On the origin of 'bloopergenes': unraveling the evolution of the balanced lethal system in Triturus newts

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Triturus *newts in a glass cuvette*

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Chapter 1 - General introduction

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Evolution by natural selection is the theory in biology that explains the origin and diversity of all life on earth [6, 7]. The process of evolution, in which natural selection favors traits that enhance the survival and reproductive output of individuals in populations, is inherently not goal-oriented, nor predetermined [8, 9]. Despite this, my observation is that adaptations are often categorized as evolutionary ‘successes' or evolutionary 'failures’, depending on their relative fitness consequences [10, 11]. Strictly speaking, this is a distinction which I think translates the randomness of the evolutionary process into understandable, human-defined constructs (since nothing in evolution is truly an achievement or a blunder: there is no end goal). Nevertheless, I will use such definitions throughout this dissertation, as it would be very hard to discuss evolutionary mechanisms otherwise.

Evolution is thoughtless, lazy and imperfect

While evolutionary successes are broadly recognized and comprehended, evolutionary failures remain misunderstood, understudied, and likely overlooked in nature [6, 10, 12, 13]. Natural selection acts on the phenotypic level: it works against individuals that are less fit to their environment, and it works in favor of individuals that are more fit to their environment [6, 7, 11]. In other words: the individuals with so-called favorable phenotypes are the ones that are more likely to survive, reproduce, and pass on their genetic makeup to the next generation (i.e., they have a higher fitness), compared to individuals with unfavorable phenotypes [14, 15].

Today it is known that, in sexually reproducing diploids that generally carry two gene copies, the mechanism of inheritance often leads to a change in frequency of the occurrence of heritable traits over the course of successive generations [16]. This shift is influenced not only by the works of natural selection, but also by the (combined) works of genetic drift, recombination, gene flow, and mutation – all of which influence the genetic variability necessary for adaptive potential [15, 17]. Overall, the favorable mutations, or more specifically the favorable ‘alleles’ (gene variants), are positively selected for, whereas deleterious (including lethal) alleles are selected against. This is referred to as directional and purifying selection, respectively [11, 14, 18].

The effect of purifying selection in particular is most strong when an unfavorable allele appears lethal, especially in the pre-reproductive phase as it prevents an individual from reproducing altogether. Therefore, dominant lethal alleles (of which only one needs to be present in a diploid organism to be expressed phenotypically) are hardly detected in nature because of their rapid elimination from the gene pool (Fig. 1A). Recessive lethal alleles, on the other hand, can only be negatively selected against if their frequency in the population is high enough that they start to appear in a homozygous state [i.e., they will

only be phenotypically expressed if a diploid organism carries two similar copies of them; 11, 14, 15]. Thus, these are the types of alleles that are easily purged in populations that contain a relatively high frequency of them, but that can really only be eliminated to the point that these mutations must linger on within the population at very low frequencies [assuming an infinite population size, or more particularly, assuming the absence of an effect of genetic drift; 15, 19, 20]. This is because they will remain concealed by the heterozygous masking of the carriers, meaning that - theoretically - the frequency of recessive, harmful alleles is subdued by natural selection, but can never reach zero (Fig. 1B). One would think that, under such natural laws that automatically lead to the suppression of disadvantageous variants and to the enhancement of beneficial ones, nature will always end up 'having the upper hand' in the long term. In other words: nature would not allow genetic anomalies or genetic diseases (i.e., evolutionary failures) to prevail in a population, at least not on abundantly. However, by means of this dissertation, I intend to show you that this is not necessarily true.

To get straight to the point: the most remarkable example of an evolutionary failure is an extremely deadly one. Imagine an extraordinary situation in which a diploid organism possesses two different versions of a chromosome with unique, recessive lethal alleles that are compensated for by the functioning gene copy on the other chromosome version (Fig. 1C). In such a situation, natural selection will be unable to purge these harmful alleles and will reach what could be considered an 'impasse', as on these terms all homozygous offspring are inviable, whereas all heterozygous offspring depend on both chromosome versions for their viability. Subsequently, these heterozygous individuals grow into adults and reproduce, repeating the cycle. This is called a 'balanced lethal system' [21]: a genetic anomaly which will always lead to the reproductive output being cut in half. While the maintenance of such a system is relatively easily explained in theory, the details on the origin of it are all the more puzzling (I will arrive at the details on this later).

In the grand scheme of things, a balanced lethal system can only be the result of evolution by natural selection being a thoughtless, lazy, and imperfect process. As the famous biologist Richard Dawkins described: *"Natural selection, the blind, unconscious, automatic process which Darwin discovered, and which we now know is the explanation for the existence and apparently purposeful form of all life, has no purpose in mind. It has no vision, no foresight, no sight at all. If it can be said to play the role of watchmaker in nature, it is the blind watchmaker"* [22]. The most famous example of a balanced lethal system has been described in the wild, in a specific group of amphibians, and forms the basis of this research dissertation.

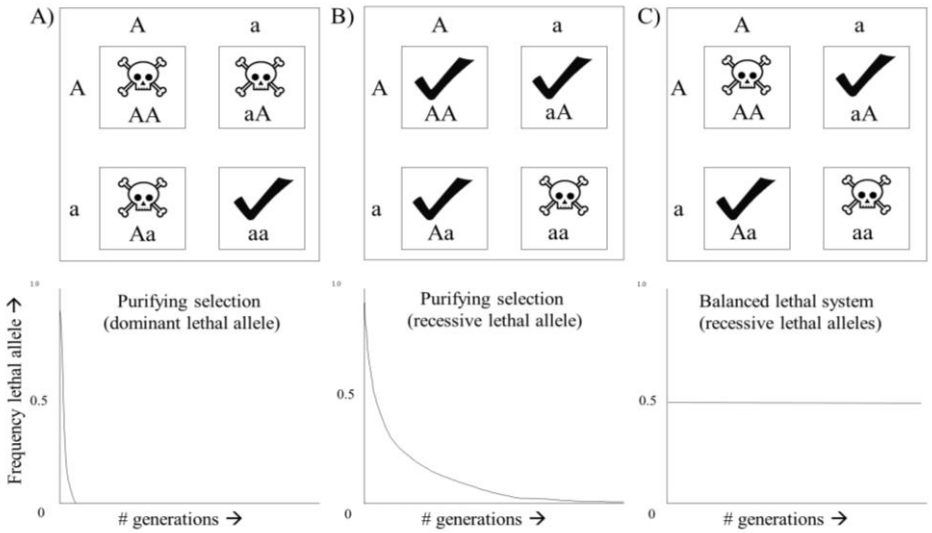

Figure 1: *Schematic representations of the frequency of harmful alleles over a certain number of generations under three different scenarios observed in nature (assuming infinite population sizes). Top: Punnett squares. Bottom: Allele frequency graphs.* ***A)*** *Purifying selection causes dominant, lethal alleles to be quickly eliminated from the gene pool (hence, they are hard to detect).* ***B)*** *Purifying selection causes recessive lethal alleles to be purged as much as possible (however, in theory they could never reach a frequency of zero).* ***C)*** *A balanced lethal system is an exceptional situation in which only heterozygotes are viable and natural selection is prevented from purging the lethal alleles (which will be maintained in the population at a frequency of 0.5).*

Of all vertebrates, amphibians are natural outcasts

Amphibians form a diverse group of vertebrates with traits that have fascinated scientists for centuries. They have clearly been evolutionarily successful in adapting to both aquatic and terrestrial environments, hence their name ("amphibian" comes from the ancient Greek word "*amphibios*," which means "both kinds of life" or "living a double life"). For example, they are generally able to respire through their lungs, as well as through their skin [23, 24]. They also show various defense strategies, an exceptional example being the epimorphic regeneration abilities displayed by some species after losing a limb, tail, or even organ, in order to avoid predation [25-27]. Despite these evolutionary successes, amphibians belong to one of the most vulnerable vertebrate groups when it comes to anthropogenic environmental change and pollution [28-31]. Semi-aquatic salamanders, for example, are particularly susceptible to habitat changes due to their limited dispersal abilities [32, 33]. This vulnerability has led to some surprising adaptive behaviors, such as

newts hibernating in unconventional places such as waterfowl nests [observed in the canals of Leiden, The Netherlands; 34]. However, much remains unknown about the mysterious terrestrial lifestyle of pond-dwelling salamanders, including their land use and hibernation habits [although a variety of methods are tested nowadays to research this, one being canine detection; 35, 36-39].

Another remarkable trait of salamanders that I would like to highlight (before I will explain more about the evolutionary mystery that is the balanced lethal system), is their genomic gigantism. Compared to other animals, salamanders have incredibly large genomes that are full of non-coding repetitive DNA and that, depending on the species, can be over ten times the size of that of a human [40-45]. So far, it seems that natural selection has simply tolerated the genomic expansion observed in the clade of the salamanders (a result of neutral evolution). However some studies do suggest that the bigger the genome of a salamander, the less successful it is at limb regeneration – even while it is simultaneously suggested that larger genomes delay cellular differentiation more effectively, which should actually facilitate the regenerative process [45, 46]. Clearly, this is paradoxical, and scientists have yet to decide whether genomic gigantism in salamanders can be considered an evolutionary success, an evolutionary failure, or perhaps something in between. One thing scientists have agreed on, however, is something that I as a salamander scientist can fully stand by: these gigantic genomes are a hurdle to investigate in-depth (a topic I will revisit later in this dissertation).

Triturus*: survival of the ~~fittest~~ *least doomed

At this juncture, I would like to revisit balanced lethal systems. Among all amphibian peculiarities, the most striking one has to be the balanced lethal system found specifically in the crested and the marbled newts of the genus *Triturus* [21, 47-49]. Known also as 'chromosome 1 syndrome', this condition in *Triturus* newts involves two distinct versions of chromosome 1 - the longest of the 12 pairs that they possess – which has been linked to extreme egg mortality rates [48, 50]. As visualized in Fig. 1C, this genetic syndrome causes half the offspring to succumb in the face of natural selection, regardless of the environmental circumstances [47, 48, 50, 51] – something that could be considered the biggest evolutionary failure of all. However, despite seeming unnatural, balanced lethal systems are observed in some other organisms besides *Triturus* newts as well, such as in certain species of fruit flies [52], flour beetles [53] and flowering plants like the rock isotome [54], sundrop [55], boat lily [56] and the evening primrose known as 'groundsmoke' [57]. In scientific literature, however, the example of *Triturus* newts is the most famous, as it is described most recently and in greatest detail as compared to the other cases known.

The case has puzzled scientists for at least two hundred years. For instance, the zoologist Mauro Rusconi already described this phenomenon in his book '*Amours des Salamandres Aquatiques*' as follows: *"…although this embryonic stage is a pleasure to look at for the researcher, it is dangerous for the little creatures themselves, because almost precisely half of them die …"* [freely translated from French; 58]. Here, Rusconi explains that half of all *Triturus* embryos perish while still inside the egg, and he refers to the specific moment in the embryonic development that is detrimental (more on that later).

Because of their high fitness disadvantage, balanced lethal systems seem to defy evolutionary theory. The selective removal of deleterious variants that occurs through the act of natural selection is usually important to rid natural populations of such detrimental fitness effects [6, 59]. Thus, in the light of evolution it is expected that a balanced lethal system can never become fixed, as individuals that do not carry the balanced lethal system at the moment of origin would simply outperform carriers. Also, any emerging balanced lethal system would, in theory, be broken down before fixation, as sexual reproduction is associated with allele segregation and recombination [11, 14, 60, 61]. Especially the effect of recombination in the homozygotes would lead to a disconnection of lethal alleles on both chromosome forms, causing the balanced lethal system to evanesce instantly – which implies that recombination must be suppressed somehow [21]. How exactly this works, is explained in the remaining Chapters of this dissertation, but the crux of the matter is as follows: the genetic structure of a balanced lethal system renders selection against it impossible, because of the compensating effects of the two chromosome versions [62-64], and this leads to an 'eternal' wasting of 50% of the reproductive output. A huge failure, that does not appear to follow the winning '*survival of the fittest*' principle, but rather a losing '*survival of the least doomed*' fallacy, and it makes one wonder: how can something this wasteful originate in nature?

The (brutally) costly courtship & oviposition of *Triturus* newts

The geographical distribution of *Triturus* species ranges from western Europe to western Asia [see Fig. 2; 1, 2]. Currently, ten *Triturus* species are recognized [see Table 1; 2]; the Anatolian crested newt (*T. anatolicus*), the Italian crested newt [*T. carnifex*, comprising of two diverged populations that might be different species, but have so far not been described as such; 4]), the northern crested newt (*T. cristatus*), the Danube crested newt (*T. dobrogicus*), the Balkan crested newt (*T. ivanbureschi*), the southern crested newt (*T. karelinii*), the Macedonian crested newt (*T. macedonicus*), the marbled newt (*T. marmoratus*), and the pygmy marbled newts [*T. pygmaeus* and the newly described *T. rudolfi*; 5]. The most recent common ancestor of *Triturus* is thought to have lived around ± 24 million years ago [1, 2, 65], suggesting that the balanced lethal system originated also

that long ago, as all known *Triturus* species suffer from chromosome 1 syndrome.
In all species of *Triturus*, reproduction starts at about 2-4 years of age [33], and it is a costly process. Every year during the aquatic phase the males develop a crest, which is highly denticulated in crested newts, while smooth in marbled newts [66]. They use this breeding 'cloak' (see Table 1) to impress the females under water in an elaborate courtship dance [described in detail in; 67]. Once a male and female *Triturus* newt meet in a breeding pond, the male immediately starts dancing: while doing this, he not only grasps her attention, but he also spreads his pheromones her way [66].

The female ultimately makes a choice: if she is not in the mood, she swims away, whereas if she is interested, she calmly waits [67]. This initiates the next stage of the courtship dance: the display. The display includes several, alternating behaviors performed by the male, such as 'rocking and whipping' [shifting between lashing out with his tail and doing a handstand kind of pose; 67], 'leaning in' [hanging over the female, showing off the shapes and colorations of his body; 33, 67] and "cat buckling" [arching his back, much like a cat does when stretching or when threatened; 67]. If the female ultimately touches his tail with her snout, the male releases a spermatophore which the female will then take up with her cloaca to fertilize her eggs internally [66, 67].

Figure 2: *Map showing the general distribution of the species of* Triturus *[adjusted from; 1, 2]. The pins refer to the populations that the initial founders of the breeding lines of which I use samples throughout the studies included in this dissertation, originated from. Note that two genetically distinct populations of* T. carnifex *are included [4], and that* T. rudolfi *was described only in 2024 as a new species [5] and therefore is not shown here, nor included in the studies of this dissertation.*

After the male has done his fair share of hard work, the female takes over: she will carefully lay her fertilized eggs – one by one – in between the leaves of the water vegetation [33, 50]. This oviposition strategy, in which she uses her hind limbs to fold, wrap, and press the

leaves around each egg, presumably serves to spread out and conceal the offspring as to hide them from external forces and predators and thus maximize fitness [and also, no further parental care exists; 67]. A *Triturus* female may lay around 200 eggs to 400 eggs in this manner per breeding season [33, 67]. It is, in a way, brutal that half of those eggs – in which both parents have invested so much resources – are not even viable.

Table 1: Species affected by chromosome 1 syndrome (genus *Triturus*). The pictures are taken by collaborator Mr. M. Fahrbach and shows the adult male morphology during the breeding season for each species [except the recently described *T. rudolfi*; 5].

Phenotype	**Common name**	**Scientific name (*Triturus*)**
	Anatolian crested newt	*T. anatolicus*
	Italian crested newt*	*T. carnifex*
	Northern/Great crested newt	*T. cristatus*
	Danube crested newt	*T. dobrogicus*
	Balkan crested newt	*T. ivanbureschi*
	Southern crested newt	*T. karelinii*
	Macedonian crested newt	*T. macedonicus*
	Marbled newt	*T. marmoratus*
	Pygmy marbled newt	*T. pygmaeus*
Not available in this series	Pygmy marbled newt	*T. rudolfi*

** Note that, in some research Chapters of this dissertation, two genetically distinct* T. carnifex *populations are included [4].*

Embryonic arrest occurs in half of all *Triturus* eggs

The two versions of chromosome 1 that *Triturus* newts possess are known to be morphologically distinct [48, 68], where in one version (called '1A') the long arm is longer than in the other version (dubbed '1B'). No chiasmata are formed between the heteromorphic regions and these do not recombine [68, 69]. Individuals that are heterozygous for the two versions survive, whereas both types of homozygotes die during the late tail-bud stage of embryonic development [51, 69-72]. In existing literature, 1A1B (/1B1A) embryos are classified as 'viable', 1B1B embryos as 'fat-tailed', and 1A1A embryos as 'slim-tailed'. These labels are based on supposed differences in tail-bud stage morphology, where fat-tailed embryos would show anomalies, whereas both slim-tailed and viable embryos would appear normal in shape [47, 51, 72].

Embryonic development is driven by strict processes that regulate pattern formation, morphogenesis, cell differentiation and growth [73]. Before homozygote *Triturus* embryos fall victim to the deadly chromosome 1 syndrome, they seem to successfully complete the embryogenic phases of cleavage, gastrulation and neurulation, although a yolk plug seems to interfere with the caudal fusion of the neural folds in some embryos [69, 71, 72].

But then, when the first organs start to develop (a phase called 'organogenesis'), more problems arise. Organogenesis is divided into what is called the 'tail-bud stage' and the 'larval stage', the latter being the last stage to complete before finalizing organogenesis and proceeding towards hatching [51, 71, 74]. During the early tail-bud stage, development of inviable embryos slows down and arrest occurs during the late tail-bud stage in all species, including interspecific hybrids [48, 51, 70-72].

Embryonic arrest kicks in during the phylotypic stage, an ontogenetic period that shows the highest level of conservation among vertebrates (Fig. 3), and is typically referred to as the thin part of the 'hourglass model' [75]. This is the ontogenetic period during which the basic body plan is laid out [73, 75-77], and it corresponds to the tail-bud stage of *Triturus*. The most apparent embryonic changes that take place during the tail-bud stage are the prolongation of the notochord, the development of somites, and the generation of the neural tube [72-74].

Although amphibian embryos are often used as a model to study developmental biology in general and to learn more about the mechanisms behind limb generation [27, 45, 78-81], nobody so far has investigated the mutations that cause chromosome 1 syndrome, and how these could have possibly evolved.

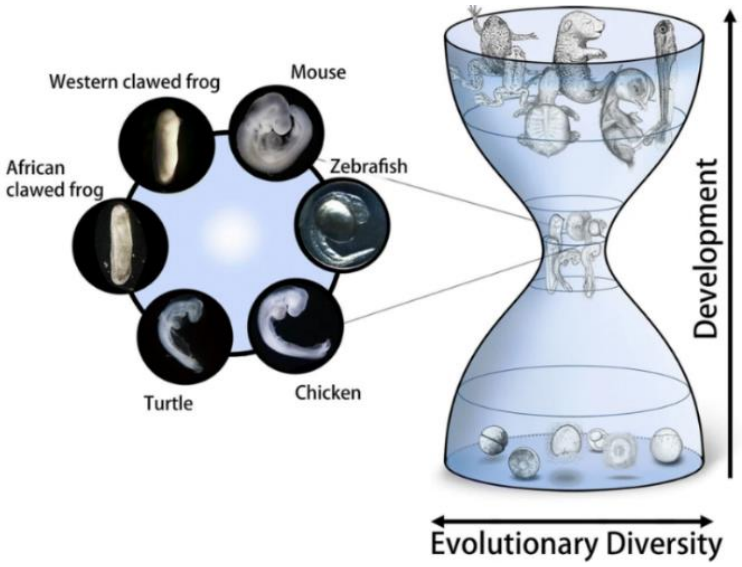

Figure 3: *The 'evo-devo hourglass model' representing embryonic development. In the midst of embryonic development, i.e. in the so-called phylotypic stage, vertebrate embryos morphologically look most similar (as opposed to earlier and later stages). During this highly conserved stage (represented by the thin part of the hourglass) embryos of all vertebrate species (example photos on the left) acquire a similar, phylum-wide body plan. It is during this stage that 50% of* Triturus *embryos succumb. [re-used image, published before by 3 under the terms of the Creative Commons Attribution 4.0 license - http://creativecommons.org/licenses/by/4.0/. The image was cropped in order to show only the photos and the hourglass visualization].*

Two hypotheses: investigating the roles of supergenes & introgression

Suppressed recombination in combination with heterozygote advantage could paves the way for the origin of a balanced lethal system [21, 63, 64]. When chiasmata are unable to form on a chromosome, for example due to chromosomal rearrangements, no physical link can be made between chromatids and cross-over of DNA is inhibited [82]. In case linkage between loci is extremely tight, supergenes comprising of multiple genes can be formed [83]. Supergenes are named as such because the linked genes are inherited together, in stretches of DNA that often do not undergo recombination and thus evolve independently of one another, which can rapidly lead to complex adaptations [82, 84-86]. Thus, supergenes may contain a set of advantageous genes and an individual can be polymorphic (i.e., can have two different versions of the supergene) if different combinations of such alleles lead to unique, advantageous outcomes – which causes balancing selection [83, 85, 87].

In case of high balancing selection and suppressed recombination, purifying selection is extremely low and deleterious mutations are accumulated rapidly by genetic drift due to Muller's Ratchett [21, 63, 85, 88], and this could continue to the point of homozygous lethality. This theory, which states that a balanced lethal system must comprise of two supergenes that got caught in a balanced polymorphism and that degraded over time due to a lack of recombination, is the working theory that was adopted by the research team of the Wielstra lab in 2019, and that forms the basis of the work presented in this dissertation: the balanced lethal system in *Triturus* posed the perfect case study to investigate this more in-depth, using modern, molecular tools. All that is necessary to figure out more about the evolution of the balanced lethal system in *Triturus* is to either use, or invent, methods that can detect the genetic differences between genomic data of heterozygote versus homozygote *Triturus* individuals, and methods that can map the differences between the DNA of *Triturus* and that of closely related, unaffected salamanders, for instance the smooth newts [of the sister genus *Lissotriton*; 89]. To summarize: all that I have to do is to, somehow, bring to light the genetic variation underlying chromosome 1 syndrome.

As mentioned before, the existence of genetic variation is paramount in nature and can be seen as the ultimate cornerstone of evolution [6, 7, 11, 15, 90, 91]. This is why it is studied so broadly, meaning that I will have (at least some) tools at my disposal to study the DNA of *Triturus*. Hybridization and introgression are currently being increasingly recognized as a source of adaptive variation in natural populations [92, 93]. In short, hybridization occurs when separated populations that are in the midst of a speciation event are still able to sexually reproduce viable offspring [94, 95]. Hence hybrids, intermediate varieties of both populations or species, are formed. In case hybrids are able to backcross with one of the parent species over many generations, introgression (also called introgressive hybridization) can take place, whereby alleles from one species are incorporated in the gene pool of the other [96, 97]. Introgression is sometimes facilitated by the act of balancing selection, which occurs as a natural result of adaptation in diploids [83, 92, 98].

Interspecific gene-flow between the *Triturus* ancestor and ancient ancestors of extant genera could have brought two uniquely adapted supergenes together into one genome. Subsequently, this could have kickstarted balancing selection in case this *Triturus* ancestor somehow benefitted from having these two forms, in turn initiating the gradual 'supergene degradation' process.

Examples of supergenes that have originated through transmission from one species into another by hybridization and introgression are becoming increasingly apparent. For example, in white-throated sparrows (*Zonotrichia albicollis*) a supergene, currently maintained in the species due to a strong, disassortative mating system, likely originated through introgressive hybridization in the past [99]. This also appears to be the

case in the Numata longwing butterfly (*Heliconius numata*), where the introgression of a divergent and inverted DNA segment has resulted in a balanced, mimicry polymorphism maintained by negative frequency-dependent selection [100]. Furthermore, rapid sex chromosome evolution constitutes an example, like the Y chromosome of the ninespine stickleback (*Pungitius pungitius*) that has been transferred from another stickleback species through ancient hybridization [101].

The hypothesis that the balanced lethal system of *Triturus* comprises two supergene variants that got caught in a balanced polymorphism by slowly degrading over time alone seems plausible, however fixation of the system is hard to explain in that case. Therefore, testing the alternative, or perhaps complementary, hypothesis that introgressive hybridization somehow played a role in bringing together chromosome 1A and chromosome 1B in *Triturus*' patient zero is not an uncanny thought. Introgressive hybridization is also not uncommon in the genus *Triturus* [102, 103] and other salamanders [104-107] and a recent study on Salamandridae supports extensive introgression at deep timescales [89]. In fact, (apart from the issue of gigantic, 'crappy' genomes) ancient episodes of hybridization that resulted in introgression are believed to be the main reason why resolving the phylogeny of salamanders has proven to be rather difficult [89, 104]. It is likely that introgressive hybridization played a causal role in the origin of chromosome 1 syndrome in *Triturus* newts, in which rapid diversification by evolutionary radiation is common [108] and in which various cases of ancient hybridization and introgression are suspected [89].

Objectives & dissertation outline

This dissertation is part of a broader research project that revolves around unraveling the evolution of balanced lethal systems. To understand why balanced lethal systems exist in nature, it is important to learn as much as possible about their genomic basis at the point of origin. To do this, the case of *Triturus* gives rise to an excellent starting point. However, as mentioned before, salamanders have gigantic genomes, which does not always make it easy to genomically study them. Thus, this dissertation comprises not only Chapters describing the most recent hypotheses and empirical insights regarding the balanced lethal system of *Triturus*, but also Chapters describing the innovative methodologies that I applied to make studying this possible in the first place.

Overall, this dissertation includes one (popular) scientific review Chapter, three research Chapters presenting new or improved methods, and a main empirical Chapter that summarizes my discoveries about the balanced lethal system of *Triturus*. As you will notice, I here highlight the key collaborators who were crucial in realizing these Chapters (in addition to the general acknowledgements), as this work was a particularly extensive

joint research effort. The Chapters are written as independent research articles and therefore they may contain some theoretical overlap. Also, the Chapters are organized chronologically to guide the reader through my scientific journey from the summer of 2019 up to the winter of 2024/2025, as summarized in Fig. 4.

CHAPTER 2 is a translated version of a Dutch (popular) scientific literature review. This Chapter focuses specifically on the main theory of the Wielstra lab that was adopted in 2019, namely that a balanced lethal system likely comprises two degraded supergene versions that got caught in a balanced polymorphism [21]. This Chapter firstly explains what a balanced lethal system is exactly, and it secondly explains in detail what the supergene theory entails. This Chapter was originally meant to be comprehensible for a non-specialist audience, and also the translated version provided here should (hopefully) interest any reader that is curious about the biology of *Triturus* newts and balanced lethal systems.

CHAPTER 3 is the first of a set of three Chapters that focus on a method that is required to start investigating the balanced lethal system in *Triturus* newts on a molecular level. Namely, to study what the differences are in the DNA of healthy versus diseased *Triturus* embryos, the initial step in the investigation is identifying which embryos are healthy (i.e. fall in the heterozygous 1A1B/1B1A class), and which ones are not (i.e., fall in one of the two diseased, homozygous classes 1A1A and 1B1B). Thus, this Chapter – *which is largely the result of a 50/50 collaboration with my co-worker Willem Meilink, and thus comprises a shared, first authorship* – introduces a molecular, laboratory procedure that can quickly tell apart the three classes of *Triturus* embryos using 1A and 1B-linked markers. This new method, which we call 'multiplex Kompetitive Allele-Specific PCR (mxKASP), is based on markers that I discovered in early tests using standard PCR methods, and it can be used to genotype *Triturus* embryos on a large scale (as classifying embryos based on morphology is a tedious and specialist task). This study explains the mxKASP method in more detail, and the broader, potential utilities of the method are described as well.

CHAPTER 4 follows naturally after Chapter 3, providing information on a target-capture method called 'NewtCap' that can be used to sequence (a part of) the DNA of *Triturus*. Namely, once it is clear to which genotypic category a *Triturus* embryo belongs (Chapter 3), the next step of the research is acquiring more in-depth genetic information about these embryos, so that the differences between the different classes (and thus, the differences between chromosomes 1A and 1B) can be mapped out and retraced to the ancestor. Moreover, this method should also be able to sequence the same (orthologous) regions of the DNA of other salamander species that do not suffer from a balanced lethal system for the sake of comparison. As whole genome sequencing of salamanders remains unattainable for now, the NewtCap tool provides an efficient solution. The study not only describes the ins and outs of the lab-protocol behind NewtCap – *which was*

initially designed by a group of the supportive co-authors, and which was impressively optimized in the laboratory by my co-worker James France – it also shows how well the method works across a wide range of species from the diverse Salamandridae family. Furthermore, instead of focusing on the usefulness of the tool for the main goal of this project (i.e., unraveling the evolution of balanced lethal systems), I emphasize that this Chapter is primarily meant to show that NewtCap in general promises to be a resourceful tool for molecular research towards Salamandridae salamanders.

CHAPTER 5 is a methodological paper that truly relies on the power of interdisciplinary research. This Chapter combines (bio)engineering and (bio)technological approaches to allow for the next step in the research process: identifying the mutations that underly chromosome 1 syndrome by searching for anomalies in the *Triturus* target capture data obtained with NewtCap (Chapter 4). The study focuses on the search for presence/absence variation, or PAV, in target capture data in general. In other words: it provides information on how to discover genes that are missing from chromosome 1A, but that are still present on chromosome 1B – and *vice versa* – using a tool called 'PAV-spotter' (validated by genotyping, see Chapter 3). Existing, bioinformatic tools were not always adequate at recognizing PAV patterns in my data due to (occasional) low coverage and low sample sizes. Thus, I reached a 'scientist's block', and for a while I ended up discussing my frustrations at home on a daily basis, over dinner. This not only unexpectedly led to the formation of the final, methodological computer-based tool required to continue my work – *something that was only possible thanks to my co-worker and husband Chris van der Ploeg, who is a software engineer* – it also led to the formation of this study. Firstly, it explains how considering the genomic position as a variable for 'signal displacement' (rather than time) enables identifying PAV in target capture data. Secondly, it provides background on potential other applications of this tool.

CHAPTER 6 comprises the main, empirical, and final research Chapter of this dissertation and focuses on the genetic background, likely origin, and evolution of the balanced lethal system in *Triturus*. Thus, from this point on the dissertation shifts from discussing theoretical frameworks and methodologies, to presenting the final research outcomes and conclusions. In a nutshell, this study compares embryos of all genotypic classes across *Triturus* species (as genotyped, see Chapter 3), based on the contents of chromosomes 1A and 1B (as sequenced, see Chapter 4), highlighting both differences and notable similarities in PAV (as determined, see Chapter 5). Additionally, the study demonstrates how detailed downstream analyses of the data at hand can uncover whether, and how, the evolutionary history of *Triturus'* chromosome 1 differs from that of the rest of the genome and that of other Salamandridae species. Lastly, the implications of all findings are described, making this Chapter the cornerstone of the dissertation.

Finally, **CHAPTER 7** concludes the dissertation with a comprehensive discussion and summary. This Chapter comprises of two parts. In the first part, the new insights into

the evolution of the balanced lethal system in *Triturus* are discussed (Chapter 2 and Chapter 6), and suggestions for further research are provided. In the second part, the various ways this dissertation contributes to both science and education are discussed, as the new insights, methods, and tools gained throughout this research extend beyond answering the specific research questions relating to chromosome 1 syndrome in *Triturus*.

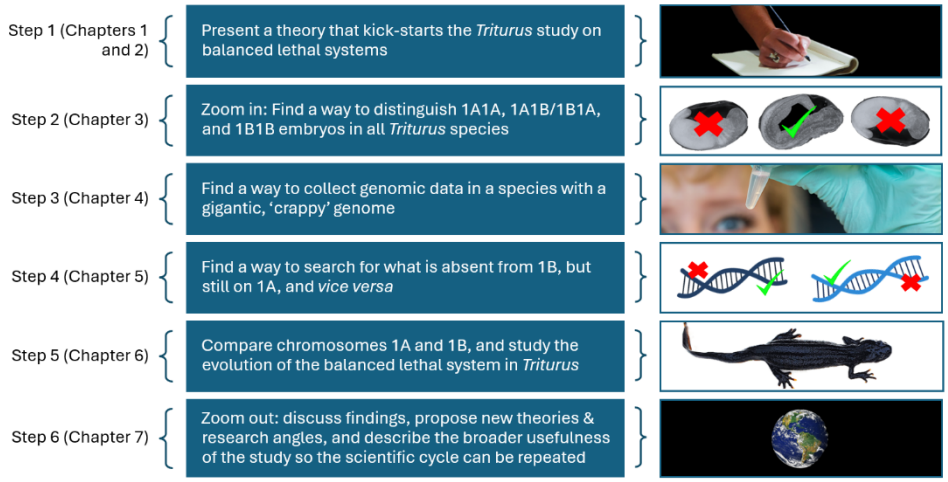

Figure 4: *A summary visualizing how the ordering of the Chapters in this dissertation corresponds to the steps undertaken in the +five year long scientific process. Explanation and sources of the photos and images: Step 1 & Step 6 – photos and images adjusted from www.pixabay.com; Step 2 – X-radia scans of* Triturus *embryos by Dr. Tijana Vučić, adjusted with symbols to indicate there are three types, namely healthy 1A1B/1B1A (checkmark) versus diseased 1A1A and 1B1B (crosses) individuals; Step 3 – photo of me in the laboratory at Leiden University, holding a test tube with freshly extracted DNA, visible as a white cloud (© Ingrid den Boer); Step 4 – representation of comparing chromosome 1A and 1B, which have unique recessive, lethal alleles, adjusted from www.pixabay.com; Step 5 – photo of a* Triturus *newt I observed in the wild during fieldwork in France (© Manon de Visser).*

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