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Andréaz Dupoué

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THESE

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Présentée par :

Andréaz DUPOUÉ

****

Importance des compromis liés à l'eau chez une espèce caractéristique des milieux bordiers, la vipère aspic (*Vipera aspis*)

****

Directeur de Thèse : Olivier LOURDAIS (CR 1)

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Soutenue le 06 Novembre 2014

devant la Commission d'Examen

****

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« Il y a une chose que l'histoire de l'Evolution nous a apprise, c'est que la vie ne peut pas être contenue. La vie prend le large, la vie conquiert de nouveaux territoires, elle renverse toutes les barrières ; c'est parfois pénible, c'est parfois dangereux, mais c'est comme ça. […] La vie trouve toujours un chemin... »

Pr Ian Malcolm

(Jurassic Park, S. Spielberg, 1993)

Vipère aspic – Acrylique sur Papier – par: **Lucie Montorio**,
http://kikikiworld.wordpress.com/

Résumé

Importance des compromis liés à l'eau chez un reptile caractéristique des milieux bordiers, la vipère aspic (*Vipera aspis*)

L'un des enjeux majeurs en écologie est de comprendre et prédire la réponse des organismes aux variations environnementales. Pour cela, la clarification des mécanismes proximaux est une étape indispensable pour comprendre des patrons écologiques généraux comme l'utilisation de l'habitat ou la distribution des espèces. Dans ce contexte, l'approche écophysiologique est particulièrement pertinente. Si la ressource trophique et les contraintes énergétiques ont attiré un intérêt considérable, les compromis liés à l'eau demeurent actuellement peu considérés. Pourtant l'eau est aussi une ressource capitale pouvant être limitante. La régulation de la balance hydrique pourrait donc jouer un rôle clé dans les compromis physiologiques et comportementaux.

L'objectif principal de ce doctorat est d'évaluer l'importance des compromis liés à l'eau chez une espèce caractéristique des milieux bordiers (haies, lisières), la vipère aspic (*Vipera aspis*). Cette espèce vivipare est particulièrement dépendante des conditions thermiques, notamment pour sa reproduction. Notre hypothèse générale est qu'au même titre que les conditions thermiques, les conditions hydriques confrontent l'organisme à des compromis physiologiques et comportementaux importants, particulièrement lors de la reproduction (i.e., gestation).

Nous avons combiné des études descriptives (thermorégulation, pertes hydriques) et expérimentales (manipulation de l'accès à l'eau) qui suggèrent l'existence d'un compromis entre la thermorégulation et la balance hydrique. Ces contraintes liées à l'eau sont associées à des ajustements physiologiques et comportementaux qui doivent être considérés pour comprendre les stratégies reproductrices et identifier de possibles conflits intergénérationnels (mère-embryons). Au même titre que les contraintes énergétiques ou thermiques, les besoins en eau sont donc essentiels à considérer pour aborder des questions écologiques et évolutives générales.

Mots-clés : Balance hydrique, conflit mère-embryons, compromis, corticostérone, métabolisme, pertes hydriques, Squamate, thermorégulation.

Summary

Importance of water-based tradeoffs in a reptile using ecotone habitats, the Aspic viper (*Vipera aspis*)

A major goal in ecology is to understand and predict species responses to environmental variations. Clarifying the proximate factors involved is a crucial step to unravel general ecological patterns such as habitat use or species distribution. In this context, the use of an ecophysiological approach can be particularly relevant. Trophic resource and energy tradeoffs attracted considerable interest but water-based tradeoffs remain relatively overlooked to date. However water is a critical, often limiting resource that must be considered. The regulation of water balance may have a key influence on physiological and behavioral tradeoffs.

The main objective in this thesis is to evaluate the importance of water-based tradeoffs in a species characteristic of "ecotone" habitats (hedgerows, edges), the Aspic viper (*Vipera aspis*). This species is viviparous and highly depends on thermal conditions during reproduction. Our general hypothesis is that, as thermal conditions, hydric conditions should expose individuals to important physiological and behavioral tradeoffs, especially during reproduction (i.e., pregnancy).

We combined descriptive (thermoregulation, water losses) and experimental studies (manipulation of water availability) that suggest a significant tradeoff between thermoregulation and water balance regulation. Water-based tradeoffs induce physiological and behavioral adjustments that are relevant to understand reproductive strategies and identify possible transgenerational (mother-embryos) conflicts. As energy or thermal resource, water should therefore also be considered to address ecological or evolutionary questions.

Keywords : Water balance, mother-offspring conflict, tradeoff, corticosterone, metabolism, water losses, Squamate, thermoregulation.

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Introduction

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1. Variabilité spatio-temporelle de l'environnement

La variabilité de l'environnement est un paramètre majeur auquel tout organisme vivant est exposé. Elle s'exprime à différentes échelles que ce soit temporelle (e.g., variations saisonnières, nycthémérales) ou spatiale (e.g., variations latitudinales, altitudinales). Elle inclue à la fois les variations de l'environnement abiotique (e.g., climat, photopériode, pression, salinité, pollution) et biotique (e.g., disponibilité des ressources, abondance de prédateurs/compétiteurs, parasites, partenaires sexuels). La variabilité environnementale impacte considérablement la dynamique démographique des animaux et des plantes, ce qui peut être déterminé à travers leur distribution et leur abondance (Brown 1984 ; Brown et al. 1996). C'est la raison pour laquelle un grand nombre d'études se sont intéressés à comprendre les facteurs déterminants de la distribution des espèces (Lawton 1991 ; Chown & Gaston 1999 ; Kearney 2006 ; Guillon 2012 ; Dunkin et al. 2013 ; Thiers et al. 2014). Actuellement, comprendre l'impact des variations environnementales sur les traits des espèces (morphologiques, physiologiques, comportementaux, ou traits d'histoire de vie) est essentiel pour appréhender et prédire efficacement les réponses des organismes en particulier face aux changements climatiques (Walther et al. 2002 ; Root et al. 2003 ; Parmesan & Yohe 2003 ; Kearney et al. 2009 ; Dillon et al. 2010 ; Angelier & Wingfield 2013 ; Todgham & Stillman 2013). Dans ce contexte, l'écophysiologie est une approche particulièrement pertinente (Feder & Block 1991). En effet, cette discipline relativement récente s'intéresse, à l'échelle de l'individu, à déterminer les mécanismes mis en jeux dans les réponses comportementales et physiologiques qui permettent à un organisme d'optimiser ses performances dans un environnement donné et d'assurer à la fois sa **survie** et sa **reproduction**.

Les adaptations développées par les organismes sont souvent liées à la résolution de compromis physiologiques (i.e., allocation des ressources ; Stearns 1992; Roff 1992) et comportementaux. Par exemple, la gestion des compromis d'allocation de l'énergie (dépenses énergétiques, réserves, etc) est un paramètre déterminant pour la survie et la reproduction des organismes (Bonnet et al. 1998 ; Post & Parkinson 2001 ; Brown et al. 2004 ; O'Connor et al. 2006 ; Kearney 2012). Bien que les compromis physiologiques liés à la gestion du budget énergétique aient été bien décrits (Roff & Fairbain 2007 ; King et al. 2011), les compromis liés au maintien de la balance hydrique ont été moins étudiés. Dans ce doctorat, nous nous sommes intéressés plus spécifiquement aux compromis liés à l'eau chez une espèce ectotherme de milieu tempéré.

La base de ces travaux résulte du compromis entre les besoins thermiques qui visent à assurer les performances physiologiques et la gestion de la balance hydrique. En effet, l'ectothermie induit une sensibilité importante et directe aux conditions climatiques (température, humidité), qui impose aux individus une thermorégulation comportementale, ce qui est associé à des contraintes spécifiques.

2. L'ectothermie et les coûts de la thermorégulation

2.1. L'ectothermie et la sensibilité thermique des performances

On distingue généralement deux grandes stratégies métaboliques, l'ectothermie et l'endothermie, qui diffèrent non seulement par leur mode de thermorégulation, par le niveau des dépenses énergétiques (i.e., métabolisme) et au final par leur sensibilité thermique. La thermorégulation correspond aux ajustements physiologiques et comportementaux qui permettent aux organismes d'atteindre une gamme de températures corporelles tolérées. En effet, la température corporelle est un paramètre déterminant étant donné qu'elle influence directement le fonctionnement enzymatique ; essentiel puisqu'au niveau cellulaire, les enzymes accélèrent la vitesse des réactions biochimiques d'un facteur 10^8 à 10^{12} (Hochachka & Somero 2002 ; Moyes & Schulte 2008). Le fonctionnement enzymatique se traduit à l'échelle de

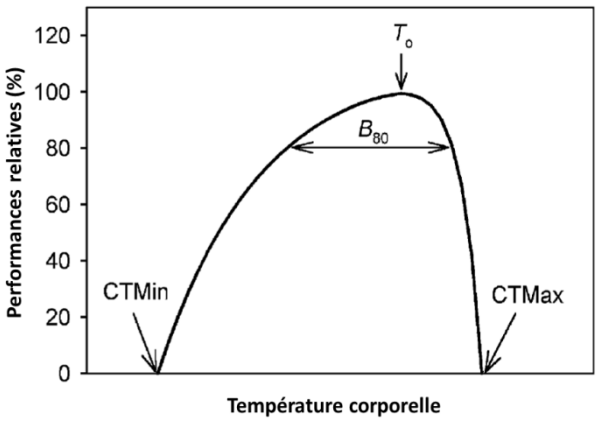

Figure 1. Relation asymétrique classique entre les performances physiologiques relatives et la température corporelle. Les performances augmentent progressivement avec la température jusqu'à une température optimale (T_o) pour laquelle la performance sera maximale. Lorsque la température augmente au-delà de T_o, la performance chute brutalement. B_{80} désigne la gamme de températures autour de T_o pour lesquelles 80% des performances sera assuré. CTMin et CTMax désignent respectivement les températures critiques minimales et maximales au-delà desquelles les performances ne sont plus biologiquement viables. D'après Angilletta et al. (2002), (originalement basé de Huey & Stevenson 1979).

l'organisme au niveau des performances physiologiques (e.g., locomotion, acquisition de la ressource, digestion, reproduction, etc) (Fig. 1).

Les **ectothermes**, qui représentent plus de 99% des espèces animales, utilisent une stratégie métabolique basée sur l'économie d'énergie avec un métabolisme standard relativement faible (i.e., 10 à 20 fois plus faible qu'un endotherme de masse équivalente ; Nagy 2005). A l'inverse, les endothermes (oiseaux et mammifères) s'affranchissent en partie des variations thermiques de l'environnement en maintenant une température corporelle élevée, relativement constante, leur permettant d'assurer un niveau de performances important (Bennett & Ruben 1979 ; Bicego et al. 2007 ; Angilletta et al. 2010 ; Clarke & Pörtner 2010). L'endothermie est basée sur une forte dépense énergétique qui impose aux animaux une alimentation fréquente. Chez les ectothermes, le niveau métabolique relativement faible ne permet pas de produire une chaleur résiduelle suffisante pour compenser les variations thermiques de l'environnement. Dans quelques cas, certaines espèces sont néanmoins capables d'élever temporairement leur température corporelle par rapport à celle de leur environnement, ce qui inclue l'hétérothermie spatiale (e.g., yeux et cerveau des espadons : Fritsches et al. 2005 ; thorax des bourdons avant le vol : Stone 1993) ou l'hétérothermie temporelle (e.g., moustiques pendant l'alimentation : Lahondère & Lazzari 2012 ; crotale après un repas : Tattersall et al. 2004 ; python frissonnant pendant la couvaison : Slip & Shine 1988a).

La thermorégulation chez les ectothermes est principalement comportementale, c'est-à-dire que ces organismes ajustent leur temps passé à se chauffer ou sous abris pour atteindre les températures corporelles cibles (Hertz et al. 1993 ; Christian et al. 2006 ; Kearney et al. 2009). Dépendamment des espèces, différents comportements (héliothermie, thigmothermie, etc) et différentes stratégies de thermorégulation (thermoconformité, thermorégulation « parfaite », etc) existent toutefois, en lien avec les différents modes de transfert de température (i.e., conduction, convection, radiation) (Angilletta 2009). Ces ajustements comportementaux permettent aux individus d'assurer un niveau de performances optimales puisque le niveau de dépenses énergétiques (i.e., métabolisme), qui corrèle avec le niveau des performances physiologiques, augmente de façon exponentielle avec la température ambiante (Angilletta 2009) (Fig. 2). La thermo-dépendance de processus physiologiques influençant la survie (locomotion, croissance, digestion, immunité, etc) ou la reproduction induit donc de façon plus ultime, un impact direct de la température sur la valeur sélective des individus (Shine 1980 ;

Slip & Shine 1988b ; Blouin-Demers & Weatherhead 2001a ; Angilletta et al. 2002 ; Angilletta 2009 ; Richards-Zawacki 2010 ; Lelièvre et al. 2011 ; Lorioux et al. 2013a ; Meylan et al. 2013), ce qui rend les ectothermes particulièrement sensibles aux conditions climatiques (Walther et al. 2002 ; Deutsch et al. 2008).

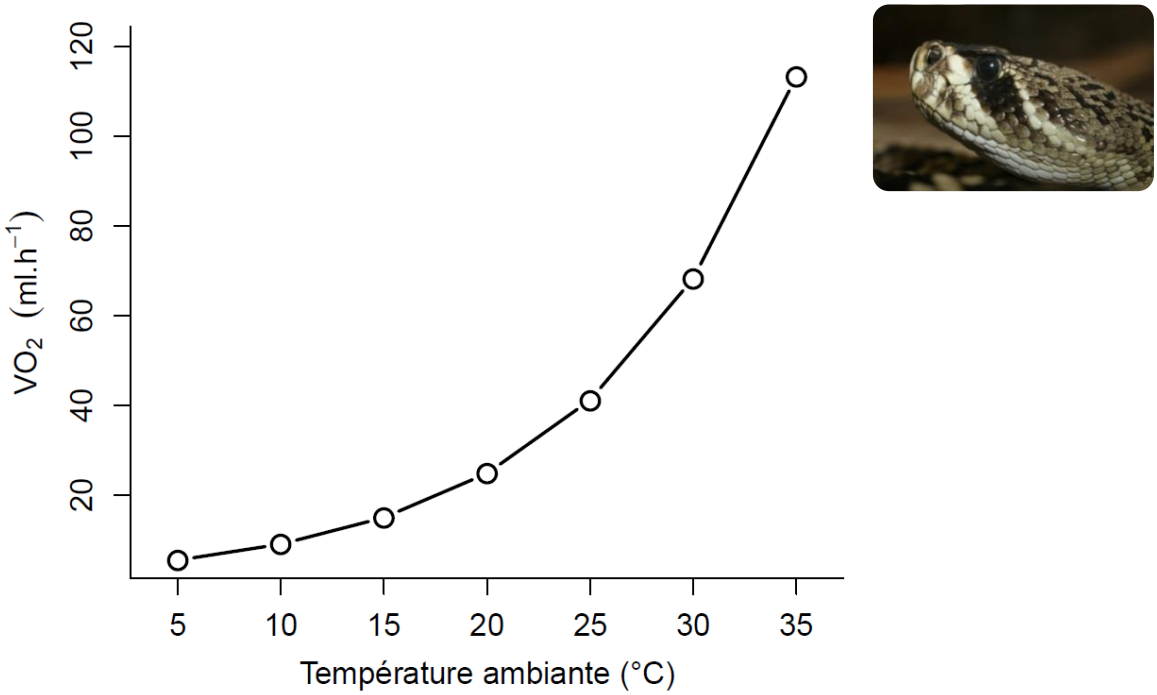

Figure 2. Relation exponentielle entre le métabolisme standard (estimé par la consommation d'oxygène : VO_2) et la température ambiante chez un ectotherme : *Crotalus adamanteus*. Les données ont été obtenues d'après Dorcas et al. (2004).

2.2. Les coûts de la thermorégulation

Bien que la thermorégulation soit essentielle puisqu'elle permet d'assurer l'ensemble des processus physiologiques (locomotion, reproduction, digestion, immunité, etc) (Stevenson et al. 1985 ; Angilletta 2006, 2009 ; Butler et al. 2013 ; Lorioux et al. 2013a ; Lourdais et al. 2013), elle est également associée à des coûts. L'étude de ces coûts est cruciale pour comprendre et modéliser des réponses écologiques ou évolutives plus générales (utilisation de l'habitat, répartition, etc) (Dunham et al. 1989 ; Huey 1991 ; Kearney et al. 2009 ; Dillon et al. 2010). Les coûts de la thermorégulation dépendent typiquement de la qualité thermique de l'habitat (i.e., opportunités thermiques) et on peut les regrouper en trois catégories (Blouin-Demers & Nadeau 2005 ; Angilletta 2009). Premièrement, l'exposition pour la thermorégulation peut entrainer des coûts d'ordre écologiques avec l'augmentation de la compétition intra- ou interspécifique, du risque de prédation, ou du parasitisme (Hertz et al. 1982 ; Downes &

Bauwens 2002 ; Webb & Whiting 2005 ; Kerr & Bull 2006). Ensuite, la thermorégulation peut induire des coûts dans la gestion du budget énergétique ou du budget temps puisque par exemple, le temps passé à thermoréguler rentre en compétition directe avec le temps disponible pour d'autres activités (alimentation, recherche de partenaires sexuels, etc) (Adolph & Porter 1993 ; Gregory et al. 1999 ; Gvozdik 2002). Enfin la thermorégulation implique des coûts d'ordre physiologiques, notamment parce qu'elle peut compromettre le maintien de la balance hydrique (Ladyman & Bradshaw 2003 ; Köhler et al. 2011 ; Guillon et al. 2014). Comme suggéré récemment (Guillon et al. 2014), ce compromis physiologique entre la thermorégulation et le maintien de la balance hydrique est encore relativement peu considéré, bien qu'il soit au cœur d'ajustements physiologiques (e.g., niveau métabolique, perméabilité cutanée, etc) et comportementaux (e.g., décisions d'exposition, température préférée, activité, etc) (Kennett & Christian 1994 ; Lillywhite 2004 ; Ladyman et al. 2006 ; Muir et al. 2007 ; Stahlschmidt et al. 2011 ; Iacarella & Helmuth 2012). C'est dans le contexte du compromis physiologique entre la thermorégulation et le maintien de la balance hydrique que se placent les travaux présentés dans ce doctorat.

3. Les compromis d'allocation

A la base de l'étendue de la variabilité dans les stratégies d'histoire de vie se trouvent les compromis d'histoire de vie (Reznick 1985 ; Stearns 1989, 1992 ; Roff 1992 ; Clobert et al. 1998 ; Zera & Harshman 2001). C'est la raison pour laquelle ces compromis d'histoire de vie continuent d'attirer un vif intérêt en écologie évolutive (Roff & Fairbain 2007 ; Monaghan et al. 2009 ; Johnston et al. 2013 ; Saino et al. 2014). Au sens général, un compromis peut se produire lorsque deux paramètres sont fonctionnellement reliés entre eux et de façon négative car dépendants de la même ressource (Zera & Harshman 2001). Les compromis d'allocation des ressources représentent ainsi la base physiologique des compromis entre traits d'histoire de vie (Dunham et al. 1989 ; Zera & Harshman 2001 ; O'Connor et al. 2006). En effet, dans l'environnement, les ressources fluctuent, ce qui les rend spatialement et/ou temporellement limitantes. Les organismes sont donc obligés d'allouer les ressources de façon préférentielle vers l'une ou l'autre des fonctions physiologiques principales (i.e., maintenance, croissance, stockage et/ou reproduction) (Fig. 3).

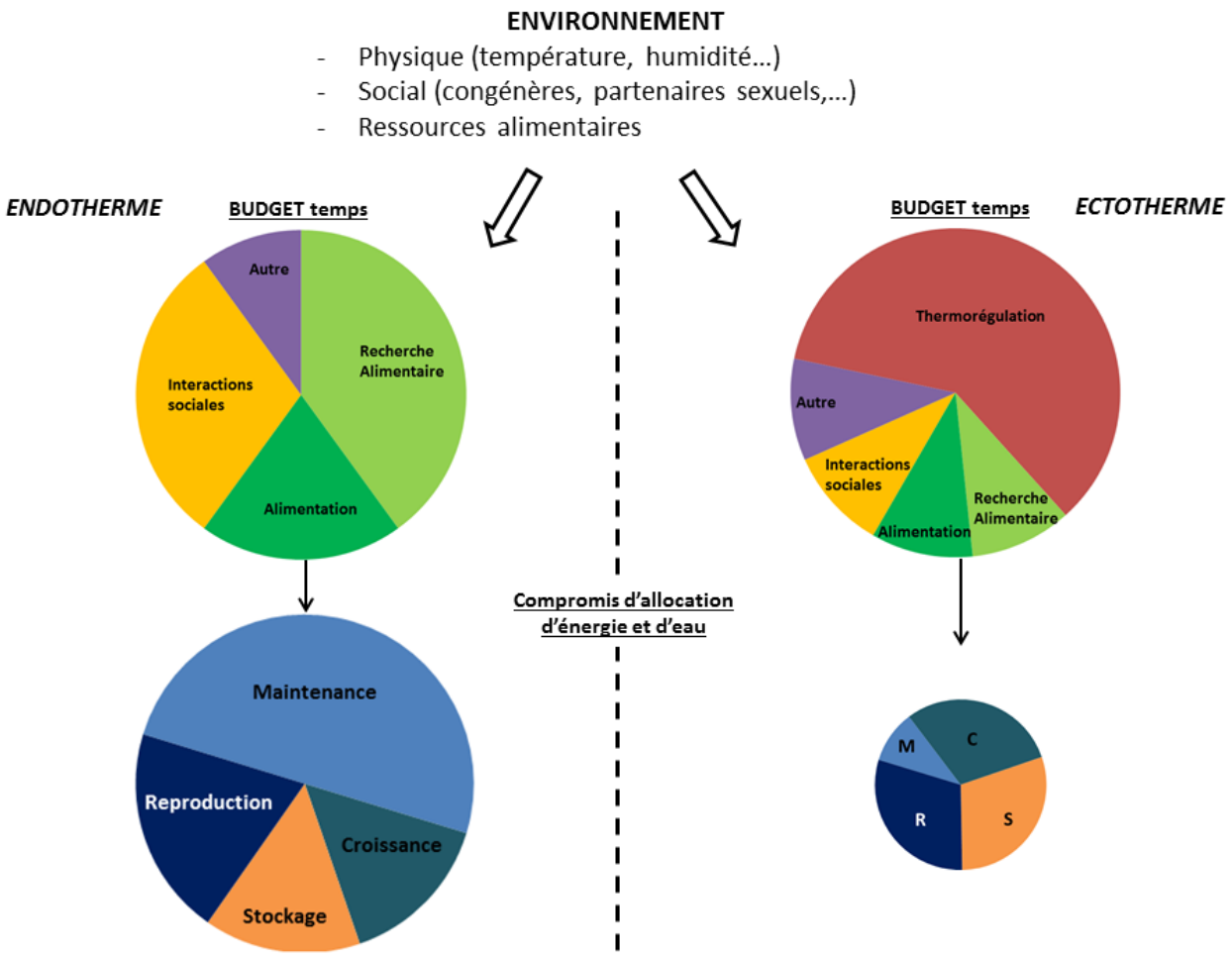

Figure 3. Contraste entre les endothermes (à gauche) et les ectothermes (à droite) dans la dynamique des compromis d'allocation des ressources (temps, énergie et eau). Les endothermes favorisent leur budget temps en recherche alimentaire et en alimentation pour assurer des coûts de fonctionnement 10 à 20 fois supérieurs à ceux d'un ectotherme. A l'inverse, un ectotherme favorise plus son temps en thermorégulation pour permettre l'accomplissement des fonctions physiologiques. De même, les priorités d'allocation ne sont pas les mêmes entre endothermes et ectothermes (e.g., maintenance). Schéma modifié d'après O'Connor et al. (2006).

Les compromis d'allocation des ressources varient donc entre les espèces (e.g., allocation du temps principalement dans la recherche alimentaire pour un endotherme *versus* principalement dans la thermorégulation pour un ectotherme) (Fig. 3). Ils peuvent également varier également au sein même d'une espèce, pour un même individu (i.e., compromis **intra-individuels**), ou bien entre un individu et sa descendance (i.e., compromis **intergénérationnels**) (Stearns 1989).

3.1. Les compromis intra-individuels

Les compromis à l'échelle de l'individu peuvent s'exprimer à travers l'alternance des différents stades d'histoire vie (Perrin & Sibly 1993 ; Piersma et al. 1999 ; Jacobs & Wingfield 2000 ; Piersma 2002 ; Ricklefs & Wikelski 2002 ; Wingfield 2008). En effet, les priorités d'allocation des ressources vers les fonctions physiologiques principales ne seront pas les mêmes à chaque stade du cycle de vie. Par exemple, les jeunes vont privilégier la croissance alors que les adultes vont plutôt privilégier leur reproduction ou le stockage de réserve pour préparer le passage de la mauvaise saison (i.e., migration automnale, puis entrée et sortie d'hivernage). Les compromis intra-individuels peuvent également s'exprimer à long terme, c'est-à-dire lorsque les décisions d'allocation des ressources à un stade de vie *n* affectent l'allocation des ressources au stade de vie *n+1* (Metcalfe & Monaghan 2001, 2003). C'est le cas par exemple du compromis associé au **coût de la reproduction** (Stearns 1989). Le coût de la reproduction a été très largement étudié dans ces dernières dizaines d'années puisqu'il intervient directement sur la fonction ultime de tous les individus (Williams 1966 ; Reznick 1985 ; Stearns 1992 ; Roff 1992). Il fait référence à la relation négative entre la reproduction courante et la reproduction future et/ou la longévité d'un individu. Empiriquement, un grand nombre d'études ont permis de mettre en évidence ce type de compromis dans plusieurs taxa chez les animaux (voir Reznick 1985 pour une review) mais aussi chez les plantes (voir Obeso 2002 pour une review). A l'échelle de l'individu, les coûts impliqués dans les compromis entre reproduction courante et reproduction future et/ou longévité peuvent être de deux sortes. Ils impliquent en effet des coûts en termes de survie (e.g., risque de prédation accru : Magnhagen 1991 ; Ghalambor & Martin 2001, dommages oxydatifs : Dowling & Simmons 2009 ; Stier et al. 2012, immunité diminuée : French et al. 2007), ou bien en termes d'investissement énergétique (i.e., effort reproducteur : Shine 1980 ; Bonnet et al. 1998).

3.2. Les compromis intergénérationnels

Les compromis d'allocation des ressources peuvent également se produire de façon intergénérationnelle, c'est-à-dire entre un parent et sa descendance (Stearns 1989 ; Rogowitz 1996). L'un des exemples les plus étudiés correspond à la quantité *versus* la qualité des jeunes à la naissance (van Noordwijk & de Jong 1986; Sinervo & Licht 1991 ; Olsson & Shine 1997 ; Walker et al. 2008). Ce compromis est classiquement décrit en corrélant (négativement) le nombre et la taille des descendants à la naissance ou à un âge précis (Fig. 4). Au cœur de ces

compromis intergénérationnels se trouvent donc les limites liées à l'allocation des ressources (i.e., effort reproducteur), mais aussi les limites imposées par la morphologie des femelles (i.e., espace disponible de la cavité abdominale) (Olsson & Shine 1997). Bien que la définition de l'effort reproducteur ait été assujettie à débat, on peut le décrire comme la proportion totale de ressources investie dans la reproduction tout au long de la vie d'un individu (Tuomi et al. 1983).

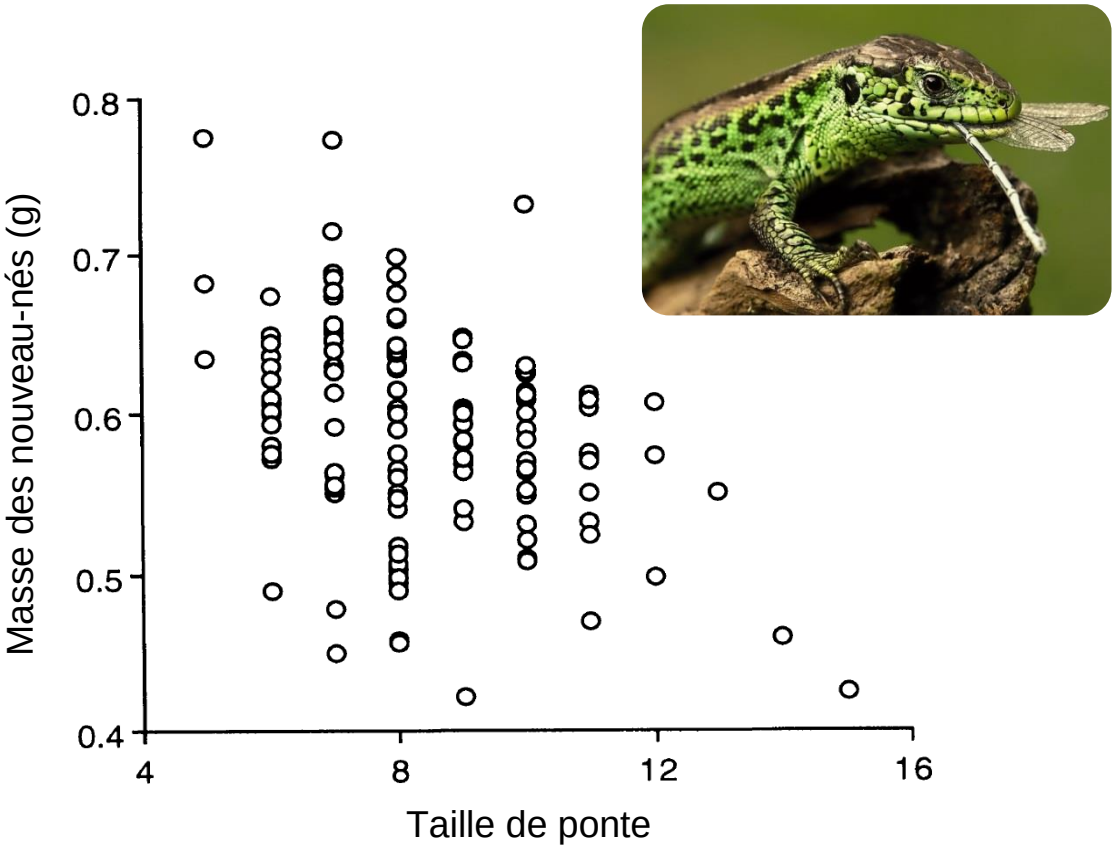

Figure 4. Relation négative entre la masse moyenne des nouveaux-nés à la naissance et le nombre d'œufs de la ponte chez le lézard des souches (*Lacerta agilis*). D'après Olsson & Shine (1997).

3.3. Du compromis au conflit

La distinction entre compromis et conflit n'est pas toujours très claire dans la littérature. Dans ce doctorat, on se réfère au conflit physiologique comme la résultante d'un compromis pour une ressource lorsque celle-ci un limitante et lorsque l'allocation de la ressource vers l'une des fonctions physiologique (i.e., compromis intra-individuel) ou vers la descendance (i.e., compromis intergénérationnel) induit un coût sur la survie ou la reproduction des individus. Par exemple, lorsque les ressources sont limitantes, les compromis intergénérationnels en lien avec l'effort reproducteur peuvent induire des conflits parents-descendants (i.e., théorie des « Parent-

Offspring Conflict » ; Trivers 1974) (Charnov 1982 ; Godfray et al. 1991 ; Godfray 1995a). Selon la théorie des conflits parents-descendants (Trivers 1974), l'inégalité génétique entre un parent et ses descendants (coefficient d'apparentement : $r = 0.50$; Hamilton 1964) induit une différence de perception de la valeur optimale de l'acquisition des ressources. Alors qu'un descendant a pour « but » de maximiser ses propres besoins, les parents doivent allouer les ressources à la fois pour leur propre survie et pour celle des autres descendants (voir Godfray 1995b pour une review). De nombreux modèles type coûts / bénéfices ont donc été développés pour déterminer l'effort reproducteur optimal des parents (voir Kölliker & Richner 2001 pour une review). Ainsi, les conflits parents-descendants suivent en général une dynamique de type « course aux armements » instable et sous sélection. Du côté des parents, les traits sous sélection sont liés à l'investissement dans la reproduction (i.e., soins parentaux) alors que du côté des descendants, la sélection influence l'intensité de manipulation ou de sollicitation des jeunes (i.e., manipulation hormonale, comportement de quémande) (Clutton-Brock 1991 ; Haig 1993, 1996 ; Crespi & Semeniuk 2004). Au final, ces conflits ont une portée évolutive importante puisqu'ils vont influencer les modes de reproduction (Zeh & Zeh 2000 ; Crespi & Semeniuk 2004), mais aussi l'histoire de vie des individus (Bossan et al. 2013 ; Fig. 5) et donc leur valeur sélective.

La dynamique des compromis d'allocation à différentes échelles (i.e., interspécifiques, intra-individuelles, ou intergénérationnelles) représentent les bases essentielles des réponses fonctionnelles des organismes face aux variations de leur environnement. En effet ces compromis vont directement influencer la valeur sélective des individus à travers leur survie et leur reproduction. Aussi, il est crucial de déterminer les mécanismes impliqués dans l'orchestration de ces compromis d'allocation. Dans ce contexte les concepts d'allostasie et de réponse au stress ont été développés pour décrire les ajustements physiologiques et comportementaux des animaux (principalement endothermes) face aux variations de l'environnement (McEwen & Wingfield 2003, 2010).

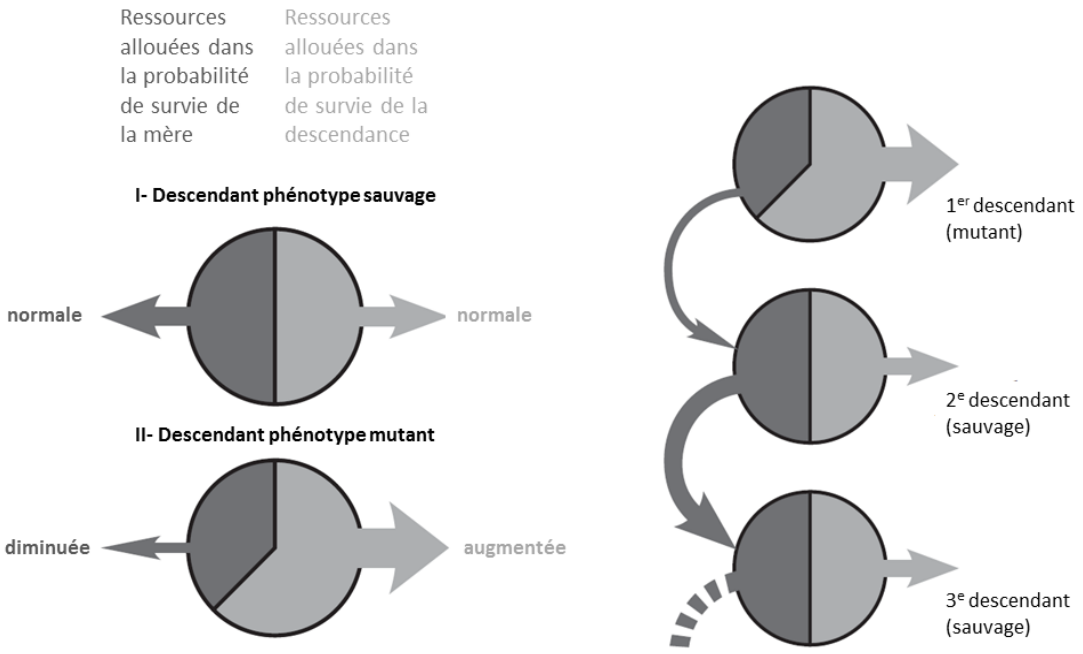

Figure 5. Modèle d'histoire de vie des conflits parents-descendants. Chaque cercle correspond à un compromis d'allocation qui affectera la survie (épaisseur des flèches) de la mère (gris foncé) ou de la descendance (gris clair). A gauche est représenté l'effet d'une mutation dans la descendance qui va augmenter le comportement de sollicitation des jeunes. Ce qui va induire un déséquilibre entre survie de la mère (diminuée) et celle de la descendance (augmentée). A droite, est représenté le même modèle mais répété au cours de l'histoire de vie hypothétique d'une femelle. Ce qui permet de visualiser l'impact des conflits parents-descendants sur l'histoire de vie des individus en affectant la probabilité de survie et donc la valeur sélective. D'après Bossan et al. (2013).

4. Allostasie et stress : médiation hormonale des compromis d'allocation

4.1. Les concepts d'allostasie et de stress

Le concept de **réponse au stress**, dont la signification a été assujettie à de nombreux débats (Buchanan 2000 ; McEwen & Wingfield 2003, 2010 ; Romero 2004) est utilisé pour comprendre la réponse des organismes face aux évènements stressants et imprévisibles. Dans cette thèse, on se référera donc au stress en suivant la définition consensus de Romero (2004). A savoir que la notion de réponse au stress fait intervenir 3 niveaux avec i) un évènement stressant (imprévisible et néfaste), ii) les réponses physiologiques et comportementales mises en place par l'organisme pour répondre à cet évènement stressant et iii) l'exposition prolongée à l'évènement qui aboutit à un stress chronique. Le concept d'**allostasie** qui a été introduit par Sterling & Eyer (1988) et repris depuis est plus intégrateur puisqu'il vise à déterminer les réponses physiologiques et comportementales des organismes à la fois face aux évènements prévisibles et imprévisibles de l'environnement (McEwen & Wingfield 2003, 2010; Romero 2004 ; Korte et al. 2005 ; Wingfield 2005 ; Landys et al. 2006 ; Romero et al. 2009). Selon ce

concept, un animal exposé à une perturbation dans son environnement ajustera sa physiologie et son comportement pour atteindre un état homéostatique stable (McEwen & Wingfield 2003). Comme pour la réponse au stress, le concept d'allostasie inclut plusieurs niveaux, avec i) l'état allostatique qui représente la modification du niveau des signaux primaires (e.g., hormones, neurotransmetteurs, immuno-cytokines, etc), ii) la charge allostatique qui correspond au résultat du cumul des demandes métaboliques classiques journalières (activité, digestion, etc), saisonnières (reproduction, migration, mue, etc) et les perturbations, et iii) la surcharge allostatique lorsque l'environnement devient limitant ou stressant et ne permet plus de maintenir la charge allostatique (McEwen & Wingfield 2010).

Cependant, le concept d'allostasie (i.e., « maintenir la stabilité à travers le changement » ; McEwen & Wingfield 2003) a été développé pour des organismes endothermes, et on peut donc se demander dans quelle mesure il s'applique à des ectothermes. C'est d'autant plus vrai quand on sait que la plupart des modèles utilisent l'énergie comme ressource limitante (McEwen & Wingfield 2003, 2010). Or, les ectothermes ajustent également leur physiologie et leur comportement aux variations prévisibles et imprévisibles de leur environnement. Ils tolèrent cependant une plus grande variation du *milieu intérieur* que les endothermes. C'est pourquoi Mrosovsky (1990) a défini le terme de « rhéostasie » pour élargir « l'homéostasie » à la régulation du milieu interne mais pour différents points de consigne (voir aussi Bradshaw 1997). Plus récemment, McEwen (2009) a repris le principe de rhéostasie pour l'intégrer au concept d'allostasie pour des organismes avec des points de consigne variables tels que les ectothermes. Aussi, ce concept se généralise à des organismes comme les ectothermes mais vis-à-vis de ressources différentes (e.g., température plutôt que disponibilité alimentaire). Une méthode adaptée pour illustrer cela est de déterminer la dynamique de sécrétion hormonale.

4.2. Les glucocorticoïdes au cœur de l'allostasie et du stress

Au niveau de l'organisme, les glucocorticoïdes (GCs) sont des médiateurs essentiels aux effets pléiotropiques, puisqu'ils orchestrent un grand nombre de fonctions physiologiques (Desantis et al. 2013), dont les compromis d'allocation (Ketterson & Nolan 1992 ; Wingfield et al. 1998 ; Zera & Harshman 2001 ; Ricklefs & Wikelski 2002 ; Martin et al. 2008). Les GCs sont donc au cœur de l'allostasie et du stress car ils représentent un intermédiaire clé entre les variations

de l'environnement et les réponses physiologiques des organismes (Sapolsky et al. 2000 ; Dufty et al. 2002 ; McEwen & Wingfield 2003, 2010 ; Romero 2004 ; Landys et al. 2006). Ils sont notamment essentiels pour permettre à l'organisme de répondre efficacement aux perturbations, mais lorsqu'ils sont sécrétés en trop forte concentration et trop fréquemment ils sont potentiellement néfastes pour l'organisme (stress chronique ou surcharge allostatique) (Wingfield et al. 1998 ; Romero et al. 2009). En effet, la sécrétion de GCs limite d'autres fonctions comme la reproduction, les défenses immunitaires, la croissance, ou la digestion (Boonstra et al. 1998 ; Sapolsky et al. 2000). Dans ce doctorat, on se concentrera sur la corticostérone qui correspond au GC principal chez la plupart des rongeurs, les oiseaux, les reptiles et les amphibiens (Greenberg & Wingfield 1987).

4.3. La corticostérone

La corticostérone (CORT) est l'hormone finale produite à la suite de l'activation de l'axe Hypothalamus – Hypophyse – Surrénales (ou HPA pour « Hypothalamus – Pituitary – Adrenal »). La CORT est un stéroïde dérivé du cholestérol et sécrété par des cellules spécialisées de la partie corticale des glandes surrénales (Norris 1997 ; Bentley 1998 ; Moyes & Schulte 2008). Une fois sécrétée dans le système circulatoire, la majorité de la CORT (90-95%) se lie à des protéines de transport (« corticosteroid binding globulins » ; CBGs) (Mendel 1992, Romero 2002). Pour interagir avec les tissus cibles, la CORT se lie à des récepteurs protéiques membranaires et intracellulaires spécifiques (Breuner & Orchinik 2001, 2009). Ces récepteurs sont de deux types : les récepteurs de type I et les récepteurs de type II avec des affinités différentes pour la CORT. En effet, les récepteurs de type I ont une affinité forte pour la CORT et sont donc rapidement saturés (Fig. 6). A l'inverse, les récepteurs de type II ont une affinité plus faible et la proportion de liaison se fait à des concentrations en CORT plus fortes (Fig. 6).

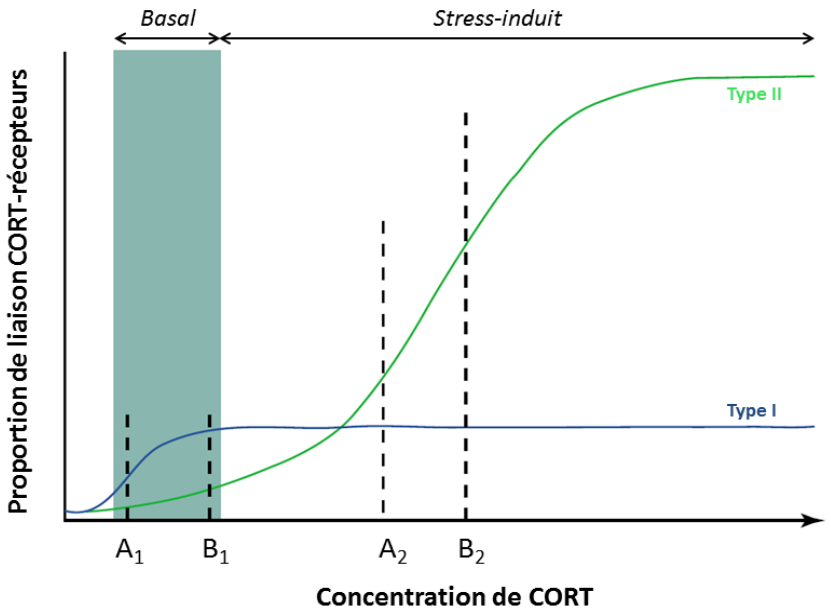

Figure 6. Interaction entre concentration plasmatique de CORT et la proportion de liaison avec les récepteurs de type I et de type II. Les récepteurs de type I ont une affinité forte pour la CORT et sont rapidement saturés (dès le taux basal) alors que les récepteurs de type II ont une affinité plus faible et commencent à être liés quand les récepteur de type I sont saturés (en stress-induit). A_1 et B_1 représentent des niveaux basaux de CORT alors A_2 et B_2 leurs niveaux respectifs de stress induit. On constate donc aisément que pour une augmentation similaire entre taux basal et taux stress-induit, les effets biologiques, dépendant de la proportion de liaisons CORT-récepteurs, seront plus prononcés pour B_2 que pour A_2. Modifié de Romero (2004).

En conséquence, on associe chaque type de récepteurs avec chacun des deux niveaux majeurs de sécrétion de CORT. En effet, les nombreux effets biologiques associés à la CORT sont notamment dépendants du niveau de sécrétion de CORT (concentration plasmatique) (Sapolsky et al. 2000 ; Romero 2004 ; Romero et al. 2009). On distingue donc classiquement le **taux basal** et le **taux induit par un stress** (Wingfield et al. 1998). Le taux basal de CORT est sécrété en concentration relativement faible en suivant un rythme journalier ou saisonnier et implique principalement les récepteurs de type I (Fig. 6). Le taux de CORT basal a en effet pour principale rôle la gestion des compromis d'allocation de l'énergie (Romero 2002 ; Landys et al. 2006 ; Angelier et al. 2007). Le taux de CORT induit par un stress est à l'inverse sécrété en concentration plus importante, suite à une perturbation et fait intervenir principalement les récepteurs de type II (Fig. 6). A cette concentration, la CORT induit des modifications physiologiques et comportementales qui ont pour but ultime de maximiser la survie immédiate au détriment de fonctions comme la croissance, l'alimentation ou la reproduction (Wingfield et al. 1998). Ces modifications incluent par exemple la mobilisation d'énergie pour fuir une perturbation (Breuner & Hahn 2003 ; Angelier et al. 2009). Etant donné le rôle central de la CORT dans les compromis d'allocation, plusieurs études se sont intéressées à mettre en évidence les perturbateurs environnementaux qui influencent la sécrétion de CORT (e.g.,

disponibilité alimentaire: Kitaysky et al. 2010, changement brutale de température: Eriksen et al. 2007, pression de prédation: Saino et al. 2005 ; Coslovsky & Richner 2011, modifications anthropogéniques: Ellenberg et al. 2007 ; Tartu et al. 2014).

En définitive, la CORT est un médiateur fonctionnel impliqué dans un grand nombre de réponses physiologiques et comportementales. Comme spécifié précédemment, un enjeu majeur en écologie est de comprendre et de prédire la réponse des organismes face aux changements globaux, et la CORT représente un indicateur physiologique essentiel à considérer pour déterminer la persistance d'espèces dans leur environnement (Meylan et al. 2012 ; Angelier & Wingfield 2013).

5. Les reptiles Squamates

Les Squamates (lézards et serpents) sont des organismes ectothermes particulièrement sensibles aux conditions climatiques. Ils ont été des modèles particulièrement étudiés pour clarifier les bénéfices et les coûts de la thermorégulation (voir Angilletta 2009). Toutefois le compromis avec la balance hydrique chez ces espèces est relativement sous-considéré.

5.1. Les Squamates et l'allostasie

Bien que le concept d'allostasie ait été défini sur des modèles endothermes type oiseaux, il s'applique également à des organismes comme les Squamates (Wingfield 2004). Pour illustrer cela, les iguanes marins offrent un bon exemple puisque les facteurs affectant la sécrétion de CORT ont été bien décrits. Chez cette espèce, la sécrétion de CORT varient à la fois en réponse aux variations prévisibles de la marée (i.e., disponibilité alimentaire), à l'alternance jour/nuit, en fonction des stades d'histoire de vie, mais aussi en réponse à différentes perturbations telles que les évènements El-Niño, les marées noires, ou le tourisme (Romero & Wikelski 2001, 2002, 2006 ; Wikelski et al. 2001 ; Woodley et al. 2003 ; French et al. 2010 ; Vitousek et al. 2010). Chez les Squamates comme chez les vertébrés en général, les variations prévisibles des stades d'histoire de vie sont associées à l'alternance de différents états physiologiques. Les **états physiologiques** sont en effet un paramètre clé dans le concept d'allostasie puisqu'ils sont

associés à différentes demandes énergétiques prévisibles des stades d'histoire de vie et à différents niveaux hormonaux tels que les niveaux de CORT (Fig. 7) (Wingfield & Kitaysky 2002 ; Landys et al. 2006 ; Romero et al. 2009).

Chez les Squamates, les états physiologiques sont associés à un effort de thermorégulation plus intense qui permet d'assurer l'ensemble du processus (i.e., thermophilie) (Naulleau 1983 ; Stevenson et al. 1985 ; Gibson et al. 1989 ; Charland & Gregory 1990 ; Lourdais et al. 2002a ; Wang et al. 2003 ; Lourdais et al. 2004). Si on prend comme exemple les serpents, on retrouve notamment 3 grands états physiologiques thermophiles : la mue, la digestion et la reproduction qui ont été associés à une augmentation des niveaux d'hormones en particulier d'hormones thyroïdiennes (i.e., T_3 et T_4), d'hormones de croissance (i.e., IGF-1) et/ou de CORT (Chiu & Lynn 1971 ; Tyrrell & Cree 1998 ; Woodley & Moore 2002 ; Secor 2009 ; Sparkman et al. 2009). Chaque état est caractérisé par une demande énergétique, une fréquence et une durée spécifique qui font donc varier la charge allostatique sur les individus (Fig. 7).

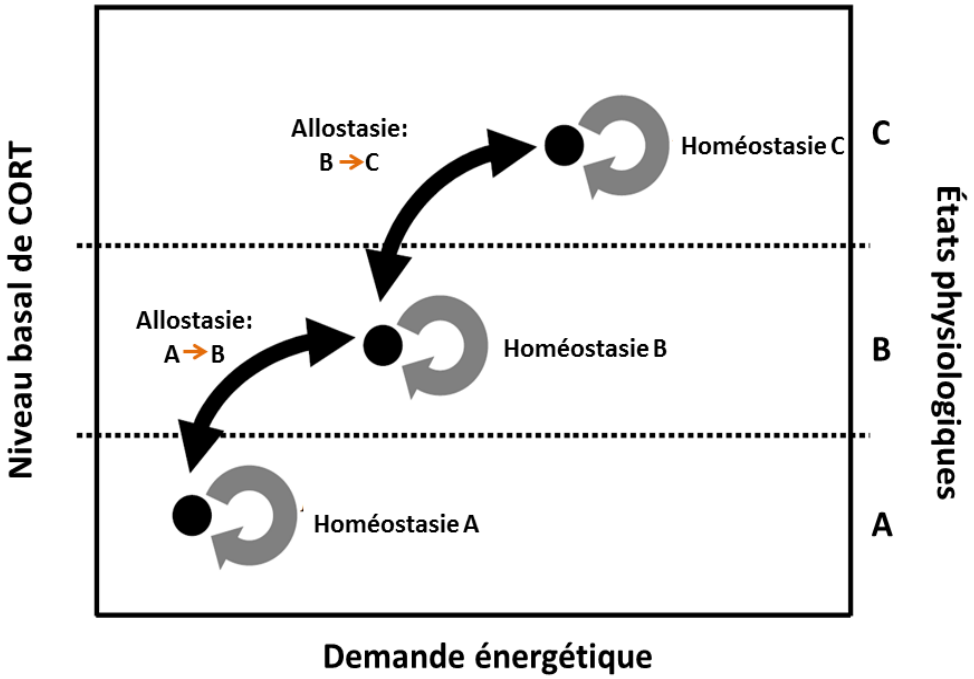

Figure 7. Variations de la demande énergétiques des stades d'histoire de vie associées d'une part aux changements d'états physiologiques et d'autre part au niveau basal de CORT. L'état physiologique A représente l'état physiologique de base d'un animal associé à un niveau basal de CORT minimal pour sa survie. L'état physiologique B représente l'augmentation prévisible de la demande d'un stade d'histoire de vie avec l'augmentation de sécrétion de CORT adaptée (i.e., charge allostatique). L'état physiologique C représente la réponse à une perturbation (i.e., le stade d'urgence) associé à une élévation importante du niveau de CORT. D'après Angelier (2006), modifié de Landys et al. (2006).

5.2. Compromis entre thermorégulation et pertes hydriques

Les Squamates sont des modèles particulièrement étudiés pour comparer les bénéfices et les coûts de la thermorégulation (Huey & Slatkin 1976 ; Huey & Pianka 1977 ; Huey 1982 ; Angilletta et al. 2002 ; Blouin-Demers & Nadeau 2005 ; Angilletta 2009). Toutefois l'intégration du compromis entre la thermorégulation et la balance hydrique reste encore sous-considérée. De plus, la grande majorité des études qui se sont intéressées aux réponses adaptatives des Squamates vis-à-vis de la disponibilité en eau de l'environnement ont été réalisées sur des espèces originaires d'environnements particulièrement desséchants. C'est le cas par exemple d'environnements secs (e.g., désert, « wet/dry tropic », etc) ou salés (e.g., océan, mangrove, etc) (Dmi'el 1972 ; Minnich 1982 ; Bradshaw 1997 ; DeNardo et al. 2004 ; Calsbeek et al. 2006 ; Davis & DeNardo 2007, 2009 ; Lillywhite et al. 2008a, 2008b, 2009, 2014 ; Bonnet & Brischoux 2008). Néanmoins, des contraintes liées à l'eau existent également en milieu tempérée (e.g., crues, sécheresse estivales) (Lorenzon et al. 1999). Des espèces comme les Squamates présentent des capacités de dispersion limitées (Aragon et al. 2010), et sont donc tributaires de périodes pendant lesquelles l'accès à l'eau est restreint voir absent. De plus, les modèles climatiques prédictifs prévoient une diminution globale de la disponibilité en eau en lien avec la diminution des précipitations et l'augmentation de la fréquence et de la durée d'épisodes climatiques extrêmes (e.g., canicule, sécheresse estivales, vague de chaleur, etc) (Easterling et al. 2000 ; IPCC 2014). C'est pourquoi il est également nécessaire de s'intéresser aux réponses physiologiques et comportementales mises en place par des espèces comme les Squamates vis-à-vis de la disponibilité en eau. C'est notamment le cas pendant la reproduction qui représente un état physiologique particulièrement demandeur pour ces espèces en raison d'importants besoins thermiques et énergétiques et d'une durée élevée (Bonnet et al. 2002 ; Ladyman et al. 2003 ; Le Galliard et al. 2003 ; Lorioux et al. 2013a). Le compromis entre thermorégulation et balance hydrique devrait donc être particulièrement contraignant pendant cette période.

5.3. Les Squamates et la reproduction

Jusqu'à présent, la majorité des études qui se sont intéressées au coût de la reproduction chez ces espèces se sont intéressé aux contraintes écologiques et énergétiques (Shine 1980 ; Doughty & Shine 1997 ; Bonnet et al. 1999 ; Lourdais et al. 2002a, 2002b ; Cox & Calsbeek 2009 ; Van Dyke & Beaupre 2011 ; Bleu et al. 2011). En effet, de nombreuses espèces assurent leur

reproduction à partir de leurs réserves énergétiques accumulées pendant plusieurs années (i.e., « capital breeders » ; Jonssön 1997 ; Bonnet et al. 1998), ce qui offre un lien fonctionnel entre reproduction courante et future (Stearns 1989 ; Doughty & Shine 1997 ; Bonnet et al. 2002). En parallèle, les contraintes liées à l'eau chez ces espèces a reçu moins d'intérêt. Or le développement des embryons nécessite une quantité considérable en eau (Dauphin-Villemant & Xavier 1986 ; Andrews 2004). Premièrement pour les femelles, cette demande importante des embryons peut représenter une véritable contrainte étant donné que la gestation est un processus long (i.e., plusieurs semaines à plusieurs mois) qui a lieu en général pendant l'été, lorsque la disponibilité en eau peut manquer. De plus, les femelles doivent assurer des besoins thermiques embryonnaires élevés en assurant une thermorégulation plus intense (Lourdais et al. 2002a ; Lourdais et al. 2004 ; Lorioux et al. 2013a), ce qui augmente la vitesse des pertes en eau (Mautz 1982; Guillon et al. 2014). Deuxièmement pour les descendants, des études expérimentales et descriptives chez des espèces ovipares suggèrent qu'un manque d'eau pendant le développement embryonnaire peut altérer la mobilisation de vitellus, et se terminer par la mort embryonnaire ou affecter la morphologie et les performances des nouveau-nés (Shine & Brown 2002; Aubret et al. 2005; Lourdais et al. 2007). De plus, des études expérimentales et sur le terrain ont montré que la disponibilité en eau pendant la gestation pouvait impacter la taille et la survie des descendants (Dauphin-Villemant & Xavier 1986; Marquis et al. 2008). L'eau est donc une ressource cruciale pendant la reproduction que ce soit pour les femelles (i.e., compromis intra-individuel) ou leurs descendants (i.e., compromis intergénérationnel). Dans ce doctorat, nous nous sommes donc intéressés à mettre en évidence un possible conflit entre la mère et les embryons résultant du compromis intergénérationnel pour l'eau.

6. Objectifs, hypothèses et prédictions

Ce doctorat s'inscrit dans un projet, le programme BOCAGE (Lourdais O. & Boissinot A., 2011-2014) qui vise à comprendre l'importance de la structure bocagère sur la richesse et la diversité de l'herpétofaune. L'un des objectifs de ce projet est notamment de comprendre l'aspect fonctionnel et proximal de la réponse des reptiles face à un bocage de plus ou moins bonne qualité. Pour ce doctorat, on s'est donc intéressé en particulier à comprendre l'importance de la ressource en eau dans les compromis physiologiques et comportementaux

liés à la thermorégulation et à la gestion de la balance hydrique pendant la gestation. Nous avons étudié une espèce de Squamate de milieu tempéré et caractéristique des milieux bordiers (haies, lisières) : la vipère aspic (*Vipera aspis*). Cette thèse se divise en 3 axes, chacun de ces axes étant associé à des objectifs, hypothèses et prédictions spécifiques.

Axe 1 – Compromis entre thermorégulation et pertes hydriques

Le premier objectif de ce doctorat est de clarifier l'influence de l'état physiologique sur les pertes hydriques. L'une des contraintes physiologiques de la thermorégulation chez les ectothermes est liée au compromis avec les pertes hydriques. Ce compromis est vraisemblablement plus contraignant lorsqu'un état physiologique demandeur en énergie impose aux individus un effort de thermorégulation plus intense. Nous avons donc d'une part cherché à quantifier les effets directs de différents états physiologiques sur les pertes hydriques ainsi que les effets indirects des conditions microclimatiques. D'autre part, nous avons voulu déterminer la réponse comportementale associée ; à savoir si les individus modèrent les contraintes physiologiques (pertes hydriques) par la sélection de microclimat.

Hypothèse. Le compromis physiologique qui existe entre les besoins thermiques et les pertes hydriques devrait dépendre de l'état physiologique et devrait être modéré par un ajustement comportemental.

Prédictions. Nous nous attendons premièrement à ce que les états physiologiques thermophiles tels que la mue, la digestion ou la gestation influencent l'intensité des pertes hydriques, à la fois directement (i.e., propriétés spécifiques ventilatoires et cutanées) et indirectement (i.e., thermorégulation plus élevée). Nous prédisons ensuite qu'un individu ajuste son choix d'exposition pour limiter les contraintes associées à un état physiologique thermophile (pertes hydriques supérieures) ; c'est-à-dire en sélectionnant une placette à la fois chaude pour assurer le processus physiologique et humide pour limiter les pertes hydriques.

Axe 2 - Contraintes physiologiques et morphologiques de la gestation

Au cœur des compromis intra-individuels et intergénérationnels se trouvent les contraintes liées à l'allocation des ressources (i.e., effort reproducteur), mais aussi les limites imposées par la morphologie des femelles (i.e., espace disponible de la cavité abdominale). Les espèces vivipares assurent l'ensemble du développement embryonnaire, ce qui impose des contraintes physiologiques et morphologiques spécifiques. Le second objectif de ce doctorat est donc d'étudier différentes contraintes physiologiques et morphologiques associées à la gestation. C'est-à-dire dans un premier temps de clarifier les contraintes physiologiques énergétiques et hydriques induites par la gestation, en lien avec le transfert d'eau vers la portée et les modifications morphologiques (distension cutanée) induites.

Hypothèse. La gestation devrait induire des contraintes énergétiques, hydriques et morphologiques importantes et influencées par l'effort reproducteur.

Prédictions. Nous nous attendons premièrement à ce que la demande énergétique (métabolisme) augmente pendant la gestation et soit influencée par l'effort reproducteur. Nous prédisons qu'en parallèle, le transfert d'eau vers la portée évolue exponentiellement avec la croissance somatique des fétus, et que cela influence le comportement des femelles (comportement de boisson). Ensuite, la prise en eau par les embryons devrait induire des modifications morphologiques (encombrement abdominal) qui influencent la distension de la peau des femelles. Au final, nous prédisons que l'augmentation du métabolisme et les modifications cutanées au cours de la gestation augmentent les pertes hydriques des femelles (ventilation et transpiration plus élevées), ce qui devrait en plus être accentué par le changement de comportement (augmentation du temps et de l'intensité de thermorégulation pendant la gestation).

Axe 3 – Manipulation des contraintes hydriques pendant la gestation

Le dernier objectif propre à ce doctorat est de déterminer l'impact d'une période de 20 jours de privation d'eau au cours de la gestation sur la physiologie des femelles et leurs performances de reproduction.

Hypothèses. La privation d'eau devrait induire un compromis intergénérationnel résultant en un possible conflit d'intérêt entre la mère et les embryons. Cela devrait déclencher un état de surcharge allostatique et affecter le phénotype des nouveau-nés.

Prédictions. Nous nous attendons à ce que les vipères soient déshydratées en réponse à une privation d'eau et que la déshydratation soit d'autant plus marquée lorsque les femelles sont gestantes comparé aux femelles non-reproductrices. Nous prédisons également que l'effort reproducteur influence l'intensité de la déshydratation. Enfin, les performances reproductrices devraient être négativement impactées par la privation d'eau en milieu de gestation. Ensuite, nous nous attendons à ce que la gestation impose un niveau de sécrétion de CORT augmentée (i.e., charge allostatique) et que ce niveau de sécrétion soit accrue dans le cas d'une privation d'eau (i.e., surcharge allostatique). Au final nous prédisons que des concentrations de CORT plus élevées en réponse à la privation d'eau impactent négativement le phénotype des jeunes.

Matériel & Méthodes

1. Le modèle d'étude : la vipère aspic

1.1. Biologie générale

La vipère aspic (*Vipera aspis* ; Linnaeus 1758) est un serpent qui appartient à la famille des Viperidae et à la sous-famille des Viperinae (i.e., « vraies » vipères). C'est un serpent relativement court avec une taille totale moyenne de 55cm et une masse moyenne de 150g (Naulleau 1997). Cette espèce est caractérisée par un polymorphisme de colorations dorsales important, allant du jaune au rouge brique, et du gris au marron clair (Fig. 8) (voir l'ouvrage de Vacher & Geniez (2010) pour une mosaïque de l'ensemble des patterns dorsaux). La vipère aspic se différencie facilement des autres vipères Européennes grâce à son museau légèrement retroussé (Fig. 8). On reconnaît aujourd'hui 4 sous-espèces (*Vipera aspis aspis*, *V. a. hugyi*, *V. a. francisciredi* et *V. a. zinnikeri*) qui se distinguent en particulier par leur répartition (Vacher & Geniez 2010). La vipère aspic est une vipère typiquement médio-européenne présente en France, Suisse, Italie et au Nord de l'Espagne (Fig. 9). On la trouve en particulier dans des

Figure 8. Vipère aspic femelle en thermorégulation.

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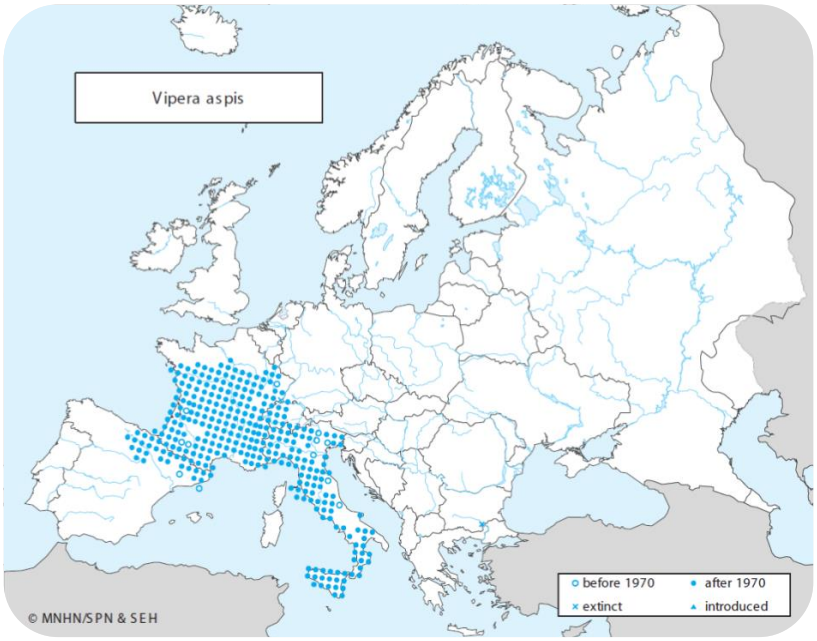

Figure 9. Répartition de la vipère aspic (*Vipera aspis*) en Europe.

D'après Gasc et al. (1997)

habitats assez ouverts offrant des possibilités de thermorégulation et des refuges. Elle occupe typiquement les milieux bordiers et fréquente les réseaux de haies dans les zones bocagères mais aussi les landes (sèches ou mésophyles), les parcelles abandonnées (friches ou anciennes vignes). On la trouve également dans des zones anthropisées (bords de lignes de chemin de fer, bords de chemin).

Les travaux de thèse de Guillon (2012) ont notamment permis de clarifier les déterminants proximaux de la répartition chez cette espèce. Des mesures physiologiques couplées aux données environnementales (température et humidité) permettent ainsi de modéliser l'occurrence de la vipère aspic en France. Ces travaux ont permis d'une part de mettre en évidence que l'espèce est principalement limitée au Nord par son niveau de performance de reproduction puisque la phénologie est contrainte par des opportunités thermiques favorables moins fréquentes. D'autre part au Sud ce sont les contraintes énergétiques et hydriques qui limitent son occurrence ; à savoir un coût de maintenance et des pertes hydriques trop élevées en réponse à des conditions estivales chaudes et sèches (Fig. 10).

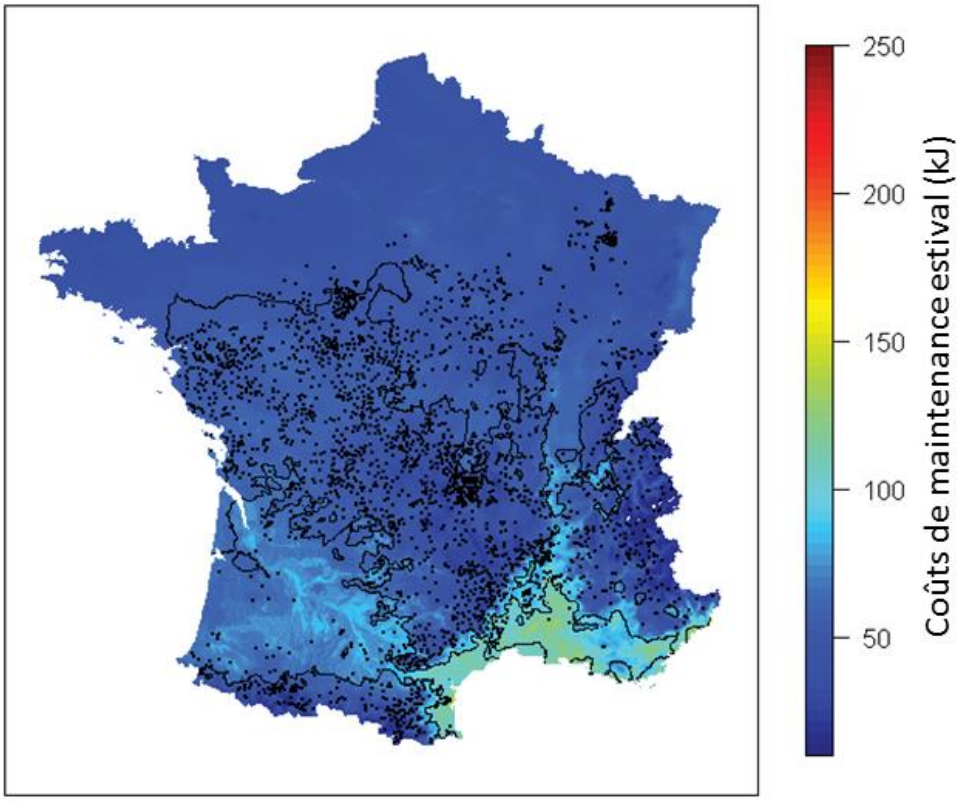

Figure 10. Prédictions des coûts de maintenance énergétiques estivales chez la vipère aspic à l'échelle de la France. Les points noirs correspondent aux données de présence observée et les lignes noires représentent les limites de la niche réalisée (modélisée à partir des données de présence). D'après Guillon (2012).

1.2. Le cycle de vie et la physiologie

De nombreuses études ont été réalisées sur cette espèce au Centre d'Etudes Biologiques de Chizé (CEBC), ce qui offre un recul conséquent sur son cycle de vie et sa physiologie. Tout au long de son cycle de vie, la vipère aspic alterne entre des phases actives de début mars à fin octobre, suivies de phases d'hibernation de fin octobre à fin février (Saint Girons 1952 ; Lourdais 2002). Pendant la phase active, les individus vont assurer différents processus physiologiques qui vont imposer aux individus à la fois une dépense d'énergie et une augmentation des préférences thermiques (i.e., thermophilie). Ces processus majeurs incluent notamment la digestion, la mue et la reproduction qui sont connus pour être des états thermophiles chez les Squamates (Taylor & Davies 1981 ; Blem & Zimmerman 1986 ; Andrade et al. 1997 ; Gibson et al. 1989 ; Ladyman et al. 2003 ; Lorioux et al. 2013a). Ces états physiologiques spécifiques sont essentiels aux performances individuelles (acquisition de la ressource, succès reproducteur, croissance). Ils ont été au cœur de mes travaux de thèse (notamment la reproduction) et sont décrits par la suite.

1.2.1. La digestion

La vipère aspic est un serpent venimeux qui chasse à l'affût, c'est-à-dire qu'elle se déplace peu en attendant qu'une proie passe à sa portée (Naulleau 1983, 1997). Elle se nourrit principalement de micromammifères (mulots sylvestre, *Apodemus sylvaticus* et campagnols des champs, *Microtus arvalis*) qui sont particulièrement vulnérables à son venin. En conditions naturelles, les vipères aspics se nourrissent tout au long de leur phase active, bien que les phases d'alimentation soient plus intenses entre fin avril et début juin (Saint Girons 1979 ; Naulleau 1983). Comme les autres processus physiologiques, l'efficacité digestive (i.e., temps de digestion et extraction d'énergie) est déterminée par la température corporelle des individus (Stevenson et al. 1985 ; Angilletta et al. 2002 ; Blouin-Demers et al. 2003 ; Lelièvre et al. 2010). En nature, la digestion chez la vipère aspic dure en général entre 5 et 6 jours (Naulleau 1983).

1.2.2. La mue

Pendant la phase active, les individus vont également alterner des phases de mue. La mue est relativement fréquente chez les jeunes puisqu'elle est liée en partie à la croissance. Chez les adultes en conditions naturelles, la mue se produit en général tous les 2 à 3 mois, et un cycle complet dure entre 2 et 3 semaines (Saint Girons 1980). Au cours du cycle de mue, différents sous-stades caractéristiques vont se succéder, et sont facilement identifiables grâce à l'activité lymphatique dans l'espace entre la future et l'ancienne peau (i.e., ventre- ou œil-bleu ; Fig. 11).

Figure 11. Vipère aspic femelle pendant le cycle de mue (œil-bleu).

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1.2.3. La gestation

La reproduction est probablement le processus physiologique le plus étudié chez la vipère aspic (Saint Girons 1957 ; Naulleau 1971, 1981, 1986, 1997 ; Bonnet et al. 1998, 1999, 2002 ; Lourdais et al. 2002a, 2002b), puisque cette espèce est un reproducteur sur capital (Bonnet et al. 1998). En effet, les femelles accumulent des réserves énergétiques pendant 2 à 3 ans avant de se reproduire (Bonnet et al. 1998, 1999, 2002 ; Lourdais et al. 2002a, 2002b). Chez les femelles, la reproduction commence en mars-avril avec l'accouplement et la vitellogénèse (synthèse de vitellus). Ensuite, elles ovulent entre fin mai et fin juin, avant d'entrer en gestation pendant environ 3 mois (jusque fin août / début septembre). L'ovulation est facilement détectable grâce à une mue pré-ovulatoire (Lorioux et al. 2013b). Les travaux précédents de thèse de Lorioux (2011) ont permis de mettre en évidence les modifications physiologiques (métabolisme, niveaux hormonaux, etc) et comportementales associées à la gestation chez la vipère aspic, et notamment l'augmentation de l'effort de thermorégulation (Lorioux et al. 2013a). L'hypothèse résultante de ces travaux est clé dans le contexte de ce doctorat puisqu'elle

vise à comprendre la dynamique des contraintes thermiques et hydriques pendant la gestation à la fois du point de vue de la femelle et des embryons (Fig. 12).

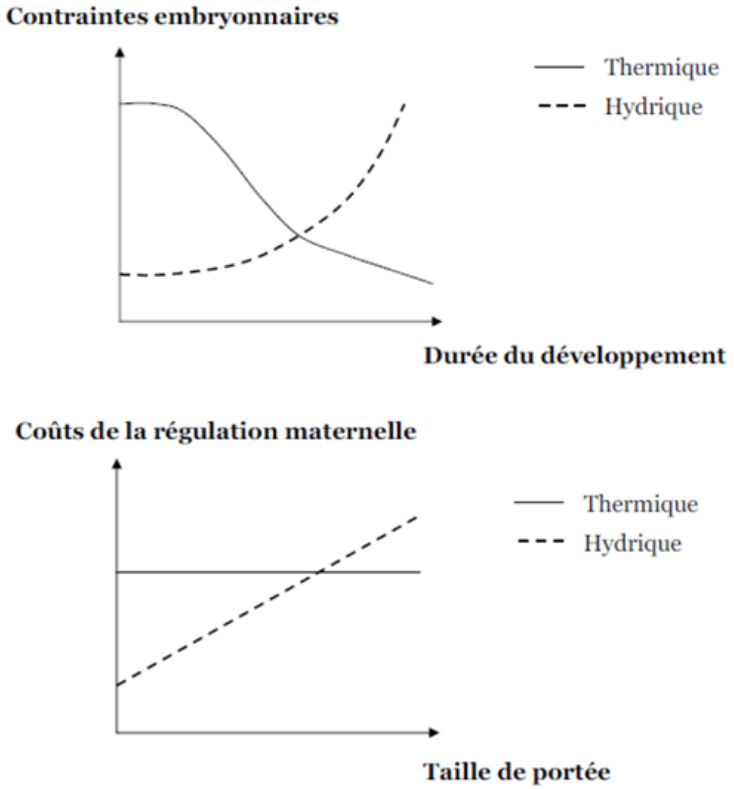


Figure 12. Dynamique des contraintes thermique et hydrique au cours de la gestation de la vipère aspic. (a) Les contraintes embryonnaires thermiques diminuent au cours du développement alors que les besoins en eau des embryons augmentent exponentiellement. (b) la nature des coûts maternelles associés varient en fonction des contraintes (thermique ou hydrique) : la régulation thermique par la mère est non-dépréciable car non divisible entre les embryons alors que la régulation hydrique est directement influencée par la fécondité. D'après Lorioux (2011).

1.3. Capture et maintenance

Au cours de ce doctorat, des individus ont été capturés de façon temporaire (avec relâché sur le lieu de capture) dans différents sites de l'Ouest de la France, dans les départements de Loire-Atlantique, du Maine et Loire, de la Vendée et des Deux-Sèvres. Au total, ce sont 62 mâles et 92 femelles qui ont été capturés entre avril et juin 2012 et entre mai et juin 2013. Sur le terrain chaque individu a été marqué temporairement avec des marqueurs colorés et la position exacte a été relevée. Une fois au laboratoire, les individus ont été marqués définitivement par une procédure classique de marquage d'écailles, puis la taille (SVL ± 0.5cm pour « snout-vent length ») et la masse corporelle (± 0.1g) ont été mesurées. Les individus ont ensuite été séparés par sexe et par statut reproducteur dans 10 enclos semi-naturels (4 x 4 x 2.5m) en extérieur du

laboratoire (Fig. 13). Ces enclos sont végétalisés et équipés de plusieurs abris (plaques ondulées en fibrociment, parpaings ou tuyaux souterrains) (Fig. 13). Un système d'arrosage automatique permet une vaporisation d'eau pendant 30min tous les jours. Dans les enclos extérieurs, les individus ont été nourris avec des souris une fois toutes les 2 semaines.

Figure 13. Enclos extérieurs dans lesquels ont été maintenues les vipères.

Deux semaines avant les séries de mesures descriptives (pertes hydriques, comportements), les individus étaient ramenés dans les salles d'élevage et placés dans des boites individuelles (30 x 16 x 10cm) avec un gradient de température (20-33°C) fourni par un câble chauffant 6h par jour, un abri et de l'eau *ad libitum* (Fig. 14a). Dans le cadre de manipulation expérimentale (disponibilité en eau), les animaux ont aussi été maintenus sur d'assez longues périodes au laboratoire. Pour cela, nous avons placé les serpents dans des cages expérimentales (100 x 30 x 35 cm) qui comportent un point de chauffe fourni par une ampoule (75W) 5h par jour (de 10:00 à 15:00), un abri principal à l'opposé de la zone de chauffe (demi cylindre PVC percé de 2 trous, diamètre = 15 cm, longueur = 37 cm), 3 abris intermédiaires (demi cylindre PVC, diamètre = 15 cm, longueur = 25 cm), et de l'eau *ad libitum* (Fig. 14b). Les serpents étaient répartis par groupe de 3-4 individus par cage étant donné la place disponible et parce que cette

espèce ne présente pas de comportement agonistique (Lorioux et al. 2013a). Une fois l'ensemble des séries mesures réalisées (novembre 2012 et octobre 2013), tous les individus ont été relâchés au site exact de capture. Les femelles vipères post-parturientes sont en général très émaciées (Bonnet et al. 1999, 2002), c'est pourquoi nous avons pris soin de les nourrir avant de les relâcher avec leur portée.

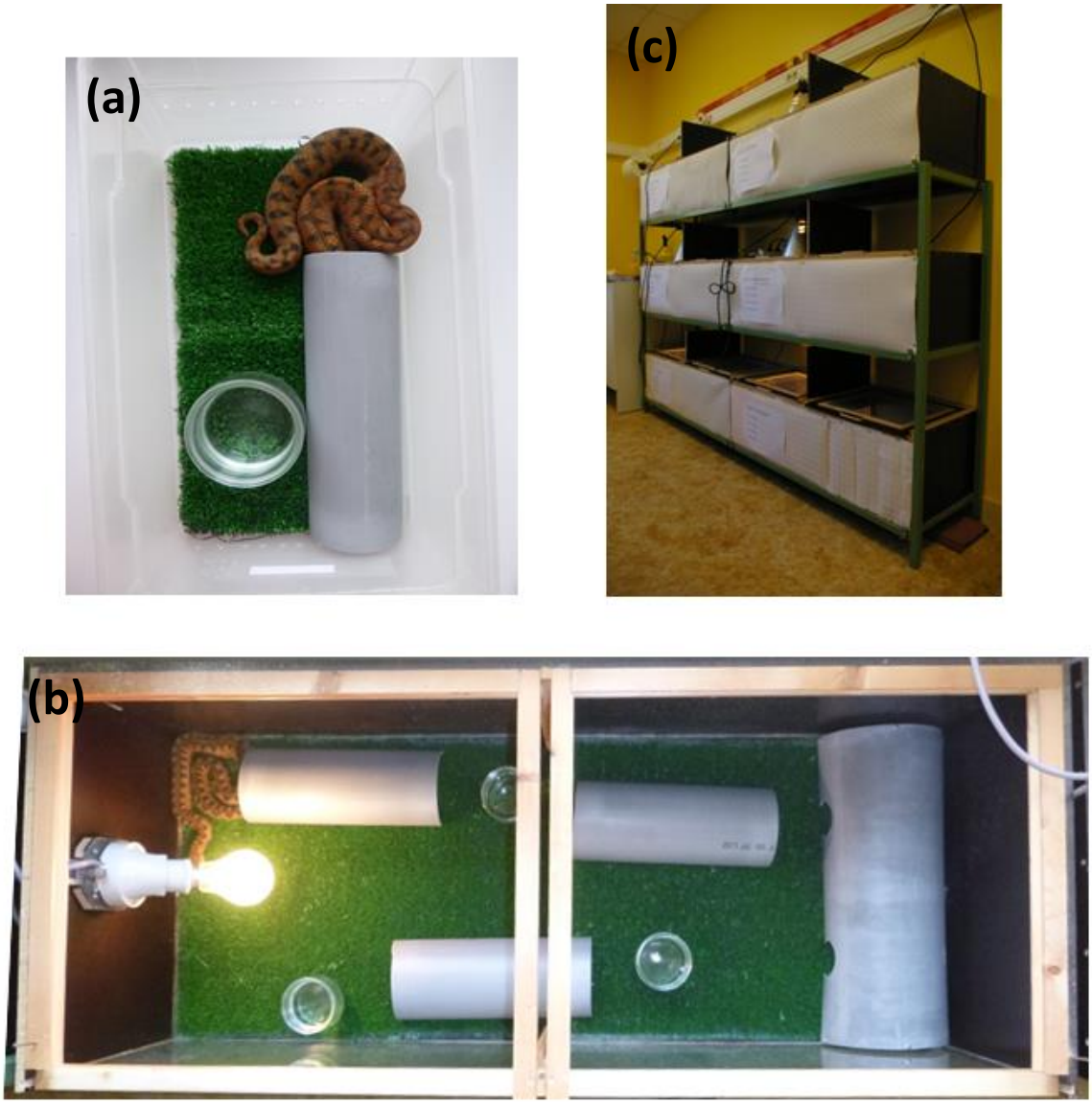


Figure 14. Maintenance des individus de vipères aspic (a) dans les cages en plastique, ou (b) dans les cages expérimentales. (c) Les 16 cages expérimentales sont rassemblées sur 3 meubles différents.

2. Compromis entre thermorégulation et pertes hydriques totales

2.1. Pertes hydriques totales et métabolisme

Pour quantifier le compromis entre thermorégulation et pertes hydriques, les 5 premiers mois de ce doctorat ont été menés par l'installation et la mise en place d'une chaîne de calorimétrie indirecte correspondant à un système de respirométrie ouvert à 8 voies. Ce système nous a permis de mesurer les pertes hydriques totales par évaporation et aussi la consommation d'oxygène (VO_2) qui est un bon indice du métabolisme (Withers 1977). Nous avons mesuré les pertes hydriques totales et la VO_2 d'individus dans les trois états physiologiques demandeurs en énergie précédemment décrits (i.e., gestation, digestion, mue) que nous avons comparés à des individus non-reproducteurs, post-digestifs et post-mues (i.e., témoins). Nous avons également déterminé l'effet des conditions microclimatiques en combinant 2 températures ambiantes (T_a = 20 et 30°C) et 2 humidités absolues (ρ = 14 ou 25 $gH_2O.m^{-3}$). Nous avons pris soin de randomiser à la fois les états physiologiques et l'ordre des conditions microclimatiques.

2.1.1. Déroulement d'une session de mesure

Chaque session de mesure suit systématiquement le même déroulement : 7 vipères sont sélectionnées au hasard et placées dans un bocal de verre étanche de 500ml équipé d'un bouchon avec une entrée et une sortie d'air (Fig. 15a). Le 8ème bocal est vide et sert de référence pour les calculs d'échanges gazeux. Les bocaux sont ensuite placés dans un caisson thermique

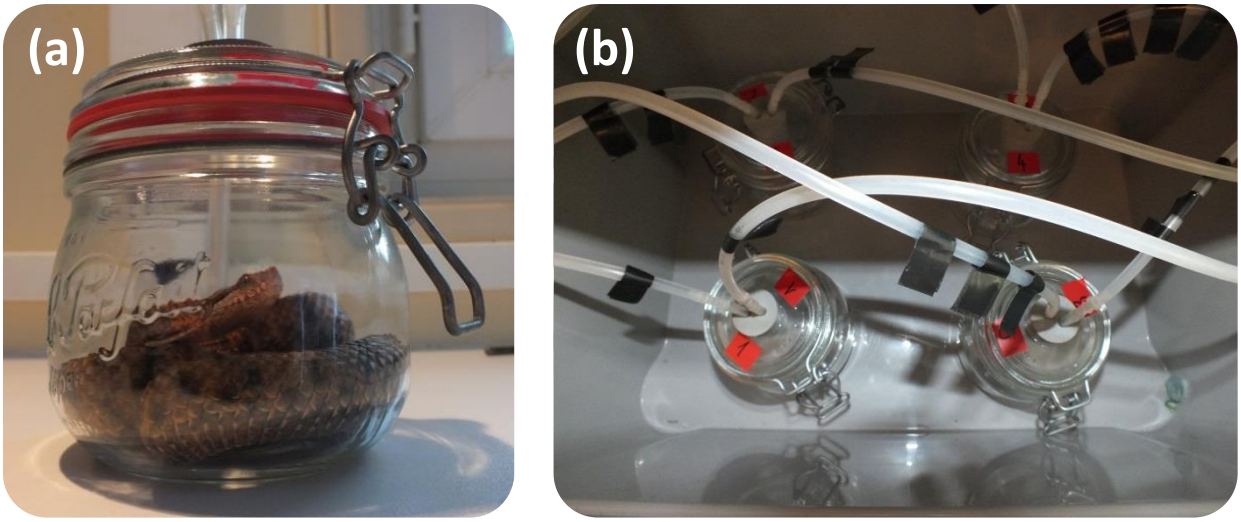


Figure 15. Mise en place des vipères (a) en bocal (500ml) et (b) connectées aux systèmes d'arrivée et de sortie d'air.

(±1°C, Cryosystem, air conditioned system Carel MasterCella) et immédiatement reliés au système d'arrivée et de sortie d'air (Fig. 15b).

Au préalable, nous produisons de l'air sec et sans dioxyde de carbone (CO_2) en utilisant un purificateur d'air industriel (#CO2F-70h, Texol Products Ltd, Dundee, Scotland). L'air est envoyé dans le système avec un débit global de 1170ml.min^{-1} contrôlé par un régulateur massique de débit (#UFC-1100, Unit Instruments, Yorba Linda, CA) et une unité 21X. Afin de contrôler l'humidité de l'air, nous utilisons un système de saturation en eau en contrôlant la température de point de rosée de l'air (T_{DP}) (Fig. 16a). L'air sec passe dans deux colonnes d'eau entourées de câbles chauffants et isolées par du Styrodur® (BASF Corporation, Ludwigshafen, Allemagne) puis dans un bocal vide qui récupère l'excès de condensat (Fig. 16a). L'ensemble du système de saturation est placé dans une enceinte thermique (#400 DG LMS ltd, Sevenoaks, Kent, UK) réglée à la valeur cible du point de condensation (Fig. 16a). L'air d'humidité connue est ensuite séparé au niveau du FlowBar-8 qui correspond à 8 régulateurs / indicateurs de débit disposés en parallèle (FlowBar-8, Sable Systems, Las Vegas, USA) (Fig. 16b). Cet appareil permettant de contrôler et de mesurer le débit de l'air envoyé vers chaque cage (moyenne ± erreur-type : 82.7 ± 6.6ml.min^{-1}), il a préalablement été calibré grâce à un système de bulles de savon et de verrerie très précise (Bubble-O-Meter, 1-10-500ml, Dublin, Ohio, USA). L'air sortant de chaque cage est ensuite séquentiellement envoyé vers les analyseurs grâce à un échangeur (RM-8 Multiplexer, Sable Systems, Las Vegas, USA) (Fig 16b) programmé selon un cycle précis (Fig. 17). La température de point de rosée de l'air sortant de chaque cage est ensuite analysée par un hygromètre (RH 300, Sable Systems, Las Vegas, USA) (Fig. 16c). Ensuite, l'air est desséché en passant à travers une colonne de driérite avant de passer dans l'analyseur CO_2 (CA10, Sable Systems, Las Vegas, USA) et pour finir dans l'analyseur O_2 (FC10A, Sable Systems, Las Vegas, USA) (Fig. 16d).

2.1.2. Calibration des analyseurs et récupération des données

L'hygromètre est calibré une fois avant la série de mesure avec un air sec (azote pur : T_{DP} = -40°C) et un air d'humidité connue (T_{DP} = 15°C). De même pour l'analyseur CO_2 qui est calibré avec un air sans CO_2 (azote pur : CO_2 = 0ppm) et un air de quantité connue et calibrée (CO_2 = 6000ppm). L'analyseur O_2 est quant à lui calibré avant chaque session de mesure en utilisant de l'air extérieur (O_2 = 20.95%).

Avant la phase d'enregistrement, les individus sont laissés dans le système pendant au moins 3h afin de stabiliser leur température corporelle et leur activité. Pendant la phase d'enregistrement, les signaux analogiques de chaque analyseur (O_2, CO_2, T_{DP}, Débit, Pression barométrique, Température des cages) sont simultanément reçus et transmis à l'ordinateur toutes les secondes, grâce à une interface spécifique (UI-2, Sable Systems, Las Vegas, USA). L'enregistrement des différents canaux analogiques des différents analyseurs se fait ensuite via le logiciel ExpeData via une interface graphique spécifique selon un cycle préalablement défini (Fig. 17). L'ensemble du cycle d'enregistrement est répété au moins 2 fois pour s'assurer que les animaux sont calmes au moment des mesures. Des équations dérivées de celles de Withers (1977) et adaptées à notre setup expérimental nous permettent ensuite de calculer les pertes hydriques totales (en $mg.h^{-1}$) et la VO_2 (en $ml.h^{-1}$).

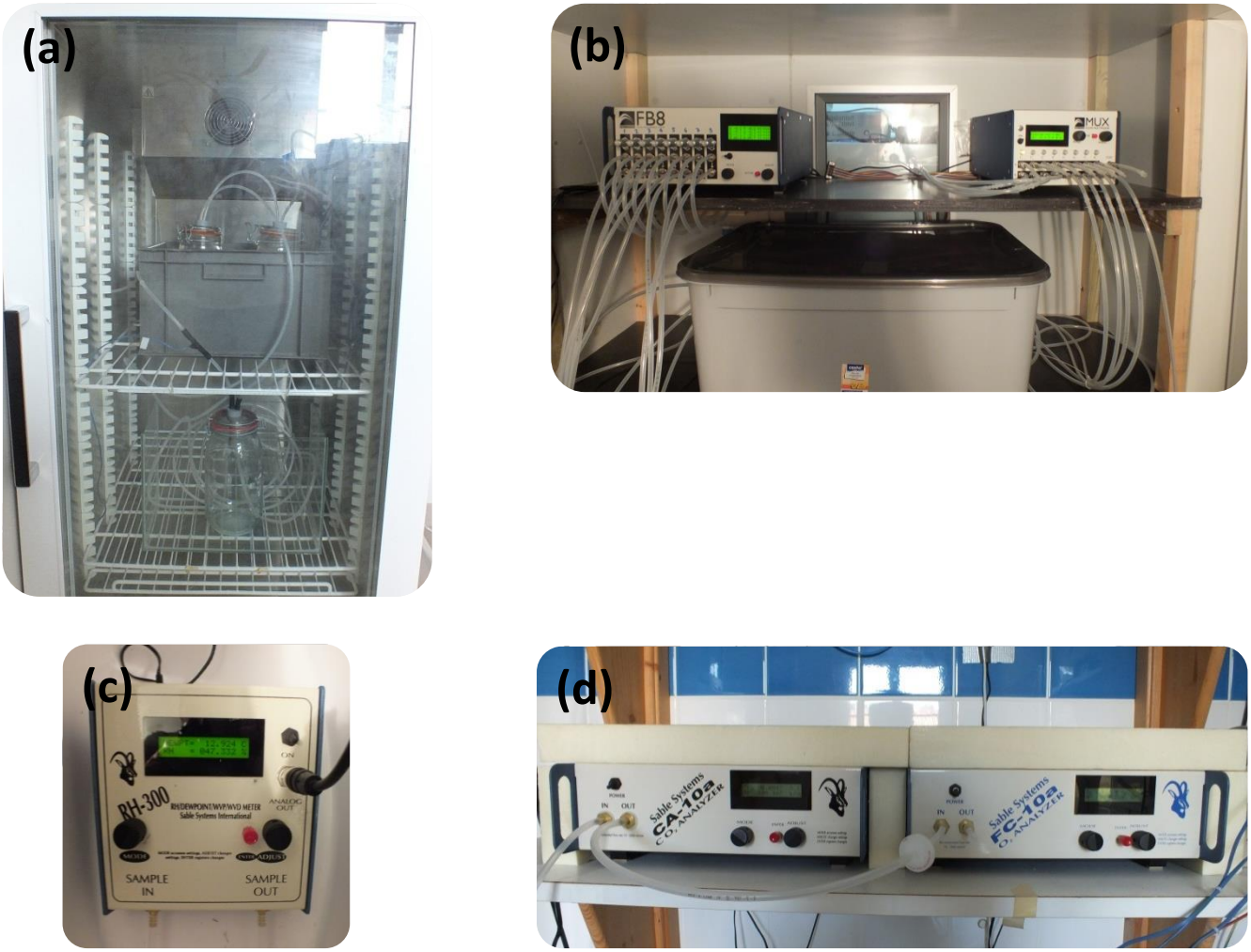


Figure 16. Composants principaux de la chaîne calorimétrique indirecte (système ouvert à 8 voies) avec (a) le système de saturation de l'air en eau, (b) à gauche le régulateur / indicateur de débit et à droite l'échangeur de gaz, (c) l'hygromètre et (d) les analyseurs CO_2 et O_2.

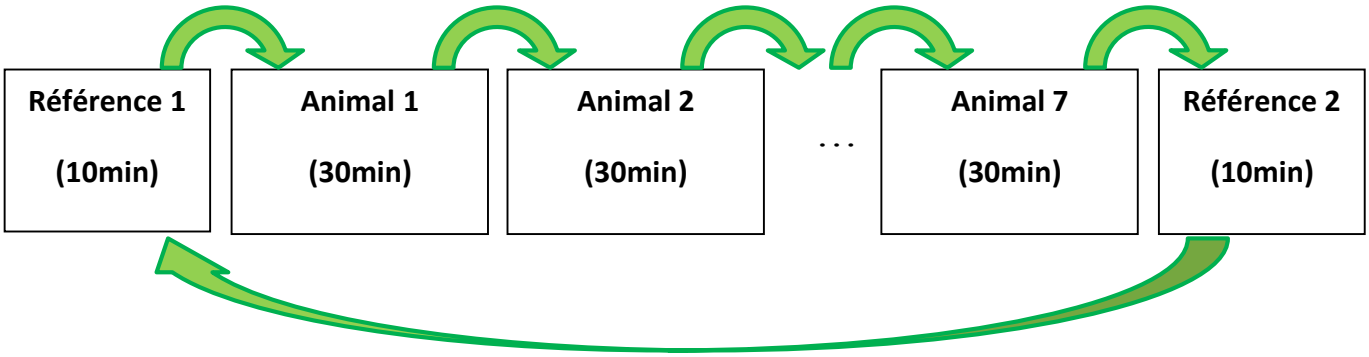


Figure 17. Cycle utilisé dans nos mesures de pertes hydriques et de métabolisme. On fait passer de l'air dans toutes les cages et on va séquentiellement analyser les gaz des différentes cages pendant un temps donné (10min pour les baselines et 30min pour les animaux).

2.1.3. Equations respirométriques

- **Pertes hydriques totales**

Les pertes hydriques totales sont calculées à partir de l'humidité absolue (ρ) sortante d'une cage avec un animal (ρ_{out}) à l'humidité absolue dans la cage vide (ρ_{in}) :

Perte hydriques totales (en mg.h^{-1}) = Débit x ($\rho_{out} - \rho_{in}$) x 1.44 x 0.001

ρ est calculée à partir de la pression de saturation de vapeur d'eau (PSV, en kPa) (voir équation au 8ème degré dans Flatau et al. (1992)), de la température de point de rosée (T_{DP}, en °C) et de la pression barométrique (Pb, en kPa) :

ρ (en gH_2O.m^{-3}) = [216.7 x PSV / (T_{DP} + 273.15)] x [1013 (T_{DP} + 273.15)] / [Pb x 273.15]

- **Consommation d'oxygène (VO_2)**

La VO_2 est calculée à partir de la fraction entrante d'O_2 et de CO_2 dans la cage vide (F_{in} O_2 et F_{in} CO_2) et la fraction sortante d'O_2 et de CO_2 de la cage avec un animal (F_{out} O_2 et F_{out} CO_2):

VO_2 (en ml.min^{-1}) = Débit x (F_{in} O_2 – F_{out} O_2) – [F_{out} O_2 x (F_{out} CO_2 – F_{in} CO_2)] / (1 – F_{out} O_2)

2.2. Dispositif comportemental de choix microclimatique

Afin de tester notre hypothèse selon laquelle les vipères optimiseraient leur choix d'exposition pour un état physiologique hydriquement contraignant, nous avons créé un dispositif comportemental (Michautron 4X). Ce dispositif comportemental correspond à quatre compartiments reliés entre eux de sorte que les animaux puissent circuler librement (Fig. 18).

Figure 18. Le Michautron 4X vu du dessus destiné à déterminer la sélection de conditions microclimatiques par les vipères. Pour cette session par exemple les conditions microclimatiques sont les suivantes pour la boite 1 (Froid – Humide), la boite 2 (Chaud – Humide), la boite 3 (Chaud – Sec) et la boite 4 (Froid – Sec).

Chaque compartiment est composé de 2 boites en plastique (30 x 20 x 10cm) encastrées l'une dans l'autre et séparées par une grille pour permettre de créer des conditions microclimatiques contrastées. Les quatre compartiments sont en effet associés à quatre conditions microclimatiques que l'on catégorise via la combinaison de 2 températures (Chaud ou Froid) et de 2 humidités (Sec ou Humide). Afin de créer des conditions de température différentes, le dessous de chaque boite a été équipé avec un câble chauffant de 25W recouvert par du papier aluminium adhésif pour homogénéiser la température dans le compartiment. Un thermostat

(Thermo Control PRO II, Lucky Reptile, Allemagne) et sa sonde permettent de déterminer si la température du compartiment est froide (i.e., T_a = 25°C) ou chaude (i.e., T_a = 30°C). Pour créer des conditions d'humidité différentes, nous avons utilisé des morceaux de 2 x 2cm d'éponges plates, bien humidifiées et posées sur le câble chauffant afin de diffuser de la vapeur d'eau dans le compartiment. Pour chaque session de mesure (i.e., un animal), la combinaison des différentes conditions microclimatiques dans chaque compartiment est déterminée aléatoirement. Chaque compartiment est équipé avec 2 thermocouples (Type T standard) tous connectés à une centrale 21X pour suivre l'évolution de conditions thermiques en direct. En parallèle, nous avons programmé 3 thermo-hygrochrons (DS1923, Maxim Integrated Products, Sunnyvale, CA, USA) dans chaque compartiment pour enregistrer les conditions thermiques et hydriques toutes les 10min. Nous avons ainsi pu estimer un coefficient de variation dans les boites à la fois pour les conditions thermiques (CV = 5,2%) et les conditions hydriques (CV = 3,2%).

Pour éviter tout processus d'apprentissage ou de communication, chaque individu a été testé pour un seul état physiologique (i.e., gestation, mue, digestion, ou témoin) et les grilles ont été nettoyées avec de l'eau et un détergent industriel entre chaque individu. De la même façon, pour éviter un effet horaire, nous avons standardisé chaque session de mesure. Pour chaque session, nous lançons le système (allumage des câbles chauffants et mise en place des éponges) à 9h du matin. Après 1h30 (temps préalablement mesuré pour que les conditions thermiques et hydriques se stabilisent), l'animal est placé dans un compartiment sélectionné au hasard. Il est laissé libre d'explorer dans le système pendant 6h. A la fin de la session (16h30), la position de l'animal dans le Michautron 4X est notée (Stahlschmidt et al. 2011) et sa température de surface est mesurée avec un pistolet thermique infrarouge (Raytek Corporation, Santa Cruz, USA), en suivant les recommandations de Hare et al. (2007). Bien que cette méthode ne donne pas une mesure précise de la température corporelle, il s'agit quand même d'un bon indicateur et non-invasif.

3. Manipulation expérimentale de l'accès à l'eau

3.1. Protocole expérimental

Nous avons cherché à déterminer les effets d'une privation d'eau sur la physiologie des femelles au cours de la gestation et sur les performances reproductrices. Nous avons pour cela utilisé une approche expérimentale en manipulant l'accès à l'eau pour des vipères femelles gestantes (n = 29) et non-reproductrices (n = 29). Les individus étaient répartis aléatoirement dans les cages expérimentales (voir description au-dessus ; Fig. 14b et 14c) avec 3-4 individus par cages. Pour chaque statut reproducteur, nous avons privé d'eau la moitié des femelles (n = 14) pendant 20 jours, ce qui correspond typiquement à la période de sécheresse estivale en milieu tempéré. En parallèle, les autres femelles avaient de l'eau *ad libitum* avec 4 ramequins d'eau par cage et une vaporisation d'eau dans la cage une fois tous les 2 jours. Pendant toute la phase expérimentale, les autres conditions d'élevage sont restées les mêmes que celles précédemment décrites.

Nous avons tout d'abord déterminé les effets d'une privation d'eau sur la physiologie des femelles. Pour ce faire, nous avons considéré différents indicateurs physiologiques de la balance hydrique. Nous avons mesuré d'une part les variations de masses corporelles, puisque la masse est connue chez les reptiles comme un très bon indicateur des changements de la balance hydrique (DeNardo et al. 2004; Lillywhite et al. 2008a, 2008b, 2012). D'autre part, nous avons mesuré différents paramètres sanguins en réalisant des prises de sang en début et en fin d'exposition au traitement hydrique (i.e., privation d'eau *versus* témoins). Nous avons considéré à la fois des indicateurs physiologiques de la balance hydrique (i.e., osmolalité plasmatique et hématocrite) et le niveau de CORT (concentration de CORT basale et induite par un stress). Pour cela, le sang est prélevé directement dans le cœur (localisé au préalable avec une légère palpation) en utilisant des seringues de 1ml et des aiguilles fines (Microlance 27G) (Fig. 19a). Nous récupérons ensuite 20µl (2 x 10µl) de sang total dans 2 microcapillaires pour mesurer l'hématocrite. Le reste du sang total est placé dans un eppendorf de 0.675ml et mis à centrifuger pendant 3min à 3000tours.min^{-1} (Fig. 19b). Le plasma est ensuite séparé du culot (cellules sanguines) en utilisant une micropipette. Les échantillons sont conservés dans un congélateur à -28°C en attendant les dosages.

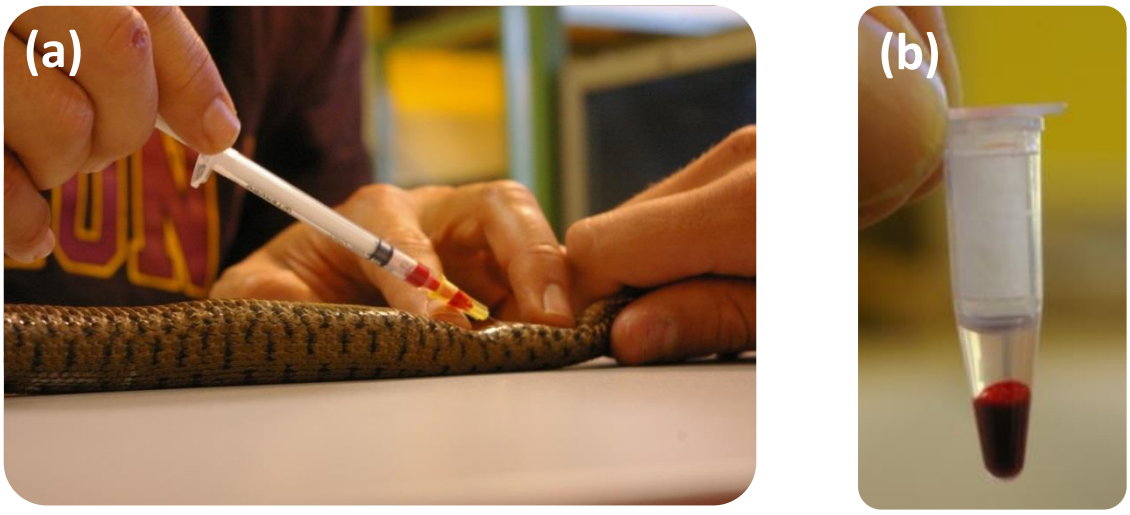


Figure 19. Prise de sang intra-cardiaque chez une vipère aspic. (a) L'avant du corps est rentré dans un tube plastique pour éviter tout risque pour l'opérateur ou pour l'animal et l'arrière du corps est maintenu. (b) Le plasma est séparé du culot après centriguation (3min à 3000tours.min^{-1}).

3.2. Mesure des indicateurs sanguins de la balance hydrique

Deux indicateurs physiologiques de la balance hydrique ont donc été mesurés, l'hématocrite et l'osmolalité plasmatique (Peterson 2002). L'hématocrite correspond à la proportion relative de cellules sanguines par rapport au plasma. Pour le mesurer, nous avons donc centrifugé les 2 microcapillaires de sang total 3min à 3000 tours.min^{-1} dans une centrigeuse adaptée. Nous avons ensuite mesuré la longueur totale de sang (L_{total}) et la longueur de la partie occupée par les cellules sanguines (L_{cell}) dans chaque microcapillaire avec un pied à coulisse (± 0.01mm). L'hématocrite (en %) a été obtenu avec le calcul suivant : Hématocrite = L_{cell} / L_{total} x 100. Pour chaque prise de sang, nous avons moyenné les deux valeurs d'hématocrite par individu. L'osmolalité correspond au nombre d'osmoles de solutés (i.e., quantité d'ions et de particules libres dans le sang) par kilogramme de plasma sanguin. Elle augmente donc lorsque le volume d'eau dans le sang diminue (i.e., déshydratation). L'osmolalité plasmatique (en mOsm.kg^{-1}) a été déterminé à partir de triplicats (10µl) de plasma sanguin à l'Arizona State University (Tempe, AZ 85287-4501, USA). Pour cela, nous avons utilisé un osmomètre à tension de vapeur (model 5500, Wescor, Logan, UT, USA), qui a préalablement été calibré à l'aide de standards d'osmolalités connues (290 et 1000mOsm.kg^{-1}).

3.3. Les concentrations de corticostérone

Pour estimer les différents niveaux de charges ou de surcharges allostatiques, nous avons mesuré la concentration plasmatique de CORT basale et de CORT induite par un stress. Les niveaux de CORT suivent un rythme journalier chez les vertébrés (Dauphin Villemant & Xavier 1987) et peuvent aussi être influencés par la température corporelle chez les Squamates (Tyrell & Cree 1998), nous avons donc pris soin de noter l'heure et de mesurer la température de surface des individus avec un pistolet thermique infrarouge (voir méthode au-dessus). Pour mesurer la CORT basale, les prises de sang ont été réalisées en moins de 3min, ce qui correspond au temps mesuré chez la plupart des vertébrés avant que la concentration de CORT n'augmente en réponse au stress de la manipulation (Romero & Wikelski 2001). Immédiatement après cette première prise de sang les vipères ont été placées dans une boite transparente 1h à température ambiante (i.e., 25°C) (Fig. 20). Ce protocole a été préalablement mis en place et documenté pour induire une réponse au stress chez la vipère aspic (Lorioux 2011). Une seconde prise de sang a donc été réalisée à la suite de ce protocole pour déterminer la concentration de CORT induite par un stress.

Figure 20. Protocole de réponse au stress chez les vipères. L'animal est maintenu dans une boite transparente pendant 1h à 25°C.

Les concentrations plasmatiques de CORT ont ensuite été mesurées par dosage radio-immunologie (RIA pour « radioimmunoassay ») au laboratoire en suivant un protocole bien établit (Lormée et al. 2003). On a tout d'abord extrait la CORT à partir de 50µl de plasma dans de l'éther. Les extraits de CORT à doser (CORT « froide ») sont mélangés à de la CORT marquée au tritium (^{3}H-CORT) et sont mis en compétition avec un anticorps spécifique de la CORT dans un milieu réactionnel avec des conditions précises (température, temps de réaction, etc.) pour former des complexes CORT-Anticorps. Ainsi, plus l'extrait est concentré en CORT « froide », moins il y aura de complexe ^{3}H-CORT-Anticorps. Une fois l'incubation terminée, les fractions libres de ^{3}H-CORT ou de CORT « froides » sont séparées des complexes CORT-Anticorps par adsorption sur charbon Dextran. Le milieu ne contenant finalement plus que les complexes CORT-Anticorps, ils sont comptés sur un compteur à scintillations. Après ajout de

scintillant, les complexes ^{3}H-CORT-Anticorps émettent un nombre de photons fluorescents proportionnel à la radioactivité du ^{3}H et donc inversement proportionnel à la concentration de CORT à doser initialement.

4. Suivi de la reproduction

4.1. Suivi échographique

La gestation est un état particulièrement contraignant pour la femelle et sur lequel j'ai porté une attention spécifique. Nous avons utilisé un suivi échographique sur les femelles reproductrices au cours de la gestation. Pour cela, nous avons utilisé un échographe (Sonosite microMaxx, Inc., Bothell, WA, USA) associé à une sonde linéaire à haute fréquence (13MHz) (Fig. 21) qui aura permis d'acquérir plusieurs données essentielles. Tout d'abord, l'échographe a permis de confirmer le statut reproducteur des femelles après l'ovulation. Ensuite, nous avons pu suivre le développement des œufs depuis l'ovulation jusqu'à la mise bas. On entend par « œuf » l'ensemble formé par l'embryon, le vitellus et les membranes extra-embryonnaires (i.e., chorion-allantoïde). Différentes mesures ont donc été réalisées sur les œufs pour les différentes expérimentations.

Figure 21. Echographie d'une vipère aspic gestante. Le premier opérateur tient l'avant de l'animal dans le tube et fait passer la sonde tout le long de l'abdomen alors que le second opérateur tient la queue de l'animal et prend les notes.

4.1.1. Fécondité

Tout d'abord, l'échographe nous permet très rapidement de connaitre la taille totale (nombre total d'œufs) et effective de la portée (nombre d'embryons vivants uniquement) tout au long de la gestation. En effet, dans certaine portée, des œufs sont non-fécondés ou bien l'embryon ne se développe pas. Ces œufs sont facilement identifiables à l'échographie car relativement petits

et ronds. Ensuite, pour les embryons en développement, l'organogénèse a lieu tôt dans le développement, en particulier la formation du cœur (Andrews 2004). Un cœur qui palpite est un critère fiable pour considérer un embryon vivant.

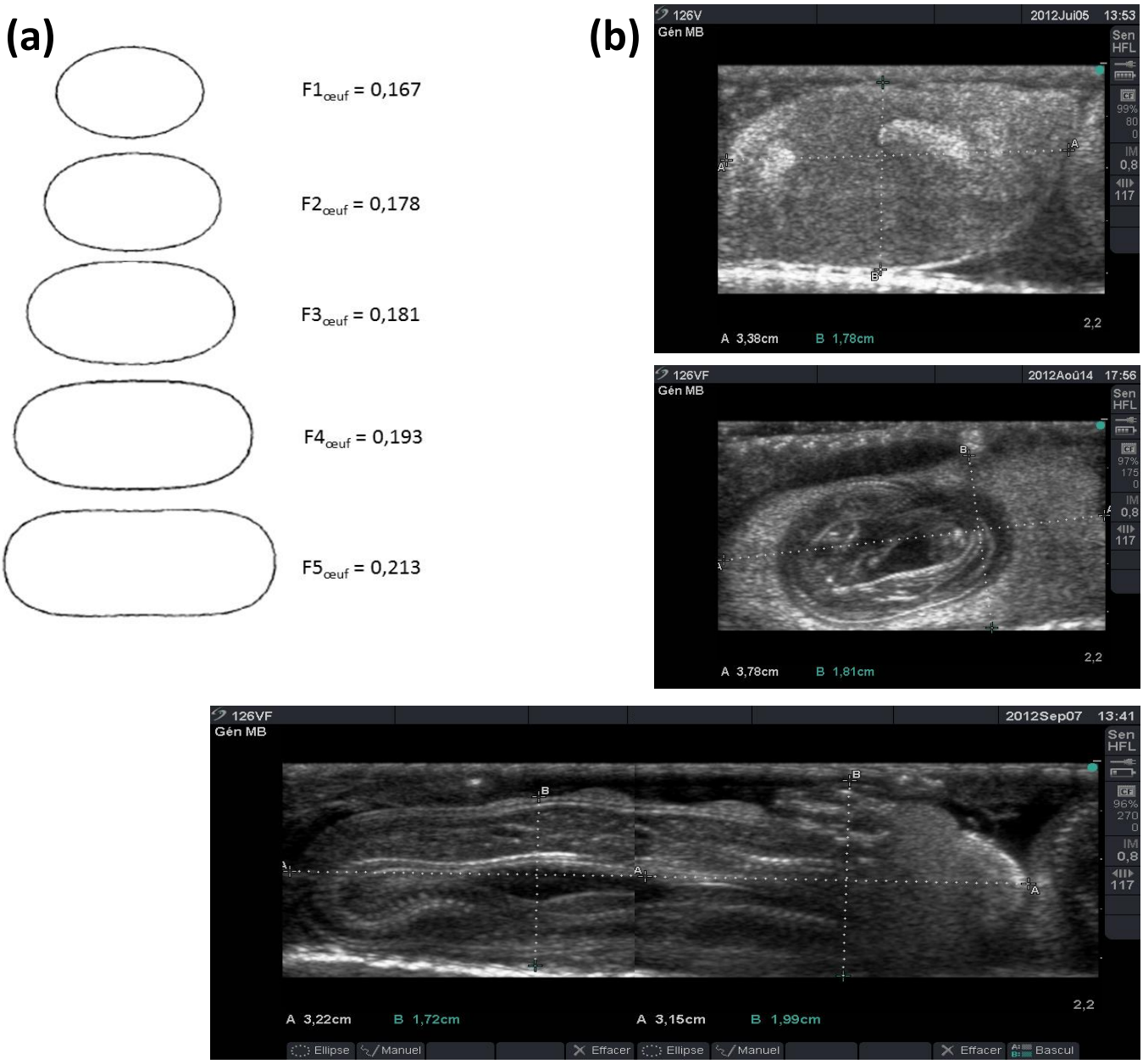


Figure 22. Méthode d'estimation du volume des œufs avec (a) les coefficients de forme ($F_{œuf}$) que nous avons considéré et (b) le suivi échographique de l'œuf crânial de la femelle 126VF et détermination de son volume en utilisant sa longueur (A) et sa hauteur (B). On distingue facilement l'évolution de la taille de l'œuf et la proportion de vitellus restant (zones gris clair) sur les images échographiques entre le début (5 juillet), le milieu (14 août) et la fin de la gestation (7 septembre). Souvent en fin de gestation, la longueur des œufs dépasse la longueur de la sonde (3,83cm), on utilise alors des images composites pour avoir sa longueur totale.

4.1.2. Croissance et allocation en eau

L'échographe nous a également permis de déterminer visuellement le stade gestationnel en se référant aux planches de développement de Hubert & Dufaure (1968). Tout au long de la croissance exponentielle des embryons, nous avons quantifié la prise en eau des œufs. Le gain de volume est en effet représentatif du gain en eau des embryons (Lorioux 2011). La méthode de calcul utilisée pour estimer le volume de l'œuf (en cm^3) nécessite de connaître sa longueur (L), sa hauteur (h) et un coefficient associé à sa forme ($F_{œuf}$) (Maritz & Douglas 1994).

$$\textbf{Volume (en cm}^3\textbf{)} = \pi \times L \times h^2 \times F_{œuf}$$

avec $F_{œuf}$ qui varie selon la forme de l'œuf (Fig. 22a). A partir des images échographiques, nous avons donc pu estimer le volume des œufs en début, milieu et fin de gestation (Fig. 22b). Nous avons pour cela estimé le volume de 2 œufs par femelle : le volume de l'œuf le plus crânial et le volume de l'œuf le plus caudal à chaque stade de gestation.

4.1.3. Contraintes morphologiques associées à la gestation

Ensuite, pour suivre les contraintes morphologiques imposées par la portée sur la femelle, nous avons mesuré la place occupée par les embryons dans l'abdomen et les conséquences sur la distension du corps de la mère. La première étape a été de quantifier le déplacement longitudinal des embryons (avancée abdominale des embryons). Nous avons donc mesuré le point de départ de la portée dans l'abdomen en début et en fin de gestation. Pour ce faire, nous avons placé la femelle sur un mètre un ruban (cloaque au niveau de la mesure de SVL) et la sonde de l'échographe au début du premier œuf. Nous avons noté la distance associée sur le mètre à ruban pour connaître le point de départ de la portée dans l'abdomen.

4.2. Suivi morphologique

En parallèle au suivi échographique, nous avons réalisé différentes mesures morphométriques tout au long de la gestation sur les femelles reproductrices. Tout d'abord un suivi de la masse corporelle qui nous a permis d'estimer la prise en eau par boisson pendant le développement embryonnaire. En effet, les femelles ne s'alimentant pas ou très occasionnellement pendant la gestation (Lourdais et al. 2002b), donc les variations de masses corporelles sont principalement représentatives des gains ou des pertes d'eau. Nous avons également déterminé si l'étirement

de la peau au cours de la gestation pouvait influencer les pertes hydriques. Pour cela, nous avons utilisé des clichés photographiques de femelles gestantes de 2010 en vue dorsale (Fig. 23a) et en vue sagittale (Fig. 23b) afin de déterminer l'espacement entre les écailles (i.e., distance inter-écaille). Pour limiter l'erreur associée à l'échelle de la distance inter-écaille (~3.6mm) et à la marge d'erreur du logiciel (± 0.1mm), nous avons mesuré l'écart entre 8 écailles avant de le rapporter à une longueur inter-écaille. A nouveau, nous avons considéré la moyenne de 3 réplicas de longueur et de largeur inter-écailles (coefficients de variations respectifs : 7.4% et 7.3%) sur les photos à l'aide du logiciel Inskcape (Inkscape, version 0.15). La somme longueur et largeur inter-écaille a été utilisée pour obtenir un indice de distension, pour tester l'influence de cette variable sur les pertes hydriques totales.

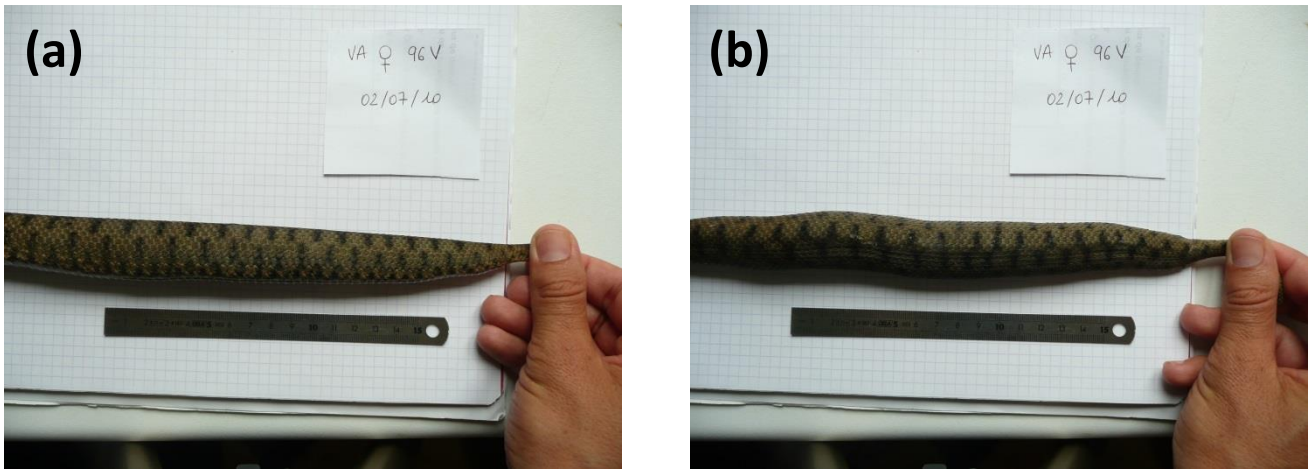


Figure 23. Photographies d'une femelle gestante réalisées sous 2 profils avec (a) une vue sagittale et (b) une vue dorsale.

4.3. Parturition

Immédiatement après la mise-bas, les femelles vipères aspics ainsi que leur portée sont systématiquement pesées (Fig. 24a), ce qui permet premièrement d'estimer le contenu en eau dans les portées. Pour cela, nous utilisons la masse des femelles avant et après la mise-bas (respectivement $M_{pre\text{-}parturition}$ et $M_{post\text{-}parturition}$) et la masse de portée ($M_{portée}$) :

Contenu en eau (en g) = ($M_{pre\text{-}parturition}$ - $M_{post\text{-}parturition}$) - $M_{portée}$

Les différentes données de masses des femelles et masses de portée sont également utilisées pour déterminer les différents indicateurs de l'effort reproducteur. En effet, de nombreux indicateurs sont en général utilisés pour estimer l'effort reproducteur chez cette espèce (Bonnet et al. 2003). En plus des données de taille de portée (taille de portée totale et effective ; voir au-dessus), nous avons donc considérée la masse totale de portée (masse des nouveau-nés, des œufs non développés et des mort-nés) et la masse effective de portée (masse de nouveau-nés viables uniquement).

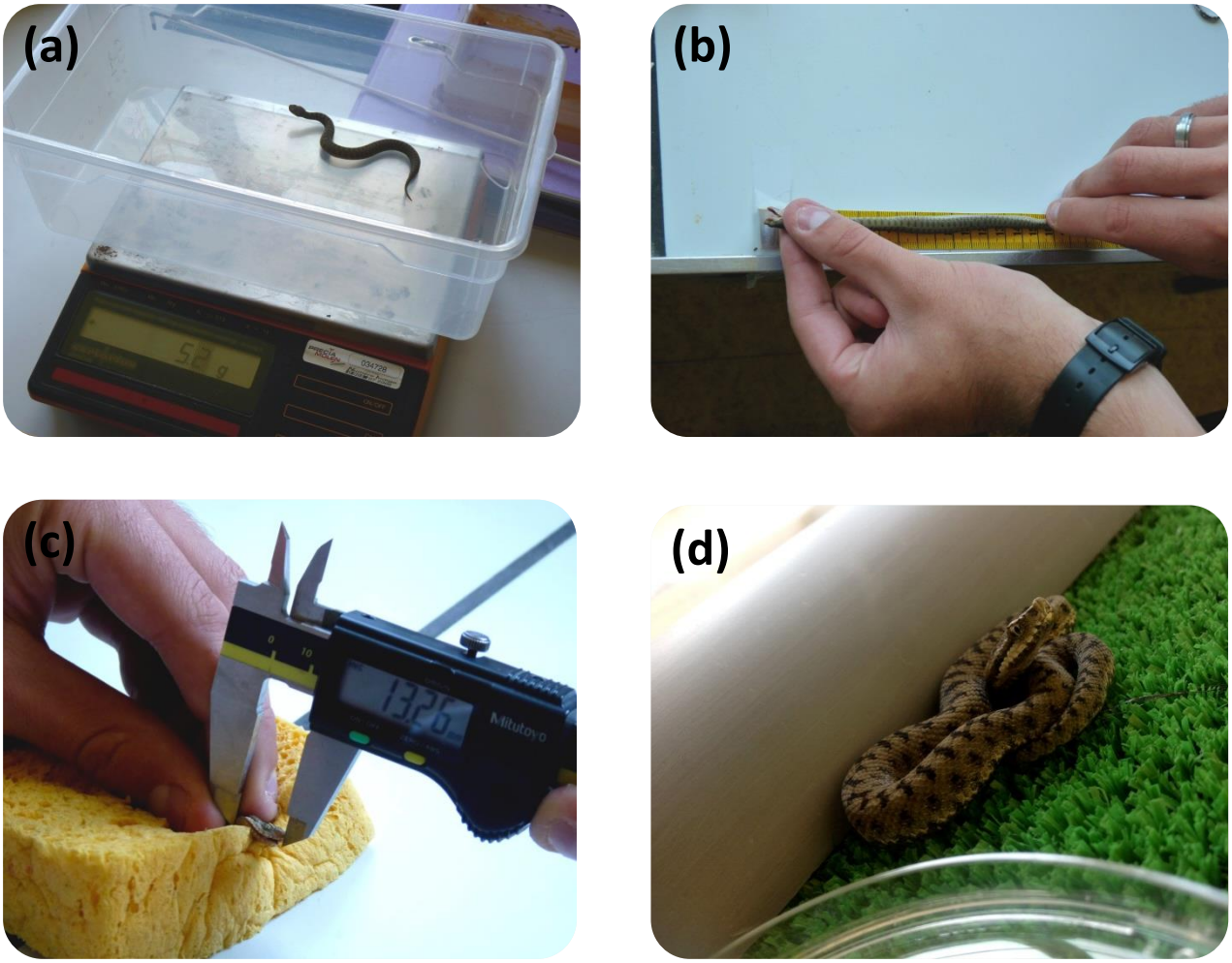


Figure 24. Mesures réalisées sur les vipéreaux à la naissance avec la mesure de (a) la masse corporelle, (b) la taille, (c) la taille des mâchoires, puis (d) l'isolement des individus en boites individuelles pour pouvoir mesurer leur croissance.

Nous avons également estimé l'effort reproducteur relatif à la taille des femelles. En effet, la taille de la mère influence directement la taille/masse de portée (Bonnet et al. 2003). Toutefois de la variation existe et certaines femelles de petite taille peuvent investir dans des portées relativement grandes (i.e., effort reproducteur relatif important), et à l'inverse des femelles de

grande taille peuvent investir dans des portées plus petites (i.e., effort reproducteur relatif faible). En ce sens, l'effort reproducteur relatif est donc un indicateur différent de l'effort reproducteur absolu. Les méthodes de calcul de l'effort reproducteur relatif sont bien décrites dans l'**Article 2**.

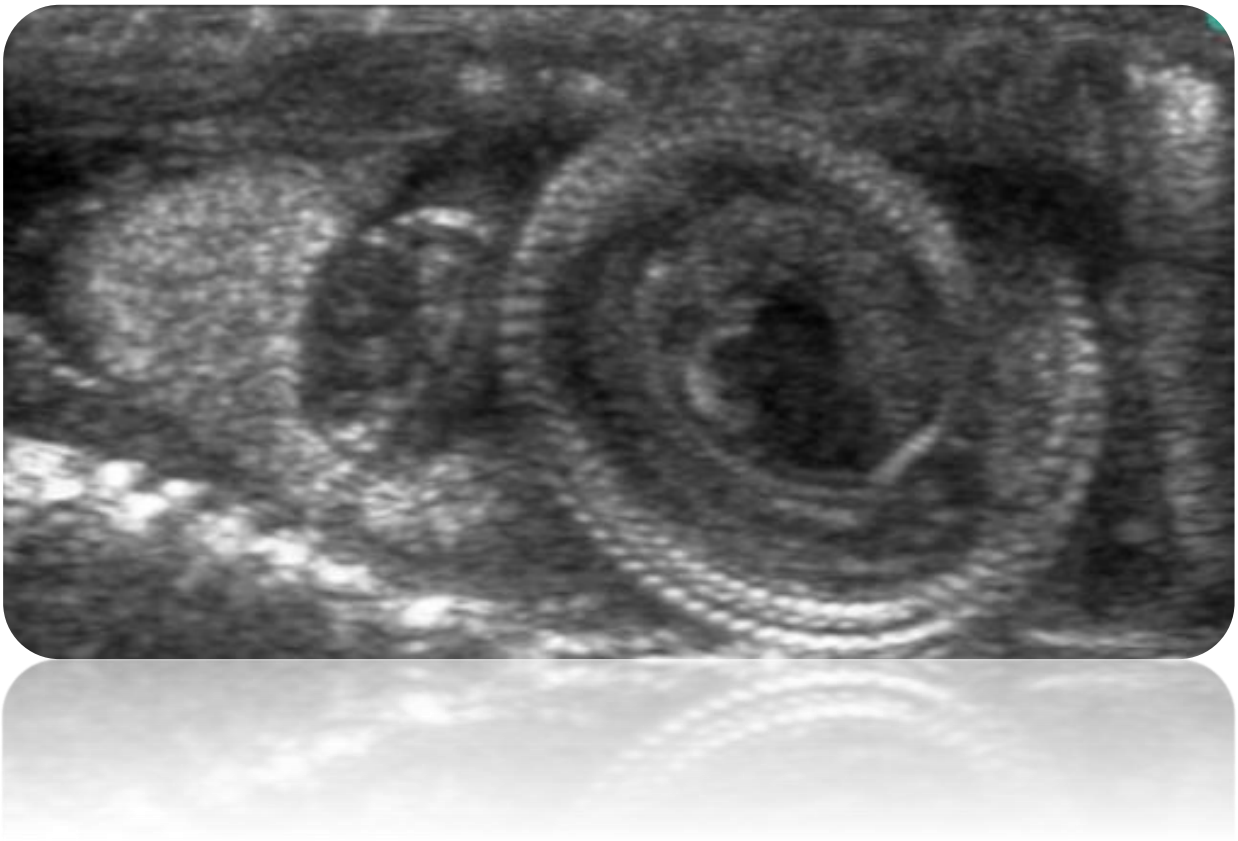
En plus de peser les nouveau-nés, nous avons également mesuré la taille corporelle (SVL ; Fig. 24b), et la taille de leur mâchoires (Fig. 24c). Une fois toutes les mesures réalisées, les vipéreaux sont isolés dans des boites individuelles (30 x 16 x 10cm) avec un abri, un gradient de température (20-33°C), et de l'eau *ad libitum* (Fig. 24d). Les mesures sont répétées 2 semaines plus tard afin de déterminer des paramètres de croissance.

4.4. Corticostérone dans les mues des vipéreaux

En plus des différentes mesures présentées dans ce doctorat, nous avons également tenté de mettre en place un protocole pour le dosage de CORT dans les mues de vipéreaux. En effet, dans les premières minutes / heures qui suivent la naissance, les vipéreaux réalisent leur première mue (Fig. 25), notamment pour s'imperméabiliser (Tu et al. 2002). Nous avons donc souhaité pouvoir quantifier les métabolites de la CORT présents dans la mue, puisqu'ils sont par exemple dosés dans des mues d'adultes (Berkvens et al. 2013) ou dans d'autres tissus cornés comme les plumes et les poils (Davenport et al. 2006 ; Fairhurst et al. 2012). La mue représente un tissu archive et permettrait de mesurer la quantité de CORT sécrétée pendant toute la période de croissance des embryons. Toutefois, le protocole est encore à ce jour en cours de développement puisque nous avons fait face à plusieurs difficultés principalement causées par la couche lipidique dans l'épiderme et les dépôts d'écailles qui interagissent avec les anticorps et faussent ainsi le dosage.

Figure 25. Première mue d'un vipéreau à la naissance.

Résultats



Chapitre 1 :
Compromis entre thermorégulation et pertes hydriques

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Résumé du chapitre 1

Chez les ectothermes, la thermorégulation comportementale est un processus majeur qui influence directement les performances physiologiques et la valeur sélective des individus. Cependant, la thermorégulation peut être associée à des coûts, et possiblement un compromis avec les pertes hydriques. En parallèle, des réponses comportementales telles que les préférences de conditions microclimatiques peuvent permettre de tamponner le compromis physiologique entre thermorégulation et pertes hydriques.

Dans ce chapitre, l'objectif a été de déterminer l'influence directe de l'état physiologique sur les pertes hydriques et sur un possible ajustement comportemental de la vipère aspic (**Article 1**). Chez les Squamates, des processus physiologiques majeurs tels que la gestation, la mue ou la digestion sont déjà connus pour être des états demandeurs en énergie. En effet, ces états physiologiques augmentent d'une part l'effort de thermorégulation des individus et d'autre part, ils sont associés à un niveau métabolique (i.e., dépenses énergétiques) plus élevé. Un métabolisme augmenté impacte les pertes hydriques (i.e., via la ventilation), ce qui est en plus amplifié par des préférences thermiques plus importantes. Nous avons tout d'abord quantifié l'effet direct de l'état physiologique et les effets indirects des conditions microclimatiques (température et humidité) sur les pertes hydriques totales par évaporation. Nous avons utilisé une chaine de calorimétrie indirecte (système ouvert à 8 voies) pour mesurer les pertes hydriques totales et la consommation d'oxygène (VO_2). Les tests ont été réalisés sur des individus en gestation, en mue, ou en digestion et comparés à des individus témoins (i.e., non-reproducteurs, post-mues et post-prandiaux). Par ailleurs, les mesures de pertes hydriques totales et de VO_2 ont été réalisées à 3 conditions microclimatiques obtenues en combinant 2 températures (T_a = 20 et 30°C) et 2 humidités absolues (ρ = 14 et 25 $gH_2O.m^{-3}$). Les résultats obtenus soutiennent notre hypothèse générale, à savoir qu'un état physiologique demandeur en énergie augmente les pertes totales en eau ainsi que le métabolisme.

Une façon pour limiter le compromis physiologique entre thermoregulation et perte hydrique est de sélectionner un microclimat à la fois chaud et humide pour optimiser le processus physiologique tout en limitant les pertes hydriques. Nous avons testé les réponses comportementales (i.e., sélection de microclimats) mises en place par les vipères pour limiter

les effets d'états physiologiques demandeurs en énergie sur les pertes hydriques. Nous avons expérimentalement testé la sélection de microclimats pour des individus dans les mêmes états physiologiques que précédemment. Les individus ont donc été placés dans le Michautron 4X et exposés à un choix de 4 conditions microclimatiques (Chaud ou Froid & Humide ou Sec). Les résultats obtenus tendent à soutenir notre hypothèse selon laquelle les individus tamponneraient le compromis entre besoins thermiques et pertes hydriques en optimisant leur sélection de microclimat. Toutefois, la différence entre états physiologiques hydriquement contraignant et état témoin est marginale. De plus la sélection d'une humidité plus élevée semble plus présente chez les vipères gestantes, ce qui suggère que la sélection de microclimat dépend des contraintes spécifiques associées aux états physiologiques.

Ces résultats soutiennent l'existence d'un compromis entre les besoins thermiques et les pertes hydriques influencé par l'état physiologique, et en partie tamponné par un ajustement comportemental. Le compromis entre thermorégulation et pertes hydriques affectent probablement la balance hydrique des individus lorsque la disponibilité en eau est restreinte (i.e., possible conflit physiologique), notamment pendant la période estivale. Les modèles climatiques actuels (scenarios IPCC) prévoient l'augmentation de la durée et de la fréquence d'épisodes climatiques contraignants (i.e., sécheresses estivales, canicules, vague de chaleur), il apparaît donc crucial d'intégrer l'impact des contraintes hydriques sur la physiologie et le comportement, pour une meilleure compréhension de la complexité des réponses associées aux changements environnementaux.

ARTICLE 1

Physiological state influences evaporative water loss and microclimate preference in the snake *Vipera aspis*

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In revision for *Physiology and Behavior*

Abstract

Environmental variation is nearly ubiquitous in nature, and animals typically respond to this variation by adjusting their physiology, behavior, or both. Ectothermic animals are particularly sensitive to climatic conditions (e.g., temperature and humidity) and, thus, behaviorally thermoregulate to optimize physiological performance. Yet, thermoregulation can be costly and may obligate a physiological tradeoff with water loss. Presumably, this tradeoff intensifies when animals undergo necessary life-history events (e.g., pregnancy or digestion) that impose significant behavioral and physiological changes, including shifts in behavioral thermoregulation and increased metabolic rate. In this context, behavioral responses, such as modified microclimatic preferences, may help mitigate the physiological tradeoff between thermoregulation and water loss. Herein, we examined the influence of major physiological states (specifically, pregnancy, ecdysis, and digestion) on evaporative water loss and on behavioral adjustments in a viviparous snake, *Vipera aspis*. First, we used open-flow respirometry to measure the effects of physiological states and microclimatic conditions (temperature and humidity) on the rate of total evaporative water loss (TEWL) and metabolic rate (rate of O_2 consumption, $\dot{V}O_2$). Then, we experimentally tested the influence of physiological state on microclimate selection. We found that energy-demanding physiological states were associated with i) an increased rate of TEWL and $\dot{V}O_2$ compared to control states and ii) a marginal preference for both warm and humid conditions compared to controls. This preference was most marked in pregnant snakes, highlighting a state-specificity in behavioral response. Overall our results underline the impact of physiological state on water loss and demonstrate the potential for behavior to mitigate the physiological tradeoff between thermoregulation and water balance.

Keywords: water loss, metabolic rate, pregnancy, ecdysis, digestion, temperature, humidity

Introduction

Temperature influences myriad physiological processes from enzymatic kinematics and metabolic rate to physiological performance (e.g., locomotion, digestion, immunity, and reproduction) (Hochachka & Somero 2002; Angilletta et al. 2002, 2010; Angilletta 2009; Butler et al. 2013). To perform critical activities (e.g., feeding, breeding, and migration), animals must adjust both their physiology and their behavior to natural temperature fluctuations. Ectotherms are not able to produce sufficient metabolic heat to compensate for ambient thermal variation; therefore, they mainly rely on behavioral thermoregulation (Huey 1982; Blouin-Demers & Weatherhead 2001a, 2001b; Blouin-Demers & Nadeau 2005). Although it benefits physiological processes (Huey & Stevenson 1979; Hertz et al. 1982; Stevenson et al. 1985; Huey & Kingsolver 1989; Angilletta et al. 2002), thermoregulation is also known to present several costs (reviewed in Angilletta 2009). Classically, ecological costs associated with thermoregulation include increased predation risks (Webb & Whiting 2005; Herczeg et al. 2008; Angilletta 2009) or reduced time available for other activities (Grant & Dunham 1988; Adolph & Porter 1993; Angilletta 2009).

Thermoregulation can also induce physiological constraints, such as a tradeoff with water balance (Ladyman & Bradshaw 2003; Ladyman et al. 2006; Angilletta 2009; Köhler et al. 2011). The maintenance of high body temperature passively increases the rate of water loss due to increased ventilation (higher respiratory water loss) and increased transpiration (lower skin resistance) (Tieleman et al. 2002; Lillywhite 2006; Guillon et al. 2014). Among ectotherms, major physiological processes related to fitness, such as digestion, gestation or immune response, induce significant shifts in thermoregulation via increased basking behavior (Vaughn et al. 1974; Shine 1980; Slip & Shine 1988; Blouin-Demers & Weatherhead 2001a; Angilletta et al. 2002; Angilletta 2009; Richards-Zawacki 2010; Lelièvre et al. 2011; Lorioux et al. 2013; Meylan et al. 2013). However, basking behavior often increases an animal's exposure to drier ambient conditions. Importantly, increases in temperature-dependent metabolic rate are also often associated with major physiological processes, such as digestion (see Secor 2009 for a review on specific dynamic action), gestation (Birchard et al. 1984; Van Dyke & Beaupre 2011; Dupoué & Lourdais 2014), and immune response (Martin et al. 2008; Ardia et al. 2012). Such metabolic shifts may, in turn, affect evaporative water loss through an increase in respiratory

gas exchange. However, the potential tradeoff between temperature-dependent physiological processes and water balance remains grossly overlooked (Guillon et al. 2014).

A diversity of studies have focused on water balance and water-conserving mechanisms, including major changes in activity (Pough et al. 1983; Crowley 1987; Lorenzon et al. 1999; Bonnet & Brischoux 2008; Davis & DeNardo 2009; Hetem et al. 2010), shifts in thermoregulatory strategies (Crowley 1987; Bradshaw 1997; Lorenzon et al. 1999; Ladyman et al. 2006; Angilletta et al. 2010; Köhler et al. 2011), and/or metabolic depression (Kennett & Christian 1994; Christian et al. 1999, 2003; Tieleman et al. 2002; Muir et al. 2007). These adjustments aim to lower the rate of total evaporative water loss (TEWL) (Moen et al. 2005) and eventually the rate of dehydration. However, as discussed above, energy-demanding physiological states often impose a preference for warmer temperatures, which limit metabolic depression. One solution to these dual constraints is the selection of a microhabitat that is both warm and humid (Köhler et al. 2011; Stahlschmidt et al. 2011). Behavioral adjustments that mitigate physiological tradeoffs are therefore also crucial to investigate when determining animals' responses to environmental changes.

In this study, we addressed the influence of physiological state on total evaporative water loss (TEWL), metabolic rate (rate of O_2 consumption, $\dot{V}O_2$) and behavior (microclimate selection) in a viviparous snake species, the aspic viper (*Vipera aspis*). Ecdysis (skin shedding), digestion, and reproduction are physiological processes that increase thermal demand and metabolism in squamate reptiles (Naulleau 1983; Stevenson et al. 1985; Gibson et al. 1989; Charland & Gregory 1990; Lourdais et al. 2002a, 2002b; Wang et al. 2003; Lourdais et al. 2004) as they do in many animals (Anger & Spindler 1987; Janes & Chappell 1995; King 2000). To determine the influence of energy-demanding physiological states on water loss, metabolic rate, and behavior, we conducted two independent experiments. In Experiment 1, we determined the rate of TEWL and $\dot{V}O_2$ of snakes in different energy-demanding physiological states (i.e., during pregnancy, ecdysis, or digestion) and compared them to snakes in a post-prandial (non-digesting), non-reproductive, and non-shedding state (i.e., control). We hypothesized that energy-demanding physiological states would impact rates of water loss and metabolism. Specifically, we expected pregnancy, ecdysis, and digestion to increase the rate of TEWL and $\dot{V}O_2$ compared to control snakes. In the Experiment 2, we determined whether physiological

state influences microclimate selection. We hypothesized that physiological state would affect snakes' microclimate selection with the prediction that energy-demanding states would orient snake preferences to a more humid microclimate. Our study integrates the impacts of two climatic variables and physiological state on physiological and behavioral responses thus, it should provide new insight into the complexity of animals' responses to environmental shifts (Todgham & Stillman 2013).

Material & Methods

Study species

We studied the aspic viper (*Vipera aspis*), a medium-sized viviparous snake abundant in western Europe (Naulleau 1997). Previous studies on this species provide background on its digestion, ecdysis, and reproduction. The aspic viper is an ambush forager that feeds mainly on rodents (Saint Girons 1979; Naulleau 1983; Lourdais et al. 2002a). In natural conditions, foraging activity occurs between April and August (Saint Girons 1979; Naulleau 1983) and the mean duration of digestion ranges from 5 - 6 days (Naulleau 1983). In adults, skin ecdysis occurs every 2 - 3 months, and a complete cycle of ecdysis approximates 2 - 3 weeks (Saint Girons 1980). The ecdysis cycle is characterized by the succession of easily detectable sub-stages (e.g., blue-belly or blue-eyes) due to lymphatic activity in the space between the old and new layers of epidermis (Mader 2005). The species is a typical capital breeder (Bonnet et al. 1998), which means that females amass energetic reserves for 2 - 3 years before reproducing (Bonnet et al. 1998, 1999, 2002; Lourdais et al. 2002a, 2002b). Vitellogenesis (i.e., synthesis of yolk) begins in spring (March-April) (Bonnet et al. 2002). Then, ovulation occurs in mid-June immediately followed by pregnancy until parturition in late August - early September (Lourdais et al. 2002a, 2002b).

Importantly, digestion, ecdysis, and pregnancy in *V. aspis* are energy-demanding physiological states that induce increased metabolic rate and a preference for warmer body temperatures (Taylor & Davies 1981; Blem & Zimmerman 1986; Andrade et al. 1997; Gibson et al. 1989; Ladyman et al. 2003; Lorioux et al. 2013; Dupoué & Lourdais 2014). Therefore, *V. aspis* is a good candidate to examine the possible physiological tradeoff between thermoregulation and water balance in the context of variable physiological states.

Capture and maintenance

We caught snakes in west-central France from neighboring populations (Vendée, Loire-Atlantique and Deux-Sèvres districts). From late April to mid-June 2012, we captured 77 individuals (31 males, 46 females) to measure TEWL and VO_2 (Experiment 1, see below). From early-May to late-June 2013, we captured 78 different individuals (32 males, 46 females) to test microclimate selection (Experiment 2, see below). We placed all snakes in a semi-natural outdoor enclosure (4 m x 4 m x 2.5 m; 10 individuals maximum per enclosure) at the Centre d'Etudes Biologiques de Chizé (CEBC). We fed snakes with mice once every two weeks, and we provided *ad libitum* access to water by misting enclosures 30 min per day. Two weeks before experimentation, we housed each viper individually in a plastic box (30 cm x 16 cm x 10 cm) in the laboratory, with a natural thermal gradient (20-33°C) provided with a heat cable, water *ad libitum* in water bowls, and an opaque polyvinyl shelter (half cylinder PVC pipe, diameter = 15 cm, length = 25 cm). In both experiments, we followed all laws and rules relating to the conservation and welfare of the animals (Permit # 792, Direction service vétérinaire des Deux-Sèvres). After each experiment, we released snakes (with their respective litter for pregnant females) at the exact site of capture.

Morphology

Before both experiments, we measured (snout-to-vent length, SVL ± 0.5cm) and weighed (body mass, BM ± 1g) all snakes. In Experiment 1, we also estimated the total skin surface area and the total body volume of each snake given the influence of these variables on TEWL. To do so, we used a non-invasive method based on morphological measures as detailed in methods from Brischoux & Shine (2011). We divided snakes into six equivalent sections, and we determined the length and width (diameter) of each section using a tapeline and a digital caliper, and the corresponding shape (i.e., truncated cone for body sections and cone for tail section). We summed the surface and volume of all sections to estimate total snake skin surface area and total snake volume. We did not measure head surface/volume for safety reasons and because cutaneous water loss is negligible compared to the rest of body (Dmi'el 1972).

Experimental design

Experiment 1: Gas exchange

We measured the rate of snake TEWL and VO_2 using an open-flow respirometry system. We distinguished thermal and hydric effects by measuring TEWL and VO_2 at two targeted ambient temperatures (T_a = 20 or 30°C) and humidities (absolute humidity, ρ = 14 or 25 $gH_2O.m^{-3}$) (Table 1) that are ecologically relevant (Guillon et al. 2014). We compared TEWL and VO_2 at three microclimatic conditions, named thereafter Cool x Humid, Warm x Dry, and Warm x Humid (see details of thermal and humidity conditions in Table 1).

We measured each individual in only one physiological state but in all three of the microclimatic conditions. We randomized the order of microclimatic conditions, snake physiological state, and sex in each measurement session. We placed each snake in a test chamber (500 ml glass jar to minimize hygroscopicity), which were housed in a temperature controlled climatic chamber (± 1°C; Cryosystem, air conditioned system Carel MasterCella). To obtain air with target humidity, we used the same method described in a previous study (Guillon et al. 2014). Briefly, we vapor-saturated the air with a double bubbling system in a climatic chamber set at a targeted temperature (i.e., dew point temperature T_{DP}). In this experiment, we used two T_{DP}s (T_{DP} = 15 or 25°C) to obtain the two targeted humidity conditions (14 or 25 $gH_2O \bullet m^{-3}$). We used an eight-channel flow controller and meter (± 1 ml • min^{-1}) (FlowBar-8, Sable Systems, Las Vegas, USA) to provide air at a constant influx (82.7 ± 6.6ml • min^{-1}) to each test chamber. We previously used a soap bubble system to calibrate the flow meter (Tieleman et al. 2002). Efflux of each test chamber was sequentially selected with a gas switcher (RM-8 Multiplexer, Sable Systems, Las Vegas, USA). The efflux was then passed through a hygrometer (RH 300, Sable Systems, Las Vegas, USA) and a column of desiccant (self-indicating drierite) before entering the CO_2 analyzer (CA10, Sable Systems, Las Vegas, USA), and O_2 analyzer (FC10A, Sable Systems, Las Vegas, USA). We left the eighth test chamber empty to serve as reference for baseline. We continuously recorded output signals from the different hardware with a specific interface (UI-2, Sable Systems, Las Vegas, USA). We analyzed each test chamber for 30 min before switching to another test chamber. We determined the Baseline influx for each test chamber from a 10 min sample of the empty test chamber before and after we measured efflux from the seven test chambers containing snakes. We repeated the sequence (10 min Baseline, 30 min each snake, 10 min Baseline) a second time

to ensure snake TEWL and VO_2 were stable during measurements. Finally, we used equations derived from Withers (1977) and adapted to our setup to calculate TEWL (mg • h^{-1}) and VO_2 (ml • h^{-1}).

	Microclimatic conditions	n	Observed conditions				
			T_a (°C)	RH (%)	ρ ($gH_2O.m^{-3}$)	TEWL ($mg.h^{-1}$)	VO_2 ($ml.h^{-1}$)
Experiment 1	Cool x Humid	76	20.8 ± 0.04[a]	53.8 ± 1.6[a]	13.8 ± 0.03[a]	10.5 ± 0.8[a]	5.9 ± 0.5[a]
	Warm x Dry	73	30.5 ± 0.04[b]	28.6 ± 1.5[b]	13.9 ± 0.03[a]	41.8 ± 2.9[b]	16.1 ± 1.4[b]
	Warm x Humid	75	30.4 ± 0.04[b]	51.4 ± 1.6[a]	25.4 ± 0.05[b]	22.1 ± 1.1[c]	16.0 ± 1.4[b]
Experiment 2	Cool x Dry	78	25.1 ± 0.1[a]	47.5 ± 1.1[a]	12.0 ± 0.3[a]	-	-
	Cool x Humid	78	25.5 ± 0.1[a]	82.0 ± 0.3[b]	21.2 ± 0.1[b]	-	-
	Warm x Dry	78	30.8 ± 0.1[b]	40.6 ± 1.0[c]	14.3 ± 0.4[c]	-	-
	Warm x Humid	78	30.8 ± 0.1[b]	65.8 ± 0.7[d]	23.2 ± 0.2[d]	-	-

Table 1. Summary of observed conditions (ambient temperature: T_a, relative humidity: RH, absolute humidity: ρ) in each microclimatic category from the two experiments. We included sample sizes (n) relative to analyses of physiological state effects in each microclimatic condition. For experiment 1, the rate of total evaporative water loss (TEWL) and oxygen consumption (VO_2) are reported regardless of physiological state. Different superscripted letters are used to show significant differences in thermal or humidity conditions and in the rate of TEWL or VO_2 between microclimatic conditions. All values are displayed as mean ± SE. See text for details.

Experiment 2: Behavioral paradigm

We designed a maze that consisted of four inter-connected compartments with different thermal and humidity conditions (Cool x Dry, Cool x Humid, Warm x Dry, Warm x Humid; see details of thermal and humidity conditions in Table 1). Contrary to Experiment 1, we included a cool and dry condition. Although this microclimate is not ecologically relevant, we used this condition as a negative control in individual microclimate selection. We created thermal conditions that vipers regularly encounter in the field and that approximate the thermal preference of control (~25°C) *vs.* energy-demanding states (>30°C) (Lorioux et al. 2013). Unlike in Experiment 1, fine control of humidity was not feasible with the design of Experiment 2 given the connectedness of compartments (described below) obligated by the choice

paradigm. Although humidity control was coarse, we ensured that absolute humidity conditions were significantly different among the four compartments (Table 1).

Each compartment consisted of two plastic boxes (30 cm x 20 cm x 10 cm), one stacked inside the other. To create the two thermal conditions (~25°C and 30°C), we equipped the bottom box of each compartment with a 25W thermal cable covered with adhesive aluminum to homogenize temperature that was controlled by a thermostat (Thermo Control PRO II, Lucky Reptile, Germany). To create humid conditions, we placed a set of five sponges (2 cm x 2 cm x 0.4 cm) in each bottom box—'Humid' compartments contained water-saturated sponges and 'Dry' compartments contained dry sponges. The floor of the upper box was comprised of a metal grid to allow heat and water vapor transfer from the bottom box (Table 1). We used two thermocouples (Type T standard) per compartment and a data logger (21X, Campbell Scientific Instruments, Logan, UT, USA) to check temperature variation before each trial. We programmed three miniaturized temperature-humidity data loggers (Hygrochron DS1923, Maxim Integrated Products, Sunnyvale, CA, USA) per compartment to record temperature (intra-box coefficient of variation = 5.2%) and humidity (intra-box coefficient of variation = 3.2%) conditions every 10 min. We used opaque plastic tube (diameter = 5 cm, length = 15 cm) to connect the four compartments to one another (each compartment was connected to two others).

We measured each individual in one physiological state to avoid any learning process. For each trial, we randomly assigned the four combinations of microclimatic conditions to the four compartments. In the morning (9:00 am), we started the system and let temperature and humidity conditions stabilize for 90 min. Then, we put each snake in one randomly chosen compartment. After 6 h of acclimation, we noted each animal's position at the end of its trial (*sensu* Stahlschmidt et al. 2011), and we measured skin surface temperature using an infrared laser thermometer (Raytek Corporation, Santa Cruz, USA). Measurement procedure was similar to Dupoué et al. (2013) as recommended by Hare et al. (2007) (emissivity set at 0.95 and the thermometer was oriented in-line with the body axis). This method did not provide an accurate measure of internal temperature. It rather provided a noninvasive relative measure of temperature variation among the individuals in our study. In this study, the species was medium-sized and skin surface temperature approximates core body temperature (T_{body})

(Lourdais unpublished data). Between each trial, we washed the maze with warm water and a mild detergent to avoid chemical communication.

Physiological states

We first identified pregnant females (Experiment 1: 19 females, SVL = 46.0 ± 0.7cm, BM = 94.4 ± 5.0g; Experiment 2: 11 females, SVL = 50.2 ± 1.2cm, BM = 127.8 ± 11.7g) in the field with manual palpation of the abdomen and then checked in the laboratory with high resolution ultrasonography (Sonosite microMaxx, Inc., Bothell, WA, USA). We experimented on females in the final trimester of pregnancy during the period of most embryonic somatic growth (Experiment 1: 19.4 ± 3.2 days before parturition; Experiment 2: 22.5 ± 2.8 days before parturition).

We selected pre-shedding snakes (Experiment 1: 10 males, 6 females, SVL = 47.0 ± 0.9cm, BM = 76.5 ± 5.2g; Experiment 2: 11 males, 12 females, SVL = 51.2 ± 1.0cm, BM = 116.3 ± 6.6g) with blue-belly, the most detectable sub-stage of ecdysis cycle. We regularly checked snakes to find pre-shedding individuals.

We measured digestive snakes (Experiment 1: 9 males, 12 females, SVL = 45.2 ± 0.9cm, BM = 80.8 ± 4.1g; Experiment 2: 11 males, 11 females, SVL = 49.4 ± 1.6cm, BM = 103.2 ± 9.8g) 24 h after the ingestion of a mouse (30% of snake BM) when specific dynamic action (SDA: post-prandial hypermetabolism) is maximal (Andrade et al. 1997).

We selected control snakes (Experiment 1: 12 males, 9 females, SVL = 45.8 ± 1.1cm, BM = 77.1 ± 5.8g; Experiment 2: 10 males, 12 females, SVL = 50.3 ± 1.3cm, BM = 105.5 ± 8.1g) in a non-reproductive, non-shedding, and post-absorptive state (> 7 days after meal).

In both experiments, snakes were not different in SVL (all $P > 0.518$) but BM was significantly higher in reproductive snakes from Experiment 1 compared to control and pre-shedding snakes (all $P < 0.023$) and the difference was marginal when compared to digestive snakes ($P = 0.058$).

Statistical analyses

All analyses were performed with R software (R Development Core Team, 2011). TEWL and VO_2 data were $\log_{10}$ transformed to achieve normality condition.

Experiment 1: We used linear mixed models (lme, package nlme) to assess the effects of physiological state and microclimatic conditions on the rate of TEWL and VO_2, with physiological state, microclimatic condition, and their interaction as explanatory factors. We included morphological parameters as covariates because they are known to influence the rate of TEWL and VO_2. In TEWL and VO_2 analyses, we respectively included both the residuals of the surface *vs.* volume relationship and SVL as explanatory variables to control for allometric variation. Although each snake was measured in one physiological state, we considered the identity of each snake as a random factor because each snake was measured in all three microclimatic conditions. We conducted pairwise *post hoc* comparisons after accounting for α-inflation with Tukey's tests to determine the effects of physiological states (package lsmeans) on TEWL and VO_2. In figures, we used the least square means method to adjust values of TEWL by residuals of the surface-volume relationship and SVL, and VO_2 by SVL. We found no impact of sex on TEWL ($F_{1,73} = 0.4$; $P = 0.525$) or on VO_2 ($F_{1,73} = 3.0$; $P = 0.087$), so we discarded this factor from analyses. We also removed from analyses individuals that were active during measurements in Experiment 1 (Cool x Humid: n = 1; Warm x Dry: n = 4; Warm x Humid: n = 2; Table 1). We used linear regression model (lm, package stats) to test relationships between absolute values of TEWL and VO_2 for each physiological state. We analyzed these relationships in each microclimatic condition, but we only presented results obtained in Warm x Humid condition because statistical effects were similar in the other two conditions.

Experiment 2: We used two types of analyses to determine if physiological state impacted snake microclimate preferences. First, we used a global analysis on final microclimate selection—

specifically, χ^2 tests to determine if snakes in energy-demanding states selected different microclimatic conditions than control snakes. Second, we performed a state-specific analysis to limit the influence of differences in sample sizes among all physiological states. We used general linear models to determine if snakes in energy-demanding states differed from controls in T_{body} and in selected humidity, with T_{body} (estimated from infrared thermometry) or humidity (measured from hygrometric data loggers) as the dependent variable and physiological state as the explanatory factor.

Results

Experiment 1

Influence of physiological state

TEWL was significantly influenced by SVL ($F_{1,133} = 21.4$, $P < 0.001$) and residuals of surface *vs*. volume relationship ($F_{1,133} = 23.3$, $P < 0.001$). VO_2 was significantly influenced by SVL ($F_{1,134} = 13.9$, $P < 0.001$). Snakes with high surface-to-volume ratios exhibited greater TEWL, and longer snakes exhibited great VO_2. We found a significant effect of the physiological state on the rate of TEWL ($F_{3,133} = 36.2$, $P < 0.001$; Fig. 1) and on VO_2 ($F_{3,134} = 111.6$, $P < 0.001$; Fig. 2). Compared to control snakes, snakes in all physiological states showed significantly higher TEWL (post hoc, all $P < 0.045$; Fig. 1) and VO_2 (post hoc, all $P < 0.001$; Fig. 2). Pre-shedding and pregnant snakes presented similar TEWL (post hoc, z = -0.83, $P = 0.948$; Fig. 1) and similar VO_2 (post hoc, z = -0.55, $P = 0.842$; Fig 2). In digesting snakes, TEWL was higher than control snakes (post hoc, z = 2.61, $P = 0.045$; Fig. 1) and lower than both pre-shedding and pregnant snakes (post hoc, all $P < 0.001$; Fig. 1). The VO_2 of digesting snakes was higher than snakes in all other states (post hoc, all $P < 0.001$; Fig. 2). We found a significant relationship between VO_2 and the rate of TEWL for control ($F_{1,18} = 44.8$, $P < 0.001$, $r^2 = 0.71$) and digesting snakes ($F_{1,18} = 48.5$, $P < 0.001$, $r^2 = 0.73$), but this relationship was not significant in pregnant ($F_{1,17} = 0.1$, $P = 0.814$, $r^2 = 0.00$), or in pre-shedding snakes ($F_{1,14} = 0.4$, $P = 0.536$, $r^2 = 0.06$).

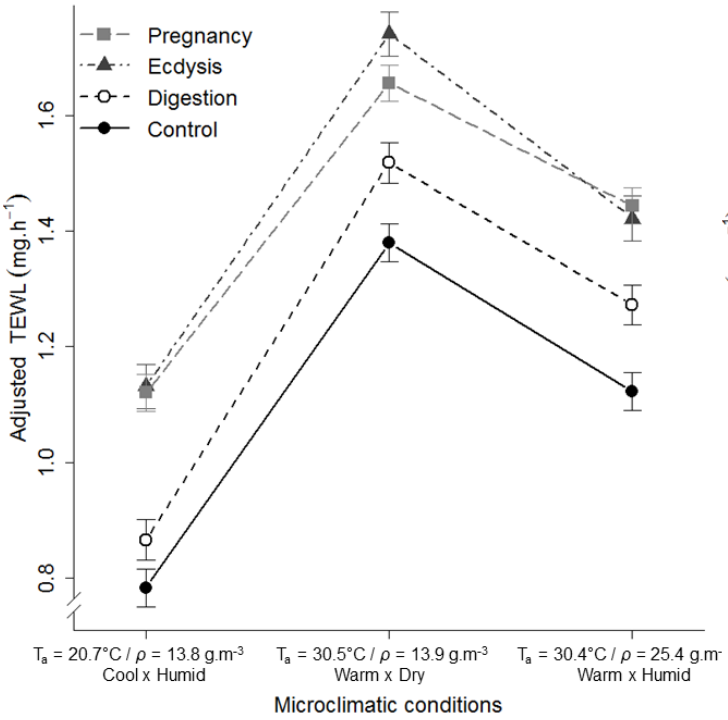

Figure 1. Effect of physiological state (Pregnancy: n = 19; Ecdysis: n = 16; Digestion: n = 20; Control: n = 22) and microclimatic condition (combination of ambient temperature T_a and absolute humidity ρ) on snake total evaporative water loss (TEWL). Points represent mean values (± SE) of $\log_{10}$ TEWL adjusted by SVL and residuals from the surface-to-volume relationship. See text for details.

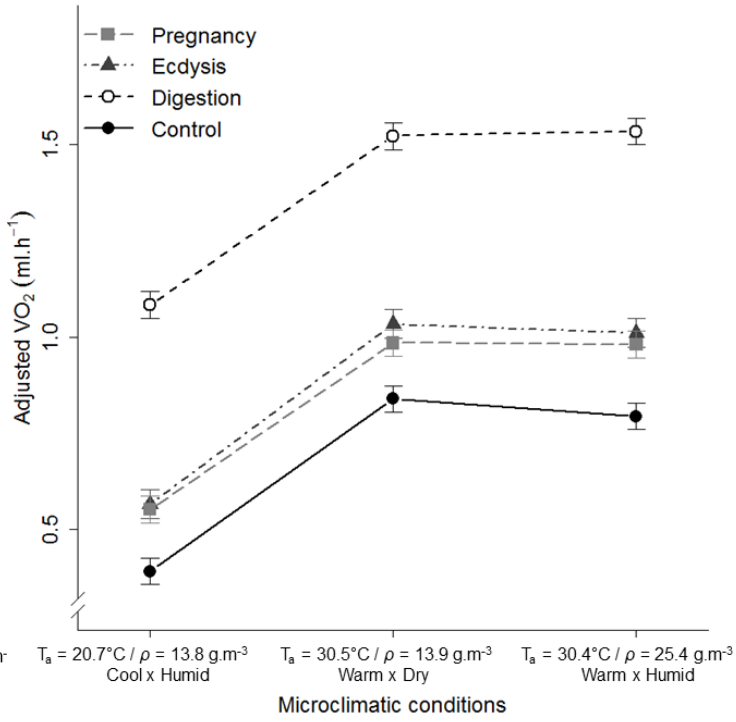

Figure 2. Effect of physiological state (Pregnancy: n = 19; Ecdysis: n = 16; Digestion: n = 20; Control: n = 22) and microclimatic condition (combination of ambient temperature T_a and absolute humidity ρ) on snake metabolic rate (VO_2). Points represent mean values (± SE) of $\log_{10}$ VO_2 adjusted by snake SVL.

Influence of microclimatic conditions

We found a significant effect of microclimatic conditions on TEWL ($F_{2,133} = 828.1$, $P < 0.001$; Table 1, Fig. 1) and on VO_2 ($F_{2,134} = 438.2$, $P < 0.0001$; Table 1, Fig. 2). Within each physiological state, TEWL was significantly different among the three microclimatic conditions (post hoc, all $P < 0.001$; Table 1, Fig. 1). At 30°C, the rate of TEWL in the dry condition was 86% higher than in the humid condition (Table 1). In equivalent humidity conditions (ρ = 14 $gH_2O.m^{-3}$), TEWL was higher in the warm than in the cool (fold-increase due to a 10°C increase in temperature: $Q_{10} = 3.98$, Table 1). The VO_2 was significantly influenced by temperature (post hoc, z = -25.73, $P < 0.001$; Fig. 2) but not by humidity (post hoc, z = -0.88, $P = 0.656$; Fig. 2). At 30°C, VO_2 was higher compared to snakes at 20°C ($Q_{10} = 2.72$, Table 1).

Interaction between physiological state and microclimatic conditions

Physiological state and microclimatic condition interactively affected TEWL ($F_{6,134} = 2.5$; $P = 0.025$; Fig. 1). For instance, microclimatic conditions affected TEWL by 22 – 43% during pre-shedding and pregnancy and by 10 – 13% during digestion (Fig. 1). We did not find a significant effect of the interaction between physiological state and microclimatic condition on VO_2 ($F_{6,134} = 0.4$, $P = 0.893$; Fig. 2).

Experiment 2

Global analysis

Snakes from all physiological states chose microclimatic conditions non-randomly ($\chi^2 = 37$, df = 3, $P < 0.001$; Fig. 3) but differences were not significant among physiological states ($\chi^2 = 8.0$, df = 3, $P = 0.540$; Fig. 3). When pooling the snakes in the three energy-demanding physiological states and comparing them to control snakes, snakes in the energy-demanding states marginally preferred Warm x Humid conditions ($\chi^2 = 3.5$, df = 1, $P = 0.061$). Although we also found that control snakes were more likely to choose Cool x Dry conditions ($\chi^2 = 4.6$, df = 1, $P = 0.032$), our sample sizes for this test were relatively low—thus, this result is less interpretable (Fig. 3).

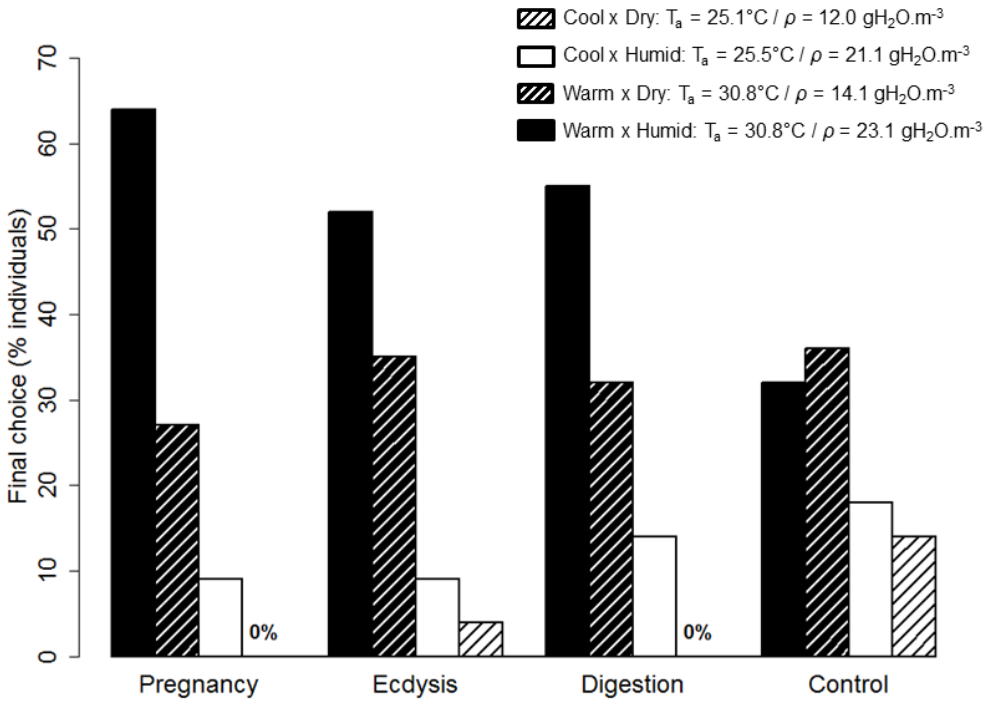

Figure 3. Proportion of snakes in different physiological states (Pregnancy: n = 11; Ecdysis: n = 23; Digestion: n = 22; Control: n = 22) selecting varied microclimatic conditions (combination of ambient temperature T_a and absolute humidity ρ).

There was no significant preference between control and energy-demanding physiological states for Cool x Humid (χ^2 = 0.79, df = 1, P = 0.38) and Warm x Dry (χ^2 = 0.13, df = 1, P = 0.72) (Fig. 3).

State-specific analysis

We found a significant effect of physiological state on snake T_{body} ($F_{3,74}$ = 3.3, P = 0.024; Fig. 4A). At the end of behavioral session, pre-shedding and digesting snakes had higher T_{body} than control snakes (post hoc, all P < 0.043; Fig. 4A). Pregnant snakes had intermediate T_{body} because they did not significantly differ either from controls (post hoc, t = -1.6, P = 0.400; Fig. 4A) or from the other energy-demanding physiological states (post hoc, all P > 0.901; Fig. 4A). We did not find a significant effect of physiological state on selected humidity ($F_{3,70}$ = 1.9, P = 0.143; Fig. 4B), although pregnant snakes tend to select higher humidity than controls (Fig. 4B).

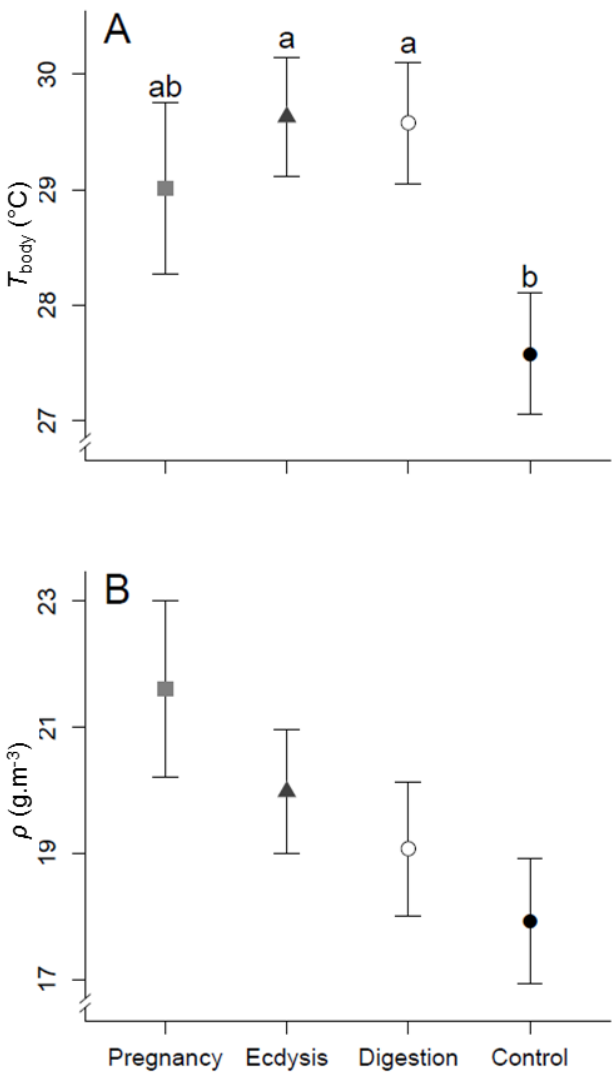

Figure 4. Effect of physiological state on selected microclimatic conditions. Points represent mean values (± SE) of A) snake T_{body}, and B) absolute humidity (ρ) in the selected compartment at the end of behavioral trials. Different letters above symbols represent significant differences among physiological states.

Discussion

Water is a vital resource, often overlooked in ecological studies (Bonnet & Brischoux 2008). Yet, habitat humidity and water availability in the environment are critical to animal survival and reproduction (Lorenzon et al. 1999; Marquis et al. 2008), and behavioral adjustments (e.g., microhabitat or microclimate selection) are therefore central to optimizing physiological tradeoffs (Köhler et al. 2011; Stahlschmidt et al. 2011). In this study, we conducted two independent experiments to assess the effects of

physiological state and microclimatic conditions on i) total evaporative water loss (TEWL) and metabolic rate (VO_2), and ii) on microclimate preferences in a viviparous snake. We first hypothesized that physiological would impact water losses and metabolic rates. Supporting our associated prediction, we found direct effects of energy-demanding states (i.e., pregnancy, ecdysis, and digestion) on physiological processes because they increased the rate of TEWL and VO_2. We then hypothesized that physiological state would drive snake microclimate selection, and we predicted that energy-demanding state will orient snake preferences to a more humid microclimate. Instead, behavioral investigations revealed only marginal preference for warm and humid conditions in energy-demanding states compared to controls.

Energy-demanding states and water balance

When compared to control snakes, we found that snakes in states of pregnancy, ecdysis, and digestion exhibited increased the rates of water loss (average, pregnancy: +31%, ecdysis: +33%, digestion: +11%) and oxygen consumption (average, pregnancy: + 27%, ecdysis: +32%, digestion: +117%). It has been previously demonstrated that metabolic rate increases during ecdysis (Taylor & Davies 1981; Blem & Zimmerman 1986), pregnancy (Birchard et al. 1984; Van Dyke & Beaupre 2011; Dupoué & Lourdais 2014), and digestion (SDA reviewed in Secor 2009) in snakes, but we are the first to report shifts in metabolic rate *and* water loss due to all three of these physiological states in a single study. Importantly, higher oxygen consumption should result in higher ventilation and thereby increase respiratory water flux (i.e., respiratory evaporative water loss; Mautz 1982). However, while digesting *V. aspis* presented the highest increase in metabolic rate due to SDA (Fig. 2), they showed a lower TEWL rate than other energy-demanding physiological states (Fig. 1). Moreover, we detected a significant correlation between VO_2 and TEWL in digesting and control snakes but not in pregnant and pre-shedding individuals. Together, these results suggest that respiratory water loss is not the main component of the observed increase in TEWL. Instead, skin surface may represent a more important avenue for water losses (cutaneous evaporative water loss, CEWL; Mautz 1982; Dmi'el 1972; Cohen 1975; Kattan & Lillywhite 1989; Lillywhite 2006). In support of this, pregnant and pre-shedding snakes undergo important skin modifications, which likely results in increased rate of CEWL. During pregnancy, offspring burden causes the distension of maternal skin (Shine 1988). This mechanic distension increases the surface-to-volume ratio and decreases skin's resistance (by reducing its thickness), which increases the rate of CEWL (e.g.,

Agugliaro & Reinert 2005). Similarly, over the ecdysis cycle, skin permeability increases in response to the loss of thickness and covering lipid (Cohen 1975; Zucker & Maderson 1980; Tu et al. 2002; Lillywhite 2006). Also, the space between the old and next skin is filled with a lymph-like fluid (Mader 2005), which also likely increases the rate of CEWL. Yet, the impact of physiological states on water balance remains understudied, and further studies should continue to investigate the functional properties of physiological states that induce water loss.

Our study allowed us to quantify the effects of microclimatic conditions on the rate of TEWL and VO_2. We found a strong effect of temperature on TEWL and VO_2 with a higher thermal dependence of water loss (Q_{10} = 3.98) than metabolic rate (Q_{10} = 2.72), which is consistent with previous assumptions that respiratory water loss is not the main component of TEWL (see above). Water loss increases with temperature because of increased metabolism (see above) but mostly because of the decrease in skin resistance (Lillywhite 2006). When thermal conditions were constant (T_a = 30°C), the rate of TEWL was 86% higher in dry (ρ = 14 gH2O.m^{-3}) than in humid conditions (ρ = 25 gH2O.m^{-3}) while VO_2 was not impacted by humidity.

Behavioral adjustment to mediate state-specific water constraint

Considering the effects of microclimatic conditions on water loss, we expected snakes to optimize their microclimatic choices (i.e., choose a warm and humid microclimate) to enhance temperature-dependent physiological processes while limiting evaporative water loss (Köhler et al. 2011; Stahlschmidt et al. 2011). Yet, we found a complex interaction between physiological state and microclimatic selection. Although marginal in our study, energy-demanding states induced a higher selection for both warm and humid conditions compared to control snakes. Marginal differences likely result from a reduced sample size that impacted the power of this test (1-β = 0.78). Ideally, we would have to test microclimate selection in at least 20 snakes for each physiological state, but we were dependent on snake capture. However, pregnant snakes tended to select higher humidity than control snakes, which might highlight state-specific physiological constraints. For instance, pregnancy has been demonstrated to be a particularly energy-demanding state (King 2000; Bonnet et al. 2002; Lourdais et al. 2002a, 2002b; Dupoué & Lourdais 2014) with a shift in maternal behavior and thermal preference (Waspra et al. 2010; Lorioux et al. 2013). Further, pregnant squamates are likely exposed to

water constraints because they exhibit higher TEWL than controls (this study) and because they provide water to their developing embryos over a prolonged period (Dauphin-Villemant & Xavier 1986). In comparison, ecdysis and digestion states may impose lower constraints on water balance because individuals are only exposed to a transient increase in water loss. Digestion is likely even less constraining for water balance because water loss is low (Fig. 1) and because snakes rely on water content of the prey to maintain water balance (but see Wright et al. 2013).

Microclimate selection buffers physiological constraints and has been reported in a number of different taxa (Lorenzo & Lazzari 1999; Patten et al. 2005; Boyles et al. 2007; Köhler et al. 2011; Stahlschmidt et al. 2011). Our results on humidity selection suggest that different physiological processes impose different constraints on water balance. In our study, snakes were provided with water *ad libitum* and therefore were not in a negative water balance. Organisms are regularly exposed to significant water restriction periods (i.e., summer droughts or heat waves). Under water deprivation, we expect that constraining physiological state will compromise water balance and likely induce dehydration. In response to negative water balance, we expect more marked microclimate selection than the response we observed here and possibly a decrease in thermal preference (Ladyman & Bradshaw 2003). Future work should investigate the interaction between microclimate selection and physiological state following water deprivation.

More generally, our results underline the impact of physiological state on water loss and demonstrate a potential tradeoff between thermoregulation and water balance. We considered the interaction of two major climatic conditions (temperature and humidity) on physiological and behavioral responses. Studies integrating the impacts of shifts in multiple environmental factors offer ecologically relevant insight because animals typically respond to multiple stressors in the field (Todgham & Stillman 2013). First, we demonstrate that energy-demanding physiological states negatively impact water balance. Second, we found that these physiological states may drive microclimatic preferences, particularly during pregnancy. Such behavioral adjustments in response to state-specific physiological constraints are important to investigate given the increasing frequency or duration of extreme climatic events (Easterling et al. 2000; IPCC 2014). Currently, a major objective of scientists is to determine and predict animal

responses to global changes (Kearney et al. 2009; Dillon et al. 2010; Angelier & Wingfield 2013; Todgham & Stillman 2013). The duration and frequency of water-constraining events are expected to increase in the near future because of global climate change (IPCC 2014). Therefore, integrating the impact of physiological states on water balance, energy use (e.g., metabolic rate or VO_2), and behavioral responses provides key insight into the complexity of animals' responses to environmental shifts (Todgham & Stillman 2013).

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Chapitre 2 :
Contraintes physiologiques et morphologiques de la gestation

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Résumé du chapitre 2

La reproduction est généralement associée à de nombreux changements d'ordre physiologiques, morphologiques, et comportementaux. La nature des contraintes (dynamique et dépendance à la fécondité) est essentielle à déterminer pour comprendre les bases fonctionnelles du coût de la reproduction (i.e., le compromis entre reproduction courante et survie et/ou reproduction future).

Dans ce chapitre, nous nous sommes intéressés à des contraintes de la gestation à l'aide d'une approche descriptive. La viviparité est un mode de reproduction qui présente des avantages (i.e., optimisation des conditions de développement) mais qui peut induire des coûts pour les femelles en termes de diminution de la survie ou de la reproduction future. A l'origine de ces coûts se trouvent des contraintes proximales imposées notamment au moment de la gestation qui peuvent être d'ordre physiologique : énergétiques (**Article 2**), hydriques (**Articles 3** et **4**), ou bien d'ordre morphologique (**résultats préliminaires**). Comme chez la plupart des espèces de Squamates vivipares, la vipère aspic présente un mode de placentation lécithotrophe, c'est-à-dire qu'avant la gestation les femelles produisent des follicules remplis de vitellus, qui représentent la source principale d'énergie pour l'ensemble du développement embryonnaire. Les femelles doivent également allouer de l'énergie pour soutenir la gestation (i.e., approvisionnement en eau et en oxygène, gestion des déchets azotés). Dans un premier temps, nous avons quantifié l'évolution du métabolisme au cours de la gestation et l'influence de l'effort reproducteur. Ensuite, contrairement à l'énergie, l'eau est difficilement stockée ; or le transfert d'eau est essentiel notamment pour la croissance des fétus. Nous avons donc examiné en détail des contraintes hydriques de la gestation. D'une part, nous avons étudié la dynamique de transfert d'eau dans la portée, et les conséquences sur les variations morphologiques des femelles (distension cutanée). D'autre part, nous avons clarifié les déterminants directs et indirects des pertes hydriques au cours de la gestation.

Les résultats indiquent que les contraintes de la gestation (énergétiques, hydriques, et morphologiques) sont très marquées et directement influencées par l'effort reproducteur. Le métabolisme standard des femelles augmente de 55% au cours de la gestation, ce qui est directement dépendant de la fécondité. Par ailleurs, le transfert d'eau au cours de la gestation

estimé par l'évolution du volume des œufs augmente exponentiellement en lien avec la croissance somatique des embryons. De façon concomitante, la masse corporelle des femelles augmente au cours de la gestation, dépendamment de la fécondité, ce qui suggère une prise en eau massive des femelles (jusqu'à 30g ; 25% de la masse initiale). L'évolution des besoins en eau en lien avec le développement exponentiel des embryons induit des modifications morphologiques conséquentes avec d'une part un avancement abdominal de 5.5cm (12%) de la portée, et d'autre part une distension abdominale de l'ordre de 25-30% dès l'ovulation. L'augmentation du métabolisme standard et la distension abdominale sont probablement les deux paramètres physiologiques principaux qui induisent l'augmentation des pertes hydriques totales. Cette cause directe de l'augmentation des pertes en eau liée aux propriétés physiologiques du statut reproducteur est de plus indirectement renforcée par les modifications comportementales pendant la gestation. En effet, les femelles gestantes augmentent leurs préférences thermiques, ce qui au vue de nos résultats est probablement la principale cause de l'augmentation des pertes hydriques totales. En définitive, lorsqu'on considère les résultats des pertes hydriques totales chez les femelles gestantes et non-reproductives couplés à leurs préférences thermiques respectives précédemment déterminées (T_{set} = 33 *vs* 25°C), la gestation induit une augmentation de 38% des pertes hydriques totales.

Les différentes contraintes associées à la gestation et l'influence de la fécondité sont essentielles à considérer lorsqu'on s'intéresse au coût de la reproduction. Contrairement aux besoins thermiques non-dépréciables (i.e., indivisibles entre les embryons), nos résultats ont démontré que d'une part les changements métaboliques et d'autre part les contraintes liées à l'eau représentent des soins maternelles dépréciables puisque les changements métaboliques, le transfert d'eau et les pertes hydriques sont dépendant de la fécondité. L'eau représente donc une ressource majeure potentiellement coûteuse pendant la gestation puisque les femelles doivent acquérir de l'eau d'une part pour la croissance des embryons et d'autre part pour compenser des pertes hydriques totales supérieures. La gestation ayant lieu pendant l'été lorsque la disponibilité en eau est réduite, l'influence significative de la fécondité sur les contraintes hydriques est un paramètre déterminant dans l'optimisation des compromis intergénérationnels (voir le chapitre suivant).

ARTICLE 2

Relative reproductive effort drives metabolic changes and maternal emaciation during pregnancy in a viviparous snake

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pregnancy; metabolic rate; fecundity; relative reproductive effort; emaciation; *Vipera aspis*.

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Abstract

Most viviparous squamates are lecithotrophic, and maternal effort during pregnancy mainly involves behavioural and thermoregulatory shifts to optimize developmental conditions. Still, pregnancy also imposes specific metabolic demands on the female, known as the metabolic cost of pregnancy (MCP). Contrary to the thermoregulatory shift, these energy constraints should be directly fecundity dependent and their evaluation is important to assess the 'costs' of viviparity. We measured the metabolic rate of aspic vipers *Vipera aspis* at three stages (early pregnancy, late pregnancy and post parturition), and we examined the determinants of maternal metabolic changes over time. We found a 55% metabolic increase over the course of pregnancy that was better explained by maternal relative reproductive effort (relative litter mass) when compared with absolute estimates (litter mass, litter size). After parturition, female metabolism dropped below values recorded at early pregnancy and this decrease was closely related to maternal relative reproductive effort. Our estimates for MCP ranged from 13.9 to 14.7% of maternal metabolic rate, suggesting that specific energetic demands of pregnancy are significant. It appears crucial to consider both direct (MCP) and indirect (thermoregulatory shift) components to evaluate overall maternal metabolic demand during pregnancy. Because females are already emaciated at the onset of pregnancy, these combined constraints are likely costly by inducing structural protein mobilization and altered performances after parturition.

Introduction

Reproduction imposes multiple demands on females that have to allocate a substantial amount of energy and time towards offspring production. Because resources are often limited, optimality models predict that females will adjust their allocation in current reproduction to optimize lifetime reproductive success (i.e. life-history traits; reviewed in Congdon, Dunham & Tinkle, 1982; Charnov, Warne & Moses, 2007). One major aspect in addressing reproductive constraints is the influence of fecundity. For example, mothers will have to allocate nutrients to support offspring development, and a fecundity increase will directly affect maternal effort. Alternatively, reproduction can involve substantial behavioural shifts in activities that are fecundity independent. This is typically the case for accessory activities such as egg guarding, breeding migration or maternal thermoregulation (Adamczewska & Morris, 2001; Steinhart *et al.*, 2005; Shine, 2012; Lorioux, Lisse & Lourdais, 2013*a*; Lorioux *et al.*, 2013*b*). The fecundity dependence of maternal allocation and associated costs is likely a major determinant of optimal reproductive effort and, more generally, reproductive strategies (Bull & Shine, 1979; Bleu *et al.*, 2011).

Viviparity has emerged on repeated occasions among vertebrate lineages including fishes (Dulvy & Reynolds, 1997), amphibians (Blackburn, 1999), reptiles (Tinkle & Gibbons, 1977; Blackburn, 1985; Shine, 1985) and mammals (Zeller, 1999). Within squamates, transition to viviparity appeared more than 100 independent times (Blackburn, 2000). Most squamates are lecithotrophic, and embryonic energy demands are supported by important yolk reserves (Stewart & Thompson, 2000). Maternal effort during pregnancy is therefore primarily linked to thermoregulation (Tinkle & Gibbons, 1977; Shine & Harlow, 1993; Shine, 1995; Bernardo, 1996). That is, pregnant females typically display prolonged basking behaviours, modified thermal preference and more stable body temperatures (Shine, 1980, 2006, 2012; Charland & Gregory, 1990; Lorioux *et al.*, 2013*a*). Maternal thermoregulation is beneficial by buffering the impact of environmental fluctuations (Shine, 1985; Stewart & Thompson, 1993; Qualls & Andrews, 1999; Crespi & Semeniuk, 2004; Lorioux *et al.*, 2013*b*) on developmental rate (Tinkle & Gibbons, 1977; Shine

& Bull, 1979; Shine, 1983) and offspring quality (Shine, 1995, 2004; Lorioux *et al.*, 2013*b*).

Viviparity also imposes substantial constraints on the female. For example, the physical burden imposed by developing embryos will alter female locomotor performance and will increase vulnerability to predators (Shine, 1988; Le Galliard, Le Bris & Clobert, 2003; Schwarzkopf & Andrews, 2012). Moreover, basking behaviour will profoundly affect maternal activity budget and will sometimes translate in reduced or absent food intake and compromised energy balance (Lourdais *et al.*, 2004; Lourdais, Lorioux & DeNardo, 2013). Modified thermal preferences will affect female metabolic rate especially in species that shift to higher thermal regimes (Ladyman *et al.*, 2003; Lorioux *et al.*, 2013*a*). Pregnancy also imposes specific metabolic demand on the female, called metabolic cost of pregnancy (MCP; Birchard *et al.*, 1984). Indeed, even in lecithotrophic species, pregnant females have to cope with embryonic oxygen demand and also the processing of embryonic waste (Van Dyke & Beaupre, 2011). For example, pregnant garter snakes display increased heart rate and modified blood parameters to facilitate oxygen delivery to the embryos (Birchard *et al.*, 1984). Although embryonic metabolic requirements are minimal at ovulation, they increase progressively along with exponential somatic growth and are likely to affect maternal demand. MCP has already been documented in various viviparous squamates species (Birchard *et al.*, 1984; Beuchat & Vleck, 1990; DeMarco & Guillette, 1992; Robert & Thompson, 2000; Schultz, Webb & Christian, 2008; Van Dyke & Beaupre, 2011; Yue *et al.*, 2012).

Clarifying the relative costs of pregnancy and the proximate determinants of metabolic changes is important to better understand the energetic implication of viviparity (Bleu *et al.*, 2011). Metabolic rate at late pregnancy will result from both maternal contribution (maintenance requirements and MCP) and embryonic metabolism that dramatically increases during somatic growth and foetal life (Robert & Thompson, 2000; Andrews, 2004). Importantly, substantial variations in reproductive output (i.e. litter size) exist within snakes (Bonnet *et al.*, 2003), which can reflect either variation in maternal body size or variation in maternal reproductive allocation (relative to maternal size or mass) (Bonnet *et al.*, 2003). Maternal relative reproductive effort is likely a relevant parameter that should help us to understand metabolic demand and the specific costs of pregnancy. If MCP is significant, we predict that maternal relative reproductive effort should be a better descriptor of metabolic changes when compared with absolute reproductive output (embryonic contribution). Yet, this parameter remains largely understudied.

In this study, we measured metabolic rate over pregnancy in a viperid snake, the aspic viper *Vipera aspis*. We considered three relevant periods: early pregnancy, late pregnancy and post parturition. We compared different measures of reproductive effort on (1) metabolic increase during pregnancy; (2) metabolic decrease associated with parturition; (3) maternal emaciation. We predicted that maternal relative reproductive effort would be a better predictor of metabolic changes and of female emaciation than absolute output (litter mass, litter size). Finally, we estimated MCP using a method previously described (Birchard *et al.*, 1984; Schultz *et al.*, 2008).

Materials and methods

Study species

We studied the aspic viper *V. aspis*, a medium-sized venomous snake of the Western Palearctic region (Naulleau, 1997). The aspic viper is known as a typical capital breeder, which means that females store important fat reserve before reproduction and often cease to feed during pregnancy (Bonnet, Bradshaw & Shine, 1998; Bonnet *et al.*, 1999; Bonnet, Naulleau & Lourdais, 2002*a*; Bonnet *et al.*, 2002*b*; Lourdais, Bonnet & Doughty, 2002*a*). Pregnancy begins after ovulation in early June (Naulleau, 1981) and parturition occurs 2–3 months later from late August to early September (Lourdais *et al.*, 2002*a*). Because of high energetic demand of reproduction, females are extremely emaciated after parturition (Bonnet *et al.*, 1999, 2002*b*).

Capture and maintenance

In late May to early June 2010, we caught 16 reproductive females [mean ± standard error (SE); body mass (BM): = 84.7 ± 4.4 g; snout-vent length (SVL): = 44.9 ± 0.9 cm] from several neighbouring population in Western-Central France (Vendée, Loire-Atlantique and Maine et Loire District). Reproductive status was first determined with manual palpation of the abdomen and then checked in the laboratory with high-resolution ultrasonography (SonoSite microMaxx, Inc., Bothell, WA, USA). All snakes were weighed (±1 g), measured (±0.5 cm) and individually housed in plastic boxes (30 × 20 × 10 cm). In this species, a skin-shedding episode occurs at the onset of pregnancy (early June) close to ovulation time (see Lorioux *et al.*, 2013*b*). For each female, we recorded skin-shedding date as an index of the onset of pregnancy. Females were maintained in climatic chambers (Vötsch, Industrietechnik, VP 600, Balinger, Germany), which allowed us to recreate natural daily temperature cycle (16–33°C). Water was provided *ad libitum*. Because females often cease feeding during pregnancy, individuals were fasted during the experimentation (Lourdais *et al.*, 2002*a*) to avoid possible bias caused by food intake (Andrade, Cruz-Neto & Abe, 1997).

At the end of pregnancy, cages were inspected daily to detect parturition. The different components of the litters (neonates, stillborns and undeveloped ova) were counted and weighed (±0.1 g). Offspring and stillborns were also measured (±0.1 cm) and sexed. These measurements allowed us to consider different estimates of reproductive output: litter size (i.e. number of all litter components), fit litter size (i.e. number of viable offspring), litter mass (i.e. mass of all litter components) and fit litter mass (i.e. mass of viable offspring); see details on methods in Lourdais *et al.* (2002*a*). Offspring were housed individually in small plastic containers (10.5 × 30.0 × 16.5 cm)

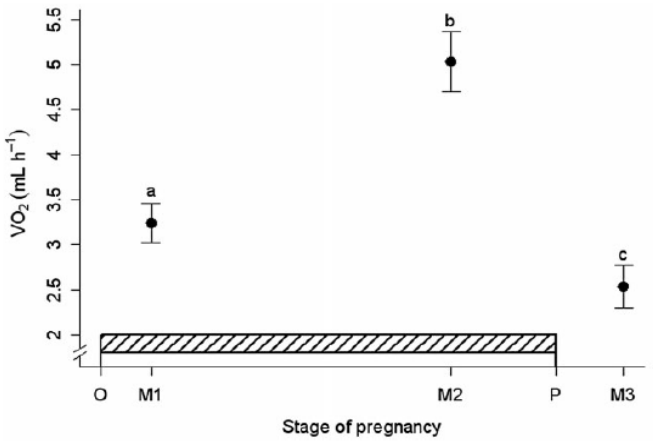

Figure 1 Oxygen consumption (VO_2) of female *Vipera aspis* ($n = 16$) measured at different stages of pregnancy (M1: early stage, 11% of pregnancy duration; M2: late stage, 77% of pregnancy duration; M3: post parturition, 115% of pregnancy duration). O and P designate Ovulation and Parturition (i.e. 0 and 100%, respectively, of pregnancy duration). Each point represents the mean (± standard error) of VO_2 (mL h^{-1}). Different letters above symbols represent significantly different values.

containing a polyvinyl chloride shelter and water *ad libitum* for 1 month before being release with their mother at the exact site of capture.

Experimental design

Metabolic rate was assessed at three stages of pregnancy (early, late pregnancy and post parturition; see Fig. 1). Using shedding (i.e. ovulation) and parturition dates it was possible to estimate accurately at what stages measures were carried. First metabolic rate was measured shortly after ovulation (mean ± SE: 6.9 ± 0.9 days since ovulation) while 11% of pregnancy duration occurred. Then a second measure was carried in late pregnancy (mean ± SE: 51.2 ± 1.2 days since ovulation) when >90% of vitellus was absorbed by embryos (see details on the method in Lorioux *et al.*, 2013*b*), while 77% of pregnancy duration occurred. A final measure was carried few days after parturition (mean ± SE days since parturition: 9.2 ± 1.0 days).

Oxygen consumption

We measured standard metabolic rate (SMR) of females using closed-system respirometry. All trials were carried at 25°C as this temperature has previously been used for metabolic measure on aspic viper (Ladyman *et al.*, 2003). Snakes were placed into test chambers (2170 mL) in a climatic chamber (±1°C) (Mastercella Cryosystem, SAS ERCO, Niort, France). Snakes acclimatized for 3 h before each run to reach thermal equilibrium and reduce activity. During acclimation, air was continuously flush with a 55 L min^{-1} pump (Bioblock Scientific, AVANTEC, Illkirch, France). A baseline air sample was collected for each individual at the onset of the trial, and the test chamber was then carefully sealed. Average trial duration (mean ± SE) was 131.6 ± 2.7 min. A final sample of air was collected with two 140-mL syringes connected to a three-way stopcock. The oxygen (O_2) proportion was then determined via an O_2 analyser (FoxBox; Sable Systems, Las Vegas, NV, USA). Before entering the analyser, air was dried by passing through a column of self-indicating Drierite and sent with a constant flow (36 mL min^{-1}) controlled with an infusion pump (KDS210; KD Scientific, Inc., Holliston, MA, USA). The O_2 analyser was calibrated before each trial using outdoor air. Finally, O_2 consumption (VO_2 in mL h^{-1}) was obtained by subtracting final from baseline values considering the volume of test chamber (mL) and run duration (h).

Reproductive effort and maternal emaciation

Multiple methods have been described to assess reproductive effort in snakes (see Bonnet *et al.*, 2003). Absolute measures of reproductive output (litter mass, litter size, or fit litter mass and fit litter size) are routinely used (Lourdais *et al.*, 2002*a*). However, it is crucial to control for allometric influences (Bonnet *et al.*, 2003). Therefore, we also considered the relative litter mass (RLM) derived from the ratio between litter mass and post parturition female BM (i.e. RLM, in %). We also used the residuals of the linear relationship between litter mass and maternal SVL (i.e. RLM, residuals; Bonnet *et al.*, 2003) as this relation was significant ($F_{1,14} = 5.2$, $P = 0.039$).

We considered the body condition (BC) after parturition (i.e. post-partum BC) as an estimate of maternal emaciation (Bleu *et al.*, 2011). Post-partum BC was obtained by extracting residuals from the relation between female BM after parturition against SVL (Jayne & Bennett, 1990).

MCP estimates

We used a previously described method to estimate MCP (Birchard *et al.*, 1984). This method consists of plotting the difference between the VO_2 of pre-parturient females and the VO_2 of post-parturient females (i.e. ΔVO_2) against the litter size. The intercept of the regression that represents the VO_2 for a hypothetical pregnant female with no offspring should indicate the MCP. Here, we also estimated MCP by plotting ΔVO_2 against the RLM (%). The RLM (residuals) could not be used as the intercept does not represent a hypothetical absence of embryos.

Statistical analyses

All statistical analyses were performed with R software (R Development Core Team, 2011). First, we used linear mixed model (package nlme) to analyse metabolic change over pregnancy with pregnancy stage (i.e. early, late and post) as a factor and female identity as a random factor to control for repeated contributions. VO_2 was log_{10} transformed so the distribution of the residuals of the model conformed to the

Table 1 Model selection comparing the relation between different estimates of reproductive effort (litter size, fit litter size, litter mass, fit litter mass or relative litter mass) and metabolic increase (late–early pregnancy) or ΔVO_2 (late pregnancy–post parturition)

	Metabolic increase		ΔVO_2		MCP	
	AIC	*P*	AIC	*P*	Value	*P*
Litter size	60.0	0.947	62.5	0.827	2.24 (44.6%)	0.062
Fit litter size	50.1	0.004	55.9	0.023	0.67 (13.3%)	0.109
Litter mass (g)	52.8	0.013	53.8	0.007	0.74 (14.7%)	0.044
Fit litter mass (g)	49.6	0.003	48.4	0.001	0.70 (13.9%)	0.016
RLM (%)	**40.3**	<0.001	48.9	0.001	0.71 (14.1%)	0.016
RLM (residuals)	43.6	<0.001	**45.4**	<0.001	–	–

Metabolic cost of pregnancy (MCP; mL h^{-1}) was estimated from the intercept of the relation of ΔVO_2 against fecundity or relative litter mass (RLM) and its statistical difference from zero. Boldfaced numbers correspond to the smallest values of AIC. Values between parentheses correspond to the MCP expressed as a proportion of late pregnancy metabolic rate. AIC, Akaike's information criterion. See text for details.

assumptions of normality (Shapiro–Wilk test, $P = 0.342$). We did not adjust VO_2 with BM of females because pregnancy is associated with dramatic variation in BM ($F_{2,30} = 247.8$, $P < 0.0001$). VO_2 was not adjusted to female body size as the relation was not significant at the different stages considered ($F_{1,30} = 2.6$, $P = 0.128$). We used pairwise *post hoc* tests of Tukey (package multcomp) to compare the three stages of pregnancy.

Second, we compared the influence of different measures of reproductive effort (i.e. litter size, fit litter size, litter mass, fit litter mass and the two estimates of RLM) on metabolic changes. We considered separately metabolic increase over pregnancy (late–early pregnancy) and metabolic drop associated with parturition (late–post; ΔVO_2). We considered the model with the smallest Akaike's information criterion (AIC) to determine the best determinant of maternal metabolic changes. When two models differed by less than two AIC, they were considered equivalent.

We used non-linear models to compare the influence of the different determinants of reproductive effort on the ΔVO_2 and to assess the associated values of MCP. A previous investigation on the dataset revealed that the best fitting curve between ΔVO_2 and the different determinants of reproductive effort followed an exponential relation. We used linear models to test the influence of the metabolic increase and RLM (residuals) on female post-partum BC.

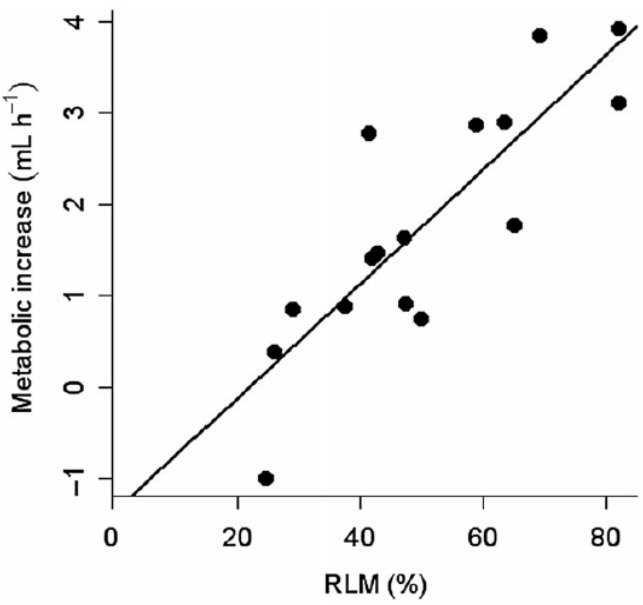

Figure 2 Influence of relative litter mass (RLM; %) on the metabolic increase during pregnancy (mL h^{-1}). Metabolic increase was derived for each individuals (n = 16) from the difference in VO_2 between early and late stages. The line represents the fitted linear regression ($P < 0.001$, $r^2 = 0.71$).

Results

Metabolic changes over pregnancy

Oxygen consumption (VO_2) was not constant throughout pregnancy ($F_{2,30} = 28.7$, $P < 0.0001$; Fig. 1). First, a 55% increase in SMR was observed between early and late pregnancy measures (Tukey's test, $P < 0.001$; Fig. 1). Then, metabolic rate significantly dropped after parturition and differed from the two other stages (Tukey's test, all $P < 0.01$; Fig. 1).

Determinants of metabolic changes and maternal emaciation

The metabolic increase between early and late pregnancy was significantly explained by the different estimates of reproductive effort except litter size (Table 1; Fig. 2). Yet, model selection indicated that RLM (%) was the best descriptor (Table 1). Similarly, the drop in metabolic rate after parturition was significantly influenced by each estimate of reproductive effort except litter size (Table 1). Model selection indicated that RLM (residuals) was the best descriptor (Table 1).

Finally, female post-partum BC was significantly influenced by the RLM (residuals) ($F_{1,14} = 6.0$, $P = 0.028$) and by the metabolic increase over pregnancy ($F_{1,14} = 5.8$, $P = 0.030$; Fig. 3).

MCP estimate

MCP estimates were derived from the intercept and were significant when using litter mass, RLM (% and residuals), but

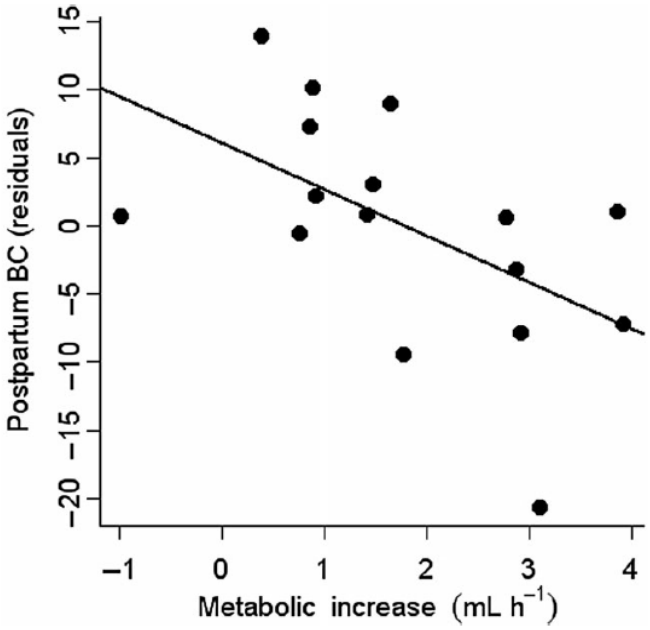

Figure 3 Relation between the metabolic increase during pregnancy and female post-partum body condition (BC; residuals). The line represents the fitted linear regression ($P = 0.030$, $r^2 = 0.29$).

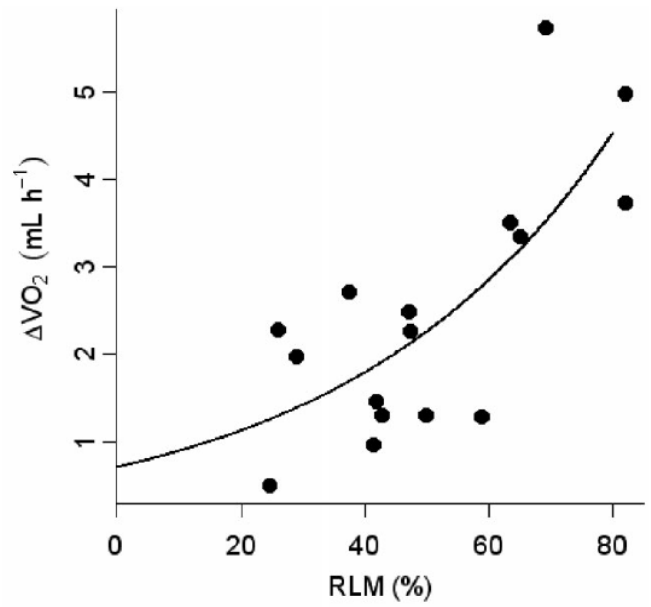

Figure 4 Relation between the relative litter mass (RLM; %) and metabolic drop (ΔVO_2) after parturition. The line represents the fitted curve from exponential regression ($y = 0.705\ e^{0.0232x}$). The metabolic cost of pregnancy corresponds to the intercept (0.705 ± 0.259 mL h^{-1}) of the regression.

not litter size as descriptive variables (Table 1; Fig. 4). Considering only significant contributions, we found close MCP values ranging from 0.70 to 0.74 mL h^{-1}, which represent 13.9 and 14.7%, respectively, of the maternal metabolic rate in late pregnancy (Table 1).

Discussion

Viviparity has emerged independently on a multitude occasions (>110 times) among squamates, and addressing associated 'costs' is critical to better understand this evolutionary transition. Our study, in a lecithotrophic species, highlights that litter mass influences maternal metabolic changes over pregnancy but relative reproductive effort is a better determinant than absolute measures of reproductive output. This component of gestational constraints may contribute to post reproductive costs (maternal emaciation) and thereby favour optimal allocation strategy at earlier stages (i.e. vitellogenesis).

We observed a 55% rise in the metabolic rate between early and late pregnancy. This increase was closely related to the different estimates of reproductive effort (except litter size), illustrating a proximate link between embryo development and maternal metabolic rate. Previous investigations on the same species did not reveal any metabolic change over pregnancy (Ladyman *et al.*, 2003). This is possibly due to methodological bias and the fact that different individuals were compared over time. In this study, we used closed-system respirometry with repeated measures, which revealed a clear metabolic increase consistent with other studies (Birchard *et al.*, 1984; Beuchat & Vleck, 1990; DeMarco & Guillette, 1992; Robert & Thompson, 2000; Schultz *et al.*, 2008; Van Dyke & Beaupre, 2011; Yue *et al.*, 2012). This pattern mainly reflects embryonic metabolism that will increase over time due to exponential somatic growth (Robert & Thompson, 2000; Andrews, 2004). Besides, specific maternal physiological adjustments occur such as an increased heart rate (Birchard *et al.*, 1984) combined with modified red cell oxygen affinity (Holland *et al.*, 1990; Ingermann, Berner & Ragsdale, 1991; Ragsdale & Ingermann, 1991). Such physiological adjustments likely aim at optimizing the transfer of oxygen and water to the embryos along with the excretion of waste (Van Dyke & Beaupre, 2011). Parturition was associated with a major metabolic drop, and the lower metabolic rate observed in post-partum females compared with ovulated ones could reflect female emaciation due to protein catabolism. Females may also already face an increased metabolism early in pregnancy although embryonic metabolic requirements shortly after ovulation are likely negligible.

Body size has a proximate influence on reproductive output (either litter size or mass) in snakes (Bonnet *et al.*, 2003), but substantial variation also exists independently of body size reflecting different levels of reproductive allocation. In this context, we expect that a female with a high output relative to its size or mass (i.e. high relative reproductive effort) should face higher costs of supporting pregnancy when compared with a female that allocates in a lower relative reproductive effort. If so, metabolic changes over pregnancy should be more closely related to relative reproductive effort than absolute reproductive output. In turn, if MCP is small or

negligible, metabolic changes should predominantly reflect the embryonic demand, and measures of reproductive output (litter size or litter mass) should then provide better descriptors. Here, we specifically compared the influence of absolute and relative reproductive effort on metabolic changes. We found that metabolic increase over pregnancy and metabolic drop after parturition were closely related to the different estimates considered (see Table 1). Interestingly, the influence of litter size (including non-viable small-sized elements such as undeveloped ova) was not significant, whereas fit litter size and mass were closely related to metabolic changes suggesting that the number or mass of developing embryos (i.e. metabolically active tissues) is more relevant. Still, metabolic changes were best explained respectively by RLM (%) and size-adjusted litter mass (residuals) (Table 1) underlying that female relative reproductive effort will profoundly affect metabolic changes. Maternal emaciation after parturition is an important life-history parameter in viviparous squamates (Bonnet *et al*., 1999; Bleu *et al*., 2011; Baron *et al*., 2012) that influences female survival after reproduction and the time to recoup energy between breeding episodes (Bonnet *et al*., 1999; Lourdais *et al*., 2002*b*). We found that post-partum BC was closely related to metabolic increase over pregnancy as well as maternal relative reproductive effort. Together, these results suggest that relative reproductive effort drives maternal metabolic changes over pregnancy and affects maternal physiological state after reproduction.

We detected significant and close MCP estimates (13.9–14.7% of late pregnancy maternal metabolic rate) with the different measures of reproductive effort. MCP has already been measured in different viviparous squamate species (Robert & Thompson, 2000; Schultz *et al*., 2008; Yue *et al*., 2012), but values derived from other studies were non-significant or negative (Birchard *et al*., 1984; Beuchat & Vleck, 1990; DeMarco & Guillette, 1992; Van Dyke & Beaupre, 2011). It is not clear whether these discrepancies result from biological differences or inherent methodological biases (Van Dyke & Beaupre, 2011). Here again, model selection suggests that maternal relative reproductive effort provides a valuable descriptor. It is worth noting that direct metabolic demands (MCP) are not sufficient to assess the overall energetic impact of pregnancy. Modified thermoregulation also contributes to increase metabolic rate (Bennett & Dawson, 1976; Ladyman *et al*., 2003) notably in species that select higher body temperature during pregnancy such as the aspic viper (Ladyman *et al*., 2003; Lorioux *et al*., 2013*b*). It has been recently demonstrated that thermoregulatory effort is independent of fecundity, and all developing embryos benefit from maternal thermal regimes (Lorioux *et al*., 2013*a*). That is, females will maintain a temperature set point that is driven by the narrow embryonic thermal tolerance. In turn, direct metabolic demand of pregnancy will depend on fecundity and, as demonstrated here, on maternal relative reproductive effort.

Clarifying the metabolic impacts of pregnancy is a key component in improving our understanding of the 'cost' of viviparity (Bleu *et al*., 2011). Van Dyke & Beaupre (2011) suggested that MCP is relatively low when compared with metabolic costs of vitellogenesis. However, although metabolic measures during vitellogenesis and pregnancy estimate energetic requirements, they are poor currencies for actual 'costs' to the mother. Assessing actual costs of reproduction requires quantifying the impact on survival and/or future reproduction (Bonnet *et al*., 2000; Bleu *et al*., 2011). In this context, the metabolic demands of pregnancy (direct and indirect) occur while females are already extensively emaciated with only residual fat reserves and already mobilized muscle protein (Bonnet *et al*., 2002*b*). Pregnant females will have to catabolize critical muscle proteins to provide amino acid for gluconeogenesis (Lourdais *et al*., 2004, 2013). Besides, direct amino acid transfer to the embryos can also occur during pregnancy in lecithotrophic species (Van Dyke & Beaupre, 2012). Compromising structural proteins will translate in altered musculature and performance after parturition (Lourdais *et al*., 2004, 2013). The actual 'costs' induced by pregnancy are likely significant, as suggested by the significant relationship between metabolic changes and maternal emaciation. A recent study on the common lizard *Zootoca vivipara* demonstrated that an experimental litter size reduction during pregnancy was beneficial to the mother resulting in higher BC after parturition (Bleu *et al*., 2011) and such results provide support for high costs induced by pregnancy.

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ARTICLE 3

Total embryonic water uptake during pregnancy is stage and fecundity dependent in the snake *Vipera aspis*

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Abstract

Water is a crucial resource that can profoundly impact the biology of terrestrial organisms. Early life stages are particularly sensitive to hydric constraints, since water uptake is an important component of embryonic development. While amniotic eggs constitute a key innovation to terrestrial life, many vertebrates are viviparous, and, in such cases, the mother must be the source of water for her developing embryos. Since most viviparous squamates are lecithotrophic (i.e., energy is supplied to the offspring as yolk deposited into the pre-ovulated follicles), water is the predominant resource allocated from the mother to the offspring during their development. Contrary to energy that can be stored (as fat reserves), water typically cannot be acquired in advance, and, therefore, the embryos' need for water can impose significant constraints on the pregnant female, especially in environments where water is limited. Thus, we detailed water flux during pregnancy in a snake, the aspic viper (*Vipera aspis*). We found that embryonic water uptake occurred mostly during the second half of pregnancy a period dominated by somatic growth. We also found that, somewhat surprisingly, changes in female plasma osmolality were negatively related to fecundity. This latter result suggests that water consumption by the female is especially important for large litter sizes, and thus may suggest an important sensitivity of reproductive females to environmental water availability.

Key words: water balance, pregnancy, osmolality, fecundity, embryo

Introduction

Variations in environmental resources can have profound influences on physiological traits, tradeoffs, and life history traits (O'Connor et al., 2006). Water is a critical resource that can show important seasonal fluctuations and is known to affect terrestrial organisms (Bradshaw, 1997; Lorenzon et al., 2001; Shine and Brown, 2008; McKechnie and Wolf, 2010). Embryonic life is particularly sensitive to water availability, and eggs are particularly sensitive to dehydration (Du, 2004; Stein and Badyaev, 2011). For instance, water supply is critical for the conversion of stored energy reserves (yolk) to embryonic mass (Vleck, 1991; Thompson and Speake, 2002). Therefore, water limitation can profoundly alter embryonic development and affect offspring quality or result in embryonic death (Brown and Shine, 2005; Lourdais et al., 2007). Such negative impacts have favored the emergence of multiple adaptations including the selection of an appropriate nesting site and/or parental care of the eggs to minimize water loss (Shine, 2004; Shine and Brown, 2008; Stahlschmidt and DeNardo, 2010). Eggs shell structure can also be modified either to minimize water loss (hard-shell eggs are laid in a desiccating atmosphere) or, conversely, favor water uptake from the environment (parchment shell eggs laid in a humid environment) (Deeming and Ferguson, 1991; Shine and Thompson, 2006).

While most terrestrial organisms are oviparous, viviparity exists in each of the major vertebrate lineages (Blackburn, 2000; Shine, 2004). These repeated transitions are associated with a diversity of embryonic nutrition strategies that have attracted considerable interest (Blackburn, 1999, 2006). Despite the focus on energy allocation, the female must also be the source of water for embryonic development, and this facet of maternal resource provisioning has multiple implications and is particularly important to consider (Oftedal, 2002). For instance, contrary to energy that can be stored (as body fat) to prepare for energy investment during reproduction (Lourdais et al., 2004), water typically cannot be accumulated in advance, and therefore water availability in the environment must match the timing of the reproductive requirement for water. In many environments, water may well be more constraining than energy during reproduction, since, gestation often occurs during dry summer months (Lourdais et al., 2004). Water limitation may alter reproductive success either by inducing embryonic mortality or affecting offspring quality (Dauphin Villemant and Xavier, 1986; Ross and Desai, 2005). Importantly, water demand during reproduction should be directly related to reproductive effort since an increase in the number of developing embryos increases water demand. Therefore, increased

fecundity should either increase female water acquisition from the environment or cause greater hydric deficit in the female.

Despite the likely importance of water in influencing female behavior, physiology, and fecundity,, maternal water flux remains largely overlooked. Squamates reptiles are unique among vertebrates for the vast number of times that viviparity has independently evolved (>100, Stewart and Thompson, 2000; Blackburn, 2006). While some species show complex placentotrophy, most lizards and snakes are lecithotrophic where yolk reserves support the energy demands during embryonic requirement (Stewart and Thompson, 2000; Thompson and Speake, 2002; Van Dyke et al., 2014). Therefore, maternal resource demand during pregnancy is primarily focused on water. This situation offers a simple context to address the implication of water allocation independent of nutritional aspects. A previous study in the common lizard, *Zootoca vivipara*, suggested that water uptake is greatest during late embryonic stages when most somatic growth occurs (Dauphin Villemant and Xavier, 1986). The same study also revealed an important impact of experimental water deprivation on reproductive success. To improve our understanding of water allocation, it is also critical to consider the impact of pregnancy and fecundity on female water balance.

We used a viviparous snake, the aspic viper, *Vipera aspis*, to describe water flux during pregnancy. This species is a medium-sized viviparous snake with a southern European distribution. Pregnancy is a long process (2 to 3 months) that occurs during summer months. Because feeding during gestation is not obligatory, mass changes during pregnancy are not confounded by foraging. We used high resolution ultrasonography to monitor embryonic volume and estimate water intake during development. We also monitored relevant maternal traits including body mass and plasma osmolality over pregnancy. Our main hypothesis was that embryonic water requirements should influence maternal water intake and water balance.

We tested the following predictions:

(1) Water uptake by the embryos should mainly occur during the second half of gestation when embryonic somatic growth occurs.

(2) Maternal mass change during pregnancy should reflect water intake and be closely related to the number of developing embryos.

Material and methods

Study species and maintenance

The aspic viper, *Vipera aspis*, is a small viviparous snake of the western Paleartic region. This species is a typical capital breeder and thus accumulates energy needed for reproduction over an extended period before engaging in a reproductive effort (Bonnet et al., 2002; Lourdais et al., 2002a). Pregnancy is a long process, lasting up to three months, and it is associated with an increase in thermal preference and precision, which necessitates an increase in thermoregulatory activities (Saint Girons, 1952; Naulleau, 1979; Ladyman et al., 2003) and modified escape tactics (Lorioux et al., 2013a). Reproduction in females is also associated with reduced movement and food intake (Saint Girons, 1952).

Experimental design

We collected 43 pregnant females (12 in 2009 and 31 in 2010) in neighboring districts (Loire Atlantique, Maine et Loire, Vendée,) of west-central France. Reproductive status (late stages of vitellogenesis) was first determined by manual palpation of the abdomen and then confirmed in the laboratory with high-resolution ultrasonography (SonoSite microMaxx, Inc.,Bothell, WA, USA). Body mass (± 1 g) and snout-vent length (± 5 mm) were recorded, and scale clipping was used to identify individuals.

Female vipers were housed in cages (100cm x 30cm x 35cm) that had a thermal gradient (18 - 41°C) created by placing a 75 W incandescent light bulb over one side of the cage. The light was on for 6 hrs per day enabling basking from 10:00 to 16:00 hr, but forcing body temperatures to drop to room temperature (18 °C) at night. Each cage contained a main shelter (half cylinder of polyvinyl chloride (PVC) pipe, diameter = 15 cm, length = 37 cm, with two 3 cm circular openings in the sides) located at one end of the cage opposite from the basking zone and three

secondary shelters (half cylinder PVC pipe, diameter = 15 cm, length = 25 cm) scattered throughout the cage to facilitate concealed movements to the basking zone.

Females were randomly assigned to one of the cages, some housing two females and some housing three as a result of space limitations. Importantly, no agonistic behavior was observed in co-housed individuals. Females were provided water *ad libitum*, but were not fed until parturition, since they typically do not eat during gestation (Lourdais et al., 2002b). Importantly, a pre-ovulation ecdysis occurs and provides a reliable indicator of the onset of gestation in this species (Lorioux et al., 2013b).

Variables measured

Embryonic volume: We monitored the change in volume of the embryonic unit (i.e., embryo, yolk, and extra-embryonic membranes) to estimate embryonic water uptake during pregnancy. The study species is lecithotrophic, and it has been shown that food intake after ovulation has no influence on reproductive output (Lourdais et al., 2002b). Using high-resolution ultrasonography (see Lorioux et al., 2013b), we determined total volume at three different stages of embryonic development (Hubert & Dufaure, 1968): "ovulation" (- 1.9 ± 0.7 days before ecdysis), mid-pregnancy (29.8 ± 0.9 days after ecdysis; 37.6 ± 1.2 % of developmental duration), and late pregnancy (55,5 ± 1.3 days after ecdysis; 70.6 ± 1.5 % of developmental duration). For each female, we collected sagittal view images of the most cranial and most caudal embryonic units and measured their heights (H), lengths (L), and form. We estimated embryonic volume (cm^3) following the method described in Maritz and Douglas (1994), which considers embryonic unit L, H, and a coefficient associated with its form (F_{emb}): Embryonic volume = π x L x H^2 x F_{emb}. For analysis, we averaged the volume of the two embryonic units measured at each stage for each female.

Body mass (BM): change in BM has been well established as an estimator of water loss in squamate reptiles that are food-restricted (DeNardo et al., 2004; Lillywhite et al., 2008a, 2008b; Dupoué et al., 2014). BM was collected during early and late pregnancy stages and also after

parturition. We calculated BM changes (g) between early and late pregnancy stages for all individuals.

Osmolality: we also measured changes in female plasma osmolality (Osmo), as this parameter is an effective indicator of hydration state (Peterson, 2002). For safety reasons, each snake was encouraged to enter its head and upper body into a clear plastic tube, and then blood was collected via cardiocentesis (Saint Girons et al., 1993). This variable was only measured in the 12 pregnant females collected in 2009, which had substantial variation in litter size (1 to 11). We collected blood samples (100 µL) using a 1 ml heparinized syringe and a 27-gauge needle. Blood samples were collected at early, mid, and late pregnancy and after parturition. Plasma osmolality ($mOsm.kg^{-1}$) was measured from 10 µl triplicates (intra-individual variation < 1%) as described in Wright et al. (2013).

Litter traits: for each female, we collected litter size (number of undeveloped ova, stillborn, and neonates), fit litter size (number of neonates only), litter mass (mass of undeveloped ova, stillborn, and neonates) and fit litter mass (mass of neonates only) (see details in methods of Lourdais et al., 2002b).

Statistics

All statistics were performed with Statistica 6.0. Changes in embryonic volume, body mass, and osmolality over time were analysed using repeated measures ANOVA using the successive measurement sessions as the repeated factor. We also analysed the variation in embryonic volume over pregnancy by fitting embryonic volume with developmental time. We tested three functions (i.e., linear, square and exponential) and used Akaike's information criterion (AIC) to select the best model. When two models differed by less than two AIC, they were considered equivalent. The relationship between mass changes and reproductive output were analysed using simple regression. A similar procedure was used to correlate osmolality changes with mass changes and reproductive output. Tukey's *post hoc* tests were conducted for 2X2 comparisons. Unless otherwise stated, values are reported as means ± standard deviation.

Results

1) Embryonic volume

We detected significant variation in embryonic volume over time ($F_{2, 80} = 151.68$, $p < 0.001$) with a clear increase over the three consecutive periods (mean embryonic volume 2.71 ± 0.10, 4.90 ± 0.25, and 8.42 ± 2.6 cm^3 for early, mid, and late pregnancy measures, respectively). All three stages were significantly different from each other (Tukey's *post hoc* tests, all p values < 0.001). Total volume change represented, on average, an increase of 210% of initial volume. When considering time from ovulation rather than period, we found that the increase in embryonic volume was non-linear and the best fit was obtained using an exponential curve (AIC = 476.5, 451.6, and 448.2, respectively for linear, square, and exponential functions, Figure 1).

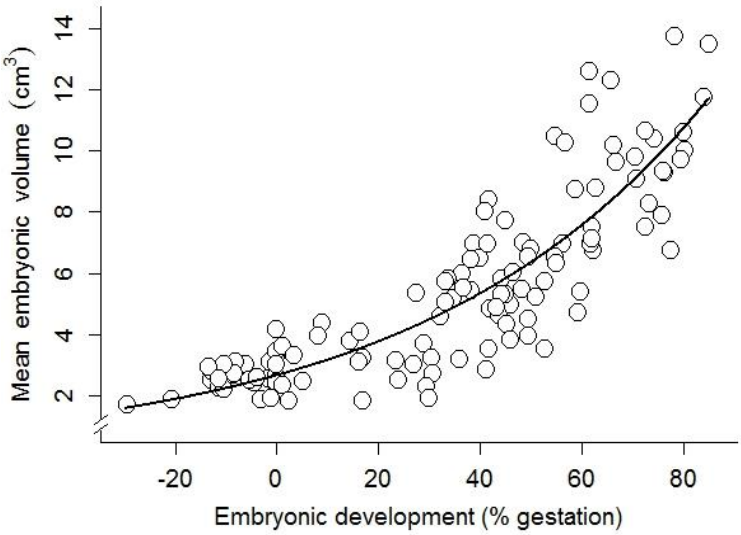

Figure 1. Relationship between embryonic volume (cm^3) and developmental time expressed as a proportion of total gestation duration (%). Variation were best described with an exponential function (equation: $y = 2.66\ e^{0.017}$). See text for statistics.

2) Body mass

We observed a significant increase in body mass over pregnancy (107.79 ± 6.49 vs. 112.24 ± 6.94 g for initial and late measures; $F_{1, 41} = 13{,}48$, $p < 0.001$). However, important variations were observed between females and mass changes ranged from -11.2 g to +30.2 g (Figure 2). Mass change was positively related to litter size ($F_{1,40} = 25.71$, $p < 0.001$, $r^2 = 0.38$), but was better explained when considering fit litter size ($F_{1,40} = 94.77$, $p = < 0.001$, $r^2 = 0.71$, Figure 3). Mass change was also closely related to litter mass ($F_{1,40} = 43.32$, $p < 0.001$, $r^2 = 0.51$) and fit litter mass ($F_{1,40} = 74.39$, $p < 0.001$, $r^2 = 0.64$).

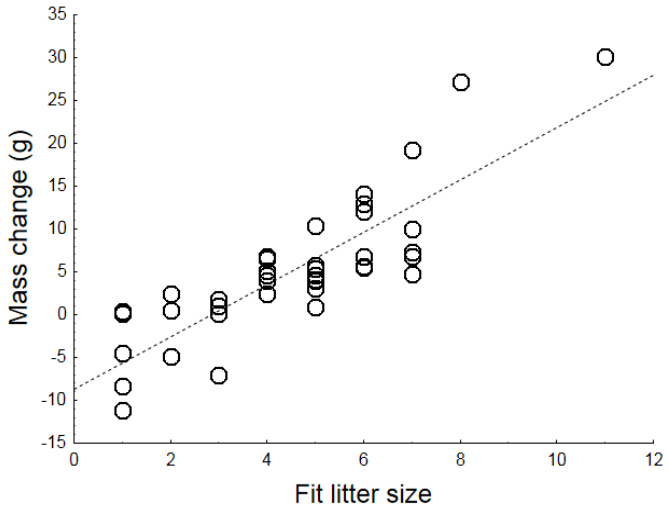

Figure 2. Relationship between mass change during pregnancy and the number of developing embryos (fit litter size).

3) Osmolality

No significant difference were observed in absolute osmolality ($F_{1,11} = 2.48$, $p = 0.14$; 302.4 ± 10.7 vs. 309.9 ± 14.4 mOsm.kg^{-1} , respectively, for early and late pregnancy). However, the change in osmolality over pregnancy was quite variable among females with values ranging from -24.0 to +37.5 mOsm.kg^{-1}. Osmolality change was negatively related to body mass change ($F_{1,40} = 8.67$, $p < 0.010$, $r^2 = 0.41$). It was also negatively related to fit litter size ($F_{1,10} = 5.54$, $p = 0.020$, $r^2 = 0.29$) and fit litter mass ($F_{1,10} = 8.97$, $p = 0.010$, $r^2 = 0.42$, Figure 3).

Discussion

Our data on the aspic viper provide significant insight into the dynamics of water transfer between mother and offspring, but also into the potential impact of embryonic water demands on maternal physiology and drinking behavior. We discuss our results in details below.

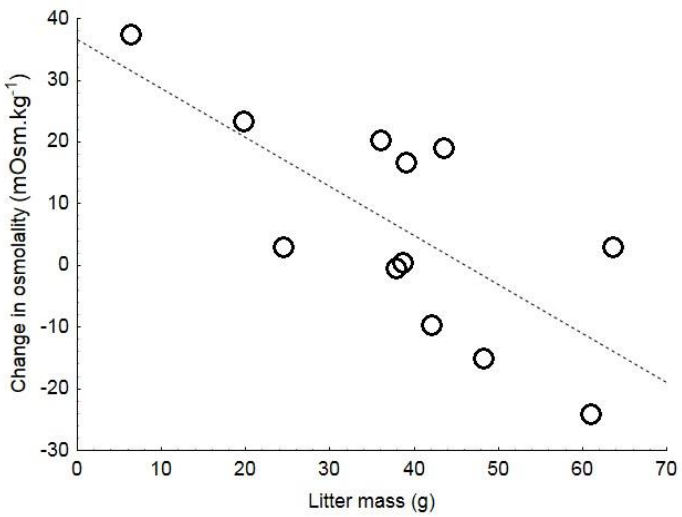

Figure 3. Relation between osmolality change (mOsm.kg^{-1}) between early and late pregnancy and litter mass (g).

Using high resolution ultrasonography, we revealed massive changes in embryonic volume over pregnancy (+210%). Because the study species is lecithotrophic and most of the nutrients are allocated prior to ovulation, embryonic volume change can therefore be attributed to water uptake. Examining embryonic volume relative to developmental time revealed that water uptake was not linear but rapidly increased during the second half of pregnancy. This pattern is likely related to exponential somatic growth that occurs mainly during the last third of development (Dauphin Villemant and Xavier, 1986; Andrews, 2004) and requires water uptake to convert yolk reserves to embryonic tissue (Vleck, 1991; Thompson and Speake, 2002). Our final measures of embryonic volume were made at, on average, 70% of developmental time, and, therefore, we likely underestimate total water uptake due to massive uptake that occurs during pre–parturition stages. In the common lizard, *Zootoca vivipara*, Dauphin Villemant and Xavier (1986) reported a + 266% increase in embryonic wet mass that was directly related to changes in water content. In six other lecithotrophic squamates, Blackburn (1999) reported somewhat smaller wet mass increases of the conceptus (from + 51 to 142%) while dry mass decreased over time. Estimates of embryonic water uptake during gestation are only available for a few other lecithotrophic vertebrates, such as the gulper shark (+99 to 101%, Guallart and Vicent, 2001). Vleck (1991) suggested that the magnitude of water uptake by reptilian embryos exceeds the amount required for somatic growth, and that water uptake is also necessary in other embryonic compartments (i.e., amniotic fluids and allantois). Our measure of total embryonic volume likely encompasses all of these components.

Changes in body mass provided additional information with a significant increase during gestation. Important among female variation was observed, and mass change was closely related to the number or mass of developing embryos (see Figure 3); females with small litter sizes tended to lose mass. Mass change during gestation likely integrates multiple and opposite processes at the maternal and embryonic levels. Water uptake by the embryos is clearly responsible for the important mass increase demonstrated here. Yet, females face important metabolic increase that results from supporting pregnancy (MCP, see Dupoué and Lourdais, 2014) and the maintenance of high body temperature (Lorioux et al., 2013a) required to optimize reproduction (Lorioux et al., 2013a, 2013b). Because food intake is reduced (or absent), pregnant females mobilize remaining fat stores and structural proteins resulting in significant structural mass loss and important emaciation after parturition (Bonnet et al., 2001; Dupoué and Lourdais, 2014). Water uptake would be related to reproductive effort and would

"mask" energy costs of gestation notably in females with large litter sizes for which mass increase reaches substantial levels (+30g, i. e.. 25% of initial gestation mass). Maternal mass changes during pregnancy should be interpreted cautiously, since this parameter reflects the balance of these opposite processes. In contrast, plasma osmolality provides a reliable index of water balance in vertebrate (Davis and DeNardo, 2009). We found that females that gained body mass also experienced a significant drop in osmolality while the opposite pattern was observed in females that lost mass. Osmolality change was also negatively related to the number and mass of developing embryos, suggesting that females with high fecundity drank relatively more during pregnancy. In turn, an important osmolality increase (+39 $mOsm.kg^{-1}$) was detected in an individual with only one developing embryo (Figure 3). These results could reflect variation in phenotypic quality (Beauplet et al., 2004; Stahlschmidt et al., 2013) with enhanced maternal water intake in more fecund individuals.

Maternal influences at early stages of development have attracted considerable attention and encompass crucial aspects such as nutrient provisioning and developmental temperature (Bernardo, 1996; Blackburn, 1999; Mousseau et al., 2009). While water partitioning is inherent to vertebrate viviparity, this facet of the female-offspring relationship remains largely understudied. Our study underlines the magnitude of water requirement and the impact of pregnancy on maternal water balance in non-constraining conditions. Contrary to energy that can be accumulated (e.g., fat reserves) to anticipate environmental variations (See Bonnet et al., 1998), long-term storage options for water are limited (Davis and DeNardo, 2007) .Water deprivation during pregnancy may induce reproductive failure and even result in maternal death (Hirschhorn et al., 1969). Therefore, a possible conflict for water may exist between maternal and embryonic needs as previously documented for energy (Crespi and Semeniuk, 2004; Dupoué et al., in press). Such conflict is more likely at the end of gestation when embryonic water requirements are peaking.

Finally, our work is relevant in the framework of understanding the impacts of ongoing climatic change. Environmental temperature increase and its impacts on activity budget and energy acquisition has attracted considerable interest (e.g., Sinervo et al. 2010). While changes in precipitations are more complex to predict, the frequency of extreme climatic hydric events such as prolonged drought is expected to increase in the coming decades (IPCC 2014). Based

on our results, we predict that reduced water availability during pregnancy will have a profound impact on reproductive performance in many viviparous vertebrates.

Acknowledgments:

We thank François Brischoux for providing valuable comments on earlier drafts of the manuscript. This research was made possible by financial support from the "Programme opérationnel plurirégional Loire FEDER" (# PRESAGE 30810), the "Etablissement Public Loire", the FYSSEN foundation, and the Centre National de la Recherche Scientifique.

Les contraintes morphologiques : résultats préliminaires

Dans cette partie, je présenterai brièvement les résultats que nous avons obtenus sur les variations morphologiques des femelles gestantes. Chez les Squamates, la taille de portée est en premier lieu influencée par la taille des femelles, ce qui est notamment dû à l'espace disponible dans la cavité abdominale. En effet, comme on l'a montré dans l'article précédent, les fétus vont se développer et progressivement occuper l'espace abdominal disponible jusqu'à ce que la portée soit limitée par les organes internes de la femelle (Fig. 1). Nous avons donc réalisé différentes mesures afin de quantifier cette progression, puis l'impact résultant du développement des embryons sur la distension abdominale des femelles. Pour l'approche corrélative, nous avons choisis d'utiliser en variable explicative l'effort reproducteur relatif (RLS = résidus de la relation entre la taille de portée effective et la taille) pour tenir compte de la taille des femelles et les limiter les problèmes d'autocorrélations.

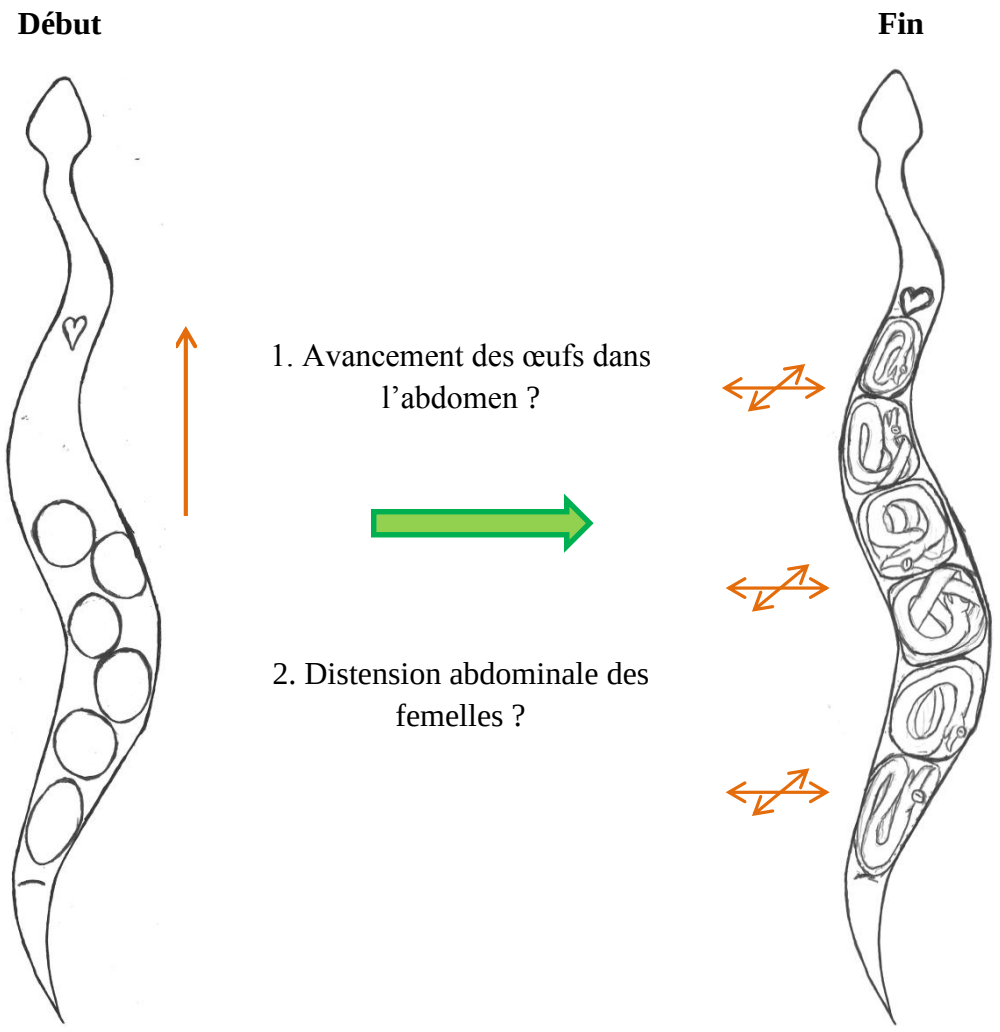

Figure 1. Représentation schématique de l'évolution des contraintes morphologiques au cours de la gestation (début et fin) chez la vipère aspic, en lien avec le développement des embryons. Le cœur symbolise la limite imposée par les organes internes des femelles.

1. Avancement des œufs dans l'abdomen

Nous avons réalisé les mesures sur 24 femelles gestantes (voir détails dans les méthodes générales). Entre le début et la fin de la gestation, on observe un avancement significatif moyen de 5.5cm de l'œuf en position crâniale (i.e., le plus proche de la tête) dans l'abdomen des femelles ($F_{1,23} = 197.0$, $P < 0.001$, Fig. 2a), ce qui représente environ 12% de la SVL moyenne. Cet avancement est de plus fortement corrélé à l'effort reproducteur ($F_{1,24} = 19.3$, $P < 0.001$, $r^2 = 0.45$, Fig. 2b).

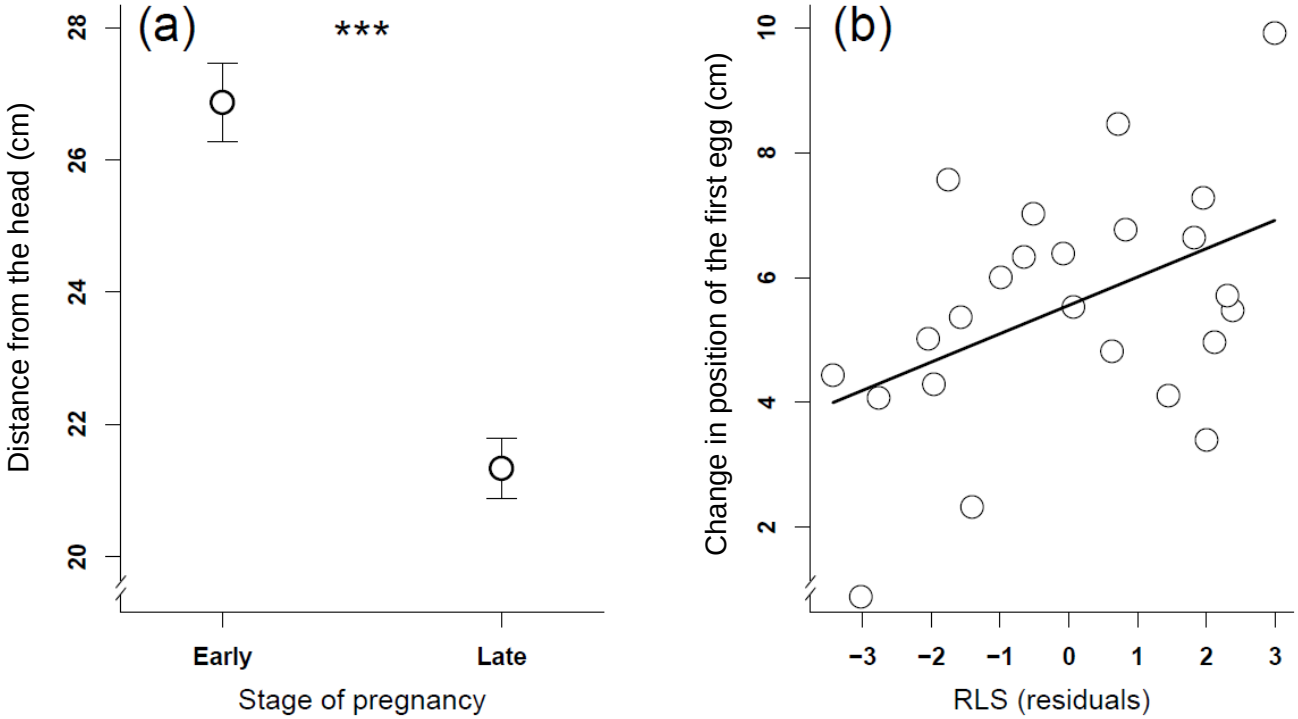

Figure 2. Avancement de la portée dans l'abdomen estimé via a) le point de départ du premier œuf dans l'abdomen et b) l'influence de l'effort reproducteur (RLS : résidus dérivés de la relation entre Fit Litter size et SVL des femelles) sur cet avancement.

2. Distension abdominale des femelles

Dans cette partie, nous avons utilisé des données d'épaisseurs corporelles obtenues pour l'expérience 1 du chapitre 1. Nous avons considéré des individus différents pour chaque statut (n = 13 femelles non-reproductrices, n = 15 en début et n = 14 en fin de gestation). Les mesures d'épaisseurs des vipères gestantes ont permis de mettre en évidence un encombrement abdominal significatif de l'ordre de 25-30% dès l'ovulation comparé aux femelles non-reproductrices (Tukey *post hoc*, all $P < 0.001$, Fig. 3). Cet encombrement est similaire entre les femelles en début et en fin de gestation (Tukey *post hoc*, all $P > 0.999$, Fig. 3). Les variations

d'épaisseurs au cours de la gestation étant probablement inférieures à la variance interindividuelle (Fig. 5), une solution efficace pour savoir si l'épaisseur augmente au cours de la gestation aurait été de réaliser des mesures répétées sur les mêmes femelles.

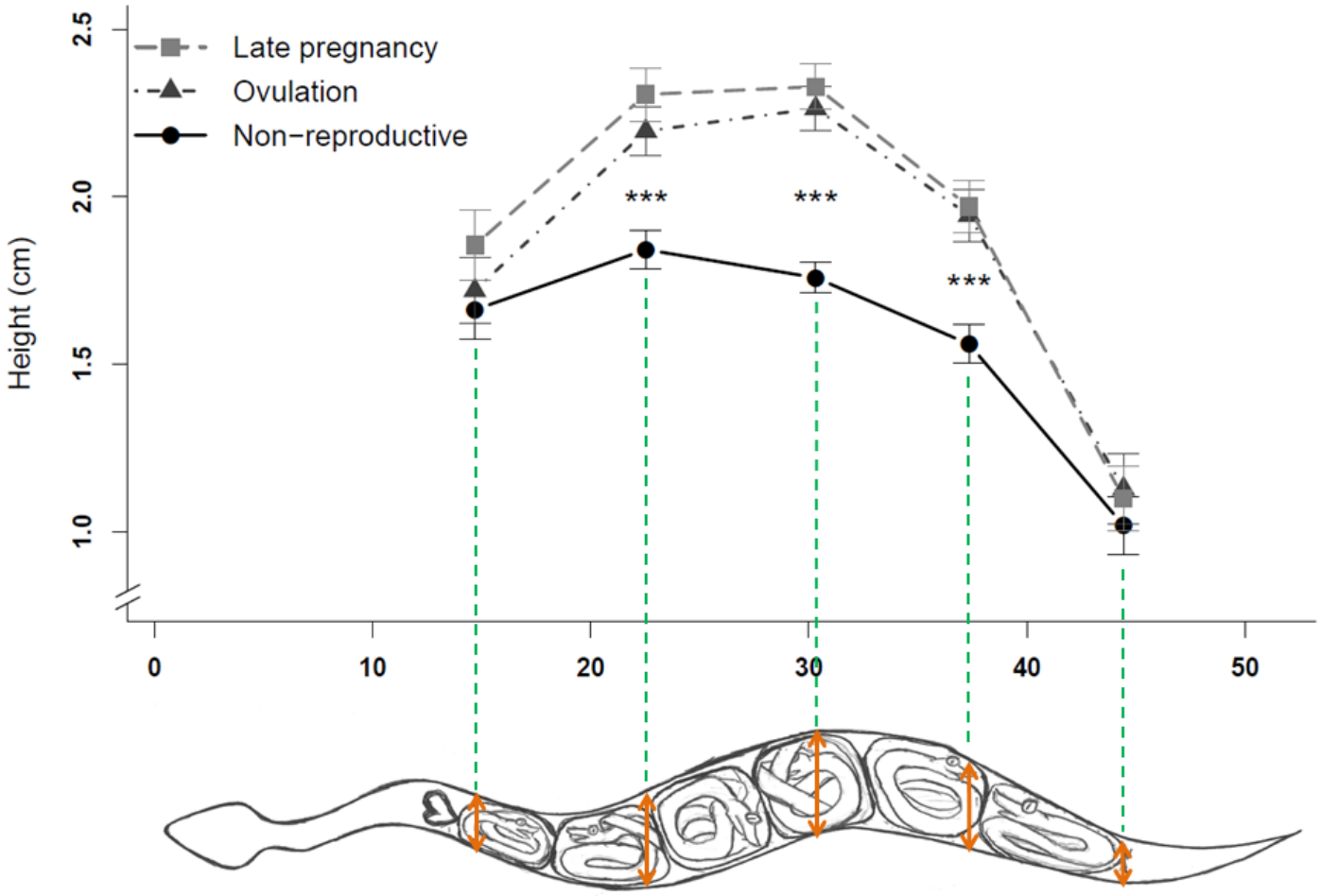

Figure 3. Mesures de largeur corporelles à différents points du corps des femelles (n = 13 non-reproductrices, n = 15 en début et n = 14 en fin de gestation) avec une première mesure thoracique, suivie de 3 mesures abdominales et pour finir par l'épaisseur cloacale. Une différence significative entre les statuts est symbolisée : *** $P < 0.001$.

ARTICLE 4

Hydric "costs" of viviparity: pregnancy increases evaporative water loss in the snake (*Vipera aspis*)

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Abstract

To date, water balance has attracted much less attention than energy constraints when addressing potential costs of reproduction or parental offspring conflict. However, water is a vital resource that is critical for reproduction and especially in viviparous species since females support embryonic water requirements. Viviparity is associated to important shift in maternal behaviour and physiology that may affect maternal water loss. For example, within squamate reptiles, pregnancy is associated to a shift in maternal thermoregulation, as well as an elevation in metabolic rates and a modified morphology. Such changes may be in conflict with maternal water balance and induce significant water constraints to the mother. Herein we examined to what extent pregnancy increases evaporative water loss in a viviparous snake, the aspic viper. We found that morphological (abdominal distention) and physiological (increased metabolism) changes resulted in increased TEWL in pregnant females. These direct effects were reinforced by the maternal thermoregulatory shift. Water constraints may affect basking site selection to mitigate the conflict between thermal needs and water loss. In support with this prediction we found that pregnant female selected warm and moist microclimates in the field when compared to non-reproductive ones. Clarifying if increased EWL constitute a real "costs" for pregnant females requires to investigate long term effect on female survival and future reproduction.

Keywords. Water loss, pregnancy, physiological conflict, behavioural mitigation

Introduction

Costs of reproduction constitute a central assumption in life-history theory (Stearns 1992). Such costs are expressed either in term of reduced survival of reduced residual reproductive success (Williams 1966). A critical aspect to understand reproductive strategies is to clarify the proximate mechanistic basis of reproductive costs. Reproduction often induces massive physiological and behavioural changes that can profoundly alter individual performances. For example, substantial energy mobilization can significantly impact individual survival of reduce energy available for future reproduction. Similarly behavioural changes can increase vulnerability to predators or conflict with other activities such as foraging (Blouin-Demers & Nadeau 2005; Angilletta 2009). Therefore it appears crucial to clarify the nature of proximate physiological or behavioural conflicts that induce a possible trade-off between current and future reproduction. Because energy resource is often fluctuation or limiting, it has attracted considerable interests and energy based trade-off are crucial to address the diversity in reproductive strategies.

Viviparity has emerged repeatedly origins among vertebrates including fishes, amphibians, reptiles and mammals. This reproductive mode likely provides multiple benefits derived either from enhanced control of developmental (thermal) condition and a diversity of foeto-maternal nutrient transfer. Meanwhile pregnancy also imposes significant constraints on the maternal organism. For example embryonic development is associated with prominent physical burden that alter female mobility and increase vulnerability (Shine 1988). Besides supporting pregnancy impose a significant energy demand to the mother that can compromise energy budget. Energy constraints and predation risks attracted significant interest and several studies reported significant emaciation or altered performances after reproduction.

Other component as physiological homeostasis (Stevenson 1985; Angilletta et al. 2002; Blouin-Demers et al. 2003) may be compromised during pregnancy. Water is also a critical resource during gestation since female allocate important amount of water to support their developing embryos. Pregnant females can be particularly sensitive to water shortage. Heretofore, this parameter remains under considered but a significant foeto-maternal conflict may exist during pregnancy (Dupoué et al. in press).

Squamate reptiles are unique for multiple (>130) independent emergences of viviparity and a diversity in nutrient transfer ranging from lecithotrophy to mammal like placentation (Blakburn 1993). Maternal effort seems is primarily linked to behavioural thermoregulation and enhanced control of developmental condition. Pregnancy is associated to a major behavioural shift with modified thermal preferences and increased basking times. Maternal thermal influences appear critical to optimize both reproductive phenology and offspring phenotype (Waptsra et al. 2010; Lorioux et al. 2013b). Evaporative water loss (EWL, e.g., ventilatory and cutaneous water losses) accounts for majority of water loss in squamates (Dmi'el & Zilber 1971; Moen et al. 2005). Basking in open sunlight, while providing needed heat to meet physiological requirements, increase EWL due to relatively high temperature and/or by exposing the organism to drier environment (Mautz 1982; Guillon et al. 2014). Besides, the massive physical burden imposed by pregnancy is likely to induce skin distention and therefore increase skin surface exposure to cutaneous water loss. Therefore we expect that a thermoregulation can be in conflict with water balance during pregnancy (Ladyman & Bradshaw 2003; Kohler et al. 2011; Guillon et al. 2014).

Herein, we examined to what extant pregnant female may be more exposed to evaporative water loss in a viviparous snakes (*Vipera aspis*) for which we have extensive knowledge on thermoregulation and gestation. We considered both TEWL and CEWL and tested the following predictions:

1) Thermoregulatory shift during pregnancy should results in increased TEWL due to high thermal preferences
2) Morphological (abdominal distention) and physiological (increased metabolism) changes associated with pregnancy should also increase TEWL
3) Pregnant females should select appropriate (warm and moist) microclimate to mitigate the conflict between thermal needs and water loss

Material & Methods

Study species

The aspic viper (*Vipera aspis*) is a small viviparous snake of the western-Paleartic region. In this species, gestation begins just after ovulation in early June (Naulleau 1981). During gestation, females have a higher preferred temperature and substantial increase basking time relative to non-reproductive females (Saint Girons 1952; Naulleau 1979; Ladyman et al. 2003; Lourdais et al. 2004). After two to three month of gestation, parturition occurs from late August to early September (Lourdais et al. 2002a).

Capture and maintenance

We caught 45 female aspic vipers in west-central France from neighboring populations (Loire Atlantique, Vendée and Maine et Loire district) between May 25 and June 17 2010. Each female was measured (snout vent length, SVL ± 0.5 cm), weighed (body mass, BM ± 0.1 g), and marked. Snakes were then housed in opaque plastic boxes (10.5 x 30 x 16.5 cm), with a shelter and water *ad libitum*. They were placed in a temperature-controlled climatic chamber (Vötsch Industrietechnik, VP 600, Balingen, Germany), exposed to a natural daily cycle of temperature (16 – 33°C). Females were not fed until parturition, since they typically do not eat during gestation (Lourdais et al. 2002b).

Females were given an ultrasound examination (Micromaxx, SonoSite, Inc., Bothell, WA, USA) to assess reproductive status. Ultrasonography was realized in a minimal time (5-10 min) to reduced disturbance and stress. 18 females were pregnant (mean ± SE, BM = 115.6 ± 8.1 g, SVL = 48.1 ± 1.1 cm) and 21 were non-reproductive (mean ± SE, BM = 91.2 ± 6.9 g, SVL = 48.6 ± 1.2 cm). Pregnant vipers presented higher BM than non reproductive females ($F_{1,37}$ = 5.4, P = 0.026) but both reproductive statuses presented similar SVL ($F_{1,37}$ = 0.1, P = 0.739). For each pregnant female, we measured different estimates of reproductive output (litter size, fit litter size, litter mass, fit litter mass; see details from methods in Lourdais et al. 2002b) and maternal reproductive effort (i.e., relative litter size, RLS; see Dupoué & Lourdais 2014). Maternal reproductive effort is derived from the relationship between litter size to maternal SVL ($F_{1,16}$ = 9.0, P < 0.01).

Since this species is a low energy specialist and infrequent feeder, long-term fasting is ecologically relevant, especially during pregnancy (Lourdais et al. 2002b). Thus, to avoid confounding effects of nutritional balance, no female (either pregnant or non-reproductive) were fed until the completion of the experiment. After the completion of the experiment, vipers were fed with a mouse and released with offspring at the exact site of capture.

Evaporative water loss

To test the effect of pregnancy on both total evaporative water loss (TEWL) and cutaneous evaporative water loss (CEWL), we realized measurements in late stages pregnancy (i.e. during fetal life, see Lorioux et al. 2013b) when water constraints are presumably higher.

1) TEWL

We used flow-through respirometry to measure the rate of TEWL (see details on methods in Lourdais et al. 2007, 2013). We measured TEWL at three trial temperatures:

15.0°C: this temperature represents thermal condition that can be experienced during inactivity period.

25.0 °C: this temperature broadly intermediate temperature frequently achieved by non pregnant females (Ladyman et al. 2003).

33.0°C: this temperature corresponds to pregnant female T set (Ladyman et al. 2003) that are extremely precise thermoregulator (Lorioux et al. 2013b).

Snakes were placed in 500 ml test chamber and housed in a walk-in, temperature-controlled environmental chamber (±1°C, Cryosystem, air conditioned system Carel MasterCella). Air supply to the test chamber was controlled by a mass-flow controller (#UFC-1100, Unit Instruments, Yorba Linda, CA). The average influx was 162.0 ± 0.5 $ml.min^{-1}$, which provided 99% air turnover within the test chamber every 15 min. We controlled absolute humidity of the influent air with the same method than Guillon et al. (2014). We first produced dry air by an industrial air purifier (#CO2F-70h, Texol Products Ltd, Dundee, Scotland). Dry air was then

saturated by bubbling it through two serially connected heated water columns. The bubbling system was held in a temperature-controlled chamber (#400 DG LMS ltd, Sevenoaks, Kent, UK) set at a target dew point (T_{DP} = 10.0°C). Saturated air circulated in an empty column collecting water condensate. The hydrated air then flowed to the test chamber. The dew point of air stream was measured using a dew-point hygrometer (#RH300, Sable Systems, Las Vegas, NV). Minimally hygroscopic tubing (Bev-A-Line IV, Thermoplastic Processes Inc., Stirling, NJ) was used throughout the system.

For each session, we measured six individuals. Prior to each session, snakes were held in the test chambers two hours prior the onset of a trial. TEWL measurements were collected after stabilization of the dew point. Baseline values for the supply air were obtained at the beginning and end of each session by flowing the supply air through an empty test chamber. The lack of urine and feces production was confirmed at the end of each trial. We used hygrometric dew points to calculate vapor pressures using an 8th order polynomial describing saturation vapor pressure as a function of air temperature (Flatau et al., 1992). Vapor pressures were used to calculate vapor densities using the Ideal Gas Law (Campbell and Norman, 1998). Finally, evaporative fluxes ($mg.h^{-1}$) were calculated by multiplying vapor densities ($mg.ml^{-1}$) by air flow rates ($ml.h^{-1}$). We calculated absolute evaporative fluxes ($mg.h^{-1}$) as well as fluxes relative to snake mass ($mg.g^{-1}.h^{-1}$).

2) CEWL

We used another experimental group of vipers caught in spring 2012 (capture and housing similar to 2010 group; see above) to measure the rate of CEWL. We compared the rate of CEWL between 8 pregnant (mean ± SE, BM = 88.7 ± 4.6 g, SVL = 45.4 ± 0.9 cm) and 8 non reproductive (mean ± SE, BM = 74.9 ± 10.6 g, SVL = 46.2 ± 2.6 cm) female aspic vipers. Measurements were carried out at room temperature (25°C), and snakes were acclimated at this temperature for at least 2 hours prior to measurement.

We measured CEWL using an AquaFlux AF200 (Biox, UK), which derives water vapor flux from measurements of a humidity gradient within a closed chamber. The Biox software

AquaFlux 6.2 was used to calibrate and compute CEWL rate ($g.m^{-2}.h^{-1}$). A standard "measurement cap" (orifice diameter of 7 mm, Biox AF005-01) was maintained in tight contact with the snake's latero-dorsal skin at mid-body. A calibration of the AquaFlux was performed at the beginning of each trial.

Morphometric measures

In pregnant females, we examined the influence of different allometric determinants on the rate of TEWL. We considered the distances between two scale apexes (i.e., inter-scale distance). This measure integrates the size of scale and therefore the size of individual, but also the inter-scale space. We used photographic pictures and adapted software (Inkscape, version 0.15) to measure these distances. For each individual, we took a picture in a lateral view and a picture in a dorsal view. We measured three lengths on the third scale row (lateral pictures) and three widths on the top of the animal (dorsal picture) at different places on the abdomen. We considered the mean length (intra-individual variation, CV = 7.4 ± 0.6%) sum with the mean width (intra-individual variation, CV = 7.3 ± 0.6%) as an estimate of inter-scale distance (cm). To compare the influence of this integrative measure with its two major components (i.e., size of animal and inter-scale space), we also tested the influence of the size of individual and the inter-scale space separately. We considered the SVL to test the influence of individual's size, and we considered the residuals from the relationship between the inter-scale distance and the SVL ($F_{1,24} = 23.4$, $P < 0.001$) to estimate the inter-scale space.

Field hygrometry

We tested the influence of pregnancy on the microclimate selection on the field. On the field, we recorded temperature and relative hygrometry on the ground at each point of capture with a multiparameter sensor from May to August in 2009 and in 2010. We noted the sex and the physiological status of vipers. In this study, we selected data on female aspic vipers in two reproductive statuses (i.e., pregnant or non-reproductive).

Statistical analyses

All statistical analyses were performed with R software (R Development Core Team, version 2.13.1). All data were $\log_{10}$ transformed in analyses to achieve normality conditions. We checked that the residuals of our models respected this condition (Shapiro-Wilk test, all $P > 0.05$).

First, we used linear mixed model (package nlme) to test the impact of temperature and pregnancy on the rate of TEWL, with the temperature (i.e., 15, 25 and 33°C), the reproductive status (i.e., pregnant *vs*. non-reproductive) and the interaction between these two parameters as factors. Female SVL was included as linear covariate to correct by animal size. We did not use animal BM because of allometric contrast between pregnant and non reproductive females (see above). We included female identity as a random factor because of repeated measures. We realized pairwise *post hoc* tests of Tukey (package lsmeans) to test differences between reproductive statuses at each temperature. We used least square means (package lsmeans) method to adjust values of TEWL by SVL in figure.

We used linear models to test the relationship between the rate of TEWL and VO_2 in pregnant and non-reproductive females separately. We used the rate of TEWL and the VO_2 measured at 33°C since the inter-individuals variations of TEWL were higher at 33°C (pregnant: 10.2%; non reproductive: 10.7%) than inter-individuals variations at 15°C (pregnant: 6.8%; non reproductive: 6.0%) or 25°C (pregnant: 3.3%; non reproductive: 2.4%); and therefore easier to explain.

We compared the influence of different allometric determinants (i.e., SVL, inter-scale space and inter-scale distance) on the rate of TEWL at 33°C. We used linear models with the different determinants allometric measures as linear variable. We also tested a model with both SVL and inter-scale space as linear variable since they are not significantly related ($F_{1,16} = 0.0$, $P = 1.0$). We considered the model with the smallest Akaike's information criterion (AIC) to determine the best determinant that influences the rate of TEWL. When two models differed by less than two AIC, they were considered equivalent.

We used linear models to test the effect of reproductive status on the rate of CEWL, with reproductive status as factor. We also used linear model to test the relationship between CEWL and inter-scale space in pregnant females.

We used linear mixed models to analyze the effect of reproductive status on temperature and water vapour pressure selected by vipers on the field with the reproductive status as factor. We

included the month (i.e., May, June, July and August) and the year (i.e., 2009 and 2010) as random factors.

Results

1) TEWL

Influence of reproductive status

The rate of TEWL was significantly influenced by SVL ($F_{1,36}$ = 7.3, P = 0.011), ambient temperature ($F_{2,67}$ = 194.1, P < 0.0001; Fig. 1), reproductive status ($F_{1,36}$ = 31.0, P < 0.0001; Fig. 1) and the interaction between temperature and reproductive status ($F_{2,67}$ = 6.7, P = 0.002; Fig. 1). The rate of TEWL of pregnant females was significantly higher than non-reproductive females at 15°C (Tukey test; P < 0.05; Fig. 1), 25°C (Tukey test; P < 0.01; Fig. 1) and 33°C (Tukey test; P < 0.001; Fig. 1).

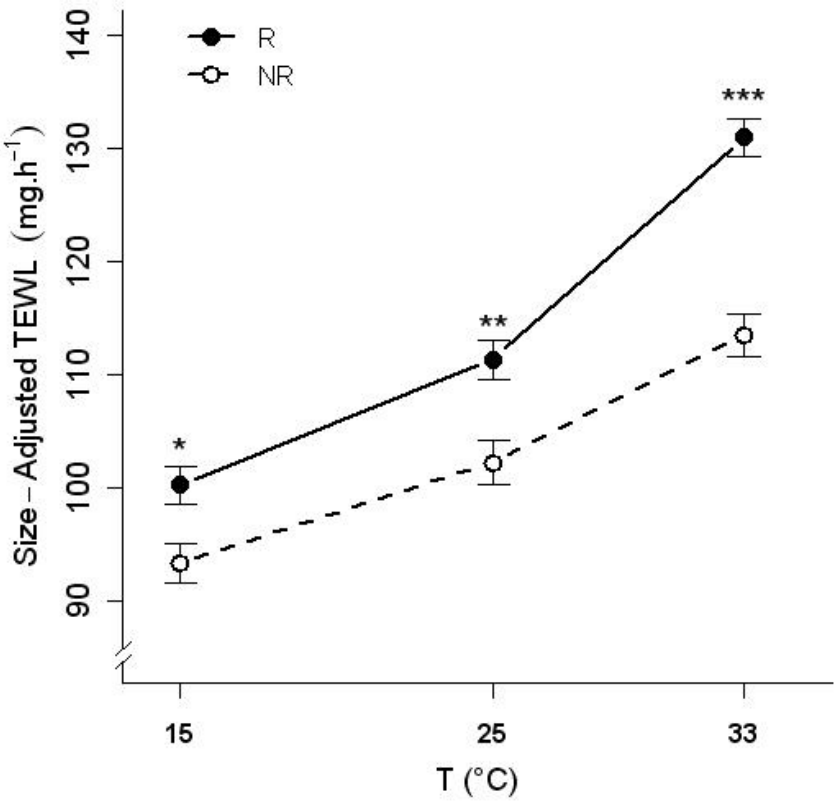

Figure 1. Comparison of the rate of total evaporative water loss (TEWL) between pregnant (n = 18, filled circle, solid line) and non-reproductive (n = 21, open circle, dashed line) females, at three temperatures. Points represent means (± SE) of TEWL ($mg.h^{-1}$) adjusted by snake SVL. *** P<0.001, ** P<0.01, * P<0.05.

Influence of metabolic rate

At 33°C, pregnant females present a higher rate of VO_2 than non reproductive females ($F_{1,34}$ = 11.4, P = 0.002). Moreover, we found significant and positive influence of the rate VO_2 on the rate of TEWL in pregnant females ($F_{1,16}$ = 5.4, P = 0.034; Fig. 2) and non-reproductive females

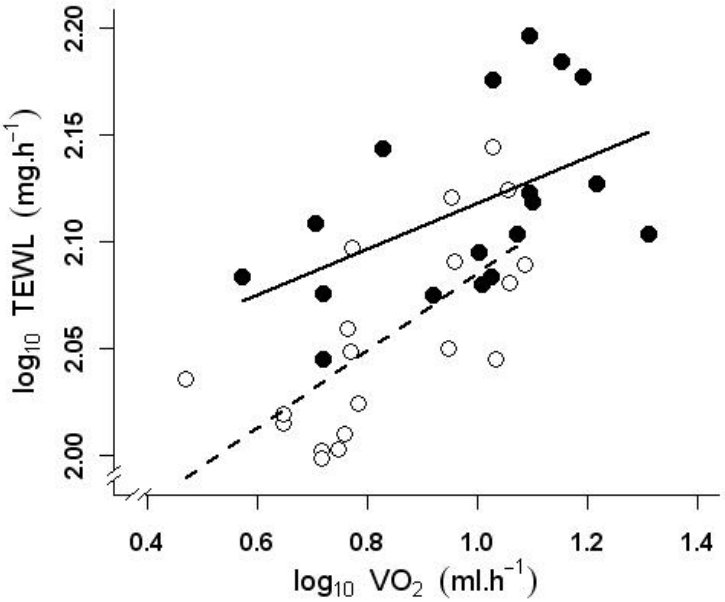

Figure 2. Relationship between the rate of TEWL (mg.h^{-1}) and VO_2 (ml.h^{-1}) in pregnant (filled circle) and non-reproductive females (open circle). Lines represent the fit linear regressions in pregnant (solid line, r^2 = 0.25) and non reproductive (dashed line, r^2 = 0.48) females.

($F_{1,16}$ = 14.4, P = 0.001; Fig. 2). In pregnant females, the VO_2 explains 25% of the variations of TEWL (Fig. 2). In non-reproductive females, VO_2 explains 48% of the variations of TEWL (Fig. 2).

Influence of allometric measures

Considering the different allometric measures, the inter-scale distance corresponds to the better determinant to explain the variation of the rate of TEWL (Table 1). The model including both animal size and inter-scale space present an equivalent AIC than the model with only the inter-scale distance (Table 1). This model explains an equivalent degree of variations but it includes two explanatory variables and therefore is less parsimonious than the model with inter-scale distance only (Table 1). Separately, inter-scale space is a better determinant of the rate of TEWL than the animal size (Table 1).

Allometric measure	df	AIC	r^2	*P*
SVL (cm)	3	-59.8	0.15	0.112
Inter-scale space (residuals)	3	-61.6	0.23	0.042
SVL + Inter-scale space	4	-63.6	0.38	0.027
Inter-scale distance (cm)	3	-65.0	0.36	< 0.01

Table 1. Model selection to compare the influence of different determinants of allometric measures on the rate of TEWL at 33°C in pregnant females (n = 18). See text for details.

2) CEWL

The rate of CEWL was significantly higher in pregnant females than in non-reproductive ones ($F_{1,14}$ = 60.9, P < 0.001; Fig. 3). Pregnant females present a rate of CEWL 46% higher than non reproductive ones. Furthermore, in pregnant females, the rate of CEWL is strongly related to the inter-scale space ($F_{1,6}$ = 17.3, P < 0.01, r^2 = 0.74).

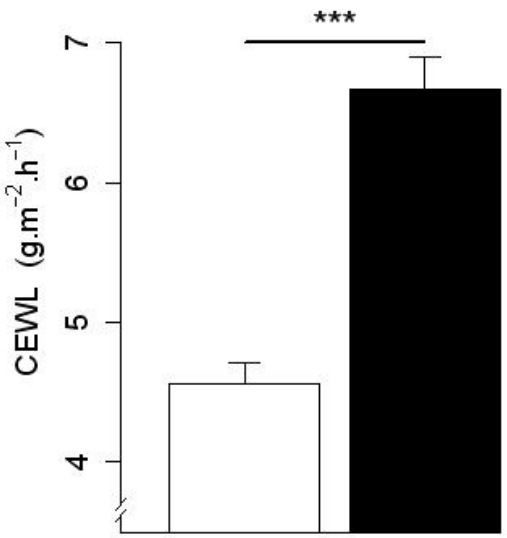

Figure 3. Comparison of the rate of cutaneous evaporative water loss (CEWL) between pregnant (n = 8, filled bar) and non-reproductive (n = 8, open bar) females. Bars represent means (± SE) of CEWL ($g.m^{-2}.h^{-1}$). *** P<0.001.

3) Field hygrometry

On the field, we found significant effect of the reproductive status on selected temperature ($F_{1,55}$ = 6.1, P = 0.016; Fig. 4A) and on selected absolute humidity ($F_{1,55}$ = 9.0, P < 0.01; Fig. 4B).

Discussion

Viviparity has emerged independently on a multitude occasions (> 110 times) among squamates and addressing associated "costs" is critical to better understand this evolutionary transition. Our study highlights indirect and direct effects of pregnancy on water losses (TEWL). As

expected, temperature strongly increases the rate of TEWL in pregnant and non-reproductive females. Specifically, pregnancy imposes higher rate of TEWL, because of modified behavior (i.e., higher selected temperature), modified physiology (i.e., increased metabolic rate) and modified morphology (i.e., increased inter-scales space). In response to these constraints on water balance, females select higher humidity to thermoregulate on the field.

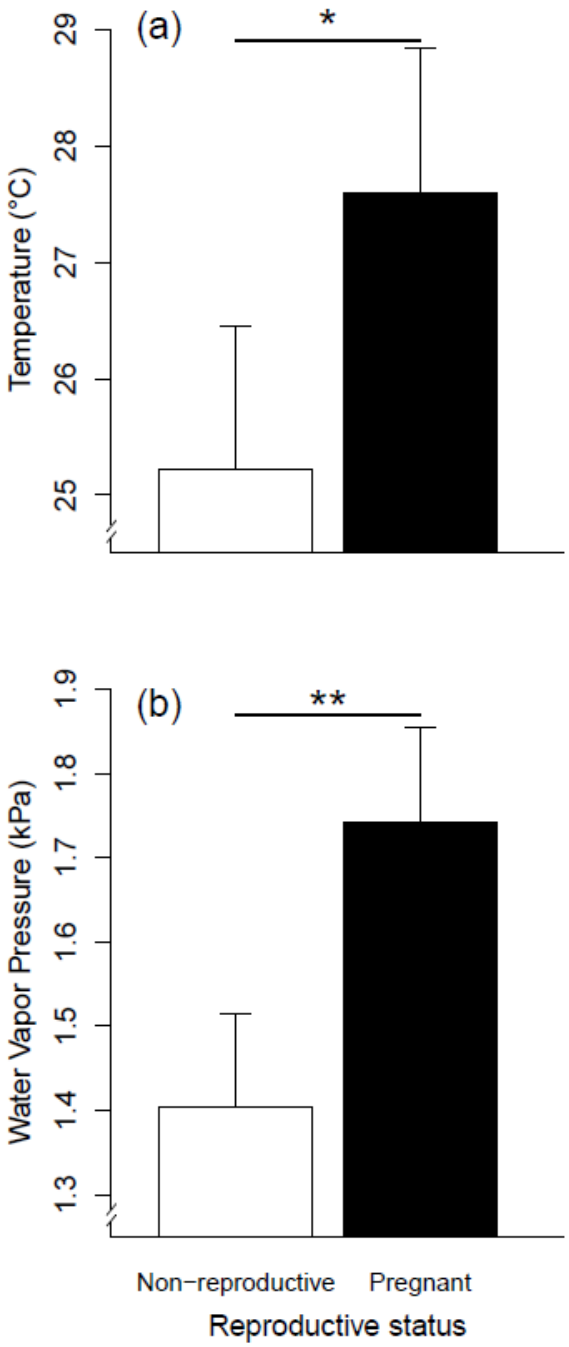

Figure 4. Micro-climates conditions selected by pregnant (filled bars) and non-reproductive females (open bars) on the field. Bars represent means (± SE) of A) temperature (°C) and B) water vapour pressure (kPa) selected by vipers.

We observed a strong increase of the rate of TEWL with the increase of temperature and this pattern is more intense in pregnant females. Considering the Tset of pregnant females (i.e., 33°C; Ladyman et al. 2003) and non-reproductive females (i.e., 25°C; Ladyman et al. 2003), the indirect effect of ambient temperature is therefore crucial to consider. According to our results, pregnant females at 33°C present a rate of TEWL 38% higher than the rate of TEWL of non-reproductive females at 25°C. As expected, in the same thermal conditions, pregnant females also present higher rate of TEWL than non-reproductive females, which correspond to direct effect of pregnancy on water losses. Direct effect of pregnancy on the rate of TEWL likely depends on specific gestational modifications (i.e., higher ventilation and higher transpiration). In support with this, we found higher rate of VO_2 and higher rate of CEWL in pregnant females compared to non-reproductive females. Moreover, the increase of the rate of TEWL is related to the increase of VO_2, what suggest a direct influence of VO_2 on the rate of TEWL. This influence of metabolic rate on water loss has previously been described in reptiles. However, the proportion of variations explained by metabolic rate in pregnant females is two times lower than in non-reproductive females.

Therefore, it suggests that other physiological or morphological characteristics of pregnant females are implied in the increase of TEWL.

Notably, in pregnant females, we observed a significant influence of inter-scale distance on the rate of TEWL. Since this measure integrates both animal size and inter-scale space, it is consistent to find a strong influence of this determinant on the rate of TEWL. Separately, we found that inter-scale space is a better determinant of the rate of TEWL than animal size. Yet, the inter-scale space corresponds to an index of skin distension (i.e., skin stretching). Increased skin distension likely increases the Surface ~ Volume ratio of the skin and therefore increases rate of transpiration. In support with this, we found that the rate of CEWL correlated with the inter-scale space. Together, these results suggest that the females that are more stretched present higher rate CEWL and therefore higher rate of TEWL.

We found a slight influence of the litter size on the rate of TEWL and no influence of other estimates of reproductive output or maternal relative reproductive effort. Increased metabolic rate during pregnancy has previously been reported to directly depend on maternal reproductive effort (i.e., litter mass adjusted with mother size) (Dupoué & Lourdais 2014). Interestingly, such difference in the influence of fecundity (i.e., litter size versus maternal reproductive effort) between hydric and energetic budget suggests different underlying mechanisms. The litter size integrates all component of the litter, including the undeveloped follicles and dead embryos (Lourdais et al. 2002b). Therefore, the litter size could be used as an index of physical burden. High litter sizes likely increase burden inside mother, influence mother's skin stretching and therefore the increase in CEWL. Such hypothesis could be directly tested with higher sample size by following morphological and structural modifications over pregnancy.

On the field, pregnant vipers selected micro-habitat with higher temperature and higher absolute humidity than non-reproductive females. The higher selected temperature by pregnant females is consistent with all previous studies that also reported higher thermal needs for pregnant females (Lorioux et al 2013a). Conversely, to our knowledge, higher selected absolute humidity due to pregnancy or gravidity in reptiles has attracted less attention (Stahlschmidt et al. 2011). High water pressure of the micro-climate passively limits the water loss of animals (Mautz 1982). Therefore, the contrast in micro-climate selection between pregnant and non-reproductive females during summer may represent a behavioural response to gestational water constraints. This result is consistent with a previous study that experimentally demonstrated the

importance of hygrometry for gravid snakes in micro-climate selection (Stahlschmidt et al. 2011). Importantly, such behavioural adjustment likely aims at buffering physiological constraints (i.e., increase TEWL), suggesting that TEWL could represent an actual cost of pregnancy. However, considering this physiological constraint on water balance as a cost for pregnant females require to investigate the effects of water shortage on fitness. Long-term field studies could specifically address this question by determining the importance of water during pregnancy on the life-time reproductive success of females.

Our study clearly demonstrated that pregnancy imposes water constraints. Pregnancy increases the rate of TEWL via indirect effect (i.e., higher selected temperature) and direct effects (i.e., physiological and morphological modifications). Pregnancy corresponds to a challenging period for mothers since they face several constraints associated with their fecundity. For instance, females have to deal energetic demand related to their reproductive effort (Dupoué & Lourdais 2014). Herein, we observed a slight relationship between the litter size and the rate of TEWL. According to our results, we hypothesize that the physical burden imposed by the litter stretches the mother skin, resulting in higher rate of CEWL. Since CEWL corresponds to the main avenue for EWL, the impact of fecundity on the rate of TEWL is therefore crucial to investigate. Indeed, such fecundity dependence would correspond to a water cost for females and possibly determine optimal reproductive effort. Importantly, this constraint may represent a cost associated with viviparity. Still, further studies are required to specifically test this hypothesis.

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Chapitre 3 :
Manipulation des contraintes hydriques de la gestation

Résumé du chapitre 3

Nous avons mis en évidence différentes contraintes liées à la gestation pour les femelles, en particulier d'ordre hydrique (i.e., transfert d'eau vers la portée et pertes hydriques supérieures). Ces résultats suggèrent des compromis d'allocation liés à l'eau à l'échelle des femelles (i.e., compromis intra-individuel). Dans ce chapitre, nous avons donc cherché à déterminer si les compromis d'allocation liés à l'eau pouvaient également s'exprimer entre la mère et les embryons (i.e., compromis intergénérationnel). Nous avons pour cela déterminé les conséquences d'une privation d'eau à la fois sur les femelles et sur leurs descendants. En réponse à la privation d'eau, le compromis intergénérationnel lié à la ressource en eau devrait induire un conflit significatif entre la mère et les embryons (**Article 5**). La privation d'eau devrait déclencher l'augmentation des concentrations de corticostérone (CORT) (i.e., surcharge allostatique) chez les femelles (**Article 6**) et possiblement le phénotype des jeunes (i.e., effets maternels).

Pour tester ces hypothèses, nous avons comparé les effets d'une période de 20 jours de privation d'eau sur des femelles gestantes et non-reproductrices. Pour mettre en évidence un compromis mère-embryons pour l'eau, nous avons comparé les effets du traitement expérimental (i.e., privation d'eau *versus* témoin) et du statut reproducteur sur l'état d'hydratation des femelles (i.e., variations de masse corporelle, d'hématocrite et d'osmolalité) et sur les concentrations de corticostérone (i.e., concentrations de CORT basale et induite par un stress). Nous avons également déterminé l'influence de l'effort reproducteur sur les variations de ces paramètres physiologiques. Enfin, nous avons comparé les conséquences sur les performances reproductrices (i.e., transfert d'eau vers la portée, succès reproducteur, nombre, qualité et croissance des nouveau-nés).

Les résultats obtenus soutiennent l'hypothèse d'un compromis significatif entre la mère et les embryons lorsque l'eau est manquante. En effet, en réponse à une privation d'eau, les femelles se déshydratent, et de façon plus marquée quand elles sont gestantes. De plus, l'effort reproducteur influence positivement l'intensité de la déshydratation des femelles gestantes privées d'eau. En parallèle, nous n'avons détecté aucun impact de la déshydratation des femelles sur le transfert d'eau au cours de la gestation, ni même après l'exposition à la privation

d'eau, ou sur les performances reproductrices. Ce résultat suggère qu'en réponse à une privation d'eau en milieu de gestation, le compromis qui résulte entre la mère et les embryons est résolu en faveur des embryons. Bien que les optimums des besoins en eau de la mère et des embryons ne soient pas connus, nous émettons l'hypothèse que ce compromis intergénérationnel induit un conflit mère-embryons significatif pour l'eau. Cependant, pour tester cette hypothèse il est nécessaire de mesurer les effets d'une privation d'eau en milieu de gestation sur la survie et la reproduction future des femelles.

En parallèle, nos résultats soutiennent l'hypothèse selon laquelle la privation d'eau influencerait les concentrations de CORT maternelles et le phénotype des jeunes. Les niveaux de CORT sont d'une part plus élevés chez les femelles gestantes comparées aux non-reproductrices (i.e., charge allostatique) et d'autre part, ils augmentent significativement en réponse à la déshydratation (i.e., surcharge allostatique). Nous avons détecté une relation négative entre l'effort reproducteur et les variations du niveau basal de CORT chez les femelles gestantes témoins. Ce résultat suit la première prédiction de l'hypothèse CORT-valeur sélective, à savoir que les femelles de bonne qualité (fécondité élevée) ont une concentration basale de CORT faible. Cette relation n'existe plus chez les femelles déshydratées, ce qui suggère que l'augmentation de CORT basale maternelle est indépendante de l'effort reproducteur. Enfin, cette étude a permis de mettre en évidence une croissance plus importante chez les nouveau-nés de femelles déshydratées *versus* témoins. Ce résultat comparatif est soutenu par les corrélations positives significatives entre la croissance des juvéniles et les variations d'osmolalité ou de CORT maternelle. Ainsi une déshydratation en milieu de gestation semble induire des effets maternels phénotypiques possiblement orchestrés par la sécrétion de CORT maternelle. En support, de cette hypothèse, plusieurs études expérimentales ont précédemment démontré l'influence significative (positive ou négative) de l'augmentation de CORT maternelle sur la croissance des nouveau-nés.

ARTICLE 5

Intergenerational trade-off for water may induce a mother-offspring conflict in favour of embryos in a viviparous snake

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Intergenerational trade-off for water may induce a mother–offspring conflict in favour of embryos in a viviparous snake

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Summary

1. Parent–offspring conflicts are likely to occur when resources are limiting either at pre- or post-natal stages due to intergenerational trade-offs over resources. Current theory posits that such conflicts may influence the evolution of parental allocation as well as reproductive modes. While energy allocation to the offspring has received considerable attention, the distribution of water – another potentially limited vital resource to both the mother and offspring – and the resulting outcomes remain grossly understudied.

2. Here, we explored the intergenerational trade-off related to water resources in the viviparous aspic viper (*Vipera aspis*) by examining the effects of water deprivation on female physiology (body mass, haematocrit and osmolality), water transfer to developing embryos and reproductive performance.

3. As a result of water deprivation, females became dehydrated, with the effects more pronounced in pregnant compared to nonreproductive females. Among pregnant females, the impacts of water deprivation on water balance were correlated with fecundity. In contrast, water deprivation had no effect on water transfer to the offspring or on reproductive performance.

4. Our results demonstrate that, under water-constraining conditions, female water balance is compromised in favour of the developing embryos, highlighting a significant intergenerational trade-off for water. Although ectothermic reptiles are particularly tolerant in water balance perturbations, our results suggest that, like energy, water can be a conflicting resource between mother and offspring. Parent–offspring conflict over water should therefore be further investigated to better understand reproductive modes and reproductive trade-offs in terrestrial organisms.

Key-words: aspic viper, dehydration, fecundity, parent–offspring conflict, pregnancy, reproductive mode, trade-off

Introduction

The parent–offspring conflict theory (Trivers 1974) aims at clarifying the determinants of the balance between parental investment and offspring requirements. Conflict occurs because parents and offspring are not genetically identical, and therefore, selection acts on each protagonist to favour its own needs (Trivers 1974). Several studies have provided empirical support to this theory (Parker & Mock 1987; Kölliker & Richner 2001), mainly in species with post-natal parental care (Parker & Mock 1987; Clutton-Brock 1991; Kölliker & Richner 2001). Surprisingly, much less attention has been paid to conflicts at prenatal stages (Haig 1993; Crespi & Semeniuk 2004) even though prenatal parent–offspring conflict may be a major selective pressure in the evolution of reproductive mode (i.e. the viviparity-conflict hypothesis, hereafter "VCH"; Crespi & Semeniuk 2004). According to the VCH, a tug-of-war for resources exists between mother and developing embryos, and there is selection for offspring traits to draw maternal

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resources (Crespi & Semeniuk 2004). Supporting this hypothesis, manipulations of the mother by offspring have previously been reported in humans (Haig 1993) and non-mammalian vertebrates (Crespi & Semeniuk 2004). While offspring are expressing traits to draw maternal resources, females adjust their resource allocation to optimize their lifetime reproductive success (Schwarzkopf & Andrews 2012), which may result in resource allocation that is suboptimal for current offspring development (Crespi & Semeniuk 2004).

While not explicit to resource type, maternal-offspring conflicts are typically evaluated in terms of energy resource allocation (i.e. nutrient supply; Crespi & Semeniuk 2004). Conflicts between a mother and offspring may occur whenever there is an intergenerational trade-off for a limited resource (Charnov 1982; Godfray & Parker 1991; Godfray 1995). Several examples of energy-based trade-offs or conflicts between mother and offspring have been previously reported in invertebrates (Mas, Haynes & Kölliker 2009), fishes (Hussey *et al.* 2010), amphibians (Kupfer *et al.* 2006), birds (Müller, Korsten & von Engelhardt 2007) and mammals (Capellini, Venditti & Barton 2011). In comparison, while water represents another depreciable resource for organisms, water allocation has not yet been considered in the framework of parent–offspring trade-offs or conflicts. Water is oftentimes readily available, but this is not the case in environments where seasonal or prolonged droughts are common.

Studies of the impact of water limitation on the parent and its offspring are challenging because water is typically a significant component of the consumed meal, and thus it is characteristically difficult to separate the impacts of water constraint from those of energy constraint. However, squamate reptiles (lizards and snakes) provide an excellent opportunity to examine water limitations free of influence from energy conflicts. Many squamates energetically support reproduction by utilizing previously accumulated energy stores (i.e. capital breeding; Bonnet, Bradshaw & Shine 1998), and these energy stores are then invested into reproduction through yolk deposition into the developing follicles (i.e. lecithotrophy; Blackburn 1993). As a result, the allocation of energy by the female to the offspring is completed prior to ovulation, and therefore, the embryos cannot influence this investment.

Developing embryos require a considerable amount of water, especially during the later foetal life stage when somatic growth is exponential (Andrews 2004; Lourdais unpublished data). In viviparous species, this water must be provided by the female. The water demands of the foetuses are especially challenging for many squamates, because pregnancy is a lengthy process that generally occurs in the summer when water resources can be most limited. Additionally, pregnant female squamates typically increase their thermal preferences during pregnancy (Lourdais *et al.* 2002a, 2004; Lorioux, Lisse & Lourdais 2013a), resulting in a concomitant increase in the rate of evaporative water loss (Mautz 1982; Guillon *et al.* 2014). Collectively, these challenges make pregnant females particularly vulnerable to water limitations. Regardless, failure to provide sufficient water to the embryos can greatly compromise their development, as water content of the litter increases from 40 to 50% at ovulation to 80% at the end of gestation (Thompson 1981). In oviparous species, experimental and correlative studies suggest that water constraints during development alter yolk mobilization, affect offspring morphology and performance, and can result in embryo death (Shine & Brown 2002; Aubret *et al.* 2005; Lourdais, Hoffman & DeNardo 2007). Additionally, both experimental and field studies conducted in a viviparous species (*Zootoca vivipara*) suggest that water restriction and rainfall regime during pregnancy can alter offspring size and survival (Dauphin-Villemant & Xavier 1986; Marquis, Massot & Le Galliard 2008).

Given the maternal and embryonic sensitivities to water limitation, a significant mother–offspring conflict for water should exist when water resources are limited. Accordingly, we manipulated access to water (control vs. water deprived for 20 days) for pregnant and nonreproductive female aspic vipers (*Vipera aspis*). We considered the impact of water deprivation on (i) the physiology of females (body mass, haematocrit and plasma osmolality), (ii) the transfer of water to the embryos during pregnancy and (iii) the reproductive performance (reproductive success, litter traits and offspring quality at birth).

We hypothesized that a significant intergenerational trade-off for water may exist and induce a mother–offspring conflict when access to water is limited. We tested the following predictions:

1. Water deprived pregnant females should be more dehydrated than water deprived nonreproductive females as a result of water demands of the embryos and physiological changes associated with reproduction (e.g. increased body temperature).
2. The number of developing embryos (i.e. fecundity) should be correlated with the effects of water deprivation on female physiology.
3. Water deprivation should alter water transfer to the embryos and therefore, compromise reproductive performance.

Material and methods

STUDY SPECIES AND HOUSING

We studied the aspic viper (*Vipera aspis*), a medium-size viviparous snake that is abundant in Western Europe (Naulleau 1981). The aspic viper is a typical capital breeder, and females mobilize their energy reserves to support yolk production during vitellogenesis. Pregnancy begins after ovulation in early June (Naulleau 1981), which is associated with a pre-ovulatory ecdysis (Lorioux *et al.* 2013b). Parturition occurs 2–3 months later from late August to early September (Lourdais *et al.* 2002a). Pregnant females often cease to feed (Bonnet, Bradshaw & Shine 1998; Lourdais, Bonnet & Doughty 2002b), mainly because of an

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important behavioural shift that emphasizes thermoregulation over foraging (Lorioux, Lisse & Lourdais 2013a).

In May and June 2012, we caught 58 females (29 pregnant, 29 nonreproductive) from neighbouring sites in western France (Vendée and Loire-Atlantique Districts). Reproductive status was determined by palpation in the field and then confirmed with high-resolution ultrasonography (Sonosite microMaxx, Inc., Bothell, WA, USA) when the animals were brought to the laboratory where all snakes were measured (snout-vent length, SVL ± 0·1 cm) and weighed (body mass, BM ± 0·1 g). Husbandry followed the protocol described in Lorioux, Lisse & Lourdais (2013a). Individuals were housed 3–4 per cage in 16 cages (100 × 30 × 35 cm). Room temperature was held constant at 20 °C. To mimic natural conditions and allow for thermoregulation, heat was provided at one end of each cage with a 75W incandescent light bulb for 5 h per day (from 1000 to 1500 h), creating a thermal gradient (20–40 °C). Water was available *ad libitum*. Individuals were fasted 2 weeks prior to the start of the experiment and remained fasted throughout the experiment, thus preventing meal consumption from contributing dietary water to the snakes' water balance (Wright, Jackson & DeNardo 2013). During the entire study, we followed all laws and rules relating to the conservation and welfare of the animals (Permit #792, Direction service vétérinaire des Deux-Sèvres).

EXPERIMENTAL PROTOCOL

Individuals from each reproductive status were randomly assigned to the control (pregnant: $n = 15$; nonreproductive: $n = 15$) or water deprived (pregnant: $n = 14$; nonreproductive: $n = 14$) hydric treatments. Within each reproductive status, water deprived individuals and controls did not differ in BM or SVL (all $P > 0{\cdot}117$). During the experiment, housing conditions remained as described above, except that water was removed for 20 days in the water deprived treatment. Water deprivation occurred at the time when pregnant females were in mid-gestation, and the duration of the deprivation represents the typical duration of a summer drought in western France.

At the end of the treatment period, water intake and mass recovery were assessed by placing the females in individual boxes (30 × 20 × 10 cm) with a thermal gradient (similar to that described earlier), a water bowl and a shelter. Pregnant females were checked daily for parturition (Lorioux *et al.* 2013b), and female and offspring traits were measured on the day of birth. Once all measurements were collected, females were fed and released with their litters at their original capture sites.

PHYSIOLOGICAL PARAMETERS

Body mass

Change in BM (Δ BM) has been well-established as an estimator of water loss in squamate reptiles (DeNardo, Zubal & Hoffman 2004; Lillywhite *et al.* 2008; Dupoué *et al.* 2014a). BM was collected after each blood sample. To account for changes in BM associated with reproductive state (pregnant or nonreproductive), we also determined Δ BM of water deprived females relative to the mean Δ BM of control females of the same reproductive state over the same period (relative Δ BM = Δ BM minus the mean Δ BM of relevant control animals).

Blood parameters

We measured the change in haematocrit (Δ Hct) and the change in osmolality (Δ Osmo), as both parameters are effective indicators of hydration state (Peterson 2002). Blood samples were collected at the beginning and end of the water deprivation period. Females were sampled in random order for both blood sampling points. Blood was immediately collected upon removing the female from its cage. We collected blood samples (150 µL) via cardiocentesis using a heparinized 1-ml syringe with a 27-gauge needle. Immediately after collection, we allocated two replicates of blood into 10-µL micro-capillary tubes to measure Hct. The microcapillary tubes were centrifuged for 3 min at 5500 × g, and we then measured the lengths of the column of cells and the total sample (i.e. cells + plasma) with a digital calliper (±0·01 mm). Hct (%) was determined as the proportion of cells to total sample. For each blood sample, we averaged the two Hct values (intra-individual CV: 3·82%). The remaining blood from each sample was placed into a 0·675 mL microcentrifuge tube and centrifuged for 3 min at 2000 × g. The plasma was separated from the cells and stored in airtight tubes at −28 °C until laboratory analyses. Plasma osmolality (mOsm.kg^{-1}) was measured from 10-µL triplicates (intra-individual CV < 1%) as described in Wright, Jackson & DeNardo (2013). In one female (pregnant from the water deprived treatment), blood collection failed during the final sampling session, so this female was not included in the analyses of maternal blood parameters.

WATER TRANSFER TO THE EMBRYOS

Because water deprivation likely impacts the transfer of water to the embryos, we monitored the change in volume of the embryonic unit (i.e. embryo, yolk and extra-embryonic membranes) using high-resolution ultrasonography (see Lorioux *et al.* 2013b). We determined total volume at three different stages of embryonic development (Hubert & Dufaure 1968): early pregnancy (mean ± SE: 12·3 ± 1·5 days since ovulation; stages 30–33), mid-pregnancy (58·8 ± 1·6 days since ovulation; stages 37–39) and late pregnancy (78·7 ± 1·4 days since ovulation; stages 42–43). The second, mid-pregnancy measurement was specifically performed at the end of the hydric treatment to assess the impact of water deprivation on water transfer to the embryos. For each female, we collected images in a sagittal view of the most cranial and caudal embryonic units and measured their heights (H), lengths (L) and form. We estimated embryonic volume (cm^3) following the method described in Maritz & Douglas (1994) which considers embryonic unit L, H and a coefficient associated with its form (F_{emb}): embryonic volume = $\pi \times L \times H^2 \times F_{emb}$. For analysis, we averaged the volume of the two embryonic units measured at each stage for each female (intra-individual variation: 15·3%).

REPRODUCTIVE PERFORMANCE

We determined the effect of hydric treatment on reproductive success (i.e. number of females with at least one viable offspring) and the duration of pregnancy. The duration of pregnancy (days) was derived from the difference between parturition and ovulation dates (Lorioux *et al.* 2013b). Additionally, we measured litter size (number of undeveloped ova, stillborn and neonates), fit litter size (number of neonates only), litter mass (mass of undeveloped ova, stillborn, and neonates) and fit litter mass (mass of neonates only) (see details in methods of Lourdais, Bonnet & Doughty 2002b). We also calculated embryonic fluid mass (g), which corresponds to the difference between mass lost by the female at parturition and total mass of the litter.

To assess offspring quality, we collected previously described morphometric traits (Lorioux *et al.* 2013b). Briefly, each individual was sexed by attempting to manually evert hemipenes. Shortly after birth (<1 day), we collected neonate BM (±0·01 g) and SVL (±0·1 cm), and we measured neonate jaw length (JL) using digital

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callipers (±0·01 mm). We collected all values in triplicate and used the mean values in analyses (intra-individual variation <1%). We estimated body condition (BC) as the residuals from the relationship of BM against SVL ($F_{1,123}$ = 69·9, P < 0·001).

STATISTICAL ANALYSES

All analyses were performed using R software (R Development Core Team, 2011). We used the Shapiro–Wilk test to determine whether residuals of our models significantly differed from a normal distribution (all P > 0·05).

We built linear models with initial values of physiological parameters (BM, Hct and osmolality) as dependent variables and with treatment assignment, reproductive status and their interaction as fixed factors (Table 1). We used pairwise *post hoc* tests of Tukey (lsmeans, package lsmeans) on the interaction to determine statistical differences between groups. We used the same analytic design to test the effects of water deprivation and reproductive status on changes in BM (Δ BM), Hct (Δ Hct) and osmolality (Δ Osmo) using the initial value as a linear covariate.

We used linear models to test the relationship between Δ BM and Δ Osmo, or between Δ Hct and Δ Osmo. Fecundity effects on physiological parameters were analysed with linear models with Δ BM, Δ Hct or Δ Osmo as the dependent variable and fecundity as the explained variable.

We used linear mixed models (lme, package nlme) to determine the effect of water deprivation on embryonic volume with embryonic volume as the dependent variable, and hydric treatment, reproductive status, stage of pregnancy, and their interaction as fixed factors. Female identity was set as a random factor considering the females' repeated contribution over gestation.

For analyses of reproductive performance, we used different type of models systematically with hydric treatment as a fixed factor. Reproductive success of the female was analysed with binomial models (i.e. success = 1; fail = 0). Duration of pregnancy, litter size, litter mass, fit litter size, fit litter mass and embryonic fluid mass were analysed with linear models. Offspring BM, SVL, BC and JL were analysed with linear mixed models with mother identity as a random factor since siblings are statistically nonindependent.

Results

FEMALE PHYSIOLOGY

Body mass

At the onset of the water deprivation period, BM was not influenced by treatment assignment, reproductive status or their interaction (Table 1). Δ BM was not influenced by initial BM, but was significantly impacted by hydric treatment ($F_{1,53}$ = 96·3, P < 0·001, Fig. 1a), reproductive status ($F_{1,53}$ = 4·5, P = 0·039, Fig. 1a) and their interaction ($F_{1,53}$ = 9·6, P = 0·003, Fig. 1a). Water deprivation induced a significant loss in BM for both pregnant females (*post hoc*, control vs. water deprived, t = 9·1, P < 0·001, Fig. 1a) and non reproductive females (*post hoc*, control vs. water deprived, t = 4·7, P < 0·001, Fig. 1a). Absolute BM loss was similar between pregnant and nonreproductive water deprived females (*post hoc*, pregnant vs. nonreproductive, t = 0·7, P = 0·897, Fig. 1a). Within the control group, pregnant females gained mass while nonreproductive females lost mass (*post hoc*, pregnant vs. nonreproductive, t = −3·7, P = 0·003, Fig. 1a). Consequently, relative BM loss in water deprived females (i.e. Δ BM minus the mean Δ BM of the control group) was 89% higher in pregnant females compared to nonreproductive females (*post hoc*, pregnant vs. nonreproductive, t = 4·1, p < 0·001, Fig. 1a).

In water deprived pregnant females, Δ BM was negatively influenced by fecundity (i.e. females with higher fecundity lost more mass; $F_{1,11}$ = 4·9, P = 0·049, Fig. 2a). Conversely, Δ BM was positively influenced by fecundity in control females (i.e. females with higher fecundity gained more mass; $F_{1,11}$ = 9·7, P < 0·01, Fig. 2a).

The day after re-exposure to water, females from the water deprived treatment gained mass and attained BM similar to that of controls in both pregnant (*post hoc*, control vs. water deprived, t = −0·7, P = 0·890) and nonreproductive females (*post hoc*, control vs. water deprived, t = −2·2, P = 0·143).

Blood parameters

At the onset of the water deprivation period, Hct was significantly lower in pregnant females than in nonreproductive females (Table 1) and was similar between treatment assignments within each reproductive status (Table 1). Δ Hct was significantly influenced by initial value ($F_{1,52}$ = 4·2, P = 0·046) and was not influenced by hydric treatment ($F_{1,52}$ = 3·4, P = 0·072), reproductive status ($F_{1,52}$ = 0·7, P = 0·403) or their interaction ($F_{1,52}$ = 0·04, P = 0·842). Δ Hct was not affected by hydric treatment in pregnant females (*post hoc*, control vs. water deprived, t = −1·4, P = 0·492, Fig. 1b) or in nonreproductive

Table 1. Body mass (BM), haematocrit (Hct) and plasma osmolality of pregnant and nonreproductive female aspic vipers (*Vipera aspis*) at the onset of the water deprivation period. Females are represented by treatment assignment, although the water deprivation period treatment had not yet begun

	Nonreproductive		Pregnant		Statistical effect of		
	Control	Water deprived	Control	Water deprived	Assignment	Status	Assignment × Status
BM (g)	83·5 ± 8·6	90·3 ± 9·5	98·5 ± 6·7	101·2 ± 6·7	n.s.	n.s.	n.s.
Hct (%)	28·2 ± 1·1	28·4 ± 1·5	21·7 ± 0·7	19·9 ± 0·9	n.s.	***	n.s.
Osmolality (mOsm.kg^{-1})	321·3 ± 3·6	307·2 ± 3·4	322·0 ± 3·6	321·8 ± 2·4	*	*	*

Significant differences are symbolized: *P < 0·05, ***P < 0·001, and n.s. (non significant). See text for details.

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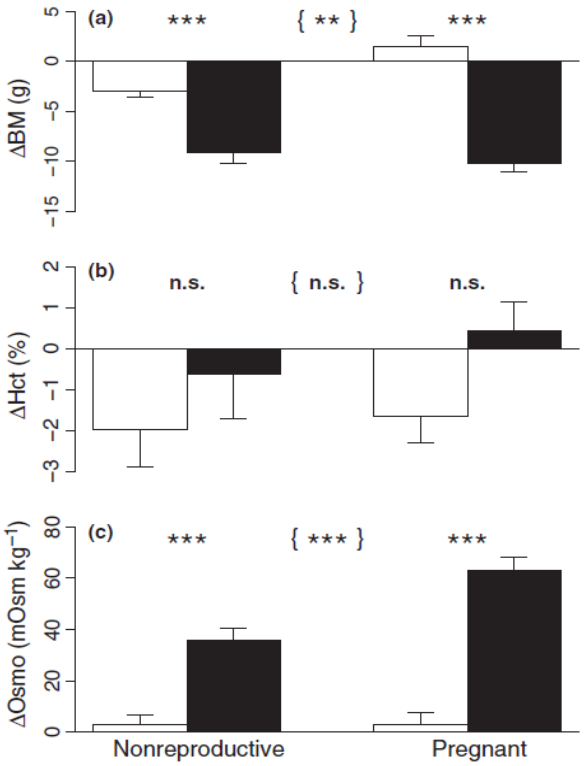

Fig. 1. Effects of hydric treatment on changes in (a) body mass (Δ BM), (b) haematocrit (Δ Hct) and (c) osmolality (Δ Osmo) between initial and final sampling points for pregnant and nonreproductive female aspic vipers (*Vipera aspis*). Bars represent means ± SE for control (open bars) and water deprived (filled bars) females. Significant differences between control and water deprived females are symbolized: ***$P < 0{\cdot}001$, and n.s. (nonsignificant), while significant effect of the interaction between hydric treatment and reproductive status are symbolized: {**}$P < 0{\cdot}01$, {***}$P < 0{\cdot}001$, and n.s.; non significant.

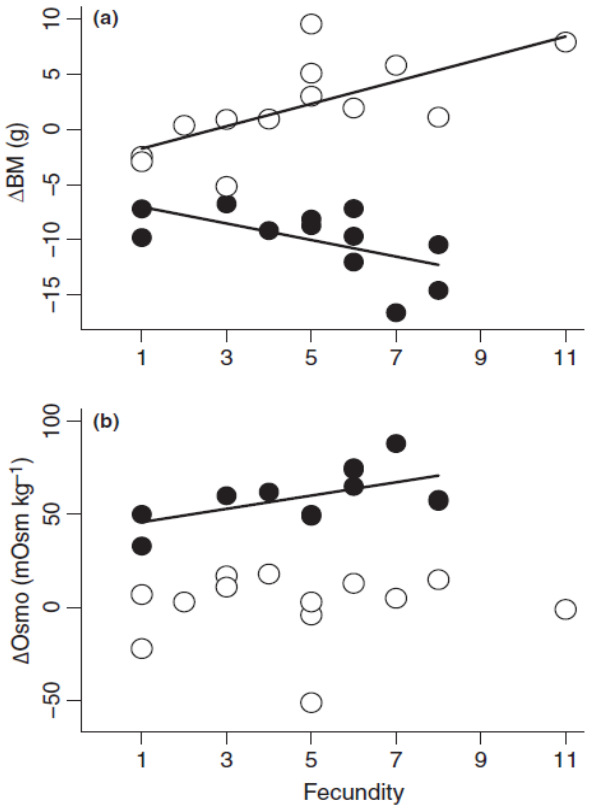

Fig. 2. Influence of fecundity on changes in (a) body mass (Δ BM) and (b) osmolality (Δ Osmo) of control (open circles) and water deprived (filled circles) pregnant female aspic vipers (*Vipera aspis*). Trend lines are included for significant relationships. Fecundity negatively influenced Δ BM of water deprived females ($r^2 = 0{\cdot}31$), but positively influences Δ BM of control females ($r^2 = 0{\cdot}47$). Fecundity positively influences Δ Osmo ($r^2 = 0{\cdot}34$) in water deprived females, but not controls ($r^2 = 0{\cdot}00$).

females (*post hoc*, control vs. water deprived, $t = -1{\cdot}2$, $P = 0{\cdot}643$, Fig. 1b). Δ Hct was not influenced by fecundity for either water deprived ($F_{1,10} = 0{\cdot}3$, $P = 0{\cdot}595$) or control females ($F_{1,11} = 0{\cdot}0$, $P = 0{\cdot}841$).

At the onset of the water deprivation period, plasma osmolality was influenced by treatment assignment, reproductive status and their interaction (Table 1) due to a lower value in nonreproductive females that were scheduled to start their water deprivation treatment (Table 1). Δ Osmo was significantly influenced by initial value ($F_{1,52} = 11{\cdot}1$, $P = 0{\cdot}002$) and was significantly impacted by hydric treatment ($F_{1,52} = 100{\cdot}4$, $P < 0{\cdot}001$, Fig. 1c), reproductive status ($F_{1,52} = 15{\cdot}1$, $P < 0{\cdot}001$, Fig. 1c) and their interaction ($F_{1,52} = 13{\cdot}9$, $P < 0{\cdot}001$, Fig. 1c). Osmolality significantly increased after water deprivation in pregnant females (*post hoc*, control vs. water deprived, $t = -9{\cdot}8$, $P < 0{\cdot}001$, Fig. 1c) and nonreproductive females (*post hoc*, control vs. water deprived, $t = -4{\cdot}2$, $P < 0{\cdot}001$, Fig. 1c). In water deprived females, Δ Osmo was 77% greater in pregnant than in nonreproductive females (*post hoc*, $t = -5{\cdot}0$, $P < 0{\cdot}001$, Fig. 1c).

When combining treatments, we found a significant negative relationship between Δ BM and Δ Osmo for pregnant (i.e. females with greater increases in osmolality had greater BM loss, $F_{1,26} = 90{\cdot}6$, $P < 0{\cdot}001$, $r^2 = 0{\cdot}78$) and nonreproductive females ($F_{1,27} = 24{\cdot}0$, $P < 0{\cdot}001$, $r^2 = 0{\cdot}47$). We did not find a significant relationship between Δ Hct and Δ Osmo for either pregnant or nonreproductive females (all $P > 0{\cdot}246$).

Finally, Δ Osmo was positively influenced by fecundity for water deprived females ($F_{1,10} = 5{\cdot}2$, $P = 0{\cdot}045$, Fig. 2b), but not for control females ($F_{1,11} = 0{\cdot}04$, $P = 0{\cdot}829$, Fig. 2b).

REPRODUCTION

Water transfer to the embryos

Embryonic volume increased significantly throughout development ($F_{2,48} = 105{\cdot}7$, $P < 0{\cdot}001$; Fig. 3), but it was not influenced by treatment ($F_{1,24} = 0{\cdot}2$, $P = 0{\cdot}644$; Fig. 3) or the interaction between treatment and stage of

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development ($F_{2,48}$ = 0·6, P = 0·544; Fig. 3). Mothers from the control and the water deprived treatments had similar embryonic volumes at all stages considered including the end of water deprivation (*post hoc*, control vs. water deprived, early pregnancy: z = −1·0, P = 0·920, mid-pregnancy: z = 0·2, P > 0·999, late pregnancy: z = −0·3, P > 0·999; Fig. 3).

Reproductive performance

The percentage of females that produced at least one viable offspring was equivalent between the control (n = 13/15; 87%) and water deprived (n = 13/14; 93%) treatments (z = 0·0, P > 0·999). Similarly, the duration of pregnancy was not impacted by hydric treatment (control: 99·9 ± 3·6 days, water deprived: 99·5 ± 2·6 days; $F_{1,24}$ = 0·01, P = 0·932).

Water deprivation did not have a significant effect on litter size (control: 5·9 ± 0·5, water deprived: 6·0 ± 0·3), fit litter size (control: 5·2 ± 0·7, water deprived: 5·2 ± 0·6), litter mass (control: 28·9 ± 3·7 g, water deprived: 30·3 ± 3·0 g), fit litter mass (control: 27·4 ± 4·1 g, water deprived: 28·0 ± 3·8 g) or embryonic fluid mass (control: 16·7 ± 1·4 g, water deprived: 16·9 ± 1·8 g) (all P > 0·774).

Finally, hydric treatment did not significantly affect offspring BM (control: 5·48 ± 0·10 g, water deprived: 5·53 ± 0·10 g), SVL (control: 16·22 ± 0·11 cm, water deprived: 16·37 ± 0·10 cm), BC (control: 0·02 ± 0·08, water deprived: −0·01 ± 0·08), or JL (control: 12·78 ± 0·07 mm, water deprived: 12·59 ± 0·10 mm) (all P > 0·574).

Discussion

In this study, we show for the first time in a viviparous vertebrate that an intergenerational trade-off exists over a limited water resource between the mother and her developing embryos. We predicted water deprived pregnant

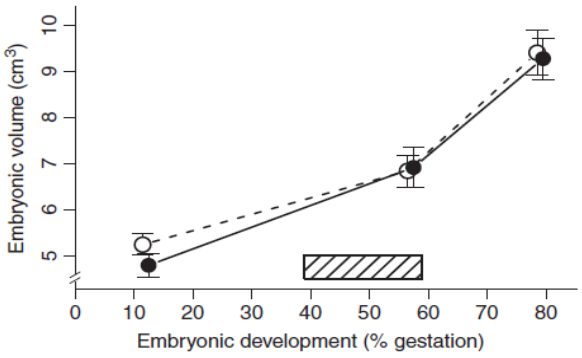

Fig. 3. Change in embryonic volume (cm^3) during development in control (open circle, dashed line) and water deprived (filled circle, solid line) pregnant female aspic vipers (*Vipera aspis*). Points represent means ± SE. Hatched space corresponds to the timing of the water deprivation. While embryonic volume increased over time, there was no effect of treatment.

females to be more dehydrated than water deprived nonreproductive females as a result of water demands of the embryos. Accordingly, we found that a 20-day water deprivation had a greater negative impact on water balance of pregnant females compared to nonreproductive ones. We found that the number of developing embryos amplified physiological impacts of water deprivation on females' hydration state (greater loss of BM and increased plasma osmolality), thereby supporting our second prediction. Finally, we predicted water deprivation would alter water transfer to the embryos and therefore, compromise reproductive performance. Conversely, we found that water transfer and reproductive performance were not affected by water deprivation, suggesting that the females' hydration state is compromised in favour of embryos water balance and development. Even though female aspic vipers tolerated dehydration, our results suggest that water is likely a limiting resource between mother and offspring and thus a likely target of conflict.

INCREASED SENSITIVITY TO WATER DEPRIVATION IN PREGNANT VIPERS

In the water deprived treatment, both pregnant and nonreproductive females showed a significant loss in BM. However and importantly, relatively to control individuals, pregnant females experienced 89% greater mass loss than nonreproductive females when water deprived. Similarly, water deprived females showed a sharp increase in plasma osmolality that was 77% higher in pregnant females compared to that of nonreproductive females. Although haematocrit is usually considered a good indicator of hydration state (Peterson 2002), there was no influence of treatment on changes in Hct or a significant relationship between changes in Hct and osmolality, thereby challenging this generality. Regardless, the patterns of BM and osmolality changes underline differing water requirements and constraints between pregnant and nonreproductive females. Indeed, during pregnancy, females actively drink to remain normosmotic (Lourdais unpublished data, this study). Water uptake is associated with an increase of BM that is positively related to fecundity (Lourdais unpublished data, this study). When exposed to water deprivation, we found that fecundity directly influenced changes in BM and osmolality in pregnant females. Therefore, when access to water is restricted, developing offspring have an additive, negative effect on water balance of pregnant females. Our results therefore suggest that, under water deprivation, female body water is transferred to developing offspring causing the female to become increasingly dehydrated. It is possible that females allocate water to their embryos and therefore, compromise their own water balance to support offspring survival. An alternative explanation is that embryos are actively acquiring water from the mother. For instance, during incubation, embryos of oviparous squamates are able to control aquaporin function or regulate the osmotic gradient to

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efficiently extract water from the nest environment (Packard 1991; Brown & Shine 2005; Shine & Thompson 2006). These observations underline significant embryonic adaptations to accumulate water from their surrounding environment and therefore, provide support for a foeto-maternal conflict. Unfortunately, the physiological mechanisms used by females or embryos of viviparous species to control the transfer of water is not yet understood like they are for the transfer of energy (e.g. hormones, cytokines, growth factor, etc.) (Crespi & Semeniuk 2004). Clearly, further work on this topic is needed.

EFFECTS OF WATER DEPRIVATION ON WATER TRANSFER AND REPRODUCTIVE PERFORMANCE

The water deprivation period had no effect on reproductive success, gestation duration and litter or offspring traits, supporting the idea that body water from the female is transferred to the developing offspring to buffer the offspring at the expense of female water balance. Amniotic fluid might have buffered the transfer of female body water, but measuring fluid volume immediately after dehydration would have required invasive techniques. To indirectly address this question, we ultrasonographically estimated embryonic volume before and after dehydration, and we estimated non-embryonic fluid volume at parturition (prepartum mass of female minus littler mass and post-partum mass). We found no difference in either of these estimates between water deprived and control females, suggesting that amniotic fluid volume was not affected by dehydration. Importantly, water deprivation in this study was imposed during mid-pregnancy, and embryo water requirements are known to dramatically depend on developmental stage. Specifically, water demands increase exponentially throughout development following the pattern of somatic growth (Dauphin-Villemant & Xavier 1986). Therefore, any foeto-maternal conflict for water would likely be stage dependent, and it would be valuable to investigate the impact of water deprivation on reproductive performance at different stages, especially during late gestation (see Lorioux *et al.* 2013b). As embryonic demand for water increases, water deprivation in late-term pregnancy could result in severe dehydration of the female that may switch the mother–offspring conflict in favour of the mother (i.e. 'selfish mother hypothesis'; Schwarzkopf & Andrews 2012).

Our study highlights an intergenerational trade-off between mother and offspring over water resources. Parent–offspring conflicts can arise when resource optimums for the different protagonists are not the same (Trivers 1974). Parents have to limit the cost of reproduction (i.e. the trade-off between current reproductive effort and survival or future reproductive success; Stearns 1989), while offspring favour their own survival (Trivers 1974). Although ectothermic reptiles are particularly tolerant to water balance perturbations, dehydration is known to affect individual performances (Wilson & Havel 1989; Lorenzon *et al.* 1999; Moeller, Butler & DeNardo 2013). Over the water deprivation period, osmolality of pregnant females raised from $321{\cdot}8 \pm 2{\cdot}4$ to $384{\cdot}9 \pm 5{\cdot}4$ mOsmol.kg^{-1}, and these females immediately drank when re-exposed to freshwater, suggesting that they were highly dehydrated (Moeller, Butler & DeNardo 2013; Lillywhite *et al.* 2014). Data on optimum water requirements are currently missing in the studied species, either for females during pregnancy or for developing embryos. However, the alteration of physiological performances is known to entail significant costs of reproduction (Shine 1980, 2003). Therefore, dehydration during pregnancy is likely an important constraint for females, and our findings advocate for a significant conflict between mother and offspring over water resource. Future studies need to investigate female and offspring optimums over water requirements and the effects of dehydration during pregnancy on female survival and future reproduction (Bonnet *et al.* 2000; Bleu *et al.* 2011).

PARENT–OFFSPRING WATER CONFLICT AND REPRODUCTIVE TRADE-OFFS

Considering the predictions of the VCH (Crespi & Semeniuk 2004), a water conflict could be, at least in squamate reptiles, an important influence during the transition to viviparity. Constraints on gas and water requirements have previously been hypothesized as a key factor in limiting egg retention in oviparous species to typically no more than the first-third of embryonic development (Shine & Thompson 2006). Here, we have demonstrated a significant foeto-maternal conflict for water when water restriction was imposed in the middle third of embryonic development. Because embryonic demand for water increases dramatically during the last third of development, we posit that water availability and foeto-maternal conflict for water may constitute important selective limitations to viviparity.

Parent–offspring conflict theory has been empirically demonstrated for parental investment of energy and time (see Kölliker & Richner 2001 for a review). To our knowledge, no study has examined parental–offspring conflict for water. Water is a critical, often unpredictable resource, and water restriction can affect both survival and reproductive success (McKechnie & Wolf 2010). Water-based parent–offspring conflict may be taxonomically widespread. For example, water is a critical resource in mammals, since pregnancy alters water balance and decreases the osmotic thresholds for thirst and antidiuretic hormone release (Davison *et al.* 1984). Importantly, in humans, severe dehydration highly increases the risk of foetal death (Hirschhorn, Chowdhury & Lindenbaum 1969). In altricial species, water might represent a conflicting resource between parents and offspring. However, water conflicts are difficult to dissociate from energy conflicts, since water is typically provided concurrently with energy (e.g. in the form of milk for mammals) (Williams & Nagy 1985). Nonetheless, some species display specific adaptations that exclusively provide water to their offspring (Mougeot *et al.*

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2014), and these species could be excellent study organisms for evaluating post-natal water-based parent–offspring conflict. Interestingly, parent–offspring conflict also applies in plants (Uma Shaanker, Ganeshaiah & Bawa 1988), and water is likely a transgenerational conflicting resource especially during fruit formation. Therefore, while energy and time resources have attracted considerable interest over past decades, we emphasize that water demands may mediate a critical parent–offspring conflict influencing reproductive trade-offs and optimal reproductive effort and thus deserve greater attention.

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Data accessibility

Data from this paper are deposited in the Dryad Digital Repository, doi: 10.5061/dryad.mn40k. Dupoué *et al.* (2014b).

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ARTICLE 6

Water deprivation during pregnancy induces higher maternal corticosterone stress-response and influences offspring growth in the viviparous *Vipera aspis*

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Abstract

Because it provides clear indicator of individual fitness, maternal effects are essential to consider when investigating organism responses to environmental changes. Such effects are often mediated by maternal hormones, such as glucocorticoids, and especially corticosterone (CORT). Therefore numerous studies were interested in investigating the effects of different environmental stressors on maternal CORT secretion and the resulting consequences on offspring phenotype. Yet, despite it ecologically relevance, the impact of water restriction on maternal CORT secretion during reproduction and the resulting effects on offspring phenotypes have not yet been clearly established. In this study, we aimed at determining the effects of water deprivation on the CORT stress response on female aspic vipers (*Vipera aspis*) and on offspring growth. We used an experimental approach previously published in which we exposed both pregnant and non-reproductive female to a 20-day period of water deprivation. As a result of the 20-day period of water deprivation, only water-deprived pregnant females showed a significant increase in the CORT stress-response. Changes in baseline CORT level were related to female dehydration in pregnant but not in non-reproductive females. Accordingly with predictions of CORT-fitness hypothesis, the changes in baseline CORT were negatively influenced by maternal reproductive effort in control females. Finally, we found that offspring born of water-deprived females displayed higher early growth rates than offspring born of controls, and these contrasted growth rates were related to both maternal dehydration and changes in baseline CORT. Together, our results suggest that dehydration induced by water deprivation may have triggered a higher maternal CORT stress response (i.e., allostatic overload) that have impacted offspring developmental trajectory. Further long-term field studies are therefore required to assess the adaptive output of this functional response.

Keywords. Corticosterone; water-deprivation; maternal effects; offspring growth; stress-response

Introduction

Maternal effects correspond to a set of maternal (or paternal) influence early in life (i.e., from gamete to postnatal parental care) that interact with genome to determine offspring phenotype (Mousseau & Fox 1998a, 1998b; Agrawal 1999; Marshall & Uller 2007). Depending on environmental context (Sinervo & DeNardo 1996), such effects could be adaptive either deleterious to offspring fitness (reviewed in Groothuis et al. 2005; Marshall & Uller 2007; Groothuis & Schwabl 2008; Monaghan 2008). For instance, during embryonic development, maternal environment will profoundly impact offspring developmental trajectory (i.e., morphology, physiology, or behaviour), which have largely been documented to be widespread in all taxa (Bernardo 1996). Signals used by mother to interact with the offspring and their adaptive output provide therefore excellent indicators of functional responses to environmental stressors. For instance, hormones, mRNAs, or yolk amount are known to impact offspring development (Mousseau & Fox 1998b).

Hormones such as glucocorticoids (GCs) are likely one of the primary maternal signal because of their pleiotropic functions (e.g., metabolic rate: Preest & Cree 2008, growth: Hayward & Wingfield 2004, behaviour: Angelier et al. 2008, etc). In vertebrates, maternal GCs secretion has been viewed as a context-dependent adaptive response (Meylan & Clobert 2005; reviewed in Breuner et al. 2008). To date, a consequent amount of literature experimentally demonstrated that maternal increase of GCs, especially corticosterone (CORT, the main GC in birds, reptiles and rodents) significantly impacted offspring traits (Meylan et al. 2002; Belliure et al. 2004; Hayward & Wingfield 2004; Meylan & Clobert 2005; Zagron & Weinstock 2006; Warner et al. 2009). Importantly, effects of maternal CORT on offspring have generally been demonstrated to result in long-lasting and sometimes transgenerational effects (reviewed in Welberg & Seckl 2001; Naguib et al. 2006). Previous studies have yet well described the effects of different environmental stressors on maternal CORT secretion and the resulting consequences on offspring phenotype (e.g., food shortage: Kitaysky et al. 2010, heat stress: Eriksen et al. 2007, predation: Saino et al. 2005; Coslovsky & Richner 2011, anthropogenic disturbances: Ellenberg et al. 2007; Tartu et al. 2014). To the best of our knowledge, the effects of water deprivation on CORT secretion during reproduction have not yet been clearly established. Though, CORT is the main mediator of allostasis (i.e., physiological and behavioural responses to predictable and unpredictable changes) and presumed to be involved

in water balance regulation and osmoregulation (i.e., mineralocorticoid function, Duggan and Lofts 1978; Dauphin-Villemant and Xavier 1986; Bentley 2002; McCormick and Bradshaw 2006).

To investigate thoroughly the effects of water deprivation on CORT secretion, it is essential to consider the entire stress response; that is the baseline and the stress-induced CORT concentrations (Dupoué et al. 2014a). Indeed, the distinction is classically made between baseline and stress-induced CORT levels because of their contrasted physiological and behavioural functions (Landys et al. 2006; Romero 2004). At baseline level, CORT have mainly permissive actions that aim at maintaining energetic balances and deal with seasonal and daily routines (Sapolsky et al. 2000; Romero 2002; Landys et al. 2006). At stress-induced level, CORT has stimulatory and inhibitory actions that aim at promoting individual's short-term survival (i.e., a life-threatening event; Angelier et al. 2009; Wingfield et al. 1998, 2011) at the expend of other functions such as growth, reproduction or immune system (Sapolsky et al. 2000; Romero 2004). It is therefore essential to consider both baseline and stress-induced CORT levels to clarify the functionality of animal responses to water deprivation (Dupoué et al. 2014a).

Squamate reptiles (lizards and snakes) represent excellent study models to investigate the effect of water deprivation on CORT secretion during pregnancy. Most viviparous species are capital breeders (Bonnet et al. 1998), which mean that females accumulate energy for an extended period before engage in a reproductive effort (Bonnet et al. 2002; Lourdais et al. 2002a). As a result, the allocation of energy by the female to the offspring is completed prior to ovulation, and therefore, the embryos cannot influence this investment. Conversely, water is transferred to embryos all over pregnancy, and water uptake from embryos increases exponentially until parturition with somatic growth (Dauphin-Villemant & Xavier 1986, Andrews 2004). Interestingly this difference in the dynamic of resource allocation (energy *vs*. water) allows isolating resource effect on the CORT stress response while avoiding confounding effects. Moreover, squamates display relatively limited dispersal ability, which regularly exposes individuals to local conditions (Aragon et al. 2010), and make them liable to face difficulties to find drinking water during drought periods.

Here we provide additional results stem from a previous experiment on a viviparous snake, *Vipera aspis* (Dupoué et al. in press). In this previous study, we demonstrated that water deprivation induces an intergenerational trade-off for water that may result in a mother-offspring conflict in favour of embryos (see details in Dupoué et al. in press). We present here the second goal of this experiment, which was to determine the effects of water deprivation and reproductive status on the CORT levels (baseline and stress-induced). Specifically, we aimed at determining if water deprivation could trigger i) a higher CORT stress response, and ii) specific maternal effects. To do so, we compared the effects of a 20-days period of water deprivation on the CORT stress response of pregnant and non-reproductive female aspic vipers (*Vipera aspis*). Then, we determined if water deprivation could induce maternal effects, that is difference in offspring traits. We expect dehydration to induce an increased stress response, with a higher baseline CORT level because of the mineralocorticoid function of CORT (Duggan and Lofts 1978; Dauphin-Villemant and Xavier 1986; Bentley 2002; McCormick and Bradshaw 2006) but also a higher stress-induced CORT level as demonstrated in another snake species (Dupoué et al. 2014a). We further predict that the increased CORT stress response in water-deprived females to be more pronounced in pregnant compared to non-reproductive ones because of higher dehydration (Dupoué et al. in press). If so, we predict that fecundity drives changes in CORT stress response. Finally we expect water deprivation to induce different early growth patterns between offspring born from control and water-deprived females.

Material & Methods

Animals

We studied the aspic viper (*Vipera aspis*), a medium-size viviparous snake that is abundant in Western Europe (Naulleau, 1981). The aspic viper is a typical capital breeder, and females mobilize their energy reserves to support reproduction and yolk production during vitellogenesis. Pregnancy begins after ovulation in early June {Naulleau, 1981 213 /id}, which is associated with a pre-ovulatory ecdysis (Lorioux et al. 2013b). Parturition occurs two to three months later from late August to early September (Lourdais et al. 2002a). Pregnant females often cease to feed (Bonnet et al. 1998; Lourdais et al. 2002b) mainly because of an important behavioral shift that emphasizes thermoregulation over foraging (Lorioux et al. 2013a).

We used the same animals described in another experiment (see Dupoué et al. in press). That is, in May and June 2012, we caught 58 females (29 pregnant, 29 non-reproductive) from neighboring sites in western France (Vendée and Loire-Atlantique Districts). Reproductive status was determined by palpation in the field and then confirmed pregnant with high resolution ultrasonography (Sonosite microMaxx, Inc., Bothell, WA, USA) when the animals were brought to the laboratory. All snakes were measured (snout-vent length, SVL ± 0.5 cm) and weighed (body mass, BM ± 1 g). Husbandry followed the protocol described in Lorioux et al. (2013a). Briefly, individuals were housed 3-4 per cage in 16 cages (100 x 30 x 35 cm) because communal housing is known not to affect snake behavior (Lorioux et al 2013a). Room temperature was held constant at 20°C. To mimic natural conditions and allow for thermoregulation, heat was provided at one end of each cage with a 75W incandescent light bulb for 5 h per day (from 10:00 am to 3:00 pm), creating a thermal gradient that mimicked natural conditions (20 - 40°C). Water was available *ad libitum* in 4 water bowls per cage. Individuals were fasted two weeks prior to the start of the experiment and remained fasted throughout the experiment, thus preventing meal consumption from contributing dietary water to the snakes' water balance (Wright et al. 2013) and to avoid any influence of digestive activity on CORT levels.

Experimental design

As described in Dupoué et al. (in press) individuals from each reproductive status were randomly assigned to the control (pregnant: n = 15; non-reproductive: n = 15) or the water-deprived (pregnant: n = 14; non-reproductive: n = 14) hydric treatment. Within each reproductive status, water-deprived females and controls did not differ in BM or SVL (all $p > 0.117$). During the experiment, housing conditions remained as described above, except that water was removed for 20 days for water-deprived treatment since this period of deprivation represents the typical duration of a summer drought in western France. Water deprivation occurred when pregnant females were in mid-gestation (Dupoué et al. in press).

At the end of the water deprivation period, water intake were assessed by placing the females in individual boxes (30 x 20 x 10 cm) with a thermal gradient (similar to that described earlier), a water bowl, and a shelter. Pregnant females were then checked daily until parturition (Lorioux

et al. 2013b), which occurred 99.7 ± 2.1 days after ovulation (control: 99.9 ± 3.6 days, water-deprived: 99.5 ± 2.6 days; Dupoué et al. in press). On the 29 pregnant females, 26 females (13 controls, 13 water-deprived) successfully achieved their pregnancy. We obtained 125 neonates (60 neonates born from control females, 65 neonates born from water-deprived females). Immediately after birth, each neonate was individually housed in a box (30 x 16 x 10cm) with a natural thermal gradient (20 to 33°C) 6 h per day (from 9:00 am to 3:00 pm) and water *ad libitum* in a water bowl. We collected measures on females and neonates the days of birth and two weeks after (see below). Once all measurements were collected, females were fed with a mouse and released with their litters at their original capture site.

Blood sampling

Blood samples were collected at the beginning and the end of the water deprivation period (i.e., sampling session). Vipers were randomly bled in order to mix the hydric treatment and the reproductive statuses. Because of communal housing and potential disturbance initiate by taking an individual for others of a same cage, we took care not to sample more than one female in each cage in the morning or in the afternoon. All blood samples were collected via cardiocentesis, using a 1 ml heparinized syringe and a 27-gauge needle. The first blood samples (i.e., baseline CORT level, 150 µL) were collected within 3 min (mean ± SE: 2.75 ± 0.11 min), since it represents the typical time before measurable changes in CORT concentration in squamates (Romero & Wikelski 2001). We also used this sample to determine plasma osmolality (see details in methods from Dupoué et al. in press). Snakes were then placed in an empty transparent box (30 x 20 x 10 cm) since this protocol has previously been showed to initiate a CORT stress response in snake (Dupoué et al. 2014a). After one hour in this stressful condition, we collected a second blood sample (i.e., stress-induced CORT level, 100 µL). Blood was collected from each animal and placed into a 0.675 ml eppendorf and centrifuged 3 min at 3000 RPM. The plasma was separated and stored at -28°C. Plasma CORT concentration ($ng.ml^{-1}$) was determined at the CEBC following a well-established radioimmunassay protocol (see Lormée et al. 2003 for details). Samples were run in two assays (intra-assay variation: 7.07%, inter-assay variation: 9.99%).

In one female (pregnant from the water-deprived treatment), blood collection failed during the final session for both sampling (baseline and stress-induced) and this female was therefore removed from corresponding analyses on CORT. For another female (pregnant from the control treatment), stress-induced blood collection failed during the final session and this female was therefore removed from analyses on stress-induced CORT. Preliminary examinations of the data revealed that another female (control and non-reproductive) displayed extremely high baseline CORT level during the final session (201.06 $ng.ml^{-1}$). Importantly this concentration was the highest of the dataset and was 80% higher than corresponding stress-induced CORT level. Therefore, we also removed this individual from the analyses. In this study, we did not find any significant influence of time of day or body temperature on CORT levels (all $p > 0.115$) and we did not include these covariates in the analyses of CORT levels.

Maternal reproductive effort

Multiple methods have been described to assess reproductive effort in snakes (see Bonnet et al. 2003). Absolute measures of reproductive output such as litter size (i.e., number of all litter components including developing embryos, stillborn and undeveloped follicles), fit litter size (i.e., number of developing embryos), litter mass (i.e., mass all litter components), and fit litter mass (i.e., mass of developing embryos) are routinely used (Lourdais et al. 2002a). However it is also crucial to control for allometric influences (Bonnet et al. 2003; Dupoué & Lourdais 2014). Therefore, we considered the relative litter size (RLS) and relative litter mass (RLM), derived from the residuals of the linear relationship respectively between fit litter size or fit litter mass and maternal SVL (Dupoué & Lourdais 2014) since these relations were significant (RLS: $F_{1,26} = 17.6$, $p < 0.001$, RLM: $F_{1,23} = 24.9$, $p < 0.001$).

Offspring growth

The day of birth, we measured offspring snout-vent length (SVL ± 0.1cm), and jaw length (JL ± 0.01mm) respectively with a tapeline and a digital caliper (Digimatic caliper 150mm, Mitutoyo, Japan). We did not observe any difference in offspring SVL or JL at birth (all $p > 0.574$; Dupoué et al. in press). We repeated these measures two weeks after birth (mean ± SE: 13.9 ± 0.3 days) in order to determine offspring growth. We calculated body and jaw growth ($mm.day^{-1}$) respectively as the difference between final SVL or JL and SVL or JL at birth,

divided by the number of days between initial and final measures. One offspring born from a control female displayed several morphological malformations and died 9 days after birth. This individual was removed from the analyses focusing on offspring growth.

Statistical analyses

All analyses were performed using R software (R Development Core Team, 2011). We checked that residuals of our models respected normality (Shapiro-Wilk test).

First, we analyzed the effect of the water deprivation on baseline CORT levels with linear mixed models with baseline CORT levels as the dependent variable, sampling session (initial *vs*. final), hydric treatment (control *vs*. water-deprived), reproductive status (pregnant *vs*. non-reproductive), double and triple interactions between these terms as explanatory factors. Female identity was set as a random factor since measures were repeated between sampling sessions. We compared inter- and intra-group differences in baseline CORT level using *post hoc* tests of Tukey on the triple interaction (package lsmeans). Second, stress-induced CORT was analysed as the same design as above. We used two sets of models: the first in which we did not adjust stress-induced CORT with baseline CORT level. The second in which we added the baseline CORT level as linear covariate.

Third, we used linear models to test the relationship between changes in baseline or absolute stress-induced CORT level and changes in osmolality within pregnant and non-reproductive females. Hydric treatment significantly impacted plasma osmolality (see Dupoué et al. in press), and we avoided co-linearity issue by not including treatment in model.

Fourth, we used linear models to test the influence of the different estimates of maternal reproductive effort (litter size, fit litter size, litter mass, fit litter mass, RLS, RLM) on changes in baseline or absolute stress-induced CORT levels within hydric treatment. Hydric treatment was treated as an explanatory factor and each estimate of maternal reproductive effort as linear covariates.

Fifth, we used linear mixed models to compare the effect of female hydric treatment on offspring growth, with body or jaw growth as dependent variable and mother treatment as explanatory factor. Female identity was set as random factor because siblings are not statistically independent (Massot et al. 1994).

Finally, we used linear mixed model to test the relationship between offspring body growth or jaw growth and changes in maternal osmolality or changes in baseline or absolute stress-induced CORT level. Hydric treatment significantly impacted offspring growth (see results), and we avoided co-linearity issue by not including treatment in model.

Results

Baseline CORT levels were significantly influenced by the sampling session ($F_{1,52} = 7.0$, $p = 0.011$) and the reproductive status ($F_{1,52} = 19.0$, $p < 0.001$) but not by the hydric treatment ($F_{1,52} = 0.01$, $p = 0.905$). We did not find significant influence of the three double interactions (all $p > 0.153$) but a significant influence of the triple interaction ($F_{1,52} = 4.5$, $p = 0.039$). At the onset of water deprivation, inter-group analyses showed similar baseline CORT levels between controls and water-deprived individuals within pregnant (*post hoc*, control *vs*. water-deprived, $z = 0.9$, $p = 0.985$, Fig. 1a) and non-reproductive females (*post hoc*, control *vs*. water-deprived, $z = -0.3$, $p > 0.999$, Fig. 1b). At the end of water deprivation, we did not find any difference in baseline CORT level between controls and water-deprived females for both reproductive statuses (all $p > 0.834$, Fig. 1). Intra-group analyses showed a significant increase in baseline CORT levels through the treatment period in pregnant females from the water-deprived treatment (*post hoc*, initial *vs*. final, $z = -3.8$, $p = 0.004$, Fig. 1a). Baseline CORT levels did not significantly change through the study period in all the other groups (all $p > 0.979$, Fig. 1).

Stress-induced CORT levels were significantly influenced by the sampling session ($F_{1,51} = 4.7$, $p = 0.036$) and slightly by the reproductive status ($F_{1,51} = 3.5$, $p = 0.066$) but they were not influenced by the hydric treatment ($F_{1,51} = 0.2$, $p = 0.624$). We found that the interaction between sampling session and treatment significantly influenced stress-induced CORT level ($F_{1,51} = 7.8$, $p = 0.007$). The two other interactions did not have significant effect of stress-induced CORT

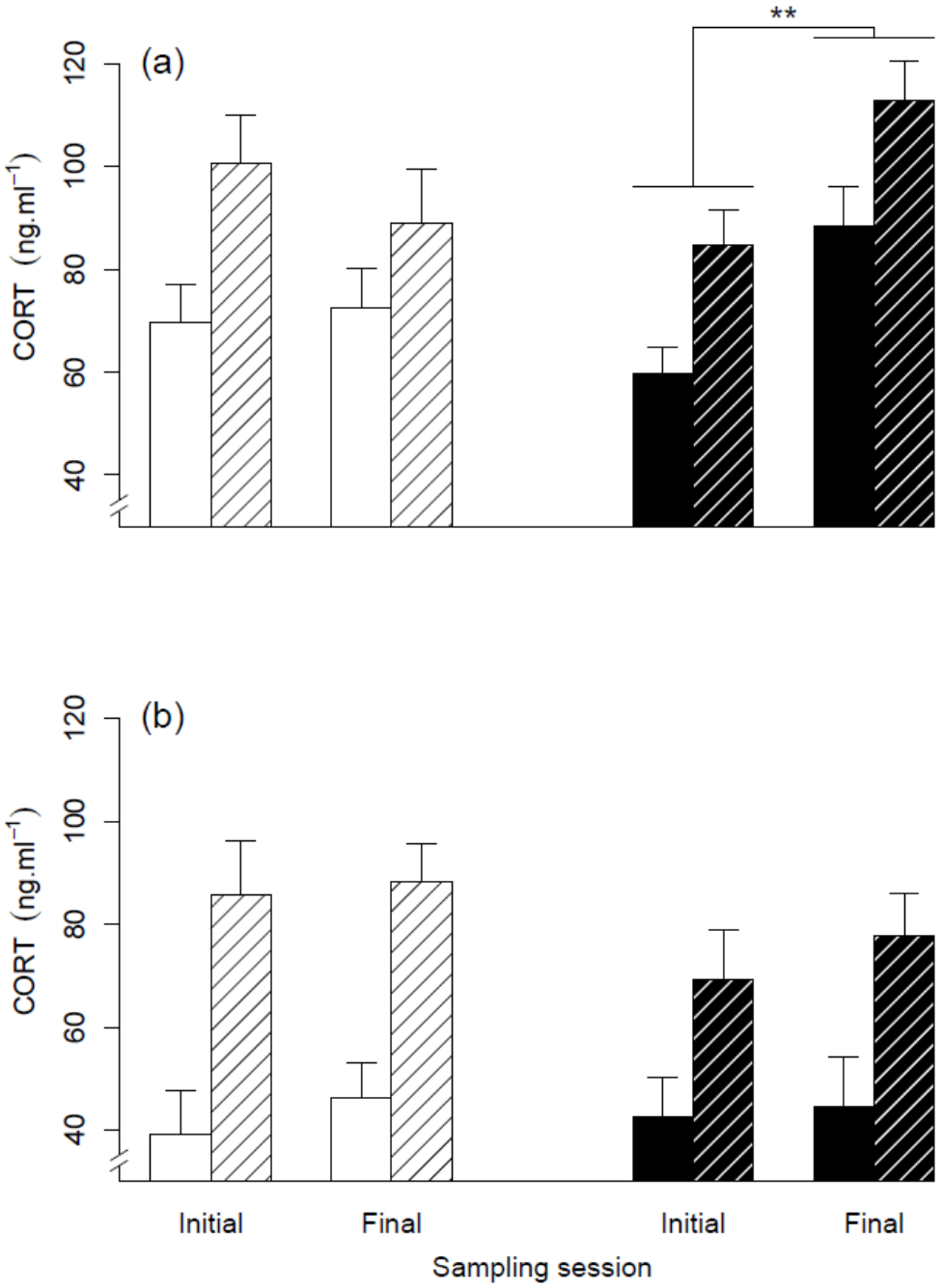

Figure 1. Effect of water deprivation and reproductive status on female aspic viper stress-response. Bars represent means (± SE) of baseline CORT (full bars) and stress-induced CORT level (hatched bars) in a) pregnant and b) non-reproductive females from the control (white bars) and the water-deprived treatment (black bars). Significant difference of stress response between initial and final sampling session is symbolised: ** $p < 0.01$.

level (all $p > 0.225$). The triple interaction significantly impacted stress-induced CORT level

($F_{1,51}$ = 4.2, p = 0.046). Inter- and intra-group analyses on stress-induced CORT level revealed the same statistical differences than the analyses on baseline CORT level. That is, no difference at the onset and at the end of water deprivation between control and water-deprived females from both reproductive statuses (all p > 0.556) and a significant increase of stress-induced

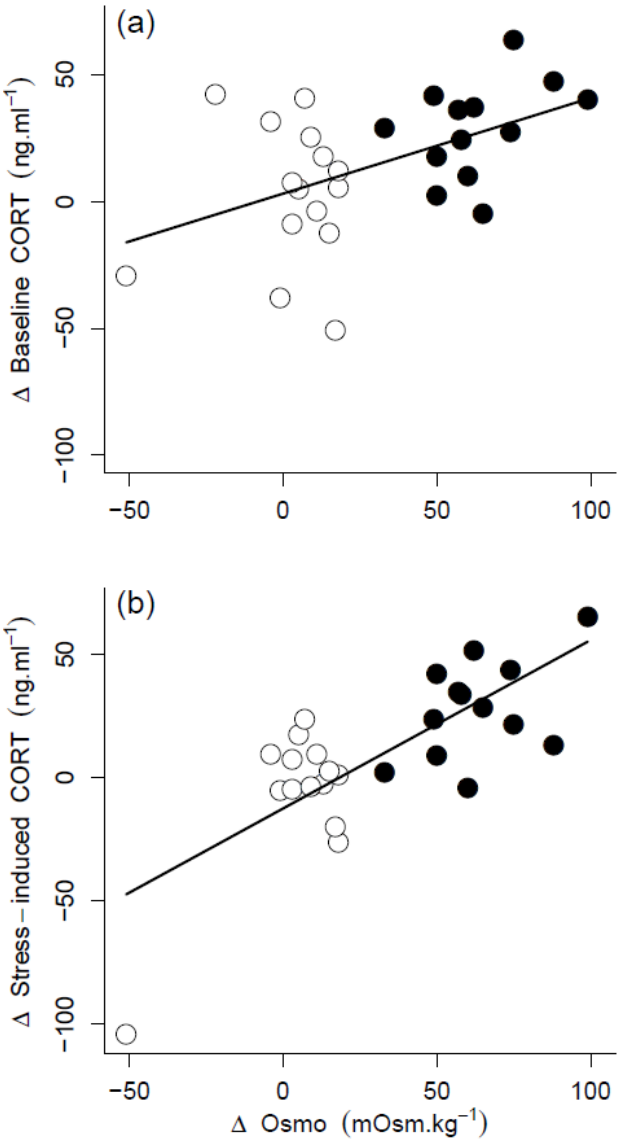

Figure 2. Relationship between changes in a) baseline and b) stress-induced CORT levels and changes in osmolality during experiment in pregnant females from the control (open circles) or the water-deprived (filled circles) treatments. Trend lines are included for significant relationship: changes in osmolality are positively related to changes in baseline CORT (r^2 = 0.25) and to changes in stress-induced CORT (r^2 = 0.56).

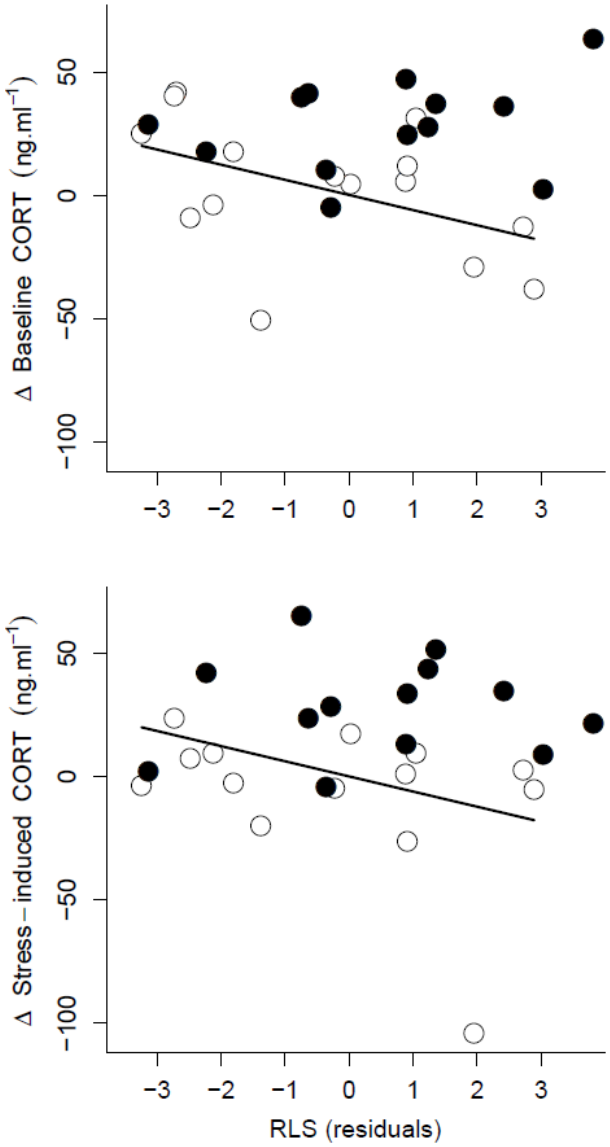

Figure 3. Relationship between changes in a) baseline or b) stress-induced CORT levels and maternal relative litter size (RLS: residuals from the linear model of fit litter size against mother SVL) in pregnant females from the control (open circles) or the water-deprived (filled circles) treatments. Trend line is included for significant relationship since RLS negatively influences changes in baseline CORT levels in control females (r^2 = 0.22) but not in water-deprived females (r^2 = 0.07). The influence of RLS on the changes in stress-induced CORT level is non-significant either for control (r^2 = 0.14) or water-deprived females (r^2 < 0.00).

CORT level only in pregnant females from the water-deprived treatment (*post hoc*, initial *vs*. final, z = -3.8, p = 0.003, Fig. 1a). When adjusted with baseline CORT level ($F_{1,50}$ = 19.5, p <

0.001), stress-induced CORT level was no more significantly influenced by any factors (all p > 0.205), excepted the interaction between sampling session and treatment ($F_{1,50}$ = 4.7, p = 0.036). Similarly, inter- and intra-group analyses were all non-significant (all p > 0.232).

We found a positive and significant relationship between changes in baseline CORT levels and changes in osmolality for pregnant females ($F_{1,26}$ = 8.4, p = 0.007, Fig. 2a) but not for non-reproductive females ($F_{1,26}$ = 0.05, p = 0.819). We found similar relationships between changes in stress-induced CORT levels and changes in osmolality for pregnant females ($F_{1,25}$ = 32.2, p < 0.001, Fig. 2b) but not for non-reproductive ($F_{1,26}$ = 0.31, p = 0.582).

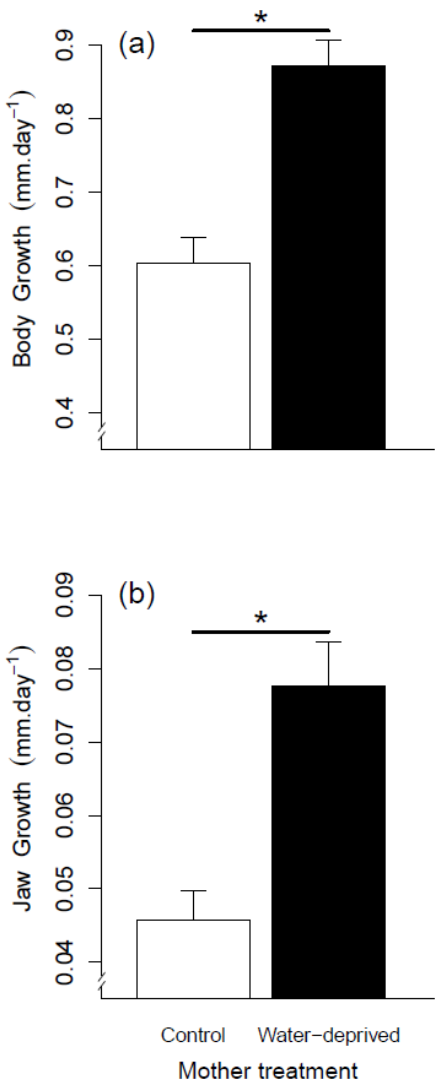

Figure 4. Effect of mother hydric treatment on offspring growth within two weeks after birth. Bars represent means (± SE) of a) body growth and b) jaw growth of offspring born of mothers from control (white bars) and water-deprived treatment (black bars). Significant effect of hydric treatment is symbolised: * p < 0.05.

In pregnant control females, we found a negative relationship between changes in baseline CORT and fit litter size (t = -2.2, p = 0.036) or RLS (t = -2.1, p = 0.045, Fig. 3a). All others estimates of maternal reproductive effort (litter size, litter mass, fit litter mass, RLM) did not significantly relate with changes in baseline CORT (all p > 0.225). In females from the water-deprived treatment, we did not find any significant relationship between changes in baseline CORT and estimates of maternal reproductive effort (all p > 0.366). We found no significant relationship between changes in stress-induced CORT and any estimates of maternal reproductive effort either for control (all p > 0.126) or water-deprived females (all p > 0.206) (Fig. 3b).

We found a significant effect of water deprivation during pregnancy on both offspring body growth ($F_{1,23} = 7.1$, $p = 0.014$, Fig. 4a) and jaw growth ($F_{1,23} = 7.8$, $p = 0.010$, Fig. 4b). Compared to offspring born from control females, offspring born from water-deprived females showed higher growth of body (Fig. 4a) and of jaws (Fig. 4b).

We found a significant and positive influence of changes in maternal osmolality through the hydric treatment on offspring body growth ($F_{1,22} = 6.7$, $p = 0.017$, Fig. 5), and jaw growth ($F_{1,22}$

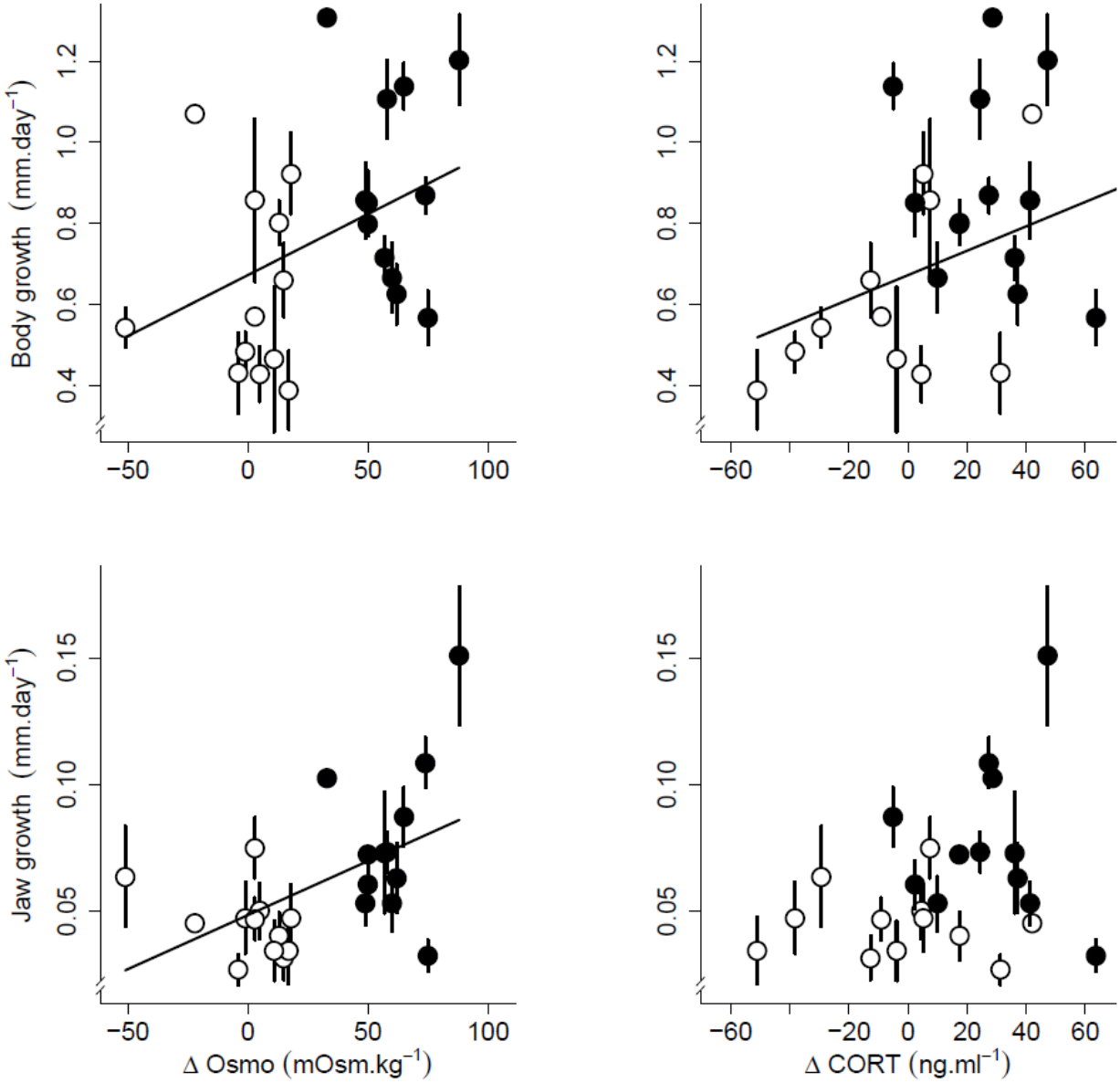

Figure 5. Relationship between offspring body growth (mean per litter ± SE, top panels) or jaw growth (mean per litter ± SE, bottom panels) and changes in mother osmolality (Δ Osmo, left panels) or changes baseline CORT level (Δ CORT, right panels) over exposition to hydric treatment. Trends lines are included for significant relationship. Δ Osmo positively influences offspring body growth ($r^2 = 0.16$) and jaw growth ($r^2 = 0.26$). Δ CORT positively influences offspring body growth ($r^2 = 0.19$) but not offspring jaw growth ($r^2 = 0.09$).

= 8.1, p = 0.009, Fig. 5). We found a similar influence of changes in baseline CORT levels on offspring body growth ($F_{1,22}$ = 4.4, p = 0.047, Fig. 5) but not on offspring jaw growth ($F_{1,22}$ = 2.1, p = 0.165, Fig. 5). We did not find any effect of changes in stress-induced CORT levels on offspring growth (all p > 0.184).

Discussion

Water is a vital resource, often readily available, although overlooked in ecological studies (Bonnet & Brischoux 2008). In this study, we investigated the effects of water deprivation on the CORT stress response and on maternal effects in a viviparous snake. In response to the 20-day period of water deprivation, females from both reproductive statuses were significantly dehydrated (see mass loss and increased of plasma osmolality in Dupoué et al. in press). Herein, we found that water deprivation induced a higher CORT stress response only in pregnant females and this higher stress response was positively related with female water balance. In non-reproductive females, we did not find any effect of water deprivation on CORT levels. In pregnant control females, changes in baseline CORT were negatively influenced by two estimates of maternal reproductive effort (fit litter size and RLS) while this relationship was missing in water-deprived females. Finally, we found that the water deprivation induced higher offspring growth, which was positively related with maternal dehydration and changes in baseline CORT.

Water deprivation induces differential maternal CORT stress response

As females become dehydrated as a result of water deprivation (Dupoué et al. in press), pregnant ones experience a significant elevation in the CORT stress response, however this response is not observed in non-reproductive females. Such contrasting effects of water restriction and reproductive status on CORT levels have been previously reported in the common lizard (Dauphin-Villemant & Xavier 1986). Importantly, this pattern likely reveal a difference related to specific physiological needs of reproductive statuses. Squamate reptiles are particularly tolerant to deviation of water balance and therefore in non-reproductive females, a 20-days period of water deprivation may have not triggered a physiologically stressful situation. Conversely in pregnant females are exposed to gestational water constraints and demands (increased water loss; embryonic requirements), and as a result the water deprivation period did

dehydrated them twice as much than non-reproductive ones (Dupoué et al. in press). Although the lack of effect of water deprivation on the CORT stress response was unexpected in non-reproductive females, baseline CORT has been suggested not to be involved in regulation of water balance *per se* (Dupoué et al. 2014a). Instead, mineralocorticoid function of CORT might correspond in salt balance regulation as demonstrated in other species (Holmes and McBean 1964; Duggan and Lofts 1978; Bradshaw and Rice 1981; Bradshaw et al. 1984). Ultimately, these results suggest that non-reproductive females are more suitable than pregnant females to endure a drought on the field. Likely, a more extended period of water deprivation would also have induced physiological stress in non-reproductive females. Since the effects of water deprivation on snake survival or reproduction are relatively unknown, we did not impose water deprivation further than 20 days for ethic matters.

Moreover, we found that changes in baseline CORT levels are positively related to changes in osmolality in pregnant females but not in non-reproductive females. We therefore posit two hypotheses (non-exclusive) to interpret the increase of CORT stress response in water-deprived pregnant females. CORT might act as a signal to allocate maternal body water to the developing embryos. At baseline level, CORT is involved in energy allocation (Angelier et al. 2009), and might have the same effect for water allocation. Alternatively, the increase in baseline CORT could reflect the acute physiological stress associated with reproduction in this species (i.e., allostatic overload). Herein, we did not find any significant influence of fecundity on changes in baseline CORT in water-deprived pregnant females and therefore, further experiments manipulating CORT levels are necessary to clarify the role of CORT in water allocation.

In pregnant females, changes in maternal baseline CORT negatively correlate with two estimates of maternal reproductive effort (i.e., fit litter size and RLS) in control females, and this relation was non-significant in water-deprived females. In control females, we expected a positive relationship since higher fecundity is generally associated with increase in maternal reproductive effort in term of energy or water investment, which could increase CORT levels (Bonier et al. 2011). However negative relationships between baseline CORT level and reproductive performance are also currently found (see Bonier et al. 2009a for a review; Angelier & Wingfield 2010). The negative relationship between reproductive effort and changes in baseline CORT level may therefore follow predictions of the CORT-fitness

hypothesis, that is the more baseline CORT level is high, the more reproductive success is low (Bonier et al. 2009a, 2009b). Alternatively, this negative relationship might represent a passive and indirect effect of water intake during pregnancy on CORT levels. Fecundity is indeed known to trigger female drinking behaviour in aspic vipers (Lourdais, unpublished data), and as shown previously (Dupoué et al. in press) control females from our experiment gained BM related to their fecundity while remaining normosmotic. The decrease in baseline CORT level related to reproductive effort may simply correspond to a dilution of plasma CORT caused by an increase of maternal body water.

In pregnant water-deprived females, stress-induced CORT levels also significantly increase after water deprivation while we observe no significant variations in the other experimental groups. This increase in pregnant water-deprived females was due to an already higher baseline CORT level since most of variation (66%) in stress-induced CORT level is explained by variations in baseline CORT levels. Indeed, including baseline CORT levels as a covariate in the models neutralise the significant increase in stress-induced CORT through water deprivation. While absolute stress-induced are important for their physiological effects (Romero 2004), our results suggest that female response to a stressor is not affected by modifications of their water balance. This result is not consistent with a previous experiment on another snake species in which we found that dehydrated male and female pythons display higher stress-induced CORT level when dehydrated (Dupoué et al. 2014a). Although we induced a large perturbation of the water balance in this study (see mass loss and osmolality increase in Dupoué et al. in press) female vipers did not seem to have perceived this intense dehydration as a stressful situation. However, elevated concentrations of absolute stress-induced CORT level in pregnant water-deprived female may negatively influences individual fitness (McEwen & Wingfield 2003; Breuner et al. 2008). Although we did not find any effect of water deprivation on female reproductive success (Dupoué et al. in press), further investigations are required to test the long-term effects of higher absolute stress-induced CORT level on female survival and reproductive performance.

Water deprivation during pregnancy induces higher postnatal offspring growth

During the first two weeks following birth, offspring born from water-deprived mothers exhibit faster growth (i.e., body and jaw growth) than offspring born from control mothers. As expected, this differential growth in offspring likely reflects differential maternal effects induced by water deprivation. Interestingly these contrasted growth patterns are related to variations of osmolality suggesting that the more the female were dehydrated during pregnancy, the more their offspring rapidly grew up. Similarly we find a significant influence of changes in maternal baseline CORT on offspring body growth. Together, our results suggest that the dehydration induced by water deprivation (Dupoué et al. in press) may have triggered a higher maternal CORT stress response (i.e., allostatic overload) that have impacted offspring developmental trajectory. In support of this, previous experimental studies already showed significant effects of maternal CORT increase on offspring growth in different vertebrate lineages (Lesage et al. 2001; Hayward & Wingfield 2004; Meylan & Clobert 2005; Chin et al. 2009; Coslovsky & Richner 2011). Furthermore, previous long-term field studies have shown positive relationship between habitat humidity (air humidity and freestanding water availability) and juvenile growth (Lorenzon et al. 1999, 2001).

Since we measured juvenile growth within two weeks after birth, our results unable we to test whether higher growth is an adaptive response to perturbation of mother water balance. In order to clearly establish the adaptive significance of the female response and offspring growth to water restricted environments, it is essential also to consider other indicators of juvenile fitness (e.g., survival, age and size at maturity; Stearns 1992; Roff 1992). Accelerated juvenile growth could promote different selective advantages (anti-predator, competitive and/or dispersal; reviewed in Arendt 1997), but it could also induce immediate or delayed costs that affect their long-term survival and/or reproductive success (Gotthard 2000; Metcalfe & Monaghan 2001). Therefore, fast-growth juveniles of aspic viper might be more advantaged than slow-growth phenotypes to evade more efficiently from a water constraining environment (Lorenzon et al. 2001). However, we were unable to measure long-term effects of mother treatment on offspring life history traits for logistical and ethical reasons. In a previous long-term field study, Marquis et al. (2008) demonstrated that level of precipitations (i.e., water availability) positively influences reproductive performance of female common lizard, *Zootoca vivipara* (i.e., litter size, offspring size and survival). Further similar and complementary studies are now required to model the effects of decreasing precipitations intensity and frequency on animal growth and survival.

Our study demonstrated that for a reproductive episode, the maternal stress response to dehydration may adjust offspring phenotype. Indeed, accordingly with previous work, we highly suspect maternal CORT to determinate offspring growth in response to water deprivation. Because of the complexity of phenotypic plasticity, it would be difficult to generalize these results to other vertebrate species. This is especially true since animal responses to environmental stressors are often species-specific (Warner et al. 2009) and context dependent (Sinervo & DeNardo 1996). However and importantly, water resource remains relatively overlooked in ecological studies (Bonnet & Brischoux 2008), and future work could specifically address this issue. It is crucial to investigate the effects of water restriction on animal responses to be able to predict the effects of increasing droughts frequency and intensity on population processes. According to our results and previous work, we predict that such changes would have deleterious effects on snake populations, either directly (e.g., diminishing performance because of dehydration: Wilson & Havel 1989; Lorenzon et al. 1999, higher chronic CORT release: allostatic overload, McEwen & Wingfield 2003) or indirectly (e.g., diminishing food resource availability: Madsen & Shine 2000).

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Discussion

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L'eau est une ressource capitale pour la survie et la reproduction des plantes et des animaux (Nelson & Desjardin 1987 ; Dudley 1996 ; Cheptou et al. 2000 ; Lorenzon et al. 1999, 2001 ; Taylor et al. 2006 ; Barnabas et al. 2008 ; Marquis et al. 2008 ; McDowell et al. 2008 ; McKechnie & Wolf 2010). Pourtant, l'eau comme ressource d'intérêt dans les études en écologie a reçu une attention relativement limitée en comparaison d'autres disciplines de la biologie (Bonnet & Brischoux 2008). Les études réalisées sur les adaptations physiologiques et comportementales des animaux vis-à-vis de la ressource en eau ont principalement ciblées des espèces qui évoluent dans des environnements desséchants, à savoir en milieu désertique/xérique, océanique ou polaire (Kennett & Christian 1994 ; Bradshaw 1997 ; DeNardo et al. 2004 ; Calsbeek et al. 2006 ; Davis & DeNardo 2007, 2009 ; Muir et al. 2007 ; Elnitsky et al. 2008 ; Lillywhite et al. 2008a, 2008b, 2012, 2014 ; Hetem et al. 2010 ; Teets et al. 2012). A l'inverse, la relation à l'eau chez des espèces de milieux tempérés a été relativement moins étudiée (mais voir Lorenzon et al. 1999). Or, ces espèces peuvent également subir des périodes où l'accès à l'eau est restreint pendant plusieurs jours/semaines consécutifs (sécheresse estivale, canicule, etc). L'étude des réponses fonctionnelles des espèces de milieux tempérés face à la disponibilité en eau est donc requise. C'est d'autant plus vrai quand on sait que les modèles climatiques prédisent notamment la diminution en durée et en fréquence des précipitations en plus de l'augmentation d'épisodes climatiques extrêmes (canicule, vague de chaleur, etc) (Easterling et al. 2000 ; IPCC 2014).

Dans ce doctorat, nous nous sommes intéressés à clarifier l'importance de l'eau dans les compromis physiologiques chez une espèce de Squamate caractéristique des milieux bordiers, la vipère aspic (*Vipera aspis*). Pour ce faire nous avons premièrement quantifié les effets d'états physiologiques demandeurs en énergie sur le compromis physiologique entre besoins thermiques et pertes hydriques ainsi que la réponse comportementale associée (i.e., sélection de microclimat) (**Article 1**). Nous nous sommes ensuite intéressés à un état physiologique particulièrement contraignant, la gestation. Nous avons tout d'abord clarifié des contraintes énergétiques et hydriques associées à cette période (**Articles 2**, **3**, **4**, et **résultats préliminaires**). Nous avons ensuite étudié l'impact d'une période de privation d'eau sur les compromis intergénérationnels (entre la mère et les embryons) (**Article 5**), et sur la sécrétion de corticostérone, médiatrice de la charge, ou de la surcharge allostatique (**Article 6**). Dans l'ensemble, nos résultats montrent bien que la disponibilité en eau est un paramètre majeur, vis-à-vis duquel les serpents ajustent leur physiologie et leur comportement. Cela suggère donc

qu'au même titre que l'énergie ou la température, il est également essentiel de considérer l'eau comme ressource d'intérêt dans les compromis d'allocation.

1. Le compromis entre thermorégulation et pertes hydriques

1.1. Des contraintes physiologiques spécifiques

Les résultats présentés dans l'**Article 1** ont permis de quantifier l'impact de l'état physiologique sur l'intensité du compromis physiologique entre les besoins thermiques et les pertes hydriques. Nous avons considéré 3 états physiologiques (la mue, la digestion et la gestation) déjà connus chez les reptiles pour augmenter les besoins thermiques et la dépense énergétique (Naulleau 1983 ; Stevenson et al. 1985 ; Gibson et al. 1989 ; Charland & Gregory 1990 ; Lourdais et al. 2002a, 2002b ; Wang et al. 2003 ; Lourdais et al. 2004), que nous avons comparé à un état

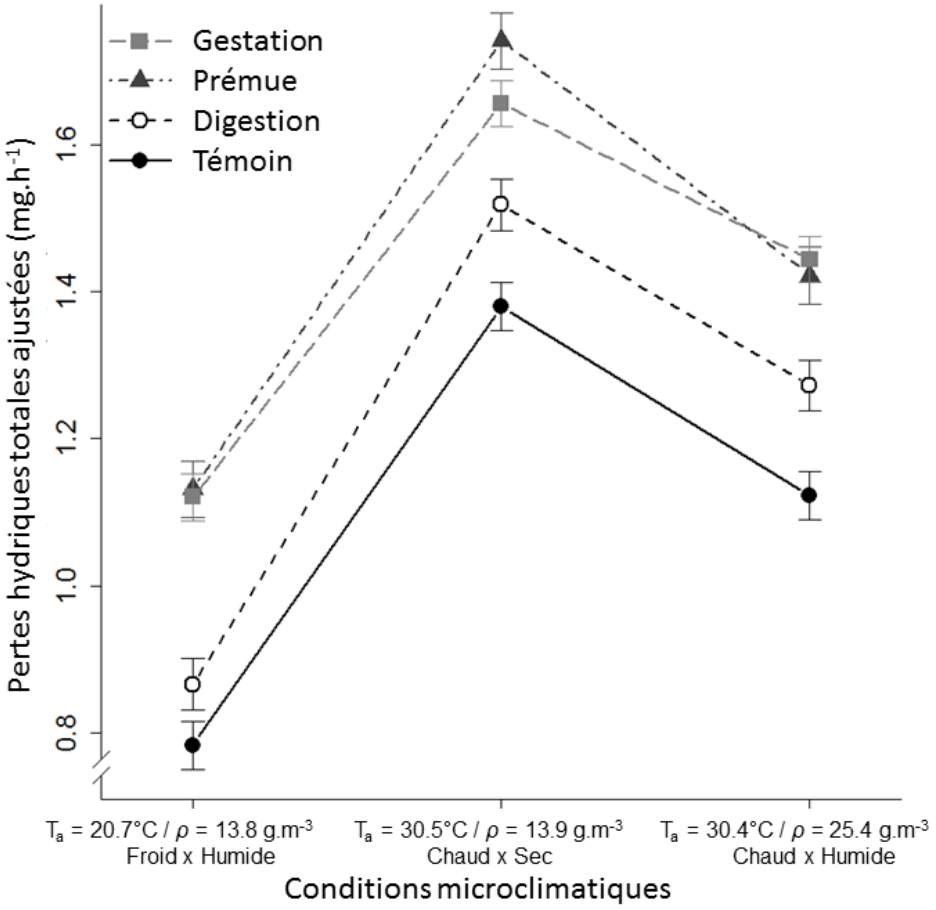

Figure 26. Pertes hydriques totales ajustées par la SVL et les résidus de la relation Surface~Volume, chez des vipères dans différents états physiologiques à différentes conditions microclimatiques : combinaison de 2 températures ambiantes (T_a) et 2 humidités absolues (ρ).

physiologique témoin (post-mue, postprandial et non-reproducteur). En accord avec nos prédictions, nous avons pu montrer que ces états physiologiques augmentent significativement les pertes hydriques totales comparées aux individus témoins (Fig. 26).

De façon proximale, les résultats comparatifs (effets de l'état physiologique) et corrélatifs (relations entre pertes totales en eau et VO_2) nous ont permis d'émettre des hypothèses quant aux bases fonctionnelles des pertes totales en eau. En particulier sur l'importance relative de la composante respiratoire *versus* cutanée dans les pertes hydriques totales. En effet, nos résultats ont montré une augmentation de la VO_2 très importante chez les serpents en digestion en comparaison avec les autres états physiologiques (mue, gestation et témoins), en lien avec l'hyper-métabolisme de la digestion (ou Action Dynamique Spécifique ; voir Secor 2009 pour une review). Malgré cette augmentation importante de la ventilation, les vipères en digestion ne montrent qu'une faible mais significative augmentation des pertes hydriques comparé aux individus témoins (Fig. 26). En parallèle, nous trouvons une relation significative et positive entre les pertes hydriques et la VO_2 pour les individus en digestion ou témoins, mais pas pour les individus en prémue ou gestants. Conjointement aux suppositions d'études précédentes (Mautz 1982 ; Dmi'el 1972 ; Cohen 1975 ; Kattan & Lillywhite 1989 ; Lillywhite 2006), nous avons donc émis l'hypothèse que la part relative des pertes hydriques via la respiration est bien inférieure en comparaison à la transpiration cutanée. En support de cette hypothèse, pendant le cycle de mue ou la gestation, les serpents présentent des modifications cutanées importantes. Pendant la phase de prémue, la transpiration cutanée augmente à cause d'une part de l'augmentation de perméabilité de la peau (diminution d'épaisseur de l'épiderme et diminution des lipides de surface) (Cohen 1975 ; Zucker & Maderson 1980 ; Tu et al. 2002 ; Lillywhite 2006), et d'autre part parce que l'espace entre la nouvelle et l'ancienne peau est remplie d'un liquide lymphatique (Mader 2005). Pendant la gestation, la peau des femelles se distant dû à l'encombrement de la portée (Shine 1988). Cette distension a vraisemblablement comme conséquence de diminuer l'épaisseur cutanée, d'accentuer le ratio surface/volume et donc d'augmenter la transpiration cutanée. Nous avons pu montrer dans les **résultats préliminaires sur les contraintes morphologiques** une distension significative causée par la portée et dans l'**Article 4** nous avons trouvé que cette distension influence positivement les pertes hydriques. Cependant, les modifications structurales associées aux différents états physiologiques et leurs effets sur les pertes hydriques sont relativement méconnus et mériteraient plus d'attention pour confirmer notre hypothèse.

Les **Article 1** et **4** ont également permis de mettre en évidence l'effet des conditions microclimatiques sur les pertes hydriques et sur la VO_2. Dans l'Article 1, nous avons tout d'abord trouvé un effet fort de la température ambiante à la fois sur les pertes hydriques (Q_{10} = 3.98) et sur la VO_2 (Q_{10} = 2.72). Cet écart dans les valeurs de Q_{10} suggère un effet plus important de la température sur les pertes hydriques que sur la VO_2, ce qui supporte à nouveau notre hypothèse d'un effet mineur de la ventilation sur les pertes hydriques. Ensuite, nos résultats permettent de mettre en évidence qu'à température ambiante égale, l'humidité de l'air impacte également les pertes hydriques mais pas la VO_2. En moyenne, pour une augmentation de la densité de vapeur d'eau de 11 $gH_2O.m^{-3}$, on trouve une diminution des pertes en eau de 86%. Partant de ce constat sur l'effet indirect des conditions microclimatiques, nous avons donc également voulu tester l'effet de l'état physiologique sur la sélection de microclimat.

1.2. Réponses comportementales

La sélection de microclimat comme un potentiel atténuateur de contraintes physiologiques a été montrée chez plusieurs espèces (Lorenzo & Lazzari 1999; Patten et al. 2005; Boyles et al. 2007; Köhler et al. 2011; Stahlschmidt et al. 2011). Notre prédiction était qu'un état physiologique hydriquement contraignant (mue, gestation ou digestion) oriente préférentiellement les individus vers un microclimat à la fois chaud et humide pour assurer le processus physiologique tout en limitant les pertes hydriques. Comme attendu, nous avons pu montrer que l'ensemble des états physiologiques contraignant induisent un choix préférentiel (bien que marginal) pour le microclimat chaud et humide. Cependant, nos résultats expérimentaux (**Article 1**) et sur le terrain (**Article 4**) semblent montrer que seules les femelles gestantes tendent à sélectionner une humidité absolue plus élevée, alors que les individus en prémue ou en digestion sélectionnent des humidités absolues intermédiaires. Cette différence de choix entre les états physiologiques est peut-être due à la spécificité des contraintes associées à chaque état. En effet, la gestation est probablement l'état physiologique le plus demandeur en eau. Nous avons vu dans les **Article 1** et **4** que les femelles sont notamment exposées à des pertes hydriques supérieures. Par ailleurs, comme quantifié dans l'**Article 3**, les femelles doivent transmettre de l'eau pour la croissance des embryons. Les contraintes en eau sont donc probablement plus importantes pour des femelles gestantes en comparaison avec des individus en mue ou en digestion qui sont uniquement exposés à des pertes hydriques supérieures. De la même façon, dans cette étude, les vipères avaient à disposition de l'eau *ad libitum*. Or, en conditions

naturelles pendant la gestation par exemple (de juin à septembre), la disponibilité en eau n'est pas continue. Une expérience intéressante serait donc de répéter le même protocole en faisant varier le niveau de l'état d'hydratation. Les pertes en hydriques supérieures pour des états physiologiques telles que la mue, la digestion ou la gestation devraient contraindre la balance hydrique plus rapidement suite à une privation d'eau (déshydratation). Or, on sait que la déshydratation induit des ajustements comportementaux chez la plupart des organismes tels que des modifications de l'activité ou des préférences thermiques (Pough et al. 1983 ; Crowley 1987 ; Bradshaw 1997 ; Lorenzon et al. 1999 ; Ladyman et al. 2006 ; Bonnet & Brischoux 2008 ; Davis & DeNardo 2009 ; Angilletta et al. 2010 ; Hetem et al. 2010 ; Köhler et al. 2011). En réponse à une privation d'eau, la sélection de microclimat à la fois chaud et humide devrait donc être plus marquée pour des états physiologiques demandeurs en énergie. A l'inverse, les individus dans un état physiologique non-contraint devraient favoriser des microclimats plus frais (Ladyman et al. 2006).

2. Les contraintes physiologiques et morphologiques de la gestation

La reproduction est connue comme un état physiologique contraignant et coûteux pour les organismes (voir Harshman & Zera 2007 pour une review). La vipère aspic est vivipare et de plus un exemple de reproducteur sur capital (Bonnet et al. 1998), ce qui fait de cette espèce un modèle particulièrement étudié pour déterminer les compromis intra-individuels en lien avec le coût de la reproduction (*sensu* Stearns 1989), mais aussi les compromis intergénérationnels. En effet, les femelles accumulent des réserves énergétiques pendant 2 à 3 ans avant d'investir dans la reproduction (Bonnet et al. 1998, 1999, 2002; Lourdais et al. 2002a, 2002b). Ainsi pendant la gestation, l'effort reproducteur maternel est principalement orienté vers l'allocation de temps dans l'intensité de thermorégulation pour assurer le développement embryonnaire (Shine 1980; Charland & Gregory 1990; Shine 2006, 2012; Lorioux et al. 2013a). Toutefois, les femelles doivent également assurer le transfert d'oxygène et d'eau vers les embryons en développement en plus d'évacuer les déchets azotés (Van Dyke & Beaupre 2011). Dans ce contexte, nous avons premièrement mesuré dans l'**Article 2** la dynamique de la dépense énergétique (i.e., VO_2) au cours de la gestation. Ensuite dans les **Articles 3** et **4**, on s'est intéressé plus en détail aux contraintes hydriques pendant la gestation.

2.1. Les contraintes énergétiques

Les résultats présentés dans l'**Article 2** ont permis de mettre en évidence une augmentation du métabolisme standard des femelles gestantes de l'ordre de 55% pendant la gestation, ce qui est suivi d'une chute du métabolisme après la parturition. Ces changements métaboliques sont significativement influencés par différents indicateurs de l'effort reproducteur. L'effort reproducteur a été estimé en considérant la fécondité absolue (nombre et masse des nouveau-nés) mais aussi la fécondité relative (fécondité absolue ajustée par la taille des femelles). Parmi les différents indicateurs de l'effort reproducteur nous nous sommes aperçus que l'effort reproducteur relatif corrèle mieux avec les changements métaboliques au cours de la gestation que l'effort reproducteur absolu. Ces résultats suggèrent donc que la gestation impose également une demande énergétique spécifique aux femelles. En support de cela, nous avons pu déterminer que pendant la gestation, le MCP (pour « Metabolic Cost of Pregnancy ») est une composante significative et représente entre 14-15% de la dépense énergétique totale des femelles gestantes (i.e., métabolisme des femelles + métabolisme des embryons + MCP).

Par ailleurs, nous avons également mesuré la condition corporelle des femelles après la

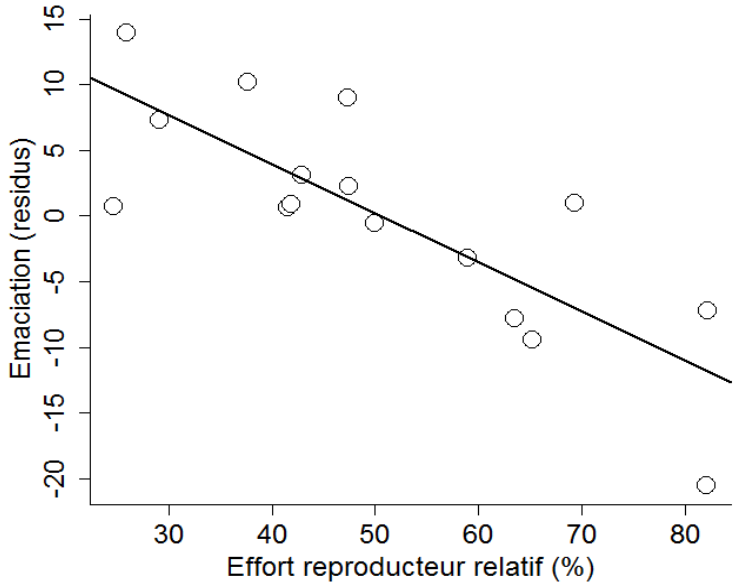

Figure 27. Relation entre l'état d'émaciation des femelles (condition corporelle post-partum) et l'effort reproducteur relatif (ratio entre masse de portée et masse post-partum des femelles) ($P < 0.001$, $r^2 = 0.64$).

parturition (masse post-partum ajustée par la taille), puisqu'il s'agit d'un bon indice de leur état d'émaciation (Bleu et al. 2011). Nous avons ainsi démontré une relation négative entre la condition corporelle post-partum des femelles et l'augmentation métabolique au cours de la gestation (plus l'augmentation métabolique est forte, plus la condition corporelle est mauvaise).

L'émaciation des femelles est également influencée par l'effort reproducteur relatif (Fig. 27). Or, l'état d'émaciation est une composante clé dans l'histoire de vie des individus puisqu'il influence directement la survie des femelles après la mise-bas, mais aussi le temps d'acquisition de l'énergie nécessaire à une reproduction future (Bonnet et al. 1999 ; Lourdais et al. 2002b ; Bleu et al. 2011 ; Baron et al. 2012).

Dans l'ensemble ces résultats révèlent des changements métaboliques majeurs au cours de la gestation, principalement représentatifs du développement embryonnaire, et qui impactent considérablement la condition corporelle post-partum des femelles. L'augmentation métabolique impose donc probablement des contraintes énergétiques spécifiques liées au coût de la reproduction (compromis intra-individuel entre reproduction courante et survie ou reproduction future), mais également des contraintes hydriques. Nous avons donc déterminé les contraintes hydriques de la gestation dans la suite de ce doctorat.

2.2. Les contraintes hydriques

Dans les **Articles 3** et **4**, on s'est intéressé au compromis liés à l'eau à l'échelle de la mère. Dans l'**Article 3**, nous avons cherché à quantifier la dynamique de transfert de l'eau au cours de la gestation. Nos résultats montrent que du côté de la mère, la masse corporelle augmente pendant la gestation, ce qui est influencé positivement par la fécondité. Etant donné que les femelles n'ont pas été nourris pendant cette période, les variations de masses sont principalement représentatives des prises en eau des embryons ou des pertes hydriques. De la même façon, l'osmolalité plasmatique diminue au cours de la gestation en lien avec la taille de portée. Du côté des embryons, le volume des œufs (i.e., embryons + vitellus + annexes embryonnaires) augmente de façon exponentielle pendant la gestation (Fig. 28), en lien avec la croissance somatique des fétus (Dauphin-Villemant & Xavier 1986 ; Andrews 2004). Nos résultats suggèrent donc une prise en eau conséquente (jusqu'à 30g) par les femelles et non

linéaire au cours de la gestation. Cette prise en eau est de plus significativement influencée par l'effort reproducteur, probablement en lien avec le transfert d'eau pour la croissance des embryons.

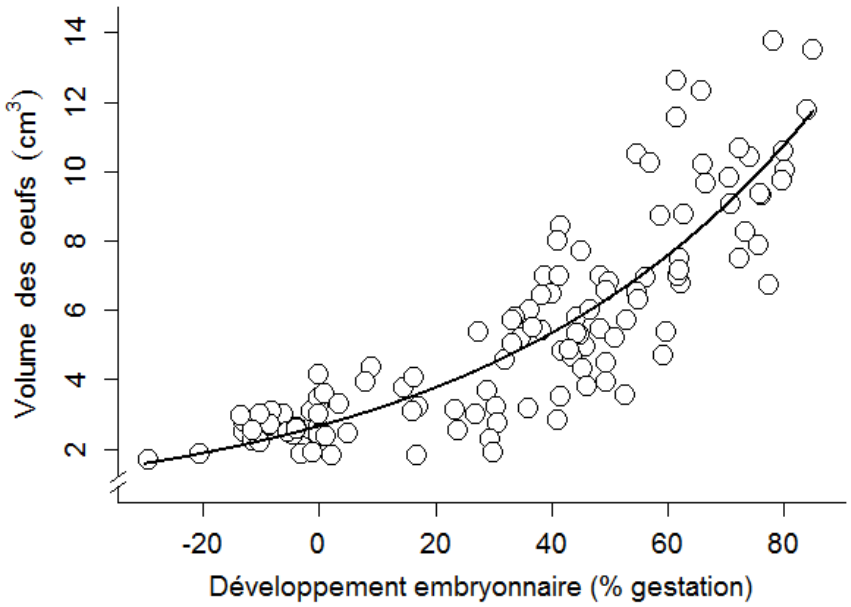

Figure 28. Evolution exponentielle du volume (moyenne par femelle) des œufs (embryons + vitellus + annexes embryonnaires) au cours de la gestation de la vipère aspic.

Nous avons pu mettre en évidence par ailleurs que cette dynamique exponentielle de croissance des fétus induit de profonds changements morphologiques pour les femelles. En effet, la portée va d'une part une avancée dans l'abdomen en moyenne de 5.5cm vers l'avant (~12% de la taille des femelles), et d'autre part elle va induire une distension abdominale des femelles de l'ordre de 25-30% dès l'ovulation. Pendant la gestation, l'augmentation du métabolisme standard et la distension abdominale sont probablement deux paramètres physiologiques majeurs qui induisent l'augmentation des pertes hydriques totales (i.e., augmentation de la ventilation et de la transpiration). Dans l'**Article 4**, nous avons en effet pu montrer que les pertes hydriques totales augmentent significativement pendant la gestation. Les pertes hydriques sont marginalement influencées par la fécondité des femelles (taille totale de portée) et positivement déterminées par la distension abdominale. Ces effets directs du développement embryonnaire sur l'augmentation des pertes hydriques des femelles sont de plus renforcés par des effets indirects liés aux besoins thermiques des embryons tout au long de la gestation. En effet, contrairement aux contraintes hydriques qui dépendent de la fécondité (pertes hydriques et allocation d'eau vers la portée), les femelles gestantes augmentent leurs préférences thermiques

(température préférée et temps de thermorégulation ; Waspra et al. 2010; Lorioux et al. 2013a) indépendamment de leur fécondité (soin maternelle non-dépréciable ; Lorioux 2011). Etant donné les résultats de l'effet de la température sur les pertes hydriques (**Article 1** et **4**), il s'agit probablement de la principale cause de l'augmentation des pertes hydriques totales pendant la gestation. En définitive, lorsqu'on considère les résultats des pertes hydriques totales chez les femelles gestantes et non-reproductives couplés à leurs préférences thermiques respectives précédemment déterminées (T_{set} = 33 *vs* 25°C), la gestation induit une augmentation de 38% des pertes hydriques totales.

Les contraintes énergétiques et hydriques décrites dans cette partie suggèrent donc un possible compromis intra-individuel en lien avec le coût de la reproduction (Stearns 1989). Toutefois, pour déterminer si ces contraintes physiologiques et morphologiques liées à l'eau induisent un réel coût pour les femelles, il aurait été nécessaire de déterminer si l'allocation en eau ou des pertes hydriques supérieures compromettent la survie ou la reproduction future des femelles (Bonnet et al. 2000 ; Bleu et al. 2011). Par ailleurs, la fécondité-dépendance des contraintes hydriques permet de supposer un compromis d'ordre intergénérationnel pour l'eau. Pour tester cette hypothèse, nous avons utilisé une approche expérimentale.

3. Manipulation des contraintes hydriques de la gestation

3.1. Un compromis intergénérationnel pour l'eau

Les résultats présentés dans l'**Article 5** ont été obtenus suite à la privation d'eau de femelles gestantes (en milieu de gestation) et de femelles non-reproductrices. Nos résultats montrent que comparativement aux témoins, les femelles privées d'eau pendant 20 jours (gestantes et non-reproductrices) perdent de la masse corporelle et subissent une augmentation de l'osmolalité plasmatique, ce qui confirme qu'elles ont été significativement déshydratées. Toutefois, comme nous l'avions prédit, les femelles gestantes privées d'eau ont une osmolalité plasmatique qui augmente deux fois plus que les non-reproductrices. De façon concordante, les changements de masses et d'osmolalité sont fortement corrélés et influencés par la fécondité. Du côté des descendants, les différentes mesures des performances de reproduction (succès reproducteur, volume des œufs, quantité et qualité des nouveaux nés, quantité de fluide dans la portée) n'ont

fait apparaître aucune différence entre les femelles gestantes privées d'eau et les femelles gestantes témoins. Ces résultats suggèrent donc qu'en réponse à une privation d'eau pendant la gestation, l'eau corporelle de la mère est transférée vers les embryons en développement, ce qui soutient l'hypothèse d'un compromis intergénérationnel significatif pour l'eau. Nos résultats ne nous permettent toutefois pas de savoir quel protagoniste contrôle le transfert de l'eau. La première hypothèse serait que la femelle contrôle activement le transfert de l'eau vers la portée (**hypothèse d'allocation maternelle**), et compromettrait ainsi sa propre balance hydrique en faveur des embryons. L'hypothèse alternative est que ce sont les embryons qui seraient capables d'extraire l'eau maternelle pour eux-mêmes (**hypothèse d'acquisition embryonnaire**). On sait par exemple que pendant l'incubation, les embryons des espèces ovipares sont capables de contrôler le fonctionnement des aquaporines et de réguler le gradient osmotique pour extraire l'eau de l'environnement (Packard 1991; Brown & Shine 2005; Shine & Thompson 2006). Ces observations mettent en évidence des adaptations embryonnaires spécifiques pour accumuler l'eau de l'environnement, ce qui supporterait l'hypothèse d'acquisition embryonnaire. Cependant, les mécanismes fonctionnels qui sont utilisés par la femelle ou les embryons pour contrôler le transfert d'eau restent méconnus chez les espèces vivipares. On peut aisément supposer que des signaux similaires à ceux impliqués dans le transfert d'énergie (e.g., hormones, cytokines, facteurs de croissance, etc) (Crespi & Semeniuk 2004) sont concernés, ce qui mériterait d'être clarifié.

3.2. Un possible conflit mère-embryons

Dans l'**Article 5**, nous avons émis l'hypothèse que ce compromis intergénérationnel pour l'eau pouvait conduire à un conflit entre la mère et la descendance. Il convient donc tout d'abord de clarifier la distinction entre compromis et conflit. Initialement le concept du conflit parent-descendant de Trivers (1974) a été utilisé pour modéliser l'investissement parental optimal dans les soins parentaux (i.e., la balance entre l'investissement dans le succès reproducteur actuel *versus* dans sa propre survie ou reproduction future). Chaque protagoniste présente un optimum différent dans l'acquisition des ressources. Alors qu'un descendant a pour but de maximiser ses propres besoins, les parents doivent allouer les ressources à la fois pour leur propre survie et pour celle des autres descendants (voir Godfray 1995b pour une review). Le conflit parent-descendant résulte donc d'un compromis intergénérationnel (Charnov 1982 ; Godfray & Parker 1991 ; Godfray 1995a), lorsque ce dernier est suffisamment poussé (ressource limitante) pour

imposer un coût en terme de survie ou reproduction future sur l'un ou les deux protagonistes. Dans notre étude, nous avons considéré que l'eau pouvait donc au même titre que l'énergie être une ressource conflictuelle entre une mère et ses embryons en développement.

En effet, bien que les Squamates soient particulièrement tolérants aux perturbations de la balance hydrique, la déshydratation est connue pour impacter les performances des individus (Wilson & Havel 1989; Lorenzon et al. 1999; Moeller et al. 2013). Dans notre expérimentation, l'osmolalité plasmatique des femelles gestantes privées d'eau a augmenté en moyenne de 321.8 ± 2.4 à 384.9 ± 5.4 $mOsmol.kg^{-1}$, et ces femelles se sont immédiatement mises à boire intensément lorsqu'elles ont été réexposées à l'eau. Ces résultats suggèrent donc qu'elles ont été fortement déshydratées suite à la privation d'eau (Moeller et al. 2013; Lillywhite et al. 2014). Les données sur les optimums dans les besoins en eau sont encore manquantes pour l'instant que ce soit pour les femelles ou les embryons. Toutefois, on sait que l'altération des performances physiologiques représente un coût de reproduction significatif (Shine 1980, 2003). C'est la raison pour laquelle nos données supportent l'idée d'un conflit significatif entre la mère et la descendance pour l'eau. Des études plus poussées (à long-terme) sont néanmoins requises pour clarifier les optimums des besoins en eau et déterminer les effets d'une déshydratation pendant la gestation sur la survie ou la reproduction future des femelles (Bonnet *et al*. 2000; Bleu *et al*. 2011).

Les conflits parent-descendants ont en effet attiré un vif intérêt (2974 citations du modèle original de Trivers 1974 sur google scholar) à la fois en écologie évolutive ou en écologie comportementale (Danchin et al. 2005), et jusqu'à des disciplines comme la psychologie (Buunk et al. 2008). Toutefois, la grande majorité des études en écologie mettent en évidence un conflit entre l'investissement parental et la sollicitation des jeunes principalement vis-à-vis des ressources en énergie ou en temps (voir Kölliker & Richner 2001 pour une review). L'hypothèse d'un conflit mère-embryon pour l'eau est à notre connaissance complètement nouvelle. Or un tel conflit n'est probablement pas restreint aux Squamates et s'applique vraisemblablement à de nombreux taxa. La difficulté majeure est d'isoler les effets des différentes ressources puisque pour plusieurs espèces, l'eau est typiquement transférée aux descendants via l'alimentation en même temps que l'énergie (Williams & Nagy 1985). Néanmoins, certaines espèces ont développé des soins parentaux postnataux spécifiques pour l'approvisionnement en eau des jeunes (e.g., eau dans le plumage chez les *Pteroclidae* ;

Mougeot et al. 2014), et ce type d'espèces représentent d'excellent modèles pour évaluer l'application d'un conflit parent-descendant postnatal pour l'eau. Les conflits parents-descendants s'appliquent également aux plantes (Uma Shaanker et al. 1988) et un tel conflit pour l'eau est probablement vrai, surtout pendant la formation des fruits. De façon plus ultime, nous émettons l'hypothèse qu'un tel conflit mère-embryons pour l'eau représente une pression de sélection importante dans l'évolution des modes reproducteurs, et de l'effort reproducteurs.

3.3. Implications dans l'évolution vers la viviparité

Que l'on considère la ressource en énergie (**Article 2**) ou en eau (**Article 3**, **4** et **5**), clarifier les contraintes associées aux compromis d'allocation pendant la gestation est nécessaire pour comprendre les coûts associés à la reproduction vivipare. Les pressions de sélection qui ont favorisé et/ou limité la transition vers la viviparité ont en effet attiré un grand intérêt en particulier chez les Squamates (Tinkle and Gibbons 1977; Shine 1985, 1995; Lorioux et al. 2013b), compte tenu de la diversité des modes reproducteurs, de la proximité phylogénétique, et du fait que la transition se soit produite une centaine de fois indépendantes (Blackburn 1993). Les précédentes études se sont soit intéressées aux bénéfices associés (Tinkle and Gibbons 1977; Shine 1985, 1995; Lorioux et al. 2013b), soit aux coûts physiologiques ou écologiques (Blackburn 1985; Shine 2004; Qualls and Andrews 1999; Andrews 2000; Crespi and Semeniuk 2004; Lourdais et al. 2004; Shine & Thompson 2006).

La sensibilité thermique embryonnaire est centrale dans les deux grandes hypothèses actuelles qui sont décrites : l'**hypothèse des environnements froids** (Tinkle and Gibbons 1977) et l'**hypothèse de la manipulation maternelle** (Shine 1995). Si on tient compte de nos résultats et des prédictions de l'hypothèse de Crespi & Semeniuk (2004) (« Viviparity-conflict hypothesis »), le conflit mère-descendant pour l'eau pourrait au moins chez les Squamates constituer un frein significatif à la transition vers la viviparité. Chez les espèces

Figure 29. Eclosion d'une céraste (*Cerastes cerastes*).

ovipares, les échanges gazeux et hydriques sont des paramètres clés qui limitent la rétention des œufs en général au premier tiers du développement embryonnaire (Shine & Thompson 2006). Le conflit mère-descendant que l'on suppose dans l'**Article 5**, ou en tout cas, le compromis intergénérationnel pour l'eau mis en évidence en milieu de développement augmente très vraisemblablement en suivant la dynamique exponentielle des besoins en eau des fœtus en croissance (**Article 3**). Aussi, dans des milieux desséchants, il est probable que le conflit pour l'eau soit une véritable limite à la transition vers la viviparité. Par exemple, les Viperinae (i.e., « vraies » vipères) sont principalement représentés par des espèces vivipares et le peu d'espèces ovipares sont uniquement présentes en zones arides ou dans les déserts (e.g., genre *Cerastes*, *Echis*, *Macrovipera* ou *Montivipera*; Blackburn 1985) (Fig. 29). La disponibilité en eau de ces environnements est très réduite et constitue très probablement une limite significative à la rétention des œufs.

4. Implication de la corticostérone dans les compromis pour l'eau

La corticostérone (CORT) représente l'un des médiateurs essentiels de l'allostasie chez les vertébrés (McEwen & Winglfield 2003, 2010 ; Romero et al. 2009). Les ectothermes étant prédisposés à tolérer des variations internes importantes (température corporelle, déshydratation, etc), on peut se demander si le concept d'allostasie s'applique à de tels organismes. Dans l'**Article 6**, nous avons présenté les résultats obtenus à partir de la même expérimentation que celle présentée dans l'**Article 5**, mais sur les concentrations de CORT ; à savoir clarifier les effets du statut reproducteur et de la privation d'eau sur la réponse au stress.

4.1. Corticostérone et balance hydrique

Dans l'**Article 6**, nous nous attendions à ce que les concentrations de CORT augmentent en réponse à une période de 20 jours de privation d'eau. Contrairement à cette prédiction, les concentrations de CORT basales et induites par un stress ne diffèrent pas entre les femelles non-reproductrices privées d'eau et leurs témoins respectifs. Pourtant les données physiologiques (masse corporelle et osmolalité plasmatique) et leur comportement de boisson une fois réexposées à de l'eau, suggèrent que les femelles non-reproductrices ont été significativement déshydratées. De plus, les relations entre les variations de CORT (basale et induite par un stress)

et les variations d'osmolalité ne sont pas significatives chez les femelles non-reproductrices. De la même façon, dans une expérimentation parallèle sur une autre espèce de serpent, le python de children (**Article 8** en annexes), les individus ne montrent aucune variation des concentrations de CORT basale suite à la déshydratation. L'ensemble de ces résultats, sur 2 expérimentations indépendantes, ne soutiendraient donc pas l'hypothèse du rôle de la CORT dans la régulation de la balance en eau *per se*. Néanmoins, ce n'est pas forcément contraire aux précédents travaux puisqu'en tout cas à notre connaissance, les études qui se sont intéressées au rôle minéralocorticoïde de la CORT chez les reptiles ont principalement montré une fonction ionorégulatrice de la CORT (régulation du sodium, du potassium, etc) (Holmes & McBean 1964 ; Duggan & Lofts 1978 ; Bradshaw & Rice 1981 ; Bradshaw et al. 1984a ; Bradshaw 1997) (Tableau 1).

Traitement	Corticostérone ($ng.dl^{-1}$)
Déshydratation	9.26 ± 1.73
Témoins	9.11 ± 2.09
Injection de Sels	19.24 ± 2.56 **
Réponse au stress	46.1 ± 1.46 **

Tableau 1. Concentrations plasmatiques de corticostérone en réponse à la déshydratation, l'hydratation, l'injection de sels ou la réponse au stress chez le varan de Gould (*Varanus gouldii*). Le tableau montre les valeurs (moyennes ± SE) présentées dans Bradshaw & Rice (1981). L'effet significatif du traitement est illustré: ** $P < 0.01$.

Il semblerait par exemple que la CORT soit impliquée dans le fonctionnement de la glande à sel chez les Squamates (Holmes & McBean 1964 ; Bradshaw et al. 1984a ; Bradshaw 1997), mais aussi chez des oiseaux marins (Allen et al. 1975 ; Harvey & Phillips 1982). De façon plus générale, les corticostéroïdes sont supposés agir sur la régulation de l'équilibre en ions (action principalement permissive) au niveau de différents organes (i.e., glande à sel, intestin, vessie, etc) chez plusieurs vertébrés (voir McCormick & Bradshaw 2006). Toutefois, l'effet de la CORT sur le fonctionnement de la glande à sel a été remis en question pour d'autres espèces (Bradshaw et al. 1984b). De plus amples expérimentations sont donc requises à ce stade pour clarifier la fonction de la CORT dans la régulation de l'équilibre en eau ou en sel.

4.2. Variations de corticostérone au cours de la gestation

4.2.1. Un possible rôle dans le transfert d'eau vers la portée

Dans l'**Article 6**, on constate premièrement un effet global du statut reproducteur sur les concentrations de CORT. Comme attendu, les femelles gestantes présentent des concentrations de CORT basales et induites par un stress plus fortes que les femelles non-reproductrices. Ces résultats sont concordants avec de précédents travaux sur la vipère aspic (voir Chapitre 3 dans Lorioux 2011), comme pour la plupart des organismes (Romero 2002 ; Anderson et al. 2014). L'augmentation des niveaux de CORT sont probablement représentatifs d'une charge allostatique induite par l'allocation maternelle des ressources vers les embryons en développement. Par ailleurs, les travaux de thèse de Lorioux (2011) ont montré une augmentation progressive des concentrations de CORT pendant la gestation, suggérant ainsi un possible rôle de la CORT dans le transfert d'eau (Dauphin-Villemant & Xavier 1986). Nos résultats soutiennent cette hypothèse puisque les concentrations de CORT (basale et induite par un stress) augmentent significativement en réponse à la privation d'eau. On observe de plus une relation positive significative entre les changements de CORT basale et d'osmolalité, contrairement aux femelles non-reproductrices.

En suivant les 2 hypothèses que nous avons émises quant au transfert d'eau de la mère vers la portée (i.e., **allocation maternelle** *vs.* **acquisition embryonnaire**), on peut interpréter l'augmentation des concentrations de CORT en réponse à la déshydratation de façon concomitante. Tout d'abord, dans le cas de l'hypothèse de l'allocation maternelle d'eau vers la portée, la CORT peut agir comme hormone d'allocation de l'eau corporelle vers les embryons en développement. Au niveau basal, la CORT est connue pour son implication dans l'allocation d'énergie (Angelier et al. 2009), et pourrait avoir un rôle similaire vis-à-vis de l'eau. Ensuite, dans l'hypothèse d'une acquisition embryonnaire, l'augmentation des concentrations de CORT peut résulter d'un stress physiologique de la mère (surcharge allostatique) causé par l'extraction de son eau corporelle par les embryons. Toutefois, des analyses supplémentaires sont nécessaires pour mieux comprendre la fonction de la CORT vis-à-vis du transfert d'eau pendant la gestation.

4.2.2. Dépendance à la fécondité

Nos résultats ont montré une différence d'influence de la fécondité sur les variations de CORT entre les femelles témoins et privées d'eau dans. Chez les femelles gestantes témoins, les changements de concentrations de CORT basale sont significativement et négativement corrélés à 2 indicateurs de l'effort reproducteur (absolu et relatif), et cette relation disparaît chez les femelles gestantes déshydratées. Nous aurions pu toutefois nous attendre à une relation positive entre CORT basale et effort reproducteur chez les femelles témoins puisque des fécondités importantes sont en général associées à un effort reproducteur plus important en termes d'investissement en eau et énergie (Bonier et al. 2011). Cependant, une autre hypothèse,

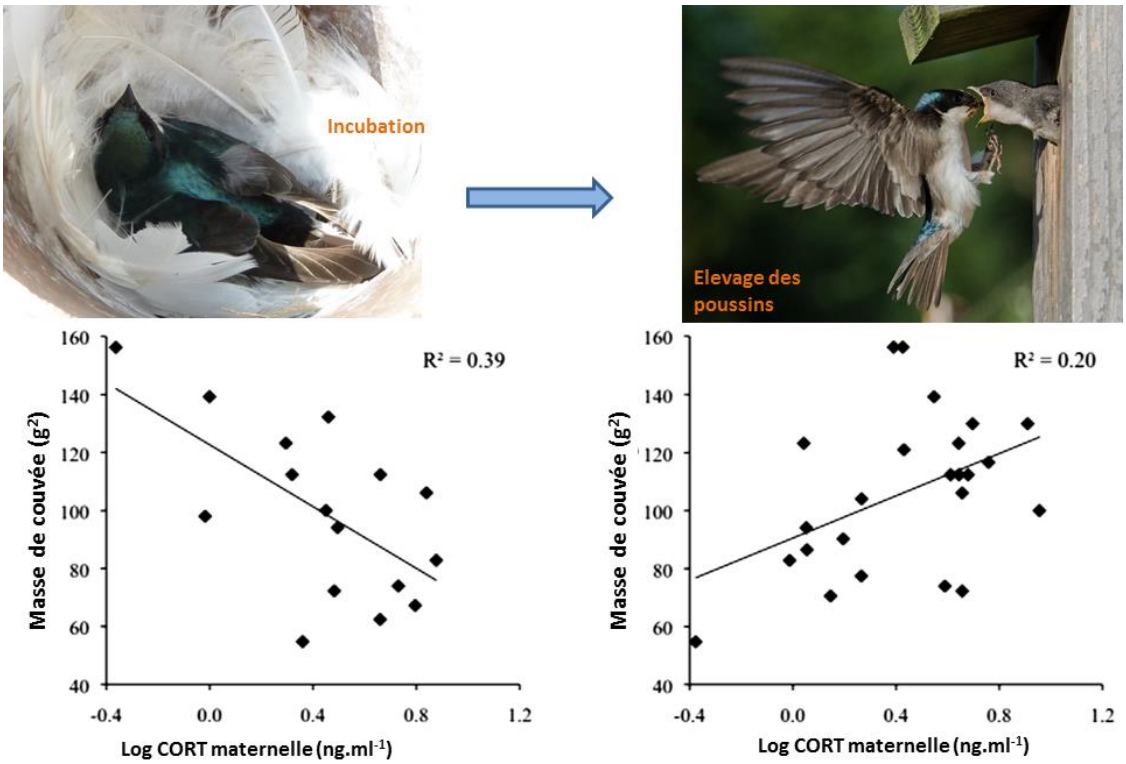

Figure 30. Variations du sens de la relation entre valeur sélective (ici la masse de la couvée) et la CORT maternelle chez des femelles d'hirondelles bicolores (*Tachycineta bicolor*). A gauche pendant l'incubation lorsque l'effort reproducteur est relativement faible, la relation est négative et suit donc la prédiction première de l'hypothèse CORT-valeur sélective : les individus de bonne qualité (i.e., CORT basale faible) investissent plus dans la reproduction. A droite, pendant l'élevage des poussins, l'effort reproducteur augmente et les animaux qui maintiennent une allocation d'énergie importante vers la reproduction (i.e., CORT basale élevée) sont ceux qui ont des meilleures couvées. D'après Bonier et al. (2009b).

l'hypothèse CORT-adaptation prédit des relations positives ou négatives selon l'intensité de la charge allostatique (Bonier et al. 2009a) (Fig. 30). Dans notre expérimentation (**Article 5** et **6**), les mesures de CORT ont été réalisées en milieu de gestation (40-60% de la gestation) lorsque la croissance somatique des embryons et donc la demande en eau est encore relativement limitée

(Andrews 2004). La relation négative que l'on observe entre l'effort reproducteur et les variations de CORT maternelle suivraient donc les prédictions de l'hypothèse CORT-adaptation quand la charge allostatique est faible, ce qui suit également la prédiction de l'hypothèse CORT-valeur sélective (Bonier et al. 2009a, 2009b). Une alternative possible à cette interprétation serait que la relation négative s'explique en raison d'effets indirects causés par l'influence de l'effort reproducteur sur les besoins en eau des femelles. Dans l'**Article 3**, nous avons montré que le comportement de boisson augmente avec l'effort reproducteur. A priori dans les **Articles 5** et **6**, les femelles témoins ont réagi de la même façon puisque la masse corporelle augmente pendant l'expérimentation, et ces variations de masses sont influencées par l'effort reproducteur. La diminution de CORT basale en lien avec l'augmentation de l'effort reproducteur s'explique donc peut-être tout simplement par un phénomène de dilution de la CORT plasmatique (i.e., augmentation du volume d'eau corporelle).

4.2.3. Médiation d'effets maternels

Bien que la période de privation d'eau n'ait pas impacté le succès reproducteur ou le phénotype des nouveau-nés à la naissance, nous avons trouvé une croissance différentielle entre les jeunes nés de mères déshydratées et ceux nés de mères témoins. Dans les 2 semaines qui ont suivies la naissance, les jeunes nés de mères déshydratés montrent une vitesse de croissance (corps et mâchoires) plus rapide que les jeunes nés de mères témoins. Comme prédit, cela suggère donc de possibles effets maternels induits par la privation d'eau pendant la gestation. En support de cette hypothèse, nous avons en plus trouvé des relations significatives entre croissance des jeunes et variations d'osmolalité ou variations de CORT maternelle. Cela suppose donc que la période de privation d'eau a significativement déshydraté les femelles, ce qui a induit une augmentation des niveaux de CORT qui a pu en retour impacter la trajectoire phénotypique des decendants. En effet, de précédents travaux ont expérimentalement permis de montrer un effet (positif, négatif ou neutre) de l'augmentation de la CORT pendant le développement embryonnaire sur la croissance des jeunes chez différentes espèces de vertébrés (Lesage et al. 2001 ; Hayward & Wingfield 2004 ; Meylan & Clobert 2005 ; Chin et al. 2009 ; Warner et al. 2009 ; Coslovsky & Richner 2011) (Fig. 31). Par ailleurs, deux études de long-terme sur le lézard vivipare en conditions naturelles ont décrits des relations positives entre la disponibilité en eau dans l'environnement et la croissance des juvéniles (Lorenzon et al. 1999, 2001).

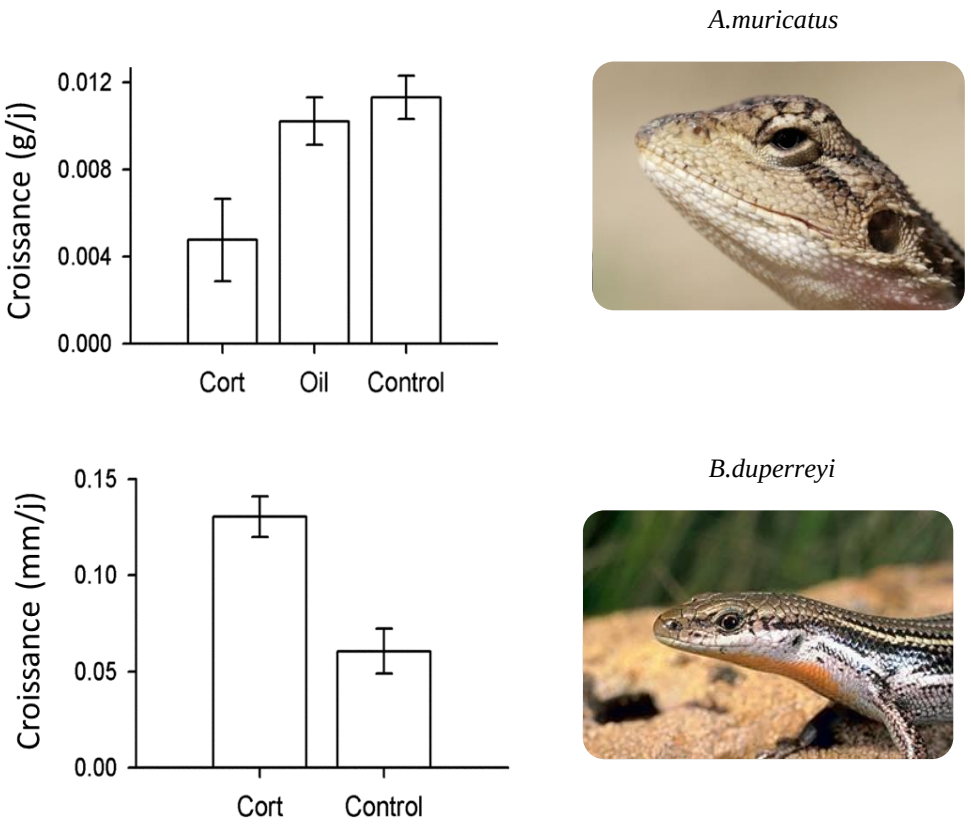

Figure 31. Vitesses de croissance différentielles chez des juvéniles de 2 espèces de lézards (*Amphibolurus muricatus* en haut et *Bassiana duperreyi* en bas) suite à l'injection dans l'œuf de corticostérone (Cort), d'huile de sésame (Oil) ou rien (Control). La croissance est différente entre les groupes Cort et Control pour les 2 espèces mais l'effet de la corticostérone sur la croissance est différent chez chaque espèce (effet stimulant ou inhibant). D'après Warner et al. (2009).

Les résultats de notre expérimentation sur la croissance différentielle des jeunes en lien avec la CORT maternelle ne nous permettent toutefois pas de conclure à un effet positif ou négatif d'une croissance accélérée sur la valeur sélective des jeunes. Pour cela, d'autres mesures sont en effet essentielles à considérer (e.g., survie, âge et taille à la maturité, etc; Stearns 1992 ; Roff 1992). Une croissance accélérée peut en effet présenter d'une part des avantages sélectifs (prédation/compétition diminuées, meilleures capacités de dispersion ; voir Arendt 1997 pour une review), et d'autre part des coûts immédiats ou décalés qui affectent la survie et la reproduction future des jeunes (Gotthard 2000 ; Metcalfe & Monaghan 2001). Ainsi, selon nos résultats une croissance plus élevée des vipéreaux peut être avantageuse pour échapper à un environnement hydriquement contraignant (Lorenzon 2001), et imposer des coûts décalés par la suite. Des études à plus long-terme sont néanmoins requises à cette étape pour déterminer les effets d'une privation d'eau pendant la gestation sur la survie et la reproduction future des

jeunes. Marquis et al. (2008) ont notamment démontré que le niveau de précipitations influence positivement les performances reproductrices du lézard vivipare.

5. Conclusion et perspectives de recherche

Les travaux originaux menés pendant ce doctorat ont permis de mettre en évidence qu'au même titre que l'énergie ou le temps, l'eau est également une ressource d'intérêt dans les compromis physiologiques et comportementaux. A l'échelle de l'individu ou de façon intergénérationnelle, les compromis pour l'eau semblent être déterminants dans les ajustements physiologiques (niveau métabolique, stress) et comportementaux (thermorégulation, activité), mais aussi dans l'évolution des stratégies de reproduction. De telles études combinées à des suivis à long-terme en nature sont essentiels à considérer aujourd'hui pour mieux comprendre les réponses fonctionnelles des animaux et prédire leurs réponses face à la diminution des précipitations et l'augmentation du rythme d'évènements climatiques extrêmes à venir (Easterling et al. 2000 ; IPCC 2014). L'absence de mesure à long-terme représente très probablement la limite principale de ce doctorat puisque nous ne pouvons pas affirmer par exemple qu'une privation d'eau pendant la gestation induise un véritable coût de reproduction (*sensu* Stearns 1989). En effet, de la même façon que les espèces sauvages, un doctorant fait également face à des compromis d'allocation de temps étant donné que cette ressource est particulièrement limitée pendant un doctorat (Fig. 32). Nos résultats nous ont donc amené à émettre différentes hypothèses explicatives, ce qui ouvre de nombreuses perspectives de recherche pour mieux comprendre l'importance de l'eau dans les ajustements physiologiques et comportementaux des organismes.

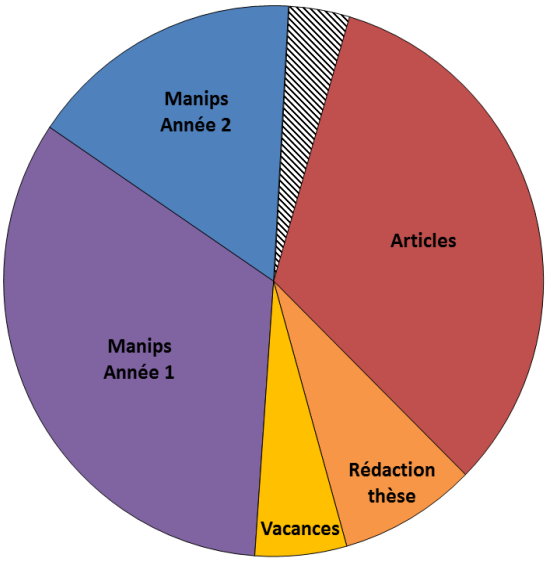

Figure 32. Exemple du budget temps d'un doctorant (A. Dupoué ; 2011-2014) au cours de sa thèse. Pour simplifier les calculs et comme il s'agit de proportions, les calculs sont réalisés sur une base totale de 3 ans (1095 jours). Les manips incluent la mise en place et la commande de matériels, la capture des individus (~100e de vipères pour >1000 km parcourus pendant ce doctorat), les mesures et les analyses statistiques. Les Manips Année 1 et 2 représentent une estimation du temps réellement alloué aux manips et la zone hachurée correspond au temps restant pour des Manips en 3e année (19 j). Le temps alloué à la rédaction d'articles a été estimé à 2 mois/article ce qui inclue la rédaction, les corrections/échanges entre les co-auteurs, et les différentes révisions pendant le processus de soumission. La thèse représente le travail final estimé à 3 mois avec : la rédaction de ce manuscrit, les échanges correctionnels, et la préparation de la soutenance. Le temps libre a été obtenu à partir d'Agate (service de congés CNRS). Sont négligés dans ce budget temps (mais pas forcément négligeables) : les formations doctorales, la participation et la préparation d'intervention (colloque, projets personnels de La Rochelle, formation pompier) et l'organisation des 8ème rencontres en écologie et comportement pour la SERL en 2012, et l'aide aux manips et papiers d'autres doctorants.

5.1. Stade dépendance du conflit mère-descendant pour l'eau

Le conflit que l'on suspecte entre la mère et les descendants pour l'eau est probablement variable pendant la gestation. En effet, contrairement à la température (Lorioux et al. 2013a), les optimums des besoins en eau augmentent probablement pendant la gestation, en lien avec la croissance exponentielle somatique des fœtus (Dauphin-Villemant & Xavier 1986 ; Andrews 2004 ; **Article 3**). Une méthode élégante pour illustrer l'évolution du conflit mère-embryons pour l'eau pendant la gestation consisterait à priver d'eau des femelles à différents stades de la gestation. L'étude récente de Lorioux et al. (2013b) sur la vipère aspic a notamment permis de mettre en évidence le stade-dépendance des besoins thermiques pendant la gestation. L'importance d'atteindre des températures optimales est ainsi particulièrement importante en début de gestation pendant l'organogénèse. Le protocole expérimental de cette étude étant robuste, une approche similaire pourrait être utilisée mais pour déterminer les effets d'une privation d'eau à différents stades : en début, milieu et fin de gestation. Il est probable qu'une

privation d'eau en fin de gestation soit bien plus compromettante qu'en début. Aussi, on peut prédire 2 alternatives en lien avec les 2 hypothèses développées dans la discussion : dans le cas de l'**hypothèse de l'allocation maternelle**, on peut s'attendre à ce que les femelles privées d'eau en fin de gestation privilégient leur propre survie et reproduction future au détriment de la reproduction en cours. Le succès reproducteur et le nombre de jeunes en vie devraient donc différer entre les femelles privées d'eau en fin de gestation de celles privées en début ou en milieu de gestation et des femelles témoins. A l'inverse, dans le cas de l'**hypothèse de l'acquisition embryonnaire**, on peut s'attendre à ce que chaque embryon tente d'extraire l'eau pour son propre développement, ce qui devrait contraindre fortement la survie des femelles privées d'eau en fin de gestation. Une telle expérimentation pourrait également permettre de déterminer des signaux essentiels probablement impliqués dans le contrôle du transfert de l'eau.

5.2. Importance de la structure de l'habitat

5.2.1. Approche expérimentale en enclos extérieurs

Initialement dans ce doctorat, un 4e objectif était prévu, à savoir utiliser des conditions semi-naturelles (10 enclos extérieurs disponibles au CEBC) pour déterminer les effets de la hauteur de la bande enherbée sur la physiologie des vipères. En effet, pendant son stage de master, A. Mathiot (2012) a pu montrer qu'en nature, la hauteur de l'ourlet herbacé influence significativement et positivement la richesse spécifique en reptiles (Fig. 33).

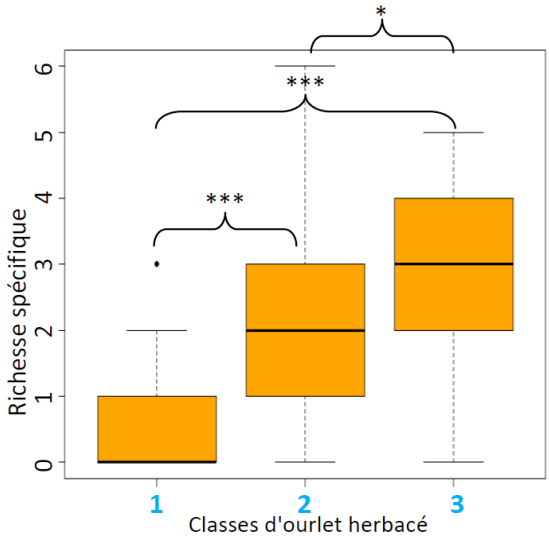

Figure 33. Effet de la classe de l'ourlet herbacé sur la richesse spécifique en reptiles. Les différences significatives sont symbolisées : * $P < 0.05$, *** $P < 0.001$.

Partant de ce constat, nous avons donc imaginé une expérimentation en enclos extérieurs afin de comparer les effets de la hauteur de végétation (« herbe haute » *vs* « herbe rase ») sur la physiologie de femelles gestantes et non-reproductrices. On peut s'attendre à ce qu'une hauteur de végétation importante soit favorable pour les individus non seulement d'un point de vue écologique (protection anti-prédation), mais

aussi d'un point de vue physiologique (favorisation des compromis thermorégulation / pertes hydriques). Cela devrait donc transparaitre au niveau des concentrations de CORT basale et induite par un stress qui devraient être plus élevées chez les femelles exposées à une hauteur de végétation plus faible. L'expérimentation devait suivre la période de gestation (début juin – fin septembre) avec dans l'idéal une période de sécheresse estivale conséquente (> 20 jours). On s'attend en effet à ce qu'une hauteur de végétation importante permette de tamponner un tel épisode climatique et que cela impacte différemment la physiologie des femelles témoins *versus* les femelles « herbe rase » et leurs descendants. Cette expérimentation était prévue initialement en 2014 mais n'a pas pu aboutir pour des raisons de temps (Fig. 32).

5.2.2. Approche descriptive *in natura*

Nos données apportent de nouveaux éléments sur les bases fonctionnelles en lien avec les ajustements des organismes aux variations de leur environnement. En Europe, le bocage représente à grande échelle un contexte d'études comparatives particulièrement intéressant. En effet, les réseaux de haies sont très fréquentés par un grand nombre d'organismes en raison de l'hétérogénéité et de la diversité de milieux. La qualité du bocage varie le long d'un gradient depuis des haies favorables et préservées jusqu'à des haies de mauvaise qualité (largeur faible, pauvre diversité faunistique/floristique, pied de haie inexistant, etc). Il est donc possible de réaliser des études comparatives pour déterminer l'utilisation de l'habitat par différentes espèces selon la disponibilité en eau. Par exemple, il pourrait être envisagé d'étudier les effets d'une supplémentation en eau pendant une période de sécheresse estivale sur l'activité et les performances reproductrices. Différentes mesures pourraient être réalisées avec des paramètres physiologiques (niveau métabolique, température corporelle, concentration de CORT, osmolalité, etc) et comportementaux (préférences thermiques, sélection de microclimat, distance de fuite, comportement de défense, etc) (voir aussi Davis & DeNardo 2009). De telles études sont requises pour améliorer nos connaissances fondamentales sur la relation à l'eau dans les compromis physiologiques, mais aussi modéliser efficacement les effets de modifications environnementales sur la réponse des organismes (Guillon 2012 ; Dunkin et al. 2013) et apporter des pistes adaptées en termes de conservation des espèces (voir Wikelski & Cooke 2006 pour une review).

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Annexes

ARTICLE 7

Influence of temperature on the corticosterone stress-response: An experiment in the Children's python (*Antaresia childreni*)

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Children's python

ABSTRACT

To cope with environmental challenges, organisms have to adjust their behaviours and their physiology to the environmental conditions they face (i.e. allostasis). In vertebrates, such adjustments are often mediated through the secretion of glucocorticoids (GCs) that are well-known to activate and/or inhibit specific physiological and behavioural traits. In ectothermic species, most processes are temperature-dependent and according to previous studies, low external temperatures should be associated with low GC concentrations (both baseline and stress-induced concentrations). In this study, we experimentally tested this hypothesis by investigating the short term influence of temperature on the GC stress response in a squamate reptile, the Children's python (*Antaresia childreni*). Snakes were maintained in contrasting conditions (warm and cold groups), and their corticosterone (CORT) stress response was measured (baseline and stress-induced CORT concentrations), within 48 h of treatment. Contrary to our prediction, baseline and stress-induced CORT concentrations were higher in the cold *versus* the warm treatment. In addition, we found a strong negative relationship between CORT concentrations (baseline and stress-induced) and temperature within the cold treatment. Although it remains unclear how cold temperatures can mechanistically result in increased CORT concentrations, we suggest that, at suboptimal temperature, high CORT concentrations may help the organism to maintain an alert state.

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1. Introduction

Organisms are exposed to predictable (seasonal or diurnal variations) or unpredictable (extreme climatic events) changes of most biotic and abiotic variables (Wingfield, 2003; Wingfield et al., 2011). To cope with such variation and maintain their performances, individuals adjust their behaviour and their physiology to the environmental conditions (the concept of allostasis; (McEwen and Wingfield, 2003, 2010; Romero et al., 2009). These adjustments will organize resource allocation between potentially competing life-history traits and, thus, mediate decisions that aim at optimizing individual's fitness (Ricklefs and Wikelski, 2002). It is therefore crucial to study the central mechanisms that govern behavioural and physiological adjustments adopted when a given environmental change occurs (Hau, 2007; McEwen and Wingfield, 2003, 2010; Ricklefs and Wikelski, 2002; Romero et al., 2009). In vertebrates, one of these central mechanisms is the secretion of glucocorticoids (GCs) that is well-known to be involved in the maintenance of homeostasis (Landys et al., 2006; McEwen and Wingfield, 2003; Romero et al., 2009; Wingfield, 2012, 2013). In response to predictable or unpredictable environmental changes, the Hypothalamic–Pituitary–Adrenal (HPA) axis is activated and this results in a rapid and intense secretion of GCs by the adrenal glands (Romero et al., 2009; Wingfield et al., 1998, 2011; Wingfield, 2003, 2012, 2013; Wingfield and Sapolsky, 2003). In turn, this increase in circulating GCs activates and/or inhibits specific physiological and behavioural traits in order to help the organism to cope with the challenge (Landys et al., 2006; Romero, 2004; Sapolsky et al., 2000).

Environmental temperature is a crucial parameter with important fluctuations at multiple time scales (days, seasons) and unpredictable cold or warm temperature extremes. Most organisms adjust their physiology (metabolic rate, evaporative water loss) and/or behaviour (activity) in order to maintain their body temperature relatively constant (Scholander et al., 1950; Tieleman et al., 2002). Terrestrial ectotherms cannot produce significant amounts of heat and their body temperature is more sensitive to environmental conditions which will influence metabolic rate according to the Q_{10} relationship (Arrhenius equation) (Bennett and Dawson, 1976). Ambient temperature will directly influence major biological processes including digestion, locomotion, and behaviour (Angilletta, 2009; Huey and Stevenson, 1979; Huey and Kingsolver,

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1989; Stevenson et al., 1985). Ectotherms are subjected to daily and seasonal temperature variations (Huey and Pianka, 1977; Peterson, 1987) and chiefly rely on behavioural means (habitat selection) to regulate their body temperature (Blouin-Demers and Weatherhead, 2001a,b,c, 2002). Because performances are reduced at low (i.e. suboptimal) temperature, one could also expect reduced GCs concentrations at low temperature (Cree et al., 2003; Tyrrell and Cree, 1998). First, low temperature should induce both decreased GCs secretion rates because of a slower activity of the HPA axis; and reduced GCs diffusion because of slower cardiac rhythm and blood circulation. Second, low temperatures are associated with a low metabolism in reptiles (Bennett and Dawson, 1976) and GCs concentrations are known to be positively correlated with metabolic activity in many vertebrate species (reviewed in Landys et al. (2006)).

The positive relation between GCs concentration and temperature has been showed in several species. For example, positive correlations between body temperature and baseline corticosterone (CORT, the primary GC in avian and non avian reptiles, (Greenberg and Wingfield, 1987) have been reported in tuatara, lizards and sea turtles (Cree et al., 2003; Jessop et al., 2000; Jones and Bell, 2004; Romero and Wikelski, 2006; Tyrrell and Cree, 1998; Woodley et al., 2003). However, this relation is not always supported among reptiles (Mathies et al., 2001; Sykes and Klukowski, 2009). While previous studies have focused on the relationship between body temperature and baseline CORT concentrations, much less attention has been paid to the thermal dependence of CORT stress response despite its ecological relevance (Cree et al., 2003; Romero and Wikelski, 2006; Sykes and Klukowski, 2009). Baseline and stress-induced CORT concentrations are known to have different physiological and behavioural actions (Landys et al., 2006; Romero, 2004). Thus, the action of CORT on behaviour and physiology depends on its circulating concentrations because CORT acts through the binding of two different receptors that have very different affinity for CORT. At baseline concentrations, CORT have mainly permissive actions that aim at maintaining energetic balances and deal with seasonal and daily routines (Landys et al., 2006; Romero, 2002; Sapolsky et al., 2000). At stress-induced concentrations, CORT has stimulatory and inhibitory actions that aim at promoting individual's short-term survival (i.e. a life-threatening event; (Angelier et al., 2009; Wingfield et al., 1998, 2011). Therefore it is essential to consider both baseline and stress-induced CORT concentrations.

In this study, we investigated the influence of temperature on both baseline and stress-induced CORT concentrations in a constricting snake, the Children's python (*Antaresia childreni*). Our aim was to test the influence of temperature on CORT stress response in response to a short term ($\leqslant$2 days) change in temperature. We created a thermal contrast between two groups of snakes by either allowing access to preferred body temperature (29 °C, "Warm" treatment) or imposing cold temperature (17 °C, "Cold" treatment). We predicted that (1) baseline CORT concentrations should be higher in individuals accessing preferred temperature because of higher metabolic rate, and (2) stress-induced CORT concentrations – the rate at which CORT is secreted following a stressor – should be higher in warm individuals because of higher CORT secretion and diffusion rates.

2. Materials and methods

2.1. Study individuals

Children's pythons (*A. childreni*) are medium-sized (up to 1.2 m snout vent length, 600 g body mass) constricting snakes that occur in Australian wet-dry tropics (Wilson and Swan, 2003). Preferred temperature has been previously studied in this species and is relatively high in non-reproductive individuals (T_{set}: ~29 °C; Lourdais et al., 2008). Snakes used in this study (16 non-reproductive females) were part of a captive colony of Children's pythons maintained in the Centre d'Etudes Biologiques de Chizé, France. Snakes were housed individually in plastic cages (35.5 × 63 × 15 cm) containing a shelter and providing a thermal gradient (25 to 35 °C). We kept experimental animals fasted before the experiment to avoid any influence of digestive activity on CORT concentrations. Individuals were kept fasted two weeks prior to the experiment and water was provided *ad libitum*.

2.2. Experimental protocol

We manipulated body temperatures applying ecologically relevant conditions. Snakes were exposed to thermal treatment and sampled (baseline and stress-induced CORT) within 48 h. The two thermal treatments were:

"Warm": 8 snakes were housed in individual transparent boxes (35 × 25 × 12.5 cm) with a shelter and equipped with heating cables providing a thermal gradient and therefore access to preferred body temperature.

"Cold": 8 snakes were kept in similar boxes without heating cables and therefore exposed to ambient room temperature (Mean ± SE, 17.03 ± 0.01 °C) well below T_{set}. This temperature is suboptimal but can be seasonally encountered in the field (Madsen and Shine, 1999).

The experiment was conducted in 2012 during two sessions (session 1: July 17–18, session 2: August 29–30). Each snake was randomly assigned to a thermal treatment. The experimental groups were switched during the second session, so that, each individual was sequentially exposed to both treatments. Snake were exposed to the usual housing condition (see above) between sessions. Individuals from both experimental sequence were similar in BM (ANOVA, $F_{1,14} = 0.13$, $P = 0.729$) and SVL (ANOVA, $F_{1,14} = 0.48$, $P = 0.498$).

2.3. Temperature sampling

From 24 to 48 h after the exposure to thermal treatment, each snake was handled to assess CORT concentrations. Skin surface temperature was recorded after capture using an infrared laser thermometer (Raytek Corporation, Santa Cruz, USA). Measurement procedure was similar to (Andrews, 2008) and recommendations from Hare et al. (2007) (emissivity set at 0.95 and the thermometer was oriented in-line with the body axis). We are fully cognizant that this method does not provide an accurate measure of internal temperature. It rather provides a simple (non invasive) measure of the thermal contrast between groups. Previous studies reported that skin surface temperature was strongly related to core temperature and these measures only differed from each other about 1.5 °C in small lizards (Carretero, 2012). In this study, the species is medium size and skin surface temperature is closely related to core temperature (Lourdais *per obs*).

2.4. Blood sampling and CORT assay

All snakes were bled according to a standardized capture/restraint stress protocol (Wingfield, 1994). Immediately after capture, an initial blood sample – 150 µl – was collected through cardiocentesis with a 1 ml heparinized syringe and a 27 gauge needle within approximately 3 min (Mean ± SE, 2.48 ± 0.17 min).

After blood collection, snakes were placed into an empty transparent plastic box without any shelter in their respective thermal conditions. For snake, the combination of being handled, bled and then placed in a new environment is known as a stressful situation

since they typically display increased CORT concentrations (Lourdais unpublished data). Two additional samples – 150 µl each – were collected respectively 30 min (Mean ± SE, 32.34 ± 0.31 min) and 60 min later (Mean ± SE, 61.81 ± 0.16 min). Immediately after sampling, the blood was centrifuged 3 min at 3000g. The plasma was separated, collected and stored at −28 °C. Plasma CORT concentrations were then determined at the CEBC by following a well-established radioimmunassay protocol (see Lormée et al., 2003 for details). Samples were run in two assays (intra-assay variation: 7.07%, inter-assay variation: 9.99%). All blood samples were collected between 8:00 and 18:00 and we included this factor (i.e. time of day) in our statistical analyzes since CORT concentrations could be subjected to nycthemeral variations (Cree et al., 2003; Dauphin-Villemant and Xavier, 1987).

2.5. Statistical analyses

All statistical analyses were performed with R software (R Development Core Team, version 2.13.1). First, we built a global model (i.e., model 1) using linear mixed model (lme, package nlme) to assess the effect of the thermal treatment on CORT concentrations. Thermal treatment (warm vs. cold) and sampling time (0, 30 and 60 min post handling) were treated as explanatory factors and the time of day as linear covariate. Female identity was taken in account as a random factor because of repeated samples (two sessions and three blood sampling per session). We used pairwise *post hoc* Tukey tests (lsmeans, package lsmeans) to compare the impact of thermal treatment for each blood sample (0, 30 and 60 min post handling). We did not adjust stress-induced CORT concentrations with baseline CORT concentrations since it is recognized as more relevant to consider absolute values (Romero, 2004).

Second, we built separate linear models (package stat) to test the influence of baseline CORT on stress-induced CORT (30 and 60 min post handling) for each treatment (i.e., model 2: cold treatment; model 3: warm treatment). Sampling time (30 and 60 min post handling) was treated as explanatory factor and baseline CORT as linear covariate.

We used the same design to test the influence of temperature on CORT concentrations. For each thermal treatment (i.e., model 4: cold treatment; model 5: warm treatment), sampling time (0, 30 and 60 min post handling) was treated as explanatory factor and time of day and temperature as linear covariates. We used the residuals of the relation between CORT concentrations and time of the day to graphically present our results. In all analyses, plasma concentration of CORT was Log_{10} transformed to normalized data (Shapiro–Wilk test all, $P > 0.05$).

3. Results

3.1. Effect of the thermal treatment on CORT concentrations

The two thermal treatment resulted in contrasted temperature (ANOVA, session 1: $F_{1,14} = 351.9$, $P < 0.001$, Mean ± SE, cold group: 16.6 ± 0.15 °C, warm group: 29.2 ± 0.65 °C, session 2: $F_{1,14} = 557.2$, $P < 0.001$, cold group: 17.3 ± 0.21 °C, warm group: 29.4 ± 0.47 °C).

CORT concentrations were significantly affected by the thermal treatment (model 1, $F_{1,72} = 29.3$, $P < 0.0001$) and sampling time (model 1, $F_{2,72} = 45.3$, $P < 0.0001$). At baseline, individuals from the cold treatment had higher CORT concentrations than those from the warm treatment (model 1, baseline: Tukey test, $P < 0.001$, Fig. 1). We found similar results with stress-induced concentrations although the difference was significant only at 30 min post handling (model 1, stress-induced, 30 min post handling: Tukey test, $P < 0.01$, 60 min post handling: Tukey test, $P = 0.149$; Fig. 1). There was no interaction between thermal treatment and sampling time on CORT concentrations (model 1, $F_{2,72} = 0.9$, $P = 0.423$), suggesting a similar kinetic of the CORT stress response for both thermal treatments. Finally, we found a significant influence of time of day on CORT concentrations (model 1, $F_{1,72} = 14.0$, $P < 0.001$), with CORT concentrations increasing during the day.

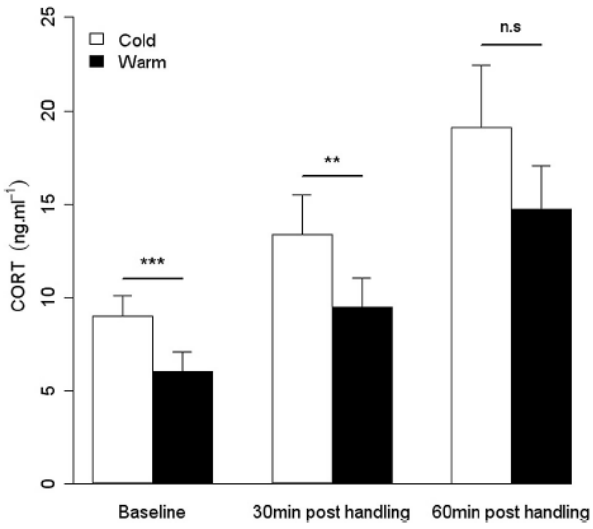

Fig. 1. Comparison of baseline and stress-induced (30 and 60 min post handling) plasma CORT concentrations between snakes maintained at cold (cold treatment: open bars, Mean ± SE, 18.0 ± 0.14 °C) and warm (warm treatment: filled bars, Mean ± SE, 29.6 ± 0.20 °C) temperatures. Bars represent means (±SE) of CORT concentrations (ng ml^{-1}). ***$P < 0.001$, **$P < 0.01$, n.s (non-significant).

Stress-induced CORT was significantly influenced by baseline CORT concentration within cold treatment (model 2, 30 min post handling: $t = 4.3$, $P < 0.001$; 60 min post handling: $t = 3.7$, $P < 0.001$; Fig. 2) and warm treatment (model 3, 30 min post handling: $t = 3.9$, $P < 0.001$; 60 min post handling: $t = 2.2$, $P = 0.035$; Fig. 2). Baseline CORT concentration positively influences stress-induced CORT concentrations (30 and 60 min post handling) for all individuals (Fig. 2).

3.2. Influence of temperature on CORT concentrations

Within the cold treatment, baseline and stress-induced CORT concentrations were negatively related to temperature (model 4, baseline: $t = -2.1$, $P = 0.045$; 30 min post handling: $t = -2.8$, $P = 0.008$; 60 min post handling: $t = -2.1$, $P = 0.043$; Fig. 3). As above, CORT concentrations increased during the day (model 4, $t = 3.4$, $P = 0.002$).

Baseline CORT concentrations were marginally positively correlated with temperature for the warm treatment (model 5, $t = 1.7$, $P = 0.089$; Fig. 4). Stress-induced CORT concentrations were not correlated with temperature (model 5, 30 min post handling: $t = 0.3$, $P = 0.747$; 60 min post handling: $t = 0.2$, $P = 0.855$; Fig. 4). As above, CORT concentrations increased during the day (model 5, $t = 2.0$, $P = 0.049$).

4. Discussion

We expected a positive relationship between temperature and CORT concentrations due to positive effect of temperature on metabolism (Cree et al., 2003; Landys et al., 2006; Tyrrell and Cree, 1998). Our study on the Children's python highlights complex effect of temperature on CORT concentrations and contradicts our predictions. First, we found that individuals from the cold treatment had

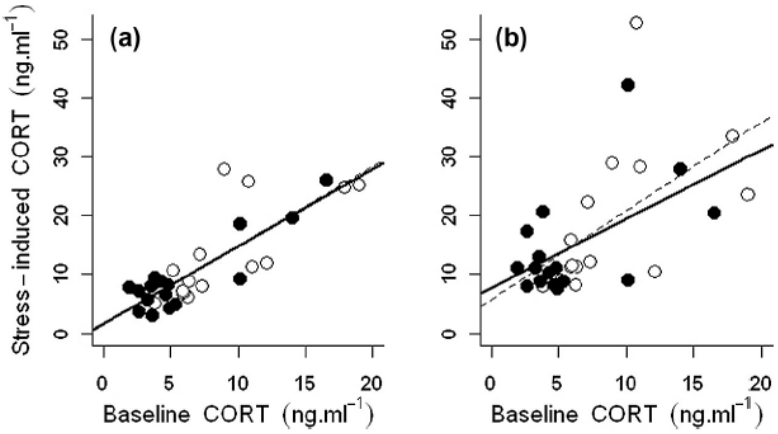

Fig. 2. Influence of baseline CORT concentration on stress-induced CORT concentration in python from cold treatment (open circle, dashed line) and warm treatment (filled circle, solid line) at (a) 30 min post handling and (b) 60 min post handling.

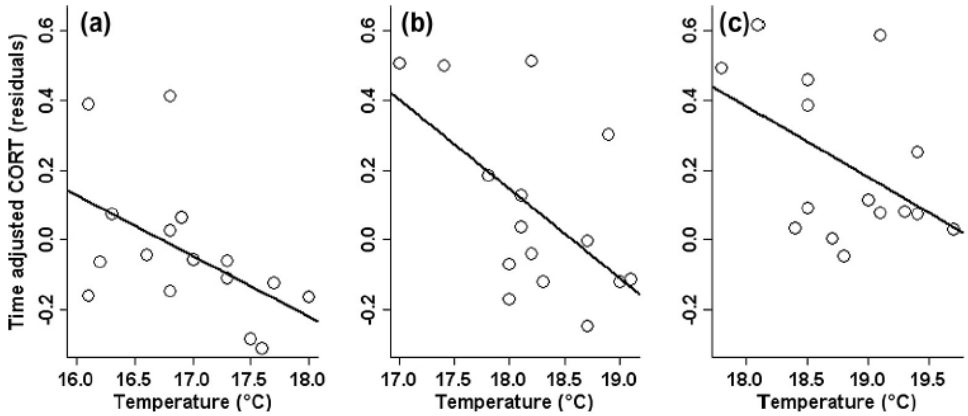

Fig. 3. Influence of temperature on time adjusted CORT concentrations (residuals from the relation between CORT and time of day), in python from cold treatment at (a) baseline concentration, (b) 30 min post handling and (c) 60 min post handling.

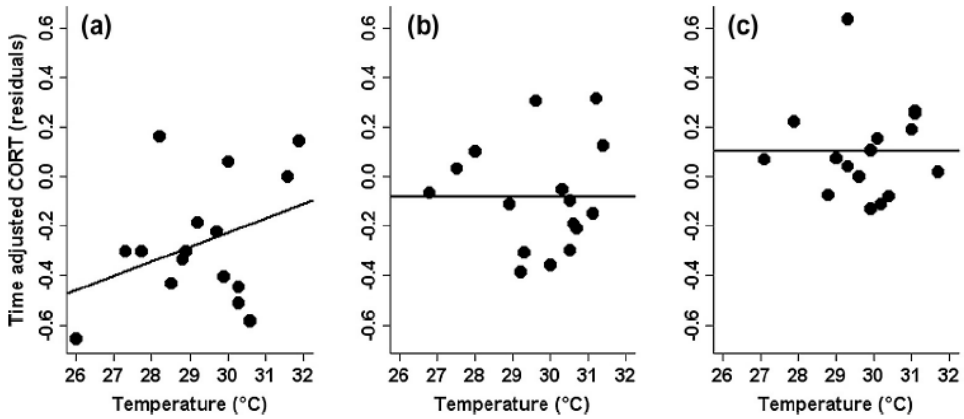

Fig. 4. Influence of temperature on time adjusted CORT concentrations (residuals from the relation between CORT and time of day), in python from warm treatment at (a) baseline concentration, (b) 30 min post handling and (c) 60 min post handling.

higher baseline CORT concentrations than those from the warm treatment. Second, the kinetic of the CORT stress response was not slowered at 17 °C. Such counter-intuitive result could be interpreted as a cold compensation mechanism that stimulates physiological and behavioural response (Landys et al., 2006; McEwen and Wingfield, 2003, 2010; Romero et al., 2009). Our study underlines that short term changes in body temperature affects absolute CORT concentrations (baseline but also stress-induced concentrations) and should therefore be considered when studying CORT concentrations in reptile species (Cree et al., 2003; Jessop et al., 2000; Jones and Bell, 2004; Romero and Wikelski, 2006; Tyrrell and Cree, 1998; Woodley et al., 2003).

How does temperature mechanistically affect the CORT stress response?

In our experiment, snakes were kept in a cold environment (17 °C) from one to two days before sampling. Our predictions were not supported which therefore advocate for alternative hypotheses to the positive relationship between CORT concentration and temperature. For example, cold temperature may have been perceived as a perturbation and, as a result, elicited an activation of the HPA axis. Although such activation might have been progressive because of reduced metabolism and physiological processes, it resulted in increased CORT concentrations within 48 h of continuous exposure. Therefore, the cold treatment may have been stressful for our captive snakes possibly because of altered locomotor performance and inducing increased in CORT concentrations.

In vertebrates, acute environmental stressors influence CORT concentrations and favour the activation of an "emergency" life-history stage (Wingfield et al., 1998) promoting immediate survival (Sapolsky et al., 2000; Wingfield et al., 1998; Wingfield and Sapolsky, 2003). We found that pythons from the cold group had higher absolute stress-induced CORT concentrations than those from the warm group. Remarkably, this suggests that the activation of the HPA axis, the secretion of CORT and its diffusion is not significantly impaired by cold temperature in this species and is less thermally sensitive than other physiological process (Angilletta, 2009; Bennett and Dawson, 1976; Stevenson et al., 1985) and all literature therein]. Accordingly, such high concentrations of CORT in response to short term exposure to low temperature have already been reported in other ectothermic organisms such as fish species (Chen et al., 2002). Temperature compensation has been largely studied in ectotherms after prolonged exposure to a temperature change (Angilletta, 2009; Huey and Berrigan, 1996). For instance, some species display a shift in metabolic rate or a modified thermal sensitivity of performance in response to low temperature (Glanville and Seebacher, 2006; Hare et al, 2010). Such adjustments aim at maintaining functional balance in a new thermal environment. Such integrated acclimation processes usually requires several days to reach a new steady state (Lucassen et al., 2003).

Alternatively, our results may simply reflect more passive effects of temperature on CORT physiology. Indeed plasma CORT concentrations do not only depend on secretion by the adrenal glands and circulation of CORT through the organism (Romero, 2004; Sapolsky et al., 2000). For example, clearance plays a major role in determining circulating CORT concentrations because it determines the rate at which CORT is catabolised. In addition, corticosteroid-binding globulin (CBG) is essential to consider: a large percentage of CORT is bound to CBGs in the blood and it seems that only free CORT can be active and catabolised (Breuner and Orchinik, 2002; Jennings et al., 2000; Mommsen et al., 1999; Siiteri et al., 1982). CORT also binds to receptors directly on tissues and such binding will affect the clearance rate. Blood concentration of CORT also depends on CORT itself through a negative feedback (Romero, 2004). Importantly, these different mechanisms are likely to be temperature-dependent but may show contrasted thermal reaction norms and therefore influence circulating concentrations. For instance, the CORT catabolism in the liver is certainly reduced when body temperature rapidly decreases due to lower enzymatic activity. Similarly, low temperature should affect the affinity of CORT to CBG and receptors and, therefore, the amount of CORT that can be cleared out of the blood. The influence of temperature on these physiological processes has never been investigated in reptiles and further studies are required to better understand the role of temperature.

Non-linear effect of temperature on CORT concentrations

Our results emphasize a complex relationship between temperature and CORT concentrations (baseline and stress-induced). That is, when snakes were imposed cold temperature, a strong negative relationship was detected between CORT concentrations (baseline and stress-induced) and temperature. In turn, this negative relationship was not supported when snakes had access to preferred body temperature (warm treatment). A positive effect of temperature on baseline CORT was found but the relation was marginal. High temperatures (upper limit) are known to be stressful in ectotherms and may therefore elicit an activation of the HPA axis. However, individuals in the warm treatment were at preferred temperature and therefore well below critical thermal maximum (Lourdais et al., 2008). Therefore, our study does not allow assessing the impact of high temperature on CORT. Still, the non-linear relationship suggests complex thermal dependence and the relation between CORT concentrations and temperature should be investigated over species thermal tolerance range.

Elevated CORT concentrations reflect suboptimal temperature in pythons?

Thermal sensitivity of performance attracted considerable interest in ectotherms (Angilletta et al., 2002; Angilletta, 2006; Martin and Huey, 2008; Somero et al., 1996). For instance, relative performance curves are typically asymmetric with a progressive increase toward optimal performance breadth (Angilletta, 2009) and then a rapid decline when body temperature gets closer to critical temperature maximum (Martin and Huey, 2008). Imposed low temperatures are likely to be a constraint resulting in impaired locomotion capacities and lower physiological performances (Angilletta, 2009; Huey and Stevenson, 1979; Huey and Kingsolver, 1989). Defensive response and ability to escape from predators are also dramatically affected by temperature (Cury de Barros et al., 2010; Herrel et al., 2007; Hertz et al., 1982). Hence, the increase of CORT concentrations in response to suboptimal temperature might stimulate compensating mechanisms, either behavioural (increase activity to evade poor thermal conditions) or physiological (increased metabolism) (AlKindi et al., 2003; Belliure et al., 2004; Preest and Cree, 2008). Importantly, this would help the individuals to maintain an alert state. In support with this hypothesis, CORT is known to influence on metabolic rate in reptiles (DuRant et al., 2008; Guillette et al., 1995; Preest and Cree, 2008) and likely stimulates locomotor activity (Belliure and Clobert, 2004; Belthoff and Dufty, 1995, 1998; Dauphin-Villemant et al., 1990; Silverin, 1997). Moreover, some reptile species display small metabolic increase at low temperature (Aleksiuk, 1971; Hare et al., 2010) and therefore at short term, CORT might be one mechanism involved in such compensation. In reptiles, metabolism is primarily driven by body temperature but CORT might allow fine-tuned regulation of metabolic rate independently of temperature.

Overall, our study demonstrates that CORT concentrations respond to short term temperature variation. Elevated baseline and stress-induced CORT concentrations may both promote immediate survival (Wingfield and Sapolsky, 2003) under constraining thermal conditions. Here, we hypothesize that the endocrine response of python could be a short term compensation to suboptimal temperature (Lance and Elsey, 1999; Li et al., 2011; Sykes and Klukowski, 2009). However, CORT concentrations should also depend on the duration, and the intensity of thermal stress. In addition, it is crucial to note that the co-occurrence of multiple stressors (cold temperature, predation risk) may be particularly demanding (very

high allostatic load *sensu* (Landys et al., 2006; McEwen and Wingfield, 2003, 2010; Romero et al., 2009) and CORT secretion may therefore depend on multiple factors. For instance, the availability of a shelter and temperature have recently been shown to interact to affect CORT concentrations in another snake species (Bonnet et al., 2013). Further studies are required to specifically test the effects of multiple stressors on the CORT stress response.

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ARTICLE 8

Effects of water deprivation on baseline and stress-induced corticosterone levels in the Children's python (*Antaresia childreni*)

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ABSTRACT

Corticosterone (CORT) secretion is influenced by endogenous factors (e.g., physiological status) and environmental stressors (e.g., ambient temperature). Heretofore, the impact of water deprivation on CORT plasma levels has not been thoroughly investigated. However, both baseline CORT and stress-induced CORT are expected to respond to water deprivation not only because of hydric stress per se, but also because CORT is an important mineralocorticoid in vertebrates. We assessed the effects of water deprivation on baseline CORT and stress-induced CORT, in Children's pythons (*Antaresia childreni*), a species that experiences seasonal droughts in natural conditions. We imposed a 52-day water deprivation on a group of unfed Children's pythons (i.e., water-deprived treatment) and provided water ad libitum to another group (i.e., control treatment). We examined body mass variations throughout the experiment, and baseline CORT and stress-induced CORT at the end of the treatments. Relative body mass loss averaged ~10% in pythons without water, a value 2 to 4 times higher compared to control snakes. Following re-exposition to water, pythons from the water-deprived treatment drank readily and abundantly and attained a body mass similar to pythons from the control treatment. Together, these results suggest a substantial dehydration as a consequence of water deprivation. Interestingly, stress-induced but not baseline CORT level was significantly higher in water-deprived snakes, suggesting that baseline CORT might not respond to this degree of dehydration. Therefore, possible mineralocorticoid role of CORT needs to be clarified in snakes. Because dehydration usually induces adjustments (reduced movements, lowered body temperature) to limit water loss, and decreases locomotor performances, elevated stress-induced CORT in water-deprived snakes might therefore compensate for altered locomotor performances. Future studies should test this hypothesis.
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1. Introduction

Organisms cope with environmental variations using behavioural and physiological adjustments that are largely mediated by endocrine regulation (McEwen and Wingfield, 2003; Romero et al., 2009; McEwen and Wingfield, 2010). For instance, when an environmental change occurs, the Hypothalamic–Pituitary–Adrenal (HPA) axis is activated which results in a rapid and important release of corticosterone (CORT) in the blood (Wingfield et al., 1998; Wingfield, 2003; Wingfield et al., 2011; Wingfield, 2012, 2013). This increase in circulating CORT levels can activate and/or inhibit specific physiological and behavioural responses to allow the organisms to cope with potentially challenging situations (Sapolsky et al., 2000; Romero, 2004; Landys et al., 2006). Diverse environmental factors, such as variations of temperatures (Tyrrell and Cree, 1998; Cree et al., 2003; Romero and Wikelski, 2006; Dupoué et al., 2013), food availability (Woodley et al., 2003; Jenni-Eiermann et al., 2008), predation pressure (Engh et al., 2006; Remage-Healey et al., 2006; Berger et al., 2007), or anthropogenic disturbances (Wikelski et al., 2001; Romero and Wikelski, 2002; Partecke et al., 2006; Crino et al., 2011), have all been shown to affect CORT levels in vertebrates (Sapolsky et al., 2000; Romero, 2002; Landys et al., 2006).

Surprisingly, the potential effect of one of the most essential resources – water – has not been investigated in this framework (Schmidt-Nielsen, 1997). Further, water acquisition and maintenance of water balance remain overlooked in ecological field studies (see Bonnet and Brischoux, 2008 for a review). Yet, deviation of the osmotic balance, especially dehydration, is a typical example of a particularly challenging situation for many organisms. Dehydration triggers behavioural and physiological adjustments to limit water loss and/or to restore water balance. Typical examples involve major changes in activity (Pough et al., 1983; Crowley, 1987; Lorenzon et al., 1999; Bonnet and Brischoux, 2008; Lillywhite et al., 2008a; Davis and DeNardo, 2009; Hetem et al., 2010), shifts in thermoregulatory strategies (Crowley, 1987; Bradshaw, 1997; Lorenzon et al., 1999; Angilletta et al., 2010; Köhler et al., 2011), or reduced metabolic rates (Kennett and Christian, 1994; Tieleman et al., 2002; Muir et al., 2007). Prolonged

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dehydration might lead to severe physiological alteration and ultimately to death (Whitehead et al., 1996). Thus, acute or long-lasting dehydration can be considered as life-threatening. In addition, CORT is presumed to act as a mineralocorticoid, and thus to play a role in the maintenance of osmotic balance (Duggan and Lofts, 1978; Dauphin-Villemant and Xavier, 1986; Bentley, 2002; McCormick and Bradshaw, 2006). Taken together, these elements suggest that water deprivation can significantly affect secretion of CORT, not only as a result of a stressful physiological state (i.e., dehydration) but also because CORT can play a role in osmotic balance.

Snakes are well suited to investigate such questions. First, limited dispersal ability regularly exposes snakes to local conditions (Aragon et al., 2010). Therefore, they would be liable to face difficulties to find fresh water during drought periods. Second, snakes display marked behavioural and physiological adjustments to limit water loss when dehydrated (Silveira et al., 1992; Ladyman and Bradshaw, 2003; Ladyman et al., 2006). Third, several snakes species inhabit (at least temporarily) environments where fresh water acquisition represents a major challenge, such as wet–dry tropics, deserts or oceanic areas. Finally, snakes are particularly tolerant to strong deviation of their water balance, and thus allow creating marked contrasts under experimental conditions.

In this study, we investigated the influence of water deprivation on both baseline CORT and stress-induced CORT in a snake species from Australian wet–dry tropics, the Children's python (*Antaresia childreni*). We experimentally created a hydric contrast between two groups of snakes (control versus water-deprived) to examine the consequences of water deprivation on CORT secretion. Specifically, we expect baseline CORT to be higher in water-deprived snakes, both as a result of hydric stress and the putative mineralocorticoid role of CORT (Duggan and Lofts, 1978; Dauphin-Villemant and Xavier, 1986; Bentley, 2002; McCormick and Bradshaw, 2006). In response to an additional stressor (capture and stress protocol), we expected both groups to show a similar stress response. However, the maximal stress-induced CORT level should be higher in water-deprived snakes, as a result of an already elevated baseline level (Dupoué et al., 2013).

2. Materials and methods

2.1. Study species and husbandry

Children's pythons (*A. childreni*) are medium-sized (up to 1.2 m snout vent length, 600 g body mass) constricting snakes that occur in Australian wet–dry tropics, areas characterised by a seasonal alternation of wet (rainfall > 200 mm per month from December to March) and dry (<5 mm per month from June to August) periods (Wilson and Swan, 2003). During dry seasons, pythons can face significant water deprivation. Snakes used in this study (15 females and 12 males) were part of a captive colony of Children's pythons maintained in the Centre d'Etudes Biologiques de Chizé, France. Snakes were housed individually in plastic cages (35.5 × 63 × 15 cm) containing a shelter and displaying a thermal gradient (25 to 35 °C) to allow free thermoregulation. We kept experimental animals fasted one month before experiments and checked that females were in a non-reproductive state to avoid any influence of digestive activity or reproductive state on CORT levels.

2.2. Experimental protocol

Pythons were assigned randomly within sex to the control or water-deprived hydric treatment (N = 14 and 13 respectively). In each treatment, we distributed males and females evenly (N = 6 males in each treatment, N = 8 and 7 females for control and water-deprived group respectively; Table 1). Individuals from the water-deprived group were exposed to a 52-day period of water deprivation while snakes from the control group were provided water ad libitum.

Snakes from both experimental groups were similar in terms of body mass (BM) (ANOVA with treatment as the predictor and BM as the dependent variable, males: $F_{1,10} = 0.2$, $P = 0.694$; females: $F_{1,13} = 0.2$, $P = 0.699$) and snout–vent length (SVL) (same design than above but with the SVL as the dependent variable, males: $F_{1,10} = 0.2$, $P = 0.666$; females: $F_{1,13} = 0.4$, $P = 0.519$).

Although not as precise as total body water or plasma osmolality quantifications, BM is an integrative variable to assess dehydration in squamate reptiles (see DeNardo et al., 2004; Lillywhite et al., 2008a,b, 2012). We recorded BM weekly (eight measures over the experiment). These data were used to compare the pattern of BM loss through time (henceforth "Time") between our two experimental treatments. At the end of treatment, water-deprived snakes were provided water ad libitum immediately after the blood sampling (see below). We subsequently recorded snake BM 4 and 6 days after re-exposition to water. These additional measures were used to calculate the amount of water drunk in water-deprived snakes, as well as the corresponding proportion of their BM deficit (%, see Lillywhite et al., 2008a).

2.3. Blood sampling and CORT assay

Pythons were randomly selected in order to mix sexes and experimental treatments during the blood sampling sessions. All blood samples were drawn by cardiocentesis using a 1 mL syringe and a 27-gauge needle that was heparinized. A first blood sample (150 μL) was collected within approximately 3 min post capture (mean ± SE, 2.12 ± 0.13 min, range: 1.02–3.67 min) in order to measure baseline CORT level (Romero and Wikelski, 2001). We used a standardized capture/restraint stress protocol, slightly modified from Wingfield (1994) (Dupoué et al., 2013): snakes were placed in a transparent plastic box (35 × 25 × 12.5 cm) without any refuges at room temperature (mean ± SE, 23.2 ± 0.1 °C). A second sample (150 μL) was collected 1 h later (mean ± SE, 61.88 ± 0.24 min, range 60.80–66.25 min). Preliminary investigations on this species suggested that this protocol allowed initiating the CORT stress response (Dupoué et al., 2013). Because body temperature can affect baseline CORT in reptile (Tyrrell and Cree, 1998; Cree et al., 2003; Dupoué et al., 2013), we recorded snake skin surface temperature with an infrared thermometer (Fluke 572 CF, Germany) before each blood sampling. Skin surface temperature should be closely related to core body temperature in this medium sized species (Dupoué et al., 2013) and the close focus of the thermometer (1 cm Ø) allows precise measurements.

Table 1
BM variation over the experiments for the two experimental treatments (control versus water-deprived). Initial BM and final BM correspond to the BM at the beginning and the end of the experiment. Absolute (g) and the relative (%) mass loss correspond to the difference between initial and final BM. Absolute (g) and relative (%) amount of water drunk was calculated in water-deprived snakes as the difference of BM before and after re-hydration. Deficit (%) corresponds to the relative proportion of water drunk compared to mass loss. Table shows means (±SE). See text for details.

Sex	Treatment	Initial BM (g)	Mass loss		Water drunk			Final BM (g)
			g	%	g	%	Deficit %	
F	Control (8)	484.8 ± 22.3	12.6 ± 2.4	2.6 ± 0.7	–	–	–	473.4 ± 21.4
F	Water-deprived (7)	495.5 ± 13.1	49.9 ± 4.6	10.1 ± 1.4	36.8 ± 3.4	8.4 ± 1.0	76.3 ± 8.7	482.3 ± 10.7
M	Control (6)	413.5 ± 27.8	23.9 ± 2.6	5.9 ± 0.5	–	–	–	387.7 ± 26.3
M	Water-deprived (6)	397.9 ± 26.7	49.1 ± 3.6	12.7 ± 1.0	26.5 ± 4.1	8.2 ± 1.7	55.1 ± 9.8	375.2 ± 24.3

Immediately after sampling, the blood was centrifuged 3 min at 3000 *g*. The plasma was collected and stored at −28 °C. Plasma CORT assays were performed at the CEBC by following a well-established radioimmunoassay protocol (see Lormée et al., 2003 for details). Samples were run in two assays (intra-assay variation: 7.07%, inter-assay variation: 9.99%). All blood samples were collected between 8:00 and 18:00 h and we took into account this factor in our analyses since CORT can be subjected to daily variations in reptiles (Dauphin-Villemant and Xavier, 1987; Cree et al., 2003).

Preliminary examinations of the data revealed that two individuals (water-deprived males) displayed extremely high baseline CORT (41.05 and 35.56 ng·mL^{-1} respectively). Baseline CORT of these individuals was >390% higher than those of the other experimental individuals, and even >240% higher than stress-induced levels of the other experimental individuals. In addition, these two snakes displayed stress-induced values lower than their baseline levels. Importantly, none of them showed any other behavioural or physiological peculiarity (e.g., BM loss was similar to other individuals). As a consequence, we removed these individuals from our CORT analyses, although in practice, including them in our analyses did not change statistical effects.

2.4. Statistical analyses

All analyses were performed using R software (R Development Core Team, version 2.13.1). We used linear models (lm, package stat) and linear mixed model (lme, package nlme) when analyses required us to take snake identity into account as a random factor. We checked that all our models respected normality (Shapiro–Wilk test, all $P > 0.05$).

We assessed the effect of the experiment on the pattern of BM loss with linear mixed models with BM as the dependent variable, initial BM as linear covariate, and experimental treatment (control vs. water-deprived), time, sex and interaction between these three parameters as explanatory factors. Individual identity was set as a random factor. BM was compared between experimental treatments for each sex at each weighing time by using post hoc tests on the interaction (Treatment × Time × Sex) with no adjustments on P-value (package lsmeans). Adjusted values of BM presented in figures were calculated using lsmeans function (package lsmeans).

We analysed the effect of the experiment on baseline CORT levels with linear models with baseline CORT levels as the dependent variable, body temperature and time of day as linear covariates, and experimental treatment, sex and the interaction between treatment and sex as explanatory factors. Stress-induced CORT was analysed as the same design as above but with baseline CORT added as a linear covariate. The difference between stress-induced CORT and baseline CORT level was assessed using a linear mixed model with CORT level as dependent variable, and sample (baseline or stress-induced), experimental treatment and the interaction between sample and treatment as explanatory factors. Individual identity was set as a random factor. CORT levels were compared between samples for each treatment using post hoc tests on the interaction (Sample × Treatment) with no adjustments on P-value.

3. Results

3.1. BM variation

A significant mass loss was observed over the experiment (Fig. 1, Tables 1 & 2), and BM loss was dependent on the initial BM (Table 2) and experimental treatment (Fig. 1, Table 2). Water-deprived snakes lost mass at a higher rate than control snakes (post hoc, $P < 0.05$; Table 1, Fig. 1). We found a significant effect of the sex on BM variations (Fig. 1, Tables 1 & 2) with males losing a higher proportion of BM than females (Fig. 1, Tables 1 & 2).

During the re-hydration phase, water-deprived snakes were observed to drink readily and abundantly despite the disturbance associated with blood sampling and capture/restraint stress protocol. These individuals rapidly gained mass and eventually attained BM similar to control snakes (post hoc, males: $P = 0.454$; females: $P = 0.987$; Table 1, Fig. 1). Overall, the amount of water drunk accounted for 69.0% of the BM deficit accumulated during the water deprivation (SE: 6.9%, range: 31.9–106.3%; Table 1).

3.2. CORT levels

Baseline CORT level was influenced solely by body temperature and time of the day (Table 3). We did not detect any effect of the sex, treatment or their interaction on baseline CORT level (Table 3).

Stress-induced CORT level was significantly influenced by baseline level and treatment (Table 3, Fig. 2). Indeed, snakes from the water-deprived treatment showed a stress-induced CORT level significantly higher than control individuals (Table 3, Fig. 2). We did not detect any influence of sex, body temperature, time of the day or interaction between sex and treatment on stress-induced CORT level (Table 3).

Stress-induced CORT levels were significantly higher than baseline CORT levels in pythons from both treatment (post hoc, control: $P = 0.022$; water-deprived: $P < 0.001$; Fig. 2).

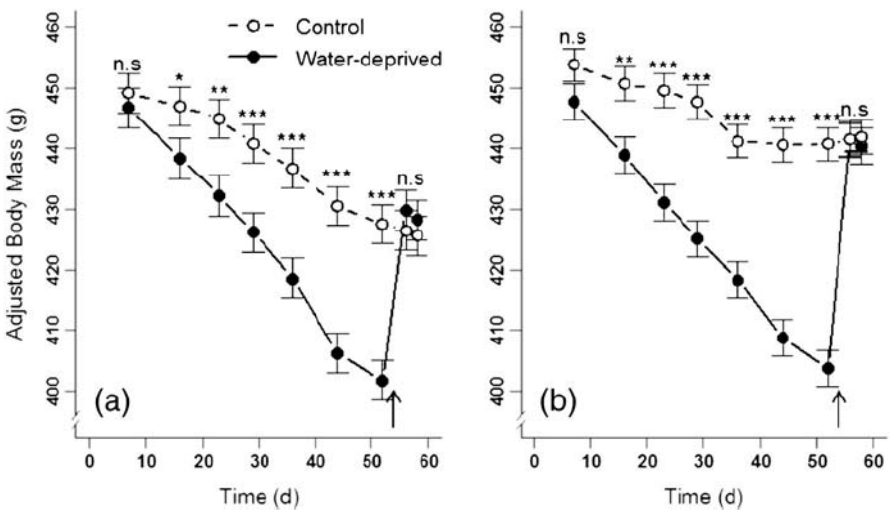

Fig. 1. Effect of the experimental treatment on BM (mean ± SE, control snakes: open circles, dashed line and water-deprived snakes: filled circles, full lines) in a) males and b) females. BM values were adjusted to initial BM. Arrows indicate the end of the dehydration period, and subsequent access to water ad libitum for water-deprived snakes. Significant effects of experimental treatment are symbolized: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and n.s (non-significant).

Table 2
Statistical model used to examine BM loss in control and water-deprived snakes. Individual identity was treated as random factor and initial BM was treated as linear covariate. See text for details.

Factor	df	F	*P*
Intercept	1	143,324.6	<0.0001
Initial BM	1	3595.8	<0.0001
Time	8	102.7	<0.0001
Treatment	1	39.5	<0.0001
Sex	1	4.7	0.042
Time × Treatment	8	39.3	<0.0001
Time × Sex	8	6.5	<0.0001
Treatment × Sex	1	1.5	0.233
Time × Treatment × Sex	8	0.5	0.845

4. Discussion

In this study, we investigated the effects of water deprivation on baseline and stress-induced CORT levels in the Children's python (*A. childreni*). Our experiment induced mass loss in both treatments, but this loss was significantly higher in water-deprived snakes (Fig. 1). Physiological (i.e., mass gain) and behavioural (i.e., drinking behaviour) patterns during re-hydration strongly suggest that most of the mass deficit was due to water loss (Fig. 1, Table 1), as suggested in other snake species (Lillywhite et al., 2008a,b, 2012). Overall, water-deprived snakes lost >10% of their initial BM, and although we did not measure total water volume or plasma osmolality, it likely resulted in a substantial dehydration (DeNardo et al., 2004). Interestingly, the dehydration caused by our experimental treatment influenced stress-induced but not baseline CORT levels.

Regardless of treatments, males tended to lose mass more rapidly than females (Table 1, Fig. 1). This pattern likely reflects sexual dimorphism in body plans and allocation strategies (Bonnet et al., 1998; Lourdais et al., 2006; Stahlschmidt et al., 2011). Indeed, *A. childreni* is a typical capital breeder (Lourdais et al., 2006). Prior to reproduction, females accumulate large lipid body reserves that allow sustaining the whole reproductive process (from vitellogenesis to egg brooding) during which these females cease feeding (Lourdais et al., 2013). In contrast, males are more muscular, and accumulate less fat stores (Lourdais et al., 2006). Therefore, during a fasting episode, males should rely on protein (muscle) catabolism and lose more mass while females should presumably rely on their lipid reserves. Still, even if males lost more mass, dehydration accounted for a significant portion of BM loss (Fig. 1, Table 1), with a similar pattern of rehydration between sexes.

We predicted baseline CORT levels to be higher in water-deprived snakes both as a result of hydric stress and the mineralocorticoid role of CORT. However and interestingly, we found similar baseline CORT levels between treatments suggesting alternative hypotheses. First, this pattern may reveal a putative caveat in our experimental design, insofar as the dehydration induced may not have triggered a physiologically stressful situation. This seems unlikely as water deprivation

Table 3
Statistical model used to examine baseline and stress-induced CORT levels in control and water-deprived snakes. "T_b" indicates body temperature. See text for details.

Variable	Factor	df	F	*P*
Baseline	T_b	1	12.7	0.002
	Time of day	1	9.0	0.007
	Treatment	1	0.0	0.958
	Sex	1	1.4	0.256
	Treatment × Sex	1	0.0	0.938
Stress-induced	Baseline	1	34.6	<0.001
	T_b	1	1.9	0.183
	Time of day	1	0.4	0.559
	Treatment	1	9.4	0.007
	Sex	1	4.1	0.058
	Treatment × Sex	1	2.7	0.116

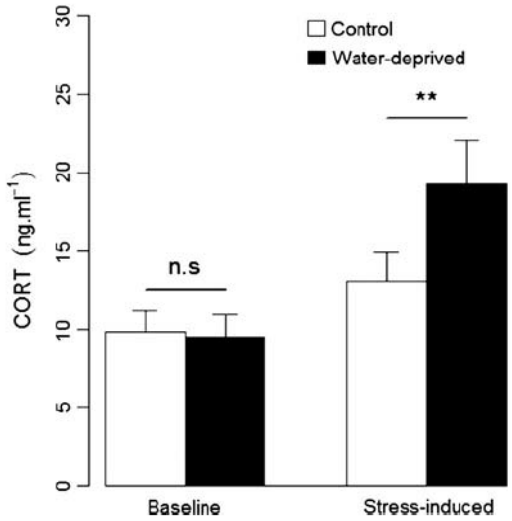

Fig. 2. Comparison of baseline and absolute stress-induced (60 min post handling) CORT levels between pythons (*A. childreni*) from control (open bars) and water-deprived (filled bars) experimental treatment. Bars represent means (±SE) of CORT levels (ng·mL^{-1}). Significant effects of experimental treatment are symbolized: ** $P < 0.01$ and n.s (non-significant).

occurred over a prolonged duration (>50 days), during which water-deprived snakes lost a significant proportion of their BM (>10%, Table 1). Relative BM loss was 2 to 4 times higher in water-deprived individuals (Table 1). In addition, the eagerness with which water-deprived snakes restored their water balance during the rehydration phase (we observed immediate drinking behaviour once water was available) strongly suggests that these individuals were in negative water balance. More likely, we suggest that water deprivation, at least at the level of dehydration induced by our experiment, does not trigger a HPA response resulting in baseline CORT level elevation. Although it is unknown whether more severe water deprivation would induce such a response, our results do not support mineralocorticoid role of CORT. Previous studies examining the relationship between CORT and hydric constraints reported conflicting results. For instance, water deprivation significantly increases baseline CORT levels in pregnant female, but not in male common lizards (Dauphin-Villemant and Xavier, 1986), suggesting complex interactions with sex and reproductive status on CORT levels. Actually, it seems that CORT is mostly involved in salt balance regulation (Holmes and McBean, 1964; Duggan and Lofts, 1978; Bradshaw and Rice, 1981; Bradshaw et al., 1984), rather than water balance per se (Bradshaw and Rice, 1981; Dauphin-Villemant and Xavier, 1986). Future studies should experimentally investigate the role of CORT in water versus salt balance regulation in order to clarify the mineralocorticoid function of CORT.

Why then did we find an increased stress-induced CORT in water-deprived snakes? The increase of stress-induced CORT concentration in pythons from the water-deprived treatment could be the result of a decrease of body fluids but this explanation seems unlikely since baseline CORT was not affected by water deprivation. More likely, we hypothesise that the reason probably lies in the behavioural and physiological adjustments known to occur in these taxa. Squamates reduce dehydration by reducing their movements (Davis and DeNardo, 2009) and secluding themselves in humid microenvironments such as subterranean caverns, burrows or underneath large rocks until environmental conditions improve (Bonnet and Brischoux, 2008; Lillywhite et al., 2008a). They also display a thermal depression and reduced metabolic rates (Ladyman and Bradshaw, 2003; Ladyman et al., 2006). All of these adjustments probably aim at reducing additional transcutaneous and ventilatory water loss. These water-saving strategies (especially lowered temperature and metabolism) likely reduce performances, including anti-predator responses (locomotion, defensive

behaviour) (Angilletta, 2009). In addition, dehydration has previously been shown to decrease locomotor performances in amphibians and lizards (Moore and Gatten, 1989; Wilson and Havel, 1989; Prates et al., 2013). To our knowledge, such effects have not yet been investigated in snakes and further studies are required to test for the effects of water deprivation on locomotor performances in snakes. According to our results, we hypothesise that the high CORT stress response we found in water-deprived snakes might positively compensate the alteration of performances by allowing these individuals to rapidly mobilize reserves, to increase metabolic rate, and thus to efficiently retaliate to a potential threat. Accordingly, CORT is known to significantly influence the metabolic rates in snakes (Guillette et al., 1995; DuRant et al., 2008; Preest and Cree, 2008), and the CORT stress response is known to increase when snakes are exposed to cold temperature (Bonnet et al., 2013; Dupoué et al., 2013).

More generally, our results show that baseline CORT might not respond to water deprivation (at least at the level of dehydration induced by our experiment), as it does for other vital resources (e.g., temperature and/or food availability, Jenni-Eiermann et al., 2008; Woodley et al., 2003). The role of CORT as a mineralocorticoid still needs to be clarified, and future studies should investigate CORT differential functions relative to water versus salt balance, two intricate aspects of the osmotic equilibrium (McCormick and Bradshaw, 2006). For instance, CORT manipulation of individuals kept in contrasted conditions (control versus water-deprived) should help to clarify these issues. Finally, the increased stress-induced CORT found in water-deprived snakes suggests a stronger stress response in reaction to lower performances, and such hypothesis can be directly tested.

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ARTICLE 9

Postglacial recolonisation in a cold climate specialist in Western Europe: patterns of genetic diversity in the adder (*Vipera berus*) support the central-marginal hypothesis

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Abstract

Understanding the impact of postglacial recolonisation on genetic diversity is of major interest. The central-marginal hypothesis (CMH) predicts a reduction in genetic diversity from the core of the distribution, or the refugium, to peripheral populations. While the CMH has received considerable empirical support, its broad applicability is still debated and alternative hypotheses predict opposite patterns. Using microsatellite markers, we analysed the genetic diversity of the adder (*Vipera berus*) in Western Europe to reconstruct postglacial recolonisation. Approximate Baysian Computation (ABC) analyses suggested a postglacial recolonisation from two routes: a western route from the Atlantic Coast up to Belgium and a central route from the Massif Central to the Alps. This cold-adapted species likely used two isolated glacial refugia in Southern France, in areas free of permafrost during the last glacial maximum. Adder populations further from putative glacial refugia have lower genetic diversity and, therefore, our results support the CMH. Finally, our study also illustrates the utility of highly variable nuclear markers, such as microsatellites, and ABC to test competing recolonisation hypotheses.

ARTICLE 10

Cold tolerance and sex-dependent hypothermia may explain winter sexual segregation in a farmland bird

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Abstract

Migration is an important event in the life history of many organisms, but considerable variation can occur within populations in the timing and final destination. A common form of sexual segregation in migratory birds is differential migration whereby females usually spend winter farther south than males. However, why sexes segregate during winter is yet to be clarified. The *body size* or *cold tolerance hypothesis* predicts that body size dimorphism modulates metabolic costs under cold climate. Using the Eurasian Skylark as a study model, we first checked that this species displays sexual differential migration the scale of France, and then we explored the body size hypothesis by experimentally testing the effect of low ambient temperature (T_a) on both metabolic rate and body temperature (T_b). Specifically we tested for sexual related differences in metabolic costs at low temperature, and energy saving mechanism (i.e. hypothermia or torpor). We found clear evidence for a differential migration strategy in Skylark wintering populations with a male-biased sex-ratio decreasing towards southern latitudes in France. Thermal treatments on captive birds resulted in a significant increase in metabolic rate when T_a decreases from thermo-neutral treatment (20°C) to thermogenesis treatments (6 and -5°C), with no evidence that the slope of the relationship differed between sexes. However, while both males and females reduced their T_b overtnight, females reduced their T_b at lower values when exposed to the lowest temperature treatment (i.e. -5°C). In addition, we found that individuals that reduced more their T_b , were those that lost less body weight during the night, suggesting that reducing T_b may help minimizing energy expenditure when nocturnal conditions become constraining. Our study therefore suggests which functional mechanisms may be involved in latitudinal segregation between sexes, and supports the hypothesis that sex-specific physiological strategies and thermal tolerance may explain latitudinal segregation between sexes.