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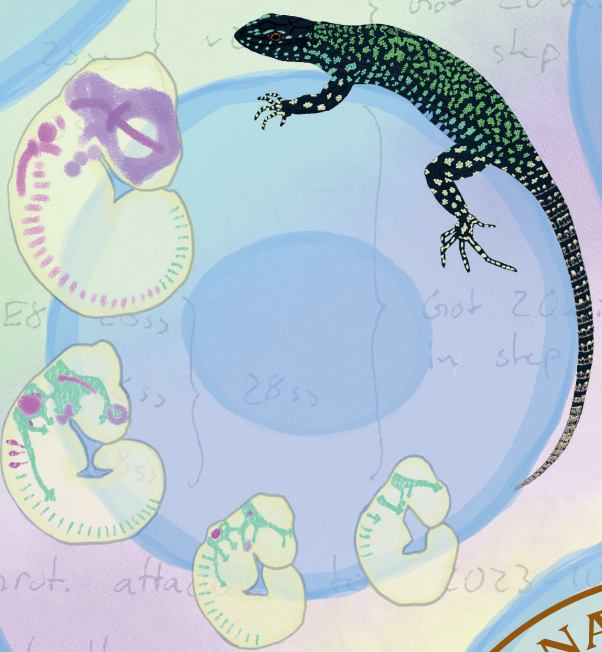

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Robin Pranter

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DOCTORAL DISSERTATION

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Abstract:

The cell is the fundamental unit of life and in multicellular animals, all cells are derived from stem cells. To understand how stem cell development may influence phenotypic evolution, this thesis studies a population of vertebrate stem cells known as neural crest cells (NCCs). With unparalleled potency and migratory behavior, NCCs affect many different traits spread over the entire vertebrate body, for example fascial morphology, coloration and behavior. It has been posited that the fact that fact that NCC-derived traits share a developmental origin wire these traits together and thus facilitate the evolution of syndromes . This thesis studies the nigriventris syndrome that has evolved in the common wall lizard (Podarcis muralis) in Italy. Lizards with this syndrome show exaggerated expression of facial morphology (larger heads), coloration (extensive body melanization, bright green dorsal coloration, and enlarged UV-blue lateral spots) and behavior (aggressive and dominant behavior) relative to ancestral phenotypes . A variety of approaches are applied – from single-cell transcriptomics to staining of individual genes in whole embryos – to trace the origination, migration and differentiation of NCCs in embryos of the common wall lizard. Building on this, I developed a methodology to isolate NCCs using flow cytometry that does not require the use of transgenics or other manipulations. To test for differences in NCC biology associated with the nigriventris syndrome, I isolated NCCs from embryos of parents of nigriventris and ancestral origins and applied single-cell transcriptomics. I found that key aspects of NCC biology such as total number, proliferation, migration, and gene expression are all found to be increased in embryos from parents with the nigriventris phenotype. This hyperfunction of NCCs in nigriventris embryos supports the idea that NCC biology facilitates the evolution of phenotypic syndromes.

Key words:

Developmental bias, stem cells, evo-devo, cell migration, cell differentiation, lizard,

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"...the separation of means and ends was, to her too, false. For her as for him, there was no end. There was process..."

-Ursula K. Le Guin, *The Dispossessed*

Table of Contents

Abstract

The cell is the fundamental unit of life and in multicellular animals, all cells are derived from stem cells. To understand how stem cell development may influence phenotypic evolution, this thesis studies a population of vertebrate stem cells known as neural crest cells (NCCs). With unparalleled potency and migratory behavior, NCCs affect many different traits spread over the entire vertebrate body, for example fascial morphology, coloration and behavior. It has been posited that the fact that fact that NCC-derived traits share a developmental origin wire these traits together and thus facilitate the evolution of syndromes . This thesis studies the nigriventris syndrome that has evolved in the common wall lizard (Podarcis muralis) in Italy. Lizards with this syndrome show exaggerated expression of facial morphology (larger heads), coloration (extensive body melanization, bright green dorsal coloration, and enlarged UV-blue lateral spots) and behavior (aggressive and dominant behavior) relative to ancestral phenotypes . A variety of approaches are applied – from single-cell transcriptomics to staining of individual genes in whole embryos – to trace the origination, migration and differentiation of NCCs in embryos of the common wall lizard. Building on this, I developed a methodology to isolate NCCs using flow cytometry that does not require the use of transgenics or other manipulations. To test for differences in NCC biology associated with the nigriventris syndrome, I isolated NCCs from embryos of parents of nigriventris and ancestral origins and applied single-cell transcriptomics. I found that key aspects of NCC biology such as total number, proliferation, migration, and gene expression are all found to be increased in embryos from parents with the nigriventris phenotype. This hyperfunction of NCCs in nigriventris embryos supports the idea that NCC biology facilitates the evolution of phenotypic syndromes.

Populärvetenskaplig sammanfattning

Om du rest i Medelhavet så är chansen stor att du sett en murödla (*Podarcis muralis*) sola sig på en stenmur. Förmodligen var den liten, brun och diskret, det är deras normala utseende. Men om du besökte Rom, Toscana eller Ligurien, så var den antagligen betydligt mer iögonfallande. Här har nämligen murödlor evolverat ett helt nytt utseende och beteende. De är större och har ett kraftigare huvud och sticker ut med ett tydligt grönt ryggmönster mot en annars nästan helt svartfärgad kropp. De svartgröna murödlorna är också betydligt mer aggressiva – mot varandra, inte mot semesterfirare.

Förr trodde man att de här svartgröna murödlorna utgjorde en egen underart, men det har visat sig vara betydligt intressantare än så. Vissa av de svartgröna murödlorna är nämligen närmre släkt med ”normala” små och bruna murödlor än de är med varandra. Det finns inte heller någon skarp gräns mellan de områden där murödlorna är svartgröna och de områden där de har det normala utseendet. I gränstrakterna har de ett utseende som ligger någonstans mitt emellan. Det fascinerande är att egenskaperna som skiljer sig mellan de svartgröna och de normala murödlorna, alltid förekommer tillsammans. En murödla kan alltså inte vara svartgrön utan att också vara stor och aggressiv. Vad kan förklara det?

Svaret verkar finnas i livets tidigaste stadium – embryot. Mer specifikt i en sorts stamceller som kallas neuralkamceller. Dessa stamceller bildas på kanten (kammen) av det blivande centrala nervsystemet, men vandrar sedan ut i resten av embryot. Väl spridda visar de sin superkraft: neuralkamceller kan nämligen ge upphov till en oöverträffad mängd olika celltyper som bygger upp skelett, nerver, pigment, adrenalinkörtlar, och mycket mer.

Det är slående att de egenskaper som skiljer svartgröna från normala murödlor så alla utvecklas från neuralkamceller. Kan det faktum att de celltyper och vävnader som reglerar färg, huvudform och beteende utvecklas från samma stamcell göre det enklare för evolutionen att förändra alla egenskaperna samtidigt? I så fall borde vi hitta skillnader mellan embryon från svartgröna och normala föräldrar i den process då neuralkamceller utvecklas, migrerar och differentierar till olika celltyper. Det är denna process som jag studerat.

Neuralkamceller har tidigare främst studerats i ett fåtal ”modellorganismer” såsom mus och zebrafisk. Därför började jag med att ta reda på var och när man kan hitta olika sorters neuralkamceller i murödlornas embryon. Det gjorde jag genom att färga in hela embryon i olika utvecklingsstadier med färgämnen som bara fäster vid neuralkamceller. Genom att studera de infärgade embryona under mikroskop kunde jag då beskriva neuralkamcellernas vandring genom embryot.

För att studera själva processen då neuralkamcellerna omvandlar sig själva till olika celltyper och vävnader så behövde jag en ny metod. Jag samlade nya embryon och

löste upp dessa till en soppa av fritt flytande celler. Genom att färga in soppan med ett av de färgämnen som bara binder till neuralkamceller, och sedan rikta en laser mot var och en av cellerna, en i taget, så kunde jag hitta och sortera ut de infärgade neuralkamcellerna från resten av soppan.

Den här metoden är enklare än äldre metoder för att samla neuralkamceller, och kan i princip användas i vilket ryggradsdjur som helst. Det gör att andra forskare nu kan studera neuralkamceller i många fler arter än vi kunde tidigare.

Med denna teknik samlade jag in neuralkamceller från embryon – med antingen svartgröna eller normala föräldrar. Jag studerade var och en av cellerna och undersökte vilka gener som användes i just den cellen. Genom att gruppera celler som använde samma gener så kunde jag identifiera flera av de celltyper som neuralkamceller ger upphov till och beskriva deras utveckling.

Jag riktade därefter uppmärksamheten mot särskilda aspekter av neuralkamcellernas utveckling – såsom deras celldelning, vandring och hur mycket de använde olika gener. Det visade sig att embryon från svartgröna ödlor har fler och mer aktiva neuralkamceller än normala embryon. Det tyder på att de svartgröna ödlorna har evolverat genom en ökad aktivitet i neuralkamcellerna – vilket skulle kunna påverka storlek, färg, beteende och många fler egenskaper, samtidigt.

Att vi hittade just högre aktivitet av neuralkamceller från murödlor med mörkare färg, kraftigare huvuden och högre aggressivitet är intressant. Det finns en gammal hypotes om att domesticerade djur – alltså gamla hederliga, grisar, kor, och hundar med flera – evolverat ljusare färg, mindre huvuden och lägre aggressivitet genom minskad aktivitet av neuralkameceller. Detta som ett svar på selektion för just lägre aggressivitet. Det är precis tvärt emot vad vi hittar i våra ödlor vilket stämmer överens med tidigare forskning som visat att de svargröna ödlorna evolverat under sexual selektion, där mer aggressiva hanar får bättre revir och därför fler ungar.

Det verkar alltså som att neuralkamcellerna kopplar ihop färg, huvudform och beteende. Och att evolutionen, genom att förändra neuralkamcellernas aktivitet, därför synkroniserat kan förändra alla dessa egenskaper samtidigt.

Det här är ett exempel på hur utvecklingen kan styra evolutionen. Ibland tänker vi att evolutionära förändringar bara är resultat av selektion, alltså vem som får flest avkommor, och av arv, alltså att avkomman liknar sina föräldrar. Men det är inte allt. För att selektion och arv ska spela någon roll, så måste det finnas variation; om alla individer är lika dana spelar det ingen roll vem som får flest barn, det är ändå samma egenskaper som skickas vidare. Utvecklingsbiologiska processer som skapar ny variation kan därför ge evolutionen riktning och fart. Genom att koppla ihop olika egenskaper, påverkar neuralkamcellerna vilken variation som är möjlig och hur ofta olika varianter uppstår. Och en förändring som uppstår oftare, den har större chans att påverka evolutionen, därför är det inte bara selektion och arv som ger evolutionen riktning och fart, utan även hur egenskaper utvecklas.

List of papers

I. **Pranter, R.** and Feiner, N., 2024, Spatiotemporal distribution of neural crest cells in the common wall lizard *Podarcis muralis*. *Developmental Dynamics*, https://doi.org/10.1002/dvdy.758

II. **Pranter, R.**, Patthey, C. and Feiner, N., Enrichment of neural crest cells by antibody labelling and flow cytometry for single-cell transcriptomics in a lizard. Manuscript submitted to *Evolution and Development*. Preprint available on *bioRxiv* https://doi.org/10.1101/2025.05.21.655068

III. **Pranter, R.**, Patthey, C. and Feiner, N., An atlas of neural crest cells in a lizard at single-cell resolution. Unpublished manuscript.

IV. **Pranter, R.**, Uller, T. and Feiner, N., The role of neural crest cells in the evolution of a sexually selected syndrome. Unpublished manuscript.

Author contributions

I. **RP** and NF planned the experiments. **RP** and NF both acquired funding. **RP** collected the data and curated, analyzed and visualized the results with input from NF. **RP** and NF wrote the first draft and the final paper.

II. **RP** and NF planned the experiments with guidance from CP. **RP** and NF both acquired funding. **RP** collected, analyzed and visualized the data with input from NF and CP. **RP** wrote the first draft, CP commented on the draft and **RP** and NF wrote the final manuscript with input from all co-authors.

III. **RP** and NF planned the experiment. NF acquired funding. **RP** collected, analyzed and visualized the data with input from CP and NF. **RP** wrote the first draft. All authors contributed to the writing of the final manuscript.

IV. **RP,** TU and NF planned the experiment. NF and TU acquired funding. **RP** collected, analyzed and visualized the data with input from TU and NF. **RP** wrote the first draft. All authors contributed to the writing of the final manuscript.

RP: Robin Pranter. NF: Nathalie Feiner. CP: Cedric Patthey. TU: Tobias Uller.

Introduction

The ultimate goal of evolutionary biology is to understand why organisms are the way they are. The way organisms are (often captured in the phenotype concept), changes within lifetimes, something known as *development*[1]; and across generations, something known as *evolution*.

Evolution can be captured by three principles. For a population to evolve, said population must vary phenotypically (*phenotypic variation*), some of those variants must pass on more offspring than others (*differential fitness* or *selection*), and offspring must resemble their parents (*heredity*) (Lewontin, 1970). Understanding each of these three principles is fundamental to understanding how the evolutionary process takes place and how it shapes the living world.

For the past 100 years, extensive focus in evolutionary biology has been given to understanding heredity and selection. However, less attention has been given to the principles that generate phenotypic variation and how those principles influence evolutionary outcomes (Amundsen, 2005). Broadly defined, the generative processes that generate variation are encapsulated in the term *development*.

Development is a lifelong process where the organism relies on resources and templates from the genotype, the environment and the initial conditions of its material substance (e.g., RNAs and proteins deposited in a zygote by the mother), and generates a phenotype (Brun-Usan et al., 2022; Oster and Alberch, 1982). The mechanisms by which development can integrate traits, how those mechanisms evolve, and their implications for adaptation and diversification are not well understood and need further investigation.

This thesis investigates the cellular developmental underpinnings of the evolution of a striking suite of traits in common wall lizards (*Podarcis muralis*) here called "the nigriventris syndrome" (figure 1). The evolution of the nigriventris syndrome in *P. muralis* offers an excellent system in which to study variation of such mechanisms on microevolutionary timescales. I hypothesize that the traits involved in the nigriventris syndrome covary because they are developmentally coupled by their origin in the same population of stem cells, known as *neural crest cells* (NCCs) and that this covariability has facilitated the evolution of the nigriventris syndrome.

[1]Development is sometimes referred to as just a synonym to embryology. This thesis uses a much wider understanding of the term.

If this is true, then it should be possible to find variation in NCC development that corresponds to the adult phenotypic variation. Before going into the specifics of the system, I will establish in general terms how knowledge about the developmental process may inform our understanding of the evolutionary process.

Figure 1. The common wall lizard *Podarcis muralis.*
Female common wall lizards of the ancestral (top) and nigriventris (bottom) phenotypes. Photo credit: Javier Ábalos Álvarez

Development as a biased novelty generator

While selection is a powerful process for evolutionary change, it can only preserve or eliminate phenotypes that already exist. For it to be effective it must be combined with a process that generates variation. On the level of the phenotype, this generative process is development. If the generation of novel variation was equally likely in any direction of phenotype space[2], this addition would not be needed for a full understanding of the evolutionary process. However, both theoretical and empirical work has demonstrated that the non-linear nature of developmental systems causes even random genetic and environmental changes to result in non-random phenotypic changes (Brakefield, 2006; Milocco and Uller, 2024; Salazar-Ciudad

[2] A phenotype space is a theoretical construct where every individual is represented as a point in space where every axis is a measurable "trait" and an individual's position along each axis is given by its trait-states.

and Jernvall, 2010; Uller et al., 2018). This phenomenon is known as developmental bias.

Since the variability (ability to vary) of developmental systems is not isotropic, there will be unequal generation of new phenotypes and hence evolvability (the ability to evolve) will not be equal in every direction. Populations are generally under the influence of both developmental bias and selection at the same time, and evolutionary outcomes will be co-determined by both processes. Depending on the relative strength of bias and selection in the system of study, either may be more explanatory for a specific evolutionary change (Uller et al., 2020).

Developmental biases themselves evolve as the result of selection, and selection always acts on preexisting biased phenotypic variation which in turn is also partially the result of previous selection and so on. This dialectical interplay of selection and developmental bias goes all the way back to the origin of life. It does not matter if one subscribes to a "metabolism first" (Doolittle and Booth, 2016) or "replicator first" (Dawkins, 2006) view of the origin of life; in either case, there will be both bias and selection co-acting on the population. But why does the developmental process bias the generation of novel phenotypic variation?

I will explain the cause of developmental bias using two heuristics, "neutral networks" and "major axes of variability". One important insight about developmental systems is that a given phenotype can be produced by many different developmental systems and that some of those developmental systems are more likely to be perturbed by mutation or environmental change than others (Oster and Alberch, 1982).

Neutral networks

A common visual representation which explains developmental bias is a graph where each node is a developmental system (sometimes represented by gene regulatory networks) and edges connect nodes which are separated by just one mutation (Fig. 2). This is called a "network of networks" and a group of nodes that are connected and produce the same phenotype is called a "neutral network". Neutral networks were first studied empirically in the context of RNA-folding (Schuster et al., 1997) but have since been generalized (see for example Wagner 2014[P18] for a discussion). Studying this model, one realizes that depending on where in the neutral network the studied system is situated, phenotypic change may be more likely in some directions than others given the same number of random mutations. This is developmental bias (Uller et al., 2018).

Consider the gene regulatory network labelled A in Figure 2. Four out of five random mutations would result in no phenotypic change and only a single one would lead to the development of the purple phenotype. The blue phenotype can only be reached by three or more mutations. This means that more purple than blue variants would be produced by random mutation. In other words, even if the underlying

genetic or environmental change is random, the dice is loaded and resulting phenotypic change will be directed.

The network of networks model is useful for understanding why the generation of new phenotypic variants is non-random. However, it can lead one to think that developmental bias is only about "jumpy" or discrete phenotypic changes (even if that is not actually implied). Another way to think about developmental bias is that the specific entities and their interactions that compose a developmental system (which proteins, cells and organ primordia etc. are involved) can couple trait variabilities that align multivariate phenotypes along a major axis of variability.

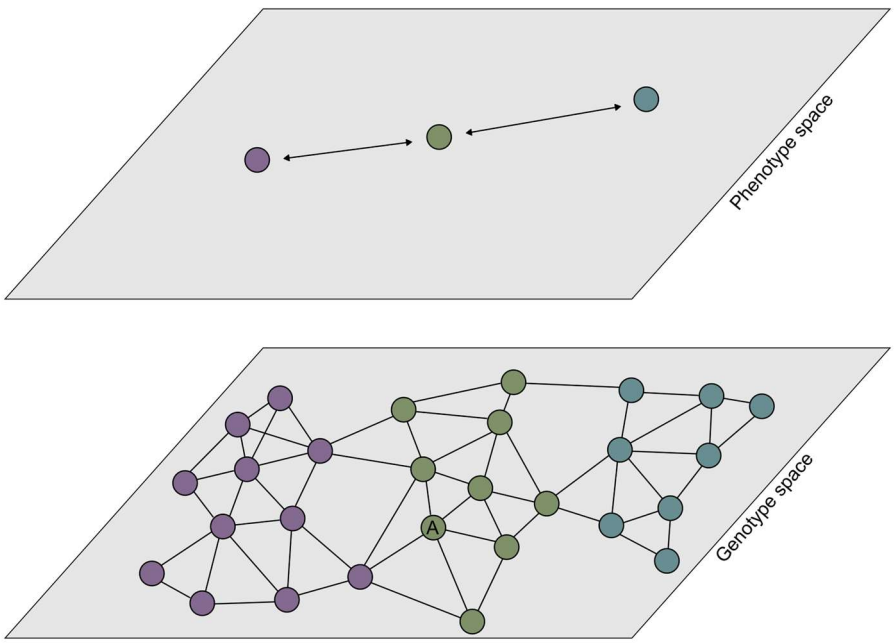


Figure 2. The neutral networks representation of developmental evolution.
The lower plane represents a genotype space simplified in two dimensions. Each node is a gene regulatory network and edges connect networks that are separated by just one mutation. Nodes of the same color are part of the same "neutral network" giving rise to the same phenotype. The upper plane represents phenotype space simplified in two dimensions. Each node is a phenotype and the arrows represent possible phenotypic transitions. The colors correspond to their respective neutral network. Starting in the neutral network labelled A, one out of five mutations will result in a phenotypic change from green to purple while four will be neutral with respect to phenotype. However, at least three mutations are required to trasition from A to the blue phenotype.

Major axes of variability

While many studies have revealed uneven distribution of phenotypic variation statistically, few empirical studies have investigated this from a developmental perspective. However, doing so makes it possible to link the mechanisms that generate phenotypic variation with its evolutionary consequences. Kavanagh et al.

(2007) studied the development of molars in mice (*Mus musculus*); they then constructed a mathematical model capturing regularities in the observed data and finally showed that this model accurately predicts relative molar size, not just in mice, but across the larger clade of murines.

In a series of studies reviewed in Brakefield, (2010), the variability of eyespots in the butterfly *Bicyclus anynana* was investigated. It was observed that size, but not color traits, responded readily to artificial divergent selection, and further found that this was due to differences in the underlying developmental systems. They then went on to look at the same traits in the wider Mycalesinae phylogeny and found that across the family, eyespot size, but not coloration, is diverse across species and occupies a large part of the phenotype space.

These studies start with developmental data, construct models of development predicting the variability of the system, and then compare those predictions with macroevolutionary divergence and find that they align. These are powerful examples of how the rules of development set up axes of variability that are utilized by adaptation and diversification. Variation in other directions in phenotype space is produced more rarely and is therefore less accessible for selection to act upon.

Such examples are reminiscent of "genetic lines of least resistance" and related concepts in quantitative genetics (Schluter, 1996). For example, the variance-covariance matrix of mutational effects on wing traits in *Drosophila melanogaster* predicts wing shape variation between species in a 40 million year old clade of flies (Houle et al., 2017). Similarly, a recent large meta-analysis found a positive scaling relationship between macroevolutionary divergence and evolvability (~variability of the heritable additive phenotypic variation) (Holstad et al., 2024). One interpretation of these results is that the variability of shared inherited developmental systems channels phenotypic evolution along the major axes of variability. However, the two latter studies were entirely based on linear relationships between adult traits without considering developmental mechanisms, which makes this hypothesis difficult to evaluate.

Development facilitates covariation

Trait covariations are ubiquitous in nature. When certain, often quite extreme, character states occur strictly together, then the "package" of character states is sometimes referred to as a *syndrome*. To some extent, such covariation of traits is needed for bringing about adaptive fit between an organism and its environment. For example, it may be useless for an organism to have cryptic coloration unless this is matched by cryptic behavior. This introduces correlational selection, which can cause traits to covary at the population level.

However, such adaptive fit can be brought about before even being exposed to selection. This is evident from scenarios where one trait is modified, and others

respond to accommodate the new state of this trait and build a coherent phenotype. A famous example is Slijpers two-legged goat that was born without functional forelegs (Slijper, 1942a, 1942b). Lacking forelegs led to the use of a new behavior – walking on two legs – which introduced new stressors on the musculoskeletal system which in turn led to considerable reshaping and reshuffling of muscles and skeletal elements as well as the development of some completely novel tendons. What this example teaches us is that developmental processes can bring about adaptive solutions even in situations that the system has not yet been selected for.

Correlational selection and developmental covariability are not mutually exclusive processes, and in most situations both act in concert to cause traits to vary and evolve together. In fact, given enough time they should become aligned and even cause each other (Brun-Usan et al., 2022; Milocco and Uller, 2024; Watson et al., 2014).

Developmental mechanisms that couple trait variabilities

Trait variabilities can be coupled in many ways. They may be coupled simply by being inherited together. For example, if different genes that affect the traits in question are physically linked by close genomic proximity, perhaps even 'locked up' in an inversion. Well known examples of this are syndromes with different mating strategies in ruff (Lamichhaney et al., 2015) or mimicry syndromes in Heliconius butterflies (Joron et al., 2006).

But trait variabilities can also be coupled because they develop together. For example, they may both be affected by the same hormone. A well-known example of that is the melanocortin system in vertebrates, which has been proposed as a mechanism explaining the frequent association between melanization and aggression (Ducrest et al., 2008). Alternatively, they can be coupled because they share a developmental origin in the same stem cell or embryonic tissue. For example, root hairs and trichomes are located in completely separate parts of the organism, but in *Arabidopsis* plants they covary because they develop from the same epidermal stem cells (Ishida et al., 2008). Understanding stem cell biology is therefore central to understanding trait variability and, *by extension, also evolution.*

Stem cells, cell types, and cellular differentiation

Stem cells differentiating to become specialized cell types is one of the central processes in development. Cellular differentiation can be viewed as a tree where the stem is the zygote and the branch tips are terminally differentiated cell types. This branching structure was first conceptualized by Haeckel and Weisman (references in Dröscher, 2014). The current mechanistic models for how differentiating cells gain their cell type identities are still being worked out (Davidson and Erwin, 2006; Graf and Enver, 2009; Hobert, 2011; Wagner, 2007).

In short, these models typically posit that cell type identity is determined by a series of signals, which commit the cell to a certain fate. These signals can be both received

from the environment, which can also be other cells, and internally inherited from the "mother" cell. The commitment is controlled by a core gene regulatory network sometimes known as a "character identity mechanism" (DiFrisco et al., 2020) and sometimes as "a core regulatory complex" (Arendt et al., 2016). The cellular function and morphology of the particular cell type is realized through the expression of a battery of effector genes that are regulated by the core regulatory network. This happens in multiple, successive and hierarchical steps by which stem cells become gradually more specified and less multipotent.

It follows from this branching understanding of cellular development that the later two cell types bifurcate (i.e., the further towards the tip of the tree they branch off), the more development they share and hence the more coupled their variabilities should be. Little is known about how such processes affect phenotype variability and evolution on the level of morphology, physiology and behavior (Albertson, 2017; Parker, 2024). To investigate this question, one would need a study system that varies phenotypically in traits that are derived from the same stem cells and that are adaptively significant.

One of the major model systems in stem cell biology is the lineage of cells emanating from the neural crest (Le Douarin & Kalcheim, 1999). The neural crest cells (NCCs) are migratory and give rise to many different cell types in many different parts of the body. To study how stem cell biology affects phenotypic evolution, this thesis investigates the role of NCCs during the evolution of a novel phenotype in the common wall lizard, a species with considerable covariation of a suite of traits derived from the neural crest. Before presenting the findings, we will first need some background on wall lizards and neural crest cells.

Study system

Wall lizards – the nigriventris syndrome

The common wall lizard is a familiar sight in much of Europe, with a distribution spanning from the Iberian peninsula in the west to the Anatolian peninsula in the east (Speybroeck et al., 2016). Throughout its range, the species can be divided into six genetic lineages that diverged 6.24–2.5 million years ago (Yang et al., 2021).

In most of its range, the common wall lizard does not vary substantially in terms of phenotype. Individuals are typically small (~50-70 mm snout-to-vent length; Böhme, 1986), with delicate body shapes and an overall brown-tan coloration and a series of small lateral UV-blue spots along the flanks. However, the lizards in the lowlands around Rome, Tuscany and Liguria stand out as they exhibit a striking new phenotype, composed of a suite of characteristic features, here referred to as *the nigriventris syndrome*. Populations with this phenotype have historically been referred to as subspecies *P. muralis nigriventris* (because of its striking black ventral color), but population genomic investigations have shown that these do not correspond to a monophyletic group, and hence cannot properly be described as a subspecies (Yang et al., 2018).

The nigriventris syndrome consists of exaggerated states of several morphological, behavioral, and physiological traits (Fig. 3). Individuals with the nigriventris phenotype are hyper-melanistic with a large proportion of black scales. They have an extensive and striking green and black dorsal pattern, and their lateral blue spots are bigger and more intense in both hue and UV-reflectance. Nigriventris lizards are also overall larger, and have longer and wider heads, stronger bite force and are more aggressive and socially dominant (Heathcote et al., 2016; MacGregor et al., 2017; While et al., 2015a; While and Uller, 2017). The nigriventris syndrome affects both males and females, but it is more extreme in males (Heathcote et al., 2016; While et al., 2015b, 2015b), which is consistent with the traits being under intra-sexual selection in male common wall lizards (Heathcote et al., 2016; MacGregor et al., 2017; While et al., 2015a, 2015b).

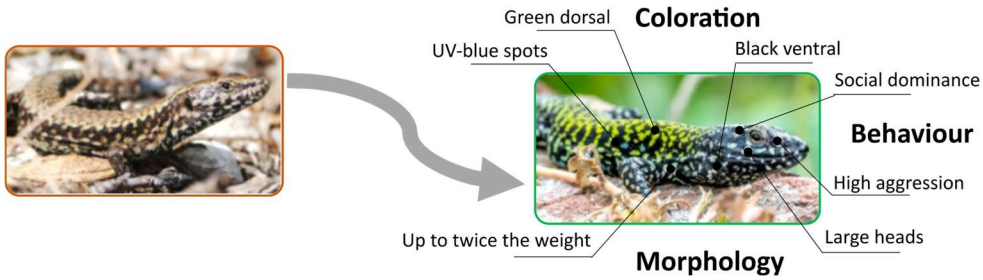


Figure 3. The nigriventris syndrome compared to the ancestral phenotype.
Lizards with the nigriventris syndrome exhibit exhagerated states of multiple characters compared to the encestral phenotype. Photo and illustration credits Nathalie Feiner.

The most striking expression of the nigriventris syndrome is found in the area around contemporary Rome (Miñano et al., 2021), which is also where the syndrome most likely originated (Yang et al. 2018). Populations throughout central Italy vary continuously from the ancestral phenotype, to the derived nigriventris phenotype (Fig. 4; Miñano et al., 2021), but the traits involved in the syndrome are strongly and consistently correlated (Feiner et al., 2024).

Furthermore, the nigriventris phenotype is spreading in northern Italy where common wall lizards of the central Italy (IT) lineage form a contact zone with common wall lizards of the southern Alps (SA) lineage (which otherwise exhibit the ancestral phenotype). The tight covariation between the syndrome traits is maintained even during introgression into the SA lineage, despite that the two lineages diverged over five million years ago (Feiner et al., 2024; While et al., 2015a). From this we can conclude that it is not just the variation, but also *the variability of the syndrome traits that is coupled*.

As expected, given the continuous nature of the phenotypic variation, genomic analyses have identified multiple loci that associate with the nigriventris syndrome. Among these is a genomic region that introgresses together with the syndrome traits into the SA lineage (Feiner et al., 2024; Yang et al., 2018). This region contains the two genes *Rab18* and *Acbd5*, and between them there is considerable structural variation including multiple copies of the genes *Pks* and *Ptchd3* (Feiner et al., 2024). It is likely that this genomic region plays an important role for the development of traits associated with the nigriventris syndrome, but that the expression of the syndrome also depends on many other genetic loci.

Intriguingly, three separate lines of evidence suggest that developmental stem cells known as neural crest cells (NCCs) are involved in structuring the trait variabilities, thereby facilitating the evolution of the nigriventris syndrome. First, it is known from other vertebrates that the traits in question are built by cell types that are derived from NCCs. I will describe this in more detail below. Second, a large proportion (53%) of the genomic loci associated with the nigriventris phenotype

contain genes that are known to be involved in NCC development (Feiner et al., 2024). And third, bones derived from the neural crest have been shown to contribute significantly more than mesoderm-derived bones to the exaggerated skull morphologies of nigriventris lizards (Horta-Lacueva et al., 2025).

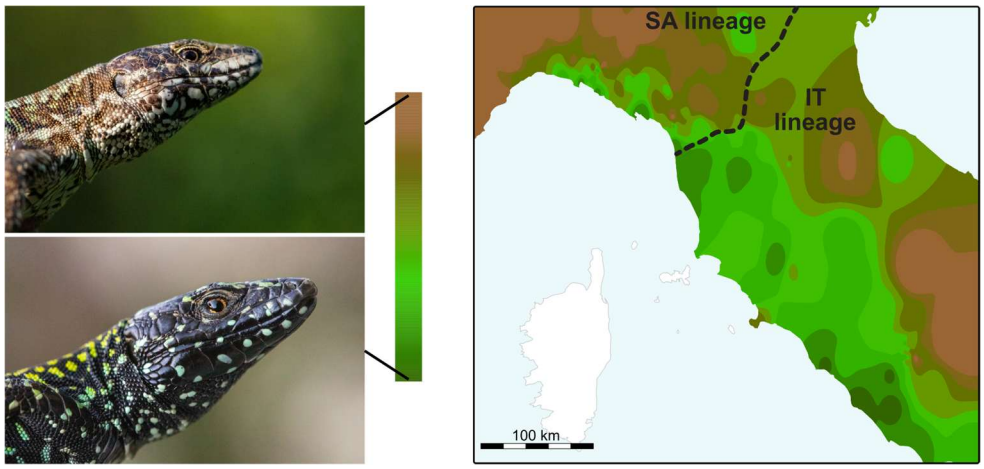


Figure 4. Distribution map of the phenotypic variation in the central Italy (IT) and southern Alps (SA) lineages of the common wall lizard.
The continous variation of common wall lizard phenotypes from the ancestral phenotype (upper left) in brown to the nigriventris phenotype (lower left) in green are plotted over parts of central and northern Italy. The map is specifically colored based on data on dorsal greenes, which is used as a proxy for the full syndrome. Map adapted from Feiner et al. (2024); photo credits Javier Ábalos Álvarez.

This makes the nigriventris syndrome in common wall lizards, and its potential relationship with the NCCs, an excellent study system to investigate how the developmental biology of stem cells affects phenotype evolution. However, most of what we know about NCCs is drawn from studies in chicken (*Gallus gallus*), mouse (*Mus musculus*), zebrafish (*Danio rerio*) and African clawed frog (*Xenopus levis*) (for some recent examples see: Howard et al., 2021; Kotov et al., 2024; Soldatov et al., 2019; Thiery et al., 2023). While this is very useful and fundamental processes in development are expected to be conserved, it is important to recognize that they are also subject to evolutionary change. As such, taxon-specific differences are not only expected but have also been documented in some cases (Eames et al., 2020; Le Douarin and Kalcheim, 1999).

The following section will review some fundamental NCC biology. As much as possible, this draws on examples in other squamates or at least non-avian reptiles, but the majority of the information stems from the four model organisms mentioned above.

Neural crest cells

Neural crest cells (NCCs) are multipotent and migratory stem cells in vertebrate embryos that originate during neurulation (Fig. 5A) and give rise to a vast number of cell types. Neurulation is the process during vertebrate embryonic development, when the central nervous system forms from a longitudinal region of the ectoderm, called the neural plate. The border between the neural plate and the non-neural ectoderm is called the *neural plate border*. During neurulation, the neural plate invaginates and forms the neural groove. At this stage, the neural borders can be found on the lateral crests of this groove and are now named the *neural crests*. As the crests close, and the neural groove forms the neural tube, the cells in the neural crest (the NCCs) undergo an epithelial-to-mesenchymal transition and delaminate from the dorsal neural tube (Le Douarin & Kalcheim, 1999).

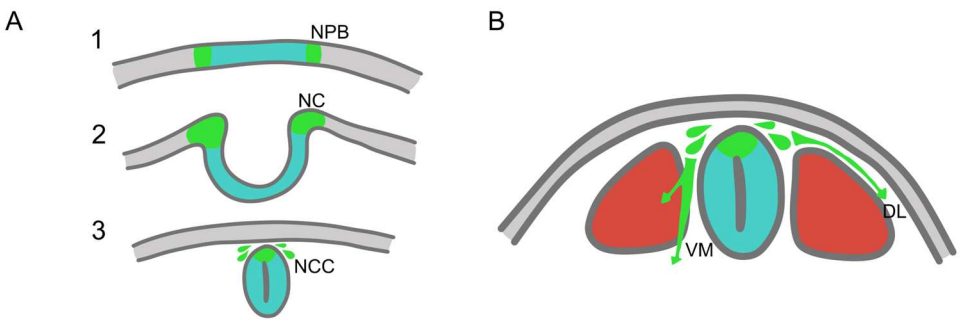


Figure 5.
A The specification, delamination and migration of NCCs durin neurulation. The neural plate border (NPB), neural crest (NC), neural crest cells (NCC) are shown in green. The neural plate, groove and tube are shown in blue. **B** Migratory streams of trunk NCC migration are shown in green. The dorsolateral stream (DL) migrates between the somites (red) and the ectoderm (gray), and the ventromedial stream (VM) migrates between the neural tube (blue) and the somites.

The delaminated NCCs migrate through the embryo and differentiate into a number of different cell types, for example, chromatophores, which give animals their coloration, osteocytes and chondrocytes that make up the facial and much of the cranial skeleton, several types of endocrinal cells such as the chromaffin cells of the adrenal gland and the neurons and glia (Schwann cells) of the peripheral nervous system (Le Douarin & Kalcheim, 1999).

The remarkable potency of NCCs (even exceeding that of the mesoderm), has led some people to refer to them as “the fourth germ layer” (Hall, 2000). However, while this seems justified given the remarkable diversity of cell types derived from the NCCs, the truly remarkable thing about NCCs is not that they constitute another germ layer. Instead, they partially break down the borders between the germ layers by giving rise to cells typically associated with all three layers.

NCCs seem to have evolved this incredible potency in two ways. Some of the cellular phenotypes that are today derived from NCCs could probably be generated from the unknown cell type in vertebrate ancestors from which NCCs evolved (Fatieieva et al., 2025; Stolfi et al., 2015). Later, the contemporary potency seem to have evolved by stepwise co-option of cellular phenotypes that were previously generated by other stem cells in vertebrate ancestors (Adameyko, 2020; Furlan and Adameyko, 2018; Green et al., 2017; Jandzik et al., 2015; Kaucka and Adameyko, 2019; Wang et al., 2025).

Specification and delamination

The specification of NCCs (i.e., the process when NCCs gain their identity) takes place at the neural plate border. The exact mechanisms of induction are not entirely understood, but it depends on the balance of signaling molecules such as WNTs and BMPs expressed by the neural and non-neural ectoderm respectively (Simões-Costa and Bronner, 2015; Thiery et al., 2023; Williams et al., 2022). These signals trigger the expression of a network of transcription factors including *Tfap2*, *Pax3*, *Pax7*, *FoxD3*, *Snai1*, *Snai2* and *Twist1*, which specify NCC identity (Simões-Costa and Bronner, 2015). Among other things, these NCC identity specifying transcription factors repress the expression of cadherins, which allows the NCCs to undergo epithelial-to-mesenchymal transition, delaminate from the neural tube and become migratory (Simões-Costa and Bronner, 2015). Some of the transcription factors that induce delamination also induce other transcription factors such as *Sox9* and most importantly *Sox10*, which are major NCC identity specifiers (Simões-Costa and Bronner, 2015).

Migration and fate determination

NCC delamination from the dorsal neural tube progresses in a wave-like fashion along the anterior-posterior axis and is followed by subsequent lateral and ventral migration. The first migration takes place in the head, and the timing of this has been studied in three non-avian reptiles by staining migratory NCCs with the antibody HNK-1. In the veiled chameleon (*Chamaeleo calyptratus*) and the softshell turtle (*Trionyx sinensis*), the first migratory NCCs are detected by somite stage 6 (6ss; Diaz et al., 2019; Hou & Takeuchi, 1994) and in the brown anole (*Anolis sagrei*) migration was found to have started before somite stage 8 (8ss; Weberling et al., 2025).

This is similar to results in chicken (6ss; Le Douarin & Kalcheim, 1999[p66]), but later than in mouse (5ss; Nichols, 1981, 1986; Serbedzija et al., 1992) and human (*Homo sapiens*, 4ss; O'Rahilly & Müller, 2007). Delamination and subsequent migration then follow at gradually more posterior positions. During migration, and once they have arrived at their final locations, NCCs differentiate to specialized cell

types. Historically, it has been debated if NCCs mainly differentiate based on external cues that they receive from the surrounding cells along their migratory path, or if their fates are determined by internal factors already when they leave the neural crest (Harris and Erickson, 2007). Both hypotheses have been shown to be partially correct. Some differences in potential exist among premigratory NCCs biasing them towards certain fates, but further differentiation appears to depend on external cues during migration and at their final positions in the developing embryo (Jacobs-Li et al., 2023; Rothstein et al., 2018; Soldatov et al., 2019).

The main source of bias in premigratory NCCs is their region of origin along the anterior-posterior axis (Rothstein et al., 2018), which is patterned by the expression of different *Hox* genes (Le Douarin, 2004; Rothstein et al., 2018; Trainor & Krumlauf, 2000). Based on these differences, NCCs have been divided into subpopulations (table 1; Brandon et al., 2023; Rothstein et al., 2018). Going from the anterior to the posterior, these are:

Table 1 NCC-derived cell types and how they relate to the nigriventris syndrome in *P. muralis*. Subpopulations of NCCs along the anterior-posterior axis, their derivatives and some relations to the nigriventris syndrome. Adapted from Brandon et al., (2022) and Rothstein et al., (2018).

NCC subpopulation	Derived cell types and/or structures	Related nigriventris syndrome trait
Cranial	Ostecytes and chondrocytes	Relative head size and head shape, bite force
	Odontoblasts of teeth	
	Sensory ganglia and cranial nerves	
	Mesenchyme of parathyroid and thymus	Body size?
	Corneal endothelium and stroma	
	Parafollicular cells of thyroid	Body size?
	Chromatophores of skin	Black and green coloration
	Carotid body	
Vagal (including cardiac)	Cardiomyocytes of the aortic pulmonary arteries	
	Portions of the heart septa	
	Neurons and glia of parasympathetic (including the enteric) nervous system	
Trunk	Chromatophores of skin and internal integuments	Black and green coloration and lateral blue spots
	Chromaffin cells of adrenal gland and sympathetic ganglia	Aggression / dominant behavior
	Neurons and glia (schann cells) of sympathetic nervous system	Aggression / dominant behavior
	Dorsal root ganglia (sensory neurons)	
Sacral	Neurons and glia of parasympathetic (including the enteric) nervous system	

(1) *Cranial NCCs* specify in the dorsal part of the developing brain. After delamination, the cranial NCCs follow superficial, dorsolateral routes of migration,

between the ectoderm and the surface of the neural epithelium (Le Douarin, 2004; Rothstein et al., 2018). Migration is channeled along a series of streams reaching towards the rostrum and the branchial arches (Le Douarin, 2004). In anterior to posterior order, the first stream originates in the anterior part of the dorsal midbrain to the first three rhombomeres of the hindbrain and migrate towards the rostrum, maxillary process and first branchial arch. The second stream originates in rhombomeres three through five (anterior of the otic vesicle) and contributes cells to the second branchial arch. The third stream originates in the fifth through seventh rhombomere and is directed to the third branchial arch (Le Douarin, 2004). There is also some migration of NCCs from the forebrain and anterior midbrain contributing cells to the rostrum, but these form a less distinct stream (Le Douarin, 2004). Cranial NCCs give rise to cartilage, bone, sensory and sympathetic cranial neurons, glia, chromatophores, muscle and connective tissues. They also contribute cells to the thymus, thyroid and parathyroid glands and odontoblasts in teeth.

(2) *Vagal NCCs* specify from the level of the first to the seventh somite pair. They migrate along either a dorsolateral pathway similar to cranial NCCs or a ventromedial one (Kuo and Erickson, 2011). Vagal NCCs give rise to neurons and glia of the *vagus nerve* and in the enteric ganglia of the parasympathetic nervous system, which innervates the gastrointestinal tract. A subpopulation of the vagal NCCs is known as the *Cardiac NCCs*. These originate in the anterior most part of the vagal NCCs and migrate along the dorsolateral route and reach the heart where they contribute to the large arteries and one of the cardiac septa.

(3) *Trunk NCCs* specify along the entire trunk and migrate either ventromedially between the neural tube and the somites or dorsolaterally between the dermomyotome and the ectoderm (Fig. 5B; Harris & Erickson, 2007). The ventromedial migration follows multiple streams separated by the posterior half of each somite, which expresses molecular signals such as ephrins that repel migrating NCCs (Harris and Erickson, 2007). Each ventromedially migrating stream enters the anterior half of its respective somite and is further split into two branches. One branch migrates only a short distance after entering the somite and differentiates into sensory neurons and glia of the dorsal root ganglia (DRG), and the other continues ventrally to differentiate into autonomic neurons, glia and chromaffin cells of the sympathetic chain and adrenal medulla. The NCCs migrating along the dorsolateral path leave the area above the neural tube later than those migrating ventromedially. The first wave of migration is stopped from entering this path by the local expression of ephrins, but the second wave expresses a different ephrin receptor that makes the signal attractive rather than repelling (Harris & Erickson, 2007). The dorsolaterally migrating NCCs in the trunk differentiate to chromatophores.

(4) The last subpopulation along the anterior-posterior axis is known as *Sacral NCCs,* a subpopulation of trunk NCCs that specify in the caudal most part of the trunk (in chicken posterior of somite 28). Sacral NCCs, like the vagal NCCs, give

rise to neurons and glia in the enteric ganglia of the gut, but they also contribute neurons and glia to the sympathetic ganglia and chromatophores.

The biggest difference in potential along the anterior-posterior axis is that between cranial and trunk NCCs. Cranial, but not trunk NCCs, give rise to ectomesenchyme, which is a pluripotent cell type from which, for example, cartilage forming chondrocytes and bone forming osteocytes are derived (Adameyko, 2020; Le Douarin, 2004; Rothstein et al., 2018). However, as modern developmental biology has expanded beyond the traditional model systems and sampled more branches of the tree of life, some of this dogma around which cell types that can be generated by which NCC subpopulation has started to blur. Most strikingly, trunk NCCs in pond slider turtles (*Trachemys scripta*) and alligators (*Alligator mississippiensis*) appear to contribute osteocytes. In the pond slider, these trunk NCC-derived osteocytes are found in the plastron bone and one of the bones in the carapace as well as in ribs and vertebrae. In alligators, NCCs contribute to the gastralia or "floating ribs" which are thought to be homologous of the posterior bones of the turtle plastron (Cebra-Thomas et al., 2007; Clark et al., 2001; Gilbert et al., 2007; Rodrigues-Da-Silva et al., 2022).

Neural crest cells in squamates

NCCs and NCC-derived cells and traits in non-avian reptiles are known to differ from those in mammals and birds in several ways. For example, mammals and birds only have one kind of chromatophore called melanocytes, while non-avian reptiles, just like fish and amphibians, have three chromatophore types known as melanophores, iridophores, and xanthophores. Furthermore, non-avian reptiles have extensive pigmentation of their internal organs (Griffing et al., 2020). This pigmentation is probably formed by NCCs following the ventromedial rather than the dorsolateral migration route (Hou and Takeuchi, 1994), but in mammals and birds chromatophores (melanocytes) are only formed from NCCs migrating along the dorsolateral pathway.

Early results suggest there might be some differences in NCC migration between squamates (lizards and snakes) and the standard models of NCC development (chicken and mouse). As described above, cranial NCCs are typically thought to migrate along three main streams from the hindbrain and anterior midbrain towards the branchial arches and rostrum. However, in the veiled chameleon (and also in the Crocodile *Crocodylus niloticus* and the ostrich *Struthio camelus*), results suggest a forth stream starting posterior to the third stream described above (Diaz et al., 2019; Kundrát, 2009, 2008). This stream was not observed in the brown anole (Weberling et al., 2025). Two studies investigating the migration of trunk NCCs in snakes (king snake *Lampropeltis getula* and Egyptian cobra *Naja haje*) report a second wave of ventromedial migration passing through the inter-somitic space rather than through the anterior half of the somite as the first wave (Khannoon et al., 2021; Reyes et al.,

2010). Outside of squamates, such a stream has also been described in the pond slider turtle (Goldberg et al., 2020). This second ventromedial migration was not found in veiled chameleons (Diaz et al., 2019), and results in the brown anole are inconclusive (Weberling et al., 2025). The fate of the NCCs in the second wave of ventromedial migration in squamates is not known. These results are partially inconclusive, and it is unknown how the potential differences affect the development and the resulting phenotypes.

Two studies compare the shape of the developing dorsal root ganglia (DRG) of king snake, banded gecko (*Coleonyx variegata*), tokay gecko (*Gecko gecko*), pond slider turtle and chicken (Goldberg et al., 2020; Reyes et al., 2010). They conclude that squamates have smaller, more spindle-shaped DRG compared to the turtle and the chicken. A similar shape is found in DRG of veiled chameleon (Diaz et al., 2019) and Egyptian cobra (Khannoon et al., 2021). This smaller size of the developing DRG in squamates should reflect either the recruitment to, or the proliferation of, trunk NCCs to the developing DRG (Goldberg et al., 2020). In a study using cross-sections of adult watersnakes (*Nerodina sipedon*), Cundall & Deufel, (2022) showed that DRG in snakes were not located in the dorsal root as in mammals, but more distally, outside of the neural canal (Cundall and Deufel, 2022).

Diaz et al. (2019) identify a population of NCCs condensing along the mesonephros of the veiled chameleon embryo, the location where the squamate adrenal glands develop (Rupik, 2002). Squamate adrenal glands are quite different from those of mammals. While mammal adrenal glands attach to the rostral ends of the kidneys (Hanemaaijer et al., 2021), squamate adrenal glands are separate from the kidneys, and rather associate to the gonads (Perry and Capaldo, 2011). Furthermore, mammal adrenal glands are divided into two spatially separated tissues, the steroidogenic adrenal cortex and the adrenergic and noradrenergic adrenal medulla. The medulla, but not the cortex, consist of NCC-derived chromaffin cells (Hanemaaijer et al., 2021). The degree of spatial separation between steroidogenic and adrenergic tissues varies considerably among reptiles and even within wall lizards (genus *Podarcis*) (Hebard and Charipper, 1955; Laforgia et al., 1991; Laforgia and Varano, 1982; Perry and Capaldo, 2011; Wright and Chester Jones, 1957). In the common wall lizard, the two tissues are largely intermingled, but some separation is found. Most chromaffin tissue is located in a thin “ribbon” along the dorsal surface of the adrenal gland with projections extending into the steroidogenic tissue where there are also scattered islets of chromaffin tissue (Fig. 6) (Capaldo, 2023; Laforgia et al., 1991). The degree of separation matters for chromaffin cell function since the enzyme PNMT which catalyzes noradrenaline into adrenaline is activated by glucocorticoids from the steroidogenic tissue. Therefore, species with more separation between the two tissues are hypothesized to have more adrenalin producing chromaffin cells relative to noradrenaline chromaffin (Laforgia et al., 1991).

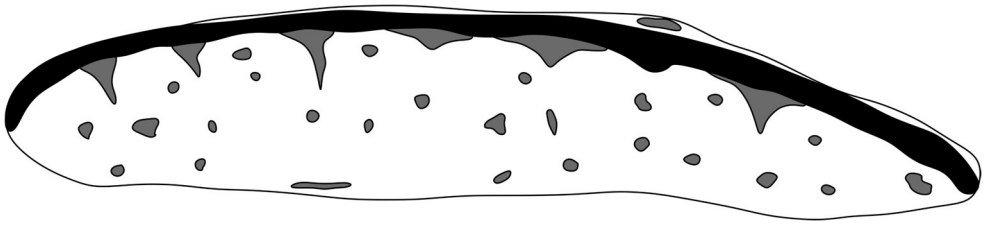


Figure 6. Adrenal gland in lacertid lizards.
Sagittal section through an adrenal gland. Chromaffin tissue (black and grey) are not located in a discrete internal zone such as the mammalian adrenal medulla. Instead, they are found in a "dorsal ribbon" with projections and islets scattered in the adrenal steroidogenic tissue. The illustration is an adaptation of a drawing of an adrenal gland from European green lizard *Lacerta viridis* in Wright and Chester Jones (1955). While there is considerable variation in adrenal gland morphology in lacertids, they are largely similar between *L. viridis* and common wall lizards (Capaldo, 2023)

The potential for NCCs to couple trait variabilities

Much of the attention that NCCs have received from evolutionary biologists has focused on a macro-evolutionary context (see Eames et al., (2020) for a review). However, recently their potential to generate micro-evolutionary variation has also received some attention (Brandon et al., 2023). This new attention was stimulated by the observation by Wilkins et al., (2014) that the covarying traits in the so-called "domestication syndrome" seem to share developmental origins in NCCs. The domestication syndrome is a pattern observed in domesticated animals first described by Darwin in *The variation of animals and plants under domestication* (Darwin, 1888). He realized that many domesticated animals share some trait states that were not observed in their respective wild counterparts. These traits include pale coat color, white blotches, reduced and floppy ears, short muzzles, small craniums and brains, curled up tails, reduced dentition, more frequent and less seasonal estrous cycles and reduced adrenal gland size. Similar trait changes have later been experimentally reproduced by simply selecting for "tameness", docility and/or the ability to live among humans in foxes, rats, minks, many other mammals and even some birds and fishes (reviewed in Arbuckle, 2002; Belyaev, 1969).

Wilkins et al., (2014) hypothesized a mechanism by which NCCs may have facilitated the evolution of this syndrome. They state that domestication was brought about through initial selection for tameness, which they traced mechanistically to a reduced size of the adrenal gland. They further stated that reduced adrenal gland activity alone is not enough to explain the other traits of the syndrome and added the observation that all these traits, including the adrenal gland, share developmental origins in NCCs. They therefore continued to hypothesize that the change in all these traits was brought about through "reduced NCC input". Reduced NCC input could be brought about through reduced induction, migratory ability, or proliferation of NCCs. These are quantitative traits most likely affected by a very large number of genes with alleles of minor effect. This is important as we know from the medical

literature on so-called “neurocristopathies” (NCC related congenital disorders) that mutations in the major NCC related genes result in dramatic and pleiotropic disease (Cerrizuela et al., 2022). While this hypothesis is clearly framed in the context of domestication, others have seen the potential in expanding it into non-domesticated animals (Brandon et al., 2023; Feiner et al., 2024; Marconi et al., 2022; San-Jose and Roulin, 2020).

Aims

As explained above, three independent lines of evidence suggest that NCCs are involved in the evolution of the nigriventris syndrome. This thesis describes NCC development in the common wall lizard and tests the hypothesis that all the characters that make up the nigriventris syndrome have evolved exaggerated states through upregulated NCC input. Such increased NCC input may have evolved as a response to sexual selection for aggression resulting in increased activity of the adrenal gland and sympathetic ganglia as well as knock-on effects on other NCC-derived structures such as increased melanization and larger heads. Increased NCC input can be brought about through increased induction, migration, proliferation and/or a changed cellular state of NCCs. Notice that this general hypothesis is largely the reverse of Wilkins hypothesis for how NCCs couple the traits in the domestication syndrome (Wilkins et al., 2014). Based on this general hypothesis, I derive four specific and testable predictions:

1. **Nigriventris embryos have higher numbers of NCCs.**
2. **NCCs from nigriventris embryos are more proliferative.**
3. **NCCs from nigriventris embryos are more migratory.**
4. **NCC-derived cell types in nigriventris embryos show altered gene expression compared to NCCs in ancestral embryos.**

To test these predictions, I use single-cell transcriptomics and flow cytometry to compare key cellular processes related to the hypotheses in NCCs collected from embryos exhibiting either nigriventris or ancestral phenotypes. However, very little was known about NCC development in squamates when this thesis started, and nothing at all in lacertid lizards.

Testing the specific predictions outlined above therefore required a detailed description of the NCCs in the common wall lizard. Accordingly, this thesis begins by describing the migration and distribution of NCCs in common wall lizards using spatial methods such as *in situ* hybridization and immunohistochemistry (Chapter 1). To allow the detailed study of expression profiles of NCCs at single-cell level, a method for isolating NCCs from whole embryos was developed, something that, to my knowledge, had not been implemented in any non-model organism. This method and its validation are described in Chapter 2 and are the basis for Chapters 3 and 4). Chapter 3 characterizes NCC differentiation and diversity using single-cell RNA-

sequencing (scRNA-seq), a task that has previously been accomplished in only a handful of well-studied model organisms (e.g., zebrafish, Howard et al., 2021; African clawed frog, Kotov et al., 2024; mouse, Soldatov et al., 2019; and chicken, Thiery et al., 2023). Only once these foundational descriptions were in place was it possible to rigorously test the hypotheses, which is presented in Chapter 4.

Methods

To study the development of NCCs in common wall lizard embryos, I have primarily used three methods which all rely on detecting specific cell types using various markers. These are *(1)* spatial methods such as *in situ* hybridization and immunohistochemistry, *(2)* flow cytometry, and *(3)* single-cell transcriptomics.

Animal husbandry and embryo collection

All embryos used in this thesis originated from a captive breeding colony of common wall lizards, established from individuals caught in the wild in central Italy in 2019. Adult lizards were maintained in cages according to conditions and methods described by Feiner et al., (2018a, and 2018b). One male and one or two females were kept in each cage. During the breeding seasons (April to July) of 2021–2023, eggs were collected daily from nesting pots filled with moist sand placed in each cage. Females lay 2–3 clutches per season with 2–10 eggs per clutch (Böhme, 1986). One egg per clutch was dissected immediately upon collection to assess the developmental stage of the clutch by counting somites. The remaining eggs were incubated in moist vermiculite at 24 °C until they reached the desired developmental stage. Based on 563 eggs, the average embryonic stage at the time of oviposition was estimated at 21.4ss (standard deviation: 5.2 ss), with the earliest embryos observed at 11ss. Somite formation proceeds at a steady rate of approximately four somites per day at 24 °C, as previously reported (Feiner et al., 2018b).

In situ detection methods

In situ detection methods (*in situ* hybridization and immunohistochemistry) were used to spatially resolve where in developing embryos different NCC-derived cell populations are at different stages of development. By staining ten selected markers of various NCC-derived cell types, the spatiotemporal distribution of NCCs was described in Chapter 1.

In situ hybridization stains cells based on their expression of certain mRNAs. This is done by exposing the tissue to a synthesized RNA-probe matching the chosen marker gene. Since the probe is the reverse complement of the targeted mRNA, they

will hybridize, binding the probe to the marker. After washing away the unbound probe, the tissue is exposed to an antibody that binds to small molecules incorporated in the backbone of the probe. The antibody is conjugated with an enzyme that catalyzes a reaction that precipitates a purple dye which can be visualized in a regular light microscope (Barresi and Gilbert, 2020). To do this, whole embryos were collected and fixed in 4% paraformaldehyde overnight to preserve tissue morphology. The fixed embryos were stored in methanol at -20°C. To synthesize the probe, total RNA was extracted from whole embryos and reverse transcribed to cDNA. The chosen marker was then amplified using PCR and transcribed back to RNA.

Immunohistochemistry stains cells based on their presentation of marker molecules such as proteins, lipids or carbohydrates by directly exposing the tissue to antibodies that bind to epitopes on the chosen markers. These (primary) antibodies are bound by a later batch of (secondary) antibodies, which are conjugated with fluorophores. These fluorophores can be visualized using a fluorescence- or confocal microscope. Immunohistochemistry with the antibody HNK-1, which recognizes a carbohydrate in the cytoplasm and cell membrane of NCCs, is one of the canonical ways to label NCCs and has been used in a wide diversity of taxa (Bronner-Fraser, 1986; Clark et al., 2001; Erickson et al., 1989; Giovannone et al., 2015; Goldberg et al., 2020; Hirata et al., 1997; Juarez et al., 2013; Kalcheim and Le Douarin, 1986; Kundrát, 2009; Olsson et al., 2002; Tucker et al., 1988; Vincent et al., 1983).

Flow cytometry and fluorescence activated cell sorting

Flow cytometry was used to identify NCCs in suspensions of dissociated cells. This was done to *(1)* enrich samples for NCCs using fluorescence activated cell sorting (FACS) and *(2)* quantify the relative abundance of NCCs in embryos.

Cell suspensions were achieved by mincing fresh embryos using a sterile scalpel followed by trypsin digestion. The dissociated cells were then stained using a Live/Dead stain to identify the cells that died during the digestion, followed by fixation using methanol. Fixed cells were stored at -80°C before further staining with the NCC-labelling antibody HNK-1 and the DNA stain 4′,6-diamidino-2-phenylindole (DAPI), which was used to discriminate singlets and doublets.

In flow cytometry, cells are passed in a buffer through a thin capillary. At the end of the capillary the buffer exits as sequential droplets, each droplet containing just one cell. Upon leaving the capillary the cell in the droplet is exposed to a laser. The light from the laser scatters, and the properties of the scattered light are detected and analyzed to ‘phenotype’ the cell. If the cells were labelled with a fluorescent marker, the marker is excited by the laser and emits light of a specific wavelength, which can be used to further phenotype and identify cells (Cossarizza et al., 2019). Furthermore, the phenotyping of cells can be used to sort them. This is done by

charging the droplet with static electricity based on the phenotype of the cell and then pull the droplet to one of two (or more) tubes using electromagnets. Alternatively, the droplets can be sorted into wells on a plate using a motorized stage. When flow cytometry is used to sort cells in this manner it is called fluorescence activated cell sorting (FACS).

Single-cell transcriptomics

There are several different platforms for single-cell RNA-sequencing (scRNA-seq); two different methods were used in the present thesis, 10X Chromium and Smart-seq3. Both are methods for the construction of cell-specific barcoded cDNA libraries that are later subjected to short-read sequencing. 10X Chromium uses an emulsion-based system and is optimized for high cell numbers, while Smart-seq3 uses plates and is optimized for deep sequencing of full-length transcripts. Either method starts from a solution of dissociated cells. In this work, FACS was used to enrich samples for NCCs prior to scRNA-seq.

10x Chromium uses a microfluidic system to encapsulate individual cells together with gel beads in oil droplets, forming gel beads-in-emulsion (Zheng et al., 2017). Each gel bead is coated with barcoded oligonucleotides that contain: *(1)* a cell-specific barcode (unique to that bead), *(2)* a unique molecular identifier (UMI) to label individual mRNA molecules, and *(3)* a poly(dT) sequence to capture polyadenylated mRNA. Within each droplet, cell lysis occurs, and mRNA is hybridized to the bead-attached oligos. Reverse transcription is performed in each droplet separately, incorporating the bead-specific barcode and UMI into the cDNA. After reverse transcription, the emulsion is broken, oil is removed, and cDNA from all droplets is mixed and amplified. The resulting barcoded cDNA is sequenced using a short-read platform (e.g., Illumina). Reads are aligned to a reference genome to assign gene identity, and the cell barcodes associate each read with a specific cell. UMIs help eliminate PCR duplicates. This results in a cell-by-gene matrix, where each entry represents the number of detected transcripts of a given gene in each individual cell.

Smart-seq3 is a plate-based full-length scRNA-seq method (Hagemann-Jensen et al., 2020). Individual cells are sorted into wells of a plate (typically 96- or 384-well plates), each pre-filled with lysis buffer, RNase inhibitors, and external RNA spike-ins (e.g., ERCCs). After cell lysis, reverse transcription is performed using poly(dT)-primers that bind to the poly(A)-tail of mRNAs, along with template-switching oligos (TSOs). The TSOs contain: *(1)* a partial Tn5 recognition motif, *(2)* an 11-base well-specific barcode, and *(3)* a UMI for each transcript. This enables UMI-based molecule counting and cell barcode-based demultiplexing. Following reverse transcription, the full-length cDNA is amplified by PCR. The resulting amplicons are then tagmented using Tn5 transposase, which fragments the cDNA and attaches sequencing adapters. The cDNA libraries from all wells are then pooled

and sequenced on a short-read platform such as Illumina. Reads containing the 5′ UMI tag and barcode are used to reconstruct transcript identities and assign them to individual cells. Full-length transcript information is recovered, and a cell-by-gene matrix is produced, incorporating UMI counts for accurate transcript quantification.

Results and discussion

Across four chapters, this thesis explores the developmental biology of NCCs in common wall lizards. To do so, a flexible method was developed for the collection of NCCs for scRNA-seq. Combining the results from all chapters provide a thorough understanding of the development of NCCs in common wall lizards, and suggest that changes in NCC regulation may be responsible for the expression of the nigriventris phenotypes.

HNK-1 reveals NCC migration in common wall lizards

The migration of NCCs was described by staining whole embryos using canonical NCC markers such as the antibody HNK-1 (Fig. 7). The antibody was conjugated to a fluorophore and the NCC distribution was visualized using confocal and fluorescence microscopy. The youngest embryos that could be collected from eggs after oviposition were around somite stage 13. At this stage, NCC migration was already ongoing in the head, and beginning in the anterior trunk. Thus, the study captured cranial NCC development from migration to differentiation towards facial mesenchyme and peripheral nervous system, and trunk NCC development from premigratory NCCs, via migration, to differentiating NCCs in the peripheral nervous system and adrenal glands. This work is part of Chapter 1.

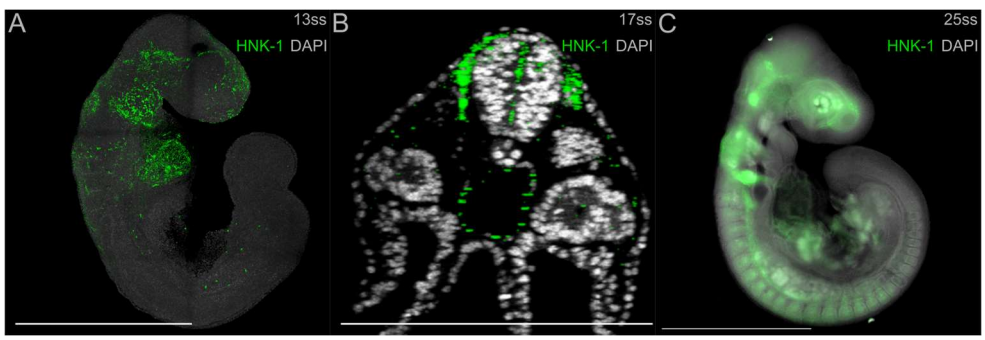
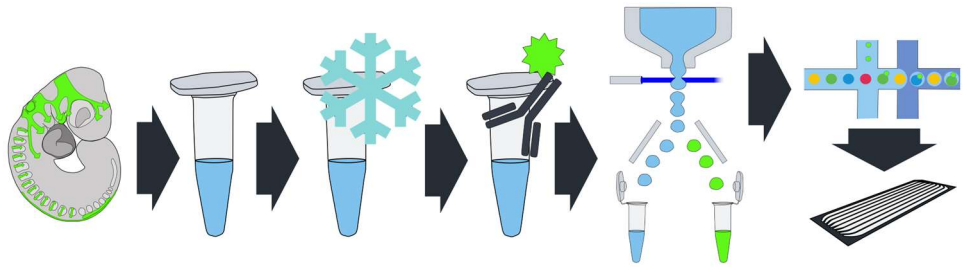


Figure 7. Common wall lizard embryos stained with HNK-1 antibodies.
The HNK-1 antibody binds to carbohydrates located in the cytosol and on the cell membrane of NCCs. Thus, staining developmental series of whole embryos in different developmental stages with HNK-1 visualizes the spatiotemporal distribution of NCCs. **A** Cranial and cardiac NCC migration visualized at 13ss. **B** Ventromedial migration of trunk NCCs seen in an optical cross-section through the trunk of a

17ss embryo. **C** Migration of trunk NCCs seen in the posterior trunk. Early differentiation towards peripheral nervous system is seen in the head and anterior trunk. Cell nuclei were stained with DAPI to visualize the overall anatomy of the embryo. Scale bars in A and C are 1 mm and the one in B is 0.5 mm.

NCC-collection by HNK-1 guided FACS

Drawing on the ability of HNK-1 to stain NCCs, we developed a method for the collection of NCCs for scRNA-seq using antibody guided FACS. In principle, this method can be applied to any vertebrate and consists of five steps: dissociation, fixation, optional storage, labelling and collection (Fig. 8A).



Figur 8. NCCs collection using HNK-1 guided FACS.
Outline of the method developed in Chapter 2. Whole embryos are dissociated and fixed using trypsin and methanol. Fixed cells can be stored at -80°C for several months. Staining the fixed cells with the NCC-binding antibody HNK-1 and sorting them using FACS, achieves a sample that is enriched for NCCs. This sample can then be prepared for single-cell sequencing. We validated the collection-method with two different scRNA-seq methods, one emulsion based (10X Chromium) and one plate based (Smart-seq3).

The dissociation of a whole embryo into a single-cell suspension is achieved by first mincing the freshly dissected embryo in a cold droplet of nuclease-free PBS, followed by trypsin digestion. The dissociated cells are then strained to remove any remaining doublets and dyed with the DNA-stain DAPI and a Live/Dead stain that stains the cytosol of dead cells.

To arrest the transcriptional activity and maintain the integrity of each cell, the suspension is fixed using ice-cold methanol. Once fixed, the suspension can be stored long-term in methanol at -80°C.

To identify NCCs among all suspended cells, the sample is exposed to the antibody HNK-1 to label NCCs. The fixed and stained cells are then analyzed using a flow cytometer. Debris and doublets are removed by filtering based on properties of the light scatter, and the DAPI signal. Cells that were dead before fixation are removed by filtering out the cells that are colored by the Live/Dead stain. This leaves just

single cells that were alive before fixation. The HNK-1 signal of the remaining cells is analyzed, and NCCs are collected from the upper distribution of this signal. Each step was validated using established techniques that are outlined in detail in a preprint (Pranter et al., 2025) and Chapter 2.

Three datasets were collected using this method and three different levels of 'strictness' in the sorting process were assessed. The first dataset consisted of 10,674 unsorted cells, representing a random sample of the whole embryo. The second dataset consisted of 3,036 cells collected above the 85th percentile of HNK-1 (FITC) signal, and the third consisted of 694 cells collected above the 95th percentile.

The effectiveness of the NCC enrichment was evaluated by comparing the three datasets with each other and with a published NCC dataset which was collected from transgenic mice and is of recognized high quality (Soldatov et al., 2019). The relative expression of the NCC marker *Sox10* (*Sox10*-UMIs per 1 million UMIs), was compared across datasets, and the sorted dataset with cells collected above the 95th percentile of HNK-1 signal was found to be at least as enriched for NCCs as the mouse NCC dataset. Similar results were achieved using three other NCC-markers (*Snai1, Snai2* and *Sox9*).

HNK-1 is a widely used antibody with documented NCC binding in a wide range of vertebrates. This new method should therefore facilitate the study of NCC development using scRNA-seq in a broad range of vertebrate taxa that lack transgenic tools.

NCC-development in common wall lizards

The newly developed method was used to generate a scRNA-seq dataset from common wall lizard consisting of 7,128 NCCs collected from common wall lizard embryos. The embryos originate from parents of the IT lineage, either with the nigriventris or the ancestral phenotype. We targeted three different developmental stages to capture different aspects of NCC development (nigriventris: 1,214 cells at 14ss, 1,531 cells at 21ss and 1,375 cells at 28ss; ancestral: 205 cells at 14ss, 1,384 cells at 21ss and 1,419 cells at 28ss).

To minimize variation, we first focused on cells derived from the nigriventris group and built a "cell atlas" that describes the diversity and development of the NCC-lineage. We selected nigriventris embryos since the sample of cells from ancestral embryos contained fewer cells at the earliest developmental stage. However, comparable diversity was later recovered in the full dataset by embedding and clustering all cells together.

The expression of selected marker genes was investigated in both the cell atlas, using uniform manifold approximation and projection (umap; Healy and McInnes,

2024), and in whole embryos, using *in situ* hybridization. This allowed the identity of the different cell clusters to be partially established. For example, the transcription factor *Snai2* was useful for identifying migratory and early differentiating NCCs (Fig. 9A-C) which are found in the largest group of cells in the umap (i.e. the 'supercluster'). Similarly, the complementary expression of mesenchyme-biasing transcription factor *Twist1* (Fig. 9G-I), and neuron-biasing transcription factors *Sox10* (Fig. 9 D-F), was informative to identify this fundamental bifurcation in the development of NCCs.

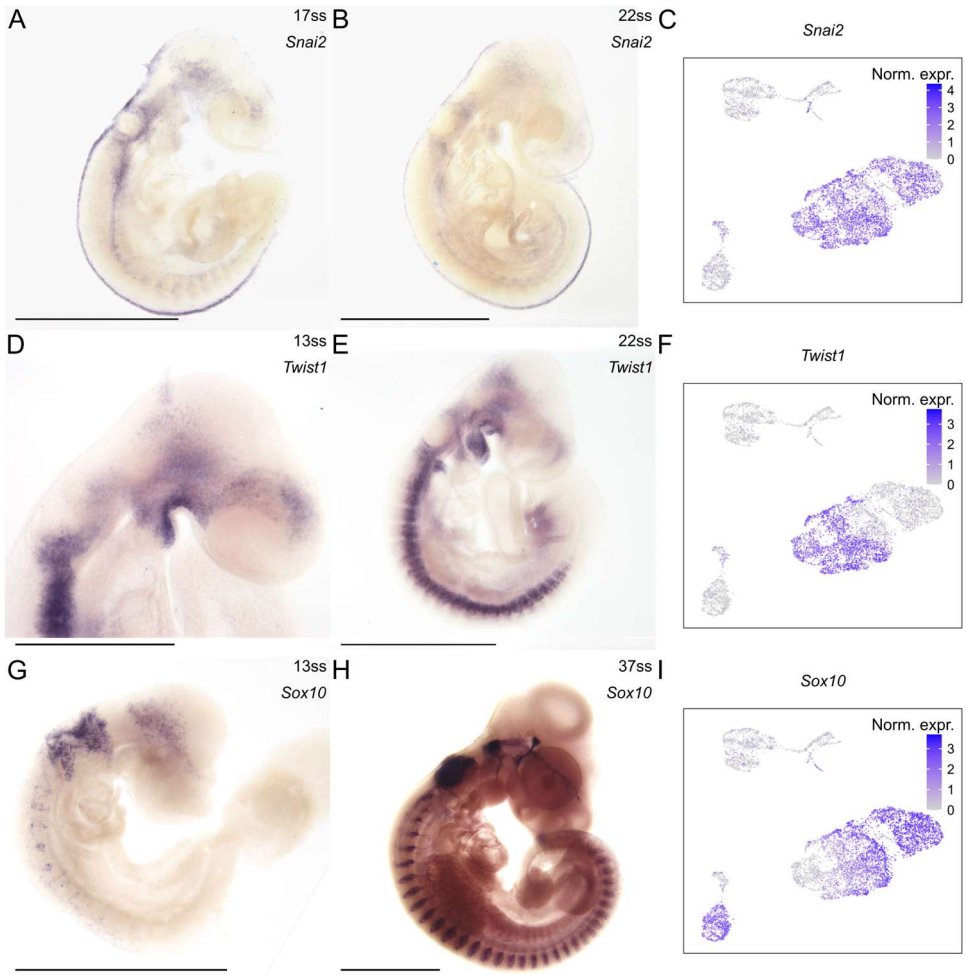


Figure 9. NCC marker gene expression visualized by *in situ* hybridization and scRNA-seq. The expression of three key NCC markers is visualized in whole embryos using *in situ* hybridization (left and center columns), and in umap visualizations of all 7,128 cells (right column). **A-B** Expression of the transcription factor *Snai2* is found in premigratory and migratory NCCs of both the head and the trunk. **C** *Snai2* expression is found in most cells throughout the entire large supercluster of the umap and in come cells in other clusters as well. **D-E** The transcription factor *Twist1* is expressed in migratory and mesenchyme-biased NCCs in the head. Some expression can also be seen in the trunk

(E), but this appears to be mesodermal cells. **F** In the single-cell data, *Twist1* expression is found in a subset of the *Snai1* expressing cells of the supercluster, suggesting that these are mesenchyme-biased cranial NCCs. **G-H** In early development, the transcription factor *Sox10*, is expressed in migratory NCCs in both the head and in the trunk, and later on it is expressed in the developing peripheral nervous system. **I** In the single-cell data, *Sox10* is expressed in a subset of the *Snai2* expressing cells of the supercluster, which is complementary to those expressing *Twist1.* This suggests that these are neuron-biased NCCs. Scale bar in D is 0.5 mm, all other scale bars are 1 mm.

To further narrow down the cell type identities of the cell clusters, this approach was complemented by extracting marker genes for each cluster by identifying differentially expressed genes between that cluster and the rest of the dataset. By reviewing the resulting marker genes, it was possible to annotate all clusters. The recovered cell types were neural tube cells, migratory cells, mesenchymal cells, autonomic neuron cells, sensory neuron cells and chromaffin cells, cardiac cells and otic vesicle cells (Fig. 10). These results are presented in Chapters 3 and form the basis for the analyses presented in Chapter 4.

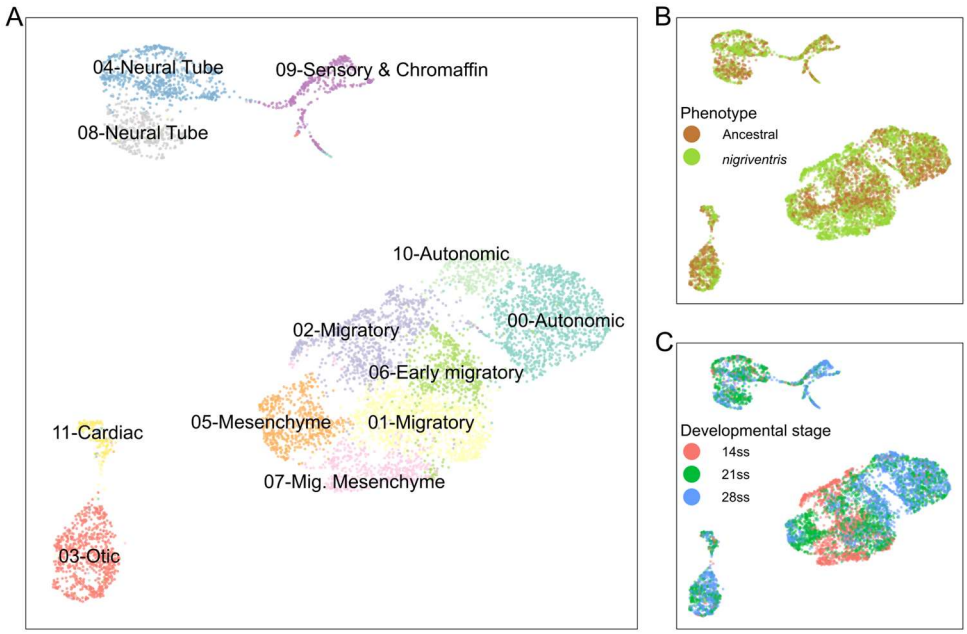


Figure 10. Transcriptomes from 7,128 NCCs collected from ancestral and nigriventris embryos. **A** Clustered and annotated cells plotted in umap. **B** The same umap as in A, but colored by phenotype (i.e., ancestral and nigriventris). **C** The same umap as in A, but colored by developmental stage.

The *in situ* hybridizations (9 different genes were analyzed), together with the immunostainings with HNK-1 described above, were also used to describe the spatiotemporal distribution of NCCs in common wall lizards (Fig. 11). NCC delamination and migratory onset progresses in a wavelike manner along the anterior-posterior axis. Cranial migration follows three main streams of migration

that later branch to populate the rostrum, maxillary process and branchial arches. In the trunk, migration follows a series of streams passing through the anterior half of each somite. Later in development, differentiation is observed towards cranial mesenchyme, peripheral nervous system and chromaffin cells. These results are published in Pranter & Feiner, (2024) and presented in Chapter 1.

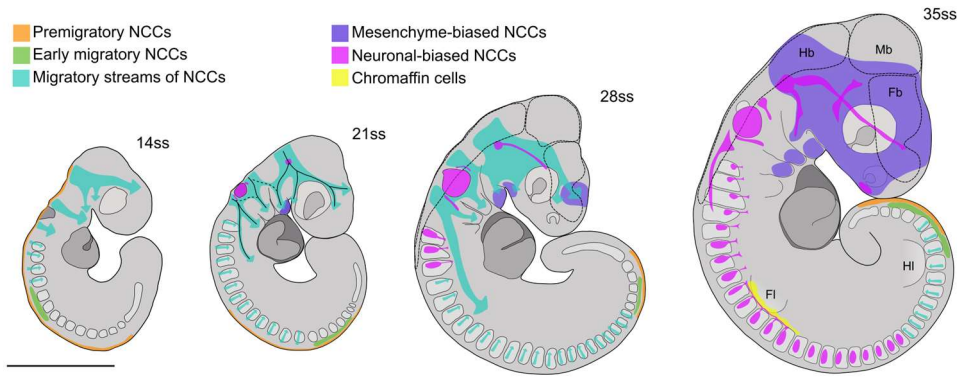


Figure 11. Spatiotemporal distribution of NCCs in common wall lizards
Schematic illustration of NCC delamination, migration and differentiation in common wall lizards. Premigratory NCCs are found along the dorsal neural tube. In a wave starting at the anterior end and progressing posteriorly the premigratory NCCs are induced to delaminate and begin their lateral and ventral migrations. In the head migration follows three main streams which later bifurcate to populate various regions. In the trunk migration can be seen in multiple consecutive streams passing through each somite. In later stages some differentiation towards fates such as mesenchyme, neurons and chromaffin cells is detected. The scale bar is 1 mm.

Hyper-function of NCCs in nigriventris embryos

With a foundational description of common wall lizard NCC development in place, this process can be compared between NCCs collected from embryos of the nigriventris and ancestral phenotypes. Two large datasets were used, one flow cytometric and one single-cell transcriptomic (the atlas described above). The datasets were used to test the hypothesis that the nigriventris syndrome is the result of increased "NCC input". The predictions derived from the hypothesis, that: *(1)* nigriventris embryos should have more NCCs, *(2)* NCCs from nigriventris embryos should be more proliferative, *(3)* NCCs from nigriventris embryos should be more migratory, and *(4)* NCCs from nigriventris embryos should have an altered transcriptional profile compared to NCCs from ancestral embryos were tested one by one.

The flow cytometric data (5,220,110 cells) was used to test if cells from nigriventris embryos had higher proportions of cells with high signal from the NCC marker HNK-1 (FITC). By comparing the signal intensity at the 95th percentile of 21 zero-centered distributions, we found a marginal effect in support of the hypothesis.

However, this experiment should be expanded with increased sampling, especially of ancestral-phenotype embryos in early developmental stages.

The scRNA-seq data was used to test if the proliferation of NCCs differed between cells collected from the two phenotypes. Two cell cycle scores were calculated representing the likelihood of each cell to be in either the S- or the G_2/M-phases of the cell cycle. The S-phase (the synthesis phase) is when the cell replicates its genome, and the G_2/M-phases (the gap two and the mitotic phases) are when the cell prepares for cell division and when it divides. The cell cycle scores were calculated from each cell's expression of marker genes for these phases (Tirosh et al., 2016). Cells that are not actively cycling should score low on both of these scores. Nigriventris cells were found to score significantly higher than cells from embryos of the ancestral phenotype (S-score $p = 0.033$, G2/M-score $p < 0.001$; Fig. 12), which implies that NCCs collected from nigriventris embryos are more proliferative than NCCs collected from embryos of the ancestral phenotype.

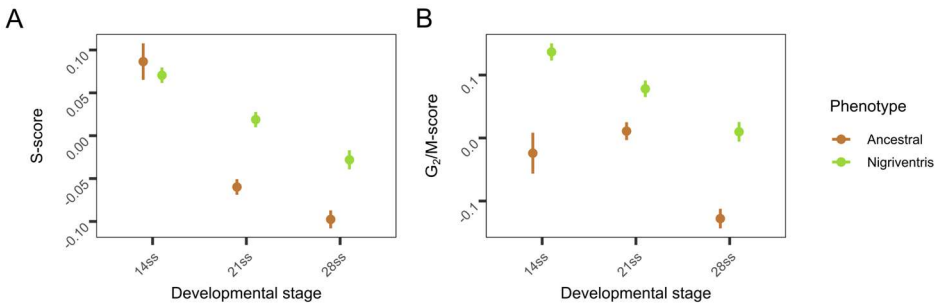


Figure 12. Cells collected from nigriventris embryos are significantly more proliferative. Cell cycle score by developmental stage and phenotype. Means are plotted as points and the bars show standard errors of the means. **A** S-scores and **B** G_2/M-scores.

To test if NCCs collected from nigriventris embryos were more likely to be in a migratory state, each cluster in the scRNA-seq data was reclassified to either 'premigratory', 'migratory' or 'postmigratory'. Across developmental stages, cells from nigriventris embryos were overrepresented in the migratory category, meaning that NCCs collected from nigriventris embryos were relatively more migratory than NCCs collected from embryos of the ancestral phenotype.

Finally, the differential gene expression between ancestral and nigriventris derived cells was analyzed cluster by cluster. In total, this identified 1,816 differentially expressed genes (DEGs). Only five DEGs overlapped with the list of genes that was previously found to be highly differentiated between nigriventris and ancestral phenotypes in genomic scans (Feiner et al., 2024). This was not significantly more than expected by chance. However, in clusters with high numbers of DEGs, an overwhelming majority of those DEGs had higher expression in nigriventris cells. Taken together, these results support the hypothesis that hyper-function of NCCs

cause the nigriventris phenotype, specifically in terms of cell abundance, cell migratory abilities and cell states.

To build on this work, future studies should focus on mechanistically establishing causal differences in the developmental processes of NCCs that led to the evolution of the nigriventris syndrome. It would also be valuable to add additional populations of embryonic cells to the comparison in order to confirm that the developmental differences are really a property of the NCCs and not of the entire embryo.

Conclusions

As one of the main model systems to study stem cell biology (Le Douarin & Kalcheim, 1999), NCC development has recently been studied with great success using modern technologies for scRNA-seq (e.g., Soldatov et al., 2019; Thiery et al., 2023). In the meantime, evolutionary developmental biologists have become increasingly interested in the role that NCCs may play in adaptation and diversification (Brandon et al., 2023; Feiner et al., 2024; Wilkins et al., 2014). However, the application of scRNA-seq to NCC development in most organisms has been held back by the lack of methods for the collection of NCCs that is independent of established transgenic lines or *in ovo* manipulation, limiting analysis to a handful of model organisms (Jacobs-Li et al., 2023; Kotov et al., 2024; Soldatov et al., 2019; Thiery et al., 2023).

The novel method for the collection of NCCs presented herein (Chapter 2), which is, in principle, applicable to any vertebrate, is therefore a significant step forward for the field. I want to put this into context by offering two examples from the field of squamate evolutionary developmental biology. The recently developed protocols for CRISPR based gene editing of lizards (Rasys et al., 2019) and the establishment of immortalized lizard cell lines (Samudra et al., 2024). Such methods can facilitate the study of complex developmental biology in organisms where this was not previously possible. It is my hope that our protocol for the collection of NCCs will contribute to the growing body of methods that facilitate a move beyond the few well-established model systems that are typically used in developmental biology.

Prior to this thesis, investigations into squamate NCCs were limited to four studies tracking NCC migration using the HNK1 antibody (Diaz et al., 2019; Khannoon et al., 2021; Reyes et al., 2010; Weberling et al., 2025), and a few studies specifically looking at the NCC-derived chromatophores in the skin (Feiner et al., 2022; Tzika et al., 2024, 2023). The thorough investigation and description of the migration, differentiation and diversity of NCCs in the common wall lizard presented in Chapters 1 and 3 of this thesis, therefore significantly increase our knowledge of this stem cell population in squamates. The developmental biology of squamates is interesting in itself, and for evolutionary biology this is also valuable as a rare point

of comparison for understanding the evolution of NCCs across the vertebrate phylogeny. Future studies should leverage this comparative potential, for example by using methods such as SAMap (Tarashansky et al., 2021) to trace cell type evolution across the vertebrate phylogeny.

The final and probably greatest contribution of this thesis is the novel application of hypothesis-testing to study how the developmental process may influence microevolution. It does so by testing the hypothesis, presented more than a decade ago that NCCs, by bias phenotypic diversification, are responsible for the evolution of some phenotypic syndromes (Brandon et al., 2023; Wilkins et al., 2014). By presenting support for the involvement of NCC hyper-function in the evolution of the sexually selected nigriventris syndrome (Chapter 4) this thesis provides an empirical example of how the developmental process influences evolutionary trajectories.

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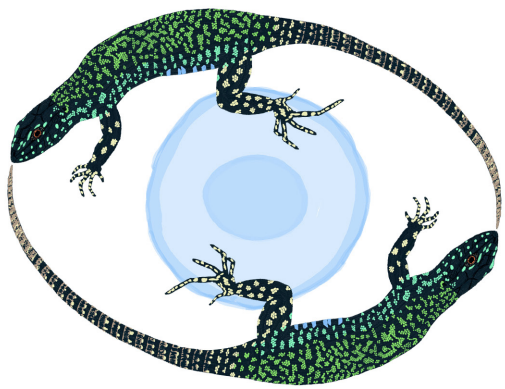
List of papers

I. Pranter, R. and Feiner, N., 2024, Spatiotemporal distribution of neural crest cells in the common wall lizard Podarcis muralis. Developmental Dynamics, https://doi.org/10.1002/dvdy.758

II. Pranter, R., Patthey, C. and Feiner, N., Enrichment of neural crest cells by antibody labelling and flow cytometry for single-cell transcriptomics in a lizard. Manuscript submitted to Evolution and Development. Preprint available on bioRxiv https://doi.org/10.1101/2025.05.21.655068

III. Pranter, R., Patthey, C. and Feiner, N., An atlas of neural crest cells in a lizard at single-cell resolution. Unpublished manuscript.

IV. Pranter, R., Uller, T. and Feiner, N., The role of neural crest cells in the evolution of a sexually selected syndrome. Unpublished manuscript.



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