Environmental constraints and genetic basis for the evolution of viviparity

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Abstract

The evolution of live-bearing (viviparity) from egg-laying (oviparity) is one of the most complex life history transitions in the animal kingdom. Yet, it has repeatedly and independently evolved across various animal groups, ranging across almost all major groups including invertebrates, fishes, amphibians, reptiles and mammals. In squamate reptiles, this transition has occurred about 100 times independently, more frequently than in any other vertebrate group. Why do transitions to viviparity occur? The evolutionary drivers for these transitions are not fully understood. Associated life-history advantages and disadvantages between parity modes are manifold, and might vary from case to case, but are difficult to compare in a controlled environment while minimizing phylogenetic effects. Transitions usually occur only into one direction, from an oviparous ancestor to a viviparous descendant. The eggshell is a complex feature, and once lost – according to Dollo's law of irreversibility – thought to be impossible to be regained. However, in squamates some cases have been discussed controversially that potentially re-evolved oviparity. Finally, in addition to understanding why transitions occur, we know very little about how transitions occur. Given that most transitions occurred in the deep evolutionary past, it is difficult to infer the genetic mechanism of how such complex traits originate. To understand how major evolutionary innovations and complex traits arise, it is indispensable to understand their genetic underpinnings.

Cold climate has been suggested to be the causal driver for transitions to viviparity. Egg clutch survival depends on the external environment, and if the environment is too cool, consequences are retarded embryonic development or even lethal freezing. Viviparous females have more control on the survival of their offspring by choosing the optimal temperature for embryonic development. The proportion of viviparous species increases with latitude, but whether viviparity evolved as a result of cooler temperatures in the first place is still unknown. I used molecular dating methods to infer at which time points transitions to viviparity occurred across the squamate tree and linked this to paleoclimatic data from the last 65 million years. I found that transitions generally occurred during cold and stable climatic conditions. This supports the prediction from the cold-climate hypothesis, but also shows for the first time that a stable environment is important for this evolutionary transition, linking life-history and cold-climate predictions.

The possibility of back-transitions from a viviparous ancestor to an oviparous descendant in squamate reptiles has been discussed rigorously. The re-evolution of a complex trait (such as the eggshell) is evolutionarily very unlikely, according to Dollo's law even impossible. However, a few exceptions in nature are known, such as the re-evolution of wings in stick insects or the re-evolution of sexuality in mites. Within common lizards, two lineages are egg-laying and four lineages are live-bearing. Earlier phylogenetic work suggested several hypotheses for parity mode evolution, including a single transition to viviparity, multiple transitions to viviparity and a reversal to oviparity. Using genome-wide SNP genotyping, I reconstructed the evolutionary history of parity modes and found that a single origin of viviparity and a reversal to oviparity was the most parsimonious phylogenetic scenario. The phylogeny was consistent with chromosomal data and supported as significantly more likely than alternative scenarios by topology testing and ancestral trait reconstructions. I suggest that common lizards represent a rare case of a reversal to oviparity, breaking Dollo's law of irreversibility.

The transition from oviparity to viviparity is complex, and comes with several changes in morphology, physiology and behaviour. Several life-history trade-offs between the two parity modes have been suggested, but analyses often suffer from confounding environmental and/or phylogenetic effects. It is predicted that viviparous species are larger in body size, produce less, but larger offspring with enhanced survival, and exhibit a larger reproductive burden. I tested if life-history traits in reproductively bimodal oviparous and viviparous common lizard (*Zootoca vivipara*) differed. I preformed this in a unique natural setting, a contact zone between both reproductive modes. The model system is almost ideal to study trade-offs between reproductive modes, as the transition is evolutionarily very young and the two parity modes occur in the same environment. I found that viviparous females have larger body sizes, smaller clutch size but higher offspring survival, and a higher reproductive burden. Contrary to predictions, offspring size and weight was smaller for viviparous females. This might indicate that viviparous common lizards are constrained for womb space. Almost all reproductive traits were significantly associated with body length in viviparous females, but not in oviparous females, suggesting a major impact of body size on reproduction in viviparous females. In general, I suggest that the link between reproductive life-history traits and reproductive mode is context dependent.

Identifying the genetic basis of complex evolutionary transitions is a major goal for evolutionary biologists. This will ultimately help us to understand how the biodiversity we observe today has been generated. However, most complex transitions occurred millions of years ago, making it difficult to discern causal genetic variants from accumulated genomic background noise. An approach to overcome this issue is admixture mapping, which makes use of the natural hybridization of lineages with different fixed phenotypes. Hybridization results in recombination of genetic variants and the disassociation of background noise from the causal genetic variation controlling a phenotype. In a unique hybrid zone between viviparous and oviparous common lizards, I detected loci associated with parity mode. I identified a few genomic regions associated with the trait, including two regions on the sex chromosome and the gene *EPAS1* on chromosome 3. A few SNPs were located next to immune response genes, possibly indicating modified immune interactions between mother and embryo in viviparous common lizards. Genome scans across all lineages supported that the sex chromosome is an important region for parity model control. Preliminary analyses suggested that more variants than expected are shared between the two oviparous lineages. However, whether the lineage with derived oviparity uses the same genetic mechanism as the lineage with basal oviparity remains to be investigated in more detail. For the first time, I identified the genetic basis of viviparity and fond it to be controlled by few genes of large effect.

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Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Abbreviations

AIC: Akaike information criterion

AICc: Corrected Akaike information criterion

ANCOVA: Analysis of covariance

ARD: All rates different

AU: Approximate Unbiased

B: Bayesian

bp: Base pairs

BSLMM: Bayesian sparse linear mixed model

CI: Confidence interval

CS: Clutch size

CM: Clutch mass

ddRADSeq: Double digest restriction-site associated DNA sequencing

EM: Average egg size

ESS: Effective sample size

ER: Equal rates

EtOH: Ethanol

GLM: Generalised linear model

GWAS: Genome-wide association study

HPD: Highest posterior density

KH: Kishino Hasegawa

LG: Linkage group

Masl: Meters above sea level

Mb: Mega bases

Mya: Million years ago

MCMC: Markov chain Monte Carlo

ML: Maximum likelihood

MP: Maximum parsimony

Mt: Mitochondrial

NGS: Next generation sequencing

Nuc: Nuclear

OS: Offspring size

OW: Offspring weight

QTL: Quantitative trait locus

PIP: Posterior inclusion probability

PVE: Phenotypic variance explained by genetic variants

PGE: Proportion of genetic variance explained by genetic variants with sparse effects

PP: Posterior probability

RCM: Relative clutch mass

ROM: Relative offspring mass

SEM: Scanning electron microscopy

SH: Shimodaira Hasegawa

SNP: Single nucleotide polymorphism

SVL: Snout vent length

SYM: Symmetrical rates

T: Temperature

TL: Tail length

Chapter 1: Introduction

1.1 How is biodiversity generated?

A major goal in biological sciences and specifically in evolutionary biology is to understand how the breadth of diversity we observe today has evolved over time. The most fascinating and complex adaptations have originated through changes in the genomic sequence. Sometimes, adaptations that had a great impact and a particular advantage over the ancestral forms not possessing that adaptation evolved in several lineages independently, such as the evolution of the eye (Gehring and Ikeo 1999) and the evolution of viviparity (Sites et al. 2011). Understanding how these key innovations arise involves several aspects: 1) Why did the adaptation evolve, or which factors drove the evolution of that adaptation? 2) How did the adaptation evolve, i.e. through which genomic changes? 3) Is there a general pattern as to why, when and how such adaptations evolve? In this thesis, using live-bearing reproduction as a striking example for a complex change that evolved independently from egg-laying across many animal groups, I want to tackle these questions.

1.2 Definition and origins of viviparity in vertebrates

Commonly, viviparity has been defined as giving birth to living young, while oviparity has been referred to as egg-laying. However, the binary categorization into viviparity and oviparity does not give justice to the complexity and variety of the different reproductive forms encountered in nature (see Figure 1). The evolution from oviparity to the most derived and complex forms of viviparity is rather a continuum and can be further classified into more categories (Shine and Bull 1979; Shine 1983; Blackburn 1992, 2006). Especially the degree of placental complexity varies substantially across vertebrate taxa, from simple water, inorganic ion and gas exchange to complex interactions and nutrient exchange between the placenta and the embryo (Thompson and Speake 2006). Since the embryonic development between different vertebrate groups varies substantially (e.g. amphibian metamorphosis vs. mammalian placentotrophic viviparity), a lot of confusion existed in the past when referring to what the terms viviparity and oviparity mean. For example, in some groups, researchers have made additional distinctions, such as differentiating between viviparity and ovoviviparity in amphibians. I believe that for the means of a global comparison, simplifying and restricting reproductive modes to oviparity and viviparity is the most sensible way (Blackburn 2000).

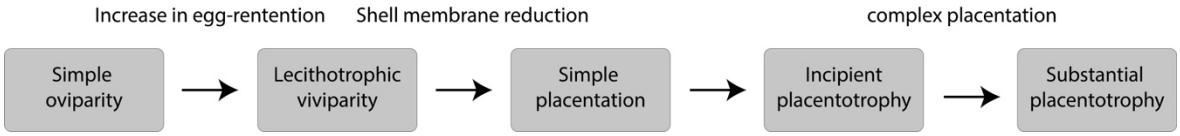

Figure 1.1 Oviparity-viviparity continuum in squamate reptiles modified from Blackburn 2006. The most simple form of viviparity is when egg-retention is prolonged, while all nutrients are still provided by the yolk within the eggshell. More complex forms of viviparity involve the loss of the eggshell and evolution of a placenta with more substantial exchange of nurtients between the embryo and its mother.

Viviparity has evolved more than a hundred times independently across vertebrates. With the exception of birds, turtles and crocodiles (Blackburn 1985; Blackburn and Evans 1986; Rafferty et al. 2013), all major vertebrate lineages evolved viviparity, including fishes (sharks, rays, ray-finned fishes, lobe-finned fishes; e.g. Wourms 1981; Wourms and Lombardi 1992), amphibians (salamanders, frogs and caecilians; e.g. Wake 1993; Kupfer et al. 2006), reptiles (snakes and lizards; e.g. Shine and Bull 1979; Blackburn 2006), and mammals (eutherians; e.g Rothchild 2003). With advances in molecular phylogenetics, the accuracy of detecting evolutionary transitions from oviparity to viviparity has improved, and thus far estimates of independent transitions from egg-laying to live-bearing go up to 132 times (Blackburn 1985, 1992, 1999b, 2006; Shine 1985).

1.3 Why did viviparity evolve?

The evolution of viviparity comes with several costs and benefits. The main advantages of viviparity are: 1) increased protection from predation, 2) more stable environment in mother's reproductive tract, 3) emancipation from finding and/or building a nest, 4) and a larger size at birth, whereas the disadvantages are 1) reduced fecundity (egg clutches are usually larger than the number of embryos that can be kept in the mother's reproductive system), 2) maternal death resulting in death of all offspring, 3) higher investment for mother (Shine et al. 1978; Wourms and Lombardi 1992). Since vertebrates encompass a wide range of different morphologies, life histories and can experience very different environments, the causes for the evolution of viviparity in particular taxa also differ. One view is that viviparity is beneficial when the environment is stable and intense competition prevails (Stearns 1976). Under these circumstances, a few well-developed offspring have higher survival chance than a lot, but much less developed offspring. However, because of the lack of clear-cut examples, this has rarely been tested (Wourms and Lombardi 1992). In ectotherm tetrapods, two main hypotheses exist to explain the evolution of viviparity. The most common explanation for the occurrence of viviparity, the cold-climate hypothesis, is based on a simple observation: The proportion of organisms that exhibit

viviparity increases with latitude and altitude (Weekes 1935; Sergeev 1940; Tinkle and Gibbons 1977; Shine 1983). Egg-layers are disfavoured in colder environments because their eggs take long to develop in the cold soil or might even die from exposure (Tinkle and Gibbons 1977), while the embryonic retention in the mother's uterus supposedly provides higher temperatures, increasing the offspring's developmental rate and reducing hatchling mortality. Temperature might be the most critical factor driving the evolution of viviparity in cold climates. However, Shine and Berry (1978) found that other factors associated with colder climates, such as measures of precipitation, evaporation and humidity, are not less likely in explaining the percentage of viviparous species in a given region. A common problem in finding the ultimate causes for the origin of any adaptation is that the environment of the extant taxa bearing that adaptation does not necessarily reflect the environmental conditions that drove the origin of the adaptation. Hence, as Shine and Berry (1978) also note, the subsequent radiation of viviparous taxa after viviparity evolved and the present-day distribution might be due to different causes than the initial causes that drove the origin of the viviparous life history.

Recent advances in phylogenetics and ancestral area reconstruction allows researchers to look into the past and reconstruct historic evolutionary events. In phrynosomatid lizards for example, viviparity evolved under cold conditions, especially in high-elevation tropical regions with throughout the year stable cold temperatures (Lambert and Wiens 2013). Using wide taxonomic sampling and high-resolution time divergence estimates in combination with climate reconstruction methodologies might give an answer to the intriguing question of which factors drive the evolution of viviparity.

Shine (1995) presented a not mutually exclusive alternative to the cold-climate hypothesis for the evolution of squamate viviparity, the maternal manipulation hypothesis. Embryonic development is highly influenced by incubation temperature and has a crucial impact on the offspring's fitness. The maternal manipulation hypothesis states that the incubation temperature within the maternal uterus is optimal for the development of the embryo. External temperatures can fluctuate substantially or just impose extreme conditions (too cold or too hot) to the developing egg, whereas internal temperatures can be behaviourally controlled in a better way and provide a more stable environment for the developing embryo. Over the last two decades, evidence for both the cold-climate hypothesis (Lynch 2009; Schulte and Moreno-Roark 2010; Rodríguez-Díaz and Braña 2012; Lambert and Wiens 2013; Pincheira-Donoso et al. 2013; Pyron and Burbrink 2014) and the maternal

manipulation hypothesis (Shine 1995; Webb et al. 2006; Rodríguez-Díaz and Braña 2011a) has accumulated. Recent molecular phylogenies (Pyron et al. 2011, 2013; Wiens et al. 2012; Pyron and Burbrink 2014; Zheng and Wiens 2016) and advanced methods in acquiring and reconstructing present and past climate will allow statistically thorough investigations on the generality of these hypotheses.

1.4 Re-evolution of oviparity – breaking Dollo's law?

Much controversy exists to whether transitions back from derived viviparity to oviparity are possible and have occurred in the animal kingdom. At any rate, transitions from oviparity to viviparity are far more common than transitions from viviparity to oviparity (Lee and Shine 1998; Griffith et al. 2015; King and Lee 2015a). This observation is in accordance with Dollo's law of irreversibility, which states that an ancestral (complex) character state that has been lost cannot be regained. Because of the loss of complex features during the evolution from oviparity to viviparity, back transitions to oviparity could be considered as an example of exception to Dollo's law (Lee and Shine 1998). Research within the last decade or so has shown that regaining complex features is indeed possible, but a rather rare phenomenon. Famous examples include the re-evolution of wings in a few wingless taxa (Whiting et al. 2003), shell coiling in gastropods (Collin and Cipriani 2003), and sexuality in asexual mites (Domes et al. 2007). Especially in squamates (snakes and lizards), in which evolutionary transitions between viviparity and oviparity are most abundant, several studies have attempted to or even claimed to find evidence for back transitions to oviparity (Fraipont et al. 1996; Lee and Shine 1998; Surget-Groba et al. 2006; Fenwick et al. 2012; Pyron and Burbrink 2014). However, except for one case (Lynch and Wagner 2010), firm evidence has not yet been presented (eg. Shine and Lee 1999; Fenwick et al. 2012; Wright et al. 2015). There also exists controversy on how back transitions are genetically achieved. In this context, time becomes an important component (Collin and Miglietta 2008). Once a phenotypic characteristic underlying a genetic basis has been lost by the occurrence of one or a few mutations, the likelihood that the character re-evolves should decrease with time, as more mutations will randomly accumulate and drive the organism's genome further away from its ancestral condition. Obviously, it is easier to rescue an ancestral condition, when the genome lacking the ancestral phenotype is still "genetically close" to the ancestral genome, and only one or a few back changes are necessary to restore the ancestral condition. Of

course, this only holds true when similar genetic pathways that lead to the ancestral phenotype are used. Alternatively - and independent of time - an organism might "invent" a new genetic pathway to reach to a similar phenotype. Such a re-evolution of a new genetic pathway might be considered as convergent evolution at the genetic level (Collin and Miglietta 2008). However, given the deep homology of key developmental pathways, this seems unlikely, even in the face of strong selection. For example, the structure of the eye has evolved several times independently in the animal kingdom; however, the genetic pathway controlling its development is highly conserved (Gehring and Ikeo 1999). A reason for this might be that complex phenotypic features often involve the recruitment and interactions of several genes. The evolution of genetic pathways used by several key morphological innovations, such as the eye, specification of the anterio-posterior body axis or the heart development (Holland 1999), seem to have arisen just once and been fixed early in the animal kingdom. Rarely are these pathways lost, and the phenotypic feature loss often probably involves small changes that disrupt the pathway. It might be evolutionarily "easier" to reactivate an existing genetic pathway, either by restoring the old genetic condition or by recruiting a new genetic element into the pathway than to evolve a new, independent genetic pathway.

Testing the re-evolution of a complex character requires a very well-resolved phylogeny with lineages exhibiting both presence and absence of the character in question and where ancestral character states can be determined with high confidence. One of the major problems in defining character states at evolutionary nodes is that if transitions occurred often, the confidence for frequent, unidirectional character-state transitions are predicted to cause parsimony to incorrectly conclude that character reversals have taken place (Cunningham 1999; Goldberg and Igić 2008). Recent advances in Maximum likelihood and Bayesian methods allow a more thorough statistical testing and should be used when characters are suggested to evolve under Dollo's law (Collin and Miglietta 2008).

1.5 How does viviparity evolve - Genetic mechanisms and developmental constraints of a major evolutionary transition

1.5.1 Genetics of complex evolutionary traits

Viviparity is a complex evolutionary transition (Thompson and Speake 2006). Until recently, it had not been possible to study the genetic basis of such major transitions in

non-model organims (Stapley et al. 2010; Ekblom and Galindo 2011; Savolainen et al. 2013). This was mainly due to the limited genomic tools available. Genetic maps and whole sequenced genomes necessary for such studies were almost exclusively available for humans and some domesticated plants and animals (Hall et al. 2010; Stranger et al. 2011; Zhang et al. 2012). However, with the development of new sequencing techniques and costs dropping dramatically, progress has been made towards understanding the genetic basis of complex traits and transitions in a few evolutionary model organisms. Examples include the adaptation to salinity tolerance in *Arabidopsis* (Baxter et al. 2010), the saltwater to freshwater transition in three-spined stickleback (Kingsley and Peichel 2007; Jones et al. 2012), adaptive divergence in fur colouration in mice (Hoekstra et al. 2006; Kingsley et al. 2009), wing pattern mimicry in *Heliconius* butterflies (Dasmahapatra et al. 2012; Huber et al. 2015), and beak shape in Darwin's finches (Lamichhaney et al. 2015). In sticklebacks, freshwater adaptation often results in the reduction of plated armour (Colosimo et al. 2005) and pelvic reduction (Shapiro et al. 2004; Chan et al. 2010); each of the traits is controlled by a single gene (plated armour reduction: *Eda*; pelvic reduction: *Pitx1*). In deer mice (*Peromyscus maniculatus*) natural selection has favoured the evolution of a lighter fur colouration on light-coloured soils, mainly by mutations in the *Agouti* gene (Kingsley et al. 2009; Linnen et al. 2013).

In the majority of cases, few genes of large effect were reported in these studies. However, the genetic architecture of the trait one seeks to infer depends on various aspects (Hoban et al. 2016), and we do not know yet whether the relatively simple genetic basis of major transitions reported so far is also reflected across a wider range of transitions in different organisms. In addition, the identification, impact and mechanism of multiple loci of small effect is much harder to achieve (Savolainen et al. 2013; Slate 2013). Two underlying issues are low sample size and low genomic resolution. These mean loci of smaller effect are difficult to detect and rare variants of large effect can be missed (Johansen et al. 2010; Stranger et al. 2011; Zeggini 2011). This is particularly true for complex quantitative traits which are controlled by tens to hundreds of loci with small effects (Flint and Mackay 2009). For example, a study using 183,727 human individuals found that 180 loci had an effect on height, but altogether only explained 20% of the heritable variation (height in humans is highly heritable, about 80%) (Lango Allen et al. 2010).

However, simple genetic architectures also have been found for some complex traits. In mice, for example, Weber et al (2013) found that burrowing behaviour is controlled by

only a few genomic regions with large effect (Weber et al. 2013). Two aspects of burrowing behaviour were measured; entrance tunnel length and the presence or absence of an escape tunnel. Entrance tunnel length was associated with three genomic regions of large effect, while the presence of an escape tunnel was associated with a single genomic region. This example demonstrates how a complex trait can be divided into separate phenotypic modules, each of them controlled by different genomic regions (Weber et al. 2013). The same has been observed for other complex phenotypic traits such as beak size in Darwin's finches (Mallarino et al. 2011), schooling behaviour in sticklebacks (Greenwood et al. 2013) and sexually dimorphic traits (Xu et al. 2012; Yang and Shah 2016).

Viviparity and oviparity are complex reproductive traits, involving several changes at the level of morphology, physiology, and behaviour (Thompson and Speake 2006; Murphy and Thompson 2011). In squamate reptiles, the two reproductive modes are not two discrete stages, but rather a continuum (Blackburn 1992, 2006). The genetic architecture for this complex evolutionary transition remains to be resolved.

1.5.1 Genetics of viviparous reproduction

Mainly through the advances coupled with Next-generation sequencing (NGS) techniques, we are just beginning to understand the general genetic and developmental mechanisms that are involved in viviparous reproduction. The evolution from oviparity to viviparity involves a few major developmental changes. The most essential adjustments to the loss of the eggshell before parturition are 1) transport of oxygen, calcium and water through the membrane that surrounds the embryo and 2) protection of the embryo from its mother's immunological response (Murphy and Thompson 2011).

In general, embryos hatched from an egg are relatively lighter in weight compared to live-born young (Thompson and Speake 2006). This implies that material transport from the mother to the embryo is upregulated in viviparous species, increasing with placental complexity. Water uptake is a main source of weight gain both in oviparous and viviparous species. While in oviparous species water is absorbed from the environment through the eggshell after oviposition (Packard et al. 1985), viviparous species supply their embryos with water via placental structures. Facilitated by aquaporins (Liu and Wintour 2005; Lindsay and Murphy 2007; Wooding et al. 2010; Brandley et al. 2012), water transport is

relatively higher in viviparous compared to oviparous species (Thompson et al. 2000; Thompson and Speake 2006). Transport of nutrients (lipids, proteins) and inorganic ions from mother to embryo are upregulated in viviparous species (Thompson et al. 2000; Thompson and Speake 2006). This is also supported by whole transcriptome studies, which show that carrier proteins transporting organic and inorganic nutrients from the mother to the embryo are upregulated during pregnancy in viviparous species (Bauersachs et al. 2008; Brandley et al. 2012). For example, solute carrier proteins (SLC gene family) are upregulated in pregnant bovines (Bauersachs et al. 2008) and also in a live-bearing pregnant lizard (Brandley et al. 2012).

A major problem that results from the loss of the protective eggshell is to avoid the embryo being repelled by its mother. Therefore, the maternal immune response must be suppressed to allow development of the allogenic embryo, a crucial step that has already been noted by Medawar in 1953. In mammals, maternal-foetal interactions are established through mechanisms of tolerance that allow cross-talk between mother and foetus (Saito 2001) and through reduced antigen presentation by the foetus during pregnancy (Moffett and Loke 2006). Cytokines and chemokines are molecules that are involved in the inflammatory response of the innate immune system. Because an excessive immune response can result in abortion, but an insufficient response might result in pathogen infestation, regulation of these molecules is crucial for the health of the mother and foetus. An essential role of cytokines and chemokines has been established for mammals (Saito 2001), and this also seems to be the case in viviparous squamates (Paulesu et al. 1995). For example, a combination of cytokines such as Interleukin-1 (IL-1) and TGF-β proteins are involved in maternal-foetal tolerance both in mammals (Simón et al. 1994; Jones et al. 2006) and in the viviparous lizard *Chalcides chalcides* (Paulesu et al. 1995; Paulesu 1997). Whereas a transcriptomic analysis of the live-bearing lizard *Chalcides ocellatus* could not detect IL1, another anti-inflammatory cytokine (IL11) was upregulated and pro-inflammatory cytokines (IL8 and IL15) were downregulated in pregnant compared to non-pregnant individuals (Brandley et al. 2012).

Another component to increase immune tolerance between embryo and mother is reduced antigen presentation by the foetus to hide it from the maternal immune response that could result in abortion (Moffett and Loke 2006). Major histocompatibility (MHC) loci encode molecules that target antigens and present these to the host's (mother's) immune system. Downregulating the transcription of these loci in embryonic tissue should therefore be

beneficial to the embryo and prevent its repulsion by the mother. A single study in the lizard *Chalcides ocellatus* has shown that MHC genes are significantly downregulated in the uterus of pregnant mothers (Brandley et al. 2012).

More general patterns of genome evolution that drive the evolution of viviparity and placentation can be acquired by the comparison of whole transcriptome data of the uterus from a range of (pregnant) animals with varying degrees of placentation and parity mode. This has been done for several mammal species such as human, cow, dog, horse, pig, armadillo, opossum, platypus (Lynch et al. 2012), Rhesus monkey (Liu et al. 2012), mouse (Chan et al. 2009), wallaby (Renfree et al. 2011), and elephant (Hou et al. 2012) as well as for the chicken (Zhang et al. 2014) and a single lizard (Brandley et al. 2012). A comparative RNA-Seq study on the evolution of pregnancy in mammals revealed that during the evolution from mammalian oviparity to more complex forms of viviparity including placentation, thousands of genes were recruited into endometrial expression (Lynch et al. 2011, 2012). The authors suggest that transposons are responsible for this vast increase in gene recruitment.

Compared to mammals, transcriptome studies in the substantially more variable reproductive biology of lizards and snakes are compulsory to get a better understanding of the underlying genetic architecture of viviparity. Reptiles offer an exciting opportunity for geneticists to study the evolution of viviparity: closely related species or even the same species vary in reproductive mode, reducing the phylogenetic noise in comparative genomic studies. In reproductively bimodal species, such as *Lerista bougainvilli*, *Saiphos equalis* and *Zootoca vivipara*, quantitative genetic approaches and/or admixture mapping and genome-wide association studies should be able to pinpoint the genetic modifications necessary for the evolution of viviparity.

1.6 Convergence and parallelism?

Viviparity is a complex life history strategy that generally does not evolve at frequent intervals. However, in some lineages viviparity seems to be more labile than in others (Blackburn 2006), especially in certain squamate and fish lineages. This raises the question whether there is a (genetic) predisposition in some lineages to evolve viviparity. Moreover, since viviparity evolved multiple times independently within closely related species, but also within distantly related groups of animals (like mammals and fishes) it is an intriguing

question whether these transitions in a) closely, and b) in distantly related species have the same genetic basis. Especially in closely related species it is tempting to speculate that -- given the rare occasions in which viviparity generally evolves -- there must be a predisposition and probably parallel genetic mechanisms that are involved in the evolution of viviparity. Genetic paths that lead to similar phenotypic outcomes can be very similar, but can also be substantially different (Elmer and Meyer 2011). While homologous changes (same mutation or same gene) are thought to be more prevalent in closely related species, non-homologous changes (different genes) are suggested to prevail in distantly related species (Elmer and Meyer 2011).

1.7 Common lizards as model organisms for the evolution of viviparity

The common lizard (*Zootoca vivipara*) is an ideal model organism to understand various aspects of the transition from oviparity to viviparity (Surget-Groba et al. 2006). It has the widest distribution of all extant reptiles, ranging from Spain to Japan from west to east and Norway to Greece from north to south (Figure 1.2). It is particularly fascinating as it is one of the only three extant vertebrate species with a bimodal reproductive mode, meaning that different evolutionary lineages within the same species are either oviparous or viviparous (Figure 1.2 and 1.3).

The most basal evolutionary lineage and all other relatives in the species' family of Lacertidae are oviparous, giving certainty to the assumption that oviparity is the ancestral state. While four evolutionary lineages are live-bearing, two are egg-laying (Mayer et al. 2000; Odierna et al. 2001, 2004, Surget-Groba et al. 2001, 2006); however, one of the egg-laying lineages seems to be nested within viviparous lineages, opening the question whether i) viviparity evolved several times independently (Odierna et al. 2004) or ii) that after evolving viviparity, a single lineage re-evolved oviparity (Surget-Groba et al. 2006). While phylogenetic analyses are not completely conclusive on this question, it offers the exciting possibility of an exception to Dollo's law, or alternatively shows how rapidly viviparity can evolve independently.

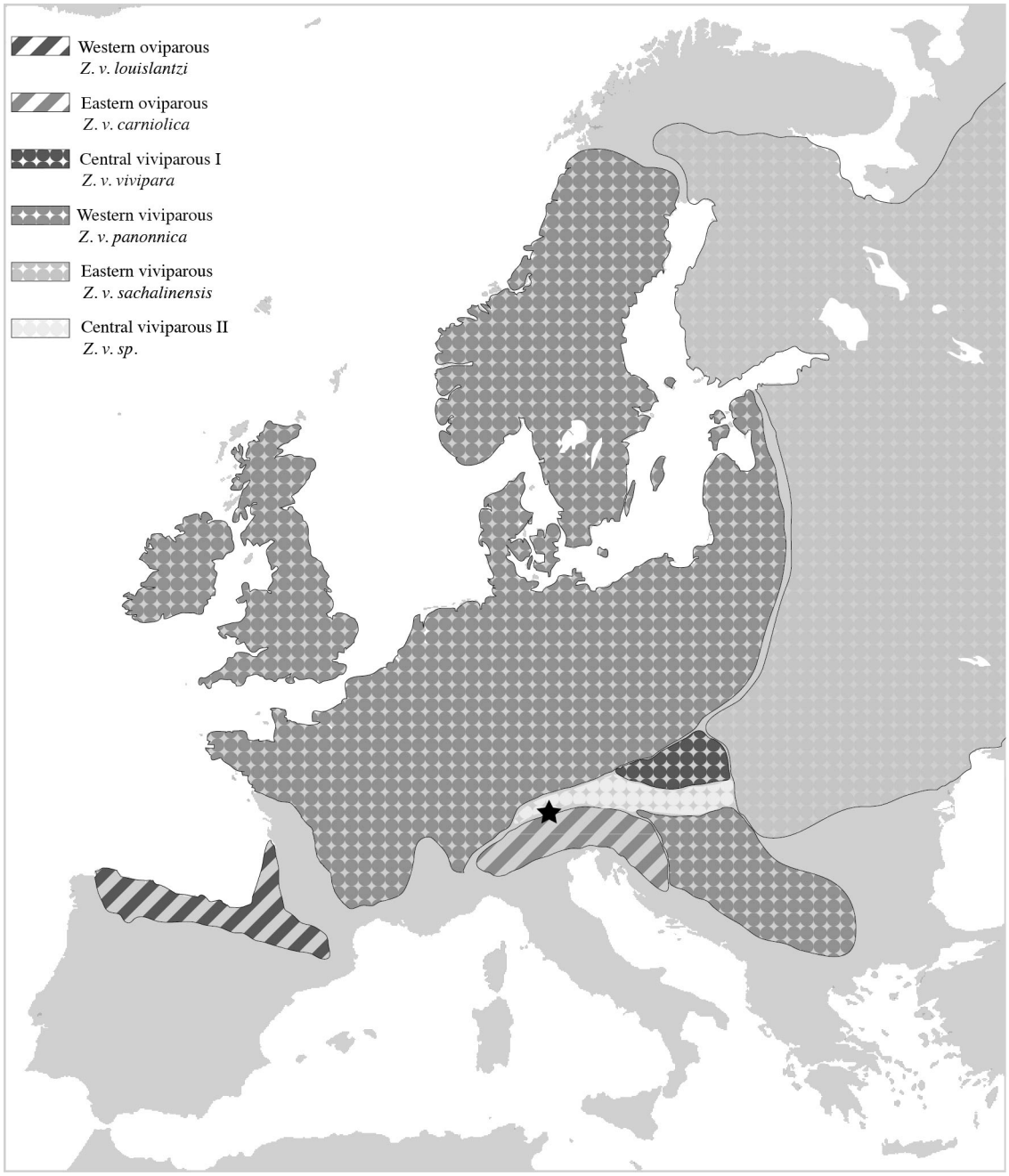

Figure 1.2 Contemporary distribution of the common lizard. Different mitochondrial lineages are indicated by different colour patterns of their range distributions on the top left of the map. Note that the eastern viviparous lineage is distributed throughout Northern Asia reaching as far as Japan. The hybrid zone between the eastern oviparous and central viviparous II lineages is indicated by a star.

With viviparous lineages mostly occurring across its northern distribution (Central Europe, UK, Scandinavia, Northern Asia, Japan) and oviparous lineages restricted to its southern distribution (Spain, Austria, Italy, Slovenia) and varying degrees of egg retention along altitudinal gradients in oviparous lineages, this also offers a test of the cold-climate hypothesis (e.g. Rodríguez-Díaz and Braña 2012). Coupling spatio-temporal geographic and climate data with phylogenetic distribution might allow us to identify those factors that favour and disfavour alternative reproductive modes. Phylogeographic distribution also suggests that oviparous lineages might have recently expanded into valleys across their

original northern distributional edge, which was presumably previously occupied by viviparous individuals (Mayer, pers. communication). This leads to the interesting question whether global warming has facilitated the expansion of oviparous individuals into areas that were too cold in the past. Further coalescent-based analyses in these populations might reveal recent demographic changes in both reproductive modes and test whether putative changes are related to temperature.

Another exiting avenue is studying the genetic basis of viviparity and oviparity. For this purpose, it first must be assessed whether the trait is heritable, i.e. that it is not a phenotypically plastic trait and influenced by certain environmental conditions. Studying the genetic basis of such key adaptations as live-bearing reproduction is often problematic because they are not variable within a single species or even at higher taxonomic levels. This makes it difficult to discern mutations responsible for a phenotype from mutations due to phylogenetic noise (genetic drift, other adaptations, etc.) (Elmer and Meyer 2011). A genetic basis of viviparity has been confirmed by crossing experiments in the western oviparous lineage (Arrayago et al. 1996), and has also been suggested for the eastern oviparous lineage (Lindtke et al. 2010; see Figure 3). Interestingly, a natural hybrid zone between oviparous and viviparous individuals has been reported recently (Lindtke et al. 2010), and it will be fascinating to explore the dynamics of selection on and fitness of different reproductive modes in this contact zone. While the study indicates that hybridization is rare, this might be either due to prezygotic (actively by phenotypic recognition of the other mode or passively by environmental barriers) or postzygotic barriers (such as genetic incompatibilities or hybrid inviability) resulting in reproductive isolation between the two reproductive modes. Behavioural observations, crossing experiments and population genetic studies should reveal the degree of admixture between reproductive modes. The natural hybrid zone also offers the possibility to perform admixture mapping (McKeigue 2005; Buerkle and Lexer 2008; Zhou and Stephens 2012) to pinpoint mutations (SNPs) that are involved in controlling whether a lizard is live-bearing or egg-laying. Admixture mapping makes use of natural recombination events between two or more lineages with divergent phenotypes. Hybridization results in recombination, and the breakup of genetic background noise resulting from the differences due to time divergence and the loci directly affecting the phenotype in question. Sequencing the whole genome and linkage mapping will be necessary to identify the regions that harbour the mutations controlling reproductive mode. Identifying loci that are associated with reproductive mode in the two lineages that hybridize will further allow us

to examine whether those same loci are associated with the reproductive mode in other common lizard lineages with an alternative reproductive mode. It will be interesting to test if – in case parity modes evolved in parallel – the shared phenotype is based on a similar genetic basis (e.g. through introgression) or a different genetic basis (Elmer and Meyer 2011; Dasmahapatra et al. 2012). Genome scans (Stapley et al. 2010) and topology weighting analyses (Martin and Van Belleghem 2017) across all lineages are tools that will help to get insights into shared genomic regions across lineages and reproductive modes. This will tell us whether selection has resulted in the evolution of similar or different genetic mechanisms underlying this complex life-history trait and about the predictability of such complex evolutionary transitions.

In addition to a genetic mapping approach, transcriptomic studies comparing the uterus of egg-laying and live-bearing females might reveal which genetic pathways are switched on during the embryonic development of the different reproductive modes. Transcriptome comparison of embryos developing with and without an eggshell might further reveal general differences in the materno-foetal communication between divergent reproductive modes. More generally, transcriptomic studies in the common lizard will allow understanding of the genetics of viviparity in a broader context by comparing these with available studies in other viviparous taxa such as mammals.

Common lizards are not just an important model for the evolution of viviparity, but a variety of other crucial adaptations such as adaptation to cold environments, life-history strategies, and colour variation (Gvoždík 2002; Sinervo et al. 2007; Vercken et al. 2007; Lepetz et al. 2009; Bleu et al. 2013). The genomic tools developed here will aid in the investigation of genetic processess invovled in those phenotypes. Moreover, research on other squamates will greatly benefit from the genomic resources developed in the common lizard, since there is a surprising lack of genomic tools available for squamate reptiles (but see Alföldi et al. 2011; Castoe et al. 2011; Shaney et al. 2014; Liu et al. 2015).

Figure 1.3 Adult females and young just born common lizards. A. Oviparous *Z. vivipara* with an only just laid clutch of eggs. B. Oviparous offspring hatching. C. Viviparous female with two just born young still within their thin membrane. D. Just borne viviparous young with yolk remaining still attached to its tail.

Testing theoretical concepts about the predictability of evolution in distantly and closely related taxa would also greatly benefit from genomic and transcriptomic research on the common lizard. While it would be expected that different evolutionary lineages of the same phenotype in common lizards (e.g. live-bearing) involve homologous changes in their genomes, it can be assumed that these differences increase with phylogenetic distance. However, depending on how conserved the genetic pathway of viviparity and oviparity is, it might also be the same across distantly related taxa.

1.8 Overall aims

The main aim of this PhD project is to contribute to the understanding of how complex phenotypic traits arise using the evolution of viviparity as an example. I tackle this question by a combination of broad comparative analyses across squamates and more specific analyses by using oviparous and viviparous common lizards as a model organism. My methods are integrative and include environmental, ecological, phylogenomic and genetic mapping analyses. More specifically, I investigate i) the environmental conditions that favour the evolution of viviparity by linking paleoclimatic data and transitions from oviparity to viviparity in squamates, ii) the reconstruction of the evolutionary history of parity mode in common lizards using phylogenomic approaches, iii) life-history trade-offs between the oviparous and viviparous reproductive strategies in sympatrically occurring oviparous and viviparous common lizards, iv) assessing the genetic architecture of viviparity and oviparity using admixture mapping and genome scans and inferring how likely the evolution of viviparity (and potentially the re-evolution of oviparity) is. These analyses aim to give us a better insight into how complex traits evolve and how the astonishing biodiversity we observe today has arisen.

1.8.1 Chapter 2: Stable and cold paleoclimate promotes the evolution of viviparity

The aim of this chapter was to identify whether paleoclimatic conditions might have favoured the evolution of viviparity across squamate reptiles. I identified 61 transitions from oviparity to viviparity that were collated from literature and ancestral character state reconstruction methods, and combined these transitions with 65 million years of paleoclimate data (Zachos et al. 2008). I first assessed whether lineage diversification in squamates increased linearly through time. I simulated a binary trait on the phylogenetic tree to test if simulated transition times and empirically derived transitions times differed. I then tested for statistical associations between the average temperature per million years (T), and the relative temperature change as variance over a window of 10 million years (ΔT_L) and emprically derived and simulated transition time estimates. Lineage diversification in squamates generally follows a linear increase in time. I found that the number of origins to viviparity significantly increased with cold and stable environmental conditions ($P < 0.001$). Frequency of the simulated binary trait did not increase with stable temperature, and was weakly associated with cold temperature conditions ($P = 0.05$). The

results were robust to differences between published time trees (Pyron and Burbrink 2014; Hedges et al. 2015; Zheng and Wiens 2016). This suggests that cold and stable climates together favoured the evolution of viviparity. This study links paleoclimatic conditions to transitional events from oviparity to viviparity in the evolutionary past on a broad phylogenetic scale. I show that stable and long-lasting cold climatic conditions have favoured the evolution of viviparity in squamates. This joins two previously disparate theories that exist to explain the evolution of viviparity: the cold-climate hypothesis (Tinkle and Gibbons 1977) and environmental stability (Stearns 1976).

1.8.2 Chapter 3: Common lizards break Dollo's law of irreversibility: genome-wide phylogenomics support a single origin of viviparity and re-evolution of oviparity

Dollo's law of irreversibility states that once a complex character has been lost in evolution, it cannot be regained. The eggshell is considered an example of such a complex trait. In this study I reconstructed the phylogeny of common lizards using 194,358 SNP loci from double digest restriction-site associated DNA sequencing (ddRADSeq). Individuals were collected in the field or acquired from museums and span a large part of the geographic distribution and all known major common lizard lineages. I performed ddRADSeq, sequencing 35 individuals on an IonProton sequencing machine using a protocol I developed in the lab (Recknagel et al. 2015) and additionally 30 individuals on a Illumina HiSeq 4000 sequencer. I reconstructed the phylogeny from these 65 individuals using maximum likelihood, maximum parsimony, and Bayesian methods to infer the evolutionary history of parity mode. The phylogeny strongly supported six main common lizard lineages that have been previously identified. Using topology testing, I found very high statistical support for a topological arrangement that suggests a single origin of viviparity and a reversal to oviparity. The topology is consistent with highly differentiated chromosomal configurations between lineages, but disagrees with previous phylogenetic studies in some nodes. While I find high support for a reversal to oviparity, more genomic and developmental data are needed to robustly test this and assess the mechanism by which a reversal might have occurred.

1.8.3 Chapter 4: Differential reproductive investment in co-occurring oviparous and viviparous common lizards (*Zootoca vivipara*) and implications for life history trade-offs with viviparity

Studying life history trade-offs between oviparous and viviparous reproductive strategies is an inherently arduous task, as most transitions to viviparity are evolutionarily old and/or are confounded by environmental effects. The common lizard (*Zootoca vivipara*) is one of a few known reproductively bimodal species, in which some populations are oviparous and others viviparous. Oviparous and viviparous populations can occur in sympatry in the same environment, making this a unique system for investigating life-history trade-offs between oviparous and viviparous reproduction. I find that viviparous females exhibit larger body size, smaller clutch sizes, a larger reproductive burden, and a higher hatching success rate than oviparous females. I find that offspring size and weight from viviparous females was lower compared to offspring from oviparous females, which may reflect space constraints during pregnancy. I suggest that the evolution of viviparity in common lizards is associated with an increased reproductive burden for viviparous females and that this promotes the evolution of larger body size to create more physical space for developing embryos. In the context of life history trade-offs for the evolution of viviparity, this research suggests that the extent of correlation between reproductive traits, or differences between reproductive modes, may also depend on the time since the transition occurred.

1.8.4 Chapter 5: The genetic basis of a major evolutionary transition: from egg-laying to live-bearing in a squamate lizard

Identifying the genetic basis of major evolutionary transitions is of great interest for evolutionary biologists to understand how todays biodiversity has arisen. While the genetics of microevolutionary processes is now relatively well understood, how major changes in morphology occur is much less well studied. A main reason for this is that it is difficult to disentangle genomic background noise from casual mutations when comparing divergent features in distant lineages. Naturally occurring hybrids of egg-laying and live-bearing common lizards open a unique opportunity to understand the genetics of live-bearing reproduction using admixture mapping. I sampled 480 females with reproductive information and used SNP genotyping to test for associations between reproductive mode and the genotype. I find a few major loci associated with reproductive modes, and more than 90% of the phenotype explained by these genotypes. Among these are a large genomic region on the sex chromosome 14, immune-related genes, and *EPAS1*, a gene that

has been previously suggested to be involved in the evolution of viviparity. Genome scans indicate that the lineage with derived oviparity shares more genetic ancestry with the lineage with basal oviparity than expected by chance alone, but no large genomic regions. Future studies should address studying the genetic basis of oviparity in this lineage.

1.8.5 Chapter 5: The genetic basis of a major evolutionary transition: from egg-laying to live-bearing in a squamate lizard

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1.9 Publication status of chapters

Chapter 2 is currently prepared for re-submission to the Journal of Evolutionary Biology. It has been reviewed by two reviewers and the Editor with encouragement to resubmit after corrections. The main change was to exclude the time-calibrated analysis performed in our study, and instead use the more complete published estimates of time divergence (e.g. Zheng and Wiens 2016) between squamate reptiles. Authors in order of appearance are: Hans Recknagel (H.R.), Nick Kamenos (N.K.), Kathryn R. Elmer (K.R.E.). All three

authors conceived the study. Analyses and writing of the manuscript were performed by H.R. All authors contributed to the version of the manuscript that had been submitted first.

Chapter 3 is currently in review in Molecular Phylogenetics and Evolution (submitted 12th of April 2018). Chapter 3 is the last submitted version of this manuscript with some minor changes, a previous version of this chapter had been reviewed by two reviewers with recommended reconsideration of the paper following major revision by the Editor. Authors in order of appearance are: Hans Recknagel (H.R.), Nick Kamenos (N.K.), Kathryn R. Elmer (K.R.E.). H.R. and K.R.E conceived the study. Main data collection, library preparation for sequencing, analyses and writing of the manuscript were performed by HR. All authors provided critical feedback and helped shape the research and the manuscript.

Chapter 4 is currently in review for the second time in Oecologia (submitted 12th of March 2018). Chapter 4 is the last submitted version of this manuscript, a previous version of this chapter had been reviewed by a Handling Editor and three reviewers with encouragement to resubmit. Authors in order of appearance are: Hans Recknagel (H.R.) and Kathryn R. Elmer (K.R.E.). H.R. and K.R.E conceived the study. H.R. performed the data collection, analysis and writing of the Both H.R. and K.R.E. authors contributed to the final version of the manuscript.

Chapter 5 is currently prepared for submission to Nature. Analyses for Chapter 5 have not been finished. Additional phenotypic and genotypic still needs to be incorporated prior to submission. I performed the data collection and main analysis and contributed to conceiving the study. Co-authors are Andrey Yurchenko (helped with construction of the whole genome and genetic mapping analyses), Mohsen Nokhbatolfoghahai (characterized embryonic stages at oviposition/parturition using microscopy), Maureen Bain (performed eggshell analyses using scanning electron microscopy) and Kathryn R. Elmer (supervised and conceived the study.

Chapter 2: Stable and cold paleoclimate promotes the evolution of viviparity

Hans Recknagel, Nick Kamenos, Kathryn R. Elmer

Preparing for resubmission to the Journal of Evolutionary Biology

2.1 Abstract

It has long been proposed but not resolved that climatic conditions influence major evolutionary transitions. Live-bearing young (viviparity) has evolved from egg-laying (oviparity) independently in many vertebrate lineages, most abundantly in lizards and snakes. While contemporary viviparous species generally occupy cold climatic regions across the globe, it is not known whether viviparity evolved as a response to cold-climate in the first place. This is the first study that links paleoclimatic conditions to transitional events from oviparity to viviparity in the evolutionary past on a broad phylogenetic scale. We used all available published time-calibrated squamate phylogenies and reconstructed a comprehensive time-tree of 61 independent evolutionary transitions from oviparity to viviparity across squamate reptiles. We compared the accumulation of transitions relative to background diversification and a simulated binary trait. Pinpointing the date of each transitions in the phylogenies and informed by 65 my of global paleoclimatic data, we tested the nonexclusive hypotheses that viviparity evolved under: i) cold, and ii) long-term stable climatic conditions. We showed that stable and long-lasting cold climatic conditions have favoured the evolution of viviparity. This joins two previously disparate theories that exist to explain the evolution of viviparity: the cold-climate hypothesis and environmental stability.

2.2 Introduction

Viviparity has arisen from oviparity more than 140 times throughout vertebrate evolutionary history (Sites et al. 2011). The factors and conditions that trigger transitions from oviparity to viviparity during evolution remain debated across taxonomic groups (Shine et al. 1978; Wourms and Lombardi 1992; Webb et al. 2006; Pollux et al. 2009; Lambert and Wiens 2013), in part because vertebrates encompass such a wide range of different morphologies, life histories, phylogenetic constraints, and environmental contexts. The advantages of viviparity include increased protection from predation, a more stable environment in the mother's reproductive tract, and a larger size at birth (Blackburn 1999b; Shine 2002). Disadvantages are reduced fecundity, maternal death resulting in death of all offspring, and a higher investment for the mother (Shine et al. 1978; Pollux et al. 2009). Climate plays a major role in modulating those advantages and disadvantages (Tinkle and Gibbons 1977).

The frequency of viviparous species increases with their distance from the equator; this led to the cold-climate hypothesis (Tinkle and Gibbons 1977). It predicts that egg-layers are disfavoured in colder environments because their eggs take long to develop in the cold soil or might die from exposure (Tinkle and Gibbons 1977). However, testing this hypothesis poses difficulties, as present and past conditions differ. A correlation between contemporary conditions in the present distribution and the proportion of viviparous species does not imply causation between these conditions and the origin of viviparity (Blackburn 2006). For example, viviparous species might have migrated to areas with a cold climate, but not originated within that area.

Squamates, the reptilian order including lizards and snakes, is the lineage in which the most transitions from oviparity to viviparity have occurred (Sites et al. 2011). The high number of origins of viviparity makes this group an ideal model system to understand which environmental factors are associated with the evolution of viviparity. In a handful of squamate species groups, recent studies have attempted to reconstruct the time when viviparity originated and they generally supported the cold-climate hypothesis (Lynch 2009; Lambert and Wiens 2013). These studies found that the majority of transitions occurred during cold periods in the Cenozoic (the last 65 million years) (Lynch 2009; Schulte and Moreno-Roark 2010; Lambert and Wiens 2013). In contrast, another recent study found no effect of an epoch-based increase in transition frequency to viviparity in reptiles and suggested that cold climate did not play a substantial role (King and Lee

2015b). However, the role of paleoclimate has not been tested at a broader phylogenetic scale in a statistical framework. This is needed because only by statistically testing a link between high resolution paleoclimate data and phylogenetically robust transitions can we identify the promoters of viviparity on geological scales.

The role of environmental stability for the evolution of viviparity is unclear. Tinkle and Gibbons (Tinkle and Gibbons 1977) suggested that viviparity is beneficial in an unpredictable environment, as females might choose an optimal time and place for the birth of the young. The maternal-manipulation hypothesis proposes that viviparity is favourable in variable environments as the female can control optimal temperatures for the embryo's development (Shine 1995; Webb et al. 2006). One recent broad-scale analysis found no support for the maternal-manipulation hypothesis (Watson et al. 2014), and another study in snakes concluded that viviparity is not necessarily adaptive in variable environments (Feldman et al. 2015). However, environmental predictability in these contexts usually relates to seasonal, small temporal scale variability in climate and environment. Stearns (Stearns 1976) proposed that the life history tactic of a few well-developed offspring (K-selection) has higher chance of survival than numerous but much less developed offspring (r-selection) in a stable environment. The impact of environmental variation on the evolution of viviparity has never been tested empirically on a geological timescale.

Here, we simultaneously assessed two hypotheses for the evolution of viviparity: i) cold-climate (as low temperature), and ii) environmental stability (as long-term stability in temperature). We did so by reconstructing a comprehensive phylogenetic analysis of 62 independent evolutionary transitions from oviparity to viviparity in lizards and snakes and testing the association with 65 million years of high-resolution global paleoclimatic data. We find that both cold and long-term stable global temperatures coincide with a significantly higher frequency of transitions to viviparity in squamate reptiles compared to the background diversification rate.

2.3 Methods and Materials

2.3.1 Identifying transitions from oviparity to viviparity

We surveyed literature to identify taxa that are viviparous and oviparous. From this initial survey, we identified a potential of 78 transitions that were subsequently individually

checked using a combination of recent molecular phylogenies and previously published parity data (Pyron and Burbrink 2014; Zheng and Wiens 2016). Each node representing a transition from oviparity to viviparity was identified.

Ancestral state reconstruction analyses were performed based on recently published time-calibrated phylogenies (Pyron and Burbrink 2014; Zheng and Wiens 2016) and parity data (Pyron and Burbrink 2014) and carried out in R using the package ape (Paradis et al. 2004). We tested alternative transition models for discrete characters implemented in the ancestral trait reconstruction ('ace') function to infer ancestral states. These included the 'equal rates' model (ER), a 'symmetric rates' model (SYM) and a model of 'all rates different' (ARD). ARD was identified as the most likely model (Δ AIC = 63.0; Δ log likelihood = 32.5; $P < 0.001$) and chosen for subsequent analysis. Transitions were included in downstream analyses when the likelihood differential between the oviparous (coded as 1) ancestor and the viviparous (coded as 0) descendant was larger than 0.5. Reversals to oviparity from viviparity involve re-evolving egg-shells and was thought to be unlikely or impossible (Shine 2005; Sites et al. 2011; Pincheira-Donoso et al. 2013); there has only been a single reported convincing example of a reversal thus far (Lynch and Wagner 2010). However, potential for reversals is still disputed and recent studies have proposed that reversals to oviparity have occurred more frequently (Fenwick et al. 2012; Pyron and Burbrink 2014; Blackburn 2015; King and Lee 2015b). Therefore we did not include potential transitions from viviparity to oviparity in our analysis. In addition, we added two transitions in New Caledonian lizards (Smith et al. 2007), transitions reported from reproductively bimodal species (Fairbairn et al. 1998; Smith et al. 2001; Surget-Groba et al. 2006), and a transition in European vipers (Wüster et al. 2008). These transitions were not available in the previously published phylogenetic datasets (either missing in phylogeny or parity data was not provided). After this quality filtering, a total of 118 taxa were included in the analysis, with 62 oviparous-viviparous taxa pairs (39 lizards and 23 snakes) representing independent origins of viviparity during squamate phylogenetic history globally (Table 2.S1). The majority of the transitions had a high likelihood differential between the oviparous parent node and the viviparous child node (mean = 0.932, median = 0.986, minimum = 0.578). Because transitions below this threshold were excluded, almost certainly there have been more transitions from oviparity to viviparity in squamates, however they could not be included with confidence in our analysis.

2.3.2 Estimation of transition times and rates

To estimate the transition time for each of the identified lineages that evolved viviparity, we obtained DNA sequences for all pairs of taxa from GenBank (Table 2.S2). These included the five mitochondrial genes 12S rRNA (12S), 16S rRNA (16S), cytochrome-b (CYTB), NADH dehydrogenase subunit 2 (ND2) and subunit 4 (ND4) and the two nuclear genes recombination-activating gene 1 (RAG1), and oocyte maturation factor (CMOS). The concatenated alignment was constructed using MEGA vers. 5 (Tamura et al. 2007) and refined by eye. The full sequence alignment comprised 8406 nucleotide positions, with 5328 mtDNA and 3078 nucDNA positions. On average, sequence data was available for 57% of all taxa per gene. The sequence alignment was partitioned into mitochondrial non-coding DNA (12S + 16S) and each gene separately (CYTB, ND1, ND4, RAG1, CMOS). The best substitution model for each partition was inferred by jModelTest 2 (Darriba et al. 2012) and selected by highest AIC (Table 2.S3). A time-tree analysis was performed in BEAST vers. 1.7.5 (Drummond et al. 2006; Drummond and Rambaut 2007) and run for 200 million generations, sampled every 20,000 generation, and the first 10% of sampled trees discarded as burn-in. Convergence was assessed with Tracer vers. 1.5 (Rambaut and Drummond 2009) based on effective sample size (ESS) values >100. Eight squamate fossil dates were drawn from the literature (ranging from 168 mya to 20 mya) and served as the minimum age of robust monophyletic groups and were used to calibrate the molecular clock rate (Table 2.S4). In each of these calibrations, the offset was set to the minimum age of the relevant group, with a log normal distribution and a mean of 1.0 and standard deviation of 1.0. The tree topology was constrained to reflect evolutionary relationships as identified by the recent comprehensive phylogeny by Pyron and Burbrink (Pyron and Burbrink 2014) to avoid topological misgroupings that can be caused by incomplete taxonomic sampling (Zwickl and Hillis 2002). For the estimation of time divergence, incomplete taxon sampling has been shown to have only a minor impact (Hug and Roger 2007). Nonetheless to avoid any artefact or bias associated with our phylogeny being heavily weighted to taxa having transitions, for all statistical tests we additionally analysed the transition time estimates for the focal oviparous-viviparous species divergences drawn from currently available phylogenies: Pyron and Burbrink (Pyron and Burbrink 2014), Hedges et al. (Hedges et al. 2015), and Zheng and Wiens (Zheng and Wiens 2016) (Table 2.S5). From the estimated transition times we inferred the transition rate to viviparity per million years using the total number of transitions and the number of nodes within a million year bin based on the phylogeny of 3498 parity-informed taxa from (Zheng and Wiens 2016) and (Pyron and Burbrink 2014).

In addition, we tested whether transition time estimates for viviparity simply reflect the background rate of lineage diversification in squamates. First, we explored the diversification pattern using a lineage-through-time plot visualizing number of squamate lineages against time using the recent time-calibrated phylogeny by Zheng and Wiens (Zheng and Wiens 2016) with the 'ltt.plot' function in ape using log transformed species numbers. Second, a binary trait was simulated 50 times based on that phylogeny with a transition rate to viviparity of 0.0015 and a much less likely transition rate to oviparity of 0.0001 using the 'rTraitDisc' function in ape (Paradis et al. 2004). These transition rates were inferred from empirically assessing a range of values (for oviparity to viviparity: 0.01, 0.005, 0.002, 0.0015, 0.001) and comparing the average number of transitions and proportion of viviparous species known in squamates (Pyron and Burbrink 2014; Zheng and Wiens 2016) (see Table 2.S6 to compare the simulations to empirical data in number of transitions and proportion of viviparous species). After the trait simulation, 'ace' was used to reconstruct ancestral traits under the all rates different (ARD) reconstruction model as above. The ggtree package in R (Yu et al. 2017) was then used to infer binary trait transitions, based on a differential in trait likelihood larger than 0.5 between any parent and child node. Background rate and simulated transitions were compared to the empirical transitions to viviparity. Lastly, we estimated the transition rate to viviparity from the number of transitions per million year time bin divided by the number of branching events in oviparous species that did not result in a transition to viviparity.

2.3.3 Statistical analysis

To examine whether transitions to viviparity are associated with temperature, we used fossil oxygen isotope ($\delta^{18}O$) measurements from benthic oceanic foraminifera, a proxy for estimating paleoclimatic temperature (Zachos et al. 2008). Measurements were averaged in bins of one million years. From these temperature estimates, we developed two different summaries: 1) the average temperature per million years (T), and 2) the relative temperature change as variance over a window of 10 million years (ΔT_L). The two temperature summaries were not correlated (Pearson correlation coefficients between -0.035 and 0.117; $P > 0.1$). Autocorrelation in temperature data should not be a problem for our analysis in principle, as there is no intrinsic causal relationship. However, we performed a test for autocorrelation for our best statistical model and found that there was no autocorrelation detected in the residuals using the 'acf' function in R. Accordingly, the regression analysis between lagged residuals (using a lag of one, which had the highest

score in acf) was not significant ($t = 1.794$, $P = 0.09$). Therefore, we conclude that autocorrelation is not an issue in our statistical analyses. Excluding data where zero transitions occurred, residuals of the best model were tested for autocorrelation using the 'acf' function in R. In addition, using linear regression we tested for an association between lagged residuals. We statistically tested for association between the two temperature parameters and the number of evolutionary transitions using R (R Core team 2015). Because the data were 'zero inflated' (58% of observations = 0, i.e. when the million year bin included no transitions to viviparity), we applied a generalized linear model with a negative binomial distribution (Crawley 2007). Transitions time estimates included i) our own inferred time-calibrated transitions, ii) transitions estimated from the Pyron and Burbrink (Pyron and Burbrink 2014) timetree, iii) the Hedges et al. (Hedges et al. 2015) timetree, iv) the Zheng and Wiens (Zheng and Wiens 2016) timetree, and v) transitions inferred from simulations of binary trait evolution using the Zheng and Wiens (Zheng and Wiens 2016) timetree. The full model included an interaction term between T and ΔT_L and was gradually simplified by AICc value and comparison to the intercept. Complementary to testing the median age for transitions, we performed further tests taking into account the highest posterior density of age estimation by i) randomly selecting a value (using R) within each estimated confidence interval, ii) using the lowest age estimate within the 95% HPD, and iii) using the highest value of the 95% HPD (Table 2.S5).

2.4 Results

2.4.1 Early Miocene burst in the origins of viviparity

Our time-calibrated phylogenetic analysis suggests that transitions from oviparity to viviparity in squamates have tended to occur during a relatively narrow window. Specifically, forty-five of 62 (73%) transitions in squamate evolution occurred between the last 5 and 25 million years (Figure 2.1; Table 2.S5). Only four transitions occurred within the last 5 million years (*Zootoca vivipara* (o/v): 3.8 mya [1.8-6.4 95%HPD]; *Sceloporus chaneyi-Sceloporus goldmani*: 4.9 mya [2.0-8.6 95%HPD]; *Liolaemus chaltin-Liolaemus puna*: 4.2 mya [2.1-6.5 95%HPD]; *Liolaemus quilmes-Liolaemus espinozai*: 4.4 mya [2.7-6.2 95%HPD]). The peak of transitions was between 13 and 16 million years ago, with 15 origins of viviparity in that short time period (Figure 2.2A). On geological scales, origins of viviparity increased markedly in frequency from the late-Oligocene (around 25 mya) and decreased in the late-Miocene (around 5 mya). The timing of transitions is broadly congruent across lizards and snakes, coinciding in the early/mid Miocene epoch; a peak

around 10 to 22 mya in lizards (25 out of 39 transitions; median = 14.4 mya) and snakes (12 out of 23 transitions; median = 19.9 mya).

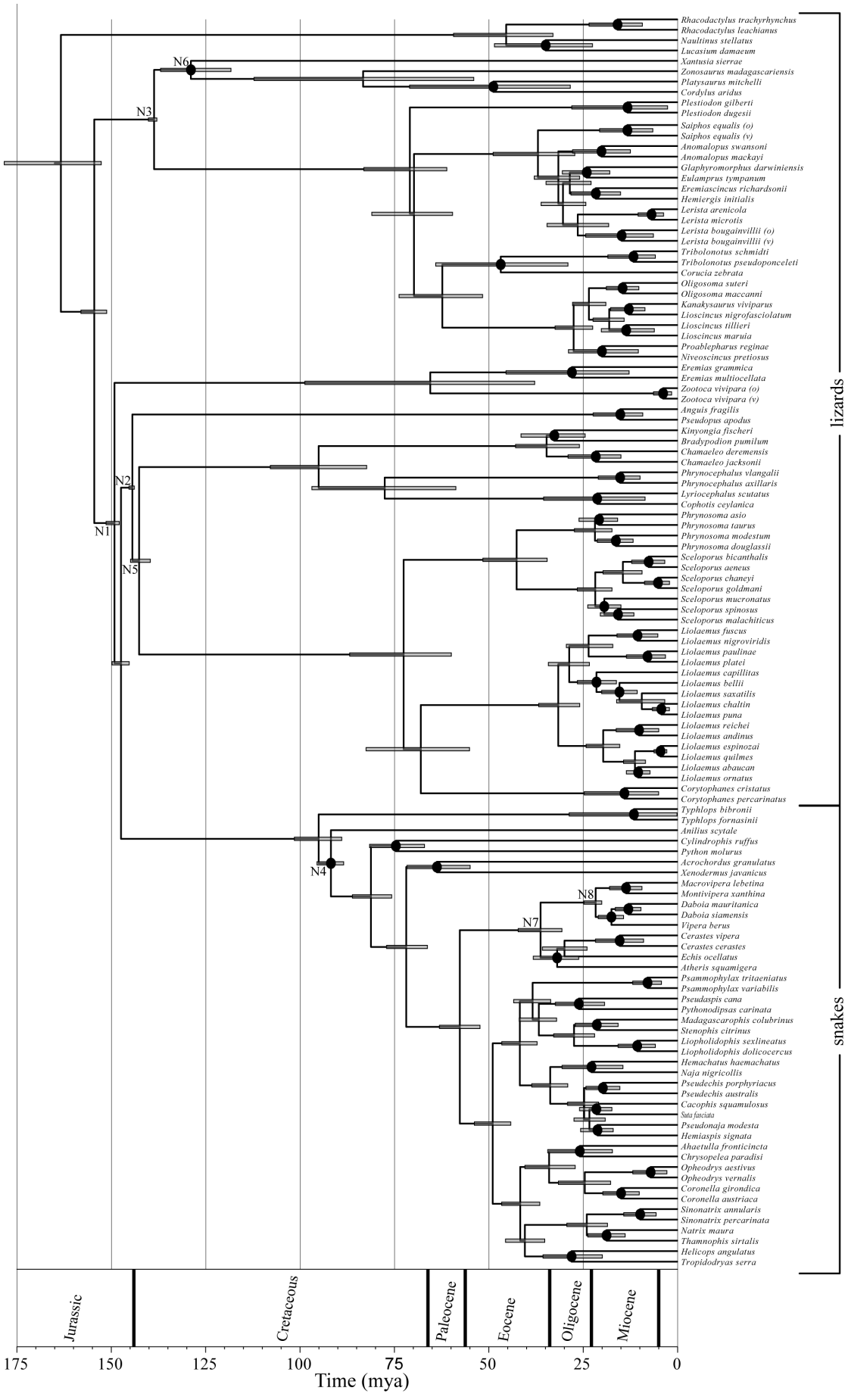

Figure 2.1 Time-tree of 118 squamate species representing transitions from oviparity to viviparity. Each parity mode transition is illustrated by an enlarged node and its respective age scaled below the tree. The 95% HPD intervals are plotted on each node as a grey bar. Fossil calibration points for a monophyletic group are indicated by magnified nodal dots (N1 = Episquamata; N2 = Anguimorpha and Iguania; N3 = Scincoidea; N4 = most Serpentes; N5 = Iguania; N6 = Cordylidae, Gerrhosauridae and Xantusiidae; N7 = Viperidae, Lamprophiidae and Colubridae; N8 = Eurasian vipers; Table 2.S4).

The pattern of transitions to viviparity we identified across the phylogeny does not reflect the general pattern of lineage diversification in squamates, suggesting they are not directly coupled. We assessed this by comparing the background rate of lineage diversification in squamates to the empirically derived transition frequencies from oviparity to viviparity based on accumulation of lineages through time. We found that the background rate of lineage diversification in squamates, based on a comprehensive phylogeny of 4162 taxa (Zheng and Wiens 2016), closely matched that of linear growth through time during the last 100 million years (Figure 2.2B). In contrast to the rate of lineage diversification, the empirically inferred transitions to viviparity (both from Zheng and Wiens 2016 and this study) differ in shape and slope, always being lower in number of lineages, and having a steep slope representing an increase approximately 10 to 23 million years ago (Figure 2.2B).

Further, the empirical transitions to viviparity differ in slope and shape to the mean transition rate of a simulated binary trait (Figure 2.2B, Table 2.S6). The more recent transition frequencies derived in this study exceed that of the simulated binary trait and were higher than those of Zheng and Wiens (Zheng and Wiens 2016), in which the transition frequency is spread over a slightly longer time duration. Both empirical estimates differed from the simulated binary trait from ca. 30 to 55 mya, during which the empirical number of transitions was substantially lower than the simulated values (Figure 2.2B, Figure S1). This time period corresponds to a warm period followed by transition to cooling in the Eocene (Figure 2.2A). Despite these temporal differences, the simulated binary trait closely matched the empirical values found for viviparous species with regard to proportions: in simulations on average 20.8% of squamate species were viviparous, which is close to the real data estimates of 19.8% of species (Zheng and Wiens 2016). Therefore, the simulations overall closely matched the empirical data in terms of number of viviparous and oviparous species, but differ in when these transitions occurred (Figure 2.S1, Table 2.S6).

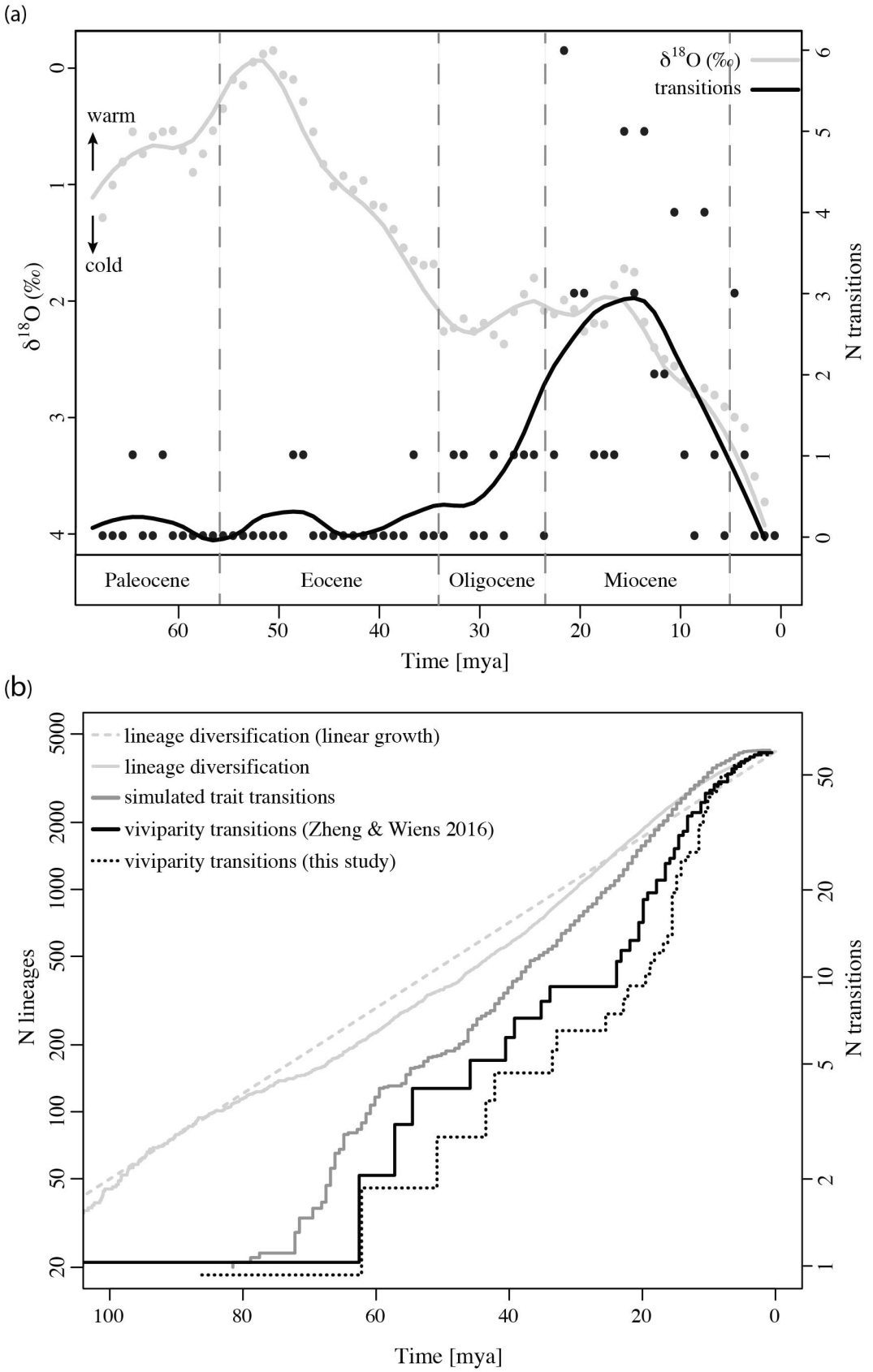

Figure 2.2 Frequency of transitions from oviparity to viviparity and paleoclimate. (a) Transitions to viviparity in squamates (dark grey) and global mean temperature (light grey) are displayed per million years from 65 million years ago to present. Temperature and number of transitions are shown as smoothed lines (span[λ] = 0.25). Oxygen isotopes from (Zachos et al. 2008) were used to determine palaeotemperature (negative relationship). Geological epochs are indicated below. In (b), Diversification rate of squamate lineages (y-axis: Number of lineages) and transition frequencies (z-axis: Number of transitions) to viviparity are compared through time. Transition frequency estimates include mean values for a simulated binary trait (of 50 independent replicates), and empirical transitions to viviparity from (Zheng and Wiens 2016) and from this study.

2.4.2 Origins of viviparity during cold and stable temperatures

We found that the increase in transitions to viviparity identified in the time-calibrated phylogeny was statistically associated with relatively cold and stable global climatic conditions (Figure 2.2; Table 2.1; Table 2.S7). The model that best explained the relationship between paleoclimate and rate of transitions to viviparity included an effect of both average temperature per million years (T) and variance in temperature (ΔT_L) (Table 2.1). Specifically, origins of viviparity significantly increased with lower temperatures (estimate = 0.84, $P < 0.001$, df = 64) and with long-term stable temperatures (estimate = -12.80, $P < 0.001$, df = 64) (Table 2.S7). This supports cold-climate (low temperatures) and environmental stability (low variance in long-term temperatures) as associated with, and perhaps promoting, the evolution of viviparity. This model was significantly better than the null model (ΔAICc = 18.78, $P < 0.001$). Excluding either T or ΔT_L from the model resulted in a significant drop in the AIC score (ΔAICc = 12.71 dropping T, ΔAICc = 10.45 dropping ΔT_L; Table 2.1), indicating that a model with either cold temperature or environmental stability alone was a poorer fit for explaining the evolution of viviparity. The transition rate (ratio of total transitions per total nodes within a million year bin) to viviparity was not significantly associated with either T (estimate = -0.066, P = 0.964) or ΔT_L (estimate = -0.94524, P = 0.955), suggesting the pattern of transition rate is driven by the low number of transitions when there are few nodes early in the phylogeny (Figure 2.S2). For this reason any inference based on transition rate should be interpreted cautiously.

Table 2.1 Statistical performance of temperature parameters explaining number of transitions to viviparity. Statistical models are sorted by AICc value and include the two climatic parameters average temperature per million year (T) and long-term change in temperature (ΔT_L). Please refer to Table 2.S7 for individual effects of paleoclimate parameters for the best ranking model.

rank	parameters	Df	theta	std err	AICc	ΔAICc
1	$T+\Delta T_L$	64	1.67	0.93	158.14	0
2	$T*\Delta T_L$	63	1.70	0.94	160.29	2.15
3	T	66	0.87	0.36	168.58	10.45
4	ΔT_L	65	0.93	0.42	170.84	12.71
5	null model	67	0.64	0.25	176.92	18.78

To test that the relationship between transitions to viviparity and climate is robust to time calibration uncertainty inherent in the phylogenies, we further analysed the relationship with paleoclimate of randomly drawn transitions dates within the 95%HPD (rather than the mean, which was used above), the maximum and the minimum 95% HPD values in our

phylogeny, mean transition age estimates drawn from the literature, and a simulated binary trait. In all three cases using HPD, the model best explaining the frequency of transitions to viviparity included a significant effect of temperature and variance in temperature (ΔAIC = 10.6, 7.8 and 10.7 respectively; in all cases T with $P < 0.001$ and ΔT_L with $P < 0.003$) with the same direction of both effects (i.e. towards cold temperature and stable conditions; Table 2.S7). Tests based on the transition time estimates drawn from Zheng and Wiens (Zheng and Wiens 2016), Hedges et al. (Hedges et al. 2015) and Pyron and Burbrink (Pyron and Burbrink 2014) all resulted in the best model having significant effects of both T and ΔT_L (Table 2.S7). In contrast, the transition times estimated from the binary trait simulation were best explained with a weak effect of cold temperature ($P = 0.05$), while variance in temperature (ΔT_L) was not significant and excluded from the best model (Table 2.S7). Therefore the association we identified between frequency of transitions and paleoclimate is robust to dating variation inherent in different phylogenies and is not explained by random evolution of a binary trait.

2.5 Discussion

We found a peak in squamate transitions to viviparity that was statistically associated with relatively cold and stable climatic conditions (Figure 2.2; Table 2.1). Specifically, we found that during the last 65 million years the number of transitions to viviparity was negatively associated with average temperature at that time. Squamate transitions increased in frequency dramatically in the late-Oligocene and decreased again in the mid-Miocene, peaking at around 13 to 16 million years ago, in the early-/mid-Miocene (Figure 2.1). The frequency of transitions to viviparity did not only increase with a cool climate alone, but multiple lines of evidence suggest it was also positively associated with long-term temperature stability. This indicates that cold and stable climates together favoured the evolution of viviparity (Figure 2.2).

While our model newly identified a role for climate stability, our support for the cold-climate hypothesis is in general agreement with previous, lineage-specific studies that found a connection between transitions to viviparity and historically cold climate conditions. For example, transitions to viviparity in vipers were associated with global cooling during the Cenozoic period, and especially in the Oligocene speciation rates for viviparous species increased substantially relative to oviparous species (Lynch 2009). In iguanid lizards, transitions to viviparity predate Pleistocene-Pliocene glaciations from the

last 4 million years and occurred within the last 65 million years, presumably during and following the cooling phase in late-Eocene (Schulte and Moreno-Roark 2010). A study by Lambert and Wiens (Lambert and Wiens 2013) has further shown that transitions to viviparity in phrynosomatid lizards were correlated with modelled cold summer temperatures. By using a larger taxonomic and temperature dataset, our study resolves a more specific window of time during which origins of viviparity occurred most frequently and assessed this generally across squamates. We found that as temperature dropped steadily from the mid-Eocene (50 mya) until around 35 mya, the frequency of transitions to viviparity did not increase notably until low temperature plateaued. Climate reconstructions show that there was limited temperature variation from the Oligocene to the mid-Miocene, which was generally characterized by a relatively stable environment (Flynn et al. 1995). This period of stability was followed by a dramatic drop in temperature from the mid-Miocene, which we found to be mirrored by a reduction in the number of transitions to viviparity to the present day (Figure 2.2).

Our findings are robust to variation inherent in deep phylogenetic inference. Specifically, we found that transition times to viviparity estimated from other recently published time trees (Pyron and Burbrink 2014; Hedges et al. 2015; Zheng and Wiens 2016) also all consistently supported the same model, with a significant effect of cold and stable temperature on transition frequency. By contrast, transition times derived from simulating the evolution of a binary trait over equivalent evolutionary time were only weakly associated with cold temperature and identified no effect of climatic stability (Figure 2.S1, Table 2.S7). The transition rate to viviparity (i.e. ratio of total transitions per total nodes within a million year bin) was not associated with any climate variable. This could be explained by the large impact of earlier transitions to viviparity, as they inflate the rate substantially due to the lower number of nodes within earlier million year time bins (one transition out of ten nodes = 0.1 transition rate versus 3 transitions out of 100 nodes = 0.03 transition rate). Therefore, a single transition closer to the base of the squamate phylogeny has a much larger effect on the transition rate than several transitions later in squamate evolution. Instead the accumulation of transitions represents an informative reflection of the evolution of viviparity in squamates, and we find this differs from background rates and is associated with cold and stable climates.

It is not well understood how evolutionary adaptations that involve a suite of aspects such as morphology, physiology, behaviour, and immunology arise (Monteiro and Podlaha

2009; Wagner and Zhang 2011). Viviparity is one example of such a complex evolutionary leap (Murphy and Thompson 2011). It has been shown that complex adaptive phenotypes often contain a complex genetic basis, meaning that they involve several mutations in several genes (McCarthy et al. 2008; Wagner and Zhang 2011). We propose that one explanation for why viviparity does not evolve during eras of changing climate might be that conditions did not favour the evolution of viviparity consistently and for long enough. Stable (and cold) environmental conditions could provide long-term directional selection pressure for the evolution of viviparity. However, the genetic architecture of the trait is also crucial and has many implications for the number of mutations necessary for the transition from oviparity to viviparity, and in squamates this remains unidentified (Murphy and Thompson 2011) and cannot be inferred by comparative reconstructions. In addition, cold and stable conditions might stabilize the viviparous reproductive state and decrease the chance of switching back to oviparous reproduction. The numerous transitions to viviparity in squamate reptiles have led to the notion that oviparity is evolutionary labile in squamates (Lee and Shine 1998). The absence of large ancient viviparous clades of squamates could be explained by a lack of climatic stability, as long-term stable environments can lead to directional, gradual changes, whereas in fluctuating environments species remain in stasis (Sheldon 1996; King and Lee 2015b). Therefore, we speculate that the complex biology of the trait together with long-term optimal conditions for viviparous reproduction could explain our finding that viviparity is most likely to evolve under stable conditions and cold temperatures.

While our results clearly support multiple origins of viviparity in the past 65 million years, this does not preclude more ancient (>100 mya) origins of viviparity in the squamate lineage. For example, one recent study argued that viviparity evolved at the stem of squamates (Pyron and Burbrink 2014). Moreover, the fossil record shows that viviparity evolved in at least some Mesozoic reptiles (O'Keefe and Chiappe 2011; Wang and Evans 2011) and as early as 245 million years ago in an archosauromorph (Liu et al. 2017). In fact, temperature reconstructions from the Triassic until the early-Cretaceous indicate that the climate during that time was relatively cold and stable (Royer et al. 2004), which according to our results would provide favorable conditions for the evolution of viviparity. Therefore, viviparity might have originated multiple times earlier in evolutionary history, however reconstructing those events with confidence would be difficult as several of those critical reptile groups are now extinct and less phylogenetic data is available.

This study links paleoclimatic conditions to transitional events from oviparity to viviparity in the evolutionary past on a broad phylogenetic scale. We show that stable and long-lasting cold climatic conditions have favoured the evolution of viviparity in squamates. This joins two previously disparate theories that exist to explain the evolution of viviparity: the cold-climate hypothesis (Tinkle and Gibbons 1977) and environmental stability (Stearns 1976).

Chapter 3: Common lizards break Dollo's law of irreversibility: genome-wide phylogenomics support a single origin of viviparity and re-evolution of oviparity

This chapter is a modified version of a manuscript that is in review in Molecular Phylogenetics and Evolution and has been published on bioRxive

3.1 Abstract

Dollo's law of irreversibility states that once a complex trait has been lost in evolution, it cannot be regained. It is thought that complex epistatic interactions and developmental constraints impede the re-emergence of such a trait. Oviparous reproduction (egg-laying) requires the formation of an eggshell and represents an example of such a complex trait. In reptiles, viviparity (live-bearing) has evolved repeatedly but it is highly disputed if oviparity has re-evolved. Here, using up to 194,358 SNP loci and 1,334,760 bp of sequence, we reconstructed the phylogeny of viviparous and oviparous lineages of common lizards and infer the evolutionary history of parity modes. Our phylogeny strongly supported six main common lizard lineages that have been previously identified. We found very high statistical support for a topological arrangement that suggests a reversal to oviparity from viviparity. Our topology was consistent with highly differentiated chromosomal configurations between lineages, but disagreed with previous phylogenetic studies in some nodes. While we found high support for a reversal to oviparity in a single lineage, more genomic and developmental data are needed to robustly test this and assess the mechanism by which a reversal might have occurred.

3.2 Introduction

There are numerous examples for the loss of a complex trait in the animal kingdom throughout evolution. Dollo's law of irreversibility states that once such a complex trait has been lost, it cannot be regained (Dollo 1893). Some exceptions to this rule have been discovered, though it remains a very rare phenomenon in evolution (Collin and Miglietta 2008; Lynch and Wagner 2010). Oviparity (egg-laying) is an example for such a complex trait and has been lost on several independent occasions throughout animal evolution (Lee and Shine 1998; Murphy and Thompson 2011). While there are more than a hundred independent transitions from oviparity to viviparity (live-bearing) in reptiles (Blackburn 2006; Sites et al. 2011), only one robust example for the re-evolution of the eggshell is known to date (Lynch and Wagner 2010). Molecular mechanisms by which reversals in complex traits such as reproductive mode occur are to date unknown.

The common lizard (*Zootoca vivipara*) is the most widespread extant terrestrial reptile species. Its distribution covers nearly the whole of Europe, northern and central Asia and as far as Japan in its easternmost range. Within this distribution, common lizards have adapted to various extreme environments. Arguably the most salient of these adaptations is the evolution of viviparous, unique within the family of 'true' (lacertid) lizards that are otherwise oviparous. As one of the youngest transitions from oviparity (egg-laying) to viviparity (live-bearing) known in vertebrates (Surget-Groba et al. 2006; Pyron and Burbrink 2014), common lizards are an emerging model system for the study of viviparity (Freire et al. 2003; Le Galliard et al. 2003; Murphy and Thompson 2011). However, not all common lizards are live-bearing: of the six currently recognized common lizard lineages, two are oviparous and four are viviparous (Surget-Groba et al. 2006; Figure 3.1). One oviparous lineage is restricted to northern Spain and southwestern France, allopatric to all other common lizard lineages. A second oviparous lineage occurs in the southern part of the Alps. Four viviparous lineages cover the rest of the Eurasian distribution (Mayer et al. 2000; Surget-Groba et al. 2006; Figure 3.2).

The phylogenetic relationships within *Zootoca* have not been fully resolved. The evolutionary history of the two different parity modes has been controversial depending on which data were used to interpret the phylogenetic relationships. In a first study using a single mitochondrial gene, both oviparous lineages were found to be basal to all other viviparous lineages, consistent with a single origin of viviparity (Surget-Groba et al. 2001; Figure 3.1A). However, subsequent analyses on the karyotype of common lizards resulted

in a more complex evolutionary scenario, arguing for two origins of viviparity based on sex-chromosome evolution (Z_1Z_2W or ZW) (Odierna et al. 2004; Surget-Groba et al. 2006; Figure 3.1B). More extensive geographic sampling and sequencing of mitochondrial genes instead favored a scenario of a single origin of viviparity followed by a reversal to oviparity in the Spanish western oviparous lineage (Surget-Groba et al. 2006; Cornetti et al. 2014; Figure 3.1C), though this phylogeny was incompatible with a single origin of the Z_1Z_2W sex chromosome system. Finally, a population inhabiting the Carpathian region in Romania was discovered recently and was found to be most closely related to the phylogenetically basal eastern oviparous lineage based on mtDNA (Velekei et al. 2015; Figure 3.1D). The reproductive mode of this lineage was not reported, but since all other common lizard populations in its geographic proximity are viviparous (Surget-Groba et al. 2006), this would suggest another independent origin of viviparity.

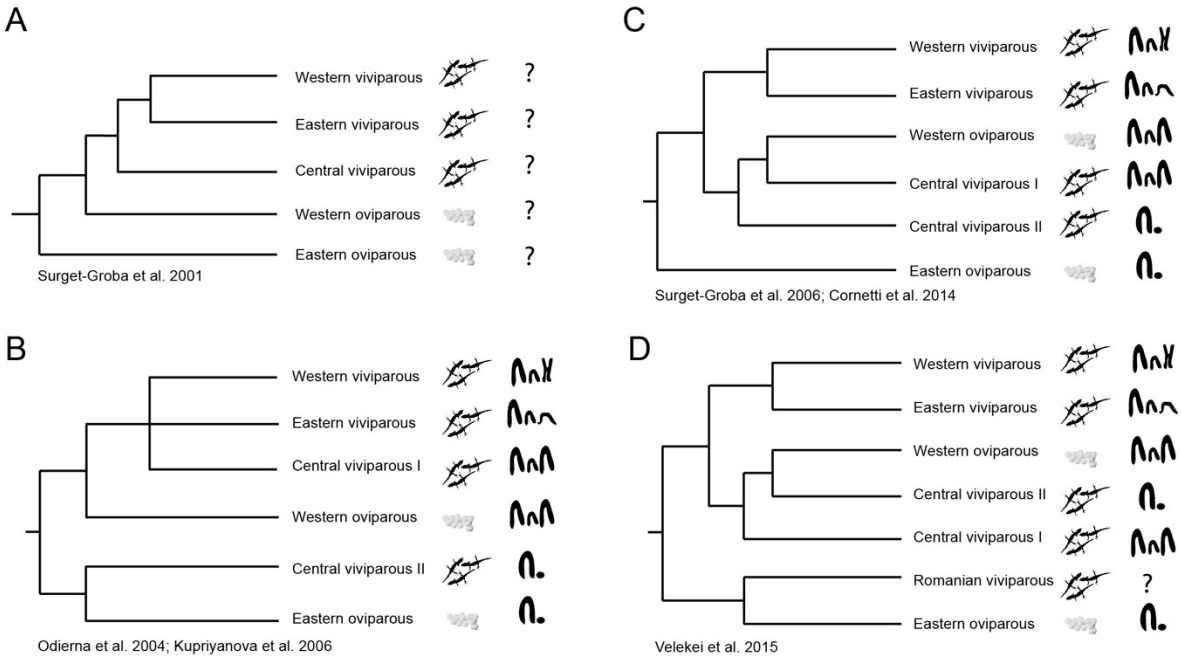

Figure 3.1 Alternative hypotheses for phylogenetic relationships of common lizards and parity mode evolution. Parity mode and sex chromosome configuration (ZW or Z_1Z_2W in that order from left to right; Odierna et al. 2004) are illustrated next to each respective lineage. A tandem fusion of the originally smaller W with an autosome has resulted in the Z_1Z_2W configuration. Phylogenetic tree A) involves a single origin of viviparity and was supported by one mtDNA gene. The second tree B) is based on karyological studies and suggests two independent origins of viviparity. Hypothesis C) suggests a reversal to oviparity as most parsimonious scenario, based on mtDNA and a few nuclear genes. The last phylogeny D) includes a recently discovered viviparous lineage in the Carpathians, which was found to be closely related to the most basal oviparous lineage. Parity mode evolution in this scenario involves two independent origins of viviparity and a reversal to oviparity.

However, all phylogenies to date have had limited support at basal nodes essential for the interpreting the evolutionary scenarios of parity mode evolution. Moreover, phylogenies reconstructed only from mitochondrial DNA have limited information and frequently misrepresent the 'true' phylogenetic relationships (Ballard and Whitlock 2004; Near and

Keck 2013; Wallis et al. 2017). Therefore, it is essential to incorporate high resolution nuclear DNA sequencing to resolve difficult topologies. Moreover, coalescent-based approaches for disentangling incomplete lineage sorting effects and hybridization have considerably advanced phylogenetic reconstruction (Pickrell and Pritchard 2012; Bouckaert et al. 2014; Posada 2016).

The evolutionary implications for models involving several origins of viviparity and/or a reversal to oviparity are significant. A reversal to oviparity from viviparity is considered a very unlikely evolutionary scenario, presumably breaking Dollo's law of irreversibility. Common lizard parity mode evolution could represent one of the very few examples for an exception to this rule (Surget-Groba et al. 2006). Further, the evolution of both oviparity and viviparity are difficult to study from a molecular genetic perspective because they have most frequently occurred at deep evolutionary time scales. Common lizards provide an example of recent parity mode changes and therefore a critical insight to usually more ancient evolutionary events.

To tackle this outstanding phylogenetic question, we use genome-wide phylogenomics with data from double-digest restriction-site associated DNA sequencing (ddRADSeq), a next generation sequencing (NGS) technique, to identify DNA polymorphisms across all common lizard lineages (Peterson et al. 2012; Recknagel et al. 2013, 2015). Using broad geographic sampling of 70 individuals, we reconstructed a nuclear phylogeny of 194,358 bp, and a mtDNA phylogeny based on cytochrome b, using Maximum Likelihood, Maximum Parsimony, and a Bayesian coalescent based approach. We performed topological tests to assess likelihoods of alternative evolutionary scenarios for parity mode evolution based on our phylogenomic dataset, which consistently supported an evolutionary scenario. Our results strongly support a single origin of viviparity in common lizards and a subsequent reversal to oviparity in one derived lineage as the most parsimonious scenario of reproductive mode evolution.

3.3 Methods and Materials

3.3.1 Sampling

Samples and specimens (N = 65 common lizards, and one individual as outgroup belonging to the species *Iberolacerta horvathi*) were obtained from the Natural History Museum in Vienna, the Royal Ontario Museum, and fieldwork during 2013-2016 (see Table 3.S1 for specimens and Figure 3.2 for a map of collecting localities). Lizards were

collected by diurnal opportunistic searches. Tail clips (up to 2 cm) were extracted and preserved in 95-99% ethanol and lizards were released thereafter. Mode of reproduction was assessed by observation of an individual retained in captivity until oviposition/parturition or from data on other individuals at the same site.

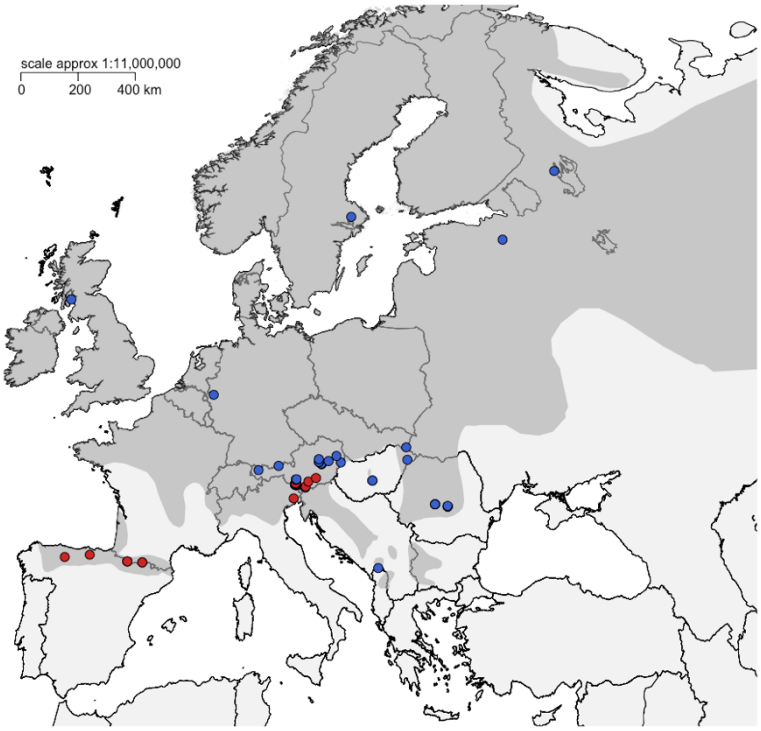

Figure 3.2 Map of common lizard (*Zootoca vivipara*) sampling locations within Europe. The dark grey shaded area marks the distribution of the common lizard in Europe. Each dot represents a single individual (red = oviparous; blue = viviparous) captured at the respective location. Note that a single individual from central Russia included in the phylogenetic analyses is outside the scope of the map (see Table 3.S1).

3.3.2 Generation of molecular data

DNA was extracted from tissue using a Dneasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. Three genomic libraries were constructed using double-digest restriction-site associated DNA sequencing (ddRADSeq). The first two libraries were run on an IonProton sequencing machine with a median of 96 bp read length (ddRADSeq-ion; Recknagel et al. 2015) and the third library was paired-end sequenced on an Illumina HiSeq 4000 with 150 bp read length (Table 3.S1). Briefly, 1 ug of starting DNA material was digested using restriction enzymes *PstI*-HF and *MspI* and subsequently cleaned with the Enzyme Reaction Cleanup kit (Qiagen). Following purification, the amount of DNA in

each individual was normalized to the sample with the lowest concentration within a library (237 ng in first, 400 ng in second, and 275 ng in third library) to minimize coverage variation. Platform specific barcoded (for IonProton: A-adapter, for Illumina: P1 adapter; binding to *PstI*-HF overhang) and global (for IonProton: P1-adapter, for Illumina: P2 adapter; binding to *MspI* overhang) adapters were ligated to the sticky ends generated by restriction enzymes. The ligated DNA fragments were then multiplexed and size-selected using a Pippin Prep (Sage Science) for a range between 175 - 225 bp for the IonProton platform and 150 – 210 bp for Illumina. To assure that the same set of loci were selected between platforms, size selection ranges were adjusted because adapter lengths are not the same between platforms. Seven separate PCR reactions (for details see Recknagel et al., 2015) were performed per genomic library and combined (Peterson et al. 2012). Following PCR purification, libraries were electrophoresed on a 1.25% agarose gel to remove any remaining adapter dimers and fragments outside the size range selected by the Pippin Prep. SYBRSafe (Life Technologies) was used for gel staining and bands in the size selected range were cut out manually and DNA was extracted from the matrix using a MinElute Gel Extraction Kit (Qiagen). Following the gel extraction, DNA was quantified using a Qubit Fluorometer with the dsDNA BR Assay. Quality and quantity of genomic libraries was assessed using a TapeStation or Bioanalyzer (Agilent Technologies). The first two libraries were sequenced at Glasgow Polyomics using an Ion PI Sequencing 200 Kit v3 on an Ion Proton PI chip at a target read size of 100 bp. The third library was sequenced at Edinburgh Genomics on an Illumina HiSeq 4000 machine with paired-end sequencing of 150 bp reads.

In addition to ddRADseq, mitochondrial DNA (mtDNA) from cytochrome b with primers MVZ04H and MVZ05L (~430 bp) was amplified (Smith and Patton 1991) and PCR products were sequenced with the forward primer (MVZ04H) on an ABI 3130x at Dundee University. Sequences were quality checked by eye, and trimmed and aligned using Geneious v. 7.1.9 (Kearse et al. 2012). Data will be deposited in NCBI.

3.3.3 Bioinformatic analysis

All NGS generated reads were analyzed using the RADseq software tool STACKS v.1.41 (Catchen et al. 2011). Reads were trimmed to a common length of 70 bp to maximize the number and length of retained reads (Recknagel et al. 2015). Libraries were de-multiplexed and all reads were sorted into individual reads. Each individual was then aligned to a *Zootoca vivipara* reference genome v. 0.9 (Yurchenko et al. in prep) using bwa (Li and

Durbin 2010) and samtools (Li et al. 2009). A catalogue of all loci identified across individuals was subsequently created using the genome referenced stacks from each individual with a minimum coverage of 3x per individual locus (using the ref_map.pl script in STACKS).

Missing data can have a substantial impact on phylogenetic inference from NGS generated data and can vary between taxonomic and phylogenetic levels (Rowe et al. 2011; Jiang et al. 2014; Streicher et al. 2016; Eaton et al. 2017). Therefore, it is crucial to first evaluate the impact of missing data before phylogenetic analysis. We filtered our data with two main options: i) using a variable minimum number of individuals that a locus had to be present in, and ii) varying the number of SNPs per locus from one to three. The amount of missing data was increased from 0% to 90% at 10% intervals. For each of these categories, loci containing only a single SNP, two SNPs, three SNPs and one to three SNPs were extracted from the whole dataset. These datasets were extracted to test the impact of missing data and number of SNPs on phylogenetic resolution and to assess optimal settings for data extraction.

3.3.4 Phylogenetic analysis

Suitability of data sets that differed in degree of missing data and number and type of SNP loci was assessed by comparing the sum of bootstrap supports (at deep, at shallow, and at all nodes combined) (Huang and Lacey Knowles 2016). The best performing dataset for inferring the evolutionary history of parity mode in common lizards was identified and chosen for more exhaustive phylogenetic and comparative analyses. This best performing dataset was assessed by constructing Maximum-likelihood (ML) phylogenies using the software RAxML vers. 8.1.20 with a GTRGAMMA substitution model of evolution (Stamatakis 2006). Conditions producing the highest bootstrap sum phylogeny were the ones chosen for all subsequent analyses.

We inferred Maximum-likelihood (ML) phylogenies using RAxML. An initial phylogenetic analysis including the outgroup species *Iberolacerta horvathi* identified the eastern oviparous clade as basal to all five other *Zootoca* lineages with high confidence (bootstrap support 100), as has been shown by previous analyses (Mayer et al. 2000; Surget-Groba et al. 2006; Cornetti et al. 2014). We further used ADMIXTURE (vers. 1.3.0; Alexander et al. 2009) to test for monophyly of the main *Zootoca* lineages. ADMIXTURE assesses the genomic ancestry of individuals according to a given set of

genetic clusters. A variable number of genetic clusters *k* was run, from 1 to 6 *k* and best fit inferred from ten-fold cross-validation. The genetic cluster with the lowest cross-validation error was chosen as optimal *k*. These analyses confirmed monophyly of the six main lineages and limited levels of admixture. Pairwise genetic differentiation between lineages was assessed using the R package diveRsity (Keenan et al. 2013).

A Maximum likelihood bootstrap search with 100 replicates using a GTRGAMMA model was performed in RAxML. Support values were drawn on the best scoring ML tree. The best ML tree was compared to four alternative pre-defined topologies, which had been proposed in previous studies. These topologies included i) both oviparous lineages basal to all viviparous lineages (Mayer et al. 2000; Surget-Groba et al. 2001; Figure 3.1A) ii) Eastern oviparous lineage basal + central viviparous II sister to all remaining viviparous and oviparous (Odierna et al. 2004; Surget-Groba et al. 2006; Figure 3.1C), iii) Eastern oviparous lineage basal + central viviparous I basal to all remaining viviparous and oviparous lineages, and iv) Romanian lineage sister to eastern oviparous and basal to all other lineages (Velekei et al. 2015; Figure 3.1D). We computed per site log likelihoods for each of the five trees and used these to perform Approximately Unbiased tests (AU tests) (Shimodaira 2002), Shimodaira-Hasegawa tests (SH tests) (Shimodaira and Hasegawa 1999), Kishino-Hasegawa tests (KH tests), and Bayesian posterior probabilities (PPs) calculated by the BIC approximation as all implemented in CONSEL vs. 0.1a (Shimodaira and Hasegawa 2001).

We performed a Bayesian approach to infer the topology in BEAST2 (Bouckaert et al. 2014). For this approach, we included a full alignment of all RAD loci (19,068 RAD loci; 1,334,760 total bp; 84,017 variant sites). The number of total SNPs differs from other analyses as loci were set to be present in at least 40% of individuals of each of the six lineages, instead of just being present in at least 40% of individuals across the whole phylogeny. We used the GTRGAMMA substitution model. The analysis was run on CIPRES (Miller et al. 2010) for 500 million generations sampling trees every 50,000 and discarded 10% as burn-in. Convergence was assessed in TRACER (Rambaut and Drummond 2009) and accepted if ESS values of all parameters were larger than 100. In addition, we reconstructed the species tree using ASTRAL (Mirarab et al. 2014), which is based on the multi-species coalescent model. The software package reconstructs evolutionary relationships between species (or deep lineages) with an algorithm integrating over all possible gene trees. Monophyletic lineages were identified from the previous

Maximum likelihood analyses as highly supported (BS=100) evolutionary deep clusters of individuals. Each clade contained a minimum number of nine individuals (ranging from 9-16 individuals). *Iberolacerta horvathi* was included as an outgroup species. ML gene trees from 375,103 RAD loci were reconstructed in RAxML under the GTRGAMMA substitution model using a window size of 100 sites. This resulted in 3,537 gene trees that were used as an input file in ASTRAL.

Additional phylogenetic analyses were carried out under the Maximum Parsimony (MP) optimality criteria. We performed a heuristic bootstrap search with 2000 replicates carried out in PAUP* (Swofford 2002) using TBR branch swapping and with ten random addition sequence replicates for each bootstrap replicate. The 50% consensus bootstrap tree was compared to phylogenies generated with ML and Bayesian analyses.

To incorporate potential past migration events and incomplete lineage sorting effects, we performed a TREEMIX v.1.3 (Pickrell and Pritchard 2012) search using only independent SNPs (one SNP per locus; 49,107 loci included) and a window size of 1000 bp. We included zero to six migration events and compared the variance explained between resulting trees with and without migration events to evaluate the impact of migration. We calculated *f3*-statistics to assess whether admixture has played a role in the evolution of common lizard lineages.

For the mitochondrial dataset, we performed a bootstrap ML search using RAxML (100 bootstrap replicates), MP using the same parameters mentioned above and Bayesian reconstruction with BEAST2 to generate the phylogeny. The best substitution model for BEAST2 was inferred from eleven different substitution schemes in JMODELTEST2 (Darriba et al. 2012) based on lowest AICc and run on CIPRES. We ran BEAST2 for 20 million generations and discarded 10% as burn-in. Convergence was inferred if ESS values in TRACER were larger than 100.

We performed an ancestral trait reconstruction using the corHMM package in R (Beaulieu et al. 2012). corHMM reconstructs ancestral states of binary characters allowing transition rates to differ and treating them as hidden states in a Markov process. We used the ML tree retrieved from RAxML as input tree for the ancestral trait reconstruction. Common lizards are the only viviparous member within the family of Lacertidae. Therefore, the root state was fixed to oviparity. First, we tested which evolutionary scenario was favoured if equal

transition rates to oviparity and to viviparity were applied. We used a transition rate of 0.001 for both directions as initial values (see Chapter 1; Pyron and Burbrink 2014). Next, we tested which evolutionary scenario was supported if the transition rate from oviparity to viviparity was 100x larger than the reverse rate. Finally, we tested which combination of transition rates resulted in a particular evolutionary scenario: i) equal certainty (50%) for single origin of viviparity and a reversal, and multiple independent origins of viviparity and no reversal, ii) 90% certainty of multiple transitions to viviparity and no reversal.

3.4 Results

3.4.1 Data evaluation and identification of optimal parameters for phylogenomic dataset

Total number of generated reads was 828,000,972 (1st library: 10,000,000 reads, 2nd library: 42,377,658 reads, 3rd library: 775,623,314 paired-end reads). After sorting reads into individual loci, mean coverage per individual was 27.6x with a standard deviation of 11.0x (range: 9.2x – 66.9x; median: 24.1x).

We found that phylogenetic resolution generally improved by accepting larger numbers of individuals with missing data (Figure 3.S1). The best summed bootstrap support was achieved using loci that were present in at least 40% of all individuals. Accepting more missing data this did not improve phylogenetic resolution. The highest number of SNPs (including up to three SNPs) resulted in the overall highest phylogenetic resolution (Figure 3.S1). Therefore, we chose the dataset with loci present in at least 40% of all individuals and including all SNPs (no restriction on number of SNPs per locus) for all subsequent analyses. Genotyping error was low (2.0-2.9% per SNP) based on three technical replicates and comparable to previous studies (Mastretta-Yanes et al. 2015; Recknagel et al. 2015).

3.4.2 Monophyletic clades in *Zootoca vivipara* and reconstruction of evolutionary history

All phylogenomic reconstructions confirmed six monophyletic evolutionary divergent lineages with high confidence (all MP and ML bootstrap supports of 100 and PP of 1.0; Figure 3.3). The eastern oviparous lineage was basal sister to all other lineages, followed by central viviparous II. The remaining four lineages are split into two groups, one with the western oviparous and central viviparous I lineages as sister and one with the eastern and western viviparous lineages. This topology is concordant with a single origin of viviparity

and a reversal to oviparity in the western oviparous lineage (see 3.2 for topological analyses). Population structure also confirmed these six genetic lineages, with high average membership values for each respective lineage (mean Q-values ranged from 92-100% identity within each lineages) (Figure 3.3). These six lineages correspond to phylogeographic clades that were previously identified. The recently reported distinct Carpathian haploclade (Velekei et al. 2015) was not confirmed as a separate genetic cluster in our phylogenomic reconstruction and was nested within the eastern viviparous lineage (individuals ELT07086-ELT07095). Our mitochondrial dataset confirmed monophyly of some of the lineages with good support (eastern oviparous, central viviparous, western oviparous), while others where not supported (Figure 3.S2). In contrast to the nuclear data, the separate Carpathian clade was strongly confirmed by mitochondrial DNA and monophyletic, sister to the eastern oviparous lineage (Figure 3.S2).

Genetic differentiation between all six lineages was substantial (Table 3.S3). *Fst* and *Jost D's* values were largest between eastern oviparous and all other lineages, and second largest between western oviparous and all other lineages, indicating that these are the most highly differentiated lineages (Table 3.S3). Compared to *Fst*, *Jost D* was weaker between the western oviparous and all other viviparous lineages (Table 3.S3). Genetic differentiation between the viviparous lineages was less pronounced.

3.4.1 Mitochondrial DNA phylogeny

The final alignment of the cytochrome b gene consisted of 428 bp (42 parsimony informative sites). HKY+I was identified as the best substitution model for BEAST2 (Table 3.S2). This phylogeny resolved eastern oviparous, central viviparous, and western oviparous each as monophyletic (Figure 3.S2). However eastern viviparous, central viviparous II, and western viviparous lineages were all polyphyletic, suggesting considerable introgression and a poor association of single gene mtDNA with the phylogeny generated from genome-wide data. Support values were generally considerably lower for both basal and terminal nodes compared to the phylogeny generated from the extensive genomic dataset. The topology also differed considerably from the topology generated from phylogenomic data (Figure 3.3; Figure 3.S2).

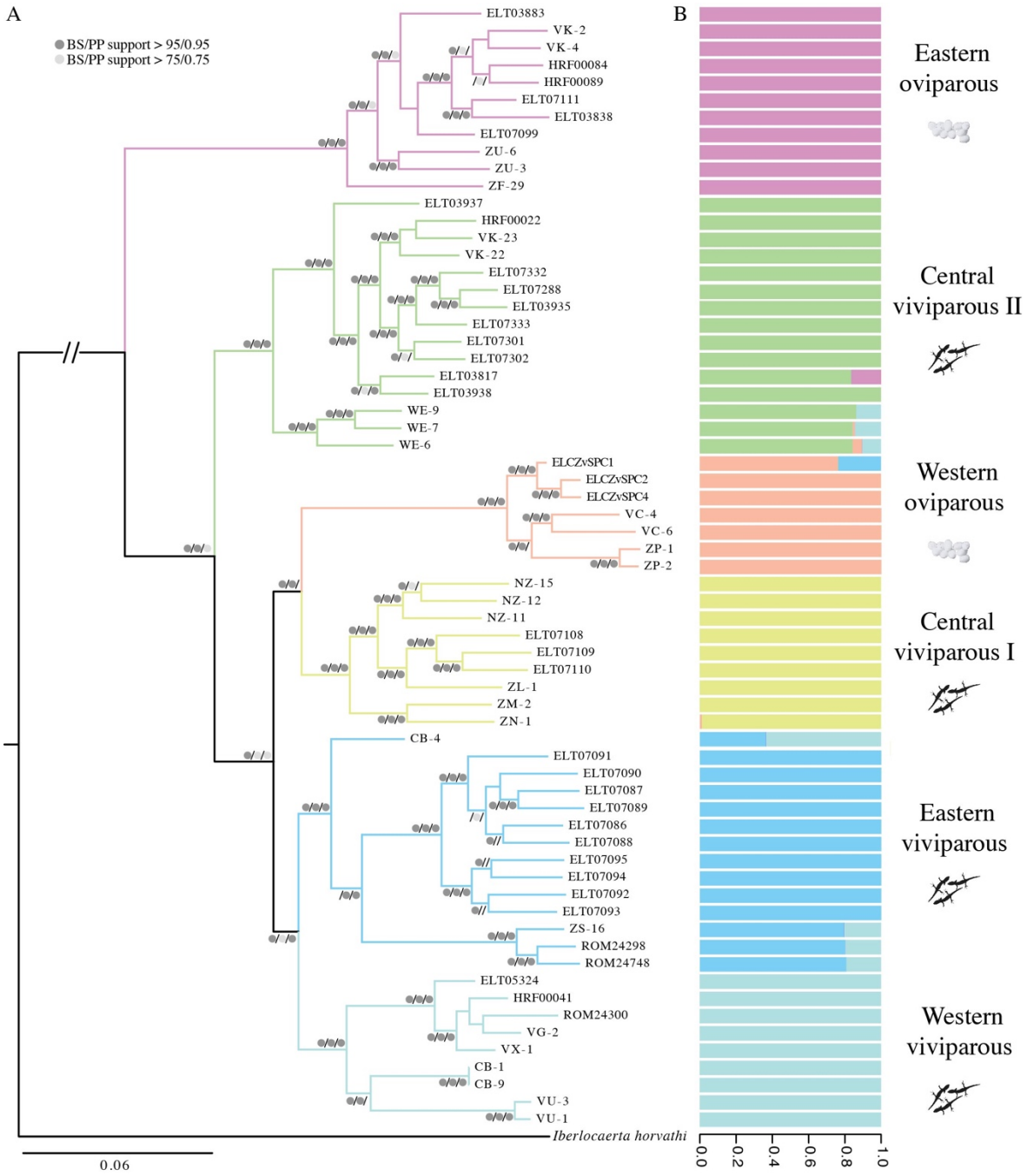

Figure 3.3 Bayesian (B), Maximum likelihood (ML) and maximum parsimony (MP) reconstruction of common lizard evolutionary relationships based on ddRADSeq data. A) The Bayesian tree was used with a full alignment using 1,334,760 sites (84,017 SNPs) and ML and MP trees were constructed with 194,358 SNPs. B posterior probabilities (BS), ML and MP bootstrap support are indicated by dark grey and light grey dots in that order (see legend). B) An ADMIXTURE analysis included the 194,358 SNPs and a k of 6 genetic clusters. Individuals are aligned vertically and respective membership values for each genetic cluster are illustrated. Node that the Carpathian clade is nested within the eastern viviparous clade and includes all individuals with a 100% genomic ancestry of the eastern viviparous cluster. Parity mode and lineage are indicated on the right. *Iberolacerta horvathi* was used as an outgroup (true branch length not shown for graphical reasons).

3.4.2 Scenarios for parity mode evolution

We found significant support for topologies associated with a single origin of viviparity and a reversal to oviparity. Bayesian, Maximum likelihood and parsimony analyses all

confirmed the same topological configuration for the six main common lizard lineages with high nodal supports (bootstraps > 100, all posterior probabilities = 1.0) (Figure 3.3). Phylogenies from all reconstruction methods support a topology in which the eastern oviparous lineage is basal to all other lineages. The following lineage splitting off is the central viviparous II lineage, sister to all remaining lineages. The western oviparous lineage is nested within the viviparous lineages, sister to the central viviparous I lineage. This topology suggests a single origin of viviparity in common lizards and a reversal to oviparity in the western oviparous lineage as the most parsimonious scenario for parity mode evolution. The species tree reconstruction also supported a single origin of viviparity and a reversal to oviparity as most parsimonious evolutionary scenario (Figure 3.S3). The topology at basal nodes was similar to the relationships recovered with other reconstruction methods, but differed in the relationship between the western oviparous, central viviparous I, western viviparous and eastern viviparous (Figure 3.S3). In the species tree, the western oviparous lineage was sister to the eastern viviparous lineage, and the central viviparous I was sister to the western viviparous lineage. All nodes had high posterior probabilities (> 0.98).

Using monophyly constraints and statistical topology testing, alternative scenarios of parity mode evolution were unlikely. Alternative scenarios included: oviparity as a basal trait and a single origin of viviparity (Figure 3.1A; Table 3.1), multiple independent origins of viviparity (Figure 3.1B; Table 3.1), a reversal to oviparity but independent sex chromosome evolution (Figure 3.1C; Table 3.1), and multiple origins of viviparity and a reversal to oviparity (Figure 3.1D; Table 3.1) and were all significantly less likely (Table 3.1) than a single origin of evolution, a reversal to oviparity and a single change in sex chromosome configuration, consistent with Figure 3.3.

Reconstructing evolutionary relationships between the six main phylogenetic lineages in TREEMIX results in a similar topology as retrieved from the other analyses, with eastern oviparous consistently sister to all other lineages. Overall likelihood and variance explained increased including more migration events, and reached a plateau after two migration events (Figure 3.S4). Topologies were unstable when more migration events were included, though these topological changes should be considered with caution since all *f3*-statistics were positive, indicating that recent admixture has not played a major role in the evolution of common lizard lineages (Table 3.S4).

Ancestral trait reconstructions confirmed that a single origin to viviparity and a reversal to oviparity was the most parsimonious evolutionary scenario if transitions rates were equal (Figure 3.S5A). If the transition rate from viviparity to oviparity was 100x lower than the transition rate from oviparity to viviparity, the likelihood for a scenario involving a reversal to oviparity was still much more likely (>90%) than a scenario with multiple transitions to viviparity (Figure 3.S5B). The transition from viviparity to oviparity had to be 315 x less likely than the transition rate from oviparity to viviparity for a scenario in which multiple transitions to viviparity being equally likely as the scenario involving a reversal to oviparity (50% likelihood of oviparous or viviparous ancestors at crucial nodes; Figure 3.S5C). If the transition rate from viviparity to oviparity was 1200x smaller than the reverse rate, likelihood for a model involving multiple transitions to viviparity had a 90% likelihood (Fig 3.S5D).

3.5 Discussion

3.5.1 Evolutionary history of parity mode transitions

Here, we show that the most parsimonious scenario for the evolution of parity mode evolution in common lizards includes a single origin of viviparity and a reversal to oviparity in a single lineage (western oviparous). Our genome-level phylogeny based on up to 194,358 nucleotides was highly supported by Bayesian ML, and MP analyses (support values >0.95). Topologies compatible with other parity mode scenarios, such as a no reversal to oviparity or multiple origins of viviparity (per Fig 3.1A, B, D) performed significantly worse in all statistical tests (Table 3.1). The topology of the species tree reconstruction supported the same evolutionary scenario of a reversal to oviparity. Ancestral trait reconstruction suggests that only with large differences in the transition rate (> 100x; see Figure 3.S5) from oviparity to viviparity relative to the reverse rate from viviparity to oviparity a scenario involving multiple transitions to viviparity becomes more likely than a single origin of viviparity and a reversal to oviparity. We found considerable differences between our high resolution phylogenomic tree and our mtDNA phylogeny.

The evolution of oviparity and viviparity in common lizards has been contentious and a range of studies, using different geographic and genetic sampling, have failed to converge on an evolutionary scenario. To date, mitochondrial DNA, nuclear DNA, and karyotypic markers have not agreed on a single topology (Figure 3.1; Surget-Groba et al. 2001, 2006; Odierna et al. 2004; Velekei et al. 2015). For example, previous research suggested that a

reversal to oviparity occurred in common lizards, however support was based on only limited genetic data (less than 5 genes) and phylogenetic support (Surget-Groba et al. 2006; Cornetti et al. 2014). It has also been proposed that viviparity evolved multiple times independently (Odierna et al. 2004; Velekei et al. 2015), however, these studies were limited to just the sex chromosomes or a single mtDNA gene as markers. Our phylogeny is the first that is consistent with nuclear genetic markers and chromosomal configuration (Figure 3.1; Figure 3.3).

In addition to our robust and well supported phylogeny and the topological statistics, our inference of a reversal to oviparity in the western oviparous lineage is also supported by other aspects of common lizard genetics and reproductive traits. These include the different morphological and physiological egg characteristics between the eastern and western oviparous lineage, such as thinner eggshells and shorter incubation time (Arrayago et al. 1996; Lindtke et al. 2010). We suggest this is compatible with our phylogeny; the derived western oviparous lineage is due to a reversal to oviparity instead of retaining the ancestral oviparous condition, and in doing so the thickness of the eggshell is reduced. Our phylogeny is consistent with the most parsimonious scenario for the derived chromosomal features in common lizards: While both the eastern oviparous and central viviparous II lineages have 36 chromosomes and a ZW sex chromosome configuration, all other lineages exhibit 35 chromosomes and a Z_1Z_2W sex chromosome configuration (Figure 3.1; Odierna et al. 2004; Kupriyanova et al. 2008). The derived Z_1Z_2W sex chromosomal configuration resulted from a tandem fusion of the original smaller W with an autosome (Odierna et al. 2004). Previous genetic studies were inconsistent with this derived sex chromosome configuration by placing central viviparous II nested within lineages exhibiting the Z_1Z_2W chromosome configuration instead of being basal to lineages with the derived configuration (Cornetti et al., 2014; Surget-Groba et al., 2001, 2006). The phylogeny presented here is the first molecular phylogeny consistent with a single transition in sex chromosome configuration, changing from the ancestral ZW system to the derived Z_1Z_2W system (Odierna et al. 2004; Kupriyanova et al. 2006).

Calcified eggshell and the associated reproductive life history traits of oviparity represent a complex character that once lost is unlikely to re-evolve, making it a trait long regarded to be subjected to Dollo's law of irreversibility (Lee and Shine 1998; Shine and Lee 1999; Sites et al. 2011). However, research on the re-evolution of insect wings (Whiting et al. 2003; Collin and Miglietta 2008), snail coiling (Collin and Cipriani 2003), or mandibular

teeth in frogs (Wiens 2011) has shown that in some cases complex characters can indeed re-evolve. In squamate reptiles, a single case arguing for the re-evolution of oviparity in sand boas has been reported (Lynch and Wagner 2010). In this example, a scenario with no reversal to oviparity required three additional evolutionary transitions compared to the most parsimonious scenario with a single reversal to oviparity. In addition to the support from parsimonious trait reconstruction from the phylogeny, sand boas lack the egg tooth, which is an important anatomical structure for hatching from eggs that is present in related oviparous snake species. This provides independent evidence for the derived state in sand boas and the re-evolution of oviparity (Lynch and Wagner 2010). In general, in addition to support from phylogenetic reconstruction, it should be best practice to assess whether the trait re-evolved is developmentally and anatomically similar to the ancestral trait. Substantially different features of the trait in the derived compared to ancestral form can be considered additional evidence for re-evolution, rather than the less plausible scenario that the ancestral form was retained but changed over time while an alternative trait was independently lost in multiple related lineages. In common lizards, the short timespan between the origin of viviparity and the re-evolution of oviparity might have facilitated the reversal, in that not many genomic changes may have been required. In general, a trait as complex as viviparity is thought to require several changes in the genome (Murphy and Thompson 2011).

Whether reversals to oviparity from viviparity occurred frequently in squamate reptiles remains a highly controversial topic. Erroneous phylogenetic reconstruction and limited assessment of characteristics of the trait in question have led to the publication of controversial examples of re-evolution (e.g. Fairbairn et al. 1998; Pyron and Burbrink 2014) that have been criticized heavily (Blackburn 1999a, 2015; Shine and Lee 1999; Griffith et al. 2015; King and Lee 2015a; Wright et al. 2015). Moreover, incomplete lineage sorting and/or introgression of the trait in question, combined with the limited information on the genetic basis of the trait, can lead to wrong conclusions in trait evolution (Hahn and Nakhleh 2016). While here we found substantial support for the re-evolution of oviparity based on the largest genomic dataset to date, more knowledge on the development and genetics of the trait is necessary to unequivocally assess whether a reversal to oviparity occurred in common lizards. In the future, more refined phylogenetic reconstructions using whole genome and phylogenomic data combined with insights into the genetic mechanisms involved in parity mode evolution should provide answers on whether reversals to oviparity occur in squamates and how common they are.

3.5.2 Evolutionary relationships between common lizard lineages and comments on taxonomic status

Our genome-wide phylogeny recovered a new topology, but this included similar clades as previously supported by mitochondrial DNA reconstructions (Surget-Groba et al. 2006; Velekei et al. 2015), except for the Carpathian clade, which we find is nested within the eastern viviparous lineage (Figure 3.1; Figure 3.3; Figure 3.S4). Incongruence between nuclear data and mitochondrial data is observed frequently (Ballard and Whitlock 2004; Near and Keck 2013; Wallis et al. 2017). Consistent with previous phylogenetic analyses (Surget-Groba et al. 2001, 2006; Cornetti et al. 2014), we found the eastern oviparous lineage is basal to all other common lizard lineages. Splitting order for the other lineages differs from previous phylogenetic reconstructions, however, the reciprocal monophyly of all remaining five lineages was highly supported by all analyses here. Most importantly, splitting order of previous molecular phylogenies were all incongruent with reconstructions based on the karyotype (Surget-Groba et al. 2001, 2006; Cornetti et al. 2014; Velekei et al. 2015). Our phylogeny is consistent with a single change in a major sex chromosome configuration in common lizards . In agreement with this, *f3*-statistics suggest that there was no significant admixture between lineages (Table 3.S3). Past mitochondrial DNA introgression and capture are a possible mechanism explaining the discordance between mitochondrial and nuclear genes (Willis et al. 2014; Leavitt et al. 2017).

Based on the strong reciprocal monophyly of the lineages, we suggest that a future revision of the subspecific taxonomy may be warranted. Some have argued that *Z v. carniolica* (= eastern oviparous lineage) should be recognized as a separate species based on limited gene flow and reproductive isolation (Cornetti et al. 2015a,b). However, while hybridization is rare and might be geographically restricted, it does occur between *Z v. carniolica* and other viviparous common lizards (Lindtke et al. 2010; pers. obs.) and phenotypic differences are generally small (Guillaume et al. 2006; Rodriguez-Prieto et al. 2017). All other main lineages (CVII, CVI, EV, WV, WO) could each be rendered a subspecific status given their clear evolutionary splits and differences in karyotype (Odierna et al. 1998, 2004; Guillaume et al. 2006; Kupriyanova et al. 2006; Surget-Groba et al. 2006). Currently, only *Z. v. louislantzi* (WO) can be recognized as a valid subspecies, while other lineages have conflicting subspecific designations (Arribas 2009; Schmidtler and Böhme 2011). While diagnostic morphological features are scarce (Guillaume et al. 2006), in-depth analyses using more levels of the phenotype (e.g. differences in

colouration, behavior, reproduction and ecology) should resolve whether the distinguished genetic lineages are supported by phenotypic data. A more balanced genetic sampling across the whole geographic range using modern molecular and phylogenetic techniques combined with morphological and ecological data collection of the group is much needed.

3.5.3 Advantages and challenges of RADSeq data for phylogenetic reconstruction

Our phylogenetic reconstruction represents the most comprehensive and robust phylogeny of common lizards to date, based on 194,358 bp of polymorphic SNPs and 67 individuals. Previous phylogenetic studies on common lizards using only mitochondrial data (Surget-Groba et al. 2006) or three nuclear markers (Cornetti et al. 2014) had only moderate congruency between different markers and weak support at basal nodes. In agreement with the challenges from previous studies, our mtDNA phylogeny of an established, informative locus was not compatible with the phylogenomic dataset, highlighting the limitations of mtDNA (Ballard and Whitlock 2004; Willis et al. 2014; Wallis et al. 2017) and suggesting it is not an appropriate marker for resolving the history of common lizards. More generally, we suggest that for groups with short internal branches and evolutionary histories of recent to several million years divergence, the type of data produced by RADSeq might be optimal to resolve difficult evolutionary splits. This is the case for adaptive radiations or more generally for short and quick speciation events and complex phylogeographic histories(Giarla and Esselstyn 2015; Rodríguez et al. 2017). This study evidences the power of fast evolving loci (loci with several SNPs) to resolve short phylogenetic branches.

A challenge of short-read phylogenomics and loci with multiple SNPs is the validity of orthology between loci. We show that topological groupings are more robustly supported when using loci with multiple SNPs (Figure 3.S1) and we present an assessment pipeline for validating the cut-offs for missing data and SNPs per locus. Without a reference genome and a large amount of duplicated and/or repetitive DNA, orthology of RAD loci is often not evaluated. Using a reference genome to map the RAD loci, and a high sequencing coverage per individual, such as done here, are important methodological measures to overcome these issues (Mastretta-Yanes et al. 2015; Shafer et al. 2017). Disadvantages of these large but informative datasets are long computational time for some analyses, in particular phylogenetic reconstructions using Bayesian coalescence based analyses (Bryant et al. 2012). Advances in phylogenomic methodologies to accommodate

these more complex datasets will be important for advancing the field (Delsuc et al. 2005; Leavitt et al. 2016; Fuentes-Pardo and Ruzzante 2017).

3.5.4 Conclusions

Our results strongly support a single origin of viviparity in common lizards and a subsequent reversal to oviparity in one derived lineage as the most parsimonious scenario of reproductive mode evolution (Figure 3.3, Table 3.1). In the light of karyological and reproductive data (Arrayago et al. 1996; Odierna et al. 1998, 2004; Heulin et al. 2002; Lindtke et al. 2010), these findings are strong evidence that a reversal to oviparity has occurred in what is now the allopatric western oviparous lineage (Figure 3.2, Figure 3.3). In addition, we propose that a taxonomic revision of this genus at the subspecific level may be needed. More generally, this suggests that Dollo's law of irreversibility is not without exceptions, and might be particularly prone to switches between characters at early stages of evolution of a new or lost trait. For the future, we suggest that common lizards represent an ideal candidate to investigate the genomic basis for evolutionary complex reversals.

Chapter 4: Differential reproductive investment in co-occurring oviparous and viviparous common lizards (*Zootoca vivipara*) and implications for the evolution of viviparity

This chapter has been reviewed in Oecologia and is currently prepared to be resubmitted.

4.1 Abstract

Live-bearing reproduction (viviparity) has evolved from egg-laying (oviparity) independently many times and most abundantly in squamate reptiles. Studying life history trade-offs between the two reproductive modes is an inherently arduous task, as most transitions to viviparity are evolutionarily old and/or are confounded by environmental effects. The common lizard (*Zootoca vivipara*) is one of a few known reproductively bimodal species, in which some populations are oviparous and others viviparous. Oviparous and viviparous populations can occur in sympatry in the same environment, making this a unique system for investigating life-history trade-offs between oviparous and viviparous reproduction. We found that viviparous females exhibit larger body size, smaller clutch sizes, a larger reproductive investment, and a higher hatching success rate than oviparous females. We found that offspring size and weight from viviparous females was lower compared to offspring from oviparous females, which may reflect space constraints during pregnancy. We suggest that the evolution of viviparity in common lizards is associated with increased reproductive burden for viviparous females and that this promotes the evolution of larger body size to create more physical space for developing embryos. In the context of life history trade-offs in the evolution of viviparity, our research suggests that the extent of correlation between reproductive traits, or differences between reproductive modes, may also depend on the time since the transition occurred.

4.2 Introduction

Live-bearing reproduction is one of the most ubiquitous life-history transitions across the animal kingdom (Sites et al. 2011). It has evolved independently from egg-laying more than 150 times across all vertebrates (Shine 2005; Blackburn 2006), and numerous times among invertebrates (Clutton-Brock 1991; Blackburn 1999b). While the causes for the evolution of viviparity are not fully understood, recent advances in phylogenetic reconstruction and environmental data collection have shed light on this question in some taxonomic groups. Several studies now suggest that squamate reptiles, the group with the largest number of transitions from oviparity to viviparity, evolved viviparity in response to cool climates (Tinkle and Gibbons 1977; Lynch 2009; Schulte and Moreno-Roark 2010; Lambert and Wiens 2013; Watson et al. 2014). Experimental case studies of squamates also support this hypothesis (Rodríguez-Díaz and Braña 2012), though many examples exist of tropical viviparous species (Tinkle and Gibbons 1977; Vitt and Blackburn 1983; Webb et al. 2006), which suggests other life history trade-offs are important (Webb et al. 2006). In other animal groups, the causes are even less well understood, mainly due to the limited number of transitions and the difficulty to separate correlative variables from causative factors (Wourms and Lombardi 1992; but see Bassar et al. 2014).

The evolution of viviparity entails dramatic changes in morphology, physiology, ecology and behaviour (Guillette 1993; Thompson and Speake 2006). Viviparity offers several potential fitness advantages, including protection of the embryo from adverse environmental conditions and predation, and higher trophic level at independence due to larger offspring size. The disadvantages of live-bearing include lower reproductive output of the female due to space constraint and reduced number of clutches, and increased female mortality due to limited locomotion and increased predation pressure (Wourms and Lombardi 1992; Blackburn 1999b; Shine 2002; Sites et al. 2011). For example, due to space constraint and a decrease in female locomotion ability with the number of offspring carried, viviparous females tend to exhibit reduced clutch sizes (Seigel and Fitch 1984; Qualls and Shine 1995). Presumably to counteract the space constraint and the increase in predation pressure, some viviparous species have evolved larger body sizes (Qualls and Shine 1995; Goodwin et al. 2002). Another adaptation that constitutes a trade-off with the decrease in clutch size is enhanced offspring survival. This can be achieved by larger offspring size at birth compared to progeny hatching from eggs, enhancing the survival of the offspring by increasing independence and avoiding predation (Goodwin et al. 2002). Viviparous species can also exhibit longer lifespans. This allows them to produce more

offspring across years and accounts for the lower reproductive output per season compared to oviparous species (Tinkle et al. 1970). Some support for these hypotheses comes from a few large-scale studies (Seigel and Fitch 1984; Stearns 1984; Meiri et al. 2012), although confounding phylogenetic and environmental effects have a substantial impact on life-history evolution associated with reproductive mode (Dunham and Miles 1985; Meiri et al. 2012; Bassar et al. 2014).

Common lizards (*Zootoca vivipara*) are one of the few known animal species that are both oviparous or viviparous, with different intraspecific lineages being fixed for either reproductive mode (Figure 4.1A; Guillette 1993; Mayer et al. 2000; Surget-Groba et al. 2006). Experimental crosses between individuals from oviparous and viviparous lineages have shown that viviparity is a genetically heritable trait (Arrayago et al. 1996). Reproductive mode is associated with distinct phylogeographic lineages across Europe that diverged between 2-4 mya (Surget-Groba et al. 2006; 3.8 mya in Chapter 2). The two parity modes are usually allopatric and interbreed exceedingly rarely (Lindtke et al. 2010; Cornetti et al. 2015a,b). Experimental studies using lizard enclosures have amassed substantial knowledge about reproductive traits and strategies within both oviparous (Heulin et al. 1997; Roig et al. 2000; Rodríguez-Díaz and Braña 2012) and viviparous populations (Avery 1975; Sorci et al. 1996; Chamaillé-Jammes et al. 2006; Eizaguirre et al. 2007; Massot et al. 2011; Bleu et al. 2013) in different geographic settings.

Because oviparous and viviparous common lizards are usually found in different geographic regions, little is known about the functional ecology of alternative reproductive strategies associated with parity modes in a controlled, similar environment. We have been studying the single known secondary contact zone between oviparous and viviparous populations, and where the two reproductive lineages are found syntopically (Lindtke et al. 2010). This allows us to directly study reproductive effort in both modes *in situ* while minimizing confounding effects of environment, phylogeny, and plasticity (Sorci et al. 1996; Sorci and Clobert 1999; Lorenzon et al. 2001; Roitberg et al. 2013). These factors make common lizards an ideal model organism to test ecological and evolutionary hypotheses on life-history trade-offs between the two alternative reproductive modes (Blackburn 2006; Murphy and Thompson 2011).

Here, we tested five predictions on the trade-offs associated with reproductive mode in female common lizards. Specifically, that viviparous individuals exhibit i) larger body size

ii) decreased clutch size iii) larger offspring at birth iv) larger reproductive investment and v) enhanced hatching success. Support for all predictions would indicate that viviparous common lizards have optimized their reproductive traits following life-history theory predictions (Tinkle 1969; Stearns 1976, 1992; Roff 1992). Alternatively, partial support for some of the predictions would indicate that factors such as cavity size (Qualls and Shine 1995) or offspring survival (Reznick 1982; Pike et al. 2008) could be limiting the viviparous common lizard's reproductive output.

4.3 Methods and Materials

4.3.1 Study site and species

The study was carried out in the Carinthian Alps in the Gailtal valley of Austria (Figure 4.1B). Co-occurring viviparous (central viviparous II lineage) and oviparous (eastern oviparous lineage) common lizards are extremely rare and to date this is the only known locality where both forms co-occur in high densities (Surget-Groba et al. 2006; Lindtke et al. 2010; Cornetti et al. 2015a). The study site covers an area of approximately 0.3 km^2 and an altitude range of 200m from 1380 – 1580 masl. Female common lizards were collected between April-August from 2013-2016 and caught by hand. Females were distinguished from males by the absence of a hemipenal bulge at the base of the tail. A female's reproductive mode was assessed based on the number of incubation days and eggshell characteristics of its clutch, and genetic ancestry (Recknagel et al. unpubl.; see Chapter 5 for details). Offspring from viviparous females are surrounded by a thin membrane after parturition, and usually emerge from the membrane within a day (Lindtke et al. 2010). In contrast, developing offspring from oviparous females are surrounded by an eggshell (about 62–94 μm; Lindtke et al. 2010). Hybrid females (number of incubation days between 4 and 28) were excluded from the analysis (Lindtke et al. 2010). For each female, the location of capture and altitude was recorded. On average, oviparous individuals are found around an altitude of 1413 masl and viviparous around 1475 masl (Lindtke et al. 2010). Presence of a biting mark on the female's belly or flank resulting from mating served to identify whether she was pregnant. All lizards were weighed using a smart weigh high precision scale (to the nearest 0.001g) and measured for snout-vent length (SVL) and tail length (TL) using digital callipers (to the nearest 0.01mm) immediately after capture. Female lizards were weighed a second time after oviposition/parturition in 2016 to measure reproductive traits relative to female weight after oviposition/parturition.

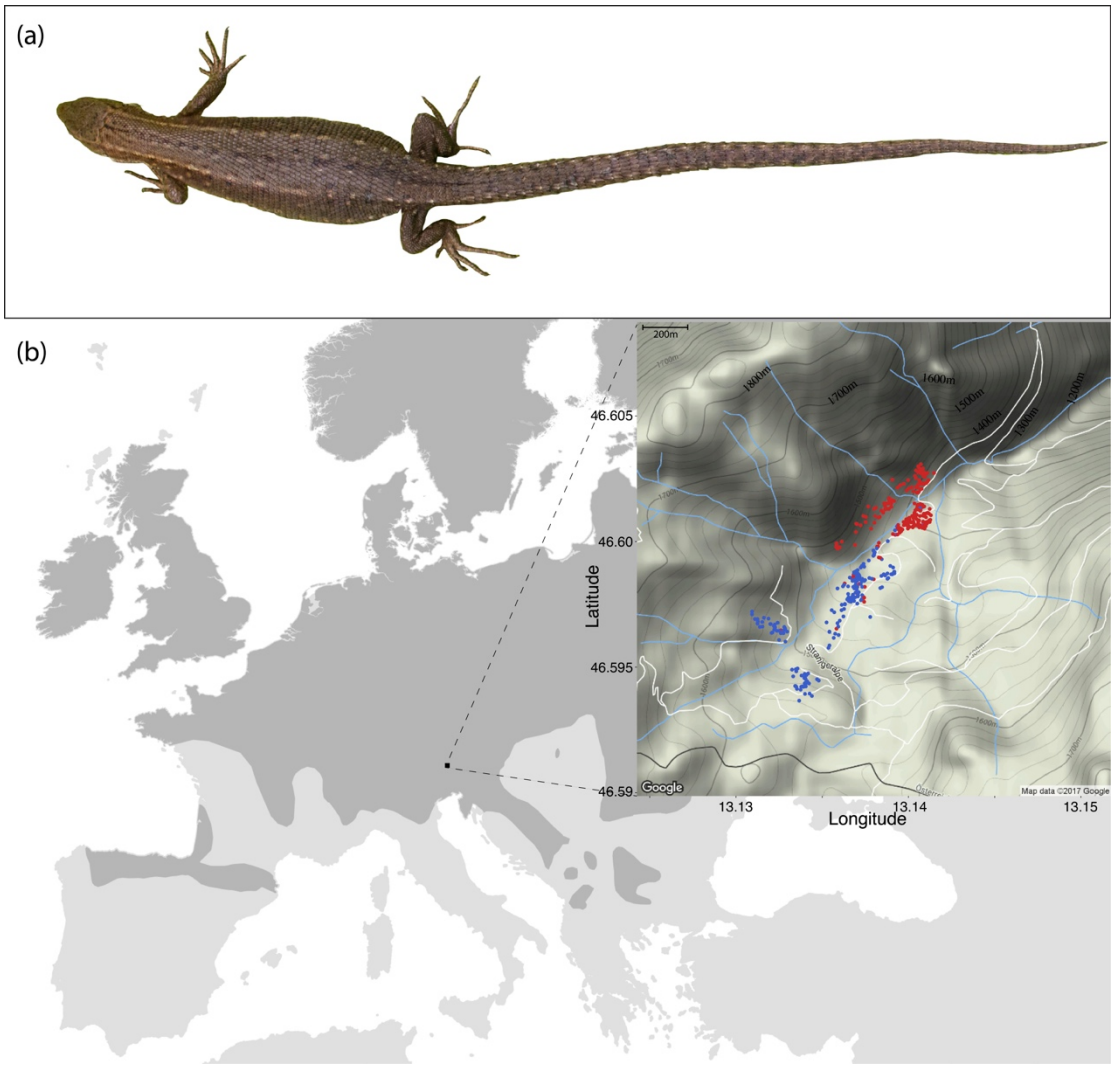

Figure 4.1 A) The study organism: a female common lizard (*Zootoca vivipara*). B) The distribution of the common lizard across Europe (dark grey shaded area, extracted from IUCN database). The sampling location situated in the Carinthian Alps in Austria is indicated in detail. The collection location for each female is indicated with a red (oviparous) or blue (viviparous) dot (N = 438).

4.3.1 Reproductive traits

Pregnant females (N = 438) were kept until oviposition or parturition to assess their reproductive mode and other reproductive traits. Females were individually housed in 56x39x28 cm plastic terraria with netting on top and one side to guarantee air flow. All terraria were set up within tents close to the study area (at ~ 900 masl), so that lizards were exposed to natural temperature variation. Tents ('Event Tent' by Vango) included plastic windows that allowed for insolation. Each terrarium contained sand as substrate, shelters (pieces of wood), moisturized moss, and a bowl of water. Insolation and shelters providing shade allowed lizards to thermoregulate, providing a temperature range close to what they would experience in their natural environment at the sampling site. Lizards were fed ad libitum with mealworms (*Tenebrio molitor*) and crickets (*Gryllus assimilis*). Females were daily checked for the presence of a clutch. All clutches were incubated at 24°C in an Exo

Terra thermoelectric reptile egg incubator until hatching (Lindtke et al. 2010; Rodríguez-Díaz et al. 2010). After oviposition/parturition, females were released at point of capture.

Here, the term 'clutch' was used for both a viviparous litter and an oviparous clutch, and the term 'hatching' was used for both live born young and oviparous hatchlings. Nine female reproductive traits were measured: clutch size (CS), clutch mass (CM), average egg mass (EM = CM/CS), relative clutch mass measured as clutch mass divided by female weight after oviposition or parturition (RCM), relative offspring mass measured as total sum of each offspring mass divided by female weight after oviposition or parturition (ROM), average offspring size (OS), and offspring mass (OM), average offspring body condition (OM/OS), and total offspring biomass. For sample sizes of each respective measured trait and reproductive mode please refer to Table 4.1. Female weight after oviposition/parturition, female weight loss, EM, RCM and ROM were only available for sampling year 2016, and therefore sample sizes were smaller for these traits (total N = 165). RCM includes the mass of the whole clutch, including eggshell, amniotic fluids, yolk and the embryo. In a few cases offspring from viviparous females had already hatched before the clutch could be weighed, these clutches were excluded from RCM measures as they were lacking amniotic fluids and eggshells. ROM is the summed mass of the hatchlings, therefore excluding eggshells, amniotic fluids and yolk remains. Here, we use the term reproductive investment to refer to an increase in pregnancy time, CZ, EM, RCM, weight loss, ROM and produced offspring biomass. The number of infertile eggs (no embryo visible) and non-hatching offspring (embryos between stage 32-40 *sensu* Dufaure and Hubert 1961) was recorded and, together with the total number of offspring, was used to calculate hatching success, which is the proportion of the total hatched offspring relative to clutch size. Non-hatching offspring was divided into two classes, embryos that died early in development (stages 32-35) and late in development (stages 36-40).

4.3.2 Statistical analyses

All statistical analyses were carried out in R vers. 3.2.3 (R Core team 2015). To test for difference in female body size (SVL) and weight between reproductive modes, we applied ANCOVAs with sampling year and altitude at point of collection as covariates. For differences in reproductive traits, female body size, number of days in captivity until parturition/gestation, sampling year, and altitude were added as covariates in a ANCOVA. Data was normalized prior to ANCOVAs and checked for heteroscedasticity using Levene's test. Interactions between sampling year and parity mode and altitude and parity

mode were included in the model to test and correct for environmental variation and parity mode. Normal distribution of model residuals was checked using a Shapiro-Wilk test. We corrected for multiple testing by applying a Bonferroni correction. To test for parity mode-specific effects of body length, models in which SVL had a significant effect were tested separately for oviparous and viviparous females.

In addition to ANCOVAs, we performed a multivariate approach using a principal component analysis (PCA) in R. Principal component (PC) loadings were compared to assess the importance of variables relative to each other for each PC. Linear regressions were performed to check if PCs differed between reproductive modes.

4.4 Results

4.4.1 Female body size and body weight

Viviparous females were significantly larger than oviparous females, as measured by body length (snout-vent length [SVL]) (N = 428, F= 93.0, η^2= 0.17, P < 0.0001; Figure 4.2A; Table 4.S1). On average, viviparous females were 4.9 mm larger (Table 4.1).

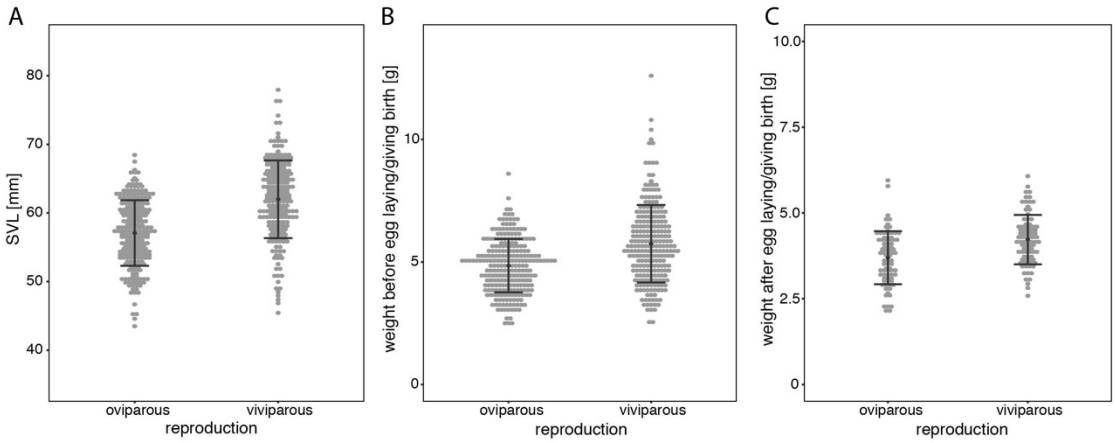

Figure 4.2 Body size (snout vent length [SVL]) and weight of oviparous and viviparous female common lizards (*Zootoca vivipara*) from the contact zone at Straniger Alm in Austria. Mean and standard error are shown for each panel. Viviparous females are larger A) and heavier before B) and after giving birth/egg-laying C) than oviparous females. Shown is the raw data, uncorrected for effects such a body size or duration of captivity. All three measures differ significantly between parity modes, also after correcting for other effects.

Viviparous females were heavier than oviparous females both before (N = 440, df = 1, F= 141.9, η^2= 0.09, P < 0.0001; Figure 4.2B; Table 4.S1) and after parturition/oviposition (N = 155, F= 37.9, η^2= 0.12, P < 0.0001; Figure 4.2C; Table 4.S1). On average, viviparous

females were 0.55 g heavier than oviparous females after giving birth/laying eggs (Table 4.1).

4.4.2 Offspring number, size and body condition at birth

Clutches laid by viviparous females had on average almost one offspring fewer (delta = 0.92) than clutches laid by oviparous females (N = 436, F= 41.2, η^2= 0.05, P < 0.0001; Table 4.1; Figure 4.3A).

Average offspring from viviparous females were smaller in body length (N = 383, F= 308.0, η^2= 0.44, P < 0.0001; Figure 4.3B; Table 4.S1) and weighed less (N = 383, F= 642.5, η^2= 0.64, P < 0.0001; Figure 4.3C; Table 4.S1) than the offspring from oviparous females (Table 4.1). Body condition (mass/SVL) was higher in oviparous offspring compared to viviparous offspring (N = 381, F = 658.3, η^2= 0.64, P < 0.0001; Table 4.1; Table 4.S1).

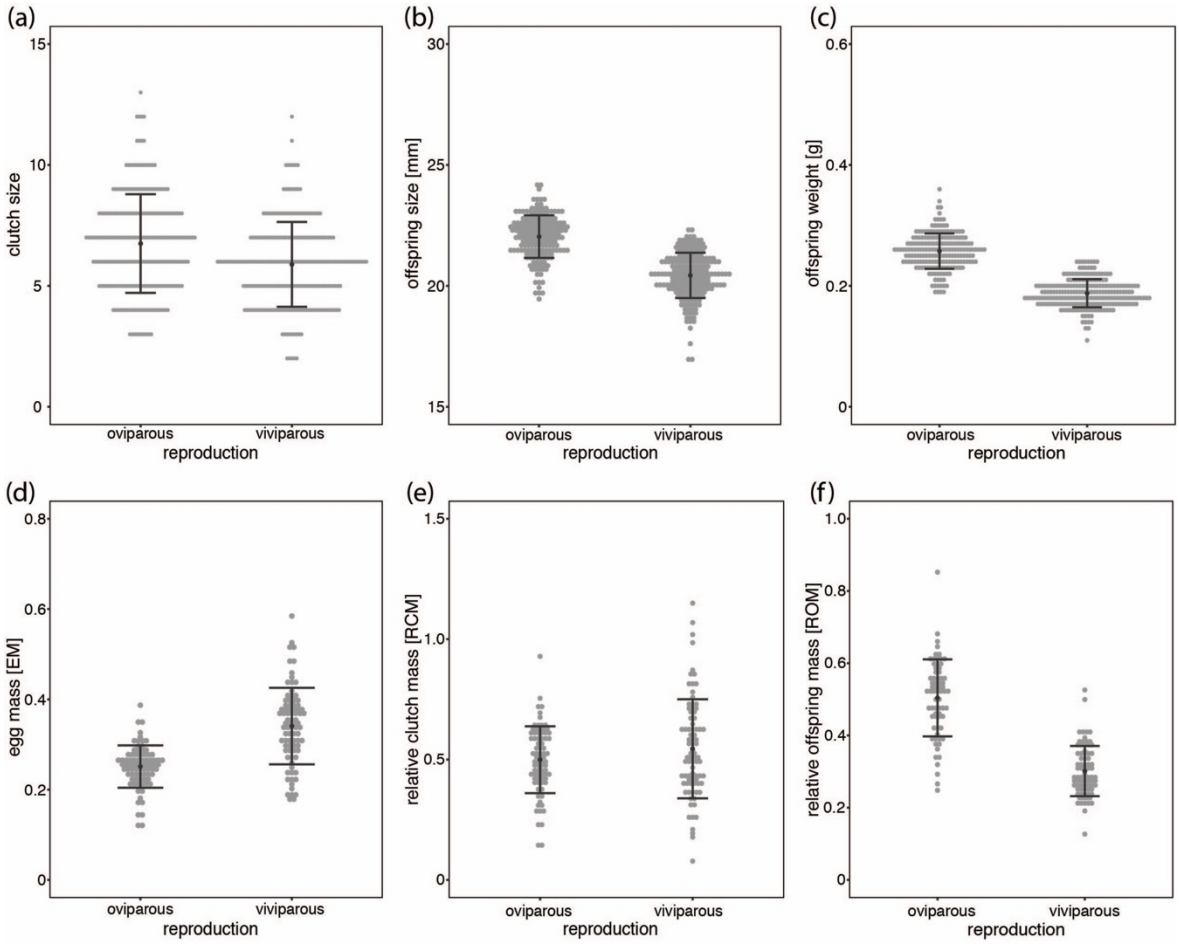

Figure 4.3 Reproductive trait variation between oviparous and viviparous common lizard females. Mean and standard error are indicated for each plot. as Oviparous females have larger clutch sizes A) and larger offspring size B) and weight C). The egg mass (EM) is larger for viviparous females D). Relative clutch mass

(RCM) is larger for viviparous females E), but does not differ significantly after Bonferroni correction (see Table 4.S1). Finally, relative offspring mass (ROM) is larger for oviparous females F).

Offspring size and weight was positively correlated with the mother's body size in viviparous females (size: N = 177, t = 3.38, R^2= 0.06, P < 0.001; weight: N = 178, t = 2.47, R^2= 0.03, P = 0.015; Table 4.S2) whereas this correlation was not significant in oviparous females (size: N = 190, t = 0.14, R^2< 0.01, P = 0.89; weight: N = 189, t = -1.36, R^2< 0.01, P = 0.18; Table 4.S2).

4.4.3 Reproductive investment

On average, parturition in viviparous females occurred 42 days later than oviposition in oviparous females. In both reproductive modes, clutch size was highly positively correlated with female SVL (oviparous: N = 228, t = 13.24, R^2 = 0.434, P < 0.0001; viviparous: N = 188, t = 12.13, R^2 = 0.44, P < 0.001). The average egg mass (EM) was significantly larger in viviparous females (N = 155, F= 87.9, η^2= 0.34, P < 0.0001; Figure 4.3D), indicating that clutches of viviparous females weighed more at the time of parturition than oviparous clutches did at the time of oviposition. EM was also positively correlated with female body size in viviparous females (N = 76, t = 3.64, R^2 = 0.15, P < 0.001), suggesting that larger females invested in clutch mass and clutch size, i.e. number of offspring per clutch (Table 4.S2).

However, at the time of oviposition/parturition, the relative clutch mass (RCM) in viviparous lizards was only slightly larger than that for oviparous lizards and did not differ significantly after Bonferroni correction between the two reproductive modes (N = 155, F = 5.7, η^2= 0.02, P = 0.018; Figure 4.3E). This might be due to the reduced clutch size produced by viviparous females and their greater weight.

The difference in weight loss before (i.e. at time of capture) and after oviposition/ parturition was higher for viviparous females than oviparous females (N = 155, F = 40.9, η^2= 0.11, P < 0.0001), also indicating a larger reproductive investment for viviparous females. Finally, the summed offspring mass relative to the female weight (ROM) was significantly smaller for viviparous females (N = 140, P < 0.0001; Figure 4.3E), indicating that oviparous females have a higher net output per clutch, also suggested by a larger total offspring biomass (N = 383, F = 105.12, P < 0.0001; Table 4.1).

4.4.4 Hatching success

Hatching success was higher for viviparous offspring compared to oviparous (N = 436, F = 12.9 P = 0.0004) (Table 4.1). This was due to a lower percentage of infertile eggs (6.0% vs. 17.1%) and lower percentage of embryos that died at an early stage of development (1.2% vs. 5.8%) in viviparous clutches (Table 4.1). The number of hatched offspring did not differ significantly between oviparous and viviparous females (N = 438, F = 2.07, P = 0.154; Table 4.1).

4.4.5 Principal component analysis (PCA) of reproductive traits

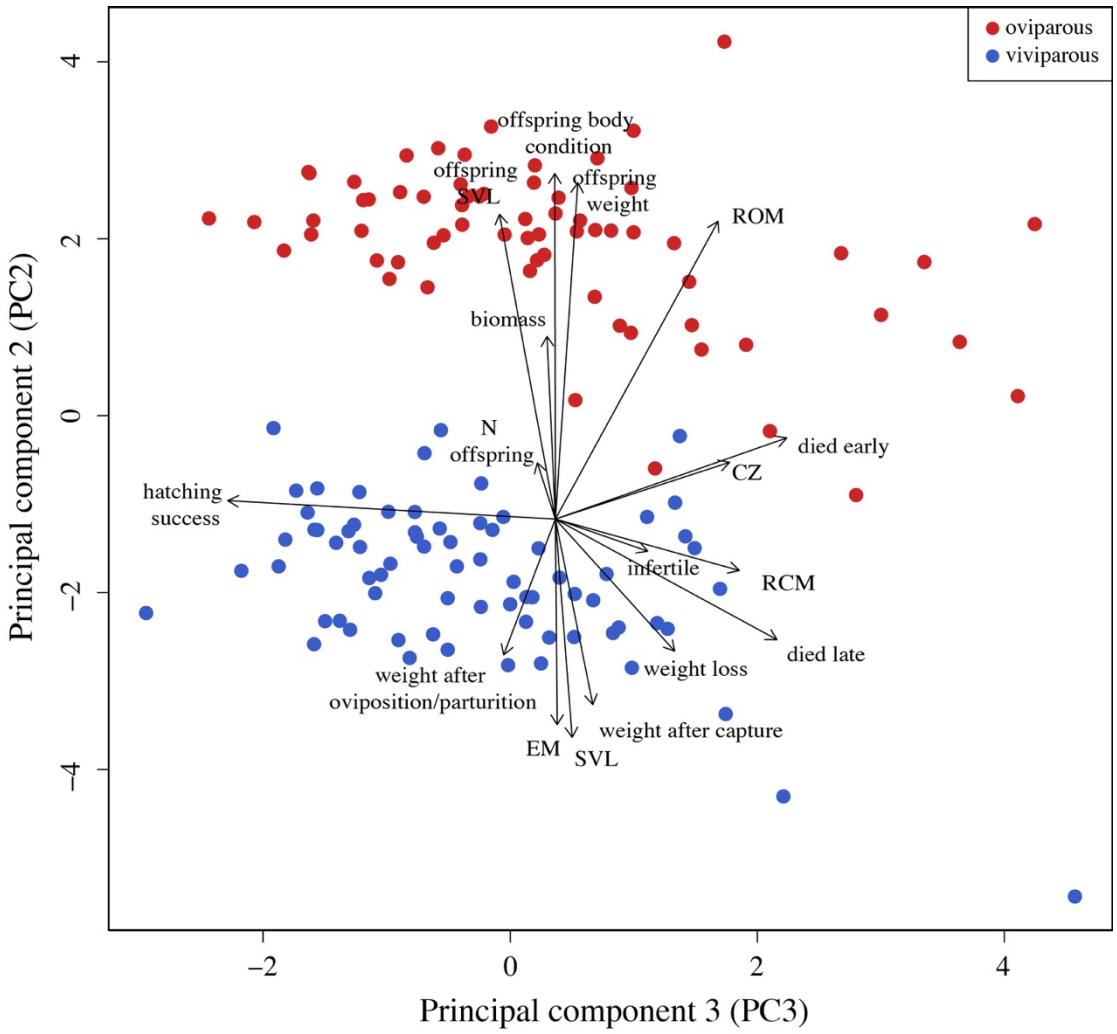

Figure 4.4 Principal component analysis (PCA) of female body size and reproductive traits. The plot shows principal components (PCs) 2 and 3. Both components significantly differ between oviparous (red dots) and viviparous (blue dots) females. Included are only individuals with complete data on body size and reproductive traits (N = 138).

The PCA summarized common directions between reproductive variables and the two reproductive modes. The first PC explained 31.4% of the variance, and mainly described

reproductive variables (CS, RCM, offspring biomass, number of offspring hatched, hatching success) increasing with body size (SVL, weight; all loadings > 0.2 or < -0.2; Table 4.S3). PC1 did not differ significantly between reproductive modes (N = 138, F = 3.06, P = 0.23), viviparous females tended to have a higher score (mean oviparous = -0.24, mean viviparous = 0.22) on that PC. Reproductive modes were significantly different on PC2, and this was the main discriminator between reproductive modes, with almost no overlap between oviparous and oviparous individuals (N = 138, F = 636.6, η^2= 0.82, P < 0.0001; Figure 4.4). PC2 explained 22.7% of the variance, and was associated with low SVL and weight, large offspring output (including large ROM, offspring SVL, weight, body condition and biomass), and low egg mass (Table 4.S3). These are also the main differences (viviparity positively correlated with PC2) associated with the two reproductive modes in the ANCOVAs. PC3, explaining 9.9% of variance, also differed significantly between reproductive modes (N = 138, F = 7.84, η^2= 0.04, P = 0.014; Figure 4.4). This PC was associated with large clutch size, RCM, weight loss, EM, ROM and a strong association with lower hatching success (with a low number of overall hatching offspring and a large proportion of non-hatching embryos) (Table 4.S3).

Table 4.1 Sample sizes, mean and standard variation for all measured traits for oviparous and viviparous females. The same individuals were used for genetic mapping in the following Chapter 5. The absolute (delta mean) and proportional difference (% difference) between reproductive modes in each trait is specified. Finally, ANCOVA statistics in each trait between the reproductive modes are shown including significance after Bonferroni correction.

	oviparous			viviparous			delta mean	% difference	F	η^2	P	
	N	mean	SD	N	mean	SD						
female SVL	235	56.96	4.78	193	61.96	5.66	5.01	8.8	93.0	0.17	<0.0001	***
female weight[1]	237	4.84	1.09	203	5.78	1.58	0.94	19.5	141.9	0.09	<0.0001	***
female weight[2]	79	3.67	0.77	76	4.22	0.72	0.55	14.9	37.5	0.13	<0.0001	***
weight loss	79	1.31	0.20	76	1.49	0.30	0.18	13.9	40.9	0.11	<0.0001	***
clutch size	232	6.73	2.04	204	5.81	1.75	0.92	15.9	41.2	0.05	<0.0001	***
offspring size	190	22.05	0.88	193	20.45	0.94	1.59	7.8	308.0	0.44	<0.0001	***
offspring weight	189	0.26	0.03	194	0.19	0.02	0.07	36.7	642.5	0.64	<0.0001	***
offspring body	189	0.12	0.01	192	0.09	0.01	0.02	26.8	658.3	0.64	<0.0001	***
EM	79	0.25	0.05	76	0.35	0.08	0.10	38.7	87.9	0.34	<0.0001	***
RCM	79	0.50	0.14	76	0.56	0.21	0.06	12.2	5.7	0.02	0.0183	NS
ROM	68	0.51	0.11	72	0.30	0.07	0.20	66.4	207.4	0.55	<0.0001	***
offspring	189	1.33	0.54	194	0.92	0.36	0.41	44.9	101.5	0.13	<0.0001	***
infertility	230	0.17	0.34	206	0.06	0.20	0.11	186.4	15.8	0.03	<0.0001	***
early mortality[3]	230	0.06	0.16	206	0.01	0.06	0.04	321.1	15.3	0.04	0.0001	**
late mortality[4]	230	0.03	0.10	206	0.09	0.22	0.05	61.5	16.3	0.03	<0.0001	***
hatching	230	0.72	0.38	206	0.84	0.30	0.12	16.1	12.9	0.03	0.0004	**
offspring	232	4.23	2.77	206	4.58	2.22	0.34	8.1	2.1	0.02	0.1514	NS

[1]weight measured at time of captivity; [2]weight measured after oviposition/parturition; [3]embryos at developmental stage 1-35; [4]embryos at developmental stage 36-40; abbreviations are: EM = average egg mass; RCM = relative clutch mass; ROM = relative offspring mass. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS = not significant.

4.5 Discussion

Here, we show that reproductive investment strategies differ substantially between syntopically occurring, reproductively bimodal oviparous and viviparous common lizards. Of our five predictions, we found empirical support for four: viviparous females exhibit larger body size, smaller clutch sizes, a larger reproductive investment, and a higher hatching success rate than oviparous females (our predictions i, ii, iv, and v). However, contrary to our prediction (prediction iii), offspring size and weight from viviparous females was lower compared to offspring from oviparous females (Table 4.1). This may suggest an effect of space constraint during pregnancy. Female body size had a major impact on reproductive output, particularly in viviparous females. All reproductive traits were significantly associated with body length in viviparous females, but not in oviparous females. The selective benefit of larger size would therefore facilitate increasing body size in the evolution of viviparous lineages. Reproductive output is lower for viviparous than for oviparous common lizards. While the production of larger offspring could offset the smaller clutch size in viviparous compared to oviparous females, this was not the case and suggests that reproductive output in viviparous common lizards is constrained by their body size. We propose an adaptive scenario for life-history trait evolution following the transition from oviparity to viviparity across vertebrates.

4.5.1 Body size evolution

We show that viviparous females have evolved larger body sizes compared to oviparous females. On average, viviparous females are almost 5 cm larger than oviparous females (Table 4.1). This agrees with the observation that viviparous species are generally larger than oviparous species (Tinkle et al. 1970; Dunham and Miles 1985; Dunham et al. 1988; Cei et al. 2003), though this has received only weak support within a phylogenetic context (Meiri 2008). In the reproductively bimodal lizard, *Lerista bougainvillii,* the viviparous form also exhibits larger body size than the oviparous (Qualls and Shine 1995, 1998). The impact of environmental factors on body size evolution has been reported for some reptile species but remains unresolved (Adolph and Porter 1993; Shine 2005; Pincheira-Donoso and Meiri 2013; Roitberg et al. 2013), however such factors are unlikely to play an important role in this analysis as the environment is the same. The distribution of oviparous and viviparous lizards within the sampling site is not associated with any habitat-specific variable (Layton et al., unpublished). The only environmental variable possibly affecting reproductive traits is altitude (as a predictor for temperature), and was

controlled for in all statistical tests (see Table 4.S1). Altitude may play a role in adult habitat origin and selection but here all females laid their clutches at the same altitude. Body size was more strongly correlated with reproductive variables in viviparous compared to oviparous females, evidenced by linear regressions of body size and reproductive variables (Table 4.S2) and also shown by a positive but not significant association of viviparous females with PC1, which was a main discriminator between several reproductive traits related to larger body size. A strong association between body size and reproductive investment in viviparous relative to oviparous common lizards confirms previous research (Horváthová et al. 2013) in this species. Here, as these common lizards are closely related geographic lineages, we show support for the body size hypothesis largely independent of phylogenetic bias.

4.5.2 Difference in clutch size and reproductive investment

Both reproductive mode show a strong association between body size and clutch size, showing that larger females generally produce larger clutch sizes. This is a well-established relationship in squamate reptiles (Dunham and Miles 1985; King 2000). Viviparous common lizards have significantly smaller clutch sizes, on average almost one individual less per clutch compared to oviparous clutches (Table 4.1). This confirms previous studies in *Zootoca vivipara* (Lindtke et al. 2010; Roitberg et al. 2013), except for one study finding the opposite pattern (Horváthová et al. 2013). Clutches laid by oviparous females are much lower in weight at the time of oviposition compared to viviparous clutches at the time of parturition, as indicated by the clutch mass to size ratio (Table 4.1). The reason for this is that during egg development, the size of the egg increases substantially, mainly due to water uptake (Mathies and Andrews 1995; Qualls and Andrews 1999; Sun et al. 2012). While for oviparous clutches, this increase in weight occurs outside of the mother's reproductive tract after oviposition, viviparous females must cope with their clutches' increase in size and weight internally.

Pregnancy poses a reproductive burden to lizards, as they are less mobile and therefore more vulnerable to predation during this time (Shine 1980; Bauwens and Thoen 1981; Van Damme et al. 1989; Itonaga et al. 2012). The time of fertilization could not be measured here, but both reproductive modes become active around the same time in spring as soon as snow melts. Viviparous females are therefore probably affected by pregnancy about a month longer than oviparous females. The increase in mass at later stages of the embryonic development poses an additional reproductive burden to viviparous common lizards, and is

compensated by smaller clutch size. In poeciliid fishes, viviparity has also been associated with smaller clutch sizes relative to oviparous (Thibault and Schultz 1978; Mank and Avise 2006). Across reptiles, the pattern is somewhat unclear, with larger-scale studies suggesting generally larger clutch sizes for viviparous reptiles (Tinkle et al. 1970; Iverson 1987). An explanation for this perhaps non-intuitive increase in viviparous clutch size might be that several oviparous species have multiple clutches per year, while viviparous species only have a single clutch per year, and single brooded species have larger clutch sizes than multi-brooded species (Tinkle et al. 1970). For example, in common lizards, oviparous lizards can lay two or more clutches (Heulin et al. 1991, 1994; Roig et al. 2000), whereas viviparous usually only lay one clutch per year (Bestion et al. 2015). There might be less selective pressure for increasing clutch size in oviparous species, while single-brooded viviparous clutch size should be maximized and might be under stronger selective pressure (Cox et al. 2003; Roitberg et al. 2013). However, at high altitudes such as studied here, oviparous common lizards populations also usually produce a single clutch per year (Rodríguez-Díaz and Braña 2012).

We found another measure of reproductive investment, the relative clutch mass (i.e. clutch mass relative to mother's weight), was only slightly larger in viviparous females. In contrast, the weight loss viviparous females experienced after parturition was significantly larger compared to oviparous females, indicating a greater reproductive investment associated with viviparity. This is in agreement with a study on the reproductively bimodal lizard *L. bougainvillii,* in which relative clutch mass (RCM) was larger for viviparous compared to oviparous females (Qualls and Shine 1998). Decreasing clutch size and increasing body size (= larger body cavity allowing for more space with developing offspring) are two ways viviparous common lizards can accommodate for the additional reproductive burden. Comparisons across oviparous and viviparous common lizard populations also showed higher reproductive investment for viviparous females (Horváthová et al. 2013; Roitberg et al. 2013). We suggest that the increase in body size and decrease in clutch size in viviparous common lizards follow from the increased reproductive burden.

4.5.3 Offspring size, weight, survival and total reproductive output

Contrary to our expectation, offspring size and weight was dramatically reduced in viviparous females, with offspring from viviparous mothers being more than 35% lighter than oviparous offspring. This strong association was also clear from the PCA, in which

PC2, which mainly differentiated the two reproductive modes, had the highest loadings for offspring size, weight and body condition (Figure 4.4). Previous research across the distribution of *Zootoca vivipara* also indicated that offspring size was smaller in viviparous populations (Lindtke et al. 2010; Roitberg et al. 2013). This is contrary to our prediction because a trade-off between clutch size and offspring size should result in either more numerous, smaller offspring or fewer, but larger offspring (Stearns 1976; Reznick 1982; Sinervo and Licht 1991; Olsson and Shine 1997); here clutch number and offspring size were both reduced in viviparous reproduction. For example, viviparous fishes usually produce fewer, but larger offspring that have increased survivorship compared to smaller offspring (Reznick 1982; Heath and Blouw 1998; Goodwin et al. 2002; Shikano and Taniguchi 2005). A relationship between offspring size and survivorship has also been demonstrated for reptile species (Sinervo 1990; Webb et al. 2006; Pike et al. 2008). However, a clear pattern of reproductive mode and offspring size has not been demonstrated in reptiles (Vitt and Blackburn 1983; Seigel and Fitch 1984; Lindtke et al. 2010; Sun et al. 2012). One study showed that viviparous offspring tend to have higher survivorship compared to oviparous offspring, though this pattern was not robust when accounting for phylogeny (Pike et al. 2008).

In accordance with other studies, we found that viviparous females had a lower reproductive output compared to oviparous females (Seigel and Fitch 1984; Meiri et al. 2012), but a higher hatching success. Indeed, we found that the greater hatching success compensated for the lower clutch size, so that between reproductive modes there was no significant difference in the number of offspring after hatching. While offspring mass and reproductive output was much greater for oviparous females, this difference may be augmented in our study because eggs were incubated at stable and slightly higher temperatures than ambient and it is known that environmental temperatures can influence size and viability of offspring (Van Damme et al. 1992; Shine and Harlow 1996; Shine 2002, 2005; Li et al. 2017). Particularly the lower temperatures at higher altitudes have a negative effect on clutch development in oviparous but can be mitigated by viviparous females (Shine 2002; Webb et al. 2006). At the sampling site (>1300m), egg incubation time under natural conditions are not known but would be substantially longer than in our study (Rodríguez-Díaz and Braña 2011b, 2012). Further, hatching success for oviparous clutches was likely higher under our incubation conditions than under strictly natural settings. We suggest that at optimal developmental temperatures oviparous reproduction has an advantage, whereas under cold environmental conditions this advantage reverses.

We found a significant effect of female body size on offspring size and weight in viviparous females, but not in oviparous (Table 4.S2). This again indicates that female cavities are size limited, and only larger females can provide enough space for the development of larger offspring. This suggests a strong selective pressure for increased body size (Shine 2005). In oviparous females, much of the offspring mass is acquired after oviposition, and is therefore not directly constrained by female size though perhaps by other environmental and physiological effects.

Differences in offspring traits can result from different temperatures during incubation, both if experienced within the mother's reproductive tract or outside. For example, it has been shown that incubation temperature affects offspring head length and survival in oviparous *Zootoca vivipara* (Heulin et al. 1994; Rodríguez-Díaz et al. 2010). Also, temperatures experienced by egg clutches in the environment usually show more than 10ºC variation (Rodríguez-Díaz et al. 2010). While we tried to minimize effects resulting from incubation temperature and keep them as close as possible to temperatures experienced in the natural environment, we cannot exclude that some of the observed differences in offspring traits include an effect of incubation temperature. In general, our observed differences between viviparous and oviparous offspring traits match those observed by other studies (Lindtke et al. 2010; Roitberg et al. 2013). We accounted for the duration of days kept in captivity for each lizard by using it as a covariate in our statistical analyses. Environmental conditions experienced in captivity were generally comparable to conditions at the sampling site, as lizards had the option to thermoregulate and bask depending on external weather conditions. However, we would like to note that rearing conditions do not (and cannot) perfectly match conditions experienced at the nearby sampling site.

In summary, our results suggest that viviparous females are substantially constrained by body size. This has a negative effect on offspring size and weight. Part of this effect can be compensated by a higher hatching success for viviparous clutches compared to oviparous clutches and costs of oviparity given the lower temperatures of high altitude sites.

4.5.4 An adaptive scenario for life-history trait evolution associated with viviparity

Alternative trade-off strategies between divergent life-histories are common across the animal kingdom (Stearns 1976; Reznick et al. 1990; Shine 2005; Pires et al. 2011). In reproductively bimodal common lizards, viviparous females must deal with a prolonged pregnancy and the increase of body mass during embryonic development (Qualls and Andrews 1999). In addition, the increase in weight and duration of pregnancy poses another problem to viviparous females: Their sprint speed and endurance is greatly reduced which increases predation risk (Shine 1980). It has been shown that gravid common lizard females behaviourally shift to a cryptic strategy rather than escape tactics when a predator approaches (Bauwens and Thoen 1981; Van Damme et al. 1989). The costs of pregnancy and space constraints for development are a reproductive burden affecting the total physical available space for reproducing viviparous females. Females adjusted to this with several adaptations that counteract this constraint: i) an increase in body size, allowing for a larger body cavity; ii) a reduction in clutch size, iii) a reduction in offspring size and mass; and iv) an increase in hatching success.

On the oviparity-viviparity continuum, the common lizard is the evolutionarily youngest transition from oviparity to viviparity known to date (Surget-Groba et al. 2006). While the benefits of being viviparous in cold environments are evident (Shine 1983), the suite of life-history traits associated with viviparous reproduction in squamates might vary across different evolutionary stages. For instance, organisms that evolved viviparity deeper in their evolutionary history have had more time to optimize their reproductive output than species with more recent transitions to viviparity. For example, the reproductively bimodal lizard, *Lerista bougainvillii* evolved viviparity earlier than common lizards (Qualls and Shine 1998; 14.7 mya (5.8-23.6 95% HPD) Recknagel et al. unpublished) and viviparous *L. bougainvillii* have increased their body size on average by 10.0% relative to oviparous *L. bougainvillii*, providing larger body cavities for a higher clutch size and/or mass (Qualls and Shine 1998). At present, viviparous common lizards have an increased body size by 8.6% relative to syntopic oviparous females (Table 4.1). Unlike viviparous common lizards and most viviparous species (Seigel and Fitch 1984; Qualls and Shine 1995), viviparous *L. bougainvillii* do not differ in clutch size compared to oviparous. Therefore we propose that in the common lizard constraint for space may be the main limiting factor decreasing reproductive output.

Another possible life history adjustment to accommodate a decreased productivity per season would be an increase in lifetime number of reproductive events. A link between longevity and reproductive mode has been suggested generally (e.g. Tinkle et al. 1970; Stearns 1976; Gunderson 1997). In support of that, a comprehensive analysis found that larger squamates with few, large offspring tend to live longer than smaller squamates that reproduce more frequently, have larger clutch sizes, and smaller offspring; however, while reproductive mode correlates with some of these life-history traits, it did not have a significant effect on lifespan (Scharf et al. 2015). It is not known whether common lizard reproductive modes differ in their lifespan, and future research should address this question.

We conclude that the link between reproductive life-history traits and reproductive mode depends on several aspects. The degree of correlation may depend on the time since the transition in reproductive mode, from oviparity to viviparity, occurred. We do not imply here that traits correlated with viviparity evolved after the origin of viviparity, some of these traits might have evolved prior to the evolution of viviparity, and facilitated the transition to viviparity. Also, even if reproductive traits had enough time to co-evolve to a more advantageous combination, different environmental conditions might favour different sets of correlated traits at different sites, not always leading to the same direction in each reproductive trait (e.g. Medina and Ibargüengoytía 2010; Meiri et al. 2012; Sun et al. 2012; Bassar et al. 2014). Reproductive traits represent a life-history trade-off that is context dependent. Finally, life-history traits can also be directly influenced by differences in incubation conditions during embryonic development, suggesting that the evolution of viviparity may have been promoted by selection on offspring phenotypes (Li et al. 2017). Testing groups at different stages along the oviparity-viviparity continuum and across different environments, with phylogenetic correction, might give a clearer picture on the ecology of reproductive traits and reproductive mode.

Chapter 5: The genetic basis of a major evolutionary transition: from egg-laying to live-bearing in a squamate lizard

In collaboration with Andrey Yurchenko, Mohsen Nokhbatolfoghahai, Maureen Bain and Kathryn R. Elmer. Currently in preparation for submission. A. Yurchenko reconstructed the common lizard genome and helped in the annotation of genes identified from admixture mapping. M. Nokhbatolfoghahai staged common lizard embryos. M. Bain is currently phenotyping eggshell characteristics of females to be included in the admixture mapping.

5.1 Abstract

Throughout vertebrate evolution, repeated major transitions from egg-laying (oviparity) to live-bearing (viviparity) have occurred, which require several complex changes in morphological, reproductive, and physiological traits. Understanding the genetic architecture of such traits is a difficult task, as sister species with alternative reproductive modes are usually very distantly related. We used an exceptional natural model, the reproductively bimodal common lizard (*Zootoca vivipara*), to explore the evolutionary history of reproductive mode and identify associated genetic changes. Common lizards can be divided into four viviparous lineages and two oviparous lineages. We sampled and phenotyped 480 females at a small-scale contact zone between the eastern oviparous and the central viviparous II lineage and performed admixture mapping using 22,988 SNPs, mapped to an annotated reference genome. Genetic variants associated with key reproductive traits were identified (embryonic stage at oviposition, incubation time, and eggshell characteristics), including a few loci of large effect (mean = 8; 95% CI = 4 - 20). These loci explained >90% of the phenotypic variation in parity mode. The major SNPs were located on four different chromosomes, including two strongly associated regions located on the sex chromosome. Genes within these regions include endothelial PAS domain protein 1 (*EPAS1*), a gene previously related to viviparity in mammals and a scincid lizard, and immunity-related genes. Genome scans across all six lineages revealed that the two oviparous lineages are not significantly differentiated from the viviparous lineages for the same focal regions, suggesting that the genetic basis for oviparity may differ between lineages. Range-wide phylogenomic data supports this finding, and together our evidence suggests that one of the oviparous lineages was a reversal to oviparity from viviparity. By identifying the genetic basis of reproductive mode, this study provides an important advance in describing a major evolutionary transition.

5.2 Introduction

The evolution of key innovations, such as eyes, feathers or live-birth, enables the utilisation of additional niches and has been found to frequently result in exceptional diversification (Hunter 1998). To understand how these traits evolved, it is of great interest to evolutionary biologists to identify the genetic basis (Stapley et al. 2010; Elmer and Meyer 2011; Wagner and Zhang 2011). However, this is an inherently difficult task because mapping quantitative trait loci (QTLs) relies on recombination events across the genome to resolve genotype-phenotype correlations (Lynch and Walsh 1998). It is rare, or impossible, for recombination events to occur between taxa with and without these major traits because they are usually deeply divergent. For example, the genetic basis of human speech cannot be inferred by merely comparing the genome of chimpanzees and humans (6.23 - 7.07 MY divergent; timetree.org; Schrago and Voloch 2013). A huge number of mutations have arisen across the two genomes of these phenotypically distinct species. It is therefore not possible to distinguish causal associations of a genotype with human speech from the overall phenotype.

However among more closely related lineages, admixture mapping is a powerful tool to elucidate genetic mechanisms associated with a phenotype that differs between two lineages with a (partially) independent evolutionary history (McKeigue 2005; Buerkle and Lexer 2008). This method makes use of natural hybridisation resulting in the recombination of the chromosomes from the two parental lineages, breaking up and reassembling genetic variants. These recombination events coupled to the phenotype make it possible to associate genetic variation to the phenotypic variation of interest (Buerkle and Lexer 2008). Using admixture mapping approaches, the difficulties associated with assessing the genetic basis of phenotypic traits from divergent genomes (i.e. between lineages or related species) can be overcome by implementing methods that correct for background divergence (population stratification) (McKeigue 2005; Buerkle and Lexer 2008; Price et al. 2010; Zhou and Stephens 2012). These methods estimate relatedness between individuals or locus-specific ancestries to correct for population stratification when estimating genotype-phenotype correlations (Hoggart et al. 2004; Zhou and Stephens 2012; Maples et al. 2013). Standard QTL approaches can be constricted by a low number of recombination events due to a fewer generations of crossing and long generation times, and in these cases admixture mapping is a more powerful genetic tool as it involves multiple generations of crossing and recombination (Gompert and Buerkle 2009; Winkler et al. 2010).

Until recently, identifying casual mutations of complex traits has been limited to humans and domesticated plants and animals, mainly because it is expensive to generate the number of genetic markers necessary for such studies (Hall et al. 2010; Stranger et al. 2011; Zhang et al. 2012). In the past five years, reduced-representation sequencing techniques have allowed researchers to quantify and identify genetic regions associated with adaptive phenotypes (Narum et al. 2013; Ashton et al. 2017). QTL and linkage mapping can identify loci associated with a complex trait, but to localize and identify causal mutations fine-mapping using a chromosome-level assembled reference genome is necessary. This is only rarely available for wild organisms of no economic importance (Stapley et al. 2010; Ekblom and Galindo 2011; Savolainen et al. 2013). Now that sequencing costs are rapidly decreasing, genome-wide association mapping (GWAS) methods and whole-genome sequencing (and re-sequencing) approaches are available for non-model organisms (Elmer and Meyer 2011; Ellegren 2014).

A few model systems in evolutionary biology have recently benefitted from a completed sequenced genome, and these have given some extraordinary insights into the genetics of complex evolutionary transitions. In general, these evolutionary transitions were attributed to a few genes with large effects (Hoekstra et al. 2006; Kingsley and Peichel 2007; Kingsley et al. 2009; Dasmahapatra et al. 2012; Jones et al. 2012; Huber et al. 2015; Lamichhaney et al. 2015). Even for complex phenotypic traits that can be separated into several phenotypic modules, simple genetic architectures have been reported (Mallarino et al. 2011; Xu et al. 2012; Greenwood et al. 2013; Weber et al. 2013; Yang and Shah 2016). At the current stage, we do not know how general this pattern is. Single loci of large effect are reported more frequently than multiple loci of small effect, presumably also because these are harder to detect (Savolainen et al. 2013; Slate 2013). This is particularly true for complex quantitative traits which are controlled by tens to hundreds of loci with small effects (Flint and Mackay 2009).

Viviparity is a non-discrete highly modular reproductive trait, involving several changes at the level of morphology, physiology and behaviour (Blackburn 1992, 2006; Arrayago et al. 1996; Thompson and Speake 2006; Lindtke et al. 2010; Murphy and Thompson 2011). The simplest form of oviparity involves the development of an eggshell, nutrient provision solely based on the yolk within the egg and no egg retention. Viviparity involves the loss of the eggshell, and the increase in mother-foetus interactions (Blackburn 2006; Moffett

and Loke 2006; Thompson and Speake 2006). Most complex forms of viviparity involve the development of a placenta providing all main nutrients for the developing embryo (Vitt and Blackburn 1983; Thompson et al. 2000; Thompson and Speake 2006). In less than a handful of examples, viviparity and oviparity are genetically fixed in different lineages of the same species, making these species prime candidates for understanding the evolution of viviparity (Murphy and Thompson 2011).

Common lizards (*Zootoca vivipara*) are one of these examples (Figure 5.1A), and the only one in which natural hybridization between an oviparous and a viviparous lineage had been documented (N = 2 hybrids from Lindtke et al. 2010; Figure 5.1B). Hybrids were identified based on a thinner eggshell and a later embryonic stage at oviposition compared to pure oviparous individuals. There has been no genetic validation of these potential hybrids so far. Within common lizards, two lineages are oviparous, and four are viviparous (Odierna et al. 2004; Surget-Groba et al. 2006). Reconstruction of the evolutionary history of parity mode suggests viviparity evolved once, with a single reversal to oviparity (Surget-Groba et al. 2006; Cornetti et al. 2014; Recknagel et al. 2017 [Chapter 3]). The lineage displaying the ancestral oviparity (eastern oviparous lineage) exhibits thicker eggshells, longer incubation time, and a lower embryonic stage at oviposition compared to the lineage which presumably re-evolved oviparity (Heulin et al. 2002; Lindtke et al. 2010). In contrast, the viviparous lineage possesses no eggshell and neonates are fully developed after parturition (see Chapter 4). While there is no substantial nutrient transport from the mother to the foetus, essential calcium – usually provided by the eggshell in common lizards – is provided by uterine expression and transport through the extra-embryonic membrane to the developing embryo (Stewart et al. 2004, 2009; Heulin et al. 2005). Therefore, while developing embryos in viviparous common lizards can be considered mostly dependent on the yolk, they also partially rely on maternal provisions through the placenta (lecithotrophy and placentotrophy respectively) (Stewart et al. 2009). Experimental hybridization between the western oviparous and the western viviparous lineages (Arrayago et al. 1996), and natural hybridization between the eastern oviparous and viviparous lineages (Lindtke et al. 2010) make common lizards an ideal model system to study the genetic basis of oviparity and viviparity.

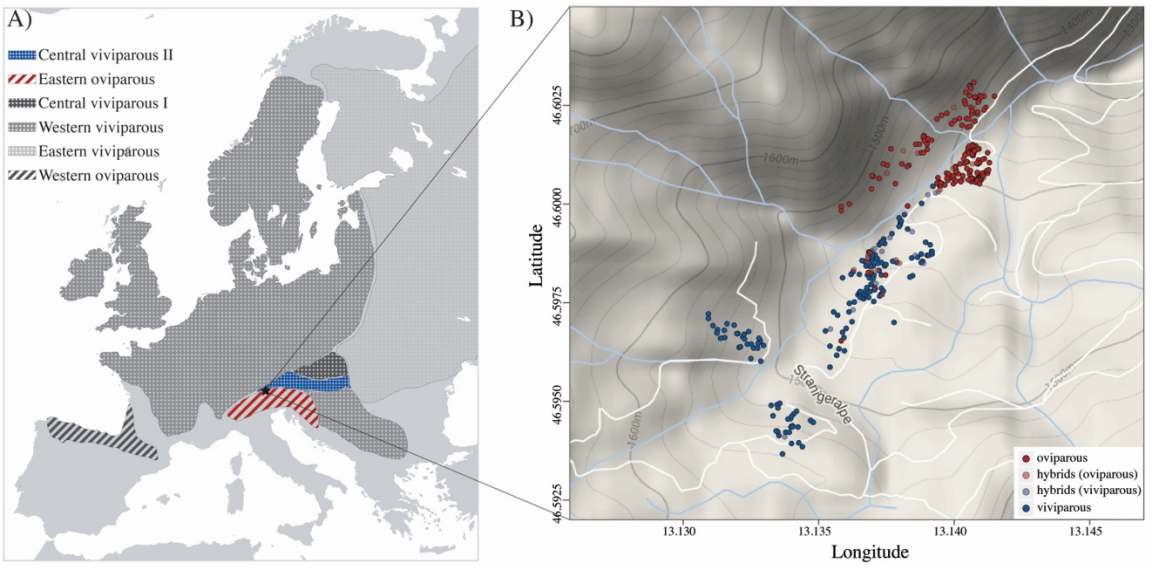

Figure 5.1 Distribution and sampling of common lizards. A) Distribution of the six main common lizard lineages. The hybridizing oviparous and viviparous lineages are indicated in red and blue, respectively. Note that the two oviparous lineages are not the most closely related. The eastern oviparous lineage inhabiting the Southern Alps is the most distant to all other lineages, including the Pyrenean western oviparous lineage that is nested within viviparous lineages. B) All samples were collected from a contact zone between the eastern oviparous and central viviparous II lineages. Individuals belonging to the oviparous lineage were found at lower altitudes, and viviparous at higher altitudes. Hybrids were found at the immediate contact zone between the two lineages (assessed by phenotype and genotype).

Here, we use the natural occurrence of hybrids of reproductively bimodal oviparous and viviparous common lizards to identify the genetic basis of reproductive mode. We do so by using an admixture mapping approach on embryonic stage at oviposition and the number of incubation days per clutch. To locally map genetic variants, we used the recently constructed common lizard genome in our lab (Yurchenko et al. in prep.). Following the evolution of viviparity, a single lineage has likely re-evolved oviparity. By expanding our analysis from the admixed population to a genome scan approach across the whole phylogeny of six lineages of common lizards, we seek to identify whether similar genetic regions are involved in controlling oviparity in the two putatively independent oviparous lineages.

5.3 Methods and Materials

5.3.1 Sampling

From May to August, 2014 to 2016, 857 common lizards (603 females, 254 males) were collected by hand in a contact zone (Figure 5.1) between oviparous (corresponding to the lineage ‘eastern oviparous’) and viviparous (corresponding to the lineage ‘central viviparous II’) lineages. Males and females were differentiated based on ventral colouration and presence of a hemipenal bulge in males. Female pregnancy was assessed by the presence of a bite mark on the female’s flank resulting from mating. Only females

were considered for the admixture analysis and detailed phenotyping (see 1.3.2), as a male's reproductive mode cannot be phenotypically assessed. All pregnant females were collected and held in terraria (20cm x 35cm x 15cm), and food was provided ad libitum. For more detailed housing conditions see Chapter 4. Here, the term 'clutch' is used for both a viviparous litter and an oviparous clutch, and the term 'hatching' is used for both live born young and oviparous hatchlings. Females were checked daily for oviposition/parturition, and then released at the capture location. Clutches were weighed before being embedded in moist vermiculite and incubated at 24ºC in an ExoTerra reptile egg incubator. From each clutch, one egg was removed for embryonic staging and eggshell analysis. All clutches were monitored daily for appropriate moisture, the presence of infertile or dead embryos, and hatching offspring. Once hatched, offspring were released at the same location as their mother. For each female, a small tail tissue sample was collected for DNA extraction.

Existing data from Chapter 3 of 65 individuals from the six divergent lineages (N = 7-15 individuals per lineage; Recknagel et al. 2017) with known reproductive mode were included for the genome scan approach to explore the genomic patterns of parity mode across all lineages.

5.3.2 Reproductive mode phenotypes

For the clutch of each pregnant female (N = 480), three traits related to reproductive mode were collected: i) the average number of incubation days for each clutch after oviposition/parturition, ii) the embryonic stage at oviposition/parturition, iii) the eggshell characteristics (thickness and chemical compounds) of one eggshell per clutch. These individuals included oviparous and viviparous females used in Chapter 4 and additional hybrid females. A single embryo per clutch was used for staging and extracted from the eggshell immediately after finding a clutch. The proportion of 'eggs' that were infertile within a clutch and the proportion of hatching offspring was also recorded for each female to infer clutch survival rate. Both the embryos and the eggshells were fixed in a 10% formalin solution for 24 hours and then transferred into a 70% ethanol solution. Embryonic stage was identified in the lab using a light microscope and a staging table (Dufaure and Hubert 1961). Embryos were only collected from sampling years 2015 and 2016, and therefore this parity mode phenotype exhibits a smaller sample size compared to the number of incubation days. Eggshells will be analysed at a later stage by collaborator Maureen Bain.

5.3.3 Library preparation and sequencing

DNA extraction and library preparation followed the same methods as Chapter 3. For the admixture mapping approach, five double-digest restriction-site associated DNA sequencing (ddRADSeq), each composed of 105 female individuals were prepared (Peterson et al. 2012; Recknagel et al. 2013, 2015) and sequenced at Edinburgh Genomics on an Illumina HiSeq 4000 with paired-end reads and lengths of 150 base pairs (bp) with around 7 million reads per individual. Technical replicates within (N = 27) and across (N = 9) libraries were included to estimate genotyping accuracy and calculate error rates. All reads were trimmed to 100 bp prior to bioinformatic processing.

For the individuals used in the broad genome scanning analysis, including all common lizard lineages, three genomic libraries were constructed using ddRADSeq (N = 67; 65 individuals and 2 technical replicates). The first two libraries were run on an IonProton sequencing machine at Glasgow Polyomics using an Ion PI Sequencing 200 Kit v3 on an Ion Proton PI chip. The target read size was 100 bp, which resulted in a median read length of 96 bp (ddRADSeq-ion; Recknagel et al. 2015). The third library was sequenced at Edinburgh Genomics on an Illumina HiSeq 4000 machine with paired-end sequencing of 150 bp reads. All reads were trimmed to 70 bp to avoid losing those reads with low quality bases towards the end of the sequence.

The software STACKS (Catchen et al. 2011) was used to process reads. After quality filtering and trimming reads, high quality reads were aligned to the *Zootoca vivipara* reference genome (Yurchenko et al. in prep.) and sorted into loci allowing up to three mutations per locus and a minimum stack depth of three reads. After building a catalogue of loci for each individuals and across all individuals, rxstacks was run to reduce the genotyping error rate (Recknagel et al. 2015). Loci that had a confounded match to the catalogue (i.e. matching more than one catalogue locus) in more than 25% of all individuals were removed and excess haplotypes pruned. After that, genotypes were called with the bounded SNP model and loci with an average log likelihood less than -10 were removed. Genotypes were extracted from STACKS with a minimum coverage of 5x, presence in at least 50% of all individuals, and a minor allele frequency of 10% (last option only applied for individuals included in the admixture mapping approach). This resulted in 26.5% missing data and 22,988 SNPs for the admixture mapping genotype matrix, and 29.1% missing data and 150,204 SNPs for the genome scan dataset.

5.3.4 Identification of hybrids

Hybrids were classified based on their phenotype (average number of incubation days per clutch and embryonic stage at oviposition/parturition) and genotype (membership value relative to the oviparous and viviparous lineage). Females with clutches that incubated for less than five days, and an embryonic stage at parturition larger than 40 were classified as viviparous. Females with clutches that incubated for at least 32 days and an embryonic stage at oviposition of equal or less than 32 were classified as oviparous (Lindtke et al. 2010). Females that were in between those values were coded as hybrids. Hybrid females were further divided into oviparous (number of incubation days: 20-32 days; embryonic stage at oviposition/parturition: 32.5 to 34.5) and viviparous hybrids (number of incubation days: 4-19 days; embryonic stage at oviposition/parturition: 35 to 40.5). Genomic ancestry was derived from ADMIXTURE v.1.3. (Alexander et al. 2009) with K=2. Females with a membership value (Q) of less than 0.01 were scored as viviparous, females with a Q between 0.01 and 0.5 as viviparous hybrids, females with a Q larger than 0.5 and up to 0.99 as oviparous hybrids and females with a Q larger than 0.99 as oviparous (Table 5.1). Generalized linear models (GLM) in R (R Core team 2015) were used to test if phenotypes and genotypes were correlated and if differences in clutch survival rate between reproductive modes and hybrids were present.

Table 5.1 Criteria used to define the reproductive mode classes.

reproductive mode	number of incubation days	embryonic stage at oviposition/parturition	membership value (Q)
oviparous	≥ 32	≤ 32	≥ 0.99
oviparous hybrids	20 - 32	32.5 - 34.5	0.5 - 0.99
viviparous hybrids	4 - 19	35 - 40.5	0.01 - 0.5
viviparous	≤ 4	≥ 41	≤ 0.01

5.3.5 Admixture mapping analyses

All females with reproductive data were selected for admixture mapping (N = 480). BEAGLE 4.1 (Browning and Browning 2007) was used to infer the phase for each individual and locus and to impute missing loci using a sliding window size of 10,000 markers and an overlap of 1,000 markers between windows. The admixture mapping software GEMMA (Zhou and Stephens 2012) was used to identify loci associated with reproductive mode phenotypes. A Bayesian sparse linear mixed model (BSLMM) was implemented in GEMMA to test for associations between genotype and the number of incubation days and embryo stage at oviposition/parturition separately. Initially a centred

relatedness matrix between all individuals was calculated to correct for population stratification. Ten BSLMM were run independently, each with 10 million sampling iterations and a burn-in of 5 million. The posterior distributions of model parameters were visually inspected to assess convergence. Runs that converged were then combined by averaging all parameters across runs. For each of the two genotype-phenotype analyses (incubation days and embryonic stage), the proportion of phenotypic variance explained by genetic variants (h, PVE) and the proportion of genetic variance explained by genetic variants with major effect (ρ, PGE) were calculated. Finally, the proportion of loci with sparse effect across all genetic variation (π) and the number of major effect loci are calculated (N (γ)). For each locus, the posterior inclusion probability (PIP) was calculated as the frequency a variant is estimated to have a sparse effect in the MCMC. In addition, the sparse effect size (parameter beta * PIP) was calculated for each locus. The outliers were separated in three classes based on confidence thresholds: present in i) 90th quantile, ii) 95th quantile and iii) 99th quantile. For SNPs with a PIP larger than 0.1, the surrounding 100 kb in the *Zootoca vivipara* draft reference genome (vers. 11.09.2017) were checked for presence of genes. The genome was constructed from a single individual of the western viviparous lineage (ELT05324) from Scotland (manuscript currently in preparation with authors A. Yurchenko, H. Recknagel and K. R. Elmer). In addition, linkage maps were constructed from ten families of the central viviparous II and ten families of the eastern oviparous lineage. Genome scaffolds were anchored to this linkage map, and designated chromosomes are therefore consistent with karyotype configuration of the central viviparous II and the eastern oviparous lineage (Odierna et al. 2004). Sex-linked markers (and the sex chromosome) were identified by estimating Fst values between female and male common lizards and detecting markers missing in one sex. Linkage group 14 was identified as the putative sex chromosome (Z).

5.3.6 Parallel genomics using genome scans

Previous phylogenomic reconstructions suggested that viviparity evolved once and in one lineage oviparity re-evolved (Chapter 3; Recknagel et al. 2017). This is also supported by morphological comparisons of eggshell structures from the two different oviparous lineages (Arrayago et al. 1996; Lindtke et al. 2010). To assess the consistency between different lineages in that framework, phylogenomic analyses were performed to test for genomic outliers between parity modes using all six major lineages. Two of these lineages were oviparous and four viviparous. Genome-wide comparisons of differentiation were performed. These contrasted reproductive modes so that outlier loci were shared by a

similar reproductive mode. Outliers were defined as those regions that exhibited elevated Fst values between the two different reproductive modes relative to the genomic background and the phylogeny. To calculate Fst values, vcftools was used with a minimum allele frequency of 5%, a window size of 250,000 bp and a step size of 125,000 bp. Three comparisons were performed: i) the oviparous lineage relative to all viviparous lineages, ii) the derived oviparous lineage (that reversed to oviparity) relative to all viviparous lineages, and finally iii) all oviparous lineages relative to all viviparous lineages. Simulations (N= 10,000) were used to test if more Fst outliers were shared between the first two contrasts than expected by chance. These were performed by randomly simulating the number of outliers found in each of the first two contrast and then testing how many times two outliers were shared.

5.3.7 Topology weighting

In addition to the outlier approach described in the previous section, weighted topology tests were executed in a custom pipeline (Martin and Van Belleghem 2017) across all 22 linkage groups using the 65 common lizard individuals representing all six major lineages. This was used to test if different genomic regions support the same or alternative tree topologies. In particular we wanted to know if a sister relationship is supported between the two oviparous lineages for some genomic regions, which could indicate a functional link to egg-laying. For example, functional loci for egg-laying might have retained their ancestral genotype in the derived oviparous lineage, or functional regions could have been introgressed from another oviparous lineage. Topology weighting consists of two main steps. First, gene trees were inferred using a window of 20 SNPs for each of the 22 linkage groups separately using PHYML vers. 3.1 with a GTR substitution model (Guindon and Gascuel 2003). This resulted in 7028 total gene trees, with an average of 320 trees per linkage group. Then, weights for each of all the possible topologies (6 lineages, resulting in 105 unrooted topologies) were estimated for each of the gene trees. These weights correspond to the percentage of subtrees supporting a topology. Weights of gene trees corresponding to similar hypothesis in parity mode evolution were combined into three sets: i) both oviparous lineages are sister to viviparous lineages, corresponding to a single origin of viviparity, ii) the western oviparous group is nested within viviparous lineages, with two origins of viviparity being equally parsimonious as a single origin of viviparity and a reversal to oviparity, iii) the western oviparous lineage is nested within viviparous lineages, but a single origin of viviparity and a reversal to oviparity (= derived oviparity) are more likely than three or more transitions to viviparity (Figure 5.2). To visualise the

distribution of topology support across linkage groups, an R script provided by Martin and Van Belleghem (2017) was used.

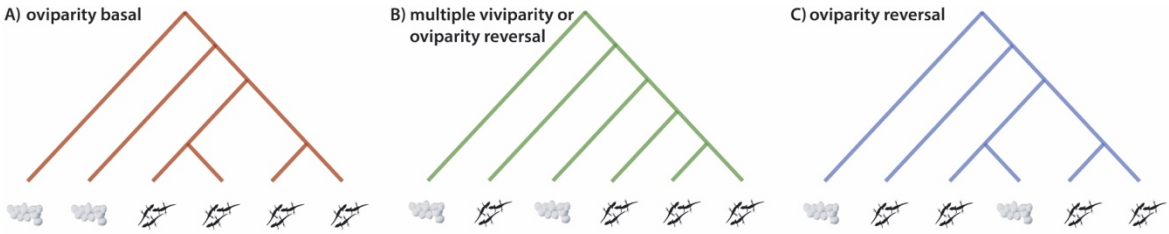

Figure 5.2 Evolutionary scenarios of parity mode evolution in a phylogenetic context. In the first scenario A), viviparous lineages are monophyletic. A common genetic basis of oviparity is plausible, if the oviparous lineage sister to all viviparous lineages retained the genetic basis of oviparity. In the second scenario B), two independent origins of viviparity or a single origin of viviparity and a reversal to oviparity might have occurred. In this phylogenetic context, whether the oviparous lineage retained oviparity or re-evolved oviparity is equally likely. In the third scenario C), a reversal to oviparity is most likely. All possible trees (N = 105) were assorted into these three topologies and their relative weights across the genome compared.

5.4 Results

5.4.1 Phenotypic and genotypic variation in the contact zone between oviparous and viviparous common lizards

Number of incubation days varied from 0 (viviparous) to a maximum of 41 (oviparous) days (Figure 5.3A) and embryonic stages at oviposition ranged from stage 26 (oviparous) to 41 (viviparous) (Figure 5.3B). Based on the number of incubation days, we identified a total of 175 viviparous, 76 hybrid, and 161 oviparous females. Therefore, we estimated individuals with an ancestry of admixture to be around 18.4% in our sample (Figure 5.4A). We identified 76 viviparous, 21 hybrid, and 116 oviparous females based on the embryonic stage at oviposition/parturition. This resulted in an estimated frequency of 18.3% of hybrids (Figure 5.4B). The number of incubation days and embryonic stage at oviposition/parturition showed a strong and significant correlation ($P < 0.0001$, $R^2 = 0.946$; Figure 5.4C).

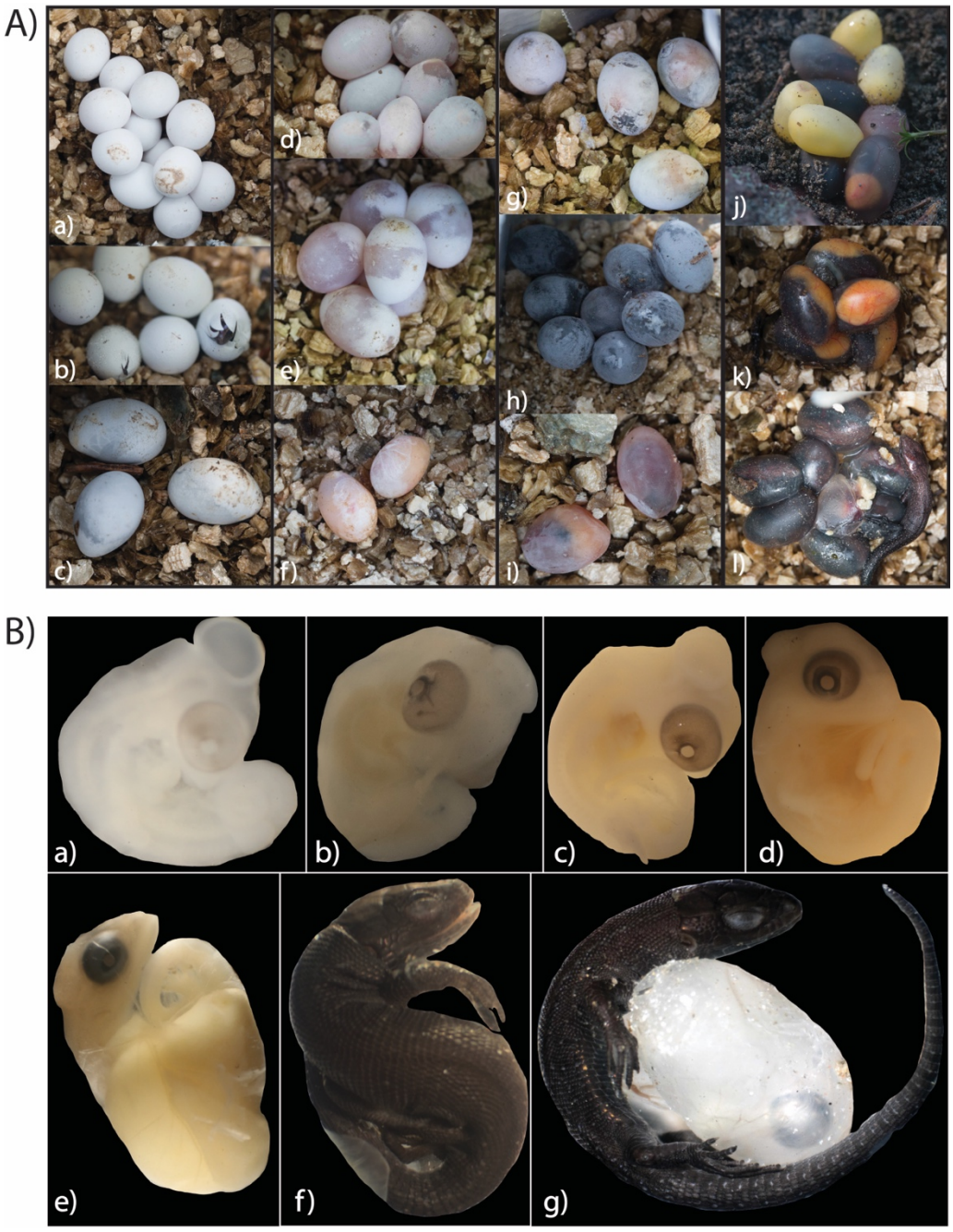

Figure 5.3 Phenotypic variation in reproductive mode phenotypes. The first panel A) shows a gradient of clutches from purely oviparous females to purely viviparous females. Images a) and b) are clutches laid by purely oviparous females. In between are examples for hybrid clutches. Images c) to j) represent a gradient of hybrid clutches. These have thinner and less calcified eggshells. At the time of oviposition, embryos are at different developmental stages. Images k) and l) are examples of embryos not possessing calcified eggshells, but were deposited at different developmental stages [note the difference in remaining yolk between k) and l)]. The second panel B) illustrates examples of embryonic stages at oviposition. The first image a) shows an example of an early stage embryo laid by an oviparous female. Images b) to e) represent embryonic stages at oviposition by hybrid females, with limbs becoming more visible. Finally, viviparous females give birth to fully developed juveniles [images f) and g)].

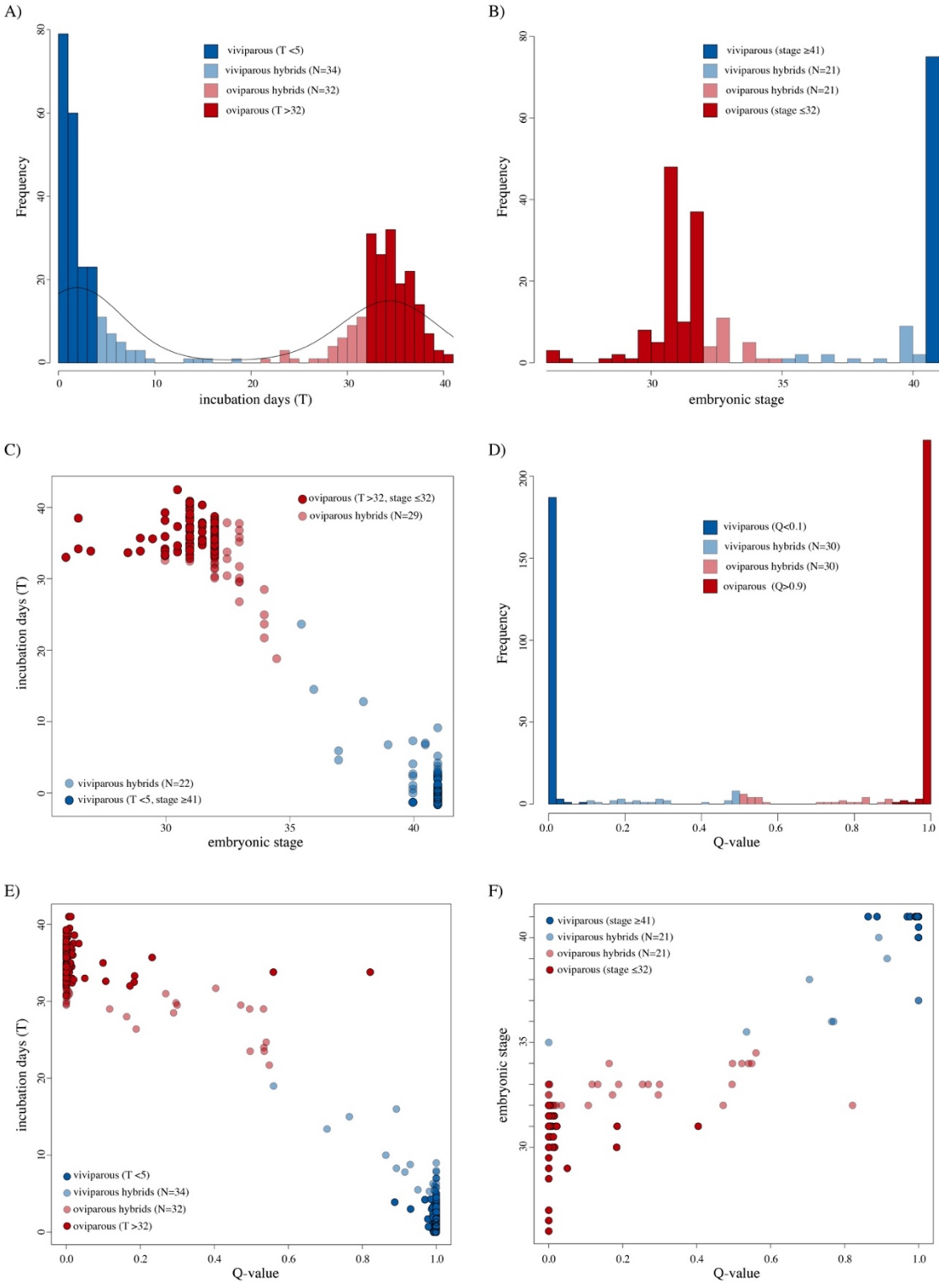

Figure 5.4 Phenotype and genotype distribution of all sampled individuals used for admixture mapping. A) Distribution of the average number of incubation days for a female's clutch (N = 412). Oviparous hybrids are classified as clutches with an average incubation time between 20 to 32 days, and viviparous hybrids between 4 to 19 days. B) Developmental stage of the embryo at the time of oviposition/parturition (N = 231). Embryos between stages 32.5 and 35 are classified as oviparous hybrids and between 35 and 40.5 as viviparous hybrids. C) Scatterplot visualising the relationship between the number of incubation days and embryonic stage at oviposition/parturition. D) Genomic background for each individual (*Q*-value: 0 = pure viviparous lineage genome, 1 = pure oviparous lineage genome) estimated from ADMIXTURE. F_1 hybrids between the two reproductive modes are expected to derive half of their genome from each of the two different lineages. Backcrossing females exhibit a range of different background values depending on the number of generations and the lineage into which backcrossing occurred. E) Relationship between the average number of incubation days per clutch and genomic background. F) Relationship between embryonic stage at oviposition/parturition and genomic background.

ADMIXTURE analysis using a k = 2 suggested that 191 individuals had a strong signature of oviparous genomic background (Q-value ≤ 0.01), 225 had a viviparous background (Q-value ≥ 0.99) and 90 individuals had some signature of hybridisation (Q-value > 0.01 and < 0.99) (Figure 5.4D). This corresponded to 18.75% of individuals with a signature of admixture. Of these admixed individuals, 43% (N = 26; 8.1% of total) had between 40% to 60% of their genome from either parental lineage, potentially being first-generation hybrids. The estimate of hybrids here was very comparable to the phenotypic estimate of hybrids (Figure 5.4A - C). A substantial part of the phenotypic variation could be explained by genomic background of a female. Correlations were significant and strong for both traits (incubation days: $P < 0.0001$, $R^2 = 0.948$; embryonic stage: $P < 0.0001$, $R^2 = 0.929$; Figure 5.4E - F).

Both viviparous and oviparous hybrid females had a higher proportion unfertilised eggs within a clutch (oviparous: 0.11; oviparous hybrids: 0.16; viviparous hybrids: 0.25; viviparous: 0.10; Figure 5.5A) and a lower hatching success (oviparous: 0.80; oviparous hybrids: 0.75; viviparous hybrids: 0.54; viviparous: 0.76; Figure 5.5B). The larger proportion of unfertilized eggs and lower hatching success was more pronounced in the viviparous hybrids and found to be significantly different compared to all other groups (N= 306, t-value = 2.23, P = 0.027; N = 306, t-value = -2.11, P = 0.036).

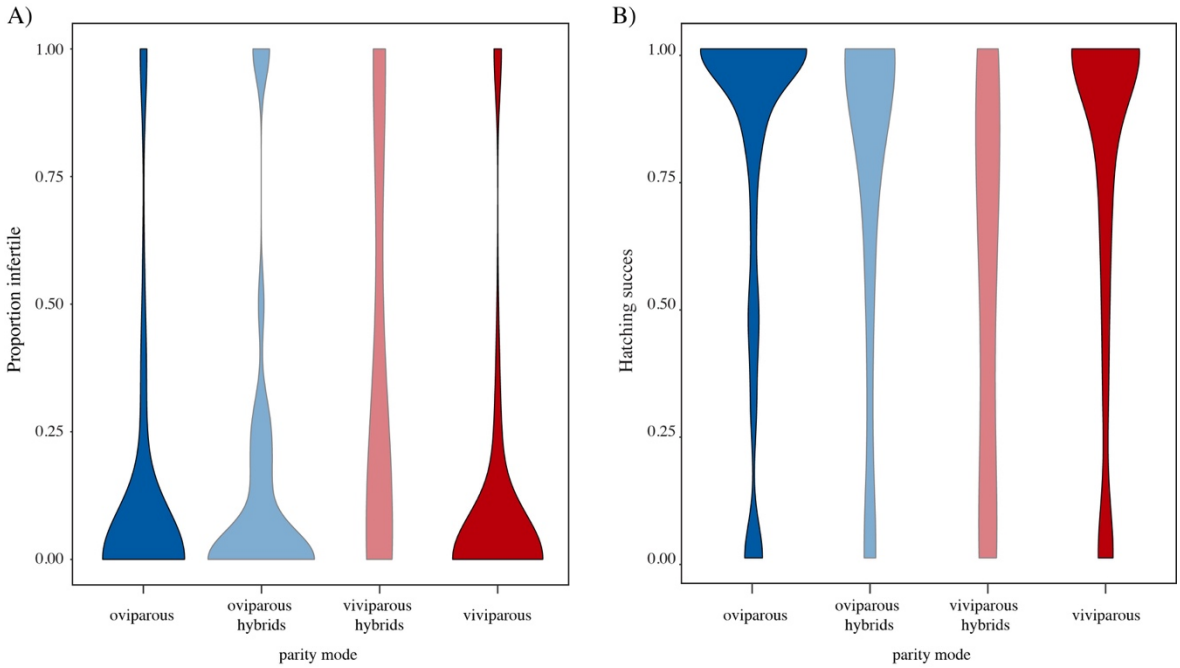

Figure 5.5 Clutch survival recorded from oviparous (N = 113), viviparous (N = 146), and hybrid (N = 48) females. Panel A) shows the proportion of unfertilised eggs within each clutch for both parity modes and the hybrids. Viviparous hybrids had significantly higher proportion of unfertilised eggs. B) shows the hatching success within a clutch. Viviparous hybrids had lower overall hatching success compared to all other classes.

5.4.2 Admixture mapping embryonic stage at oviposition and number of incubation days per female clutch

A total of 22,988 SNPs were recovered that could be mapped to the common lizard reference genome using the sequenced females with reproductive data. Of these, 21,350 SNPs mapped to one of the 22 linkage groups.

Table 5.2 Parameters estimated from the Bayesian sparse linear mixed model (BSLMM) in GEMMA for A) number of incubation days and B) embryonic stage at oviposition. The first two parameters (h, PVE) are measures of the proportion of phenotypic variation explained by genetic variation. The third parameter (ρ) indicates the proportion of genetic variants explained by major effect loci. PGE describes the proportion of genetic variance explained by sparse effects. The proportion of loci with sparse effects across all genetic variation is specified by π. The number of detected major effect markers are shown by N (γ).

A) Number of incubation days

parameter	mean	median	2.50%	97.50%
h	0.987	0.990	0.960	0.997
PVE	0.987	0.987	0.978	0.994
ρ	0.964	0.971	0.894	0.993
PGE	0.939	0.970	0.720	0.989
π	0.000	0.000	0.000	0.001
N (γ)	8.693	8	6	13

B) Embryonic stage at oviposition

parameter	mean	median	2.50%	97.50%
h	0.917	0.931	0.775	0.981
PVE	0.964	0.964	0.942	0.982
ρ	0.928	0.955	0.703	0.998
PGE	0.906	0.969	0.424	0.999
π	0.000	0.000	0.000	0.001
N (γ)	8.512	7	4	20

Abbreviations: PVE = proportion of variance in phenotypes explained; PGE = proportion of genetic variance explained by sparse effects.

First, the association between the number of incubation days and all SNP markers was assessed (N = 412). A high proportion of phenotypic variance was explained by genetic variance (99%), and this was mainly due to SNPs with large effects (ρ = 96%; Table 5.2A). We identified 24 SNPs with a PIP larger than 0.1 and these were distributed across six linkage groups (Figure 5.6A; Table 5.3). The majority of these SNPs (58%) were located on linkage group (LG) 14, with one region at the start of the linkage group (between 7-21 million base pairs from start) and one region at the end of the linkage group (between 101-107 million base pairs). Linkage mapping and whole genome data showed

that this linkage group is the sex chromosome in common lizards (Yurchenko et al. in prep). Three out of the 24 SNPs were found within genes. On LG 3, the SNP with both the largest PIP overall (1.0) and the largest effect (8.2) was located within the intragenic region of the gene Endothelial PAS Domain Protein 1 (*EPAS1*). This gene regulates the vascular endothelial factor (*VEGF*) and is involved in blood vessel development (Table 5.S1; Van Dyke et al. 2014). A second SNP on LG 3 matched to the Protein Tyrosine Phosphatase Receptor Type K (*PTPRK*). On LG 4, one SNP matched to Dynactin Subunit 1 (*DCTN1*).

Table 5.3 SNPs associated with the number of incubation days after oviposition/parturition. This table only includes loci with a posterior inclusion probability (PIP) larger than 0.1. Location of the SNP is specified by the linkage group and the position on that linkage group in base pairs (bp). Associations with the phenotype are described by the PIP and the effect size.

Linkage group	SNP	position	PIP	effect
3	34899_22	49124670	0.304	0.975
3	25552_48	55100504	0.611	1.372
3	9017_46	100240387	1.000	8.167
4	20365_7	2362891	0.212	1.233
4	10243_77	2410488	0.342	3.901
4	10243_37	2410528	0.287	3.129
10	1543_27	8134780	0.121	0.420
14	40560_84	6947884	0.115	0.704
14	40560_35	6947933	0.359	3.610
14	3421_39	13924631	0.169	0.610
14	24756_47	16207700	0.124	0.660
14	3456_77	16984720	0.196	2.997
14	3456_12	16984785	0.150	0.819
14	29515_29	18822431	0.105	0.234
14	3505_75	20978589	0.122	0.394
14	3297_82	101308299	0.152	1.594
14	3297_72	101308309	0.118	1.323
14	3297_53	101308328	0.178	1.960
14	3299_17	101433655	0.361	1.794
14	3364_46	107031983	0.115	0.391
14	3364_33	107031996	0.345	1.614
16	44804_62	32412	0.113	0.188
19	31718_65	933935	0.119	0.439
19	31718_64	933936	0.126	0.490

The other two SNPs on this linkage group did not have any exact matches with a gene, but were found in the proximity of genes (Table 5.S1). No exact matches were found for markers on LG 14, however several genes were identified with proximity to some of these SNPs (Table 5.S1). One of these was a Wnt Family Member 11 gene (*WNT11B*), located

around the middle of the linkage group. A SNP at the end of LG 14 was located in proximity to Attractin (*ATRN*), a gene involved in cell clustering during inflammatory response.

Second, the association between embryonic stage of oviposition and all SNP markers was assessed (N = 231). Similar to the analysis of incubation days, a large proportion of phenotypic variance was explained by genetic variation (> 96%). Most of the variance was explained by major effect loci (mean: 93%; CI: 70%-100%; Table 5.2B).

Table 5.4 SNPs associated with embryonic stage at oviposition. This table only includes loci with a posterior inclusion probability (PIP) larger than 0.1. Location of the SNP is specified by the linkage group and the position on that linkage group in base pairs (bp). Associations with the phenotype are described by the PIP and the effect size. Note that the last SNP was located to an unplaced scaffold.

Linkage group	SNP	position	PIP	effect
1	32380_95	53165865	0.498	0.611
4	47007_69	64290264	0.113	0.073
6	21085_97	103046890	0.128	0.301
6	11609_15	103194351	0.130	0.301
6	11609_18	103194354	0.151	0.369
6	36727_8	103767595	0.119	0.277
6	11615_99	103857043	0.159	0.379
6	11615_40	103857102	0.140	0.333
6	21089_52	104300519	0.146	0.339
8	38695_85	40060467	0.164	0.303
10	15692_45	33365072	0.207	0.409
11	28225_34	71964339	0.435	0.733
12	28866_27	55091047	0.620	0.315
14	3486_81	19202083	0.178	0.405
14	3491_20	19577617	0.169	0.383
14	4045_49	65930071	0.741	2.362
226	26671_19	37498	0.108	0.163

We identified 17 SNPs with a sparse effect using a posterior probability inclusion threshold of 0.1 over 8 linkage groups and one unplaced scaffold. Two out of the 17 total SNPs were found within genes. Seven SNPs were located within a small genomic region of 1.25 Mb on LG 6 (Table 5.4). On LG 14, three markers were associated with embryonic stage at oviposition/parturition (Figure 5.6B; Table 5.4). Two of these mapped to the start of the LG (between 19.2 – 19.5 Mbp from start of LG), and one was placed close to the middle section of the LG (at 66 Mbp from start). Eight genes were identified close to a SNP on LG 1, including two genes that have previously been linked to the establishment of

pregnancy in mammals, lysophosphatidic acid receptor 6 (*LPAR6*) and G protein-coupled receptor kinase 6 (*GRK6*) (Grotegut et al. 2016; Sadam et al. 2017; see Table 5.S2).

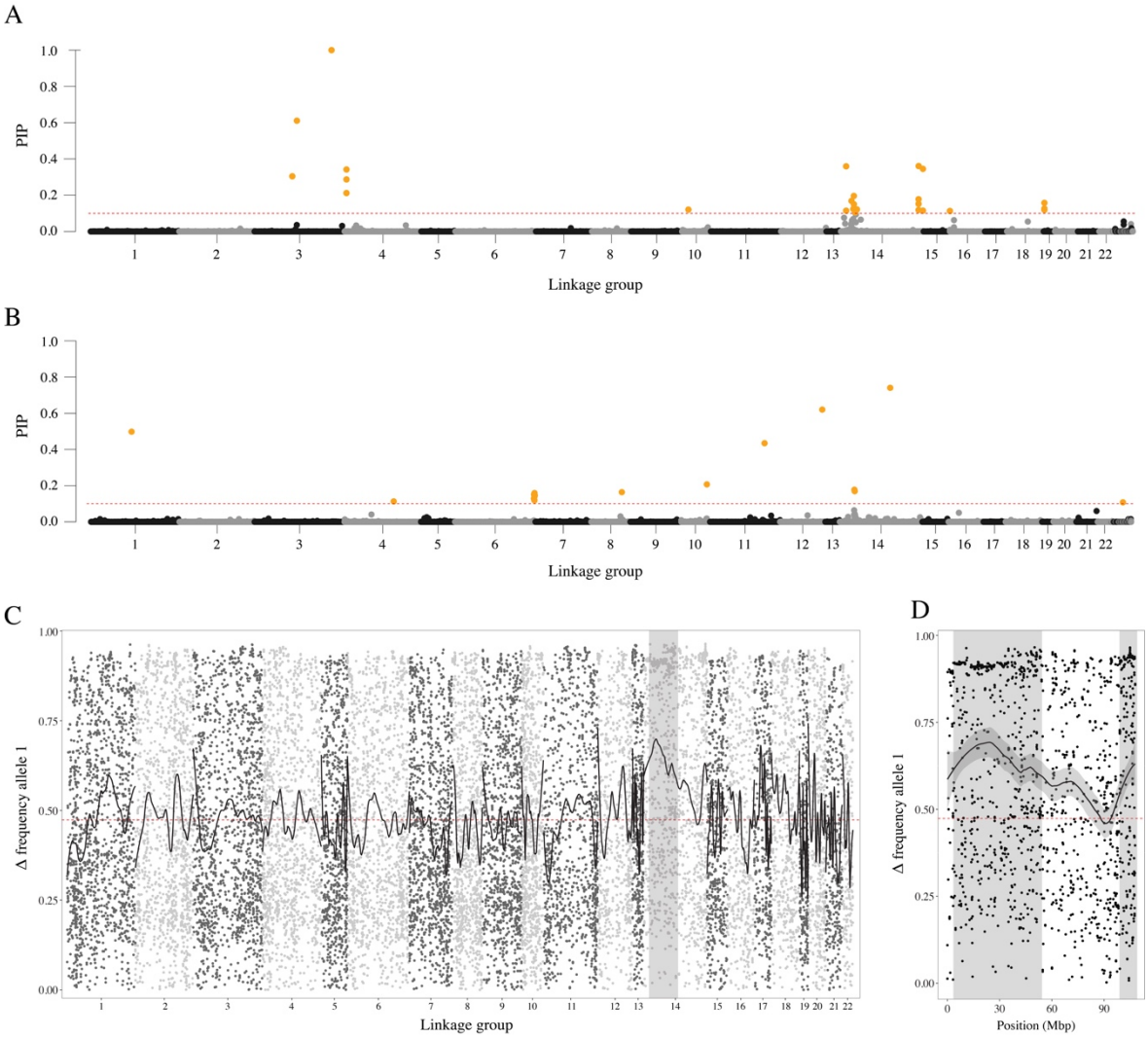

Figure 5.6 SNP association between phenotypes and genotypes within the contact zone of oviparous and viviparous common lizards. A) illustrates SNPs associated with the number of incubation days. SNPs that have a higher posterior inclusion probability (PIP) than 0.10 were marked as orange. B) shows associations between embryonic stage at oviposition/parturition and SNPs. While markers on LG 14 (the sex chromosome) were recovered in both analyses, other associated SNPs differed between the two analyses. C shows an allele frequency plot with the absolute difference in allele frequency between genetically purely oviparous (N = 183) and viviparous (N = 224) females across the genome. The red dotted line represents the average difference in allele frequency (delta = 0.47). LG 14 shows a peak of the delta allele frequency close to the start of the linkage group and another at the end of the linkage group. These peaks in LG 14 were also recovered by the admixture mapping analysis using a Bayesian sparse linear mixed model (BSLMM) combining hybrid phenotypic and genotypic information.

On LG 4, the closest gene from the associated SNP was synaptotagmin 7 (*SYT7*), a gene involved in calcium-dependent membrane trafficking (Table 5.S2). The six SNPs associated with reproductive mode on LG 6 were also found near genes. A variety of functions could be attributed to these genes, several involved in early embryonic development. None of these genes were found to have a direct link to previous studies on the placenta or parity mode phenotypes (Table 5.S2).

Two of the three SNPs associated with embryonic stage at oviposition and LG 14 overlapped with the same region (though were not the same SNPs) as identified with the number of incubation days. Allele frequency differentiation confirmed that the two reproductive modes differed substantially in the two identified regions on LG 14 and showed a significant elevation in allele frequency differentiation compared to the rest of the genome (Figure 5.6C - D).

The SNP on LG 8 was located in a gene-rich region, surrounded by a total of nine genes within the 100 kb search region. Genes associated included cadherin 11 (*CDH11*), involved in cell-cell adhesion and preeclampsia in mammals (Anton et al. 2014), and proteasome subunit beta 5 (*PSMB5*), suggested to be involved in the interaction between uterus and mother (Forde et al. 2015). A SNP on LG 11 and a SNP on LG 12 were associated with two different genes involved in immune response (Table 5.S2). Two SNPs found on the LG 14 were found in the neighbourhood of a total of eight genes. These included the genes Acyl-CoA synthetase long chain family member 4 (*ACSL4*) and potassium voltage-gated channel subfamily E regulatory subunit 5 (*KCNE5*). *ACSL4* is suggested to regulate fatty acid uptake from the uterus to the embryo (Johnsen et al. 2009), and has been associated with preeclampsia in pregnant women (Mistry et al. 2014).

5.4.3 Genetics of an evolutionary reversal – Genome scans

Due to the relatively old divergence time (~4.2 mya according to Surget-Groba et al. 2006; ~3.8 according to Chapter 2), the background Fst between the eastern oviparous lineage and all viviparous lineages was substantial (mean = 0.26, median = 0.23; Figure 5.7A). Out of the 11,532 analysed Fst windows, 683 (5.9%) were detected as outliers using the 99th quantile threshold. For the 95th and the 90th quantile, 1418 (12.3%) and 1981 (17.2%) windows were detected as having outliers, respectively. Several regions distributed across the genome showed elevated Fst. Notable regions, with an accumulation of outliers, were found in the middle of LG 3, the start of LG 6, the second half of LG 11, and finally in several regions of LG 14 (Figure 5.7A).

In comparison to viviparous lineages, the western oviparous group (presumed to have re-evolved oviparity; see Figure 5.2C and Chapter 3 for phylogeny) showed less background divergence (mean = 0.16, median = 0.14; Figure 5.7B). Out of the 11,417 Fst windows, 286 (2.5%) windows were identified as 99th quantile outliers, 829 (7.2%) as 95th quantile, and 1355 (11.9%) as 90th quantile outliers. Notable regions with outliers were the middle of LG 3, the start of LG 4, the second half of LG11, the end of LG 12, the first half of LG 14, and a few outliers in LG 16, LG 17 and LG 18 (Figure 5.7B).

When pooling both oviparous lineages and comparing them to all viviparous lineages, average Fst was moderate (mean = 0.19, median = 0.17; Figure 5.7C). The number of outlier windows were comparable to the first analysis, with 680 (5.9%), 1405 (12.1%), and 2017 (17.5%) regions detected as outliers using the 99th, 95th and 90th quantile, indicating that the pattern might be driven by the eastern oviparous lineage. Moreover, the pattern was somewhat similar to the pattern observed when using only the eastern oviparous lineage. However, there are some exceptions where both lineages showing higher Fst values compared to the background, including a region around the middle of LG 3, the second half of LG 11 and the first half of LG 14. In general, we found that Fst outliers between the two lineages were not randomly distributed across the genome (Figure 5.7C; Figure 5.8).

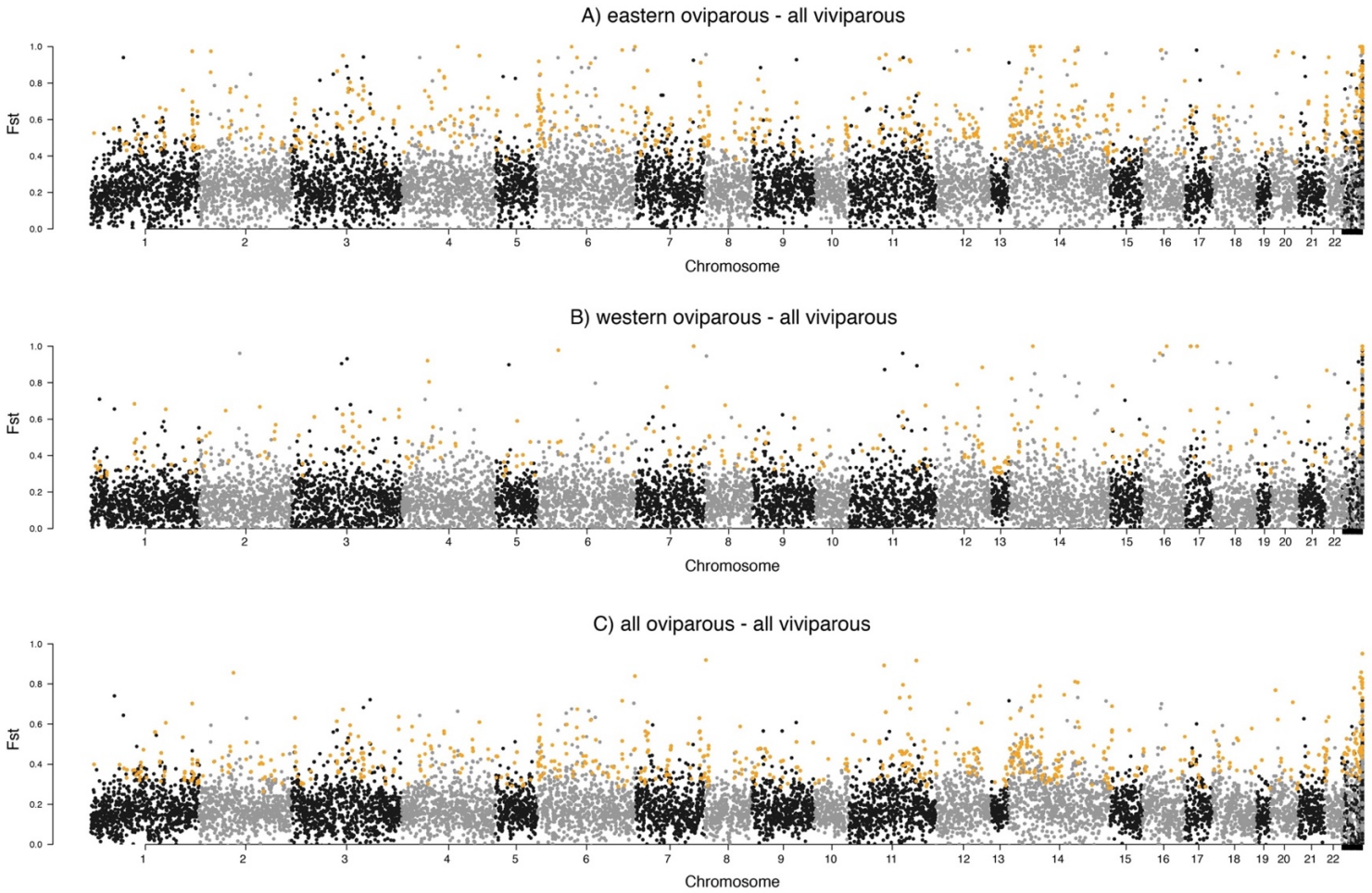

Figure 5.7 Fst plots illustrating genetic differentiation across the genome. A) shows the eastern oviparous group and all viviparous lineages, B) shows the western oviparous lineage in relation to all viviparous lineages and C) shows all combined oviparous lineages compared to all combined viviparous lineages. Outliers (99th quantile) are highlighted in orange. Note that some outliers are located on unplaced scaffolds that have not been able to be placed on any linkage group so far.

More specifically, the number of outliers that overlapped was around twice as many as expected by chance based on the simulations (Figure 5.8). The number of shared outliers for the 90th quantile outlier windows was 368, which is 1.5x more outliers than expected by chance (Figure 5.8A). For 95th quantile outlier windows, 212 outlier windows overlapped, 1.9x more than expected (Figure 5.8B). When considering the most conservative threshold for outliers using only the 99th quantile, 47 outliers overlapped between the two oviparous lineages, which is 2.7x more outliers than expected by chance (Figure 5.8C). Therefore, using more stringent thresholds results in a larger overlap of outlier windows than expected.

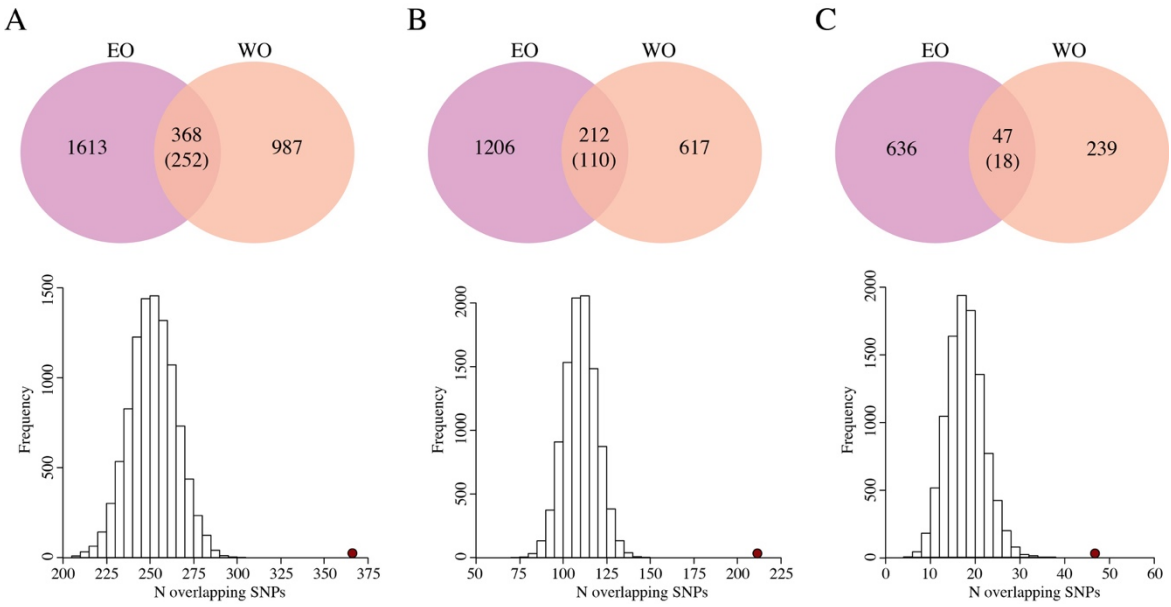

Figure 5.8 Overlap of Fst outliers between the two oviparous lineages (eastern oviparous = EO; western oviparous = WO) in relation to viviparous lineages. Panels A), B) and C) show the 90th, 95th, and 99th quantile of SNP outliers, respectively. Venn Diagrams show the number of private outliers for each lineage and the overlapping outliers. In brackets, the average number of expected outliers is specified. The frequency distributions show the number of overlapping SNPs expected by chance based on 10,000 simulations. For each of the quantiles, outliers overlapped more often than expected by chance ($P < 0.001$), as illustrated by the red dots laying outside the simulated distribution of overlapping outliers.

5.4.1 Genetics of an evolutionary reversal – Topology weighting

To test for genetic regions associated with a common genetic basis for reproductive mode, three topologies were tested in the topology-weighting method (Martin and Van Belleghem 2017). These topologies included: a closer relationship between the two oviparous lineages with all viviparous lineages being monophyletic (common genetic basis of oviparity more likely; basal oviparity); a relationship with the western oviparous lineage nested within the viviparous lineages (genetic basis for oviparity not shared more likely); and a third

topology that could not differentiate between the former two hypotheses (both hypotheses equally likely; Figure 5.9).

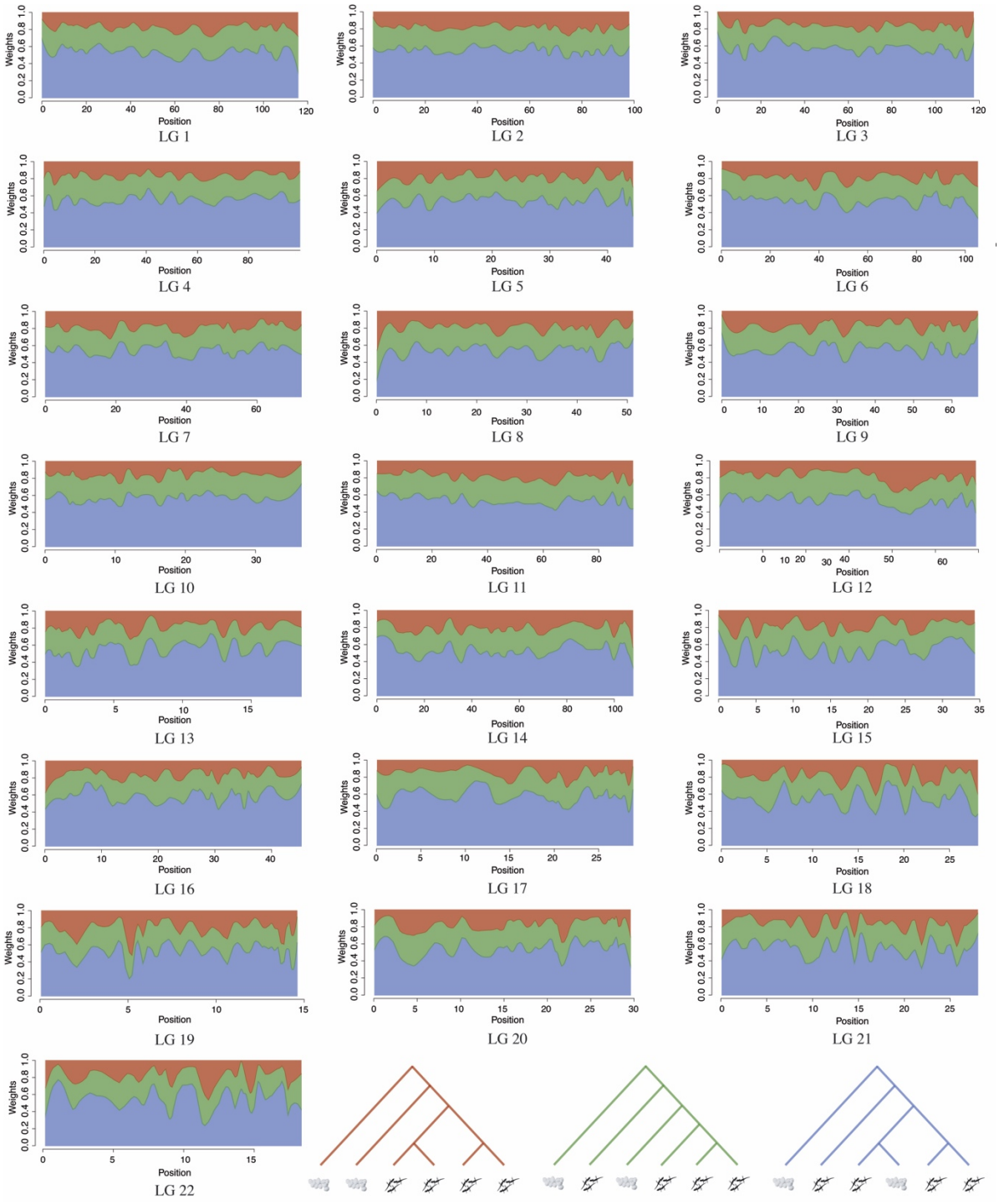

Figure 5.9 Topology weighting analysis across the 22 linkage groups. Topologies were summarized into three hypotheses: viviparous lineages monophyletic (red), reversal to (=derived) oviparity equally parsimonious as two origins of viviparity (green), single origin of viviparity and derived oviparity most parsimonious scenario (blue) (also see Figure 5.2). Topologies are all based on 65 individuals corresponding to the six common lizard lineages, two oviparous and four viviparous. Position on linkage group is in Mbp. Relative weights indicate the support for each of the three scenarios based on the gene trees.

The density of gene trees per Mb of sequence in the genome was 4.99 (N = 6899 gene trees in total; mean = 314 gene trees per LG; range = 103–597 gene trees). Across the whole genome, average weight support for a scenario with basal oviparity was 17.9%, while on average the reversal to oviparity scenario was supported by 55.5% of the weights. Larger regions across the genome supporting a closer relationship between the two oviparous modes were absent (Table 5.S1; Figure 5.9). No gene trees adjacent to each other along the genome strongly supported a scenario with basal oviparity. However, individual gene trees supporting a monophyletic viviparous clade and basal oviparity were observed, but scattered across the genome (Table 5.S1). Weights larger than 0.99 were observed in two gene trees on LG 11 and LG 9. Further gene trees on LG 3, LG 12 and LG 19 had a weight larger than 0.95. Including all gene trees with weights larger than 0.9 for the topology supporting basal oviparity, LG 3 was supported by three gene trees and LG 6, LG 9 and LG 14 were supported by two gene trees (Table 5.S1).

5.5 Discussion

5.5.1 Natural hybridization between oviparous and viviparous common lizards

For the first time, here we show the presence of natural hybrids between oviparous and viviparous common lizards supported by phenotypic and genetics analyses. Based on the two phenotypes, number of incubation days and embryonic stage at oviposition, we estimated a frequency of individuals with mixed ancestry of about 18%. This estimate was confirmed by genetic analyses. First generation hybridisation (F_1) between two different reproductive lineages was generally rare, with less than 8% across all samples (Figure 5.4). This suggests that premating barriers between the two reproductive modes exist to an extent, as the distribution of the two reproduction modes at the sampling site allows for high encounter rates and a higher degree of hybridization (Figure 5.1). More research is necessary to identify whether common lizards mate assortatively, for example through intraspecific communication via chemoreception (Gabirot et al. 2008) and sex chromosome-linked species recognition (Saether et al. 2007). We find a strong correlation between the phenotype and the genetic background (Figure 5.4C and E), and F_1-hybrids expressing a phenotype somewhat intermediate between the two reproductive modes, suggesting a single dominant SNP is not controlling the phenotype. Backcrossing does not seem to occur at a large scale, as only 10% of all individuals show admixture proportions between 1% to 40% (Figure 5.4D). It is remarkable that hybrids of such disparate

reproductive strategies survive, and as backcrossing occurs to some extent, are also fertile. However, we find a larger proportion of unfertilised eggs within a hybrid clutch and an overall lower hatching success, indicating that some postmating barriers (e.g. genetic incompatibilities) exist between the two lineages (Figure 5.5). In addition to potential premating barriers, this might explain why hybridization and backcrossing is limited. Lower fertility in oviparous-viviparous hybrids has been previously suggested but not quantified (Lindtke et al. 2010). The transition from oviparity to viviparity represents a dramatic shift in life history, so intermediate stages between oviparity and viviparity, particularly in terms of eggshell thickness and embryonic stage at oviposition, are very rarely observed in nature (Arrayago et al. 1996) and therefore presumably exhibit lower fitness. In summary, we document the natural hybridization between oviparous and viviparous common lizards. Our data shows that hybridization is rare, but that hybrids are viable and fertile to some extent.

5.5.2 Genetic architecture of oviparity and viviparity in common lizards

Here, we use the naturally occurring hybridisation between an oviparous and a viviparous common lizard lineage to investigate the genetic architecture of parity modes using admixture mapping. We identified few genomic loci of large effect for both the number of incubation days and the embryonic stage at oviposition (Figure 5.6; Table 5.2). These large effect loci (on average 8-9) accounted for over 90% of the phenotypic variance for both phenotypes. The high proportion explained by so few loci may be somewhat surprising, as the change from oviparous to viviparous reproduction is a dramatic transition accompanied by several changes in morphology, physiology and behaviour (Murphy and Thompson 2011). However, the number of incubation days and the embryonic stage at oviposition are traits directly linked to prolonged egg retention, a main requirement for evolving viviparity, often thought of as the most fundamental change associated with the transition alongside the loss of the eggshell (Guillette 1993). Many other morphological and physiological changes associated with viviparity occur at later stages, particularly during placental complexity evolution (Murphy and Thompson 2011; Van Dyke et al. 2014). Therefore, the very recent and young transition in common lizards can be considered an ideal model organism, exemplifying the minimum requirements for evolving viviparity. No direct comparison in genome-wide gene expression between a closely related oviparous and a viviparous species (or lineage) exists to date. Comparisons are usually restricted to pregnant and non-pregnant viviparous lizards, and with one exception (Brandley et al. 2012) are limited to a few genes (Murphy et al. 2010; Griffith et al. 2013). Therefore, this

study is the first aiming to infer the genetic basis of parity mode using a forward genetic approach.

Since reproductive traits are female specific, it is conceivable that some of the genetic determinants of parity mode are sex-linked (Qvarnström and Bailey 2009). For example, eggshell patterning in the great tit has been shown to be inherited by female sex-linkage (Gosler et al. 2000). Consistent with this prediction, this study found that overlap in SNPs associated with the number of incubation days and embryonic stage at oviposition/parturition was exclusive to a region on sex chromosome 14 (Figure 5.6A and 6B). From the 24 SNPs that were highly associated (PIP > 0.1, effect > 1) with the number of incubation days, most mapped to two regions on sex chromosome 14. Three other regions with more than one SNP were found on chromosomes 3, 4 and 19. Sex chromosome 14 generally exhibits a large number of differentially fixed alleles in those two regions (Figure 5.6C and D). Several lines of research suggest that sex-linked genes are effective in avoiding hybridisation and sex chromosomes generally show lower levels of admixture (Saether et al. 2007; Qvarnström and Bailey 2009; Martin et al. 2013). While some of the increased differentiation seen on the sex chromosome here could be attributed to the smaller effective population size of sex chromosomes, the association with parity mode phenotypes suggests that part of the trait is inherited via female sex-linked genes and therefore potentially suppressing recombination and hybridisation.

The strongest genetic association with the number of incubation days was shown by a SNP in the endothelial PAS domain-containing protein 1 (*EPAS1*) gene on LG 3. *EPAS1* is a transcription factor involved in the regulation of angiogenesis. It is expressed in the mammalian placenta (Sood et al. 2006; Duttaroy and Basak 2016) and is involved in uterine vascularisation in scincid lizards (Brandley et al. 2012; Van Dyke et al. 2014). *EPAS1* has been found to be highly upregulated in the uterus of pregnant females of the lizard *Chalicdes ocellatus* (Brandley et al. 2012). Therefore it has been previously been suggested to be important for the evolution of viviparity (Van Dyke et al. 2014). In function, *EPAS1* is related to vascular endothelial growth factors (*VEGF*). *VEGFs* are part of another genetic pathway, but also function as growth factors responsible for uterine vascularisation in mammals and in pregnant viviparous skinks (Murphy et al. 2010; Murphy and Thompson 2011; Van Dyke et al. 2014). Here, we show the first strong line of evidence that *EPAS1* is essential for the transition from oviparity to viviparity, supporting

literature that suggests it might have evolved multiple times in parallel across transitions to viviparity in squamates and mammals.

We found 42 genes located in seven linkage groups that could be associated with the embryonic stage at oviposition (Table 5.S2). Among the most interesting of these genes was lysophosphatidic acid receptor 6 (*LPAR6*) on LG 1, which is involved in embryo implantation and establishment of the placenta in mammals (Lin et al. 2010; Sadam et al. 2017). LPAs act through G protein-coupled receptors that alter several cellular responses; knockouts have been shown to influence some reproductive disorders. In mice, it has been demonstrated that the timing and spacing of embryos was severely disrupted by knock-outs, leading to delayed implantation and complete loss of normal spacing of embryos (Lin et al. 2010). Another gene in the vicinity of the same SNP is G protein-coupled receptor kinase 6 (*GRK6*), which is expressed in the uterus during pregnancy. Lack of *GRK6* expression can produce a phenotype of enhanced uterine contractility, leading to oxytocin-induced labour and stillbirth (Grotegut et al. 2016).

A SNP on LG 8 associated with embryonic stage was located nearby several genes, including the genes cadherin 11 (*CDH11*) and proteasome subunit beta 5 (*PSMB5*). Cadherins are important components of adherent junctions between adjacent cells, allowing the interaction between different types of cells and tissues (Takeichi 1990). In a few viviparous lizards, the uterus and cadherin proteins undergo substantial redistribution during the pregnancy, allowing the rearrangement of the uterus to accommodate the rapidly growing embryo (Murphy and Thompson 2011; Wu et al. 2011; Brandley et al. 2012). This could also be the case in common lizards, since embryos grow substantially over the pregnancy period (see Chapter 4). *CDH11* has also been associated with early pregnancy and preeclampsia in humans (Anton et al. 2014). *PSMB5* is one of several genes expressed in the uterine luminal fluid during early pregnancy in pregnant heifers, and has been proposed to facilitate interactions between the embryo and the uterus during the pregnancy recognition period (Forde et al. 2015).

Finally, a locus on LG 14 was located near two genes associated with human pregnancy (Table 5.S2). These were, first, Acyl-CoA synthetase long chain family member 4 (*ACSL4*), which has been previously shown to control transport and uptake of fatty acids in human placentas (Johnsen et al. 2009). It could therefore impact the growth and development of the embryo, although the extent to which fatty acid transport and provision

generally occurs between the embryo and mother is not known in squamates. Upregulation of voltage-dependent potassium channels (and in particular of *KCNE5*) in the placenta may lead to preeclampsia in pregnant women (Mistry et al. 2011, 2014). Placental function may be detrimentally affected by this, and impacts could range from placental proliferation, ion transport to steroidogenesis (Mistry et al. 2011).

In addition to these individual genes, some SNPs were locally associated with genes involved in neurotransmission and neural development (N = 5; Table 5.S2) and immune response (N = 3; Table 5.S2). For example, Interferon induced protein with tetratricopeptide repeats 5 (*IFIT5*), a gene close to the SNP on LG 12, is upregulated in the endometrium (a mucous membrane inside the uterus of mammals) of several mammal species (Bazer et al. 2008). Interferons are thought to be crucial for establishment of pregnancy, as they suppress immune recognition of the embryo (Bazer et al. 2008; Forde et al. 2015). In general, modification of gene regulation related to immunity might result from the loss of the eggshell, which allows direct contact between mother and embryo. Suppression of the maternal immune system might be crucial in preventing miscarriage. However, as an insufficient immune response can result in pathogen infestation, a fine-tuned regulation of the immune system is necessary to establish the health of mother and embryo (Saito 2001; Moffett and Loke 2006).

The two reproductive phenotypes, number of incubation days and embryonic stage at oviposition, are part of the same complex trait, parity mode. While more SNPS were associated with the number of incubation days (24 vs. 17), more regions (9 vs. 3 LGs) and more associated genes (15 vs. 45) were identified for embryonic stage at oviposition/parturition relative to the number of incubation days per clutch. The genomic regions associated with the two traits only partially overlap with regions of the sex chromosome, but are otherwise associated with different genomic regions. As observed in other complex traits that involve multiple differences in morphology, physiology and behaviour (Xu et al. 2012; Greenwood et al. 2013; Weber et al. 2013; Xu 2013), parity mode might be a highly modular phenotype associated with multiple genomic regions. While the two reproductive phenotypes are highly correlated (Figure 5.4C), embryonic stage at oviposition/parturition is a measure of egg retention, whereas the number of incubation days might also depend on other aspects, such as the interaction and exchange of ions, gas and nutrients between mother and embryo. For example, embryos will develop more slowly and less efficiently outside of the uterus, if they rely on interactions with the

mother. Several SNPs close to genes associated with the establishment of pregnancy and early pregnancy were identified for embryonic stage at oviposition. The main gene identified for the number of incubation days, *EPAS1*, which might be important throughout embryonic development (Van Dyke et al. 2014).

Fundamentally it is difficult to assess some traits in hybrids. For example, embryos did not always hatch as a result of the lower survival of hybrids (Figure 5.5), so the number of incubation days could not be measured for every individual. In addition, measurement of the embryonic stage at oviposition/parturition began in 2015, while number of incubation days was recorded from 2014. Therefore both phenotypic traits were not always measured in the same individuals and results are based on different sample sizes. Integration of more samples and the integration of the eggshell as a phenotype should improve our current data. Despite these limitations, a strong case linking certain genomic regions to the different parity modes in the common lizard has been built here.

5.5.3 Genetic architecture of a reversal from viviparity to oviparity

The eastern oviparous lineage presumably displays the ancestral state of oviparity within common lizards, while phylogenetic analyses suggest the western oviparous lineage has likely re-evolved oviparity from a viviparous ancestor (Chapter 3; Recknagel et al. 2017). The genetic mechanisms controlling oviparity in these two lineages could therefore be shared, independent, or a mix of shared and independent mechanisms. Eggshell characteristics and egg retention time differ considerably between the two oviparous lineages (Heulin et al. 2002; Lindtke et al. 2010), so we expect *a priori* that the genetic mechanism is not identical.

The genome scan and topology weighting analyses show that there is no large genomic region shared between the two oviparous lineages (Figure 5.7; Figure 5.9). This indicates that introgression of a large region with a gene (or several genes) crucial for oviparous reproduction has not occurred from the eastern oviparous into the western oviparous lineage. However, if introgression occurred shortly after the western oviparous lineage split from the other viviparous lineages, the signal would be difficult to detect as it could have been diluted by recombination and eroded after time (Plagnol and Wall 2006; Zhang et al. 2016). At smaller scales, we do find that more genetic regions show a signal of a close relationship between the two oviparous modes than expected, suggesting some proportion of shared genetic history (Figure 5.6; Figure 5.7; Table 5.S1). These regions

could be shared through incomplete lineage sorting, ancient introgression, similar selective pressures unrelated to parity mode (e.g. alleles adaptive to a warmer climate) or a similar genetic basis of parity mode.

The admixture mapping approach indicated that only a few loci with large effect are controlling oviparity in the eastern oviparous lineage with basal oviparity. Main candidate regions for parity mode control were LG 3, the end of LG 6, and LG 14. These regions had high support for common oviparous ancestry in the topology weighting analysis, were common Fst outliers in the western and eastern oviparous lineage relative to viviparous lineages, and showed strong signals in the admixture mapping (Figure 5.6; Figure 5.7; Figure 5.9). These regions show a strong signal of shared genetic history (Table 5.S3). With the data presented here, we cannot resolve the fine-scale association between the divergent eggshell phenotypes. More in-depth genome scans with an increased sample size for the western oviparous lineages, and ideally genetic crosses between the two oviparous lineages, and between the western oviparous and viviparous lineages, should resolve if the genetic basis between the two oviparous lineages is similar and which genes are responsible for the common basis of oviparous reproduction. However, the weight of evidence from this analysis suggests that only part of the genetic basis of oviparity between the eastern and western oviparous lineage is shared.

5.5.4 Conclusion

Rare, naturally occurring hybridisation between oviparous and viviparous common lizards allowed us to identify the genetic architecture of reproductive mode using admixture mapping. We found several SNPs that were associated with reproductive mode phenotypes in common lizards. Most of these SNPs were of large effect, explaining a large proportion of the phenotype (>90%). Two larger regions on LG 14 contained several SNPs associated with reproductive mode, and harboured higher Fst values and large differences in allele frequencies, with several nearly fixed alleles. This LG has been identified as the sex chromosome and is likely to harbour important genes for reproductive mode. Genes locally associated with SNPs identified in the admixture mapping are highly biologically relevant, included a prominent candidate gene for the evolution of viviparity, *EPAS1*, a few immune response genes, and genes that have been related to the establishment of the placenta and embryo development. In addition, we show that the common lizard lineage that putatively secondarily re-evolved oviparity shares more genomic ancestry with the lineage which retained oviparity than expected by chance. However, we did not identify large genomic

regions that showed signals of introgression or retained ancestry. Future research should address the genetic basis of oviparity in this lineage and the extent of shared genetic mechanisms. Gene knock-outs or modifications by CRISPR/Cas manipulation (Cong et al. 2013) would be necessary to robustly provide the functional significance of the identified genes. In summary, this is the first study to identify the genetic basis of viviparity and finds it to be controlled by few genes of large effect.

Chapter 6: Discussion

6.1 Main Aims and Objectives

The main aim of this PhD thesis was to examine how complex traits evolve using the two reproductive strategies, viviparity and oviparity, as a model system. I performed a combination of broad comparative analyses and ecological, phenotypic, phylogenetic and genomic analyses in common lizards (*Zootoca vivipara*) to address specific questions related to the major themes of my thesis.

In Chapter 2, by linking paleoclimatic data from the last 65 million years and transitions from oviparity to viviparity in squamates, I found that the evolution of viviparity was favoured by cold and stable climatic conditions. Diversification in squamates followed a pattern of linear growth during the last 100 million years, and a binary simulated trait differed from the pattern of the empirical transition estimates, rendering this a robust result. This study links the prediction from the cold-climate hypothesis (incidents of viviparity increase with colder conditions) and life-history theory (viviparity is favoured under stable environmental conditions).

In Chapter 3, I reconstructed the evolutionary history of parity mode evolution in common lizards. Based on the topology I found that the most parsimonious scenario for parity mode evolution was one origin of viviparity and a reversal to oviparity. Topologies that favoured other scenarios were significantly less likely. The phylogeny was also consistent with differences in sex chromsomal configuration between distinct lineages. Overall, the result supports re-evolution of the eggshell after the evolution of viviparity in common lizards, a rare and exceptional example that breaks Dollo's law of irreversibility.

In Chapter 4, I investigated the differences between life-history strategies in syntopically occurring viviparous and oviparous common lizards. I found that viviparous females exhibit larger body size, smaller clutch sizes, a larger reproductive burden, and a higher hatching success rate than oviparous females. I also found that offspring size and weight from viviparous females was lower compared to offspring from oviparous females, which may reflect space constraints during pregnancy. I suggest that the evolution of viviparity in common lizards is associated with an increased reproductive burden for viviparous females and that this promotes the evolution of larger body size to create more physical space for developing embryos.

In Chapter 5, I investigate the genetic architecture of parity mode in common lizards, using an admixture mapping approach within a hybrid zone between a single oviparous and viviparous lineage and a genome scan approach between all viviparous and oviparous lineages. In the admixture mapping approach, parity mode phenotypes were associated with only a few strongly selected markers with large effects. I find a region on chromsome 14 that is a strongly associated with oviparity in the lineage exhibiting basal oviparity, specifically for the number of incubation days and the embryonic stage. A few other genomic regions show strong signals of association with reproductive mode, including the gene *EPAS1*, which had been previously proposed to be involved in the evolution of viviparity. In the genome scan, no strong signal of common outliers between the two oviparous lineages was observed. However, more outliers were shared than expected by chance. This might suggest only a partial common genetic basis for oviparity, and corroborates the hypothesis of an independent re-evolution of the eggshell in one oviparous lineage instead of a retained ancestral genetic mechanism.

6.2 Project limitations

6.2.1 Phylogenetic methods

In Chapter 2, there were two main limitations in reconstructing the time points at which transitions occurred: i) uncertainty around the estimates (i.e. large confidence intervals), and ii) incomplete taxon sampling. Time divergence estimates generally suffer from high uncertainty, due to limited availability of precisely timed fossil data and limited sequence data (Thorne et al. 2002; Dos Reis and Yang 2013). While the amount of sequencing data available from different organisms is increasing rapidly, fossil data is still scarce. Incorporation of ancient DNA could increase the accuracy of age estimates, but this data is rarely available for older (>10,000 years) fossils (Ho et al. 2011). Incomplete taxon sampling was overcome by comparing the time estimates to those from datasets with more complete taxon sampling.

In Chapter 3, Bayesian coalescent-based reconstruction methods were either too computationally time consuming (in the case of the software SNAPP; (Bryant et al. 2012) or impossible to run (in the case of STARBEAST2 BEAUTI crashes as the input data, full sequence lengths, is too large; (Ogilvie et al. 2017)). Future software packages should address this issue as the size of sequence data will continue to increase.

6.2.2 Sample sizes

Discovering the genetic architecture of complex traits is always challenging, as it requires both large sample sizes and fine scale genomic resolution (Carlson et al. 2004). For the admixture mapping approach in Chapter 5, the most informative individuals are those which exhibit different levels of hybridization. Hybrids and particularly later generations that backcross into one of their parental lineages are highly informative, as recombination breaks up genetic varaints that might be responsible for the complex phenotype (Buerkle and Lexer 2008). Therefore, a larger number of hybrids might have increased the power to detect loci of particularly small effect. However, hybridization was generally low (see Chapter 5, Figure 5.3) so these individuals were the rarest within this contact zone, and are completely absent in other locations. In general, admixture mapping has a higher power and requires fewer genetic markers to detect causal loci compared to genome-wide association studies (Tian et al. 2006). To increase the statistical power to detect significant effects of genetic variants, the investigation of more hybrid zones would be beneficial. Ideally, obtain data from a contact zone containing the same oviparous lineage but different viviparous lineages should be obtained. Although this a realistic scenario based on the distribution of viviparous common lizard lineages, other potential hybrid zones have not yet been properly investigated. One potential contact zone between the eastern oviparous and the central viviparous I lineage has been identified in Semmering, a small region at the boarder of Lower Austria and Styria.

Larger individual sample sizes for the genome scan, maximising within lineage variation (i.e. including individuals from several different locations within a lineage's distribution) could improve the power of detecting putative candidates genetically controlling parity mode (Korte and Farlow 2013). Currently, some lineages (e.g. the western oviparous lineage) were not well represented in the genome scan.

6.3 The future of common lizard research - a new model organism for evolutionary biology

6.3.1 The genetic basis of parity mode in the common lizard

Here, I identified SNPs associated with the embryonic stage at oviposition/parturition and the number of incubation days. However, the loss of the eggshell is also an important feature for evolving viviparity. I have collected eggshells for phenotyping chemical characteristics and eggshell thickness. At this stage these phenotypes have not yet been

measured. The addition of this phenotypic information to the admixture mapping will be extremely interesting and should add valuable insight into the evolution of viviparity.

In addition, whole transcriptome studies of the uterus of non-pregnant and pregnant common lizards across all lineages would substantially improve our understanding of the developmental pathways that lead to viviparous and oviparous reproduction. A reference transcriptome is already available from a viviparous female whose whole genome has been sequenced. Ideally, transcriptome samples should be taken from females of both reproductive modes during different stages of pregnancy and gene expression compared. Transcriptomics will complement the admixture mapping and genome scan analyses, and provide a methodologically independent evaluation to compare with current results. By sequencing the whole genome of a single viviparous common lizard from Scotland (Yurchenko et al. in prep.), our lab has substantially increased the array of methods and tools that can be used for research in the genetics of viviparity. However, expanding this to the level of population-wide analyses using whole genome resequencing would improve the power, resolution, confidence and completeness of the genotype-phenotype association analyses (Bentley 2006; Shekhar Pareek et al. 2011).

The ability to find genetic variation associated with reproductive modes could be substantially improved for both of the approaches applied here (genome scanning and admixture mapping) by generating individuals from crosses between lineages with similar and different reproductive modes. It would be particularly helpful to identify the genetic basis of oviparity in the western oviparous lineage, in which a reversal to oviparity is believed to have occurred. Crosses between i) both oviparous lineages and ii) the western oviparous and a viviparous lineage are crucial to identify causal genetic variation. On a practical level, these crosses require established outdoor enclosures at a big enough scale to allow for large breeding schemes. However, my attempts to cross different lineages of common lizards have not been very successful, mainly restricted by limited available space, long generation times, suboptimal rearing conditions and difficulty in recreating the correct hibernation conditions for common lizards.

More contact zones with hybridization between different viviparous lineages should clarify whether viviparity evolved independently, or if different viviparous lineages represent different stages along the oviparity-viviparity continuum, with different sets of genes related to viviparity. We are developing a genotyping technique (http://biomeme.com)

using my data from the admixture mapping and genome scans that will allow us to test if an individual is oviparous, viviparous or a hybrid in the field. This will greatly improve the ability to identify more contact zones between oviparous and viviparous individuals; the current method for phenotyping requires a large effort in sampling time and housing space. With time-effective field-based genotyping (sequencing results available within hours), some of these can be overcome.

To robustly link genetic variants to phenotype, a functional evaluation of the candidate genomic regions for parity mode should be performed. While in the past this would have been almost impossible, if the causal loci are identified, genome editing using CRISPR/Cas9 could change a viviparous common lizard into an oviparous, and vice versa (Cong et al. 2013; Sander and Joung 2014). Genome editing could be performed on cultured tissue cells of the uterus (Cong et al. 2013; Jinek et al. 2013), at the embryonic stage (Hwang et al. 2013; Shen et al. 2013), into adult gonads (Lo et al. 2013), or potentially even on adult lizards *in vivo* (Yin et al. 2014). I will shortly begin exploring these possibilities.

6.3.2 Common lizards as a model system for studying adaptive traits

Common lizards are an excellent model system for evolutionary biologists. Not only do they represent a very rare case of a reproductively bimodal species, but they also have other astonishing adaptations. At first sight common lizards might appear 'boring', with their generally dark brown background colouration (for example Figure 1.3). However, a closer look will provide a fascinating diversity in colouration. There is a great variety of dorsal patterns (e.g. Arribas 2009; Lepetz et al. 2009), completely white to brightly coloured orange/red bellies (Vercken et al. 2007, 2010), which can be homogenously coloured or textured with spots (Cote et al. 2010), furthermore, melanic individuals exist (e.g. San-Jose et al. 2008; Jambrich and Jandzik 2012). Some of these traits have been linked to environmental variation and genetic heritability. Sequencing the genome has opened up the possibility to reveal the genetic basis of colouration in common lizards.

Some populations of common lizards are outstanding examples of frequency-dependent selection acting on alternative reproductive strategies coupled with differences in colouration (Sinervo et al. 2007; Vercken et al. 2010). The genetic basis of such evolutionary stable strategies are of great interest but remain elusive across the animal kingdom. Therefore, common lizards are an excellent model system for investigating how

frequency-dependent selection evolved, and with the tools that have been built up here (e.g. the whole genome sequence) it can be explored how it is controlled at the level of the genome.

Common lizards are a cold-adapted reptile species, and alongside the adder, are the most northerly distributed reptile species in the world (Packard 1966). Not only do they have a wide distribution, covering large parts of Eurasia, but they are also distributed across altitudinal gradients in the Alps, Pyrenees, Carpathians, Ural, Scandinavian mountains, Dinaric Alps and the Altai mountains. Therefore they are an excellent model organism for detecting the genetic basis of adaptation to cold environments and high altitudes, including the study of patterns of repeated adaptation to altitude. In summary, advances in the genomic toolkit development for common lizards will allow us to study a wide range fascinating traits displayed by this widely distributed terrestrial reptile.

6.4 Transitions to viviparity – the current state of knowledge and an outlook for future studies

The past few years have seen technological advances that have substantially increased our understanding of viviparous reproduction, a major evolutionary transition. However, the drivers and mechanisms by which viviparity evolved are still not fully understood. At the beginning of this PhD, cold-climate had been suggested to act as a driver for transitions to viviparity based on current distributions and a positive correlation between the proportion of viviparous taxa and latitude. However, not knowing the initial conditions driving the transitions remained a major problem, and factors other than temperature were also found to correlate with viviparity, so the issue remained unsatisfactorily resolved (Shine et al. 1978; Wourms and Lombardi 1992). The constant increase in sequence datasets, advances in phylogenetic and time divergence reconstructions, and long-term paleoclimate data have improved our ability to tackle this question (Zachos et al. 2008; Pyron and Burbrink 2014; Hedges et al. 2015; Zheng and Wiens 2016). For example, at the start of this PhD no broad time divergence estimates were available for the breadth of viviparous and oviparous squamates, and information on reproductive mode phenotypes was also limited. Recent studies using past climatic conditions and time divergence estimates of evolutionary transitions to viviparity have all supported the cold climate hypothesis (Lynch 2009; Schulte and Moreno-Roark 2010; Lambert and Wiens 2013). Chapter 2 of this Thesis represents the broadest analysis to date, and provides strong evidence for a role of cold climate in promoting transitions to viviparity. Stable environmental conditions may also

play a role in facilitating transitions to viviparity. Although, this pattern has not been investigated in other major animal groups, terrestrial vertebrates may not be that helpful in untangling this idea, as the number of transitions are limited. A possible exception could be caecilians, an order within amphibians (Kupfer et al. 2006), but biological information for this group is scarce and difficult to obtain. The most promising system are cartilaginous fishes, as transitions occurred relatively frequently in this group (Dulvy and Reynolds 1997).

Despite the potential of additional animal groups to increase our understanding of the transition to viviparity, it is likely that the processes driving transitions actually differ. Whether viviparity evolves might also depend on the evolution of other life-history traits, and these might or might not correlate with the transition to viviparity. Chapter 4 of this Thesis has shown that several life-history traits are correlated with viviparity, but that individual trait correlations differ across animal groups and are context-dependent (Bassar et al. 2014). More general insights on correlations between parity mode and other life-history traits could be gained from meta-analyses. This has been done to some extent in lizards (Meiri et al. 2012; Scharf et al. 2015), but a comprehensive study across all squamates is lacking so far. Ideally, this should be extended to all vertebrates to identify general patterns of viviparity and associated life-history evolution.

While research on *why* viviparity evolved has a long-standing history in evolutionary biology (Weekes 1935; Tinkle and Gibbons 1977; Goodwin et al. 2002; Blackburn 2006; Pires et al. 2011), we have only recently begun to study *how* viviparity evolves. This is due to the recent technological advances of cost-effective sequencing in non-model organisms (Stapley et al. 2010; Elmer and Meyer 2011; Ellegren 2014). These have made it possible to link reproductive mode to casual genetic variation. Candidate gene approaches have identified a range of differentially expressed genes in viviparous and oviparous animal groups (Paulesu et al. 2005; Stewart et al. 2011; Van Dyke et al. 2014). A breadth of studies now exist on mammalian transcriptomes of the placenta, providing candidate genes involved in establishing pregnancy and potentially involved in the evolution of viviparity (e.g. Hou et al. 2012; Kim et al. 2012; Lynch et al. 2012). In 2012, a study on pregnant and non-pregnant scincid lizards has provided a first large-scale transcriptome-wide analysis of genes associated with viviparity in a lizard (Brandley et al. 2012). These studies have substantially improved our knowledge on pregnancy and provided us with a long list of potential candidate genes involved in the transition from oviparity to viviparity (Murphy

and Thompson 2011; Van Dyke et al. 2014). However, a causal link between genes and reproductive mode cannot be established by these reverse genetic approaches.

In Chapter 5, I provide a direct link between phenotype and genotype by using admixture mapping on naturally occurring hybrids of oviparous and viviparous common lizards. I show that one gene, *EPAS1*, has a particularly strong association with reproductive mode. This gene is involved in uterine vascularization, and has been previously associated with pregnancy in mammals and in viviparous skinks (Murphy et al. 2010; Murphy and Thompson 2011; Van Dyke et al. 2014). Convergence on the whole-genome level could be addressed by comparing more transcriptomes and more genomes of oviparous-viviparous sister species or groups. For example, whole genome sequencing and comparison of marine mammals and terrestrial sister groups have resulted in the identification of some convergent substitutions for the major life-history transition from land to water (Foote et al. 2015; Zhou et al. 2015). Using multiple replicate transitions from oviparity to viviparity will be crucial for assessing how predictable evolution is at the genomic level, but also regarding phenotypic and genomic patterns of traits co-evolving with reproductive mode. The intense study and development of genomic tools in common lizards should trigger research on more representative organisms (such as other reproductively bimodal species, including lizards, snakes, and salamanders) in the near future to get a better understanding on the evolution of viviparity. This will provide very valuable insights into the evolution of complex traits and their predictability.

As a concluding remark, we have made substantial progress in understanding why and how viviparity evolves. Exemplary case studies of some animal groups have provided a solid base line for future research in identifying the broad causes and genetic mechanisms of this major evolutionary transition. For the first time, I have identified the genetic architecture of viviparity, and future research will hopefully show how comparable this genetic mechanism is across animal groups.

Appendix A: Publications/Press Articles

A.1 Recknagel, H. Jacobs, A., Herzyk, P. Elmer, K. R. 2015. Double-digest RAD sequencing using Ion Proton semiconductor platform (ddRADSeq-ion) with nonmodel organisms. doi:10.1111/1755-0998.12406.

MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2015) doi: 10.1111/1755-0998.12406

Double-digest RAD sequencing using Ion Proton semiconductor platform (ddRADseq-ion) with nonmodel organisms

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Abstract

Research in evolutionary biology involving nonmodel organisms is rapidly shifting from using traditional molecular markers such as mtDNA and microsatellites to higher throughput SNP genotyping methodologies to address questions in population genetics, phylogenetics and genetic mapping. Restriction site associated DNA sequencing (RAD sequencing or RADseq) has become an established method for SNP genotyping on Illumina sequencing platforms. Here, we developed a protocol and adapters for double-digest RAD sequencing for Ion Torrent (Life Technologies; Ion Proton, Ion PGM) semiconductor sequencing. We sequenced thirteen genomic libraries of three different nonmodel vertebrate species on Ion Proton with PI chips: Arctic charr *Salvelinus alpinus*, European whitefish *Coregonus lavaretus* and common lizard *Zootoca vivipara*. This resulted in ~962 million single-end reads overall and a mean of ~74 million reads per library. We filtered the genomic data using *Stacks*, a bioinformatic tool to process RAD sequencing data. On average, we obtained ~11 000 polymorphic loci per library of 6–30 individuals. We validate our new method by technical and biological replication, by reconstructing phylogenetic relationships, and using a hybrid genetic cross to track genomic variants. Finally, we discuss the differences between using the different sequencing platforms in the context of RAD sequencing, assessing possible advantages and disadvantages. We show that our protocol can be used for Ion semiconductor sequencing platforms for the rapid and cost-effective generation of variable and reproducible genetic markers.

Keywords: double-digest, genotyping by sequencing, Ion Proton, Ion Torrent, RAD sequencing, semiconductor sequencing, single nucleotide polymorphism (SNP) genotyping

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Introduction

Recent technical advances in genomics have propelled research in ecology and evolution and promoted the integration of these two fields. In particular, the development of next-generation sequencing technologies, which have massively parallelized DNA sequencing, has had a major impact (Stapley *et al.* 2010). High-throughput genotyping of wild populations of nonmodel organisms opens new possibilities to unravel the genetic material leading to phenotypic change and adaptation (Barrett & Hoekstra 2011). Only by the accumulation of such research, we will be able to understand the genetics of adaptation and gain an integrative view of the environment, the phenotype and the genotype.

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While next-generation sequencing generates a vast amount of genomic data, the interpretation of such data constitutes a major challenge to scientists. The limit is usually no longer technical, but rather a combination of time, effort and money. The analysis of complex whole genomes is costly and time-consuming and often unnecessary for understanding evolution and genetics. Hence, several methods for reducing the genome to a representative, but more manageable, fraction have been developed recently (Baird *et al.* 2008; Andolfatto *et al.* 2011; Elshire *et al.* 2011; Peterson *et al.* 2012; Narum *et al.* 2013). These reduced genome representation methods make use of restriction enzymes to digest and fragment the genome, followed by targeted sequencing of those fragments. From mutations identified in the sequences of these fragments, hundreds to tens of thousands of single nucleotide polymorphisms (SNPs) can be detected and

A.2 Recknagel, H. Hooker, O., Adams, C., Elmer, K. R. 2017. Ecosystem size predicts eco-morphological variability in a postglacial diversification. doi: 10.1002/ece3.3013

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ORIGINAL RESEARCH

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Ecosystem size predicts eco-morphological variability in a postglacial diversification

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Abstract

Identifying the processes by which new phenotypes and species emerge has been a long-standing effort in evolutionary biology. Young adaptive radiations provide a model to study patterns of morphological and ecological diversification in environmental context. Here, we use the recent radiation (ca. 12k years old) of the freshwater fish Arctic charr (*Salvelinus alpinus*) to identify abiotic and biotic environmental factors associated with adaptive morphological variation. Arctic charr are exceptionally diverse, and in postglacial lakes there is strong evidence of repeated parallel evolution of similar morphologies associated with foraging. We measured head depth (a trait reflecting general eco-morphology and foraging ecology) of 1,091 individuals across 30 lake populations to test whether fish morphological variation was associated with lake bathymetry and/or ecological parameters. Across populations, we found a significant relationship between the variation in head depth of the charr and abiotic environmental characteristics: positively with ecosystem size (i.e., lake volume, surface area, depth) and negatively with the amount of littoral zone. In addition, extremely robust-headed phenotypes tended to be associated with larger and deeper lakes. We identified no influence of co-existing biotic community on Arctic charr trophic morphology. This study evidences the role of the extrinsic environment as a facilitator of rapid eco-morphological diversification.

KEYWORDS
adaptive morphology, Arctic charr, benthic–limnetic, ecological opportunity, environmental heterogeneity, freshwater fish, trophic morphology

1 | INTRODUCTION

Identifying the environmental agents of natural selection has proven difficult because organisms live in environments that are profoundly complex, with multiple and potentially conflicting selection pressures. Some lineages, but not all, diversify rapidly in new environments, suggesting that a combination of extrinsic and intrinsic factors determine adaptive potential (Elmer, Lehtonen, Fan, & Meyer, 2013; Losos & Mahler, 2010; Stein, Gerstner, & Kreft, 2014). It is increasingly recognized that phenotypic change can arise surprisingly fast. This has been proven experimentally (Blount, Borland, & Lenski, 2008; Kawecki et al., 2012), through artificial selection such as crop modification and animal breeding (Conner, 2003; Meyer, DuVal, & Jensen, 2012; Neff & Rine, 2006), and shown in some naturally occurring populations as a response to diversifying selection (Elmer, Lehtonen, Kautt, Harrod, & Meyer, 2010; Franks, Sim, & Weis, 2007; Hendry, Nosil, & Rieseberg, 2007).

Rapid adaptive radiations across isolated islands and lakes are well recognized as important models for disentangling how diversity arises in nature, as they provide relatively simple replicated environments

A.3 Recknagel, H. Kamenos, N. Elmer, K. R. 2017. Common lizards break Dollo's law of irreversibility: genome-wide phylogenomics support a single origin of viviparity and re-evolution of oviparity. bioRxiv doi:10.1101/225086.

New Results

Common lizards break Dollo's law of irreversibility: genome-wide phylogenomics support a single origin of viviparity and re-evolution of oviparity

Hans Recknagel, Nick Kamenos, Kathryn R Elmer

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Abstract | Info/History | Metrics | Supplementary material | Preview PDF

Abstract

Dollo's law of irreversibility states that once a complex trait has been lost in evolution, it cannot be regained. It is thought that complex epistatic interactions and developmental constraints impede the re-emergence of such a trait. Oviparous reproduction (egg-laying) requires the formation of an eggshell and represents an example of such a complex trait. In reptiles, viviparity (live-bearing) has evolved repeatedly but it is highly disputed if oviparity has re-evolved. Here, using up to 194,358 SNP loci and 1,334,760 bp of sequence, we reconstruct the phylogeny of viviparous and oviparous lineages of common lizards and infer the evolutionary history of parity modes. Our phylogeny strongly supports six main common lizard lineages that have been previously identified. We find very high statistical support for a topological arrangement that suggests a reversal to oviparity from viviparity. Our topology is consistent with highly differentiated chromosomal configurations between lineages, but disagrees with previous phylogenetic studies in some nodes. While we find high support for a reversal to oviparity, more genomic and developmental data are needed to robustly test this and assess the mechanism by which a reversal might have occurred.

A.4 Recknagel, H. Kamenos, N. Elmer, K. R. 2017. Common lizards break Dollo's law of irreversibility: genome-wide phylogenomics support a single origin of viviparity and re-evolution of oviparity. bioRxiv doi:10.1101/225086; Featured in *New Scientist* **2017**, 3155: 11.

Laurie Campbell/naturepl.com

Which came first, the lizard or the egg? In the case of at least one lizard, we have an answer: the live-bearing lizard came first and only later evolved the ability to lay eggs. It's a rare example of a species re-evolving a complex trait that had been lost.

The common lizard is just that. It is found across a broad swathe of Eurasia, from Ireland in the west to Japan in the east. Its name *Zootoca vivipara* means "live-bearing" in both Greek and Latin, and as you might expect it gives birth to its young.

But there are exceptions. Two small populations on the edge of the common lizard's range lay eggs. One of these subspecies is found around the border between Spain and France, and the other in the southern Alps.

Biologists had assumed these subspecies were the last remnants of an egg-laying ancestral population from which the live-bearers evolved – something that seems to have happened over 100 times in reptiles. But when they started doing simple genetic tests around a decade ago, the data didn't fit this simple story.

One explanation for the genetic results was that live-bearing evolved at least twice. Another was that egg-laying re-evolved in one population, but this was dismissed by many as unlikely.

Making some eggs

"There is not really any consensus," says Kathryn Elmer of the University of Glasgow, UK. So her team collected 76 lizards from around Europe and carried out more detailed genetic studies, looking at over 200,000 sites in the genome. They used this data to build a detailed family tree of common lizards.

This tree shows that the egg-laying lizards in the southern Alps are a remnant of the ancestral population. Live-bearing lizards evolved once from this ancestral population and split into several different groups. In one of these, evolution went backwards and egg-laying re-evolved, giving rise to the Spanish population.

"I consider this strong evidence of regaining egg-laying," says April Wright of Southeastern Louisiana University. In 2015 she found hints of three other cases of lizards re-evolving egg-laying. But what we don't know, she says, is why or how eggs re-evolved in common lizards.

It must have happened relatively recently, as these lizards only became live-bearing around 2 million years ago. Perhaps the genetic programme involved in egg-laying lay dormant for that time, then was simply re-activated. Elmer's team is now working out what genetic changes occurred.

However, in the snakes known as sand boas, it has been suggested that egg-laying re-evolved after 60 million years. It is unlikely that the original genetic program survived this long, so the sand boas may have re-evolved eggs from scratch.

Making a U-turn

It is becoming clear that it is very common for evolution for change direction. For instance, on one island on the Galapagos ground finches evolved thicker beaks during a period of dry years when small seeds were rare, then re-evolved thinner beaks when the climate became wetter.

But it is a different matter for evolution to go into reverse long enough for a trait as complex as egg-laying to reappear. "I think it's quite uncommon," says Elmer. "I don't know of many other cases."

But it undoubtedly does happen, contrary to a claim by 19th-century biologist Louis Dollo. His assertion that such reversions are impossible, although only ever a hypothesis, is wrongly called Dollo's law.

Other examples include metamorphosis re-evolving in salamanders that had lived their entire lives as juveniles, toes reappearing in snake-like lizards that had lost them, wings re-evolving in several stick insects and parasitic mites evolving into free-living dust-mites. More examples may emerge from genetic studies.

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Supporting information

Supporting Information for Chapter 2

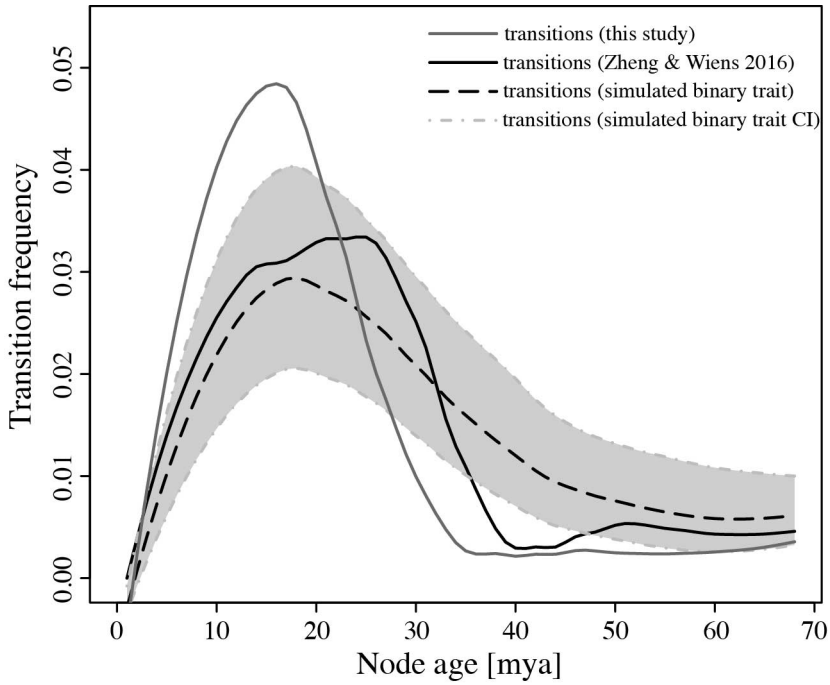

Figure 2.S1. Empirical and simulated transition frequencies. Binary trait simulation transition estimates compared to empirical transitions to viviparity across node ages from 0 to 65 mya. The grey solid line represents transition frequency to viviparity estimated in this study (normalized to transitions per million years by total transitions). The black solid line is the viviparity transition frequency estimated from Zheng and Wiens (Zheng and Wiens 2016). The dashed black line illustrates the mean transition frequency of the simulated binary trait, with the grey shaded area being the 95% confidence interval (CI) in transition frequency for each time bin from 50 simulations.

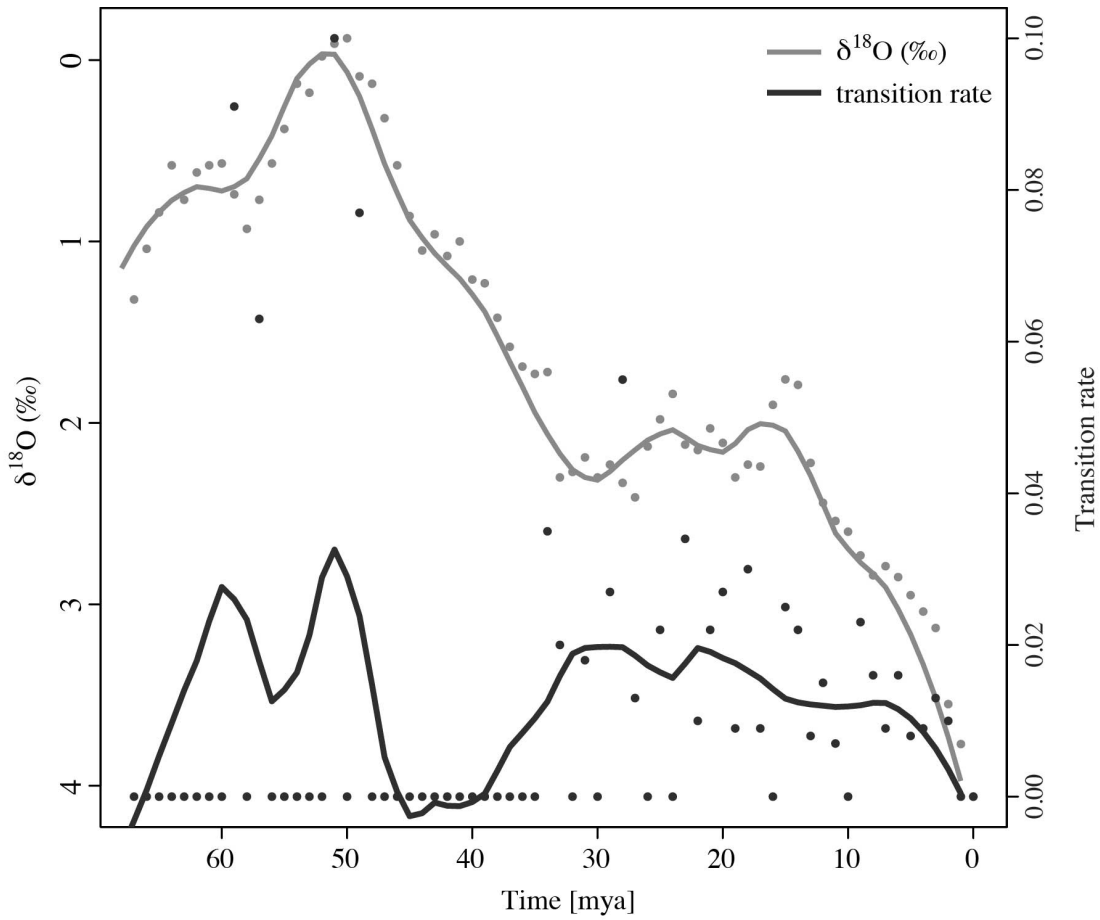

Figure 2.S2. Transition rate to viviparity and paleoclimate. Transition rate was estimated as ratio between the number of transition events and the number of total branching events per million years from 65 million years ago to present. Temperature and number of transitions are shown as smoothed lines (span[λ] = 0.25). Oxygen isotopes from (Zachos et al. 2001) were used to determine palaeotemperature (negative relationship).

Table 2.S1. Oviparous-viviparous taxon pairs included in the analysis. Each node numbers corresponds to an independent transitional event from oviparity to viviparity. In bimodal species, letters (o) and (v) refer to oviparous and viviparous lineages, respectively. References that provided information on reproductive mode and phylogeny are indicated. 'Delta transition' refers to the differential in likelihood between an ancestral lineage being oviparous and the likelihood of a descendant lineage being oviparous (a number close to 1 indicates a high likelihood for a transition from oviparity to viviparity).

Node	oviparous species	viviparous species	Ref (reproduction)	Ref (phylogeny)	delta transition
1	*Rhacodactylus leachianus*	*Rhacodactylus trachyrhynchus*	(Blackburn 1982; Bauer et al. 2012)	(Pyron and Burbrink 2014)	0.997
2	*Lucasium damaeus*	*Naultinus stellatus*	(Blackburn 1982; Nielsen et al. 2011)	(Pyron and Burbrink 2014)	0.995
3	*Plestiodon gilberti*	*Plestiodon dugesii*	(Blackburn 1982; Shine 1985)	(Pyron and Burbrink 2014)	0.996
4	*Tribolonotus pseudoponceleti*	*Corucia zebrata*	(Blackburn 1982)	(Pyron and Burbrink 2014)	0.944
5	*Tribolonotus pseudoponceleti*	*Tribolonotus schmidti*	(Blackburn 1982)	(Pyron and Burbrink 2014)	0.997
6	*Proablepharus reginae*	*Niveoscincus pretiosus*	(Stewart and Thompson 2009)	(Pyron and Burbrink 2014)	0.994
7	*Oligosoma suteri*	*Oligosoma maccanni*	(Patterson and Daugherty 1995)	(Pyron and Burbrink 2014)	0.578
8	*Lioscincus nigrofasciolatum*	*Kanakysaurus viviparus*	(Sadlier et al. 2004)	(Pyron and Burbrink 2014)	NA
9	*Lioscincus maruia*	*Lioscincus tillieri*	(Sadlier and Bauer 1999)	(Pyron and Burbrink 2014)	NA
10	*Saiphos equalis (o)*	*Saiphos equalis (v)*	(Blackburn 1982; Shine 1985)	(Pyron and Burbrink 2014)	NA
11	*Anomalopus mackayi*	*Anomalopus swansoni*	(Blackburn 1982)	(Pyron and Burbrink 2014)	0.99
12	*Eremiascincus richardsonii*	*Hemiergis initialis*	(Blackburn 1982)	(Pyron and Burbrink 2014)	0.991

13	*Glaphyromorphus darwiniensis*	*Eulamprus tympanum*	(Hosie et al. 2003)	(Pyron and Burbrink 2014)	0.99
14	*Lerista arenicola*	*Lerista microtis*	(Greer 1989)	(Pyron and Burbrink 2014)	0.979
15	*Lerista bougainvillii (o)*	*Lerista bougainvillii (v)*	(Greer 1989)	(Fairbairn et al. 1998)	NA
16	*Zonosaurus madagascariensis*	*Xantusia sierrae*	(Blackburn 1982)	(Pyron and Burbrink 2014)	0.611
17	*Platysaurus mitchelli*	*Cordylus aridus*	(Blackburn 1982)	(Pyron and Burbrink 2014)	0.826
18	*Eremias grammica*	*Eremias multiocellata*	(Blackburn 1982)	(Pyron and Burbrink 2014)	0.998
19	*Zootoca vivipara (o)*	*Zootoca vivipara (v)*	(Mayer et al. 2000)	(Surget-Groba et al. 2006)	NA
20	*Pseudopus apodus*	*Anguis fragilis*	(Blackburn 1982)	(Pyron and Burbrink 2014)	0.985
21	*Kinyongia fischeri*	*Bradypodion pumilum*	(Andrews and Karsten 2010)	(Pyron and Burbrink 2014)	0.999
22	*Chamaleo deremensis*	*Chamaleo jacksonii*	(Andrews and Karsten 2010)	(Pyron and Burbrink 2014)	0.963
23	*Phrynocephalus axillaris*	*Phrynocephalus vlangalii*	(Pang et al. 2003)	(Pyron and Burbrink 2014)	0.992
24	*Lyriocephalus scutatus*	*Cophotis ceylanica*	(Blackburn 1982)	(Pyron and Burbrink 2014)	0.982
25	*Phrynosoma asio*	*Phrynosoma taurus*	(Lambert and Wiens 2013)	(Pyron and Burbrink 2014)	0.887
26	*Phrynosoma modestum*	*Phrynosoma douglasii*	(Lambert and Wiens 2013)	(Pyron and Burbrink 2014)	0.88
27	*Sceloporus spinosus*	*Sceloporus mucronatus*	(Lambert and Wiens 2013)	(Pyron and Burbrink 2014)	0.969
28	*Sceloporus spinosus*	*Sceloporus malachiticus*	(Lambert and Wiens 2013)	(Pyron and Burbrink 2014)	0.96
29	*Sceloporus chaneyi*	*Sceloporus goldmani*	(Lambert and Wiens 2013)	(Pyron and Burbrink 2014)	0.99

30	*Sceloporus aeneus*	*Sceloporus bicanthalis*	(Lambert and Wiens 2013)	(Pyron and Burbrink 2014)	0.988
31	*Corytophanes cristatus*	*Corytophanes percarinatus*	(Blackburn 1982)	(Pyron and Burbrink 2014)	0.965
32	*Liolaemus fuscus*	*Liolaemus nigroviridis*	(Pincheira-Donoso et al. 2013)	(Pyron and Burbrink 2014)	0.994
33	*Liolaemus platei*	*Liolaemus paulinae*	(Pincheira-Donoso et al. 2013)	(Pyron and Burbrink 2014)	0.987
34	*Liolaemus chaltin*	*Liolaemus capillitas*	(Pincheira-Donoso et al. 2013)	(Pyron and Burbrink 2014)	0.973
35	*Liolaemus saxatilis*	*Liolaemus bellii*	(Pincheira-Donoso et al. 2013)	(Pyron and Burbrink 2014)	0.948
36	*Liolaemus chaltin*	*Liolaemus puna*	(Pincheira-Donoso et al. 2013)	(Pyron and Burbrink 2014)	0.992
37	*Liolaemus reichei*	*Liolaemus andinus*	(Pincheira-Donoso et al. 2013)	(Pyron and Burbrink 2014)	0.847
38	*Liolaemus quilmes*	*Liolaemus espinozai*	(Pincheira-Donoso et al. 2013)	(Pyron and Burbrink 2014)	0.992
39	*Liolaemus abaucan*	*Liolaemus ornatus*	(Pincheira-Donoso et al. 2013)	(Pyron and Burbrink 2014)	0.996
40	*Typhlops fornasinii*	*Typhlops bibronii*	(Shine and Lee 1999)	(Pyron and Burbrink 2014)	0.991
41	*Python molurus*	*Anilius scytale*	(Blackburn 1985)	(Pyron and Burbrink 2014)	0.608
42	*Python molurus*	*Cylindrophis ruffus*	(Blackburn 1985)	(Pyron and Burbrink 2014)	0.809
43	*Xenodermus javanicus*	*Acrochordus granulatus*	(Blackburn 1985)	(Pyron and Burbrink 2014)	0.968
44	*Macrovipera lebetina*	*Montivipera xanthina*	(Fenwick et al. 2012)	(Pyron and Burbrink 2014)	0.792
45	*Daboia mauritanica*	*Daboia siamensis*	(Fenwick et al. 2012)	(Wüster et al. 2008)	NA

46	*Daboia mauritanica*	*Vipera berus*	(Fenwick et al. 2012)	(Pyron and Burbrink 2014)	0.795
47	*Cerastes cerastes*	*Cerastes vipera*	(Fenwick et al. 2012)	(Pyron and Burbrink 2014)	0.671
48	*Echis ocellatus*	*Atheris squamigera*	(Fenwick et al. 2012)	(Pyron and Burbrink 2014)	0.702
49	*Naja nigricollis*	*Hemachatus hemachatus*	(Shine and Lee 1999)	(Pyron and Burbrink 2014)	1
50	*Pseudechis australis*	*Pseudechis porphyriacus*	(Shine and Lee 1999)	(Pyron and Burbrink 2014)	0.996
51	*Cacophis squamulosus*	*Suta fasciata*	(Blackburn 1985)	(Pyron and Burbrink 2014)	0.986
52	*Pseudonaja modesta*	*Hemiaspis signata*	(Blackburn 1985)	(Pyron and Burbrink 2014)	0.989
53	*Psammophylax tritaeniatus*	*Psammophylax variabilis*	(Shine and Lee 1999)	(Pyron and Burbrink 2014)	0.997
54	*Pythonodipsas carinata*	*Pseudaspis cana*	(Blackburn 1985)	(Pyron and Burbrink 2014)	0.983
55	*Madagascarophis colubrinus*	*Stenophis citrinus*	(Cadle 2009)	(Pyron and Burbrink 2014)	0.977
56	*Liopholidophis dolicocercus*	*Liopholidophis sexlineatus*	(Cadle 2009)	(Pyron and Burbrink 2014)	0.989
57	*Chrysopelea paradisi*	*Ahaetulla fronticincta*	(Blackburn 1985)	(Pyron and Burbrink 2014)	0.991
58	*Coronella girondica*	*Coronella austriaca*	(Shine and Lee 1999)	(Pyron and Burbrink 2014)	0.889
59	*Opheodrys aestivus*	*Opheodrys vernalis*	(Shine and Lee 1999)	(Pyron and Burbrink 2014)	1
60	*Tropidodryas serra*	*Helicops angulatus*	(Blackburn 1985)	(Pyron and Burbrink 2014)	0.897
61	*Natrix maura*	*Thamnophis sirtalis*	(Blackburn 1985)	(Pyron and Burbrink 2014)	0.992

62	*Sinonatrix percarinata*	*Sinonatrix annularis*	(Blackburn 1985)	(Pyron and Burbrink 2014)	0.996

Table 2.S2. GenBank accession numbers for all included mitochondrial and nuclear gene sequences.

Taxon	Family	12S	16S	cytb	ND2	ND4	c-mos	RAG1
Acrochordus granulatus	Acrochordidae	NC_7400.1	NC_7400.1	AF217841.1	NC_7400.1	NC_7400.1	HM234057.1	EU402831.1
Xenodermus javanicus	Acrochordidae	AF544781.1	AF544810.1	AY425810.1	-	U49320.1	AF544711.1	EU402869.1
Cophotis ceylanica	Agamidae	-	-	-	AF128493.1	-	-	-
Lyriocephalus scutatus	Agamidae	-	-	-	AF128494.1	-	-	-
Phrynocephalus axillaris	Agamidae	AY053662.1	AY053784.1	AY053905.1	-	KC551344.1	-	KC551432.1
Phrynocephalus vlangalii	Agamidae	AY053756.1	AY053880.1	AY053989.1	-	AY054113.1	-	KC551430.1
Anguis fragilis	Anguidae	NC_12431.1	NC_12431.1	NC_12431.1	NC_12431.1	NC_12431.1	AY099972.1	-
Pseudopus apodus	Anguidae	AF380954.1	JX987420.1	AF380965.1	AF085623.1	-	-	GU457974.1
Anilius scytale	Aniliidae	NC_14343.1	NC_14343.1	NC_14343.1	NC_14343.1	NC_14343.1	AF544722.1	EU402834.1
Bradypodion pumilum	Chamaeleonidae	-	AY289856.1	-	AF448729.1	AF443237.1	HQ130540.1	HF570731.1
Chamaeleo deremensis	Chamaeleonidae	DQ397273.1	DQ397300.1	-	AF448747.1	HF570607.1	HF570705.1	FJ746599.1
Chamaeleo jacksonii	Chamaeleonidae	DQ397240.1	JN165401.1	-	AF448753.1	AF443229.1	FJ984258.1	FJ984187.1
Kinyongia fischeri	Chamaeleonidae	AB474917.1	AB474917.1	AB474917.1	AB474917.1	AB474917.1	HF570685.1	GQ221960.1
Ahaetulla fronticincta	Colubridae	-	-	AF471072.1	-	-	AF471161.1	-
Chrysopelea paradisi	Colubridae	-	-	GQ895858.1	-	-	GQ895802.1	-
Coronella austriaca	Colubridae	AY122836.1	JQ904299.1	AY486930.1	AY486987.1	AY487065.1	AY486954.1	-
Coronella girondica	Colubridae	AY122835.1	JQ837564.1	AF471088.1	AY486988.1	AY487066.1	AF471113.1	-
Helicops angulatus	Colubridae	GQ457797.1	GQ457738.1	AF471037.1	FJ416751.1	-	AF471160.1	-
Liopholidophis dolicocercus	Colubridae	-	DQ979968.1	DQ979990.1	-	-	DQ979975.1	-
Liopholidophis sexlineatus	Colubridae	FJ404174.1	AY188063.1	AY188024.1	-	FJ404373.1	AY187985.1	-
Madagascarophis colubrinus	Colubridae	-	AY586211.1	AY188028.1	-	U49313.1	AY187989.1	-
Natrix maura	Colubridae	AF402623.1	-	AY487689.1	AF420078.1	EU437551.1	-	-
Opheodrys aestivus	Colubridae	-	-	AF471057.1	-	-	AF471147.1	-
Opheodrys vernalis	Colubridae	-	-	GQ927322.1	-	-	GQ927317.1	-

Psammophylax tritaeniatus	Colubridae	-	-	DQ486451.1	-	DQ486287.1	DQ486190.1	-
Psammophylax variabilis	Colubridae	AF544774.1	AY611864.1	AY612046.1	-	DQ486274.1	AF544709.1	AY487380.1
Pseudaspis cana	Colubridae	FJ404187.1	AY611898.1	AY612080.1	AY058962.1	DQ486319.1	DQ486167.1	-
Pythonodipsas carinata	Colubridae	FJ404189.1	AY188075.1	AY188036.1	-	FJ404386.1	AY187997.1	-
Sinonatrix annularis	Colubridae	AF544778.1	AF544807.1	JQ687431.1	-	JQ687424.1	AF544712.1	-
Sinonatrix percarinata	Colubridae	-	-	GQ281784.1	-	JQ687426.1	JQ687439.1	-
Stenophis citrinus	Colubridae	FJ404191.1	GU994843.1	AY612047.1	-	HE798413.1	AY611956.1	JQ073197.1
Thamnophis sirtalis	Colubridae	AF402647.1	-	AF420193.1	AF420194.1	AY136252.1	DQ902094.1	-
Tropidodryas serra	Colubridae	-	-	JQ598961.1	-	-	-	-
Corytophanes cristatus	Corytophanidae	-	-	-	AF528717.1	-	AF315390.1	JF806205.1
Corytophanes percarinatus	Corytophanidae	-	-	-	AF528718.1	-	-	-
Lucasium damaeum	Diplodactylidae	-	AY134534.1	-	GU459953.1	JQ398448.1	AY172927.1	GU459552.1
Naultinus stellatus	Diplodactylidae	-	GU459973.1	-	GU459775.1	-	-	GU459372.1
Rhacodactylus leachianus	Diplodactylidae	AF090176.1	GU460148.1	-	JX024451.1	-	FJ855467.1	JX024558.1
Rhacodactylus trachyrhynchus	Diplodactylidae	AB028745.1	AF215258.1	-	JX024465.1	-	-	JX024561.1
Cacophis squamulosus	Elapidae	EU547101.1	EU547150.1	EU547052.1	-	EU547007.1	EU366451.1	EU366440.1
Hemachatus haemachatus	Elapidae	U96797.1	-	AF217821.1	-	-	-	-
Hemiaspis signata	Elapidae	EU547123.1	EU547172.1	EU547074.1	-	EU547026.1	FJ587162.1	EU546897.1
Naja nigricollis	Elapidae	EU624237.1	GQ359754.1	GQ359505.1	-	DQ897703.1	-	-
Pseudechis australis	Elapidae	AJ749361.1	AJ749377.1	AF217824.1	-	AJ830278.1	EU546912.1	EU546873.1
Pseudechis porphyriacus	Elapidae	EU547096.1	-	EU547047.1	-	AY340170.1	EU546913.1	EU546874.1
Pseudonaja modesta	Elapidae	EU547098.1	EU547147.1	EU547049.1	-	DQ098503.1	EU546915.1	EU546876.1
Suta fasciata	Elapidae	EU547113.1	EU547162.1	EU547064.1	-	EU547016.1	EU546927.1	EU546888.1
Cordylus aridus	Gerrhosauridae	HQ167059.1	HQ167169.1	-	HQ166959.1	-	-	-
Platysaurus mitchelli	Gerrhosauridae	HQ167140.1	HQ167251.1	-	HQ167029.1	-	-	-
Zonosaurus madagascariensis	Gerrhosauridae	AJ416928.1	AF215240.1	EU621713.1	-	-	EU571697.1	JQ073185.1
Liolaemus abaucan	Iguanidae	EU795754.1	-	JN683128.1	AF099263.1	-	JN683081.1	-

Liolaemus andinus	Iguanidae	KF968999.1	-	KF968823.1	AF099245.1	-	KF968630.1	-
Liolaemus bellii	Iguanidae	AY662069.1	-	-	AF099223.1	-	-	HQ876436.1
Liolaemus capillitas	Iguanidae	AY367841.1	-	AY367811.1	AF099234.1	AY367869.1	AY367897.1	-
Liolaemus chaltin	Iguanidae	AY662061.1	-	-	AF099218.1	-	-	-
Liolaemus espinozai	Iguanidae	EU795765.1	-	JN683146.1	KP190034.1	-	JN683099.1	-
Liolaemus fuscus	Iguanidae	-	-	-	AF099232.1	-	-	-
Liolaemus nigroviridis	Iguanidae	-	-	KC313257.1	AF099233.1	-	-	-
Liolaemus ornatus	Iguanidae	-	-	JN683168.1	AF099266.1	-	JN683119.1	-
Liolaemus paulinae	Iguanidae	-	-	-	AY297531.1	-	-	-
Liolaemus platei	Iguanidae	-	-	AY850634.1	KJ452324.1	-	-	-
Liolaemus puna	Iguanidae	AY662059.1	-	-	AF305790.1	-	-	-
Liolaemus quilmes	Iguanidae	DQ237596.1	-	JN683164.1	AF099265.1	DQ237834.1	JN683122.1	-
Liolaemus reichei	Iguanidae	-	-	-	AF305794.1	-	-	-
Liolaemus saxatilis	Iguanidae	-	-	DQ989773.1	-	-	-	-
Eremias grammica	Lacertidae	-	DQ494822.1	-	AY607272.1	-	-	-
Eremias multiocellata	Lacertidae	DQ658793.1	HQ615623.1	-	-	-	-	-
Zootoca vivipara ovipar	Lacertidae	-	AY714977.1	AY714929.1	-	-	-	-
Zootoca vivipara vivipar	Lacertidae	-	AY714944.1	AY714896.1	-	-	EF632292.1	EF632249.1
Phrynosoma asio	Phrynosomatidae	KJ124076.1	L41452.1	AY141086.1	GQ502772.1	KJ124124.1	-	DQ385409.1
Phrynosoma douglassii	Phrynosomatidae	L40448.1	L41454.1	AY141089.1	U82686.1	AY141052.1	-	-
Phrynosoma modestum	Phrynosomatidae	DQ385397.1	L41455.1	AY141091.1	AY297484.1	JN809351.1	-	DQ385413.1
Phrynosoma taurus	Phrynosomatidae	DQ385403.1	-	AY141095.1	GQ502769.1	JN809344.1	-	-
Sceloporus aeneus	Phrynosomatidae	-	-	-	-	JN985732.1	-	-
Sceloporus bicanthalis	Phrynosomatidae	GQ464525.1	-	-	GQ464469.1	JN985728.1	-	GQ464715.1
Sceloporus chaneyi	Phrynosomatidae	-	-	-	-	JN985676.1	-	-
Sceloporus goldmani	Phrynosomatidae	-	-	-	-	U88290.1	-	-
Sceloporus malachiticus	Phrynosomatidae	GQ464547.1	L41467.1	-	AY297518.1	GQ895852.1	-	GQ464697.1

Sceloporus mucronatus	Phrynosomatidae	AF154139.1	AF440094.1	JF317190.1	AF440094.1	AF154233.1	-	GQ464723.1
Sceloporus spinosus	Phrynosomatidae	EF025756.1	L41475.1	-	AY297525.1	EF025750.1	-	GQ464718.1
Python molurus	Pythonidae	NC_15812.1	NC_15812.1	U69853.1	NC_15812.1	NC_15812.1	GQ225667.1	-
Anomalopus mackayi	Scincidae	-	AY169612.1	-	-	AY169650.1	-	-
Anomalopus swansoni	Scincidae	AY169576.1	AY169613.1	-	-	AY169651.1	HQ655196.1	-
Corucia zebrata	Scincidae	AY308334.1	AY308185.1	-	JN204259.1	JQ898450.1	HQ655201.1	-
Eremiascincus richardsonii	Scincidae	AY169582.1	AY308193.1	-	-	AY169657.1	HQ655204.1	-
Eulamprus tympanum	Scincidae	KC575658.1	KC575645.1	-	-	KC575720.1	KC575687.1	-
Glaphyromorphus darwiniensis	Scincidae	AY169586.1	DQ915310.1	-	-	DQ915334.1	-	-
Hemiergis initialis	Scincidae	HM852473.1	DQ915314.1	-	-	DQ915338.1	-	-
Kanakysaurus viviparus	Scincidae	-	-	-	DQ675209.1	DQ675209.1	DQ675413.1	DQ675288.1
Lerista arenicola	Scincidae	EF672758.1	EF672829.1	-	-	EF672970.1	-	-
Lerista bougainvillii ovipar	Scincidae	-	-	AF020021.1	-	-	-	-
Lerista bougainvillii vivipar	Scincidae	-	-	AF020032.1	-	-	-	-
Lerista microtis	Scincidae	EF672794.1	EF672865.1	AF020036.1	-	EF673006.1	-	-
Lioscincus maruia	Scincidae	-	-	-	-	-	DQ675354.1	DQ675293.1
Lioscincus nigrofasciolatum	Scincidae	EU837125.1	EU837121.1	-	EU837083.1	EU837100.1	DQ675356.1	EU837128.1
Lioscincus tillieri	Scincidae	EU567929.1	EU567923.1	EU567833.1	DQ675220.1	EU567729.1	DQ675360.1	-
Niveoscincus pretiosus	Scincidae	EU568019.1	EU567927.1	AY818820.1	DQ675234.1	EU567768.1	DQ675374.1	EU568110.1
Oligosoma maccanni	Scincidae	EU567948.1	AY308284.1	EU567794.1	EF447145.1	EF081222.1	-	EU568032.1
Oligosoma suteri	Scincidae	EU567968.1	EU567837.1	EU567773.1	EF567259.1	EU567751.1	DQ675387.1	EU568105.1
Plestiodon dugesii	Scincidae	-	-	-	-	-	-	HM161185.1
Plestiodon gilberti	Scincidae	AY308352.1	AY308203.1	-	AY607290.1	-	-	HM161193.1
Proablepharus reginae	Scincidae	-	-	-	-	-	HQ655220.1	-
Saiphos equalis ovipar	Scincidae	-	-	AF373247.1	AF373277.1	-	-	-
Saiphos equalis vivipar	Scincidae	-	-	AF373238.1	AF373268.1	-	-	-
Tribolonotus pseudoponceleti	Scincidae	-	-	HM229503.1	HM229454.1	-	HM229540.	-

Tribolonotus schmidti	Scincidae	-	-	HM229526.1	HM229481.1	-	HM229571.1	-
Typhlops bibronii	Typhlopidae	-	-	-	-	-	-	GU902696.1
Typhlops fornasinii	Typhlopidae	-	-	-	-	-	-	GU902693.1
Cylindrophis ruffus	Uropeltidae	NC_7401.1	NC_7401.1	AF471032.1	NC_7401.1	NC_7401.1	AF471133.1	EU402842.1
Atheris squamigera	Viperidae	AF544762.1	EU624279.1	EU624303.1	-	EU624212.1	AF544734.1	-
Cerastes cerastes	Viperidae	HQ658445.1	HQ267812.1	EU852299.1	-	EU624222.1	AF471131.1	EU852329.1
Cerastes vipera	Viperidae	-	AJ275757.1	AJ275705.1	-	-	-	-
Daboia siamensis	Viperidae	DQ305413.1	DQ305436.1	DQ305459.1	-	DQ305477.1	-	-
Echis ocellatus	Viperidae	EU852312.1	GQ359668.1	EU852294.1	-	AF292607.1	-	EU852324.1
Macrovipera lebetina	Viperidae	EU624260.1	EU624294.1	AJ275713.1	-	DQ897729.1	-	-
Macrovipera mauritanica	Viperidae	EU624261.1	EU624295.1	EU624313.1	-	EU624229.1	-	-
Vipera berus	Viperidae	EU624267.1	DQ186081.1	FR727104.1	AY321075.1	FR727036.1	-	-
Vipera xanthina	Viperidae	EU624268.1	AJ275777.1	AJ275724.1	-	EU624234.1	-	-
Xantusia sierrae	Xantusiidae	-	KC621453.1	EU116640.1	EU130280.1	AY584398.1	EU116809.1	EU108661.1

Table 2.S3. Most likely substitution models as inferred by jModeltest 2 and used as models in the BEAST time-tree analysis. The first two partitions are nuclear coding genes. The third partition is non-coding mtDNA and the last three partitions are mtDNA coding genes.

Gene partition	**Model with highest AIC**
RAG1	GTR+I+G
CMOS	HKY+I+G
12S+16S	GTR+I+G
CYTB	GTR+I+G
ND2	GTR+I+G
ND4	GTR+I+G

Table 2.S4. Minimum ages of fossil calibration points derived from the earliest found fossil in each respective monophyletic clade. See Figure 2.1 for their relative position and age in the time-tree analysis.

Calibration	Clade 1	Clade 2	Monophyly of	Fossil	Age (mya)	Reference
N1	Lacertoidea	Toxicofera	Episquamata	*Becklesius, Dorsetisaurus, Paramacellodus, and Pseudosaurilius*	148	(Conrad 2008)
N2	Anguimorpha	Iguanidae	Anguimorpha + Iguania	*Parviraptor estesi*	144	(Rieppel 1994)
N3	Scincidae	Cordylidae	Scincoidea	*Sakurasaurus*	138	(Evans and Manabe 1999)
N4	Viperidae	Boidae	most Serpentes	*Coniophis*	92.7	(Marsh 1871)
N5	Iguanidae	Chamaelonidae	Iguania	*Priscagamines, iguanines*, and *Isodontosaurus*	70	(Keqin and Norell 2009)
N6	*Xantusia*	*Cordylus*	Cordylidae + Gerrhosauridae + Xantusiidae	*Konkasaurus*	65.2	(Krause et al. 2003)
N7	Viperidae	Colubridae	Viperidae + Lamprophiidae + Colubridae	Ponduang snakes	40	(Head et al. 2005)
N8	*Daboia*	*Vipera*	Eurasian vipers	*Vipera* cf. *V. antiqua*	20	(Szyndlar and Rage 1999)

Table 2.S5. Divergence time for each oviparous-viviparous taxon pair including the 95% highest posterior density. Additional divergence times were extracted from recent time-calibrated phylogenies (Pyron and Burbrink 2014; Hedges et al. 2015; Zheng and Wiens 2016).

Node	oviparous species	viviparous species	mean age [mya]	95% HPD height		Pyron & Burbrink 2014	Hedges et al. 2015	Zheng & Wiens 2016
1	*Rhacodactylus leachianus*	*Rhacodactylus trachyrhynchus*	15.8	9.1	23.7	34.2	40.4	28.3
2	*Lucasium damaeus*	*Naultinus stellatus*	36.0	23.5	49.0	46.1	53.5	57.4
3	*Plestiodon gilberti*	*Plestiodon dugesii*	13.5	2.9	27.8	78.5	94.0	84.2
4	*Tribolonotus pseudoponceleti*	*Corucia zebrata*	47.1	28.7	65.1	65.9	61.4	51.2
5	*Tribolonotus pseudoponceleti*	*Tribolonotus schmidti*	11.7	5.8	18.4	21.7	20.0	14.1
6	*Proablepharus reginae*	*Niveoscincus pretiosus*	20.3	10.7	29.1	35.0	30.8	33.5
7	*Oligosoma suteri*	*Oligosoma maccanni*	14.5	10.3	19.0	16.7	17.4	15.6
8	*Lioscincus nigrofasciolatum*	*Kanakysaurus viviparus*	12.9	8.5	17.7	24.2	28.4	22.4
9	*Lioscincus maruia*	*Lioscincus tillieri*	13.8	6.5	20.5	18.8	28.4	18.3
10	*Saiphos equalis (o)*	*Saiphos equalis (v)*	13.1	6.4	20.8	NA	NA	NA
11	*Anomalopus mackayi*	*Anomalopus swansoni*	19.9	12.7	27.7	19.6	19.6	18.0
12	*Eremiascincus richardsonii*	*Hemiergis initialis*	21.7	15.4	28.2	27.4	26.9	29.6
13	*Glaphyromorphus darwiniensis*	*Eulamprus tympanum*	24.0	17.8	30.6	24.0	33.5	29.6
14	*Lerista arenicola*	*Lerista microtis*	6.8	3.5	10.3	6.0	6.2	6.4
15	*Lerista bougainvillii (o)*	*Lerista bougainvillii (v)*	14.7	5.8	23.6	NA	NA	NA
16	*Zonosaurus madagascariensis*	*Xantusia sierrae*	128.8	117.9	137.5	144.0	158.2	157.1
17	*Platysaurus mitchelli*	*Cordylus aridus*	48.9	28.7	69.7	67.2	73.0	59.7
18	*Eremias grammica*	*Eremias multiocellata*	27.9	12.8	45.4	22.6	20.8	20.6
19	*Zootoca vivipara (o)*	*Zootoca vivipara (v)*	3.8	1.8	6.4	NA	NA	NA
20	*Pseudopus apodus*	*Anguis fragilis*	15.5	9.3	22.3	16.1	13.7	12.5
21	*Kinyongia fischeri*	*Bradypodion pumilum*	32.2	25.3	39.9	44.3	32.7	49.6
22	*Chamaleo deremensis*	*Chamaleo jacksonii*	21.5	15.2	28.5	27.5	18.9	25.0

23	*Phrynocephalus axillaris*	*Phrynocephalus vlangalii*	15.0	10.0	21.1	23.2	15.5	21.8
24	*Lyriocephalus scutatus*	*Cophotis ceylanica*	20.7	8.9	34.8	23.7	16.4	23.8
25	*Phrynosoma asio*	*Phrynosoma taurus*	20.5	15.9	25.8	NA	NA	20.5
26	*Phrynosoma modestum*	*Phrynosoma douglasii*	16.0	11.3	20.9	19.4	21.9	17.2
27	*Sceloporus spinosus*	*Sceloporus mucronatus*	19.4	14.9	23.7	19.8	22.6	23.1
28	*Sceloporus spinosus*	*Sceloporus malachiticus*	15.8	11.3	19.9	12.5	13.6	15.1
29	*Sceloporus chaneyi*	*Sceloporus goldmani*	4.9	2.0	8.6	3.5	3.4	4.1
30	*Sceloporus aeneus*	*Sceloporus bicanthalis*	7.6	3.2	12.3	5.4	6.2	6.2
31	*Corytophanes cristatus*	*Corytophanes percarinatus*	12.8	4.7	22.3	12.8	13.3	8.3
32	*Liolaemus fuscus*	*Liolaemus nigroviridis*	10.5	5.4	16.1	11.7	12.2	12.5
33	*Liolaemus platei*	*Liolaemus paulinae*	7.8	3.2	13.6	8.2	8.8	8.7
34	*Liolaemus chaltin*	*Liolaemus capillitas*	21.3	16.3	26.3	21.1	23.3	19.7
35	*Liolaemus saxatilis*	*Liolaemus bellii*	15.3	10.6	19.8	15.0	16.6	15.1
36	*Liolaemus chaltin*	*Liolaemus puna*	4.2	2.1	6.5	2.9	2.5	3.2
37	*Liolaemus reichei*	*Liolaemus andinus*	10.0	4.8	15.5	11.8	13.0	NA
38	*Liolaemus quilmes*	*Liolaemus espinozai*	4.4	2.7	6.2	1.9	1.8	2.2
39	*Liolaemus abaucan*	*Liolaemus ornatus*	10.4	7.3	13.5	8.4	7.5	9.9
40	*Typhlops fornasinii*	*Typhlops bibronii*	11.7	0.5	29.3	7.5	4.3	9.3
41	*Python molurus*	*Anilius scytale*	91.9	89.5	102.5	90.3	53.9	92.7
42	*Python molurus*	*Cylindrophis ruffus*	74.8	66.8	82.2	72.9	45.1	67.7
43	*Xenodermus javanicus*	*Acrochordus granulatus*	64.0	55.4	71.9	65.6	53.9	80.6
44	*Macrovipera lebetina*	*Montivipera xanthina*	13.5	9.1	18.1	13.8	6.9	13.2
45	*Daboia mauritanica*	*Daboia siamensis*	13.0	9.7	16.6	NA	11.6	NA
46	*Daboia mauritanica*	*Vipera berus*	17.5	14.5	21.4	22.0	10.3	23.0
47	*Cerastes cerastes*	*Cerastes vipera*	15.3	9.2	22.1	15.5	6.9	18.2
48	*Echis ocellatus*	*Atheris squamigera*	31.8	26.0	37.9	32.1	14.2	34.9
49	*Naja nigricollis*	*Hemachatus hemachatus*	22.7	14.2	30.5	22.7	17.8	21.2

50	*Pseudechis australis*	*Pseudechis porphyriacus*	19.9	15.4	24.2	18.3	7.0	20.8
51	*Cacophis squamulosus*	*Suta fasciata*	21.6	17.5	25.8	23.4	9.2	27.7
52	*Pseudonaja modesta*	*Hemiaspis signata*	21.3	17.1	25.7	23.5	15.2	28.2
53	*Psammophylax tritaeniatus*	*Psammophylax variabilis*	7.9	4.5	12.1	6.0	2.7	5.9
54	*Pythonodipsas carinata*	*Pseudaspis cana*	26.1	19.7	32.8	29.7	12.8	28.4
55	*Madagascarophis colubrinus*	*Stenophis citrinus*	21.3	15.4	26.7	25.5	12.2	18.2
56	*Liopholidophis dolicocercus*	*Liopholidophis sexlineatus*	10.8	6.1	16.0	12.3	5.4	11.5
57	*Chrysopelea paradisi*	*Ahaetulla fronticincta*	25.9	16.8	34.1	33.8	14.4	31.4
58	*Coronella girondica*	*Coronella austriaca*	14.9	10.7	19.8	18.3	7.8	14.3
59	*Opheodrys aestivus*	*Opheodrys vernalis*	7.3	3.3	11.9	8.8	3.7	7.5
60	*Tropidodryas serra*	*Helicops angulatus*	28.0	19.8	35.8	32.3	22.6	34.7
61	*Natrix maura*	*Thamnophis sirtalis*	18.7	13.5	23.6	25.1	24.5	25.1
62	*Sinonatrix percarinata*	*Sinonatrix annularis*	9.8	5.6	14.3	10.0	4.1	9.4

Table 2.S6. Binary trait simulations. A binary trait was simulated (transition rate oviparity to viviparity: 0.0015, and viviparity to oviparity: 0.0001) 50 times on the time-calibrated squamate phylogeny of Zheng and Wiens (Zheng and Wiens 2016). The number of transitions to viviparity and number and proportion of viviparous species (total number of species in phylogeny: 4162) were calculated for each simulation and across all simulations on average. Empirical estimates from the phylogeny by Pyron and Burbrink (Pyron and Burbrink 2014) and Zheng and Wiens (Zheng and Wiens 2016) were included for comparison.

N simulation	N transitions	N viviparous species	% viviparous species
1	52	1616	0.388
2	67	837	0.201
3	61	323	0.077
4	63	216	0.052
5	61	828	0.199
6	51	1013	0.243
7	65	896	0.215
8	64	226	0.054
9	84	661	0.159
10	69	426	0.102
11	55	1437	0.345
12	88	511	0.123
13	35	2802	0.411
14	63	515	0.124
15	68	478	0.115
16	73	462	0.111
17	87	185	0.044
18	89	569	0.136
19	83	742	0.178
20	63	139	0.033
21	65	257	0.062
22	67	177	0.042
23	79	1195	0.287
24	74	214	0.051
25	69	162	0.039
26	91	253	0.061
27	92	205	0.049
28	2	4159	0.998
29	76	884	0.212
30	77	1657	0.397
31	67	1271	0.305
32	52	244	0.059
33	41	272	0.065
34	63	201	0.048
35	12	4158	0.998
36	61	409	0.098
37	64	764	0.183
38	53	380	0.091

39	59	810	0.194
40	51	406	0.097
41	84	347	0.083
42	26	4125	0.990
43	72	340	0.082
44	65	260	0.062
45	93	739	0.177
46	95	163	0.039
47	115	422	0.101
48	82	163	0.039
49	103	778	0.187
50	32	4161	0.998
average	66.5	889	0.208
Pyron and Burbrink (2014)	58	857	0.216
Zheng and Wiens (2016)	58	691	0.198

Table 2.S7. Models of trait and climate. Statistical overview of statistical models for transition frequency to viviparity estimates from this study and others. In all but one case (simulated binary trait model), the most likely model for explaining transitions to viviparity included a significant negative effect of temperature (T, as $\delta^{18}O$) and variance in temperature (ΔT_L; var. $\delta^{18}O$).

dataset	parameter	estimate	std. error	*z* value	*p* value	
this study	T	0.84	0.21	4.04	<0.001	***
	ΔT_Λ	-12.80	3.80	-3.37	<0.001	***
95% HPD random	T	0.91	0.20	4.48	<0.001	***
	ΔT_Λ	-10.81	3.56	-3.04	0.002	**
95% HPD low	T	1.39	0.24	5.80	<0.001	***
	ΔT_Λ	-11.18	3.63	-3.08	0.002	**
95% HPD high	T	0.69	0.17	4.01	<0.001	***
	ΔT_Λ	-11.30	3.23	-3.50	<0.001	***
Pyron & Burbrink (2014)	T	0.81	0.18	4.46	<0.001	***
	ΔT_Λ	-9.52	3.19	-2.98	0.003	**
Hedges et al. (2015)	T	0.87	0.16	5.56	<0.001	***
	ΔT_Λ	-7.20	2.63	-2.74	0.006	**
Zheng & Wiens (2016)	T	0.57	0.16	3.53	<0.001	***
	ΔT_Λ	-7.19	2.95	-2.44	0.015	*
simulated binary trait	T	0.29	0.13	2.19	0.029	*
	ΔT_Λ	-3.75	2.43	-1.54	0.123	NS

Abbreviations are: HPD = Highest posterior density.

Supporting Information for Chapter 3

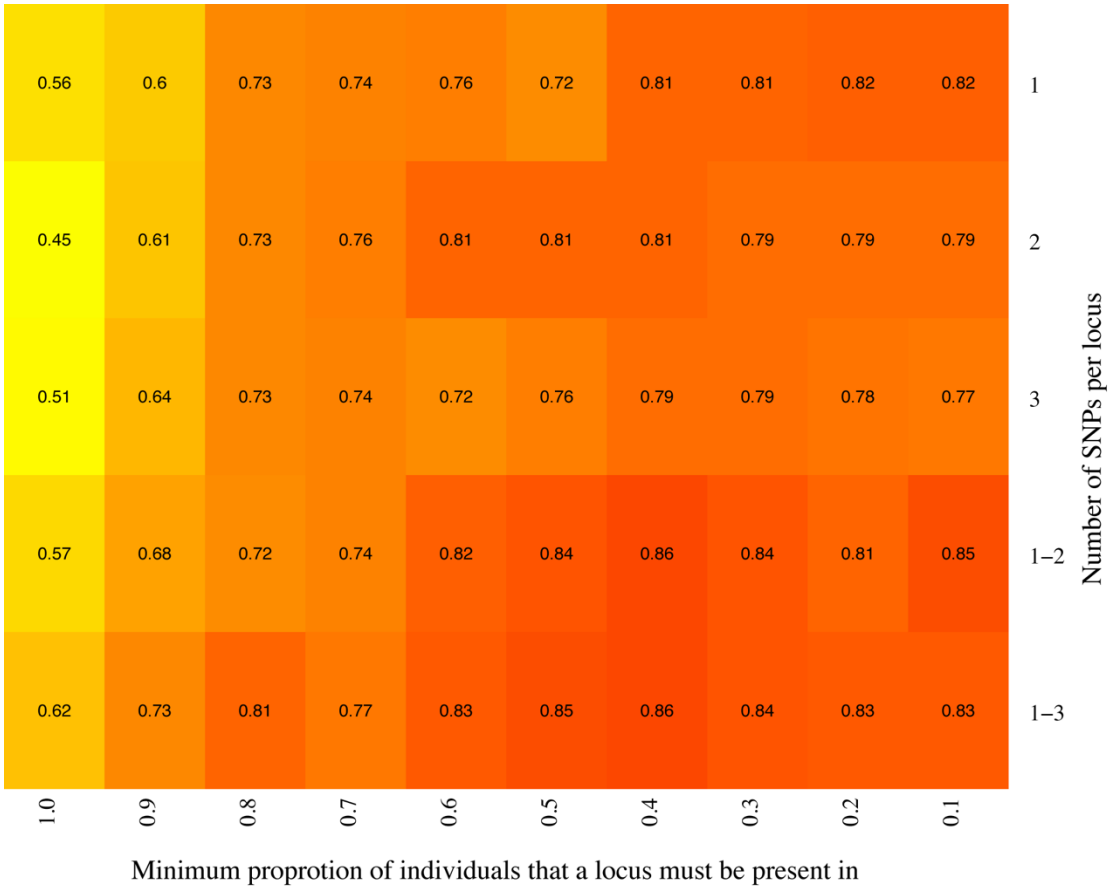

Figure 3.S1. Heat map of summed bootstrap support values across phylogenies created from different datasets and filtering conditions. Minimum proportion of individuals ranged from 0.1 to 1 and number of SNPs per locus retained either 1, 2, 3, 1 to 2 or 1 to 3 SNPs. Note that support values increased using a smaller proportion of individuals that a locus had to be present in, until a plateau was reached at a proportion of 0.4 individuals. Using more SNPs per locus generally resulted in higher bootstrap supports.

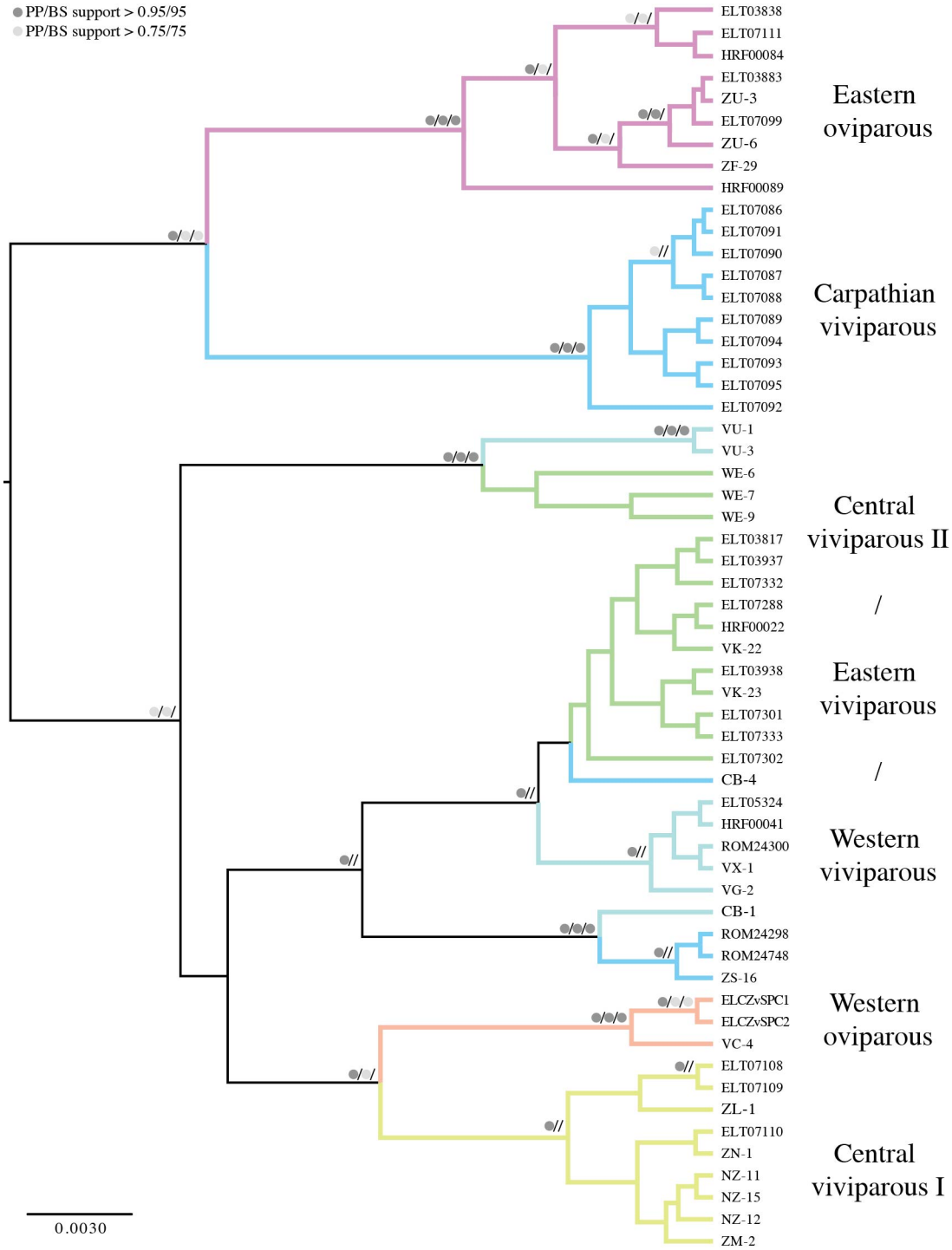

Figure 3.S2. Bayesian reconstruction of common lizard evolutionary relationships based on cytochrome b mtDNA data (427 bp). Bayesian, Maximum likelihood and Maximum Parsimony supports are drawn on the tree and indicated as dark grey (Bayesian posterior > 0.95; bootstraps support > 95) or light grey (Bayesian posterior > 0.75; bootstraps support > 75) dots on each node. Lineages are represented by different colored branches: eastern oviparous (purple), central viviparous II (green), western oviparous (peach), central viviparous I (yellow), eastern viviparous (blue), western viviparous (pale blue). The paraphyletic lineages are separated by slashes.

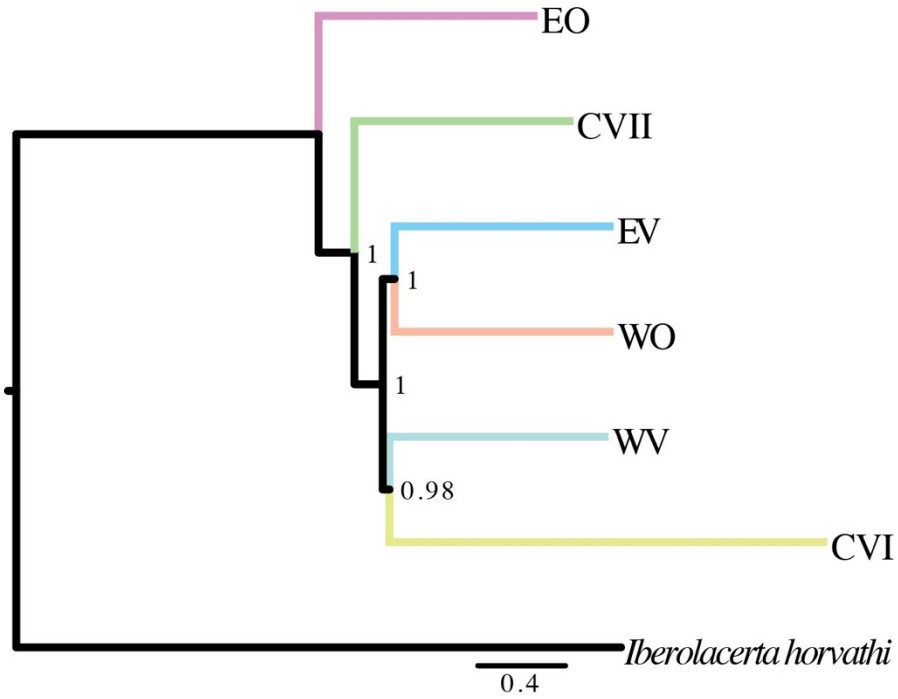

Figure 3.S3. Species tree reconstruction using lineages as identified by ADMIXTURE. The tree was estimated in ASTRAL from 3,537 gene trees. Nodes contain posterior probabilities. *Iberolacertha horvathi* was used as an outgroup species.

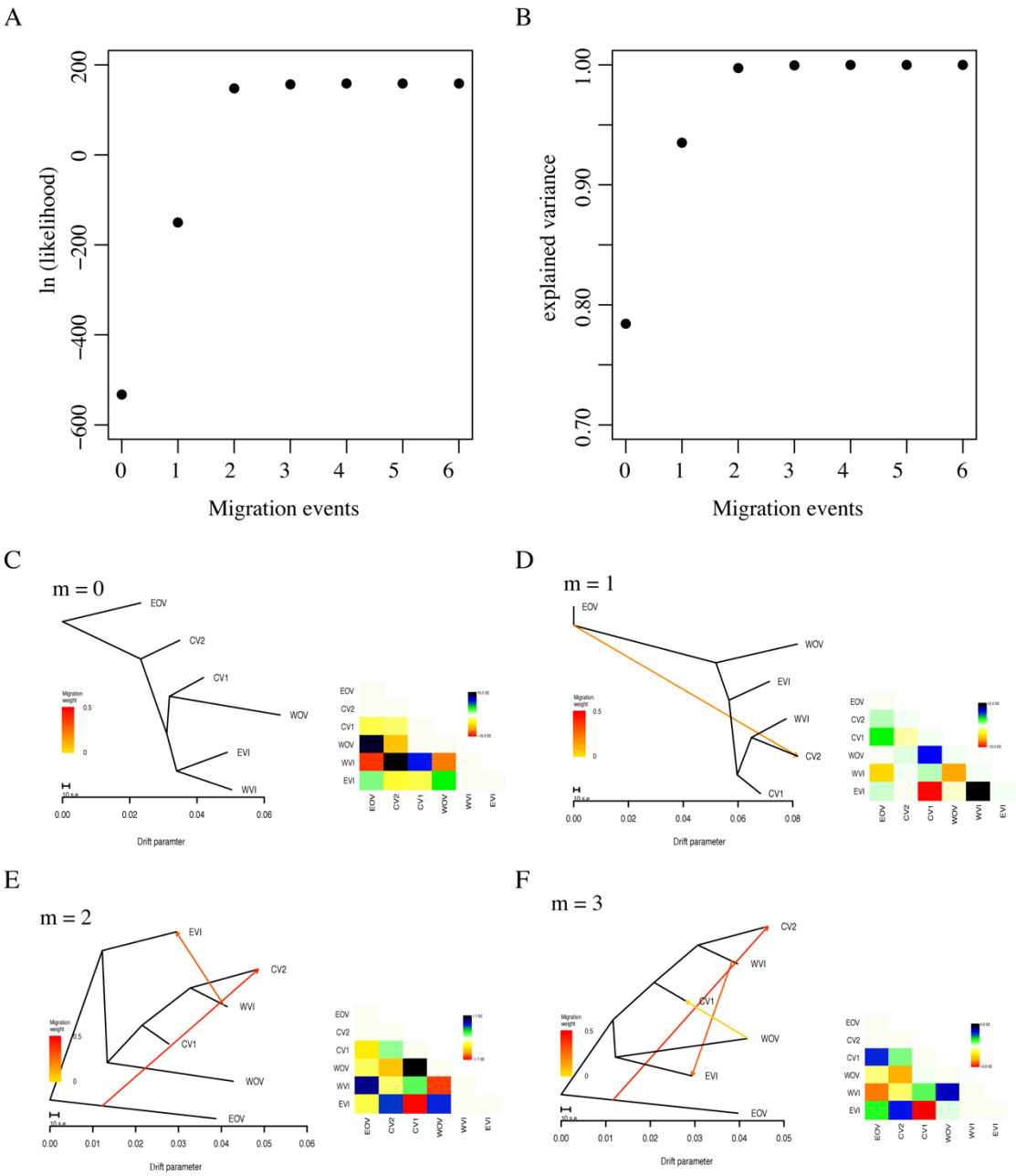

Figure 3.S4. Number of past migration events modeled on the TREEMIX phylogeny. Likelihood A) and explained variance B) do not substantially increase after two migration events. C) reflects the ML phylogeny with 0 migration events, and panels D-F an increasing number of migration events from 1 to 3. Migration events are plotted as lines from source to the lineage experiencing introgression. Next to each phylogeny, residuals are plotted for each scenario, with darker colors illustrating larger residuals. Darker residuals indicate a larger proportion of unexplained variation. CVI: central viviparous I, CV2: central viviparous II, EOV: eastern oviparous; EVI: eastern viviparous, WOV: western oviparous, WVI: western viviparous.

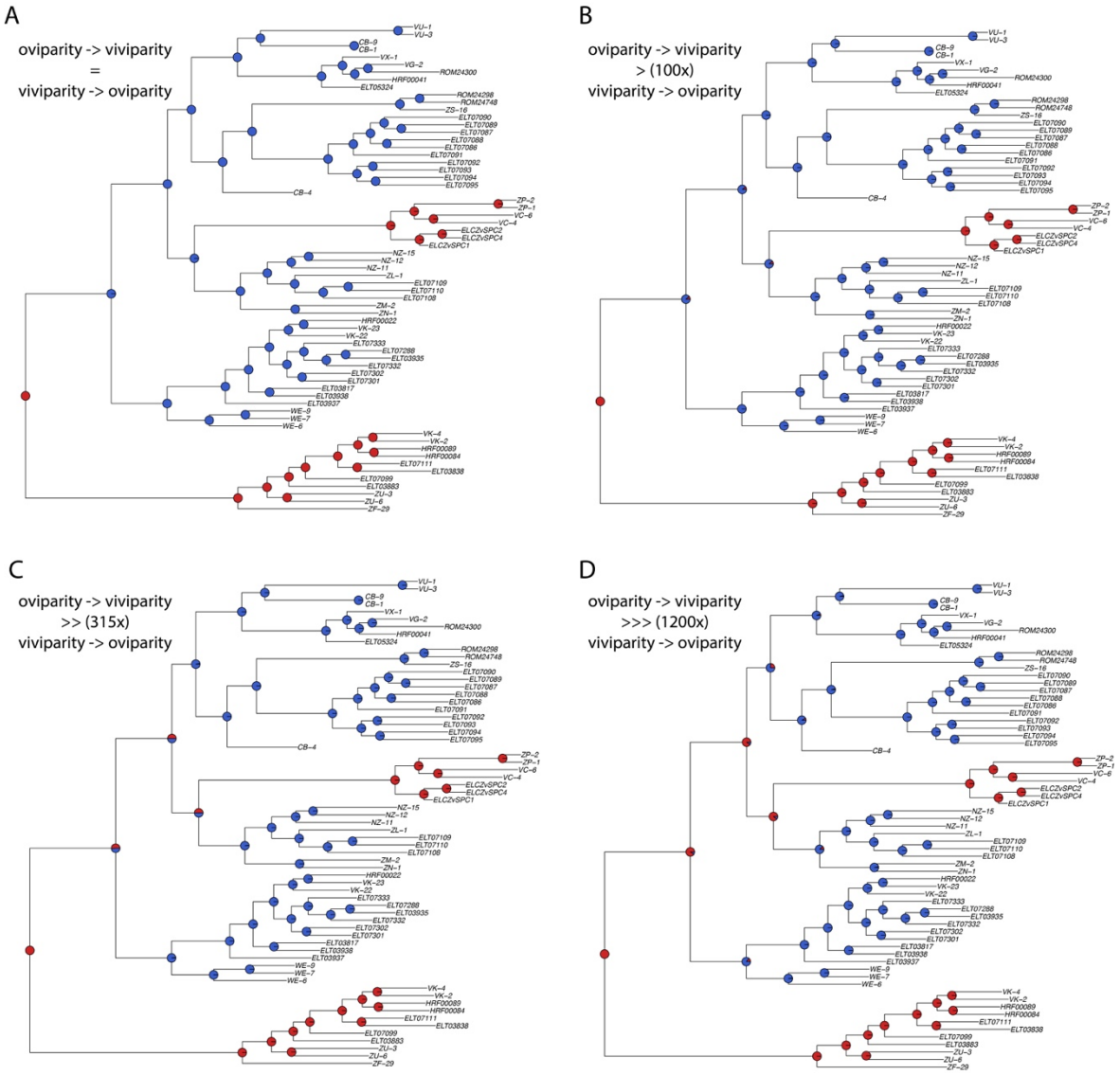

Figure 3.S5. Ancestral trait reconstruction of parity modes in common lizards. Likelihood for an oviparous ancestor is indicated by red, and viviparous by blue. The root was fixed to oviparity. First, transition rates from oviparity to viviparity and viviparity to oviparity were set as equal (A). This resulted in a model that supported a single origin of viviparity and a reversal to oviparity. Subsequently, the difference in transition rate was increased, by decreasing the transition rate from viviparity to oviparity. If this rate was 100x lower than the rate from oviparity to viviparity (B), the ancestral trait reconstruction still resulted in a high likelihood for the model supported a reversal to oviparity. If the difference in rate was change to 315x (C), a model of multiple independent transitions to viviparity were equally likely as the reversal model (crucial nodes had a likelihood of 50% viviparity and oviparity as character states. With a 1200x difference in transition rate (D), the likelihood for multiple transitions to viviparity increased to 90% probability.

Table 3.S1. Phylogenetic lineage and sampling locality listed for all individuals included in the study.

ID	Country	Location	Lineage	Latitude	Longitude	Altitude (m a.s.l.)	Sequencing platform
HRF00010	A	Straniger Alm	-	46.61	13.15	1337	Ion Proton
ELT07108	A	Breitenstein	CVI	47.65	15.25	1110	Illumina
ELT07109	A	Breitenstein	CVI	47.63	15.25	1474	Illumina
ZN-1	A	Illmitz	CVI	47.7	16.8	110	Ion Proton
NZ-11	A	Lassingtal	CVI	47.73	15.03	870	Ion Proton
NZ-12	A	Lassingtal	CVI	47.73	15.03	870	Ion Proton
NZ-15	A	Lunz	CVI	47.86	15.03	662	Ion Proton
ZM-2	A	Moosbrunn	CVI	48.02	16.45	180	Ion Proton
ZL-1	A	Schneeberg	CVI	47.77	15.81	1100	Ion Proton
ELT07110	A	Semmering	CVI	47.62	15.25	1334	Illumina
ELT03938	A	Gundersheimer Alm	CVII	46.63	13.1	1472	Illumina
ELT07332	A	Hochwipfel	CVII	46.6	13.22	2120	Illumina
ELT07333	A	Kleinkordin Alm	CVII	46.6	13.22	1643	Illumina
ELT03935	SLO	Mjostrana	CVII	46.45	13.92	694	Illumina
ELT03817	A	Oberbuhbacher Alm	CVII	46.62	13.12	1622	Illumina
ELT07288	A	Rattendorfer Alm	CVII	46.58	13.22	1404	Illumina
HRF00022	A	Straniger Alm	CVII	46.58	13.13	1463	Ion Proton
VK-22	A	Straniger Alm	CVII	46.59	13.13	1551	Ion Proton
VK-23	A	Straniger Alm	CVII	46.59	13.13	1564	Ion Proton
WE-6	A	Teuchl	CVII	46.87	13.22	1334	Ion Proton
WE-7	A	Teuchl	CVII	46.85	13.18	1500	Ion Proton
WE-9	A	Teuchl	CVII	46.85	13.18	1500	Ion Proton
ELT03937	A	Unterbuchacher Alm	CVII	46.62	13.15	1421	Illumina
ELT07301	I	Valbertat Alta	CVII	46.58	13.22	1501	Illumina
ELT07302	I	Valbertat Alta	CVII	46.58	13.22	1501	Illumina
ELT03883	A	Bertahütte	EO	46.51	13.96	1453	Illumina
ELT07099	A	Camping Reisach	EO	46.65	13.22	808	Illumina
ELT03838	A	Jochalm	EO	46.67	13.16	1480	Illumina
ZU-3	A	Preblau	EO	46.93	14.8	900	Ion Proton
ZF-29	I	Rivignano	EO	45.92	13	15	Ion Proton
ELT07111	A	Semmering	EO	47.62	15.25	1332	Illumina
ZU-6	A	St. Urban	EO	46.75	14.18	750	Ion Proton
HRF00084	A	Straniger Alm	EO	46.6	13.14	1461	Ion Proton
HRF00089	A	Straniger Alm	EO	46.6	13.14	1445	Ion Proton
VK-2	A	Straniger Alm	EO	46.6	13.14	1400	Ion Proton
VK-4	A	Straniger Alm	EO	46.6	13.13	1400	Ion Proton
CB-4	SK	Botany	EV	48.45	22.08	100	Ion Proton
ELT07092	RO	Făgăraș Mountains	EV	45.63	24.4	1250	Illumina
ELT07093	RO	Făgăraș Mountains	EV	45.63	24.4	1250	Illumina
ELT07094	RO	Făgăraș Mountains	EV	45.63	24.4	1250	Illumina
ELT07095	RO	Făgăraș Mountains	EV	45.63	24.4	1250	Illumina
ROM24298	RUS	Kivach	EV	62.13	34.01	55	Ion Proton

ROM24748	RUS	Luga	EV	58.73	29.85	51	Ion Proton
ZS-16	RUS	Novosibirsk	EV	55.01	82.92	120	Ion Proton
ELT07086	RO	Predeal	EV	45.52	25.42	1088	Illumina
ELT07087	RO	Predeal	EV	45.52	25.42	1079	Illumina
ELT07088	RO	Predeal	EV	45.52	25.42	1081	Illumina
ELT07089	RO	Predeal	EV	45.52	25.42	1083	Illumina
ELT07090	RO	Predeal	EV	45.52	25.42	1087	Illumina
ELT07091	RO	Timișu de Jus	EV	45.55	25.42	866	Illumina
ZP-1	E	Aran Valley	WO	42.75	0.79	2200	Ion Proton
ZP-2	E	Aran Valley	WO	42.75	0.79	2200	Ion Proton
ELCZvSPC1	E	El Portalet	WO	42.8	-0.42	1769	Illumina
ELCZvSPC2	E	El Portalet	WO	42.8	-0.42	1769	Illumina
ELCZvSPC4	E	El Portalet	WO	42.8	-0.42	1769	Illumina
VC-4	E	Santander	WO	43.13	-3.45	860	Ion Proton
VC-6	E	Vergarada	WO	43.02	-5.47	1740	Ion Proton
VG-2	D	Duisburg	WV	51.05	6.55	28	Ion Proton
ELT05324	SCO	Isle of Cumbrae	WV	55.77	-4.934	8	Illumina
VU-1	H	Izsák	WV	46.8	19.35	97	Ion Proton
VU-3	H	Izsák	WV	46.8	19.35	100	Ion Proton
HRF00041	A	Mittelberg	WV	47.32	10.16	1387	Ion Proton
CB-1	H	Nyirbator	WV	47.83	22.17	150	Ion Proton
CB-9	AL	Prokletije	WV	42.47	19.82	1831	Ion Proton
VX-1	A	Steinberg	WV	47.53	11.78	1080	Ion Proton
ROM24300	S	Uppsala	WV	59.86	17.64	23	Ion Proton

Abbreviations are: A = Austria, AL = Albania, D = Germany, E = Spain, H = Hungary, I = Italy, RO = Romania, RUS = Russia, S = Sweden, SCO = Scotland, SK = Slovakia, SLO = Slovenia.

Table 3.S2. Best substitution models for mitochondrial DNA (427 bp) inferred from JModeltest2 by AICc, BIC, AIC, and DT.

Method	Model	f(a)	f(c)	f(g)	f(t)	kappa	titv	Ra	Rb	Rc	Rd	Re	Rf	pInv
AICc	HKY+I	0.36	0.12	0.23	0.29	9.34	4.59	1	9.3	1	1	9.3	1	0.68
BIC	HKY+I	0.36	0.12	0.23	0.29	9.34	4.59	1	9.3	1	1	9.3	1	0.68
AIC	TrN+I	0.36	0.12	0.23	0.29	0	0	1	11.4	1	1	5.7	1	0.67
DT	HKY+I	0.36	0.12	0.23	0.29	9.34	4.59	1	9.3	1	1	9.3	1	0.68

Abbreviations are: f = frequency, titv = transition/transversion ratio, R = rate parameter; pinv = proportion of invariable sites.

Table 3.S3. Pairwise population genetic differentiation between phylogenetic lineages. Fst values are indicated below the diagonal and Jost D's values above the diagonal. CVI: central viviparous I, CVII: central viviparous II, EO: eastern oviparous; EV: eastern viviparous, WO: western oviparous, WV: western viviparous.

lineage	CVI	CVII	EO	EV	WO	WV
CVI		0.006	0.015	0.006	0.007	0.004
CVII	0.284		0.013	0.008	0.011	0.005
EO	0.429	0.420		0.016	0.016	0.018
EV	0.239	0.315	0.432		0.008	0.005
WO	0.336	0.442	0.509	0.345		0.010
WV	0.226	0.293	0.515	0.249	0.462	

Table 3.S4. *f3*-statistics for all lineage comparisons. Positive z-scores indicate that the first focal lineage has not experienced substantial introgression from the other two respective lineages. CVI: central viviparous I, CVII: central viviparous II, EO: eastern oviparous; EV: eastern viviparous, WO: western oviparous, WV: western viviparous.

Lineages	*f3*-statistics	std. error	z-score
CVII;WV,EO	0.00787	0.000521	15.114
CVI;WO,WV	0.00875	0.000470	18.626
CVI;WV,EO	0.01022	0.000496	20.611
CVI;WO,CVII	0.01341	0.000556	24.113
CVII;EV,EO	0.01820	0.000729	24.952
CVII;CVI,EO	0.01611	0.000571	28.239
CVI;WO,EO	0.02031	0.000707	28.713
EV;WV,EO	0.01642	0.000567	28.947
EV;WO,WV	0.01627	0.000551	29.556
CVI;EV,EO	0.01884	0.000624	30.210
CVII;WO,EO	0.02302	0.000748	30.783
CVI;EV,WO	0.01752	0.000527	33.266
CVI;EV,CVII	0.01675	0.000481	34.855
CVI;EV,WV	0.01815	0.000512	35.478
WV;CVII,CVI	0.01879	0.000528	35.572
EV;WO,EO	0.02714	0.000749	36.233
CVI;CVII,WV	0.01846	0.000485	38.096
WV;WO,CVII	0.02344	0.000602	38.966
EV;WO,CVI	0.02567	0.000654	39.253
WV;EV,CVII	0.01739	0.000438	39.688
EV;WO,CVII	0.02232	0.000559	39.951
WV;EV,CVI	0.01910	0.000477	40.034
EV;CVI,EO	0.02435	0.000608	40.060
CVII;WO,WV	0.02139	0.000528	40.532
WO;WV,EO	0.04151	0.001004	41.329
CVII;CVI,WV	0.02605	0.000616	42.294
EV;CVII,CVI	0.02644	0.000621	42.604
WO;EV,EO	0.04136	0.000966	42.824
EV;CVII,WV	0.02675	0.000608	43.989
CVI;CVII,EO	0.02839	0.000645	44.048
WV;WO,CVI	0.02849	0.000643	44.292
WO;EV,CVII	0.04618	0.001039	44.447
EV;CVI,WV	0.02504	0.000561	44.610

WO;CVI,EO	0.04004	0.000879	45.542
CVII;WO,CVI	0.03110	0.000682	45.582
WV;EV,WO	0.02787	0.000605	46.053
WO;CVII,CVI	0.04694	0.001015	46.230
CVII;EV,CVI	0.02775	0.000595	46.657
WO;EV,CVI	0.04283	0.000917	46.699
WO;CVII,WV	0.05665	0.001190	47.623
CVII;EV,WV	0.02744	0.000571	48.031
WV;CVII,EO	0.03696	0.000761	48.545
WV;CVI,EO	0.02703	0.000553	48.887
WV;EV,EO	0.02772	0.000565	49.024
WO;EV,WV	0.05223	0.001060	49.260
WO;CVII,EO	0.05503	0.001098	50.131
EO;WO,CVII	0.05703	0.001124	50.755
CVII;EV,WO	0.03187	0.000617	51.641
WV;WO,EO	0.03859	0.000730	52.850
WO;CVI,WV	0.05160	0.000971	53.130
EV;CVII,EO	0.03599	0.000651	55.279
EO;CVII,CVI	0.06394	0.001076	59.409
EO;EV,CVII	0.06185	0.001015	60.908
EO;EV,WO	0.07070	0.001061	66.607
EO;WO,WV	0.07055	0.001030	68.522
EO;WO,CVI	0.07202	0.001029	70.016
EO;CVII,WV	0.07218	0.001023	70.562
EO;EV,CVI	0.07349	0.000987	74.438
EO;EV,WV	0.08142	0.000970	83.937
EO;CVI,WV	0.08211	0.000920	89.250

Supporting Information for Chapter 4

Table 4.S1. Summary statistics of all statistical models including all effects tested. Response variables are listed first from a) to l). Note that year was only included as an effect when data was collected from more than one sampling year. Significant p-values (P< 0.05) are indicated in italics, and significance after Bonferroni correction is specified in the last column.

effect	Df	Mean sq	F	η^2	P-value	sign.
a) body size						
parity mode	1	2533.1	92.97	0.17	*<0.0001*	***
year	1	95.8	3.52	0.01	0.0614	NS
captivity duration	1	124.8	4.58	0.01	*0.0329*	NS
altitude	1	223.3	8.19	0.02	*0.0044*	NS
year x parity mode	1	24	0.882	0.00	0.3483	NS
altitude x parity mode	1	20.4	0.75	0.00	0.3873	NS
b) body weight before egg laying/giving						
parity mode	1	82.6	141.93	0.09	*<0.0001*	***
SVL	1	464.7	797.99	0.56	*<0.0001*	***
year	1	0.4	0.63	0.00	0.4263	NS
captivity duration	1	55.5	95.25	0.07	*<0.0001*	***
altitude	1	1.9	3.33	0.00	0.0687	NS
year x parity mode	1	0.3	0.54	0.00	0.4643	NS
altitude x parity mode	1	3.3	5.70	0.00	*0.0174*	NS
c) body weight after egg laying/giving birth						
parity mode	1	11.07	37.51	0.13	*<0.0001*	***
SVL	1	34.34	116.37	0.39	*<0.0001*	***
captivity duration	1	6.31	21.39	0.06	*<0.0001*	***

effect	Df	Mean sq	F	η^2	P-value	sign.
j) RCM						
parity mode	1	0.148	5.69	0.02	*0.0183*	NS
SVL	1	0.518	19.90	0.13	*<0.0001*	***
captivity duration	1	0.220	8.45	0.05	*0.0042*	NS
altitude	1	0.002	0.09	0.00	0.7678	NS
altitude x parity mode	1	0.016	0.62	0.01	0.4324	NS
k) ROM						
parity mode	1	1.388	207.37	0.55	*<0.0001*	***
SVL	1	0.159	23.69	0.08	*<0.0001*	***
captivity duration	1	0.046	6.80	0.01	*0.0101*	NS
altitude	1	0.003	0.49	0.00	0.4869	NS
altitude x parity mode	1	0.005	0.69	0.00	0.4092	NS
l) offspring biomass						
parity mode	1	15.57	101.46	0.13	*<0.0001*	***
SVL	1	20.41	133.03	0.21	*<0.0001*	***
year	1	0.02	0.11	0.00	0.7441	NS
captivity duration	1	1.54	10.07	0.02	*0.0016*	*
altitude	1	0.38	2.45	0.00	0.1186	NS
year x parity mode	1	0.04	0.27	0.00	0.6021	NS

altitude	1	0.03	0.11	0.00	0.7420	NS
altitude x parity mode	1	0.16	0.54	0.00	0.4650	NS
d) weight loss						
parity mode	1	1.33	40.89	0.11	*<0.0001*	***
SVL	1	1.60	49.28	0.16	*<0.0001*	***
captivity duration	1	2.82	86.62	0.28	*<0.0001*	***
altitude	1	0.00	0.15	0.00	0.6991	NS
altitude x parity mode	1	0.13	3.95	0.01	*0.0488*	NS
e) clutch size						
parity mode	1	85.6	41.19	0.05	*<0.0001*	***
SVL	1	667.9	321.31	0.41	*<0.0001*	***
year	1	23.8	11.47	0.01	*0.0008*	*
captivity duration	1	1.5	0.73	0.00	0.3932	NS
altitude	1	10.7	5.16	0.00	*0.0237*	NS
year x parity mode	1	0.2	0.09	0.00	0.7702	NS
altitude x parity mode	1	2.5	1.20	0.00	0.2738	NS
f) offspring size						
parity mode	1	241.25	308.02	0.44	*<0.0001*	***
SVL	1	5.37	6.86	0.01	*0.0092*	NS
year	1	0.04	0.05	0.00	0.8179	NS
captivity duration	1	21.1	26.94	0.04	*<0.0001*	***
altitude	1	0	0.00	0.00	0.9596	NS
year x parity mode	1	0.59	0.75	0.00	0.3872	NS
altitude x parity mode	1	1	1.28	0.00	0.2589	NS
altitude x parity mode	1	0.10	0.63	0.00	0.4271	NS
m) infertility						
parity mode	1	1.270	15.79	0.03	*<0.0001*	***
SVL	1	0.018	0.22	0.00	0.6397	NS
year	1	0.485	6.03	0.00	*0.0144*	NS
captivity duration	1	0.713	8.87	0.02	*0.0031*	NS
altitude	1	0.069	0.85	0.02	0.3565	NS
year x parity mode	1	0.290	3.63	0.00	0.0574	NS
altitude x parity mode	1	0.016	0.20	0.01	0.6569	NS
n) early embryo mortality (stage 32-35)						
parity mode	1	0.241	15.25	0.04	*0.0001*	**
SVL	1	0.025	1.61	0.00	0.2057	NS
year	1	0.191	12.08	0.03	*0.0006*	*
captivity duration	1	0.097	6.12	0.01	*0.0138*	NS
altitude	1	0.005	0.31	0.00	0.5785	NS
year x parity mode	1	0.120	7.75	0.00	*0.0056*	NS
altitude x parity mode	1	0.008	0.52	0.02	0.4697	NS
o) late embryo mortality (stage 36-						
parity mode	1	0.351	16.25	0.03	*<0.0001*	***
SVL	1	0.003	0.13	0.00	0.7234	NS
year	1	0.356	16.45	0.03	*<0.0001*	***
captivity duration	1	0.590	27.28	0.05	*<0.0001*	***
altitude	1	0.044	2.05	0.00	0.1526	NS
year x parity mode	1	0.122	5.70	0.00	*0.0174*	NS

g) offspring weight						
parity mode	1	0.442	642.52	0.64	*<0.0001*	***
SVL	1	0.000	0.24	0.00	0.6260	NS
year	1	0.000	0.00	0.00	0.9780	NS
captivity duration	1	0.011	15.72	0.01	*<0.0001*	***
altitude	1	0.000	0.21	0.00	0.6450	NS
year x parity mode	1	0.006	8.46	0.00	*0.0039*	NS
altitude x parity mode	1	0.001	1.89	0.01	0.1700	NS
h) offspring body						
parity mode	1	0.00	658.3	0.64	*<0.0001*	***
SVL	1	0.00	0.1	0.00	0.8167	NS
year	1	0.00	0.0	0.00	0.9522	NS
captivity duration	1	0.00	7.1	0.01	*0.0083*	NS
altitude	1	0.00	0.2	0.00	0.6428	NS
year x parity mode	1	0.00	10.7	0.00	*0.0012*	*
altitude x parity mode	1	0.00	1.1	0.01	0.2882	NS
i) EM						
parity mode	1	0.37	87.85	0.34	*<0.0001*	***
SVL	1	0.05	10.80	0.04	*0.0013*	*
captivity duration	1	0.02	4.68	0.02	*0.0322*	NS
altitude	1	0.00	0.47	0.00	0.4946	NS
altitude x parity mode	1	0.00	0.15	0.00	0.6978	NS
altitude x parity mode	1	0.088	4.05	0.01	*0.0447*	NS
p) hatching success						
parity mode	1	1.374	12.86	0.03	*0.0004*	**
SVL	1	0.049	0.46	0.00	0.4989	NS
year	1	0.649	6.07	0.01	*0.0142*	NS
captivity duration	1	3.932	36.80	0.07	*<0.0001*	***
altitude	1	0.283	2.65	0.01	0.1043	NS
year x parity mode	1	0.031	0.29	0.00	0.5878	NS
altitude x parity mode	1	0.111	1.04	0.00	0.3078	NS
q) offspring hatched						
parity mode	1	10.80	2.07	0.02	0.1514	NS
SVL	1	343.60	66.03	0.06	*<0.0001*	***
year	1	14.00	2.69	0.01	0.1019	NS
captivity duration	1	92.70	17.82	0.05	*<0.0001*	***
altitude	1	24.80	4.76	0.01	*0.0296*	NS
year x parity mode	1	0.60	0.11	0.00	0.7442	NS
altitude x parity mode	1	0.80	0.15	0.00	0.7017	NS

* P < 0.003; ** P < 0.0006; *** P < 0.00006; NS = not significant.

Table 4.S2. Effect of SVL (snout vent length) on female weight and reproductive traits. The estimate indicates the slope and direction of the trend. Significant correlations are indicated with an asterisk (see below).

	oviparous SVL				**viviparous SVL**			
	N	estimate	P		N	estimate	P	
female weight	234	0.155	<0.001	***	187	0.234	<0.001	***
clutch size	228	0.286	<0.001	***	188	0.200	<0.001	***
offspring size	188	0.001	0.964	NS	177	0.040	<0.001	***
offspring weight	186	-0.001	0.108	NS	178	0.001	0.015	*
offspring body condition	186	0.000	0.070	NS	178	0.000	0.133	NS
EM	76	0.001	0.339	NS	75	0.008	<0.001	***
RCM	77	0.009	0.007	**	75	0.020	<0.001	***
ROM	66	0.009	0.001	**	71	0.006	0.001	**
offspring biomass	186	0.058	<0.001	***	178	0.036	<0.001	***
offspring survival	227	-0.002	0.761	NS	190	0.005	0.164	NS

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS = not significant.

Table 4.S3. Loadings for the first six principal components. These explained a total variance of 90%. PC1 was strongly associated with allometric effects and direct correlations between body size and reproductive traits. PC2 strongly differentiated between traits that differed between reproductive mode, such as female body size, offspring size, and reproductive investment (EM, weight loss). PC3 also differed significantly between reproductive modes, and had the highest loadings for hatching success, clutch size, RCM and EM.

trait	PC1	PC2	PC3	PC4	PC5	PC6
SVL	0.305	-0.273	0.029	-0.161	-0.226	0.007
weight (capture)	0.334	-0.232	0.065	-0.169	-0.231	0.008
weight (oviposition/parturition)	0.224	-0.170	-0.090	-0.561	-0.212	-0.024
weight loss	0.262	-0.165	0.207	0.352	-0.089	0.075
clutch size	0.339	0.071	0.303	-0.237	0.277	-0.067
offspring size	0.100	0.381	-0.097	-0.001	-0.327	0.028
offspring weight	0.060	0.432	-0.001	-0.002	-0.309	0.099
offspring body condition	0.038	0.421	0.039	-0.007	-0.287	0.115
EM	0.140	-0.257	0.003	0.329	-0.470	0.028
RCM	0.273	-0.064	0.320	0.452	-0.024	-0.008
ROM	0.173	0.372	0.282	0.128	0.185	0.031
offspring biomass	0.373	0.229	-0.014	-0.090	0.147	0.033
infertility	-0.091	-0.040	0.160	-0.171	-0.038	0.869
early embryo mortality	-0.176	0.102	0.402	-0.190	-0.279	-0.443
late embryo mortality	-0.143	-0.151	0.385	-0.011	0.178	0.100
hatching success	0.242	0.023	-0.569	0.199	0.117	0.012
offspring hatched	0.406	0.070	-0.031	-0.092	0.283	-0.040

Supporting Information for Chapter 5

Table 5.S1. Genes and their function found within 100 kb of SNP markers associated with the number of incubation days. Only SNP markers with a posterior inclusion probability larger than 0.1 were included. Gene function was extracted from www.genecards.org and from UniProt (The Uniprot Consortium 2017).

Linkage group	SNP	position	PIP	effect	start	end	Gene symbol	Description
3	25552_48	55100504	0.611	1.372	55067887	55272794	PTPRK	cell growth and differentiation, mitotic cycle
3	9017_46	100240387	1.000	8.167	100212313	100281607	EPAS1	development of blood vessels
4	20365_7	2362891	0.212	1.233	2382242	2426723	DCTN1	microtubule binding, chromosome movement
4					2361595	2366592	FBXL2	ubiquitination, proteasomal degradation
4	10243_77	2410488	0.342	3.901	2428683	2433494	NOP56	pre-rRNA processing
4	10243_37	2410528	0.287	3.129	2446138	2460772	IDH3B	tricarboxylic acid cycle
4					2481602	2490154	CYP2H2	NADPH-dependent electron transport pathway
4					2503402	2518483	ZN420	negatively-regulates p53-mediated apoptosis
14	40560_35	6947933	0.359	3.610	6897092	6900124	WNT11B	oncogenesis, cell fate and patterning during embryogenesis
14					6903727	6910423	IGBP1	signal transduction
14					6920860	6940648	ARR3	retina-specific signal transduction
14					6972609	7003179	MFFA	mitochondrial and peroxisomal fission
14					7010034	7021523	CNGA2	calmodulin binding, odorant signal transduction
14					7044254	7045311	ELOB	transcription elongation factor, proteasomal degradation
14	3364_33	107031996	0.345	1.614	107112906	107263082	ATRN	immune cell clustering, inflammatory response

Table 5.S2. Genes and their function found within 100 kb of SNP markers associated with the embryonic stage at oviposition/parturition. Only SNP markers with an effect larger than 1 were included. Gene function was extracted from www.genecards.org and from UniProt (The Uniprot Consortium 2017).

Linkage group	SNP	position	PIP	effect	start	end	Gene symbol	Description
1	32380_95	53165865	0.498	0.611	53212885	53250893	PDLIM7	bone formation, embryonic flat bones mandible and cranium
1					53263364	53274520	DOK3	B-cell receptor signalling and innate immune system
1					53068420	53069622	ADRA2A	regulating neurotransmitter release
1					53080790	53089247	HGFAC	Development HGF signalling pathway and MET promotes cell motility
1					53098720	53099748	LPAR6	involved in embryo implantation and pregnancy establishment in mammals
1					53109755	53137110	GRK6	initiates beta-arrestin-mediated receptor desensitization; chemotaxis; uterine contractility
1					53165680	53167677	PRR7	unknown
1					53169452	53208528	DBN1	cell migration, extension of neuronal processes and plasticity of dendrites
4	47007_69	64290264	0.113	0.073	64371260	64466065	SYT7	calcium-dependent regulation of membrane trafficking in synaptic transmission
6	21085_97	103046890	0.128	0.301	103064669	103065568	STX1A	in ion channel regulation and neurotransmitter exocytosis
6					103047242	103056765	GIN1	nucleic acid binding
6					102983217	102984395	FAM46D	unknown; antibodies against protein only found in plasma from cancer patients
6					102944712	102949060	ERCC6L	DNA damage and cell cycle; mitotic

6	11609_15	103194351	0.13	0.301	103097082	103173777	BRWD3	regulation of cell morphology and cytoskeletal organization
6	11609_18	103194354	0.151	0.369	103290063	103310630	SLC9A2	regulation of cell pH and volume by sodium-ion transport
6					103267925	103268839	CXorf21	unknown
6					103214606	103248924	SH3BGRL	H3 domain binding and SH3/SH2 adaptor activity
6	36727_8	103767595	0.119	0.277	103778207	103823969	RPS6KA6	cell growth arrest signalling and play an inhibitory role during embryogenesis
6					103674671	103675774	POU3F4	transcription factor for early neural development; inner ear development
6	11615_40	103857102	0.14	0.333	103900696	103934931	HDX	unknown
6	21089_52	104300519	0.146	0.339	104391524	104410840	ZNF711	neuron development; brain development
6					104336041	104358264	SAT1	acetylation of spermidine and spermine; regulation of intracellular concentration of polyamines
6					104323905	104334045	APOOL	crista junction formation and mitochondrial function
6					104076238	104296957	CNKSR2	Ras signalling pathway; cellular signal transduction
8	38695_85	40060467	0.164	0.303	39930975	39965688	ACIN1	apoptotic chromatin condensation
8					39986965	40005119	CDH11	calcium-dependent cell-cell adhesion; preeclampsia
8					40018897	40019661	cpsmb7	unknown
8					40026680	40031936	PSMB5	cleaves peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway; facilitates interaction between embryo and uterus
8					40037137	40042829	C14orf93	poly(A) RNA binding
8					40049484	40058195	HAUS1	mitotic spindle assembly; completion of cytokinesis

8					40060710	40082017	PRMT5	catalyzes the transfer of methyl groups to the amino acid arginine; transcriptional regulation
8					40088909	40102950	RBM39	steroid hormone receptor-mediated transcription and alternative splicing
8					40114572	40120031	RRAD	calcium channel regulation; cardiac antiarrhythmia
11	28225_34	71964339	0.435	0.733	71562483	71919435	LRBA	secretion and/or membrane deposition of immune effector molecules
12	28866_27	55091047	0.62	0.315	55147149	55148917	IFIT5	mediator in innate immunity; antiviral defence
14	3486_81	19202083	0.178	0.405	19175863	19209841	CHRDL1	embryonic bone formation; eye development; neuronal differentiation of neural stem cells
14					19271410	19366121	AMMECR1	unknown; gene deletion results in Alport syndrome
14	3491_20	19577617	0.169	0.383	19511712	19529422	ACSL4	regulation of fatty acid uptake in embryo from placenta
14					19540713	19541168	KCNE5	potassium channel regulation; development of the early pregnancy placenta
14					19548564	19553351	NXT2	trafficking of molecules and ions between the cytoplasm and nucleus; mRNA nuclear transport
14					19661532	19663624	IRS4	role in growth, reproduction and glucose homeostasis
14					19665404	19672023	VMA21	chaperone for assembly of lysosomal vacuolar ATPase

Table 5.S3. Gene tree weights of the three topologies tested. Shown are those gene trees with an extremely high weight (between 0.9-1.0) supporting a monophyletic relationship between all viviparous lineages relative to the background across the genome (mean = 0.18). Linkage group (LG) and relative position within the linkage group is indicated.

topo1	topo2	topo3	LG	position
0.000	0.000	1.000	11	0.480
0.000	0.003	0.998	9	0.251
0.000	0.043	0.958	3	0.951
0.003	0.040	0.956	12	0.621
0.003	0.043	0.955	19	0.273
0.015	0.038	0.948	16	0.010
0.018	0.045	0.938	4	0.410
0.002	0.064	0.934	8	0.604
0.015	0.052	0.933	5	0.759
0.010	0.060	0.930	14	0.991
0.062	0.009	0.929	6	0.996
0.003	0.070	0.928	7	0.440
0.043	0.033	0.924	14	0.608
0.021	0.066	0.913	3	0.748
0.018	0.073	0.910	9	0.402
0.031	0.063	0.905	3	0.701
0.010	0.087	0.904	6	0.683