were ranked within each clutch from lightest to heaviest and alternately assigned to treatments in order to ensure no difference in egg mass between treatments (Kruskal-Wallis test: H = 0.082, Df = 1, P = 0.774). LE and EHE treatment half clutches were placed in a plastic container (20 cm x 15 cm x 5 cm) on a 2 cm layer of wet vermiculite (1:5 water to vermiculite by volume) and incubated in two identical incubation chambers (ExoTerra Model PT-2445, Rolf C. Hagen Inc., USA) set at a constant 28°C, a temperature successfully used for artificially incubating eggs of the viperine snake (Aubret, 2013a; Aubret et al., 2016a, 2017). Water bowls placed within each incubator ensured ambient humidity remained at 100% throughout incubation.

Out of 90 eggs, 65 embryos from ten females successfully hatched (72.2% hatching success rate) while 25 died at various stages during incubation. Another five neonates died shortly after hatching. We measured morphology (see below) and performance (see below) first on all 60 hatchlings at their altitude of incubation (LE or EHE). Nine days post-hatching (after all yolk was assimilated: Ji et al., 1999) hatchlings were tested for swimming performance and at 10 days for apnea performance (see below). At 13 days post-hatching, LE treatment hatchlings were transferred to EHE while hatchlings from the EHE treatment were brought down to LE. After a 24-hour acclimation period, snakes were tested for swimming and apnea performance at age 15 days and 16 days (Figure 1). Water temperature was 25°C for both performance measures because it is within the range of optimal temperature for swimming speed in this species (Aubret et al., 2015; Hailey and Davies, 1986b). Once tests were completed, young snakes were fed and released at the maternal capture site.

3.4.2. Egg mass and heart rate measurements

We weighed each egg using a digital scale (to the nearest 0.01 g) within 12 hours of oviposition, and then every 7 days until hatching (Figure 1; test 1). Embryo heart rates were first measured at 7 days post-oviposition and then every 7 days until hatching (Figure 1; test 1). To measure embryo heart rates, we used the Buddy digital egg monitor (MK2, Avitronics, Cornwall, UK) under the standardized protocol described for eggs. We conducted the measures at the same temperature as incubation (28°C). Each egg was gently placed on the sensor pad for heart rate reading (a stable reading was obtained after approximately 30 seconds) and then returned to its clutch. All eggs were only briefly (≤ 1 min) placed in the digital egg monitor to mitigate potential temperature changes owing to exposure to infrared sensors (Sartori et al., 2015; Hulbert et al., 2017). While embryonic heart rates are correlated with rates of oxygen consumption in snake and lizard embryos (Souchet J. and Gangloff E. J., unpubl. data; Kouyoumdjian et al., 2019), we note that change in heart rate is but one

of several physiological mechanisms important for the maintenance of energy flux (Sartori et al., 2017).

3.4.3. Hatchling measurements

Hatching occurred between 20 August 2016 and 8 September 2016 and hatchlings were individually marked for identification by the hot branding technique on the ventral scales (Winne et al., 2006) within 24 hours of emergence. Hatchlings were weighed using a digital scale (to the nearest 0.01 g), measured for snout-vent length (SVL) using a measuring tape (to the nearest 0.1 mm), and sexed via hemipene eversion (Figure 1; test 2). While sex is genetically determined in snakes and so we did not expect an effect of treatment on sex determination, we tested for differential effects of treatments between the sexes in developing embryos which could result in skewed hatchling sex ratio. We also weighed the yolk leftover in the eggshell (residual egg yolk) using a digital scale (to the nearest 0.01 g). Juveniles were housed together by hatching date in plastic containers (15 cm x 10 cm x 5 cm) with a water dish, shelter, and paper towel as a substrate in incubation chambers (ExoTerra Model PT-2445, Rolf C. Hagen Inc.) set at constant 20°C. While below the optimum temperature for performance, this temperature was chosen because it provides high levels of survival and growth for juveniles of this species (J.S., unpublished personal data). Juveniles were measured again at 9 days post-hatching for SVL and body mass prior to performance testing. We also calculated body condition as the residual of the $\log_{10}$-mass on $\log_{10}$-SVL linear regression at hatching day and at 9 days post-hatching.

3.4.4. Swimming performance

For this test, we were interested in measuring the maximal sprint swimming speed to evaluate the potential limitation of hypoxia on this ecologically-relevant performance. To estimate sprint swimming performance, we used a procedure that has been validated for snakes (Aubret, 2004; Aubret et al., 2005; Shine and Shetty, 2001), modified for juvenile viperine snakes. A high-definition wide-angle digital camera (25 fps, Sony Model HDR-XR160E, Sony Corporation) was fitted above a linear 100 cm x 20 cm x 20 cm swimming track and used to record swimming trials (Figure 1; test 3). The tank was filled to a depth of 5 cm with water maintained at 25°C using aquarium heaters. Each snake swam ten consecutive lengths. Raw data were extracted from video files with the software Tracker (Brown, 2019). The fastest performance over 10 cm from all trials (sprint swimming speed) was utilized for swimming analysis.

3.4.5. Apnea performance

To test for maximum voluntary breath-holding (Figure 1; test 4), we used the procedure described in Aubret et al. (2015). Briefly, a glass aquarium (25 cm x 15 cm x 20 cm) was filled with 20 cm of water maintained at 25°C. Up to four snakes were tested simultaneously. Snakes were presented to the open end of a tube (opaque PVC tubes 10 cm in length and 2 cm in diameter, closed at one end and ballasted to ensure stability under water). As soon as the snake voluntarily entered the tube, the unit was fully immersed in the water and tilted upward to make sure no air bubbles remained trapped. The tubes were then oriented towards the side of the aquarium, facing the observer, with the tube opening in direct contact with the glass. This allowed the observer to monitor the movement of the snakes inside the tube. When snakes made contact between their snout and the glass, the observer gently knocked the glass with the tip of a finger to scare them back down into the tube. This stimulus, repeated as long as necessary, encouraged the animal to prolong the duration of its time in the safety of the tube, presumably until its need to breathe overcame the perceived risk of predation imposed by the observer. At this point, the juvenile pushed against the glass with its snout and moved the tube away from the glass, allowing the snake to exit. The time taken from immersion to surface was recorded with digital chronometers (± 1 s).

3.4.6. Data analysis

We first assessed the influence of LE and EHE treatments and time on two measures of embryo development (test 1): egg mass and heart rate. We used linear mixed-effect models, including as main effects treatment (LE or EHE), age at measurement (in days after hatching, treated as a categorical effect), and their interaction. Then we assessed the influence of both treatments on ten measures of hatchling phenotypes (test 2): survival to hatching, sex ratio, incubation time, residual egg yolk, body mass at 1 day and 9 days post-hatching, body size (SVL) at 1 day and 9 days post-hatching, and body condition at 1 day and 9 days post-hatching. For survival to hatching and sex ratio, we used generalized linear mixed models and for the eight other tests we used linear mixed-effect models, including in all models the main effects of treatment. We also assessed the influence of treatment on the two measures of performance: sprint swimming speed (test 3) and apnea time (test 4). We used linear mixed-effect models, including the main effects of treatment (LE or EHE), the location of the test (low elevation or extreme high elevation), sex (male or female), and the covariates of body size (SVL) for swimming performance or body mass for apnea performance.

To meet assumptions of normal distribution of residuals, we square root transformed egg mass and apnea time. To account for the non-independence of siblings we included the clutch of origin as a random effect in all models. In models for which we measured individuals repeatedly (egg mass, heart rates, sprint swimming speed, and apnea time), we also included individual as a random effect. We used type III sums of squares to assess the significance of main effects, incorporating a Kenward-Roger denominator degree of freedom approximation (Kenward and Roger, 1997). All analyses were conducted with the lme4 package (Bates et al., 2014) and figures were made with the ggplot2 package (Wickham, 2016) in the programming language R 3.4.3 (R Development Core Team, 2017).

3.5. Results

3.5.1. Egg mass variation and embryonic heart rates

Elevation treatments significantly altered egg mass trajectories (Table 1, Figure 2A). Eggs incubated at LE maintained higher mass across the incubation period compared to eggs incubated at EHE. Further, the drop in egg mass prior to hatching was sharper in the LE treatment (-8.69% mass change between 28 days and 35 days) compared to eggs in the EHE treatment (-6.34% mass change between 28 days and 35 days; Figure 2A). Eggs incubated at LE gained mass until 28 days before decreasing, while eggs incubated at EHE maintained their initial mass until 21 days before decreasing (Table 1). Nevertheless, the eggs from EHE lost more mass between oviposition and hatching compared with eggs from LE (mass loss of -7.69% and -2.38%, respectively). Heart rates from both incubation treatments followed a similar trend across the incubation period (Figure 2B). Heart rates decreased throughout incubation but remained consistently and significantly higher in embryos incubated at EHE (Table 1).

3.5.2. Hatching success and morphological measurements

Hatching success (test 2) did not differ significantly between embryos incubated at LE versus EHE (68.2% versus 76.1% success respectively; $\chi^2 = 2.31$, Df = 1, P = 0.128). Hatchling sex ratio did not differ significantly between embryos incubated at LE versus EHE (50% versus 62.9% females respectively; $\chi^2 = 1.11$, Df = 1, P = 0.293). Embryos incubated at LE had on average a longer incubation time (by 2%) compared to embryos incubated at EHE (Table 2). LE eggs also produced heavier hatchlings (by 9%; Table 2), although hatchlings did not differ in length or body condition (Table 2). LE embryos assimilated more yolk than embryos incubated at EHE (*i.e.* had 44% less

residual yolk; Table 2). At 9 days post-hatching, juveniles from embryos incubated at LE were significantly longer (by 3%; Table 2) than juveniles incubated at EHE. On the other hand, body mass and body condition at 9 days did not differ between treatments (Table 2).

3.5.3. Effects of incubation and translocation on swimming performance of juveniles

All snakes showed higher sprint swimming speed (test 3) at LE rather than EHE (Table 3, Figure 3A). The swimming speed of snakes incubated at EHE increased after being translocated to LE (5% faster) while the swimming speed of snakes incubated at LE decreased after translocation to EHE (13% slower). This is demonstrated by the significant interaction of incubation treatment and test location (Table 3): snakes incubated at LE exhibited higher performance at LE, but groups did not differ at EHE. As expected based on other studies of snake swimming speed (Shine and Shetty, 2001), longer snakes swam faster than smaller snakes (Table 3).

3.5.4. Effects of incubation and translocation on apnea performance of juveniles

There was no effect of treatment on apnea performance (test 4), while snakes for both groups showed higher apnea performance at LE rather than EHE (15% longer; Table 3, Figure 3B). Additionally, body mass influenced apnea performance, with lighter snakes holding their breath for longer durations (Table 3).

3.6. Discussion

Our study is intended to quantify the restrictions imposed by transplantation to extreme high elevation and the potential limits of organismal responses to these constraints, relevant in the current context of global warming. We explored the way egg incubation and hatching success (primary components of successful population establishment during colonization processes) were affected by extreme high elevation (*i.e.*, hypoxia) compared to control eggs (incubated at low elevation) in the viperine snake. Although the EHE treatment did not significantly alter hatching success, it generated significant differences in egg development and affected hatchling phenotypes, including performance decrements that persisted after translocation back to the native elevation.

3.6.1. Embryo development and hatchling measurements

Typical physiological adjustments to hypoxia in other taxa include suppressed embryo metabolism, often measured as reduced heart rate (Cordero et al., 2017a, 2017b; Crossley and Altimiras, 2005; Crossley and Burggren, 2009; Du et al., 2011; Kouyoumdjian et al., 2019; Laughlin, 1978; Monge and Leon-Velarde, 1991). However, heart rates of developing viperine snake embryos exhibited the opposite trend: their heart rates increased at EHE (Figure 2B). This is a puzzling result and a physiological response that is opposite to what is observed in other taxa (see above references). Further, while eggs incubated at LE tended to gain mass during incubation, eggs incubated at EHE maintained their mass over the same period (Figure 2A), suggesting a low efficiency of water or carbon dioxide diffusion (Cunningham and Hurwitz, 1936). Excessive water loss in snake eggs may gradually increase yolk viscosity and impede absorption by the developing embryo (Aubret et al., 2005; Cunningham and Hurwitz, 1936). Eggs exposed to EHE, by losing excessive water, may have exposed the embryo to a similar constraint, leading to lesser yolk intake (and higher amounts of residual yolk post-hatching) and consequently smaller body size at hatching (Table 2). These results collectively suggest that either higher metabolic rates (*i.e.*, heart rates), excessive water loss (rendering the yolk hard to assimilate), or a combination of both, generated early hatching at EHE (Spencer et al., 2001; Du et al., 2009) compared to sibling eggs incubated at LE. Further investigations will also be needed to ascertain whether higher heart rates in EHE embryos resulted from exposure to hypoxia (a counter-intuitive finding, see references above) or from excessive water loss causing physiological stress to the embryos.

Because the difference in incubation time was minimal between the two treatment groups (*i.e.*, < 24 h; Table 2), one could question the biological relevance of this effect on hatching fitness and long term survival prospects. While further investigations are needed to address this question, there is evidence that incubation times (at 28°C) are heavily constrained in the viperine snake (*i.e.*, always remain within a 24-hour boundary, irrespective of experimental treatments; Aubret et al., 2017, 2016a, 2016b) and early hatching may entail deleterious effects. For example, early hatched Japanese quail chicks (*Coturnix coturnix japonica*) take 1–2 h longer to stand than normal chicks (Vince and Chinn, 1971), while early hatched turtles (*Chrysemys picta*) showed reduced neuromuscular function for at least 9 months after hatching (Colbert et al., 2010). Nevertheless, in areas where growing seasons are short (such as at high elevation), hatching early can be advantageous to respond to temporal constraints on food acquisition (Edge et al. 2017). In our study, however, early hatching is combined with a lesser ability to absorb egg yolk, smaller body size at hatching, poorer body condition at hatching and slower growth rates (Table 2). These results are

consistent with metabolic compensation, a physiological mechanism whereby stressful incubation conditions generate faster paces of development (McGlashan et al., 2012; McGlashan et al., 2015; Aubret et al., 2016b). Further, or alternatively, low partial pressure of O_2 at high altitude (> 2000 m ASL) is known to render embryonic development challenging, due to aerobic energetic restrictions in converting egg energy (yolk) into tissue (Bouverot, 1985; León-Velarde and Monge, 2004; Monge and Leon-Velarde, 1991; Noble, 1991; Rahn et al., 1977; Vleck and Hoyt, 1991; Vleck and Vleck, 1996; Wangensteen et al., 1974). Importantly, change in heart rate is one of many possible compensatory physiological mechanisms to accommodate abiotic limitations and may, in itself, not represent increased metabolism (Sartori et al., 2017). Whether or not metabolic compensation or a comparable physiological mechanism operated in embryos incubated at EHE remains unclear at this stage and will warrant future investigation. Importantly though, EHE did not prevent eggs from developing and hatching altogether, as hatching success did not differ between the two treatments groups (LE: 68.2% versus EHE: 76.1%; see Results). Nevertheless, EHE altered body size in neonate snakes as well as post-hatching growth rates, both important fitness proxies in squamates (Gangloff et al., 2018; Kissner and Weatherhead, 2005; Mayer et al., 2016). However, the long-term adaptive potential for observed changes in development and physiology has yet to be tested.

3.6.2. Swimming and apnea performance

Our results show that EHE significantly affected hatchling swimming performance, but not apnea performance. This difference persisted even after translocation to low elevation, suggesting a genuine long-term change of physiological and performance capacity. EHE juveniles, when transferred back to LE, did not recover full performance compared to their siblings from the LE treatment. Further, juveniles incubated at EHE did not perform better than the LE siblings when tested at EHE (Figure 3A). These findings suggest that (1) snakes' physiology was impaired during development (muscle function, locomotion, or cardiorespiratory capacity) beyond a simple reduction of body size at birth and that (2) physiology and body size were affected in a way that did not enhance organismal function in hypoxic conditions. As a result, such morphological and physiological shifts are likely a mechanistic consequence of development in hypoxic conditions, considered developmental constraints rather than an acclimation effect and thus non adaptive (Bennett, 1997; Forsman, 2015).

3.6.3. General conclusion

It should be kept in mind that our experiment did not aim at mimicking a biologically relevant situation: these organisms are unlikely to climb over 2500 m (*i.e.*, the distance separating origin populations from the extreme high elevation treatment) along the altitudinal gradient to breed. Any range shift driven by climate change is likely to be gradual, potentially allowing for animals to adjust their physiology and behavior by means of phenotypic plasticity and natural selection acting on advantageous genetic variants (mixed selection on plastic and non-plastic attributes over different time scales, eventually leading to local adaptation; Beall, 2006; Hammond et al., 2006; Mueller et al., 2015; Powell and Hopkins, 2010; Rezende et al., 2005; Storz et al., 2010). Indeed, several squamate species have adapted to permanent life at extreme high elevations (*i.e.*, Atlas Day gecko, *Quedenfeldtia trachyblepharus:* Bouazza et al., 2016*; Liolaemus* lizards: Marquet et al., 1989; Qinghai toad-headed lizards, *Phrynocephalus vlangalii:* Wu et al., 2018; western fence lizard, *Sceloporus occidentalis* and sagebrush lizard, *Sceloporus graciosus*: Adolph, 1990; Pyrnean rock lizard, *Iberolacerta bonnali;* Pottier, 2012). These records are testimony that colonization and life at extreme high altitude is possible for oviparous ectotherm amniotes, although the interactions between elevation, colonization dynamics, warming speed, plasticity, and local adaptation remain to be understood. Our study shows that extreme high elevation colonization by the viperine snake will not be prevented, but likely slowed down by hypoxia. Notably, in the context of global warming, it will be essential to measure how a combination of different environmental factors might interact to affect development and performance. For example, the effects of oxygen level may manifest differently depending on temperatures, with the effects of reduced oxygen availability stronger at high temperatures (Gangloff and Telemeco, 2018). These impacts could in turn limit the ability to colonize higher elevation. As a start, our study demonstrates that extreme high elevation has significant effects on embryo development and hatchling phenotypes, prompting further research on the matter, specifically on the interactive effects of oxygen levels and temperature.

3.7. Acknowledgements

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3.8. Tables and figures

Table 1

Results of linear mixed-effect models testing for the effect of incubation treatment (LE or EHE), age at measurement (day post-hatching), and their interaction on embryo developmental parameters in eggs of the viperine snake. Sample numbers (N) for low elevation (LE) and extreme high elevation (EHE) treatments are indicated under the developmental parameters. Significant factors shown in bold with one ($P < 0.05$), two ($P < 0.01$) or three ($P < 0.001$) asterisks.

	Egg mass *LE (N=44); EHE (N=46)*	**Heart rates** *LE (N=44); EHE (N=46)*
Day	$F_{5;\ 437.43} = 2707$; **P < 0.001*** **	$F_{4,\ 348.50} = 24.71$; **P < 0.001*** **
Treatment	$F_{1,\ 78.37} = 7.62$; **P = 0.007** **	$F_{1,\ 77.92} = 15.36$; **P < 0.001*** **
Day x Treatment	$F_{5,\ 437.06} = 4.59$; **P < 0.001*** **	$F_{4,\ 348.46} = 0.26$; P = 0.924

Table 2

Differences in hatchling traits over the first 9 days of post-hatching life between juvenile viperine snakes incubated at low elevation (LE) and at extreme high elevation (EHE). Linear mixed-effect models were used to test the effects of treatment on the relevant traits. Raw means ± SD are given. Significant factors shown in bold with one (P < 0.05), two (P < 0.01) or three (P < 0.001) asterisks.

	LE	**EHE**	**F (dfn, dfd)**	**P**
Incubation time (days) *LE (N=30); EHE (N=35)*	44.77 ± 1.27	44.03 ± 1.29	20.43 (1, 54.50)	**< 0.001*** **
Body mass (g) at 1 day *LE (N=30); EHE (N=35)*	2.95 ± 0.50	2.71 ± 0.52	10.12 (1, 55.04)	**0.002****
Body size (cm) at 1 day *LE (N=30); EHE (N=35)*	14.83 ± 0.73	14.53 ± 1.14	2.03 (1, 56.57)	0.159
Body condition at 1 day *LE (N=30); EHE (N=35)*	0.01 ± 0.05	-0.01 ± 0.04	3.08 (1, 56.64)	0.084
Residual egg yolk (g) *LE (N=30); EHE (N=35)*	0.25 ± 0.15	0.45 ± 0.49	4.64 (1, 58.88)	**0.035***
Body size (cm) at 9 days *LE (N=30); EHE (N=34)*	15.52 ± 0.79	15.09 ± 0.91	8.77 (1, 54.13)	**0.005***
Body mass (g) at 9 days *LE (N=30); EHE (N=34)*	2.07 ± 0.41	1.98 ± 0.35	2.16 (1, 54.45)	0.147
Body condition at 9 days *LE (N=30); EHE (N=34)*	0.008 ± 0.049	-0.005 ± 0.042	0.52 (1, 54.23)	0.472

Table 3

Results of linear mixed-effect models testing the determinants of performance in juvenile Viperine snakes. Sample numbers (N) for both low elevation (LE) and extreme high elevation (EHE) treatments are indicated under the performance tested. Significant factors shown in bold with one ($P < 0.05$), two ($P < 0.01$) or three ($P < 0.001$) asterisks.

	Sprint swimming speed *LE (N=29); EHE (N=31)*	**Apnea time** *LE (N=29); EHE (N=31)*
Test location	$F_{1, 58.00} = 16.82$; **P < 0.001*** **	$F_{1, 58.00} = 4.49$; **P = 0.038***
Treatment	$F_{1, 51.94} = 1.01$; P = 0.319	$F_{1, 49.92} = 0.01$; P = 0.920
Test location x Treatment	$F_{1, 58.00} = 4.06$; **P = 0.048***	$F_{1, 58.00} = 0.01$; P = 0.912
Sex	$F_{1, 52.22} = 0.74$; P = 0.392	$F_{1, 51.60} = 0.64$; P = 0.428
Body size (cm) at 9 days	$F_{1, 42.83} = 15.86$; **P < 0.001*** **	-
Body mass (g) at 9 days	-	$F_{1, 53.86} = 4.72$; **P = 0.034***

Figure 1

Experimental design. Eggs were collected from gravid females sampled from low elevation viperine snake populations in the foothills of the Pyrenees (432 m to 518 m ASL). Within 24 hours of oviposition, clutches were evenly split into two groups with equal average egg masses. For each clutch, one half-clutch was transplanted to the extreme high elevation (EHE) laboratory at 2877 m ASL, while the second half-clutch underwent incubation at low elevation (LE) 436 m ASL. Eggs mass and embryo heart rate were measured throughout incubation (test 1). At hatching, a number of morphometric traits were measured in juveniles (test 2). All hatchlings were tested for swimming and apnea performance in the environment their eggs were incubated (test 3 & 4) and then again after being translocated to the alternative treatment (test 3 & 4).

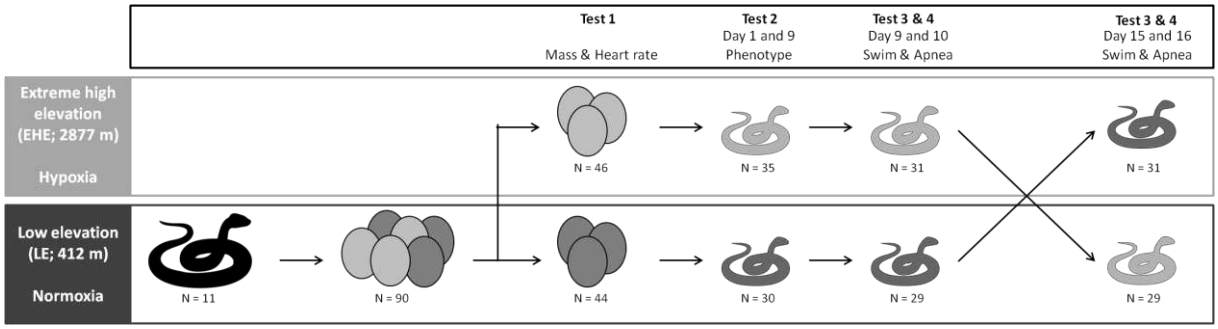


Figure 2

Egg mass (A) and embryo heart rate (B) through incubation time in viperine snakes at low elevation (LE; N = 44; circle) and extreme high elevation (EHE; N = 46; triangle). Least-squares means ± SE estimated by linear mixed models are plotted.

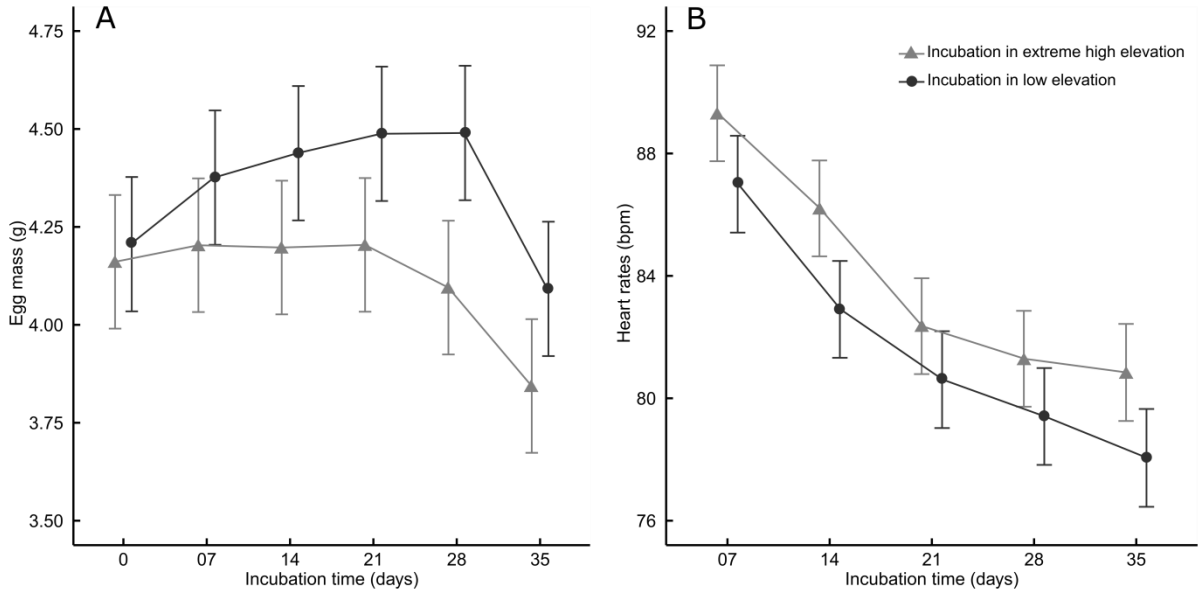
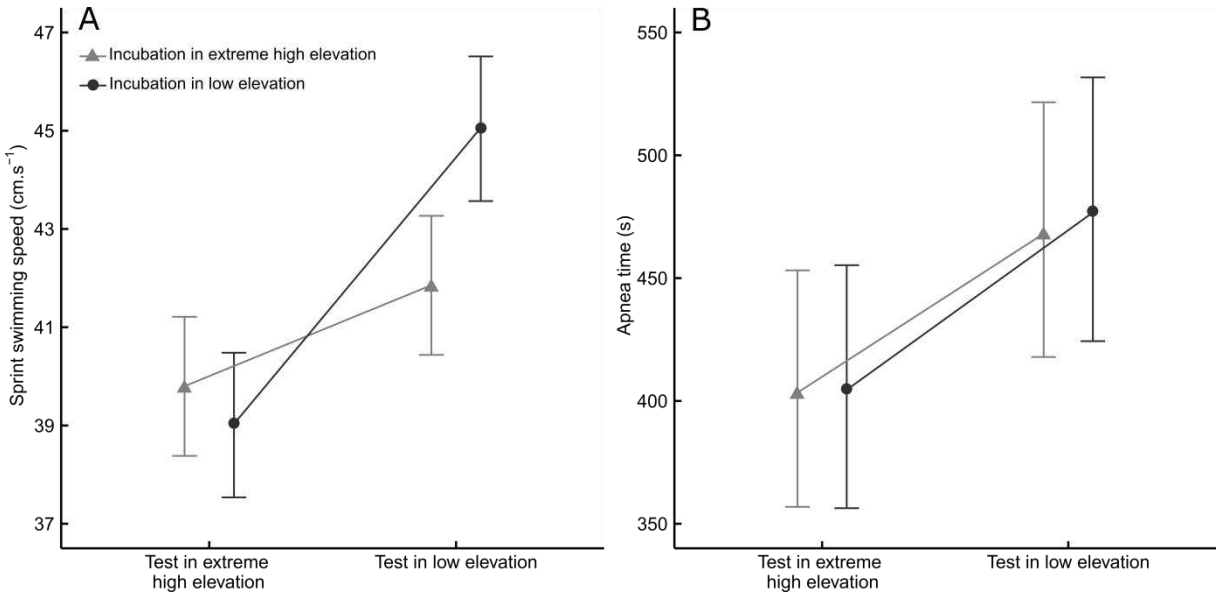


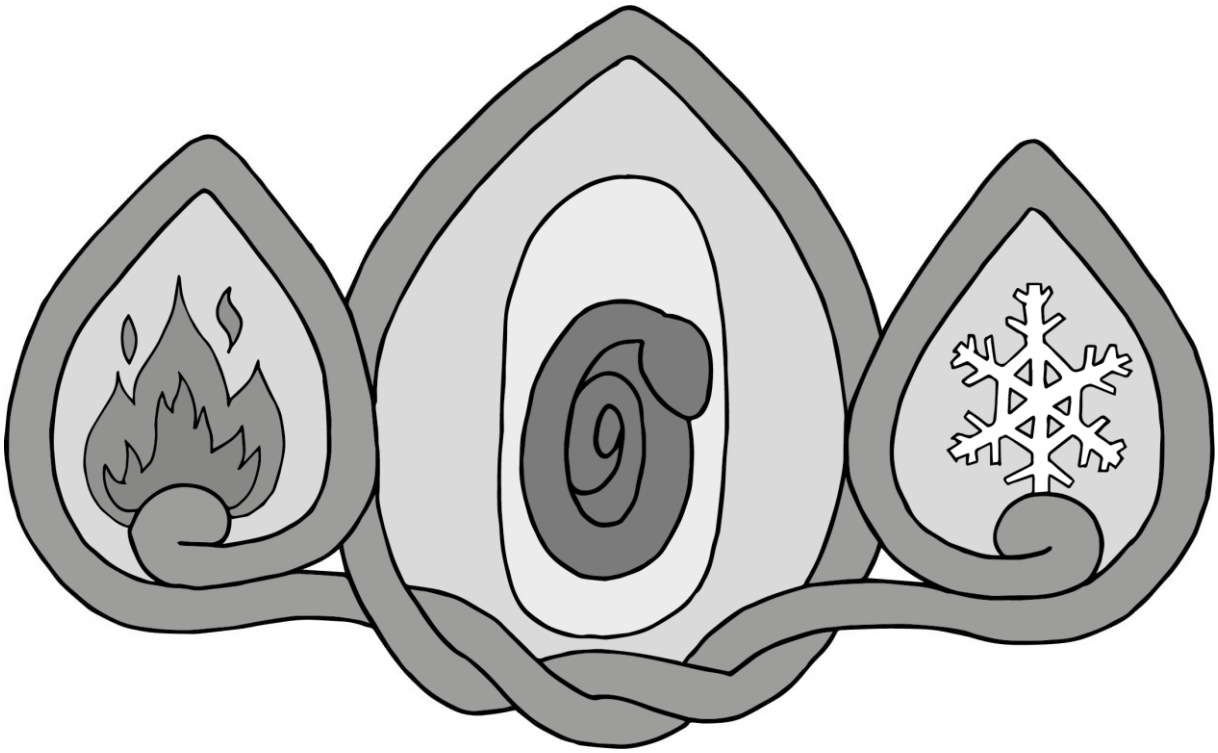
Figure 3

Sprint swimming speed (A) and apnea performance (B) by incubation treatment (LE; N = 29; circle and EHE; N = 31; triangle) and test location (low elevation and extreme high elevation) in viperine snakes. Least-squares means ± SE estimated by linear mixed models are plotted.



Chapitre 4.

EFFETS DE L'HYPOXIE D'ALTITUDE ET DES VARIATIONS DE TEMPÉRATURES SUR LE DÉVELOPPEMENT EMBRYONNAIRE ET LES PERFORMANCES JUVÉNILES



4.1. Présentation et hypothèses du chapitre

Ce chapitre s'intéresse dans un premier temps aux effets croisés de la température et de l'hypoxie sur le développement embryonnaire chez la Couleuvre vipérine. Compte tenu des conséquences physiologiques connues des contraintes thermiques et d'oxygène simultanées (*cf. section 1.5.3*), cette premiere partie tente d'évaluer différents scénario possible induit par le réchaufement climatique : 1) une haute disponibilité en oxygène (*i.e.* 95% à 436 m d'altitude) et une température d'incubation froide (*i.e.* 24°C, 4°C au dessous de l'optimum thermique pour l'incubation) ce qui correspond à une condition passée récente; 2) une haute disponibilité en oxygène (*i.e.* 95% à 436 m d'altitude) et une température d'incubation chaude (*i.e.* 32°C, 4°C au dessus de l'optimum thermique pour l'incubation) ce qui correspond à une condition actuelle pour des populations qui subissent sans déplacement d'aire de répartition le changement climatique; 3) une faible disponibilité d'oxygène (*i.e.* 70% à 2 877 m d'altitude), et une température d'incubation froide (*i.e.* 24°C, 4°C au dessous de l'optimum thermique pour l'incubation) ce qui correspond à une condition actuelle pour des populations qui se sont déplacées en altitude; 4) une faible disponibilité d'oxygène (*i.e.* 70% à 2 877 m d'altitude), et une température d'incubation chaude (*i.e.* 32°C, 4°C au dessus de l'optimum thermique pour l'incubation) ce qui correspond pour des populations qui aurait migrées en altitude à une condition future proche selon les scénarios du GIEC. Cette conception experimentale permet de distinguer les effets individuels et combinés de la température d'incubation et des niveaux d'oxygène sur le développement de l'embryon et les phénotypes d'éclosion. Dans un deuxième temps, ce chaptire s'interresse aux performances physiques des juvéniles à la température optimale de performance connue pour cette espèce (*i.e.* 25°C). Pour cela, la vitesse de nage, un indicateur de la capcité de fuite face aux prédateurs et de l'acquisition de nourriture chez les serpents semi aquatique, est mesurée. Dans un premier temps les vitesses de nage sont mesurées à l'altitude d'incubation des juvéniles. L'objectif est de determiner si les conditions d'incubation influence les performances des juvéniles. Dans un second temps, la moitié des juvéniles de chaque groupe sont de nouveaux testés à court et moyen long terme à leur altitude d'origine alors que l'autre moitié est transféré à l'altitude opposée pour etre également testé à court et moyen long terme. L'objectif est de connaitre plus précisément les effets croisés des niveaux d'oxygène et des températures sur le stade précoce de l'espèce, mais aussi de connaitre l'impact potentiellement négatif de la double contrainte environnementale durant l'incubation. Dans ces contextes, nous prédisons que :

- Les fréquences cardiaques (*i.e.* un proxy du métabolisme *cf. section 2.3.2*) seront similaires pour les embryons incubés en hypoxie et en normoxie à des températures d'incubation froides.

- Une température d'incubation chaude va augmenter la fréquence cardiaque des embryons avec une fréquence cardiaque superieure pour les embryons incubés en hypoxie.
- La durée de développement sera augmenté et similaire entre les groupes à température d'incubation froide alors qu'elle sera réduite à la température d'incubation chaude et d'autant plus pour les embryons incubés en hypoxie.
- Le phénotype des embryons sera maintenu et similaire entre les groupes à température d'incubation froide alors que les juvéniles seront de plus petites tailles et plus légers à la température d'incubation chaude d'autant plus pour les embryons incubés en hypoxie.
- La vitesse de nages des juvéniles qui ont été incubés et qui ont été testés en hypoxie sera réduite et d'autant plus pour les juvéniles qui ont été incubés à la température chaude.
- Peu importe la température d'incubation, après avoir été transférés en basse altitude, la vitesse de nage des juvéniles sera améliorée alors que pour les juvéniles incubés en normoxie puis tranférés en haute altitude elle sera réduite.

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4.2. Résumé

Le changement climatique génère des déplacements chez de nombreux organismes, notamment le long du gradient altitudinal. Cependant, monter en altitude expose les organismes à une diminution de la disponibilité en oxygène, ce qui peut négativement affecter le développement et l'aptitude des organismes, en particulier à des températures élevées. Pour tester cette possibilité chez une espèce ectotherme qui monte en altitude, nous avons artificiellement incubé des embryons de Couleuvre vipérine (*Natrix maura*, Linnaeus 1758), en utilisant un plan expérimental où chaque ponte est séparée en quatre traitements à conditions croisées. Les conditions de ces traitements sont une haute altitude (hypoxie) ou une basse altitude (normoxie) et deux températures d'incubation importantes sur le plan écologique (24°C et 32°C). Les individus incubés à basse ou haute altitude et à des températures fraîches ne différaient pas en termes de temps de développement, de phénotype d'éclosion ou de performances de nage. Toutefois, pour les indiidus incubés à la température d'incubation plus chaude associée à une haute altitude, le succès d'éclosion était réduit. De plus, le rythme cardiaque embryonnaire était plus faible, la durée d'incubation plus courte et les juvéniles plus petits. Néanmoins, les serpents de ce traitement nageaient plus vite que les juveniles des autres traitements, ce qui suggère un compromis de développement entre la taille et la performance. Les contraintes de développement peuvent être compensées par le maintien d'importantes mesures de performance, permettant ainsi une colonisation réussie de l'habitat de haute altitude même sous la double limitation d'une réduction de l'oxygène et d'une augmentation de la température.

***Mots clés** : changement climatique - développement embryonnaire - plasticité du développement - métabolisme embryonnaire - rythme cardiaque - hypoxie de haute altitude - température d'incubation - performance de nage*

High temperatures limit developmental resilience to high-elevation hypoxia in the snake *Natrix maura* (Squamata: Colubridae)

Short running title: Snake development in hypoxia and high temperature

Jérémie Souchet[1]*, Coralie Bossu[1], Elodie Darnet[1], Hugo Le Chevalier[1], Manon Poignet[1], Audrey Trochet[2], Romain Bertrand[3], Olivier Calvez[1], Albert Martinez-Silvestre[4], Marc Mossoll-Torres[5,6], Olivier Guillaume[1], Jean Clobert[1], Laurent Barthe[2,7], Gilles Pottier[7], Hervé Philippe[1,8], Eric J. Gangloff[1,9] & Fabien Aubret[1,10]

1- *Station d'Ecologie Théorique et Expérimentale du Centre National de la Recherche Scientifique, UMR 5321 CNRS – Université Paul Sabatier, 09200 Moulis, France*
2- *Société Herpétologique de France, Muséum National d'Histoire Naturelle, CP41, 57 rue Cuvier, 75005 Paris, France*
3- *Laboratoire Évolution et Diversité Biologique, UMR 5174 Université de Toulouse III Paul Sabatier, CNRS, IRD, 31062 Toulouse, France*
4- *Catalonia Reptile and Amphibian Rescue Center (CRARC), 08783 Masquefa, Barcelona, Spain*
5- *Bomosa, Pl. Parc de la Mola, 10 Torre Caldea 7º, AD700 Les Escaldes, Andorra*
6- *Pirenalia, c/ de la rectoria, 2 Casa Cintet, AD200 Encamp, Andorra*
7- *Nature En Occitanie, 14 rue de Tivoli, 31 000 Toulouse, France*
8- *Département de Biochimie, Centre Robert-Cedergren, Université de Montréal, Montréal, Canada*
9- *Department of Zoology, Ohio Wesleyan University, Delaware, Ohio, USA*
10- *School of Molecular and Life Sciences, Curtin University, Brand Drive, Bentley, WA 6102, Australia*

* Corresponding author. E-mail: *jeremie.souchet@gmail.com*

Author contributions

JS, HP and FA contributed to experimental design and logistics. JS, CB, ED, HLC, and MP conducted experiments. JS and EJG conducted statistical analyses. JS, EJG, and FA drafted the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

4.3. Introduction

The effects of global warming on biodiversity are a growing concern at all levels of ecosystem functioning (Parmesan, 2006; Scheffers et al., 2016; Pecl et al., 2017). Range shifts toward higher latitudes and elevations are now commonly observed as organisms and populations alter their geographic distributions to track their thermal requirements (Sinervo et al., 2018). The rapid contemporary pace of global warming has resulted in a process coined "thermophilization", where community compositions shift in favour of warm-affinity species (Devictor et al., 2012; De Frenne et al., 2013; Stuart-Smith et al., 2015; Fadrique et al., 2018). Many organisms, however, are unable to track the temperature changes due to habitat fragmentation, slow dispersal, and long life spans (Sinervo et al., 2010; Bertrand et al., 2016; Lenoir et al., 2020), leading to climatic debts in community responses to macroclimate warming (Devictor et al., 2012; Alexander et al., 2018; Zellweger et al., 2020). Consequently, many organisms' and populations' ecological requirements are rapidly becoming mismatched with their thermal environment, potentially leading to local extirpation (Whitfield et al., 2007; Sinervo et al., 2010; Bestion et al., 2015a), unless successful range shifts occur towards more suitable thermal latitudes or altitudes (Lenoir et al., 2020). While altitudinal range shifts have been reported in a wide range of organisms (Walther et al., 2002; Parmesan and Yohe, 2003; Bässler et al., 2013; Pauchard et al., 2016; Freeman et al., 2018), moving up in elevation exposes organisms to lower oxygen availability, potentially impacting reproduction, dispersal and overall range shift dynamics (Powell and Hopkins, 2010; Storz et al., 2010; Jacobsen, 2020). Moreover, under IPCC's current projection (Mokhov and Eliseev, 2012), temperatures are expected to keep rising, even at high elevation (Jacobsen, 2020). This may eventually expose newly established populations at high-elevation to the double constraints of low oxygen availability and sub-optimal temperatures.

Ectotherm physiology and behavioural processes are strongly dependent on environmental temperatures (Huey and Stevenson, 1979; Angilletta et al., 2002; Gillooly et al., 2002; Deutsch et al., 2008; Huey et al., 2012), and therefore they are often utilized as a model in climate change related experiments and studies (Pen et al., 2010; Sinervo et al., 2010; Bestion et al., 2015a; Dahlhoff et al., 2019). Our current study focuses on embryo development and hatching success, because the production of viable and fit offspring is a required condition for successful dispersal and population establishment in novel environments (Baguette et al., 2012). Further, incubation temperature is the main driver of embryonic development and hatchling phenotype in ectotherms such as reptiles (Deeming and Ferguson, 1991; Deeming, 2004; Booth, 2006; Goodman, 2008; Warner, 2014; Refsnider et al., 2019). The influence of incubation temperatures (notably above the optimal range;

Andrews and Schwarzkopf, 2012) on hatchling phenotype is especially well known in reptiles, affecting development, sex determination, incubation duration, body size, growth rate, locomotor performance, cognitive abilities, and post-natal behaviour (Shine, 2004; Deutsch et al., 2008; Daufresne et al., 2009; Gardner et al., 2011; Sheridan and Bickford, 2011; Bestion et al., 2015a; Cunningham et al., 2017; Noble et al., 2018; Pellerin et al., 2019; Refsnider et al., 2019). Additionally, the effects of low oxygen availably on physiology have attracted recent attention, including in the context of altitudinal range shifts driven by climate change (Powell and Hopkins, 2010; Storz et al., 2010; Jacobsen, 2020). For instance, it was shown that common wall lizards (*Podarcis muralis*, Laurenti, 1768) transplanted to high elevation enhanced oxygen-carrying capacity by increasing hematocrit and blood hemoglobin concentration, though transplanted lizards still suffered a reduction in running endurance (Gangloff et al., 2019). Further, reptile embryos exposed to hypoxia increased heart rates in some studies (Du et al., 2010a; Souchet et al., 2020), while in other cases hypoxia leads to decreased heart rates and cardiac hypertrophy (Cordero et al., 2017a; Kouyoumdjian et al., 2019). In viperine snakes, exposure to hypoxia during incubation resulted in hatchlings that were smaller in body size and slower swimmers (a proxy for predator avoidance and food acquisition in snakes; Jayne and Bennett, 1990; Kingsolver et al., 2001) compared to their siblings incubated at lower elevation (Souchet et al., 2020).

Recent work suggests that the interaction of high temperature and oxygen limitation will alter embryo development (Jackson, 2007; Flewelling and Parker, 2015; Smith et al., 2015; Gangloff and Telemeco, 2018; Hall and Warner, 2020; Li et al., 2020). Here we experimentally tested the effect of high temperature (*i.e.*, current populations caught in the climatic debt), low oxygen availability (*i.e*., populations having shifted their range in altitude in the near future), and the combined effect of high temperature and low oxygen (*i.e*., extreme high elevation in the year 2070) on the development, hatching success and hatchling phenotype in a temperate snake species (viperine snake, *Natrix maura,* Linnaeus 1758). This is a first step toward assessing the colonization potential to high elevation in a potentially upward-migrating species. We used a split-clutch design and incubated eggs in four ecologically relevant treatments: 1) oxygen availability at native elevation (normoxia; 436m above sea level [ASL]) and 32°C incubation temperature (*i.e*., populations lagging behind climate change); 2) low oxygen availability (2877 m ASL), 24°C incubation (*i.e*., range shifted in altitude); 3) low oxygen availability, 32°C incubation (*i.e*., high altitude in the year 2070), and 4) a normal oxygen availability, 24°C incubation control treatment (*i.e*., recent past conditions). We monitored embryo heart rates (a proxy for metabolism and cardiovascular function; Crossley and Burggren, 2009) and egg mass throughout the incubation and measured fitness-relevant aspects of hatchling phenotypes (body size and swimming performance) at hatching. This factorial design allowed us to tease apart

the individual and combined effects of incubation temperature and oxygen levels on embryo development and hatchling phenotypes. Based on our previous work (Souchet et al., 2020), we expected that the extreme high elevation will decrease egg mass and induce higher heart rates throughout incubation. Moreover, we predicted incubation duration will be shorter and the hatchlings will be smaller in high-elevation hypoxia. Further, we predicted that the combined constraints imposed by higher metabolic rates induced by warmer incubation temperature (Huey, 1982; Angilletta, 2009; Dillon et al., 2010) and oxygen limitation on juveniles will result in a reduced performance capacity. Specifically, we predicted that embryos incubated at extreme high elevation will produce slower-swimming juveniles and that embryos developing under conditions of both high temperature and high-elevation hypoxia will be the slowest. Finally, we further partitioned treatment groups to test whether the effects of embryonic environment would be ontogenetically stable even after hatchlings were transplanted to the alternative elevation.

4.4. Materials and methods

4.3.1. Experimental design

We captured 17 gravid females viperine snake along the banks of the Lez River (Department of Ariège, France), between May and July 2017. This aquatic species (Vacher and Geniez, 2010) has been recorded up to 1000 m above sea level [ASL] in France (Aubret et al., 2015; Pottier, 2016) and 1500 m ASL in Spain (Martinez-Rica and Reiné-Viñales, 1988; Santos, 2015). The viperine snake has been exposed to fluctuating temperatures and has migrated along the elevational gradient throughout its evolutionary history, colonizing mountainous environments repeatedly in conjunction with historical warming and cooling cycles (Gómez and Lunt, 2007). Capture sites spanned from 412m to 715m ASL. Each female was maintained in the Station d'Ecologie Théorique et Expérimentale du Centre National de la Recherche Scientifique (SETE-CNRS; 42.958394 N, 1.086440 E) and laid a single clutch for a total of 205 eggs between 21 June 2017 and 22 July 2017 (mean clutch size± SD = 11.9 ± 4.9 eggs). Three eggs were infertile, leaving 202 eggs for the experiment. All females were returned to their exact site of capture within two weeks of egg-laying.

We first investigated how temperature (cool temperature at constant 24°C; and hot temperature at constant 32°C) and oxygen availability interact to influence embryonic development. Oxygen treatments were normoxia at the SETE-CNRS (low elevation at 436 m ASL, 95% sea-level equivalent O_2 availability, PO_2 ~20.1 kPa) and high-elevation hypoxia at the Observatory Midi-Pyrénées of the Pic du Midi de Bigorre (42.936389 N, 0.142472 E, above current range limits at 2877 m ASL, 72% sea-

level equivalent O_2 availability, PO_2 ~15.3 kPa). This difference in elevation results in a decrease in atmospheric pressure, with associated reduction in the partial pressure of gases, including oxygen, carbon dioxide, and water vapor (Millet and Debevec, 2020; Richalet, 2020). Most relevant to our hypotheses is the 25% reduction in oxygen availability at the Pic du Midi de Bigorre lab in comparison to sea level (Bouverot, 2012). Eggs were weighed using a digital scale (to the nearest 0.01 g) within 12 hours of oviposition and individually marked for identification with a pencil. We used a split-clutch design and allocated eggs to four incubation treatments within 24 hours of oviposition (Figure 1): Low Elevation and Cool temperature (LEC; normoxia at constant 24°C), Low Elevation and Hot temperature (LEH; normoxia at constant 32°C), Extreme High Elevation and Cool temperature (EHEC; hypoxia at constant 24°C) and Extreme High Elevation and Hot temperature (EHEH; hypoxia at constant 32°C). Because egg mass influences both embryo metabolism and hatching phenotype (Nelson et al., 2004; Aubret, 2013a), and egg mass varied among clutches (Kruskal-Wallis test: H= 148.42, Df= 15, P < 0.001), eggs were ranked within each clutch from lightest to heaviest and alternately assigned to treatments in order to ensure no difference in egg mass between treatments (Kruskal-Wallis test: H=0.151, Df= 3, P = 0.985). LEC, LEH, EHEC and EHEH treatment quarter-clutches were placed in a plastic container (20 cm x 15 cm x 5 cm) on a 2 cm layer of wet vermiculite (1:5 water to vermiculite by volume) and incubated in four identical incubation chambers (ExoTerra Model PT-2445, Rolf C. Hagen Inc., USA). Water bowls placed within each incubator, directly under the incubator's fan, ensured high levels of humidity throughout incubation (indicated by condensation on the incubator walls).

Out of 202 eggs, 177 embryos from 16 females successfully hatched (87.6% hatching success rate) while 25 died at various stages during incubation. Another 17 neonates died shortly after hatching (between 24 hours to two weeks). We measured morphology (Test2, Figure1; see below) first on all 177 hatchlings at their incubation location (low or extreme high elevation). Our experimental design allowed us to measure the effects of temperature and hypoxia during incubation on juvenile development and performance. It also allowed us to measure the short-term effects on juvenile development and performance in acute high-elevation hypoxia after translocation to extreme high elevation. In order to assess these questions, at nine days post-hatching (after all yolk was assimilated; Ji et al., 1999) we measured morphology and swimming performance (Test 3, Figure1; see below) first on all 160 hatchlings at their incubation elevation (low or extreme high elevation). After this first measurement, half of the hatchlings in the LEC and LEH treatments were transferred to extreme high elevation while half of the hatchlings from the EHEC and EHEH treatments were brought down to the low elevation site. All juveniles were then tested for swimming performance and morphology at 11 days, 25 days, and at 40 days post-hatching (respectively one day, two weeks

and one month of acclimation for transferred juveniles; Test 4, Figure 1). Once tests were completed, young snakes were fed with small dead minnows (0.5 g to 1 g) and released between 42 and 45 days post-hatching at the maternal capture site.

4.3.2. Egg mass and heart rate measurements

We weighed each egg using a digital scale (to the nearest 0.01 g) within 12 hours of oviposition, and then every 7 days until hatching (Figure 1; Test 1). Embryo heart rates were first measured at 7 days of incubation and then every 7 days until hatching (Figure 1; Test 1) at the same temperature as incubation. To measure embryo heart rates, we used the Buddy digital egg monitor (MK2, Avitronics, Cornwall, UK) under the standardized protocol described for eggs (Aubret, 2013a; Cordero et al., 2017a; Souchet et al., 2020). Each egg was gently placed onto the sensor pad for heart rate reading (a stable reading was obtained after approximately 30 seconds) and then returned to its clutch. All eggs were only briefly (≤ 1min) placed in the digital egg monitor to mitigate potential temperature changes owing to exposure to infrared sensors (Sartori et al., 2015; Hulbert et al., 2017). Heart rates can be influenced by a variety of factors (Clark et al., 2006; Du et al., 2010a) and are linked to metabolic rate in some circumstances (Kouyoumdjian et al., 2019), though this relationship may become less clear especially late in development (Sartori et al., 2017). We also calculated the total number of heart beats (THB) of embryos throughout embryonic development using the formula THB = average heart rate × total minutes of developmental duration (Du et al., 2009, 2011).

4.3.3. Hatchling measurements

Hatching occurred between 8 August 2017 and 29 September 2017 (Figure 1; Test 2) and hatchlings were individually marked for identification with a medical cauterizer (Model HIT0, Bovie, USA) on the ventral scales (Winne et al., 2006) within 24 hours of emergence. Hatchlings were weighed using a digital scale (to the nearest 0.01 g), measured for snout-vent length (SVL) and total body length (TL) using a measuring tape (to the nearest 0.1 cm), and sexed via hemipene eversion. Since sex is genetically determined in snakes, we did not expect an effect of treatment on sex determination, but tested for differential effects between the sexes in developing embryos which could result in skewed hatchling sex ratios. We calculated body condition as the residual of the $\log_{10}$-mass on $\log_{10}$-SVL linear regression at hatching day. Finally, we weighed the yolk leftover in the eggshell (residual egg yolk) using a digital scale (to the nearest 0.01 g). Juveniles were housed together by hatching date in plastic containers (15 cm x 10 cm x 5 cm) with a water dish, shelter and paper towel as a substrate in incubation chambers (ExoTerra Model PT-2445, Rolf C. Hagen Inc. Canada) set at constant 20°C. Our

experience with viperine snake shows that cooler temperatures (below thermal optimum for performance or preferred temperatures) results in higher juvenile survivorship (93% survival at one month in this species; J.S. and F.A. unpubl. data). Juveniles were measured again at 9 days, 11 days, 25 days and 40 days post-hatching for SVL, TL and body mass prior to performance testing.

4.3.4. Swimming performance

For this test, we were interested in measuring the maximal swimming speed to evaluate the potential limitation of hypoxia on this ecologically-relevant performance. To estimate the swimming speed we used a procedure that has been validated for snakes (Shine and Shetty, 2001; Aubret, 2004; Aubret et al., 2005), modified effectively for juveniles (Souchet et al., 2020). A high-definition wide-angle digital camera (25 fps, Sony Model HDR-XR160E, Sony Corporation) was fitted above a linear swimming track (100 cm x 20 cm x 20 cm) and used to record swimming trials. The tank was filled to a depth of 5 cm with water maintained at 25°C using aquarium heaters. A standard testing temperature of water at 25°C was used because it approximates the optimal temperature for swimming speed of the viperine snake (Hailey and Davies, 1986a; Aubret et al., 2015). At 9 days, 11 days, 25 days and 40 days post-hatching, each snake was acclimated to 25°C for 30 minutes and swam 10 consecutive lengths. Raw data were extracted from video files by measuring swimming speed ($cm.s^{-1}$) for each length (ten per individual and day of measurement) with the software Tracker (Brown, 2019). The fastest performance from all trials was utilized for swimming analysis. Analyzing the average swimming speed of the 10 trials gives the same qualitative results; however, since our focus is performance capacity, we include results for maximum swimming speed here.

4.3.5. Data analysis

We first assessed the influence of the temperature and elevation of incubation, and time of development on egg mass and embryo heart rate (Test 1). We used linear mixed-effect models, including as main effects the temperature of incubation (cool: 24°C;warm: 32°C), the elevation of incubation (low elevation: normoxia; extreme high elevation: high-elevation hypoxia), the age at measurement (0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 days post-laying) treated as a categorical effect to account for the expected nonlinear response over time (Burggren and Warburton, 1994; Cordero et al., 2017a; Sartori et al., 2017), and all three- and two-way interactions. We then assessed the influence of temperature and elevation of incubation on eight measures of hatchling phenotype at hatching (Test 2): survival to hatching, sex, incubation time, total number of heartbeats (THB), body mass, body size (SVL), body condition, and residual egg yolk. We used linear mixed-effect models,

including in all models the same main effects of the temperature of incubation (cool: 24°C; warm: 32°C), the elevation of incubation (low elevation: normoxia; extreme high elevation: high-elevation hypoxia), and interactions as above. Finally, we assessed the influence of the temperature and elevation of incubation on swimming performance of juveniles (Test 3 and Test 4). We used linear mixed-effect models, including as the main effects the temperature of incubation (cool: 24°C; warm: 32°C), the elevation of incubation (low elevation: normoxia; extreme high elevation: high-elevation hypoxia), the age at measurement (9, 11, 25 and 40 days post-hatching), the location of test (low elevation or extreme high elevation), and all four-, three- and two-way interactions. We also include as covariates the total body length (TL) and the sex of juveniles.

To account for the non-independence of siblings we included the clutch of origin as a random effect (intercept) in all models. In models for which we measured individuals repeatedly (egg mass, embryo heart rates, and swimming performance), we also included individual as a random effect (intercept), nested within clutch. We used type III sums of squares to assess the significance of main effects, incorporating a Kenward-Roger denominator degree of freedom approximation (Kenward and Roger, 1997). We also conducted a pairwise comparison of least-squares means and adjusted p-values for multiple comparisons with the Tukey method. All analyses were conducted with the lme4 package (Bates et al., 2014) and the emmeans package (Lenth, 2016) and figures were made with the ggplot2 package (Wickham, 2016) in the programming language R 3.6.1 (R Development Core Team, 2019).

4.5. Results

4.4.1. Test 1: Egg mass and embryonic heart rates

The main effects of elevation, temperature, and time of measurement (days post-laying) and their interaction significantly altered egg mass trajectories (Table 1, Figure 2A). Eggs incubated at 24°C (*i.e*., LEC and EHEC) gained mass for 35 days post-oviposition before decreasing (Table 1, Figure 2A), while the mass of eggs incubated at 32°C (*i.e*., LEH and EHEH) decreased throughout the incubation (Table 1, Figure 2A). The post-hoc comparison of least-squares means from the model (Table S1) indicates that eggs masses were similar in eggs incubated at the same incubation temperature whatever the oxygen availability. Nevertheless, at the last day of measurement (28 days post-hatching for LEH and EHEH; 63 days post-hatching for LEC and EHEC) the egg mass of both treatments in extreme high elevation were significantly less than those of the low elevation treatments (Figure 2A; Table S1). Eggs incubated at 24°C (*i.e*., at LEC and EHEC) maintained higher

mass (mean difference ± SE: 0.36 ± 0.08 g) across the incubation period compared to eggs incubated at 32°C (*i.e*., at LEH and EHEH).

Heart rate trajectories were also significantly altered by elevation and temperature, time of measurement (days post-laying), and by the interaction between temperature and both elevation and time of measurement (Table 1, Figure 2B). Heart rates of embryos incubated at 32°C (*i.e*., at LEH and EHEH) increased rapidly during the first 7 days of incubation before decreasing for the remainder of the incubation (Table 1, Figure 2B), while embryos incubated at 24°C (*i.e*., at LEC and EHEC) maintained stable heart rates throughout incubation (Table 1, Figure 2B). Post-hoc comparison of least-squares means (Table S1) indicates that embryos from LEH treatment maintained higher heart rates (mean difference ± SD: 7.25 ± 1.17 bpm) across the incubation period compared to EHEH. Further, eggs in the EHEH treatment exhibited much higher heart rates (mean difference ± SE: 33.39 ± 1.00 bpm) compared to both embryo groups incubated at 24°C (*i.e*., at LEC and EHEC).

4.4.2. Test 2: Hatching success and morphological measurements

Hatching success of embryos was dependent on incubation temperature and the interaction with the elevation (LEC = 90.2%, LEH = 91.8%, EHEC = 94.1% and EHEH = 74.5%; Table 2). Post-hoc comparison of least-squares means (Table S2) indicates that hatching success differed between eggs in the EHEC and EHEH treatments. We observed than of the 25 dead embryos, half of them are from EHEH. Moreover, in this treatment, 92% of the death appeared in the last stage of development. Elevation and temperature levels did not affect the hatchling sex ratio (LEC = 56.5%; LEH = 57.8%; EHEC = 47.9%; and EHEH = 42.1% females; Table 2). Incubation duration differed between embryos incubated in the four treatments as a function of temperature and its interaction with the elevation (Table 2, Figure 3A). All treatment groups are significantly different from each other (Table S2). Snakes in the LEC treatment incubated 2.25 ± 0.28 days longer than EHEC, EHEC treatment incubated 29.01 ± 0.29 days longer than EHEH, and EHEH treatment incubated 2.66 ± 0.30 days longer than LEH. Only the temperature of incubation affected THB of embryos (Table 2, Figure 3B). THB did not differ in embryos from the same incubation temperature (Table S2) and THB were greater in the cool incubation temperature treatments (*i.e*., LEC and EHEC) compared to warm (*i.e*. LEH and EHEH). Moreover, the residual egg yolk was also significantly affected by the temperature of incubation (Table 2, Figure 3F). Comparison of least-squares means from the model (Table S2) indicates that residual egg yolk was similar for the treatments within an incubation temperature (*i.e*., LEC *vs* EHEC and LEH *vs* EHEH), but that snakes in the LEC and EHEC treatments retained an average of 0.29 g (33.6%) more residual egg yolk compared to the LEH and EHEH treatments.

Elevation and temperature and their interaction influenced body mass and body size (SVL) at hatching (Tables 2 & S2, Figures 3C & 3D). Elevation and temperature influenced hatchlings' body condition (Tables 2 & S2, Figure 3E). In all cases, the two cool treatments (*i.e.*, at LEC and EHEC) did not significantly differ from each other. For the body mass at one day post-hatching, LEH treatment did not differ from either LEC or EHEC treatments. However, the EHEH treatment was 0.39 ± 0.12 g (13.2%) lighter compared to the three other treatments. For body size at one day post-hatching, cool-temperature treatments were 0.72 ± 0.17 cm (5.1%) longer than snakes in the LEH treatment, which in turn were 0.49 ± 0.18 cm (4.7%) longer than snakes in the EHEH treatment. Finally, the different treatments also influenced the body condition at one day post-hatching (Tables 2 & S2, Figure 3E), with snakes in the LEH treatment having a 35.4% higher body condition compared to the three other treatments.

4.4.3. Tests 3 & 4: Swimming performance

Globally, maximum swimming speed (Table 3) was influenced by the effect of incubation temperature (24°C and 32°C), the time of measurement (9, 11, 25, and 40 days post-hatching), and their interaction with test location (low elevation and extreme high elevation). Moreover, size positively influenced swimming speed within each treatment group, with longer snakes swimming faster (slope estimate ± SE: 2.71 ± 0.31; Table 3, Figure 4).

At nine days post-hatching, for the first swimming performance measurement (Test 3) conducted at the elevation of incubation, the post-hoc comparison of least-squares means (Table S3) indicates that maximum swimming speed was similar for both treatments at the cool incubation temperature (*i.e.*, LEC *vs* EHEC; Figure 5A). Juveniles from LEH treatments swam significantly faster (by 22.0%) than LEC and EHEC (Figure 5A; Table S3). Finally, the juveniles from the EHEH treatment swam significantly faster (by 10.4%) compared to the LEC treatment (Figure 5A; Table S3). After translocation to the opposite oxygen level treatment, maximum swimming speed was only significantly altered in the EHEH treatment at 25 days post hatching (Figure 5B; Table S3). That is, individuals translocated to low elevation (EHEH-LE) swam faster (by 18.7%) compared to siblings retained at extreme high elevation (EHEH-EHE). These results remained qualitatively unchanged when measuring swimming speed expressed as body length per second (analysis not shown).

The proportion of residual variance attributed to clutch was up to 67% (for egg mass) and the inclusion of this random effect significantly improved model fit for most traits measured (Table S4).

Siblings most strongly covaried for traits related to offspring size (egg mass and body mass at hatching) as well as developmental duration (incubation duration and total heart beats). Only heart rate and sex ratio were not influenced by significant maternal effects.

4.6. Discussion

Our study demonstrates the impact of high-elevation hypoxia coupled with temperature regime on development, physiology, and early-life performance in an oviparous ectotherm. Irrespective of oxygen availability during incubation, eggs incubated at cool temperature (*i.e*., LEC and EHEC) maintained higher mass and much lower heart rates throughout incubation compared to siblings incubated at a warmer temperature (Figure 2; Table 1). The longer incubation duration combined with reduced heart rate at the cool incubation temperature suggests a lowered metabolic rate (Table 1), as expected (Deeming and Ferguson, 1991; Deeming, 2004; Booth, 2006; Goodman, 2008; Warner, 2014). At warm incubation temperatures (*i.e*., LEH and EHEH), viperine snake embryos in extreme high-elevation hypoxia exhibited typical physiological adjustments to hypoxia found in other taxa, including reduced heart rate (Table 1, Figure 2; Laughlin, 1978; Monge and Leon-Velarde, 1991; Crossley and Altimiras, 2005; Crossley and Burggren, 2009; Du et al., 2011; Cordero et al., 2017a; b; Kouyoumdjian et al., 2019). Importantly, this trend was not exhibited in snakes incubated at extreme high elevation and low temperatures and furthermore is counter to that we demonstrated in our previous study conducted at an intermediate incubation temperature of 28°C (Souchet et al., 2020), suggesting that the this is an effect of combined increased metabolism and reduced oxygen availability. Reduced heart rates were observed only in embryos incubated at the warmer temperature and extreme high elevation. The interaction of temperature and oxygen availability also influenced other important fitness-related parameters, including offspring development times, hatching success, body size at birth, and swimming performance. Notably, the potential negative consequences of reduced oxygen availability were exacerbated by high incubation temperatures.

We observed the strongest effects on development in embryos incubated at extreme high elevation and at high temperature, suggesting that these factors interact to limit the functional capacity of ectotherms. Gas exchange in embryos is diffusion-limited, likely constraining their ability to compensate for reduced oxygen availability through increased oxygen transport capacity (Vitt and Caldwell, 2013). These effects are then exacerbated by the increased demand induced by high temperatures. Under conditions of high temperature and low oxygen availability, we expect reductions in maximal performance, limitations on physiological processes generally, and potentially

reductions of critical thermal limits (Gangloff and Telemeco, 2018). For example, recent work demonstrated that lizard embryos suffer a mismatch between oxygen supply and demand at high temperatures, which may serve as the proximal cause of death (Hall and Warner, 2020). Our results demonstrate for the first time these effects in snake embryos, in concordance with previous work studying embryonic development *in ovo* under varying temperatures and levels of oxygen availability in other reptile taxa (Birds: Vimmerstedt et al., 2019; Crocodiles: Iungman and Piña, 2013; Lizards: Flewelling and Parker, 2015; Smith et al., 2015; Li et al., 2020; Turtles: Liang et al., 2015). For example, embryos of the lizard *Podarcis muralis* increase incubation times in conditions of hypoxia when incubated at 28°C, but not at 24°C (Cordero et al., 2017a; Kouyoumdjian et al., 2019). In this study, we found that snake embryos incubated in warm temperature and in hypoxia were less likely to survive to hatching, especially because the last-stage embryos have higher oxygen demand (Dmi'el, 1970; Sartori et al., 2017), and, when they did survive, were smaller than snakes in other treatment groups (Table 2, Figure 3D). In accordance with previous work (Shine, 2004; Daufresne et al., 2009; Du et al., 2009; Gardner et al., 2011; Sheridan and Bickford, 2011; Noble et al., 2018; Refsnider et al., 2019), our results show that snakes incubated at warm temperatures were smaller and shorter than their counterparts, and hatched after fewer total heartbeats, regardless of oxygen availability (Table 2, Figure 3C & 3D). Moreover, there was less residual egg yolk in both warm treatments (*i.e.*, LEH and EHEH) compared to cool treatments (*i.e.*, LEC and EHEC) and yet these animals were also smaller, suggesting higher basal metabolic demands associated with high-temperature incubation may reduce growth efficiency (conversion of yolk to body mass). Hatchlings incubated at cool temperatures in hypoxia did not exhibit reduced body size or mass (Table 2; Figure 3C & 3D). This result demonstrates that reduced metabolic rates and increased ability to assimilate energy stores associated with cool temperatures mitigate the negative impacts of reduced oxygen availability (Jackson, 2007; Gangloff and Telemeco, 2018).

Swimming speed is an ecologically relevant trait important to predator avoidance and food acquisition in snakes (Jayne and Bennett, 1990; Kingsolver et al., 2001), that typically correlates (positively) with body length (Shine and Shetty, 2001; Aubret et al., 2015). Although this trend was found within each treatment group (Figure 4), it was not observed across treatments: snakes incubated under both hypoxia and high temperatures demonstrated the fastest swimming speeds compared to all other treatment groups, despite exhibiting the smallest body size on average (Table 3, Figure 4). Previous studies in other ectothermic species demonstrate that cool incubation temperatures produce faster swimmers (Shine, 1999; Angilletta and Dunham, 2003; Watkins and Vraspir, 2006; Gahm et al., 2020). At nine days post hatching, juveniles in this experiment did not follow this trend: juveniles from warm treatments (*i.e.*, LEH and EHEH) were faster swimmers than

their siblings from cool treatments (*i.e.*, LEC and EHEC) in both absolute and relative swimming speed. Most surprisingly, juveniles from the EHEH treatment were also faster than juveniles from LEH despite smaller body size and conditions of oxygen limitation (Figure 5A). One potential explanation for this finding is that warm incubation temperature and oxygen limitation may reduce the optimal temperature for performance (Gangloff and Telemeco, 2018). We suggest that juveniles from EHEH potentially reduced their optimal temperature for performance, thus swimming faster than the other groups at the test temperature of 25°C. Alternatively, exposure to hypoxia during development may have induced plastic changes in cardiovascular, muscular, or mitochondrial function to increase performance capacity (Eme et al., 2013; Sun et al., 2015; Galli et al., 2016). Further experiments directed towards quantifying the effects of incubation temperature on the entire thermal performance curve are necessary to fully characterize how incubation temperature influences both physiology and performance across a range of temperatures (Taylor et al., 2020).

After relocation to low elevation, juveniles from the EHEH treatment swam faster than siblings remaining at extreme high elevation, which maintained swimming speeds similar to other treatment groups measured at both extreme high and low elevation (Figure 5B). In birds and mammals the acclimation to high-elevation hypoxia can include an alteration of cardio-respiratory pathways, a modification of blood composition, and increased muscle performance (Monge and Leon-Velarde, 1991; Beall et al., 2002; Storz et al., 2004; Lague et al., 2016). Similar effects have been demonstrated in other reptiles (Iungman and Piña, 2013; González-Morales et al., 2015; Lu et al., 2015; Wearing et al., 2015; Jochmans-Lemoine and Joseph, 2018; Gangloff et al., 2019). These modifications may allow the maintenance of locomotor performance such as swimming. Furthermore, these physiological and anatomical changes due to development in chronic hypoxia serve to improve performance under normoxic conditions, similar to athletes training at high altitudes for competition at sea level (*e.g.*, Khodaee et al., 2016). Repeated measurements throughout ontogeny are necessary to quantify the time frame over which such compensatory mechanisms remain relevant (Mitchell et al., 2018a). Finally, although we cannot speculate on the adaptive value of such behavior at this stage, this response to a double constraint (high incubation temperature and low oxygen level) may be yet another case of informed dispersal in reptiles (as in *Zootoca vivipara,* Lichtenstein, 1823 and *Natrix maura*; Clobert et al., 2009; Bestion et al., 2015c; Aubret et al., 2016a): environmental clues may convey important information about the quality of the natal environment and foster dispersal behaviour and/or dispersal enhancing traits (*i.e.*, high locomotor performance). Importantly, the high level of observed maternal effects (Table S4) indicates the necessity of a split-clutch design in any experiment measuring similar traits in Squamate reptiles. Future work directed towards partitioning

this estimate into narrow-sense heritability and maternal effects will be important to predict the evolutionary response to novel conditions within populations, especially at the colonization front.

Our results suggest that even though body size, development, and physiology are altered, and hatching success is lowered, the majority of embryos developing in high-elevation hypoxia produced viable young snakes. Furthermore, these snakes were able to equal or exceed the swimming performance of snakes incubated under native conditions. We stress that the results of this experiment represent an extreme case of abiotic limitation (exposing developing embryos from low elevation to a 32°C incubation temperature and 72% sea-level equivalent O_2 availability). Such approaches are important to identify patterns among

4.7. Acknowledgements

We are grateful to the staff of Observatoire Midi-Pyrénées for logistical support in Pic du Midi de Bigorre as well as Isabel Verdaguer, Joaquim Soler and Zuleica Alonso for their help in the laboratory. This work was supported by the French Laboratory of Excellence project "TULIP" (ANR-10-LABX-41; ANR-11-IDEX-0002-02), INTERREG POCTEFA ECTOPYR (no. EFA031/15), and the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 752299. All experimental protocols (including animal collection, housing, experimentation and release) were approved by the DREAL Midi-Pyrénées (Direction Régionale de l'Environnement, de l'Aménagement et du Logement) and by the Préfectures of Ariège , Aude, Haute-Garonne, Hautes-Pyrénées and Pyrénées Orientales districts (Arrêté Préfectoral No. 2017-s-02 du 30 mars 2017) and ethical committee (APAFIS#16359-201808011445465 v4). All experiments were carried out in accordance with the approved guidelines. Animal caretakers and handlers were trained to use wildlife in scientific purposes (Decree No. 2013-118 du 01 février 2013 and approval of the Ministry of Agriculture under No. I-75-MNHN-F1-15 du 17 juin 2015).

4.8. Tables and figures

Table 1

Results of linear mixed-effect model testing for the effects of incubation temperature, incubation elevation, age at measurement (days post-oviposition), and their interaction on embryo developmental parameters in the snake *Natrix maura* (Test 1 Figure 2). The four incubation treatments are extreme high elevation at 24°C (EHEC; N = 51), extreme high elevation at 32°C (EHEH; N = 51), low elevation at 24°C (LEC; N = 51), and low elevation at 32°C (LEH; N = 49). Significant factors shown in bold with two (P < 0.01) or three (P < 0.001) asterisks.

	Egg mass	**Embryo heart rates**
Temperature	$F_{1,426.8}$= 2602.33; **P < 0.001*** **	$F_{1,1142.3}$= 635.04; **P < 0.001*** **
Elevation	$F_{1,283.7}$ = 8.02;**P = 0.003 ** **	$F_{1,1144.2}$ = 13.22; **P < 0.001*** **
Day	$F_{9,1130.0}$ = 30.92; **P < 0.001*** **	$F_{9,1179.6}$ = 11.82; **P < 0.001*** **
Temperature x Elevation	$F_{1,426.1}$ = 22.57;P = 0.500	$F_{1,1143.0}$ = 9.61; **P = 0.002 ** **
Temperature x Day	$F_{4,1138.8}$ = 97.60; **P < 0.001*** **	$F_{4,1194.3}$ = 7.21; **P = 0.007 ** **
Elevation x Day	$F_{9,1139.8}$ = 5.66; **P < 0.001*** **	$F_{9,1191.2}$ = 2.38;P = 0.123
Temperature x Elevation x Day	$F_{4,1138.5}$ = 5.75; **P < 0.001*** **	$F_{4,1191.8}$ = 0.24;P = 0.626

Table 2

Results of linear mixed-effect model testing for the effect of incubation temperature, incubation elevation, and their interaction on the juvenile traits at hatching in the snake *Natrix maura* (Test 2, Figure 3). The four incubation treatments are low elevation at 24°C (LEC; N = 46), low elevation at 32°C (LEH; N = 45), extreme high elevation at 24°C (EHEC; N = 48), and extreme high elevation at 32°C (EHEH; N = 38). Least-squares means ± SE are given. Significant factors shown in bold with one (P < 0.05) two (P < 0.01) or three (P < 0.001) asterisks.

	EHEC	**EHEH**	**LEC**	**LEH**	**Temperature effect**	**Elevation effect**	**Temperature x Elevation effect**
	LSM ± SE	*LSM± SE*	*LSM± SE*	*LSM± SE*	*F (dfn, dfd) P-value*	*F (dfn, dfd) P-value*	*F (dfn, dfd) P-value*
Hatching success	-	-	-	-	4.04 (1, 184.4) **P = 0.046 ***	2.43 (1, 185.2) P = 0.120	5.72 (1, 185.2) **P = 0.018 ***
Sex	-	-	-	-	0.09 (1, 163.7) P = 0.764	2.56 (1, 165.0) P = 0.112	0.22 (1, 166.9) P = 0.643
Incubation duration (days)	65.59 ± 0.42	36.59 ± 0.44	67.85 ± 0.43	33.93 ± 0.43	24415.32 (1, 158.4) **P < 0.001*** **	0.99 (1, 158.8) P = 0.321	145.83 (1, 159.1) **P < 0.001*** **
Total number of embryo heart beats	7650391 ±105481	6954688 ±111257	7689242 ±105997	7036319 ±106591	108.49 (1, 158.8) **P < 0.001*** **	0.86 (1, 159.4) P = 0.355	0.11 (1, 159.9) P = 0.744
Body mass (g) at hatching	2.87 ± 0.12	2.56 ± 0. 12	3.00 ± 0. 12	2.97 ± 0. 12	11.87 (1, 158.7) **P < 0.001*** **	24.39 (1, 158.7) **P < 0.001*** **	7.20 (1, 158.9) **P = 0.008 ** **
Body length (cm) at hatching	15.30 ± 0.20	14.09 ± 0.21	15.37 ± 0. 20	14.58 ± 0.20	67.12 (1, 158.8) **P < 0.001*** **	5.27 (1, 159.3) **P = 0.023 ***	2.83 (1, 159.8) P = 0.094
Body condition at hatching	-0.029 ± 0.010	-0.007 ± 0.010	-0.015 ± 0.010	0.031 ± 0.010	30.95 (1, 158.8) **P < 0.001*** **	18.47 (1, 159.3) **P < 0.001*** **	3.69 (1, 159.9) P = 0.056
Residual egg yolk (g)	0.655 ± 0.055	0.431 ± 0.061	0.815 ± 0.056	0.456 ± 0.56	34.61 (1, 60.8) **P < 0.001*** **	3.46 (1, 162.0) P = 0.065	1.81 (1, 163.4) P = 0.180

Table 3

Results of linear mixed-effect model testing for the effect of incubation temperature, incubation elevation, age at measurement (days post-hatching), test elevation, and their interaction on the maximum swimming performance in juveniles in the snake *Natrix maura* (Tests 3 & 4, Figure 4 & 5). Sex and total body length (TL) were included as covariates. The four incubation treatments are extreme high elevation at 24°C (EHEC; N = 41), extreme high elevation at 32°C (EHEH; N = 37), low elevation at 24°C (LEC; N = 41), and low elevation at 32°C (LEH; N = 41). Significant factors shown in bold with one (P < 0.05) two (P < 0.01) or three (P < 0.001) asterisks.

	F (dfn, dfd)	**P-value**
Sex	0.70 (1,145.5)	P = 0.401
Total body length (cm)	73.81 (1,169.2)	**P < 0.001 *****
Temperature	49.80 (1,156.4)	**P < 0.001 *****
Elevation	0.45 (1,165.5)	P = 0.501
Day	12.62 (3,500.6)	**P < 0.001 *****
Location of test	0.02 (1,442.8)	P = 0.964
Temperature x Elevation	0.002 (1,163.5)	P = 0.962
Temperature x Day	4.84 (3,506.1)	**P = 0.003 ****
Temperature x Location of test	0.01 (1,442.8)	P = 0.909
Elevation x Day	1.14 (3,487.8)	P = 0.331
Elevation x Location of test	9.80 (1,440.7)	**P = 0.002 ****
Day x Location of test	13.85 (2,460.9)	**P < 0.001 *****
Temperature x Elevation x Day effect	0.18 (3,487.1)	P = 0.913
Temperature x Elevation x Location of test	0.04 (1,446.9)	P = 0.839
Temperature x Day x Location of test	3.25 (2,457.5)	**P = 0.040 ***
Elevation x Day x Location of test	0.04 (2,456.9)	P = 0.961
Temperature x Elevation x Day x Location of test	0.79 (2,456.6)	P = 0.456

1

Figure 1

Experimental design. Eggs were collected from gravid females (represented by different colors) sampled from low-elevation population of the snake *Natrix maura* in the foothills of the Pyrenees, France (412 m to 715 m ASL). Within 24hr of oviposition clutches were evenly split into four groups of eggs with similar average egg mass. For each clutch two quarter-clutches were transplanted to the extreme high elevation site (2877 m ASL, Observatoire Midi-Pyrénées du Pic du Midi de Bigorre), with one quarter-clutch incubated at 24°C and the other at 32°C. The two remaining quarter-clutches were incubated at 24°C and at 32°Cat the low-elevation site (436 m ASL, Station d'Ecologie Théorique et Expérimentale du Centre National de la Recherche Scientifique). Egg mass and embryo heart rate were measured throughout incubation (Test 1). At hatching a number of phenotypic traits were measured in juveniles (Test 2). All hatchlings were first tested for swimming performance in the environment their eggs were incubated (Test 3). Each treatment was then again split in half with half of each treatment group translocated to the alternative environment for additional swimming measures (Test 4). Snake color represents incubation temperature treatment (cool or warm) and snake pattern represents incubation elevation treatment (low or extreme high elevation).

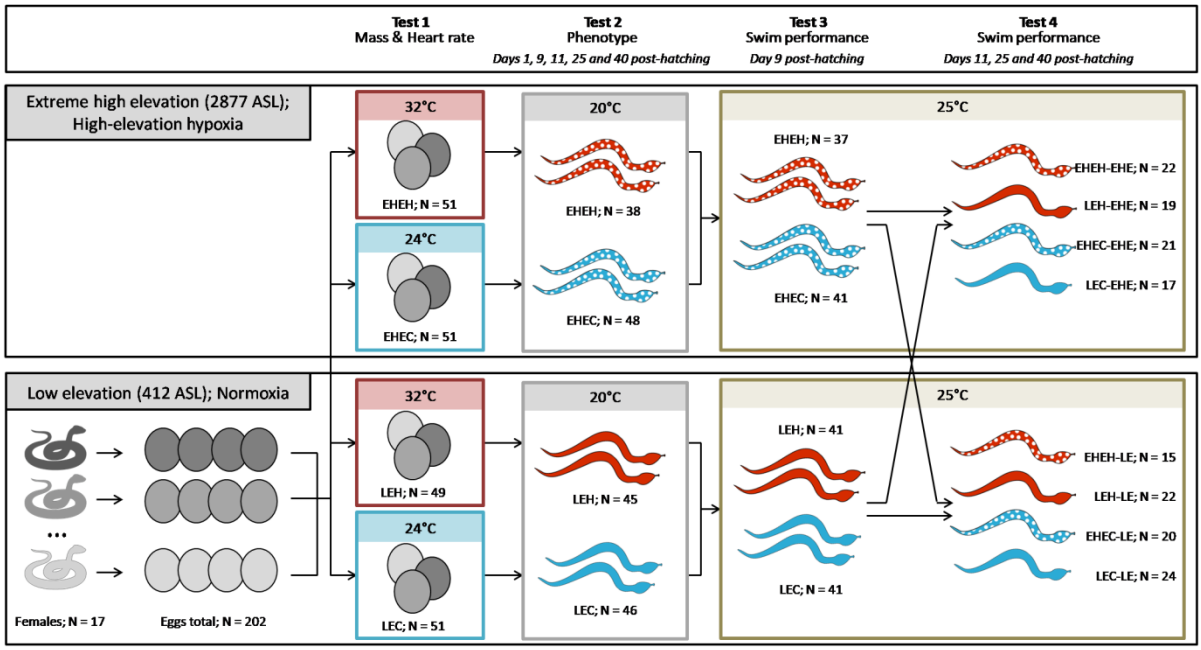


Figure 2

Egg mass (A) and embryo heart rate (B) measured at the same temperature as the incubation temperature through incubation duration in the snake *Natrix maura* at extreme high elevation at 24°C (EHEC; N = 51; blue triangle), extreme high elevation at 32°C (EHEH; N = 51; red triangle), low elevation at 24°C (LEC; N = 51; blue circle), and low elevation at 32°C (LEH; N = 49;red circle). Least-squares means ± SE estimated by linear mixed-effect models are plotted.

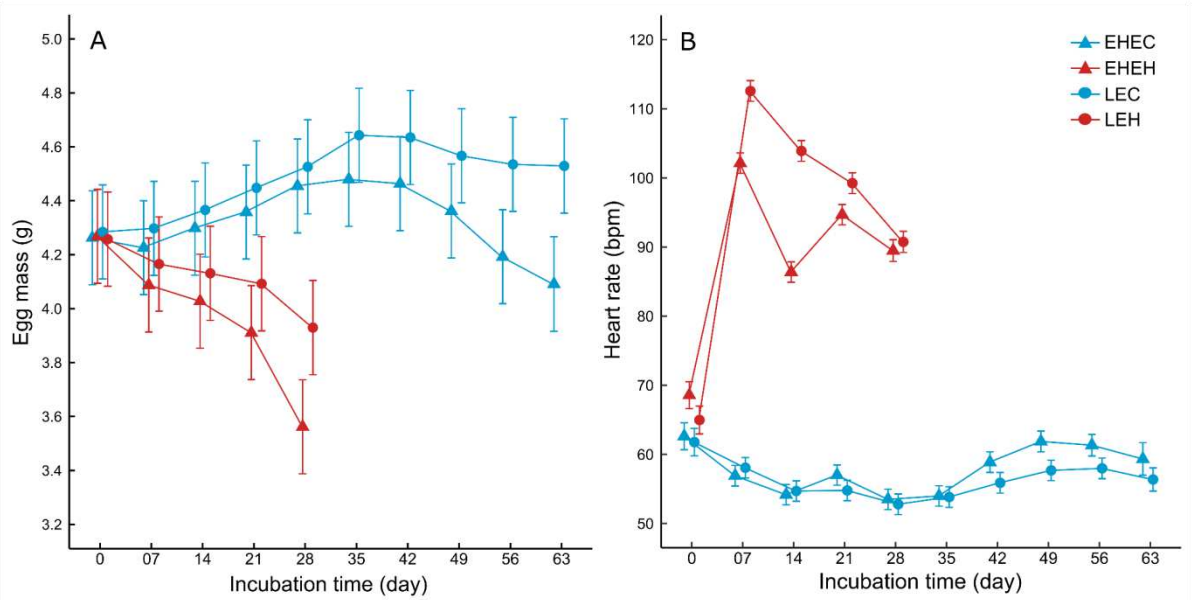


Figure 3

Hatching phenotypes in juveniles of the snake *Natrix maura:* incubation duration (A), total number of embryo heartbeats (B), body mass (C), body size (D), body condition (E), and residual egg yolk (F) for each incubation treatment: low elevation at 24°C (LEC; N = 46; blue circle), extreme high elevation at 24°C (EHEC; N = 48; blue triangle), low elevation at 32°C (LEH; N = 45; red circle), and extreme high elevation at 32°C (EHEH; N = 38; red triangle).Least-squares means ± SE estimated by linear mixed-effect models are plotted. Significant differences between least-squares means are shown with one ($P < 0.05$) or three ($P < 0.001$) asterisks.

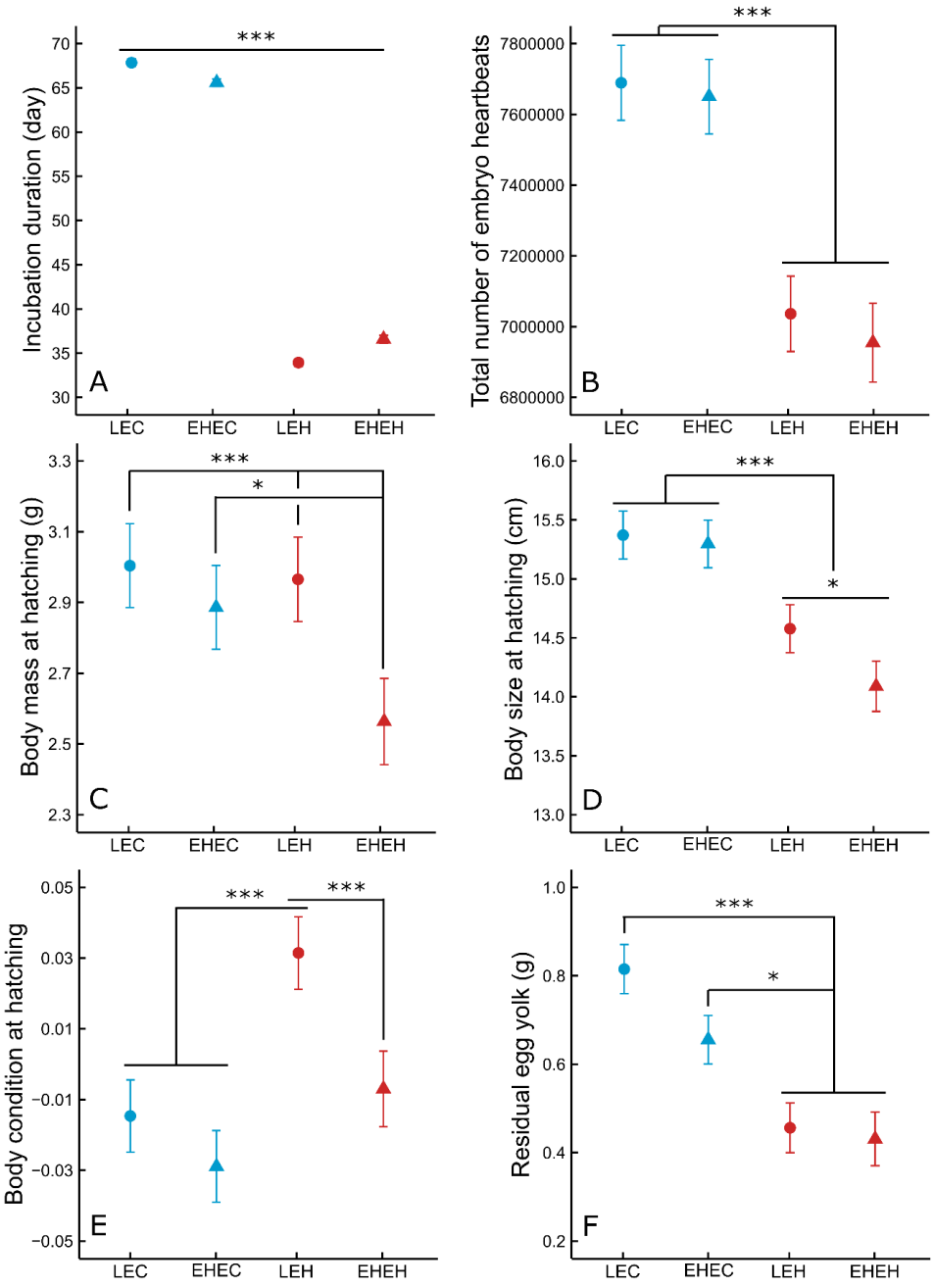


Figure 4

Maximum swimming speed as a function of body length in juveniles of the snake *Natrix maura* incubated in four treatments: low elevation at 24°C (LEC; N = 41; blue circle), extreme high elevation at 24°C (EHEC; N = 41; blue triangle), low elevation at 32°C (LEH; N = 41; red circle), and extreme high elevation at 32°C (EHEH; N = 37; red triangle). Raw data for each individual are plotted with regression lines and 95% CI.

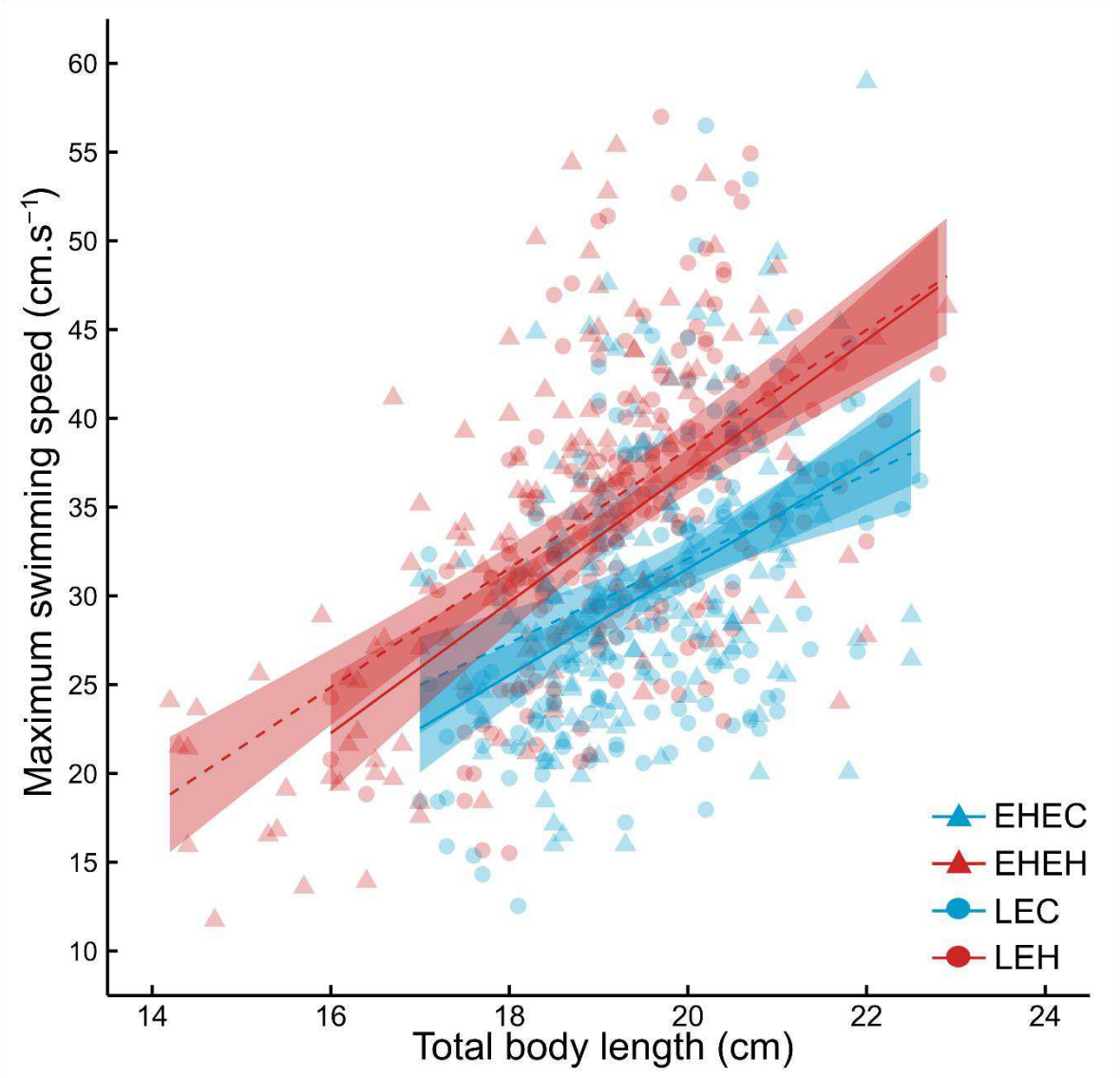
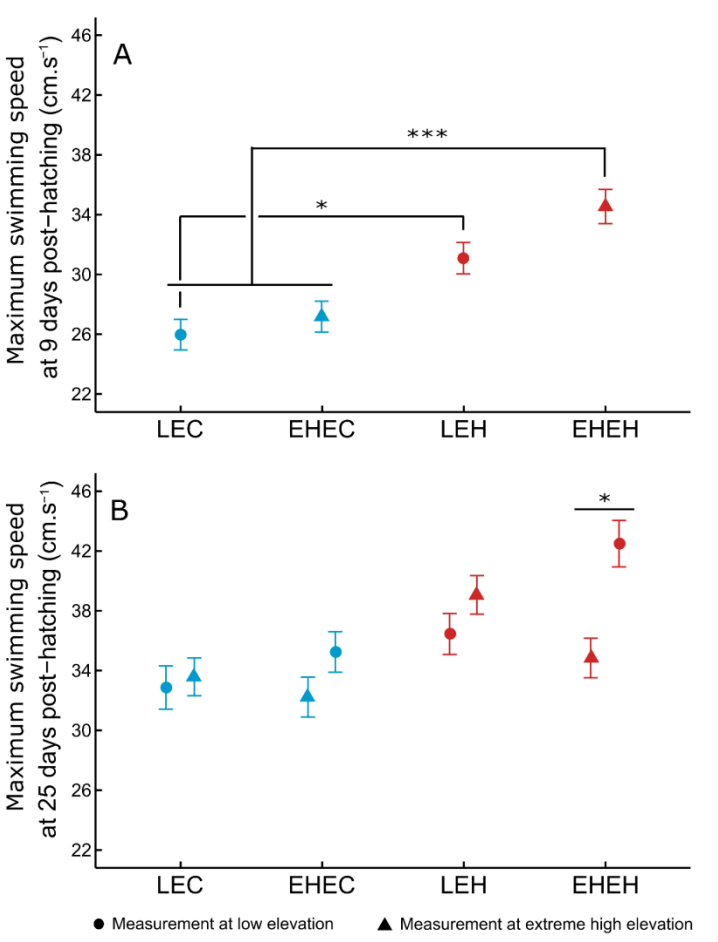


Figure 5

Maximum swimming speed in juveniles of the snake *Natrix maura* for each incubation treatment. First at 9 days post-hatching (A) at elevation of incubation (low elevation or extreme high elevation) for all juveniles of the four incubation treatments: low elevation at 24°C (LEC; N = 41; blue circle) extreme high elevation at 24°C (EHEC; N = 41; blue triangle), low elevation at 32°C (LEH; N = 41; red circle),and extreme high elevation at 32°C (EHEH; N = 37; red triangle). Second, at 11 days post-hatching (B) at the same elevation as incubation for half of the juveniles: low elevation at 24°C (LEC-LE; N = 24; blue circle), extreme high elevation at 24°C (EHEC-EHE; N = 21; blue triangle),low elevation at 32°C (LEH-LE; N = 22; red circle), and extreme high elevation at 32°C (EHEH-EHE; N = 22; red triangle). Also at 11 days post-hatching (B) at opposite elevation as incubation for the other half of the juveniles: low elevation at 24°C (LEC-EHE; N = 17; blue triangle), extreme high elevation at 24°C (EHEC-LE; N = 20; blue circle), low elevation at 32°C (LEH-EHE; N = 19; red triangle), and extreme high elevation at 32°C (EHEH-LE; N = 15; red circle). Least-squares means ± SE estimated by linear mixed-effect models are plotted. Significant differences between least-squares means are shown with one (P < 0.05) or three (P < 0.001) asterisks.



Supplementary table 1

Results of the pairwise comparison of least-squares means and adjusted p-values for multiple comparisons with the Tukey method from the linear mixed-effect model testing for the effect of incubation temperature, incubation elevation, age at measurement (day post hatching), and their interaction on embryo traits in the snake *Natrix maura*. The four incubation treatments are extreme high elevation at 24°C (EHEC; N = 51), extreme high elevation at 32°C (EHEH; N = 51), low elevation at 24°C (LEC; N = 51), and low elevation at 32°C (LEH; N = 49). Significant differences between least-squares means shown in bold with one (P <0.05) two (P < 0.01) or three (P < 0.001) asterisks.

	Egg mass			**Embryo heart rate**		
	Estimate	*SE*	*P-value*	*Estimate*	*SE*	*P-value*
EHEC D0 - EHEH D0	-0.005	0.090	1.000	-5.941	2.650	0.971
EHEC D0 - LEC D0	-0.022	0.090	1.000	0.851	2.676	1.000
EHEC D0 - LEH D0	0.005	0.091	1.000	-2.348	2.703	1.000
EHEH D0 - LEC D0	-0.016	0.090	1.000	6.792	2.675	0.868
EHEH D0 - LEH D0	0.010	0.091	1.000	3.593	2.702	1.000
LEC D0 - LEH D0	0.027	0.091	1.000	-3.199	2.726	1.000
EHEC D0 - EHEC D7	0.037	0.040	1.000	5.708	2.352	0.920
EHEH D0 - EHEH D7	0.180	0.040	**0.005 ****	-33.564	2.343	**< 0.001 *****
LEC D0 - LEC D7	-0.013	0.041	1.000	3.706	2.372	1.000
LEH D0 - LEH D7	0.092	0.041	0.971	-47.589	2.411	**< 0.001 *****
EHEC D7 - EHEH D7	0.138	0.090	1.000	-45.213	1.976	**< 0.001 *****
EHEC D7 - LEC D7	-0.072	0.091	1.000	-1.150	1.976	1.000
EHEC D7 - LEH D7	0.061	0.091	1.000	-55.645	1.986	**< 0.001 *****
EHEH D7 - LEC D7	-0.210	0.090	0.949	44.063	1.965	**< 0.001 *****
EHEH D7 - LEH D7	-0.078	0.091	1.000	-10.432	1.975	**< 0.001 *****
LEC D7 - LEH D7	0.132	0.091	1.000	-54.494	1.975	**< 0.001 *****
EHEC D7 - EHEC D14	-0.072	0.040	0.999	2.747	1.975	1.000
EHEH D7 - EHEH D14	0.060	0.041	1.000	15.755	1.964	**< 0.001 *****
LEC D7 - LEC D14	-0.068	0.041	1.000	3.367	1.964	1.000
LEH D7 - LEH D14	0.034	0.041	1.000	8.671	1.995	**0.009 ****
EHEC D14 - EHEH D14	0.271	0.090	0.528	-32.205	1.965	**< 0.001 *****
EHEC D14 - LEC D14	-0.068	0.091	1.000	-0.530	1.966	1.000
EHEC D14 - LEH D14	0.167	0.091	0.999	-49.721	1.986	**< 0.001 *****
EHEH D14 - LEC D14	-0.338	0.091	0.095	31.675	1.965	**< 0.001 *****
EHEH D14 - LEH D14	-0.103	0.091	1.000	-17.516	1.986	**< 0.001 *****

LEC D14 - LEH D14	0.235	0.091	0.847	-49.191	1.986	**< 0.001** ***
EHEC D14 - EHEC D21	-0.060	0.041	1.000	-2.838	1.955	1.000
EHEH D14 - EHEH D21	0.117	0.041	0.634	-8.294	1.955	**0.014** *
LEC D14 - LEC D21	-0.082	0.041	0.996	-0.082	1.964	1.000
LEH D14 - LEH D21	0.039	0.042	1.000	4.660	2.006	0.953
EHEC D21 - EHEH D21	0.447	0.090	**0.001** ***	-37.661	1.946	**< 0.001** ***
EHEC D21 - LEC D21	-0.090	0.091	1.000	2.226	1.956	1.000
EHEC D21 - LEH D21	0.266	0.091	0.597	-42.224	1.976	**< 0.001** ***
EHEH D21 - LEC D21	-0.537	0.091	**< 0.001** ***	39.887	1.956	**< 0.001** ***
EHEH D21 - LEH D21	-0.181	0.091	0.995	-4.563	1.977	0.957
LEC D21 - LEH D21	0.356	0.091	0.059	-44.450	1.986	**< 0.001** ***
EHEC D21 - EHEC D28	-0.097	0.041	0.937	3.511	1.955	0.999
EHEH D21 - EHEH D28	0.349	0.041	**< 0.001** ***	5.196	2.024	0.852
LEC D21 - LEC D28	-0.078	0.041	0.998	1.992	1.975	1.000
LEH D21 - LEH D28	0.163	0.042	**0.047** *	8.500	2.028	**0.017** *
EHEC D28 - EHEH D28	0.893	0.090	**< 0.001** ***	-35.976	2.034	**< 0.001** ***
EHEC D28 - LEC D28	-0.071	0.091	1.000	0.707	1.977	1.000
EHEC D28 - LEH D28	0.525	0.091	**< 0.001** ***	-37.235	2.009	**< 0.001** ***
EHEH D28 - LEC D28	-0.964	0.091	**< 0.001** ***	36.683	2.045	**< 0.001** ***
EHEH D28 - LEH D28	-0.368	0.091	**0.037** *	-1.259	2.076	1.000
LEC D28 - LEH D28	0.596	0.091	**< 0.001** ***	-37.942	2.019	**< 0.001** ***
EHEC D28 - EHEC D35	-0.025	0.041	1.000	-0.490	1.964	1.000
LEC D28 - LEC D35	-0.117	0.041	0.651	36.683	2.045	**< 0.001** ***
EHEC D35 - LEC D35	-0.163	0.091	0.999	-1.259	2.076	1.000
EHEC D35 - EHEC D42	0.016	0.041	1.000	-37.942	2.019	**< 0.001** ***
LEC D35 - LEC D42	0.008	0.041	1.000	-4.892	1.975	0.898
EHEC D42 - LEC D42	-0.171	0.091	0.998	2.991	1.986	1.000
EHEC D42 - EHEC D49	0.101	0.041	0.903	-2.988	1.996	1.000
LEC D42 - LEC D49	0.068	0.041	1.000	-1.792	1.975	1.000
EHEC D49 - LEC D49	-0.205	0.091	0.966	4.187	1.987	0.989
EHEC D49 - EHEC D56	0.170	0.041	**0.022** *	0.534	2.054	1.000
LEC D49 - LEC D56	0.032	0.041	1.000	-0.299	1.975	1.000
EHEC D56 - LEC D56	-0.342	0.091	0.086	3.355	2.046	1.000
EHEC D56 - EHEC D63	0.101	0.046	0.977	1.994	2.741	1.000
LEC D56 - LEC D63	0.006	0.043	1.000	1.614	2.130	1.000
EHEC D63 - LEC D63	-0.438	0.094	**0.003** **	2.974	2.805	1.000

Supplementary table 2

Results of the pairwise comparison of least-squares means and adjusted p-values for multiple comparisons with the Tukey method from the linear mixed-effect model testing for the effect of incubation temperature, incubation elevation, and their interaction on the juvenile traits in the snake *Natrix maura*. The four incubation treatments are low elevation at 24°C (LEC), low elevation at 32°C (LEH), extreme high elevation at 24°C (EHEC), and extreme high elevation at 32°C (EHEH). Significant differences between least-squares means shown in bold with one (P < 0.05) or three (P < 0.001) asterisks.

	Hatching success	**Sex**	**Incubation duration (day)**	**Total number of heart beats**	**Body mass at hatching (g)**	**Body size at hatching (cm)**	**Body condition at hatching**	**Residual egg yolk (g)**
EHEC - EHEH	0.194 ± 0.062 **P = 0.011 ***	0.058 ± 0.011 P = 0.951	29.01 ± 0.29 **P < 0.001 *****	695703 ± 94019 **P < 0.001 *****	0.323 ± 0.076 **P = 0.011 ***	1.21 ± 0.18 **P < 0.001 *****	-0.022 ± 0.009 P = 0.064	0.225 ± 0.072 **P = 0.011 ***
EHEC - LEC	0.037 ± 0.062 P = 0.935	-0.086 ± 0.010 P = 0.840	-2.25 ± 0.28 **P < 0.001 *****	-38851 ± 88811 P = 0.972	-0.118 ± 0.072 P = 0.359	-0.07 ± 0.17 P = 0.971	-0.015 ± 0.008 P = 0.311	-0.160 ± 0.068 P = 0.091
EHEC - LEH	0.020 ± 0.063 P = 0.989	-0.099 ± 0.010 P = 0.780	31.66 ± 0.28 **P < 0.001 *****	614072 ± 88776 **P < 0.001 *****	-0.079 ± 0.072 P = 0.687	0.72 ± 0.17 **P < 0.001 *****	-0.061 ± 0.008 **P < 0.001 *****	0.199 ± 0.068 **P = 0.020 ***
EHEH - LEC	-0.157 ± 0.062 P = 0.058	-0.144 ± 0.011 P = 0.559	-31.26 ± 0.29 **P < 0.001 *****	-734554 ± 94583 **P < 0.001 *****	-0.441 ± 0.077 **P < 0.001 *****	-1.28 ± 0.18 **P < 0.001 *****	0.008 ± 0.009 P = 0.828	-0.384 ± 0.072 **P < 0.001 *****
EHEH - LEH	-0.174 ± 0.063 **P = 0.030 ***	-0.157 ± 0.011 P = 0.493	2.66 ± 0.30 **P < 0.001 *****	-81631 ± 95322 P = 0.827	-0.402 ± 0.077 **P < 0.001 *****	-0.49 ± 0.18 **P = 0.036 ***	-0.038 ± 0.009 **P < 0.001 *****	-0.025 ± 0.073 P = 0.985
LEC - LEH	-0.017 ± 0.063 P = 0.993	-0.013 ± 0.011 P = 0.999	33.91 ± 0.28 **P < 0.001 *****	652923 ± 89836 **P < 0.001 *****	0.039 ± 0.073 P = 0.951	0.79 ± 0.17 **P < 0.001 *****	0.046 ± 0.008 **P < 0.001 *****	0.359 ± 0.069 **P < 0.001 *****

Supplementary table 3

Results of the pairwise comparison of least-squares means and adjusted p-values for multiple comparisons with the Tukey method from the linear mixed-effect model testing for the effect of incubation temperature, incubation elevation, age at measurement (days post-hatching), test location, and their interaction on the maximum swimming performance in juveniles of the snake *Natrix maura*. The four incubation treatments are extreme high elevation at 24°C (EHEC; N = 41), extreme high elevation at 32°C (EHEH; N = 37), low elevation at 24°C (LEC; N = 41), and low elevation at 32°C (LEH; N = 41). Significant factors shown in bold with one (P < 0.05) or three (P < 0.001) asterisks.

	Estimate	*SE*	*P-value*
EHEC D9 - EHEH D9	-7.358	1.386	**< 0.001 *****
EHEC D9 - LEC D9	1.211	1.290	1.000
EHEC D9 - LEH D9	-3.905	1.305	0.423
EHEH D9 - LEC D9	8.568	1.396	**< 0.001 *****
EHEH D9 - LEH D9	3.453	1.339	0.752
LEC D9 - LEH D9	-5.115	1.308	**0.035 ***
EHEC D9 - EHEC-EHE D11	3.416	1.330	0.761
EHEC D9 - EHEC-LE D11	1.872	1.356	1.000
EHEH D9 - EHEH-EHE D11	3.237	1.320	0.836
EHEH D9 - EHEH-LE D11	4.531	1.541	0.463
LEC D9 - LEC-LE D11	2.853	1.262	0.925
LEC D9 - LEC-EHE D11	-0.586	1.450	1.000
LEH D9 - LEH-LE D11	1.436	1.307	1.000
LEH D9 - LEH-EHE D11	-2.419	1.397	0.998
EHEC-EHE D11 - EHEC-LE D11	-1.543	1.710	1.000
EHEH-EHE D11 - EHEC-LE D11	5.993	1.785	0.187
LEC-EHE D11 - LEC-LE D11	3.439	1.733	0.985
LEH-EHE D11 - LEH-LE D11	3.855	1.718	0.932
EHEC-EHE D11 - EHEC-EHE D25	-8.464	1.450	**< 0.001 *****
EHEC-LE D11 - EHEC-LE D25	-9.940	1.489	**< 0.001 *****
EHEH-EHE D11 - EHEH-EHE D25	-3.538	1.420	0.811
EHEH-LE D11 - EHEH-LE D25	-12.488	1.733	**< 0.001 *****
LEC-EHE D11 - LEC-EHE D25	-6.313	1.610	**0.034 ***
LEC-LE D11 - LEC-LE D25	-10.467	1.357	**< 0.001 *****
LEH-EHE D11 - LEH-EHE D25	-2.962	1.532	0.990

LEH-LE D11 - LEH-LE D25	-9.403	1.443	**< 0.001 *** **
EHEC-EHE D25 - EHEC-LE D25	-3.020	1.709	0.998
EHEH-EHE D25 - EHEH-LE D25	-7.656	1.831	**0.013 ***
LEC-EHE D25 - LEC-LE D25	-0.714	1.734	1.000
LEH-EHE D25 - LEH-LE D25	-2.586	1.714	1.000
EHEC-EHE D25 - EHEC-EHE D40	-2.137	1.448	1.000
EHEC-LE D25 - EHEC-LE D40	-0.724	1.485	1.000
EHEH-EHE D25 - EHEH-EHE D40	-2.375	1.417	0.999
EHEH-LE D25 - EHEH-LE D40	4.279	1.719	0.813
LEC-EHE D25 - LEC-EHE D40	-2.154	1.609	1.000
LEC-LE D25 - LEC-LE D40	0.607	1.355	1.000
LEH-EHE D25 - LEH-EHE D40	-2.342	1.525	1.000
LEH-LE D25 - LEH-LE D40	4.080	1.419	0.517
EHEC-EHE D40 - EHEC-LE D40	-1.607	1.709	1.000
EHEH-EHE D40 - EHEH-LE D40	-1.002	1.835	1.000
LEC-EHE D40 - LEC-LE D40	2.046	1.735	1.000
LEH-EHE D40 - LEH-LE D40	3.836	1.714	0.934

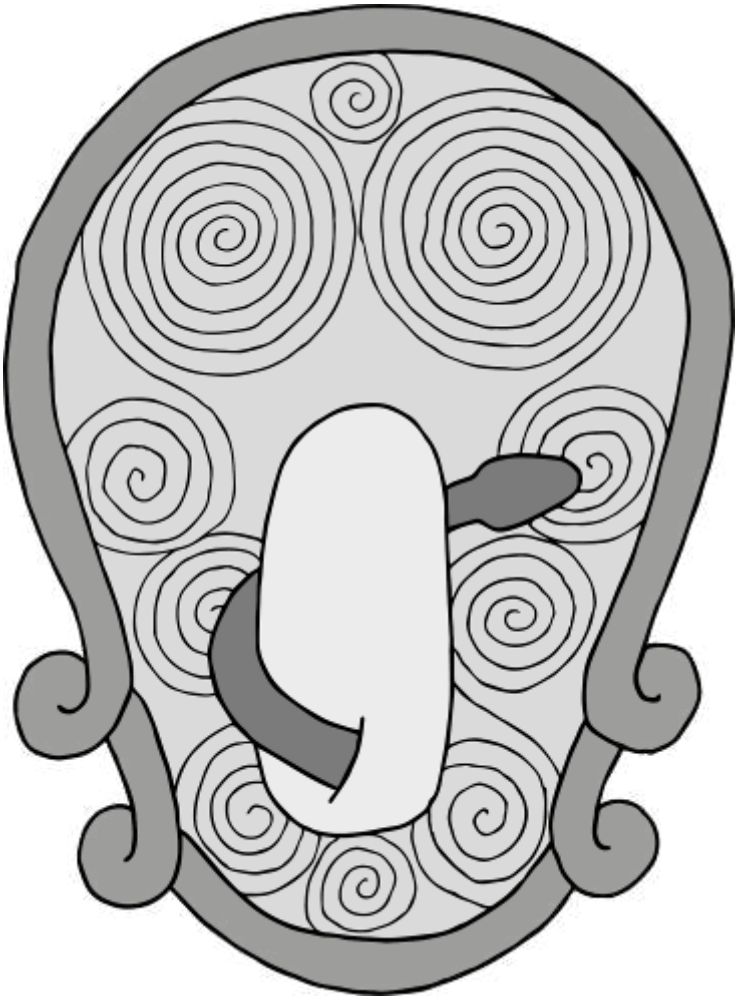
Supplemantary table 4

Residual variance estimates from linear and generalized linear mixed models. Traits were measured in embryos and in juveniles of the snake *Natrix maura*. The significance of model fit improved by inclusion of clutch as a random effect is indicated by one (P < 0.05) two (P < 0.01) or three (P < 0.001) asterisks. See text for model details.

	Residual	**Individual**	**Clutch**				
	variance	***variance***	***variance***	***Proportion of residual variance***	***χ^2***	***Df***	***P-value***
Egg mass	0.041	0.165	0.418	0.67	197.48	1	**< 0.001 *****
Embryo heart rate	94.550	0.000	3.765	0.038	13.50	1	0.999
Hatching success	0.252	-	0.000	~ 0	4.23	1	**0.040 ***
Sex	0.097	-	0.007	0.067	2.27e-13	1	> 0.99
Incubation duration	1.754	-	2.252	0.56	104.20	1	**< 0.001 *****
Total number of heart beats	1.815×10^{11}	-	1.137×10^{11}	0.39	51.59	1	**< 0.001 *****
Residual egg yolk	0.107	-	0.011	0.093	5.95	1	**0.015 ***
Body mass at hatching	0.119	-	0.182	0.60	120.86	1	**< 0.001 *****
Body size at hatching	0.646	-	0.426	0.40	53.71	1	**< 0.001 *****
Body condition at hatching	0.002	-	0.001	0.33	59.78	1	**< 0.001 *****
Swimming speed	22.010	11.697	3.186	0.086	9.72	1	**0.002 ****

Chapitre 5.

EFFETS DE L'HYPOXIE D'ALTITUDE SUR LE MÉTABOLISME RESPIRATOIRE DES EMBRYONS ET DES JUVÉNILES



5.1. Présentation et hypothèses du chapitre

Ce chapitre tente de mettre en évidenceles effets de l'hypoxie d'altitude sur le métabolisme des embryons peandant l'incubation puis chez les juvéniles. Chez les reptiles, Les niveaux métaboliques peuvent être reliés aux battements cardiaques mais peuvent aussi être estimés à travers des mesuresde la consommation d'oxygène et de la production de dioxyde de carbone. Les réponses physiologiques du métabolisme respiratoire mesurées chez les embryons puis chez les juvéniles pourraient indiquer des modifications physiques du système cardiovasculaire ou des modifications biochimiques de la composition du sang. Dans le contexte du réchauffement climatique, si une remontée altitudinal de l'espèce pourrait dans un premier temps être envisageable (*cf*. *Chapitre 3*), elle pourrait être limitée par un métabolisme réduit ou améliorer par la plasticité développementale des embryons. Pour cela, l'activité respiratoire ainsi que les battements cardiaques ont été mesurés chez des embryons de Couleuvre vipérine à la température optimale d'incubation connue pour cette espèce (*i.e.* 28°C). Un lot était placé en normoxie et un deuxième lot en hypoxie (*i.e.* 70% de l'oxygène disponible à 2 877 m d'altitude). A l'éclosion, les mesures de l'activité respiratoire des juvéniles s'est poursuivie à une temperature de repos de 20°C. Ce chapitre tente également à définir si une plasticité comportementale maternelle, en plaçant les femelles gravides en hypoxie, pourrait améliorer l'adaptation développementale des embryons dans ces conditions.En effet, bien qu'il n'y ait pas de soins parentaux après la ponte, les comportements de thermorégulation des femelles peuvent modifier les premieres étapes du developpement (*cf*. *section 1.3.3*) permettant aux embryons de se préparer aux conditions futures qu'ils rencontreront durant l'incubation. L'activité respiratoire des embryons et des juvéniles de ce troisème lot à également été mesurée.

Dans ce contexte, nous prédisons qu'en condition hypoxique et à une température optimale de developpement ou de maintien:

- La fréquence cardiaque des embryons, la consomation d'oxygène et la production de dioxyde de carbone seront diminuéesindiquant une réduction du métabolisme.
- La durée de développement ne sera pas impactée.
- La réduction du métabolisme durant le développement va modifier le phénotype à l'éclosion avec des juvéniles plus petits
- La consomation d'oxygène et la production de dioxyde de carbone des juvéniles seront diminuéesindiquantégalement une réduction du métabolisme.
- Néanmoins, pour les embryons issus des mères gravides maintenues en condition d'hypoxie, la réduction du métabolisme embryonnaire et juvénile ainsi que les modifications du phénotype devrait être moins marquée

L'article présenté dans ce chapitre est en actuellement en préparation :

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5.2. Résumé

La colonisation d'un nouvel environnement implique, pour les espèces, de s'adapter à des conditions rarement rencontrées dans l'histoire récente des populations. En milieu montagneux, les individus doivent faire face à une pression atmosphérique plus faible en altitude et donc à une disponibilité réduite en dioxygène. Les réponses physiologiques induites par ce stress pourraient favoriser la survie de la progéniture durant une hypoxie chronique, contribuant ainsi à l'invasion des écosystèmes alpins en réponse au réchauffement climatique. Pour tester cette réponse à l'hypoxie de haute altitude chez la couleuvre vipérine, *Natrix Maura*, nous avons maintenu artificiellement des femelles gravides de populations de basse altitude en haute altitude (*i.e.* hypoxie d'altitude) et leurs embryons ont également été incubés en haute altitude. Un autre groupe de femelles gravides a été maintenu en basse altitude (*i.e*. normoxie) et la moitié de leurs embryons ont été incubés à cette altitude, l'autre moitié ont été transplantés en haute altitude. Les embryons incubés en haute altitude ont présenté une réduction du métabolisme à la fin de l'incubation. Ces réactions ont pû contribué à maintenir une durée d'incubation, un succès d'éclosion ou un phénotype d'éclosion similaire aux juveniles incubés à basse altitude. Néanmoins, après avoir été maintenus en haute altitude, les juvéniles présentent un métabolisme réduit et une hyperventilation par rapport aux juvéniles maintenus à basse altitude. Enfin, la gestation des femelles en haute altitude n'a pas affectée les réponses des embryons et des juvéniles à l'hypoxie d'altitude. Ces résultats soulignent le rôle de la plasticité physiologique dans le maintien des phénotypes pertinents pour la forme physique dans les environnements de haute altitude.

Mots-clés *: Métabolisme embryonnaire; Métabolisme juvénile; Natrix maura; Hypoxie d'altitude; Consommation de dioxygène; Production de dioxyde de carbone*

High-elevation hypoxia alters embryonic and juvenile metabolism in the viperine snake

Jérémie Souchet[1,*], Alicia Josserand[1], Elodie Darnet[1], Hugo Le Chevalier[1], Audrey Trochet[2], Romain Bertrand[1], Jean Clobert[1], Olivier Calvez[1], Albert Martinez-Silvestre[3], Olivier Guillaume[1], Marc Mossoll-Torres[4,5], Laurent Barthe[2,6], Gilles Pottier[6], Hervé Philippe[1,7], Fabien Aubret[1,8] & Eric J.Gangloff[1,9]

1- Station d'Ecologie Théorique et Expérimentale du Centre National de la Recherche Scientifique, UMR 5321 CNRS – Université Paul Sabatier, 09200 Moulis, France.
2- Société Herpétologique de France, Muséum National d'Histoire Naturelle, CP41, 57 rue Cuvier, 75005 Paris, France
3- Catalonia Reptile and Amphibian Rescue Center (CRARC), 08783 Masquefa, Barcelona, Spain
4- Bomosa, Pl. Parc de la Mola, 10 Torre Caldea 7º, AD700 Les Escaldes, Andorra
5- Pirenalia, c/ de la rectoria, 2 Casa Cintet, AD200 Encamp, Andorra*
6- Nature En Occitanie, 14 rue de Tivoli, 31 000 Toulouse
7- Département de Biochimie, Centre Robert-Cedergren, Université de Montréal, Montréal, Canada
8- School of Molecular and Life Sciences, Curtin University, Brand Drive, Bentley, WA 6102, Australia
9- Department of Zoology, Ohio Wesleyan University, Delaware, Ohio, USA

*Corresponding author. E-mail: *jeremie.souchet@gmail.com*

Authors contributions

JS, EJG and FA contributed to experimental design and logistics. JS and AJ conducted experiments. JS and EJG conducted statistical analyses. JS, AJ, FA and EJG drafted the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

5.3. Introduction

In mountainous regions, the impacts of climate change are particularly pronounced (Nogués-Bravo et al., 2008; Chen et al., 2011; Dirnböck et al., 2011). Formerly inhospitable habitats at high elevations have warmed up and become thus thermally suitable for some low-altitude species (Parmesan, 2006; Sinervo et al., 2010, 2018; Pauchard et al., 2016). Nevertheless, the colonization of this novel environment depends on the species ability to adapt to the specific conditions at high altitude, which have been rarely encountered in the recent history of populations (Schluter, 2000; Aubret, 2013). In particular, individuals need to cope with low levels of atmospheric pressure and thus reduced oxygen availability (*i.e.* high-elevation hypoxia; (Bouverot, 1985; Powell and Hopkins, 2010). In oviparous reptiles, most of the embryonic development occurs after the eggs are laid in unattended subterranean nests (Packard and Packard, 1988; Ackerman and Lott, 2004). As a result, embryos must adjust to adverse soil conditions, notably to consistent low oxygen levels and fluctuations in gas concentrations (Packard and Packard, 1988; Deeming and Thompson, 1991; Ackerman and Lott, 2004). For instance, for reptiles embryo the gas exchange is diffusion-limited, likely constraining their ability to compensate for reduced oxygen availability through increased oxygen transport capacity (Vitt and Caldwell, 2013). Nevertheless, exposure to hypoxia (not only high-elevation hypoxia) during development may have induced plastic changes in cardiovascular, muscular, or mitochondrial function to increase performance capacity (Eme et al., 2013; Sun et al., 2015; Galli et al., 2016). Such stress-induced physiological responses might promote offspring survival when the partial pressure of O_2 is consistently low. Therefore, through rapid adaptive evolutionary responses to life at high elevation (Rezende et al., 2005), oviparous species may be able to colonize alpine ecosystems in response to climate warming (Storz et al., 2010; Ortega et al., 2016).

The acclimatation of reptiles to hypoxia vary depending on the Order (*i.e., Testudines, Squamata, Crocodilia*; Porteus et al., 2011) and include an alteration of cardio-respiratory pathways, a modification of blood composition, and increased muscle performance (Iungman and Piña, 2013; González-Morales et al., 2015; Lu et al., 2015; Wearing et al., 2015; Jochmans-Lemoine and Joseph, 2018; Gangloff et al., 2019). However, those responses are related to the hypoxia during the embryo development induced by the burial of eggs in subterranean nests (Kam, 1993; Crossley and Altimiras, 2005; Iungman and Piña, 2013; Cordero et al., 2017b; Wearing et al., 2017; Williamson et al., 2017). The effects of high-elevation hypoxia during this stage have only received recent interest, especially on Squamata (lizards: Cordero et al., 2017a; Kouyoumdjian et al., 2019; Li et al., 2020; snakes: Souchet et al., 2020). In general, the embryos of lizards' species respond to the low level of oxygen at high-elevations by a reduction of the metabolic rates without modifying the embryonic development

or, the phenotype of juveniles at hatching (Cordero et al., 2017a; Kouyoumdjian et al., 2019; Li et al., 2020). Conversely , the embryo of viperine snake showed higher metabolic rates with as results the reduction of hatchling phenotype (Souchet et al., 2020).

In order to assess the potential impact of high-elevation hypoxia on the cardio respiratory system of snake and the effect of maternal parental care before laying in high-elevation hypoxia, we exposed gravid females and eggs of viperine snake, *Natrix maura* (Colubridae), to three alternative incubation treatments: gestation of females and incubation of their eggs in hypoxia (*i.e.* extreme high elevation, above current range limits; 72% O_2 availability compared to sea level equivalent [ASL]), gestation of female in normoxia (*i.e.* low elevation, native elevation; 95% O_2 availability compared to ASL) and incubation of eggs at extreme high elevation, and gestation of females and incubation of their eggs in normoxia. We collected gravid females from low elevation population (583.5 m ± 161.5 m ASL) and we maintained them in high-elevation hypoxia or normoxia during gestation. After they lay, we monitored embryo heart rate and egg mass throughout the incubation, we measured the metabolic rates across development (from embryo to juvenile) and we measured important aspects of hatchling phenotype. We predict that high-elevation hypoxia will reduce the metabolism of embryo and juveniles, leading to decreases in hatchling size, and mass. Nevertheless, maternal parental care in extreme high elevation can probably prepare the embryo and reduce the impact of this low level of oxygen availability during incubation.

5.4. Materials and methods

5.4.1. Females capture and housing

Twenty-two gravid females *Natrix maura* were captured along the banks of the Lez River (Department of Ariège, France) in June 2018. Capture sites spanned from 422 m to 745 m ASL. Fifteen gravid females were maintained at low elevation at the Theoretical and Experimental Ecology Station of Moulis, Scientific Research National Center (SETE-CNRS; 42.958394 N, 1.086440 E; 436; normoxia; low elevation at 436 m ASL; native elevation; 95% sea-level equivalent O_2availability; PO_2 ~20.1 kPa). The seven others gravid females were maintained in extreme high elevation at the Observatoire Midi-Pyrénées at Pic du Midi de Bigorre (42.936389 N, 0.142472 E; hypoxia; extreme high elevation at 2877 m ASL, above current range limits; 72% sea-level equivalent O_2availability; PO_2 ~15.3 kPa). This difference in elevations results in a decrease in atmospheric pressure, with associated reduction in the partial pressure of gases, including oxygen, carbon dioxide, and water

vapor (Millet and Debevec, 2020; Richalet, 2020). The reduction in oxygen partial pressure at this extreme high elevation provides a useful experimental environment to test the physiological and developmental responses to reduced oxygen availability (Cordero et al., 2017a; Kouyoumdjian et al., 2019; Souchet et al., 2020). All females were fed and returned to their exact site of capture within two weeks after egg-laying.

5.4.2. Embryos incubation and measurements

A total of 257 eggs were obtained between 03 July 2018 and 28 July 2018 (mean clutch size ± SD = 11.7 ± 5.0 eggs). Among those eggs, 28 eggs were infertile or died within the first 7 days of post-laying, leaving 229 eggs from 22 females for the experiments (Figure 1). Eggs were individually marked for identification purposes with a pencil and allocated to three different (i) treatments. 97 eggs laid by seven females at extreme high elevation were maintained at extreme high elevation (EHE), (ii) 65 eggs laid by eight females at low elevation were maintained at low elevation (LE) and (iii) 67 eggs laid by seven females at low elevation were transplanted at extreme high elevation (LE-EHE; Figure 1). All eggs were incubated at 28°C under conditions identical to that described in Souchet et al. 2020. During incubation, we weighed each egg using a digital scale (to the nearest 0.01 g) within 12 hours of oviposition, and then every 7 days until hatching (Figure 1; test 1). Embryo heart rates were also measured at 28°C using a Buddy digital egg monitor (MK2, Avitronics, Cornwall, UK) under the standardized protocol described for eggs by Aubret et al. (2016b), Cordero et al. (2017a), and Souchet et al. (2020), first at 7 days post-oviposition and then every 7 days until hatching (Figure 1; test 1). During incubation, at 14 and 28 days post-laying, we measured at 28°C the metabolic rate in a subset of 155 embryos (Figure 1; test 2). Each egg was individually placed in a 250 ml metabolic chamber and replaced the chamber in the incubator. We used closed-system respirometry (Foxbox-C Field O_2 and CO_2 Analysis System, Sable Systems, Inc., Las Vegas, NV, USA) to measure gas exchange [oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$), both corrected for barometric pressure]. We flushed the chamber for 10 min at a flow rate of 400 ml min^{-1} and closed valves to seal the chamber for 60 min. We then opened the valves tore-establish air flow and dried air from water vapour with Drierite, and measured O_2 and CO_2 as above. Data were analysed with the ExpeData software (v.1.7.30,Sable Systems, Inc.) to calculate $\dot{V}O_2$ and $\dot{V}CO_2$ by integrating the change in instantaneous gas concentrations during the period in which the chamber was sealed (Lighton, 2018). We also calculated the respirometry quotient (RQ) as the ratio of the $\dot{V}CO_2$ to the $\dot{V}O_2$ at 14 and 28 days post-hatching

5.4.3. Juveniles housing and measurements

While 3 embryos out of 229 eggs died at various stages during incubation, 226 embryos from 22 females successfully hatched between 18 August 2018 and 21 September 2018 (98.7% hatching success rate). Shortly after hatching, 4 neonates died shortly after hatching, leaving 222 hatchlings for morphologic measurements. Hatchlings were sexed via hemipene eversion, individually marked for identification with medical cauterizer (low temperature power handle Model HIT0 with 0.05 mm tip Model H100, Bovie®, USA) by hot branding technique (Winne et al., 2006) within 24 hours of emergence (Figure 1; test 3) and raised at 20°C under conditions identical to that described in Souchet et al., 2020. At hatching, the yolk leftover in the eggshell (residual egg yolk) were weighed and juveniles were weighed using a digital scale (to the nearest 0.01 g) and, measured for snout-vent length (SVL) using a measuring tape (to the nearest 0.1 mm). At 14 and 28 days post-hatching, all juveniles were measured again for SVL and body mass. The resting metabolic rate at 20°C of a subset of 148 juveniles (from eggs tested before; Figure 1; test 4) was under the same protocol than eggs. We also calculated body condition as the residuals of the linear regression $\log_{10}$-mass against $\log_{10}$-SVL at hatching day, 14 and 28 days post-hatching. Once tests were completed, young snakes were fed and released at the maternal capture site.

5.4.4. Data analysis

We first assessed the influence of treatment and time on two measures of embryo development (test 1): egg mass and embryo heart rate. We used a linear mixed-effect model, including the main effects of treatment (LE, LE-EHE and EHE) and the time (post-laying). For the egg mass, the model includes the initial egg mass at laying time as covariate. Then we assessed the influence of treatment on seven measures of phenotypes (test 3): hatching success, sex ratio, incubation duration, residual egg yolk, body mass, body size (SVL) and body condition. We used a linear mixed-effect model, including the main effect of treatment (LE, LE-EHE and EHE). Finally, we assessed the influence of treatment and time on the respirometry metabolism (RQ) of embryos and juveniles (tests 2 and 4,). We used a linear mixed-effect model, including the main effect of treatment (LE, LE-EHE and EHE) and the time (post-hatching), and the heart rate (for embryos) or the body mass (for juveniles) as a covariate.

To account for the non-independence of siblings we included the clutch of origin as a random effect in all models. In the models for which we measured individuals repeatedly (egg mass, embryo heart rates and respirometry metabolism), we also included individual as a random effect. We used type III

sums of squares to assess the significance of main effects, incorporating a Kenward-Roger denominator degree of freedom approximation (Kenward and Roger, 1997). We also conducted a pairwise comparison of least-squares means and adjusted p-values for multiple comparisons with the Tukey method. All analyses were conducted with the lme4 package (Bates et al., 2014) and lsmeans package (Lenth, 2016). Figures were made with the ggplot2 package (Wickham, 2016) in the programming language R 3.6.1 (R Development Core Team, 2017).

5.5. Results

5.5.1. Egg mass variation and embryonic heart rates

Egg mass trajectories differed among the three treatment groups. For all treatments (Figure 2; Table 1), the egg mass increased from the laying day to the days 28 of incubation. Then, the LE egg mass continued to increase until the end of incubation whereas the egg mass decreased for the groups incubated in high-elevation hypoxia (Figure 2). Embryo heart rates trajectories differed between the three treatments (Figure 2; Table 1). For all treatments, heart rates decreased similarly between days 7 and 21, before remaining stable between days 21 and 28. Finally, embryos' heart rates from the LE-EHE treatment remained stable until the end of incubation while in LE and EHE treatment heart rates increased (Figure 3).

5.5.2. Hatching and morphological measurements

Hatching success and hatchling sex ratio did not differ between embryos incubated in the three treatments (Table 2). The different treatments did not influence incubation duration and residual egg yolk (Table 2). They also did not alter the body size (SVL), the body mass and the body condition at hatching day and at 14 and 28 days post-hatching (Table 2). For each trait measured, the post-hoc comparison of least-squares means from the models did not indicates any significant difference between all treatments.

5.5.3. Embryos and juveniles metabolic rate

The embryonic respirometry quotient was significantly affected by the incubation duration and its interaction with the treatment (Figure 3A; Table 3). At 14 days of incubation, the post-hoc comparison of least-squares means from the model (Figure 3A; Table S2) indicated that the

embryonic RQ were similar between all treatments. At 28 days post-laying, the embryonic RQ of the LE treatment was maintained, while the embryonic RQ of both LE-EHE and EHE treatments decreased identically (Figure 3A; Table S2).

The juveniles' respirometry quotient was significantly affected by the treatment, the time and their interaction (Figure 3B; Table 3). At 14 days post-hatching, the post-hoc comparison of least-squares means from the model (Figure 3B; Table S2) indicatedthat the juveniles' RQ were similar between EHE and LE-EHE treatments and also significantly lower compared to the LE treatment. At 28 days post-hatching, the juveniles' RQ of LE and LE-EHE treatments were maintained, whereas the embryonic RQ of the EHE treatment significantly increased (Figure 3B; Table S2).

5.6. Discussion

Our study aims to examine how low oxygen partial pressure in extreme high-elevation (*i.e.* high-altitude hypoxia) during incubation affects both embryos and juveniles' metabolism in the viperine snake. We also explored if maintaining gravid females in extreme high-elevation can prevent and help embryos to be acclimated to this environmental condition. Extreme high-elevation affected the egg mass trajectories which decreased at the end of the incubation (Figure 2A; Table 1). Additionally, the treatments affected the embryo heart rate trajectories that increased in the end of incubation for both low elevation and extreme high-elevation treatment (Figure 2B; Table 1). Regardless of the elevation of the mother during gestation, the incubation at extreme high-elevation did not affect hatchling phenotype (Table 2). Nevertheless, this condition induced a reduction of metabolism of embryo (Figure 3A; Table 3). These responses might have contributed to maintain similar incubation duration, hatching success and body phenotype of juvenile, relative to those in low elevation (Table 2). Nevertheless, after being maintained in high-elevation hypoxia, juveniles show a hyperventilation compared to juvenile maintained in normoxia (Figure 3B; Table 3).

5.6.1. Maternal effect and hatchling phenotype

In our experiment, we have no impact of maternal translocation of gravid females maintained to extreme high-elevation. This can be explained by the fact that most part of embryonic development will occur after eggs are laid (Packard and Packard, 1988; Ackerman and Lott, 2004). Nevertheless, hypoxia is expected to affect ATP demand and supply pathways, which ultimately decrease cellular respiration rates by down regulating ion-pumping and protein synthesis (Hochachka et al., 1996;

Bickler and Buck, 2007). This common homeostatic response ensures survival without necessarily compromising embryonic development if O_2 delivery to tissues is enhanced (Crossley and Burggren, 2009). Our results corroborate this expectation, though we did not directly measure compensatory biochemical changes in blood, for example enhanced O_2 affinity to hemoglobin or hemoglobin and hematocrit concentration (Storz et al., 2010; Lu et al., 2015; Storz, 2016; Gangloff et al., 2019). Indeed, and contrary to what we expected (Kam, 1993; León-Velarde and Monge, 2004; Du et al., 2010a; Cordero et al., 2017a; Crossley et al., 2017), incubation duration, phenotype at hatching and phenotype after being maintained one month at their elevation of incubation (*e.g.* body mass, body size and body condition; Table 2) were comparable between juveniles from all treatments. Furthermore, we did not found any effect of hypoxic condition on the egg resources (*e.g*. residual egg yolk, Table 2).

5.6.2. Embryos' and juveniles' metabolism

Developing viperine snake embryos exposed to high-elevation hypoxia exhibited typical vertebrate physiological adjustments responses. Firstly, the suppressed embryo metabolism reflected by the reduction of the heart rate throughout incubation (Laughlin, 1978; Crossley and Burggren, 2009; Cordero et al., 2017a; Kouyoumdjian et al., 2019; Figure 2B, Table 1). Secondly, is also confirmed by the decreasing RQ in the end of incubation (Figure 3A, Table 3), which results in an augmentation of O_2 consumption and CO_2 production (Gardner, 1996). These results are strongly correlated in snakes and lizards (Greenwald, 1971; Bennett, 1972; Butler et al., 2004; Du et al., 2010a; Kouyoumdjian et al., 2019) and indicate that high-elevation hypoxia induces hyperventilation in the end of incubation (Bouverot, 1985; Peacock, 1998; Powell and Hopkins, 2010; Storz et al., 2010; Cordero et al., 2017a).

After hatching, the juveniles' RQ were also lower for both group incubated and maintained in extreme high-elevation (*i.e*., treatment LE-EHE and EHE; Figure 3B; Table 3). This results also indicates that high-elevation hypoxia induces hyperventilation (Bouverot, 1985; Peacock, 1998; Powell and Hopkins, 2010; Storz et al., 2010; Cordero et al., 2017a). Metabolic plasticity during development may have allowed juveniles to maintain or improve their metabolism compared to juveniles in normoxic conditions. It is possible that the development in hypoxia changes the blood composition of individuals (Storz, 2007; Storz et al., 2010), thereby improving the oxygen-carrying capacity of the blood. This biochemical change, which is well known in high-elevation reptile populations, results in higher haemoglobin concentrations and higher haematocrit counts in the blood compared to low-altitude populations (Vinegar and Hillyard, 1972; Weathers and White, 1972; Newlin and Ballinger, 1976; González-Morales et al., 2015; Lu et al., 2015; Megía-Palma et al., 2020).

However, while this could maintain the performance of juveniles in high-elevation hypoxia, this phenotypic plasticity could have significant costs in the long term. Indeed, increasing red blood cell density can increase blood viscosity and the energy cost of blood circulation (Hedrick et al., 1986; Dunlap, 2006).

5.6.3. General conclusion

Collectively, our findings support the hypothesis that plastic physiological responses to high-elevation hypoxia may facilitate the maintenance of fitness-related phenotypes in *Natrix maura*. Furthermore, this plastic physiological response could be increase by plastic behavior of the gravid females in their nest choice to increase the hatchlings survival (Burger and Zappalorti, 1986; Escalona et al., 2009; Pike et al., 2010; Refsnider et al., 2010; Peet-Paré and Blouin-Demers, 2012). Even though, population establishment will depend on the long-termcosts associated with life in reduced oxygen availability and the consequences of reduced performance (Gangloff et al., 2019), we advocate that metabolic plasticity in embryos should facilitate altitudinal range expansion in *Natrix maura*, in response to climate warming.

5.7. Acknowledgements

We are grateful to the staff of Observatoire Midi-Pyrénées for logistical support at Pic du Midi de Bigorre as well as Isabel Verdaguer, Joaquim Soler and Zuleica Alonso for their help in the laboratory. This work was supported by the French Laboratory of Excellence project "TULIP" (ANR-10-LABX-41; ANR-11-IDEX-0002-02), INTERREG POCTEFA ECTOPYR (no. EFA031/15), and the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 752299. All experimental protocols (including animal collection, housing, experimentation and release) were approved by the DREAL Midi-Pyrénées (Direction Régionale de l'Environnement, de l'Aménagement et du Logement) and by the Préfecture of Ariège , Aude, Haute-Garonne, Hautes-Pyrénées and Pyrénées Orientales districts (Arrêté Préfectoral No. 2017-s-02 du 30 mars 2017) and ethical committee (APAFIS#16359-201808011445465 v4). All experiments were carried out in accordance with the approved guidelines. Animal caretakers and handlers were trained to use wildlife in scientific purposes (Decree No. 2013-118 du 01 février 2013 and approval of the Ministry of Agriculture under No. I-75-MNHN-F1-15 du 17 juin 2015).

5.8. Tables and figures

Table 1

Results of linear mixed-effect model testing for the effect of incubation treatment, age at measurement (days post-laying), and their interaction on the egg mass and on the embryo heart rates during embryo development of the snake *Natrix maura* (test 1, Figure 1; Figure 2A and 2B). The egg mass at laying time was included as covariate. The three treatments are gestation at extreme high elevation and incubation at extreme high elevation (EHE; N = 97), gestation at low elevation and incubation at extreme high elevation (LE-EHE; N = 67) and gestation at low elevation and incubation at low elevation (LE; N = 65). Significant factors shown in bold with three (P < 0.001) asterisks.

	Egg mass	**Embryo heart rates**
Day	$F_{6, 1346.36}$ = 241.93; **P < 0.001 *****	$F_{5,}$ 1095.92 = 38.47; **P < 0.001 *****
Treatment	$F_{2, 18.73}$ = 0.29; P = 0.755	$F_{2, 18.88}$ = 0.57; P = 0.578
Treatment x Day	$F_{12, 1346.35}$ = 14.18; **P < 0.001 *****	$F_{10, 1096.02}$ = 6.13; **P < 0.001 *****
Egg mass at laying time	$F_{1,170.36}$ = 672.11; **P < 0.001 *****	-

Table 2

Results of linear mixed-effect model testing for the effect of treatment at hatching day and at 14 and 28 days post-hatching on phenotype in juvenile of the snake *Natrix maura* (test 3, Figure 1). The 3 treatments are gestation at extreme high elevation and incubation at extreme high elevation (EHE; N = 95), gestation at low elevation and incubation at extreme high elevation (LE-EHE; N = 63) and gestation at low elevation and incubation at low elevation (LE; N = 64). Least-squares mean ± SE or percentages are given.

	LE	**LE-EHE**	**EHE**	**F (dfn, dfd)**	**P-value**
Incubation duration (day)	52.72 ± 3.11	52.08 ± 3.97	50.40 ± 2.75	2.01 (2, 35.49)	P = 0.149
Hatching success (% of success)	98.96	96.92	100.00	0.58 (2, 18.20)	P = 0.571
Sex ratio (% of females)	51.58	52.38	50.00	0.04 (2, 15.66)	P = 0.965
Body mass (g) at hatching	2.72 ± 0.48	2.82 ± 0.60	2.71 ± 0.34	0.42 (2, 33.67)	P = 0.663
Body size (cm) at hatching	15.35 ± 0.69	15.06 ± 1.10	15.16 ± 0.85	1.27 (2, 29.42)	P = 0.295
Body condition at hatching	-0.016 ± 0.051	0.018 ± 0.042	-0.001 ± 0.032	0.99 (2, 24.75)	P = 0.386
Residual egg yolk (g)	0.88 ± 0.50	0.84 ± 0.47	0.60 ± 0.44	2.39 (2, 20.21)	P = 0.117
Body mass (g) at 14 days post-hatching	2.53 ± 0.46	2.64 ± 0.54	2.52 ± 0.32	0.31 (2, 33.74)	P = 0.739
Body size (cm) at 14 days post-hatching	15.70 ± 0.74	15.41 ± 1.08	15.55 ± 0.85	1.15 (2, 29.53)	P = 0.331
Body condition at 14 days post-hatching	-0.016 ± 0.043	0.0.22 ± 0.034	-0.004 ± 0.033	2.63 (2, 22.80)	P = 0.094
Body mass (g) at 28 days post-hatching	2.29 ± 0.42	2.37 ± 0.50	2.27 ± 0.28	0.28 (2, 32.55)	P = 0.756
Body size (cm) at 28 days post-hatching	15.87 ± 0.79	15.51 ± 1.13	15.76 ± 0.87	1.06 (2, 29.95)	P = 0.378
Body condition at 28 days post-hatching	-0.016 ± 0.43	0.025 ± 0.039	-0.006 ± 0.032	3.02 (2, 22.06)	P = 0.069

Table 3

Result of linear mixed-effect model testing for the effect of incubation treatment, the age at measurement (days post-laying) and their interaction on the respirometry quotient (RQ; test 2 and 4, Figure 1; Figure 3A and 3B) in embryos and juveniles of the snake *Natrix maura*. The embryo heart rates or juvenile body mass were included as covariate. The three treatments are gestation at extreme high elevation and incubation at extreme high elevation (EHE; triangle; embryo: N = 59; and juvenile: N = 58), gestation at low elevation and incubation at extreme high elevation (LE-EHE; squares; embryo: N = 51; and juvenile: N = 48) and gestation at low elevation and incubation at low elevation (LE; circle; embryo: N = 45; and juvenile: N = 42).

	Embryo RQ	**Juvenile RQ**
Day	$F_{1, 148.60}$ = 37.92; **P < 0.001 *****	$F_{1, 436.89}$ = 4.40; **P = 0.037 ***
Treatment	$F_{2, 18.16}$ = 2.26; P = 0.133	$F_{2, 17.51}$ = 76.23; **P < 0.001 *****
Day x Treatment	$F_{2, 146.12}$ = 9.31; **P < 0.001 *****	$F_{2, 428.69}$ = 7.04; **P < 0.001 *****
Heart rates	$F_{1, 229.28}$ = 1.03; P = 0.312	-
Body mass	-	$F_{1, 44.76}$ = 0.20; P = 0.655

Figure 1

Experimental design. Gravid *Natrix maura* females were captured at low elevation in viperine snake populations in the foothills of the Pyrenees (422 m to 745 m ASL). A third of them were transplanted and maintained for gestation to the extreme high elevation laboratory at 2877 m ASL, while two thirds of them were maintained for gestation at low elevation laboratory at 436 m ASL. Within 48 hours of oviposition, half of clutch from low elevation of females gestation were transplanted and maintained for incubation to the extreme high elevation laboratory, while the other half were maintained for incubation at low elevation laboratory. Clutches from females in gestation to the extreme high elevation laboratory where maintained at this elevation. Eggs mass and embryo heart rate were measured throughout incubation (test 1). At hatching, a number of morphologic traits were measured in juveniles (test 3). A subset of eggs and juveniles in each treatment were tested for VO_2 consumption and VCO_2 production in the environment where eggs were incubated (test 2 and test4).

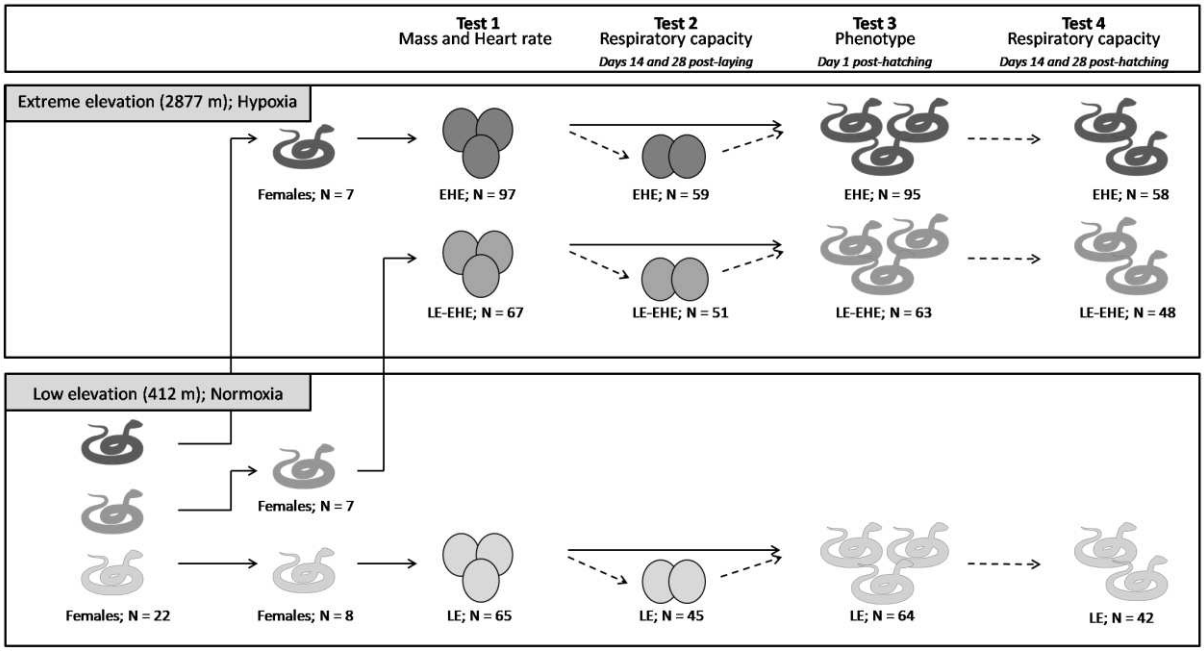


Figure 2

Egg mass (A) and embryo heart rates (B) through incubation time at 28°C in embryos of the snake *Natrix maura* (test 1, Figure 1). The three treatments are gestation at extreme high elevation and incubation at extreme high elevation (EHE; N = 97; triangle), gestation at low elevation and incubation at extreme high elevation (LE-EHE; N = 67; squares) and gestation at low elevation and incubation at low elevation (LE; N = 65; circle). Least-squares means ± SE estimated by linear mixed models are plotted.

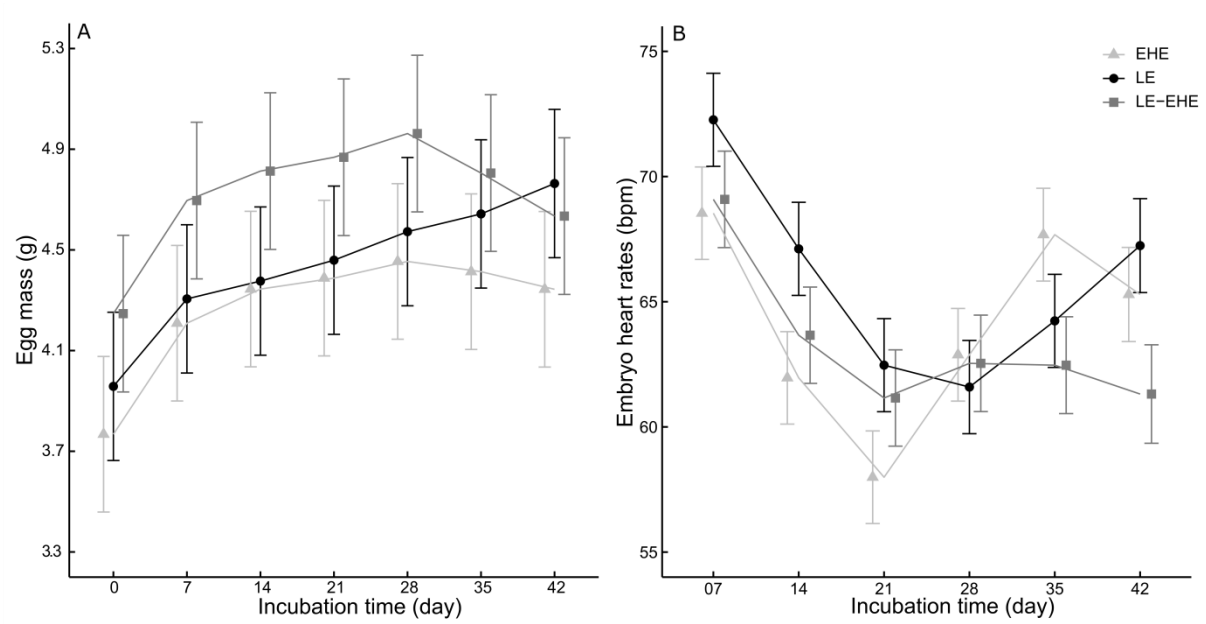
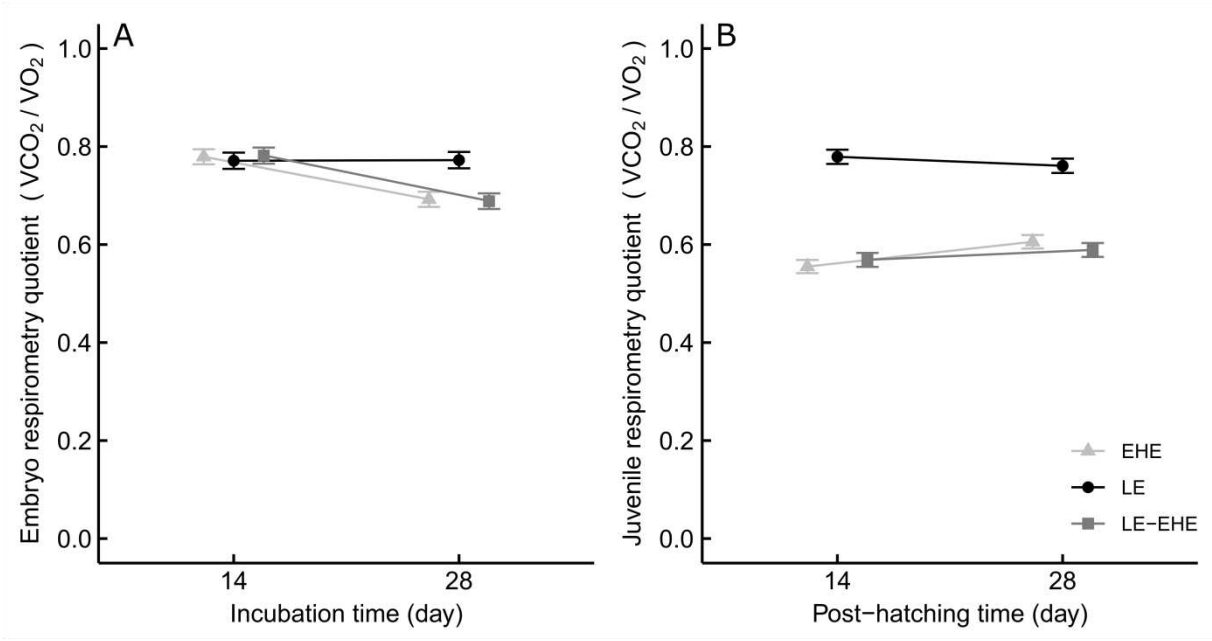


Figure 3

Embryo respirometry quotient (A) and juvenile respirometry quotient (B) in each treatment at 14 and 28 days post-laying in embryos and post-hatching in juvenile of the snake *Natrix maura*. The three treatments are gestation at extreme high elevation and incubation at extreme high elevation (EHE; triangle; A: N = 59; and B: N = 58), gestation at low elevation and incubation at extreme high elevation (LE-EHE; squares; A: N = 51; and B: N = 48) and gestation at low elevation and incubation at low elevation (LE; circle; A: N = 45; and B: N = 42). Least-squares means ± SE estimated by linear mixed models are plotted.



Supplemantary table 1

Results of the pairwise comparison of least-squares means and adjusted p-values for multiple comparisons with the Tukey method from the linear mixed-effect model testing for the effect of incubation treatment, the age at measurement (days post-laying) and their interaction on the egg mass and on the embryo heart rates during embryo development of the snake *Natrix maura* (Figure 2A & 2B; Table 1). The three treatments are gestation at extreme high elevation and incubation at extreme high elevation (EHE; N = 97), gestation at low elevation and incubation at extreme high elevation (LE-EHE; N = 67) and gestation at low elevation and incubation at low elevation (LE; N = 65). Significant differences between least-squares means shown in bold with one (P < 0.05), two (P < 0.01) or three (P < 0.001) asterisks.

	Egg mass			**Embryo heart rate**		
	Estimate	*SE*	*P-value*	*Estimate*	*SE*	*P-value*
EHE D0 - LE-EHE D0	-0.479	0.439	1.000	-	-	-
EHE D0 - LE D0	-0.190	0.427	1.000	-	-	-
LE-EHE D0 - LE D0	0.289	0.428	1.000	-	-	-
EHE D0 - EHE D7	-0.441	0.032	**< 0.001 *** **	-	-	-
LE-EHE D0 - LE-EHE D7	-0.449	0.038	**< 0.001 *** **	-	-	-
LE D0 - LE D7	-0.348	0.039	**< 0.001 *** **	-	-	-
EHE D7 - LE-EHE D7	-0.487	0.439	1.000	-0.552	2.668	1.000
EHE D7 - LE D7	-0.097	0.427	1.000	-3.730	2.621	0.990
LE-EHE D7 - LE D7	0.391	0.428	1.000	-3.178	2.678	0.999
EHE D7 - EHE D14	-0.136	0.032	**0.003 ****	6.577	1.089	**< 0.001 *** **
LE-EHE D7 - LE-EHE D14	-0.117	0.038	0.206	5.426	1.315	**0.005 ****
LE D7 - LE D14	-0.071	0.039	0.963	5.154	1.330	**0.013 ***
EHE D14 - LE-EHE D14	-0.469	0.439	1.000	-1.703	2.666	1.000
EHE D14 - LE D14	-0.032	0.427	1.000	-5.153	2.621	0.861
LE-EHE D14 - LE D14	0.437	0.428	1.000	-3.450	2.676	0.997
EHE D14 - EHE D21	-0.043	0.032	0.999	3.969	1.094	**0.033 ***
LE-EHE D14 - LE-EHE D21	-0.055	0.038	0.998	2.507	1.310	0.906
LE D14 - LE D21	-0.083	0.039	0.848	4.646	1.330	**0.050 ***
EHE D21 - LE-EHE D21	-0.480	0.439	1.000	-3.165	2.668	0.999
EHE D21 - LE D21	-0.071	0.427	1.000	-4.476	2.623	0.949
LE-EHE D21 - LE D21	0.409	0.429	1.000	-1.311	2.676	1.000

EHE D21 - EHE D28	-0.066	0.032	0.879	-4.890	1.100	**< 0.001 *** **
LE-EHE D21 - LE-EHE D28	-0.094	0.038	0.634	-1.384	1.315	1.000
LE D21 - LE D28	-0.113	0.039	0.292	0.875	1.335	1.000
EHE D28 - LE-EHE D28	-0.508	0.439	0.999	0.342	2.671	1.000
EHE D28 - LE D28	-0.118	0.427	1.000	1.289	2.625	1.000
LE-EHE D28 - LE D28	0.390	0.428	1.000	0.947	2.680	1.000
EHE D28 - EHE D35	0.041	0.032	1.000	-4.797	1.106	**0.002 ** **
LE-EHE D28 - LE-EHE D35	0.157	0.038	**0.007 ** **	0.074	1.331	1.000
LE D28 - LE D35	-0.070	0.039	0.966	-2.644	1.335	0.878
EHE D35 - LE-EHE D35	-0.392	0.439	1.000	5.213	2.679	0.869
EHE D35 - LE D35	-0.229	0.427	1.000	3.443	2.626	0.996
LE-EHE D35 - LE D35	0.162	0.428	1.000	-1.770	2.683	1.000
EHE D35 - EHE D42	0.070	0.032	0.810	2.389	1.156	0.835
LE-EHE D35 - LE-EHE D42	0.171	0.038	**< 0.001 *** **	1.153	1.384	1.000
LE D35 - LE D42	-0.121	0.039	0.217	-3.006	1.352	0.738
EHE D42 - LE-EHE D42	-0.291	0.439	1.000	3.976	2.721	0.987
EHE D42 - LE D42	-0.420	0.427	1.000	-1.953	2.657	1.000
LE-EHE D42 - LE D42	-0.129	0.429	1.000	-5.929	2.719	0.752

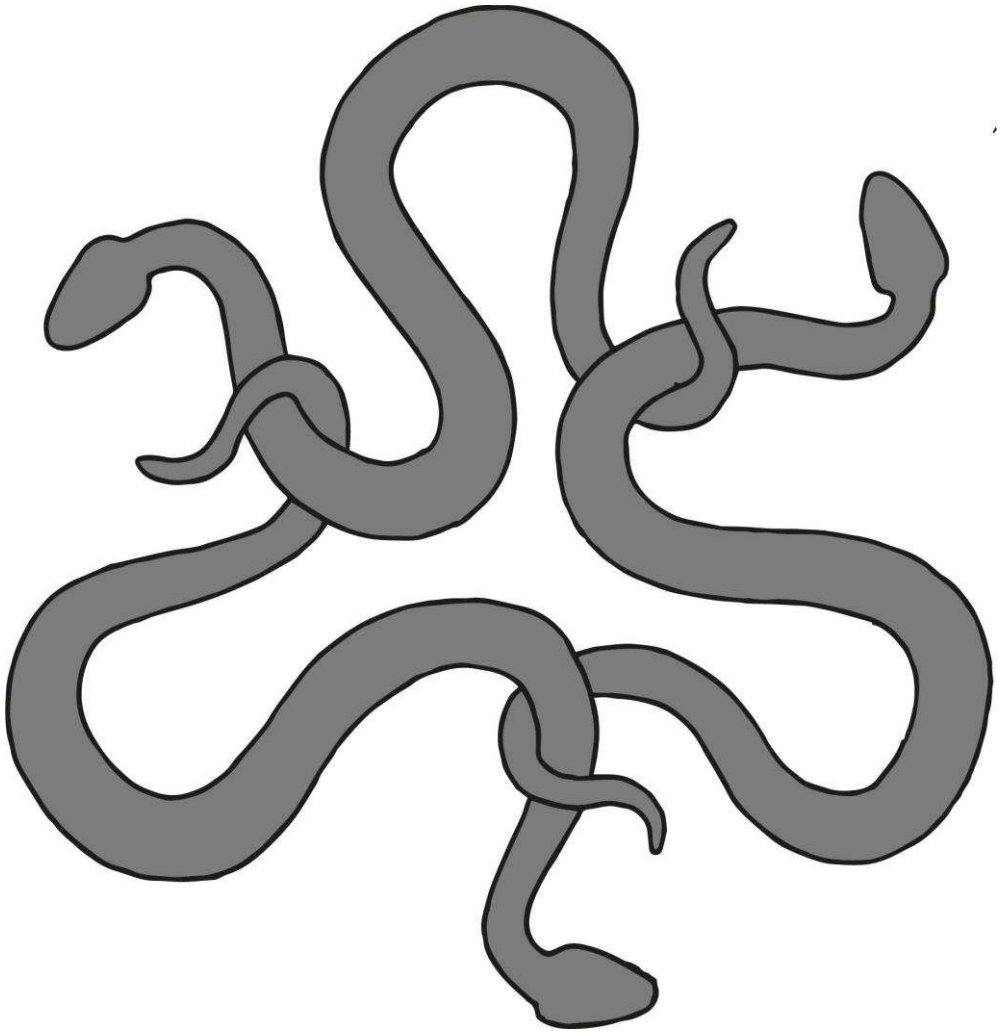
Supplemantary table 2

Results of the pairwise comparison of least-squares means and adjusted p-values for multiple comparisons with the Tukey method from the linear mixed-effect model testing for the effect of incubation treatment. the age at measurement (days post-laying or days post-hatching) and their interaction on the respirometry quotient (RQ) in the embryos and juveniles on the snake of *Natrix maura* (Figure 3A & 3B; Table 3). The three treatments are gestation at extreme high elevation and incubation at extreme high elevation (EHE; embryo: N = 59; and juvenile: N = 58), gestation at low elevation and incubation at extreme high elevation (LE-EHE; embryo: N = 51; and juvenile: N = 48) and gestation at low elevation and incubation at low elevation (LE; embryo: N = 45; and juvenile: N = 42). Significant differences between least-squares means shown in bold with one (P < 0.05) or three (P < 0.001) asterisks.

	Embryo RQ			**Juvenile RQ**		
	Estimate	*SE*	*P-value*	*Estimate*	*SE*	*P-value*
EHE D14 - LE-EHE D14	-0.002	0.023	1.000	-0.014	0.020	0.981
EHE D14 - LE D14	0.008	0.023	0.999	-0.224	0.020	**< 0.001** ***
LE-EHE D14 - LE D14	0.010	0.023	0.998	-0.210	0.020	**< 0.001** ***
EHE D14 - EHE D28	0.087	0.015	**< 0.001** ***	-0.051	0.012	**< 0.001** ***
LE-EHE D14 - LE-EHE D28	0.093	0.017	**< 0.001** ***	-0.020	0.014	0.670
LE D14 - LE D28	-0.001	0.018	1.000	0.018	0.014	0.802
EHE D28 - LE-EHE D28	0.004	0.022	1.000	0.017	0.020	0.954
EHE D28 - LE D28	-0.080	0.023	**0.015** *	-0.155	0.020	**< 0.001** ***
LE-EHE D28 - LE D28	-0.084	0.023	**0.010** *	-0.172	0.020	**< 0.001** ***

Chapitre 6.

DISCUSSION GÉNÉRALE



6.1. Synthèse générale

6.1.1. Remise en contexte

Selon l'hypothèse de la remonté altitudinale, on peut s'attendre à ce que la Couleuvre vipérine, *Natrix maura* (Linnaeus, 1758), bien présente dans les Pyrénées jusqu'à 1000 m en France et 1500 m en Espagne (*cf. section 2.1.1*), remonte en altitude avec l'augmentation des températures, induite par le réchauffement climatique (*cf. section 1.1.5*). La colonisation des milieux de haute altitude par cette espèce pourrait néanmoins être limitée par le manque de dioxygène entrainant à long terme une réduction de l'aire de répartition de l'espèce dans les Pyrénées. En outre, la colonisation et l'établissement des populations dans un nouveau milieu passe par le succès reproducteur des individus (Grevstad, 1999; Yeh, 2004). Pour savoir si les populations de Couleuvre vipérine vont pouvoir s'établir en haute altitude, nous avons émis différentes hypothèses des effets de l'hypoxie d'altitude sur le succès du développement embryonnaire et les performances juvéniles. Les hypothèses de travail étaient que l'hypoxie d'altitude pourrait à la fois influencer de manière négative le développement embryonnaire et réduirait également les performances des juvéniles. Elles proposaient également que les effets négatifs, dus à un développement en hypoxie, se maintiendraient même après un retour des individus en condition de normoxie. De plus, les hypothèses prédisaient qu'une augmentation des températures pendant l'incubation aggraverait les effets de l'hypoxie d'altitude. Cependant, nos hypothèses proposaient également que les effets négatifs d'une incubation en haute altitude seraient réduits par les effets maternels, c'est à dire par une « préparation aux conditions environnementales » par les femelles gravides, si la première partie du développement embryonnaire (*i.e.* avant la ponte) était effectué en condition d'hypoxie de haute altitude. En effet, à travers l'histoire de cette espèce (*cf. section 2.1.1*), qui a colonisé les environnements montagneux des Pyrénées à travers les cycles historiques de réchauffement et de refroidissement (Gómez and Lunt, 2007), il est possible que des formes de plasticité adaptative se soient développées en réponse à l'hypoxie de haute altitude.

Les travaux de recherche présentés dans ce manuscrit s'inscrivant dans le contexte du réchauffement climatique, plusieurs scénarios climatiques ont été utilisés pour déterminer les effets de l'hypoxie d'altitude couplés à une augmentation des températures sur le métabolisme de l'embryon et du juvénile chez la Couleuvre vipérine. Compte tenu des prédictions de réchauffement proposés par le GIEC *(cf. section 1.1.3*) et considérant le concept de la remontée altitudinale des espèces (*cf*. *section 1.3*), quatre scénarios possibles ont été considérés (Figure 1) :

- Scénario 1 : Un environnement normoxique (*i.e.* équivalent à 21% d'O_2 au niveau de la mer) avec des températures favorables (*i.e.* 24°C et 28°C), qui correspond, pour des populations de basse altitude, aux conditions actuelles, sans augmentation des températures.
- Scénario 2 : Un environnement normoxique (*i.e.* équivalent à 21% d'O_2 au niveau de la mer) avec une température élevée (*i.e.* 32°C), qui correspond au scénario du réchauffement climatique pour des populations de basse altitude qui ne migrent pas.
- Scénario 3 : Un environnement hypoxique (*i.e.* équivalent à 15% d'O_2 au niveau de la mer) avec des températures favorables (*i.e.* 24°C et 28°C), qui correspond, pour des populations de basse altitude, aux conditions environnementales qu'elles pourront rencontrer en migrant en haute altitude.
- Scénario 4 : Un environnement hypoxique (*i.e.* équivalent à 15% d'O_2 au niveau de la mer) avec une température élevée (*i.e.* 32°C), qui correspond aux conditions environnementales futures en haute altitude, si le changement climatique perdure, et qui pourront être rencontrées par des populations ayant migré et qui se seraient maintenues en haute altitude.

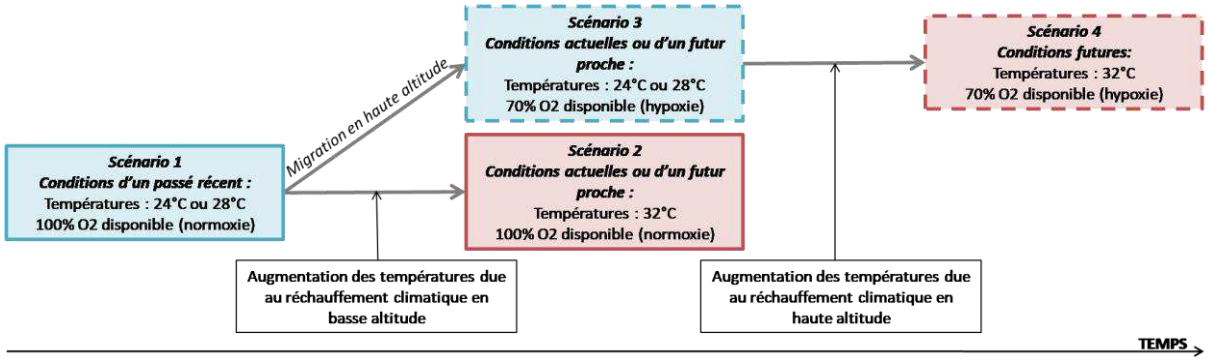


Figure 1 *: Résumé temporel schématique des différents scénarios considérés.*

Deux tableaux résumant les résultats comparatifs des mesures du développement embryonnaire (Tableau 1) et des phénotypes juvéniles (Tableau 2) en fonction des différentes combinaisons de températures (*i.e.* 24°C, 28°C et 32°C) et de niveaux de dioxygène disponible (*i.e.* normoxie et hypoxie) sont présentés ci-dessous. Ces différents résultats vont être remis en contexte et discutés selon les 4 scénarios décrits.

***Tableau 1** : Comparatif des principaux résultats des mesures du développement embryonnaire en fonction des différentes conditions d'incubation des œufs de Couleuvre vipérine.*

Traitement initial	*Traitement de comparaison*			
	Normoxie à 24°C	**Hypoxie à 24°C**	**Normoxie à 28°C**	**Normoxie à 32°C**
Hypoxie à 24°C	= Masse des œufs = Rythme cardiaque - Durée d'incubation = Succès d'éclosion	/	/	+ Masse des œufs - Rythme cardiaque + Durée d'incubation = Succès d'éclosion
Normoxie à 24°C	/	/	/	+ Masse des œufs - Rythme cardiaque + Durée d'incubation = Succès d'éclosion
Hypoxie à 28°C	/	/	= / - Masse des œufs - / + Rythme cardiaque - Métabolisme respiratoire = / - Durée d'incubation = Succès d'éclosion	/
Hypoxie à 32°C	- Masse des œufs + Rythme cardiaque - Durée d'incubation - Succès d'éclosion	- Masse des œufs + Rythme cardiaque - Durée d'incubation - Succès d'éclosion	/	= Masse des œufs - Rythme cardiaque + Durée d'incubation - Succès d'éclosion

***Tableau 2** : Comparatif des principaux résultats des mesures du phénotype juvénile en fonction des différentes conditions d'incubation des œufs de Couleuvre vipérine.*

Traitement initial	*Traitement de comparaison*			
	Normoxie à 24°C	**Hypoxie à 24°C**	**Normoxie à 28°C**	**Normoxie à 32°C**
Hypoxie à 24°C	= Résidu de l'œuf = Masse corporelle = Longueur du corps = Condition corporelle = Capacité de nage	/	/	+ Résidu de l'œuf = Masse corporelle + Longueur du corps - Condition corporelle = Capacité de nage
Normoxie à 24°C	/	/	/	+ Résidu de l'œuf = Masse corporelle + Longueur du corps - Condition corporelle - Capacité de nage
Hypoxie à 28°C	/	/	= / + Résidu de l'œuf = / - Masse corporelle = / - Longueur du corps = / - Condition corporelle - Métabolisme respiratoire - Capacité denage = Capacité d'apnée	/
Hypoxie à 32°C	- Résidu de l'œuf - Masse corporelle - Longueur du corps = Condition corporelle + Capacité de nage	- Résidu de l'œuf - Masse corporelle - Longueur du corps = Condition corporelle + Capacité de nage	/	= Résidu de l'œuf - Masse corporelle - Longueur du corps - Condition corporelle = Capacité de nage

6.1.2. Scénario 1 : normoxie et températures favorables

Le scénario 1 représente des conditions d'incubation actuelles de températures (*i.e*. 24°C et 28°C) et de niveau de dioxygène disponible (*i.e*. équivalent à 21% d'O_2 au niveau de la mer, normoxie) pour des populations de Couleuvre vipérine qui ne subiraient pas les effets du réchauffement climatique (Figure 1). Dans cette thèse, ce scénario est considéré comme le scénario contrôle.

Nos résultats montrent que la masse des œufs reste stable ou augmente légèrement durant l'incubation avant de chuter dans le dernier stade du développement. Ils montrent également pour une température d'incubation de 28°C, comparée à celle de 24°C, une augmentation du rythme cardiaque et une diminution de la durée d'éclosion. Ces résultats sont en accord avec d'autres études portant sur cette espèce dans des conditions d'incubation similaires (Aubret, 2013a; Aubret et al., 2016a; b, 2017). Ils sont aussi en accord avec une littérature plus générale chez les ectothermes qui spécifie que le rythme cardiaque s'accélère avec l'augmentation de l'activité métabolique (Greenwald, 1971; Du et al., 2010b; c) qui est elle-même est positivement corrélée à la température (Bull, 1980; Shine and Harlow, 1993; Angilletta et al., 2002). Enfin, nos résultats corroborent le fait que la température soit le facteur déterminant de la durée d'incubation chez les reptiles non-aviens (Shine, 2004; Noble et al., 2018). Durant l'incubation, le métabolisme respiratoire des embryons est resté stable. Cependant, selon la littérature, en condition de normoxie et à des températures de 28°C, le métabolisme devrait augmenter en fin d'incubation (Kouyoumdjian et al., 2019). En effet, la dernière phase du développement embryonnaire est celle où la demande en dioxygène est la plus importante (Dmi'el, 1970; Sartori et al., 2017). Par conséquent, le métabolisme respiratoire de l'embryon augmente pour assurer un apport en dioxygène suffisant. Dans le cas de cette thèse, il est possible que la deuxième mesure du métabolisme ait été effectuée trop tôt dans le développement embryonnaire pour remarquer cette augmentation. Dans l'ensemble, le succès d'éclosion (≥ 90%) et le sexe ratio équilibré sont comparables à ceux de la littérature pour une condition d'incubation similaire (Aubret, 2013a; Aubret et al., 2016a; b, 2017).

A l'éclosion, les juvéniles de Couleuvre vipérine sont de longueur corporelle (≈ 15,36 cm) et de masse corporelle (≈ 2,86 g) comparables à ce qui est connus chez cette espèce (Aubret, 2013a; Aubret et al., 2016a; b, 2017). Durant le premier mois post éclosion, le métabolisme respiratoire des juvéniles reste stable. Vu que l'augmentation de la consommation en dioxygène est positivement corrélée à la masse (Graham, 1974), il n'est pas étonnant de constater que les juvéniles ne grossissent pas durant le premier mois. Enfin, la vitesse de nage des juvéniles, un trait écologique important pour échapper aux prédateurs et acquérir de la nourriture (Jayne and Bennett, 1990; Kingsolver et al., 2001),

augmente progressivement durant les 40 jours post-éclosion, car elle est corrélée positivement à la longueur corporelle des individus (Shine and Shetty, 2001; Aubret et al., 2015).

Avec l'augmentation des températures de plusieurs degrés d'ici 2100 prévu par le GIEC (Pachauri et al., 2014; IPPC, 2018), notamment au niveau des surfaces émergées du globe (Arneth et al., 2019), l'espèce va certainement devoir faire face à un changement environnemental majeur. La Couleuvre vipérine étant ectotherme, l'ensemble de leur cycle biologique est dépendant de la température environnementale (*cf. section 1.2*). Ainsi, les populations de Couleuvre vipérine qui resteront à basse altitude vont devoir s'adapter, si cela est possible, à l'augmentation des températures due au réchauffement climatique, au travers de mécanismes tels que la plasticité phénotypique, notamment durant le développement embryonnaire et/ou durant le stade juvénile.

6.1.3. Scénario 2 : normoxie et réchauffement climatique

Pour tester la plasticité phénotypique des embryons et des juvéniles de Couleuvre vipérine en réponse à l'augmentation des températures en basse altitude, nous nous sommes intéressés à un second scénario (scénario 2 ; Figure 1) représentant des conditions d'incubation normoxique (*i.e.* équivalent à 21% d'O_2 au niveau de la mer) mais à une température élevée (*i.e.* 32°C).

Le développement embryonnaire des reptiles non-aviens est principalement contrôlé par la température d'incubation (Deeming and Ferguson, 1991; Deeming, 2004; Booth, 2006; Goodman, 2008; Warner, 2014). Le rythme cardiaque des embryons, fortement corrélé à la température d'incubation (Greenwald, 1971; Du et al., 2010a; c), ainsi qu'à la durée d'incubation (Shine, 2004; Noble et al., 2018) en sont des bons exemples. Des températures élevées durant l'incubation augmentent l'activité métabolique des individus (Bull, 1980; Shine and Harlow, 1993; Angilletta et al., 2002), ce qui peut augmenter la conversion de l'énergie métabolique de l'embryon. À ces températures, les œufs évacuent la chaleur produite par cette augmentation de l'activité métabolique par l'intermédiaire de la perte de l'eau du milieu interne de l'œuf, réduisant ainsi leur masse (Ackerman et al., 1985). Conformément à ces prédictions, nos résultats (Tableau 1), dans le cadre du scenario 2, montrent un rythme cardiaque plus élevé, une durée d'incubation plus courte et une prise de masse des œufs plus faible comparés aux résultats de ces mêmes traits en condition de normoxie à des températures plus basses (*i.e.* 24°C et 28°C). Bien que la température d'incubation élevée (*i.e.* 32°C) ait des effets non négligeables sur le développement embryonnaire, cela ne semble pas influencer ni le succès d'éclosion, ni le sexe ratio. Nos résultats mettent en évidence une plasticité du développement embryonnaire chez la Couleuvre vipérine, mais cette plasticité peut

avoir un coût. En effet, la perte d'eau dans le milieu interne de l'œuf en réponse à l'augmentation de la température d'incubation peut progressivement augmenter la viscosité du jaune et entraver l'absorption par l'embryon en développement (Cunningham and Hurwitz, 1936; Aubret et al., 2005) ce qui pourrait affecter négativement le phénotype à l'éclosion.

Nos résultats, en accord avec la littérature (Deeming and Ferguson, 1991; Shine et al., 1997; Wapstra, 2000; Blouin-Demers et al., 2004; Booth, 2006; Watkins and Vraspir, 2006; Warner, 2014) montrent qu'à l'éclosion les juvéniles ont une masse corporelle équivalente mais toutefois d'une longueur corporelle réduite comparées à celles des juvéniles issus d'une incubation en normoxie à des températures basses. Néanmoins, alors que la littérature s'accorde sur le fait que chez les ectothermes, une incubation à des températures froides produit des nageurs plus rapides (Shine, 1999; Angilletta and Dunham, 2003; Watkins and Vraspir, 2006), nos résultats montrent que les juvéniles de Couleuvre vipérine issus d'une incubation à une température élevée (*i.e.* 32°C) nagent plus rapidement que ceux incubés aux basses températures (*i.e.* 24°C et 28°C). Ce résultat s'explique potentiellement par le fait que chez les reptiles, les conditions d'incubation peuvent indiquerla qualité de l'environnement et donc favoriser, dans le cas où celui-ci ne semble pas favorable, des comportements de dispersions efficaces, comme l'augmentation des performances locomotrices (Clobert et al., 2009; Bestion et al., 2015c; Aubret et al., 2016a).

Nos résultats montrent que la température d'incubation élevée impacte le développement embryonnaire mais pas le succès d'éclosion ni le sexe ratio. Cela confirme l'hypothèse que l'augmentation des températures, induite par le réchauffement climatique, pourrait avoir un impact négatif sur le développement embryonnaire (Andrews and Schwarzkopf, 2012; Mitchell et al., 2018b), entrainant une diminution de la taille corporelle des juvéniles à l'éclosion (Daufresne et al., 2009; Gardner et al., 2011; Sheridan and Bickford, 2011). La règle de la TSR (*cf. section 1.2.2*) nous permet de supposer que l'augmentation des températures pourrait également altérer le développement juvénile et donc diminuer la taille corporelle des individus à l'âge adulte (Invertébrés: Frazier et al., 2006; Brans and Meester, 2018; Poissons: Loisel et al., 2019). Bien que cela n'a pas été mesuré durant cette thèse, il est ainsi possible que l'augmentation des températures lors du développement embryonnaire entraine des coûts énergétique à long terme chez les individus, diminuant le taux de survie à l'âge adulte (Frazier et al., 2006; Santos, 2007; Bestion et al., 2015a). Comme chez de nombreuses espèces ectothermes, les changements phénotypiques pourront avoir pour conséquences des modifications dans la structure et la dynamique des populations de Couleuvre vipérine (Daufresne et al., 2009; Le Galliard et al., 2010; Cunningham et al., 2017) et risqueront d'impacter négativement la démographie des populations,

quand elles n'entraineront pas leur extinction (Whitfield et al., 2007; Sinervo et al., 2010; Bestion et al., 2015a).

Par conséquent, en réaction au réchauffement climatique, certaines espèces déplacent progressivement leur étendue géographique vers des environnements aux conditions climatiques pour lesquelles elles sont pré-adaptées (Sorte et al., 2010; Wernberg et al., 2011). Elles peuvent notamment migrer en altitude (*cf. section 1.3*) pour retrouver des températures environnementales favorables (Bässler et al., 2013; Pauchard et al., 2016; Freeman et al., 2018; Sinervo et al., 2018), ce que nous avons testé dans le cadre du scénario 3.

6.1.4. Scénario 3 : hypoxie d'altitude et températures favorables.

Pour tester la plasticité phénotypique du développement embryonnaire de la Couleuvre vipérine en réponse à l'hypoxie de haute altitude, cette thèse s'est attachée à réaliser un scénario probable de migration en haute altitude. Le scénario 3 (Figure 1) représente des conditions d'incubation qui seraient rencontrées, aujourd'hui ou dans un futur proche, par des populations de Couleuvre vipérine qui migreraient en altitude, avec des températures basses (*i.e*. 24°C et 28°C) et un niveau de dioxygène disponible faible (*i.e*. équivalent à 15% d'O_2 au niveau de la mer, hypoxie d'altitude de 30%).

Chez les serpents et les lézards, la fréquence cardiaque est fortement corrélée au taux de consommation du dioxygène (Greenwald, 1971; Bennett, 1972; Butler et al., 2004; Du et al., 2010a; Kouyoumdjian et al., 2019). Conformément à d'autres études réalisées sur le Lézard des murailles dans les mêmes conditions (Cordero et al., 2017a; Kouyoumdjian et al., 2019), nous nous attendions en effet à une réduction du rythme cardiaque en réponse à l'hypoxie chronique, comme cela a été montré chez d'autres Vertébrés endothermes (Monge and Leon-Velarde, 1991; Peacock, 1998).Nos résultats ne sont pas aussi tranchés. À une température d'incubation de 24°C, le rythme cardiaque des embryons incubés en hypoxie est resté similaire à celui des embryons incubés en normoxie (Tableau 1). En effet, à cette température d'incubation basse, l'activité métabolique est réduite (Greenwald, 1971; Bull, 1980; Shine and Harlow, 1993; Angilletta et al., 2002; Du et al., 2010b; c), limitant probablement le besoin en dioxygène et limitant ainsi les effets de l'hypoxie d'altitude. À une température d'incubation légèrement plus élevée (*i.e.* 28°C), nous avons constaté que, selon les années d'expérimentation, l'hypoxie d'altitude pouvait soit augmenter, soit diminuer le rythme cardiaque des embryons par rapport à celui des embryons incubés en basse altitude (Tableau 1). Tous les embryons répondant de la même manière au cours d'une même expérimentation, cette

variation interannuelle n'est pas vraiment expliquée. Elle pourrait découler de paramètres parfaitement non-contrôlés selon l'année d'expérimentation, tels que des modifications dans la physiologie des femelles gravides capturées dues à des variations des conditions environnementales. Si l'effet de l'hypoxie sur le rythme cardiaque des embryons de Couleuvre vipérine n'a pas pu être réellement mis en évidence, son effet sur la masse des œufs et sur la durée d'incubation s'est avéré beaucoup plus sensible. La masse des œufs incubés en hypoxie est restée stable (ou a augmenté légèrement durant l'incubation), mais la masse moyenne des œufs est restée inférieure à celle des œufs incubés en basse altitude à la même température (*i.e.* 24°C et 28°C; Tableau 1). De plus, nos résultats montrent qu'en fin d'incubation, la masse des œufs chute de manière plus importante qu'en condition de normoxie à basses températures. Ce résultat peut s'expliquer par l'incompatibilité entre la demande en dioxygène des embryons et l'apport disponible en hypoxie de haute altitude dans la dernière phase de développement embryonnaire, au moment où la demande en dioxygène de l'embryon augmente (Dmi'el, 1970; Sartori et al., 2017). Cela peut entrainer une diminution de la fixation du carbone et donc de la transformation des réserves lipidiques en glucides (Cunningham and Hurwitz, 1936), réduisant ainsi la prise de masse de l'embryon. En effet, l'hypoxie est connue pour rendre le développement embryonnaire difficile, en raison des restrictions énergétiques aérobies dans la transformation des réserves lipidiques (oiseaux: Wangensteen et al., 1974; Rahn et al., 1977; Monge and Leon-Velarde, 1991; Noble, 1991; Vleck and Hoyt, 1991; Vleck and Vleck, 1996; León-Velarde and Monge, 2004; Mammifères: Bouverot, 1985; Monge and Leon-Velarde, 1991; reptiles: Noble, 1991; Vleck and Hoyt, 1991), susceptible de mener à une réduction de la taille des individus à la naissance (Douglas et al., 2005; Du et al., 2010a; Harrison et al., 2015; Cordero et al., 2017a; Kouyoumdjian et al., 2019; Parker and Dimkovikj, 2019; Li et al., 2020). Parallèlement à la réduction de la masse des œufs en fin d'incubation, nos résultats montrent les embryons de Couleuvre vipérine incubés en hypoxie présentent une hyperventilation, qui se caractérise par une augmentation de la consommation de dioxygène et une diminution de la production de dioxyde de carbone (Gardner, 1996). Ce résultat est en accord avec de nombreuses études chez les Vertébrés qui indique l'hyperventilation comme une réponse commune à l'hypoxie (Bouverot, 1985; Faraci, 1991; Monge and Leon-Velarde, 1991; Peacock, 1998; Scott and Milsom, 2006; Powell and Hopkins, 2010; Storz et al., 2010). Pour autant, le success à l'éclosion ainsi que la masse et la taille corporelle des juvéniles (Tableau 2) issus d'une incubation en hypoxie d'altitude à une température de 24°C ou 28°C sont restés similaires à ceux des juvéniles issus d'une incubation en normoxie aux mêmes températures. Ces résultats sont en partie contraire à la littérature, où plusieurs études ont montré qu'à l'éclosion, la taille corporelle des juvéniles était réduite (alligators: Owerkowicz et al., 2009a; Iungman and Piña, 2013; crustacés: Peck and Chapelle, 2003; lézards: Du et al., 2010a; Cordero et al., 2017a; Kouyoumdjian et al., 2019; Li et al., 2020). Dans nos

expérimentations, la perte de masse des œufs en fin d'incubation en condition hypoxique ne se traduit donc pas par une augmentation de la mortalité embryonnaire ou par une réduction de la masse corporelle des juvéniles à l'éclosion. Couplés à la réduction observée du métabolisme durant le développement des embryons en hypoxie (Tableau 1), notamment à travers la diminution du nombre de battements cardiaques par minute (Du et al., 2009, 2011) et se traduisant par une augmentation de la durée d'incubation des embryons, ces résultats suggèrent que la présence d'une plasticité développementale embryonnaire en réponse à l'hypoxie d'altitude favoriseraient le succès d'éclosion de la Couleuvre vipérine.

Au stade juvénile, nos résultats montrent que le métabolisme respiratoire au repos des Couleuvre vipérine, issus d'une incubation en hypoxie d'altitude, est inférieur à celui des juvéniles issus d'une incubation en normoxie (Tableau 2) et qu'ils présentent une hyperventilation. Ces résultats sont en accord avec la littérature, où il est montré qu'en hypoxie le métabolisme est réduit aussi bien chez des espèces ectothermes (Insects: Hoback and Stanley, 2001; lézards: González-Morales et al., 2015; Lu et al., 2015; Gangloff et al., 2019; Li et al., 2020; tortues: Altland and Parker, 1955; Boyer, 1963; Jackson, 1973; Stone et al., 1992; Herman and Smatresk, 1999), que chez des espèces endothermes (Mammifères: Ramirez et al., 2007; oiseaux: Ramirez et al., 2007; Lague et al., 2016). En accord avec de récents travaux (Dahlhoff et al., 2019; Gangloff et al., 2019), nos résultats montrent également que la réduction du métabolisme des juvéniles issus d'une incubation en condition d'hypoxie à une température de 28°C réduit aussi les performances physiques (*i.e.* vitesse de nage) des individus (Tableau 2). Cependant, les juvéniles issus d'une incubation en hypoxie à une température de 24°C maintiennent des vitesses de nage similaire aux juvéniles incubés en normoxie à la même température, comme cela a été montré dans de précédentes études (Du et al., 2010a; Li et al., 2020). Dans ce cas, il est possible que le développement en hypoxie ait modifié la composition sanguine des individus (Storz, 2007; Storz et al., 2010), améliorant ainsi la capacité du sang à transporter l'oxygène et permettant le maintien des performances physiques. Ce changement biochimique, bien connu des populations de reptiles non-aviens vivant en haute altitude, se traduit par une concentration d'hémoglobine plus grande et un nombre d'hématocrites dans le sang plus élevés que dans les populations de basse altitude (Vinegar and Hillyard, 1972; Weathers and White, 1972; Newlin and Ballinger, 1976; González-Morales et al., 2015; Lu et al., 2015), suggérant un certain niveau de plasticité phénotypique chez les juvéniles. Enfin, les embryons incubés en haute altitude et issus des femelles gravides qui ont également réalisé leur gestation en haute altitude n'ont montré aucune différence avec les embryons incubés en haute altitude mais issus des femelles gravides qui ont réalisé leur gestation en basse altitude.

Pour résumé, nos résultats montrent que, comme chez d'autres espèces de Squamates, les embryons de Couleuvre vipérine incubés en haute altitude ne subissent pas de réduction du succès d'éclosion mais présentent des modifications phénotypiques à la naissance (Du et al., 2010a; Cordero et al., 2017a; Kouyoumdjian et al., 2019; Li et al., 2020). Cette plasticité développementale embryonnaire, bien qu'elle favorise l'acclimatation à l'hypoxie d'altitude pourrait entrainer des coûts à long terme pour les individus (i.e. hyperventilation, réduction des performances locomotrices, modification de la composition sanguine). Par exemple, les modifications de la composition sanguine des juvéniles, qui permet le maintien des performances est surtout due à l'augmentation de la densité des globules rouges qui à long terme, pourrait augmenter la viscosité du sang et donc les dépenses énergétiques liées à la circulation sanguine (Hedrick et al., 1986; Dunlap, 2006). Néanmoins, dans un contexte de migration en altitude pour retrouver des températures environnementales favorables, nos résultats suggèrent que le stade embryonnaire ne sera pas le facteur limitant dans la capacité à coloniser les milieux de hautes altitudes. En effet, les réponses physiologiques plastiques pourraient atténuer le stress environnemental, favorisant ainsi la survie de la progéniture et modifiant l'évolution ultérieure des populations colonisatrices (Atkinson and Thorndyke, 2001; McNab, 2002; West-Eberhard, 2003; Hammond et al., 2006; Ghalambor et al., 2007).

Cependant, bien que plusieurs espèces de reptiles soient déjà adaptées à la vie à des altitudes extrêmement élevées (*i.e. les Lézards du genre Liolaemus*: Marquet et al., 1989; le Lézards des palissades, *Sceloporus occidentalis* et le Lézard de Sagebrush, *Sceloporus graciosus*: Adolph, 1990; le Lézard de Bonnal, *Iberolacerta bonnali;* Pottier, 2012; *Quedenfeldtia trachyblepharus:* Bouazza et al., 2016; les serpents du genre *Thermophis* : Li et al., 2018; *Phrynocephalus vlangalii:* Wu et al., 2018),si l'augmentation des températures, à travers le réchauffement climatique, continue (scénario 4 ; Figure 1), le mécanisme de production énergétique aérobie des tétrapodes ectothermes pourrait être limité par la diminution de la disponibilité en dioxygène (*i.e.* OCLTT ; *cf. section 1.4.3* (Pörtner, 2002; Pörtner et al., 2017; Gangloff and Telemeco, 2018).

6.1.5. Scénario 4 : hypoxie d'altitude et réchauffement climatique.

Le quatrième scénario envisagé (Figure 1) concerne le devenir des populations de Couleuvre vipérine qui auraient migré et se seraient maintenues en hypoxie d'altitude (*i.e*. équivalent à 15% d'O_2 au niveau de la mer, hypoxie d'altitude de 30%), mais qui subiraient malgré tout, à terme, une augmentation des températures (*i.e*. 32°C) en réponse au changement climatique.

Nos résultats indiquent que l'incubation des embryons dans une condition hypoxique à une température de 32°C, comparée à une incubation hypoxique à une température de 24°C, entraine une augmentation du rythme cardiaque, réduit la masse moyenne des œufs et réduit la durée d'incubation (Tableaux 1). A l'éclosion, les juvéniles sont de taille réduite et la vitesse de nage est supérieure (Tableau 2). Ces résultats sont similaires à la comparaison des résultats entre une incubation en normoxie à une température de 24°C et 32°C (*cf. section 6.1.3*). Cela indiquent que même si l'hypoxie d'altitude modifie le développement embryonnaire (Cordero et al., 2017a; Kouyoumdjian et al., 2019; Parker and Dimkovikj, 2019; Li et al., 2020), le facteur principale du développement des embryons reste la température d'incubation (Deeming and Ferguson, 1991; Deeming, 2004; Booth, 2006; Goodman, 2008; Warner, 2014). Pour autant, on constate également une diminution du succès d'éclosion, ce qui suggère globalement un effet délétère de la double contrainte hypoxie d'altitude – température élevée durant l'incubation.

Concernant le développement embryonnaire à une température de 32°C en hypoxie, nos résultats montrent que, comparé une incubation à 32°C en normoxie, le rythme cardiaque des embryons est réduit (Tableau 1). Ce résultat est conforme à la littérature et est une réponse connue à l'hypoxie chronique chez les Vertébrés (Monge and Leon-Velarde, 1991; Peacock, 1998; Cordero et al., 2017a; Kouyoumdjian et al., 2019). En effet, l'augmentation des températures accélère la fréquence cardiaque des embryons et augmente leur besoin en dioxygène (Du et al., 2010a; b; Hall and Warner, 2020). Avec une disponibilité en dioxygène réduite, les embryons ne peuvent pas maintenir des niveaux métaboliques maximum (*i.e.* quand les niveaux de dioxygène ne sont pas limitant).Avec cette réduction du rythme cardiaque, la durée d'incubation est plus longue (Tableau 1). Ce résultat est en accord avec la littérature si l'on considère que le nombre de battements cardiaques est le facteur déterminant de la durée d'incubation (Du et al., 2009, 2011). Nos résultats soulignent également que la masse des œufs est similaire à celle des œufs incubés en normoxie pour la même température d'incubation de 32°C (Tableau 1), avec une chute de la masse en fin d'incubation plus importante. Cette chute plus importante en hypoxie est due à l'incompatibilité entre la demande en dioxygène des embryons et l'apport disponible en hypoxie de haute altitude, réduisant la fixation du carbone et diminuant la transformation des réserves lipidiques en glucides (Cunningham and Hurwitz, 1936; *cf. section 1.6.4*). Nos résultats montrent que dans cette condition d'incubation le succès d'éclosion est inférieur (-15%) à celui de chacune des trois autres conditions d'incubation (Tableau 1). Cette réduction du succès d'éclosion en condition d'hypoxie et d'une haute température est connue (Iungman and Piña, 2013; Flewelling and Parker, 2015; Smith et al., 2015; Hall and Warner, 2020). Plus précisément, 92% des embryons qui n'ont pas éclos sont mort dans le dernier quart du développement, là où la demande en oxygène est la plus haute (Dmi'el, 1970; Sartori et al., 2017).

A l'éclosion, les juvéniles (Tableau 2) incubés en hypoxie à 32°C, ont une masse et une longueur corporelle plus petites que ceux incubés à une température de 32°C en normoxie. Ces résultats soulignent un effet direct de la réduction du développement embryonnaire (Douglas et al., 2005; Harrison et al., 2015). Malgré ces résultats, les mesures de vitesse de nage montrent que les juvéniles issus de cette incubation sont les plus rapides (Tableau 2). Cela n'est pas en accord avec la littérature qui stipule que dans cette condition d'incubation les performances physiques des juvéniles sont réduites (Iungman and Piña, 2013; Liang et al., 2015). En effet, selon la théorie de l'OCLTT (*cf. section 1.4.3*), l'augmentation de la température, et donc du métabolisme, augmente la demande en dioxygène(Pörtner, 2002; Jackson, 2007; Pörtner and Knust, 2007; Verberk et al., 2016; Pörtner et al., 2017). Ainsi l'inadéquation entre la demande en dioxygène de l'organisme et sa capacité à la fournir réduira les performances des individus (Pörtner, 2002; Pörtner et al., 2017; Gangloff and Telemeco, 2018). Néanmoins, les performances de nage de ces juvéniles maintenu en condition hypoxique diminue rapidement après l'éclosion. La vitesse de nage étant, chez les serpents, un trait écologique important pour échapper aux prédateurs et acquérir de la nourriture (Jayne and Bennett, 1990; Kingsolver et al., 2001), il est probable que la survie des juvéniles soient réduites.

Nos résultats montrent que dans une condition d'incubation hypoxique à une température élevée, le développement embryonnaire est impacté et le succès d'éclosion réduit. Cela suggère que la plasticité développementale embryonnaire en réponse à l'augmentation des températures (scénario 2) et à l'hypoxie d'altitude (scénario 3) ne permet pas, dans un contexte de double contraintes, aux embryons de produire des juvéniles acclimatés. De plus, les juvéniles ont une longueur corporelle réduite, et, bien que lesperformances de nage soient dans un premier temps supérieures, elles diminuent rapidement après l'éclosion, indiquant une probable diminution du taux de survie des juvéniles. Qui plus est, si dans le futur, les températures environnementales s'approchent des températures d'incubation létales, en accord avec l'OCLTT (Pörtner, 2002; Pörtner et al., 2017; Gangloff and Telemeco, 2018), les besoins en dioxygène de l'embryon ne pourront pas être assurés ce qui diminuera encore les succès d'éclosion (Iungman and Piña, 2013; Flewelling and Parker, 2015; Smith et al., 2015; Hall and Warner, 2020). De plus, tout comme l'effet de l'augmentation des températures en basse altitude, les changements phénotypiques pourraient avoir pour conséquences des modifications dans la structure et la dynamique des populations (Daufresne et al., 2009; Le Galliard et al., 2010; Cunningham et al., 2017) ce qui impactera négativement la démographie des populations, quand elles n'entraineront pas leur extinction (Whitfield et al., 2007; Sinervo et al., 2010; Bestion et al., 2015a).

6.2. Critiques et perspectives

Avec le réchauffement climatique, de nombreuses espèces migrent le long du gradient altitudinal des zones montagneuses pour retrouver des conditions environnementales favorables (Walther et al., 2002; Parmesan and Yohe, 2003; Hickling et al., 2006; Lenoir et al., 2008; Chen et al., 2011; Pottier, 2012; Bässler et al., 2013; Pauchard et al., 2016; Freeman et al., 2018; Bani et al., 2019). Cependant, les réponses physiologiques et comportementales à l'hypoxie de haute altitude chez les reptiles non-aviens sont encore mal connues et n'ont reçu qu'une attention récente (González-Morales et al., 2015; Lu et al., 2015; Cordero et al., 2017a; Li et al., 2018, 2020; Gangloff et al., 2019; Kouyoumdjian et al., 2019; Parker and Dimkovikj, 2019). Néanmoins, il est probable que, tout comme l'hypoxie liée à l'enfouissement des œufs ou à l'immersion prolongée dans l'eau, les réponses varient en fonction des différents ordres (*i.e.* Crocodiliens, Chéloniens et Squamates; Porteus et al., 2011). Des travaux plus poussés sont nécessaires pour mieux comprendre les mécanismes sous-jacents de la plasticité embryonnaire, mais aussi pour évaluer la plasticité comportementale potentielle des juvéniles qui se sont développés dans des milieux de haute altitude. De plus, les aspects maternels (*i.e.* choix de site de ponte) pourraient, dans un premier temps, prendre une part importante dans la réussite de la colonisation de ces milieux, actuellement considérés comme des refuges potentielsface au changement climatique (Sinervo et al., 2018). Dans un deuxième temps, il semble important de définir si ces espèces pourront se maintenir à ces altitudes si les températures continuent d'augmenter, même dans ces zones refuges. Bien que nos résultats aient montré que les embryons de Couleuvre vipérine soient en mesure de se développer en hypoxie de haute altitude et d'être viables à la naissance, beaucoup de paramètres ne sont pas abordés dans nos expériences. Malgré le succès du développement embryonnaire en condition d'hypoxie, à travers la plasticité phénotypique, nous ne connaissons pas les conséquences sur le long terme, notamment sur la survie des juvéniles. Si ces modifications sont bénéfiques à l'individu et améliorent son acclimatation à l'hypoxie, alors la plasticité phénotypique embryonnaire sera considéré comme adaptative. A l'inverse, si les coûts réduisent l'acclimatation à long terme, alors la plasticité phénotypique embryonnaire sera considérée comme non-adaptative (Noble et al., 2018). En effet, nous savons que des températures élevées durant le développement peuvent imposer des contraintes à la croissance qui ne seront visibles que tardivement dans la vie de l'individu (Angilletta and Dunham, 2003). Il serait donc intéressant de continuer les mesures des performances des phénotypes des juvéniles à plus long terme. Enfin, afin de limiter le nombre de variables durant l'incubation et extraire des mesures précises des réponses des embryons aux différentes conditions testées, il était indispensable de simplifier les conditions d'incubation. Pour cela, nous nous sommes affranchi de plusieurs paramètres comportementaux naturels présents chez la Couleuvre vipérine. En effet, chez cette

espèce es femelles pondent dans un site de ponte adéquat pour le développement des embryons, et disposent les œufs accolés ou non. Ainsi, chez cette espèce qui pond dans des sites dits communautaires, l'éclosion des juvéniles peut se faire de manière synchrone au sein d'une même pontes mais aussi entre plusieurs pontes. En s'affranchissant des paramètres comportementaux de choix maternel et de l'éclosion synchrone, nous avons probablement modifié en partie les réponses à l'hypoxie du développement embryonnaire et donc de la durée d'incubation. De plus, en utilisant, la normoxie de basse altitude (*i.e*. équivalent à 21% d'O_2 au niveau de la mer) comme scénario contrôle, nous surévaluons potentiellement les différences entre les réponses en normoxie et en hypoxie. En effet, les nids des Couleuvres vipérines étant souterrains, il n'est pas impossible que le développement embryonnaire se fasse en condition d'hypoxie légère.

6.2.1. Le phénomène de synchronie à l'éclosion

La durée d'incubation des œufs bien que réduite de façon significative en condition d'hypoxie pourrait n'être due, chez les embryons de Couleuvre vipérine, qu'à la variation interindividuelle dans les durées d'incubation au sein d'une même ponte (Aubret et al., 2016a; b, 2017). Cependant, chez de nombreuses espèces ovipares, les juvéniles éclosent de manière synchrone (insectes : Nishide and Tanaka, 2016; Tanaka, 2017; poissons : Bradbury et al., 2004; amphibiens : Sih and Moore, 1993; Warkentin, 1995, 2000; oiseaux : Lack, 1968; Vince, 1969; Stoleson and Beissinger, 1999; crocodiles : Ferguson, 1985; tortues : Spencer et al., 2001; Colbert et al., 2010; Spencer and Janzen, 2011; lézards : Vitt, 1991; serpents : Aubret et al., 2017). Ce phénomène d'éclosion synchrone est également présent chez la Couleuvre vipérine (Aubret, 2013a; Aubret et al., 2016b), notamment à travers la ponte dans des sites de ponte communautaire (Vacher and Santos, 2010). L'objectif de ce cette éclosion synchrone est de diluer le risque de prédation individuel par le nombre (Arnold and Wassersug, 1978; Delm, 1990; Spencer et al., 2001; Spencer and Janzen, 2011; Santos et al., 2016). Chez les tortues par exemple, le nid subit un gradient de température vertical, avec des températures plus élevées en haut. Cette température plus élevée va accélérer le métabolisme des embryons qui vont donc éclore plus rapidement. Les embryons des œufs situés plus bas dans le nid, où les températures sont plus froides, vont palier un développement plus lent par l'augmentation de leur rythme cardiaque et accélérer leur développement ou éclore prématurément pour assurer la synchronisation de leur éclosion (Spencer et al., 2001). Cependant, les mécanismes sous-jacents de cette communication ne sont pas encore bien définis (Spencer et al., 2001; Aubret et al., 2016b). Chez les insectes ovipares, des études ont montré que des œufs maintenus en grappe synchronisaient leur éclosion contrairement à ceux maintenus isolés, avec l'hypothèse que cette synchronisation se réalise via la perception d'informations reçues des œufs voisins (Nishide and

Tanaka, 2016; Tanaka, 2017). De plus, l'information transmise nécessaire à la synchronisation ne serait ni auditive ni due à des phéromones mais vibratoire (Nishide and Tanaka, 2016). Il semblerait que chez la Couleuvre vipérine, un mécanisme informatif similaire, à travers les battements cardiaques des embryons, favorise la synchronie à l'éclosion. En effet, le rythme cardiaque des œufs plus jeunes placés à côté d'œufs plus vieux va accélérer afin qu'ils éclosent en même temps que ces œufs plus âgés (Aubret et al., 2016b). De plus, des travaux chez les oiseaux ont montré que les oisillons éclosent généralement après un nombre cumulatif fixe, mais spécifique à l'espèce, de battements cardiaques depuis le début de l'incubation (Ar and Tazawa, 1999; Tazawa, 2005). Chez les reptiles, des travaux similaires ont permis de définir que le nombre total de battements cardiaques pendant l'embryogenèse était relativement constant dans des conditions d'incubation chaudes et que pour des incubations à basse température le nombre total de battements requis pour compléter l'embryogenèse était augmenté (Du et al., 2009, 2011). Ces études démontrent que la durée d'incubation chez les reptiles non-aviens est probablement déterminée par le nombre de battements cardiaques, lui-même déterminé par la température d'incubation (Greenwald, 1971; Du et al., 2010a; c). Néanmoins, dans nos expériences, les œufs de chaque groupe de traitement n'étaient pas physiquement en contact et ne pouvaient donc pas partager d'information pour permettre une synchronisation de l'éclosion.

Compte tenu de ces connaissances, il serait peut-être nécessaire de mesurer les réponses du développement embryonnaire en condition d'hypoxie d'altitude en tenant compte de ce phénomène d'éclosion synchrone. Cela pourrait réduire la variabilité interannuelle, notamment au niveau du rythme cardiaque des embryons mais aussi modifier l'écart entre la durée d'incubation des groupes d'œufs incubés en normoxie ou en hypoxie d'altitude, à une même température.

6.2.2. L'importance des données sur le long terme

Les expérimentations menées durant cette thèse avaient pour objectif des fournir des réponses sur les capacités de la Couleuvre vipérine à coloniser les zones de haute altitude. Nous nous sommes uniquement intéressé à mesurer les effets de l'hypoxie et des températures sur le développement embryonnaire et sur les performances juvéniles jusqu'à un mois après l'éclosion. Ce choix expérimental ne nous permet pas d'obtenir des informations sur les modifications à plus long terme des conditions physiques des juvéniles, sur leurs comportements ou encore sur leur survie, en réponse à une hypoxie chronique avec des températures plus ou moins importantes. Comme nous avons pu le constater durant nos expériences, la température durant le développement embryonnaire peut avoir des effets sur le phénotype des juvéniles (Deeming and Ferguson, 1991;

Shine et al., 1997; Wapstra, 2000; Blouin-Demers et al., 2004; Booth, 2006; Watkins and Vraspir, 2006; Warner, 2014). Des études montrent que, pour de nombreuses espèces ectothermes, des conditions froides seraient favorables en condition hypoxique afin de réduire la température corporelle des individus, diminuant ainsi la demande en dioxygène des organismes (Dupré and Wood, 1988; Dupré et al., 1988; Jackson, 2007) et limitant la déshydratation (Dupoué et al., 2017). De plus, les conditions de température rencontrées au début de la vie pourraient également entrainer des modifications du comportement de thermorégulation des individus à plus long terme : la préférence thermique des serpent à l'âge adulte dépend d'ailleurs plus des conditions thermiques vécue pendant le développement que des conditions thermiques actuelles, démontrant les limites de la plasticité phénotypiques (Aubret and Shine, 2010). Connaître les effets de l'hypoxie d'altitude sur ces paramètres semblent d'autant plus importantes que l'hypoxie d'altitude modifie de manière importante les réponses phénotypique des organismes ectothermes, notamment en limitant la gamme des températures optimums pour les performances (Gangloff and Telemeco, 2018).

Pour mesurer, les effets de l'hypoxie d'altitude sur les individus, nous pourrions à travers différentes expérimentations contrôlées en laboratoire mesurer les préférences thermiques des individus au repos et en activité. Les individus de Couleuvre vipérine nés et maintenus en haute altitude pourraient être placés dans un terrarium où un gradient thermique serait artificiellement maintenu et nous pourrions relever ces choix de thermorégulation. Si une telle plasticité comportementale existe chez les individus de Couleuvre vipérine, nous devrions nous attendre à ce que les individus choisissent de se maintenir à des températures plus froides que des individus nés et maintenus en basse altitude. Nous pourrions également comparer les taux de croissance ainsi que la survie des individus jusqu'à ce qu'ils soient en âge de se reproduire.

6.2.3. Choix maternel et plasticité comportementale

La plasticité comportementale peut être un mécanisme particulièrement efficace pour atténuer les effets des variations environnementales car ces comportements peuvent changer rapidement tout en étant réversibles (Charmantier et al., 2008; Huey et al., 2012; Zuk et al., 2014; Muñoz et al., 2015). Par exemple, avec le changement climatique, un modèle prédictif chez un lézard, *Sceloporus undulatus*, prévoit des échecs de reproduction sur 35 % de l'aire de répartition d'ici 2100, en réponse à la température du sol, et donc des nids, qui deviendrait létale pour les embryons. Toutefois, si les femelles pondent leurs œufs dans des microhabitats 25 % plus ombragés ou 3 cm plus profonds, les prévisions s'inversent et les populations pourraient bénéficier des modifications climatiques (Levy et al., 2015). De façon empirique, des études se sont intéressées à des comportements de sélection de

site de ponte par des femelles gravides de plusieurs espèces de reptiles qui avaient pour résultat une amélioration du phénotype physique des juvéniles (Burger and Zappalorti, 1986; Escalona et al., 2009; Pike et al., 2010; Refsnider et al., 2010; Peet-Paré and Blouin-Demers, 2012). Chez certaines espèces, quand plusieurs sites de ponte sont disponibles, les femelles choisissent des sites relativement ouverts et chauds pour pondre leurs œufs, ce qui a un effet positif sur le succès d'éclosion (Angilletta et al., 2009; Pruett et al., 2019). De même, les femelles vont chercher des sites de ponte suffisamment humides pour éviter la dessiccation des œufs et favoriser le phénotype des juvéniles (Brown and Shine, 2004). Chez des lézards dont le sexe est déterminé par la température, une plasticité comportementale des femelles dans le choix des sites de ponte compense les effets du changement climatique sur le sex-ratio des juvéniles (Doody et al., 2006). À l'inverse d'autres études ont montré que des femelles de lézard qui ont été soumises à une augmentation des températures environnementales n'ont pas ou trop peu modifié leur comportement de nidification, ce qui réduit la fitness des juvéniles à la naissance (Telemeco et al., 2009, 2016). Dans le contexte actuel du changement climatique, les femelles de Couleuvre vipérine pourraient monter en altitude pour y trouver des températures plus fraîches et s'y pondre. Les embryons seront donc soumis à des contraintes environnementales nouvelles. Dans le cadre de mes travaux, les expériences menées sur l'incubation des embryons de Couleuvre vipérine en condition d'hypoxie à basses températures ont induit une réduction des performances de nage chez les juvéniles. Cependant, l'incubation en condition d'hypoxie et à des températures plus élevées a permis d'augmenter la vitesse de nage des juvéniles, un trait écologique qui améliore l'acquisition de nourriture et l'évitement des prédateurs (Jayne and Bennett, 1990; Kingsolver et al., 2001). Afin de maximiser les performances de nage, les femelles gravides de Couleuvre vipérine en condition d'hypoxie pourraient, à travers de la plasticité comportementale, pondre leurs œufs dans des sites de ponte avec des températures plus élevées quand les sites de pontes de basse altitude. Cette plasticité pourra être considérée adaptative ce qui pourrait entrainer à terme, par la sélection naturelle, le maintien de ce comportement dans la population(Aubret and Shine, 2009).

Pour tester cette hypothèse plasticité comportementale dans la sélection de site de ponte, les femelles gravides de Couleuvre vipérine capturées en basse altitude devront être séparées en deux groupes. Un groupe de femelles gravides restera en gestation à basse altitude en condition de normoxie et un autre groupe sera transféré en haute altitude en condition d'hypoxie. Les femelles gravides seront placées individuellement dans des terrariums offrant plusieurs boites de ponte à différentes températures, comme par exemple les trois températures d'incubation utilisées dans cette thèse (i.e. 24°C, 28°C et 32°C ; Figure 1). À la ponte, les œufs de chaque ponte pourront être équitablement répartis dans les trois températures d'incubation. Différents paramètres du

développement embryonnaire, comme le rythme cardiaque, la masse des œufs ou encore le métabolisme respiratoire pourront être suivis. A l'éclosion différentes paramètres du phénotype des juvéniles pourront être mesurés comme la durée d'incubation, la taille et la masse corporelle, la vitesse de nage ou encore le métabolisme respiratoire. Les individus juvéniles pourront être maintenus en haute altitude (*i.e.* hypoxie d'altitude) et les mesures répétées à moyen et long terme. Avec cette expérience, nous devrions être en mesure de déterminer si la femelle de Couleuvre vipérine à préalablement pondu ses œufs dans le site de ponte qui maximisaient le phénotype des individus en condition d'hypoxie.

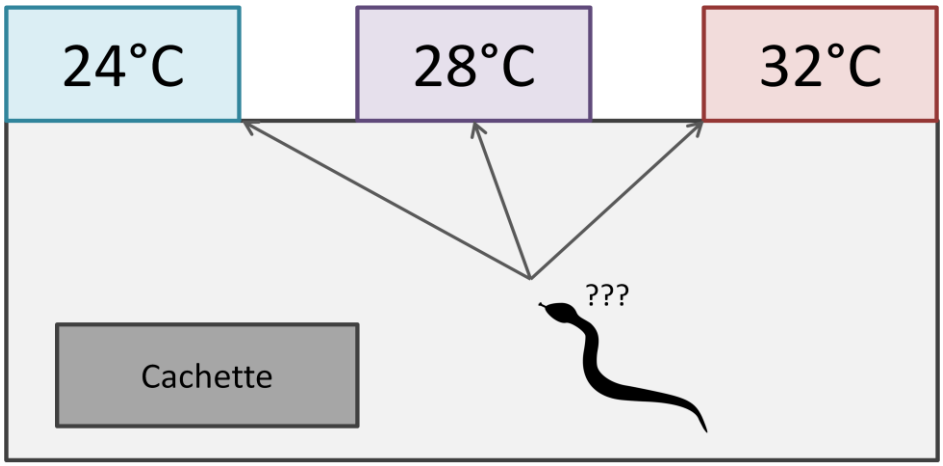


Figure 1 : Représentation schématique d'un terrarium de femelles gravides de Couleuvre vipérine, *Natrix maura*, avec trois boites de ponte disponibles avec chacune une température de d'incubation différente.

6.2.4. L'hypoxie d'altitude similaire à l'hypoxie des sites de pontes ?

Chez les reptiles non-aviens, la similarité entre les réponses développementales et phénotypiques à l'hypoxie d'altitude et celles à l'hypoxie liée à l'enfouissement dans des nids souterrains (Annexe 1) soulèvent une question importante quant à l'utilisation de la condition normoxique (*i.e.* équivalent à 21% d'O_2 au niveau de la mer) comme contrôle. En effet, dans les nids souterrains, les embryons doivent ajustés leur développement en fonction des fluctuations de la concentration des gaz (Packard and Packard, 1988; Deeming and Thompson, 1991; Ackerman and Lott, 2004). Par exemple, dans des nids de tortues (Prange and Ackerman, 1974; Ackerman, 1977) et de crocodiles (Lutz and Dunbar-Cooper, 1984; Whitehead, 1987), des mesures de la concentration en dioxygène chutaient progressivement durant l'incubation. Cette diminution pourrait être due à la difficulté de circulation de l'air dans les nids profonds mais aussi en fin d'incubation à la consommation en dioxygène importante des embryons. En effet, à la fin de l'incubation la demande en dioxygène des embryons augmentent (Dmi'el, 1970; Sartori et al., 2017). De plus, les tortues et les crocodiles pondent

relativement beaucoup d'œufs et de taille conséquente ce qui augmente la diminution de la concentration de dioxygène disponible dans les nids. Dans des sites de ponte moins profonds, comme chez l'Iguane terrestre de Cuba, *Cyclura nubila*, des mesures de la concentration en dioxygène ont montré que les concentrations de dioxygène variaient entre 17% et 20% (équivalent au niveau de la mer), en fonction des conditions de température et d'humidité (Christian and Lawrence, 1991). Dans ces nids, aucune chute de la concentration en dioxygène n'a été enregistrée en fin d'incubation, ce qui s'explique par une taille et un nombre d'œufs réduits. La Couleuvre vipérine est une espèce qui, tout comme les iguanes, pond ses œufs, peu nombreux et de petites tailles, dans des nids souterrains peu profonds. Cependant, ces sites de pontes sont dit communautaires, c'est-à-dire que plusieurs femelles y pondent (Vacher and Geniez, 2010; Pottier, 2016). Malgré une taille et un nombre d'œufs assez faible par femelle (Vacher and Geniez, 2010), le regroupement de plusieurs pontes peu constituer une masse et un nombre d'œufs suffisant pour entrainer des diminutions de la concentration en dioxygène dans les nids entrainant une hypoxie locale. Néanmoins, aucune étude n'a actuellement mesuré les niveaux d'hypoxie dans les nids communautaires de couleuvres européennes. Bien que cela soit spéculatif, il est possible que la condition d'hypoxie d'altitude durant l'incubation utilisée dans nos expériences soit plus proche de la condition d'incubation des sites de ponte naturel que le groupe contrôle utilisé (*i.e.* normoxie en basse altitude). Si cela s'avère exacte, l'ensemble des résultats de cette thèse, ainsi que de nombreuses études traitant des effets de l'hypoxie d'altitude doivent être réévalués. Il semble donc nécessaire de mesurer différents paramètres du développement embryonnaire (*i.e.* rythme cardiaque, durée d'incubation…) et des phénotypes juvéniles à l'éclosion dans les sites de pontes naturels et de les comparer aux résultats obtenus durant une incubation en normoxie et en hypoxie d'altitude.

6.3. Conclusion générale

Avant tout chose, il est important de rappeler que les expériences menées durant cette thèse ne visaient pas à reproduire une situation biologiquement pertinente à court terme, mais plutôt à simuler des scénarios futurs qui associent une modification d'un facteur environnemental (*i.e.* le réchauffement climatique) à la remonté altitudinale d'une espèce en réponse à cette modification. En effet, nous avons dans un premier temps directement déplacé des œufs de Couleuvre vipérine de basse altitude vers des zones de haute altitude avec des écarts d'altitude allant de 2100m à 2500m, en fonction de l'origine des femelles gravides. Dans un second temps, nous avons déplacé des juvéniles de Couleuvre vipérine nés à basse altitude à haute altitude et inversement. Néanmoins, de

telles approches sont nécessaires pour pressentir les capacités des espèces (*i.e.* plasticité phénotypique) à s'acclimater à cette potentielle future contrainte environnementale qu'est l'hypoxie d'altitude. De nombreuses espèces de Squamates sont présentes à des altitudes élevées (lézards : Huey, 1977; Marquet et al., 1989; Adolph, 1990; Pottier, 2012; Bouazza et al., 2016; Wu et al., 2018; serpent : Lue et al., 1999; Luiselli et al., 2007; Huang et al., 2013), et témoignent que la colonisation et l'établissement y sont possibles pour les amniotes ectothermes ovipares. Ces colonisations ancestrales suggèrent que les espèces d'ectothermes de basse altitude pourront, en réponse à l'augmentation des températures (Pachauri et al., 2014; IPPC, 2018), atteindre ces zones de haute altitude pour y trouver un refuge thermique (Sinervo et al., in prep., 2018; Pottier, 2012; Bässler et al., 2013). Cependant, les processus naturels de colonisation sont graduels et permettent aux organismes d'ajuster leur physiologie et leur comportement au moyen de la plasticité phénotypique (Munday et al., 2017), de la sélection naturelle(Aubret and Shine, 2009) et de la sélection spatiale (Phillips and Perkins, 2019) conduisant éventuellement, si les conditions le favorisent, à une adaptation locale (Bouverot, 1985; Rezende et al., 2005; Beall, 2006; Hammond et al., 2006; Powell and Hopkins, 2010; Storz et al., 2010; Mueller et al., 2015).

Globalement, les résultats de ces travaux de thèse confirment que la température d'incubation est le facteur clé du développement embryonnaire chez les reptiles non-aviens (Deeming and Ferguson, 1991; Deeming, 2004; Booth, 2006; Goodman, 2008; Warner, 2014). Ils ont démontré que les variations de températures modifient de manière importante les réponses à l'hypoxie d'altitude du développement embryonnaires et des phénotypes juvéniles. En effet, nos résultats ont montré que, dans un premier temps, la Couleuvre vipérine serait capable de se reproduire en condition d'hypoxie, un des éléments indispensable à l'établissement des populations (Grevstad, 1999; Yeh, 2004). Les embryons, à travers la plasticité développementale, seraient capables de se développer en condition d'hypoxie et pourraient maintenir des phénotypes leur permettant de conserver des performances similaires à celles des individus de l'espèce en basse altitude (Bodensteiner et al., 2020). Dans un second temps, si le changement climatique s'intensifie, l'augmentation des températures dans les milieux de haute altitude modifiera de manière importante les conditions de développement des embryons et devrait influencer la direction de l'évolution ultérieure des populations colonisatrices (Atkinson and Thorndyke, 2001; McNab, 2002; Hammond et al., 2006; Ghalambor et al., 2007). Nos résultats montrent que dans ce contexte, les réponses physiologiques plastiques des embryons de Couleuvre vipérine serraient limités (*i.e*. diminution du succès d'éclosion et taille corporelle réduite) mais favorisant à court terme les performances des juvéniles (*i.e*. diminution rapide des performances).

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Annexe I.

Synthèses des effets de l'hypoxie aigue et de l'hypoxie chronique sur les Reptiles non-aviens

Tableau 1

Synthèse des effets de l'hypoxie aigue et chronique durant l'incubation sur des traits phénotypiques embryonnaires et juvéniles comparés à ces mêmes trait en normoxie (i.e. 21% d'O_2 au niveau de la mer). Une hypoxie de 30%, 40% ou 50% représente respectivement un équivalent d'environ 15%, 12% et 10% d'O_2 au niveau de la mer. Le symbole "-" représente une réduction du trait, le "+" une augmentation et le "=" qu'il n'y a pas de modification.

	Niveau d'hypoxie durant l'incubation des oeufs					Ordres	Références
	Hypoxie aigue 30%	Hypoxie aigue 50%	Hypoxie chronique 30%	Hypoxie chronique 40%	Hypoxie chronique 50%		
Métabolisme embryonnaire							
Fréquence cardiaque		-	=	-	-	Crocodiliens	Crossley and Altimiras, 2005
	+		- / +			Squamates	Du et al., 2010a; Cordero et al., 2017b; Kouyoumdjian et al., 2019
Consommation de dioxygène			-			Squamates	Cordero et al., 2017b;
					-	Chéloniens	Kam, 1993, Cordero et al., 2017c
Pression artérielle			-		-	Crocodiliens	Crossley and Altimiras, 2005
Développement embryonnaire							
Masse des embryons		=	=	-	-	Crocodiliens	Crossley and Altimiras, 2005; Ilungman and Piña, 2013
			=	-		Crocodiliens	Ilungman and Piña, 2013
Durée de l'incubation	=	=	= / +			Squamates	Du et al., 2010a; Cordero et al., 2017b; Kouyoumdjian et al., 2019; Li et al., 2020
					=	Chéloniens	Kam, 1993
			=	=		Crocodiliens	Ilungman and Piña, 2013
Succès d'éclosion	=		=			Squamates	Du et al., 2010a; Cordero et al., 2017b; Kouyoumdjian et al., 2019; Li et al., 2020
					=	Chéloniens	Kam, 1993
Phénotype juvénile à l'éclosion							
			=	-	-	Crocodiliens	Owerkowicz et al, 2009; Ilungman and Piña, 2013
Longueur du corps	=		-			Squamates	Du et al., 2010a; Cordero et al., 2017a; Kouyoumdjian et al., 2019; Li et al., 2020
					-	Chéloniens	Wearing et al, 2015
Volume du coeur et des poumons				+	+	Crocodiliens	Owerkowicz et al, 2009; Crossley and Altimiras, 2005
	=		+			Squamates	Du et al., 2010a; Cordero et al., 2017a
				-	-	Crocodiliens	Owerkowicz et al, 2009; Crossley and Altimiras, 2005
Taux de croissance	=		=			Squamates	Du et al., 2010a
					-	Chéloniens	Wearing et al, 2015
Taux de survie	=		=			Squamates	Du et al., 2010a
Performance locomotrice	=		=			Squamates	Du et al., 2010a; Li et al., 2020

Tableau 2

Synthèse des effets de l'hypoxie aigue et chronique durant la croissance juvénile et adulte sur différents traits phénotypiques comparés à ces mêmes trait en normoxie (i.e. 21% d'O_2 au niveau de la mer). Une hypoxie de 30%, 40% ou 50% représente respectivement un équivalent d'environ 15%, 12% et 10% d'O_2 au niveau de la mer. Le symbole "-" représente une réduction du trait, le "+" une augmentation et le "=" qu'il n'y a pas de modification.

	Niveau d'hypoxie durant le stade adulte					Ordres	Références
	Hypoxie aigue 30%	Hypoxie aigue 50%	Hypoxie aigue 80%	Hypoxie chronique 30%	Hypoxie chronique 50%		
Métabolisme							
				+	+	Crocodiliens	Boyer, 1966
Fréquence respiratoire	-	-	- / +	+	+	Squamates	Boyer, 1966; Pough, 1973; Gratz, 1979; Bartlett and Birchard, 1983
				+	+	Chéloniens	Boyer, 1966
				-	-	Crocodiliens	Boyer, 1966
Consommation de dioxygène	+		+	-	-	Squamates	Boyer, 1966; Gratz, 1979
	=	=	=	=	=	Chéloniens	Boyer, 1963; Boyer, 1966
				=	=	Crocodiliens	Boyer, 1966
Fréquence cardiaque	+	+	+	+	+	Squamates	Boyer, 1966; Pough, 1973; Gratz, 1979
	-	-	-	+	+	Chéloniens	Boyer, 1963; Boyer, 1966
Température corporelle préférée				-		Squamates	Megía-Palma et al., 2020
Phénotype							
Volume pulmonaire			+			Squamates	Bartlett and Birchard, 1983
Condition corporelle				-		Squamates	Gangloff et al, 2019; Megía-Palma et al., 2020
Performance locomotrice				-		Squamates	Gangloff et al, 2019
Composition sanguine							
Concentration d'hémoglobine	+			+ / =	+	Squamates	Vinegar and Hillyard, 1972; Weathers and White, 1972; González-Morales et al., 2015; Lu et al, 2015; Gangloff et al, 2019; Megía-Palma et al., 2020
Nombre d'hématocrites	+			+ / =	+	Squamates	Vinegar and Hillyard, 1972; Weathers and White, 1972; González-Morales et al., 2015; Lu et al, 2015; Gangloff et al, 2019
Affinité des globules rouges pour le dioxygène				+	+	Squamates	Vinegar and Hillyard, 1972; Herman and Ingermann, 1996; Lu et al, 2015

Annexe II.

Isolation and characterization of fourteen polymorphic microsatellite markers in the viperine snake *natrix maura*

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Isolation and characterization of fourteen polymorphic microsatellite markers in the viperine snake *Natrix maura*

Hugo Le Chevalier[1] | Neus Marí-Mena[2,3] | Belén Carro[3] | Jérôme G. Prunier[1] | Coralie Bossu[1] | Elodie Darnet[1] | Jérémie Souchet[1] | Olivier Guillaume[1] | Olivier Calvez[1] | Romain Bertrand[1] | Laurent Barthe[4] | Gilles Pottier[4] | Albert Martínez-Sylvestre[5] | Isabel Verdaguer-Foz[5] | Marc Mossoll-Torres[6,7] | Audrey Trochet[1] | Fabien Aubret[1]

[1]Station d'Ecologie Théorique et Expérimentale (SETE), UMR5321, Centre National de la Recherche Scientifique (CNRS), Moulis, France

[2]Department of Molecular and Cell Biology, Evolutionary Biology Group (GIBE), Universidade da Coruña, A Coruña, Spain

[3]All Genetics & Biology, SL, A Coruña, Spain

[4]Association Nature en Occitanie (NEO), Maison de l'Environnement de Midi-Pyrénées, Toulouse, France

[5]Catalonia Reptile and Amphibian Rescue Center (CRARC), C/Santa Clara, s/n Masquefa, Spain

[6]Pirenalia, Encamp, Andorra

[7]Bomosa, Les Escaldes, Andorra

Correspondence
Hugo Le Chevalier, SETE CNRS 09200 Moulis, France.
Email: hugo.lechevalier@sete.cnrs.fr

Abstract

Nineteen polymorphic microsatellite loci were identified and developed for *Natrix maura*. Polymorphism was assessed for 120 individuals sampled across four sampling sites from the French Pyrenees Mountains. The number of alleles per locus ranged from 3 to 15, and expected heterozygosity per locus ranged from 0.227 to 0.863. We tested for deviation from Hardy–Weinberg equilibrium and linkage disequilibrium and assessed the presence of null alleles for all loci, resulting in a selection of 14 high-quality polymorphic markers. These markers will be extremely useful in identifying fine-scale genetic structures and providing insight into conservation management plans of this species.

KEYWORDS

Natrix maura, polymorphic microsatellites, population genetics, viperine snake

1 | INTRODUCTION

Anthropogenic activities have already led to massive species extinction, and this loss of biodiversity is expected to continue at an unprecedented pace (Ceballos, Ehrlich, & Dirzo, 2017). Global warming is likely the most preoccupying threat given the potential synergy with many other environmental changes (Cahill et al., 2013; Thomas et al., 2004), impacting organisms at both the individual and population levels and resulting in local increase in extinction risks, species redistribution and community reshuffling (Aubret & Shine, 2010; Pauls, Nowak, Bálint, & Pfenninger, 2013; Walther et al., 2002). Ectotherms represent more than 98% of animal species and are the more likely to be affected because of direct physiological sensitivity to climate conditions (Deutsch et al., 2008; Dupoué et al., 2017; Sinervo et al., 2010). When the conservation status of a given population is uncertain, genetic studies constitute an indirect and valuable approach to assess the impacts of these environmental threats on levels of population genetic diversity and structure, effective dispersal, demographic status and possible past and future responses to global change (Segelbacher et al., 2010).

The viperine snake (*Natrix maura*) is a common Mediterranean snake inhabiting natural and artificial aquatic environments in Northwestern Africa, Iberian Peninsula, Southern France and Northern Italy. Although some localities may exhibit high snake densities, populations are generally considered as declining (Santos & Llorente, 2009). The viperine snake is threatened by multiple factors, such as aquatic pollution, habitat loss and fragmentation, direct destruction by humans because of confusion with venomous vipers and climate change (Santos & Fernández Cardenete, 2015; Santos & Llorente, 2009). All of these environmental threats are likely to interact significantly, impacting viperine snake populations (Gangloff, Sorlin, Cordero, Souchet, & Aubret, 2019; Muthoni, 2010). In this context, the development of polymorphic genetic markers is critical for this species in order to study patterns of genetic diversity and understand population structure and functioning. Here, we isolated and characterized 19 new polymorphic microsatellite markers for *N. maura* using Illumina high-throughput sequencing.

2 | MATERIAL & METHODS

We sampled DNA from 120 viperine snakes from four populations in the southwestern France (Ariège, Table 1), using buccal swabs as a noninvasive sampling method (Beebee, 2008). Swabs were suspended in 1X TE buffer for DNA conservation and DNA extraction was performed using the RealPure MicroSpin DNA Isolation Kit following manufacturers' instructions (Durviz). Microsatellite development was performed at AllGenetics (www.allgenetics.eu). A single DNA sample belonging to a female viperine snake was used to generate a library with the Nextera XT DNA Library Preparation Kit (Illumina). The library was then enriched in fragments with microsatellite motifs by hybridization to four groups of biotinylated oligo repeats (i.e., AC, AG, ACG, and ATCT) that were captured with Dynabeads/M280 Streptavidin (Invitrogen, Thermo Fisher Scientific). The enriched library was sequenced in the Illumina MiSeq PE300 platform (Macrogen Inc.). Reads were processed in Geneious 10.2.2 (Biomatters Ltd). Primer design was carried out in Primer3 software (Koressaar & Remm, 2007; Untergrasser et al., 2012) implemented in Geneious 10.2.2.

A total of 108 primer pairs, each targeting a different locus, were identified and organized into 31 multiplexes using Multiplex Manager (Holleley & Geerts, 2009). The computer-designed multiplexes were validated and checked for polymorphism using DNA samples from an additional set of seven individuals. The polymerase chain reactions (PCRs) were carried out following Schuelke (2000). As oligonucleotide tails, we used the universal sequences M13 (GGA AAC AGC TAT GAC CAT), CAG (CAG TCG GGC GTC ATC), and T3 (AATTAA CCC TCA CTA AAGGG) labeled with the HEX dye, the FAM dye, and the TAMRA dye, respectively. PCRs were performed in a final reaction volume of 12.5 µl, containing around 10 ng of DNA, Type-it Multiplex PCR Master Mix (Qiagen), and Primer Mix 1× (0.2 µM forward primers and labeled tails, and 0.02 µM reverse primers). The optimal PCR protocol consisted in an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 57°C for 90 s, 72°C for 30 s; 8 cycles of 95°C for 30 s, 53°C for 90 s, 72°C for 30 s; and a final extension step at 68°C for 30 min. All PCR rounds included a negative control to check for potential cross-contamination. PCR products were subsequently subjected to fragment analysis. Allele calling was performed using Geneious 11.1.2 (Biomatters). Finally, the 19 primer pairs with the highest polymorphism were organized into seven multiplexes according to dye colors and expected amplicon sizes (Table 2). We finally applied this genotyping protocol to the 120 viperine snake samples to assess markers' quality.

To avoid any bias in further analyses, we first identified populations showing HWE across the maximum number of markers. We used the *test_HW* function from the R-distribution of the Genepop software (Rousset, 2008) to assess HWE for each locus and each population, and only retained loci showing HWE.

Considering each populations independently, we then estimated for each locus the number of alleles (n_a), observed and expected heterozygosity (H_O and H_E, respectively) using FSTAT 2.9.3.2 (Goudet, 1995). We also computed null allele frequency along with 95% confidence intervals using the *null.all* function from the R-package *PopGenReport* (Adamack & Gruber, 2014). Loci showing a lower bound exceeding a null allele frequency of 5% where discarded. We finally assessed linkage disequilibrium across populations using the *test_LD* function (Rousset, 2008). Tests for HWE and linkage disequilibrium were all conducted using false discovery rate FDR-correction to account for multiple-related tests (Benjamini & Hochberg, 1995).

TABLE 1 Characteristics of sampled sites: name of the sampling site, geographic coordinates (WG584), number of sampled individuals (n_{ind}) per site

Sampling site	X	Y	n_{ind}
Alas	42°57′00.208″N	1°02′46.365″E	32
Audressein	42°55′33.665″N	1°01′36.659″E	34
Augirein	42°55′53.390″N	0°54′58.164″E	22
Moulis	42°57′37.694″N	1°05′16.735″E	32

3 | RESULTS AND DISCUSSION

All loci amplified well (from 0% to 5.8% of missing values). Five loci (NM_013, NM_170, NM_346, NM_384, and NM_462) did not conform to HWE. Using a subset of the 14 remaining markers, we found that all populations conformed to HWE.

In each considered population, the 19 loci were found to be polymorphic with n_a ranging from 3 to 15 and H_E ranging from 0.227 to 0.863 (Table 2). Yet, the presence of null alleles was detected in five loci (NM_013, NM_170, NM_245, NM_346, and NM_384). These markers should therefore be used with caution as they can significantly affect the results of genetic analyses (Pompanon, Bonin, Bellemain, & Taberlet, 2005; Wen et al., 2013). They were discarded from further analyses, resulting in a new set of 14 polymorphic

TABLE 2 Characteristics of the 19 microsatellites developed in the viperine snake (*Natrix maura*). The table provides multiplex and locus names, primer sequences, repeat motif and number, allelic size range (in base pairs), number of alleles (n_a), observed and expected heterozygosity (H_O and H_E, respectively), fluorescent label, and rationale for discarding (null alleles [NA] and linkage disequilibrium [LD]). The 14 high-quality markers are indicated in bold

Multiplex	Locus	Primer sequence (5′–3′)	Repeat	Size range (bp)	n_a	H_O	H_E	Fluorescent label	Rationale
1	**NM_064**	F: GCAAAGCTTCAACTGGCCAA	$(AC)_{12}$	185–235	11	0.547	0.572	6-FAM	LD (with NM_465)
		R: CCACAGGGTGACTATGGCTG							
	NM_368	F: CTGTGAAATGTTGGTGGCGC	$(ATC)_{15}$	198–243	11	0.848	0.799	HEX	LD (with NM_321)
		R: CACATTGAAGTCCCGGGTGA							
2	**NM_268**	F: ACGGAAGTGACCCTCCAGTA	$(AC)_{14}$	127–134	3	0.218	0.267	6-FAM	–
		R: CGAAACGGTGGCACTGGATA							
	NM_085	F: GCTGGTTCCAGAAGGGTCTC	$(AG)_{15}$	185–213	5	0.213	0.227	HEX	–
		R: TCCTTGGTGGGTCAAACTGG							
	NM_170	F: GCATCTTGAGCTCGTGAGGT	$(AC)_{30}$	204–238	11	0.492	0.704	TAMRA	NA
		R: TCCGCCGATTCCAATTCCTT							
3	NM_384	F: GCCAAGGAACTGCTGAACCT	$(AC)_{17}$	102–121	7	0.495	0.685	6-FAM	NA
		R: CATTTGGGACTGGCAGCATG							
	NM_462	F: CACTAGTGGCAGCAGAGTGT	$(AG)_{12}$	98–106	7	0.521	0.587	HEX	–
		R: TGGGCTGCAGAGATTCAGAG							
	NM_497	F: TTGCTTGCTGTGATGTGCTG	$(AC)_{17}$	124–158	7	0.697	0.636	TAMRA	–
		R: ACGAAGTGTTGAGCGGAAGG							
	NM_364	F: AGAAGCAACCCAACACCAGA	$(AC)_{29}$	191–214	13	0.778	0.778	HEX	LD (with NM_054)
		R: CTGCCATGGGTGTAGGACTG							
4	NM_013	F: GTCCTTTGGGAGAAGGGTGG	$(AAAC)_{15}$	125–156	6	0.499	0.723	HEX	NA
		R: CCTTCTCCAGTGGTGGGTTC							
	NM_214	F: TATCTTTCCGGCTTTGCGGA	$(AC)_{24}$	108–135	13	0.653	0.750	TAMRA	–
		R: TGCACAGTCACATGGAACCA							
	NM_465	F: TGCTTCTCTTGCCTCTTCGT	$(AC)_{17}$	253–276	6	0.543	0.565	TAMRA	LD (with NM_064)
		R: AGCCACCACTCTGAGAGTCA							
5	NM_245	F: TGCGCCAAGAACAATCACAC	$(AATAG)_{11}$	140–195	11	0.489	0.678	6-FAM	NA
		R: TGCCACTCCACAACCAATCA							
	NM_051	F: CTTGCAACACAACGGAGTCG	$(AC)_{15}$	126–132	3	0.486	0.528	TAMRA	–
		R: ACAACATCTGTGACGGCAGT							
6	NM_346	F: ATTGCTTGGCTTGGTTTGGC	$(AAGG)_{14}$	190–292	15	0.445	0.863	6-FAM	NA
		R: CCTAGAAATGAGGGCGGGAG							
	NM_054	F: GCCGCAAACCCAAACACTAG	$(AC)_{12}$	138–229	11	0.519	0.627	HEX	LD (with NM_364)
		R: ACCAGTGATGGCGAACCTTT							
7	**NM_321**	F: TCGTGACAGTGAGTTGGCAG	$(AAAG)_{18}$	129–183	12	0.771	0.774	6-FAM	LD (with NM_368)
		R: TCTTTCCTCCTCTCCCTCCC							
	NM_093	F: CATGTGTCTGCCTGCATTGG	$(AC)_7$	75–133	4	0.715	0.497	HEX	–
		R: CTTCATGTGGGATTGCGCTG							
	NM_076	F: ACCAGTTCACAAGTCCACGG	$(ACCT)_{18}$	243–275	9	0.770	0.798	TAMRA	–
		R: AAAGAAGGATGCAGCGTGGA							

markers. Finally, we found significant linkage disequilibrium in three pairs of loci: NM_054/NM_364, NM_064/NM_465, and NM_321/NM_368. Some genetic analyses do not require linkage equilibrium (e.g., sPCA; Jombart, Devillard, Dufour, & Pontier, 2008), and we here provide a useful set of 14 polymorphic microsatellite markers for the viperine snake. For genetic analyses requiring independent loci, we recommend using makers showing highest levels of polymorphism (notably NM_465 in place of NM_064).

The viperine snake *N. maura* is a well-suited model species as it is both common in Southwestern Europe while being threatened by multiple environmental factors, inducing distribution shifts and individual perturbations (Aubret & Shine, 2010; Muthoni, 2010). The new set of 14 high-quality polymorphic markers developed in this study (Table 2, in bold) may be used in several scientific contexts from conservation surveys to population genetic studies.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

All authors contribute significantly to the present study and to the revision of the manuscript. H.L.C., with support from A.T., J.G.P., and F.A., wrote the manuscript. H.L.C. and E.D. performed DNA extractions and PCR. N.M.-M. and B.C. performed sequencing, primer identification and selection. Statistical and genetic analyses were performed by J.G.P, A.T., H.L.C., and E.D. Animal captures and DNA sampling on the field were performed by H.L.C., E.D., C.B., J.S., O.G., O.C., R.B., L.B., G.P., A.M.-S., I.V.-F., M.M.-T. Research project was leaded by F.A.

DATA AVAILABILITY STATEMENT

The microsatellite data are available on Dryad: https://doi.org/10.5061/dryad.0vd1fj3

ORCID

Hugo Le Chevalier https://orcid.org/0000-0002-3695-6819

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Annexe III.

Transplanting gravid lizards to high elevation alters maternal and embryonic oxygen physiology, but not reproductive success or hatchling phenotype or hatchling phenotype

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RESEARCH ARTICLE

Transplanting gravid lizards to high elevation alters maternal and embryonic oxygen physiology, but not reproductive success or hatchling phenotype

Laura Kouyoumdjian[1,*], Eric J. Gangloff[1,*,‡], Jérémie Souchet[1], Gerardo A. Cordero[2], Andréaz Dupoué[1,3] and Fabien Aubret[1,4]

ABSTRACT

Increased global temperatures have opened previously inhospitable habitats, such as at higher elevations. However, the reduction of oxygen partial pressure with increase in elevation represents an important physiological constraint that may limit colonization of such habitats, even if the thermal niche is appropriate. To test the mechanisms underlying the response to ecologically relevant levels of hypoxia, we performed a translocation experiment with the common wall lizard (*Podarcis muralis*), a widespread European lizard amenable to establishing populations outside its natural range. We investigated the impacts of hypoxia on the oxygen physiology and reproductive output of gravid common wall lizards and the subsequent development and morphology of their offspring. Lowland females transplanted to high elevations increased their haematocrit and haemoglobin concentration within days and maintained routine metabolism compared with lizards kept at native elevations. However, transplanted lizards suffered from increased reactive oxygen metabolite production near the oviposition date, suggesting a cost of reproduction at high elevation. Transplanted females and females native to different elevations did not differ in reproductive output (clutch size, egg mass, relative clutch mass or embryonic stage at oviposition) or in post-oviposition body condition. Developing embryos reduced heart rates and prolonged incubation times at high elevations within the native range and at extreme high elevations beyond the current range, but this reduced oxygen availability did not affect metabolic rate, hatching success or hatchling size. These results suggest that this opportunistic colonizer is capable of successfully responding to novel environmental constraints in these important life-history stages.

KEY WORDS: Climate change, Development, Embryo, Hypoxia, Metabolic rate, Physiological plasticity, *Podarcis muralis*, Reactive oxygen metabolites, Reproductive output

[1]Station d'Ecologie Théorique et Expérimentale du CNRS–UMR 5321, Moulis, France. [2]Fachbereich Geowissenschaften, Eberhard Karls Universität Tübingen, Tübingen, Germany. [3]iEES Paris, Sorbonne Université, CNRS, UMR 7618, Tours 44-45, 4 place Jussieu, 75005 Paris, France. [4]School of Molecular and Life Sciences, Curtin University, 6102 WA, Australia.
*These authors contributed equally to this work

‡Author for correspondence (eric.gangloff@sete.cnrs.fr)

L.K., 0000-0003-3201-7862; E.J.G., 0000-0002-9074-8009; J.S., 0000-0001-9296-1332; G.A.C., 0000-0002-9137-1741; A.D., 0000-0002-2501-464X; F.A., 0000-0002-7783-8659

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INTRODUCTION

In mountainous regions, the impacts of climate change are particularly pronounced owing to shifts in the altitudinal climate envelope (Chen et al., 2011; Dirnböck et al., 2011; Nogués-Bravo et al., 2008; Walther et al., 2002). Such shifts may invoke biological changes through the processes of adaptation, plasticity and migration; or may result in extirpation (Parmesan, 2006; Sinervo et al., 2010). Just as low-lying valleys provided refuge for a number of organisms during periods of glaciation, it is possible that high-elevation areas will play a similar role in times of global warming (Hewitt, 1999; Tzedakis, 2004). Indeed, the migration of some populations to higher elevations is currently observed in mountainous landscapes (Bani et al., 2019; Bassler et al., 2013; Chen et al., 2011; Freeman et al., 2018; Hickling et al., 2006; Pottier, 2012; Pauchard et al., 2015) and the preservation of such habitats has been proposed specifically as a refuge for lizards (Sinervo et al., 2018). As a result, it is important to identify factors that both permit and limit colonization of higher-elevation habitats. Terrestrial ectothermic vertebrates, such as non-avian reptiles, are sensitive to variation in environmental temperatures and exhibit substantial metabolic plasticity (Huey, 1982). As such, they provide excellent models to understand the impacts of climate change across geographic scales (Diele-Viegas and Rocha, 2018; Huey et al., 2018; Le Galliard et al., 2012; Walther et al., 2002). However, their flexible physiologies and complex interactions among pathways when responding to many environmental factors make broad predictions difficult as the effects of changing habitats may vary dramatically across taxa (Levy et al., 2017; Moore et al., 2018; Pontes-da-Silva et al., 2018; Sinervo et al., 2010; Weatherhead et al., 2012).

One potential constraint to upslope migration is the reduced oxygen partial pressure at high elevations, which causes high-altitude hypoxia (Bouverot, 1985; Powell and Hopkins, 2010). This phenomenon imposes important physiological challenges on a variety of organisms (reviewed in Storz et al., 2010). Importantly, oxygen availability interacts with temperature in a context-dependent manner to influence thermal performance curves and thermal limits in terrestrial ectotherms (Gangloff and Telemeco, 2018; Jackson, 2007). Acute and chronic effects of hypoxia have been fairly well studied in some mammals (humans in particular), birds and some reptiles (Monge and Leon-Velarde, 1991; Weathers and McGrath, 1972), and the responses can be diverse (Storz et al., 2010). Acute effects usually include hyperventilation and tachycardia, as for example in humans brought to high altitude (Saito et al., 1988). Chronic effects may involve changes to the cardiorespiratory systems, such as increased size of the lungs, changes to cardiac morphology, and increased blood pressure (Cunningham et al., 1974; He et al., 2013; Hillyard, 1980; Powell and Hopkins, 2010). In lizards specifically, chronic hypoxia may also result in hematological and muscle composition changes

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(Gonzáles-Morales et al., 2015; Lu et al., 2015; Weathers and McGrath, 1972). Such physiological shifts in oxygen-carrying capacity, however, may be insufficient to maintain performance traits or compensate for reduced oxygen in the long term. Hypoxia exposure during early development, such as embryos *in ovo*, can also dramatically affect physiology and development. Most common responses include mechanisms to facilitate oxygen diffusion and transport, such as increased vascularization of chorioallontoric membranes, increased haematocrit and cardiac hypertrophy (Corona and Warburton, 2000; Crossley and Altimiras, 2005; Jochmans-Lemoine and Joseph, 2018; Kam, 1993; Nechaeva, 2011; Warburton et al., 1995). Under certain conditions, oxygen restrictions can also result in depressed metabolism, decreased growth and reduced survivorship to hatching (Iungman and Piña, 2013; Warburton et al., 1995). These early developmental effects can persist into later life phenotypes (Sun et al., 2014; Wearing et al., 2017).

Previous work demonstrates the impacts of hypoxia on adult male and embryo common wall lizards (*Podarcis muralis*) transplanted to extreme high elevation beyond the current range. Transplanted adult males increased haematocrit and haemoglobin within 3 weeks, but were unable to sustain this response for longer. Despite these changes in oxygen-carrying parameters, lizards transplanted to high elevation suffered reduced running performance and a reduction in body condition compared to lizards kept at lowland native elevation (Gangloff et al., 2019). Embryos of this species developing in high-altitude hypoxia, however, demonstrate a potential to sustain growth and development at least until hatching. High-altitude hypoxia affected embryo physiology, resulting in reduced metabolism, cardiac hypertrophy, and hyperventilation. These physiological and morphological adjustments seemed to buffer embryos from negative impacts of hypoxia, resulting in similar hatching success and hatchling body size compared with embryos developing at native lowland elevation (Cordero et al., 2017a). While studies such as these provide important data on the effects of high-altitude hypoxia across life stages, relatively little is known about the effects of high-altitude hypoxia exposure on maternal energetics and reproductive allocation in natural populations. If reduced oxygen availability imposes a physiological constraint, we might expect that gestating females will be less able to provide adequate resources or environments for developing embryos. This is particularly important in the context of responses to climate change, given the potential for transgenerational plasticity (e.g. maternal effects) to buffer developing offspring from the impacts of novel environmental conditions (Sinervo et al., 2018; Warner, 2014). These effects have the potential to promote offspring success in future environments, such as when maternal exposure to warmer temperatures increases offspring survival in future warm environments (Shama et al., 2014; Sun et al., 2018).

With this study, we quantify physiology and reproduction at different levels of oxygen availability on native and transplanted common wall lizards (*P. muralis*). Such data are needed to assess the potential importance of the effects of high-altitude hypoxia on reproduction and development, which, in turn, will determine the colonization potential of this species with a wide and expanding range (Pottier, 2012; Speybroeck et al., 2016). Our goals are to quantify how reproducing females physiologically respond to changes in oxygen availability; how these responses can influence reproductive output and capacity; how reduced oxygen availability affects embryo development; and how such responses might differ between lizards native to different elevations. We first measured aspects of maternal physiology and reproduction from replicate low- and high-elevation populations at native elevations and after translocation to high elevations. Then, we measured embryo development and physiology at native, high (within the natural range) and extreme high (beyond current range limit) elevations, to test five primary hypotheses.

Energetic limitations imposed by hypoxia will force a trade-off resulting in reduced relative reproductive investment

Because reduced oxygen availability affects the shape of performance curves (Gangloff and Telemeco, 2018), we expected that short-term (i.e. weeks) exposure to hypoxic conditions might reduce efficiency of physiological functions involved in energy processing for both gravid females and developing embryos. If lizards are unable to compensate, we expected that a maternal allocation trade-off would reduce egg mass of lizards transplanted to high elevation, resulting in a smaller relative clutch mass (RCM), a metric of reproductive investment. Because clutch size is likely determined before our experimental manipulations and females are unlikely to resorb eggs (Blackburn, 1998), we did not expect to see an effect of transplant on clutch size. If female lizards at high elevation are able to maintain the same level of reproductive investment (egg size and RCM), we expected to observe prolonged egg retention and thus laying of eggs at a later developmental stage, as a consequence of reduced oxygen availability on gravid mothers (see Mathies and Andrews, 1995). Alternatively, if oxygen limitation imposes a constraint on reproduction, mothers may sustain investment into offspring at a physiological cost to themselves, for instance resulting in reduced post-oviposition body condition.

Hypoxia and reproductive stage will interact to affect reactive oxygen metabolite production

Reactive oxygen metabolites (ROMs) are produced as by-products of oxidative metabolism and can damage a variety of subcellular molecules. ROMs have been implicated in senescence and as a by-product of reproduction in reptiles (Costantini, 2016; Dowling and Simmons, 2009; Robert et al., 2007; Stahlschmidt et al., 2013; Stier et al., 2017; Webb et al., 2018). ROM production generally increases as a result of cellular hypoxia (reviewed in Guzy and Schumacker, 2006; Harrison et al., 2015; Solaini et al., 2010). Exploring the interaction of reproduction and hypoxia on ROM production is essential to both understand short-term physiological changes and potential long-term consequences of reproduction at altitude. We expected that the reactive oxygen metabolite profile of lizards would shift across reproduction and that transplanted lizards would suffer from relatively increased levels compared with lizards kept at native elevation.

Developing embryos will exhibit changes in physiology in response to hypoxia to meet metabolic demands important to development and differentiation

Reptilian embryos *in ovo* often experience naturally occurring hypoxia due to flooding events or limited gas exchange in subterranean nests (reviewed in Packard and Packard, 1987; Ackerman and Lott, 2004; Booth, 1998). Given the resilience to these conditions exhibited by a variety of taxa, we predicted that embryos would adjust physiology and development, such as through changes in heart rate and incubation time. With these responses, embryos can sustain differentiation, growth, and organogenesis, critical processes that must be fuelled by aerobic metabolism and are likely buffered from moderate hypoxia (Crossley et al., 2017; Iungman, and Piña, 2013; Warburton et al., 1995). Physiological or morphological responses will facilitate the maintenance of oxygen consumption ($\dot{V}_{O_2}$, an integrated index of

general metabolism), although late-period increases in demand might present challenges at high elevation. Furthermore, we predicted that these adjustments would allow embryos to maintain important fitness-related traits such as size and mass at birth; if energetic constraints are present this would be demonstrated by reduced body size of embryos developing at extreme high elevation.

Effects of hypoxia on post-oviposition development will be reduced in embryos that are exposed to hypoxia during gestation

Exposure to hypoxia earlier in development will provide both more time for embryos to respond and a potential window for anticipatory maternal effects. We expected that the change in heart rate and incubation time predicted above would be less pronounced in embryos from females that were transplanted to high altitudes compared with embryos translocated after oviposition. Furthermore, maternal effects in response to hypoxia may provide a mechanism to prime embryos for subsequent development in low-oxygen environments, thus reducing the potentially negative impacts on development in embryos transplanted to high or extreme high elevation.

Lizards from high-elevation populations will demonstrate local adaptation in traits related to oxygen capacity

We predicted that the responses to hypoxia of transplanted females would differ from that of lizards native to high elevation, while embryos from high-elevation populations would show reduced effects on development at extreme high elevations compared to embryos from low-elevation populations. As has been found in previous studies of transplanted lizards (He et al., 2013; Gangloff et al., 2019; Weathers and McGrath, 1972) we predicted physiological plasticity in parameters related to oxygen-carrying capacity in blood chemistry (haematocrit and haemoglobin concentration) in reproductive females from low elevations transplanted to high elevations. Importantly, we did not expect the physiological profile of lowland lizards transplanted to high elevation to match those of lizards from high-elevation populations, as often the short-term plastic response differs from that of locally adapted populations (He et al., 2013; Jochmans-Lemoine et al., 2015; Reyes et al., 2018; Storz et al., 2010; Velotta et al., 2018). We predicted that such shifts in oxygen capacity would allow lizards to maintain resting metabolic rates but perhaps bear other physiological or reproductive consequences given the increased energetic demands of reproduction (Angilletta and Sears, 2000; Foucart et al., 2014). Furthermore, we predicted that embryos from high-elevation populations would respond less dramatically to development at extreme high elevations compared with lizard embryos native to low elevations.

Research objectives

Taken together, these observations allow us to evaluate how hypoxia may constrain physiology in reproducing and developing lizards and how these responses may differ between populations native to different elevations. Given the dependence of successful colonization on recruitment (Aubret, 2013; Warner, 2014; While et al., 2015), testing these effects in reproductive females and their developing offspring is critical to predict the dynamics of colonization in common wall lizards in mountainous environments affected by climate change.

MATERIALS AND METHODS

Source populations and husbandry

The common wall lizard [Lacertidae: *Podarcis muralis* (Laurenti 1768)] is widespread across southern Europe in a variety of habitats (Arnold et al., 1988; Speybroeck et al., 2016). Its geographic distribution is restricted by the thermal environment because embryos are incapable of completing development at cold temperatures (Strijbosch et al., 1980; Van Damme et al., 1991; While et al., 2015). However, wall lizards have recently been observed at higher areas of mountainous regions in the south of France, extending up to 2600 m above sea level (ASL) (Pottier, 2012). At low elevations, females produce two or three clutches per season with an average of 6 eggs per clutch (Le Hénanff, 2011). We monitored populations in the Pyrénées Ariégeoises (France) from the beginning of the active season and sampled adult female lizards during their first reproductive event of the season in April and May 2018 at low (382–472 m ASL) and high (1402–1795 m ASL) elevations. A total of 47 gravid females were sampled from three low-elevation and two high-elevation populations (see Table S1 for sampling details). Individuals were caught by using the lasso method (Blomberg and Shine, 1996; Fitzgerald, 2012) and marked using a cautery pen (Vervust and Van Damme, 2009). On the day of capture, we measured mass to the nearest 0.01 g with a precision balance (mean±s.d.: 5.73±1.06 g) and snout–vent length (SVL) with a digital caliper to the nearest 0.01 mm (mean±s.d.: 61.97±4.08 mm). Neither M_b nor SVL at capture differed between lizards from low- and high-elevation populations (Wilcoxon rank-sum test, M_b: W=206, P=0.28; SVL: W=176, P=0.082). On the day of capture, we transported animals to facilities either at the Station d'Ecologie Théorique et Expérimentale du CNRS à Moulis (low-elevation treatment: 42°57′26.8″N, 1°05′08.3″E; 436 m ASL; P_{O_2} ~20.1 kPa) or in the commune of La Mongie (high-elevation treatment: 42°54′34.8″N, 0°10′53.6″E; 1735 m ASL; P_{O_2} ~17.4 kPa; see Fig. 1 for experimental design). We maintained females individually in identical plastic enclosures (38×26×23 cm) containing a thin layer of substrate, a water bowl, a plastic shelter/basking platform (15×5×3.5 cm) and a nest box (18×14×10 cm) filled with wet sand. Enclosures were misted with water 3–4 times a week. Every 2 days, five live mealworms (*Tenebrio* sp. larvae) were distributed to each female and standing water was supplied *ad libitum*. Light was provided by UV lamps for 12 h per day and the enclosures were warmed by heat lamps (42 W) for 5 h per day at 1 h intervals, providing a gradient of ~25–40°C. Additionally, we monitored temperatures in the enclosures with iButton thermal data loggers (Model DS1921G-F5, Maxim Integrated, San Jose, CA, USA) placed in the cool end of two of the enclosures in each treatment. Temperature regimes were nearly identical across labs, with a diel range from 15°C at night to 25°C during the day. Furthermore, we rotated the enclosures on their shelves every several days to ensure no position effects.

Maternal metabolic rate

We measured resting metabolic rate after 2 days in captivity and approximately every week thereafter until 1 week after egg-laying (measures were skipped when lizards were actively nesting; mean time between measures±s.d.: 8.5±2.9 days) Lizards were offered their first meal after their initial measurement (2 days after capture) and food was withheld for 2 days before each subsequent measure. Each individual was placed in a custom-made 250 ml opaque metabolic chamber, which was then placed in an incubator set at 32°C for 1 h before measurement, a temperature intermediate between thermal preference (32.6°C) and field body temperatures (30.6°C) for gravid females in this area (Trochet et al., 2018; F.A., unpublished data). This acclimation time allows body temperature to equilibrate and reduces effects of handling stress (Braña, 1993; Tosini and Avery, 1996). We used pull-mode respirometry (Foxbox-C Field O_2 and CO_2 Analysis System, Sable Systems, Inc., Las Vegas, NV, USA) to measure gas

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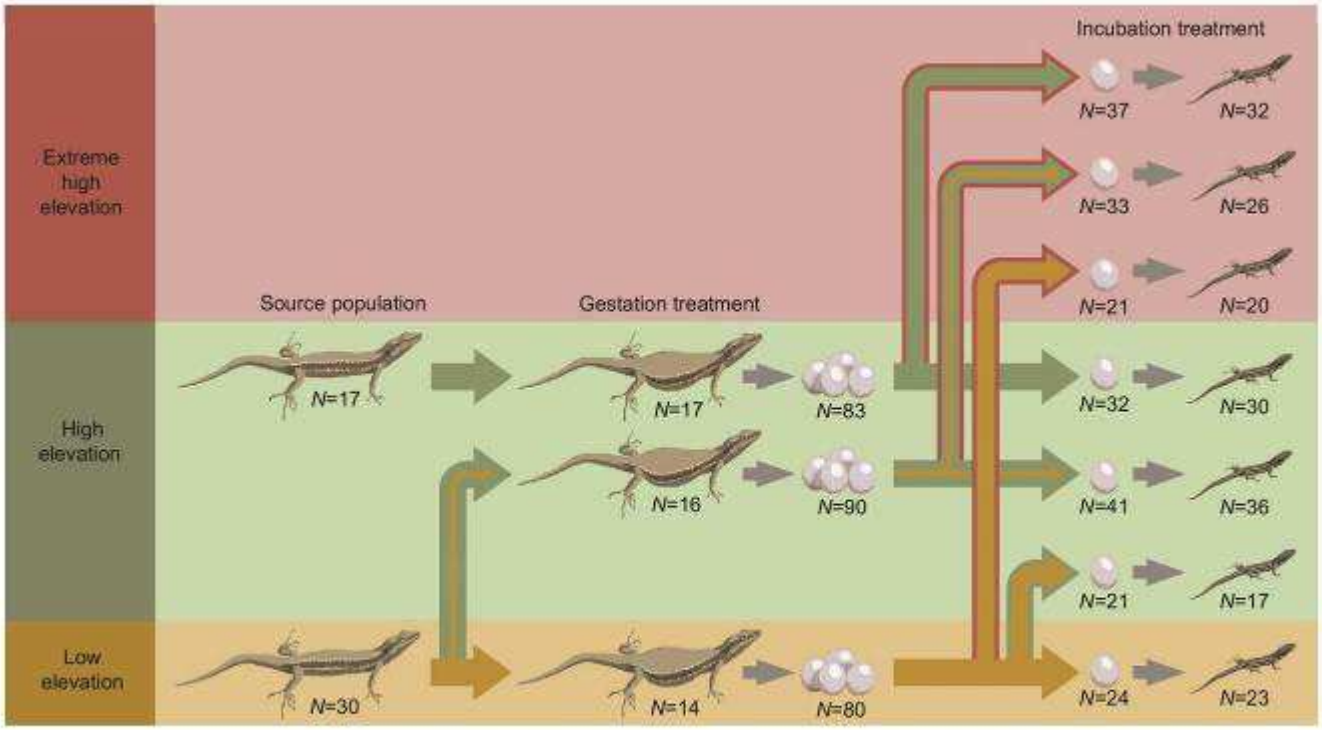

Fig. 1. Experimental design. Gravid lizards were collected from three low-elevation (382–472 m ASL) and two high elevation (1402–1795 m ASL) populations in the Pyrénées of southern France (see Table S1 for details). We maintained all lizards in common-garden conditions in labs at low elevation (436 m ASL; PO_2 ~20.1 kPa) or high elevation (1735 m ASL; PO_2 ~17.4 kPa), where they completed gestation and laid eggs. Egg clutches were then split and incubated at low, high or extreme high elevation (2877 m ASL; PO_2 ~15.3 kPa). Illustration credit: Bea Angelica Andersson.

exchange [oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$), corrected for barometric pressure] continuously for 30 min. Air was pumped at a rate of 500 ml min^{-1} through the metabolic chamber, dried of water vapour with Drierite, then measured for both CO_2 and O_2 content. Data were analysed with ExpeData software (v.1.7.30, Sable Systems, Inc.) to calculate $\dot{V}_{CO_2}$ and $\dot{V}_{O_2}$. We extracted the average of the lowest values of gas exchange over a 10 min interval for our analysis of metabolic rate, which allows the elimination of elevated metabolic rates during the measurement period (presumably because of activity). We recorded the body temperature of each lizard immediately following measurement using a cloacal thermometer (Miller & Weber T-6000, Ridgewood, NY, USA). All metabolic rate measures were conducted during the normal activity period for these diurnal lizards (07:30–20:00 h), and so represent resting metabolic rate (Andrews and Pough, 1985).

Blood sampling and hematological measures

The day after metabolic rate measurements, blood samples were collected by placing a heparinized glass capillary in the retro-orbital sinus of lizards (MacLean et al., 1973; Meylan et al., 2003). We collected 15–25 µl of blood in less than 3 min (bleed time mean±s.d.: 1.66±0.62 min), which was stored on ice until processing. Haematocrit values represent the volume of packed red blood cells relative to the total volume as measured with a caliper after spinning 10–15 µl of whole blood for 5 min at 5000 ***g***. Haemoglobin concentration was measured with the colorimetric cyanmethemoglobin method using 5 µl whole blood, following the manufacturer's instructions (Drabkin's reagent, Sigma-Aldrich, St Louis, MO, USA; cat. no. D5941). Samples were run in duplicate on five plates, with a pooled sample run repeatedly on each plate to provide estimates of intra- and interplate variation of 6.5% and 5.7%, respectively. Additionally, we quantified the amount of hyperoxides in blood plasma with a reactive oxygen metabolite (d-ROM) test kit (MC001, Diacron International, Italy) as a proxy for overall plasma oxidative status (Costantini, 2016). After centrifugation of whole blood at 3000 ***g*** for 5 min, we removed plasma by pipette and stored plasma at −80°C until the time of assay. We followed the manufacturer's instructions modified for use with a 4 µl lizard plasma sample (A.D., unpublished data). We calculated the amount of ROMs corrected for control blanks and report values in equivalents of mg H_2O_2 dl^{-1}. Samples were run on three plates, with a pooled sample run repeatedly on each plate to provide estimates of intra- and interplate variation of 7.0% and 8.6%, respectively. In some cases, plasma volume limitations and other logistical constraints precluded all measurements on each sample, so the number of observations varies slightly for each measure (see Table 1).

Reproductive measures

The nest boxes were checked every 24–48 h and eggs were immediately removed upon discovery. Eggs were individually weighed with a digital scale and the female was immediately re-weighed to obtain a post-oviposition mass. We calculated the relative clutch mass (RCM) as the ratio of total clutch mass to female post-oviposition mass. One egg from each clutch was randomly selected for dissection and staging. This egg was kept in moist vermiculite at room temperature for 1–4 days before we removed approximately one-third of the yolk with a syringe needle to facilitate embryo isolation and fixed the remaining embryo and tissue in 10% buffered formalin. Fixed eggs were sequentially transferred to 40 and 70% ethanol and then washed in a Tween 20/phosphate-buffered saline solution (PBS-T), following the embryo dissection protocol of Cordero and Janzen (2014). Incisions were

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Table 1. Results of linear mixed model analyses of physiological measures in gravid female *Podarcis muralis* lizards

Source of variation		Hct (*N*=186)	[Hb] (*N*=187)	Resting $\dot{V}_{O_2}$ (*N*=175)	ROMs (*N*=155)
Treatment		Low–high>high–high>low–low	Low–high>high–high>low–low		High–high>low–high>low–low
	F (d.f.$_n$, d.f.$_d$)	9.91 (2, 6.9)	18.6 (2, 6.9)	1.37 (2, 9.68)	3.49 (2, 23.8)
	Pr>F	0.009**	0.0017**	0.30	0.047*
Relative oviposition date linear		Negative	Negative		Negative
	F (d.f.$_n$, d.f.$_d$)	6.30 (1, 159.1)	28.0 (1, 161.4)	–	43.9 (1, 106.1)
	Pr>F	0.013*	<0.0001**		<0.0001**
Relative oviposition date quadratic		See Fig. 2A			
	F (d.f.$_n$, d.f.$_d$)	8.43 (1, 154.5)	–	–	–
	Pr>F	0.0042*			
Treatment×relative oviposition date linear					See Fig. 2D
	F (d.f.$_n$, d.f.$_d$)	–	–	–	4.48 (2, 144.7)
	Pr>F				0.013*
$\log_{10}$(Mass)				Positive	
	F (d.f.$_n$, d.f.$_d$)	–	–	9.97 (1, 82.7)	–
	Pr>F			0.0022*	
Pre-oviposition		Pre>post		Post>pre	Post>pre
	F (d.f.$_n$, d.f.$_d$)	14.88 (1, 157.2)	–	13.33 (1, 157.3)	41.9 (1, 128.5)
	Pr>F	0.00017**		0.0004**	<0.0001**
Body temperature				Positive	
	F (d.f.$_n$, d.f.$_d$)	–	–	6.51 (1, 156.1)	–
	Pr>F			0.012*	
Size-corrected clutch mass					Negative
	F (d.f.$_n$, d.f.$_d$)	–	–	–	13.98 (1, 33.9)
	Pr>F				0.0007**

See Materials and Methods for statistical details. Significant factors are indicated by *P<0.05 and **P<0.001 along with directionality. N indicates the number of observations; d.f.$_n$ is the numerator degrees of freedom; d.f.$_d$ is the denominator degrees of freedom. Hct, haematocrit; [Hb], haemoglobin concentration; $\dot{V}_{O_2}$, oxygen consumption rate; ROMs, reactive oxygen metabolite concentration.

made on extreme longitudinal poles of the egg and egg contents were gently removed and placed in PBS-T. The embryo was then located, transferred to 70% ethanol, and placed on a dissecting microscope for observation and staging, following the criteria of Dufaure and Hubert (1961). Since stage at oviposition is generally invariable within a clutch (Mathies and Andrews, 1995), we can infer the stage of the randomly selected embryo to be representative of all eggs in the clutch.

We recognize that an ideal design would have included more frequent checks of nest boxes and an immediate fixing of the randomly selected embryo. However, this was impossible due to logistical constraints. We combated bias by checking nest-boxes in both treatments at the same intervals and through an analysis of the potential effect of the number of possible days the eggs were incubating in the nest boxes before being discovered. The number of potential days in the nest box did not affect mass of eggs at oviposition ($F_{1,46.1}$=2.99, P=0.09) nor did the number of days before fixation affect embryo stage at oviposition ($F_{1,37}$=0.57, P=0.46).

Egg incubation and treatments

The remaining eggs of each clutch (N=209) were individually incubated in plastic cups containing moist vermiculite (1:5 water to vermiculate by volume; Cordero et al., 2017a; While et al., 2015) and sealed with plastic film to retain moisture. Incubators contained an open tray of water to maintain high humidity. We verified the total cup mass weekly and added water to maintain this ratio throughout incubation. Eggs were incubated at a constant 28°C, which provides optimal hatching success in this species (Van Damme et al., 1991). Within the first week after oviposition (mean±s.d.: 1.8±2.2 days), eggs were dispatched to the three different incubation treatments (low elevation, high elevation and extreme high elevation). Within each maternal treatment, mass of eggs allocated to different incubation treatments did not differ (all P>0.15). Low- and high-elevation treatments matched the locations of the maternal treatments (see above) while the extreme high-elevation treatment was conducted at the laboratory of Pic du Midi de Bigorre (42°56′11.0″N, 0°08′32.9″E; 2877 m ASL; P_{O_2}: ~15.3 kPa; see Fig. 1 for complete experimental design and sample sizes). We rotated egg position in incubators twice a week to ensure no position effects.

Embryo heart and metabolic rates

Beginning 1 week post-oviposition, we measured the heart rate of all embryos repeatedly throughout incubation (up to four times per embryo, mean±s.d. interval between measures: 12.8±4.2 days), spacing our measurements to ensure an equal sampling across the incubation period and amongst treatment groups. We measured the heart rate with a Buddy digital egg monitor (MK2; Avitronics, Truro, UK) placed inside the incubator at 28°C. Eggs were quickly moved from their incubation cup to the instrument and we used a stopwatch to measure the time from egg removal to when a consistent heart rate was observed (mean±s.d. time: 121.6±73.7 s). If a reliable measurement could not be made in under 5 min, we replaced the egg in the incubator and re-measured later in the day to reduce any effects of handling or temperature change (Cordero et al., 2017a; Hulbert et al., 2017).

In a subset of eggs, we also measured metabolic rate on the same day as we measured heart rate. Each egg was placed in a small cup with moist vermiculite and inside a 250 ml metabolic chamber. We replaced the chamber in the incubator at 28°C and used closed-system respirometry to measure gas exchange. We flushed the chamber for 15 min at a flow rate of 300 ml min^{-1} then closed valves to seal the chamber for 90 min. We then opened the valves to re-establish air flow, dried air of water vapour with Drierite, and measured O_2 and CO_2 as above. We used ExpeData software to calculate $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ by integrating the change in instantaneous gas concentrations over the period the chamber was sealed (Lighton, 2008).

Hatchling morphology

Eggs were checked for hatching every 48 h. After emergence from the egg, we measured SVL using digital calipers (mean±s.d.: 25.1±1.2 mm) and mass using an electronic balance (mean±s.d.: 0.363±0.053 g). Of the 209 eggs incubated, 184 hatched (88.0% hatching success rate).

Field sampling and experimental protocols were conducted under permit provided by the Direction régionale de l'environnement, de l'aménagement et du logement (DREAL) Midi-Pyrénées (Arrêté Préfectoral no. 2017-s-02 30 March 2017), under current ethical committee approval (APAFIS#16359-201808011445465 v4), and in accordance with Directive 2010/63/EU on protection of animals used for scientific purposes.

Statistics

For our measures of the reproducing females, we assessed the influence of native elevation and gestation elevation treatments, time relative to oviposition and reproductive investment on five physiological parameters (haematocrit, haemoglobin concentration, $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and ROMs) using linear mixed models with the lme4 package (Douglas et al., 2015) in the programming language R (https://www.r-project.org/). We verified normal distributions of residuals in all cases and assessed the relative importance of fixed effects using type III sums of squares, correcting denominator degrees of freedom for *F*-tests (Kenward and Roger, 1997). Models included the fixed effect of experimental treatment group, which combined origin elevation and gestation elevation (low–low, low–high or high–high), relative oviposition date (both linear and quadratic to account for non-linear changes over this time period), and the interaction between treatment group and relative oviposition date (both linear and quadratic). We also included a categorical fixed effect indicating whether the measure occurred before or after oviposition (Y/N). To test for the effect of reproductive investment, we included the covariate of size-corrected clutch mass (calculated as the residuals of the total clutch mass on SVL regression). Initial models also included the covariate of body size (SVL for haematocrit, haemoglobin concentration and ROMs; $\log_{10}$-transformed mass for $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$). We accounted for non-independence of samples from each population and the repeated measures on individuals by including population of origin and individual as additive random effects. To simplify models, we used a backward selection procedure, removing non-significant interactions and main effects (all $P>0.10$) except for the factor of treatment, which addresses our main biological hypotheses. To meet assumptions of normal distribution of residuals, $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and ROMs data were $\log_{10}$-transformed before analysis.

For the reproductive parameters (stage at oviposition, clutch size, egg mass, relative clutch mass, post-oviposition body condition), we used linear mixed models with a fixed effect of treatment (as above) and size (SVL) as a covariate. We included the random effects of population of origin for all models and also maternal identity in the model of egg size to account for covariance among eggs in the same clutch. Body condition was calculated as the residual of the linear regression of $\log_{10}$-transformed mass on $\log_{10}$-transformed SVL. We reduced models following a backward-selection process as above. Because of the limited range of embryonic stages observed at oviposition, we also assessed the effect of treatment on stage at oviposition with an ordinal logistic regression using the polr function of the MASS package (Venables and Ripley, 2002), which gave results qualitatively identical to the linear mixed model analysis.

We analysed aspects of embryo physiology (heart rate and oxygen consumption throughout incubation), development (incubation time and survival) and hatchling morphology (mass and SVL) using the same linear model structure and backward selection approach as described above. The analysis of survival utilized a generalized linear mixed model with a binomial error distribution. We categorized embryos into one of seven treatment groups based on the combination of population of origin, maternal gestation treatment, and egg incubation treatment (low–low–low, low–low–high, low–low–extreme high, low–high–high, low–high–extreme high, high–high–high or high–high–extreme high). All models included the fixed effect of treatment group. Initial models also contained the covariate of egg mass at the time of measurement (for heart rate and oxygen consumption) or egg mass at oviposition (for survival, hatchling mass and hatchling size) to account for variation in maternal energetic investment (Uller and While, 2015; Van Damme et al., 1991; Warner and Lovern, 2014), as well as the interaction of egg mass with treatment group. We also included the covariate of stage at oviposition in initial models of incubation duration to test if the developmental stage at which an egg was laid affected time to hatching. Initial models of embryo heart rate and oxygen consumption also included the linear and quadratic effects of time (days post-oviposition) to account for the expected non-linearity of these parameters over time (Burggren and Warburton, 1994; Cordero et al., 2017a; Sartori et al., 2017), as well as the interaction of treatment and the linear and quadratic effects of time. As above, models included the random effect of population of origin and maternal identity. Models of heart rate and oxygen consumption also included the random effect of individual to account for covariation of measures on the same individual. For models that demonstrated a clear effect of treatment, we then tested our specific biological hypotheses by using linear contrasts of estimated marginal means using the emmeans package (Russell, 2016, 2019), correcting for multiple comparisons with the Šidák method (see Results for details). All data figures were created with the ggplot2 package (Wickham, 2009).

RESULTS

Maternal physiology

Reproducing lizards from low-elevation populations increased both haematocrit and haemoglobin concentration in response to translocation to high elevation (by 23.9% and 31.8% compared with low-elevation values, respectively) and values for both parameters decreased over time in all groups. Additionally, haematocrit changed non-linearly over time and was greater before oviposition (Table 1, Fig. 2A,B).

As the results of the $\dot{V}_{CO_2}$ and $\dot{V}_{O_2}$ analyses are qualitatively identical, we present and discuss the results of $\dot{V}_{O_2}$ only. Resting metabolic rate increased after oviposition and did not differ among the treatment groups (Table 1, Fig. 2C). As expected, resting metabolic rates were positively dependent on both body mass and body temperature. Reactive oxygen metabolites in blood plasma were affected by time differently among the treatment groups: ROMs decreased linearly over time for lizards from low- and high-elevation populations kept at native elevations, whereas they remained elevated for lizards from low elevation transplanted to high elevation. Despite the fact that lizards at native elevations exhibited higher levels of ROMs early in reproduction, this interaction resulted in transplanted lowland lizards exhibiting higher ROMs later in reproduction (Table 1, Fig. 2D). Additionally, ROMs after oviposition were higher than predicted by the change across oviposition date. Finally, ROMS were negatively correlated with size-corrected total clutch mass, indicating that individuals investing more in reproduction also produced less reactive oxygen metabolites (Table 1, Fig. 3).

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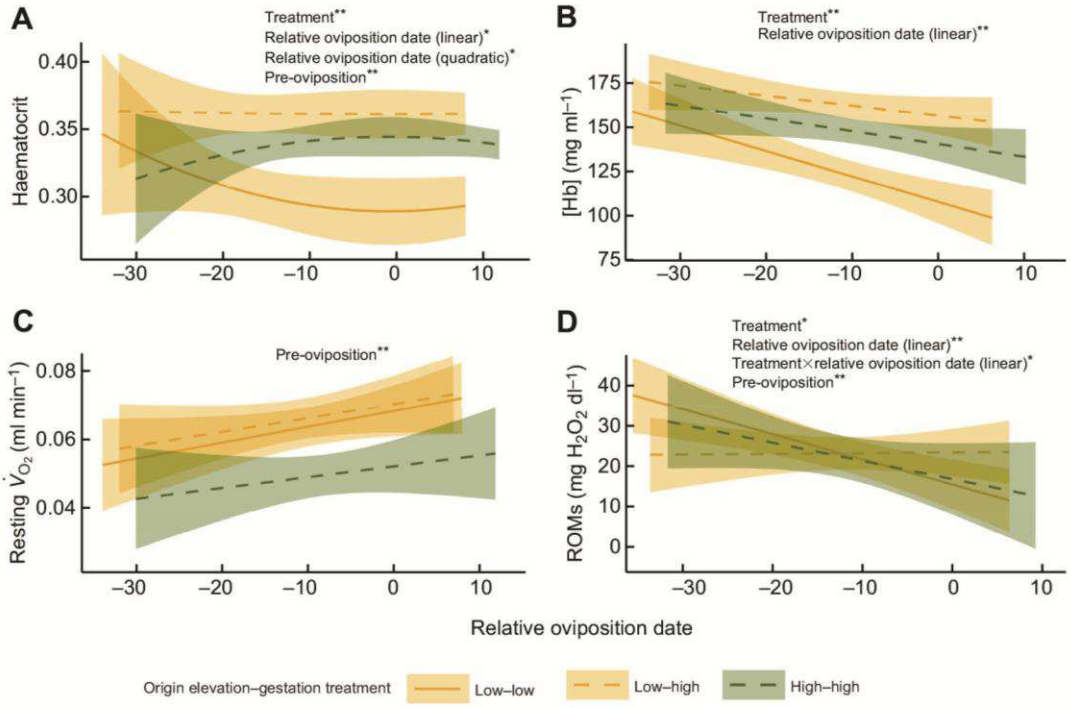

Fig. 2. Response of haematocrit, haemoglobin concentration, resting oxygen consumption rate ($\dot{V}_{O_2}$) and reactive oxygen metabolites over time in reproducing lizards from each experimental treatment. Haematocrit (A), haemoglobin concentration (B), $\dot{V}_{O_2}$ (C) and reactive oxygen metabolites (ROMs; D) in reproducing lizards from different elevation treatments. Lines are linear or quadratic regressions depending on the results of mixed effect models with shaded areas representing standard error of the estimate. Significant effects shown in inset with one (P<0.05) or two (P<0.001) asterisks (see Table 1 and Materials and Methods for statistical details). Relative oviposition date 0=date of oviposition. Low–low, N=14 individuals; low–high, N=16 individuals; high–high, N=17 individuals.

Reproductive measures

Larger lizards had larger clutches and invested more in reproduction. After accounting for this variation, egg mass at oviposition differed slightly among the maternal origin treatment groups, although none of the pairwise differences was significant. Lizards originating from populations at different elevations did not differ in any other reproductive parameter, nor did transplanting low-elevation lizards to high elevation affect reproduction. Female *P. muralis* laid eggs with embryos that were on average at stage 27 (range: 25–28.5) of Dufaure and Hubert (1961). Finally, we saw no effect of treatment group on post-oviposition body condition (Table 2, Fig. 4A–D).

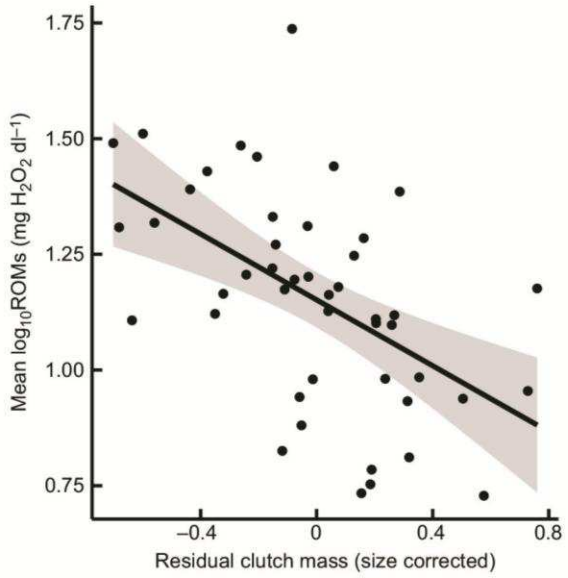

Fig. 3. Scatterplot showing relationship between total clutch mass (corrected for body size) and mean ROMs for all females (N=47). Black line represents linear regression with standard error in grey shading. After accounting for other effects, ROMs decrease with increased relative clutch mass (see Table 1 for details).

Embryo heart and metabolic rates

Embryonic heart rates varied amongst treatment groups in a manner that was dependent upon the interactions with both linear and quadratic age post-oviposition (Table 3, Fig. 5A). Embryos from low-elevation populations generally showed a concave response, whereby heart rates were lowest early and late in incubation. Conversely, embryos from high elevations showed a convex response, whereby heart rates were highest early and late in incubation. Using linear contrasts from the mixed models to test our specific *a priori* hypotheses demonstrates that embryos from low-elevation populations exhibited higher heart rates at their native elevation compared with embryos from high-elevation populations at native elevation, on average by 15.3 beats min^{-1} (Table 4). Furthermore, embryos from low-elevation populations reduced heart rates when incubated at high elevation (by 18.9 beats min^{-1}) or extreme high elevation (by 9.6 beats min^{-1}). Notably, the change was greatest in the high-elevation treatment, in which heart rates were lower than the extreme high treatment by 9.3 beats min^{-1}. Embryos from high elevation populations increased heart rates when incubated at extreme high elevation compared with native (high) elevation, on average by 9.6 beats min^{-1}.

Table 2. Results of linear mixed model analyses of reproductive parameters in gravid female *P. muralis* lizards

Source of variation		Stage at oviposition (*N*=39)	Clutch size (*N*=47)	Egg mass (*N*=253)	Relative clutch mass (*N*=47)	Post-oviposition body condition (*N*=47)
Treatment				See Fig. 4C		
	F (d.f.$_n$, d.f.$_d$)	0.32 (2, 9.1)	0.37 (2, 8.4)	4.80 (2, 7.5)	1.32 (2, 8.3)	1.32 (2, 7.2)
	Pr>F	0.73	0.70	0.046*	0.32	0.33
Snout–vent length			Positive	Positive	Positive	
	F (d.f.$_n$, d.f.$_d$)	–	14.17 (1, 43.0)	5.58 (1, 43.2)	5.48 (1, 43.0)	–
	Pr>F		0.0005**	0.023*	0.024*	

See Materials and Methods for statistical details. Significant factors indicated as *P<0.05 or **P<0.001 along with directionality.

Embryo metabolic rate (oxygen consumption; $\dot{V}_{O_2}$) increased with time post-oviposition, although this response was non-linear and dependent on a quadratic function of embryo age (Table 3; Fig. 5B). Embryos from the different treatments exhibited different patterns of oxygen consumption, with this response dependent on a linear interaction with embryo age post-oviposition. The interaction of treatment and embryo age is driven by differences in embryos incubated at extreme high elevation: embryos from high-elevation populations increased oxygen consumption across incubation, whereas embryos from low-elevation populations exhibited lower levels later in incubation. No test of our *a priori* hypotheses for oxygen consumption demonstrated a clear difference between treatment groups. Furthermore, oxygen consumption was not clearly dependent on egg mass as we would expect (Vleck and Hoyt, 1991), most likely because of the strong effect of days post-oviposition, which is correlated with egg mass.

Embryo survival and hatchling morphology

We found no difference among treatment groups in probability of hatching (χ^2_6=5.45, P=0.49) or an effect of oviposition egg mass on survival to hatching probability (χ^2_1=2.83, P=0.09). Among embryos that survived to hatching, incubation duration was affected by incubation treatment (Table 3, Fig. 6A): embryos native to low-elevation populations took an average of 1.5 days longer to hatch at high elevation and 1.4 days longer to hatch at extreme high elevation. Egg mass at oviposition strongly and positively influenced hatchling mass and body size, although these were not affected by treatment (Table 3, Fig. 6B,C).

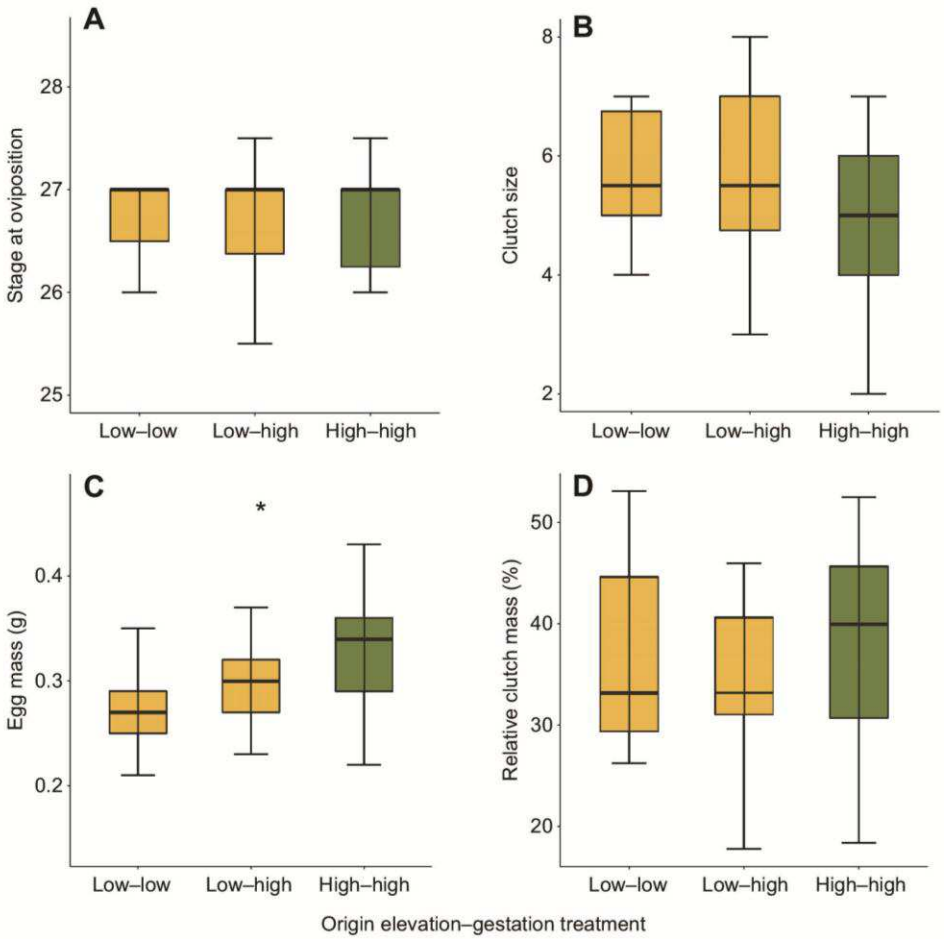

Fig. 4. Reproductive parameters in females from three experimental treatments (combining origin elevation and gestation treatment). (A) Stage at oviposition. (B) Clutch size. (C) Egg mass at oviposition. (D) Relative clutch mass. Plots are Tukey box plots showing median, interquartile range and range of raw data values. Significant differences among treatment groups shown with one asterisk (P<0.05; see Table 2 and Materials and Methods for statistical details). Low–low, N=14 individuals; low–high, N=16 individuals; high–high, N=17 individuals.

Table 3. Results of linear mixed model analyses of embryo *P. muralis* heart rate and oxygen consumption rate during development, incubation duration and hatchling morphology

Source of variation		Heart rate (N=414)	$\dot{V}_{O_2}$ (N=136)	Incubation duration (N=184)	Hatchling mass (N=184)	Hatchling body size (SVL) (N=184)
Treatment		See Fig. 5A	See Fig. 5B	See Fig. 6A		
	F (d.f.$_n$, d.f.$_d$)	4.02 (6, 306.6)	6.14 (6, 91.3)	3.01 (6, 131.9)	1.97 (6, 144.4)	1.53 (6, 40.6)
	Pr>F	0.0007**	<0.0001**	0.009*	0.074	0.19
Oviposition egg mass					Positive	Positive
	F (d.f.$_n$, d.f.$_d$)	–	–	1.33 (1, 71.2)	42.5 (1, 79.3)	36.0 (1, 63.1)
	Pr>F			0.25	<0.0001**	<0.0001**
Oviposition mass×treatment						
	F (d.f.$_n$, d.f.$_d$)	–	–	2.10 (6, 136.4)	2.12 (6, 146.0)	–
	Pr>F			0.057	0.055	
Days post-oviposition linear			Positive			
	F (d.f.$_n$, d.f.$_d$)	1.35 (1, 300.1)	29.7 (1, 81.6)	–	–	–
	Pr>F	0.25	<0.0001**			
Days post-oviposition quadratic			See Fig. 5B			
	F (d.f.$_n$, d.f.$_d$)	1.41 (1, 305.0)	7.52 (1, 81.8)	–	–	–
	Pr>F	0.24	0.008*			
Treatment×days post-oviposition linear		See Fig. 5A	See Fig. 5B			
	F (d.f.$_n$, d.f.$_d$)	2.33 (6, 302.4)	6.55 (6, 89.9)	–	–	–
	Pr>F	0.033*	<0.0001**	–	–	–
Treatment×days post-oviposition quadratic		See Fig. 5A				
	F (d.f.$_n$, d.f.$_d$)	2.35 (6, 307.1)	–	–	–	–
	Pr>F	0.031*				

See Materials and Methods for statistical details. Significant factors indicated as *P<0.05 or **P<0.001 along with directionality. $\dot{V}_{O_2}$, oxygen consumption rate; SVL, snout–vent length. N indicates the number of observations.

DISCUSSION

Our results demonstrate that translocation to high elevation alters the physiology of lowland gravid lizards compared with that of lizards kept at native elevations (from both low- and high-elevation populations) but does not affect short-term reproductive investment or output. Importantly, the concentration of ROMs did not decrease during the reproductive period in transplanted lizards, as it did in lizards kept at native elevation. This suggests that there may be a deleterious effect of gestation at high elevation for non-native lizards, even if that cost is not evident in current reproduction. Interestingly,

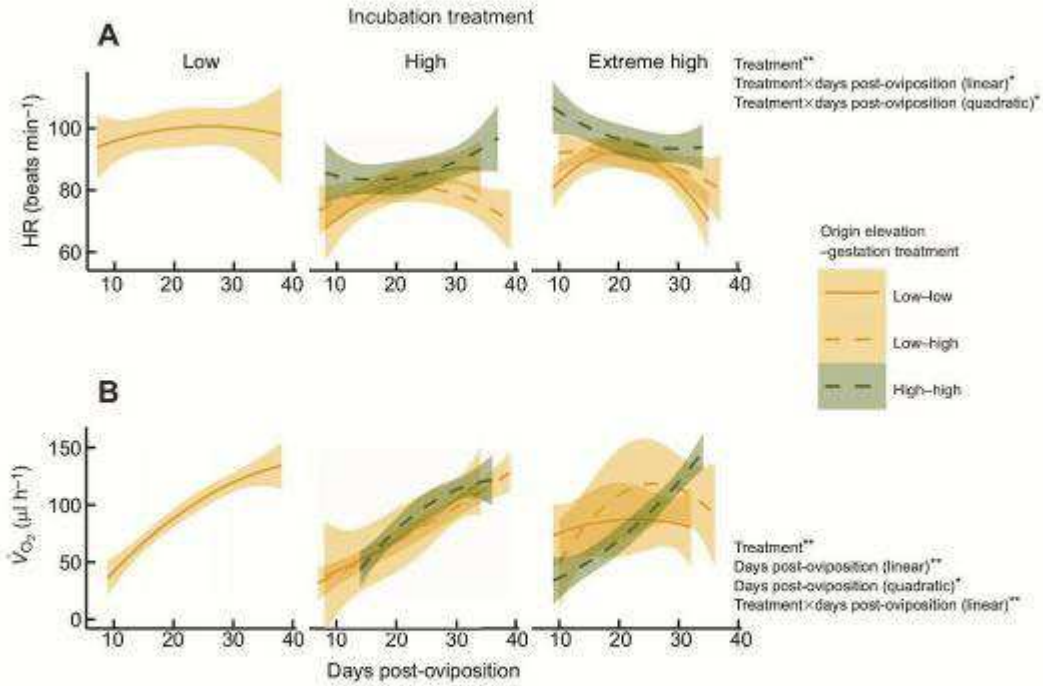

Fig. 5. Heart rate and $\dot{V}_{O_2}$ over time in lizard embryos from each experimental treatment. (A) Heart rate and (B) $\dot{V}_{O_2}$ quadratic regressions (lines) with shaded areas representing standard error of the estimate. Significant effects shown in inset with one (P<0.05) or two (P<0.001) asterisks (see Table 3 and Materials and Methods for statistical details). Embryos are from three maternal treatments (low–low, N=14 clutches; low–high, N=16 clutches; high–high, N=17 clutches) incubated at three elevations. See Fig. 1 for complete sample size details.

Table 4. Results of *a priori* hypothesis tests

Hypothesis test		Heart rate (N=414)	$\dot{V}_{O_2}$ ($\log_{10}$-transformed) (N=136)	Incubation duration (N=184)
Embryos native to low elevation versus embryos native to high elevation incubated at native elevations	Estimate (s.e.)	−15.29 (4.11)	−0.047 (0.04)	0.058 (1.00)
	t-Statistic (d.f.)	−3.72 (53.7)	−1.10 (6.27)	0.058 (4.4)
	Pr>*t*	0.0029*	0.89	>0.99
Embryos native to low elevation incubated at native elevation versus incubated at high elevation	Estimate (s.e.)	−18.85 (3.58)	−0.076 (0.03)	1.49 (0.42)
	t-Statistic (d.f.)	−5.26 (375.4)	−2.71 (56.7)	3.52 (166.6)
	Pr>*t*	<0.0001**	0.052	0.003*
Embryos native to low elevation incubated at native elevation versus incubated at extreme high elevation	Estimate (s.e.)	−9.60 (3.49)	−0.039 (0.03)	1.35 (0.47)
	t-Statistic (d.f.)	−2.75 (363.1)	−1.35 (33.1)	2.86 (167.3)
	Pr>*t*	0.037*	0.71	0.028*
Embryos native to low elevation incubated at high elevation versus incubated at extreme high elevation	Estimate (s.e.)	9.25 (2.92)	0.037 (0.03)	−0.14 (0.36)
	t-Statistic (d.f.)	3.17 (338.3)	1.25 (42.5)	−0.38 (162.3)
	Pr>*t*	0.0098*	0.77	>0.99
Embryos native to high elevation incubated at native elevation versus incubated at extreme high elevation	Estimate (s.e.)	9.55 (3.60)	−0.009 (0.03)	−0.80 (0.41)
	t-Statistic (d.f.)	2.65 (389.0)	−0.27 (66.6)	−1.95 (156.1)
	Pr>*t*	0.049*	>0.99	0.28
Embryos native to low elevation gestated at native elevation versus gestated at high elevation	Estimate (s.e.)	0.63 (3.41)	−0.001 (0.03)	−0.56 (0.41)
	t-Statistic (d.f.)	0.19 (126.1)	−0.043 (35.4)	−1.37 (62.4)
	Pr>*t*	>0.99	>0.99	0.69

Tests were constructed using linear contrasts of estimated marginal means. Estimate represents the differences in observed values between groups, taking into account covariates included in the model (see Table 3 for full model results and Materials and Methods for statistical details).
Significant factors indicated as *P<0.05 or **P<0.001. $\dot{V}_{O_2}$, oxygen consumption rate. N indicates the number of observations.

reactive oxygen metabolite concentration was highest in females exhibiting the lowest relative clutch mass, contrary to assumptions of a reactive metabolite cost of reproduction. Nonetheless, at least in the short-term, there appears to be no indication that reproduction in lowland *P. muralis* is limited at higher elevations within the range of the species, assuming the environment is otherwise suitable. Furthermore, the conditions experienced by females during gestation did not affect the physiology, development or hatchling morphology of embryos post-oviposition. Thus, we did not find evidence that maternal hypoxia exposure may 'prime' embryos for the constraints of post-oviposition hypoxia. Embryos post-oviposition demonstrated a clear physiological and developmental response to hypoxia, including reduced heart rate at high elevation, altered patterns of oxygen consumption – indicating diminished late-stage metabolic rate – and increased incubation duration. These adjustments in embryo physiology and developmental timing at least partially buffer these lizards from potentially negative effects of reduced oxygen availability; development at these elevations did not affect fitness-related traits, including embryo survivorship to hatching or hatchling morphology. Taken together, these results suggest that this widespread and common species, a successful invader elsewhere (Beninde et al., 2018; Deichsel and Gist, 2001; Michaelides et al., 2015), will be likely to colonize higher-elevation habitats in future climate scenarios.

Maternal physiology and reproduction

As predicted, gravid females from low-elevation populations transplanted to high elevation had higher haematocrit and haemoglobin concentration compared with low-elevation lizards kept at low elevation. This response is concordant with other studies showing that lizards from high-elevation populations feature higher haematocrit and haemoglobin concentration compared with lowland populations (González-Morales et al., 2015; Lu et al., 2015). Haematocrit and haemoglobin concentration was increased in lowland lizards transplanted to high elevation to match lizards native to high elevations, which does not support the prediction that lizards native to different elevations would exhibit different blood chemistry profiles. The current data do not allow us to distinguish between plasticity and local adaptation in these traits in lizards native to high elevations, but in either case, this could illustrate an adaptive response of lizards to facilitate oxygen transport at high elevation.

In parallel with these results, we did not observe differences in resting metabolic rate among the treatment groups. Even in a state of elevated energy exchange, such as occurs during reproduction (Angilletta and Sears, 2000; Foucart et al., 2014), oxygen demands are met at high elevation, possibly due to the observed plasticity in blood biochemistry. While energetic demands are increased in reproductive compared with non-reproducing females, it is

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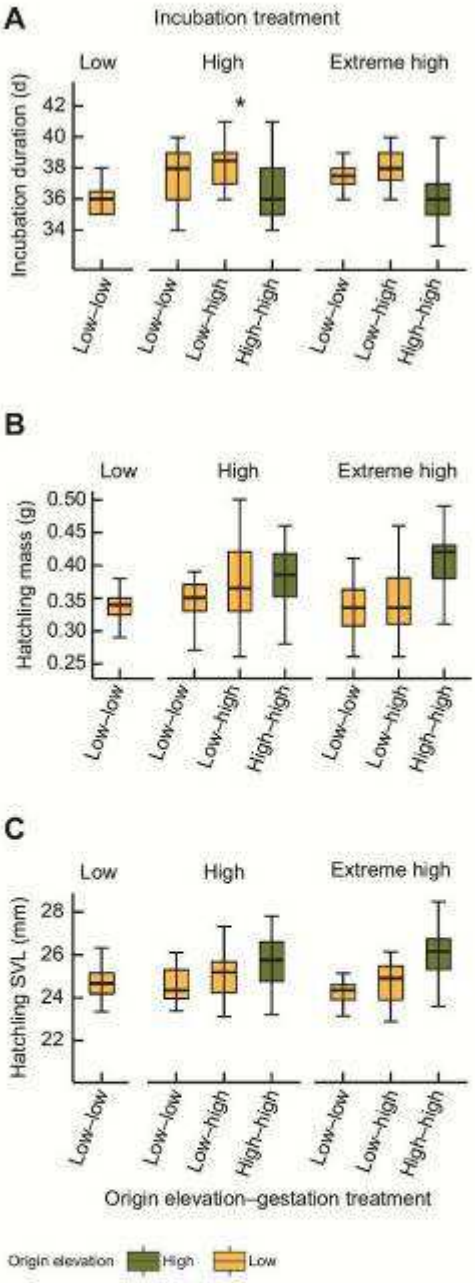

Fig. 6. Incubation duration and hatchling morphology for lizard embryos from each experimental treatment. Incubation duration (A), hatchling mass (B) and hatchling snout–vent length (SVL) (C). Plots are Tukey box plots showing median, interquartile range and range of raw data values. Significant differences among treatment groups shown with one asterisk ($P<0.05$; see Table 3 and Materials and Methods for statistical details). Hatchlings are from three maternal treatments (low–low, $N=14$ clutches; low–high, $N=16$ clutches; high–high, $N=17$ clutches) incubated at three elevations. See Fig. 1 for complete sample size details.

important to note that this increased demand occurs during vitellogenesis (Foucart et al., 2014; Van Dyke and Beaupre, 2011), before the start of this experiment. Nonetheless, the question of long-term effects of increased haemoglobin concentration and haematocrit remains because a higher concentration of red blood cells increases blood viscosity. This phenomenon could then carry an important cost for blood circulation (Dunlap, 2006; Hedrick et al., 1986) and may not be maintained, as observed in lowland males of this species transplanted to extreme high elevation (Gangloff et al., 2019). Increasing red blood cell concentration is especially constraining during gravidity when haematocrit drops, probably to reduce viscosity, and facilitate circulation and gas transport to developing embryos (e.g. Dupoué et al., 2015).

We found little evidence of important differences among treatment groups in indicators of reproductive investment, including clutch size and relative clutch mass. After accounting for variation due to maternal size, we identified a trend whereby lizards at high elevations (either native or transplanted) produced eggs with a greater mass at oviposition. This finding is counter to our prediction that lizards at higher elevations would demonstrate reduced reproductive output. Indeed, through changes in physiological parameters, females appeared to compensate for reduced oxygen availability and maintained reproductive output across treatments. It is important to recognize that this experiment encompassed only the first reproductive event of a single year. Lowland lizards transplanted to high elevation were able to maintain reproductive output without an apparent cost in terms of body condition in this bout, but there may be a trade-off with future reproduction such as via ROM damage. Although stage at oviposition did not differ among treatment groups and was within the expected range for most oviparous lizards (reviewed in Mathies and Andrews, 1999), our findings broadly support the prediction that gestation duration is a highly stable trait whose population mean might undergo gradual intergenerational change as an adaptive evolutionary response to high-altitude conditions (e.g. temperature, Mathies and Andrews, 1995). Thus, because stage at oviposition might be highly canalized in *P. muralis*, variation in this trait may not manifest until several generations are exposed to oxygen-limited conditions, similar to the adaptive evolution of prolonged egg retention in cool environments (Telemeco et al., 2010; While et al., 2015).

We provide two important results regarding ROM production in response to both reproduction and hypoxia. First, we found that females with increased reproductive investment (size-corrected total clutch mass) had lower levels of ROMs in blood plasma. Broadly, this result does not support the common assumption that reproduction incurs an oxidative cost and thereby mediates life-history trade-offs (Dowling and Simmons, 2009; Metcalfe and Monaghan, 2013; Speakman and Garratt, 2014). Previous work in lizards concurs with our results: no such oxidative cost of reproduction was found in two other lizard species (*Niveoscincus ocellatus*, Isaksson et al., 2011; *Ctenophorus pictus*, Olsson et al., 2009, 2012). This result suggests that both ROM production and reproductive investment could be correlated via some unmeasured physiological parameter representing overall 'individual quality': higher-quality individuals are able to both invest more in reproduction and meet energetic demands while producing fewer potentially detrimental by-products (Wilson and Nussey, 2010). On the contrary, Webb et al. (2018) found that increased reproductive investment (as measured by follicle number) was associated with increased ROMs in the lizard *Cyclura cychlura inornata*. Importantly, Webb et al. (2018) measured this effect during vitellogenesis, thus pointing to this period as being key to understanding the relationship between reproductive investment and ROM production. A similar pattern of higher reactive oxygen metabolite production earlier in the reproductive cycle is also found in snakes (*Antaresia childreni*, Stahlschmidt et al., 2013) and birds (*Sula leucogaster*, Montoya et al., 2016). Our results agree with this pattern, whereby ROMs decreased during the gestation period, indicating that such potentially harmful by-products are greatest earlier in the reproductive process.

Our second significant finding is that both low- and high-elevation lizards kept at native elevations decreased reactive oxygen metabolite production across the reproductive period, while lowland lizards transplanted to high elevations did not. We might expect that the reactive oxygen cost of reproduction will likely be context dependent, perhaps because of resource limitations and subsequent allocation decisions (Dowling and Simmons, 2009). In our case, it appears that the reduced oxygen availability at higher elevations elicits such a constraint and prohibits females from decreasing ROM production across the reproductive period. Our results demonstrate higher reactive oxygen metabolite production later in gravidity and after oviposition in transplanted lizards. These elevated levels of ROMs have the potential to bear costs for future fitness in both the mothers and their offspring, given the potential damaging effects on proteins, membranes and DNA (Blount et al., 2016; Costantini, 2016; Speakman and Garratt, 2014). Unfortunately, plasma volume limitations precluded us from also measuring antioxidant capacity, which would provide valuable data to quantify the response to these damaging molecules. For example, antioxidant capacity may change across the reproductive period, thus mitigating the potential negative impacts of increased ROM production. Future work is needed to understand patterns of ROMs mediating physiological trade-offs at high elevations by measuring additional tissues, measuring antioxidant and repair capacity, and quantifying the long-term impacts of increased ROM levels (Speakman and Garratt, 2014).

Embryo physiology and development

Developing *P. muralis* embryos appear to be robust to the effects of ecologically relevant hypoxia, at least until hatching and the beginning of the free-living stage. The most dramatic physiological response is the decreased embryonic heart rate in naturally occurring hypoxia at high elevation (Cordero et al., 2017a), which is similar to results seen in many oviparous species exposed to laboratory-simulated hypoxia (Crossley and Altimiras, 2005; Du et al., 2010; Nechaeva and Vladimirova, 2008; VanGolde et al., 1997). This response is coupled with a relative increase in heart size in *P. muralis* (Cordero et al., 2017a), which may facilitate the maintenance of oxygen delivery via increased stroke volume. This response is predicted under a scenario where hypervolemia increases the optimal haematocrit: oxygen delivery is maintained, despite reductions in heart rate, via increases in haematocrit and stroke volume (Birchard, 2015; Weibel et al., 1991). While work is needed to test these specific relationships among parameters important to efficient oxygen delivery under hypoxia, we found that embryos at high and extreme high elevations maintained comparable metabolic rates (as measured by oxygen consumption). This concurs with previous studies finding that incubation under hypoxia had little effect on the metabolic rates of developing alligator embryos (*Alligator mississippiensis*, Crossley et al., 2017; Warburton et al., 1995).

While oxygen consumption generally increased during incubation, the pattern was dependent on treatment. Notably, lowland embryos incubated at extreme high elevation exhibited a pattern whereby oxygen consumption decreased late in incubation. This pattern is mirrored in the pattern of heart rate variation across incubation, suggesting a late-stage metabolic suppression resulting from oxygen limitation. This metabolic constraint on low-elevation *P. muralis* embryos developing at high and extreme high elevations could also explain the increased incubation duration of these embryos. That late-stage embryos native to high elevation did not experience this metabolic suppression and did not alter incubation times at extreme high elevation suggests a role for local adaptation in maintaining physiological and developmental robustness in hypoxia. Although late-stage embryos of sea-level chicken populations also exhibited reduced oxygen consumption when initially transplanted to high elevation, oxygen consumption rates exceeded sea-level values after >9 generations of selective breeding (based on hatching success) at 3100 m ASL (Beattie and Smith, 1975; Wangensteen et al., 1974). The fitness consequences of hypoxia-induced metabolic suppression in non-avian reptilian embryos awaits further experimentation.

While embryo physiology and development responded to high-elevation hypoxia, neither survival to hatching nor hatchling morphology were affected. This corroborates our previous work in this system in demonstrating that early-life stages are relatively resilient to hypoxia and therefore would not limit future colonization of high-elevation habitats beyond the current species range. We emphasize that even our extreme high elevation treatment represents a moderate and biologically relevant reduction in oxygen availability (approximately 72% of sea level equivalent), which explains why we did not find the clear negative effects of hypoxia as have other studies in reptiles exposed to more severe hypoxia, even for short periods of time (Andrews, 2002; Cordero et al., 2017b; Iungman and Piña, 2013; Kam, 1993). The lack of effect of our maternal gestation treatments implies that embryos pre-oviposition did not face a mismatch between oxygen supply and demand. Our data suggest a maternal ability to maintain oxygen supply to tissues, including developing embryos, by adjusting oxygen-carrying capacity (haematocrit, haemoglobin) to maintain metabolic rates. Furthermore, the early stages of embryonic development in lizards require low oxygen consumption (Thompson and Stewart, 1997), as during this time embryos only constitute ~10% of their hatchling body mass (Andrews, 2004). A fruitful direction for future experiments is to transplant reproductive females earlier in the reproductive process (i.e. before vitellogenesis) to test for trade-offs in reproductive investment between offspring size and number and also for earlier maternal exposure to affect offspring development, either via adaptive maternal effects (e.g. hormonal mechanisms) or because of energetic constraints imposed by hypoxia.

We report this apparent resilience of embryos to hypoxia with the important caveat that oxygen availability interacts with temperature to exacerbate the performance limitations and negative effects of hypoxia at higher temperatures (Gangloff and Telemeco, 2018). This may be most notable in the late embryonic stages, in which oxygen uptake depends on diffusion across the chorioallantoic membrane and behavioural responses to the environment are highly restricted (Cordero et al., 2018; Telemeco et al., 2016; but see Shine and Du, 2018). Work in other reptile taxa has demonstrated a clear pattern whereby the detrimental effects of hypoxia are more severe at higher temperatures (Flewelling and Parker, 2015; Iungman and Piña, 2013; Liang et al., 2015; Smith et al., 2015). The interaction of temperature and hypoxia likely also explains why we found longer incubation periods for low-elevation embryos incubated at higher elevations, while our previous work did not find such an effect. In the current study, we incubated eggs at 28°C, whereas previously we used 24°C (Cordero et al., 2017a). The impact of hypoxia on prolonging development times was more pronounced at higher temperatures, which is probably due to limitations of the embryo to meet the corresponding increased oxygen demand, such as found previously in caimans (*Caiman latirostris*; Iungman and Piña, 2013). While the effects of reduced oxygen availability and high temperatures interact to impact physiology, it is also possible that shared response pathways offer protection against these simultaneous stressors (e.g. Teague et al., 2017). Exploring the inter-relation of these response

pathways, as well as how responses may change across life-history stages, remains an important avenue for research.

Our results concur with other recent work demonstrating the apparent flexibility of reptilian embryos in response to novel environments (e.g. Tiatragul et al., 2017). This is not surprising, given that reptile nests are frequently exposed to low-oxygen conditions in natural settings (Ackerman and Lott, 2004; Booth, 1998; Packard and Packard, 1987). Thus, evolution may have promoted physiological mechanisms to deal with hypoxia in embryos that can then facilitate development under situations of reduced oxygen partial pressure, as found at high elevations. Physiological plasticity to adjust to hypoxia could thus be stage-specific, explaining the apparent resilience of *P. muralis* embryos but not adults (Cordero et al., 2017a; Gangloff et al., 2019). The long-term effects of hypoxia exposure during development on phenotype and fitness at later life-history stages remain important research questions. For example, development in hypoxia has long-term effects on post-hatching cardiovascular phenotypes in snapping turtles (*Chelydra serpentina*; Wearing et al., 2016, 2017), decreases swimming performance in snakes (*Natrix maura*; F.A. and J.S., unpublished results), and impacts cognitive ability in lizards (*Eremias argus*; Sun et al., 2014). The adaptive significance of embryonic plasticity in response to hypoxia can only be evaluated by quantifying the long-term effects on individual fitness (Mitchell et al., 2018).

Conclusion

This study addresses two important dimensions of reproduction, with implications for understanding both life histories and range expansion in mountainous regions in common wall lizards: ROM production and the effects of hypoxia. Females transplanted to high elevation matched the reproductive output of lizards native to both low-elevation and high-elevation populations, and embryos are physiologically resilient to development at high elevations, even above the current range limits. This suggests that these life stages will not limit initial colonization of high-elevation habitats. However, population establishment will depend on the long-term costs associated with life in reduced oxygen availability on adults (such as damage from increased ROM production) and the consequences of reduced performance (Gangloff et al., 2019). Colonization of high-altitude areas is important for conservation efforts as it will likely put high-elevation specialist lizards endemic to small mountaintop ranges, such as *Iberolacerta* spp., at further risk via competition for resources and direct interactions (Žagar et al., 2015, 2017). With our work in this system, we hope to quantify the relative potential of physiological plasticity and local adaptation across life history stages to facilitate range expansion in a species likely to benefit under climate change scenarios.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.K., E.J.G., J.S., G.A.C., F.A.; Methodology: E.J.G., A.D.; Investigation: L.K., E.J.G., J.S., G.A.C., A.D., F.A.; Resources: F.A.; Data curation: L.K., E.J.G.; Writing - original draft: L.K., E.J.G.; Writing - review & editing: L.K., E.J.G., J.S., G.A.C., A.D., F.A.; Supervision: E.J.G., F.A.; Project administration: F.A.; Funding acquisition: E.J.G., F.A.

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Data availability

Data are archived in Mendeley at DOI: 10.17632/94xsf6wv6c.1.

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.206839.supplemental

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Annexe IV.

Lizards at the peak: physiological plasticity does not maintain performance in Lizards transplanted to high altitude

Lizards at the Peak: Physiological Plasticity Does Not Maintain Performance in Lizards Transplanted to High Altitude

Eric J. Gangloff[1,*]
Mahaut Sorlin[1]
Gerardo A. Cordero[2]
Jérémie Souchet[1]
Fabien Aubret[1]
[1]Station d'Ecologie Théorique et Expérimentale du Centre National de la Recherche Scientifique–Unités Mixtes de Recherche 5321, Moulis, France; [2]Fachbereich Geowissenschaften, Eberhard Karls Universität Tübingen, Tübingen, Germany

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ABSTRACT

Warming climates are facilitating the range expansion of many taxa to habitats that were formerly thermally inhospitable, including to higher latitudes and elevations. The potential for such colonization, however, varies widely among taxa. Because environmental factors may interact to affect colonization potential, an understanding of underlying physiological and behavioral mechanisms is necessary to predict how species will respond to potentially suitable habitats. For example, temperature and oxygen availability will interact to shape physiological and performance traits. Our model species, the wall lizard, *Podarcis muralis*, is a widely distributed ectotherm that continues to expand its range in Europe despite being limited by cold temperatures at high elevations and latitudes. To test the potential for organisms to expand to warming high-altitude environments, we conducted a transplant experiment to quantify the within-individual effects of high-altitude hypoxia on physiological and performance traits. Transplanted lizards maintained individual differences in physiological traits related to oxygen capacity and metabolism (hemoglobin concentration, hematocrit, and peak postexhaustion metabolic rate), as well as performance traits tied to fitness (sprint speed and running endurance). Although lizards altered blood biochemistry to increase oxygen-carrying capacity, their performance was reduced at high altitude. Furthermore, lizards at high altitude suffered a rapid loss of body condition over the 6-wk experiment, suggesting an energetic cost to hypoxia. Taken together, this demonstrates a limited potential for within-individual plasticity to facilitate colonization of novel high-altitude environments.

Keywords: climate change, colonization, high-altitude hypoxia, metabolic rate, performance, physiological plasticity, *Podarcis muralis*, range expansion.

*Corresponding author; email: eric.gangloff@sete.cnrs.fr.

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Introduction

Formerly inhospitable habitats at high elevations have warmed to become thermally suitable for some low-altitude species (Parmesan 2006; Sinervo et al. 2010; Pauchard et al. 2015). This has resulted in range expansion to higher elevations in some cases, but species vary widely in their response to new potentially suitable habitat (Walther et al. 2002; Chen et al. 2011). In mountainous regions, such upward migration is dependent not only on warming thermal environments but also on the ability of colonizing individuals to cope with lower atmospheric pressure at altitude and thus reduced oxygen availability (hereafter, high-altitude hypoxia). In terrestrial ectothermic vertebrates, thermal performance curves and, potentially, thermal limits are closely tied to the capacity for an individual to obtain oxygen from the environment and deliver it to tissues in sufficient quantities (reviewed in Jackson 2007; Gangloff and Telemeco 2018). Given the interdependence of physiological systems on temperature regimes and oxygen availability, an understanding of the physiological mechanisms by which ectotherms respond to high-altitude hypoxia is necessary to characterize the ability of individuals, and by extension populations, to move up in elevation (Storz et al. 2010).

Acute exposure to hypoxia will limit aerobically dependent performance and recovery, while physiological acclimation can serve to alleviate potentially negative repercussions. However, short-term responses to hypoxia may compensate for only some consequences of hypoxia, may only partially compensate, or may bear energetic or trade-off costs (Storz et al. 2010). Thus, measuring traits related to aerobic capacity and oxygen transport, as well as performance traits, is necessary to examine the short-term response, consequences, and costs of oxygen limitation. Compensatory mechanisms generally include increased oxygen diffusion and transport capacity, reduced metabolic demand for oxygen, and a shift toward greater dependence on anaerobiosis (reviewed in Hochachka et al. 1996; Gangloff and Telemeco 2018). Thus, we expect that individuals demonstrating greater physiological plasticity will also be able to maintain performance traits in hypoxic conditions. In general, within-individual plas-

ticity is expected to be a primary mechanism by which many taxa respond to novel environments (Hoffmann and Sgrò 2011; Urban et al. 2014; Llewelyn et al. 2018). Therefore, an integrative whole-organism approach is required to describe the complex relationships among physiological parameters, and their plasticity, with outcomes and fitness proxies (Forsman 2015).

The goal of this study is to assess the physiological response of adult lizards exposed to acute hypoxia beyond what is experienced in natural populations. We test the hypothesis that altitude-induced hypoxia will promote plastic physiological responses to maintain oxygen delivery and compensate for lower O_2 partial pressure (P_{O_2}). Specifically, we predict that lizards transplanted to high elevations will experience performance limitations in endurance capacity and reduced peak postexhaustion metabolic rates compared to lizards kept in normoxic conditions. Over several weeks at altitude, we expect that blood chemistry will shift to favor greater oxygen-carrying capacity, including increases in hematocrit and hemoglobin concentration. This physiological compensation will then result in at least a partial recovery of performance traits relative to lizards maintained at low altitude. Because burst speed in lizards is generally dependent on anaerobic respiration (Bennett and Licht 1972; Gleeson 1982, 1991), we do not predict differences in absolute sprint speed between lizards at low and high altitudes, but we do predict that hypoxia will result in a performance decrement over short-term repeated trials, as well as a reduction in running endurance. Sprint speed and running endurance are well-established performance traits in small lizard species, with direct links to survival, reproduction, dispersal, and, ultimately, fitness (Huey and Bennett 1987; Irschick and Garland 2001; Miles 2004; Hoskins et al. 2017). Finally, we predict that potential energetic trade-offs involved with physiological remodeling will result in reduced body condition over time in hypoxia. Taken together, we test the within-individual plastic response to high-altitude hypoxia in a widespread vertebrate currently expanding its range into warming alpine environments.

Methods

Source Populations and Husbandry

The common wall lizard, *Podarcis muralis*, is cosmopolitan and often conspicuous in a variety of habitats across its broad geographic range (Speybroeck et al. 2016). Importantly, its geographic distribution is restricted by thermal environment, since recruitment is reduced at lower temperatures because of an inability of embryos to complete development (Strijbosch et al. 1980; Van Damme et al. 1992; While et al. 2015). However, as a result of the acceleration of climate warming, wall lizards have recently been observed colonizing higher parts of the mountainous environment beyond their previous range, extending up to ~2,600 m asl (in southern France, notably; Pottier 2012). We sampled lizards from six populations in the area around Moulis, France (421–522 m asl; for sampling details and habitat description, see table A1), between August 13 and August 30, 2017. Adult male lizards (all snout-vent length [SVL] > 50 mm; $N = 82$) were collected with looped thread attached to a telescopic fishing pole (McDiarmid 2012). Four lizards that were initially included dropped their tails during the experiment and are therefore excluded from analyses. On the day of capture, we measured SVL to the nearest 0.01 mm using digital calipers (mean ± SD: 62.88 ± 4.13 mm) and weighed lizards to the nearest 0.01 g using a digital scale (mean ± SD: 6.63 ± 1.18 g). We maintained groups of three to six lizards from a single population in plastic enclosures (26 cm × 38 cm × 23 cm) containing wood mulch bedding and two plastic shelters (15 cm × 5 cm × 3.5 cm) that also served as basking platforms. Ambient room temperature fluctuated between approximately 15°C (night) and 20°C (day), ambient light was provided with fluorescent bulbs for 14 h/d, and heat lamps provided a temperature gradient of ca. 25°–40°C for 6 h/d in 1-h intervals. We provided water ad lib. via a small water bowl, and cages were misted three or four times per week. Lizards were fed mealworms (*Tenebrio* spp. larvae) ad lib., with fresh worms added three or four times per week, with the exception that food was withheld for 48 h before performance measures to ensure a postabsorptive state (Van Damme et al. 1991; Angilletta 2001).

After an acclimation period (mean: 9.1 d; range: 7–12 d), lizards were either transferred to identical housing at Observatoire Midi-Pyrénées in Pic du Midi de Bigorre (42°56′11.0″N, 0°08′32.9″E; 2,877 m asl; P_{O_2}: ~15.3 kPa) or moved into a new enclosure at the Station d'Ecologie Théorique et Expérimentale du Centre National de la Recherche Scientifique à Moulis (42°57′26.8″N, 1°05′08.3″E; 436 m asl; P_{O_2}: ~20.1 kPa). The difference in atmospheric pressure between locations results in approximately 25% reduced oxygen availability at the Pic du Midi lab (Bouverot 1985; Cordero et al. 2017). After an overnight acclimation period, we began the experiment with performance trials the next day and blood collection the day after. We maintained animals under uniform conditions, conducting performance trials and collecting blood samples on consecutive days at two additional time points, after 3 wk and 6 wk. Lizards were weighed and measured as above after each performance trial (see table A3). We calculated lizard body condition as the residual of the linear regression of $\log_{10}$ mass on $\log_{10}$ SVL, a common metric of relative energy stores used in numerous taxa, including lizards. Although this provides a measure of mass corrected for size, it does not describe the component driving variation in relative mass (e.g., muscle, fat stores, water; Warner et al. 2016). Because of logistical constraints and occasional mistrials, sample size varied slightly among measures at each time point, with measures of metabolic rate conducted on a subset of lizards (for sample size details, see tables 2, A2).

Performance and Postexhaustion Metabolic Rate

At three time points (1 d, 3 wk, and 6 wk), we employed a novel assay to efficiently quantify multiple aspects of lizard locomotor performance (sprint speed, speed decrement, and running endurance), as well as peak postexhaustion metabolic rate. This approach allowed us to streamline repeated measures of these traits while minimizing handling stress on the animals. Trials were conducted on a 1-m level racetrack of artificial grass with lines drawn at intervals of 25 cm. Racetracks were constructed to identical

specifications with the same materials at both laboratories. All performance trials were conducted during daylight active hours (between 0800 and 1930 hours). Before each trial, we placed lizards in a shelter identical to that in their enclosures, modified with secure flaps. This was placed in an incubator set to 31°C, the average body temperature of active lizards during the summer (F. Aubret, unpublished data; Osojnik et al. 2013), for 30 min to allow body temperature to equilibrate (Zajitschek et al. 2012). This provided an ecologically relevant temperature at which we could test performance traits, while also being below the maximum at which temperature effects would be compounded with reduced oxygen availability in hypoxia (Gangloff and Telemeco 2018). Lizards were placed at one end of the racetrack and raced 12 times with exactly 30 s rest between each run. This allowed us to measure both maximum sprint performance and performance decrement over repeated trials. If lizards were reluctant to sprint, we gently chased them with a soft paintbrush. To maintain consistency, the same researcher (E.J.G.) acted as lizard motivator for all trials. Room temperature was maintained at 25°C with electric space heaters (ambient temperature mean ± SD: 25.70° ± 0.90°C). Furthermore, we provided lighting at either end of the racetrack with portable LED and halogen light stands, positioned identically at the same height for each track. Immediately after the final trial, we recorded lizard body temperature using a cloacal thermometer (model T-6000, Miller and Weber, Ridgewood, NY; body temperature mean ± SD: 29.69° ± 0.69°C). We video recorded the trials and used Solomon Coder software (Péter 2017) and the programming language R (R Core Team 2017) to extract the fastest speed attained over a 50-cm interval as the top velocity for each trial, a distance within the range of *P. muralis* movements in the field (Braña 2003) and that has been used in similar-sized lizards (Huey and Bennett 1987; Van Damme et al. 1989). In >90% of trials, lizards achieved their top speed before reaching the end of the track, indicating that the track length was sufficient for lizards to achieve top performance. Corruption of data files and inconsistencies in protocols precluded the inclusion of some sprint trials, resulting in a total of $N = 2,393$ sprint speed values for analysis. A single author (M.S.) scored all videos, with high intra-rater reliability on a randomly selected subset of trials scored twice (Kendall's $W = 0.98$, $F_{136,136} = 47.92$, $P < 0.0001$).

After the twelfth sprint for each lizard, one researcher (E.J.G.) continually chased the lizard with a soft paintbrush back and forth without stopping on the racetrack. We quantified endurance as the time that lizards continually moved until exhaustion. Lizards were considered exhausted when further stimulation with the paintbrush elicited no locomotor response for 5 s and the lizard then did not further attempt to flee (Gleeson and Dalessio 1989). As a measure of the maximum postexhaustion rate of oxidative metabolism in lizards, we quantified the peak postexhaustion rate of carbon dioxide production ($\dot{V}_{CO_{2peak}}$) in a subset of lizards (for sample size for each measure, see table A2). Immediately after being run to exhaustion (within approximately 10 s), we placed lizards in a modified shelter with secure flaps (as above) and with many holes drilled along the length to ensure gas circulation. This shelter was placed in a cylindrical acrylic metabolic chamber (700 mL), which was then placed in the incubator set to 31°C. We utilized pull-mode respirometry to quantify gas concentrations (Foxbox-C Field O_2 and CO_2 Analysis System, Sable Systems, Las Vegas, NV; Lighton 2008). Air was circulated through the metabolic chamber at a rate of 500 mL/min, dried of water vapor with Drierite, and then measured for both CO_2 and O_2 content, corrected for barometric pressure. Data were analyzed with ExpeData software (ver. 1.7.30, Sable Systems) to calculate the rates of carbon dioxide production ($\dot{V}_{CO_2}$) and oxygen consumption ($\dot{V}_{O_2}$). We report results for $\dot{V}_{CO_2}$ because O_2 sensor drift precluded reliable extraction of data and resulted in the loss of $\dot{V}_{O_2}$ from many trials. Both $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ are elevated simultaneously after exercise, with measures of postexhaustion $\dot{V}_{CO_2}$ representing an important physiological parameter that comprises both the oxidative capacity to replenish energy substrates and the maintenance of acid-base balance, including lactate processing (Gleeson and Bennett 1982; Hailey et al. 1987; Jackson et al. 2015). Metabolic rates peaked for lizards in both treatment groups within the first several minutes of measurement (fig. 1; Hailey et al. 1987). We measured each lizard continuously for 45 min after exhaustion and extracted the highest average rate of carbon dioxide production over a 15-s interval as our measure of $\dot{V}_{CO_{2peak}}$.

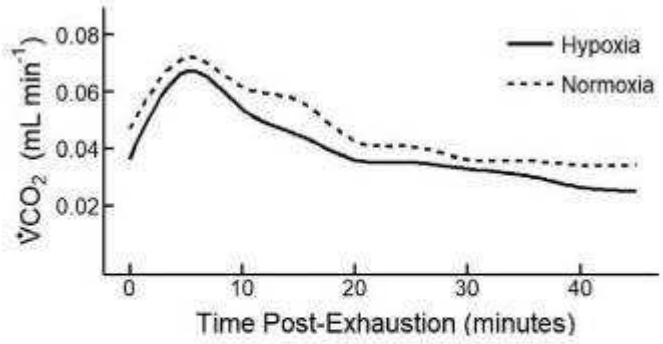

Figure 1. Postexhaustion metabolic response of adult male *Podarcis muralis* lizards. Curves are general additive models fitted to raw data from lizards in hypoxia and normoxia treatments. $\dot{V}_{CO_2}$, rate of carbon dioxide production.

Blood Sampling and Measures

On the day after performance measures, we collected blood samples from the retro-orbital sinus with a heparinized glass capillary tube (MacLean et al. 1973; Meylan et al. 2003). Lizards were removed from their enclosures under normal husbandry conditions and blood was collected generally within 3 min (bleed time mean ± SD: 1.85 ± 1.22 min). We collected 25–40 μL of whole blood and stored blood on ice until processing (generally 4–6 h). To measure hematocrit, we spun 10–15 μL of whole blood in a capillary tube (capacity: 19 μL) for 5 min at 5,000 *g* and then measured the volume of packed red blood cells relative to total volume (%Hct). Hemoglobin concentration was measured in 5 μL whole blood utilizing the colorimetric cynamethemoglobin method, following manufacturer's instructions (Drabkin's reagent, Sigma-Aldrich, St. Louis, MO).

Lizards were kept over winter and released the next spring at the site of capture. Field sampling and experimental protocols were

conducted under current permit (Arrêté Préfectoral 2017-s-02). All procedures are in accordance with Directive 2010/63/EU on Protection of Animals Used for Scientific Purposes (http://ec.europa.eu/environment/chemicals/lab_animals/pdf/guidance/directive/en.pdf) and the Guidelines for Use of Live Amphibians and Reptiles in Field and Laboratory Research (http://www.asih.org/sites/default/files/documents/resources/guidelinesherpsresearch2004.pdf).

Statistics

We used linear mixed models with proc mixed in SAS 9.4 (SAS Institute, Cary, NC) to assess repeatability of physiological and performance measures, as well as the relative influences of treatment (normoxia/hypoxia) and acclimation over time. To better meet assumptions of normality, measures of $\dot{V}_{CO_{2peak}}$ and time to exhaustion were $\log_{10}$ transformed before analysis. We first estimated repeatability of measures within each treatment group. Because the mean values of our measured traits changed over time, we included time as a factor in our repeatability models and report constancy repeatability (R_c), which provides a measure of among-individual variation after accounting for changes in such mean values over time (Biro and Stamps 2015). We created models with the fixed effect of time point and the random effect of individual, modeled with a compound symmetric covariance structure, and calculated constancy repeatability as the ratio of variance explained by individual to total variance. We assessed significance with a likelihood ratio test, using the difference in log likelihoods between models with and without individual as a χ^2 statistic with 1 and 0 df (mathematically equivalent to halving the P value obtained from a χ^2 test with 1 df; $\chi^2_{0:1}$ in "Results"), which accounts for the fact that variance component estimates are bounded by 0 (Stram and Lee 1994; Bolker et al. 2009; Snijders and Bosker 2012). For measures of sprint speed, we assessed constancy repeatability at two timescales: across measurement time points (as with other traits) and within time point (using the 12 repeated sprints from each lizard at each time point).

To assess the impact of hypoxia treatment on traits across time, we specified models with the fixed effects of treatment (hypoxia/normoxia), time point (1 d, 3 wk, 6 wk), and their interaction. To account for heterogeneity among sampled populations, we also included population of origin as a fixed effect. Except for analyses of body mass and body condition, initial models contained lizard size (SVL) as a covariate. This effect did not approach significance in any model and was removed from further analysis (all $P > 0.35$), with the exception of sprint speed, in which it was retained because of the known effect of size on sprint speed (e.g., Zajitschek et al. 2012). Sprint speed was analyzed using data from all trials (12 trials at each time point); therefore, models also included the fixed effect of trial as well as all two-way interactions between trial, time point, and treatment. Because of its positive influence on sprint speed, body temperature was retained (taken immediately after sprinting trials) in models of sprint speed. Models of $\dot{V}_{CO_{2peak}}$ included log-transformed mass as a covariate; initial models also included body temperature, but this was removed because it did not influence metabolic rate ($P > 0.93$). For all mixed models, we estimated denominator degrees of freedom for F-tests using the Kenward-Roger degrees of freedom approximation (Kenward and Roger 1997).

To further describe the within-individual relationships among physiology and performance, we utilized a path analysis with the *lavaan* package (Rosseel 2012) in the programming language R (R Core Team 2017). Path analyses describe the magnitude and significance of potential causal relationships among variables within an a priori specified model. Our model includes the influence of hematocrit on hemoglobin concentration, the influence hemoglobin on $\dot{V}_{CO_{2peak}}$, running endurance and sprint speed, and, finally, the influence of $\dot{V}_{CO_{2peak}}$ on running endurance and sprint speed (fig. 3). We specified the model in this way, assuming that increased hematocrit is the primary driver of increased hemoglobin, which in turn would permit greater oxygen-carrying capacity and thus greater maximum aerobic metabolic rate. Further, we tested whether this increase in aerobic capacity would improve sprint speed over repeated trials, which relies on aerobic respiration to replenish ATP stores once anaerobic capacity is expended (Gleeson 1991), and running endurance, which is linked to activity metabolism (Garland and Else 1987; Clemente et al. 2009). Because of its effect on blood viscosity, hematocrit may also directly influence physiological and performance measures, independent of the effect on oxygen-carrying capacity. However, preliminary models demonstrated that hematocrit was not a significant predictor of either $\dot{V}_{CO_{2peak}}$ or running endurance (all $P > 0.11$); therefore, we excluded this relationship from subsequent models. Path analysis requires that all variables are measured for an individual at a given time point, resulting in a reduction in the number of observations that were available to analyze (because time constraints precluded the measurement of metabolic rates for all animals; $N = 84$ total observations included in path analysis). Because we combined data across all three time points, this analysis does not account for the correlation of repeated measures within individuals. Nonetheless, our goal is to examine the relationship among these physiological and performance traits over time; thus, including all available observations allows us to quantify within-individual relationships of these parameters for lizards in both treatment groups.

Results

Repeatability

Constancy repeatability of physiological and performance traits ranged from 0.168 to 0.949, with all estimates except for hemoglobin under hypoxia statistically significant (table 1). Sprint speed was highly repeatable in both experimental treatments, both across the 6 wk of the experiment and within each measurement day, indicating among-individual variation in sprint performance across multiple timescales.

Treatment and Acclimation

All seven physiological and performance measures were influenced either by treatment (normoxia/hypoxia) or by the interaction of treatment and time point (fig. 2; table 2). Peak

Table 1: Constancy repeatabilities of physiological and performance traits in adult male *Podarcis muralis* lizards in normoxia and hypoxia

	Normoxia			Hypoxia		
Trait	R_c	$\chi^2_{0.1}$	P	R_c	$\chi^2_{0.1}$	P
[Hemoglobin]	**.181**	3.2	.037	.178	2.1	.074
Hematocrit	**.265**	6.7	.0048	**.168**	3.0	.042
$\dot{V}_{CO_{2peak}}$	**.577**	7.7	.0028	**.292**	2.8	.047
Running endurance	**.691**	30.5	<.0001	**.313**	5.2	.011
Body mass	**.949**	337.2	<.0001	**.946**	353.2	<.0001
Body condition	**.690**	53.9	<.0001	**.709**	64.8	<.0001
Overall	**.403**	549.9	<.0001	**.413**	620.8	<.0001
Sprint 1 d	**.487**	195.9	<.0001	**.539**	269.4	<.0001
Speed 3 wk	**.535**	223.6	<.0001	**.516**	243.6	<.0001
6 wk	**.505**	210.6	<.0001	**.500**	214.7	<.0001

Note. Traits were measured in lizards at three time points over the 6 wk of the experiment. Additionally, sprint speed was assessed in 12 consecutive trials at each time point. Significant estimates ($P < 0.05$) are shown in bold. R_c, constancy repeatability; $\dot{V}_{CO_{2peak}}$, peak postexhaustion rate of carbon dioxide production.

postexhaustion rate of carbon dioxide production ($\dot{V}_{CO_{2peak}}$) decreased by 10% and running endurance was reduced by 21% in hypoxic conditions across all time points (effect of treatment: $P = 0.04$ and $P = 0.0035$, respectively), though overall $\dot{V}_{CO_{2peak}}$ decreased and running endurance increased across the time of the experiment (effect of time point: $P = 0.0093$ and $P < 0.0001$, respectively). Models of maximal postexhaustion metabolic rate include mass and thus account for changes in individual mass over the course of the experiment; as expected, mass exerted a positive effect on $\dot{V}_{CO_{2peak}}$ (scaling exponent ± SE: 0.60 ± 0.12). Lizards in the normoxic treatment began with higher values of hemoglobin concentration and hematocrit, but this pattern was reversed after 3 wk, and then values converged after 6 wk (treatment × time point interaction: $P = 0.041$). Body mass was identical between treatments to start and then increased after the first measurement in the normoxia treatment and remained elevated compared to starting values (effect of time and treatment × time point interaction: both $P < 0.0001$). Similarly, body condition was not different to start but diverged across the duration of the experiment (significant treatment × time point interaction: $P = 0.0037$). Sprint speed decreased with short-term repetition (within the 12 consecutive trials run by a lizard in a day) but increased over the duration of the experiment (effect of trial and time point: both $P < 0.0001$). Performance decrement over the repeated sprints was not affected by treatment (treatment × trial number interaction: $P = 0.32$), though improvement over the time of the experiment was more pronounced in lizards in the normoxia treatment (treatment × time point interaction: $P = 0.027$). Finally, we found significant among-population heterogeneity in running endurance ($P = 0.0049$), largely driven by lizards from one population running for longer times before exhaustion (Engomer; see table A1).

Path Analysis

The path analysis demonstrates that for lizards in both normoxic and hypoxic conditions, hemoglobin concentration is largely determined by hematocrit. In turn, hemoglobin positively influenced $\dot{V}_{CO_{2peak}}$ in normoxia but not in hypoxia. Hemoglobin also positively influenced sprint speed in normoxia and running endurance in hypoxia. Interestingly, $\dot{V}_{CO_{2peak}}$ exerted a negative influence on sprint speed in both treatments, though the effect was significant only in hypoxia. Counter to our expectations, $\dot{V}_{CO_{2peak}}$ did not influence running endurance in either treatment group.

Discussion

Our results demonstrate a limited capacity of adult male *Podarcis muralis* lizards to acclimate to high-altitude hypoxia over a time frame of several weeks. Although hypoxia induced shifts in blood biochemistry to increase oxygen-carrying capacity, measures of sprint speed and running endurance were not maintained by lizards in hypoxia relative to lizards at low altitudes. Further, we found evidence that exposure to hypoxia over this amount of time results in a rapid decrease in body condition and reduced mass gain, likely due to negative consequences for energy balance. We also found strong evidence that lizards maintain individual differences over time in physiological and performance traits, both in normoxia and under conditions of high-altitude hypoxia.

Our repeatability estimates are within the reported range for physiological traits in wild vertebrates generally and for lizard performance specifically (e.g., Huey and Dunham 1987; Tsuji et al. 1989; Zajitschek et al. 2012; Goulet et al. 2017; Hoskins et al. 2017). All physiological traits were significantly repeatable, except for hemoglobin concentration in hypoxia, with estimates indicating moderate to high levels of among-individual variation. Among-individual differences in performance were also present in both treatments, corroborating previous studies of both running endurance and sprint speed in this and other lizard species (e.g., Garland and Else 1987; Huey and Dunham 1987; Sorci et al. 1995; Zajitschek et al. 2012). Furthermore, sprint speed was repeatable across multiple timescales, indicating significant among-individual variation in this trait both under rapid repeated trials and across the

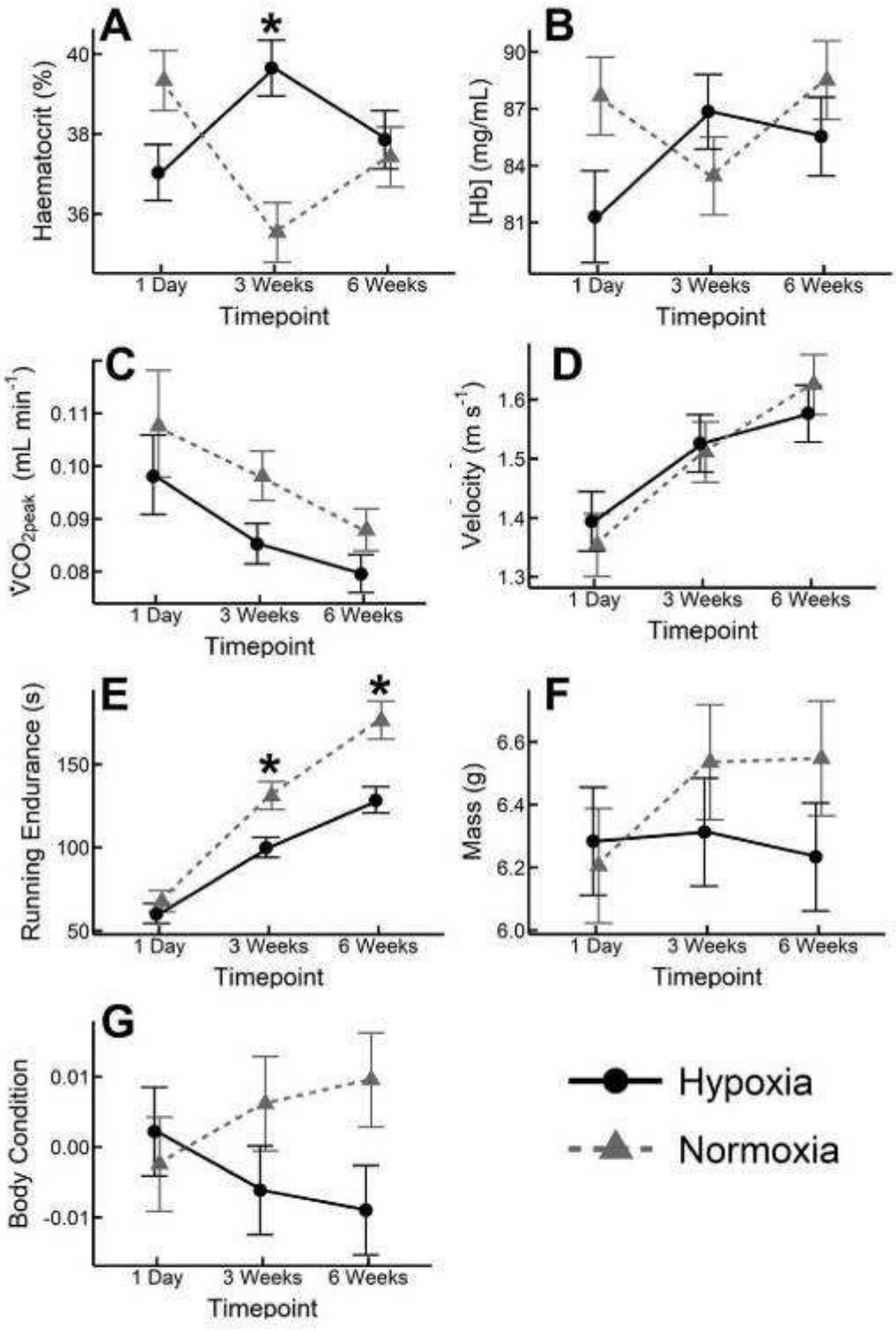

Figure 2. Effects of high-altitude hypoxia and time on physiological and performance traits in adult male *Podarcis muralis* lizards. *A*, Hematocrit (%). *B*, Hemoglobin concentration (mg/mL). *C*, Peak postexhaustion rate of carbon dioxide production ($\dot{V}_{CO_{2peak}}$; mL/min). *D*, Sprint velocity (m/s). *E*, Running endurance (s). *F*, Body mass (g). *G*, Body condition. Data are least squares means from linear mixed models ± SE. Significantly different estimates between treatments at a given time point are indicated with asterisk (adjusted $P < 0.05$). [Hb], hemoglobin concentration.

several weeks of the experiment. Sprint speed is commonly tested in lizard species because of its established ecological and fitness importance (see above), but seldom is the stability of this trait tested over multiple timescales. Our results further support the use of sprint speed as proxy for individual performance capacity in this lizard species, as has been established in some fish species (e.g., Oufiero and Garland 2009). To our knowledge, this is the first study to establish the repeatability of sprint speed under hypoxic con-

Table 2: Results of linear mixed-model analyses of physiological and performance measures in adult male *Podarcis muralis* lizards measured at three time points over the 6-wk experiment

Source of variation	[Hb] ($N = 207$)	Hct ($N = 228$)	$\dot{V}_{CO_{2peak}}$ ($N = 91$)	Endurance ($N = 172$)	Body mass ($N = 232$)	Body condition ($N = 232$)	Sprint speed ($N = 226$)
Treatment	$1.03_{1, 69.3}$, .313	$1.14_{1, 70.5}$, .288	$4.48_{1, 42.1}$, **.0402**	$9.02_{1, 83.8}$, **.0035**	$.38_{1, 71}$, .534	$1.12_{1, 71.1}$, .295	$.1_{1, 92.9}$, .752
Time point	$.89_{2, 134}$, .412	$.52_{2, 148}$, .598	$5.14_{2, 49.7}$, **.0093**	$74.87_{2, 95}$, **<.0001**	$11.46_{2, 150}$, **<.0001**	$.01_{2, 150}$, .993	$24.93_{2, 2,355}$, **<.0001**
Treatment × time point	$3.26_{2, 134}$, **.0413**	$12.2_{2, 148}$, **<.0001**	$.14_{2, 57}$, .869	$1.06_{2, 103}$, .351	$13.23_{2, 150}$, **<.0001**	$5.81_{2, 150}$, **.0037**	$3.61_{2, 2,325}$, **.0273**
Population	$1.24_{5, 70.7}$, .302	$2.23_{5, 70.6}$, .0612	$.92_{5, 37.7}$, .479	$3.69_{5, 74.3}$, **.0049**	$.65_{5, 71}$, .663	$.61_{5, 71.1}$, .695	$1.03_{5, 71.5}$, .406
Body size: $\log_{10}$(mass) for $\dot{V}_{CO_{2peak}}$, SVL for sprint speed	...	...	$22.96_{1, 32.4}$, **<.0001**	...	...	...	$.51_{1, 484}$, .474
Trial number	...	...	...	...	...	...	$85.98_{1, 2,305}$, **<.0001**
Treatment × trial number	...	...	...	...	...	...	$.98_{1, 2,305}$, .323
Time point × trial number	...	...	...	...	...	...	$2.75_{2, 2,305}$, .0643
Body temperature	...	...	...	...	...	...	$8.43_{1, 2,375}$, **.0037**

Note. Values shown are $F_{df1, df2}$ Pr > F. Significant effects are shown in bold. See table A2 for additional details. [Hb], hemoglobin concentration; Hct, hematocrit; $\dot{V}_{CO_{2peak}}$, postexhaustion rate of carbon dioxide production.

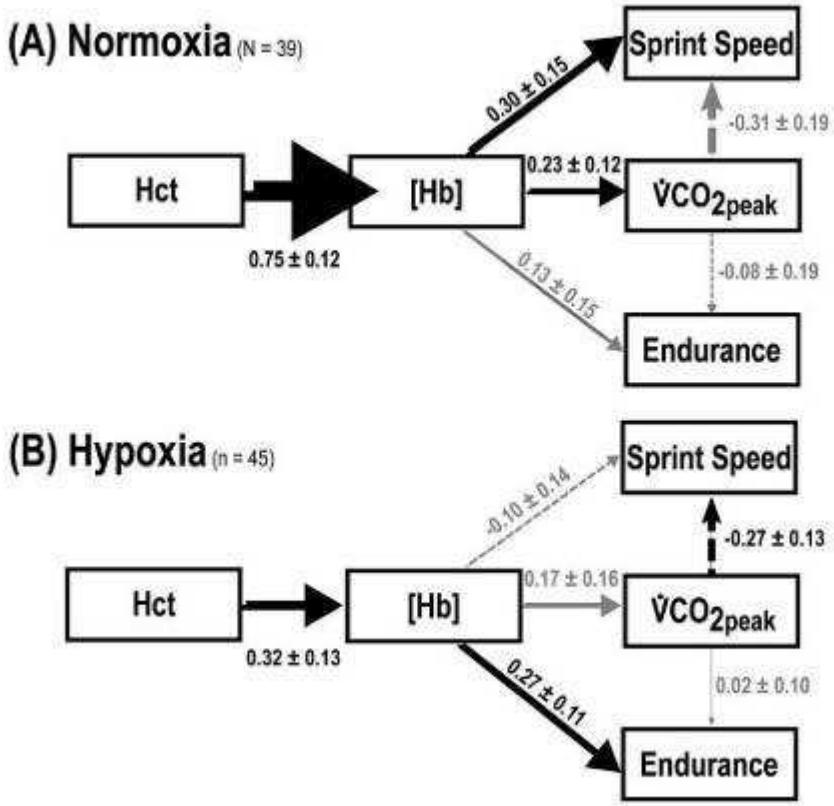

Figure 3. Path analysis results of relationships among physiological and performance measures in adult male *Podarcis muralis* lizards in normoxia and hypoxia. Coefficient estimates are shown ±SE. Path thickness is proportional to the estimated effect, with statistically significant paths ($P < 0.05$) shown in black and nonsignificant paths in gray. Positive relationships are shown with solid lines and negative relationships with dashed lines. Hct, hematocrit; [Hb], hemoglobin concentration; $\dot{V}_{CO_{2peak}}$, peak postexhaustion rate of carbon dioxide production.

ditions in lizards, corroborating a recent study in striped bass (*Morone saxatilis*) that found individual consistency in swimming speed under hypoxic conditions (Kraskura and Nelson 2018).

As we predicted based on previous work in a variety of taxa, lizards adjusted blood chemistry in response to high-altitude hypoxia (Storz 2007; Storz et al. 2010). Two important parameters related to blood-oxygen capacity—hematocrit and hemoglobin concentration—were correlated and increased within the first 3 wk of the experiment (increases of 12% and 4%, respectively). This response is concordant with numerous studies demonstrating that lizards from high-altitude populations have higher hemoglobin concentration and hematocrit compared to lowland populations (Vinegar and Hillyard 1972; Weathers and White 1972; Newlin and Ballinger 1976; González-Morales et al. 2015; Lu et al. 2015). Lowland lizards acclimated to simulated high-altitude hypoxia increased hemoglobin concentration and hematocrit and changed other parameters related to oxygen transport and use, but these responses were not identical to those in lizards native to high-altitude populations (Weathers and McGrath 1972; He et al. 2013). As demonstrated in mammals and birds, the acute plastic response may differ from changes resulting from natural selection and may, in fact, be maladaptive (reviewed in Storz et al. 2010). After 6 wk, the values of hemoglobin concentration and hematocrit converged between the treatment groups. This could be due to the potential energetic costs of maintaining high levels of these parameters. For example, increased red blood cell density can increase blood viscosity and the energetic cost of circulation (Hedrick et al. 1986; Dunlap 2006), which could negatively impact an individual's energy balance. Correspondingly, there are several potential nonexclusive reasons for the observed decline in body condition and failure to regain mass of lizards in hypoxia. These could include an energetic cost associated with such physiological shifts, reduced efficiency of overall energy processing, or reduced food intake. Future work will benefit from examining hypoxia-induced shifts in other aspects of blood biochemistry, such as hemoglobin oxygen-binding affinity and hemoglobin isoforms, as well as their energetic consequences (Storz 2007, 2016; Lu et al. 2015).

The plasticity in parameters important for oxygen-carrying capacity in blood impacted postexhaustion metabolic rate only in normoxia (fig. 3). After exhaustion, CO_2 production is elevated as a result of both increased oxidative metabolism and the maintenance of acid-base balance in response to elevated lactic acid concentrations (Gleeson and Bennett 1982). This implies that the postexhaustion increase in metabolism in hypoxia may be more strongly driven by the need to regulate acid-base balance due to anaerobiosis during exercise rather than oxidative capacity after exhaustion. Previous work in lizards describes a weak but significant relationship between hematocrit and maximum metabolic rates

(Garland et al. 1987), though intermediate levels of blood parameters related to oxygen capacity are optimal in mammals (Villafuerte et al. 2004; Schuler et al. 2010). Furthermore, we found a negative relationship between postexhaustion metabolic rate and sprint speed under hypoxic conditions, suggesting that individuals that had the greatest capacity for burst speed were less rapidly able to resupply energetic capacity via oxidative metabolism. Given the known dependence of lizard sprinting on anaerobiosis (Bennett and Licht 1972; Gleeson 1991), this relationship suggests that hypoxic conditions evoke a trade-off between anaerobic and aerobic capacity. In our study, this trade-off is further evidenced by the effect of acclimation in both treatment groups: during their time in captivity, lizards decreased maximum metabolic rates while increasing running endurance. Additionally, lizards in normoxia increased their sprint speed during the course of the experiment to a greater extent than hypoxic lizards. That time in captivity has such a strong effect on these measures is surprising, though consistent with previous studies in the lizard *Amphibolurus nuchalis* kept in captivity over similar time spans (Garland et al. 1987). It may be that the strong effect of time on performance is due not to physiological changes but to habituation effects after exposure to repeated test stimuli (e.g., Rodríguez-Prieto et al. 2010). These results collectively suggest a great acclimation potential for plasticity in the physiological and biochemical traits underlying performance, though not sufficient to compensate for low oxygen availability. Future studies should examine the biochemical basis for differences in oxidative and glycolytic capacity of muscle, as these pathways likely determine variation in running endurance and trade-offs between aerobic and anaerobic capacity (Garland et al. 1987).

We note that our experimental treatment represents an extreme situation: individual lizards are unlikely to naturally migrate up 2,500 m in elevation. However, *P. muralis* is an exemplar species to illustrate the potential for human-assisted transport to facilitate range expansion. For example, this species is well established in areas in both the United States and England, likely because of isolated incidents whereby small numbers of individuals were relocated (Deichsel and Gist 2001; Michaelides et al. 2015). Though human-assisted movement to high-elevation sites is plausible, our results indicate that relocation to 2,877 m asl is beyond the upper limit of acclimation capacity for adult male lizards. Although this does not preclude the possibility that this lizard species is capable of colonizing habitat at higher altitudes, it does suggest that other processes—such as developmental plasticity triggered during earlier life-history stages or local adaptation—will play a role in facilitating such range expansion. Three non–mutually exclusive mechanisms may, individually or concurrently, permit the colonization of high-altitude environments: within-individual reversible plasticity (or flexibility), developmental plasticity in response to early-life environments and/or resulting from maternal effects, or local adaptation (selection on currently existing genetically determined variation within populations). Our previous work in this system indicates that embryos can adjust their physiology to compensate for high-altitude hypoxia, thus pointing to an important role for developmental plasticity in the colonization of habitats at higher elevation (Cordero et al. 2017).

Despite the observed short-term shifts in hematocrit and hemoglobin concentration observed in this study, adult lizards in high-altitude hypoxia exhibited lower maximum metabolic rates across all time points and impaired sprint speed and running endurance over the duration of the experiment. This suggests that within-individual plasticity of physiology is not sufficient to maintain important performance traits in adult male lizards. Furthermore, this level of hypoxia appears to bear important long-term health consequences for lizards, demonstrated by the rapid decrease in body condition after just 6 wk at altitude. Thus, we hypothesize that early-life exposure to trigger developmental plasticity may be necessary for lizards to respond successfully to high-altitude environments. We are currently conducting studies directed toward assessing the relative roles of local adaptation, maternal effects, and long-term developmental plasticity in facilitating range expansion of this widespread lizard under continually increasing temperature regimes (Bravo et al. 2008; IPCC 2014).

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APPENDIX

Table A1: Habitat description and sample sizes from sampled populations of *Podarcis muralis*

Name	Latitude, longitude	Altitude (m asl)	Habitat type	*N* hypoxia, *N* normoxia
Alas	42°56′58″N, 001°2′47″E	472	Cemetery	6, 8
Astien	42°56′20″N, 001°3′53″E	522	Rock wall, stone buildings	7, 6
Aubert	42°57′52″N, 001°6′10″E	425	Rock wall and bridge	8, 6
Engomer	42°56′46″N, 001°3′20″E	473	Cemetery	7, 8
Lambège	42°58′41″N, 001°7′18″E	425	Rock wall	7, 4
Luzenac[a]	42°57′22″N, 001°5′1″E	443	Cemetery	6, 5

[a]This population is also studied in Calsbeek et al. (2010).

Table A2: Sample sizes for each physiological and performance measure

	[Hb]	Hct	$\dot{V}_{CO_{2peak}}$	Endurance	Body mass	Condition	Sprint speed
1 d:							
Normoxia	36	36	5	14	37	37	36
Hypoxia	26	41	8	12	41	41	41
3 wk:							
Normoxia	36	36	17	34	36	36	35
Hypoxia	39	41	21	39	41	41	40
6 wk:							
Normoxia	35	36	20	35	37	37	36
Hypoxia	35	38	20	38	40	40	38

Note. [Hb], hemoglobin concentration; Hct, hematocrit; $\dot{V}_{CO_{2peak}}$, peak postexhaustion rate of carbon dioxide production.

Table A3: Mean ± SD for body mass at time of capture (feeding status unknown) and at three experiment time points (fasted) for experimental lizards (*Podarcis muralis*)

	Capture	1 d	3 wk	6 wk
Normoxia	6.74 ± 1.32	6.20 ± 1.13	6.52 ± 1.11	6.54 ± 1.17
Hypoxia	6.53 ± 1.06	6.28 ± 1.06	6.31 ± 1.05	6.23 ± 1.02

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Annexe V.

Physiological plasticity in lizard embryos exposed to high-altitude hypoxia

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RESEARCH ARTICLE

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Physiological plasticity in lizard embryos exposed to high-altitude hypoxia

Gerardo A. Cordero[1] | Bea Angelica Andersson[1] | Jeremie Souchet[2] | Gaëlle Micheli[2] | Daniel W.A. Noble[3] | Eric J. Gangloff[2,4] | Tobias Uller[1*] | Fabien Aubret[2*]

[1]Department of Biology, Lund University, Lund, Sweden

[2]Station d'Ecologie Théorique et Expérimentale du CNRS à Moulis, Moulis, France

[3]Ecology & Evolution Research Centre, School of Biological, Earth and Environmental Sciences, The University of New South Wales, Sydney, NSW, Australia

[4]Department of Ecology, Evolution, and Organismal Biology, Iowa State University, Ames, Iowa, USA

Correspondence
Gerardo Antonio Cordero, Department of Biology, Lund University, Sölvegatan 37, SE-223 62 Lund, Sweden.
Email: antonio.cordero@biol.lu.se

*Tobias Uller and Fabien Aubret have shared senior authorship.

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Abstract

Coping with novel environments may be facilitated by plastic physiological responses that enable survival during environmentally sensitive life stages. We tested the capacity for embryos of the common wall lizard (*Podarcis muralis*) from low altitude to cope with low-oxygen partial pressure (hypoxia) in an alpine environment. Developing embryos subjected to hypoxic atmospheric conditions (15–16% O_2 sea-level equivalent) at 2,877 m above sea level exhibited responses common to vertebrates acclimatized to or evolutionarily adapted to high altitude: suppressed metabolism, cardiac hypertrophy, and hyperventilation. These responses might have contributed to the unaltered incubation duration and hatching success relative to the ancestral, low-altitude, condition. Even so, hypoxia constrained egg energy utilization such that larger eggs produced hatchlings with relatively low mass. These findings highlight the role of physiological plasticity in maintaining fitness-relevant phenotypes in high-altitude environments, providing impetus to further explore altitudinal limits to ecological diversification in ectothermic vertebrates.

1 | INTRODUCTION

The geographic expansion of species often involves colonization of novel environments featuring conditions rarely encountered or not experienced in the recent history of populations (Schluter, 2000; Aubert, 2013). This requires organisms to cope with conditions to which they are not expected to be adapted, which may be particularly challenging to developing offspring. Assuming the occurrence of heritable variants suited to sub-optimal conditions, natural selection should favor the persistence of individuals best able to cope with environmental stress during sensitive life stages (Schmalhausen, 1949; Waddington, 1957; Levins, 1968; West-Eberhard, 2003; Atkinson, & Thorndyke, 2001). On the other hand, plastic physiological responses might mitigate environmental stress, thereby promoting offspring survival and influencing the direction of subsequent evolution of colonizing populations (Hammond, Cardullo, & Ghalambor, 2006; Atkinson, & Thorndyke, 2001; Ghalambor, McKay, Carroll, & Reznick, 2007; McNab, 2002).

Physiological traits are well suited to examine phenotypic shifts in response to new environments (Hammond et al., 2006; Storz, Scott, & Cheviron, 2010; Storz, 2016; Piersna, & van Gils, 2011; Rezende, Gomes, Ghalambor, Russell, & Chappell, 2005; Chown et al., 2010). Indeed, physiological processes often comprise evolvable reaction norms (Mueller, Eme, Burggren, Roghair, & Rundle, 2015), such as in responses to altered atmospheric oxygen (O_2) conditions in stressful high-altitude environments (Rezende et al., 2005; Hammond et al., 2006; Bouverot, 1985; Powell, & Hopkins, 2010; Storz et al., 2010; Beall, 2006). In addition to low temperature, the low partial pressure of O_2 (hypoxia) at high altitudes (> 2,000 m above sea level [ASL]) renders embryonic development challenging, as fewer O_2 molecules may be available to convert egg energy into tissue (Vleck, & Vleck, 1996; Noble, 1991; Wangensteen, Rahn, Burton, & Smith, 1974; Rahn, Carey,

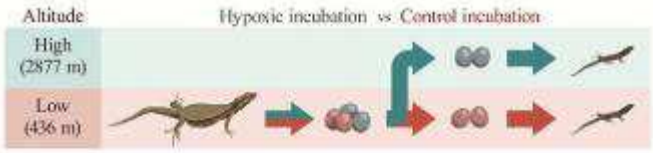

FIGURE 1 Experimental eggs were collected from gravid females sampled from low-altitude *Podarcis muralis* populations in the foothills of the Pyrenees (300–500 m above sea level). Within 48 hr of oviposition, clutches of eggs were evenly split in two batches of eggs (half-clutches). For each clutch, one half-clutch was transplanted to a high-altitude laboratory at 2,877 m where atmospheric PO_2 was 70–73 kPa (equivalent to 15–16% O_2 at sea level); while the second half-clutch underwent incubation at low altitude (436 m; control) where PO_2 (93–96 kPa; 20.8% O_2) approximated sea level conditions (101.3 kPa; 20.95% O_2). *Illustration credit*: Bea Angelica Andersson [Color figure can be viewed at wileyonlinelibrary.com]

Balmas, Bhatia, & Paganelli, 1977; Carey, Larson, Hoyt, & Bucher, 1984; Bouverot, 1985; Monge, & Leon-Velarde, 1991; Leon-Velarde, & Monge, 2004). Thus, altitudinal hypoxia is thought to impose a limit on the geographic distribution of vertebrate species (Powell, & Hopkins, 2010; Storz et al., 2010).

In birds, the suppression of embryonic metabolism is a common and effective means of reducing O_2 demand while undergoing egg incubation at high altitude (Monge, Leon-Velarde, & Gomez de la Torre, 1988; Leon-Velarde, & Monge, 2004; Lague, Chua, Farrell, Wang, & Milsom, 2016). This suppression is often associated with reduced heart rate and slower growth (Beattie, & Smith, 1975; Wangensteen et al., 1974; Leon-Velarde, & Monge, 2004). Artificial selection and comparison of high- versus low-altitude avian populations revealed that these physiological responses can evolve (Beattie, & Smith, 1975). Similarly, physiological processes that enhance O_2 transport have the potential to evolve in birds (Rahn et al., 1977; Hammond et al., 2006; Storz, 2016; Monge, & Leon-Velarde, 1991). Whether responses to cope with hypoxia in high-altitude environments are general to non-avian reptiles is unclear (McNab, 2002; Powell, & Hopkins, 2010), though laboratory manipulations at low altitude would support this assumption (Kam, 1993; Warburton, Hastings, & Wang, 1995; Andrews, 2002; Du, Thompson, & Shine, 2010; Eme, Altimiras, Hicks, & Crossley, 2011; Harrison, Shingleton, & Callier, 2015; Liang, Sun, Ma, & Du, 2015; Smith, Telemeco, Angilletta, & VandenBrooks, 2015; Cordero, Karnatz, Svendsen, & Gangloff, 2017; Crossley, Ling, Nelson, Gillium, Conner et al., 2017).

Oviparous reptiles display little or simple parental care (While, Halliwell, & Uller, 2015), and most embryonic development will occur after eggs are laid in unattended subterranean nests (Packard, & Packard, 1988; Ackerman, & Lott, 2004). As a result, embryos must adjust to fluctuations in nest temperature, moisture, and gas concentrations (e.g., acute hypoxia) (Packard, & Packard, 1988; Ackerman, & Lott, 2004; Deeming, & Thompson, 1991). Such stress-induced physiological responses might promote offspring survival when the partial pressure of O_2 is consistently low (i.e., chronic hypoxia), contributing to the invasion of alpine ecosystems in response to climate warming (Storz et al., 2010; Ortega, Mencia, & Perez-Mellado, 2016) and, perhaps, rapid adaptive evolutionary responses to life at high altitude (Rezende et al., 2005).

We incubated eggs from low-altitude populations of the common wall lizard (*Podarcis muralis*) to test the hypothesis that embryos employ metabolic adjustments known to facilitate survival in hypoxic conditions. We predicted reduced growth via metabolic suppression, leading to decreases in hatchling size, mass, and energy content in hypoxia at 2,877 m ASL. We also tested the hypothesis that cardiovascular compensatory changes induce heart enlargement in hatchlings (Crossley, & Burggren, 2009).

2 | MATERIALS AND METHODS

2.1 | Egg collection and experimental design

Podarcis muralis is widely distributed across southern and central Europe from sea level to approximately 2,500 m ASL (Speybroeck, Beukema, Bok, & Van Der Voort, 2016). Breeding occurs during April–June with females producing up to three clutches of eggs (Speybroeck et al., 2016). In May–June 2016, we captured gravid *P. muralis* females in the foothills of the French Pyrenees (Department of Ariège; 300–500 m ASL) and transported them to the laboratory of Station d'Ecologie Théorique et Expérimentale du CNRS à Moulis (436 m ASL; 38.898556 N, – 77.037852 E). The partial pressure of O_2 (PO_2) at this locality was 93–96 kPa, which translates to a sea level atmospheric O_2 concentration of 20.8% (Bouverot, 1985).

Females were housed individually in plastic enclosures (26 × 38 × 23 cm) with heat lamps, water, and sand boxes for nesting. Food was provided ad libitum. Sand boxes were visually inspected daily for signs of nesting, in which case eggs were immediately removed, weighed, labeled, and individually placed in plastic cups filled with moist vermiculite (1:5 water to vermiculite). Eggs from 22 clutches were then transferred to an environmental chamber set to a constant 24°C (100% air humidity).

We implemented a split-clutch design with eggs assigned to low altitude (control) and high altitude (hypoxia) treatments (Figure 1). The group assignment of the first egg in a clutch was randomly chosen with subsequent eggs alternating between treatment groups. Within 48 hr of oviposition, hypoxia eggs were transported to the Observatoire Midi-Pyrénées in Pic du Midi de Bigorre (2,877 m ASL; 38.898556 N, – 77.037852 E). Atmospheric conditions in this alpine environment were hypoxic as PO_2 was 70–73 kPa (sea level equivalent: 15–16% O_2) (Bouverot, 1985). Hypoxia eggs were otherwise incubated under the same temperature and moisture laboratory regime as in the control (normoxia) treatment in Moulis (24°C; 100% air humidity).

2.2 | Embryonic metabolism

Embryonic heart rate is a reliable indicator of metabolism and cardiovascular function in non-avian reptiles (Crossley, & Burggren, 2009). Thus, we measured heart rate weekly (6.8 d ± 2 SD) during egg incubation in *P. muralis* using the Buddy® digital egg monitor (MK2; Avitronics, Cornwall, UK) (Aubret, Tort, & Blanvillain, 2013). Briefly, Buddy® directs infrared light onto the egg and registers the net amount of

infrared light absorbed by blood, thus blood flow changes caused by heart beats can be used to estimate heart rate (Lierz, Gooss, & Hafez, 2006). Eggs were briefly (≤5 min) placed in the digital egg monitor to control for potential temperature changes owing to exposure to infrared sensors (Sartori, Taylor, Abe, & Crossley, 2015). Egg temperature did not increase and ambient temperature remained stable (~24°C) during sampling.

Carbon dioxide (CO_2) production ($\dot{V}CO_2$) was measured during the last third of the egg incubation period. In reptiles, physiological compensation for reduced growth rate might be employed during this time (discussed in Spencer and Janzen (2011)). Following a stop-flow respirometry protocol (Lighton, 2008; Lighton, & Halsey, 2011), 16 eggs drawn from eight split clutches (control: $N = 8$; hypoxia: $N = 8$) were individually placed in 65-ml sealed glass jar chambers with moist vermiculite (50 ml) held at a constant 24°C, for example, Cordero et al. (2017). The egg chamber was then flushed and sealed for 180 min, incurrent air flow (200 ml/min; from chamber to respirometer) was restored and water scrubbed from air using drierite desiccant (Hammond Drierite, Xenia, Ohio, USA). Carbon dioxide was measured using the FC-10 analyzer (Sable Systems International, Las Vegas, Nevada, USA). Using Sable Systems ExpeData software, CO_2 production rate ($\dot{V}CO_2$ in ml/hr corrected for water vapor pressure of excurrent air) was calculated by integrating the change in $\dot{V}CO_2$ instantaneous level over the period the chamber was sealed (Lighton, & Halsey, 2011). Repeated respirometry measurements alternated (within 24 hr) between control and hypoxia eggs on days 38, 45, and 52 post-oviposition. Heart rate was measured immediately before each trial.

2.3 | Hatchling morphology and physiology

Field sampling and experimental protocols were approved by the Préfecture de l'Ariège (Arrêté #09-2016-01). Following guidelines for the use of live reptiles in laboratory research (HACC, 2004), hatchlings were humanely euthanized by blunt force trauma to the head (AVMA, 2013). This method has proven effective for experimental reptiles (reviewed in Nevarez, Strain, da Cunha, & Beaufrere, 2014). Hatchling carcasses were weighed before preserving in 10% buffered formalin according to standard methodology for preservation of reptile specimens (McDiarmid, Foster, Guyer, Gibbons, & Chernoff, 2012).

The ventral surface of preserved hatchlings was imaged using a scanner (CanoScan LiDE 120) and snout–vent length (SVL) was measured in ImageJ 1.5 (U.S. National Institutes of Health). Hearts were then excised and dried to a constant mass by placing on absorbent paper while exposed to room temperature (25°C) air for 1 hr. Only the ventricular region of dissected hearts was examined. Carcasses were dried to a constant mass in an oven set to 60°C for 24 hr. All dry tissue measurements were recorded to the nearest 0.00001 g using an analytical balance (R200D Sartorius Research, Germany).

To estimate the energetic cost of hatchling tissue production, dry hatchling carcasses were subjected to standard calorimetric procedures (Lighton, 2008), using an oxygen bomb calorimeter (Parr 6200 Calorimeter). Energy density of hatchling tissue was initially estimated by pooling samples by clutch and treatment. The mean energy density per treatment (control: 22.005 kJ/g ± 0.208 SE, $N = 20$; hypoxia: 21.912 kJ/g ± 0.158 SE, $N = 20$) was multiplied by dry hatchling mass to obtain per individual values of total hatchling energy content (Vitt, 1978). Note that yolk sacs were no longer herniated in hatchlings, thus energetic estimates are inclusive of any potentially internalized residual yolk mass. This potential source of variation was likely negligible because energy content values were within the expected range for *P. muralis* hatchlings that had yolk sacs removed (Ji, & Braña, 1999).

2.4 | Statistical analyses

Treatment effects on physiology and morphology were tested using mixed-effect linear and non-linear models (Zuur, Ieno, Walker, Saveliev, & Smith, 2009). Incubation duration, hatchling mass (wet and dry), SVL, dry heart mass, and hatchling energy content were analyzed as response variables in univariate models. Initial egg mass was entered as a covariate with clutch of origin as a random effect. Survival probability (i.e., hatching success) was tested using a generalized linear mixed effect model (with a Bernoulli error distribution) with initial egg mass as a covariate and clutch of origin as a random effect.

Dry heart mass models included dry hatchling mass to control for potential body mass effects. We also used Pearson's correlation tests to explore the relationship of dry heart mass versus heart rate (averaged across egg incubation) and hatchling water content. Decreased heart rate is generally associated with ventricular enlargement (heart hypertrophy) (Crossley, & Burggren, 2009), and body fluid mass should correlate with increased ventricular stroke volume caused by hypertrophy (Faber, Green, & Thornburg, 1974; Wagman, Hu, & Clark, 1999; Convertino, 1991). Models for heart rate and CO_2 production included an AR1 (autoregressive order of 1) covariance structure that accounted for temporal autocorrelation due to repeated measures. Heart rate was fitted with a third-order polynomial model according to expected heart rate trends in embryos (Burggren, & Warburton, 1994).

Mixed-effect models were evaluated with type III sums of squares analysis of variance (ANOVA), with main effects considered statistically significant if $P < 0.05$. Factors with $P < 0.10$ and their interactions were retained in final models. Models were validated by examining plots of residual distributions and residual versus fitted values (Zuur et al., 2009). The residuals for all models were normally distributed (Shapiro-Wilk's tests, $P > 0.10$), with the exception of incubation duration and initial egg mass. Thus, to meet model assumptions, analyses on incubation duration and initial egg mass were performed on $\log_{10}$-transformed data (Zuur et al., 2009). Note that initial egg mass for control (0.298 g ± 0.010 SE, $N = 50$) versus hypoxia (0.314 g ± 0.010 SE, $N = 52$) treatments differed at the onset of experimentation (ANOVA: $F_{1,78} = 4.43$, $P = 0.038$). We therefore included initial egg mass ($\log_{10}$-transformed) as a covariate in most analyses on hatchling traits, which also accounted for variation in maternal energetic investment. To interpret main effects in the presence of these interactions, model inputs were centered and standardized (mean of zero and unit standard deviation) and models were compared with likelihood ratio tests (Schielzeth, 2010). Analyses were conducted using the *lme4* package of the R programming language (R Development Core Team, 2017).

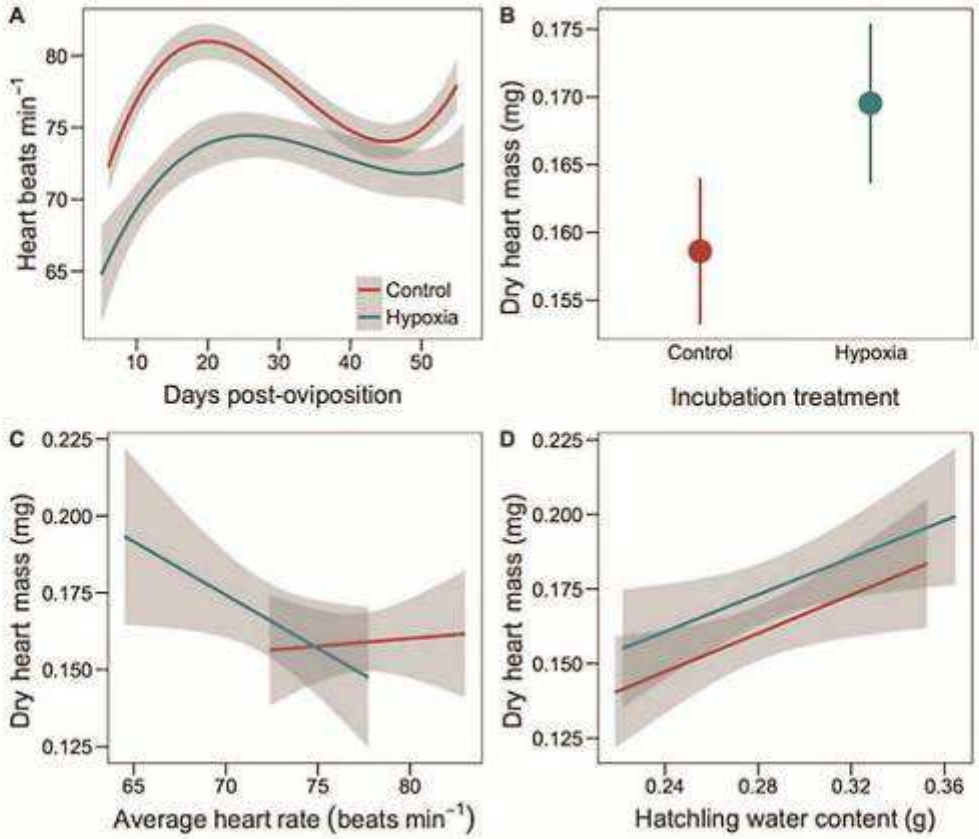

FIGURE 2 Embryonic *Podarcis muralis* from control (low altitude) and hypoxia (high altitude) groups differed in heart rate (**A**). Dry heart mass (covariate-adjusted means ± SE) was slightly reduced in hypoxia hatchlings (**B**). Dry heart mass was negatively correlated with embryonic heart rate (averaged across egg incubation) in hypoxia but not in control hatchlings (**C**). There was a positive correlation of dry heart mass and hatchling water content in both groups (**D**); shaded regression intervals represent the standard error of the fit. [Color figure can be viewed at wileyonlinelibrary.com]

3 | RESULTS

3.1 | Embryonic metabolism

Embryos from the hypoxia group exhibited lower heart rate values (beats per minute) during egg incubation than embryos from the control group (ANOVA: $F_{1,100} = 30.8$, $P < 0.0001$; Figure 2A). Dry heart mass was lower in control hatchlings, although this did not reach statistical significance (LRT: $\chi^2 = 2.23$, df = 1, $P = 0.134$) (Figure 2B). Dry heart mass was negatively correlated with heart rate (averaged across egg incubation) in hatchlings from the hypoxia treatment ($r = -0.33$, $P = 0.049$), but there was no correlation in the control group ($r = 0.05$, $P = 0.753$) (Figure 2C). Dry heart mass was positively correlated with hatchling water content in hypoxia and control groups (control: $r = 0.38$, $P = 0.019$; hypoxia: $r = 0.43$, $P = 0.022$) (Figure 2D).

Embryos from hypoxia and control groups differed in CO_2 production (ANOVA; $F_{1,14} = 7.16$, $P = 0.018$). Treatment and sampling intervals interacted such that embryos of the hypoxia treatment initially displayed a lower mean for CO_2 production (days 38.5 and 45.5 post-oviposition), but exceeded that of the control group when near hatching (day 52.5, Figure 3A; treatment by day interaction: $F_{1,30} = 6.54$, $P = 0.015$). During CO_2 sampling intervals, heart rate in hypoxia embryos remained lower than in the control group ($F_{1,14} = 22.1$, $P = 0.0003$) (Figure 3B).

3.2 | Hatching success, hatchling energy content, and body size

Raw means for hatchling success, incubation duration, and morphological and physiological variables are listed in Table 1. Although hatching success was marginally affected by initial egg mass, there was a treatment by initial egg mass interaction that approached significance (LRT: $\chi^2 = 3.828$, df = 1, $P = 0.050$). This interaction contributed to a slight decrease in hatching success in the Hypoxia treatment that featured larger initial egg mass (Tables 1 and 2). Both hatchling dry mass (LRT: $\chi^2 = 12.7$, df = 1, $P = 0.0003$) and total energy content (LRT: $\chi^2 = 4.43$, df = 1, $P = 0.035$) were significantly affected by the interaction between incubation treatment and egg mass, such that lizards from large eggs were lighter at hatching if incubated under hypoxic conditions (Figure 4A and B and Table 2). In contrast, hypoxic conditions did not affect incubation duration, wet hatchling mass, or SVL (Table 2).

4 | DISCUSSION

Developing *P. muralis* embryos exposed to hypoxic atmospheric conditions at 2,877 m ASL exhibited physiological adjustments that mirrored typical acclimation responses in vertebrates. These included:

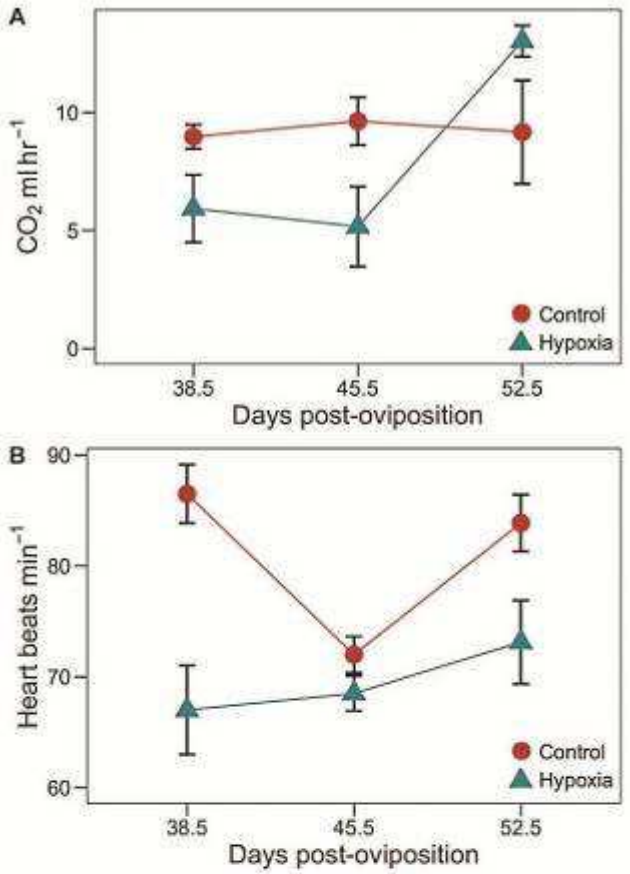

FIGURE 3 Embryonic *Podarcis muralis* from control (low altitude; $N = 8$) and hypoxia (high altitude; $N = 8$) groups differed in CO_2 production rate (**A**) and heart rate (**B**) during the last third of the egg incubation period; means ± SE are displayed. The marked increase in $\dot{V}CO_2$ (**A**), relative to heart rate (**B**), in hypoxia embryos on day 52.5 post-oviposition is indicative of a hyperventilatory response [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Hatching Success and Raw Morphological and Physiological Means (±SE) for Hatchling *Podarcis muralis* Incubated in Low-Altitude (Control) versus High-Altitude (Hypoxia) Environments

	Control	Hypoxia
Hatchling success	88%	77%
Initial egg mass (g)	0.294 ± 0.007	0.312 ± 0.008
	$N = 50$	$N = 52$
Incubation duration (d)	60.045 ± 0.315	60.682 ± 0.273
	$N = 43$	$N = 38$
Snout-vent length (cm)	2.282 ± 0.015	2.298 ± 0.017
	$N = 43$	$N = 40$
Wet hatchling mass (g)	0.327 ± 0.006	0.334 ± 0.007
	$N = 43$	$N = 38$
Dry hatchling mass (g)	0.053 ± 0.001	0.052 ± 0.001
	$N = 42$	$N = 35$
Dry Heart mass (mg)	0.158 ± 0.005	0.167 ± 0.006
	$N = 42$	$N = 35$
Total hatchling energy content (kJ)	1.149 ± 0.027	1.133 ± 0.026
	$N = 38$	$N = 35$

TABLE 2 Estimates and 95% Profiled Confidence Intervals from Mixed Effects Models on Hatchling Traits

Variable	Estimate	2.5% CI	97.5% CI
Hatching success ($N_T = 102$, $N_C = 23$) (log odds)			
Intercept	**−4.255**	**−11.016**	**−2.049**
Treatment (hypoxia)	*1.519*	*0.008*	*3.496*
Egg mass (log scale)	0.054	−2.213	1.786
Treatment × initial egg mass	*−1.706*	*−3.948*	*0.003*
Incubation duration (log_{10}d) ($N_T = 81$, $N_C = 21$)			
Intercept (log scale)	**4.096**	**4.083**	**4.11**
Treatment (Hypoxia)	0.004	−0.004	0.013
Egg mass (log scale)	−0.005	−0.011	0.002
Dry mass (mg) ($N_T = 62, N_C = 21$)			
Intercept	**52.992**	**50.154**	**55.749**
Treatment (hypoxia)	**−2.37**	**−4.688**	**−0.077**
Egg mass (log scale)	**5.079**	**3.113**	**7.107**
Treatment × initial egg mass	**−4.886**	**−7.436**	**−2.335**
Wet mass (g) ($N_T = 77, N_C = 21$)			
Intercept	0.332	0.315	0.349
Treatment (hypoxia)	0.002	−0.011	0.013
Egg mass (log scale)	**0.014**	**0.002**	**0.027**
Treatment × initial egg mass	*−0.013*	*−0.025*	*0*
Dry heart mass (mg) ($N_T = 66, N_C = 22$)			
Intercept	**0.159**	**0.148**	**0.169**
Treatment (hypoxia)	0.011	−0.003	0.025
Dry mass	*0.007*	*0.001*	*0.014*
Snout-vent length (cm) ($N_T = 79$, $N_C = 21$)			
Intercept	**2.29**	**2.261**	**2.32**
Treatment (hypoxia)	−0.007	−0.045	0.03
Egg mass (log scale)	**0.049**	**0.024**	**0.074**
Energy content (kJ) ($N_T = 70, N_C = 21$)			
Intercept	**1.166**	**1.111**	**1.219**
Egg mass (log scale)	**0.108**	**0.064**	**0.154**
Treatment (hypoxia)	**−0.056**	**−0.109**	**−0.004**
Treatment × initial egg mass	**−0.062**	**−0.119**	**−0.004**

All continuous predictor variables are centered at zero and normalized to unit standard deviation. Initial egg mass was not included in the final model on heart mass, as it was not statistically significant ($P > 0.10$). Bolded estimates are significant ($P < 0.05$), whereas italics is are $P < 0.10$. N_T, total number of observations; N_C, total number of clutches.

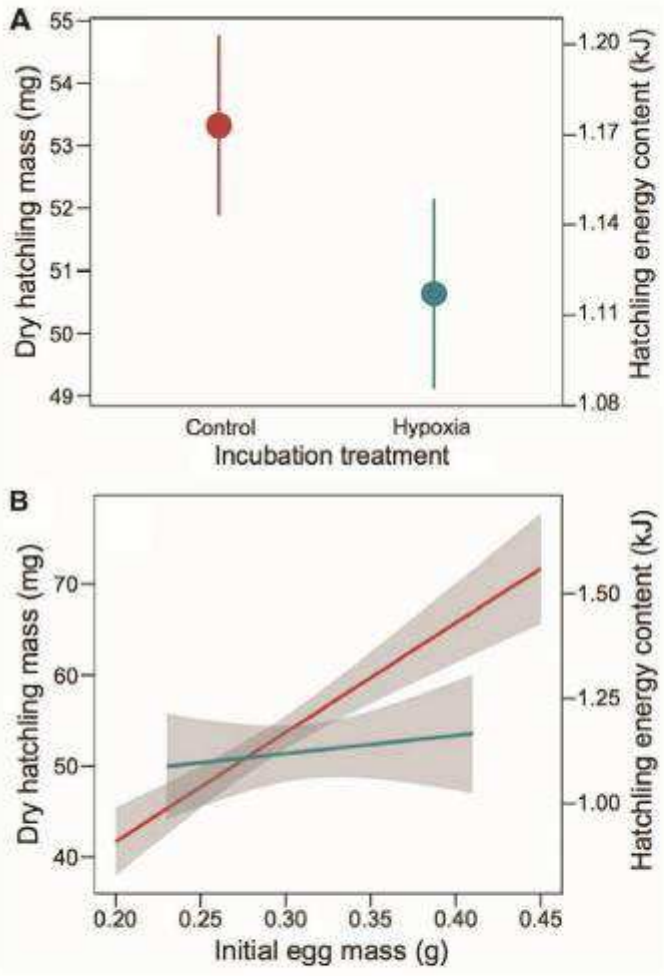

FIGURE 4 The relationships of dry hatchling mass, hatchling energetic content, and initial egg mass were altered in *Podarcis muralis* subjected to high-altitude hypoxia (**A and B**). Means adjusted for initial egg mass are displayed (range bars and shaded regression intervals = ± SE) [Color figure can be viewed at wileyonlinelibrary.com]

(i) suppressed embryo metabolism, as indicated by reduced heart rate (Laughlin, 1978; Crossley, & Altimiras, 2005), (ii) elevated CO_2 production, that is, hypoxia-induced hyperventilation (Peacock, 1998; Powell, & Hopkins, 2010; Bouverot, 1985; Storz et al., 2010), and (iii) some evidence for cardiac hypertrophy, that is, heart enlargement (Du et al., 2010; Crossley, & Burggren, 2009). These physiological responses supported our predictions to some extent. However, contrary to what we expected, SVL and incubation duration were comparable to siblings that underwent egg incubation in ancestral atmospheric conditions (i.e., low-altitude environments), whereas effects of hypoxia on mass depended on the initial egg mass. Furthermore, we found that the effects of egg resources on hatchling traits were moderated by hypoxic incubation (more below).

Hypoxic environments suppressed embryonic metabolism in *P. muralis*, which is a common physiological response to hypoxia in vertebrates (Laughlin, 1978; Monge, & Leon-Velarde, 1991; Crossley, & Burggren, 2009). Hypoxia is expected to affect ATP demand and supply pathways, which ultimately decrease cellular respiration rates by downregulating ion-pumping and protein synthesis (Hochachka, Buck, Doll, & Land, 1996; Bickler, & Buck, 2007). This common homeostatic response ensures survival without necessarily compromising embryonic development if O_2 delivery to tissues is enhanced (Crossley, & Burggren, 2009). Our results corroborate this expectation, though we did not directly measure compensatory biochemical changes in blood, for example enhanced O_2 affinity to hemoglobin (Storz et al., 2010; Storz, 2016). Still, we demonstrated that hatchling heart mass was negatively correlated with embryonic heart rate in *P. muralis* incubated under hypoxic conditions, suggesting that suppressed metabolism coincided with predicted cardiovascular alterations that enhance blood volume and circulation (discussed below).

While we provide evidence of suppressed metabolism throughout incubation, we did observe a spike in CO_2 production without a concomitant increase in heart rate when near hatching at high altitude. This might have been the result of excess arterial CO_2 that accumulated as a consequence suppressing metabolism during development in hypoxia (see Wei et al., 2007). Whether this hyperventilatory response in *P. muralis* was caused by compensatory growth in hypoxia is difficult to ascertain, as embryos might have initiated the transition from chorioallantoic gas exchange to air breathing (i.e., lung use) in preparation for hatching (Baumann, 1984; Thompson, 2007). Thus, pulmonary hyperventilation, a well-known acclimation response to high-altitude hypoxia (Bouverot, 1985; Peacock, 1998; Powell, & Hopkins, 2010; Swenson, & Bärtsch, 2014), probably reflected elevated $\dot{V}CO_2$ in pre-hatching *P. muralis*. Specifically, hypoxia will stimulate arterial chemoreceptors that promote the release of CO_2 (reviewed in Storz et al. (2010)), enabling maintenance of normal blood pH and O_2 in post-embryonic life stages at high altitude (reviewed in Bouverot (1985)).

Across many birds and reptiles, hypoxia has been shown to reduce hatchling size, mass, and in some cases, incubation duration (reviewed in Hempleman, Adamson, and Bebout (1993); Kam (1993); Leon-Velarde and Monge (2004); Du et al. (2010); Cordero et al. (2017); Crossley et al. (2017)). Altered body composition and lower growth rates are predicted from metabolic suppression because mitosis will be slowed (Douglas et al., 2005; Harrison et al., 2015), which may lead to extended incubation duration under chronic hypoxia (Sun, Wang, Pike, Liang, & Du, 2014). Indeed, *P. muralis* had lower dry mass and total energy content, suggesting that the conversion from yolk to tissue was affected by hypoxia. Even so, these changes did not elicit the expected shift in skeletal size (SVL) and incubation duration, suggesting that total cardiac output was similar to in low altitude if larger hearts afforded greater ventricular stroke in hypoxia (Burggren, & Warburton, 1994; Crossley, & Burggren, 2009; Warburton et al., 1995). In general, developing animals, including reptiles (see Crossley et al. (2017)), may match mass-specific metabolic demands to survive and grow in hypoxic incubation environments (Harrison et al., 2015).

We also showed that *P. muralis* incubated in hypoxia did have a slight increase in total hatchling water content, which may compensate for any tissue-specific changes. An increase in total body water (hypervolemia) is a common cardiovascular response associated with physiological stress and may require a larger heart to pump out more blood (i.e., greater stroke volume) (Gregg, & Wiggers, 1933;

Convertino, 1991; Wagman et al., 1999; Faber et al., 1974). Even though hypoxia induced slightly larger dry heart mass, we did not observe a strong response. To test these hypotheses, future work should address how cellular metabolism, including blood circulation and biochemistry, are affected in embryos exposed to hypoxic conditions. Note that normal growth in *P. muralis* might have simply been able to ensue because hypoxic treatments were not extreme, as suggested by montane lizards that successfully develop at < 10% O_2 (Andrews, 2002). Still, we demonstrated physiological strategies that might be employed to achieve this.

The effect of hypoxia on mass (dry) and energy content of embryos depended on the initial egg mass. *P. muralis* embryos from larger eggs under normal developmental conditions were able to assimilate more energy from yolk reserves than those from smaller eggs, but this relationship was constrained in siblings developing under hypoxic conditions. This response is consistent with lower energy utilization of embryos under hypoxia, suggesting that less energy could be converted into tissue to begin with. In fact, hatchling energy content in the hypoxia treatment was lower than expected for the given incubation temperature (Ji, & Braña, 1999). Of potential interest would be to test how *P. muralis* respond to hypoxia across diverse thermal regimes, as developmental temperatures may set the tolerance limit for low O_2 availability in lizard species of alpine ecosystems (e.g., *Sceloporus*; Smith et al., 2015).

Whether physiological adjustments that promote successful development in hypoxia-incubated embryos affect post-hatching performance and survival is as of yet unclear (but see Wearing, Conner, Nelson, Crossley, and Crossley (2017)). However, constraints on yolk utilization, and hence hatchling size, may have important fitness consequences (Sinervo, 1993; Warner, 2014). In alligators, laboratory-simulated hypoxia has been shown to cause a reduction in both embryonic and post-hatching growth rates (Owerkowicz, Elsey, & Hicks, 2009; Crossley, & Altimiras, 2005) and may even compromise cognitive capacity in some hatchling lizards (Sun et al., 2014). As adults, reptiles are expected to cope well with high-altitude hypoxia owing to their low basal metabolic rates (McNab, 2002; Jackson, 2007), though empirical evidence to test this generalization is lacking (Powell, & Hopkins, 2010). Future work that examines the impact of hypoxia on long-term fitness traits will be important in establishing whether this is the case.

In addition to the general physiological responses that are likely to explain the ability of embryos to respond functionally to high-altitude hypoxia, low-altitude populations may also be adapted to acute hypoxia (e.g., during flooding of nests (Deeming, & Thompson, 1991; Ackerman, & Lott, 2004)) (Rezende et al., 2005). Moreover, the oviparous ancestor of modern lizards evolved in an atmosphere with 15–16% O_2 at sea level (Huey, & Ward, 2005). If so, resilience to hypoxia might be facilitated by the activation of latent physiological variation inherent to most reptiles. Although we did not sample high-altitude *P. muralis*, we succeeded in demonstrating that the capacity to cope with hypoxia already exists in low-altitude populations. This is foundational to future research aiming to directly compare the capacity of physiological responses across altitudinal gradients. To the best of our knowledge, our study is the first to examine physiological responses to high-altitude hypoxia in situ in developing non-avian reptiles.

5 | CONCLUSIONS

Collectively, our findings support the hypothesis that plastic physiological responses to high-altitude hypoxia may facilitate the maintenance of fitness-related phenotypes in *P. muralis*. Although potential trade-offs and effects of other environmental parameters warrant further examination, we propose that cardiovascular and metabolic plasticity in embryos should facilitate altitudinal range expansion in *P. muralis*, and other non-avian reptiles, in response to climate warming.

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ORCID

Gerardo A. Cordero http://orcid.org/0000-0002-9137-1741

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Effets de l'hypoxie d'altitude sur le développement embryonnaire et les performances juvéniles chez la Couleuvre vipérine, *Natrix maura*, dans le contexte actuel du changement climatique.

Le changement climatique pourrait entrainer, d'ici 2100, une hausse de la température moyenne à la surface de la Terre de 1°C à 6.5°C par rapport à la température moyenne estimée entre 1986 et 2005. Cela est susceptible d'augmenter le risque d'extinction des espèces, de modifier leur aire de répartition, en impactant la phénologie de reproduction et de migration des organismes, entraînant un changement des schémas de biodiversité à l'échelle mondiale. Les ectothermes, dont l'ensemble des traits physiologiques et comportementaux sont dépendants des températures environnementales, vont d'autant plus être affectés par le changement climatique et devront migrer vers des zones thermiques plus favorables, comme les zones de haute altitude. Cependant, en altitude, la diminution de la pression partielle de l'air réduit la quantité d'oxygène disponible. Cette nouvelle contrainte environnementale, l'hypoxie d'altitude, pourrait limiter leurs chances de coloniser ces milieux. Cette thèse cherche à mettre en évidence les réponses physiologiques à l'hypoxie d'altitude chez la Couleuvre vipérine, *Natrix maura*, une colonisatrice historique qui subit une expansion de gamme vers le haut, et à définir sa capacité à utiliser les espaces montagnards comme refuge face au changement climatique. Les objectifs sont, d'abord mesurer les effets de l'hypoxie d'altitude et de l'interaction qu'elle peut avoir avec la température sur le développement par l'intermédiaire du suivi de l'activité métabolique embryonnaire et des taux de développement. Puis, d'observer la persistance potentielle de ces effets sur les performances et le métabolisme des juvéniles. Les résultats de ces travaux suggèrent que, chez la Couleuvre vipérine, les réponses physiologiques plastiques des embryons à l'hypoxie de haute-altitude pourraient faciliter l'expansion de l'aire de répartition altitudinale à travers le maintien des phénotypes corporels et des performances physiques des juvéniles.

***Mots-clés** : Changement climatique, Hypoxie d'altitude, Développement embryonnaire, Taux métabolique, Plasticité phénotypique, Natrix maura.*

Effects of high-elevation hypoxia on embryonic development and juvenile performance in the Viperine snake, *Natrix maura*, under the current context of climate change.

By 2100, climate change could lead to an increase in the average temperature on the Earth's surface of 1°C to 6.5°C compared to the average temperature estimated between 1986 and 2005. This is likely to increase the risk of species extinction or change species ranges by impacting the reproductive phenology and the migration of organisms, leading to a change in biodiversity patterns on a global level. Ectotherms, whose set of physiological and behavioural traits are dependent on environmental temperatures, will be further affected by climate change and will have to migrate to more favourable thermal zones, such as to high altitude. However, at higher altitudes, the decrease in the partial pressure of the air reduces the availability of oxygen. This new environmental constraint, high-elevation hypoxia, could limit organisms' chances of colonizing these environments. This thesis seeks to highlight the physiological responses to high-elevation hypoxia in the Viperine snake, *Natrix maura*, a historical colonizer currently undergoing an upward range expansion, and to define its capacity to use mountain areas as a refuge in the context of climate change. The objectives are, in the first instance, to measure the effects of high-elevation hypoxia and the interaction it may have with temperature on development through monitoring embryonic metabolic activity and development rates. The second objective is to observe the potential persistence of these effects on the performance and metabolism of juveniles. The results of this work suggest that, in the Viperine Snake, the plastic physiological responses of embryos to high-elevation hypoxia could facilitate the expansion of the altitudinal range through the maintenance of body phenotypes and physical performance of juveniles.

***Keywords**: Climate Change, High-altitude hypoxia, Embryonic development, Metabolic rate, Phenotypic plasticity Natrix maura.*