Amphibians in a changing world

An ecophysiological perspective on amphibian metamorphosis

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"Wie alles sich zum Ganzen webt, // Eins in dem andern wirkt und lebt!" — *Vers 447 f. / Faust*

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Für all diejenigen, die mich in meiner Begeisterung für die *Welt in der wir leben* unterstützt haben.

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Summary

Alterations in abiotic and biotic environmental factors caused by drivers of global change expose wildlife to an array of additional chemical, physical, and biological stressors. Some environmental stressors have the ability to alter endocrine function and are thus, characterized as endocrine disruptors. Many endocrine disruptors impair the hypothalamus-pituitary-thyroid axis resulting in altered thyroid hormone (TH) levels, which is of special concern in larval amphibians since amphibian metamorphosis is the classical and unique example of thyroid hormone-regulated development. Larval amphibians have been shown to respond to stressful conditions in their larval habitat by exhibiting developmental and physiological plasticity which provides a means for increasing fitness in later life stages. Nevertheless, ongoing global change will result in multiple simultaneously occurring environmental stressors in larval habitats, but studies investigating interactive effects of those environmental stressors are still rare. Considering the current worldwide decline of amphibians it is of major interest to investigate whether and how anuran larvae adjust their metamorphic and physiological traits to new thermal challenges and to altered TH levels, which are caused by natural or anthropogenic stressors in their larval habitat.

In my dissertation, I investigated the capacity for developmental and physiological plasticity at the onset of metamorphosis in larvae of two anuran species, the common frog (*Rana temporaria*) and the African clawed frog (*Xenopus laevis*), which differ in their ecology and thermal adaptation. In particular, I analyzed whether developmental temperature, altered TH levels, and the interactive effect of both affect survival to and age, size, body condition, thermal tolerance, and standard metabolic rate at the onset of metamorphosis. I further evaluated whether the endocrine-disrupting effect of environmental stressors modifies this capacity and influences energetic costs and energy allocation during the metamorphic climax. Beyond that, I examined possible carry-over effects on energetics and performance in later life stages caused by endocrine disruption experienced during the larval stage. This study provides a comprehensive investigation of several fitness related traits and the potential and limitations of adaptability to environmental stress during the larval stage in two ecologically different species.

A prerequisite for a comprehensive investigation of two ecologically different species in terms of their capacity for stress-induced phenotypic plasticity is an understanding of general interspecific patterns of phenotypic plasticity and how the species' thermal adaptation may influence this pattern. Therefore, I analyzed 25 populations based on 18 different anuran

species in respect of their capacity to exhibit temperature induced developmental plasticity as well as the question of how their thermal adaptation influences this capacity. All included populations developed faster and the majority was smaller at the onset of metamorphosis when developmental temperatures increased. Warm adapted populations revealed a reduced capacity for temperature induced developmental plasticity and therefore, those species may be more vulnerable to the impacts of climate change.

On the basis of that interspecific pattern, I examined the capacity for a plastic response in metamorphic traits to variation in developmental temperature and whether altered TH levels modify this capacity in the two study species, the tropical *X. laevis* and the temperate *R. temporaria*. In both studied species altered TH levels modified the capacity for developmental plasticity to a temperature-given change independent of their thermal adaptation. Proximate effects of several environmental stressors due to global change may therefore result in ramifications for survival and fitness in later lifer stages.

Since THs also determine energy metabolism, exposure to environmental stressors may have potential implications for the acclimation capacity to new thermal challenges resulting from climate change. I assessed the acclimation capacity to warmer developmental temperatures in both study species, demonstrating that the temperate *R. temporaria* in contrast to the tropical *X. laevis* is able to exhibit a plastic response in metabolism to new thermal challenges whereas both species are able to acclimate their thermal tolerance. Altered TH levels affected this capacity for physiological plasticity in both species. Consequently, warm-acclimated and/or stressed larvae may be more vulnerable to the impacts of climate change in terms of lacking the capacity for an acclimation in physiological traits.

If the capacity for an acclimation of metabolic rate is impaired by environmental stress and global warming which is resulting in increased energetic requirements, the capacity to store energy which is needed for the complex reorganization during metamorphic climax may also be reduced. In both study species, body condition (i.e. size of energy stores) was reduced by warmer developmental temperatures and high TH levels. Thus, environmental stress and global warming may determine metamorphic success and thus, individual fitness in later life stages.

Finally, I assessed effects of altered TH levels on energetic costs of the metamorphic climax and juvenile performance in *R. temporaria* to demonstrate how environmental stressors may affect energy allocation to developmental costs during the metamorphic climax and fitness in

later life stages. The energy budget available for metamorphosis was reduced in tadpoles which experienced stressful larval environments as well as the juvenile performance. Therefore, ongoing climate change and anthropogenic disturbances of larval habitats will result in altered phenotypes at metamorphosis and relative higher energetic costs during climax making a completion of metamorphosis energetically ineffective. Any changes in metamorphic and physiological traits caused by larval stress exposure that affect post-metamorphic performance and therefore, survival and growth or delay time to maturity could have important impacts on fitness and population persistence.

Overall, my dissertation emphasizes that anuran larvae independent of their ecology and thermal adaptation are threatened by environmental stressors but to different extent. These stressors impair the capacity for developmental and physiological plasticity with potential ramifications for a successful metamorphosis, energy budgets, and juvenile performance. However, tropical species are predicted to be worstly affected by the negative impacts of global change in contrast to temperate species as demonstrated in the present study for tropical *X. laevis* and temperate *R. temporaria*.

The results of my study contribute to a better understanding of the complex and various effects of global change on amphibian metamorphosis and possible impacts in later life stages and thus, long-lasting effects on amphibian populations. This knowledge helps to predict the vulnerability of both study species and anuran larvae in general to the impacts of environmental change and temperature variation, as predicted in global and climate change scenarios. Hence, my study provides a better knowledge on relationships between amphibian declines and the interactions of impacts due to global change and other environmental stressors.

Zusammenfassung

Veränderungen von abiotischen und biotischen Faktoren, die vor allem auf anthropogen induzierte Einflüsse auf die Umwelt zurückzuführen sind, konfrontieren Wildtiere mit einer Vielzahl an zusätzlichen chemischen, physikalischen und biologischen Stressoren. Einige dieser Umweltstressoren können sich auf die endokrine Funktion auswirken und werden somit als endokrine Disruptoren bezeichnet. Ein Großteil dieser endokrinen Disruptoren beeinflusst die Hypothalamus-Hypophysen-Schilddrüsen-Achse, was zu einer Veränderung des Schilddrüsenhormonspiegels führt. Dieser Einfluss ist vor allem in Amphibienlarven von spezieller Bedeutung, da die Metamorphose der Amphibien das klassische und einzigartige Beispiel für eine schilddrüsenhormonregulierte Entwicklung ist. Es konnte gezeigt werden, dass Amphibienlarven auf stressvolle Umweltbedingungen in ihrem larvalen Habitat durch die Ausbildung phänotypischer Plastizität in Bezug auf Entwicklungsrate und Physiologie reagieren. Die Ausbildung von phänotypischer Plastizität trägt zur Erhöhung der Fitness in späteren Lebensphasen bei.

Nichtsdestotrotz wird die fortschreitende Veränderung der Umwelt durch natürliche, aber vor allem anthropogene Einflüsse dazu führen, dass sich das Auftreten von multiplen, simultanen Umweltstressoren in Habitaten von Amphibienlarven erhöht. Studien, die diese interaktiven Effekte von Umweltstressoren untersuchen, sind dennoch kaum vorhanden. In Anbetracht des weltweiten Rückgangs der Amphibien ist es von großem Interesse zu untersuchen, ob und wie Froschlurchlarven ihre Entwicklung und Physiologie an neue thermische Herausforderungen und veränderte Schilddrüsenhormonspiegel anpassen, welche durch natürliche oder anthropogene Stressoren in ihrem Larvenhabitat verursacht werden.

In meiner Dissertation habe ich die Larven zweier Froschlurcharten, dem Grasfrosch (*Rana temporaria*) und dem Afrikanischen Krallenfrosch (*Xenopus laevis)*, in Hinblick auf ihre Fähigkeit zur Ausbildung von phänotypischer Plastizität in Bezug auf Entwicklung und Physiologie zu Beginn der Metamorphose untersucht. Die beiden ausgewählten Arten unterscheiden sich in ihrer allgemeinen Ökologie und ihrer Temperaturanpassung. Im Speziellen habe ich untersucht, ob die Temperatur während der Entwicklung, veränderte Schilddrüsenhormonspiegel und der interaktive Effekt aus beiden einen Einfluss auf Alter, Körpermasse und -größe, physiologische Konstitution, Temperaturtoleranzbereich und Grundumsatz haben. Außerdem habe ich evaluiert, inwieweit der endokrin-disruptive Effekt von Umweltstressoren diese Fähigkeit verändert und die energetischen Kosten und

Energiezuweisung während der Metamorphoseklimax beeinflusst. Darüber hinaus habe ich untersucht, ob endokrine Disruption während der Larvalentwicklung zu möglichen Carry-Over-Effekten auf Energetik und Performance in späten Lebensphasen führt. Diese Studie bietet in ihrer Gesamtheit eine umfassende Untersuchung verschiedener fitnessbezogener Merkmale und der Möglichkeiten und Grenzen der Anpassungsfähigkeit an Umweltstress während des Larvenstadiums in zwei ökologisch unterschiedlichen Arten.

Eine Grundvoraussetzung für eine umfassende Untersuchung zweier ökologisch unterschiedlicher Arten hinsichtlich ihrer Fähigkeit zur stressinduzierten phänotypischen Plastizität ist ein Verständnis über allgemeine interspezifische Muster zur Ausbildung phänotypischer Plastizität und inwiefern die Temperaturanpassung einer Art dieses Muster beeinflussen kann. Dafür habe ich 25 Populationen basierend auf 18 verschiedenen Froschlurcharten hinsichtlich ihrer Fähigkeit temperaturinduzierte Entwicklungsplastizität zu zeigen untersucht. Des Weiteren habe ich untersucht, wie die entsprechende Temperaturanpassung der Population diese Fähigkeit beeinflusst. Alle eingeschlossenen Populationen entwickelten sich schneller und die Mehrzahl war zu Beginn der Metamorphose kleiner, wenn die Entwicklungstemperaturen anstiegen. Warmadaptierte Populationen zeigten eine verminderte Fähigkeit zur temperaturinduzierten Entwicklungsplastizität, weshalb diese Arten anfälliger für die Auswirkungen des Klimawandels sein könnten.

Auf der Grundlage dieses interspezifischen Musters habe ich die Fähigkeit zur Ausbildung temperaturinduzierter Entwicklungsplastizität in den beiden Versuchsarten, dem tropischen *X. laevis* und dem gemäßigten *R. temporaria*, untersucht. Des Weiteren habe ich evaluiert, ob und in welchem Ausmaß sich veränderte Schilddrüsenhormonspiegel auf diese Fähigkeit auswirken. In beiden Versuchsarten beeinflussten veränderte Schilddrüsenhormonspiegel diese Fähigkeit, unabhängig von ihrer Temperaturanpassung. Dementsprechend können proximale Effekte verschiedener Umweltstressoren zu Konsequenzen für das Überleben und die Fitness in späteren Lebensstadien der Amphibien führen.

Da Schilddrüsenhormone ebenfalls an der Regulation des Energiestoffwechsels beteiligt sind, können Umweltstressoren durch ihre endokrin-disruptive Wirkung potenzielle Auswirkungen auf die Fähigkeit zur Akklimatisierung an neue Temperaturbedingungen im Zuge des Klimawandels haben. In dieser Arbeit habe ich deshalb beide Versuchsarten auf ihre Fähigkeit zur Akklimatisierung an wärmere Entwicklungstemperaturen untersucht. Ich konnte zeigen, dass der an gemäßigtes Klima angepasste *R. temporaria* im Gegensatz zum an

tropisches Klima angepasste *X. laevis* in der Lage ist, seinen Grundumsatz an neue Temperaturbedingungen anzupassen und diese somit zu kompensieren. Beide Arten konnten hingegen ihren Temperaturtoleranzbereich akklimatisieren. Veränderte Schilddrüsenhormonspiegel beeinträchtigten diese Fähigkeit zur physiologischen Plastizität bei beiden Arten. Folglich können warm-adaptierte und/oder gestresste Larven anfälliger für die Auswirkungen des Klimawandels sein, da ihnen die Fähigkeit zur Akklimatisierung fehlt bzw. diese nur geringfügig ausgebildet ist.

Wenn die Fähigkeit zur Akklimatisierung der Stoffwechselrate durch Umweltstress und globale Erwärmung beeinträchtigt und mehr Energie zur Deckung des Grundumsatzes benötigt wird, kann ebenfalls die Fähigkeit Energiereserven anzulegen beeinflusst werden. Da diese Energiereserven für die komplexe Reorganisation während der Metamorphoseklimax im Zuge der Entwicklung von der Kaulquappe zum juvenilen Frosch benötigt werden, können unzulängliche Energiereserven zu Entwicklungsdefiziten und somit zu einer verringerten Fitness in späteren Lebensstadien führen. Wärmere Entwicklungstemperaturen und hohe Schilddrüsenhormonspiegel führten in beiden Versuchsarten zu verringerten Energiespeichern und somit zu einer geringeren körperlichen Konstitution. Somit können Umweltstress und globale Erwärmung ultimativ eine erfolgreiche Entwicklung und damit die individuelle Fitness in späteren Lebensphasen beeinflussen.

Im letzten Teil dieser Studie habe ich untersucht, wie sich veränderte Schilddrüsenhormonspiegel auf die energetischen Kosten während der Metamorphoseklimax und die juvenile Bewegungsleistung in *R. temporaria* auswirken. Dadurch zeige ich, wie Umweltstressoren die Energiebereitstellung während der Metamorphoseklimax und die Fitness in späteren Lebensstadien von Froschlurchen beeinflussen können.

Das für die Metamorphose zur Verfügung stehende Energiebudget sowie die juvenile Bewegungsleistung war bei den Individuen reduziert, die während ihrer Entwicklung Umweltstressoren ausgesetzt waren, welche sich stimulierend auf den Schilddrüsenhormonspiegel auswirken. Dementsprechend werden der fortschreitende Klimawandel und die anthropogenen Störungen in Habitaten von Amphibienlarven über die Auswirkungen auf den morphologischen und physiologischen Phänotyp zu Beginn der Metamorphose und relativ höheren Energiekosten während der Metamorphoseklimax führen. Die gesamte Metamorphose wird dadurch in ihrer energetischen Effizienz herabgesetzt. Jegliche Veränderungen von Entwicklungsrate und Physiologie, die durch Umweltstressoren

verursacht werden, können somit auch die juvenile Bewegungsleistung, das Überleben und das Wachstum bzw. die Zeit bis zur Geschlechtsreife in juvenilen Froschlurchen beeinflussen und dementsprechend Auswirkungen auf die individuelle Fitness und das Bestehen ganzer Amphibienpopulationen haben.

Insgesamt betonen die Ergebnisse meiner Dissertation, dass Froschlurchlarven unabhängig von ihrer Ökologie und ihrer Temperaturanpassung von Umweltstressoren jeglicher Herkunft bedroht sind, da diese die Fähigkeit zur Ausbildung phänotypischer Plastizität in Bezug auf Entwicklungsrate und Physiologie vermindern. Dies kann wiederum zu negativen Konsequenzen für den erfolgreichen Abschluss der Metamorphose, die larvalen Energiebudgets und juvenile Bewegungsleistung führen. Diese Arbeit bestätigt zudem, dass tropisch-adaptierte Arten stärker von den negativen Auswirkungen des globalen (Klima-)wandels betroffen sein werden als temperat-adaptierte Arten. Die vorliegende Studie zeigt dies am Beispiel des an das tropische Klima adaptierten *X. laevis* und den an das temperate Klima adaptierten *R. temporaria*.

Die Ergebnisse meiner Arbeit tragen zu einem besseren Verständnis der komplexen und vielfältigen Auswirkungen der globalen Umweltveränderungen auf die Metamorphose von Amphibienlarven bei und zeigen mögliche Auswirkungen auf spätere Lebensstadien und damit verbundene langfristige Auswirkungen auf Amphibienpopulationen auf. Dieses Wissen kann dazu beitragen, die Anfälligkeit der beiden untersuchten Arten und Froschlurchlarven im Allgemeinen in Bezug auf die Auswirkungen von Umweltveränderungen und Temperaturschwankungen vorherzusagen. Daher liefern die Ergebnisse meiner Studie wichtige Erkenntnisse über die Zusammenhänge zwischen dem globalen Rückgang der Amphibien und den multiplen, simultan auftretenden Auswirkungen verschiedener Umweltstressoren.

General Introduction

Global change and climate warming

Species are declining worldwide due to environmental variation caused by both natural and anthropogenic global environmental change (Stuart et al. 2004; Strong et al. 2017). Land use change due to urbanization and agricultural expansion, chemical pollution, the introduction of invasive alien species, and climate change are considered to be the major drivers of global change affecting biodiversity and ecosystem functioning worldwide (Pacifici et al. 2015; Franklin et al. 2016; Bernhardt et al. 2017). These human-induced changes, habitat fragmentation, novel species assemblages, reduced habitat quality due to toxic contamination, and global warming present wildlife with complex new challenges (Sala et al. 2000).

The effects of climate change in particular are a serious threat to biodiversity and ecosystems and present major challenges to organisms (Huey et al. 2012). In the last 100 years, the Earth has warmed by about 0.74 °C, and global mean temperatures are projected to increase further by 4.3 ± 0.7 °C by 2100 (Pachauri et al. 2014; Pacifici et al. 2015). Furthermore, increased ultraviolet-B radiation, acidification of marine and freshwater systems, and increased frequency of some forms of extreme events such as those associated with heat, drought or flooding, are also predicted in future climate change scenarios (Sheridan and Bickford 2011; Palmer et al. 2017). During the past half-century, climate change altered seasonal timing, geographic range, and population abundance in many species around the world (Parmesan 2006; Kingsolver et al. 2013), and between 20 and 30 % of all species are likely to be at an increasing high risk of extinction in the face of increasing global warming (Pachauri et al. 2014; Pacifici et al. 2015).

Ectotherms in a changing world

Ectothermic animals are considered to be particularly vulnerable to climate warming (Paaijmans et al. 2013) because their basic physiological functions, such as locomotion, growth, and reproduction, are strongly influenced by environmental temperature (Deutsch et al. 2008; Angilletta 2009). This can be directly related to the thermal sensitivities of the rates of biochemical and physiological processes (Little and Seebacher 2016). The relationships between ectotherm performance and fitness, and temperature is typically characterized by a thermal performance curve, which defines the optimum temperature and thermal range of tolerance (i.e. thermal window; Pörtner et al. 2006) between critical minimum and maximum temperatures (Deutsch et al. 2008; Tewksbury et al. 2008; Paaijmans et al. 2013) (Fig. 0.1). The thermal window is generally related to the geographic and altitudinal distribution of a

species (Turriago et al. 2015) and is relatively broad in temperate species, narrow in tropical species and narrowest in species found only in polar areas (Huey and Kingsolver 1993; Angilletta et al. 2002; Pörtner and Peck 2010). The breadth of a species' thermal window determines its sensitivity to temperature variation and, thus, to climate change (Oyamaguchi et al. 2017).

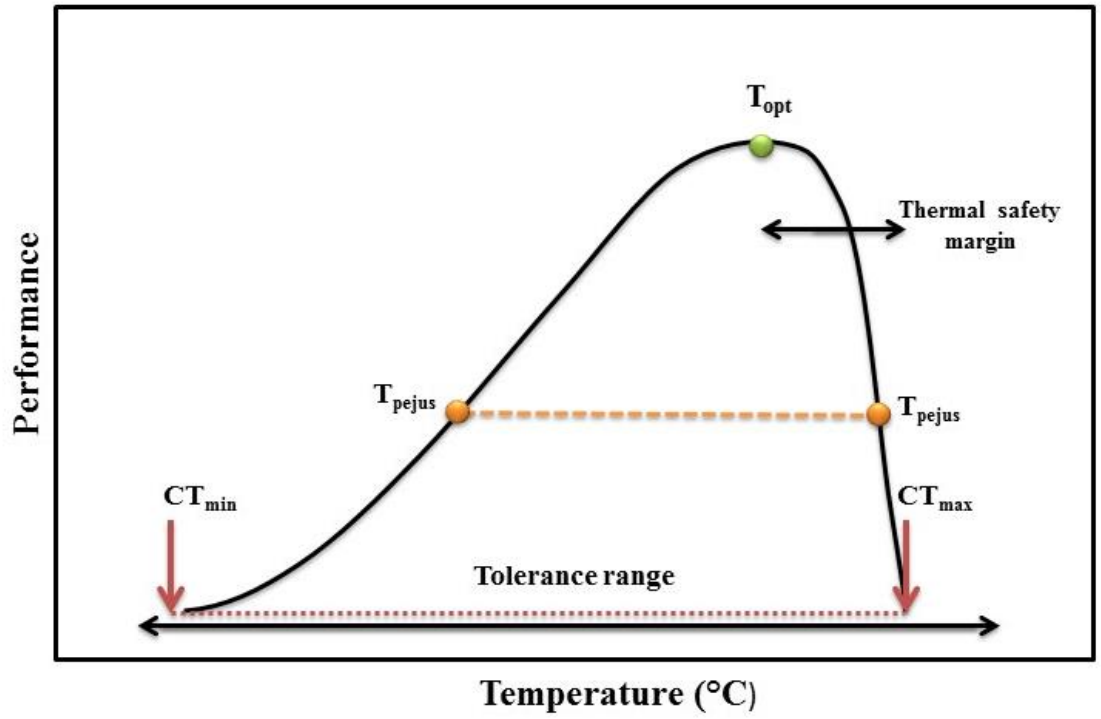

Fig. 0.1 Hypothetical performance curve of an ectothermic animal as a function of (body) temperature (modified from Sinclair et al. 2016). CT_{min}=minimum critical temperature (i.e. lower thermal limit). CT_{max}=maximum critical temperature (i.e. upper thermal limit). T_{opt}=thermal optimum at which performance is maximized.T_{pejus}=onset of limitation in (aerobic) scope. The thermal range of tolerance is bordered by the lower and upper thermal limits (CT_{min} and CT_{max}). Thermal safety margin=Temperature difference between T_{opt} and CT_{max}.

Paleontological records, as well as the recent history of global warming, demonstrate that even modest changes in environmental temperature of only a few degrees can result in major shifts in ectotherm distribution and mass population extinction (Hochachka and Somero 2002; Sokolova and Lannig 2008). Therefore, climate change represents a selection pressure that results either in migration, genetic (thermal) adaptation or in the evolution of phenotypic plasticity (Hoffmann and Sgró 2011; Seebacher et al. 2015). However, the ability for migration is often reduced due to life history, species interactions, or increasing habitat fragmentation (Urban et al. 2013). Additionally, genetic (thermal) adaptation is considered to be inefficient and not fast enough under rapid human-induced climate change, which can occur across few a generations or even within a generation (Seebacher et al. 2015). In relation to the rapid speed of ongoing climate change, behavioral responses are the fastest and most flexible option (Wong and Candolin 2015), followed by physiological and developmental plasticity, which should be favored (Seebacher et al. 2015) if the potential for behavioral adjustment is reduced (Gunderson and Stillman 2015; Berg et al. 2017).

Phenotypic plasticity is key to cope with global warming

Phenotypic plasticity is the ability of a single genotype to produce more than one phenotype, e.g., a form of morphology, behavior, development, and physiological state in response to environmental conditions (West-Eberhard 1989; Agrawal 2001; Miner et al. 2005), and is adaptive in heterogeneous environments (Ghalambor et al. 2007). As a result of the increase in mean environmental temperatures and frequency of extreme thermal events, ectotherms across the globe will be more likely to experience temperatures beyond their physiological limits (Gunderson and Stillman 2015). Therefore, the ability to show physiological plasticity to changing thermal conditions is expected to be a primary factor dictating the vulnerability of ectotherms to climate change (Gunderson and Stillman 2015; Kern et al. 2015). Thus, it is expected to mitigate fitness decrease (Duputié et al. 2015).

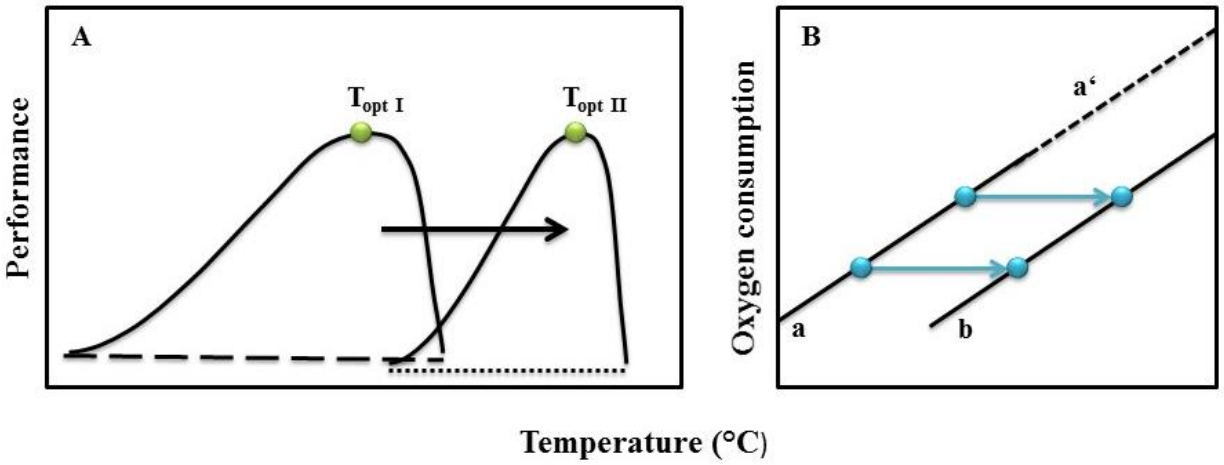

Fig. 0.2 Acclimatization=compensation of **A** the hypothetical performance curve and **B** the temperature dependence of oxygen consumption in ectothermic animals. **A** The acclimatization of the thermal limits (CT_{min} and CT_{max}) leads to an altered thermal range of tolerance and thermal optimum (T_{opt}). **B** Complete compensation of oxygen consumption at temperature **a** resulting in a constant oxygen consumption at temperature **b** (i.e. blue arrow). Without this compensation, oxygen consumtion at temperature **a** would increase with increasing temperature **a'** (i.e. dashed line; modified from Heldmaier and Neuweiler 2004).

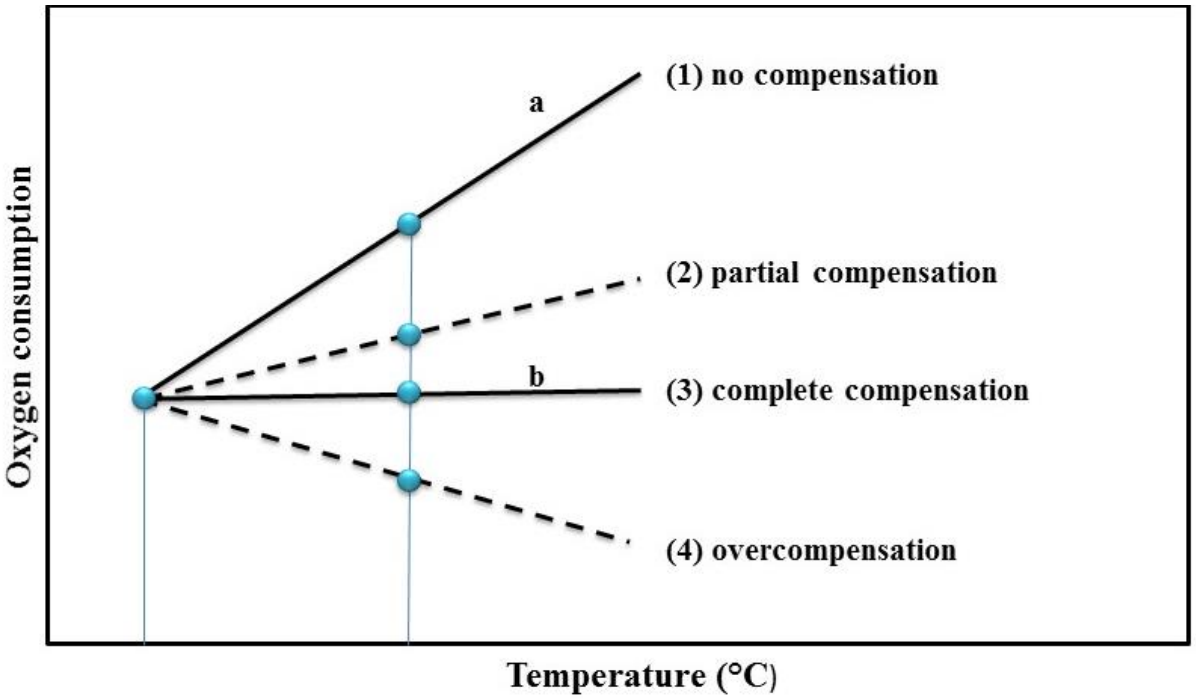

Fig. 0.3 Different patterns of acclimatization (i.e. compensation) in oxygen consumption with increasing ambient temperature in ectothermic animals. **1** no compensation=oxygen consumption increases significantly at warmer temperatures. **2** partial compensation=oxygen consumption increases insignificantly at warmer temperatures. A partial compensation in oxygen consumption is reversible and is commonly found in terrestrial ectotherms. **3** complete compensation=oxygen consumption is constant after acclimatization to warmer temperatures. A complete compensation is commonly found in aquatic ectotherms. 4 overcompensation=oxygen consumption decreases after acllimatization to warmer temperatures (modified from Heldmaier and Neuweiler 2004).

Physiological responses to changes in the thermal environment that are based on plasticity are referred as “acclimation” in controlled laboratory experiments, or “acclimatization” if they occur under natural conditions (Somero 2010; Berg et al. 2017). Thermal acclimation encompasses the alteration of thermal tolerance and thermal limits (Gunderson & Stillman 2015; Fig. 0.2) and the maintenance of thermal reaction norms of physiological processes such as metabolic rate, muscle function, and locomotor performance at different temperatures by biochemical adjustments (e.g. changes in enzyme concentration, activity and efficiency, mitochondrial biogenesis and modification of cellular and/or mitochondrial membranes (Seebacher 2005; Kern et al. 2015; Little and Seebacher 2016; Fig. 0.2 and 0.3). If thermal compensation were perfect, physiological rates would remain constant across environmental conditions, so that animals could maintain fitness across a broader temperature range compared to animals that show little or no physiological plasticity (Seebacher et al. 2015). However, in most instances the magnitude of the change outstrips this compensatory capacity of animals (Seebacher et al. 2015).

A species’ thermal tolerance range and ability to acclimate are proportional to the amount of environmental temperature variation that they experience in the wild (Janzen 1967). As tropical environments are relatively stable, tropical ectotherms generally have a low tolerance to extreme fluctuations in temperature (Deutsch et al. 2008; Oyamaguchi et al. 2017) and a limited acclimation capacity (Janzen 1967; Gunderson and Leal 2015). As a consequence, the negative impacts of climate change should be greatest on tropical ectothermic species (Tewksbury et al. 2008). However, recent meta-analyses revealed a contradicting pattern of increasing acclimatization capacity towards the tropics in ectotherms in general (Seebacher et al. 2015; Berg et al. 2017; Oyamaguchi et al. 2017).

Temperature-induced developmental plasticity

Despite physiological acclimation, changing thermal conditions induce plastic responses in the timing of metamorphosis (i.e. plastic growth and developmental rate) in ectotherms with complex life cycles such as holometabolous insects, marine invertebrates, parasites, most teleost fish, and amphibians (Wilbur 1980; Pechenik et al. 1998; Rudolf and Rödel 2007; Laudet 2011). As organisms must divide energy between physiological maintenance, growth, and development, they might limit growth in favor of development and basic maintenance physiology (Sheridan and Bickford 2011). Plasticity in growth and development can be explained by the intraspecific "temperature-size rule", which predicts that ectothermic species reared at relatively higher temperatures display higher growth and developmental rates due to an increased metabolism and thus, typically mature at a smaller size and younger age, when compared with conspecifics reared at lower temperatures (Atkinson 1994; Angilletta et al. 2004; Courtney Jones et al. 2015). Recent studies have shown a decrease in the size of ectotherms with global warming, suggesting that they are responding to increased temperatures with higher metabolism, quicker development, and shrinking body size (reviewed in Sheridan and Bickford 2011; Forster et al. 2012). For organisms that live in temporally and spatially heterogeneous environments, phenotypic plasticity in age and size at metamorphosis may provide a means for increasing fitness in later life stages (Schlichting and Pigliucci 1998; Boorse and Denver 2004; Rudolf and Rödel 2007).

Environmental stress as an endocrine disruptor

Beside temperature variation related to climate change, alterations in abiotic and biotic environmental factors caused by other drivers of global change expose wildlife to an array of additional chemical, physical, and biological stressors (Noyes et al. 2009) (Fig. 0.2). A wide range of these environmental stressors have the ability to alter endocrine function in wildlife (Carr and Patino 2011) and are characterized as endocrine disruptors (Kloas and Lutz 2006; Kloas et al. 2009). Alteration of endocrine function caused by an endocrine disruptor may be through interference with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior (Crisp et al. 1998; Vos et al. 2000). Environmental stressors can be endocrine disruptors that mimic, enhance (an agonist), or inhibit (an antagonist) the action of hormones (Vos et al. 2000; Mann et al. 2009).

Many endocrine disruptors impair the hypothalamus-pituitary-thyroid axis, which is of special concern in aquatic ectotherms with a critical thyroid hormone-regulated development, such as teleost fish and amphibians (Kloas et al. 2009; Carr and Patino 2011). In fish, dramatic developmental changes requiring thyroid hormones (TH) are less pronounced (examples include metamorphosis in flatfish and obligatory smoltification in anadromic fish such as salmon) (Carr and Patino 2011) but amphibian metamorphosis is the classical and unique example of endocrine regulation of development by the thyroid system (Kloas et al. 2009; Shi 2000; Tata 2006).

Amphibian metamorphosis

In the beginning of the 20th century, Gundernatsch (1912) made the remarkable discovery that equine thyroid extracts could accelerate the metamorphosis of tadpoles into juvenile frogs and subsequent studies demonstrated that removal of the tadpole thyroid gland or treatments with inhibitors of TH synthesis prevents metamorphosis (Furlow and Neff 2006). Consequently, THs could be identified as the major causative agents of amphibian metamorphosis (Shi 2000).

There are two naturally occurring THs regulating amphibian metamorphosis: 3,5,3´,5´-tetraiodothyronine (T4), commonly known as thyroxine, and 3,5,3´-triiodothyronine (T3) (Shi 2000; Tata 2006). T4 is the precursor for T3 and can be converted to T3, which is the more biologically active form. During pre- and prometamorphosis, concentrations of both THs increase until metamorphic climax where THs are at peak levels and the tadpole stops feeding and undergoes rapid metamorphic transitions (Shi 2000) (Fig. 0.4). In the growing larva, the action of THs is inhibited by the growth hormone prolactin (Beachy et al. 1999). Upon the completion of metamorphosis, TH levels are also reduced (Shi 2000; Brown and Cai 2007). The hypothalamic-pituitary-thyroid axis controls the production of THs: The corticotrophin-releasing factor from the hypothalamus regulates the release of thyroid-stimulating hormone (TSH) from the pituitary (Denver 1997a,b; Carr and Patino 2011) (Fig. 0.4). The release of THs from thyroid follicles is regulated by pituitary TSH (Denver 1997a,b). Circulating THs negatively influence the activity of the hypothalamus and the pituitary (Shi 2000). Thus, the activity of the hypothalamic-pituitary-thyroid axis is regulated by negative feedback. As the synthesis of THs in the thyroid gland is under complex neuroendocrine control, environmental factors that stimulate the central nervous system can interact with the hypothalamic-pituitary-thyroid axis (Denver 1997a,b; Shi 2000).

The period between the hatching of an egg and the end of metamorphosis is divided into three stages in anuran larvae (Etkin 1932; Gosner 1960; Dodd and Dodd 1976) (Fig. 0.5): Premetamorphosis, prometamorphosis, and metamorphic climax. Premetamorphosis (Gosner stage 23-34) is the truly larval period and is characterized by considerable growth and development of larval structures (e.g. limb bud growth and length growth) but no metamorphic changes (Dodd and Dodd 1976; Shi 2000). Metamorphosis, the second stage, is subdivided into prometamorphosis (Gosner stage 35-41) and metamorphic climax (Gosner stage 42- 46). During prometamorphosis length and hind limb growth and toe differentation continues and minor metamorphic transformations are initiated (Dodd and Dodd 1976). Metamorphic climax begins when at least one forelimb emerges and the cloacal tail piece begins to shrink (Shi 2000). During the relatively short period of their life, anuran larvae undergo a phase of extremely complex events with morphological and physiological changes. There are three major types of changes that take place: the complete resorption of tadpole-specific organs (e.g. tail, gills), the de novo development of frog-specific organs (e.g. limb development), and the remodeling of existing organs into their adult forms (e.g liver, nervous system, intestine) (Shi 2000; Tata 2006; Brown and Cai 2007). Age, size, and energetics of the larva at the onset of metamorphosis are of key importance for the successful completion of metamorphic climax and fitness of later life stages (e.g. Berven 1990; Metcalfe and Monaghan 2001; Rudolf and Rödel 2007; Scott et al. 2007; Orlofske and Hopkins 2009).

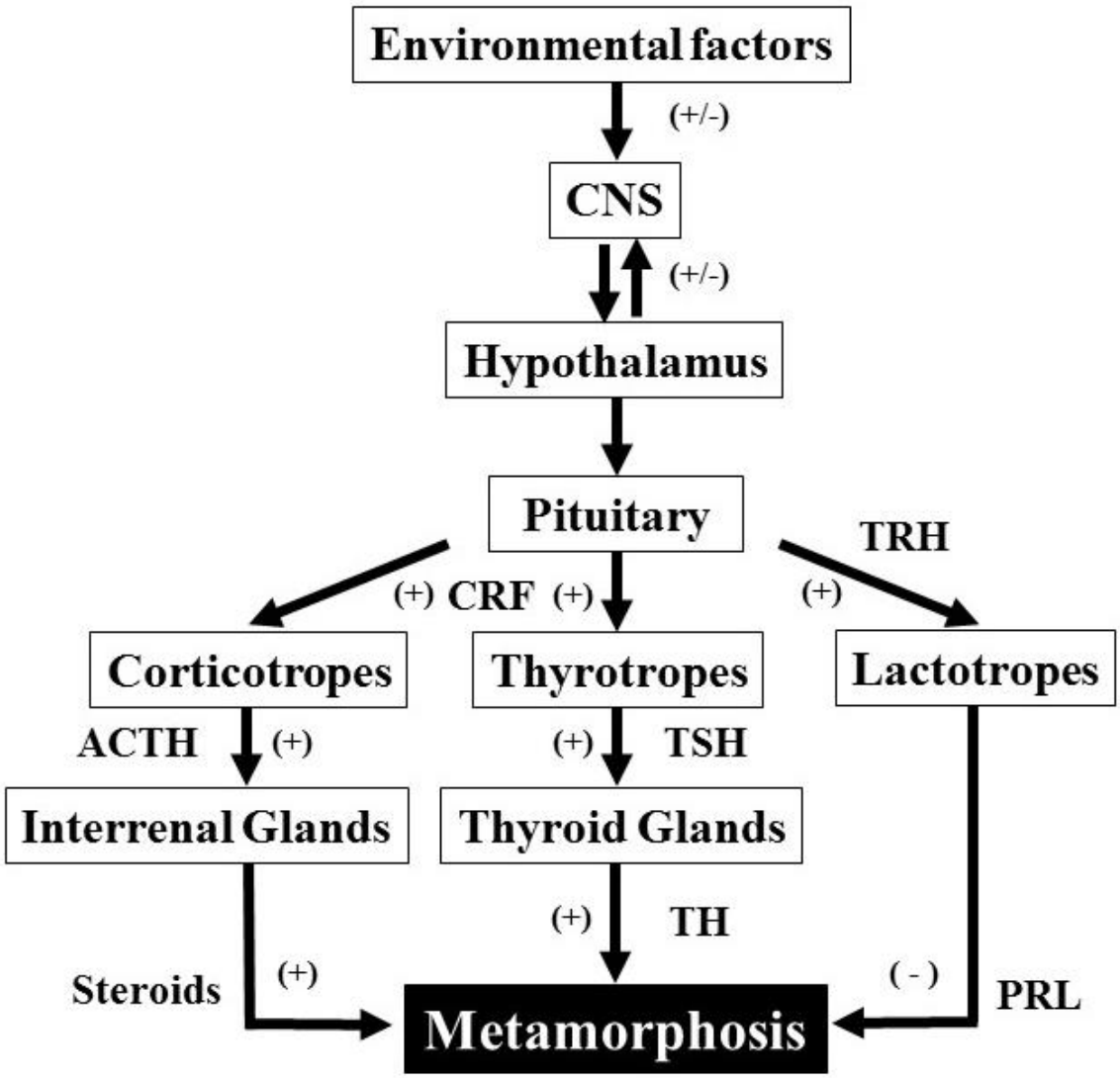

Fig. 0.4 Neuroendocrine control of amphibian metamorphosis. Environmental factors affect amphibian metamorphosis by impacting the hypothalamus-pituitary-thyroid axis. A plus sign indicates a positive or stimulatory action and a minus sign indicates a negative or inhibitory action. THs and steroids (corticoids) exert positive effects on metamorphosis whereas prolactin inhibits metamorphosis. Their synthesis and secretion are under complex neuroendocrine regulation involving both positive and negative feedbacks. CNS: central nervous system; CRF: corticotropin releasing factor; TRH: thyrotropin-releasing hormone; TSH: thyrotropin; ACTH: adrenocorticotropin; TH: thyroid hormone (T4); PRL: prolactin (modified after Shi 2000).

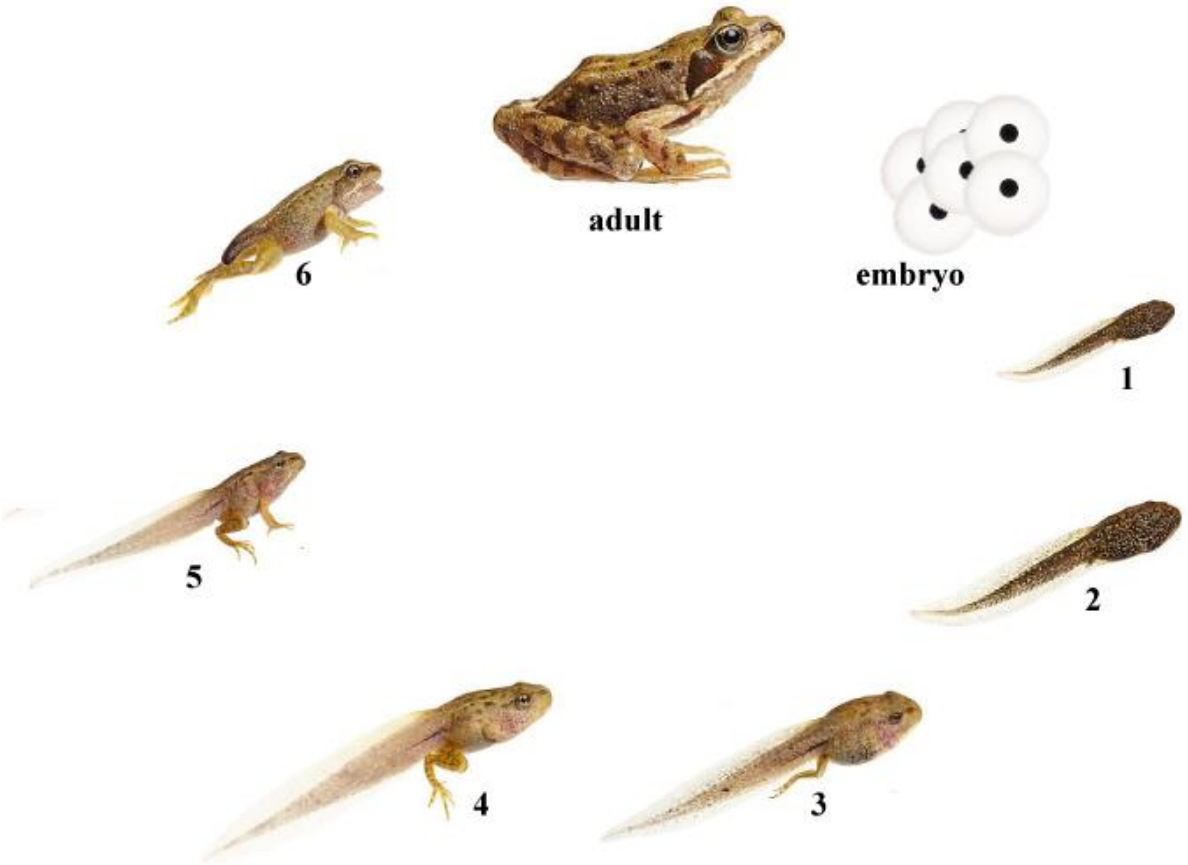

Fig. 0.5 Life cycle of the common frog (*Rana temporaria*), a typical anuran with metamorphosis which changing a larval tadpole (**1-5**) into a juvenile froglet (**6**). Developmental stages according to Gosner (1960) and Ortiz-Santaliestra and Sparling (2007). Gosner stage group **1-5**: **1**. pre-limb (absence of hind limbs, Gosner stages 24-26), **2**. limb bud (hind limb visible, but no clear joint formed, Gosner stages 27-34), **3**. middle hind limb (knee joint apparent, but toes not completely separated, Gosner stages 35-37), **4**. late hind limbs (hind limb tubercles and subarticular patches formed, Gosner stages 38-41), and **5**. metamorph (at least one forelimb present, Gosner stage 42). The period between the hatching of an egg and the end of metamorphosis is divided into three stages in anuran larvae: Premetamorphosis, prometamorphosis, and metamorphic climax. Premetamorphosis (Gosner stage 23-34, **1-2**) is the truly larval period and is characterized by considerable growth and development of larval structures (e.g. limb bud growth and length growth) but no metamorphic changes. During prometamorphosis length and hind limb growth and toe differentation continues and minor metamorphic transformations are initiated. Metamorphosis, the second stage, is subdivided into prometamorphosis (Gosner stage 35-41, **3-4**) and metamorphic climax (Gosner stage 42-46, **5-6**). Metamorphic climax begins when at least one forelimb emerges and the cloacal tail piece begins to shrink (Gosner stage 42, **5**). Climax is completed when the tail is fully resorbed (i.e. juvenile froglet; Gosner stage 46, **6**). Pictures: See pictorial sources.

Endocrine disruption of amphibian metamorphosis

Since the findings of Gundernatsch (1912) and others established THs as the developmental signal that triggers the onset of metamorphosis, ongoing research has investigated whether and how altered abiotic and biotic environmental factors due to global change influence amphibian metamorphosis by interacting and disrupting TH production pathways (e.g. Denver et al. 1998; Kiesecker 2002; Tietge et al. 2005; Hayes et al. 2010; Burraco and Gomez-Mestre 2016; Fig. 0.6 and 0.7). As they are limited in their capacity for habitat selection (Sanzo and Hecnar 2006; Yu et al. 2013), amphibian larvae are especially sensitive to the impact of changing environmental factors, which can affect the TH system through inhibitory or stimulatory action.

Inhibition of TH production pathways results in decreased TH levels and thus, decreased developmental rates (Carr et al. 2003; Bulaeva et al. 2015; Fig. 0.6 and 0.7) and decelerated energy metabolism (Carr and Patino 2011; Kashiwagi et al. 2009; Ortiz- Santaliestra and Sparling 2007; Fort et al. 2017), with tadpoles consequently metamorphosing at a larger size and older age (Shi 2000). A large number of aquatic contaminants have been shown to inhibit or disrupt the normal action of THs in amphibians, leading to changes in growth, development, and regulation of energy metabolism (Brown and Cai 2007; Kashiwagi et al. 2009). Pesticides and herbicides, road salt, fertilizers, heavy metals, and active pharmaceutical ingredients are known to disrupt TH production pathways and production, resulting in decreased TH levels (Carr and Patino 2011; Kashiwagi et al. 2009; Ortiz-Santaliestra and Sparling 2007; Fort et al. 2017) (Fig. 0.6 and 0.7). A relevant environmental endocrine disruptor is perchlorate (ClO_4^-), which is a goitrogen that inhibits TH synthesis via competitive inhibition of the sodium-iodide symporter (Ortiz-Santaliestra and Sparling 2007). Because iodide is essential for the production of both T4 and T3, perchlorate may therefore act as a disrupter of amphibian metamorphosis (Ortiz-Santaliestra and Sparling 2007).

Perchlorate salts are strong oxidizers and are widely used as components of fireworks, airbags, and currently applied fertilizers (Trumpolt et al. 2005; Carr and Patino 2011; Schmidt et al. 2012). Contamination of surface and ground water occurs from military, aerospace, and other commercial sources, but perchlorate also occurs naturally in arid places on the surface of the earth (Carr and Patino, 2011). In the United States, concentrations from 3 µg to 30 mg $\times L^{-1}$ have been found in surface and ground waters (U.S. Environmental Protection Agency [USEPA], 2004; Carr and Patino, 2011), which are levels high enough to disrupt the histology of thyroid follicles and decrease thyroid hormone concentration in several aquatic vertebrates

including the African clawed frog, *Xenopus laevis* (Goleman et al. 2002 a,b; Hu et al. 2006), fathead minnow, *Pimephales promelas* (Crane et al. 2005), zebrafish, *Danio rerio* (Mukhi et al. 2005), and rabbits (York et al. 2001) (reviewed in Mukhi and Patino 2007).

Stimulation of TH production pathways results in increased TH levels and thus, increased developmental and metabolic rates and decreased growth rates (Rowe et al. 1998; Tata 2006; Brown and Cai 2007). This results in shorter larval periods, smaller size at the onset of metamorphosis and higher energetic maintenance costs (Denver 1998; 2009; Orlofske and Hopkins 2009). Perception of environmental stressors by the central nervous system activates the hypothalamic–pituitary–interrenal axis, which is the neuroendocrine stress axis in amphibians (Shi 2000; Sapolsky 2002; Dantzer et al. 2014; Fig. 0.6 and 0.7). As a result, the corticotropin-releasing factor from the hypothalamus regulates the release of adrenocorticotropin from the pituitary (Denver 1998; 2013), and also stimulates TH production, since the corticotrophin-releasing factor controls both the thyroid and interrenal axes (Glennemeier and Denver 2002; Laudet, 2011; Kulkarni and Buchholz, 2012). The latter produces corticosteroid stress hormones which can synergize with THs and promote metamorphosis (Denver 1997; 1998; Beachy et al. 1999). Abiotic and biotic environmental stressors such as crowding (Ding et al. 2015), the presence of (novel) predators (Relyea 2007), food quality and quantity (Courtney Jones et al. 2015), pathogens (Warne et al. 2011), extreme (water) temperatures (Vences et al 2002), UV-B radiation (Belden et al. 2003), and desiccation risk (Gervasi and Foufopoulos 2008) may act as indirect endocrine disruptors by the activation of the neuroendocrine stress axis (Mann et al. 2009; Dantzer et al. 2014) and an increase of stress hormone levels (Denver 1997a,b) (Fig. 0.6).

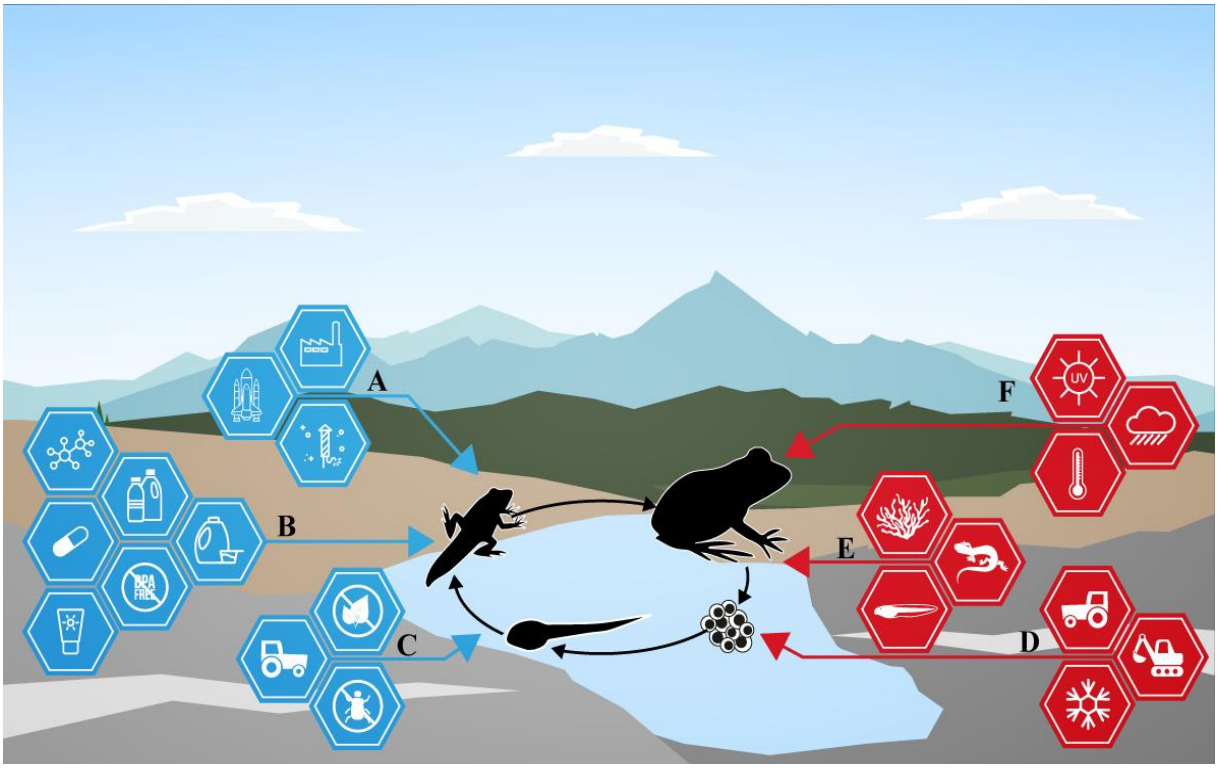

Fig. 0.6 Environmental stressors in the larval habitat of anuran larvae affecting developmental rate and thus, age and size at the onset of metamorphic climax by influencing endogenous thyroid hormone (TH) levels (Kashiwagi et al. 2008; Mann et al. 2009; Carr and Patino 2011; Boas et al. 2012). Altered TH levels also influence the thermal reaction norm (i.e. sensitivity) of growth and developmental rate during metamorphosis. Blue symbols: stressors acting as endocrine disruptors inhibiting TH production pathways resulting in low endogenous TH levels. Red symbols: stressors increasing TH production by the activation of the neuroendocrine stress axis. **A** Byproducts of industry (persistent organic products), aerospace (ClO_4^-), and fireworks (ClO_4^-). **B** Household chemicals and pharmaceuticals: Artificial steroid hormones (testosterone) and hormonal contraceptives (oestrogen and gestagen), analgesic agents (e.g., ibuprofen, diclofenac), chemicals from sunscreen, microplastics from packaging and clothes, bisphenol A (BPA) from packaging, phosphates from washing agents. **C** Chemicals from agriculture: fertilizer (NO3-), herbicides, pesticides (Atrazine, Malathion). **D** Habitat fragmentation and road salt. **E** Biotic stressors: Food availability, competition (i.e. crowding), predator pressure. **F** Climatic stressors: UV-radiation, temperature variation, precipitation and desiccation risk.

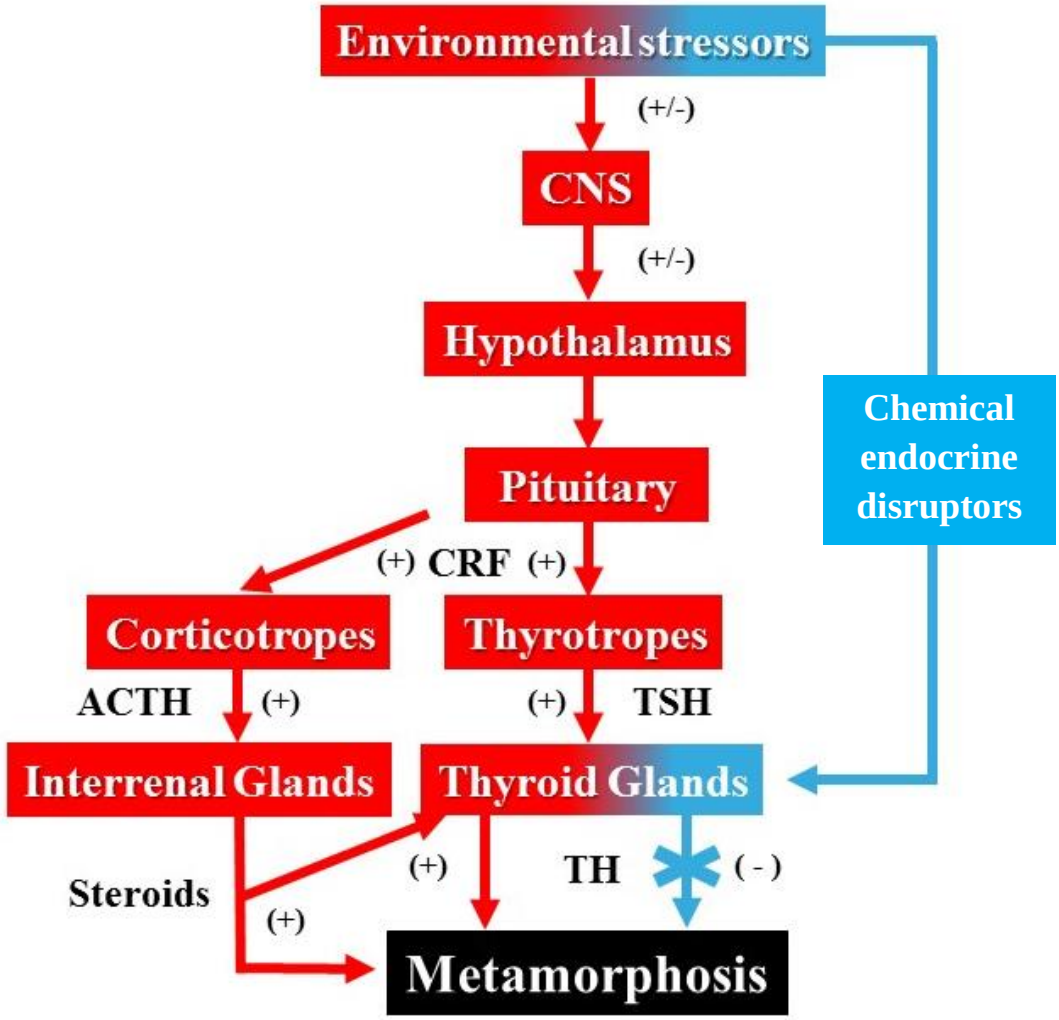

Fig. 0.7 Neuroendocrine control of amphibian metamorphosis under stressful environmental consitions. Environmental stressors affect amphibian metamorphosis by impacting the hypothalamus-pituitary-thyroid axis in a stimulatory (red) or inhibitory (blue) way. A plus sign indicates a positive or stimulatory action and a minus sign indicates a negative or inhibitory action. CNS: central nervous system; CRF: corticotropin releasing factor; TRH: thyrotropin-releasing hormone; TSH: thyrotropin; ACTH: adrenocorticotropin; TH: thyroid hormone (T4). **Red pathway**: Perception of environmental stressors by the central nervous system activates the hypothalamic–pituitary–interrenal axis, which is the neuroendocrine stress axis in amphibians. As a result, the CRF from the hypothalamus regulates the release of adrenocorticotropin from the pituitary, and also stimulates TH production, since the CRF controls both the thyroid and interrenal axes.The latter produces corticosteroid stress hormones (steroids) which can synergize with THs and promote metamorphosis. **Blue pathway**: Chemical endocrine disruptors usually act as goitrogens that inhibit TH synthesis via competitive inhibition of the sodium-iodide symporater in thy thyroid gland. Because iodide is essential for the production of both T4 and T3, endocrine disruptors of amphibian metamorphosis (modified after Shi 2000).

Environmental stress and amphibian decline?

The impact of changing environmental factors and environmental stressors as endocrine disruptors of metamorphosis is of special concern for amphibians owing to their worldwide decline (Stuart et al. 2004; Hayes et al. 2010). Currently, 41% of amphibian species are

threatened with extinction due to multiple environmental stressors associated with global change (Stuart et al. 2004; Hayes et al. 2006; Alroy 2015). There is evidence suggesting that endocrine disruption - caused by environmental stressors - impairs fitness in later life stages of amphibians through carry-over effects to post-metamorphic stages.

In particular, environmental stressors which activate the neuroendocrine stress axis during the larval stage are known to negatively affect the immune system of post-metamorphic amphibians (Gervasi and Foufopoulos 2008), resulting in more sensitive juveniles to the three major pathogens linked to amphibian decline: ranaviruses and two species of chytrid fungi in the genus *Batrachochytrium* (Stuart et al. 2004; Rollins-Smith 2017). Larval exposure to pesticides can also lead to suppression of the immune system, thereby preventing amphibians from developing a normal and adequate response against pathogens (reviewed in Mann et al. 2009). Furthermore, altered sex ratios and gonadal abnormalities are examples of physical manifestations of hormone disruptions during the events leading up to metamorphosis (reviewed in Mann et al. 2009). Many environmental contaminants, and increasing mean temperature due to global warming, are known to affect sex ratios during larval life resulting in distorted sex ratios at or soon after metamorphosis (reviewed in Hayes 1998; Noriega and Hayes 2000). Pesticides used in agricultural production around the world may adversely affect the reproductive capacity of anurans via endocrine disruption (Hayes et al. 2002a,b; 2003; Oka et al. 2008) and, as a consequence, potentially contribute to population declines.

Owing to many contributing factors, causes for amphibian population decline are complex (Blaustein et al. 2011), but also the effect of environmental stressors as endocrine disruptors in amphibian metamorphosis is complex, as several stressors may act in synergy in larval habitat (Blaustein and Kiesecker 2002; Ficetola et al. 2015). Synergistic interactions may lead to a reduced capacity for developmental and physiological plasticity, which, in turn, is of key importance for larval amphibians to cope with environmental change. However, knowledge about how endocrine disruption caused by global change affects the capacity of larval amphibians to cope with temperature variation as predicted in climate change scenarios are rare. To predict the vulnerability of amphibian populations to impacts of global change, more research is needed to investigate whether endocrine disruption modifies this capacity of larval amphibians to cope with ongoing climate change.

Objectives

My dissertation investigates the capacity for developmental and physiological plasticity of age and size in anuran larvae and their energetics at the onset of metamorphosis. I evaluate whether the endocrine-disrupting effect of environmental stress modifies this capacity and influences energetic costs and energy allocation during metamorphic climax. Furthermore, I examine possible carry-over effects on energetics and performance in later life stages caused by endocrine disruption experienced during the larval stage. I chose two anuran species, the common frog (*Rana temporaria*) and the African clawed frog (*Xenopus laevis*), which differ in their ecology and thermal background (Fig. 0.8).

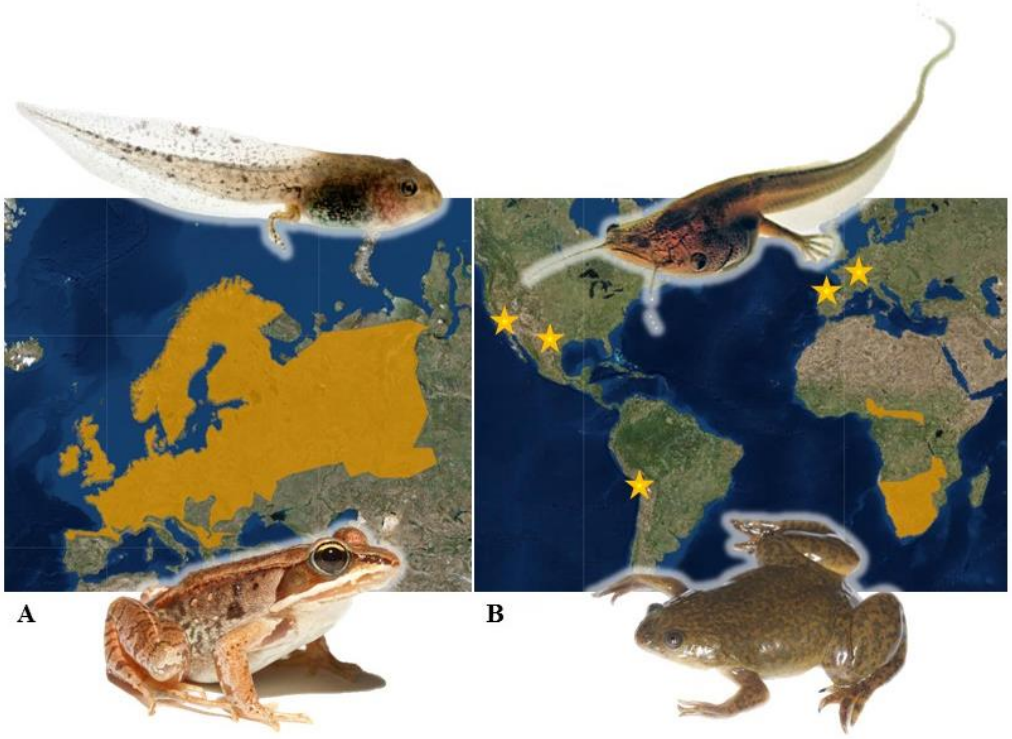

Fig. 0.8 Larval and adult phenotypes and distribution of species studied. **A** common frog (*Rana temporaria*). It is widespread throughout most of Europe, ranging from northern Spain to the Urals (absent from southern and central Iberia, much of southern Italy and the Caucasus), and eastwards to the western part of West Siberia and northern Kazakhstan through northern Greece and Bulgaria. It has a patchy distribution in the mountainous parts of the Balkans. Recorded from sea level to elevations approaching 2,700m asl (Pyrenees). **B** African clawed frog (*Xenopus laevis*). *X. laevis* are native to wetlands, ponds, and lakes across arid/semiarid regions of Sub-Saharan Africa. It is introduced (asterisks) in several places outside its native range, including the USA where it was first introduced in the 1930s and 1940s for laboratory use and later as an aquarium pet. It was introduced and established locally in California and Arizona. It has also been introduced to Chile, parts of the United Kingdom [not mapped here], in France and there is a large invasive population in Sicily [not mapped here]. This species ranges from sea-level up to 3,000 m asl (IUCN; see pictorial sources).

While *X. laevis* is a species with a solely aquatic lifestyle, *R. temporaria* represents the typical amphibian life history with a wide distribution in Europe. Because *R. temporaria* occurs in a variety of habitats at both high and low altitudes, from subtropical Mediterranean to subpolar Scandinavian regions, it is ideal for studies on phenotypic plasticity. *X. laevis* is the most well investigated amphibian species in regards to the TH system and development, and, thus, provides the physiological background for the patterns investigated in this study.

I place morphological and physiological measurements of these two species in an ecological context and discuss my results in relation to predicting the vulnerability of both species to the impacts of environmental change and temperature variation, as predicted in global and climate change scenarios. This approach provides a comprehensive investigation of several fitness related traits and the potential and limitations of adaptability to environmental stress during the larval stage in two ecologically different species. The results will contribute to a better understanding of the complex and various effects of global change on amphibian metamorphosis and possible impacts in later life stages. My research will answer specific questions on this topic in six chapters:

1. Patterns of temperature-induced developmental plasticity in anuran larvae

As is the case with most ectotherms, larval anurans show developmental plasticity in age and size at metamorphosis as a response to temperature variation according to Atkinson's (1994) intraspecific "temperature-size rule". This developmental plasticity to changing thermal conditions is expected to be a primary factor that dictates the vulnerability of amphibians to increasing ambient temperatures associated with climate change. However, the capacity for temperature-induced plasticity is known to be related to the thermal adaptation of an organism (Janzen 1967). Therefore, in the first chapter of my dissertation, I analyzed thermal effects on age and size at metamorphosis in anuran larvae to investigate whether the intraspecific "temperature-size rule" is applicable for interspecies comparisons. I did this by carrying out a combined analysis based on the data from 25 studies performed on 18 different anuran species. I tested whether the thermal background of respective populations impacted the capacity for a plastic response in metamorphic traits. Furthermore, I reviewed possible fitness consequences of temperature-induced developmental plasticity. Chapter One investigated the following questions:

- Is the intraspecific "temperature-size rule" applicable over a broad range of anuran species?

- Does the thermal background and spawning ground temperature of anuran populations impact their capacity for a plastic response in metamorphic traits?

2. Developmental plasticity in amphibian larvae as a key to coping with the proximate impacts of global change

The second chapter of my dissertation compared the capacity for developmental plasticity to variation in developmental temperatures in the two study species. As variation in developmental temperature is often accompanied by several other environmental stressors, which impact amphibian metamorphosis in different ways, I further investigated whether altered TH levels as caused by environmental stress modified the capacity for temperature-induced developmental plasticity. Chapter Two therefore investigated the following questions:

- Do larvae of *R. temporaria* and *X. laevis* show a capacity for temperature-induced developmental plasticity?
- Do these two species differ in their capacity for developmental plasticity?
- Does the endocrine disrupting effect of environmental stress modify the capacity for developmental plasticity in both species?

3. Thyroid hormone levels and temperature during development affect the thermal tolerance and energetics in larvae of *Xenopus laevis*

The third chapter of my dissertation examined whether altered TH levels caused by environmental stress, variation in developmental temperature, and their interaction influenced the capacity for physiological plasticity in larvae of *X. laevis*. I therefore investigated if standard metabolic rate and thermal tolerance range were influenced by these environmental stressors. Possible plastic responses in physiological traits provide insight into their capacity for acclimation and, thus, the compensatory ability of anuran larvae to environmental change. Moreover, testing the interactive effect of both altered TH levels and developmental

temperature may provide information about a species' vulnerability to the complex habitat alterations caused by global and climate change. Furthermore, I generated a developmental thermal window for larvae of *X. laevis* by combining critical thermal limits at a range of five different developmental temperatures. Chapter Three investigated the following questions:

- Are larvae of *X. laevis* able to show temperature-induced physiological plasticity?
- Do altered TH levels and developmental temperatures, as caused by global change, affect the capacity for physiological plasticity in *X. laevis* larvae?
- Does the interactive effect of altered TH levels and developmental temperature impact the capacity for physiological acclimation?
- Do altered TH levels affect the size of the developmental thermal window in *X. laevis*?

4. Multiple environmental stressors reduce physiological plasticity and body condition during and after metamorphosis in the common frog (*Rana temporaria*)

As the capacity for temperature-induced (physiological) plasticity is known to be related to the thermal adaptation of an organism and to be proportional to the amount of environmental temperature variation they are adapted to (Janzen 1967), temperate species such as *R. temporaria* are expected to have a high acclimation capacity to temperature fluctuations. I therefore investigated the capacity for temperature-induced physiological plasticity in larvae of *R. temporaria*. I further examined whether altered TH levels as caused by environmental stress modified this capacity. In addition, I generated a developmental thermal window for larvae of *R. temporaria* by combining critical thermal limits at a range of six different developmental temperatures and compare this with the developmental thermal window of *X. laevis*. Chapter Four investigated the following questions:

- Do temperate species such as *R. temporaria* have a high acclimation capacity during larval stage?
- Do altered TH levels as caused by environmental stress affect the capacity for temperature-induced physiological plasticity in larvae of *R. temporaria*?
- Does the interactive effect of altered TH levels and developmental temperature impact the capacity for physiological acclimation?
- Do altered TH levels affect the size of the developmental thermal window in *R.*

temporaria?

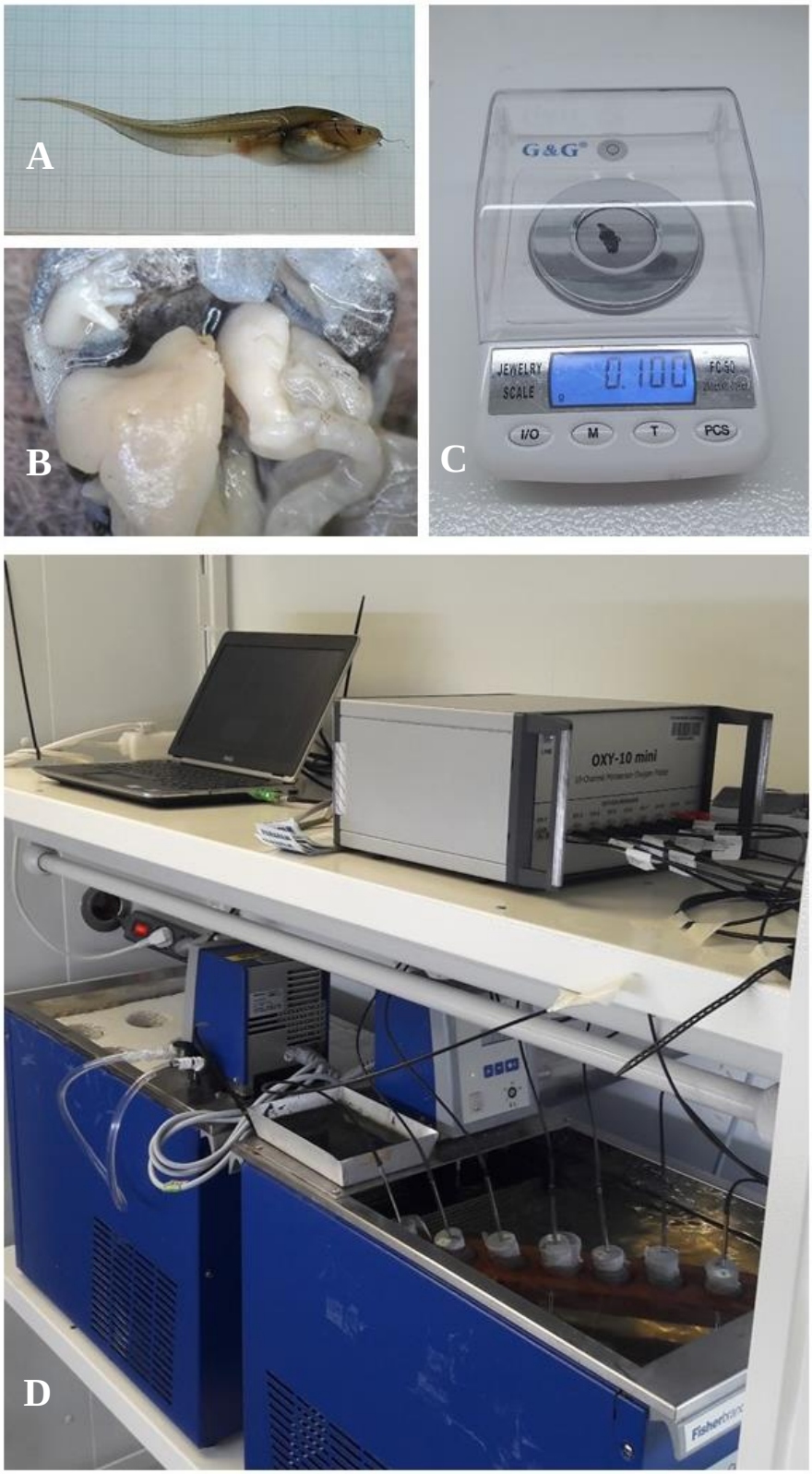

Fig. 0.9 Different measurements during the experiments on tadpoles of the common frog (*Rana temporaria*) and the African clawed frog (*Xenopus laevis*). **A** Snout-vent length (mm) and total length (mm) measurements by use of millimeter paper (picture) or an electronic caliper (not shown). **B** Dissection of the liver for calculating the hepatosomatic index. **C** Mass (mg) measurements with an electronic scale. **D** Oxygen consumption (ml O_2/h) measurements for calculation of standard metabolic rate ml (O_2/h/mg) and determination of critical thermal limits (°C). Pictures: K. Ruthsatz.

5. Altered thyroid hormone levels affect body condition at metamorphosis in larvae of *Xenopus laevis*

Environmental stressors may affect the thyroid hormone axis in larval amphibians via endocrine disruption, with consequences for energy partitioning among development, growth, and metabolism. Body condition and energy storages at the onset of metamorphosis determine the successful completion of metamorphosis in amphibian larvae. In the fifth chapter of my dissertation, I addressed the endocrine-disrupting effects of different environmental stressors on body condition at the onset of metamorphosis. I used a combination of three different body condition indices to measure energy storages and estimate fitness. Furthermore, I investigated whether altered TH levels, as caused by environmental stress, alter metamorphic traits, survival rate, and body condition at metamorphosis in *X. laevis*. Chapter Five investigated the following questions:

- Does endocrine disruption as caused by environmental stress affect metamorphic traits and survival rate?
- Is body condition influenced by altered TH levels?

6. Proximate effects of environmental stress affect energy allocation during amphibian metamorphosis and alter post-metamorphic performance in larvae of the Common frog (*Rana temporaria*)

Because environmental stress-induced endocrine disruption may affect metamorphic and physiological traits and alter body condition at the onset of metamorphosis, energy expenditure and energy allocation during metamorphic climax may be influenced as well. In the final chapter of my dissertation, I investigated the impact of altered TH levels as caused by environmental stressors on energy allocation for growth, development, and energetics at the onset of metamorphosis, during metamorphic climax and after successful completion of metamorphosis in *R. temporaria*. I measured energy expenditure during metamorphic climax and calculated maintenance and developmental cost. Moreover, I estimated fitness by examining whether altered TH status experienced during the larval stage affects energetics and performance in later life stages. Finally, Chapter Six investigated the following questions:

- Does environmental stress alter metabolic rate and body condition at the onset of metamorphosis?
- Does environmental stress influence energetic costs and energy allocation

during metamorphosis?

- Do possible differences in energetics (i.e. metabolic rate and body condition) persist beyond the metamorphic boundary?
- Do altered TH levels as caused by environmental stress during larval stage lead to carry-over effects on post- metamorphic performance?

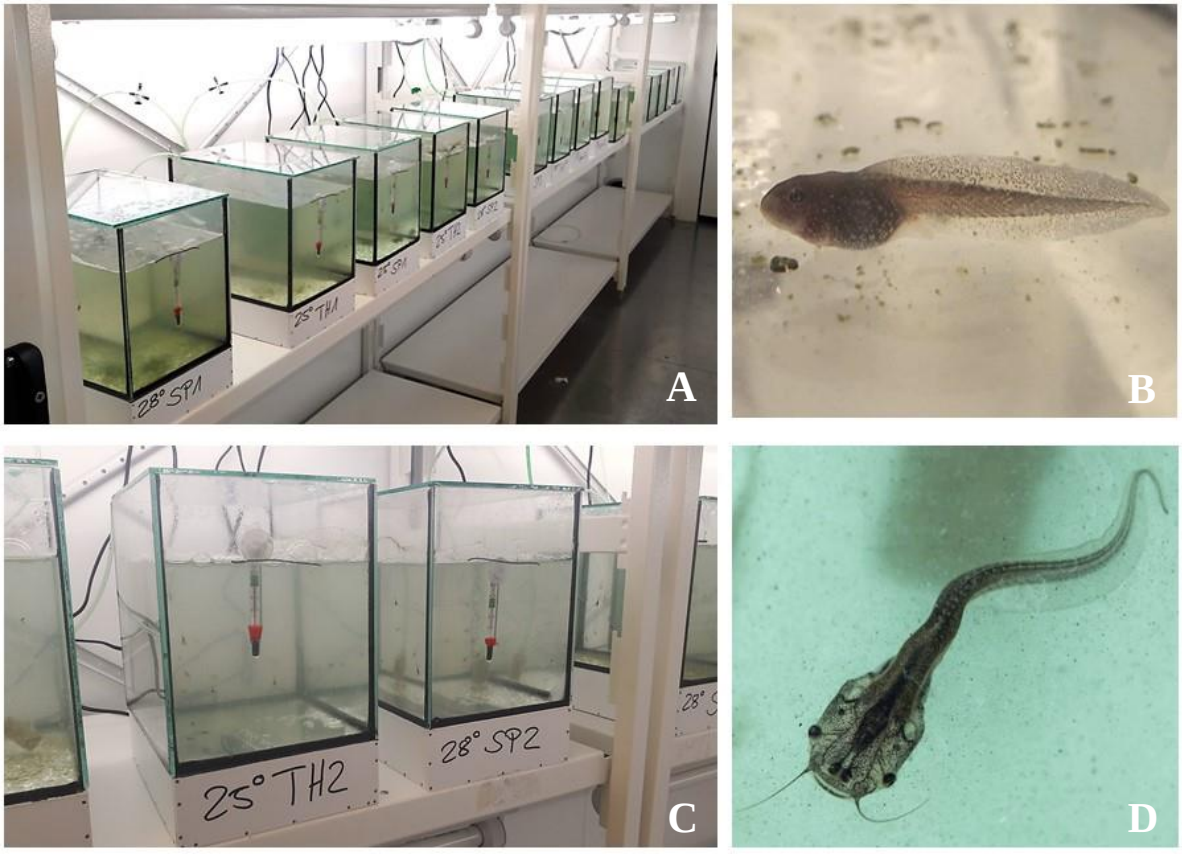

Fig. 0.10 Animal husbandry during the experiments conducted in two separate climate chambers. **A** and **C** Aquaria filled with 8 L of water at different experimental tempeatures in a range from 10°-28°C (*Rana temporaria*) and 16°-28°C (*Xenopus laevis*). SP (i.e. sodium perchlorate) and TH (i.e. thyroid hormone L-thyroxine, T4) for different exogenous hormone treatments during the experiments to achieve altered endogenous thyroid hormone levels in the tadpoles. SP=dereased TH levels. TH=increased TH levels. **B** Larvae of the common frog (*R. temporaria*) and **D** the African clawed frog (*X. laevis*) during the experiments. Pictures: K. Ruthsatz.

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Chapter One

Patterns of temperature induced developmental plasticity in anuran larvae

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Abstract

Anurans exhibit plasticity in the timing of metamorphosis and tadpoles show phenotypic plasticity in age and size at metamorphosis as a response to temperature variation. This developmental plasticity to changing thermal conditions is expected to be a primary factor that dictates the vulnerability of amphibians to increasing ambient temperatures such as are predicted in climate change scenarios. We analyzed the patterns of thermal effects on size and age at metamorphosis to investigate whether the intraspecific "temperature-size rule" is applicable over a broad range of anuran species by carrying out a combined analysis based on the data from 25 studies per- formed on 18 anuran species. Furthermore, we tested whether the thermal background of respective populations impacts the capacity for a plastic response in metamorphic traits. We could confirm this pattern for across- population comparisons. All included populations developed faster and 75% were smaller at the onset of me- tamorphosis when developmental temperatures were warmer, but the sensitivity of growth and developmental rate to a given temperature change was different. We found that the thermal background of a population in- fluences the sensitivity of metamorphic traits and thus, the capacity for a plastic response in growth and de- velopmental rate. Warm adapted populations were less sensitive to temperature variation indicating a reduced capacity for developmental plasticity and therefore, those species may be more vulnerable to the impacts of climate change. Future studies should include a broader range of rearing temperatures and temperature fluc- tuations to determine full knowledge of the capacity for developmental plasticity within a species-specific thermal window.

Keywords

Metamorphosis, Thermal tolerance, Climate change, Temperature-size rule, Thermal adaptation, Tadpoles

1. Introduction

Species are declining worldwide due to habitat loss, disease, and environmental variation as caused by both natural and anthropogenic global environmental change (Stuart et al. 2004; IUCN 2013; Strong et al. 2017). In organisms with complex life cycles such as holometabolous insects, marine invertebrates, parasites, most teleost fish, and amphibians changing environmental conditions lead to a plastic response in their timing of metamorphosis (i.e. plastic growth and developmental rate) (Wilbur 1980; Pechenik et al. 1998; Rudolf & Rödel 2007; Laudet 2011). Depending on the taxa, metamorphosis can cause a life-history transition that involves radical changes in habitat, morphology, and physiology. Thus, the timing of metamorphosis is of key importance for the entire life history and population dynamics (Walters & Hassall 2006; Rudolf & Rödel 2007). For organisms that live in temporally and spatially heterogeneous environments, phenotypic plasticity in age and size at metamorphosis may provide a means for increasing fitness in later life stages (Schlichting & Pigliucci 1998; Boorse & Denver 2004). For example, in amphibians, traits such as short larval period and large size at metamorphosis are assumed to confer greater fitness (Wilbur & Collins 1973; Beck & Congdon 2000).

Larval amphibians are especially sensitive to environmental variation due to their life history (Searcy et al. 2015), their highly permeable skin (Yu et al. 2013; Strong et al. 2017), and their limited capacity for habitat selection (Sanzo & Hecnar 2006; Yu et al. 2013). Amphibians exhibit plasticity in the timing of metamorphosis and tadpoles show phenotypic plasticity in the larval stage in general, but especially in age and size at metamorphosis (Wilbur & Collins 1973; Newman 1992; Denver et al. 1998; Boorse & Denver 2004). Different studies demonstrated a plastic response of metamorphic traits in anuran larvae to changes in environmental conditions such as crowding (Ding et al. 2015), presence of predators (Relyea & Hoverman 2003; Relyea 2007), food quality and quantity (Courtney Jones et al. 2015), photoperiod (Laurila et al. 2001), desiccation (Gervasi & Foufopoulos 2008), water quality (Calich & Wassersug 2012), and temperature (Vences et al. 2001).

As amphibians are ectotherms, temperature is one of the major abiotic factors influencing metamorphosis. This can be directly related to the thermal sensitivities of the rate of biochemical and physiological processes that underlie morphogenesis (Smith-Gill & Berven 1979; Hayes et al. 1993; Denver et al. 1998; Little & Seebacher 2016). Plasticity in age and size at the onset metamorphosis results from plastic responses of somatic growth of existing

tissues and of the developmental rate of new tissues (Newman 1992; Chambers & Leggett 1992). These two processes can, to some extent, be decoupled by environmental factors (Zuo et al. 2012; Walters & Hassall 2006; Gomez-Mestre et al. 2010).

The plasticity in growth and development can be explained by the intraspecific "temperature-size rule" (TSR), which predicts that ectothermic species, including amphibians, reared at relatively lower temperatures display slower growth rates but a prolonged larval period and thus, typically mature later at larger sizes when compared with conspecifics reared at higher temperatures (Atkinson 1994; Angilletta et al. 2004; Courtney Jones et al. 2015). Walters & Hassall (2006) emphasized that developmental rate is more sensitive to increasing temperatures than growth rate due to differential effects on anabolism and catabolism (von Bertalanffy 1960; Angilletta & Dunham 2003). Therefore, a higher temperature affects the development stronger than the growth rate (Gomez-Mestre et al. 2010).

For the TSR an optimal thermal range exists and this is bordered by a suboptimal range in which age and size plasticity does not occur (i.e. extreme conditions at which size decreases significantly) (Walczynska et al. 2016) and thermal limits, which are usually defined by the critical thermal minimum (CT_{min}) and maximum (CT_{max}) in amphibians (Cowles & Bogert 1944; Lutterschmidt & Hutchison 1997; Turriago et al. 2015). The magnitude and direction of the response to temperature is species- and population-specific and depends on the range in thermal tolerance (Freitas et al. 2010). Adaptive shifts in the thermal range of tolerance (i.e. thermal adaptation) can result from biological processes that occur over longer time scales (Angilletta at al. 2002). Pörtner et al. (2006) uses "thermal windows" as an alternative term for the range of thermal tolerance of growth and development for all aquatic taxa but not for anurans which usually have a semiaquatic life-history. However, in most cases a tadpole's mode of life is entirely aquatic. Therefore, we suggest equally using the term "thermal windows" to describe the range of temperatures suitable for the development of anuran larvae. The thermal window in amphibians is generally related to the geographic and altitudinal distribution of the species (Turriago et al. 2015). In ectotherms, it is relatively broad in temperate species, narrower in tropical species and most narrow in species found only in polar areas (Huey & Kingsolver 1993; Angilletta et al. 2002; Pörtner & Peck 2010). In tadpoles, the width of thermal windows increases from tropical to temperate latitudes due to an increasing cold tolerance (Gutiérrez-Pesquera et al. 2016).

Temperatures beyond this species- or population-specific thermal window proximately cause stress which alters tadpoles' hormonal balance by activating the neuroendocrine stress axis

(Wilbur & Collins 1973; Berven & Chadra 1988; Laudet 2011; Navas et al. 2016). As metamorphosis is a process driven by thyroid hormones (TH), stress hormones may interact with TH resulting in increased TH production (Laudet 2011; Glennemeier & Denver 2002) and thus, lead to an increased developmental rate. Consequently, the rate of metamorphosis is in two respects influenced by the ambient temperature: through physiological and endocrine mechanisms which result in plastic responses of growth and developmental rate (Smith-Gill & Berven 1979; Denver et al. 1998; Courtney & Jones et al. 2015).

This considerable impact of temperature on growth and development during the larval stage and therefore, on fitness in later life stages, takes on greater significance in terms of the ongoing global climate change: The frequency of extreme thermal events (temperature peaks beyond CT_{max} of many species, increased desiccation risk, and increased mean annual temperatures) will increase in the future in all climate zones (Pachauri et al. 2014; Gutiérrez-Pesquera et al. 2016). Rijnsdorp et al. (2009) and Mehner et al. (2011) emphasize that knowledge on the thermal window of organisms is fundamental to understand the response of populations to global warming. Numerous studies on anuran larvae investigated the effect of temperature on growth and developmental rate and thus, on age and size at the onset of metamorphosis. However, these studies refer to the species-specific effect of temperature during the larval period (Smith-Gill & Berven 1979; Àlvarez & Nicieza 2002; Walsh et al. 2008; Dittrich et al. 2016; Courtney Jones et al. 2015; Gutiérrez-Pesquera et al. 2016). Even if amphibian larvae are generally known to develop at different rates and metamorphose at different sizes within their thermal windows, across-species comparisons, which allow for projections on the impact of climate change, are rare.

In this paper, we examine whether there is a general pattern of thermal effects on age and body size at the onset of metamorphosis in anuran larvae. We perform a combined analysis based on a total of 25 studies from 18 articles published between 1988 and 2016. This analysis aims to examine whether the "temperature-size rule" is not only applicable to intraspecific but also to interspecific comparisons of different anuran species. We specifically investigated (1) the effect of rearing temperature on metamorphic traits within and across all included populations, and (2) how the thermal background of the respective populations impacts the sensitivity of growth and developmental rates to different rearing temperatures. Furthermore, we review the potential consequences of temperature-driven plastic responses in rates of growth and development of pre- and pro-metamorphic larvae to post-metamorphic and adult life stages. This synthesis reveals whether common patterns exist among species-

specific thermal effects on metamorphic traits which would allow more robust projections on the impacts of climate change at individual and population level.

2. Material & Methods

2.1 Systematic literature review

We did a systematic literature review using ISI Web of Science in January 2017 (searched for: "**TOPIC**"; search term: (("amphibian larvae" OR "anuran larvae" OR "tadpoles") AND ("thermal" OR "temperature" OR "environment*" OR "abiotic" OR "biotic" OR "climat* change" OR "climat* shift" OR "acidification" OR "pH" OR "predator" OR "density" OR "desiccation") AND ("effect*" OR "impact*" OR "cause") AND ("growth" OR "development*") AND (rate OR time) AND ("larval time" OR "larval duration" OR "larval period") AND (development* window OR "thermal window") AND ("development* plasticity" OR "growth plasticity" OR "plasticity metamorphosis") AND ("size" OR "time" OR "age") AND ("metamorphosis")); Timespan**:** All years.) (Pullin & Stewart 2006). The systematic literature review returned 1236 articles into an unfiltered reference library. After examining titles and abstracts, 523 articles were left as possibly relevant in the filtered reference library. Examining the full text of the filtered reference library led to 18 articles accepted in the reference library. The following selection criteria had to be fulfilled by the experimental design of the included studies: (1) experiments were conducted in the laboratory (no field studies), and (2) at least at two different rearing temperatures for the tadpoles. These articles were published between 1988 and 2016 (with 55% of the studies published during the past 10 years) and comprised 25 individual studies representing 25 amphibian populations, which were included as replicated in the analysis. These 25 populations were from 18 species, 12 genera and 7 families according to the Linnean classification.

2.2 Data collection

For each population we extracted results for age (days after hatching to onset of metamorphosis), and size at onset of metamorphosis and T_{rear} as rearing temperature (i.e. tested temperature points) used in the respective studies. Size was measured by mass (mg), and snout-vent length (SVL; mm). Rearing temperatures reached from 10 to 33 °C (Appendix Table A.1). The onset of metamorphosis was defined as the emerging of at least one forelimb according to Gosner developmental stage 42 (Gosner 1960). For studies which use figures instead of tabularization of their results we used Engauge Digitizer 9.7 to extract data from

the graphs. Different sample sizes for independent variables mass, SVL, and age at metamorphosis result from studies which include not all of the three variables.

2.3 Thermal background

From each study we extracted information on the respective spawn collection site as detailed as possible. To investigate the thermal adaption of the population to the climate of spawn collection site we listed average annual temperature ($T_{average}$), temperature of the coldest (T_{CM}) and hottest (T_{HM}) month of the respective spawn collection site using the database of Climate-Data.org. We used macroclimate data (i.e. non-buffered air temperature; Steffens et al. 2016) as a proxy to estimate the thermal adaptation for the respective populations due to the lack of the microclimate data (e.g., actual water temperatures in the breeding pond) in original articles. We are aware of the restriction when predicting the impacts of climatic change using macroclimate data, because understanding the way that microenvironments filter environmental fluctuations is crucial for amphibian larvae inhabiting fluctuating microhabitats (Woods et al. 2015; Oyamaguchi et al. 2017). Temperatures at spawn collection sites reached from 0.5 to 26.5 °C for the average annual temperature, from 13 to 31 °C for the hottest month, and -13 to 25 °C for the coldest month (Appendix Table A.1).

2.4 Statistical analysis

All statistical tests were carried out in the R environment (R 3.4.1; R Development Core Team, 2007) for Windows and plots were constructed using ggplot2 (Wickham 2009) and Adobe Illustrator CS6.

2.5 Effect of temperature on metamorphic traits

We used a plasticity index (PIX) that describes the change in in metamorphic traits with a given change in rearing temperature (Appendix Table A.1). This PIX was determined by performing linear regressions of rearing temperature (independent variable) and metamorphic traits (dependent variables) for individual populations (Fig.1) and using the slope to determine the effect of rearing temperature on metamorphic traits (as measured by SVL, mass, and age). PIX values indicate the sensitivity of growth and developmental rate to different rearing temperatures, and thus, the ability for a plastic response in metamorphic traits. Values for SVL, mass, and age were log-transformed to account for high levels of regression residuals.

2.6 Effect of thermal adaptation on metamorphic traits

To examine the effect of the thermal background on metamorphic traits (i.e. the plasticity index of SVL, mass, and age at the onset of metamorphosis) data were analyzed using linear mixed-effect models [lme, Type III model, covariance type: variance components, REML (restricted maximum likelihood) method for parameter estimation, 100 iterations (Bates & Sarkar 2007)], using the covariates 'T_{CM}' and 'T_{HM}' as fixed factor. The plasticity indices for log-transformed SVL, mass, and age at the onset of metamorphosis were used as dependent variables in three separate models (Table 1). P-Values were obtained from likelihood-ratio tests, which compared the models with the respective null-model (Crawley 2007). To avoid pseudoreplication and to correct for phylogenetic differences, the variables 'population', 'species', 'genus', and 'family' were included as a nested random factor. Although the number of species is moderate (N=18) this is close to the 20 species key value to have statistical power to distinguish a significant phylogenetic signal value (Blomberg et al. 2003). The nesting followed the Linnean classification (i.e. family/genus/species/population) and addresses the interrelation of datapoints due to varying distances of phylogenetic relatedness.

Before the analysis, the covariates T_{CM} and T_{HM} (i.e. explanatory variables) in the models were tested for covariation using Spearman's rank correlation. Consequentially, all covariates were included in statistical analysis as the correlation was significant but well below the suggested threshold of 0.7 for eliminating variables (N=69, R^2=0.349, P=<0.001) (Chin 1998).

3. Results

We tested the effect of rearing temperature on age and size at the onset of metamorphosis in anuran larvae performing a combined analysis based on a total of 25 studies. Furthermore, we tested how the thermal background of a population at respective spawn collection site affects the plasticity index (PIX) of metamorphic traits.

3.1 Effect of rearing temperature on age and size at the onset of metamorphosis (PIX)

3.1.1 Age at metamorphosis

Across all studies, log-transformed age at the onset of metamorphosis (AOM) was significantly, linearly related to temperature (T) according to: AOM = -0.02(T)+2.11, (N = 23, R^2=0.218, P<0.001) indicating that AOM decreased by 0.95 days with every 1°C increase in rearing temperature (Fig. 1). The highest rearing temperatures led to the youngest age at the onset of metamorphosis and vice versa. On individual study level AOM also decreased as with increasing rearing temperature in 100% of the studies but to different extent (Appendix Table A.1).

3.1.1 Size at metamorphosis

Across all studies neither log-transformed mass nor SVL at the onset of metamorphosis decreased or increased significantly with rearing temperature (mass: y=-1.01x+2.65, N = 15, R^2=0.11, P=0.66; SVL y=-0.48x+1.34, N = 6, R^2=0.16, P=0.11) (Fig. 1). On individual study level slopes of mass at metamorphosis reveal a decrease with increasing rearing temperature in 75 % of the studies, whereas SVL decreased in 50 % of the studies (Appendix Table A.1).

3.1 Effects of the population-specific thermal background on the PIX of age and size at metamorphosis

The population-specific thermal background influences the PIX of age, mass, and SVL at the onset of metamorphosis to different extents (Table 1). Generally, the warmer the climate at the spawn collection site, the higher the PIX of age and size at metamorphosis (Fig. 2).

Whereas the T_{HM} marginal significantly affected the PIX of age and mass at the onset of metamorphosis, T_{CM} had a significant effect on the PIX of mass and a highly significant effect on SVL (Fig. 2). The higher the temperatures of the warmest month the higher the PIX of age

and mass, whereas the lower the temperatures of the coldest month the lower is the PIX of mass and SVL (Fig. 2).

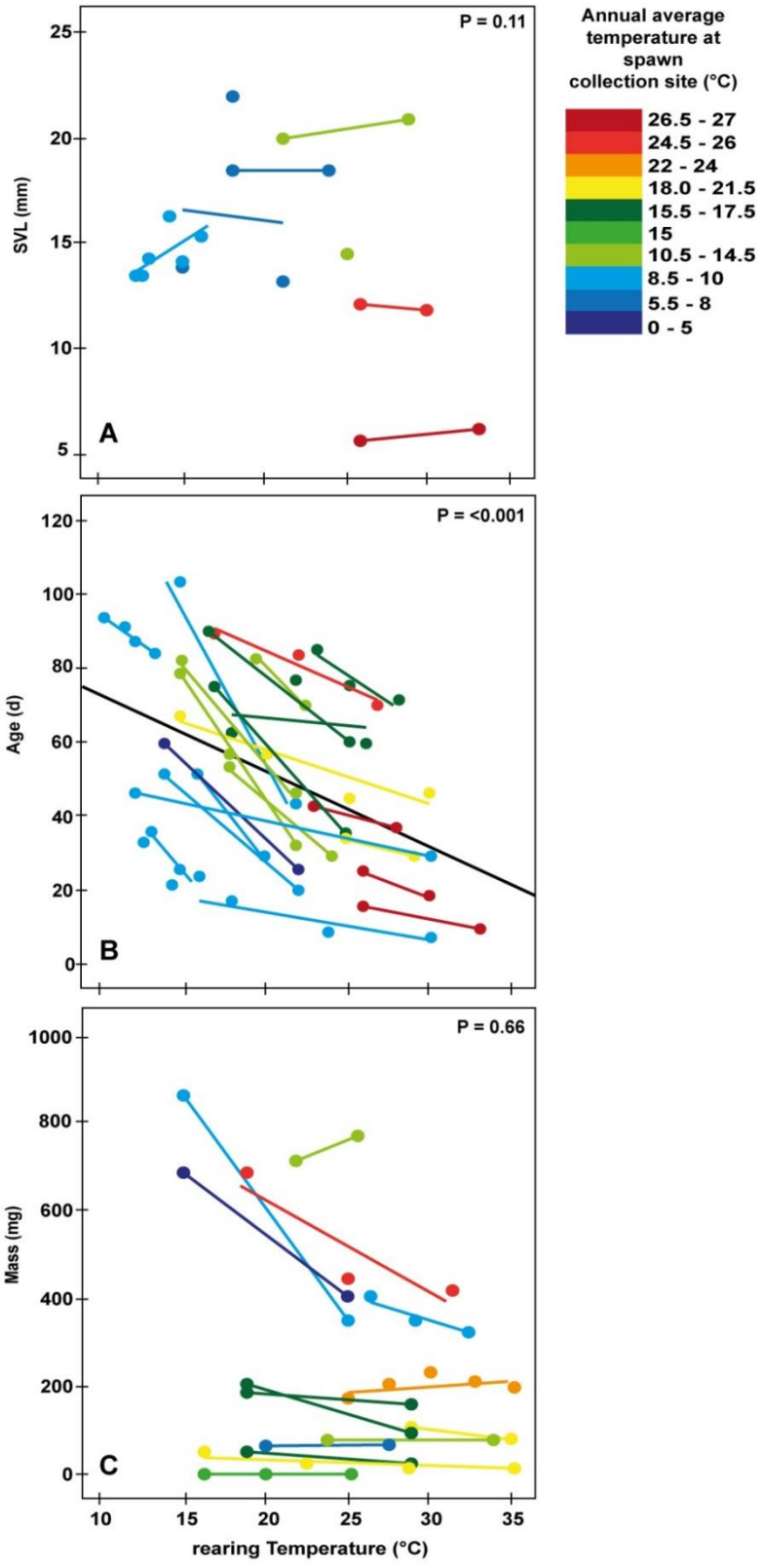

Fig. 1. The effect of rearing temperature (T_{rear}) on absolute values of **A** snout-vent length (SVL), **B** age, and **C** mass at the onset of metamorphosis. Dots and respective regression lines refer to the individual studies. Black regression line shows the general effect of T_{rear} on dependent variables of all included studies if regression is significant. The color code refers to the annual average temperature (°C) at spawn collection site of respective populations.

3.2 Effects of the population-specific thermal background on the PIX of age and size at metamorphosis

The population-specific thermal background influences the PIX of age, mass, and SVL at the onset of metamorphosis to different extents (Table 1). Generally, the warmer the climate at the spawn collection site, the higher the PIX of age and size at metamorphosis (Fig. 2). Whereas the T_{HM} marginal significantly affected the PIX of age and mass at the onset of metamorphosis, T_{CM} had a significant effect on the PIX of mass and a highly significant effect on SVL (Fig. 2). The higher the temperatures of the warmest month the higher the PIX of age and mass, whereas the lower the temperatures of the coldest month the lower is the PIX of mass and SVL (Fig. 2).

Table 1. Effects of the thermal background (T_{CM} and T_{HM}) on plasticity index (PIX) of log-transformed snout-vent length (SVL), mass, and age at the onset of metamorphosis of different anuran species. The plasticity index describes the change in metamorphic traits with a given change in rearing temperature. T_{CM} = Temperature of the coldest month. T_{HM} = Temperature of the hottest month at spawn collection site. N is the total number of included studies, n refers to the number of groups in nested random effects. Random effects were nested for phylogenetic correction.

Dependent variable	**Fixed factor**	**Linear mixed-effect model**						**Nested random effects (n)**		
		Estimate	**SE**	**Chi²**	**Df**	**P**	**N**	**Species**	**Genus**	**Family**
PIX Age (d)	T_{HM}	-0.071	0.001	3.521	1	0.06	23	18	13	8
	T_{CM}	-0.073	0.001	0.172	1	0.677				
PIX Mass (mg)	T_{HM}	-0.068	0.001	3.388	1	0.065	15	13	9	5
	T_{CM}	-0.073	0.007	4.303	1	**0.038**				
PIX SVL (mm)	T_{HM}	-0.074	0.004	0.325	1	0.568	6	6	5	5
	T_{CM}	-0.079	0.001	27.799	1	**<0.001**				

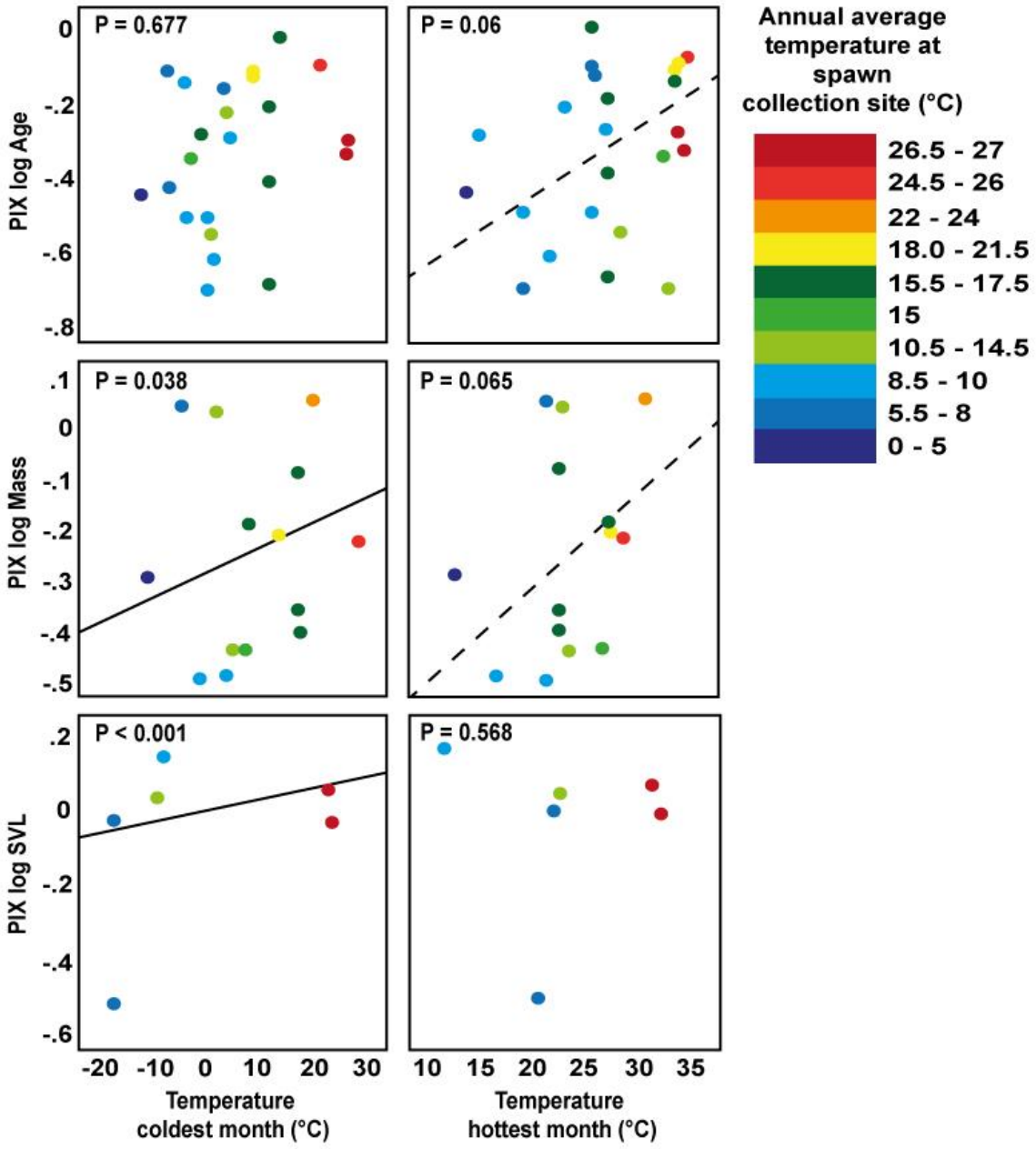

Fig. 2. Effects of thermal adaptation (T_{CM} and T_{HM}) on plasticity index (PIX) of log-transformed snout-vent length (SVL), mass, and age at the onset of metamorphosis of different anuran species. The plasticity index describes the change in metamorphic traits with a given change in rearing temperature. T_{CM} = Temperature of the coldest month. T_{HM} = Temperature of the hottest month at spawn collection site. The color code refers to the annual average temperature (°C) at spawn collection site of respective populations.

4. Discussion

Temperature has a profound influence on rates of development and growth of ectotherms and, with the ongoing warming of aquatic habitats, a better understanding is needed of the thermal

windows supporting growth and survival of organisms. Surprising little work has been made on anurans and our study compiles the available data from 25 different studies to examine general, interspecific relationships between metamorphic traits and temperature. Our results demonstrated that age and size at the onset of metamorphosis are lowest at warmest rearing temperatures and highest at coldest rearing temperatures. Thus, warmer developmental temperatures due to climate change may result in smaller and younger metamorphic larvae. Furthermore, we detected an effect of the population-specific thermal background on the sensitivity of the metamorphic traits with a given change in rearing temperature. Metamorphic traits are less sensitive to changing developmental temperatures in populations adapted to warmer mean temperatures.

4.1 Thermal effects on age and size at metamorphosis

According to Atkinson (1994) there is a very clear instraspecific pattern between growth, size and temperature: most ectotherms at colder temperatures grow slower but reach a larger size than at higher temperatures. Our results confirm this pattern also for different species adapted to different climates but to different extent. Hence, we show that TSR (Atkinson 1994) is also applicable for across species comparisons, but with some constraints.

In our analysis, age at the onset of metamorphosis was lower at warmer developmental temperatures in all included populations indicating that developmental rate responds plastically to temperature variation independent from respective thermal adaptation. This is obviously due to the general accelerating effects of increasing temperature on physiological and biochemical processes in general and especially on the intensity of THs which are the major triggers of amphibian metamorphosis (Smith-Gill & Berven 1979; Tata 2006; Little & Seebacher 2016). THs are more effective at warmer temperatures during development and their intensity on developmental rate is positively correlated with developmental temperature (Ceusters et al. 1978). This impact of temperature on the TH affects all populations despite their respective thermal adaptation and any stress induced endocrine disruption which may arise due to temperature variation.

Unlike age, no consistent decrease at warmer temperatures was observed in mass or SVL at the onset of metamorphosis. These results for body size demonstrate that the reaction and sensitivity of growth rate are not independent from thermal adaptation. Nevertheless, growth rate is accelerated at warmer temperatures due to the effects of temperature on physiological and biochemical processes (Smith-Gill & Berven 1979; Tata 2006; Little & Seebacher 2016).

However, the effecet of temperature on TH appears to have a greater impact on developmental rate as opposed to growth rate (Hayes et al. 1993; Shi 2000). These two rates can, to some extent, be decoupled by this thermal effect on the intensity of TH (Zuo et al. 2012; Walters & Hassall 2006; Gomez-Mestre et al. 2010). Furthermore, growth rate was generally less sensitive than developmental rate within our analysis as shown with the PIX. Hence, growth rate is less plastic in response to different constant temperatures than development rate (Atkinson 1994; Gomez-Mestre et al. 2010). This could be due to the fact that tadpoles must reach a minimal size in order to become metamorphosed (Morey & Reznick 2000; Rot-Nikcevic & Wassersug 2004). However, there is no minimum larval duration before metamorphosis sets on as the onset of metamorphosis depends on TH concentration, which has to reach a specific, threshold level (Morey & Reznick 2000; Buchholz 2017).

4.2 Thermal adaptation affects thermal sensitivity of metamorphic traits

In this analysis, we also investigated whether the population-specific thermal background accounts for the different sensitivity of metamorphic traits to temperature variation as shown by the different plasticity indices for age and size of respective populations. We found that both temperature of the coldest and of the hottest month impact the sensitivity of age and size at the onset of metamorphosis to temperature variation and thus, require the ability for a plastic response in growth and developmental rate. We assume that tadpoles are adapted to the thermal background at respective spawn collection sites.

Our results revealed that populations from colder climates (i.e. low temperatures in the coldest and the hottest month) show lower plasticity indices indicating that development and growth rate are more sensitive to a given change in temperature. Therefore, those populations from colder climates, such as temperate anurans, are more likely to respond plastically in both rates to developmental temperature variation. In contrast, populations adapted to warm temperatures in the coldest and hottest month revealed a lower sensitivity of metamorphic traits to temperature variation indicating a reduced capacity for a plastic response in both growth and developmental rate. Less sensitive growth and developmental rate to changing developmental temperature are common in populations from warmer climates (i.e. tropical climates) due to the relatively stable thermal environments in the tropics (Janzen 1967; Gunderson & Leal 2015; Oyamaguchi et al. 2017). However, temperate populations experience more heterogenous thermal environments during their larval stage. Therefore,

selection favors a high sensitivity of growth and developmental rate due to temperature variation resulting in a high capacity for a plastic response in both rates (Seebacher et al. 2015).

4.3 Fitness consequences

Ecologists generally view the relationship between growth and development in anuran larvae as an adaptive strategy for coping with selection pressures such as temperature variation (Rose 2005). In larvae with a reduced capacity for a plastic response in growth and developmental rate as shown for populations from warm climates in our analysis this coping with temperature variations is complicated and limited. As a consequence, negative impacts of temperature variation and extreme thermal events are greatest in warm adapted populations due to the lack of accelerating their developmental rate to avoid overheating or to escape from increased desiccation risk of ephemeral ponds (Gunderson & Stillman 2015).

Developmental plasticity is adaptive in variable environments (Newman 1992), but the capacity for a plastic response in growth and developmental rate as a result of temperature variation in larval history leads to different ages and sizes at the onset of metamorphosis which in turn are known to influence fitness in later life stages (i.e. age and size at first reproduction, breeding success, and jumping ability) (Smith-Gill & Berven 1987; Denver 1997; Altwegg & Reyer 2003). Individuals with larger body size will tend to have greater performance and fitness than smaller individuals (Berven 1990; Kingsolver & Huey 2008). Consequently, a decrease in body size as a result of higher temperatures may be disadvantaged compared to individuals that experience colder temperatures and that reach larger body sizes.

However, growing to and sustaining large size also entails significant costs and risks, as well as delayed maturation and increased energy demands (Kingsolver & Huey 2008). Only few studies could show the disadvantage of a large body size (but see Wassersug 1975; van Buskirk 2017). In terms of energy demand, Beck & Congdon (2000) found that mass at metamorphosis was positively related to metabolic rate, whereas age at metamorphosis was negatively correlated with metabolic rate. Thus, smaller individuals need less energy for energy maintenance (Beck & Congdon 2000). Particularly under resource limitation smaller individuals are relatively efficient in maintaining somatic functions due to their lower energy demand (Wassersug 1975). Van Buskirk (2017) emphasizes that consequences of body size depend on the way competing individuals of a group interact. Individuals that metamorphose

at a smaller size (i.e. due to higher water temperatures in ponds) may benefit by an earlier escape from predation pressure in their larval habitats but may incur costs such as reduced juvenile survivorship (Smith 1987; Berven 1990; Zhao et al. 2014), physiological performance (e.g. the ability to withstand starvation and tolerance to dehydration or to escape predators (Zhao et al. 2014), as well as reduced fecundity and reduced size at first reproduction (Smith 1987; Semlitsch et al. 1988; Berven 1990).

4.4 Potential impacts of climate-driven warming

Climate change is increasing mean environmental temperatures and the frequency of extreme thermal events (Seebacher et al. 2015; Pachauri et al. 2014; Gutiérrez-Pesquera et al. 2016). The ability to show developmental plasticity to changing thermal conditions is expected to be a primary factor that dictates the vulnerability of amphibians to rising temperatures (Huey et al. 2012; Stillman 2003; Gunderson & Stillman 2015). As we demonstrated in this study, populations developing at warmer mean temperatures show a reduced capacity for a plastic response in growth and developmental rate and therefore, the impacts of climate change may be more severe on those populations or species (Tewsbury et al. 2008, Somero 2010; Oyamaguchi et al. 2017). However, to predict the vulnerability of a species, we need to determine not only its sensitivity of metamorphic traits, but also the magnitude of exposure to climate-driven warming.

In addition to developmental plasticity, the capacity for physiological acclimation is crucial for organisms to compensate for climate-driven changes in temperature. As shown for growth and developmental rate in our analysis, the capacity for a plastic response in physiological traits (e.g. metabolic rate and thermal limits) is also reduced in warm adapted or rather tropical species due to their narrower thermal window (Huey & Kingsolver 1993; Angilletta et al. 2002; Pörtner & Peck 2010). Due to their higher capacity for plastic responses in developmental and physiological traits, temperate species may be less vulnerable to the impact of climate-driven warming (within limits).

Nevertheless, Kingsolver et al. (2015) emphasized that using constant temperature studies to model the consequences of variable thermal environment as caused by to climate change is precarious. Most terrestrial and aquatic ectotherms experience daily and seasonal variation in temperature and then, mean organismal performance can differ in fluctuating and constant thermal environments, an effect due to Jensen's inequality for non-linear functions (Ruel & Ayres 1999; Martin & Huey 2008; Kingsolver et al. 2015). Thus, models of ectotherm

responses to climate change that are parameterized from data sets gathered under constant (or that assume no change in the variance of thermal regimes) may contain systematic errors when compared with the real world (Paaijmans et al. 2013).

5. Conclusion

Climate-driven warming in terms of global change will affect larval anurans, which are especially sensitive to environmental variation due to their life history, their highly permeable skin, and their limited capacity for dispersal. Animals, which are able to exhibit phenotypic plasticity in the timing of metamorphosis within their thermal windows, may escape from those heterogeneous and unpredictable larval habitats. Our effort highlights the current lack of data required to thoroughly test how the effect of temperature on developmental traits and data on temperature given developmental plasticity from temperate and tropical amphibians are, however, still too limited to allow for generalized projections on the impacts of climate change at individual and population level. Furthermore, the numerous studies investigating the effect of temperature on metamorphic traits and the sensitivity of metamorphic traits to temperature in terms of thermal adaptation use only 2-3 constant rearing temperatures. These studies are not sufficient to resolve the true shape of the thermal window and the capacity for developmental plasticity according to Kingsolver & Huey (2008). Future studies need to include a broader range of rearing temperatures in order to make robust comments on the developmental plasticity within the thermal window of specific populations or species. In addition, studies need to examine the difference between the effects of temperature fluctuation and different constant temperatures on developmental plasticity in the context of climate change.

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8. Appendices

Table A.1 Summary table of 18 studies reporting the effect of temperature on the age and size at metamorphosis in larval anurans. Studies were separated by species as well as by different spawn collection site. Spawn collection site is given as detailed as mentioned in references. T_{rear} = rearing temperature in respective study. T_{CM}= coldest month, T_{HM}= hottest month, $T_{average}$= annual average temperature at spawn collection site. Plasticity index (PIX) of age and size at the onset of metamorphosis (log-transformed) in different anuran species describing the change in metamorphic traits with a given change in rearing temperature. PIX (i.e., regression slope) was calculated from linear regressions. P and R^2 for linear regressions.

Species	T_{rear}	Spawn collection site					Plasticity Index					Reference
									Metamorphic traits			
		Spawn collection site	Coordinates	T_{CM}	T_{HM}	$T_{average}$	Number of T_{rear} (°C)	Linear Regression	Age (d)	Mass (mg)	Snout-vent length (mm)	
Bufo terrestris	25 30	Savannah River Site. USA	32°10'42.7''N 81°04'43.9''W	8.0	28.0	18.25	2	**PIX**	-0.013	-0.021		Beck & Congdon 2000
Pelophylax saharicus	22 24 26 28 30	Medenine. Tunisia	33°20'25.4''N 10°29'41.2''E	13.0	31.0	22.00	5	**PIX**		0.006		Bellakhal et al. 2014
								R^2		0.185		
								P		0.441		
Rana sylvatica	10.5 11.75 12.25 13.37	Dreadful Hollow Pond. Ilex Pond. Southwest Woods Pond. Star Pond. West Marsh Pond. and West Woods Big Pond Pinckney. USA	42°36'25.6''N 83°55'50.0''W	-4.0	22.0	9.00	4	**PIX**	-0.015			Benard 2015
								R^2	0.979			
								P	**0.009**			
Rana arvalis	16 20	Greifswald. Germany	54°05'07.5''N 13°23'23.9''E	1.0	19.0	10.00	2	**PIX**	-0.062			Burmeister 2015
Discoglossus galganoi	17 25	Grândola. Portugal	38°11'29.6''N 8°33'59.1"W	11.0	23.0	17.00	2	**PIX**	-0.021	-0.036		Carreira et al. 2016
Hyla arborea	17 25	Grândola. Portugal	38°11'29.6''N	11.0	23.0	17.00	2	**PIX**	-0.06	-0.04		Carreira et al.

			8°33'59.1" W						8	0		2016
Hyla meridionalis	17 25	Grândola. Portugal	38°11'29.6" N 8°33'59.1" W	11.0	23.0	17.00	2	**PIX**	-0.041	-0.008		Carreira et al. 2016
Rhacophorus moltrechti	17 22 27	Farmlands in the Wushan area Tainan. Taiwan	23°02'27.6" N 120°31'52.8"E	20.0	29.0	24.50	3	**PIX**	-0.010	-0.022		Chang et al. 2014
								R²	0.918	0.805		
								P	0.17	0.279		
Lithobates pipiens	23 28	Artificial breeding; Nasco - Fort Atkinson. USA	56°20'29.9" N 2°47'33.6" W	-7.0	22.0	7.50	2	**PIX**	-0.012			Freitas et al. 2017
Xenopus laevis	18 24	Artificial breeding; Nasco - Fort Atkinson. USA	56°20'29.9" N 2°47'33.6" W	-1.0	23.0	11.00	2	**PIX**	-0.029	0.004	0.003	Gomez-Mestre et al. 2010
Agalychnis callidryas	21 29	Artificial breeding; Boston University	42°21'02.1" N 71°06'33.8" W	-7.0	220	7.50	2	**PIX**	-0.043	0.005	-0.052	Gomez-Mestre et al. 2010
Pseudacris ornata	15 20 25 30	Several breeding ponds on the Savannah River Plant Aiken County. USA	32°10'42.7" N 81°04'43.9" W	8.0	28.0	18.00	4	**PIX**	-0.012	-0.03		Harkey & Semlitsch 1988
								R²	0.882	0.447		
								P	0.061	0.331		
Limnodynastes peronii	13 14.2 15	Greater Illawarra region of south-eastern New South Wales. Australia	34°26'00.0" S 150°51'00.0"E	13.0	22.0	17.50	3	**PIX**	-0.002			Courtney Jones et al. 2015
								R²	0.027			
								P	0.906			
Rana temporaria	12.2 12.7 13.1 14.5 14.9 16.1	Ponds in southwestern and central Scania. province Skåne Lund. Sweden	55°42'18.1" N 13°11'29.3" E	0.0	17.0	8.50	6	**PIX**	-0.071		0.015	Loman 2002
								R²	0.746		0.488	
								P	**0.032**		0.129	
Rhinella granulosa	26 33	Temporary ponds at the campus of the Universidade Estadual	12°15'11.9" S 38°57'53.6" W	25.0	28.5	26.75	2	**PIX**	-0.034		-0.003	Maciel & Juncá 2009

		de Feira de Santana Feira de Santana. Brazil										
Pleurodema diplolister	26 30	Temporary ponds at the campus of the Universidade Estadual de Feira de Santana Feira de Santana. Brazil	12°15'11.9''S 38°57'53.6''W	25.0	28.0	26.50	2	**PIX**	-0.030		0.005	Maciel & Juncá 2009
Rana perezi	19.5 22.5	Pond in Chozas de Arriba. Spain	42°31'17.5''N 5°42'01.0"W	3.0	20.0	11.5	2	**PIX**	-0.023			Martinez et al. 1996
Rana temporaria	14 22	Lund. Sweden	55°42'18.1''N 13°11'29.3''E	0.0	17.0	8.5	2	**PIX**	-0.051	-0.049		Merilä et al. 2000
Rana temporaria	14 22	Kiruna. Sweden	67°51'34.7''N 20°13'25.7''E	-12.0	13.0	0.5	2	**PIX**	-0.052	-0.029		Merilä et al. 2000
Rana sylvatica	15 18 22	Saginaw Forest. University of Michigan. USA	42°16'13.2''N 83°48'23.4''W	-4.0	22.0	9.0	3	**PIX**	-0.056	-0.05		Riha & Berven 1991
								R^2	0.994	0.957		
								P	0.262	**0.05**		
Rana sylvatica	15 18 22	The Shenandoah Mountains. USA	38°29'28.2''N 78°50'52.0''W	1.0	24.0	12.5	3	**PIX**	-0.036	-0.044		Riha & Berven 1991
								R^2	0.995	0.972		
								P	**0.026**	0.107		
Rana sylvatica	15 18 22	Beltsville Agricultural Research Station. USA	39°01'32.2''N 76°55'23.1''W	3.0	27.0	15.0	3	**PIX**	-0.03	-0.044		Riha & Berven 1991
								R^2	0.873	0.998		
								P	0.098	**0.03**		
Xenopus laevis	18 24 30	Artificial breeding; St Andrews University. Scotland	56°20'29.9''N 2°47'33.6"W	4.0	14.0	9.0	3	**PIX**	-0.015			Walsh et al. 2008
								R^2	0.979			
								P	0.265			
Rana sylvatica	15 18 21	Tzfardeyah Pond at Warner Nature Center Marine on	45°10'21.6''N 92°49'54.2''W	-8.0	23.0	7.5	3	**PIX**			-0.003	Watkins & Vraspir 2005
								R^2			0.4	
								P			0.95	

		St. Croix. USA									0	
Rana chensinensis	23.2 25.3 28.1	Xinyang. China	32°09'04.5''N 114°05'05.2"E	3.0	28.0	15.5	3	**PIX**	-0.016	-0.019		Yu et al. 2015
								R^2	0.91	0.930		
								P	0.207	0.171		

Author contribution

I hereby confirm that Katharina Ruthsatz conceived, designed and performed the experiments, analyzed the data and wrote the paper.

Hamburg,

Prof. Dr. Kathrin Dausmann

Chapter Two

Developmental plasticity in amphibian larvae as a key to coping with the proximate impacts of global change

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Abstract

In anuran larvae, thyroid hormone (TH) levels control growth and developmental rate and changes in TH status are a well-known stress response to sub-optimal environmental conditions. We investigated how temperature and altered TH levels affected metamorphic traits and survival at the onset of metamorphosis in larvae of the tropical African clawed frog (*Xenopus laevis*) and the temperate common frog (*Rana temporaria*). The capacity of these larvae to exhibit developmental plasticity among different developmental temperatures was examined and related to their thermal ecology. In both species, altered TH levels influenced survival, growth, and development and modified the capacity for temperature-induced developmental plasticity. High TH levels reduced thermal sensitivity of metamorphic traits and vice versa. At the onset of metamorphosis, size and age was more plastic in *X. laevis* and *R. temporaria*, respectively. Age was twice as plastic at high TH levels in *R. temporaria*. Plasticity in age (developmental rate) is likely more adaptive than growth rate in *R. temporaria* as an increase in developmental rate allows larvae to emerge more rapidly from larval ponds if conditions are sub-optimal. Since TH status also influences metabolism, future studies should investigate whether reductions in physiological plasticity is increasing the vulnerability of tadpoles to global change.

Keywords

Temperature, metamorphosis, amphibian decline, climate change, temperature-size-rule, tadpoles

Introduction

Anthropogenic alterations in abiotic and biotic environmental factors cause species and population declines worldwide due to, among others, habitat loss, disease, pollution, and increasing mean and extreme temperatures (Stuart et al., 2004; Gunderson & Stillman, 2015; Strong et al., 2017). Aquatic organisms, especially those with complex life cycles such as anuran larvae, often have a limited capacity to search for new, more favorable microhabitats as larvae (Yu et al., 2013; Searcy et al., 2015; Gutiérrez-Pesquera et al., 2016) and, hence, must cope with the stress of being exposed to unfavorable environmental conditions (Schulte, 2014; Dantzer et al., 2014; Burraco & Gomez-Mestre, 2016). Plasticity in growth and development in response to changes in abiotic factors can be an effective mechanism to avoid negative impacts on organism fitness (Schlichting & Pigliucci, 1998; Boorse & Denver, 2004). Especially in anurans, individuals risk a significant reduction in fitness if they fail to reach a specific stage (i.e. the onset of metamorphosis) before a set time, especially in ephemeral waters (Rudolf & Rödel, 2007).

A multitude of environmental stressors affect growth and development of larval anurans by influencing physiological processes and hormone systems (Calich & Wassersug, 2012; Beachy et al., 1999). Environmental stress results in the activation of stress hormones (Dantzer et al., 2014; Denver et al., 1998). Amphibian metamorphosis is under endocrine control by thyroid hormones (TH) governing the timing and speed of the morphological reorganization (Shi, 2000; Tata, 2006) and stress hormones can act to increase the production of TH (Laudet, 2011; Glennemeier & Denver, 2002) and thus, can accelerate developmental rate. However, environmental stressors may also decrease TH production in anuran larvae by obstructing TH production pathways resulting in decreased developmental rates (Ortiz-Santaliestra & Sparling, 2007; Bulaeva et al., 2015). Thus, environmental stress can lead to different ages and sizes at the onset of metamorphosis in anuran larvae by disrupting the endocrine control of metamorphosis (Wilbur & Collins, 1973; Denver et al., 1998). Whereas many natural changes in environmental conditions, such as crowding (Ding et al., 2015), the presence of predators (Relyea, 2007), food quality and quantity (Courtney Jones et al., 2015; Carabio et al., 2017), photoperiod (Laurila et al., 2001), desiccation risk (Gervasi & Foufopoulos, 2008), and temperature (Vences et al., 2002), are known to stimulate the production of TH, several studies have reported a decreased in production of TH in larvae exposed to municipal effluent, pesticides and herbicides, road salt, fertilizers, heavy metals

and active pharmaceutical ingredients during amphibian metamorphosis (Lefcort et al., 1998; Ortiz-Santaliestra & Sparling, 2007; Fong et al., 2016).

From all environmental stressors affecting growth and developmental rate in ectotherms such as anuran larvae, temperature is the most decisive (Dalvi et al., 2009; Berg et al., 2017), as body temperatures fluctuate with environmental temperatures and the rates of most biochemical reactions and biological processes increase approximately exponentially with temperature (Harkey & Semlitsch, 1988; Zuo et al., 2012; Theisinger et al., 2017). Larval amphibians are especially likely to encounter variation in temperature because they live in a variety of aquatic habitats and often in shallow ephemeral ponds (Walsh et al., 2008; Yu et al., 2015). However, an increase in temperature will influence development more than growth (Smith-Gill & Berven, 1979; Zhao et al., 2014), which leads to a reduced size at metamorphosis under higher temperatures (Alvarez & Nicieza, 2002; Tejedo et al., 2010; Ruthsatz et al., 2018). This is consistent with the temperature-size rule (TSR; Atkinson, 1994) which anurans are known to follow (Ashton, 2002; Olalla-Tárraga & Rodriguez, 2007; Ruthsatz et al., 2018). Ectotherms reared at relatively lower temperatures typically mature later at larger sizes than conspecifics reared at higher temperatures (Atkinson, 1994; Laugen et al., 2005; Courtney Jones et al., 2015). Thus, there is an optimal thermal range for the development of amphibians which is buffered by a suboptimal range in which plasticity in age and size at metamorphosis does not occur (Walczynska et al., 2016; Ruthsatz et al., 2018).

Considering the current worldwide decline of amphibians (Alroy, 2015; Stuart et al., 2004), it is critical to investigate whether and how anuran larvae adjust their metamorphic traits in response to new thermal challenges and to endocrine disruption caused by natural or anthropogenic stressors (Strong et al., 2017). The role of environmental stress in amphibians and amphibian life-histories has recently received attention (Räsänen et al., 2003; Gabor et al., 2013, 2017; Kaiser et al., 2015) but little is known regarding the interactive effects of simultaneously and sequentially occurring stressors. Furthermore, global change is projected to continue and will generat novel combinations and severities of stressors (Williams and Jackson, 2007; Niinemets et al., 2017).

In this study, we examined how developmental temperature (T_{dev}) and altered TH level as caused by environmental stressors acted to influence the survival and mean and plasticity in metamorphic traits (size and age) at the onset of metamorphosis in larvae of two anuran species, the tropical African clawed frog (*Xenopus laevis*) and the temperate common frog

(*Rana temporaria*). These species differ in their thermal ecology as they represent a common temperate (*R. temporaria*) and tropical (*X. laevis*) species which allows for a comparison of how thermal ecology influences temperature-induced developmental plasticity. Investigating the capacity for developmental plasticity of tropical and temperate anuran larvae may allow more robust projections on the impacts of global change at both the individual and population levels.

Material & Methods

Study species and experimental design

Rana temporaria and *X. laevis* were chosen as study species. *Xenopus laevis* is the best studied amphibian species in terms of the TH system and development (Buchholz, 2017), providing solid physiological background for the patterns investigated in this study. Whereas *X. laevis* completes its entire life cycle in aquatic habitats, *R. temporaria* switches to terrestrial habitats as juveniles and adults, representing the typical amphibian life history strategy in contrast to *X. laevis*. *R. temporaria* is widely distributed throughout Europe and the duration of its larval period is known to be highly dependent on environmental conditions (Laurila et al., 2002). The plasticity of responses in age and size at metamorphosis of *R. temporaria* to variation in environmental factors is also known to be higher than in other anuran species (Laurila & Kujasalo, 1999).

Five clutches of *R. temporaria* were obtained from Waldpark Marienhöhe in Hamburg, Germany (53° 34'37.4" N, 9° 46'57.5" E). Three clutches of *X. laevis* were obtained from the Universitätsklinikum Hamburg Eppendorf (UKE, Hamburg). Amphibian larvae were fed high-protein flaked fish food (Sera micron breeding feed for fish and amphibians, Sera, Heinsberg, Germany) and spirulina algae *ad libitum*. The flakes were free of perchlorate according to the manufacturer. Larvae were allowed to develop to Gosner stage 25 (free-swimming larvae; Gosner 1960). Before the start of the experiment, 675 (*X. laevis*) and 840 (*R. temporaria*) larvae were randomly selected and assigned to different treatment and the control groups. Fifteen larvae were introduced into standard 9.5-L aquaria containing 8 L of dechlorinated tap water. Each treatment was replicated three times at five (*X. laevis*) and six (*R. temporaria*) different temperatures with respective replicates of a control treatment (*X.*

laevis: 3 replicates x 3 treatments x 5 temperatures = 45 aquaria in total; *R. temporaria*: 3 replicates x 3 treatments x 6 temperatures = 56 aquaria in total).

The experiment was conducted in two climate chambers (Weiss Umwelttechnik GmbH, 35447 Reiskirchen, Germany) with a 12:12 light:dark (0900 to 2100) light regime and a mean ± SD temperature of 16 ± 0.4°C and 22 ± 0.1°C for *X. laevis* and 10 ± 0.2°C and 22 ± 0.1°C for *R. temporaria*. All other temperatures were achieved by indirect heating elements (Tetra GmbH, Melle, Germany, adjustable heating element, Tetra HT100, 100W) beneath the aquaria. The mean (± range) water temperatures were 16 (0.4), 19 (0.5), 22 (0.1), 25 (0.5) and 28 (0.3) °C for *X. laevis* and 10 (0.2), 14 (0.5), 18 (0.1), 22 (0.1), 25 (0.2) and 28 (0.3) °C for *R. temporaria*.

T4 and sodium perchlorate exposures

To achieve a decrease in TH levels we used sodium perchlorate (SP), an environmental relevant endocrine disruptor, which is a goitrogen that inhibits TH synthesis via competitive inhibition of the sodium-iodide symporter (Ortiz-Santaliestra & Sparling, 2007). Because iodide is essential for the production of both T4 and T3, perchlorate may act as a disrupter of amphibian metamorphosis (Ortiz-Santaliestra & Sparling 2007). Perchlorate salts are strong oxidizers and are widely used as components of fireworks, airbags, and currently applied fertilizers (Trumpolt et al., 2005; Carr & Patino, 2011; Schmidt et al., 2012, Fig. 1). Contamination of surface and ground water occurs from military, aerospace, agriculture, and other commercial sources, but perchlorate also occurs naturally in arid places on the surface of the earth (Carr & Patino, 2011). We used a concentration of 250 µg/L SP (99.99% trace metals basis, Aldrich, Sigma-Aldrich, St. Louis, USA) to achieve a decrease in TH levels. This selected concentration of SP is within environmental ranges measured in surface and ground waters of many industrial nations (Motzer, 2001; Tietge et al., 2005; Carr & Theodorakis, 2006; Mukhi & Patino, 2007) and in bodies of water in which amphibians breed (Smith et al., 2001; Ortiz-Santaliestra & Sparling, 2007).

We achieved increased TH levels by exposing larvae to 10 µg/L exogenous L-thyroxine (T4, IRMM468 Sigma-Aldrich**,** Sigma-Aldrich, St. Louis, MO, USA), a concentration which is known to significantly influence amphibian metamorphosis (Lucas & Reynolds, 1967; Mann et al., 2009) and is concordant to increases in T4 observed in anuran larvae responding to stress (Denver, 1997; 1998). Anuran larvae absorb exogenous T4 directly through their

permeable skin (Shi, 2000; Tata, 2006; Coady et al., 2010). Exposing larvae to exogenous THs is an established method to simulate the proximate effects of environmental stressors on the TH system (Denver et al., 2002; Tata, 2006; Denver, 2009, Fig. 1).

T4 and SP treatments were prepared in 0.1 N sodium hydroxide solutions (0.1 N, S2770 SIGMA, Sigma-Aldrich, St. Louis, USA) buffered with 0.1 N muriatic acid solutions as solvents. Solutions were added to the aquaria. To control for any effect of solvents addition, a pure solution of 0.1 M sodium hydroxide solution buffered with 0.1 M muriatic acid solution was added to the control aquaria. Water was changed every second day and fresh SP and T4 were added, which is frequent enough to maintain a constant hormone and perchlorate level, in accordance with the standard procedure for chemical and hormonal addition (Miwa & Inui, 1987; Goleman et al., 2002 a,b; Iwamuro et al., 2003; Rot-Nikcevic & Wassersug, 2004; Tietge et al., 2005; Ortiz-Santaliestra & Sparling, 2007; Bulaeva et al., 2015).

Growth and development measurements

Developmental stage was determined by evaluating the status of key morphological features typical of specific developmental stages, as detailed in Gosner (1960). The developmental stage of each tadpole was recorded according to the procedure of Ortiz-Santaliestra & Sparling (2007): Gosner stage group 1-5: 1. pre-limb (absence of hind limbs, Gosner stages 24 to 26), 2. limb bud (hind limb visible, but no clear joint formed, Gosner stages 27 to 34), 3. middle hind limb (knee joint apparent, but toes not completely separated, Gosner stages 35 to 37), 4. late hind limbs (hind limb tubercles and subarticular patches formed, Gosner stages 38 to 41), and 5. metamorph (at least one forelimb present, Gosner stage 42 and above) (Gosner, 1960; Ortiz-Santaliestra & Sparling 2007). Onset of metamorphosis was defined by the emergence of at least one forelimb (Gosner stage 42; Gosner 1960). The snout vent length (SVL) and total length (TL) of the larvae were measured with a caliper to the nearest 0.5 mm. Larvae were weighed to the nearest 0.001 g with an electronic balance (digital gold scale, Smart Weigh). Growth rate (mg/d) was calculated from mass at the onset of metamorphosis minus the mass directly after hatching, divided by the days from hatching to metamorphosis (i.e. ‘age’).

Statistical analyses

All statistical tests were carried out using R (R 3.4.3; R Development Core Team, 2007) and plots were constructed using ggplot2 (Wickham, 2009) and Adobe Illustrator CS6.

Effects of altered TH levels and T_{dev} on metamorphic traits

For each species, data were analyzed using linear mixed-effect models [lme, Type III model, covariance type: variance components, REML (restricted maximum likelihood) method for parameter estimation, 100 iterations (Bates & Sarkar, 2007)]. Probability (p) values were obtained from likelihood-ratio tests, which compared the models with the respective null-model (Crawley, 2007). To address dependencies in the data, the variable 'aquarium' was included as a random factor. Residuals of each model were visually checked for normal distribution. N refers to the total number of analyzed tadpoles.

As all larvae entered metamorphosis on the same day in *X. laevis*, 'age' could not be statistically tested due to the missing variance within the groups. The confidence interval was 0 for all tested groups. However, the differences between all tested treatment and temperature groups are obvious, thus allowing for a descriptive analysis (Table 1).

For testing the effect of altered TH levels ('treatment': T4, SP, and control), developmental temperatures ('T_{dev}'), and the interaction of both within all possible treatment × T_{dev} groups, we used 'T_{dev}' as a covariate and 'treatment: T_{dev}' as fixed factors. Body mass, SVL, TL, and survival were used as dependent variables in separate models (Appendix Table 1).

Temperature effects on metamorphic traits & plasticity index (PIX)

To determine the thermal reaction norm (i.e. sensitivity) of metamorphic traits (as measured by age, mass, and SVL) to temperature variation, we performed linear regressions of T_{dev} (independent variable) and metamorphic traits (dependent variables) for both species (Table 2, Fig. 2). The slopes of the regressions for individual populations were defined as a plasticity index (PIX) describing the change in metamorphic traits with a given change in developmental temperature according to Ruthsatz et al. (2018). Linear regressions were performed on log-transformed values of SVL, mass, and age to account for the high levels of heteroscedasticity before the linear regressions.

Results

The experiments were conducted over four weeks (*X. laevis*) and eleven weeks (*R. temporaria*), by which time all surviving larvae had reached the onset of metamorphosis (Gosner, 1960). At 10° none of the *R. temporaria* anuran larvae survived until the onset of metamorphosis and, from this point on, only the 14 to 28°C treatments will be discussed.

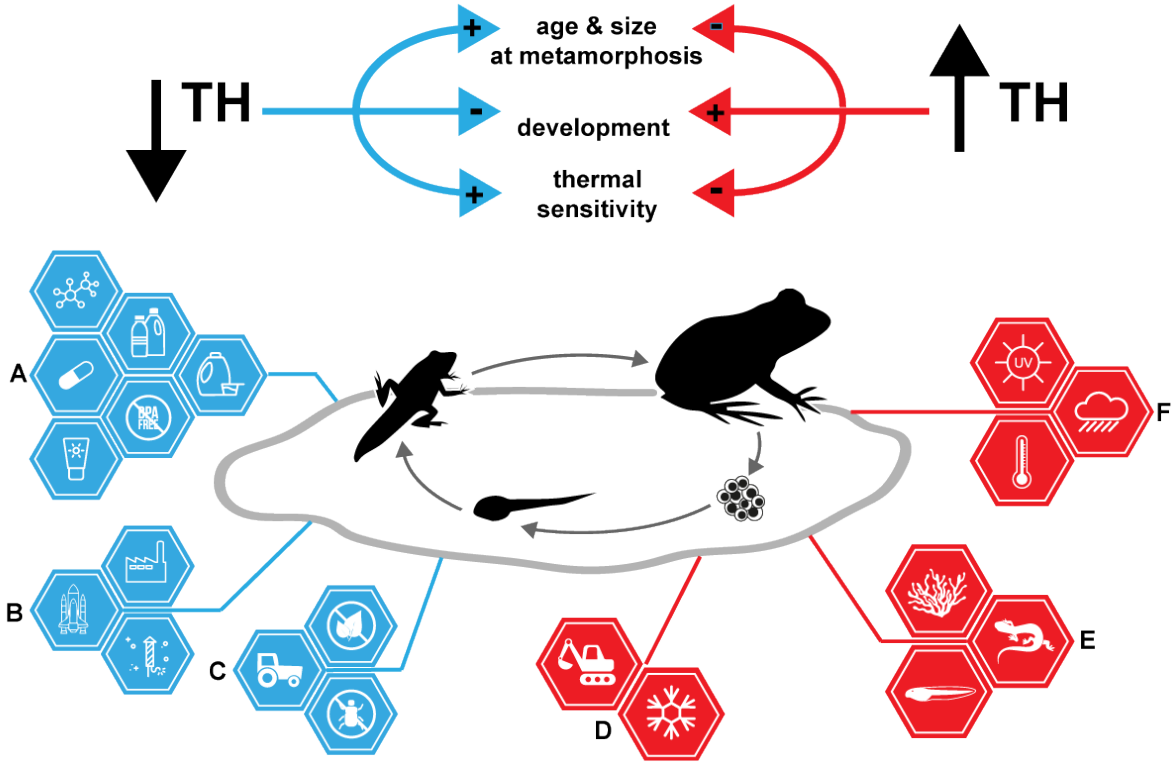

Fig. 1 Environmental stressors in the larval habitat of anuran larvae affecting developmental rate and thus, age and size at the onset of metamorphic climax by influencing endogenous thyroid hormone (TH) levels (Kashiwagi et al. 2008; Mann et al. 2009; Carr & Patino 2011; Boas et al. 2012). Altered TH levels influence the thermal reaction norm (i.e. sensitivity) of growth and developmental rate during metamorphosis. Blue symbols: stressors acting as endocrine disruptors inhibiting TH production pathways resulting in low endogenous TH levels. Red symbols: stressors increasing TH production by the activation of the neuroendocrine stress axis. **A** Household chemicals and pharmaceuticals: Artificial steroid hormones (testosterone) and hormonal contraceptives (oestrogen and gestagen), analgesic agents (e.g., ibuprofen, diclofenac), chemicals from sunscreen, microplastics from packaging and clothes, bisphenol A (BPA) from packaging, phosphates from washing agents. **B** Byproducts of industry (persistent organic products), aerospace (ClO_4^-), and fireworks (ClO_4^-). **C** Chemicals from agriculture: fertilizer (NO_3^-), herbicides, pesticides (Atrazine, Malathion). **D** Habitat fragmentation and road salt. **E** Biotic stressors: Food availability, competition (i.e. crowding), predator pressure. **F** Climatic stressors: UV-radiation, temperature variation, precipitation and desiccation risk.

Effects of altered TH levels and temperature on survival, age, and size at the onset of metamorphosis

Whereas *R. temporaria* larvae survived significantly less at the lower as well as the higher end of the T_{dev} spectrum, survival of *X. laevis* larvae was also significantly affected by treatment, as well as by the interactive effect of both (Table 1). In *X. laevis*, mean (± SD) survival from the start of the experiment (Gosner stage 25) to the onset of metamorphosis (Gosner stage 42) in the control, SP and T4 treatment groups was 92.0 (10.0), 65.7 (13.3) and 53.3(13.3) %, respectively. Thus, high levels of TH reduced survival nearly by half compared to the control group. This effect was intensified by the interactive effect of TH level and temperature. Larvae from 19°C-control, 25°C-SP and 28°C-T4 treatments experienced the lowest survival among all *X. laevis* tadpoles. Larvae survived best in 25°C-control, 28°C-SP, and 19°C-T4 treatments. In *R. temporaria* mean (± SD) survival in the control, SP, and T4 treatments was 76.4 (11.7), 77.1 (11.4), and 66.3 (13.5) %, respectively, and none of the larvae reared at 10°C survived until the onset of metamorphosis (Table 1).

Table 1 Survival (%) and age (days after hatching) at the onset of metamorphosis in anuran larvae of *R. temporaria* and *X. laevis* exposed to different combinations of developmental temperature (T_{dev}) and altered thyroid hormone (TH) levels. T4 = increased TH levels**.** SP = decreased TH levels.

		Rana temporaria						*Xenopus laevis*				
		10°C	14°C	18°C	22°C	25°C	28°C	16°C	19°C	22°C	25°C	28°C
Survival (%) ± SD	SP	0	95.5 ± 3.1	91.1 ± 3.1	97.7 ± 3.1	77.7 ± 3.3	93.3 ± 6.6	66.6 ± 0	60.6 ± 6.6	68.8 ± 4.4	57.7 ± 8.8	75.5 ± 4.4
	Control	0	91.1 ± 3.3	95.5 ± 3.3	91.1 ± 3.3	84.4 ± 3.3	91.1 ± 6.6	91.1 ± 8.8	86.6 ± 6.6	91.1 ± 8.8	97.7 ± 2.2	93.3 ± 0
	T4	0	86.6 ± 0	93.3 ± 0	84.4 ± 3.3	68.8 ± 3.3	62.2 ± 3.3	51.1 ± 2.2	68.8 ± 4.4	48.8 ± 4.4	55.5 ± 4.4	42.2 ± 4.4
Age (days) ± SD	SP	NA	59.2 ± 9.7	48.6 ± 6.7	26.0 ± 3.2	24.9± 1.3	18.0 ± 1.3	31	30	27	23	18
	Control	NA	35.8 ± 2.7	28.4 ± 1.7	20.9 ± 2.0	15.0 ± 1.2	13.1 ± 1.5	28	25	17	15	13
	T4	NA	30.0 ± 2.0	22.4 ± 1.8	16.0 ± 2.3	12.2 ± 1.7	10.3 ± 1.8	21	16	14	11	10

Age at the onset of metamorphosis was significantly different among all groups and, thus, influenced by both TH and temperature treatment during development. In *X. laevis*, larvae from the 16°C-SP group developed slowest (31 days until the onset of metamorphosis), whereas larvae from the 28°C-T4 group revealed the shortest larval period (10 days) (Table 1). In *R. temporaria*, larvae from the 14°C-SP group developed slowest (59.2 ± 9.7 days), whereas larvae from the 28°C-T4 group were found to have the shortest larval period (10.3 ± 1.8 days) (Table 1).

In both species, development was delayed by the SP treatment, whereas T4 individuals developed faster (Table A1, Fig. 1, 2). These treatment effects were more pronounced at extreme temperatures. The interactive effect of treatment and temperature was strongest at the coldest temperature and SP treatment and at the warmest temperature and T4 treatment (Fig. 2, 3). Size at the onset of metamorphosis was expressed as mass, SVL, and TL. Mass as a dependent variable was excluded from our model due to the distribution of residuals in *X. laevis*. SVL and TL were significantly influenced by the treatment (Table A1), but not by T_{dev} and the interactive effect of both in *X. laevis*. In *R. temporaria* mass, SVL, and TL were significantly affected by the treatment, T_{dev}, and the interactive effect of both (Table A1). Growth rate was only significantly affected by T_{dev} in R. *temporaria* but not in *X. laevis*. Growth was delayed in the T4 individuals (Fig. 3), whereas SP individuals grew faster in both species (Fig. 3).

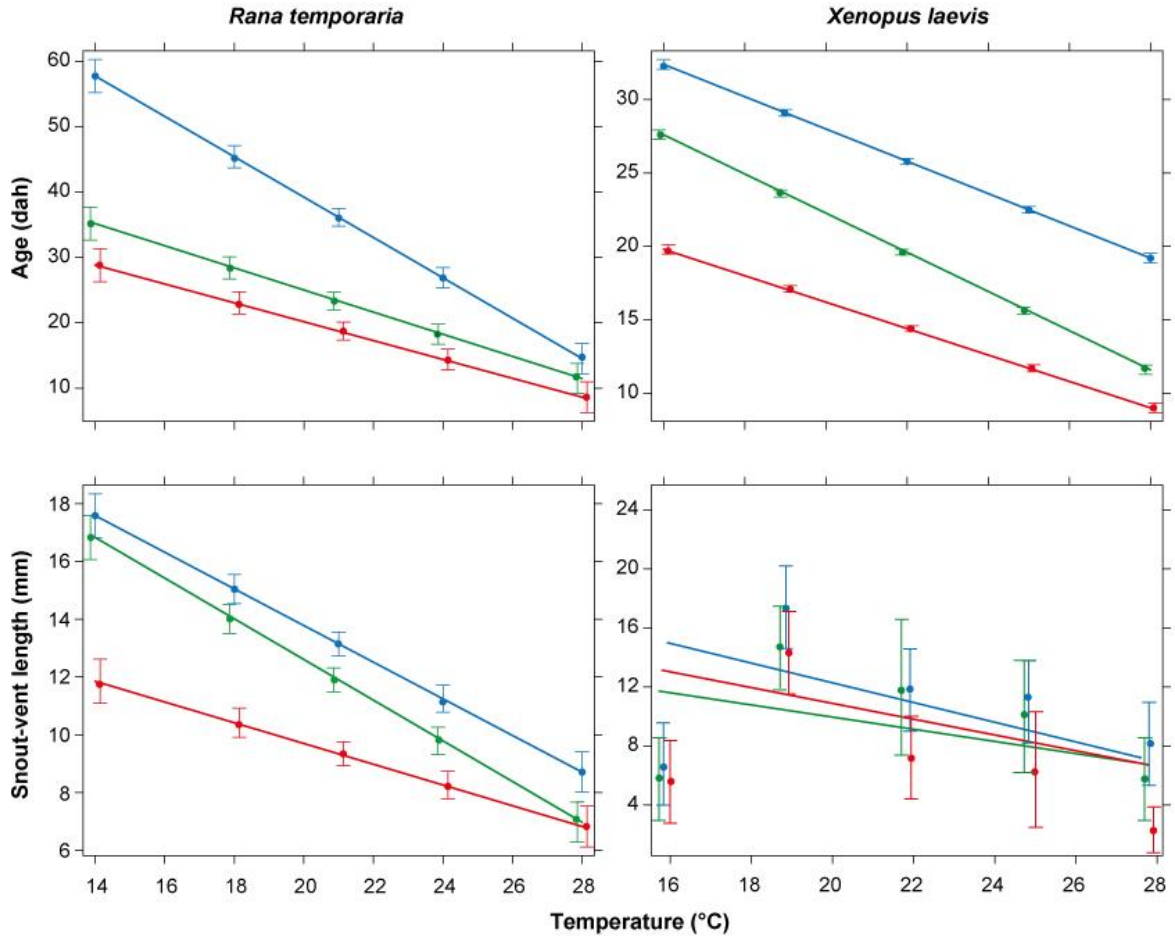

Fig. 2 Effects of altered TH level and developmental temperature on the age (days after hatching) and snout-vent length (mm; SVL) at the onset of metamorphosis in *Rana temporaria* and *Xenopus laevis*. Points show mean ± 95% confidence intervals values. Trend line for interactive effect of developmental temperature and altered TH levels calculated without data collected on *X. laevis* larvae reared at 16°C. Data were excluded from the analysis presumably because this temperature is outside the species-specific thermal range for development as larvae showed a lack of plasticity. Blue: SP = low TH levels. Green: Control group. Red: T4 = high TH levels**.**

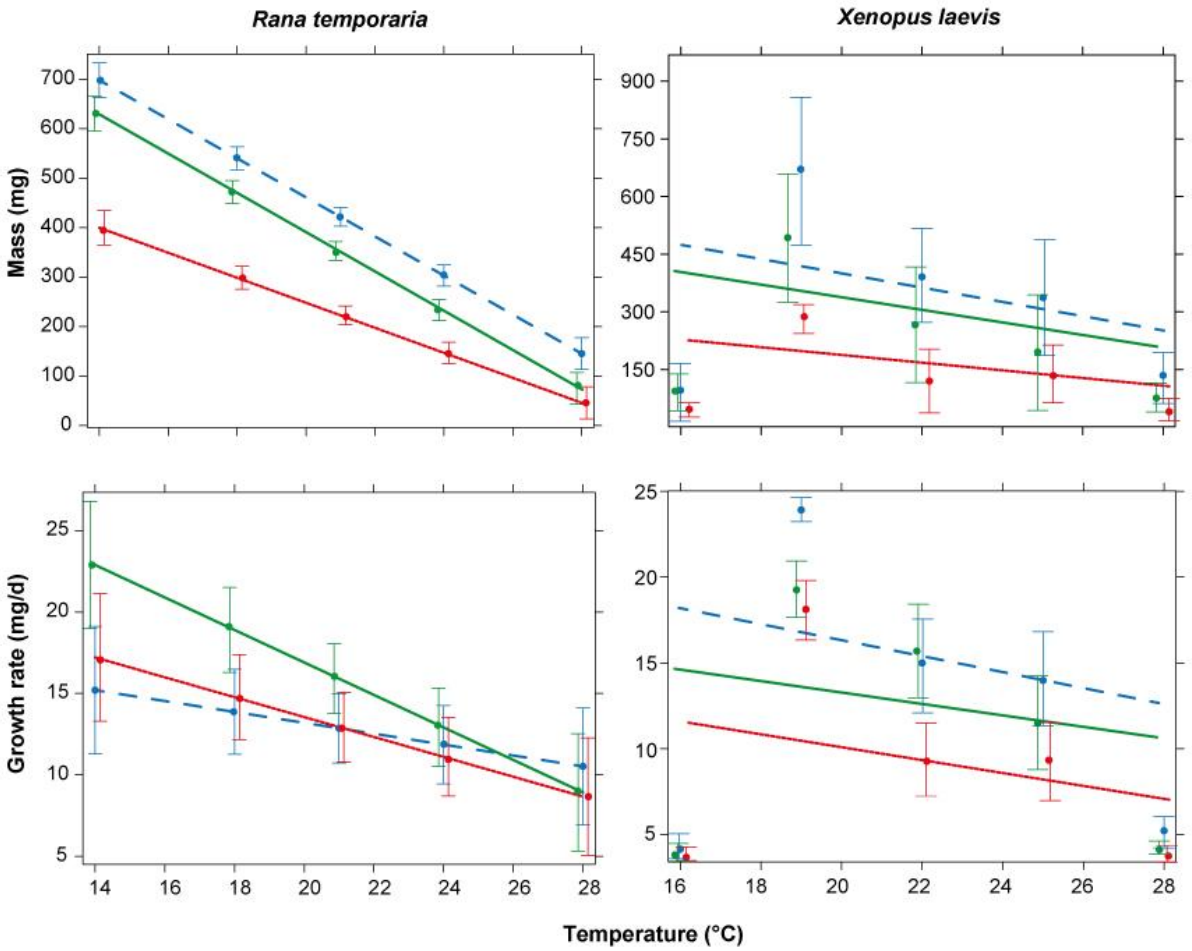

Fig. 3 Effects of altered TH levels and different developmental temperatures on mass (mg) at and growth rate (mg/d) before the onset of metamorphosis in *Rana temporaria* and *Xenopus laevis*. Circles show mean values and bars the confidence interval. Trend line for interactive effect of developmental temperature and altered TH levels calculated without data collected on *X. laevis* larvae reared at 16°C. Data were excluded from the analysis presumably because this temperature is outside the species-specific thermal range for development as larvae showed a lack of plasticity. Blue: SP = low TH levels. Green: Control group. Red: T4 = high TH levels**.**

Plasticity of age and size at the onset of metamorphosis

The data collected on *X. laevis* larvae reared at 16°C were excluded from the analysis presumably because this temperature is outside the species-specific thermal range for development as larvae showed a lack of plasticity. Thus, results presented for *X. laevis* refer to the temperature range from 19° to 28°C (Table 2).

In both species, the highest developmental temperatures led to the youngest age, lowest weight and shortest SVL at the onset of metamorphosis and vice versa in both species (Table 2). Within all treatments, age, mass and SVL at the onset of metamorphosis were

significantly, linearly correlated to temperature (Table 2). Plasticity indices of age and mass were lowest in animals from SP treatment animals, followed by animals from control treatment. Animals from T4 treatments revealed the highest PIX. For SVL, PIX was lowest in control animals, followed by T4 animals in *X. laevis* and by SP animals in *R. temporaria*. Exposure to SP increased the thermal reaction norm of metamorphic traits whereas exposure to T4 decreased the thermal sensitivity, respectively (Fig. 4). PIX for body size (snout-vent length and mass) at the onset of metamorphosis was lower in larvae of tropical *X. laevis*, whereas PIX for age was lower in larvae of temperate *R. temporaria* within the selected temperature range (Table 2).

Table 2 Thermal reaction norm of age and size at the onset of metamorphosis in *Rana temporaria* and *Xenopus laevis* exposed to different combinations of developmental temperature (T_{dev}) and endocrine disruptive treatments. Plasticity indices (PIX) were equal to the slopes of trait versus temperature regressions. T4 = increased TH levels**.** SP = decreased TH levels. P<0.001 for all linear regressions.

Dependent Variable	**Treatment**	***Rana temporaria***					***Xenopus laevis***				
		PIX	**Intercept**	**F**	**df**	**R^2**	**PIX**	**Intercept**	**F**	**df**	**R^2**
Age (d)	Control	-1.7	58.82	2994.5	216	0.9	-1.2	46.9	1089.2	165	0.8
	T4	-1.4	49.3	1733.8	187	0.9	-0.7	29.0	3246.0	95	0.9
	SP	-3.0	101.0	1023.2	218	0.8	-1.3	56.17	9976.6	117	0.9
Mass (mg)	Control	-39.5	1184.1	5166.0	216	0.9	-41.9	1209.2	4305.6	165	0.9
	T4	-25.5	758.4	7163.1	187	0.9	-26.6	749.9	837.6	95	0.8
	SP	-39.2	1247.3	3101.0	218	0.9	-62.1	1823.4	1171.1	117	0.9
SVL (mm)	Control	-0.7	26.7	1960.3	216	0.9	-1.0	35.2	988.5	165	0.8
	T4	-0.3	17.0	644.7	187	0.7	-0.9	29.5	825.8	95	0.8
	SP	-0.6	26.4	1295.5	218	0.8	-0.8	33.4	612.5	117	0.8

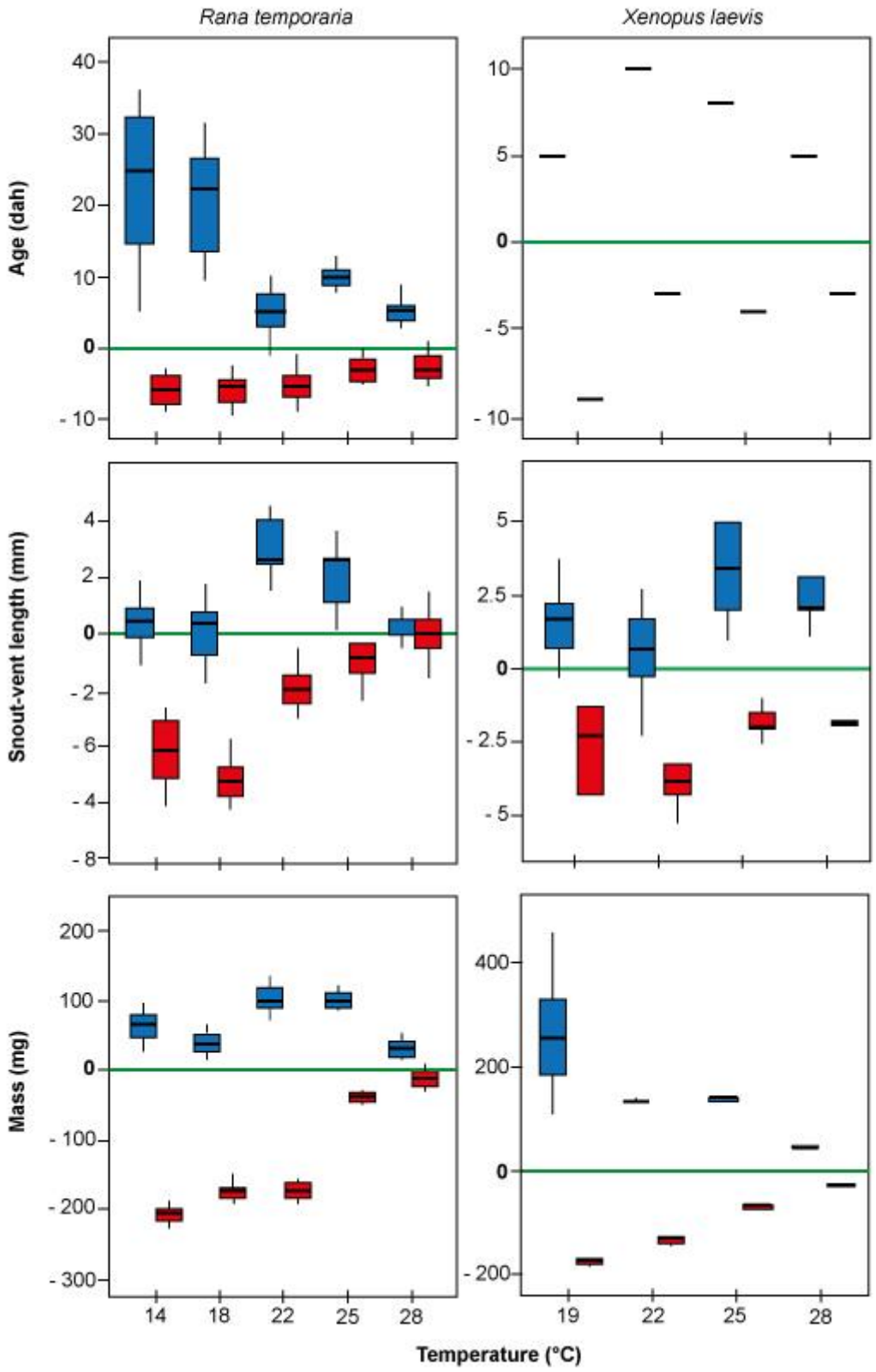

Fig. 4 Species-specific differences in thermal reaction norm of age, mass, and snout-vent length at the onset of metamorphosis in *Rana temporaria* and *Xenopus laevis* exposed to different endocrine disruptive treatments. Shown is the mean deviation for each hormone treatment from the control group in age (days after hatching), snout-vent length (mm), and mass (mg) at the onset of metamorphosis. The data collected on *X. laevis* larvae reared at 16°C were excluded from the analysis as larvae showed a lack of plasticity. Thus, results presented for *X. laevis* refer to the temperature range from 19° to 28°C. Positive and negative values indicate an increase and decrease in thermal sensitivity of

metamorphic traits, respectively. Blue boxes: SP treatment = low TH levels; the green line indicates the level of the control group. Red boxes: T4 treatment = high TH levels.

Discussion

Changing environmental conditions elicit plastic responses in most organisms, especially in those living in temporally and spatially heterogeneous environments. In amphibians, for example, the timing of and size at metamorphosis (i.e. plastic growth and developmental rate) may be adjusted (Wilbur, 1980; Pechenik et al., 1998; Rudolf & Rödel, 2007; Laudet, 2011; Ruthsatz et al., 2018), allowing for increased fitness in later life stages (Schlichting & Pigliucci, 1998; Boorse & Denver, 2004) as a short larval period and a large size at metamorphosis are assumed to confer greater fitness (Wilbur & Collins, 1973; Beck & Congdon, 2000). Most studies of phenotypic plasticity investigate the effects of single environmental factors on organism phenotypes (Stillwell et al., 2007). However, organisms exist in an ecologically complex world where multiple environmental factors interact to affect growth, development and life histories. Furthermore, ongoing (anthropogenically induced) global change will result in multiple, simultaneously occurring environmental stressors. Nevertheless, studies investigating interactive effects of those environmental stressors are still rare. In this study, we examine the independent and interactive effects of two impactful environmental factors, temperature and altered TH levels (i.e. proximate effects of different thyroid-affecting stressors), using a multifactorial experimental design, on life history traits and survival in two anuran species which differ in their thermal ecology. Our results suggest that altered TH levels as caused by environmental stressors in *R. temporaria* and *X. laevis* larvae alter survival, growth and developmental rate and modify the capacity for plasticity in metamorphic traits to a given temperature change. Effects of altered TH levels were more pronounced at extreme temperatures.

Effects of altered TH levels during metamorphosis go from bad to worse at extreme temperatures – but one stressor is not like the other

Environmental stress is of great impact for anuran larvae in ephemeral habitats, proximately causing alterations in the endocrine control of metamorphosis (Räsänen et al., 2003; Burraco & Gomez-Mestre, 2016) and influencing growth, development, and survival. In the present study, larvae exposed to high TH levels at extremely high temperatures revealed the highest developmental rate. As biochemical processes speed up at higher temperatures, high TH

levels also have a greater effect on TH dependent processes during larval development (e.g. limb development) (Little & Seebacher, 2016; Shi, 2000) at higher temperatures. In contrast, SP, a TH inhibiting factor (Sparling & Fellers, 2007; Goleman et al., 2002a, b) caused reduced developmental rates in the larvae resulting in older and larger metamorphs in both species over the whole temperature range. These results are in accordance with studies on endocrine disruption and TH control of amphibian metamorphosis (Denver, 1997; Heimeier, 2010; Bulaeva et al., 2015; Li et al., 2016) indicating an obvious disruptive effect of environmental stressors on the TH system in larval anurans with ramifications for growth, development, and survival. Altered growth and developmental rate due to environmental stressors may therefore result in differences in age and size at the onset of metamorphosis. Large body size at metamorphosis is associated with high fitness in amphibians and it is beneficial to reach a large size quickly, e.g. to reduce the time spent in more predation and desiccation sensitive larval habitats (Stählberg et al., 2001). Consequently, a reduced growth and developmental rate as caused by endocrine disruptors such as SP may expose larvae longer to the risks of a larval habitat but, if larvae survive the larval stage, may increase survival to maturity. Moreover, a large body size due to a prolonged growth time is associated with reduced predator pressure during juvenile and adult stage and a better performance (Berven, 1990; Stählberg et al., 2011). In contrast, larvae exposed to TH level increasing environmental stressors may suffer from the disadvantage of a small body size as a result of increased developmental rates but also minimize the risk of a heterogeneous larval habitat (Richter-Boix et al., 2011; Orizaola et al., 2013). However, any imbalance of growth and developmental rate due to environmental stressor may impair survival, and thus fitness in later life stages. Furthermore, a shorter larval period allows for a longer accumulation of sufficient energy reserves in juveniles which is especially crucial in hibernating species such as *R. temporaria* in the present study.

In our study, survival of larvae was reduced by changes in TH levels, regardless of the direction of TH alteration. However, specific responses differed between the two species. The effects were generally most pronounced at the highest temperatures (25° and 28°C). Whereas larvae of *X. laevis* as a tropical species frequently experience these temperatures in their natural habitats, breeding ponds of the temperate *R. temporaria* rarely become this warm (Drakulic et al., 2017). Interestingly, Rühmekorf (1958) reported that pond temperatures between 21 and 26 °C are favorable for growth and development in *R. temporaria* larvae in nature which is in contrast to our results. Nevertheless, Kingsolver et al. (2015) emphasized

that aquatic ectotherms such as anuran larvae experience daily variation in temperature and that general performance can differ between fluctuating and constant thermal environments. Thus, the constantly high T_{dev} used in the present study might be more stressful and result in greater reductions in survival compared to temporarily high temperatures more similar to the natural variation in breeding ponds. The reduced survival before metamorphosis may be also caused by a low toxicity of SP itself (Coady et al., 2009) whereas the reduced survival in larvae exposed to TH is probably due to metabolic stress (Sheridan, 1994; Coady et al., 2009) as THs are also responsible for energetic homeostasis (Frieden, 1981; McNabb & King, 1993).

High endogenous TH levels lead to high standard metabolic rates and high basal energy expenditure (Rowe et al., 1998; Steyermark et al., 2005; K. Ruthsatz, unpublished data). Thus, when endogenous TH levels are increased in anuran larvae as a consequence of endocrine disruption, larvae need a higher energy intake to cover their energetic needs (i.e. maintenance costs) (Beck & Congdon, 2000; Orlofske & Hopkins, 2009), even more so at warm temperatures. On the other hand, larvae exposed to TH inhibitors are therefore likely to have lower maintenance costs (Orlofske & Hopkins, 2009) even at warm temperatures. As anuran larvae stop eating during metamorphic climax due to the remodeling of oral and intestinal structures (Orlofske & Hopkins, 2009), high TH levels predict a lower probability of completing metamorphosis, because high maintenance costs reduce the energy available for developmental costs (Beck & Congdon, 2000). Consequently, when environmental factors cause a rise in TH within the larvae, they are less likely to succeed in completing metamorphosis especially at warmer temperatures.

Proximate effects of environmental stress modify the thermal reaction norm of metamorphic traits

Anthropogenic activities including climate change not only alter temperature regimes, but also lead to multitude of interacting environmental stressors such as higher desiccation risks (Richter-Boix, 2011; Gervasi & Foufopoulos, 2008), chemical contamination of larval habitats (Shenoy et al., 2009; Mann et al., 2014), and altered presence of predators (Mikó et al., 2017; Blaustein et al., 2011). Hence, understanding how temperature and endocrine-induced stress responses interact to affect the growth and development of anuran larvae is critical if we hope to understand and project future impacts on populations.

As the TH system is related to the stress hormone system (Dantzer et al., 2014; Denver, 1997; Bonett et al., 2010), most environmental stressors lead to an increase in endogenous TH level (in anuran larvae). However, our results show that altered TH levels change the capacity of our two study species to express temperature-induced developmental plasticity as larvae exposed to high levels of THs were less able to adjust their rates of growth and developmental at higher temperatures. Since a plastic response in growth and developmental rate is beneficial in heterogeneous larval habitats as it allows for an faster escape, environmental stressors which increase endogenous TH levels may result in more vulnerable anuran larvae due to a reduced capacity for temperature-induced developmental plasticity. If habitat temperatures increase through global change, these larvae can only show a reduced plastic response in growth and developmental rate and thus, are faced with a higher vulnerability to climate change effects. On the other hand, if environmental stressors inhibit or delay TH production during metamorphosis, the capacity for temperature-induced developmental plasticity can increase as demonstrated in our study in larvae exposed to the endocrine disruptor SP. Inhibition of TH pathways is usually caused by environmental pollution and, therefore, chemical contamination of the larval habitat. Consequently, larvae in habitats polluted by agricultural fertilizers and pesticides (Garriga et al., 2017; Mikó et al., 2017), industrial chemicals (Lefcort et al., 1998, Ossana et al., 2017) or fireworks (Sparling & Harvey, 2006; Bulaeva et al., 2015), often have a slower rate of development, are larger at the onset of metamorphosis but maintain a higher developmental plasticity and, would, therefore, be less vulnerable to the impacts of climate change. Although a large size at the onset of metamorphosis is associated with higher individual fitness in later life stages (Berven, 1990), longer larval durations are concomitant with increased pressures by predators and desiccation risk (Lefcort et al., 1998; Kloas & Lutz, 2006).

Size (i.e. SVL and mass) at the onset of metamorphosis was more plastic in larvae of tropical *X. laevis*, whereas age was more plastic in larvae temperate *R. temporaria* within the selected temperature range. Since metamorphs of *R. temporaria* switch to terrestrial habitat, we suggest that plasticity in developmental rate is more adaptive than plasticity in growth rate in *R. temporaria* as it allows for an quicker emerge from larval pond if habitat quality decreases. Consequently, selection may favor developmental plasticity more than growth rate in *R. temporaria* (Newman, 1992; Van Buskirk & Relyea, 1998; Laurila et al., 2002). Since *X. laevis* completes its entire life cycle in aquatic habitats, we suggest that plasticity in developmental rate is less important compared to *R. temporaria* to escape from larval ponds.

Moreover, tropical species are known to respond generally less plastic to temperature variation compared with temperate species (Janzen 1967; Ghalambor et al., 2006). However, the results for *X. laevis* refer to a substantially narrower range of experimental rearing temperatures within a probably narrower species-specific developmental thermal window. Larvae reared at 16°C did not follow the TSR (Walczynska et al., 2016), presumably because this temperature is outside the species-specific thermal range for development and thus, larvae showed a lack of plasticity. In contrast, the range of selected experimental rearing temperatures for *R. temporaria* was broader as temperate populations experience more heterogeneous thermal environments during their larval stage. Accordingly, the high thermal sensitivity of growth and developmental rate in larval *R. temporaria* was not surprising as selection may favor a high sensitivity of both rates due to temperature variation resulting in a high capacity for a plastic response in both rates in temperate species (Seebacher et al., 2015).

The capacity for temperature-induced developmental plasticity is known to be related to local adaptations in anuran larvae (Laugen et al., 2003; Muir et al. 2014; Drakulic et al., 2016) and thus, is population-specific. Usually, the capacity for temperature-induced plasticity is highest in populations from heterogeneous environments and/or high latitudes (Stählberg et al., 2001). In this study, we tested individuals of a single population in both species. Therefore, we would expect both different capacities for temperature-induced developmental plasticity and different impacts of altered TH levels on this capacity in different populations of *R. temporaria* and *X. laevis* related to local adaptations. In *R. temporaria*, numerous studies could demonstrate that the capacity for temperature-induced developmental plasticity is population-specific and arises from local adaptations due to geographic differences in mean temperature and temperature variation (Laugen et al., 2002, 2003; Drakulic et al., 2016; Grözinger et al., 2018). Such differences might be especially important in the light of ongoing (and predicted) environmental change (Drakulic et al., 2016). As plasticity may play a key role in the initial steps of the adaptation to rapid environmental change when genetic adaptation, a typically slower process that may span many generations, is unable to generate optimal phenotypes at required pace. Not accounting for variation in plasticity within a species can lead to inaccurate predictions about the vulnerability of populations to environmental change (Orizaola & Laurila, 2016). We therefore call for more studies on geographic variation in capacity for temperature-induced plasticity and how environmental stressors affect this capacity in different populations to make more precise predictions in the light of global (climate) change in both species studied here and other anuran species.

Conclusions

Although several studies on environmental stressors on anuran larvae have demonstrated that altered TH levels impact growth, development and survival, few studies have investigated whether these proximate effects of global (climate) change influence the capacity for temperature-induced developmental plasticity in tadpoles. In this study, high levels of TH impaired the ability of the larvae of both a tropical and temperate anuran species to display temperature-induced developmental plasticity. Therefore, environmental stressors leading to high levels of TH via the activation of stress-hormones are likely to make anuran larvae less able to cope with warmer developmental temperatures. Although some larval habitats may be contaminated with TH inhibiting substances, the majority of environmental factors activate stress-hormones and thus, cause high TH levels in anuran larvae. The present findings emphasize that the larvae of both species may suffer from the interactive effect of higher temperatures and a disrupted TH system caused by global change. However, the strength of impacts of environmental stressors on the capacity for temperature-induced plasticity may differ between populations in both species studied here and anurans in general. As THs also manage energy expenditure, future studies should focus on investigating if altered TH levels also impair physiological plasticity, as a balanced energy budget obviously is crucial for survival. Species that cannot compensate for long-term (e.g. average warming) or short-term (e.g. increased variability) changes in abiotic factors by buffering metamorphic and physiological traits will be most affected by global change.

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Appendix

Table A1 Effects of altered TH levels and temperature during development on age (days after hatching; dah), snout-vent length (SVL), total length (TL), survival, mass, and growth rate in tadpoles of the common frog (*Rana temporaria*) and the African clawed frog (*Xenopus laevis*) at the onset of metamorphosis (Gosner stage 42) (Gosner, 1960). Chi^2 and P for linear mixed-effects models, using the covariates 'Treatment' (T4, SP, and Control), 'T_{dev}' and the interactions of 'Treatment*T_{dev}' as fixed factors; 'aquarium' as random factor. SP = low TH levels. T4 = high TH levels**.** Significance was set at $P < 0.05$.

Dependent variable	Fixed effects	*Rana temporaria*						*Xenopus laevis*					
		Estimate	**SE**	**Chi²**	**Df**	**P**	**N (n)**	**Estimate**	**SE**	**Chi²**	**Df**	**P**	**N (n)**
Age (dah)	Treatment [Control]	58.8	3.18	49.0	2	**<0.001**	627 (48)	-	-	-	-	-	475 (45)
	Treatment [SP]	100.9	7.67	49.0	2	**<0.001**	627 (48)	-	-	-	-	-	475 (45)
	Treatment [T4]	49.0	7.69	49.0	2	**<0.001**	627 (48)	-	-	-	-	-	475 (45)
	T_{dev}	57.1	3.32	49.0	2	**<0.001**	627 (48)	-	-	-	-	-	475 (45)
	T_{dev}* Treatment [SP]	57.4	3.38	49.0	2	**<0.001**	627 (48)	-	-	-	-	-	475 (45)
	T_{dev}* Treatment [T4]	59.1	3.38	49.0	2	**<0.001**	627 (48)	-	-	-	-	-	475 (45)
SVL (mm)	Treatment [Control]	26.6	0.95	28.9	2	**<0.001**	627 (48)	9.6	0.93	8.4	2	**0.01**	475 (45)
	Treatment [SP]	26.4	2.3	28.9	2	**<0.001**	627 (48)	11.3	1.32	8.4	2	**0.01**	475 (45)
	Treatment [T4]	16.8	2.3	28.9	2	**<0.001**	627 (48)	7.4	1.32	8.4	2	**0.01**	475 (45)
	T_{dev}	25.9	0.99	28.9	2	**<0.001**	627 (48)	13.3	0.22	1.6	1	0.19	475 (45)
	T_{dev}* Treatment [SP]	26.6	1.01	28.9	2	**<0.001**	627 (48)	14.4	0.31	0.9	2	0.63	475 (45)
	T_{dev}* Treatment [T4]	27.0	1.01	28.9	2	**<0.001**	627 (48)	14.1	0.93	0.9	2	0.63	475 (45)
TL (mm)	Treatment [Control]	72.6	3.22	6.0	2	**0.048**	627 (48)	34.8	3.05	9.9	2	**0.01**	475 (45)
	Treatment [SP]	70.4	7.78	6.0	2	**0.048**	627 (48)	39.8	4.32	9.9	2	**0.01**	475 (45)
	Treatment [T4]	55.5	7.79	6.0	2	**0.048**	627 (48)	25.4	4.32	9.9	2	**0.01**	475 (45)
	T_{dev}	70.6	3.36	6.0	2	**0.048**	627 (48)	48.4	0.72	2.2	1	0.13	475 (45)
	T_{dev}* Treatment [SP]	72.9	3.42	6.0	2	**0.048**	627 (48)	49.4	1.02	0.2	2	0.89	475 (45)

	T_{dev}* Treatment [T4]	73.1	3.42	6.0	2	**0.048**	627 (48)	48.9	1.02	0.2	2	0.89	475 (45)
Survival	Treatment [Control]	6.1	21.84	1.9	2	0.384	(57)	-0.2	0.11	11.0	2	**0.003**	(45)
	Treatment [SP]	8.0	30.88	1.9	2	0.384	(57)	0.3	0.16	11.0	2	**0.003**	(45)
	Treatment [T4]	23.5	30.88	1.9	2	0.384	(57)	0.6	0.16	11.0	2	**0.003**	(45)
	T_{dev}	9.7	1.06	22.6	2	**<0.001**	(57)	0.0	0.01	11.0	1	**0.003**	(45)
	T_{dev}* Treatment [SP]	6.1	1.50	1.2	2	0.538	(57)	-0.0	0.01	11.0	2	**0.003**	(45)
	T_{dev}* Treatment [T4]	0.1	1.50	1.2	2	0.538	(57)	-0.0	0.01	11.0	2	**0.003**	(45)
Mass (mg)	Treatment [Control]	1185.5	44.12	28.3	2	**<0.001**	627 (48)	-	-	-	-	-	475 (45)
	Treatment [SP]	1249.8	106.52	28.3	2	**<0.001**	627 (48)	-	-	-	-	-	475 (45)
	Treatment [T4]	753.1	106.54	28.3	2	**<0.001**	627 (48)	-	-	-	-	-	475 (45)
	T_{dev}	1145.8	46.12	28.3	2	**<0.001**	627 (48)	-	-	-	-	-	475 (45)
	T_{dev}* Treatment [SP]	1185.7	46.96	28.3	2	**<0.001**	627 (48)	-	-	-	-	-	475 (45)
	T_{dev}* Treatment [T4]	1199.9	46.96	28.3	2	**<0.001**	627 (48)	-	-	-	-	-	475 (45)
Growth rate (mg/d)	Treatment [Control]	36.8	4.93	4.3	2	0.113	627 (48)	10.1	1.85	3.2	2	0.20	475 (45)
	Treatment [SP]	19.8	11.91	4.3	2	0.113	627 (48)	11.9	2.62	3.2	2	0.20	475 (45)
	Treatment [T4]	25.7	11.91	4.3	2	0.113	627 (48)	7.3	2.62	3.2	2	0.20	475 (45)
	T_{dev}	35.8	5.15	20.5	1	**<0.001**	627 (48)	13.9	0.44	0.8	1	0.34	475 (45)
	T_{dev}* Treatment [SP]	37.4	5.24	4.7	2	0.092	627 (48)	14.08	0.63	0.1	2	0.95	475 (45)
	T_{dev}* Treatment [T4]	37.2	5.24	4.7	2	0.092	627 (48)	13.93	0.63	0.1	2	0.95	475 (45)

Author contribution

I hereby confirm that Katharina Ruthsatz conceived, designed and performed the experiments, analysed the data and wrote the paper.

Hamburg,

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Chapter Three

Thyroid hormone levels and temperature during development alter thermal tolerance and energetics of *Xenopus laevis* larvae

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Abstract

Environmental variation induced by natural and anthropogenic processes including climate change may threaten species by causing environmental stress. Anuran larvae experiencing environmental stress may display altered thyroid hormone (TH) status with potential implications for physiological traits. Therefore, any capacity to adapt to environmental changes through plastic responses provides a key to determine species vulnerability to environmental variation. We investigated whether developmental temperature (T_{dev}), altered TH levels, and the interactive effect of both affect standard metabolic rate (SMR), body condition (BC), survival, and thermal tolerance in larvae of the African clawed frog (*Xenopus laevis*) reared at five temperatures with experimentally altered TH levels. At metamorphosis, SMR, BC, and survival were significantly affected by T_{dev}, TH status and their interaction with the latter often intensifying impacts. Larvae developing at warmer temperatures exhibited significantly higher SMRs and BC was reduced at warm T_{dev} and high TH levels suggesting decreased ability to acclimate to variation in temperature. Accordingly, tadpoles that developed at warm temperatures had higher maximum thermal limits but more narrow thermal tolerance windows. High and low TH levels decreased and increased upper thermal limits, respectively. Thus, when experiencing both warmer temperatures and environmental stress, larvae may be less able to compensate for changes in T_{dev}. Our results demonstrate that physiological traits in larvae of *X. laevis* are strongly affected by increased TH levels and warmer temperatures. Altered TH levels and increasing T_{dev} due to global change may result in a reduced capacity for physiological plasticity. This has far reaching consequences since the energetic requirement at the onset of metamorphosis is known to determine metamorphic success and thus, is indirectly linked to individual fitness in later life stages.

Key words

Standard metabolic rate (SMR), metabolic costs, thermal tolerance, climate change, metamorphosis

Introduction

Environmental variation exposes wildlife to multiple chemical, physical, and biological stressors that arise partly from anthropogenic activity (e.g. climate change, pollution), but also from natural sources (Noyes *et al.*, 2009). Many environmental stressors have the ability to impair endocrine function in wildlife (Carr and Patino, 2011). Stressors which alter or disturb endocrine systems are characterized as endocrine disruptors (EDs) (Kloas and Lutz, 2006; Kloas *et al.*, 2009). The impact of EDs in the environment is of special concern in amphibians, which are the most threatened class of vertebrates on the planet (Stuart *et al.*, 2004; Hayes *et al.*, 2006; Gabor *et al.*, 2018). Particularly vulnerable are larval stages of amphibians due to the inability of this life stage to select or avoid habitats (Sanzo and Hecnar, 2006; Yu *et al.*, 2013) and their increased risk of exposure to chemical contaminants due to their highly permeable skin (Hayes *et al.*, 2006; Strong *et al.*, 2017). Furthermore, amphibian larvae are particularly sensitive to EDs since metamorphosis is linked to a reorganization of several organ systems, and this complex change underlies complicated and tight hormonal control (Hayes *et al.*, 2010; Searcy *et al.*, 2015).

Amphibian metamorphosis is a crucial event in amphibian life history due to the complex reorganization of larval to juvenile structures which is mainly regulated by thyroid hormones (TH) (i.e. T3 and T4) (Tata, 2006; Furlow and Neff, 2006). THs increase in concentration during metamorphosis and determine the developmental rate (Brown and Cai, 2007; Shi, 2000). Many EDs influence the hypothalamus-pituitary-thyroid axis, which is responsible for production of THs (Carr and Patino, 2011). A large number of aquatic contaminants such as pesticides and herbicides, road salt, fertilizers, heavy metals, and active pharmaceutical ingredients have been shown to disrupt and inhibit the normal action of THs in amphibians, leading to changes in growth, development, and metabolism (Kashiwagi *et al.*, 2009; Carr and Patino, 2011). Inhibition or a decrease of TH production pathways slows the rate of development (Carr *et al.*, 2003; Bulaeva *et al.*, 2015) and decreases metabolic rates (Carr and Patino, 2011; Ortiz- Santaliestra and Sparling, 2007) causing tadpoles to metamorphose at a larger size and older age (Shi, 2000). An environmentally relevant ED is perchlorate (ClO_4^-) which is a goitrogen that inhibits TH synthesis (Ortiz-Santaliestra and Sparling, 2007). Concentrations of perchlorate measured in the field are often high enough to inhibit amphibian metamorphosis (Goleman *et al.*, 2002; Ortiz-Santaliestra and Sparling, 2007; Tietge *et al.*, 2005).

Whereas most environmental contaminants inhibit TH activity or production pathways, some contaminants and other abiotic and biotic environmental factors appear to enhance TH activity or increase TH levels by the activation of the neuroendocrine stress axis (Mann *et al.*, 2009; Dantzer *et al.*, 2014) and increase of stress hormone levels (Denver, 1997). These stress hormones may lead to a synergistic increase in TH production (Glennemeier and Denver, 2002; Laudet, 2011; Kulkarni and Buchholz, 2012). The presence of predators (Relyea, 2002; Capellán and Nicieza, 2007), crowding (Morey and Reznick, 2001), desiccation risk (Gervasi and Foufopoulos, 2008), food scarcity (Kupferberg, 1997), and extreme temperature (Smith-Gill and Berven, 1979) may increase TH production by activating the neuroendocrine stress axis. Anuran larvae with high TH levels display increased developmental and metabolic rates and decreased growth rates (Rowe *et al.*, 1998; Brown and Cai, 2007), which results in shorter larval periods, smaller size at the onset of metamorphosis and higher energetic maintenance costs in addition to energetic developmental costs (Denver, 1998, 2009; Orlofske and Hopkins, 2009). In this study, we simulated ecological stress by exposing larvae to L-Thyroxin for an increase in TH levels and by inhibiting TH production by the environmental relevant endocrine disruptor sodium perchlorate.

In all vertebrates, THs are not only critical for regulating growth and development, but also for regulating energy metabolism (Sheridan, 1994; Choi *et al.*, 2017). If the TH concentration changes due to environmental stress, a whole suite of physiological processes may be affected (Steyermark *et al.*, 2005; Hulbert and Else, 2004). Even if the effect of THs on metabolic heat production in ectotherms such as amphibians is negligible (John-Alder, 1983), THs increase the standard metabolic rate (SMR) which is estimated by measuring rates of O_2 consumption at rest and represents the energy required to cover basic physiological functions (Rowe *et al.*, 1998; Beck and Congdon, 2003). In amphibians, elevated SMR due to increased TH level manifests in increased activities of enzymes and densities of mitochondria in metabolic relevant tissues such as liver and red skeletal muscle (Chiu and Woo, 1988; Rowe *et al.*, 1998; Steyermark *et al.*, 2005). In individuals with a low SMR but not reduced metabolic scope (the difference between active metabolic rate and SMR), more energy is available for physical performance or development (Steyermark *et al.*, 2005; Orlofske and Hopkins, 2009). As metamorphosis is an energy-consuming process (Sheridan and Kao, 1998; Beck and Congdon, 2003), it may be advantageous to maintain a low SMR. Tadpoles which have larger energy reserves at the onset of metamorphosis are more likely to successfully complete metamorphosis and become juvenile froglets with larger energy stores and higher rates of survival (Orlofske and Hopkins, 2009). Therefore, the SMR and body condition at the onset

and after completion of metamorphosis are important fitness variables (Steyermark *et al.*, 2005; Muir *et al.*, 2014, Ruthsatz *et al.*, 2018).

Through its impact on physiology, temperature is considered to be the 'abiotic master factor' for ectotherms (Dalvi *et al.*, 2009; Turriago *et al.*, 2015; Sunday *et al.*, 2014; Berg *et al.*, 2017; Theisinger *et al.*, 2017). The tolerable thermal window of ectotherms is bracketed by critical temperatures (CT_{min} and CT_{max}) beyond which survival is not possible and these limits occur where aerobic scope is either zero or negative (Holzman and McManus, 1973). Therefore, environmental factors that either load (increase) or unload (decrease) SMR will impact aerobic scope and, thus, the width of tolerable thermal windows (Burraco and Gomez-Mestre, 2016). Climate change is expected to not only result in long-term warming of aquatic habitats but also increased variability in temperature leading to new thermal challenges for tadpoles in their larval habitats (Gutiérrez-Pesquera *et al.*, 2016) with likely impacts on growth, development and survival (Pörtner, 2001; Dalvi *et al.*, 2009). Altered TH levels, through their impact on SMR, may exacerbate these thermal challenges experienced prior to and at the onset of metamorphosis (Formicki *et al.*, 2003). As THs have recently been shown to play a key regulatory role in thermal acclimation in fish (Little and Seebacher, 2014; 2016) and several studies provide an indication on thyroid-regulated acclimation in amphibians and reptiles (Locker and Weish, 1966; Packard and Packard, 1975; Little and Seebacher, 2016).

Although previous studies have examined the impact of stress-induced alteration of TH levels on metamorphic and physiological traits of anuran larvae, studies have rarely examined the interaction of different stressors which is known to affect amphibian metamorphosis under natural conditions (Rowe *et al.*, 1998; Freitas *et al.*, 2017). This study examined the interactive effects of temperature and altered TH levels on the capacity for physiological acclimation (SMR and thermal tolerance) at the onset of metamorphosis in larvae of *Xenopus laevis*. For larvae acclimated to five different temperatures and experimentally enhanced or lowered TH levels, we tested the following hypotheses: (1) High and low levels of TH, as caused by the thyroid altering effect of several environmental stressors, increase and decrease SMR of tadpoles, respectively. (2) Changes in TH will be reflected in changes in body condition and survival. (3) Developmental temperature (T_{dev}) correlates positively with CT_{min}, CT_{max}, and negatively with the thermal range of tolerance. (4) T_{dev} will interact with altered TH levels to intensify the effect on larval physiological traits.

Material and Methods

Animal husbandry and experimental design

Three, unrelated clutches of *X. laevis* were obtained from the captive breeding facility of the Universitätsklinikum Hamburg Eppendorf (Martinistr. 52, 20246 Hamburg, Germany). Larvae were allowed to develop to Gosner stage 25 (free-swimming larvae; Gosner, 1960). The experiment consisted of three replicate aquaria at each of three treatments and five temperatures. From these larvae, 675 individuals originating from different families were intermixed before allocating them randomly to 45 aquaria. Fifteen larvae of *X. laevis* were kept each in a standard 9.5-L aquarium filled with 8 L of water. The experiment was conducted in two controlled climate chambers (Weiss Umwelttechnik GmbH, 35447 Reiskirchen, Germany) with a light regime of 12:12 L:D. The mean (±SD) water temperatures were 16 (±0.4), 19 (±0.5), 22 (±0.1), 25 (±0.5) and 28 (±0.3) °C. Temperature was maintained using ambient chamber temperature or via heaters and waterbaths. The experiments were conducted over four weeks, until all surviving larvae had reached the onset of metamorphosis (Gosner stage 42; Gosner, 1960). Amphibian larvae were fed high-protein flaked food (Sera, 52518 Heinsberg, Germany) and spirulina algae *ad libitum*. The flakes were free of perchlorate according to the manufacturer. Dead or abnormal tadpoles were removed each day. All temperature measurements were made using a digital thermometer (Amarell, Maxi-Pen, -50 - 200°C: 0.1°C). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The experiments were approved by the *Amt für Verbraucherschutz, Lebensmittelsicherheit und Veterinärwesen* in Hamburg, Germany (Billstraße 80, 20539 Hamburg, Germany; Gz. V1305 / 591 – 00.33, Nr. 03/16).

Thyroxine and sodium perchlorate exposures

We used a concentration of 250 µg/L SP (Sodium perchlorate hydrate 99.99% trace metals basis, 381225 Aldrich, Sigma-Aldrich, St. Louis, MO, USA) to achieve a decrease in TH levels (Tietge *et al.*, 2005). This concentration of SP is within environmental ranges measured in surface and ground waters of many industrial nations (Motzer, 2001; Tietge *et al.*, 2005; Carr & Theodorakis, 2006; Mukhi & Patino, 2007) and in bodies of water in which amphibians breed (Smith *et al.*, 2001; Ortiz-Santaliestra & Sparling, 2007).

We achieved increased TH levels by exposing tadpoles to 10 µg/L exogenous T4 (Thyroxine T4, IRMM468 Sigma-Aldrich**,** Sigma-Aldrich, St. Louis, MO, USA), a concentration which is known to influence amphibian metamorphosis (Lucas & Reynolds 1967; Mann *et al.*, 2009)

and is related to increases in T4 observed in tadpoles responding to stress (Denver, 1997; 1998). Tadpoles absorb exogenous T4 directly through their permeable skin (Shi, 2000; Tata, 2006; Coady *et al.*, 2010).

T4 and SP treatments were prepared in 0.1 N sodium hydroxide solutions (Sodium hydroxide solution 0.1 N, S2770 SIGMA, Sigma-Aldrich, St. Louis, MO, USA) buffered with 0.1 N muriatic acid solutions as solvents. A clean solution of 0.1 M sodium hydroxide solution buffered with 0.1 M muriatic acid solution was added to the control aquaria to control for any effect of solvents addition. Each treatment and the control set-up was replicated three times (i.e. 45 larvae, 15 larvae per aquarium, per treatment and control in total). Water was changed every second day and fresh SP and T4 were added, which is frequent enough to maintain a constant hormone and perchlorate level under given experimental temperatures, in accordance with the standard procedure for chemical and hormonal addition (Miwa & Inui, 1987; Goleman et al., 2002 a, b; Iwamuro et al., 2003; Rot-Nikcevic & Wassersug, 2004; Tietge et al. 2005; Ortiz-Santaliestra & Sparling, 2007; Bulaeva et al., 2015).

Processing of specimens

Developmental stage was determined by evaluating the status of key morphological features typical of specific developmental stages, as detailed in Gosner (1960). The developmental stage of each tadpole was recorded according to the procedure of Ortiz-Santaliestra & Sparling (2007): Gosner stage group **1**-**5**: **1.** pre-limb (absence of hind limbs, Gosner stages 24 to 26), **2.** limb bud (hind limb visible, but no clear joint formed, Gosner stages 27 to 34), **3.** middle hind limb (knee joint apparent, but toes not completely separated, Gosner stages 35 to 37), **4.** late hind limbs (hind limb tubercles and subarticular patches formed, Gosner stages 38 to 41), and **5.** metamorph (at least one forelimb present, Gosner stage 42 and above) (Gosner, 1960; Ortiz-Santaliestra & Sparling 2007). The onset of metamorphosis was defined by the emergence of at least one forelimb (Gosner stage 42). The snout vent length (SVL) and total length of each larva was measured with a caliper to the nearest 0.5 mm. Larvae were blotted and weighed to the nearest 0.001 g with an electronic balance (digital gold scale, Smart Weigh). At the end of the experiment tadpoles were euthanized with 200 mg/L of tricaine methanesulfonate ([MS-222], Ethyl 3-aminobenzoate methanesulfonate, E10521 ALDRICH, Sigma-Aldrich, St. Louis, MO, USA) buffered with 200 mg/L of sodium bicarbonate (Sodium bicarbonate, S5761 SIGMA, Sigma-Aldrich, St. Louis, MO, USA) (Stuart *et al.*, 2007) and transferred into ethanol (70 %).

Body condition

We estimated the body condition (i.e. energy stores) at the onset of metamorphosis by calculating the scaled mass index (SMI). This is a measure of the entire body condition of an individual as it accounts for the allometric relationship between mass and a body structure measure. It standardizes each measure so that direct comparisons among individuals can be made (Peig & Green, 2009, 2010; MacCracken & Stebbings, 2012). The SMI was considered as an accurate condition index in anuran larvae (MacCracken & Stebbings, 2012; Dittrich et al., 2016; Ruthsatz et al., 2018). A high BC suggests larger energy storages and thus, a good body condition. We followed the procedure outlined by Peig and Green (2009) to calculate the SMI for each individual.

Respiration measurements

Respiration measurements were made at the onset of metamorphosis on 45 individuals, three randomly chosen tadpoles from each aquarium. Animals were not fed for 48 h prior to and during the measurement of SMR such that tadpoles were in a post-absorptive state (Orlofske *et al.*, 2017). Oxygen consumption was measured by closed respirometry conducted between 0900 and 2100h to control for the influence of natural circadian rhythms on respiration (Orlofske *et al.*, 2017). Larvae were placed in respirometers consisting of 30-ml beakers containing 30 ml (minus the volume of the animals) of autoclaved tap water to exclude microbial oxygen consumption. The water was at 100% O_2 saturation at the start. Each respirometer was equipped with a fiber optic sensor (Oxygen Dipping Probe DP-PSt7; PreSens Precision Sensing GmbH, Regensburg, Germany) connected to a multichannel oxygen measuring system (Oxy 4 mini; PreSens Precision Sensing GmbH, Regensburg, Germany) and sealed with an air tight rubber plug. O_2 concentration was recorded every 15 seconds and measured as ml $O_2 \times L^{-1}$. Measurements were conducted at the developmental temperature of the individual. Prior to each trial, fiber optic O_2 sensors were calibrated using air-saturated water and a factory-set zero oxygen calibration point at the respective measurement temperature. Water temperature was maintained by the continuous mixing of the waterbath. Oxygen consumption was measured for every tadpole for 20 min at each of five temperatures. Empty (control) chambers were run simultaneously in every trial and values were adjusted accordingly. We took care that less than 10% of total O_2 was removed during the measurements to avoid impediment of respiration at low saturation levels. At the end of the measurements, each larva was removed and its TL, SVL and blotted wet body mass was determined.

Standard metabolic rate calculations

Prior to statistical analysis, we plotted the O_2 consumption rate of each tadpole over time and visually assessed activity peaks to exclude them for the determination of SMR (Orlofske and Hopkins, 2009). The SMR was expressed in ml $O_2 \times h^{-1} \times mg^{-1}$ wet body mass and was determined from the slope of linear least squares regressions of O_2 concentration vs. time (Hastings and Burggren, 1995; Rowe and Funk, 2017).

Values for SMR and mass were log transformed because metabolism is a power function of mass (Orlofske and Hopkins, 2009; Orlofske *et al.*, 2017). To exclude the mass-specific effect (Hulbert and Else, 2004) on SMR we did a linear regression of log transformed mass and log transformed SMR to calculate residuals. Residuals obtained from this regression ($SMR_{residuals}$) were entered into the analyses instead of SMR. We performed a multiple linear regression of log transformed SMR (dependent variable), log transformed mass (independent variable), and developmental temperature (independent) to describe the mass and temperature dependence of SMR.

Thermal tolerance

Thermal tolerance of *X. laevis* was evaluated when tadpoles reached the onset of metamorphosis (Gosner stage 42) using the critical thermal methodology (CTM) (Holzmann and McManus, 1973). Both critical thermal maximum (CT_{max}) and minimum (CT_{min}) endpoints are defined as the thermal point at which locomotor activity becomes disorganized and the animal loses the ability to right itself (Lutterschmidt and Hutchison, 1997; Turriago *et al.*, 2015). A total of 90 tadpoles were used for determination of thermal tolerance. From each aquarium six tadpoles (n = 3, CT_{max}; and n = 3, CT_{min}) were tested at set time intervals. CT_{min} and CT_{max} were determined by using the dynamic method according to Cowles and Bogert (1944) and Hutchison (1961) except for the end point (Wu and Kam, 2005). This method involves increasing (for CT_{max}) or decreasing (for CT_{min}) test temperatures by a specific rate until an appropriate endpoint is reached (Lutterschmidt and Hutchison, 1997). Tadpoles were placed individually in a 250-ml flask with 200 ml of water which was then placed in a temperature-controlled water bath. The heating and cooling rates were ± 0.1°C $\times min^{-1}$, and the water temperature served as a proxy of body temperature (Hutchison, 1961). The initial temperature in the water bath was set at the respective developmental temperature. In tadpoles, the occurrence of spasms is difficult to determine, and thus we decided to use the loss of the righting response after being flipped on its back in the water with a probe as our criterion for the endpoint (Lutterschmidt and Hutchison, 1997; Wu and Kam, 2005) for both,

CT_{min} and CT_{max} determinations (Turriago *et al.*, 2015). A time limit of 30 sec between flipping the animal and righting was adopted (Layne Jr and Claussen, 1982). All thermal tolerance tests were performed between 1100h and 1500h. After the experiments, we euthanized the tadpoles with MS-222, weighed them and measured SVL and TL, and finally transferred them into ethanol (70 %).

We adopted the method of Dalvi et al. (2009) used in fish to generate a thermal tolerance window (TW) for *X. laevis* by calculating the difference between CT_{max} and CT_{min} estimates obtained at various acclimation temperatures. To simulate long-term changes in environmental temperature, *X. laevis* were reared at a range of different temperatures. The thermal tolerance polygon was generated by plotting the five developmental temperatures for each treatment (T4, SP, and Control) on the X-axis and the mean CT_{min} and CT_{max} values on the Y-axis. The TW was calculated from the polygon and expressed as $°C^2$ (Dalvi *et al.*, 2009). We performed a linear regression for developmental temperature and thermal tolerance (as measured by CT_{min}, CT_{max}, and thermal range of tolerance). The slope of the regression for CT_{max} and CT_{min} defined the effect of developmental temperature on critical thermal limits of *X. laevis*.

Statistical analyses

For all statistical tests R 3.4.1 (R Development Core Team, 2007) for Windows was used. All plots were constructed using ggplot2 (Wickham 2009) and Adobe Illustrator CS6. Data were analyzed using linear mixed-effect models [lme, Type III model, covariance type: variance components, REML (restricted maximum likelihood) method for parameter estimation, 100 iterations (Bates and Sarkar, 2007)], using the covariate 'T_{dev}', and 'treatment' (T4, SP, and Control) and the interactions of 'treatment' and 'T_{dev}' as fixed factors. '$SMR_{residuals}$', 'body condition', 'survival', and 'thermal tolerance' (as measured by CT_{min}, CT_{max}, and the thermal range of tolerance) were used as dependent variables in separate models. P-values were obtained from likelihood-ratio tests (Crawley, 2007). To address dependencies in the data, the variable 'aquarium' was included as a random factor. Residuals of each model were visually checked for normal distribution. N refers to the total number of analyzed tadpoles. Linear mixed-effect models were followed by post hoc comparisons (Tukey's test; Tukey HSP function, multcomp package, vers. 1.2-13) to compare all possible pairwise combinations of treatments when overall tests were significant.

'Thermal tolerance' (as measured by CT_{min}, CT_{max}, and the thermal range of tolerance) was correlated with SMR and T_{dev} using Spearman's rank correlation (Table 1). Correlations were

performed on subgroups according to TH-treatment and Control. The slope of the linear regression for CT_{max} and CT_{min} with T_{dev} defined the effect of temperature during development on the thermal tolerance of *X. laevis* (Table 1) (Dalvi *et al.*, 2009).

Results

Standard metabolic rate

SMR at T_{dev} of tadpoles was significantly influenced by the hormone treatment, by T_{dev} and the interactive effect of both (Table 1). There was no consistent effect of time of day on SMR tested during this experiment. At all developmental temperatures tadpoles from the SP treatment were the largest at the onset of metamorphosis followed by control animals and tadpoles from the T4 treatment being the smallest (Table A1, Fig. 1). Mass-specific SMR decreased with increasing mass (Fig. 1) with the highest SMR observed in tadpoles from the T4 treatment followed by individuals in the SP treatment. The lowest SMR was observed in the largest tadpoles (SP treatment), followed by control group animals, and T4 treated animals (Fig. 1). At all developmental temperatures, tadpoles exposed to T4 had the highest SMR.

Table 1 Effects of altered TH levels and temperature during development on SMR, body condition, and survival in tadpoles of the African clawed frog (X. laevis) at the onset of metamorphosis (Gosner stage 42; Gosner, 1960). Chi^2 and P for linear mixed-effects models, using the covariates 'Treatment' (T4, SP, and Control), 'T_{dev}' and the interactions of 'Treatment*T_{dev}' as fixed factors; 'aquarium' as random factor. T4 = increased TH concentration**.** SP = decreased TH concentration. Significance was set at $P < 0.05$. SMR was taken as the residuals from the regression of log transformed mass on log transformed SMR.

Dependent variable	**Fixed effects**	*Xenopus laevis*					
		Estimate	**SE**	**Chi^2**	**Df**	**P**	**N (n)**
$SMR_{residuals}$	Treatment [Control]	-0.08	0.04	39.63	2	< **0.001**	119 (9)
	Treatment [SP]	-0.10	0.08	39.63	2	< **0.001**	119 (9)
	Treatment [T4]	0.13	0.06	39.63	2	< **0.001**	119 (9)
	T_{dev}	-0.08	0.06	39.63	1	< **0.001**	119 (9)
	T_{dev}* Treatment [SP]	-0.15	0.09	39.63	2	< **0.001**	119 (9)
	T_{dev}* Treatment [T4]	0.33	0.09	39.63	2	< **0.001**	119 (9)
Body condition (SMI)	Treatment [Control]	275.56	36.11	11.42	2	**0.003**	475 (45)
	Treatment [SP]	69.48	53.23	11.42	2	**0.003**	475 (45)
	Treatment [T4]	-156.08	55.72	11.42	2	**0.003**	475 (45)
	T_{dev}	-3.98	1.61	11.42	1	**0.003**	475 (45)
	T_{dev}* Treatment [SP]	-3.09	2.36	11.42	2	**0.003**	475 (45)
	T_{dev}* Treatment [T4]	-5.52	2.49	11.42	2	**0.003**	475 (45)
Survival (%)	Treatment [Control]	-0.23	0.11	11.05	2	**0.003**	(45)
	Treatment [SP]	0.32	0.16	11.05	2	**0.003**	(45)
	Treatment [T4]	0.61	0.16	11.05	2	**0.003**	(45)
	T_{dev}	0.01	0.01	11.05	1	**0.003**	(45)
	T_{dev}* Treatment [SP]	-0.01	0.01	11.05	2	**0.003**	(45)

	T_{dev}* Treatment [T4]	-0.02	0.01	11.05	2	**0.003**	(45)
CT_{min} (°C)	Treatment [Control]	-11.26	0.96	37.02	2	< **0.001**	135 (45)
	Treatment [SP]	0.76	1.35	37.02	2	< **0.001**	135 (45)
	Treatment [T4]	0.39	1.35	37.02	2	< **0.001**	135 (45)
	T_{dev}	0.99	0.04	343.16	1	< **0.001**	135 (45)
	T_{dev}* Treatment [SP]	-0.01	0.06	1.41	2	0.49	135 (45)
	T_{dev}* Treatment [T4]	-0.01	0.06	1.41	2	0.49	135 (45)
CT_{max} (°C)	Treatment [Control]	28.15	2.23	8.59	2	**0.01**	135 (45)
	Treatment [SP]	1.64	3.16	8.59	2	**0.01**	135 (45)
	Treatment [T4]	-0.7	3.16	8.59	2	**0.01**	135 (45)
	T_{dev}	-0.28	0.09	24.02	1	< **0.001**	135 (45)
	T_{dev}* Treatment [SP]	-0.02	0.14	0.05	2	0.97	135 (45)
	T_{dev}* Treatment [T4]	-0.00	0.14	0.05	2	0.97	135 (45)
Thermal range of tolerance	Treatment [Control]	39.41	1.73	0.89	2	0.63	135 (45)
	Treatment [SP]	0.87	2.44	0.89	2	0.63	135 (45)
	Treatment [T4]	-1.09	2.44	0.89	2	0.63	135 (45)
	T_{dev}	-0.69	0.07	138.08	1	< **0.001**	135 (45)
	T_{dev}* Treatment [SP]	-0.02	0.1	0.68	2	0.71	135 (45)
	T_{dev}* Treatment [T4]	0.06	0.1	0.68	2	0.71	135 (45)

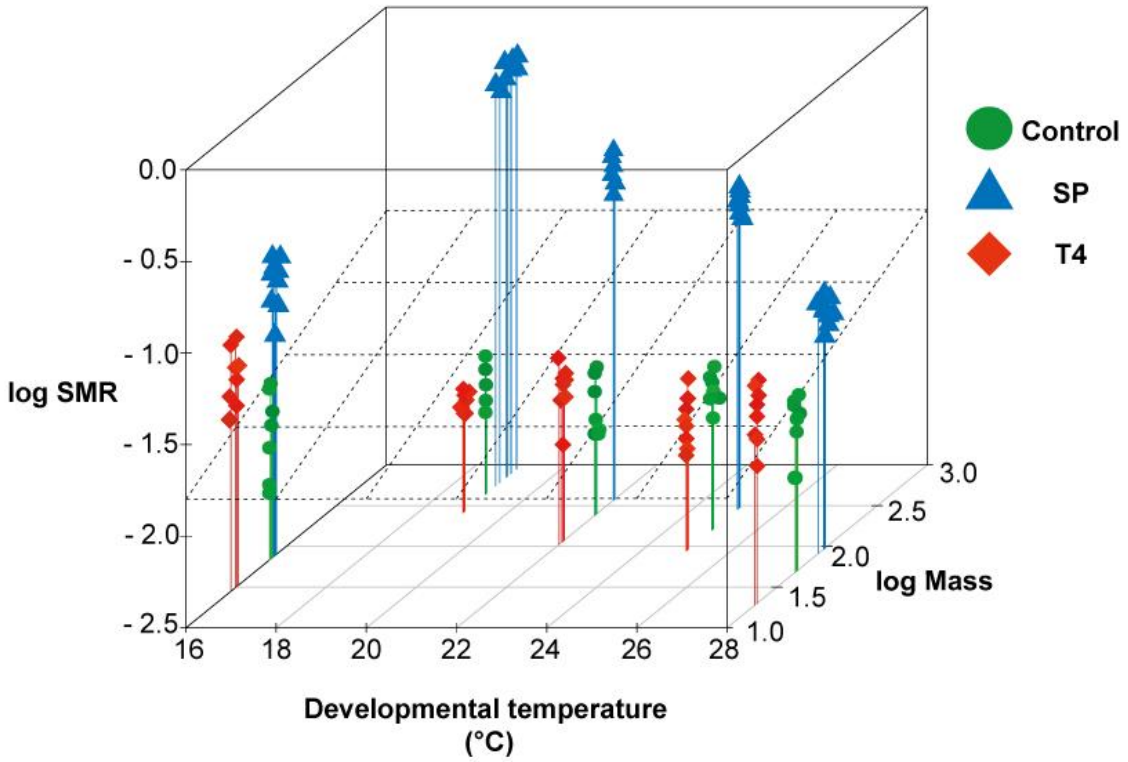

Fig. 1. Mass and temperature dependence of standard metabolic rate (SMR) in *Xenopus laevis*. Multiple linear regression of SMR (ml $O_2 \times h^{-1} \times mg^{-1}$) on developmental temperature and mass (mg; log transformed) for larvae from all three treatment groups (n = 119 animals). Green lines and dots: control animals. Blue lines and triangle: Low thyroid hormone levels (SP treatment). Red lines and diamonds: High thyroid hormone levels (T4 treatment). Dotted plane is the average multiple linear regression.

Body condition and survival

Body condition of *X. laevis* was significantly affected by treatment, temperature during development, as well as by the interactive effect of both (Table 1, Fig. 2). High TH levels, T_{dev}, and the interactive effect of altered TH levels and T_{dev} lead to a reduced body condition and thus, energy stores in tadpoles of *X. laevis* whereas low TH levels increased body condition.

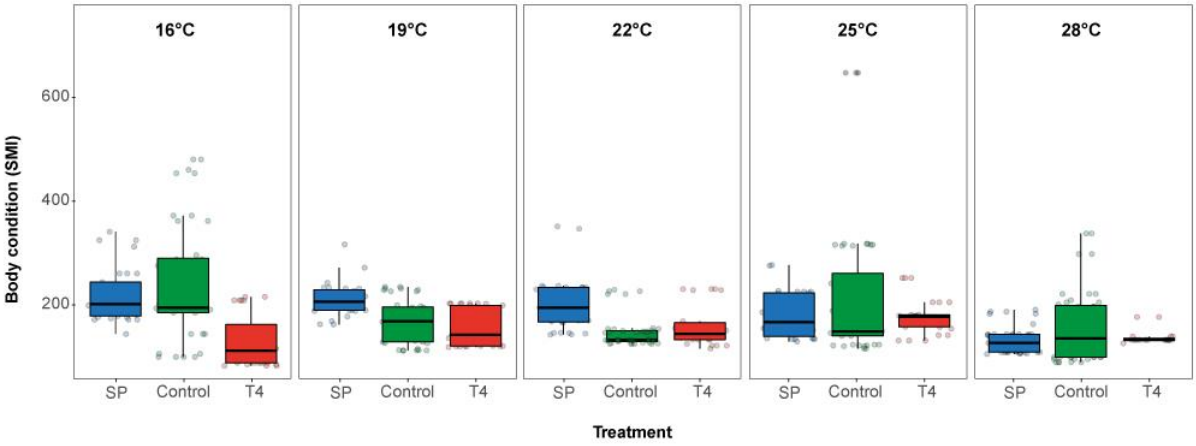

Fig. 2 Interactive effect of altered thyroid hormone levels and five developmental temperatures on body condition in tadpoles of the African clawed frog (*X. laevis*) at the onset of metamorphosis (Gosner stage 42) (Gosner, 1960). Body condition was determined by the scaled mass index (SMI).

Survival of *X. laevis* was significantly affected by hormone treatment, developmental temperature, as well as by the interactive effect of both (Table 1). Mean (± SD) survival from the start of the experiment (Gosner stage 25) to the onset of metamorphosis (Gosner stage 42) in the Control, SP and T4 treatment groups was 92.0(± 10.0), 65.7 (± 13.3) and 53.3(± 13.3) %, respectively. High levels of TH reduced survival to nearly half of that observed in the Control. This effect was intensified by the interactive effect of TH level and temperature. Larvae from 19°C-Control, 25°C-SP and 28°C-T4 treatments/groups revealed the lowest survival among all *X. laevis* tadpoles (Table A2). Most larvae survived in 25°C-Control, 28°C-SP, and 19°C-T4 treatments/groups (Table A2).

Thermal tolerance and thermal window

There were significant effects of T_{dev} on CT_{min}, CT_{max}, and the thermal range of tolerance within all three treatment groups (Table 1). There was no interactive effect of altered TH status and temperature during development on CT_{min}, CT_{max}, and the thermal range of tolerance. Whereas TH status affected CT_{min} and CT_{max}, there was no effect on the thermal

range of tolerance. CT_{max} was reduced at high TH levels and increased at low TH levels, whereas low TH levels increased both CT_{min} and CT_{max}. The thermal range of tolerance was only affected by temperature during development.

The CT_{min} and CT_{max} increased significantly with increasing developmental temperature, whereas the thermal range of tolerance decreased with increasing developmental temperature (Table A3). The SMR did not correlate with CT_{min}, CTmax, or the thermal range of tolerance (Table A3).

The regression slope for CT_{min} and CT_{max} of *X. laevis* shows that for every 1°C increase in T_{dev} the CT_{min} and CT_{max} increased by 0.98 °C and 0.31 °C, respectively, in the control group, by 0.96 °C and 0.28 °C in the T4 treatment, and by 0.95 °C and 0.27 °C in the SP treatment (Table A4). In all treatments and control group developmental temperature had a greater effect on CT_{min} than on CT_{max} (Table A4).

The thermal window polygon area (TW) for *X. laevis* reared at five different temperatures between 16 and 28 °C was calculated as 290.76 °C² for the control group, 297.86 °C² for the SP treatment, and 292.87 °C² for the T4 treatment (Fig. 3). Table 1

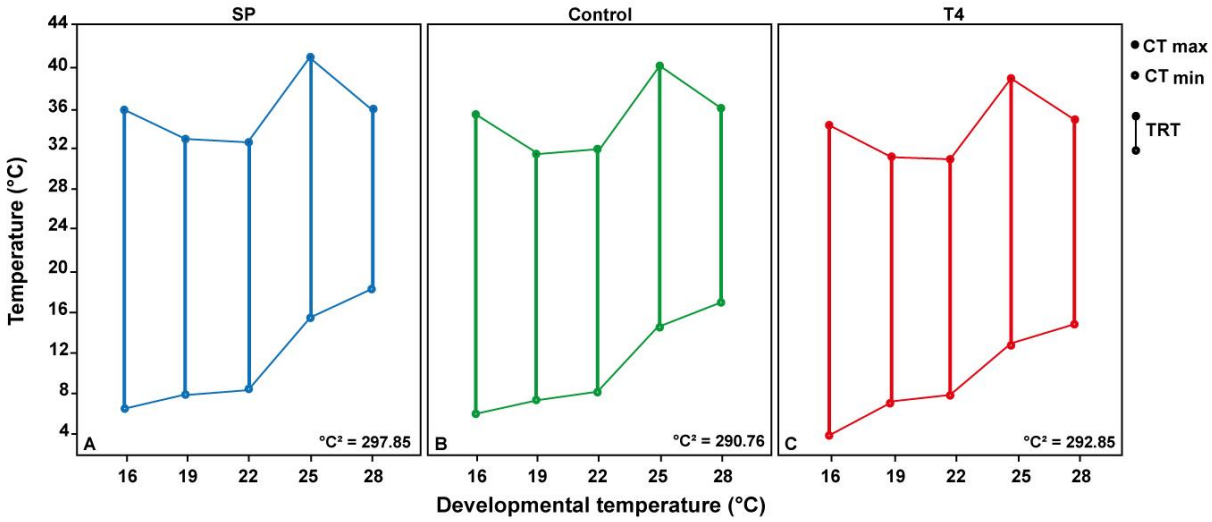

Fig. 3. Developmental thermal windows of *X. laevis*. Thermal tolerance polygons generated from the critical thermal limits (CT_{min} and CT_{max}) at five developmental temperatures in a total of 45 animals. **A** Control animals. **B** Low thyroid hormone levels (SP treatment). **C** High thyroid hormone levels (T4 treatment).

Discussion

Aquatic organisms such as anuran larvae are limited in their ability to search for favorable microhabitats (Gutiérrez-Pesquera *et al.*, 2016). Therefore, knowing the capacity of species to react to environmental change through plastic responses is a key to determining how vulnerable species with limited mobility will be to environmental variation and global climate change (Martinez *et al.*, 2016; Gutiérrez-Pesquera *et al.*, 2016; Berg *et al.*, 2017). Species that cannot compensate for long-term (e.g. average warming) or short-term (e.g. increased variability) changes in abiotic factors by buffering metamorphic and physiological traits will be most affected (Kern *et al.*, 2015). Therefore, we investigated whether T_{dev}, altered TH levels, and the interactive effect of both affect physiological traits in larvae of *X. laevis* as a model system and determined their capacity for physiological plasticity. Our results demonstrate that physiological traits in larvae of *X. laevis* are strongly affected by increased TH levels and warmer developmental temperatures. Our results for *X. laevis* suggest that altered TH levels, which can result from various environmental stressors, and warmer developmental temperatures will result in a reduced capacity for physiological plasticity in anuran larvae.

Altered TH levels and warmer temperatures increase SMR and affect body condition

Amphibians are ectothermic animals and ambient temperature regulates the rates of all physiological and biochemical processes (Smith-Gill and Berven, 1979; Tata, 2006; Little and Seebacher, 2016) impacting growth, development and metabolism. An increase in ambient temperature may, therefore, increase the SMR but animals may compensate for those thermal changes through acclimation (Angilletta *et al.*, 2006; Berg *et al.*, 2017). In this study, the SMR of larval *X. laevis* markedly increased at warmer temperatures suggesting limited capacity to compensate for warming through physiological acclimation.

As climate change is increasing mean environmental temperatures and the frequency of extreme thermal events (Pachauri *et al.*, 2014; Gutiérrez-Pesquera *et al.*, 2016; Theisinger *et al.*, 2017), species with a limited capacity for acclimation will suffer from high maintenance costs as caused by the high SMRs. Especially in anuran larvae, having a low energy expenditure before and during metamorphosis is favorable since metamorphosis is a highly energy consuming process (Orlofske and Hopkins, 2009). A high SMR before and at the onset of metamorphosis may reduce the ability of larvae to store energy (Sheridan and Kao, 1998; Orlfoske and Hopkins, 2009; own unpublished data). Energy stores are used during metamorphic climax when larvae stop feeding due to the rebuilding of the gastro-intestinal

tract and changes in oral morphology (Beck and Congdon, 2003, Orlofske and Hopkins, 2009). Depending on how much of the accumulated energy is needed for covering maintenance costs (i.e. high or low SMR), more or less of this stored energy is available for covering the costs of development (Steyermark *et al.*, 2005; Beck and Congdon, 2003, Orlofske and Hopkins, 2009) and, consequently, the larvae will have a higher or lower probability of successfully completing metamorphosis.

Anuran larvae which show limited capacity for an acclimation in SMR, such as *X. laevis* in the present study but especially tropical amphibians in general (Janzen, 1967), may be least able to tolerate additional, climate-driven warming of their habitat. Under natural conditions, any trait, such as increased SMR, that increases metamorphic rates and reduces the transition time through this vulnerable life history stage, would be preferentially selected. However, we observed reduced body condition (lower energy stores) in tadpoles reared at warmer temperatures and experiencing high levels of THs as caused by environmental stress. Environmental stress and global warming, thus, may result in increased SMRs which, in turn, impair the capacity to store energy needed for the complex reorganization during metamorphic climax. Metabolic acclimation (decreasing SMR) may be advantageous when the risk of desiccation and predation is low as it allows more energy to be accumulated and subsequently allocated to development during metamorphic climax. In addition to increases in mean environmental temperatures, altered TH levels frequently accompanies the thermal effects on developmental and metabolic rate in anuran larvae. In this study, larvae of *X. laevis* revealed a higher SMR when exposed to T4 and a reduced SMR when exposed to SP. This effect was intensified at warmer temperatures. Hence, our results confirmed that the level of TH determines the metabolic rate in *X. laevis* as shown for lizards (*Dipsosaurus dorsalis* and *Sceloporus occidentalis*; John-Alder 1983, 1990), snakes (*Thamnophis sirtalis*; Etheridge, 1993), and the leopard frog (*Rana pipiens*; Steyermark *et al.*, 2015), but had been negated for juvenile *X. laevis* (Dupre *et al.*, 1986). Therefore, larvae exposed to warmer temperatures are likely to be affected more by environmental stressors than larvae at colder temperatures before and during metamorphic climax.

Long-term temperature changes: Developmental temperatures determine CTM, thermal range of tolerance, and the thermal window

Apart from metabolic acclimation concomitant with a low sensitivity of SMR to short-term temperature variation, changes in thermal tolerance may provide a key mechanism to help amphibian larvae cope with the longer-term impacts of climate-driven warming and increased

frequency of extreme environmental events (Seebacher *et al.*, 2015; Gutiérrez-Pesquera *et al.*, 2016). In this study, tadpoles from warm developmental temperatures had higher thermal limits, but a narrower thermal range of tolerance than tadpoles from colder treatments. Consequently, larvae of *X. laevis* have the ability to compensate for changes in developmental temperature as they increased their thermal limits at warmer developmental temperatures (Schaefer and Ryan, 2006; Gunderson and Stillman, 2015; Little and Seebacher, 2016). The driver for this adjustment are changes in the thermal reaction norm and hence of physiological nature (Little and Seebacher, 2016; Theisinger *et al.*, 2017).

Although our results demonstrate the ability of *X. laevis* tadpoles to acclimate to different temperatures by changing their critical thermal limits and thermal range of tolerance, the latter was narrower when tadpoles were raised at warmer temperatures. Those warm-acclimated larvae may be more vulnerable to the impacts of climate change in terms of lacking the capacity for an acclimation in other physiological traits (Gunderson and Stillman, 2015) as larvae in the present study were not able to acclimate their SMR to warmer temperatures.

We found a significanteffect of altered TH levels on the thermal limits in *X. laevis* indicating that the thyroid systems affects the capacity to acclimate to temperature variation.Therefore, altered TH levels as caused by environmental stress affect the capacity for an acclimation in thermal limits but not in thethermal range of tolerance. Accordingly, larvae under environmental stress are constrained in their ability to compensate for changes in developmental temperature. Furthermore, they might suffer from the consequences of high SMR on body condition and thus, energy stores.

Conclusions

Considering the current worldwide decline of amphibians (Alroy, 2015; Stuart *et al.*, 2004) it is of major interest to investigate whether and how anuran larvae adjust their physiological traits to new thermal challenges and to altered TH status, as caused by natural or anthropogenic stressors in their larval habitat (Strong *et al.*, 2017). Increased metabolic rates are the expected future responses of ectotherms (Seebacher *et al.*, 2015; Berg *et al.*, 2017), especially in species with reduced physiological plasticity due to extreme but stable environments as common in tropical species (Janzen, 1967; Huey *et al.*, 2012; Oyamaguchi *et al.*, 2017). Even though *X. laevis* is often used for laboratory experiments and, thus, cultured under constant thermal conditions, this is a tropical (Sub-Saharan Africa) species adapted to warm temperatures. Our findings emphasize how environmental stress and climate-driven warming may be detrimental to tadpoles of *X. laevis* under natural conditions by causing

limits to the acclimation of physiological traits leading to reduced body condition. The lack of comparative data on SMR at different temperatures and critical thermal limits necessitates future work to explore how environmental stress may influence physiological traits at the onset of metamorphosis in other species of amphibians. Comparative studies across populations and species would help to identify the potential for local adaptation or inter-specific differences, respectively, on physiological traits affecting the age and size at metamorphosis. Furthermore, as TH levels alter metabolism and body condition in both larval and adult amphibians (Tata *et al.*, 1962), environmental stress may affect froglets and frogs alike, having long-lasting effects on amphibian populations. Long-term studies are needed to understand the consequences of various stressors during larval stages on the phenotype and on the fitness of juveniles and the adults.

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Appendices

Table A1. Descriptive statistics of mass (mg) at the onset of metamorphosis in tadpoles of the African clawed frog *Xenopus laevis* at five different developmental temperatures exposed at different TH levels. T4 = high TH levels**.** SP = low TH levels.

	Temperature	Treatment	Maximum	Minimum	Mean	SE	N
Mass (mg)	16	Control	68	74	71.27	0.312	40
		SP	73	84	77.76	0.574	29
		T4	28	33	31.26	0.399	23
	19	Control	410	452	442.64	1.190	39
		SP	551	897	695.07	18.186	27
		T4	259	275	267.87	0.698	31
	22	Control	234	254	243.37	0.930	41
		SP	369	381	375.45	0.681	31
		T4	100	119	110.45	1.309	22
	25	Control	152	164	159.89	0.455	44
		SP	284	303	296.12	1.144	26
		T4	85	95	89.88	0.441	25
	28	Control	45	54	48.81	0.357	43
		SP	82	96	93.14	0.600	35
		T4	19	21	20.11	0.105	19

Table A2. Survival (%) at the onset of metamorphosis in tadpoles of *Xenopus laevis* exposed to different combinations of developmental temperature (T_{dev}) and altered TH levels. T4 = increased TH levels**.** SP = decreased TH levels.

Group		**Survival (%)** ± SD
T_{dev}	Treatment	*Xenopus laevis*
16	Control	91.11 ± 8.89
	T4	51.11 ± 2.22
	SP	66.67 ± 0
19	Control	86.67 ± 6.67
	T4	68.89 ± 4.44
	SP	60.6 ± 6.67
22	Control	91.11 ± 8.89
	T4	48.89 ± 4.44
	SP	68.89 ± 4.41
25	Control	97.78 ± 2.22
	T4	55.56 ± 4.44
	SP	57.78 ± 8.89
28	Control	93.33 ± 0
	T4	42.22 ± 4.45
	SP	75.56 ± 4.44

Table A3 Correlation of critical thermal limits (as measured by CT_{min} and CT_{max}) and thermal range of tolerance with temperature during development and standard metabolic rate in *X. laevis* tadpoles. ρ (correlation coefficient) and P for Spearman's rank correlation. Significance was set at $P < 0.05$. T4 = high TH levels**.** SP = low TH levels.

Dependent variable	**Treatment**	**Temperature during development** (°C)		**SMR** (ml $O_2 \times h^{-1} \times mg^{-1}$)	
		ρ	**P**	**ρ**	**P**
CT_{min}	Control	0.96	< **0.001**	0.09	0.54
	T4	0.96	< **0.001**	0.08	0.57
	SP	0.97	< **0.001**	0.03	0.84

CT_{max}	Control	0.36	**0.014**	0.07	0.61
	T4	0.43	**0.003**	0.42	0.40
	SP	0.38	**0.009**	0.07	0.61
Thermal range of tolerance	Control	-0.66	< **0.001**	-0.14	0.34
	T4	-0.68	< **0.001**	0.08	0.57
	SP	-0.67	< **0.001**	-0.12	0.41

Bold indicates significant P-values.

Table A4. Critical thermal minima (CT_{min}), critical thermal maxima (CT_{max}), thermal range of tolerance, and thermal window (TW) (± SD) of tadpoles of the African clawed frog *Xenopus laevis* at five different temperatures during development exposed at different TH levels. T4 = high TH levels**.** SP = low TH levels. Regression slopes show the increase of CT_{min} and CT_{max} for every 1°C increase in $T_{dev.}$

Developmental temperature (°C)	**Treatment**	**CT_{min} (°C)** ±SD	**CT_{max} (°C)** ±SD	**Thermal range of tolerance** ±SD	**TW ($°C^2$)**	**Regression slope CT_{min} (R^2)**	**Regression slope CT_{max} (R^2)**
16	**Control**	**5.5** ±0.4	**35.1** ±0.8	**29.6** ±0.4	290.76	Y = 0.98x-11.28 (0.891)	Y = 0.31x+28.04 (0.161)
19		**7.3** ±0.1	**31.5** ±0.5	**24.1** ±0.3			
22		**8.4** ±0.1	**31.6** ±0.3	**23.1** ±0.8			
25		**14.2** ±0.2	**39.7** ±0.2	**25.5** ±0.5			
28		**16.9** ±0.1	**35.5** ±0.5	**18.5** ±0.4			
16	**SP**	**4.2** ±0.7	**34.3** ±0.6	**30.1** ±0.3	297.85	Y = 0.96x-10.66 (0.893)	Y = 0.28x+28.58 (0.122)
19		**7.1** ±0.9	**30.9** ±0.1	**23.8** ±0.6			
22		**7.8** ±0.1	**30.8** ±0.1	**23** ±0.5			
25		**13.1** ±0.8	**39.0** ±0.5	**25.8** ±0.6			
28		**15.1** ±0.4	**34.8** ±0.1	**19.7** ±0.7			
16	**T4**	**6.5** ±0.5	**35.9** ±1.1	**29.4** ±1.1	292.875	Y = 0.95x-10.68 (0.907)	Y = 0.27x+28.77 (0.124)
19		**7.9** ±0.5	**32.7** ±0.2	**24.8** ±0.1			
22		**8.5** ±0.5	**33.1** ±0.9	**24.5** ±0.4			
25		**15** ±0.5	**41.1** ±0.3	**26.1** ±0.3			
28		**17.7** ±0.7	**35.8** ±0.1	**18.1** ±1.9			

Author contribution

I hereby confirm that Katharina Ruthsatz conceived, designed and performed the experiments, analysed the data and wrote the paper.

Hamburg, 18.10.2018

Prof. Dr. Kathrin Dausmann

Chapter Four

Multiple environmental stressors reduce physiological plasticity and body condition during and after metamorphosis in the common frog (*Rana temporaria*)

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Abstract

Environmental variation and stress induced by natural and anthropogenic processes including climate change may threaten the productivity of species and persistence of populations. Ectotherms can potentially cope with stressful conditions such as extremes in temperature by exhibiting physiological plasticity. Amphibian larvae experiencing stressful environments display altered thyroid hormone (TH) status with potential implications for physiological traits and acclimation capacity. We investigated how developmental temperature (Tdev) and altered TH levels (simulating proximate effects of environmental stress) influence the standard metabolic rate (SMR), body condition (BC), and thermal tolerance in metamorphic and post-metamorphic anuran larvae of the common frog (Rana temporaria) reared at five constant temperatures (14°-28°C) with experimentally altered TH levels. At metamorphosis, tadpoles that developed at warmer temperatures had higher maximum thermal limits but narrower ranges in thermal tolerance. Mean CTmax was 37.63°C ± 0.14 (low TH levels), 36.49°C ± 0.31 (control), and 36.43°C ± 0.68 (high TH levels) in tadpoles acclimated to different temperatures. Tadpoles were able to acclimate to warmer Tdev by adjusting their SMR and thermal tolerance and these capacities were not impaired by altered TH levels. BC was best in tadpoles reared at 22°C and worst when reared at 18°C and 28°C. The effect of stressful larval conditions on SMR and BC at the onset of metamorphosis was carried over to froglets at the end of metamorphic climax. This has far reaching consequences since body condition at metamorphosis is known to determine metamorphic success and, thus, is indirectly linked to individual fitness in later life stages.

Keywords

thermal tolerance, endocrine disruption, standard metabolic rate, acclimation, CT_{max}, thermal window

Introduction

Climate change has profound and diverse effects on organisms and is altering aquatic and terrestrial systems worldwide (IPCC 2014; Deutsch et al. 2015). Changes in temperature can prove challenging for wildlife (Rowe and Crandall 2018), particularly ectotherms whose body temperature fluctuates with environmental temperature with consequences for changes in the rates of most biochemical reactions and biological processes (Harkey and Semlitsch 1988; Zuo et al. 2012; reviewed in Little and Seebacher 2016). All animals have a thermal range of tolerance that is set by upper and lower critical threshold temperatures (CT_{min} and CT_{max}) beyond which survival is not possible (Holzman and McManus 1973; Little and Seebacher 2016). Thermal stress as a result of global warming and extreme thermal events such as heat waves could, therefore, affect the performance and fitness traits of ectotherms (reviewed in Narayan 2016).

Ectotherms may respond to stressful variation in environmental temperature by exhibiting physiological plasticity. This process can include specific types of responses, including heat hardening, thermal compensation, and acclimation (Bullock 1955; Prosser 1955; Hazel and Prosser 1974; Huey et al. 1999; Angilletta et al. 2006). These different forms of thermally induced plasticity allow individuals to potentially acclimate to the complex temporal or spatial heterogeneity in environmental temperatures (Angilletta 2009; Angilletta et al. 2010; Jessop et al. 2018) and increases resilience of ectothermic animals to climate change (Seebacher et al. 2015; Little and Seebacher 2016; Lillywhite 2016). A major challenge that ectotherms face in variable thermal environments is the maintenance of energy metabolism (i.e. the standard metabolic rate, SMR) (Angilletta et al. 2002; Little and Seebacher 2016) which is determined by measuring rates of O_2 consumption at rest and represents the energy required to cover basic physiological functions (Rowe et al. 1998; Beck and Congdon 2003).

Global warming impacts wildlife in complex ways through synergistic interactions with other environmental stressors that arise either from anthropogenic activity or natural sources (Noyes et al. 2009; Narayan 2016). Sub-optimal levels of environmental factors can activate the neuroendocrine system and increase stress hormone levels (Denver 1997; Mann et al. 2009; Dantzer et al. 2014) which also target the hypothalamus-pituitary-thyroid axis, responsible for production of thyroid hormones (THs) (Carr and Patino 2011). Stress hormones may synergize with THs resulting in increased TH production (Glennemeier and Denver 2002; Laudet 2011; Kulkarni and Buchholz 2012). In all vertebrates, THs are critical for regulating energy metabolism (Sheridan 1994; Choi et al. 2017). If the TH concentration changes due to

environmental stress, a whole suite of physiological processes may be influenced (Steyermark et al. 2005; Hulbert and Else 2004). Any impact on the hypothalamus-pituitary-thyroid axis is of special concern in amphibians, as metamorphosis is mainly regulated by THs (Tata 2006; Furlow and Neff 2006). The presence of predators (Relyea 2002; Capellán and Nicieza 2007), crowding (Morey and Reznick 2001), desiccation risk (Gervasi and Foufopoulos 2008), food scarcity (Kupferberg 1997), and temperature (Smith-Gill and Berven 1979; reviewed in Ruthsatz et al. 2018a) are known to increase TH production by activating the neuroendocrine stress axis. Anuran larvae with high TH levels display increased rates of development and standard metabolism and decreased rates of growth (Rowe et al. 1998; Brown and Cai 2007), which results in shorter larval periods and a smaller size at the onset of metamorphosis (Denver 1998, 2009; Orlofske and Hopkins 2009). Whereas most environmental stressors lead to increased TH activity or production by the activation of stress hormones, a large number of aquatic contaminants have also been shown to inhibit the normal action of THs in amphibians, leading to changes in growth, development, and metabolism (reviewed in Mann et al. 2009; Kashiwagi et al. 2009; Carr and Patino 2011). Inhibition or a decrease of TH production pathways slows the rate of development (Carr et al. 2003; Bulaeva et al. 2015) and decreases SMRs (Carr and Patino 2011; Ortiz- Santaliestra and Sparling 2007) causing tadpoles to metamorphose at a larger size and older age (Shi 2000).

Metamorphic and physiological traits such as size, metabolic rate, and body condition are often used as indicators of the effects of the larval stage on future fitness for organisms with complex life cycles (Alford and Harris 1988; Pechenick et al. 1998; Van Allen et al. 2010). As metamorphosis is an energy-consuming process (Sheridan and Kao 1998; Beck and Congdon 2003), it is advantageous to maintain a low SMR. Tadpoles which are able to maintain their energy metabolism at a low level at the onset of metamorphosis are more likely to successfully complete metamorphosis and become juvenile froglets with a capacity for physiological plasticity and higher rates of survival (Orlofske and Hopkins 2009). Therefore, energetics (i.e. SMR and size of energy stores) at the onset and after completion of metamorphosis are important fitness proxies (Steyermark et al. 2005; Muir et al. 2014; Ruthsatz et al. 2018b).

Climate change is expected to not only result in long-term warming of aquatic habitats but also increased variability in temperature leading to new thermal challenges for tadpoles in their larval habitats (Gutiérrez-Pesquera et al. 2016) with likely impacts on growth, development and survival (Pörtner 2001; Dalvi et al. 2009). Additional stress leading to

altered TH levels, through their impact on energy metabolism, may exacerbate these thermal challenges experienced during metamorphosis (Formicki et al. 2003). Since THs have recently been shown to play a key regulatory role in thermal acclimation in fish (Little and Seebacher 2014, 2016) and very early studies suggest that at least some aspects of this pathway are conserved in amphibians (reviewed in Little and Seebacher 2016) alteration of TH status may impact acclimation capacity in larval and juvenile anurans especially in combination with thermal stress. Although previous studies have examined the impact of stress-induced alteration of TH levels on physiological traits of anuran larvae, studies have rarely examined interactions of different stressors which are known to affect amphibian metamorphosis in nature (Rowe et al. 1998; Rowe and Crandall 2018).

The aim of this study was to examine the interactive effects of temperature and altered TH levels on the capacity for physiological acclimation (SMR and thermal tolerance) at the onset of metamorphosis and after completion of metamorphosis in larvae and froglets of *Rana temporaria*. Furthermore, we tested whether the impact of altered TH levels on SMR is temperature-dependent. For larvae acclimated to five different temperatures, we tested the following hypotheses: (1) Developmental temperature (T_{dev}) correlates positively with CT_{min} and CT_{max}, and negatively with the thermal range of tolerance in tadpoles. (2) High and low levels of TH, as caused by the thyroid altering effect of several environmental stressors, increase and decrease SMR of tadpoles, respectively. (3) T_{dev} interacts with altered TH levels and intensifies the effect of altered TH levels on physiological traits. (4) The effect of altered TH level is more pronounced at warmer temperatures during development. (5) Effects of T_{dev} and TH levels persist beyond the metamorphic boundary resulting in froglets with reduced acclimation capacity to warmer temperatures.

Material and Methods

Study species and experimental design

Rana temporaria represents the typical amphibian life history with aquatic embryonic and larval development and terrestrial froglets and frogs. It is widely distributed throughout Europe and occurs in variety of habitats and altitudes indicating a broad thermal niche. Five clutches of *R. temporaria* were obtained from the Waldpark Marienhöhe in western Hamburg (53°34'37.4"N 9°46'57.5"E, Hamburg, Germany). Larvae were allowed to hatch and develop to developmental stage 25 (free-swimming larvae; Gosner 1960). From these larvae, 840

individuals originating from the five clutches were intermixed before allocating them randomly to the different treatments (L-thyroxine and sodium perchlorate) and the control group. Fifteen larvae of *R. temporaria* were kept each in a standard 9.5 L aquarium filled with 8 L of water (i.e., a total of 45 aquaria: 3 × T4, 3 × SP, 3 × Control). The experiment was conducted in two climate chambers (Weiss Umwelttechnik GmbH, 35447 Reiskirchen, Germany) with a 12 to 12 light:dark (0900 to 2100) photoperiod and an air temperature of 10 ± 0.2°C and 22 ± 0.1°C (mean ± SD). Water temperatures were achieved by indirect heating elements beneath the aquaria (Tetra GmbH, Melle, Germany, adjustable heating element, Tetra HT100, 100W). The mean (± SD) water temperatures were 14 (± 0.5), 18 (± 0.1), 22 (± 0.1), 25 (± 0.2) and 28 (± 0.3) °C. The experiments ran for eleven weeks. All surviving larvae had reached the end of metamorphic climax at that time (Gosner, 1960). Amphibian larvae were fed high-protein flaked fish food (Sera micron breeding feed for fish and amphibians, Sera, 52518 Heinsberg, Germany) and spirulina algae twice a day *ad libitum*. The amount of food was continuously adjusted during the entire experiment to control for differences in tadpole size and density between the aquaria since Miyata and Ose (2012) indicated that a restricted feeding condition cause an atrophy of thyroid tissue similarly to TH agonists. The flakes were free of perchlorate according to the manufacturer. The aquaria were checked daily for dead or abnormal tadpoles, which were removed (Tietge et al. 2005). At 10°C none of the tadpoles survived until the onset of metamorphosis. Therefore, we refer to tadpoles reared in a temperature range from 14° to 28°C hereafter.

L-thyroxine and sodium perchlorate exposures

We increased internal TH levels by exposing tadpoles to 10 µg/L exogenous L-thyroxine (T4, IRMM468 Sigma-Aldrich**,** Sigma-Aldrich, St. Louis, USA), a concentration which is known to influence amphibian metamorphosis (Lucas and Reynolds 1967; Mann et al. 2009) and is related to increases in T4 observed in tadpoles responding to stress (Denver 1997; 1998). Tadpoles absorb exogenous T4 directly through their permeable skin (Shi 2000; Tata 2006; Coady et al. 2010). Exposing tadpoles to exogenous THs is an established method to simulate the proximate effects of environmental stressors on the TH system (Denver et al. 2002; Tata 2006; Denver 2009).

We used a concentration of 250 µg/L sodium perchlorate (SP, 99.99% trace metals basis, 381225 Aldrich, Sigma-Aldrich, St. Louis, USA) to decrease internal? TH levels. This concentration of SP is within environmental ranges measured in surface and ground waters of many industrial nations (Motzer 2001; Tietge et al. 2005; Carr and Theodorakis 2006; Mukhi

and Patino 2007) and in bodies of water in which amphibians breed (Smith et al. 2001; Ortiz-Santaliestra and Sparling 2007).

T4 and SP treatments were prepared in 0.1 N sodium hydroxide solutions (0.1 N, S2770 SIGMA, Sigma-Aldrich, St. Louis, USA) buffered with 0.1 N muriatic acid solutions as solvents. Solutions were added to the aquaria. To control for any effect of solvents addition, a solution of only 0.1 M sodium hydroxide solution buffered with 0.1 M muriatic acid solution was added to the control aquaria. Water was changed every second day and fresh SP and T4 were added, which is frequent enough to maintain a constant hormone and perchlorate level, in accordance with the standard procedure for chemical and hormonal addition (Miwa & Inui, 1987; Goleman et al., 2002 a,b; Iwamuro et al., 2003; Rot-Nikcevic & Wassersug, 2004; Tietge et al. 2005; Ortiz-Santaliestra & Sparling, 2007; Bulaeva et al., 2015).

Processing of specimens

Developmental stage was determined by evaluating the status of key morphological features typical of specific developmental stages, as detailed in Gosner (1960). The developmental stage of each tadpole was recorded according to the procedure of Ortiz-Santaliestra and Sparling (2007). The age which describes the larval duration in days after hatching until the onset of metamorphosis was defined by the emergence of at least one forelimb (Gosner stage 42; Gosner, 1960). End of metamorphic climax was defined by the complete resorption of the tail (Gosner stage 46; Gosner 1960).

The snout-vent length (SVL) of the larvae was measured with a caliper to the nearest 0.5 mm. Larvae were weighed to the nearest 0.001 g with an electronic balance (digital gold scale, Smart Weigh). At the end of the experiment froglets were euthanized with 200 mg/L of tricaine methanesulfonate ([MS-222], Ethyl 3-aminobenzoate methanesulfonate**,** E10521 ALDRICH, Sigma-Aldrich, St. Louis, USA) buffered with 200 mg/L of sodium bicarbonate (S5761 SIGMA, Sigma-Aldrich, St. Louis, USA) (Stuart et al. 2007) and transferred into ethanol (70 %) for further analyses.

Body condition

We estimated the body condition (i.e. energy stores) at the onset of metamorphosis by calculating the scaled mass index (SMI). The SMI accounts for the allometric relationship between mass and a body structure and is a standardized measure of the body condition that

can be directly compared among individuals (Peig and Green 2009, 2010; MacCracken and Stebbings 2012). The SMI has been previously employed as a condition index in anuran larvae (MacCracken and Stebbings 2012; Dittrich et al. 2016; Ruthsatz et al. 2018). A high SMI suggests larger energy storages and thus, a good body condition. We followed the procedure outlined by Peig and Green (2009) to calculate the SMI for each individual.

Respiration measurements

Respiration measurements were made at the onset of metamorphosis and at the end of metamorphic climax on eight randomly chosen tadpoles from each aquarium, in total on 720 individuals (n= 360, onset of metamorphosis; n=360, end of metamorphic climax). No fasting prior to the respiratory measurements was needed because tadpoles stop feeding due to the remodeling of mouthparts and digestive tract during metamorphosis (Hourdry et al. 1996). Oxygen consumption was measured by closed respirometry conducted between 0900 and 2100h to control for the influence of natural circadian rhythms on respiration (Orlofske et al. 2017). Larvae were placed in respirometers consisting of 30 ml beakers containing 30 ml (minus the volume of the animals) of autoclaved tap water to exclude microbial oxygen consumption. Froglets were placed in air-filled respirometers consisting of 30 ml beakers (minus the volume of the animals) due to their transition to lung respiration. Each respirometer was equipped with a fiber optic sensor (Oxygen Dipping Probe DP-PSt7; PreSens Precision Sensing GmbH, Regensburg, Germany) connected to a multichannel oxygen measuring system (Oxy 4 mini; PreSens Precision Sensing GmbH, Regensburg, Germany) and sealed with an air tight rubber plug. O_2 concentration was recorded every 15 seconds and measured as ml $O_2 \times L^{-1}$. Prior to each trial, O_2-fiber optic sensors were calibrated using air-saturated water and a factory-set zero oxygen calibration point at the respective developmental temperature of measured larvae. Water temperature and continuous mixing were controlled by a waterbath. Oxygen consumption was measured for every tadpole for 20 min at each of five temperatures. Empty (control) chambers were run simultaneously in every trial and values were adjusted accordingly. We ensured that less than 10% of total O_2 was removed during the measurements to avoid impediment of respiration at low saturation levels. At the end of the measurements, each larva was removed and its TL, SVL and blotted wet body mass was determined.

Standard metabolic rate calculations

Prior to statistical analysis, we plotted O_2 consumption of each animal over time and visually assessed activity peaks to exclude them for the determination of standard metabolic rate (SMR) (Orlofske and Hopkins 2009). The total rate of O_2 consumption (SMR in ml O_2 $\times h^{-1} \times mg^{-1}$ wet body mass) was equal to the slope of linear least squares regression of O_2 concentration vs. time (Hastings and Burggren 1995; Rowe and Funk 2017). Values for SMR and mass were log transformed because metabolism is a power function of mass (Orlofske and Hopkins 2009; Orlofske et al. 2017).

Thermal tolerance

Thermal tolerance of *R. temporaria* was evaluated when tadpoles reached the onset of metamorphosis (Gosner stage 42) using the critical thermal methodology (Holzmann and McManus 1973). Both critical thermal minimum (CT_{min}) and maximum (CT_{max}) endpoints are defined as the thermal point at which locomotor activity becomes disorganized and the animal loses the ability to right itself (Lutterschmidt and Hutchison 1997; Turriago et al. 2015). A total of 450 tadpoles were used for determination of thermal tolerance. From each aquarium ten tadpoles (n = 5, CT_{min}; and n = 5, CT_{max}) were tested at set time intervals. CT_{min} and CT_{max} were determined by using the dynamic method according to Cowles and Bogert (1944) and Hutchison (1961) except for the end point (Wu and Kam 2005). This method involves linearly decreasing (for CT_{min}) or increasing (for CT_{max}) test temperatures by a specific rate until an appropriate endpoint is reached (Lutterschmidt and Hutchison 1997). Tadpoles were placed individually in a 250-ml flask with 200 ml of water which was then placed in a temperature-controlled water bath. The heating and cooling rates were ± 0.1°C $\times min^{-1}$, and the water temperature served as a proxy of body temperature (Hutchison 1961). The initial temperature in the water bath was set at the respective developmental temperature. In tadpoles, the occurrence of spasms is difficult to determine, and thus we decided to use the loss of the righting response after being flipped on its back in the water with a probe as our criterion for the endpoint (Lutterschmidt and Hutchison 1997; Wu and Kam 2005) for both, CT_{min} and CT_{max} determinations (Turriago et al. 2015). A time limit of 30 sec between flipping the animal and righting was adopted (Layne Jr and Claussen 1982). All thermal tolerance tests were performed between 1100h and 1500h. After the experiments, we euthanized the tadpoles with MS-222, weighed them and measured SVL and TL, and finally transferred them into ethanol (70 %).

We adopted the method of Dalvi et al. (2009) used in fish to generate a thermal tolerance window (TW) for *R. temporaria* by calculating the difference between CT_{max} and CT_{min} estimates obtained at various acclimation temperatures. To simulate long-term changes in environmental temperature, *R. temporaria* were reared at a range of different temperatures. The thermal tolerance polygon was generated by plotting the five developmental temperatures for each treatment (T4, SP, and Control) on the X-axis and the mean CT_{min} and CT_{max} values on the Y-axis. The TW was calculated from the polygon and expressed as $°C^2$ (Dalvi et al. 2009). We performed a linear regression for developmental temperature and thermal tolerance (as measured by CT_{min}, CT_{max}, and thermal range of tolerance). The slope of the regression for CT_{max} and CT_{min} defined the effect of developmental temperature on critical thermal limits of *R. temporaria.*

Statistical analysis

For all statistical tests R 3.4.1 (R Development Core Team, 2007) for Windows was used. All plots were constructed using ggplot2 (Wickham 2009) and Adobe Illustrator CS6. Data were analyzed using linear mixed-effect models [lme, Type III model, covariance type: variance components, REML (restricted maximum likelihood) method for parameter estimation, 100 iterations (Bates and Sarkar, 2007)], using the covariate 'T_{dev}', and 'treatment' (T4, SP, and Control) and the interactions of 'treatment' and 'T_{dev}' as fixed factors. 'SMR_{larvae}', 'body condition', 'thermal tolerance' (as measured by CT_{min}, CT_{max}, and the thermal range of tolerance), and '$SMR_{froglet}$' were used as dependent variables in separate models. P-values were obtained from likelihood-ratio tests (Crawley, 2007). To address dependencies in the data, the variable 'aquarium' was included as a random factor. Residuals of each model were visually checked for normal distribution. N refers to the total number of analyzed tadpoles.

Results

There was no consistent effect of time of day on SMR tested during this experiment. At all developmental temperatures tadpoles and froglets from the SP treatment were the largest at the onset of metamorphosis followed by control animals and tadpoles from the T4 treatment being the smallest (Table A1).

Standard metabolic rate and body condition

Onset of metamorphosis

SMR of tadpoles was significantly influenced by the hormone treatment, by T_{dev} and the interactive effect of both (Table 1). With increasing developmental temperature, SMR increased in all treatments except of animals which were reared at 14°C. SMR was highest at altered TH levels and warm T_{dev} whereas the interactive effect of altered TH levels and T_{dev} decreased SMR. With increasing temperature, variance of SMR in all treatment groups increased (Fig. 1). Animals from SP treatment revealed the highest SMR at 14°C, and 28°C, and the lowest SMR at 18°C, 22°C, and 25°C. In control treatment, larvae reared at 14°, 18°, and 22°C revealed the lowest SMR, which increased significantly at 25°C and 28°C. Larvae exposed to T4 revealed the lowest SMR when reared at 18°C. Below and above 18°C, SMR was significantly higher in larvae from T4 treatment.

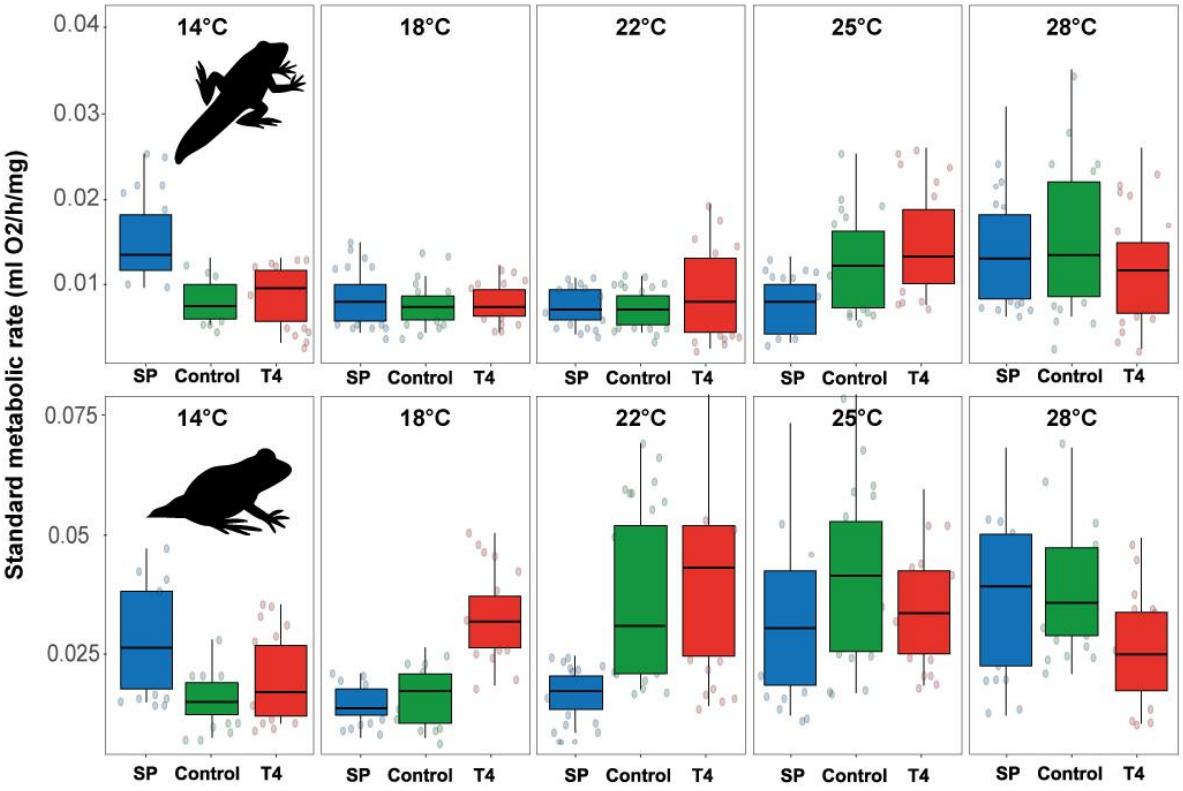

Fig. 1. Interactive effect of altered thyroid hormone levels and five developmental temperatures on SMR (ml O_2/h/mg) in **upper graphs:** tadpoles at the onset of metamorphosis and **lower graphs:** froglets after completion of metamorphic climax of the common frog (*Rana temporaria*). Blue: SP = low TH levels. Green: control treatment. Red: T4 = high TH levels. Dots show the respective data points at each hormone * temperature treatment.

Body condition at the onset of metamorphosis was affected by the hormone treatment, T_{dev}, and the interactive effect of both (Table 1, Fig. 2). Warmer temperatures during development, exposure to SP, and the interactive effect of T4 and warmer T_{dev} decreased body condition whereas exposure to T4 and the interactive effect of SP and warmer T_{dev} increased body condition. Body condition was best in larvae reared at 22°C in all hormone treatments, whereas body condition was worst in larvae reared at lowest and warmest temperature.

Table 1. Effects of altered TH levels and developmental temperature on larval and juvenile SMR (ml $O_2 \times h^{-1} \times mg^{-1}$), larval and juvenile body condition, and larval thermal tolerance (as measured by CT_{min}, CT_{max}, and thermal range of tolerance) in tadpoles and froglets of the common frog (*R. temporaria)* at the onset of metamorphosis (Gosner stage 42) and after completion of metamorphosis (Gosner stage 46, Gosner 1960). Body condition was determined by the scaled mass index (SMI). Chi^2 and P for linear mixed-effects models, using 'Treatment' (T4, SP, and Control), 'T_{dev}' and the interactions of 'Treatment*T_{dev}' as fixed factors; 'aquarium' as random factor. SP = low TH levels. T4 = high TH levels. Significance was set at $P < 0.05$. Bold indicates significant P-values. N(n)=total number of studied individuals (total number of aquaria).

Dependent variable	**N**	**Fixed effects**	**Estimate**	**SE**	**Chi^2**	**df**	**P**
SMR_{larvae} (ml $O_2 \times h^{-1} \times mg^{-1}$)	384 (45)	Treatment [Control]	-0.002	0.003	10.78	2	**0.004**
		T_{dev}	0.001	0.00	10.78	2	**0.004**
		Treatment [SP]	0.014	0.004	10.78	2	**0.004**
		Treatment [T4]	0.004	0.004	10.78	2	**0.004**
		Treatment [SP] * T_{dev}	-0.001	0.000	10.78	2	**0.004**
		Treatment [T4] * T_{dev}	-0.000	0.000	10.78	2	**0.004**
$Body\ condition_{larvae}$ (SMI)	582 (45)	Treatment [Control]	278.20	73.47	10.81	2	**0.004**
		T_{dev}	-1.04	3.34	10.81	1	**0.004**
		Treatment [SP]	-45.78	103.87	10.81	2	**0.004**
		Treatment [T4]	19.87	104.17	10.81	2	**0.004**
		Treatment [SP] * T_{dev}	1.53	4.73	10.81	2	**0.004**
		Treatment [T4] * T_{dev}	-12.56	4.74	10.81	2	**0.004**
CT_{min} (°C)	450 (45)	Treatment [Control]	1.01	0.41	21.418	2	**<0.001**
		T_{dev}	-0.11	0.59	113.29	1	**<0.001**
		Treatment [SP]	-0.05	0.59	21.41	2	**<0.001**
		Treatment [T4]	0.22	0.01	21.41	2	**<0.001**
		Treatment [SP] * T_{dev}	0.01	0.02	1.63	2	0.441
		Treatment [T4] * T_{dev}	-0.01	0.02	1.63	2	0.441
CT_{max} (°C)	450 (45)	Treatment [Control]	24.20	1.83	3.59	2	0.166
		T_{dev}	-0.36	2.60	76.19	1	**<0.001**
		Treatment [SP]	-1.29	2.60	3.59	2	0.166
		Treatment [T4]	0.57	0.08	3.59	2	0.166
		Treatment [SP] * T_{dev}	0.05	0.11	0.40	2	0.818
		Treatment [T4] * T_{dev}	0.06	0.11	0.40	2	0.818

Thermal range of tolerance	450 (45)	Treatment [Control]	23.19	1.84	1.40	2	0.495
		T_{dev}	-0.24	2.61	45.34	1	**<0.001**
		Treatment [SP]	-1.24	2.61	1.40	2	0.495
		Treatment [T4]	0.35	0.08	1.40	2	0.495
		Treatment [SP] * T_{dev}	0.04	0.11	0.47	2	0.789
		Treatment [T4] * T_{dev}	0.07	0.11	0.47	2	0.789
$SMR_{froglets}$ (ml $O_2 \times h^{-1} \times mg^{-1}$)	359(45)	Treatment [Control]	-0.69	0.47	9.64	2	**0.008**
		T_{dev}	0.03	0.02	9.64	2	**0.008**
		Treatment [SP]	1.90	0.67	9.64	2	**0.008**
		Treatment [T4]	0.95	0.67	9.64	2	**0.008**
		Treatment [SP] * T_{dev}	-0.09	0.03	9.64	2	**0.008**
		Treatment [T4] * T_{dev}	-0.05	0.03	9.64	2	**0.008**
Body $condition_{froglets}$ (SMI)	525 (45)	Treatment [Control]	252.83	30.46	20.44	2	**<0.001**
		T_{dev}	-5.27	-1.38	39.79	1	**<0.001**
		Treatment [SP]	30.46	43.14	20.44	2	**<0.001**
		Treatment [T4]	40.10	43.51	20.44	2	**<0.001**
		Treatment [SP] * T_{dev}	-0.001	1.96	2.49	2	0.287
		Treatment [T4] * T_{dev}	-2.58	1.98	2.49	2	0.287

End of metamorphic climax

At the end of metamorphic climax, SMR of froglets was significantly influenced by the hormone treatment, by T_{dev}, and the interactive effect of both (Table 1, Fig. 2). In general, altered TH levels and temperature increased SMR whereas the interactive effect of both decreased SMR. With increasing temperature, variance of SMR in all treatment groups increased (Fig. 1). In SP treatment, froglets revealed the lowest SMR at 18°C and 22°C and the highest at 14° and 28°C. Froglets from control treatment had the lowest SMR when reared at 14° and 18°C and the highest SMR at 28°C. Froglets exposed to T4 during development had the lowest SMR at 14° and the highest SMR at warmest temperatures during development.

Body condition of newly metamorphosed froglets was affected by T_{dev} and altered TH levels whereas the interactive effect of warmer temperatures and altered TH levels did not affect body condition (Table 1). In particular, warmer T_{dev} reduced body condition, whereas altered TH levels increased body condition (Fig. 2). In control and T4 treatments, body condition of froglets was best at lowest T_{dev} and decreased at warmer temperatures. In froglets, which were exposed to SP during development, body condition was best at 22°C. In froglets, which were exposed to T4 during development, body condition was worst at 22°C, whereas froglets from SP and control treatment revealed the worst body condition at warmest temperatures.

Thermal tolerance and thermal window at the onset of metamorphosis

The results for linear regressions show a significant relation between thermal limits (i.e. CT_{min} and CT_{max}) and T_{dev}. CT_{min} and CT_{max} increased significantly with increasing developmental temperature except for tadpoles reared at 28°C (Table 2, Fig. 3). For every 1°C increase in T_{dev} the CT_{min} and CT_{max} increased by 0.24 °C and 0.58 °C, respectively, in the SP treatment, by 0.22 °C and 0.57 °C in the control treatment, and by 0.20 °C and 0.63 °C in the T4 treatment. Slopes from linear regressions revealed a greater effect of T_{dev} on CT_{max} than on CT_{min} within all hormone treatments. TRT increased at warmer temperatures as CT_{min} increased less than CT_{max} at warmer temperatures. The thermal window polygon area (TW) for *R. temporaria* reared at five different temperatures between 14 and 28 °C was 440.48 °C² for the SP treatment, 429.37 °C² for the control treatment, and 435.60 °C² for the T4 treatment (Table A2, Fig. 3).

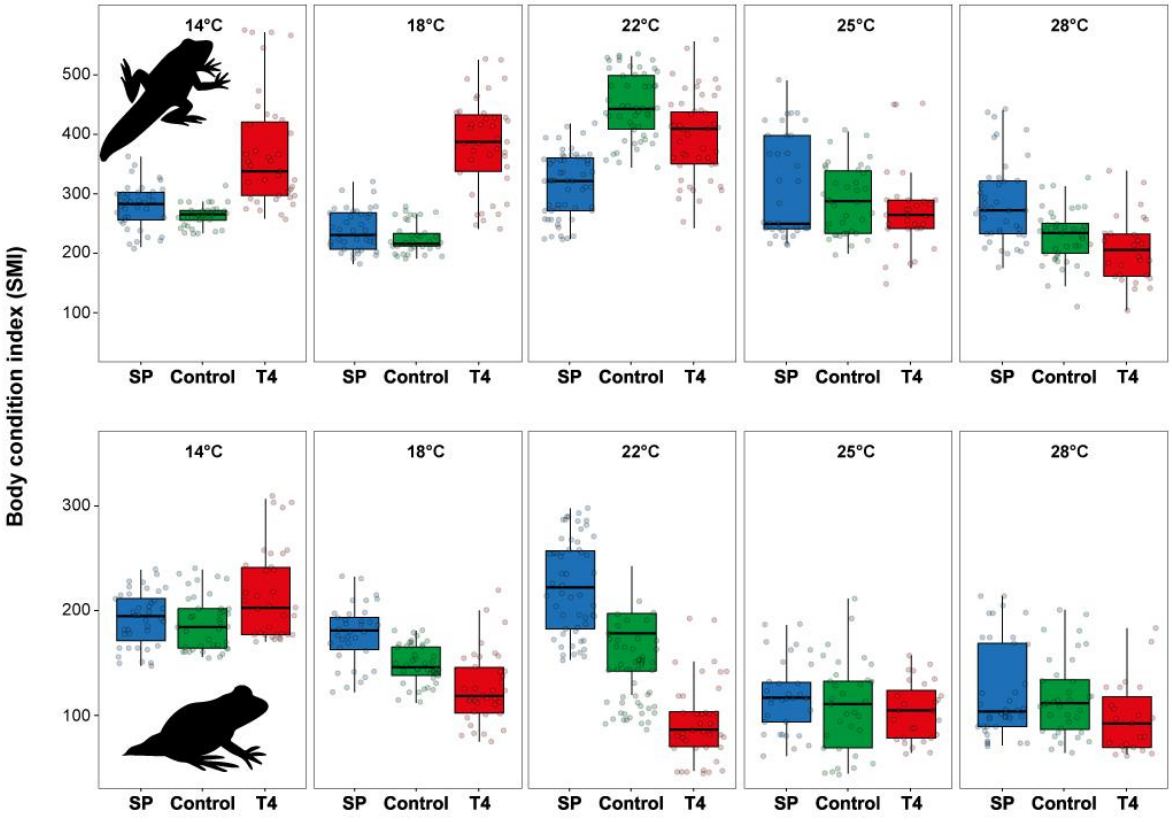

Fig. 2 Interactive effect of altered thyroid hormone levels and five developmental temperatures on body condition in **upper graphs:** tadpoles at the onset of metamorphosis and **lower graphs:** froglets after completion of metamorphic climax of the common frog (*Rana temporaria*). Body condition was determined by the scaled mass index (SMI). Blue: SP = low TH levels. Green: control treatment. Red: T4 = high TH levels. Dots show the respective data points at each hormone * temperature treatment.

Table 2. Linear regressions of critical thermal limits (as measured by CT_{min} and CT_{max}) with developmental temperature in *R. temporaria* tadpoles. Significance was set at $P < 0.05$. N= 75. SP = low TH levels. T4 = high TH levels**.** P was <0.001 for all linear regressions.

Dependent variable	**Treatment**	**Developmental temperature** (°C)	
		Slope	**R^2**
CT_{min}	SP	0.24	**0.92**
	Control	0.22	**0.96**
	T4	0.20	**0.80**
CT_{max}	SP	0.58	**0.89**
	Control	0.57	**0.39**
	T4	0.63	**0.73**

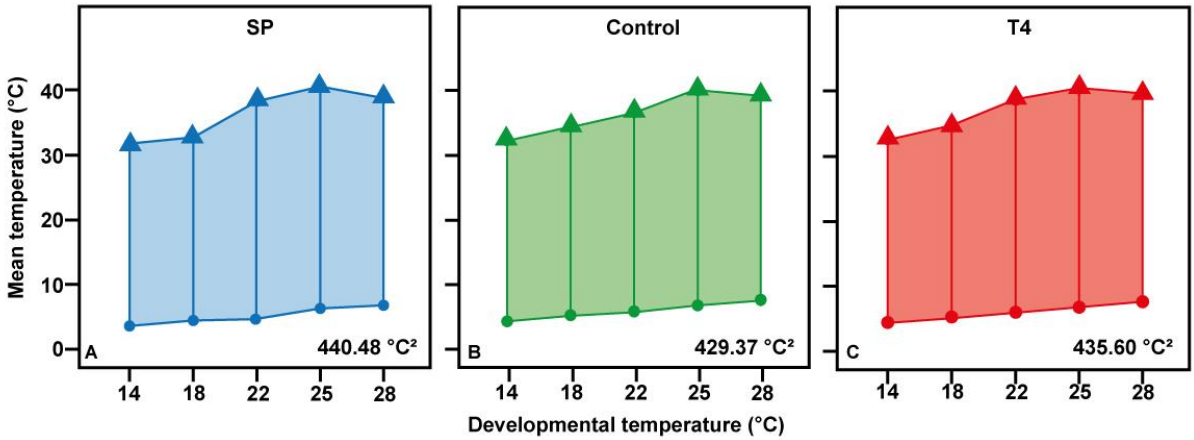

Fig. 3. Developmental thermal windows of *R. temporaria* at different thyroid hormone (TH) levels. Thermal tolerance polygons generated from the critical thermal limits (CT_{min} and CT_{max}) at five developmental temperatures in a total of 450 animals. **A** SP treatment = low TH levels. **B** Control treatment **C** T4 treatment = high TH levels. Triangles = CT_{max}, circles = CT_{min}. Line between CT_{min} and CT_{max} = Thermal range of tolerance.

Discussion

The capacity for species to express developmental (i.e. in age and size at metamorphosis) and physiological plasticity (i.e. in thermal tolerance and energetics) in response to temperature change is expected to help ectothermic species such as amphibians coping with some of the major drivers of biodiversity loss, such as climate-driven warming and other natural and anthropogenic environmental stressors (reviewed in Nowakowski et al. 2018; Seebacher et al. 2015). The interactions of warmer and more variable temperatures and different stressors are not well known (Boone et al. 2007; Polo-Cavia et al. 2017) and knowledge on their impact on

amphibian declines is still rare (Blaustein et al. 2011). Our results suggest that both tadpoles and froglets of *R. temporaria* are able to acclimate to warmer developmental temperatures by adjusting their maintenance energy costs (SMR) and that this acclimation capacity is not impaired by the interaction between warmer temperature and altered TH status as often elicited by environmental stressors. Furthermore, larvae at the onset of metamorphosis were able to acclimate to increased developmental temperature by increasing their thermal limits and broadened their range in thermal tolerance. The capacity for physiological plasticity and body condition, however, decreased at warmers temperatures, an effect which persisted across the metamorphic boundary resulting in froglets with a low body condition.

Altered TH levels and warmer temperatures increase SMR and affect body condition

In amphibian larvae as in all ectothermic animals ambient temperature regulates the rates of all physiological and biochemical processes (Smith-Gill and Berven 1979; Tata 2006; Little and Seebacher 2016) impacting growth, development and metabolism. An increase in ambient temperature may, therefore, substantially increase the SMR but larvae may compensate for those thermal changes through acclimation and thus, reduce the increase of SMR due to warmer temperatures (Angilletta et al. 2006; Seebacher et al. 2015; Berg et al. 2017). In this study, the SMR of larval *R. temporaria* was higher when larvae developed at warmer temperatures but increased only slightly indicating a capacity to compensate for warming through physiological acclimation. Moreover, SMR was relatively stable in all hormone treatments across temperatures treatments from 14° to 22°C. Not until 25°C, SMR increased significantly compared to other temperature treatments independent from TH level. As the variability in SMR increased at warmer temperatures, we suggest that larvae differed in their capacity for physiological plasticity at individual level with some still maintaining a relative stable SMR as shown for temperatures from 14°C to 22°C by acclimation to warmer temperatures during development and some lacking this capacity resulting in significantly increased SMRs. Furthermore, our results indicate that the optimum temperature to maintain SMR during development is around 18°C independent from TH levels. Thus, environmental stress may not influence the optimum temperature for maintaining a constant SMR. In natural breeding ponds of *R. temporaria*, temperatures during development are within this optimum temperature range and may outstripe this range under short-term temperature fluctuations (i.e. diurnal change) and in late developmental stages due to a seasonal mean temperature increase. However, Drakulic et al. (2017) demonstrated that mean seasonal temperature in Germany and southern Europe was 20°C and lower suggesting that larvae of *R. temporaria* may

experience mean temperatures within their thermal optimum for maintaining a stable SMR under natural conditions.

Climate change is altering patterns of environmental temperature with potentially important repercussions for ectotherms that must simultaneously cope with contaminants or other environmental stressors (Hallman and Brooks 2015). In this study, larvae of *R. temporaria* revealed a higher SMR when exposed to T4 and SP. Hence, our results confirmed that high levels of TH (i.e. by exposure to T4) determine the metabolic rate in *R. temporaria* as shown for lizards (*Dipsosaurus dorsalis* and *Sceloporus occidentalis*; John-Alder 1983, 1990), snakes (*Thamnophis sirtalis*; Etheridge 1993), the leopard frog (*Rana pipiens*; Steyermark et al. 2005), and the African clawed frog (*Xenopus laevis*; Ruthsatz et al. 2018c). Thus, environmental stressors which increase TH levels (e.g. increased desiccation risk, crowding, presence of predators) may result in slightly increased SMRs. As SMR was increased also at low TH levels through the exposure to SP, we suggest that aquatic contaminants alone may result in increased metabolism due their toxicity independent from the thyroid metabolism. Chronically higher metabolism, from contaminants alone, occurs in fish (Beyers et al. 1999), larval amphibians (McDaniel et al. 2004), and bivalves (Lannig et al. 2008; reviewed in Hallman and Brooks 2015). Thus, researchers are raising concern that organisms living in chronically polluted water might be at greater risk from global warming if they cannot provide the additional energy needed to cope with simultaneous thermal stress (Rowe and Crandall 2018). Therefore, it has also been suggested that not climate change itself may affect amphibians, but rather will act in combination with biotic and abiotic factors increasing their effects (Lopez-Alcaide and Macip-Rios 2011; Baier et al. 2016). In the present study, the effect of altered TH level was not intensified at warmer temperatures. Therefore, larvae exposed to warmer temperatures are not likely to be affected more by environmental stressors than larvae at colder temperatures experienced before and during metamorphic climax.

In general, ectothermic species from temperate latitudes display a greater capacity for physiological acclimation than species from tropical climates (reviewed in Vo and Gridi-Papp 2017). Therefore, a capacity to respond plastically in physiological traits should be high in temperate species such as *R. temporaria* (Janzen 1967). In particular, larvae of *R. temporaria* are known to respond more plastic to environmental variation than other species (Laurila and Kujasalo 1999; Grözinger et al. 2018) especially in terms of growth and developmental rate as they are exposed to large inter- and intraannual habitat variability. Therefore, temperate anuran species in general but especially larvae of *R. temporaria* may compensate for direct

(i.e. temperature increase) and indirect effects (i.e. shortening of hydroperiod, increasing tadpole density, and the concentration of contaminants as the pond desiccates; Egea-Serrano and Van Buskirk 2016) of global warming through temperature induced developmental and physiological plasticity and may be less vulnerable to the thermal impacts of climate change than tropical species. Otherwise, a high SMR is likely to allow for quicker metamorphosis and, thus, for passing this highly vulnerable phase. If so, a high SMR may even be selected for under natural circumstances. However, the capacity for temperature-induced plasticity is known to be related to local adaptations in anuran larvae (Laugen et al. 2003; Muir et al. 2014; Drakulic et al. 2016) and thus, is population-specific. In contrast to the numerous studies on developmental plasticity demonstrating a higher individual growth efficiency in high-latitude populations (Stahlberg et al. 2001; Laugen et al. 2003; Lindgren and Laurila 2005), population differentiation in SMR is not linked to latitudinal variation in developmental plasticity in *R. temporaria* (Lindgren and Laurila 2009). Nevertheless, variation in metabolic scope and maximum metabolic rate, coupled with changes in growth efficiency may result in population-specific differences in physiological plasticity (and vulnerability to global climate) change, along latitudinal gradients (Lindgren and Laurila 2009).

Despite an acclimation capacity in SMR, reductions in body condition (i.e. the ability to store energy) were noted in tadpoles having low levels of THs (i.e. as caused by the endocrine disruptor SP) that were reared at warmer temperatures. Since (anthropogenic) global change will introduce multiple environmental stressors (e.g. changes in temperature and additional factors) to natural larval habitats of anuran larvae, the capacity to store energy during the larval stage may be reduced with possible ramifications for the energetic efficiency of metamorphosis. Independent from TH status, body condition was highest and SMR was relatively low in tadpoles reared at 22°C and SMR was also relatively low at this temperature, therefore our results underscore that the optimum temperature during development is around 22°C. This is in accordance with previous studies (Rühmekopf 1958; Drakulic et al. 2017). Any increase in temperatures above this optimum increases SMR and may lead to a small but constant increase in energetic demands. Potential consequences of increased metabolic expenditures in a resource-limited, high density system are reduced growth, developmental rates, or larval survival, which could ultimately affect recruitment to the terrestrial habitat (Rowe and Crandall 2018; Ruthsatz et al. 2018b).

We further demonstrated that larval exposure to warmer developmental temperatures and environmental stress not only affects SMR and body condition at the onset of metamorphosis but also at the end of metamorphic climax in froglets of *R. temporaria*. We follow Pechenik (2006) in suggesting that metamorphosis is not "a new beginning" since effects of stressful larval environments may persist beyond the metamorphic boundary and result in strong carry-over effects on juveniles and adults (Smith 1987; Berven 1990; Goater 1994; Pechenik 2006; Altwegg and Reyer 2003; 2003; Scott et al., 2007, Morey and Reznick 2001; Van Allen et al. 2010). Carry-over effects may occur in terms of acclimation capacity to temperature warming but also in terms of performance and finally on survival (Goater et al. 1993; Beck and Congdon 2000; Chelgren et al. 2006). Consequently, future studies on acclimation capacity of larval anurans should focus on carry-over effects on juvenile and adult performance.

Thermal tolerance is determined by developmental temperature and SMR

In addition to a capacity for acclimation of SMR, understanding the plasticity in the range of thermal tolerance and how this plasticity is altered by other environmental stressors may be crucial to predicting how physiological variation can influence a species' response to global change (Katzenberger et al. 2014; Seebacher et al. 2015). In this study, tadpoles exhibited thermal acclimation such that individuals from warmer developmental temperatures increased their CT_{max} and broadened their range in tolerable temperatures, mechanisms previously observed in other amphibians (Schaefer and Ryan 2006; Gunderson and Stillman 2015; Little and Seebacher 2016). We could not report any significant effects of altered TH levels on the upper thermal limit and the thermal range of tolerance in *R. temporaria* indicating that these physiological traits are not under the control of the thyroid system. Therefore, altered TH levels as caused by environmental stressors may not affect the capacity for an acclimation in upper thermal limits and thermal range of tolerance. Thus, larvae under environmental stress are still able to compensate for changes in developmental temperature. In temperate climates, amphibians tend to have a broader range of thermal tolerance, centered at lower temperatures than in tropical climates (Janzen 1967). Therefore, the species-specific thermal window is also broader in temperate, eurythermic species than in stenothermic arctic or tropical species (Dalvi et al. 2009; Pörtner 2002). In this study, we generated the developmental thermal window for larvae of *R. temporaria* acclimated to five different temperatures. Compared to a previous study in tropical *X. laevis* (Ruthsatz et al. 2018c), we found that the developmental thermal window was substantially broader in *R. temporaria* indicating its adaptation to a temperate climate.

Even if a long-term increase in annual average temperature is the consequence of global climate change, environmental change also results in short-term, both stochastic and predictable, extreme thermal events, particularly in temperate freshwater habitats (Seebacher et al. 2015; Gutiérrez-Pesquera et al. 2016; Burggren 2018). Therefore, laboratory studies on the capacity for physiological plasticity in thermal tolerance (especially in CT_{max}) as the present study are of special relevance for predictions on vulnerability of anuran larvae to those short-term thermal challenges. Current IPCC models for global temperature in the coming century (IPCC, 2014) do not suggest warming of the magnitude necessary to threaten temperate larvae such as *R. temporaria* by exceeding their CT_{max} (Rowe and Crandall 2018). In contrast, positive effects of climate-driven warming are expected in populations of many organisms that occur at temperatures colder than their thermal optima (Deutsch et al. 2008; Kingsolver et al. 2013; reviewed in Egea-Serrano and Van Buskirk 2016). This appears to be the case for *R. temporaria* in central and northern Europe (Duarte et al. 2012) but may not for populations in southern Europe as they are locally adapted to warmer temperatures (Lindgren & Laurila 2009). However, climatic anomalies as recorded in Europe for summer temperatures in 2003 (Neveu 2009) and recent spring and summer in 2018 (Deutscher Wetterdienst) representing the warmest seasonal temperatures since weather was recorded (Luterbacher et al. 2004; Neveu 2009; Deutscher Wetterdienst) may lead to sub-optimal temperatures accompanied by an increased desiccation risk and decreased food availability in larval habitats of *R. temporaria*. So far, it is challenging to project winners and losers in terms of climate change in light of the fact that multiple factors, beyond mere warming or increased variance in temperature, may simultaneously occur.

Conclusions

Environmental change due to global warming and other environmental stressors is complex and often unpredictable (Burggren 2018) and anuran larvae will therefore be exposed to multiple environmental stressors. Our study combines the effects of the multiple stressors accompanying direct effects of climate change in a multifactorial design. By directly altering the TH levels we simulate the proximate effects of various environmental stressors and may conclude how proximate effects of multiple stressors may affect the acclimation capacity of amphibians across life stages. Our results emphasize that of *R. temporaria* might cope with short- and long-term changes as larvae and froglets are able to compensate for new thermal challenges independent from interactions with other environmental stressors impairing growth

and development by altering endogenous TH levels. Thus, warmer temperatures during development could result in increased mean fitness in temperate species such as *R. temporaria* compared to tropical species. As environmental temperatures greatly influence the behavior and physiology but also the distribution of aquatic ectotherms (Narum et al. 2013) the capacity for acclimation to warmer temperatures may influence the frogs range expansion into hotter climates (Seebacher and Franklin 2011; Jessop et al. 2018). However, *R. temporaria* is distributed over a wide geographic range with large differences in climatic conditions (Stahlberg et al. 200; Lindgren and Laurila 2009) and thus, populations may differ in their local adaptations to thermal environments and their acclimation capacity. Comparative studies across populations and species would help to identify the potential for acclimation or inter-specific differences, respectively. The present findings also emphasize that physiological traits (i.e. SMR and body condition) at the onset of metamorphosis persist beyond metamorphic climax. As phenotypic traits are often used as indicators of the effects of the larval stage on future fitness for organisms with complex life cycles we suggest that multiple environmental stressors associated with global change may affect all life stages of amphibians with possible consequences for individual fitness and long-lasting effects on amphibian populations. Future studies on physiological plasticity of larval anurans should investigate whether the ability for acclimation changes across life stages and should focus on carry-over effects on juvenile and adult performance.

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Appendices

Table A1 Descriptive statistics of mass (mg) at the onset of metamorphosis (Gosner stage 42) and after completion of metamorphic climax (Gosner stage 46) in tadpoles and froglets of the common frog *R. temporaria* at five different developmental temperatures exposed at different TH levels. T4 = high TH level**.** SP = low TH level.

	Temperature	Treatment	Maximum	Minimum	Mean	SE	N
Mass (mg) at the onset of metamorphosis (Gosner stage 42)	14	Control	578	640	605.9	2.2	41
		SP	632	702	671.3	2.9	43
		T4	380	419	400.2	1.7	39
	18	Control	471	500	484.2	1.2	43
		SP	499	549	521.2	2.23	41
		T4	296	335	312.4	1.9	42
	22	Control	346	384	362.0	1.5	55
		SP	433	496	462.4	2.5	59
		T4	172	206	188.7	1.6	49
	25	Control	112	152	133.3	1.7	38
		SP	219	254	233.3	1.8	35
		T4	83	104	94.7	1.1	31
	28	Control	62	99	81.1	1.4	41
		SP	96	134	113.1	1.8	42
		T4	51	89	69.6	2.1	28
Mass (mg) after completion of metamorphosis (Gosner stage 46)	14	Control	379	417	401.1	1.4	41
		SP	412	469	435.5	2.6	39
		T4	217	248	228.5	1.5	34
	18	Control	284	315	302.8	1.5	42
		SP	349	405	373.3	2.8	36
		T4	80	113	94.5	1.4	37
	22	Control	93	124	105.2	1.3	53
		SP	274	314	293.6	1.4	55
		T4	42	67	52.2	1.2	42
	25	Control	42	65	50.8	1.3	33
		SP	59	96	75.0	2.3	34
		T4	37	54	46.1	1.0	27
	28	Control	34	49	41.3	0.7	36
		SP	40	58	49.0	0.9	38
		T4	29	42	36.4	0.8	23

Table A2 Critical thermal minima (CT_{min}), critical thermal maxima (CT_{max}), thermal range of tolerance (TRT), and thermal window (TW) (± SD) of tadpoles of the common frog Rana temporaria at five different developmental temperatures exposed at different TH levels caused by environmental stress. T4 = high TH level**.** SP = low TH level.

Developmental temperature (°C)	**Treatment**	**CT_{min} (°C)** ±SD	**CT_{max} (°C)** ±SD	**TRT** ±SD	**TW (°C²)**
14	**Control**	**4.2** ± 0.1	**32.0** ± 0.1	**27.8** ± 0.1	429.37
18		**5.0** ± 0.1	**34.3** ± 0.1	**29.2** ± 0.2	
22		**5.6** ± 0.1	**36.6** ± 0.9	**31.0** ± 0.0	
25		**6.8** ± 0.1	**40.3** ± 0.1	**33.4** ± 0.2	
28		**7.2** ± 0.2	**39.1** ± 0.1	**31.8** ± 0.2	
14	**SP**	**4.6** ± 0.1	**33.0** ± 0.1	**28.3** ± 0.1	440.48
18		**5.5** ± 0.1	**35.0** ± 0.1	**29.5** ± 0.1	
22		**5.9** ± 0.1	**39.1** ± 0.1	**33.2** ± 0.2	
25		**7.2** ± 0.2	**40.7** ± 0.1	**33.5** ± 0.3	
28		**7.6** ± 0.2	**40.1** ± 0.1	**32.5** ± 0.2	
14	**T4**	**4.0** ± 0.1	**31.7** ± 0.2	**27.6** ± 0.3	435.60
18		**4.5** ± 0.1	**32.8** ± 0.1	**28.2** ± 0.3	
22		**4.8** ± 0.1	**38.3** ± 0.2	**33.5** ± 0.2	
25		**6.4** ± 0.1	**40.4** ± 2.6	**34.0** ± 2.6	
28		**6.8** ± 2.1	**38.7** ± 0.1	**31.9** ± 0.1	

Author contribution

I hereby confirm that Katharina Ruthsatz conceived, designed and performed the experiments, analyzed the data and wrote the paper.

Hamburg, 18.10.2018

Prof. Dr. Kathrin Dausmann

Chapter Five

Altered thyroid hormone levels affect body condition at metamorphosis in larvae of *Xenopus laevis*

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Abstract

Chemical, physical, and biological environmental stressors may affect the endocrine system such as the thyroid hormone axis in larval amphibians with consequences for energy partitioning among development, growth, and metabolism. We studied the effects of two thyroid hormone (TH) level affecting compounds, exogenous L-thyroxine (T4) and sodium perchlorate (SP), on various measures of development and body condition in larvae of the African clawed frog (*Xenopus laevis*). We calculated the scaled mass index, the hepatosomatic index, and the relative tail muscle mass as body condition indices to estimate fitness. Altered TH levels significantly altered the growth, development, survival, and body condition in metamorphic larvae in different directions. While exogenous T4 reduced growth and accelerated development, SP treatment increased growth but slowed down development. Altered TH levels improved body conditions in both treatments and especially in larvae of the SP treatment but to the detriment of lower survival rates in both TH level altering treatments. The hepatosomatic index was negatively affected by exogenous T4, but not by SP treatment indicating a lower lipid reserve in the liver in larvae of T4 treatment. These altered TH levels as caused by several environmental stressors may have influence on individual fitness across life since body condition at the onset of metamorphosis determines metamorphic and juvenile survival. Further research is needed to determine synergetic effects of environmental stressors on TH levels and its effects on physiological traits such as metabolic rate.

Short abstract

We studied the effects of two model thyroid hormone level affecting compounds, L-thyroxine (T4) and sodium perchlorate (SP), on various measures of development and body condition in larvae of the African clawed frog (*Xenopus laevis*). The scaled mass index, the hepatosomatic index, and the relative tail muscle mass were calculated as body condition indices to estimate fitness. Altered thyroid hormone levels reduced survival rate, altered growth and development and improved body condition of larvae at the onset of metamorphosis.

Key words

thyrotoxicity, fitness, amphibian decline, thyroid hormones, hepatosomatic index, L-thyroxine, scaled mass index, condition index, endocrine disruption, developmental plasticity

1. Introduction

Global change exposes wildlife to an array of chemical, physical, and biological stressors that arise largely from anthropogenic activity, but also from natural sources (Noyes et al., 2009). A wide range of these stressors have the ability to alter endocrine function in wildlife (Carr & Patino, 2011). Stressors which alter or disturb endocrine systems are characterized as endocrine disruptors (EDs) (Kloas & Lutz, 2006; Kloas et al., 2009). The impact of EDs in the environment is of special concern in amphibians, which are declining worldwide (Stuart et al., 2004; Hayes et al., 2006). Amphibians have highly permeable skin, which makes them particularly vulnerable to chemical contaminants (Hayes et al., 2006; Strong et al., 2017). Furthermore, especially larval amphibians are limited in their capacity for habitat selection (Sanzo & Hecnar, 2006; Yu et al., 2013), and are particularly sensitive to EDs due to their critical hormone-regulated development (Hayes et al., 2010; Searcy et al. 2015).

Amphibian metamorphosis is a crucial event in amphibian life history and is driven by several hormones, especially thyroid hormones (TH) (i.e. T3 and T4) (Shi, 2000; Bulaeva et al., 2015). Many EDs target the hypothalamus-pituitary-thyroid axis, which is required for production of THs (Carr & Patino, 2011). A large number of aquatic contaminants have been shown to disrupt the normal action of THs in amphibians, leading to changes in growth, development, and regulation of energy metabolism (Brown & Cai, 2007; Kashiwagi et al., 2009). Pesticides and herbicides, road salt, fertilizers, heavy metals, and active pharmaceutical ingredients are known to disrupt TH pathways and production resulting in decreased TH levels (Carr & Patino, 2011; Kashiwagi et al., 2009; Ortiz- Santaliestra & Sparling, 2007; Fort et al., 2017). Inhibition of TH production pathways results in decreased developmental rates (Carr et al., 2003; Bulaeva et al., 2015) and decelerated energy metabolism (Carr & Patino, 2011; Kashiwagi et al., 2009; Ortiz- Santaliestra & Sparling, 2007; Fort et al., 2017), with tadpoles metamorphosing at a larger size and older age (Shi, 2000).

An environmental relevant endocrine disruptor is perchlorate (ClO_4^-) which is a goitrogen that inhibits TH synthesis via competitive inhibition of the sodium-iodide symporter (Ortiz-Santaliestra & Sparling 2007). Because iodide is essential for the production of both T4 and T3, perchlorate may act as a disrupter of amphibian metamorphosis (Ortiz-Santaliestra & Sparling 2007). Perchlorate salts are strong oxidizers and are widely used as components of fireworks, airbags, and currently applied fertilizers (Trumpolt et al., 2005; Carr & Patino, 2011; Schmidt et al., 2012). Contamination of surface and ground water occurs from military,

aerospace, agriculture, and other commercial sources, but perchlorate also occurs naturally in arid places on the surface of the earth (Carr & Patino, 2011). Environmental concentrations of perchlorate measured in the field are high enough to inhibit amphibian metamorphosis (Goleman et al., 2002a, b; Ortiz-Santaliestra & Sparling, 2007; Tietge et al., 2005). In the United States concentrations from 3 µg to 30 mg × L^{-1} have been found in surface and ground waters (U.S. Environmental Protection Agency [USEPA], 2004; Carr & Patino, 2011).

Although most environmental contaminants inhibit TH activity or production pathways, some contaminants and other abiotic and biotic environmental stressors appear to enhance TH activity or increase TH levels by the activation of the neuroendocrine stress axis (Mann et al., 2009; Dantzer et al., 2014) and increase of stress hormone levels (Denver, 1997). These stress hormones may synergize with TH resulting in increased TH production (Glennemeier & Denver, 2002; Laudet, 2011; Kulkarni & Buchholz, 2012). Crowding (Ding et al., 2015), the presence of predators (Relyea, 2007), food quality and quantity (Courtney Jones et al., 2015), photoperiod (Laurila et al., 2001), temperature (Vences et al., 2002), and desiccation risk (Gervasi & Foufopoulos, 2008) may act as environmental stressors which activate the neuroendocrine stress axis resulting in increased TH levels. Therefore, the endocrine system allows for adaptation to stressful conditions resulting in the expression of developmental plasticity. Anuran larvae with high TH levels show increased developmental and metabolic rates and decreased growth rates (Rowe et al., 1998; Tata, 2006; Brown & Cai, 2007), which results in shorter larval periods, smaller size at the onset of metamorphosis and higher energetic maintenance costs (Denver, 1998, 2009; Orlofske & Hopkins, 2009). Exposing tadpoles to exogenous THs is an established method to simulate the proximate effects of environmental stressors on the TH system ultimately inducing developmental plasticity (Denver et al., 2002; Tata, 2006; Denver, 2009).

Since TH is not only critical for amphibian metamorphosis but also for the regulation of energy metabolism (Frieden, 1981; Sheridan, 1994; McNabb & King, 1993), altered TH levels as caused by several environmental stressors may also affect energetic body condition (i.e. maintenance costs and energy stores) at metamorphosis. Larval anurans fuel the energy required for metamorphosis by resorbing their tail muscle as well as by using stores of fat in the liver, a key organ for lipid storage in ectothermic animals (Sheridan & Kao, 1998; Jelodar & Fazli, 2012; Bouchard et al., 2015). The higher the energy metabolism, and thus, animals' maintenance costs, the less energy can be stored in tail and liver tissue (Sheridan & Kao, 1998; Orlofske & Hopkins, 2009). Anuran larvae with lower tail muscle and liver masses at

the onset of metamorphosis have lower energy reserves to cover developmental costs during the metamorphic climax and thus, lower individual fitness in later life stages (Berven, 1990; Bellakhal, 2003; Orlofske & Hopkins, 2009).

Whereas numerous studies demonstrated the effect of endocrine disruption on growth and development, only a few studies investigated the effect of altered TH levels as caused by environmental stressors on body condition and energy storages in larval amphibians to estimate fitness. We investigated age, size, survival, and body condition at the onset of metamorphosis in larvae of the African clawed frog (*Xenopus laevis*). Body condition was examined using the scaled mass index, the hepatosomatic index (i.e. relative liver mass), and relative mass of the tail muscle (rTMM). This combination of different condition indices provides insight into different aspects of energy mobilization and thus, energy storages during larval development. We assume that the alteration of TH levels by the environmentally relevant ED sodium perchlorate (SP; inhibitory) and by exposure to exogenous L-thyroxin (T4; stimulatory) alters growth, developmental and survival rate and body condition at metamorphosis in *X. laevis*.

7. Material and Methods

2.1 Experimental design and study species

Three clutches of *X. laevis* were obtained from the captive breeding facility of the Universitätsklinikum Hamburg Eppendorf (UKE, Martinistr. 52, 20246 Hamburg, Germany). Larvae were allowed to hatch and develop to developmental stage 25 (free-swimming larvae; Gosner 1960). From these larvae, 135 individuals originating from different families were intermixed before allocating them randomly to the different treatments (T4 and SP) and the control group. Fifteen larvae of *X. laevis* were kept each in a standard 9.5 L aquarium filled with 8 L of water (i.e., a total of nine aquaria: 3 × T4, 3 × SP, 3 × Control). The experiment was conducted in a climate chamber (Weiss Umwelttechnik GmbH, 35447 Reiskirchen, Germany) with a 12:12 h light:dark cycle at 19 ± 0.5 °C. The experiments ran for three weeks. All surviving larvae had reached the onset of metamorphosis at that time (Gosner, 1960). Amphibian larvae were fed high-protein flaked fish food (Sera micron breeding feed for fish and amphibians, Sera, 52518 Heinsberg, Germany) and spirulina algae *ad libitum*. This food was provided twice a day to guarantee that food was available in abundance. Furthermore, the amount of provided food was continuously adjusted during the entire experiment to control for differences in tadpole size and density between the aquaria since Miyata & Ose (2012)

indicated that a restricted feeding condition cause an atrophy of thyroid tissue similarly to TH agonists. The flakes were free of perchlorate according to the manufacturer. The aquaria were checked daily for dead or abnormal tadpoles, which were removed (Tietge et al., 2005).

Xenopus laevis was chosen as the study species (Fig. 1), because it is the best investigated amphibian species in terms of TH system and development (Buchholz, 2017), providing physiological background knowledge for the patterns investigated in this study. Although *X. laevis* is unusual among frogs because the adults remain primarily aquatic, results from the aquatic tadpole-stage are transferable to more typical frog species in which adults are terrestrial (Sullivan & Spence, 2003).

Fig. 1 Tadpole of the African clawed frog (*X. laevis*) with characteristic barbels lateral to the mouthpart.

2.2 T4 and sodium perchlorate exposures

We used the environmentally relevant concentration of 250 µg/L SP (Sodium perchlorate hydrate 99.99 % trace metals basis, 381225 Aldrich**,** Sigma-Aldrich, St. Louis, MO, USA) to achieve a decrease in TH levels in tadpoles. This selected concentration of SP is within environmental ranges measured in surface and ground waters of many industrial nations (Motzer, 2001; Tietge et al., 2005; Carr & Theodorakis, 2006; Mukhi & Patino, 2007) and in

bodies of water in which amphibians breed (Smith et al., 2001; Ortiz-Santaliestra & Sparling, 2007).

We achieved increased TH levels by exposing tadpoles to 10 µg/L exogenous T4 (Thyroxine T4, IRMM468 Sigma-Aldrich**,** Sigma-Aldrich, St. Louis, MO, USA), a concentration which is known to influence amphibian metamorphosis (Lucas & Reynolds 1967; Mann et al., 2009) and is related to increases in T4 observed in tadpoles responding to stress (Denver, 1997; 1998). Tadpoles absorb exogenous T4 directly through their permeable skin (Shi, 2000; Tata, 2006; Coady et al., 2010).

T4 and SP treatments were prepared in 0.1 N sodium hydroxide solutions (Sodium hydroxide solution 0.1 N, S2770 SIGMA, Sigma-Aldrich, St. Louis, MO, USA) buffered with 0.1 N muriatic acid solutions as solvents. Solutions were added to the aquaria. A clean solution of 0.1 M sodium hydroxide solution buffered with 0.1 M muriatic acid solution was added to the control aquaria to control for any effect of solvents addition. Each treatment and the control set-up was replicated three times (i.e. 45 larvae, 15 larvae per aquarium, per treatment and control in total). Water was changed every second day and fresh SP and T4 were added, which is frequent enough to maintain a constant hormone and perchlorate level, in accordance with the standard procedure for chemical and hormonal addition (Miwa & Inui, 1987; Goleman et al., 2002 a, b; Iwamuro et al., 2003; Rot-Nikcevic & Wassersug, 2004; Tietge et al. 2005; Ortiz-Santaliestra & Sparling, 2007; Bulaeva et al., 2015).

2.3 Processing of specimens

Developmental stage was determined by evaluating the status of key morphological features typical of specific developmental stages, as detailed in Gosner (1960). The developmental stage of each tadpole was recorded according to the procedure of Ortiz-Santaliestra & Sparling (2007). The age describes the larval duration in days after hatching to the onset of metamorphosis. Onset of metamorphosis was defined by the emergence of at least one forelimb (Gosner stage 42; Gosner, 1960). The snout vent length (SVL) and total length of the larvae were measured with a caliper to the nearest 0.5 mm. Larvae were weighed to the nearest 0.001 g with an electronic balance (digital gold scale, Smart Weigh). At the end of the experiment tadpoles were euthanized with 200 mg/L of tricaine methanesulfonate ([MS-222], Ethyl 3-aminobenzoate methanesulfonate, E10521 ALDRICH**,** Sigma-Aldrich, St. Louis, MO, USA) buffered with 200 mg/L of sodium bicarbonate (Sodium bicarbonate, S5761 SIGMA**,** Sigma-Aldrich, St. Louis, MO, USA) (Stuart et al. 2007) and transferred into

ethanol (70 %) for liver and tail muscle dissections. The ethanol-preserved specimens were rehydrated in a decreasing ethanol series (70, 50, 30 %, and water) in order to achieve their original wet weight.

Liver and tail muscle dissections were made using digital stereo-microscope (Keyence VHX-500F) (Fig. 2). Livers were dabbed and weighed with an electronic balance (digital gold scale, Smart Weigh) and dried for further analysis. After tail muscle dissection, fins were removed and tail muscle was dabbed and weighed.

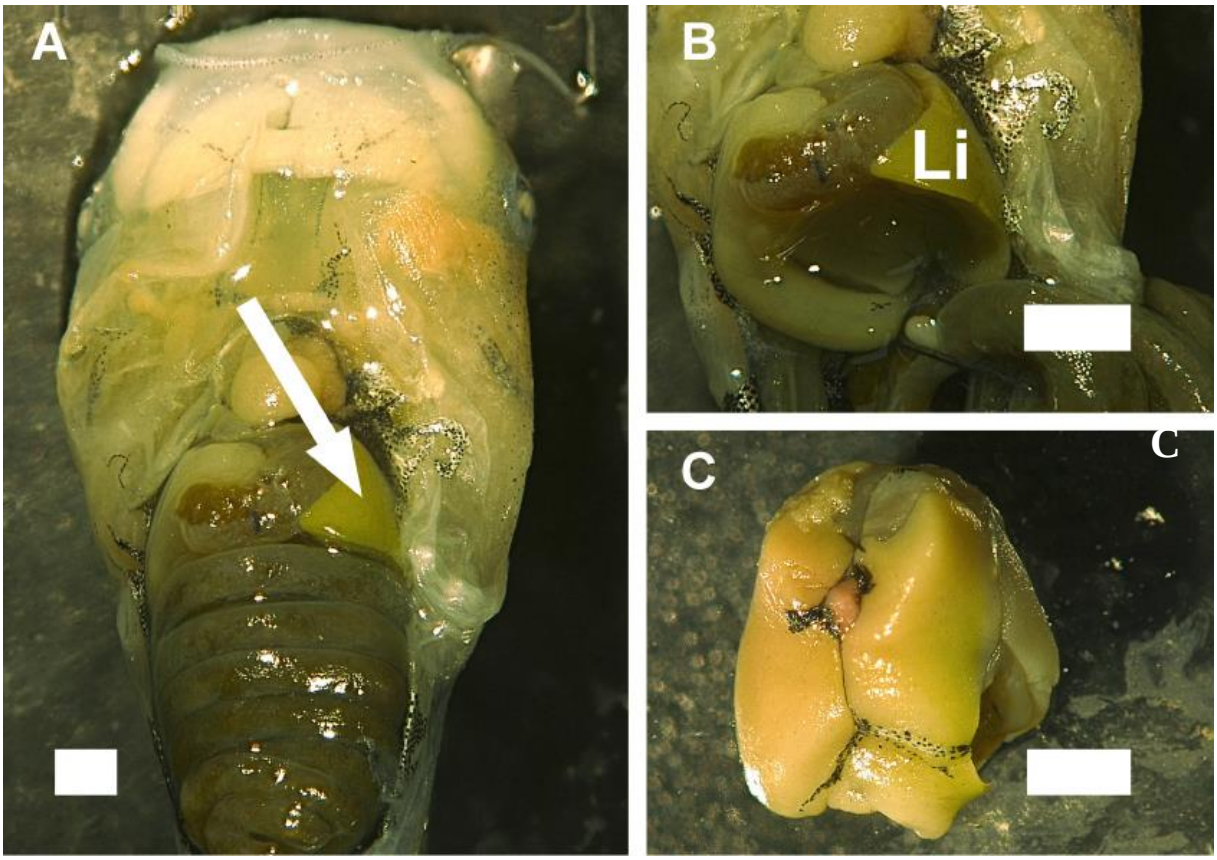

Fig. 2 Liver dissection of a *X. laevis* larvae from sodium perchlorate treatment at Gosner stage 42 (forelimb emergence) using the digital binocular Keyence VHX-500F. White square length: 1.00 mm. **A** Abdominal cavity with 20x magnification. Arrow: liver. **B** Abdominal cavity with dissected spiral gut. Li: liver with 30x magnification. **C** Dissected liver with 30x magnification.

2.4 Condition indices

We estimated the body condition (i.e. energy stores) at the onset of metamorphosis by calculating a combination of three different condition indices. The scaled mass index (SMI) is a measure of the entire body condition of an individual as it accounts for the allometric

relationship between mass and a body structure measure and standardizes each measure so that direct comparisons among individuals can be made (Peig & Green, 2009, 2010; MacCracken & Stebbings, 2012). The SMI was considered as an accurate condition index in anuran larvae (MacCracken & Stebbings, 2012; Dittrich et al., 2016). A high SMI suggests larger energy storages and thus, a good body condition. We followed the procedure outlined by Peig and Green (2009) to calculate the SMI for each individual.

The hepatosomatic index (HSI) describes the status of energy stored in the liver of animals, which is a good indicator of recent fat storage in the animal (Htun-Han, 1978; Bolger & Connolly, 1989). A decrease in HSI suggests the mobilization of the liver reserves toward the metabolic requirements (Jelodar & Fazli, 2012). The HSI was calculated according to the method of Htun-Han (1978):

$$HSI = \frac{\text{liver wet weight}}{\text{whole body wet weight}}\ x\ 100$$ (Htun-Han, 1978; Jelodar & Fazli, 2012).

The relative tail muscle mass (rTMM) was used as an index for energy condition in anuran larvae as tails are major sites of fat storage in many species (Sheridan & Kao, 1998; Scott et al., 2007) and probably provide a source of protein energy for tadpoles during metamorphosis (Hourdry et al., 1996; MacCracken & Stebbings, 2012). rTMM was calculated from absolute wet tail muscle mass as a percentage from total wet body mass.

2.5 Statistical analyses

For all statistical tests R 3.4.1 (R Development Core Team, 2007) for Windows was used. Before the analysis, all dependent variables in the models were tested for possible correlations using Spearman's rank correlation (cor.test function). Subsequently, variables were included in statistical analysis when the correlation was significant but well below the suggested threshold of 0.7 for eliminating variables (Fielding & Haworth, 1995; Chin, 1998) or not significant (Table S1).

Data were analyzed using linear mixed-effect models [lmer function, lme4 package, vers. 1.1-16, Type III model, covariance type: variance components, REML (restricted maximum likelihood) method for parameter estimation, 100 iterations (Bates & Sarkar, 2007)], entering 'Treatment' (T4, SP, and control) as fixed factor. 'Size at metamorphosis' (as measured by SVL and body mass), 'age at metamorphosis' (as measured by age in days after hatching), 'body condition' (as measured by SMI, rTMM, and HSI), and 'survival' were used as dependent variables in separate models (Table 1). P-Values were obtained from likelihood-

ratio tests, which compared the models with the respective null-model (Crawley, 2007). To address dependencies in the data, the variable 'aquarium' was included as a random factor. Residuals of each model were visually checked for normal distribution. N refers to the total number of individual analyzed tadpoles. Linear mixed-effect models were followed by post hoc comparisons (Tukey's test; Tukey HSD function, multcomp package, vers. 1.2-13) to compare all possible pairwise combinations of treatments when overall tests were significant (Table 1).

8. Results

We only calculated a model for one size measure (SVL) as the variables SVL and TL were highly correlated (Table S1). Body mass and SVL were correlated as well, but were included in the analysis for better comparisons with related studies.

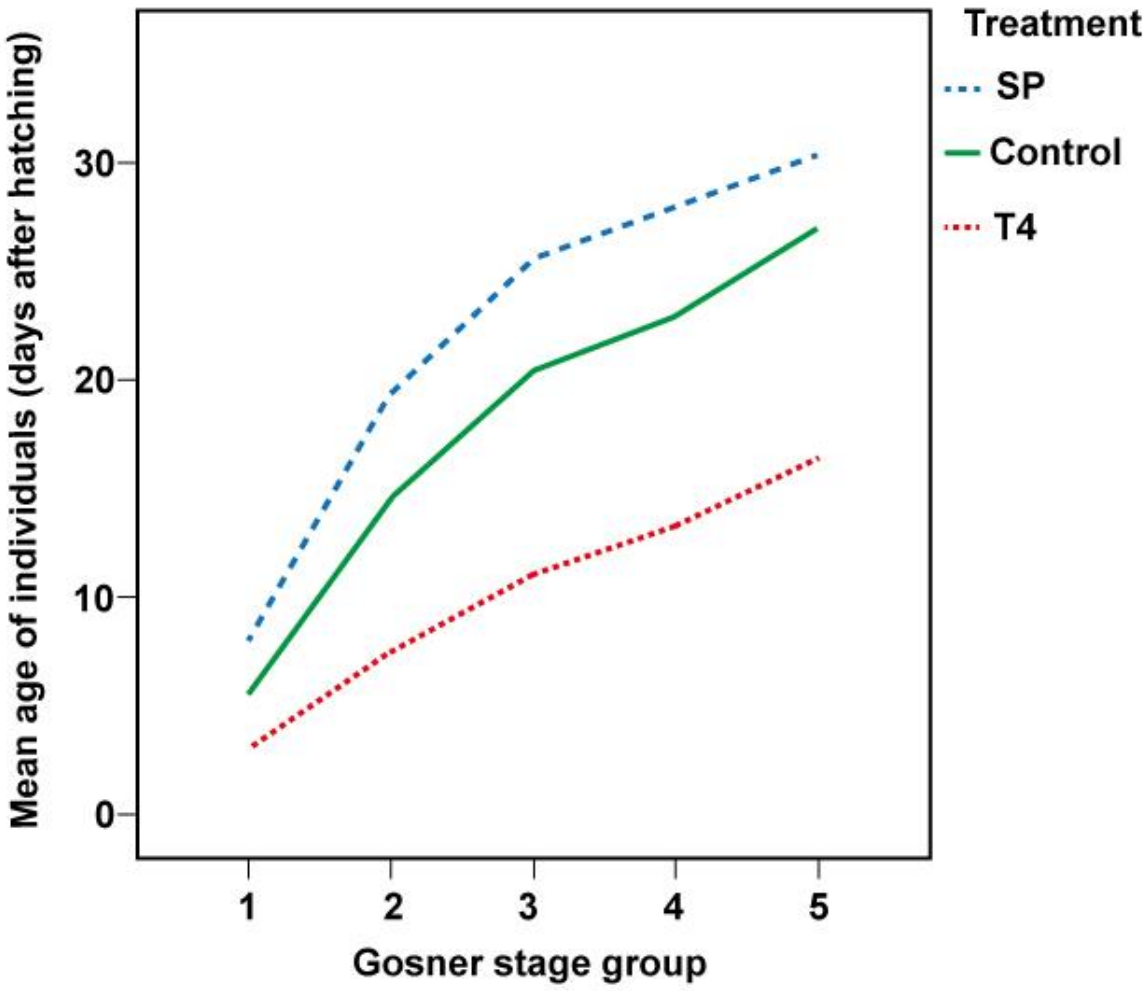

Fig. 3 Treatment effects ('Control', 'SP', and 'T4') on mean age in tadpoles of the African clawed frog (X. laevis) at Gosner stage group **1-5**: **1.** pre-limb (absence of hind limbs, Gosner stages 24 to 26), **2.** limb bud (hind limb visible, but no clear joint formed, Gosner stages 27 to 34), **3.** middle hind limb

(knee joint apparent, but toes not completely separated, Gosner stages 35 to 37), **4.** late hind limbs (hind limb tubercles and subarticular patches formed, Gosner stages 38 to 41), and **5.** metamorph (at least one forelimb present, Gosner stage 42) (Gosner, 1960; Ortiz-Santaliestra & Sparling 2007). Control = solid line; SP = dashed line; T4 = dotted line. Mean age at Gosner stage group was calculated from mean of summed up mean age at every Gosner stage within one Gosner group.

Survival from the start of the experiments (Gosner stage 25) to onset of metamorphosis (Gosner stage 42) in the treatment groups was: control: 86.7 ± 6.7 %; SP: 60.0 ± 6.7 % and T4: 68.9 ± 4.4 %. Therefore, altered TH levels in both treatments led to significantly reduced survival as compared to control treatments (Table 1). There was no variability in the age at metamorphosis and thus, length of the larval period. All tadpoles of one treatment group entered metamorphosis on the same day, 25 days after hatching in the control, 30 days in the SP treatment, and 16 days in the T4 treatment. Thus, the larval period of the larvae in the T4 treatment was only half as long as that of the SP treatment, and 2/3 of that of the T4 treatment (Fig. 3). Development was delayed in the SP individuals compared to controls during developmental period. T4 individuals showed a faster development compared to controls.

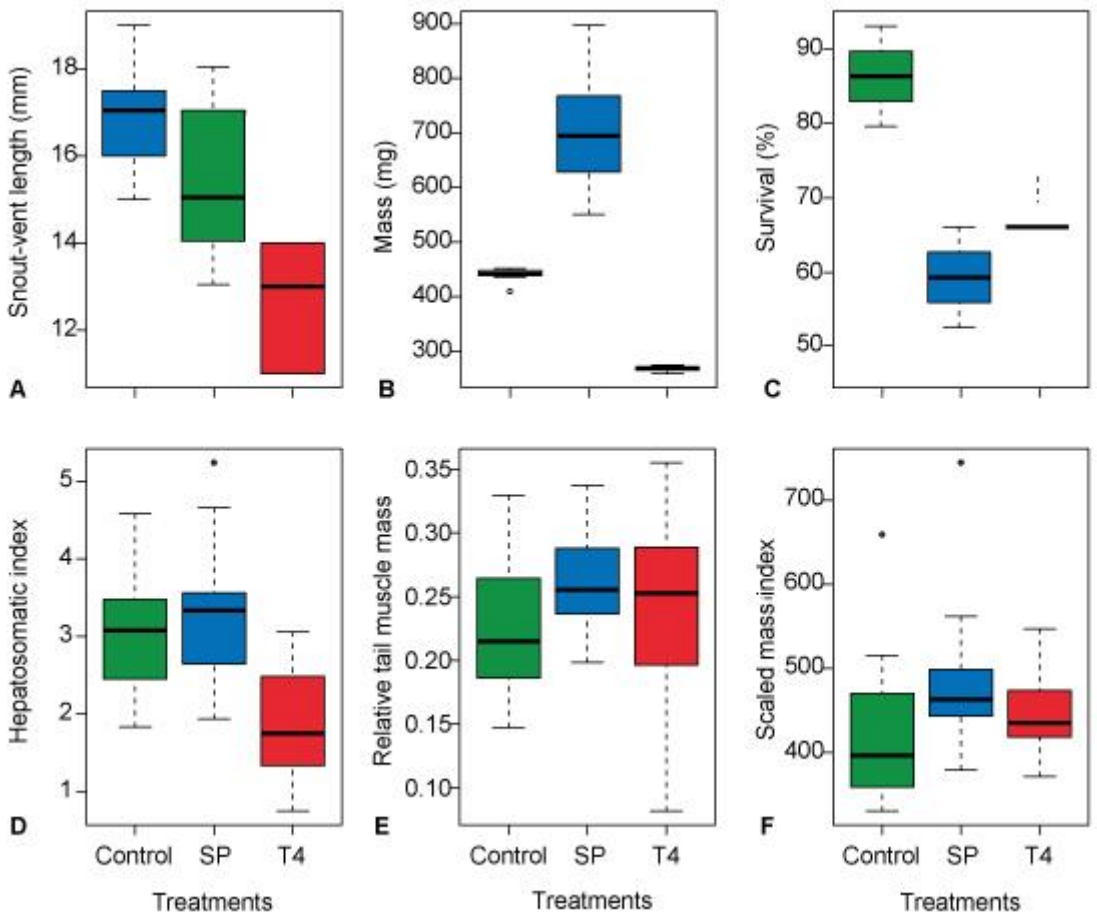

Fig. 4 Effects of altered TH levels on metamorphic traits (**A-B**), survival rate (**C**), and body condition (**D-F**) in tadpoles of the African clawed frog (*X. laevis*) at the onset of metamorphosis. Boxplot characteristics: Error bar = median. Box = 1. and 3. quartiles. Dots = outliers, minimum and maximum values. Whiskers = 1.5-fold interquartile range.

Table 1 Effects of altered TH levels on metamorphic traits, survival, and body condition in larvae of the African clawed frog (X. laevis) at the onset of metamorphosis (at least one forelimb present, Gosner stage 42) (Gosner, 1960). Chi² and P for linear mixed-effects models (LMM), using 'treatment' (Control, SP, T4) as the fixed factor; 'aquarium' as the random factor. N is the total number of analyzed individual animals, and n is the total number of tested aquaria. Pairwise multiple comparisons were made using Tukey's Test as post hoc test. Significance was set at $P < 0.05$.

Dependent variable	**LMM**							**Tukey's Test (pairwise comparisons)**								
	Estimate			**Chi²**	**df**	**P**	**N (n)**	**Control - SP**			**SP - T4**			**Control - T4**		
	Control	**SP**	**T4**					**Estimate**	**z**	**P**	**Estimate**	**z**	**P**	**Estimate**	**z**	**P**
Snout-vent length (mm)	15.30	16.8	12.55	32.8	2	< **0.001**	97 (9)	1.50	4.23	< **0.001**	-4.26	-11.39	< **0.001**	-2.75	-8.06	< **0.001**
Body mass (mg)	442.60	696.06	267.86	42.79	2	< **0.001**	97 (9)	2.35	15.88	< **0.001**	-428.20	-26.04	< **0.001**	-174.74	-11.21	< **0.001**
Survival (%)	86.66	60	68.89	16.61	2	< **0.001**	(9)	-26.67	-5.55	< **0.001**	8.89	1.85	0.15	-17.78	-3.70	< **0.001**
Scaled mass index	417.34	477.02	441.86	12.45	2	**0.002**	97 (9)	59.68	3.77	< **0.001**	24.52	1.61	0.24	-35.16	-2.11	0.08
Hepatosomatic index	3.00	3.19	1.90	24.15	2	< **0.001**	95 (9)	0.19	1.07	0.52	-1.29	-6.82	< **0.001**	-1.10	-6.34	< **0.001**
Relative tail muscle mass	0.22	0.25	0.23	5.01	2	0.08	97 (9)	-	-	-	-	-	-	-	-	-

There were significant treatment effects on SVL, body mass, SMI, and HSI, but not on rTMM (Table 1). Thus, altered TH levels have an effect on metamorphic traits, entire body condition and especially on lipid reserves in the liver. Larvae exposed to SP were significantly the largest, heaviest, and oldest animals at the onset of metamorphosis compared to control group and T4 animals (Table 1) (Fig. 4). SMI was significantly highest in SP animals compared to control animals. The T4 treated larvae differed significantly in HSI as compared to the SP

treated and control animals. None of the larvae showed any significant difference related to rTMM. Consequently, altered TH levels as caused by environmental stressors affected body condition and thus, energy storages in the larvae at the onset of metamorphosis to different extent (Table 1) (Fig. 4).

9. Discussion

Amphibians play an important role in both aquatic and terrestrial ecosystems, and are currently the most threatened group among all vertebrates (Stuart et al., 2004). Therefore, it is essential to understand how different environmental stressors affect amphibian metamorphosis and body condition by altering TH levels (Cary Coyle & Karasov, 2009). In this study, we demonstrate that in- or decreased TH levels as caused by different environmental stressors affect metamorphic traits and body condition in tadpoles of *X. laevis* in different ways. When tadpoles were exposed to the environmentally relevant endocrine disruptor SP, they revealed the best body condition and the largest size at but also the longest larval duration until the onset of metamorphosis. In contrast, tadpoles exposed to exogenous T4 (i.e. simulating the proximate effect of environmental stressors inducing the expression of developmental plasticity) revealed smaller energy reserves in the liver and the smallest size at metamorphosis, which they reached after the shortest larval duration. In both instances of altered TH levels, survival rate was substantially reduced.

4.1 Alteration of TH level affects metamorphic traits and reduces survival

Altered TH levels affected growth and developmental rates in various ways. Because the metamorphic process in amphibians is controlled primarily by the thyroid system, any alteration of growth and developmental rates suggests an impact on the thyroid system homeostasis (Sowers et al., 2009). However, the tested compounds may assert their effects via several pathways also influencing growth, development, body condition, and especially survival rate. Nevertheless, the thyroid axis is arguably the most crucial one influencing energetics and the expression of developmental plasticity. Differences in growth and developmental rate between the treatments occurred already early in larval development indicating that alteration of TH levels proceeds with immediate effect from the beginning of the exposure to SP and T4.

When aquatic contaminants such as SP affect the TH system, endocrine disruption is usually inhibitory as also confirmed in the present study (Calow, 1990; Fleeger et al., 2003; Mann et al., 2009). Beside SP as endocrine disruptor, Karaoglu & Kutrup (2010) could show the effect

of environmentally relevant concentrations of ammonium nitrate fertilizer on metamorphic traits in tadpoles of the marsh frog (*Pelophylax ridibundus*). Cary Coyle & Karasov (2009) showed the inhibiting effect of polybrominated diphenyl ethers on anuran metamorphosis. Consequently, contaminated breeding ponds may lead to large but old metamorphs. Although a large size at the onset of metamorphosis is associated with higher individual fitness in later life stages (Berven, 1990), longer larval durations are concomitant with increased pressures by predators in the aquatic habitat and desiccation risk (Lefcort et al., 1998; Kloas & Lutz, 2006).

Besides aquatic contamination, changes in abiotic and biotic factors are known to accelerate developmental rate through increased TH levels mediated by stress hormones (Denver, 1997; Gervasi & Foufopoulos, 2008; Dantzer et al., 2014). We simulated the stimulatory effect of such environmental stressors on the TH system by exposing tadpoles to exogenous T4 (Mann et al., 2009; Coady et al., 2010). Smaller metamorphs are known to have lower fitness since they are older at first reproduction and have smaller clutch sizes (Semlitsch et al., 1988; Berven, 1990; Edwards et al., 2006). Therefore, increased TH levels as caused by environmental stressors may lead to early metamorphosed juveniles, which are likely to be undersized compared to non-stressed conspecifics (Cauble & Wagner, 2005; Mann et al., 2009). However, for species which stay aquatic both as juveniles and adults, such as *X. laevis*, metamorphosing earlier at a smaller size may be favorable in terms of avoiding predators specialized on tadpoles.

Larvae exposed to T4 and SP exhibited reduced survival rates until the onset of metamorphosis indicating that any alteration of the TH level negatively affects survival. However, alterations in survival rate could also be indicative of general toxicity of both chemicals. Given the lower survival of tadpoles exposed to T4 and SP relative to the controls, it seems that the results obtained could have reflected a general toxicity, rather than an alteration in the thyroid system. However, the OECD (2007) reported the results of interlaboratory survey among five international laboratories in which exposure studies for three weeks were conducted for SP at 62.5, 125, 250 and 500 µg/L as nominal. At 500 µg/L SP (measured concentrations were ranged within 440-600 µg/L), no mortality was observed in four studies and mortality of 4 % was reported from one laboratory. These results suggest that a concentration of 250 µg/L might be too low for causing 40 % mortality in the present study. Therefore, the cause of the mortalities at 250 µg/L was not only the direct toxicity of

SP. Measures of TH, thyroid histology or TH related gene expression would help confirm that thyroid disruption did indeed occur in the exposed organisms.

4.2 Energy storages and body condition

Since THs are the major triggers of energy metabolism and are positively correlated with metabolic rate (McNabb & King, 1993; Rowe et al., 1998; Burraco & Gomez-Mestre, 2016) and thus, maintenance costs (Orlofske & Hopkins, 2009). Larvae with altered TH levels as caused by environmental stressors may differ in energy storages and thus, body condition at the onset of metamorphosis. In this study, we used a combination of three different condition indices to provide a complex insight into different energy storage organs (i.e. liver, tail muscle, and entire energy storages) and thus, to generate an overall pattern of how altered TH levels influence body condition in larval amphibians. Body condition as measured by SMI was higher in larvae exposed to SP and T4 suggesting that length growth and mass increase seem to be equally affected by the metamorphic and metabolic effects of both TH level altering compounds. However, higher body conditions do not generally indicate that altered TH levels may not affect the ability to fuel energy storages. Individuals in bad condition may be missing due to a possible toxic effect of both compounds.

As high levels of TH increase energetic maintenance costs, larvae may require more energy to fuel their increased metabolism and less energy remains to be accumulated in energy storages until metamorphosis (Dupre et al., 1986; Schmidt et al., 2012). Contrary to these expectations, body condition of larvae exposed to T4 was not reduced but larvae revealed smaller fat storages in the liver, probably due to the increasing effect of TH on maintenance costs. Otherwise, changes in HSI can be indicative of alterations in hepatic lipid mobilization, which can also be indicative of alterations in the metabolic activity (specifically associated with xenobiotic metabolism) of the liver or liver pathologies, both of which can occur in response to chemical exposures, or general toxicity associated with both compounds. Zaya et al. (2011) demonstrated that endocrine disruption induced by atrazine lead to a reduced liver weight in tadpoles of *X. laevis* but did not affect lipid stores in liver and fat body. Further studies are needed to investigate liver metabolism in tadpoles exposed to different environmental stressors.

Despite the liver as a storage organ for fat, the tail muscle is known as the main fat and protein storage, as it is resorbed during metamorphosis when anuran larvae stop eating due to tissue reorganization (Hourdry et al., 1996; Shi, 2000). In this study, altered TH levels did not

affect the tail muscle mass and thus, the size of fat and protein storages in *X. laevis*. In contrast, Yu et al. (2013) demonstrated the effect of the endocrine disrupter chlorothalonil (a broad spectrum agricultural fungicide) on tail length (tail degeneration) in larvae of the African clawed frog (*X. laevis*) and the New Mexico spadefoot toad (*Spea multiplicata*). Our results are not in agreement with those of Yu et al. (2013), which may be explained in two different ways: Either, only the lipid storages are affected by the calorigenic effect of TH or xenobiotic metabolism of the liver and not the storage of lipids and amino acids in muscle tissue, which comprises most of the tail tissue in anuran larvae. Or, the energy requirement from tail tissue starts to differ between treatments at later developmental stages, after metamorphosis has set in. At these later developmental stages (Gosner stage > 42) aquatic contaminants, such as chlorothalonil and perchlorate, may possibly interfere with pathways that trigger tail resorption by altering expression of genes involved in tail cell death as is occurs at tail absorption during metamorphosis (Yu et al., 2013).

4.3 Ecological significance

In this study, we demonstrated that altered TH levels affect body condition, but impairs survival rate and results in either large but old or young but small metamorphs which are both known to be disadvantaged in comparison with conspecifics developing in undisturbed larval habitats. Both a prolonged remaining in larval development and a small size at metamorphosis are known to reduce fitness and may outweigh the advantage of an increased body condition. As environmental stressors in a larval habitat may influence the reproductive outcome of a whole population, altered TH levels as caused by environmental stressors may subsequently lead to declining amphibian populations.

10. Conclusions

In the near future, the combination of naturally and anthropogenically induced environmental stressors will occur in natural habitats of amphibian populations more frequently and all environmental stressors will affect larval anurans by their effect on the TH system and other pathways. In case of chemical stressors, tadpoles may additionally suffer from possible toxic effects. Despite the impact on metamorphic traits, we could demonstrate that altered TH levels as caused by aquatic contaminants and environmental stressors reduces survival and affects entire body condition and energy storages probably due to the metabolic function of THs. Future studies should focus on the combined effects that different environmental stressors have on energy storages and body condition. Moreover, stress experienced during

early life stages may have long-lasting effects in later life stages and thus, on the survival of amphibian populations. Therefore, long-term studies are needed to completely understand the consequences of altered TH levels as caused by environmental stressors during the larval stages on the phenotype and fitness of the adults.

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13. Supporting information

Table S1. Spearman's rank correlation of dependent variables. N = 97 (95 for hepatosomatic index). Regular: Coefficient of correlation (ρ). Italic: P- values. Bold: Significant high correlations.

	snout-vent length (mm)	total length (mm)	body mass (mg)	hepatosomatic index	scaled mass index	relative tail muscle mass	survival (%)
Snout-vent length (mm)	-	**0.70**	0.67	0.09	0.48	0.50	0.56
Total length (mm)	*<0.001*	-	**0.85**	0.63	0.26	0.49	0.39
Body mass (mg)	*<0.001*	*<0.001*	-	0.09	0.28	0.45	0.48
Hepatosomatic index	*0.031*	*0.014*	*0.042*	-	0.48	0.53	0.23
Scaled mass index	*<0.001*	*0.002*	*0.002*	*<0.001*	-	0.43	0.21
Relative tail muscle mass	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.006*	-	0.21
Survival (%)	*0.021*	*0.052*	*0.008*	*0.002*	*0.041*	*0.021*	-

Author contribution

I hereby confirm that Katharina Ruthsatz conceived, designed and performed the experiments, analyzed the data and wrote the paper.

Hamburg, 18.10.2018

Prof. Dr. Kathrin Dausmann

Chapter Six

Environmental stress alters costs of amphibian metamorphosis in the common frog (*Rana temporaria*)

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Abstract

Global change exposes wildlife to an array of environmental stressors that arise from both anthropogenic and natural sources. Many environmental stressors have the ability to alter endocrine function and thus, cause physiological consequences to wildlife. Anuran larvae experiencing environmental stress may display altered thyroid hormone (TH) status with consequences for energy partitioning among development, growth, and metabolism during amphibian metamorphosis. In this study, we investigated how the alteration of TH levels due to exposure to the environmentally relevant endocrine disruptor sodium perchlorate (inhibitory) and exogenous L-thyroxin (T4; stimulatory) affects energy costs and allocation during and after metamorphosis in a common amphibian (*R. temporaria).* Tadpoles exposed to T4 allocated 24 % less energy to development during metamorphic climax. Therefore, the energy available for metamorphosis was reduced in tadpoles experiencing stressful conditions. Differences in size and energetics persisted beyond the metamorphic boundary and impacted on juvenile performance. We could also demonstrate the poorest performance in T4 animals. Consequently, altered TH levels as caused by environmental stressors lead to persisting effects on metamorphic traits and energetics and, thus, caused carry-over effects on performance of froglets. We demonstrate the mechanisms through which climate change and other anthropogenic disturbance of larval habitats can altered phenotypes at metamorphosis and reduce lifetime fitness in these and likely other amphibians.

Key words

Metabolic rate, L-thyroxine, body condition, physiological plasticity, endocrine disruption, carry-over effects, neuro-endocrine stress axis, energetic costs

1. Introduction

Environmental change exposes wildlife to an array of environmental stressors that arise from anthropogenic activities (e.g. climate change, pollution) as well as natural sources (Noyes et al., 2009). Many environmental stressors have the ability to alter endocrine function with physiological consequences to wildlife (Carr and Patino, 2011). In most cases, activation of the neuroendocrine stress axis (Mann et al., 2009; Dantzer et al., 2014) increases stress hormone levels (Denver, 1997). These stress hormones also target the hypothalamus-pituitary-thyroid axis, which is responsible for production of thyroid hormones (TH) (Carr and Patino, 2011), and may synergize with THs resulting in increased TH production (Glennemeier and Denver, 2002; Laudet, 2011; Kulkarni and Buchholz, 2012).

The impact of environmental stress on the hypothalamus-pituitary-thyroid axis is of special concern for amphibians as their metamorphosis is mainly regulated by thyroid hormones (i.e. T3 and T4) (Tata, 2006; Furlow and Neff, 2006) which increase in concentration during this process and determine the developmental rate (Brown and Cai, 2007; Shi, 2000). The presence of predators (Relyea, 2002; Capellán and Nicieza, 2007), crowding (Morey and Reznick, 2001), desiccation risk (Gervasi and Foufopoulos, 2008), food scarcity (Kupferberg, 1997), and temperature (Smith-Gill and Berven, 1979; reviewed in Ruthsatz et al., 2018a) are known to increase TH production by activating the neuroendocrine stress axis. Anuran larvae with high TH levels display increased developmental and metabolic rates and decreased growth rates (Rowe et al., 1998; Brown and Cai, 2007), which results in shorter larval periods, smaller size at the onset of metamorphosis and higher energetic maintenance and developmental costs (Denver, 1998, 2009; Orlofske and Hopkins, 2009). Exposing tadpoles to exogenous THs is an established method to simulate the proximate effects of environmental stressors on the TH system (Denver et al., 2002; Tata, 2006; Denver, 2009).

Whereas most environmental stressors lead to increased TH activity or production by the activation of stress hormones, a large number of aquatic contaminants such as pesticides and herbicides, road salt, fertilizers, heavy metals, and active pharmaceutical ingredients have been shown to disrupt and inhibit the normal action of THs in amphibians, leading to changes in growth, development, and metabolism (Kashiwagi et al., 2009; Carr and Patino, 2011). Inhibition or a decrease of TH production pathways slows the rate of development (Carr et al., 2003; Bulaeva et al., 2015) and decreases metabolic rates (Carr and Patino, 2011; Ortiz-Santaliestra and Sparling, 2007) causing tadpoles to metamorphose at a larger size and older age (Shi, 2000). (Chemical) stressors which alter or disturb endocrine systems are

characterized as endocrine disruptors (Kloas and Lutz, 2006; Kloas et al., 2009). Larval stages of amphibians are particularly vulnerable to endocrine disruptors due to the inability of this life stage to select or avoid habitats (Sanzo and Hecnar, 2006; Yu et al., 2013) and their increased risk of exposure due to their highly permeable skin (Hayes et al., 2006; Strong et al., 2017). An environmentally relevant endocrine disruptor is perchlorate (ClO_4^-) which is a goitrogen that inhibits TH synthesis (Ortiz-Santaliestra and Sparling, 2007). Concentrations of perchlorate measured in the field are often high enough to inhibit amphibian metamorphosis (Goleman et al., 2002; Ortiz-Santaliestra and Sparling, 2007; Tietge et al., 2005).

Since TH is not only critical for amphibian metamorphosis but also for the regulation of metabolic processes (Frieden, 1981; Sheridan, 1994; McNabb and King, 1993), altered TH levels as caused by environmental stressors may also affect energy budgeting (i.e. metabolic rate and energy stores) at metamorphosis and during metamorphic climax (Sheridan, 1994; Orlofske and Hopkins, 2009; Choi et al., 2017). THs are known to increase the standard metabolic rate (SMR) which represents the energy required to cover basic physiological functions (i.e. maintenance costs) (Rowe et al., 1998; Beck and Congdon, 2003, Orlofske and Hopkins, 2009). In larval amphibians, increased metabolic rate and maintenance costs constrain the amount of energy that can be stored in the tail and liver tissue to fuel metamorphosis and, hence, less energy can be allocated towards paying costs of development incurred during metamorphic climax (Sheridan and Kao, 1998; Orlofske and Hopkins, 2009).

During metamorphic climax several larval tissues degenerate (e.g. gills and tail), are rebuilt (e.g. gastro-intestinal tract, brain, and the liver) or regenerated (e.g lungs and limbs) (Shi, 2000; Tata, 2006). Due to the rebuilding of the gastro-intestinal tract accompanied with changes in oral morphology, anuran larvae must rely on stored energy (Beck and Congdon 2003, Orlofske and Hopkins, 2009). Tadpoles, which have larger energy reserves and a low metabolic rate at the onset of metamorphosis, are more likely to successfully complete metamorphosis and become juvenile froglets with larger energy stores and higher probabilities of survival (Orlofske and Hopkins, 2009). Therefore, energetics (i.e. SMR and size of energy stores) at the onset and after completion of metamorphosis are important fitness variables (Steyermark et al., 2005; Muir et al., 2014, Ruthsatz et al., 2018b).

Numerous studies have characterized metabolism during larval stages, before and during metamorphic climax (Sivula et al., 1972; reviewed in Beck and Congdon, 2003; Ruthsatz et al. 2018c) and few studies quantified energetics across life history stages (Pandian and Marian, 1985, Beck and Congdon, 2003, Orlofske and Hopkins, 2009, Orlofske et al., 2017).

Only one previous study has quantified the energetic costs of metamorphosis as well as the entirety of larval development using an experimental approach for testing the effect of exogenous stress hormones (Kirschman et al., 2017). However, no study to date has investigated the impact of altered TH levels as caused by environmental stress on growth, development, and energetics (i.e. metabolic rate and body condition) during metamorphosis, after successful completion of metamorphosis, and in early juvenile froglets. Furthermore, there is no knowledge on how environmental stress may affect energy portioning between growth, development, and metabolism during metamorphic climax. As altered TH levels impact physiological processes, proximate effects of environmental stress may therefore affect energy allocation for development and growth during metamorphic climax resulting in serious effects for later life stages.

The purpose of this study was to investigate the impact of altered TH levels as caused by environmental stressors on energy allocation for growth, development, and energetics at the onset of metamorphosis, during metamorphic climax and after successful completion of metamorphosis in the common frog (*Rana temporaria*). Furthermore, we estimated fitness of juvenile froglets by examining whether an altered TH status experienced during the larval stage affects energetics and performance in later life stages. This study provides a framework for quantifying how environmental stressors impact amphibian metamorphosis allowing more robust projections of how stressful environmental conditions may affect across-life stage survival and fitness in the future.

2. Material and Methods

2.1 Study species and experimental design

Rana temporaria was chosen as the model species because it represents the typical amphibian life history and it is widely distributed throughout Europe. Five clutches of *R. temporaria* were obtained from Waldpark Marienhöhe in western Hamburg, Germany (53°34'37.4"N 9°46'57.5"E). Larvae were allowed to hatch and develop to stage 25 (free-swimming larvae; Gosner, 1960). From these larvae, 180 individuals originating from different families were intermixed before allocating them randomly to the different treatments (T4 and SP) and the control group. Fifteen larvae of *R. temporaria* were kept each in a standard 9.5-L aquarium filled with 8 L of water (i.e., a total of twelve aquaria: 4 × T4, 4 × SP, 4 × Control). The experiment was conducted in a climate chamber (Weiss Umwelttechnik GmbH, 35447 Reiskirchen, Germany) with a 12:12 h light:dark cycle at 22 ± 0.1 °C. The experiment was conducted over five weeks. All surviving larvae had reached the end of metamorphic climax

and seven days after completion of metamorphic climax (Gosner, 1960). Amphibian larvae were fed high-protein flaked fish food (Sera micron breeding feed for fish and amphibians, Sera, 52518 Heinsberg, Germany) and spirulina algae. *Ad libitum* rations were provided twice a day to guarantee that food was available in abundance. The size of the rations was continuously adjusted to account for changes in the size of tadpoles and the number of individuals in each aquarium since Miyata and Ose (2012) indicated that restricted feeding conditions cause an atrophy of thyroid tissue in a similar manner as TH agonists. The flakes were free of perchlorate according to the manufacturer. Each day, any dead or abnormal tadpoles were removed from the aquaria (Tietge et al., 2005).

2.2 T4 and sodium perchlorate exposures

We used a concentration of 250 µg/L sodium perchlorate hydrate (SP, 99.99% trace metals basis, 381225 Aldrich, Sigma-Aldrich, St. Louis, USA) to decrease TH levels. This concentration is within environmental ranges measured in surface and ground waters of many industrial nations (Motzer, 2001; Tietge et al., 2005; Carr and Theodorakis, 2006; Mukhi and Patino, 2007) and in bodies of water in which amphibians breed (Smith et al., 2001; Ortiz-Santaliestra and Sparling, 2007).

We increased TH levels by exposing tadpoles to 10 µg/L exogenous L-thyroxine (T4, IRMM468 Sigma-Aldrich**,** Sigma-Aldrich, St. Louis, USA), a concentration which is known to influence amphibian metamorphosis (Lucas and Reynolds, 1967; Mann et al., 2009) and is related to increases in T4 observed in tadpoles responding to stress (Denver, 1997; 1998). Tadpoles absorb exogenous T4 directly through their permeable skin (Shi, 2000; Tata, 2006; Coady et al., 2010).

T4 and SP treatments were prepared in 0.1 N sodium hydroxide solutions (0.1 N, S2770 SIGMA, Sigma-Aldrich, St. Louis, USA) buffered with 0.1 N muriatic acid solutions as solvents. Solutions were added to the aquaria. A clean solution of 0.1 M sodium hydroxide solution buffered with 0.1 M muriatic acid solution was added to the control aquaria to control for any effect of solvents addition. Water was changed every second day and fresh SP and T4 were added, which is frequent enough to maintain a constant hormone and perchlorate level, in accordance with the standard procedure for chemical and hormonal addition (Goleman et al., 2002 a,b; Iwamuro et al., 2003; Rot-Nikcevic and Wassersug, 2004).

2.3 Processing of specimens

Developmental stage was determined by evaluating the status of key morphological features typical of specific developmental stages, as detailed in Gosner (1960). The developmental stage of each tadpole was recorded according to the procedure of Ortiz-Santaliestra and Sparling (2007). The age describes the larval duration in days after hatching. Onset of metamorphosis was defined by the emergence of at least one forelimb (Gosner stage 42; Gosner, 1960). End of metamorphic climax was defined by the complete resorption of the tail (Gosner stage 46; Gosner, 1960).

The snout vent length (SVL) of the larvae was measured with a caliper to the nearest 0.5 mm. Larvae were weighed to the nearest 0.001 g with an electronic balance (digital gold scale, Smart Weigh). At the end of the experiment tadpoles were euthanized with 200 mg/L of tricaine methanesulfonate ([MS-222], Ethyl 3-aminobenzoate methanesulfonate, E10521 ALDRICH**,** Sigma-Aldrich, St. Louis, MO, USA) buffered with 200 mg/L of sodium bicarbonate (Sodium bicarbonate, S5761 SIGMA**,** Sigma-Aldrich, St. Louis, MO, USA) (Stuart et al., 2007) and transferred into ethanol (70 %) for further experiments.

2.4 Body condition

We estimated the body condition (i.e. energy stores) at the onset of metamorphosis and in juvenile froglets by calculating the scaled mass index (SMI). The SMI is a measure of the entire body condition of an individual as it accounts for the allometric relationship between mass and a body structure measures and standardizes each measure so that direct comparisons among individuals can be made (Peig and Green, 2009, 2010; MacCracken and Stebbings, 2012). The SMI was considered as an accurate condition index in anuran larvae (MacCracken and Stebbings, 2012; Dittrich et al., 2016; Ruthsatz et al., 2018b). A high SMI suggests larger energy storages and thus, a good body condition. We followed the procedure outlined by Peig and Green (2009) to calculate the SMI for each individual.

2.5 Standard metabolic rate (SMR)

Respiration measurements were made on eight randomly chosen, late-stage tadpoles (Gosner stage 39-41) and post-metamorphic froglets from each aquarium, in total on 192 individuals (n = 96, late stage tadpoles; n = 96, post-metamorphic froglets) (Orlofske and Hopkins, 2009). Animals were not fed 48 h prior to and during the measurement of SMR the tadpoles were in a post-absorptive state (Orlofske and Hopkins, 2009). Oxygen consumption was measured by closed respirometry conducted during the natural activity phase between 0900 and 2100h to

control for the influence of natural circadian rhythms on respiration (Orlofske and Hopkins, 2009). Larvae were placed in respirometers consisting of 30 ml beakers containing 30 ml (minus the volume of the animals) of autoclaved tap water to exclude microbial oxygen consumption with respective T4 and SP concentrations. Froglets were placed in air-filled respirometers consisting of 30 ml beakers (minus the volume of the animals) due to their transition to lung respiration. Each respirometer was equipped with a fiber optic sensor (Oxygen Dipping Probe DP-PSt7; PreSens Precision Sensing GmbH, Regensburg, Germany) connected to a multichannel oxygen measuring system (Oxy 4 mini; PreSens Precision Sensing GmbH, Regensburg, Germany) and sealed with an air tight rubber plug. O_2 concentration was recorded every 15 seconds and measured as ml $O_2 \times L^{-1}$. Prior to each trial, the O_2fiber optic sensors were calibrated using air-saturated water and a factory-set zero oxygen calibration point at the developmental temperature. Water temperature and continuous mixing were controlled by a water bath. Oxygen consumption was measured for every animal for 20 min. Empty (control) chambers were run simultaneously in every trial and values were adjusted accordingly. We ensured that less than 10% of total O_2 was removed during the measurements to avoid impediment of respiration at low O_2 saturation levels. At the end of the measurements, each animal was removed and its SVL and blotted wet body mass was determined.

2.6 Energetic costs during metamorphic climax

After reaching the onset of metamorphosis at stage 42 (Gosner, 1960) two metamorphosing tadpoles were collected from each tank for respirometry using the same equipment and software parameters described above. In total, 24 individuals (3 treatments × 4 aquaria × 2 individuals) were measured during metamorphic climax. Unlike the previous SMR measurements, 12 h respirometry trials were repeated until the completion of metamorphosis when the tail was fully resorbed at stage 46 (Gosner, 1960; Orlofske and Hopskins, 2009). No fasting prior to the O_2 ml $\times h^{-1}$ measurements was needed because tadpoles stop feeding due to the remodeling of mouthparts and digestive tract during metamorphosis (Hourdry et al., 1996). Prior to the measurements, tadpoles were rinsed and drained to remove excess moisture and wet mass was recorded to the nearest ± 0.001 g. Respirometer chambers consisted of 1000 ml sealed glass culture bottles filled with 950 ml (minus the volume of the animals) well aerated, autoclaved, dechlorinated tap water with respective T4 and SP concentrations. To provide a ramp for the tadpoles to emerge from the water to facilitate air breathing, a 3.0 cm x 6.5 cm piece of glass was placed against the inner side of the chamber.

After each 12 h respirometry trial, tadpole developmental stage and mass, SVL, and TL were recorded. Before the start of the next 12 h trial, the water in each chamber was replaced. Each trial was started at approximately the same time (0900-1000 h and 2100-2200 h). When tadpoles completed metamorphosis at Gosner stage 46 (complete resorption of the tail; froglets sit on the ramp), they were removed from the chamber. After removing the tadpoles from the chambers, they were rinsed and drained to remove excess moisture and finally weighed to the nearest ± 0.001 g for body condition calculations. The total duration of metamorphic climax was recorded in hours.

2.7 Post-metamorphic performance

After completing metamorphosis at Gosner stage 46, all surviving tadpoles were transferred into separate aquaria containing a small amount of water to avoid desiccation, placed in a climate chamber maintained at 22 ± 0.1 °C, representing an average temperature commonly experienced in the field. Froglets were fed *ad libitum* with adult *Drosophila melanogaster* for seven days prior to being subjected to the performance trails. Exposure to T4 and SP was stopped after completing metamorphic climax. Prior to the performance tests, the metamorphs were measured for SVL to the nearest 0.5 mm and blotted wet body mass to the nearest ± 0.001 g for body condition calculations.

We measured jumping ability and sprint speed of newly metamorphosed frogs to test if the alteration of TH levels experienced during the larval stage would affect juvenile locomotory performance. Both traits can influence escape from predators and foraging success in this species, and hence can provide a functional link between selection of diet and thermal environment and fitness (Àlvarez and Nicieza, 2002). We placed froglets on a clean, flat surface and chased them with a probe to induce an escape response (Beck and Congdon, 2000; Àlvarez and Nicieza, 2002). Measurements of jumping ability were conducted according to the procedure of Àlvarez and Nicieza (2002): To assess jumping ability, we recorded a total of five jumps per individual using two response variables: (1) maximum jump distance, defined as the length of the longest leap, and (2) mean jump distance, defined as the mean length of the five leaps. Measurements of sprint speed were conducted according to the procedure of Beck and Congdon (2000): The sprint speed, i.e. the distance covered in the first 30s of a trial, was measured twice on every individual. The mean of the two measurements was used in the analysis. In the end of the experiments, froglets were euthanized with MS-222 and mass and SVL were measured. Froglets were transferred into ethanol (70 %) for further analyses.

2.8 Data preparations

2.8.1 SMR

Prior to statistical analysis, we plotted the O_2 consumption rate of each tadpole over time and visually assessed activity peaks to exclude them for the determination of SMR (Orlofske and Hopkins, 2009). The SMR was expressed in ml $O_2 \times h^{-1} \times mg^{-1}$ (blotted wet body mass) and was determined from the slope of linear least squares regressions of O_2 concentration vs. time (Hastings and Burggren, 1995; Rowe and Funk, 2017). The relationship between O_2 consumption rate (ml $O_2 \times h^{-1}$) and blotted wet body mass (mg) was described using a simple linear regression according to Orlofske and Hopkins (2009): ln(O_2rate) = a + b ln(mass), where O_2 rate was given in ml $O_2 \times h^{-1}$, mass in mg and a and b are regression coefficients.

2.8.2 Oxygen consumption rate during metamorphic climax

The first 10 minutes of each trial (i.e. after starting and after every water change) were excluded from the analyses because tadpoles may have still been recovering from handling. Oxygen consumption rates were interpolated between consecutive respiratory trials for each individual to generate a continuous respiration profile that covered the entire metamorphic period. The mean (± SD) O_2 consumption rate of all tadpoles was 5.136 (± 0.271) ml O2 $\times h^{-1}$in SP treatment, 4.323 (± 0.385) ml $O_2 \times h^{-1}$ in the control group, and 2.719 (± 0.493) ml $O_2 \times h^{-1}$ in T4 treatment. To estimate the total O_2 consumed (ml) throughout metamorphic climax, the O_2 consumption rate was assumed to be constant between consecutive trials and was calculated by multiplying the O_2 consumption rate (ml $\times h^{-1}$) by the corresponding time interval (h). Total oxygen consumption was converted to Joule (J) using a conversion factor of 18.8 J × ml^{-1} O_2 according to Schmidt-Nielsen (1990) to determine total energy expenditure.

2.8.3 Energy expenditure and allocation during metamorphic climax

Total energy expenditure was divided into maintenance costs and developmental costs following the procedure according to Beck and Congdon (2003): The slope and intercept of the regression of the O_2 and blotted wet body mass of late stage tadpoles (Gosner stage 39-41) provided the values for the constants used in an integration to calculate maintenance costs over time. The integration assumed a linear decrease in mass over the course of metamorphosis and an exponential relationship between mass and metabolic rate (Orlofske

and Hopkins, 2009). Energy allocated to development was calculated by substracting maintenance costs from total energy expenditure during metamorphic climax.

2.9 Statistical analyses

For all statistical tests R 3.4.1 (R Development Core Team, 2007) for Windows was used. Data were analyzed using linear mixed-effect models [lme, Type III model, covariance type: variance components, REML (restricted maximum likelihood) method for parameter estimation, 100 iterations (Bates and Sarkar, 2007)], entering 'Treatment' (T4, SP, and Control) as fixed factor. 'Growth, development, and energetics at onset of metamorphosis' (as measured by age, body mass, SVL, SMR, and SMI), 'Energetics during metamorphic climax' (as measured by % change in wet mass, duration, metabolic rate, total energy used, maintenance costs, and % developmental costs), 'Post-metamorphic performance and body condition' (as measured by maximum jump distance, average jump distance, mean sprint speed, SMI, SMR, body mass, and SVL) were used as dependent variables in separate models (Table A1). P-Values were obtained from likelihood-ratio tests, which compared the models with the respective null-model (Crawley, 2007). To address dependencies in the data, the variable 'aquarium' was included as a random factor. Residuals of each model were visually checked for normal distribution. N refers to the total number of analyzed tadpoles. Linear mixed-effect models were followed by post hoc comparisons (Tukey's test; Tuckey HSD function, multcomp package, vers. 1.2-13) to compare all possible pairwise combinations of treatments when overall tests were significant (Table A1). We performed linear regressions on variables of metamorphic climax according to Orlofske and Hopkins (2009) and on variables of post-metamorphic stage (Fig. A2).

3. Results

3.1 O_2 consumption rate

Tadpoles in late stages (Gosner stages 39-41) ranging in mass from 375 to 425 mg (Control), 467 to 588 mg (SP), and 172 to 251 mg (T4) were used for O_2 consumption rate measurements. Mass and O_2 consumption rate were negatively correlated in all treatments. The equation approximating the relationship between mass and O_2 consumption rate was: $\ln(O_2)=7.82–1.04\ln(\text{mass})$ in SP treatment ($R^2=0.435$, $p=<0.001$, $n=8$), $\ln(O_2)=1.81-0.07\ln(\text{mass})$ in the control group ($R^2=0.404$, $p=0.005$, $n=8$), and $\ln(O_2)= 0.99-0.02\ln(\text{mass})$ in T4 treatment ($R^2=0.491$, $p= <0.001$, $n=8$).

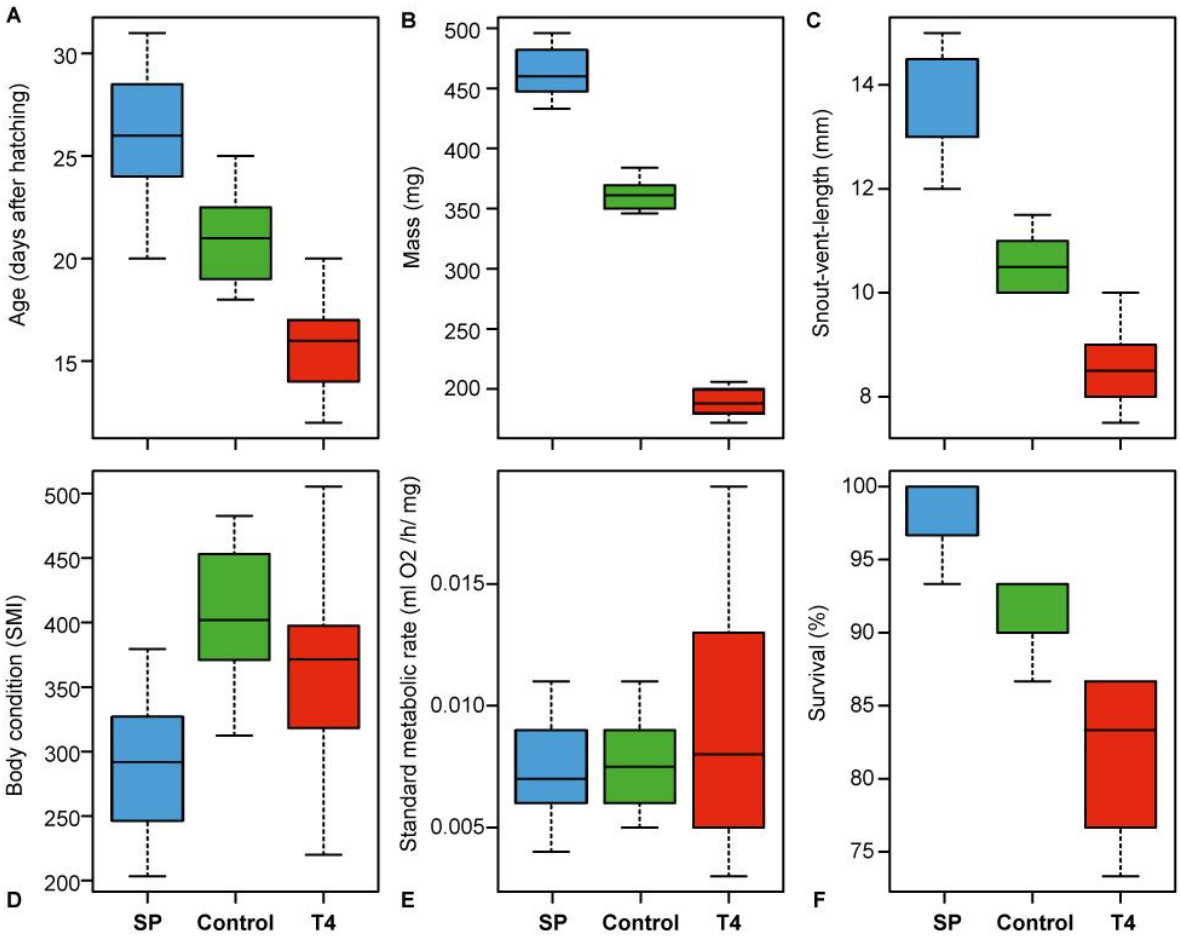

Fig. 1 Effects of altered TH levels on **A-C** metamorphic traits N(n)=163(12), **D** body condition N(n)=163(12), **E** standard metabolic rate (SMR) N(n)=96(12), and **F** survival (n=12) in tadpoles of the Common frog (*R. temporaria*) at the onset of metamorphosis. Blue boxes: SP, low TH levels. Green boxes: Control. Red boxes: T4, high TH levels. Boxplot characteristics: Bar=median. Box=1. and 3. quartiles. Whiskers = 1.5-fold interquartile range. N(n)=total number of studied individuals (total number of aquaria).

3.2 Metamorphic and physiological traits at the onset of metamorphosis

There were significant treatment effects on age, SVL, body mass, SMI, and variance of SMR, but only marginal effects on (mean) SMR (Table A1). Thus, altered TH levels have an effect on metamorphic traits and body condition at the onset of metamorphosis. Larvae exposed to SP were significantly the largest, heaviest, and oldest animals at the onset of metamorphosis compared to Control and T4 animals (Table A1, Fig. 1A-E). SMI was significantly highest in control animals compared to T4 and SP animals. Consequently, altered TH levels as caused by environmental stressors reduced body condition and thus, energy storages in the larvae at the onset of metamorphosis to different extent (Table A1, Fig. 1D).

Survival (± SD) from the start of the experiments (Gosner stage 25) to onset of metamorphosis (Gosner stage 42) in the treatment groups was: Control: 91.66 ± 2.49 %; SP: 98.32 ± 3.33 % and T4: 81.67 ± 6.67 %. Therefore, only high TH levels led to significantly reduced survival as compared to control and SP treatments (Table A1, Fig. 1F).

3.3 Energetic costs and energy allocation during metamorphic climax

The duration (h) of metamorphic climax differed significantly between all treatments and control groups (Table A1, Fig. 2A) with animals from the SP treatments having the longest metamorphic climax (mean duration: 203.8 ± 9.6 h) and those from T4 the shortest (mean duration: 62.5 ± 6.6 h). SMR (ml $O_2 \times h^{-1} \times mg^{-1}$) during climax was significantly higher in animals in the T4 treatment and revealed a higher variance compared to the SP treatment and the control animals (Table A1, Fig. 2B). Mass loss during metamorphic climax was highest in T4 and control animals and was significantly lower in SP treatment (Fig. 2C, A1).

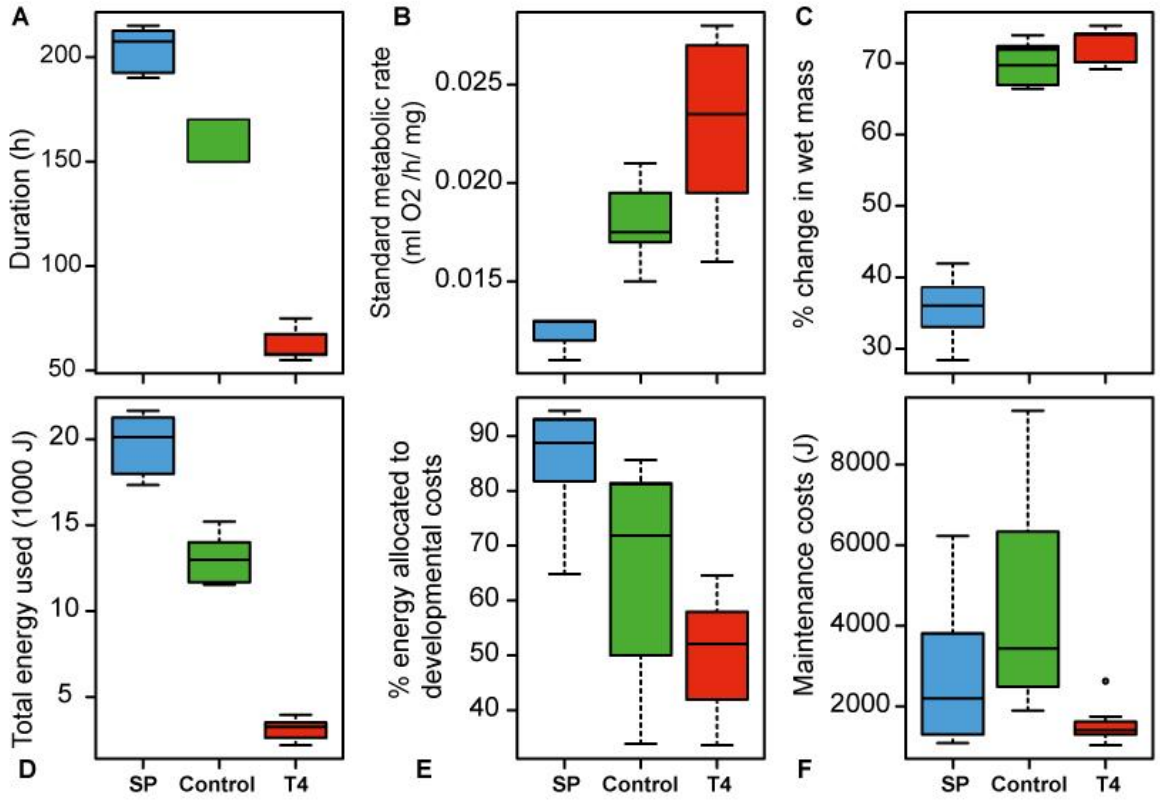

Fig. 2 Effects of altered TH levels on energetics during metamorphic climax in tadpoles of the common frog (*R. temporaria*). **A** Duration of metamorphic climax (h), **B** Mean standard metabolic rate (SMR) during metamorphic climax (ml O_2/h/mg), **C** % change in wet mass, **D** total energy used during metamorphic climax (1000 J), **E** % of total energy used during metamorphic climax allocated to developmental costs, and **F** maintenance costs (J) during metamorphic climax. Blue bars: SP, low TH levels. Green boxes: Control. Red boxes: T4, high TH levels. Boxplot characteristics: Bar=median. Box=1. and 3. quartiles. Whiskers = 1.5-fold interquartile range. N(n)=total number of studied

individuals (total number of aquaria). N(n)=24(12). N(n)=total number of studied individuals (total number of aquaria).

The O_2 consumption rate of individual tadpoles and treatments varied throughout climax, but showed no definitive pattern with respect to the developmental stage or body size (Fig. 3). In contrast, total O_2 consumption increased linearly throughout climax for all treatments and in the control group (Fig. 3). However, the slopes of the relationships varied among treatments and control group (Fig. 3). As a result, the total amount of energy used to complete metamorphic climax also varied considerably (Table A1, Fig. 2D). Animals from SP treatment used six times as much energy during metamorphic climax than animals from T4 treatment. Compared to control animals, tadpoles exposed to T4 could allocate 24 % less energy to development during metamorphic climax. Therefore, the energy reserves available for metamorphosis are reduced in tadpoles which experience larval stressful conditions.

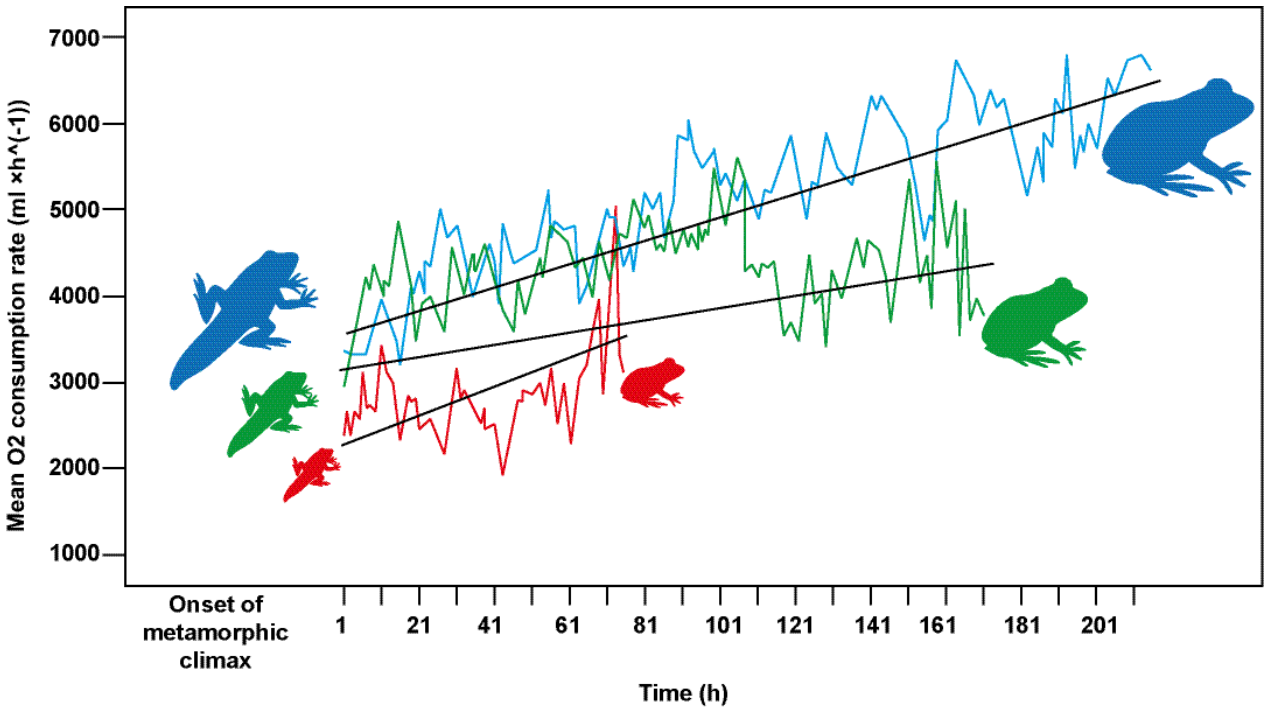

Fig. 3 Energy metabolism during metamorphic climax in tadpoles of the common frog (*R. temporaria*) and comparative body size at the onset and after completion of metamorphic climax at different TH levels (blue: SP treatment (low TH levels); green: control group; red: T4 treatment (high TH levels). Mean O_2 consumption rate (ml $\times h^{-1}$). Blue line: SP. Green line: control group. Red line: T4. N(n)=24(12). Regression lines for linear increase of total O_2 consumption rate (ml $\times h^{-1}$) during metamorphic climax: SP: Total O_2 = 4.44 × time – 14.56, R^2= 0.989, P <0.001. Control: Total O_2 = 5.23 × time – 53.41, R^2= 0.987, P <0.001. T4: Total O_2 = 2.67 × time + 0.382, R^2= 0.897, P <0.001. N(n)=total number of studied individuals (total number of aquaria).

When total energy used during metamorphic climax was portioned into maintenance costs and development, we found that, on average, the majority of energy used during metamorphic climax was used for development in all groups. Whereby individuals from SP treatment allocated significantly the largest percentage and those from T4 treatment allocated the smallest percentage to developmental costs (Table A1, Fig. 2F, 4). Animals from SP treatment allocated 40 % more energy to development than those from the T4 treatment. Compared to control animals, tadpoles exposed to T4 used 76 % less energy during metamorphic climax due to the 61% reduction in the duration of metamorphosis.

In the SP treatment, the total energy used during metamorphic climax increased linearly with increasing duration of metamorphic climax but this was not the case in T4 animals. Mass at the onset of metamorphosis was negatively related to percent of energy allocated to development in T4 treatment, but not in SP animals nor in the control group (Fig. A2). Mass at the onset of metamorphosis was not related to total energy used during climax in any group (Fig. A2).

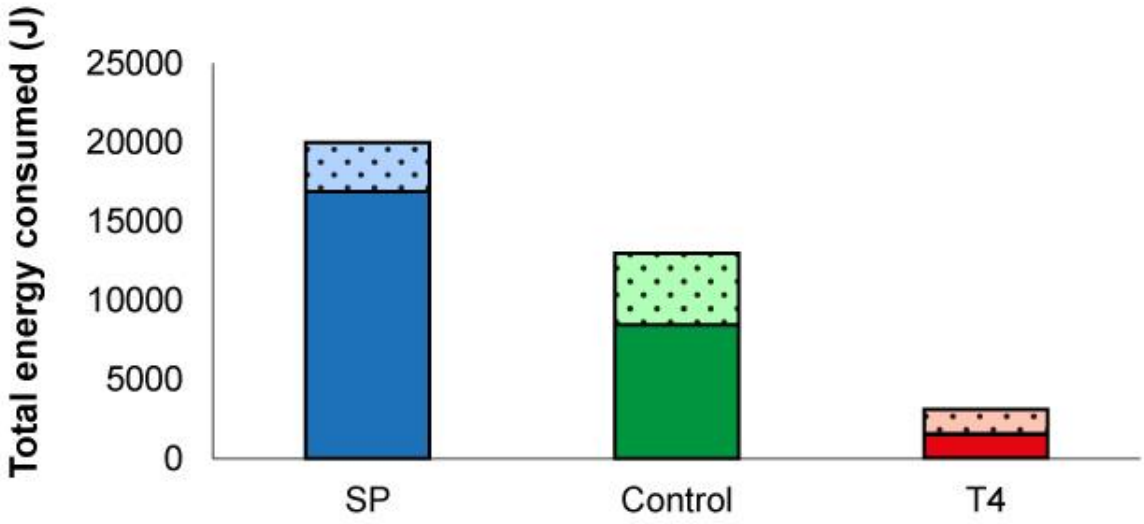

Fig. 4 Mean total energy consumed during metamorphic climax in tadpoles of the common frog (*R. temporaria*) at altered thyroid hormone (TH) levels. Blue bar: SP treatment = low TH levels. Green bar = control group. Red symbol: T4 treatment = high TH levels. Filled segments for energy allocated to developmental costs (DC). Dotted segments for energy allocated to maintenance costs. Energy for DC: SP = 85.8%, Control = 65.8%, and T4 = 50.3%.

3.4 Post-metamorphic performance and body condition

In post-metamorphic froglets there were significant differences between the treatments relating to age, SVL, body mass, and SMI as they have been at the onset of metamorphosis (Table A1, Fig.4). Compared with SMR at the onset of metamorphosis, SMR of post-metamorphic froglets was significantly reduced in SP compared to Control and T4 animals (Table A1, Fig. 5E). Froglets which experienced an altered TH status as larvae had a significantly reduced body condition compared to control froglets (Table A1, Fig.5D). Therefore, altered TH levels reduced body condition and thus, energy stores in both larvae at the onset of metamorphosis and post-metamorphic froglets, indicating a carry-over effect in relation to size of energy stores. The mean (±SD) survival from the start of the experiment (Gosner stage 25) to seven days after completion of metamorphosis was: SP: 76.7 ± 3.3 %, Control: 63.3 ± 3.3 %, and T4: 56.7 ± 3.3 %. As well as at the onset of metamorphosis only increased TH levels led to significantly reduced survival as compared to Control and SP treatments (Table A1, Fig. 5F).

Mass and SVL were significantly correlated. Therefore, we used mass for linear regressions on performance traits according to Àlvarez and Nicieza (2002). Post-metamorphic performance was significantly affected by treatment (Table A1). Animals exposed to T4 had decreased jumping ability (i.e. average and maximum jump distance) and a reduced sprint speed compared to Control and SP animals (Table A1, Fig. 5A-C). Mass was not related with performance traits within the treatments (Fig. A3A-C) but across all tested individuals (Fig. A4A-C) indicating that differences in performance may have proceeded from size differences caused by altered TH levels during the larval stage. Besides this strong size effect, there might be size independent effects which we are not able to evaluate as animals within treatment groups had highly diverse body sizes. Froglets which experienced decreased TH levels as larvae performed better than those within Control and T4 groups. We found the poorest performance in T4 animals. Consequently, altered TH levels caused persistent effects on metamorphic and energetic traits and, thus, led to carry-over effects on performance of juvenile froglets.

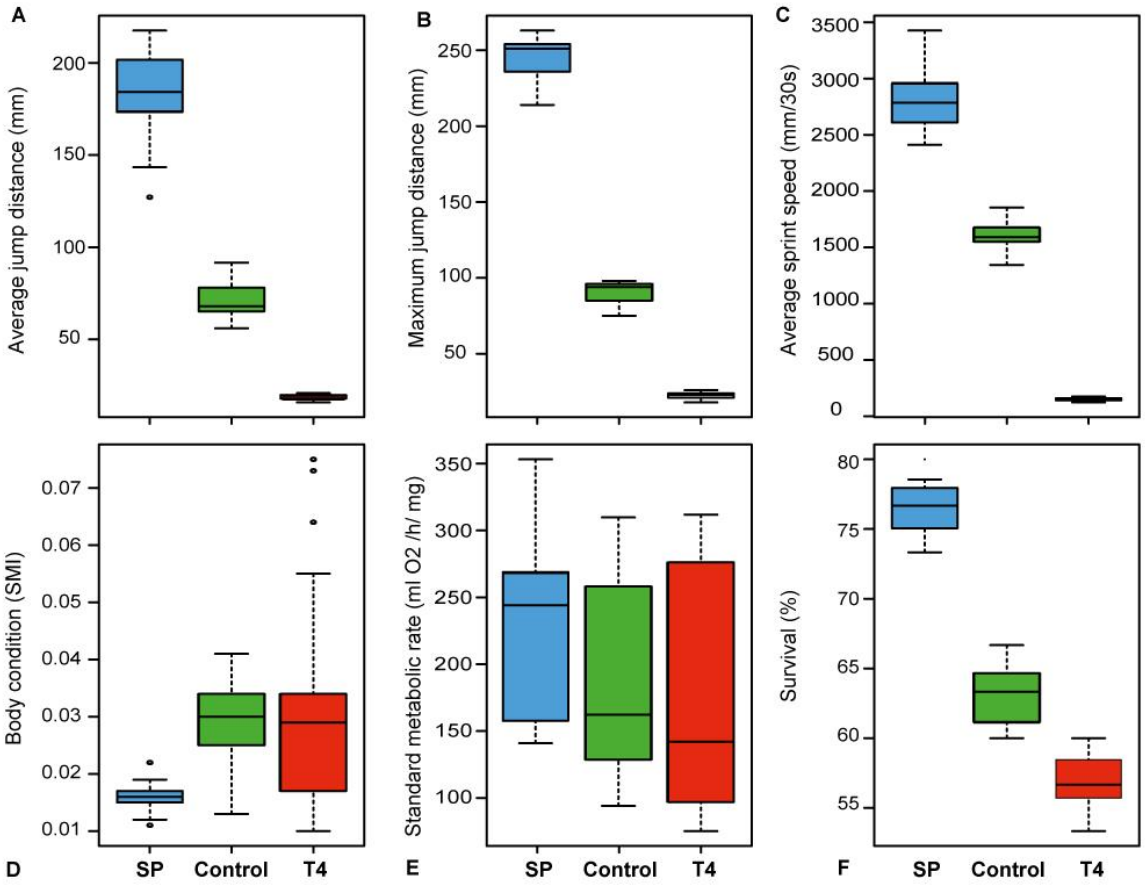

Fig. 5 Effects of altered TH levels on **A-C** post-metamorphic performance N(n)= 119(12), **D** body condition N(n)=119(12), **E** standard metabolic rate N(n)=96(12), and **F** survival n=12 in froglets of the common frog (*R. temporaria*) seven days after completion of metamorphosis. Blue bars: SP, low TH levels. Green bars: Control group. Red bars: T4, high TH levels. Boxplot characteristics: Error bar = median. Box = 1. and 3. quartiles. Dots = outliers, minimum and maximum values. Whiskers = 1.5-fold interquartile range.

4. Discussion

Amphibians are the sentinels of global change because of their sensitivity and their need to use both aquatic and terrestrial environments to complete their life cycle (James and Semlitsch, 2011). Environmental stressors have been shown to alter TH levels in amphibian larvae with consequences for energy portioning among development, growth, and metabolism (Ruthsatz et al. 2018b). Differences in energy allocation to development during metamorphosis may reduce juvenile performance and body condition which, in turn, impact survival and thus, fitness. Therefore, it is essential to understand how different environmental stressors affect energetics during and after amphibian metamorphosis and juvenile fitness by altering TH levels (Cary Coyle & Karasov, 2009). This study demonstrated how changes in energy allocation caused by altered TH levels associated with poor conditions in a larval

habitat, can carry over across metamorphosis and alter juvenile performance, with significant influence on fitness.

4.1 Getting off to a bad start: Altered TH status affects metamorphic traits, survival, and energy stores at the onset of metamorphosis

Our results confirm those from previous studies demonstrating that high (low) TH levels increase (decrease) developmental rate in amphibians resulting in younger (older) but smaller (larger) individuals at the onset of metamorphosis (Shi, 2000; Bulaeva et al., 2015). Very few studies, however, have investigated the physiological mechanisms (i.e. SMR and energy stores) associated with altered TH levels as caused by environmental stressors. Since THs are the major triggers of energy metabolism and are positively correlated with metabolic rate (McNabb & King, 1993; Rowe et al., 1998; Burraco & Gomez-Mestre, 2016) including maintenance costs (Orlofske & Hopkins, 2009) larvae experiencing environmental stress may differ in their capacity to store energy which impacts on their body condition at the onset of metamorphosis. Surprisingly, we demonstrate that differences in age and size at the onset of metamorphosis were not related to SMR but may result from direct impacts of TH status on rates of growth and development. Moreover, altered TH status generally reduced the ability of larvae to fuel energy stores until the onset of metamorphosis. Several studies found that slower developing larvae had higher lipid reserves (Alvarez and Nicieza, 2002; Scott et al., 2007; Kirschman et al., 2017) and thus, better body condition. In the present study, slow developmental rates in tadpoles exposed to SP did not increase body condition. Changes in body condition may be indicative of alterations in hepatic lipid mobilization, which can occur in response to chemical exposures, or general toxicity associated with both compounds (Kirschman et al., 2017). As in both instances altered TH levels affected growth, development, and body condition, environmental stressors causing alteration of TH status may lead to disadvantaged starting conditions into metamorphic climax.

4.2 Energy allocation during metamorphic climax

Metamorphic climax is a period of profound change in the morphology and physiology of anuran larvae that is entirely supported by stored energy (Crump, 1981, Beck and Congdon, 2003; Orlofske and Hopkins, 2009; Wright et al., 2011; Kirschman et al., 2017) as tadpoles fast due to a reorganisation of the digestive system (Pandian and Marian, 1985; Hourdry et al., 1996; Beck and Congdon, 2003; Orlofske and Hopkins, 2009). We suggest that exposure to environmental stressors (the myriad of those causing an alteration in TH status) can alter the

allocation of stored energy to development during metamorphic climax and thus, may alter energetic costs of entire climax.

As developmental rate determines the total energy needed for physiological and morphological reorganization and maintenance energy expenditure during metamorphic climax, tadpoles with high TH levels in total spend least energy whereas tadpoles with low TH status spend most. However, depending on the TH status the percentage allocated to development differs. For instance, tadpoles with low TH status could allocate about sixty percent more energy to development than those with high TH status and roughly one third more than tadpoles in the control group. Tadpoles with high TH levels need more energy to cover their maintenance costs even though as much energy as possible should be allocated to development during this stage of reorganization. Differences in energetic costs may be related to the significant differences in size between animals in the different treatments. Beck and Congdon (2003) and Orlofske and Hopkins (2009) found similar, negative relationships between developmental costs and size at the initiation of metamorphic climax in the southern toad (*Bufo terrestris)* and in the pickerel frog (*Lithobathes palustris*). Therefore, individuals with a large body size at the onset of metamorphosis as found in tadpoles exposed to SP have a significant physiological advantage over smaller tadpoles during the metamorphic climax because they complete metamorphosis more efficiently (i.e. in total use proportionally less energy for metamorphic climax and have a lower SMR) than their smaller conspecifics exposed to T4 (Pandian and Marian, 1985, Orlofske and Hopkins, 2009). This prolonged larval development and more efficient use of energy stores comes at the ecological cost of increased risks of mortality due to predation in their aquatic habitat as well as increased risks of desiccation (Lefcort et al., 1998; Kloas & Lutz, 2006).

Differences in energetic costs and efficiency of metamorphosis may also be due to the status of energy reserves since metamorphosis is fueled by internal macronutrient stores (Beck & Congdon, 2003). To meet the energetic demands of these complex reorganizations, larvae oxidize glycogen stores first (Sawant and Varute, 1973), followed by lipid stores (from fat body, liver, and tail), and protein stores (primarily from tail; Wright et al., 2011). Scott et al. (2007) emphasized that energy stores acquired in the larval stage are an important contributor to post-metamorphic success and, ultimately, fitness. Therefore, accumulating large lipid stores before the onset of metamorphic climax should be advantageous. In this study, we determined body energy stores by calculating the SMI for body condition indicating that altered TH levels decrease internal energy stores. However, measurements of the fat body or

the liver may be more precise in terms of lipid store size. In a previous study on *Xenopus laevis*, we found that high TH levels reduced the amount of fat stored in the liver (Ruthsatz et al., 2018b). We suggest that differences in SMR caused by altered TH levels during metamorphic climax and thus, energy allocation to maintenance costs might have contributed to a reduced energetic efficiency in tadpoles with high TH levels. In particular, internal lipid stores may be reduced in larvae exposed to unfavorable conditions as stress (i.e. high stress hormone and TH levels) mobilizes macronutrients from energy stores to meet increased energy demands before the onset of metamorphosis (Crump, 1981; Sapolsky et al., 2000; Kirschman et al., 2017). Moreover, Kirschman et al. (2017) showed that stressed larvae oxidized greater amounts of protein stores early in metamorphic climax, as they may need to shift to protein stores to fuel metabolism when lipids stores were exhausted due to an increased lipid catabolism by T4 (Picon, 1968; Sawant and Varute, 1973), which is unusal for amphibians (Scott et al. 2007).

4.3 Post-metamorphic performance

Pechenik (2006) emphasized that metamorphosis is not "a new beginning". In organisms with complex life cycles such as anurans, factors in the larval environment have strong carry-over effects on juveniles and adults (Smith, 1987; Berven, 1990; Goater, 1994; Pechenik, 2006; Altwegg and Reyer, 2003; Scott et al., 2007, Morey and Reznick, 2001) and thus, important fitness consequences and population-level impacts (Bouchard et al., 2016). One of the most important traits affecting success (survival and reproduction) in terrestrial habitats for anurans is body size (reviewed in Chelgren et al., 2006). Juvenile size is positively linked to both juvenile survivorship and adult fitness. Smaller size at emergence from larval ponds often leads to lower post-metamorphic survival (Smith, 1987; Berven, 1990, Goater, 1994; Relyea and Hoverman, 2003; Scott et al., 2007; Tarvin et al., 2015), whereas a larger size at emergence has been correlated with increased dispersal and survival and earlier or larger size at first breeding (reviewed in van Allen et al., 2010 and Chelgren et al., 2006). The consequences of size at metamorphosis for subsequent growth and survival can be mediated, at least in part, by locomotor performance and storage of energy reserves (Alvarez and Nicieza, 2002). Moreover, the consequences of variation in these two traits are most likely interdependent.

In our experiment, carry-over effects on juvenile performance were mainly mediated by variation in body size at metamorphic climax due to the alteration of TH levels during larval stage. Juvenile body mass was positively related to jumping ability and sprint speed. Our

findings are in accordance with those of previous studies indicating that size at metamorphosis determines locomotor performance in juvenile frogs: John-Alder and Morin (1990) found that size at metamorphosis was positively related with jumping ability in the Fowler's toad (*Anaxyrus woodhousii*, formerly *Bufo woodhousii*). Furthermore, Goater et al. (1993) found a positive relationship of size and burst speed in the common toad (*Bufo bufo*). Beck and Congdon (1999) demonstrated a positive relationship of size and sprint speed, and endurance in the southern toad (*Bufo terrestris*). Nevertheless, we assume that juvenile performance may also be influenced by a size-independent effect of achieved TH status. *As discussed above,* energetic efficiency differed between treatments due to differences in size but probably also due to differences in allocation of internal macronutrient stores. Nevertheless, we assume that juvenile performance may also be influenced by a size-independent effect of achieved TH status. Under increased maintenance costs due to high TH levels, larvae reduce tissue production during climax (i.e. energy allocated to developmental costs) and may instead allocate energy to maintenance. Furthermore, Kirschman et al. (2017) demonstrated that stressed larvae deplete their lipid stores very fast and proceed by depleting protein stores for meeting energy demand during climax. Sustained protein catabolism may present challenges for anuran larvae, because protein stores available for oxidation include muscle and newly formed adult structures crucial for terrestrial survival and juvenile locomotor performance (Beck & Congdon, 2003; Orlofske and Hopkins, 2009; Pandian and Marian, 1985; Wright et al., 2011; Kirschman et al., 2017). Differences in TH status on energy allocation of macronutrients may act synergistically with a strong size effect to alter juvenile performance but, due to differences in body size within and among treatments, this remains untested.

Locomotor performance can have a positive influence on dispersal from natal ponds, food acquisition and predator avoidance (Beck and Congdon, 2000; reviewed in Alvarez and Nicieza, 2002). Therefore, tadpoles exposed to environmental stressors which increase TH levels may suffer from difficulties in food acquisition and predator avoidance associated with their poor locomotor performance. Those difficulties may inhibit compensatory growth and storage of energy reserves. Furthermore, Morey and Reznick (2001) found that smaller metamorphs sustained higher mortality rates due their increased foraging activity to compensate for growth differences and, thus, a reduction of poor larval environment as in froglets exposed to exogenous T4 during larval stage. In contrast, froglets which experienced low TH levels during larval stage due to aquatic contamination may indeed reveal a strong juvenile performance and thus, may display an advantage in food acquisition and predator

avoidance. Nevertheless, the number of froglets which emerge from the natal pond may be extremely reduced under natural conditions due to their substantially longer duration of metamorphosis.

5. Conclusion

The present findings for *R. temporaria* emphasize that impact of environmental stressors on the energy allocation to development, growth, and metabolism during and after metamorphosis results in altered costs associated with metamorphic climax and lead to carry-over effects on juvenile performance. Therefore, ongoing climate change and anthropogenic disturbances of larval habitats will result in altered phenotypes at the onset of metamorphosis and relative higher energetic costs during climax with deleterious consequences for juvenile froglets. Any changes in metamorphic traits and energy allocation caused by exposure of larvae to stress that affect post-metamorphic performance and therefore, survival and growth or delay time to maturity could have important impacts on fitness and population persistence (James and Semlitsch, 2011). In addition, in temperate anurans such as the common frog (*R. temporaria*) a strong locomotor performance is also extremely beneficial for accumulating reserves of energy which are used during hibernation for survival and gonad development (Reading & Clarke, 1995). Energy reserves are built up after emergence before the onset of the next winter (reviewed in Reading and Clarke, 1995; Chen et al., 2011). Therefore, froglets which experienced environmental stress during larval stage may suffer from stressful larval conditions in two different ways: A small body size reduces locomotor performance which in turn impedes foraging and thus, storage of energy reserves essential for successful hibernation. Our effort highlights the current lack of studies which investigate how different environmental stressors impact energy allocation to development, growth, and metabolism across life stages resulting in carry-over effects. However, our results suggest that alteration of the TH levels, especially during the critical developmental window of metamorphic climax may have direct fitness consequences by impairing juvenile locomotor performance. Indeed, these results provide mechanistic evidence for the often-cited fitness cost of increased developmental rates. Since very little is known about the behavior or mortality of juvenile anurans in the wild (Tarvin et al., 2015), more long-term across-life studies are needed to understand the consequences of altered TH levels as caused by environmental stressors during the larval and early juvenile stages on the phenotype and fitness of the adults. A subsequent physiological-based understanding of how environmental stressors affect the efficiency of

anuran metamorphosis and survival in terrestrial stage will help to make better projections of anthropogenic impacts and to develop conservation strategies.

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8. Appendix

Table A1 Effects of altered TH levels on metamorphic traits, energetics, survival, and performance in larvae and froglets of the Common frog (*R. temporaria*) at the onset of metamorphosis (at least one forelimb present, Gosner stage 42) (Gosner, 1960), during metamorphic climax (until complete resorbtion of the tail, Gosner stage 46), and seven days after completion of metamorphosis. Chi² and P for linear mixed-effects models (LMM), using 'treatment' (Control, SP, T4) as the fixed factor; 'aquarium' as the random factor. N is the total number of analyzed individual animals, and n is the total number of tested aquaria. Pairwise multiple comparisons were made using Tukey's Test as post hoc test. Significance was set at $P < 0.05$. SP: decreased TH levels, T4: increased TH levels.

Developmental stage	Dependent variable	LMM							Tukey's Test (pairwise comparisons)								
		Estimate (SE)			Chi²	df	P	N (n)	Control - SP			SP - T4			Control - T4		
		Control	SP	T4					Estimate (SE)	z	P	Estimate (SE)	z	P	Estimate (SE)	z	P
Onset of metamorphosis	Mass (mg)	362.00 (1.99)	100.45 (2.76)	-173.20 (2.90)	90.311	2	**< 0.001**	163 (12)	100.45 (2.767)	36.31	**< 0.001**	-273.66 (2.85)	-95.92	**< 0.001**	-173.20 (2.9)	-59.73	**< 0.001**
	Age (dah)	20.96 (0.34)	5.12 (0.48)	-4.90 (0.50)	53.611	2	**< 0.001**	163 (12)	5.12 (0.48)	10.53	**< 0.001**	-10.02 (0.50)	-20.00	**< 0.001**	-4.90 (0.50)	-9.62	**< 0.001**
	SVL (mm)	10.5 (0.10)	2.99 (0.14)	-1.97 (0.14)	64.63	2	**< 0.001**	163 (12)	2.99 (0.14)	21.08	**< 0.001**	-4.97 (0.14)	-33.97	**< 0.001**	-1.97 (0.14)	-13.31	**< 0.001**
	SMI	408.13 (7.34)	-121.39 (10.21)	-50.17 (10.70)	39.44	2	**< 0.001**	163 (12)	-121.39 (10.22)	-11.88	**< 0.001**	71.22 (10.53)	6.76	**< 0.001**	-50.17 (10.71)	-4.68	**< 0.001**
	SMR (ml O2/h/mg)	0.007 (0.001)	-0.00004 (0.001)	0.001 (0.001)	5.14	2	**0.076**	96 (12)	-	-	-	-	-	-	-	-	-
	Survival (%)	91.66 (2.29)	6.66 (3.2)	-9.99 (3.24)	16.57	2	**< 0.001**	(12)	6.66 (3.24)	2.05	0.98	-16.66 (3.24)	-5.14	**< 0.001**	-9.997 (3.24)	-3.08	**0.006**
Metamorphic climax	% change in wet mass	69.78 (1.29)	-34.07 (1.83)	2.25 (1.83)	48.30	2	**< 0.001**	24 (12)	-34.07 (1.83)	-18.62	**< 0.001**	36.33 (1.83)	19.85	**< 0.001**	2.25 (1.83)	1.23	0.433

	Duration of metamorphosis (h)	160.00 (3.21)	43.75 (4.54)	-97.50 (4.54)	59.70	2	**< 0.001**	24 (12)	43.75 (4.54)	9.62	**< 0.001**	-141.25 (4.54)	-31.06	**< 0.001**	-97.5 (4.54)	-21.44	**< 0.001**
	Mean metabolic rate (ml O2/h) during metamorphosis	0.018 (0.001)	-0.005 (0.001)	0.005 (0.001)	20.66	2	**< 0.001**	24 (12)	-0.005 (0.001)	-3.36	**0.002**	0.01 (0.001)	6.43	**< 0.001**	0.005 (0.001)	3.06	**0.006**
	Total energy used (J)	13004.1 (463.4)	6701.9 (655.3)	-9898.2 (655.3)	55.06	2	**< 0.001**	24 (12)	6701.9 (655.3)	10.23	**< 0.001**	-16600.1 (655.3)	-25.33	**< 0.001**	-9898.2 (655.3)	-15.1	**< 0.001**
	Maintenance costs (J)	4466.9 (653.2)	-1725.5 (923.8)	-2925.2 (923.8)	8.98	2	**0.011**	24 (12)	-1725.5 (923.8)	-1.86	**0.148**	-1199.7 (923.8)	-1.299	0.395	-2925.2 (923.8)	-3.16	**0.004**
	% developmental costs	65.75 (4.834)	20.06 (6.83)	-15.46 (6.83)	17.58	2	**< 0.001**	24 (12)	20.06 (6.83)	2.93	**0.009**	-35.53 (6.83)	-5.19	**< 0.001**	-15.46 (6.83)	-2.26	0.06
Post-metamorphosis (7 days after completion of metamorphosis)	Average jump distance (mm)	70.90 (5.07)	112.80 (7.14)	-52.11 (7.20)	49.71	2	**< 0.001**	119 (12)	112.803 (7.13)	15.80	**< 0.001**	-164.91 (7.17)	-22.99	**< 0.001**	-52.11 (7.20)	-7.23	**< 0.001**
	Maximum jump distance (mm)	90.15 (1.51)	156.08 (2.05)	-67.59 (2.21)	89.31	2	**< 0.001**	119 (12)	156.08 (2.05)	75.89	**< 0.001**	-223.68 (2.13)	-104.66	**< 0.001**	-67.59 (2.21)	-30.49	**< 0.001**
	Mean sprint speed (mm/30s)	1610.09 (25.44)	1186.17 (34.58)	-1460.51 (37.57)	81.595	2	**< 0.001**	119 (12)	1186.17 (34.58)	34.30	**< 0.001**	-2646.69 (36.24)	-73.03	**< 0.001**	-1460.51 (37.57)	-38.87	**< 0.001**
	Age (dah)	35.82 (0.37)	5.092 (0.514)	-10.64 (0.55	61.23	2	**< 0.001**	119 (12)	5.092 (0.514)	9.89	**< 0.001**	-15.73 (0.53)	-29.43	**< 0.001**	-10.64 (0.55)	-19.18	**< 0.001**

				)													
	SVL (mm)	11.41 (0.18)	2.28 (0.24)	-1.14 (0.26)	43.34	2	< **0.001**	119 (12)	2.28 (0.24)	9.23	< **0.001**	-3.43 (0.25)	-13.35	< **0.001**	-1.14 (0.26).	-4.29	< **0.001**
	Mass (mg)	143.12 (2.22)	209.91 (3.03)	-55.30 (3.26)	88.88	2	< **0.001**	119 (12)	209.91 (3.03)	69.26	< **0.001**	-265.22 (3.14)	-84.22	< **0.001**	-55.31 (3.26)	-16.93	< **0.001**
	SMI	187.29 (11.65)	34.52 (15.88)	-7.519 (17.12)	7.79	2	**0.02**	119 (12)	34.528 (15.88)	2.174	0.075	-50.17 (16.50)	-2.548	**0.029**	-7.519 (17.12)	-0.439	0.899
	SMR (ml O2/h/mg)	0.028 (0.002)	-0.012 (0.003)	0.002 (0.003)	23.66	2	< **0.001**	96 (12)	-0.01 (0.002)	-4.62	< **0.001**	0.01 (0.003)	5.40	< **0.001**	0.002 (0.003)	0.77	0.718
	Survival (%)	63.33 (1.92)	13.33 (2.22)	-6.67 (2.22)	24.91	2	< **0.001**	(12)	13.33 (2.22)	5.99	< **0.001**	-20.00 (2.22)	-8.99	< **0.001**	-6.67 (2.22)	-3.00	**0.007**

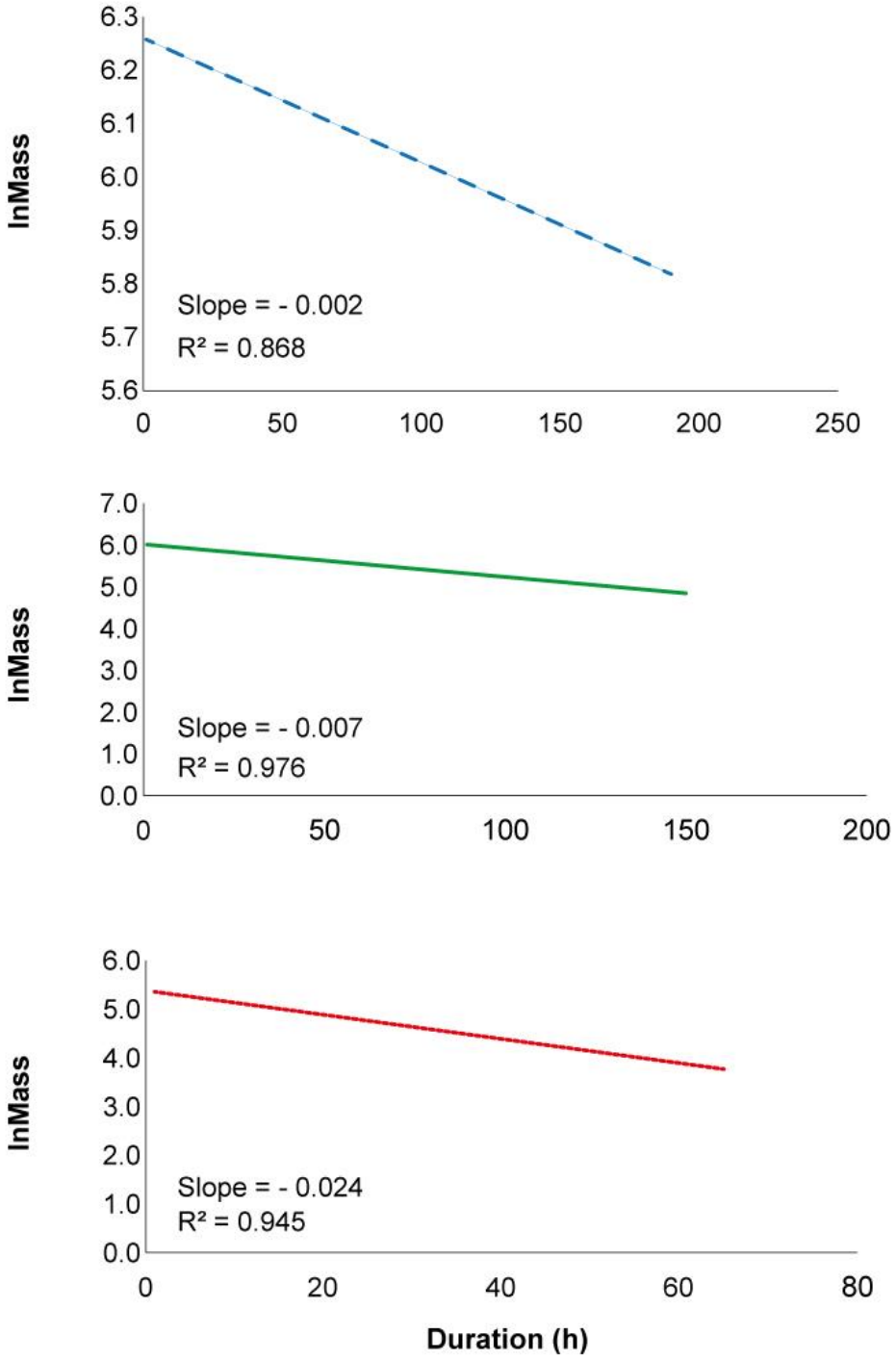

Fig. A1 Mass loss during the duration (h) of metamorphic climax in tadpoles of the Common frog (*R. temporaria*) at altered thyroid hormone (TH) levels. Regression lines for linear decrease of mass (mg) during metamorphic climax. Blue: SP treatment = low TH levels. N=24. Green = Control group. Red symbol: T4 treatment = high TH levels.

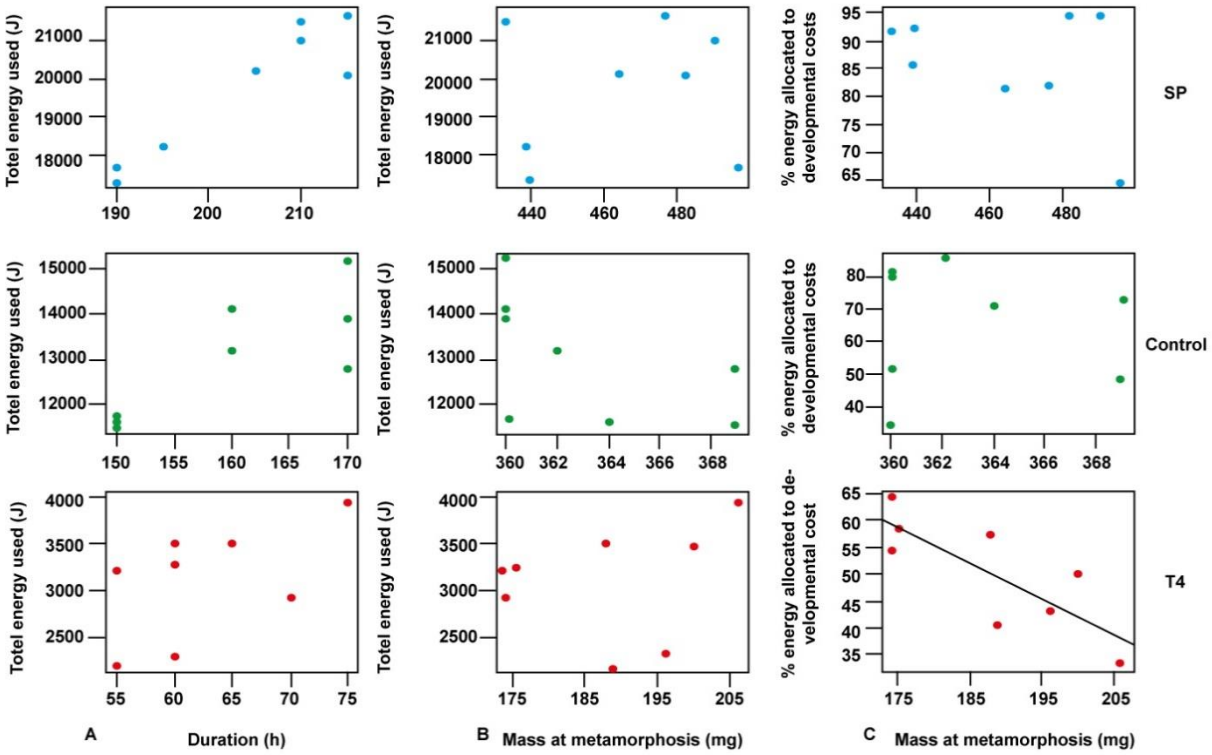

Fig. A2 Linear regressions of variables of metamorphic climax in tadpoles of the Common frog (*R. temporaria*) at the onset of metamorphosis (at least one forelimb present, Gosner stage 42) (Gosner, 1960). **A** Duration (h) of metamorphic climax and total energy used during metamorphic climax (J). **B** Mass at the onset of metamorphosis (mg) and total energy used during metamorphic climax (J). **C** Mass at the onset of metamorphosis (mg) and % of total energy used allocated to developmental costs during metamorphic climax. Blue dots: SP, low TH levels. Red dots: T4, high TH levels. Green dots: control group. Significance was set at $P < 0.05$. Regression lines show the significant relationship between two variables. N=24.

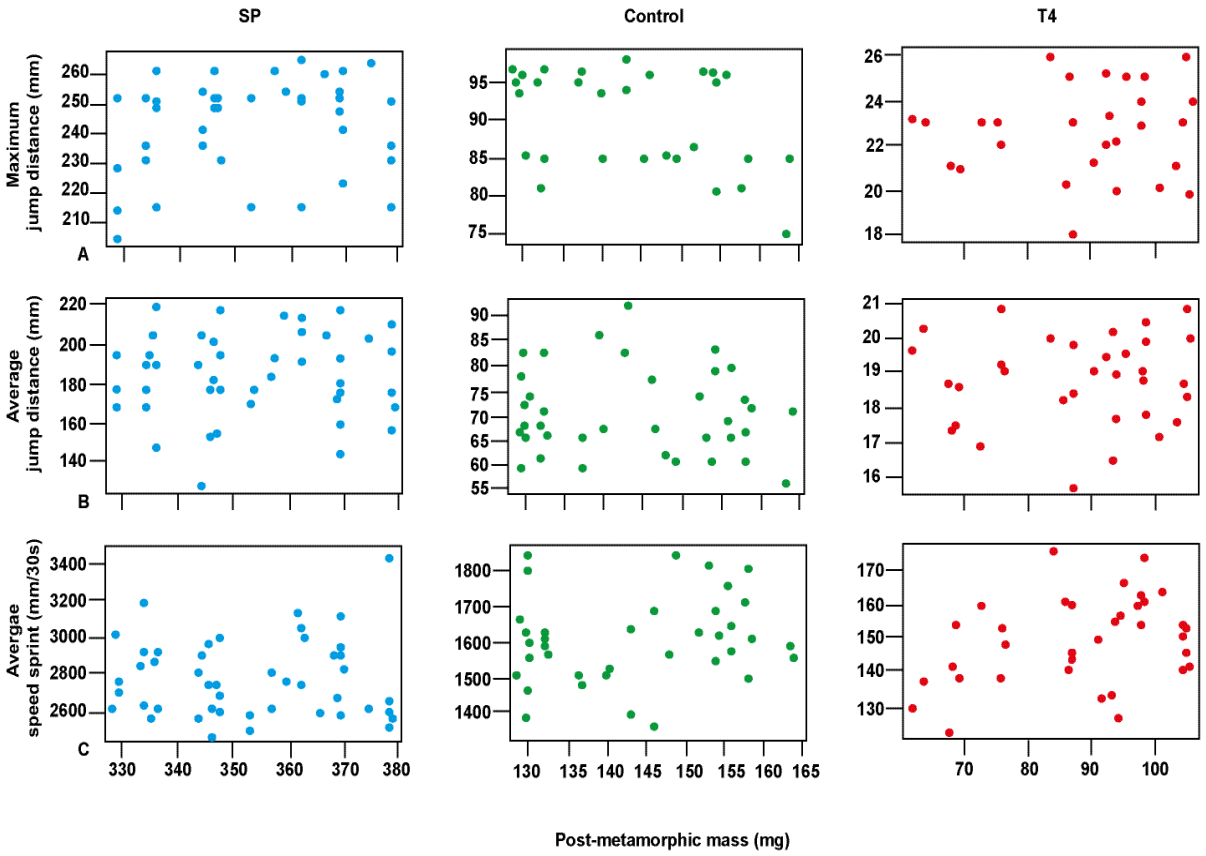

Fig. A3 Linear regressions of variables of post-metamorphic mass (mg) and performance (i.e. **A** maximum jump distance (mm), **B** average jump distance (mm), and **C** average sprint speed (mm/30 s)) in froglets of the Common frog (*R. temporaria*) seven days after completion of metamorphosis. Blue dots: SP, low TH levels, N=46. Red dots: T4, high TH levels, N=34. Green dots: control group, N=39. Significance was set at $P < 0.05$. Regression lines show the significant relationship between two variables.

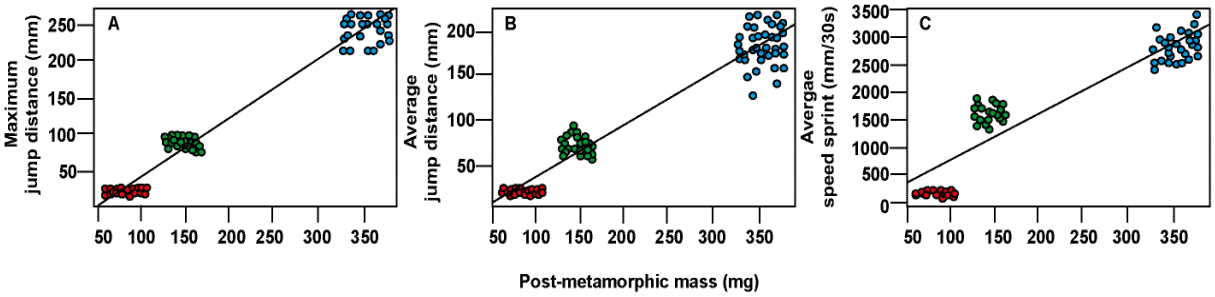

Fig. A4 Linear regressions of variables of post-metamorphic mass (mg) and performance (i.e. maximum jump distance (mm), average jump distance (mm), and average sprint speed (mm/30 s)) stage in froglets of the Common frog (*R. temporaria*) seven days after completion of metamorphosis across treatments. Blue dots: SP, low TH levels, N=46. Red dots: T4, high TH levels, N=34. Green dots: control group, N=39. Significance was set at $P < 0.05$. Regression lines show the significant relationship between two variables.

Author contribution

I hereby confirm that Katharina Ruthsatz conceived, designed and performed the experiments, analyzed the data and wrote the paper.

Hamburg, 18.10.2018

Prof. Dr. Kathrin Dausmann

General Discussion

Species-specific capacity for developmental and physiological plasticity is expected to mediate the responses of amphibians to major drivers of biodiversity loss, such as climate warming and both natural and anthropogenic environmental stressors (reviewed in Nowakowski et al. 2018). Considering the current worldwide decline of amphibians, it is of major interest to investigate whether and how anuran larvae are able to adjust their metamorphic and physiological traits to new thermal challenges and to altered TH levels, as caused by natural or anthropogenic stressors in their larval habitat. Although various anuran species have been shown to exhibit phenotypic plasticity to environmental variation at the onset of metamorphosis (reviewed in **Chapter 1**), knowledge of how multiple and/or simultaneously occurring environmental stressors may modify this capacity through their endocrine-disruptive effects on the TH system is still rare.

In my dissertation, I investigated the capacity for developmental and physiological plasticity at the onset of metamorphosis in larvae of two ecologically different anuran species, the common frog (*R. temporaria*) and the African clawed frog (*X. laevis*). Furthermore, I evaluated whether the proximate endocrine-disrupting effects of environmental stressors modify this capacity and influence energetic costs and energy allocation during metamorphic climax. Beyond that, I examined possible carry-over effects on energetics and performance in later life stages caused by endocrine disruption experienced during the larval stage. This thesis provides a comprehensive investigation of several fitness-related traits and the potential and limitations of adaptability to new thermal challenges and to environmental stressors during the larval stage in two ecologically different species. Thus, the results of the present study contribute to a better prediction of the vulnerability of both species to the impacts of environmental change and temperature variation, as predicted in global and climate change scenarios.

Temperature-induced developmental plasticity

Global (climate) change will likely have a variety of impacts on amphibian development as warmer temperatures and other natural and anthropogenic environmental stressors directly and indirectly determine physiological rates and thus, set growth, developmental, and metabolic rate. Since climate change is increasing mean environmental temperatures and the frequency of extreme thermal events (Seebacher et al. 2015; Pachauri et al. 2014; Gutiérrez-Pesquera et al. 2016), the ability to show developmental plasticity to changing thermal conditions is expected to be a primary factor dictating the vulnerability of amphibians to rising temperature (Huey et al. 2012; Stillman 2003; Gunderson and Stillman 2015).

According to Atkinson's (1994) TSR, amphibians reared at relatively higher temperatures may display higher growth rates but a shortened larval period and thus typically mature earlier at smaller sizes when compared with conspecifics reared at colder temperatures (Atkinson 1994; Angilletta et al. 2004; Courtney Jones et al. 2015). Numerous studies on anuran larvae investigated this effect of temperature on growth and developmental rate and, thus, on age and size at the onset of metamorphosis in anuran larvae. However, these studies refer to the species-specific effect of temperature during the larval period (Smith-Gill and Berven 1979; Àlvarez and Nicieza 2002; Walsh et al. 2008; Dittrich et al. 2016; Courtney Jones et al. 2015; Gutiérrez-Pesquera et al. 2016).

Interspecific patterns of temperature-induced developmental plasticity

A prerequisite for a comprehensive investigation of two ecologically different species in terms of their capacity for stress-induced phenotypic plasticity is an understanding of general interspecific patterns of temperature-induced phenotypic plasticity and how the species' thermal background may influence this pattern. Therefore, **Chapter 1** investigates whether the intraspecific TSR is applicable over a broad range of anuran species by carrying out a combined analysis based on the data from 25 studies performed on 18 anuran species in respect of their capacity to exhibit temperature-induced developmental plasticity and how their thermal background influences this capacity. All included populations developed faster and the majority were smaller at the onset of metamorphosis when developmental temperatures increased, indicating a general capacity for developmental plasticity in anuran larvae. Hence, I show that TSR (Atkinson 1994) is also applicable for comparisons across species, but with some constraints.

Whereas there was a consistent (decreasing) pattern for age at the onset of metamorphosis with increasing temperature, no consistent decrease at warmer temperatures was observed in mass or SVL (i.e. body size). Hence, growth rate is less plastic in response to different constant temperatures than development rate (Atkinson 1994; Gomez-Mestre et al. 2010). My results indicate that developmental rate responds plastically to temperature variation independent from respective thermal adaptation. This is obviously due to the general accelerating effects of increasing temperature on physiological and biochemical processes in general and especially on the intensity of THs, as they are the major triggers of amphibian metamorphosis (Smith-Gill and Berven 1979; Tata 2006; Little and Seebacher 2016), and are more effective at warmer temperatures (Ceusters et al. 1978). However, the effect of temperature on THs appears to have a greater impact on developmental rate as opposed to

growth rate (Hayes et al. 1993; Shi 2000). These two rates can, to some extent, be decoupled by this thermal effect on the intensity of THs (Zuo et al. 2012; Walters and Hassall 2006; Gomez-Mestre et al. 2010). Furthermore, growth rate is not only regulated by TH level but also by other hormones such as somatropin (i.e. growth hormone), corticosterone, melatonin, prolactin, and sexual hormones (i.e. estrogen and testosterone) (Hayes 1995, 1997; Glennemeier und Denver 2002; Denver et al. 2002; Joshi und Mohinuddin 2003; Delidow 1989; Huang and Brown 2000).
These hormones may be less sensitive to temperature than THs, resulting in a smaller effect of temperature on growth rate. Moreover, it is conceivable that via the activation of THs by increasing temperature, the growth-regulating hormones‘ modes of action might be disbalanced in various species to different extent. Imbalances of growth regulating hormones may thus result in inconsistent patterns of temperature-induced plasticity in growth rate in comparison to strongly TH dependent developmental rate.

Since all included populations demonstrated the capacity for temperature-induced developmental plasticity, albeit to different extent depending on the thermal background, my results take on greater significance in terms of the ongoing global climate change: Anuran larvae may reduce their vulnerability to global warming by a plastic response in developmental rate which allows for an earlier metamorphosing but at a smaller size. Although individuals that metamorphose earlier may benefit from an earlier escape from thermal challenges in their larval habitats, a smaller size may incur costs such as reduced juvenile survivorship (Smith 1987; Berven 1990), physiological performance (e.g. the ability to withstand starvation and tolerance to dehydration or to escape predators) (Zhao et al. 2014), as well as reduced fecundity and reduced size at first reproduction (Smith 1987; Semlitsch et al. 1988; Berven 1990).

Thermal adaptation affects thermal reaction norm of metamorphic traits

My analysis in **Chapter 1** also demonstrates that the thermal background of a population influences the thermal reaction norm (i.e. sensitivity) of metamorphic traits and thus, the capacity for a plastic response in growth and developmental rate. Warm-adapted populations were less sensitive to temperature variation, indicating a reduced capacity for developmental plasticity. Those species may be therefore more vulnerable to the impacts of climate change.

As this work is based on a comparison of two ecologically different species, which differ in their thermal adaptation, the results of **Chapter 1** indicate that *R. temporaria* and *X. laevis*

may display a different capacity for temperature-induced phenotypic plasticity. Based on Janzen's (1967) postulation that an organism's ability to acclimatize is related to the degree of variation that this animal experiences in its environment, I expect *X. laevis* as a model organism for tropical species to react less plastically to environmental variation such as new thermal challenges than *R. temporaria* as a model organism for temperate species. Nevertheless, all populations of both species analyzed in **Chapter 1** demonstrate a not significant decrease in age at the onset of metamorphosis with increasing rearing temperature and an inconsistent pattern of variation in size at metamorphosis (see Merilä et al. 2000; Loman 2002; Walsh et al. 2008; Gomez-Mestre et al. 2010). However, except from Loman's (2002) study, these studies use only 2-3 constant rearing temperatures. Hence, these studies are not sufficient to resolve the true shape of the thermal window and the capacity for developmental plasticity according to Kingsolver and Huey (2008). To determine the capacity for developmental and physiological plasticity and to illustrate the species-specific developmental thermal window, a broader range of more rearing temperatures is crucial. Therefore, I used a range of 18°C with six different rearing temperatures for *R. temporaria* and five rearing temperatures over a range of 12° for *X. laevis* (see **Chapter 2**, **3**, **4**).

Developmental plasticity in R. temporaria and X. laevis

Anuran larvae often occur in temporally and spatially heterogeneous environments and are limited in their ability to search for favorable microhabitats (Gutiérrez-Pesquera et al. 2016), and are therefore especially sensitive to environmental variation (Sanzo and Hecnar 2006; Yu et al. 2013). Larvae of temperate *R. temporaria* frequently occur in ephemeral or temporal ponds which are likely to increase in temperature or to dry out especially during later developmental stages. In tropical ponds of *X. laevis*, larvae are likely to be exposed to extreme temperatures (temperature peaks beyond CT_{max}) and also increased desiccation risk (Pachauri et al. 2014; Gutiérrez-Pesquera et al. 2016; Narayan 2016). Thus, the capacity for developmental plasticity may therefore reduce risks such as increasing desiccation risk or increasing temperatures in unfavorable larval habitats in both species.

Since climate change may face both temperate and tropical anuran species with new thermal challenges, **Chapter 2** examines whether and to what extent both species studied here reveal a capacity for temperature-induced developmental plasticity. Furthermore, **Chapter 2** investigates if these ecologically different species differ in this capacity since I could demonstrate a generally lower capacity in warm adapted species (see **Chapter 1**). My results

demonstrate that the capacity for phenotypic plasticity in age and size at the onset of metamorphosis is given in both species studied here within the species-specific developmental thermal window. Surprisingly, R. *temporaria* and *X. laevis* hardly differ in this capacity. Moreover, I illustrate the developmental thermal window of both species.

The developmental thermal window of R. temporaria and X. laevis

In contrast to the results of the combined analysis in **Chapter 1**, I found that both species reveal a consistent decreasing pattern for both age and body size (i.e. mass and SVL) at the onset of metamorphosis with increasing temperature (**Chapter 2**). However, the shown pattern refers to the rearing temperatures between 14° and 28°C in larvae of *R. temporaria* and to a temperature range from 19° to 28°C in larvae of *X. laevis* suggesting that these temperature ranges are within the respective species-specific developmental thermal window. In *R. temporaria,* all tadpoles constantly reared at 10°C died indicating that 10°C are beyond the critical thermal minimum for development and growth. However, the developmental thermal window is known to be population-specific due to local adaptations (e.g. Hjernquist et al. 2012). Laugen et al. (2003) and Stählberg et al. (2001) successfully raised larvae of *R. temporaria* from northern Sweden at constant temperatures of 10°C and lower indicating lower limits of the developmental windows compared with the present population. Thus, the lower temperature limit for development in larvae of the present *R. temporaria* population is between 10°C und 14°C. Rühmekopf (1958) reported that water temperatures ranging between 21.0°C and 26.0 °C are favorable for *R. temporaria* tadpoles (reviewed in Drakulic et al. 2017) which is within my selected experimental range of temperatures (see **Chapter 2, 3,** and **4**). However, in temperate breeding ponds of *R. temporaria* temperatures as high as 25°C are not commonly available especially during the earliest developmental stages (Drakulic et al. 2017), whereas tadpoles of *X. laevis* as a tropical species may frequently experience temperatures between 25°C and 28°C.

In *X. laevis* only the tadpoles reared at a range of 19°C to 28°C revealed the consistent decreasing pattern for age and size at metamorphosis with increasing temperature. Tadpoles reared at 16°C did not follow TSR according to Atkinson (1994) as there was a lack of a plastic response. Since the optimal thermal range for the TSR is bordered by a suboptimal range in which age and size plasticity do not occur (i.e. extreme conditions at which size decreases significantly; Walczynska et al. 2016), a constant rearing temperature of 16°C seems to be at the lower suboptimal range of the developmental thermal window in *X. laevis* accordingly. However, this does not apply to age (i.e. developmental rate), because age at

metamorphosis was highest in tadpoles reared at 16°C. Hence, growth rate was less plastic in response to different constant temperatures than developmental rate (Atkinson 1994; Gomez-Mestre et al. 2010). I suggest that the thermal window for growth is narrower than the developmental thermal window. Future studies should determine the entire species-specific developmental thermal windows and optimal temperatures for growth and development to predict more precisely how new thermal challenges as expected in scenarios of climate change may affect amphibian metamorphosis.

Thermal sensitivity of metamorphic traits in R. temporaria and X. laevis

Based on the results of **Chapter 1**, I expected both species studied here to differ in their capacity for temperature-induced developmental plasticity due to a different thermal sensitivity of growth and developmental rate in temperate and tropical species. Surprisingly, both species barely differed in the thermal sensitivity within the range of selected experimental temperatures (**Chapter 2**). Size (i.e. SVL and mass) at the onset of metamorphosis was more plastic in larvae of tropical *X. laevis*, whereas age was more plastic in larvae temperate *R. temporaria* within the selected temperature range. Since metamorphs of *R. temporaria* switch to terrestrial habitat, I suggest that plasticity in developmental rate is more adaptive than plasticity in growth rate in *R. temporaria* as it allows for an quicker emerge from larval pond if habitat quality decreases. Consequently, selection may favor developmental plasticity more than growth rate in *R. temporaria* (Newman 1992; Van Buskirk and Relyea 1998; Laurila et al. 2002). Since *X. laevis* completes its entire life cycle in aquatic habitats, we suggest that plasticity in developmental rate is less important compared to *R. temporaria* to escape from larval ponds. Furthermore, tropical species such as *X. laevis* usually show a reduced thermal sensitivity of growth and developmental rate due to the relatively stable thermal environments in the tropics (Janzen 1967; Gunderson and Leal 2015; Oyamaguchi et al. 2017). However, the results for *X. laevis* refer to a substantially narrower range of experimental rearing temperatures within a probably narrower species-specific developmental thermal window. In contrast, the range of selected experimental rearing temperatures for *R. temporaria* was broader as temperate populations experience more heterogeneous thermal environments during their larval stage. Accordingly, the high thermal sensitivity of growth and developmental rate in larval *R. temporaria* was not surprising as selection may favor a high sensitivity of both rates due to temperature variation resulting in a high capacity for a plastic response in both rates in temperate species (Seebacher et al. 2015). I suggest that at a broader range of experimental rearing temperatures, the sensitivity of

growth and developmental rate in tadpoles of *X. laevis* may decrease. Future studies should measure the thermal reaction norm of growth and developmental rate over the entire species-specific developmental thermal window in temperate and tropical species.

Temperature-induced physiological plasticity

In addition to the capacity for developmental plasticity, the ability to compensate for temperature variation by exhibiting physiological plasticity is of major importance since ectotherms will be more likely to experience temperatures beyond their physiological limits as a result of climate change (Gunderson and Stillman 2015). Without the capacity for physiological plasticity (i.e. thermal acclimation) thermal stress as a result of global warming and extreme thermal events such as heat waves could therefore affect the performance and fitness traits of ectotherms such as amphibians (reviewed in Narayan 2016). Therefore, **Chapter 3** and **4** assess the acclimation capacity to warmer developmental temperatures in both species. Furthermore, I generated a species-specific thermal tolerance zone area (i.e. thermal window; TW) to characterize the thermal range of tolerance for temperature variation. Moreover, I determined the effect of increasing developmental temperatures on the body condition at the onset of metamorphosis in both species and thus, evaluated whether warmer temperatures impair the ability to store energy. My results demonstrate that the temperate *R. temporaria* in contrast to the tropical *X. laevis* is able to respond plastically in its metabolism to new thermal challenges whereas both species are able to acclimate their thermal tolerance.

Physiological plasticity in metabolic rate in R. temporaria and X. laevis

Climate change is increasing average environmental temperatures and the frequency of extreme thermal events (Pachauri et al. 2014; Gutiérrez-Pesquera et al. 2016; Theisinger et al. 2017) resulting in increased SMRs in anuran larvae since ambient temperature regulates the rates of all physiological and biochemical processes in ectothermic animals (Smith-Gill and Berven 1979; Little and Seebacher 2016). Nevertheless, animals may compensate for those thermal changes through physiological plasticity in SMR (Angilletta et al. 2006; Berg et al. 2017). Consequently, species with a limited capacity for physiological plasticity in SMR will suffer from high maintenance costs as caused by the high SMRs at constant or decreasing food availability. My results suggest that tadpoles of temperate *R. temporaria* are able to compensate for warmer developmental temperatures by adjusting their metabolism as the SMR of larval *R. temporaria* did not significantly increase at warmer rearing temperatures (**Chapter 4**; Fig. 8.1). In contrast, the SMR of tropical *X. laevis* markedly increased at

warmer rearing temperatures indicating a limited capacity to compensate for warming through physiological acclimation (**Chapter 3**; Fig. 8.1).

Although recent meta-analyses revealed a contradicting pattern (Seebacher et al. 2015; Berg et al. 2017; Oyamaguchi et al. 2017), my results support the hypothesis by Janzen (1967) that tropical ectothermic species, in contrast to temperate species, have a limited capacity for physiological plasticity including acclimation of SMR. Consequently, anuran larvae which show limited capacity for physiological plasticity in SMR, such as *X. laevis* in the present study but especially tropical amphibians in general (Janzen 1967), may be most vulnerable to the thermal impacts of climate change. However, as long as the risk of desiccation and predators is low and food availability remains constant in the habitat of larval anurans at warmer temperatures physiological plasticity in SMR as shown for *R. temporaria* (**Chapter 4**) may be advantageous as it allows for energy accumulation and thus, supports a successful completion of metamorphosis. If desiccation risk and predation pressure increases or food availability decreases a high SMR due to a reduced capacity for physiological plasticity as shown for *X. laevis* (**Chapter 3**) is likely to allow for a quicker metamorphosis and, thus, for passing this highly vulnerable phase. If so, a high SMR may even be selected for under natural circumstances. Accordingly, a reduced acclimation capacity of SMR is not per se a disadvantage in larval anurans independent from their thermal adaptation. However, determining the full capacity for acclimation may require additional measures such as measuring the thermal reaction norm (i.e. sensitivity) of SMR by calculating Q_{10} values. If the Q_{10} values between the coldest temperatures were lower than those at the warmer temperatures, this would demonstrate some loss of acclimation potential (i.e., SMR increased more between two warmer as opposed to colder temperatures).

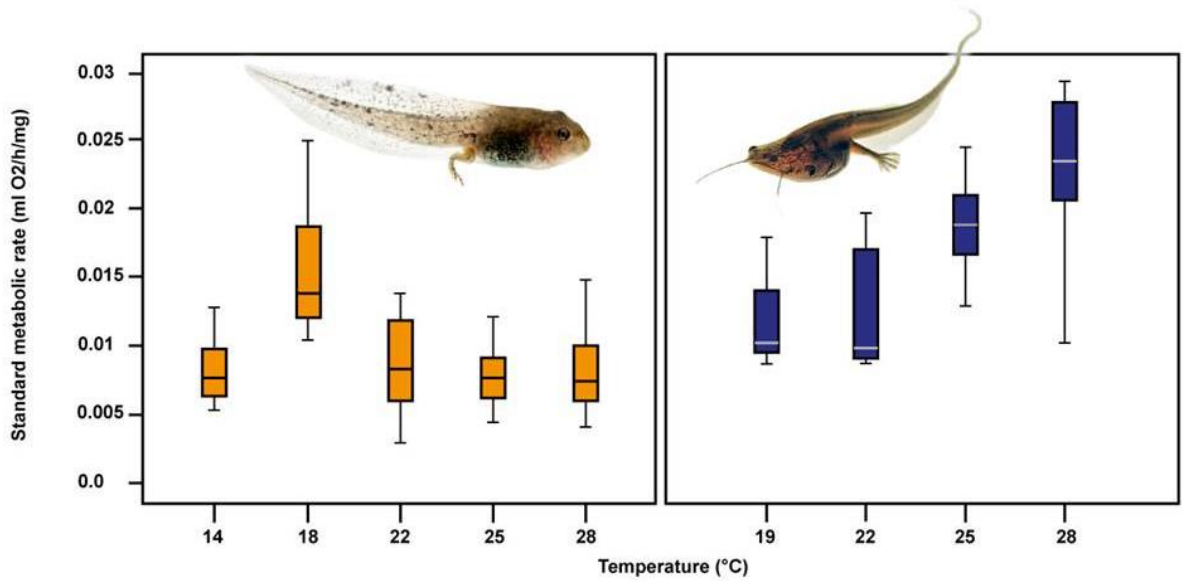

Fig. 8.1 Standard metabolic rate at five/four constant rearing temperatures in tadpoles of **left:** the common frog (*Rana temporaria*) and **right:** the African clawed frog (*Xenopus laevis*) at the onset of metamorphosis (Gosner stage 42; Gosner 1960).

Implications of warmer developmental temperatures on body condition

Increased SMRs and/or exhibition of developmental plasticity until the onset of metamorphosis may impede accumulating energy in larval energy stores (Sheridan and Kao, 1998; Orlofske and Hopkins, 2009) due to increased energetic demands. Therefore, the capacity for an acclimation of SMR may benefit the accumulation of energy stores available for covering the developmental costs during metamorphosis (Steyermark et al. 2005; Beck and Congdon 2003; Orlofske and Hopkins 2009) and ultimately supports a successful completion of metamorphosis. My results demonstrate that body condition and thus, size of energy stores was reduced in both species studied at warmer temperatures (**Chapter 3** and **4**). Therefore, warmer temperatures impair the ability to accumulate energy stores in both species probably due to an increase in SMR (*X. laevis*) or as energetic costs of phenotypic plasticity (*R. temporaria*). Although SMR of *R. temporaria* was relatively constant over the experimental temperature range, exhibiting developmental and physiological plasticity may increase energetic demands and decrease the ability to store energy even though larvae were fed *ad libitum*. Global warming may thus directly or indirectly result in increased energetic demands, albeit to different extent, independent from an acclimation capacity in SMR which in turn impairs the capacity to store energy.
For a better understanding of how global warming may impact across-life stage survival in

amphibians, future studies should examine to what extent a successful completion of metamorphosis is affected by body condition at metamorphosis.

Acclimation of thermal tolerance in R. temporaria and X. laevis

The acclimation of the thermal tolerance may provide a further key in coping with the impacts of climate change for amphibian larvae, especially in terms of long-term temperature changes and the occurrence of short-term thermal peaks beyond a species' upper thermal tolerance such as heatwaves (Katzenberger et al. 2014; Seebacher et al. 2015; Gutiérrez-Pesquera et al. 2016). In this study, tadpoles from warm developmental temperatures had higher thermal limits in both species (**Chapter 3** and **4**). Whereas tadpoles of *R. temporaria* revealed a broader thermal range of tolerance at warmer developmental temperatures (**Chapter 4**), the thermal range of tolerance in larvae of *X. laevis* was narrower at warmer temperatures (**Chapter 3**). Consequently, larvae of both species have the ability to compensate for changes in developmental temperature as they increased their thermal limits at warmer developmental temperatures but to different extent (Schaefer and Ryan 2006; Gunderson and Stillman 2015; Little and Seebacher 2016). Although the ability to acclimate to different temperatures by changing their critical thermal limits were given in both species, only tadpoles of *R. temporaria* were able to broaden their thermal range of tolerance, accordingly. Therefore, my results support Janzen's (1967) hypothesis once again and agree with my findings for temperature-induced developmental plasticity in anuran larvae (**Chapter 1**) indicating a general reduced capacity for phenotypic plasticity in warm-adapted or tropical species.

Thermal windows of R. temporaria and X. laevis

As this work is based on a comparison of two ecologically different species, which are supposed to differ in their thermal adaptation, **Chapter 3** and **4** assess the thermal window of both species studied illustrating the thermal range of tolerance to temperature variation (Dalvi et al. 2009). In temperate climates, amphibians tend to have a broader range of thermal tolerance, centered at lower temperatures than in tropical climates (Janzen 1967). Therefore, the species-specific thermal window is also broader in temperate, eurythermic species than in stenothermic artic or tropical species (Dalvi et al. 2009; Pörtner 2002). Since I found a substantially broader thermal window for *R. temporaria* (*R. temporaria*: 429.37°C²; *X. laevis*: 290.76°C; Fig. 8.2), I characterize *R. temporaria* as an eurythermic and *X. laevis* as a (poly-)stenothermic species in accordance with my results for the species-specific developmental thermal window (**Chapter 2**). The breadth of a species' thermal window determines its sensitivity to temperature variation and, thus, to climate change (Oyamaguchi et al. 2017)

indicating a different vulnerability of both species studied to the impacts of global warming with *X. laevis* being the loser in this particular comparison.

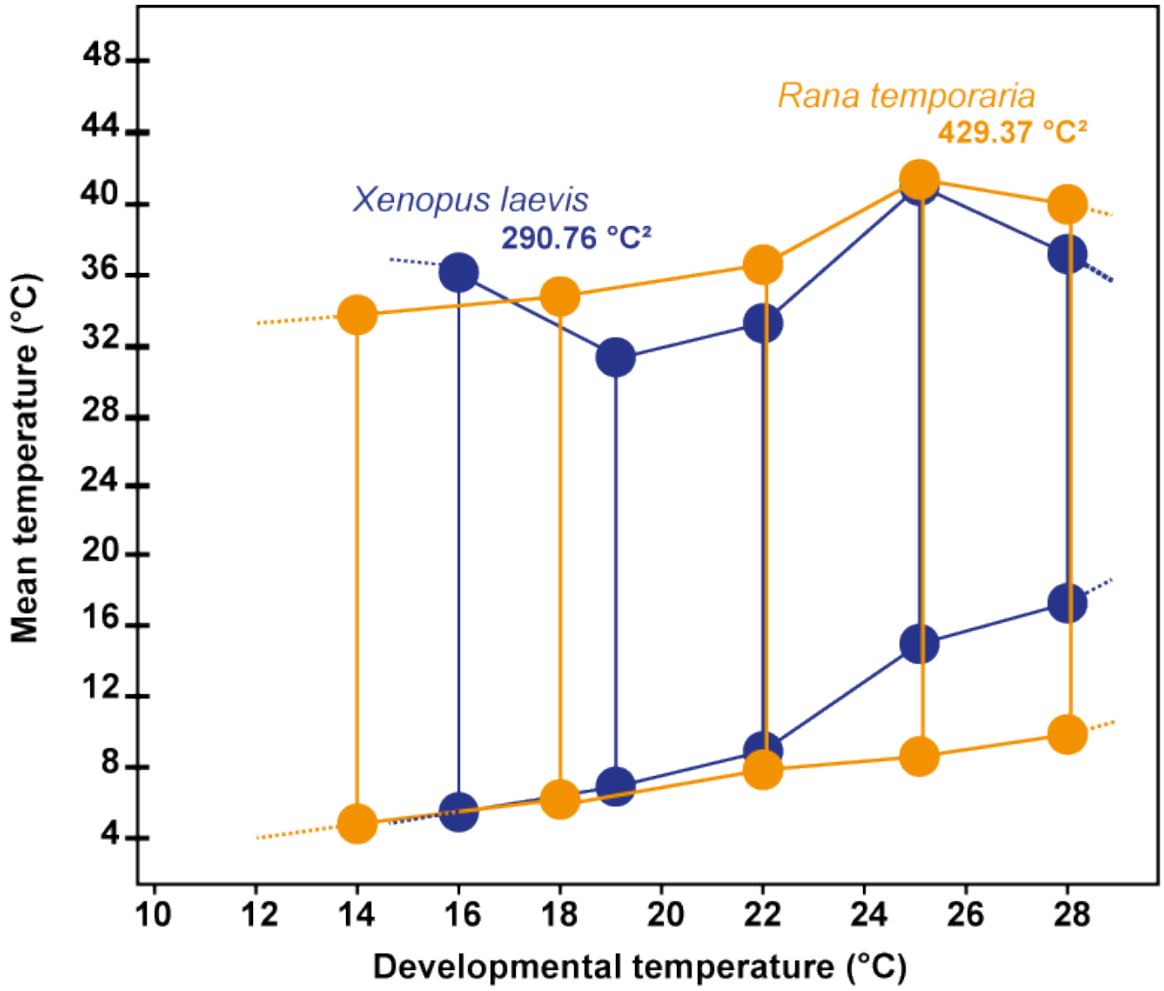

Fig. 8.2 Larval thermal windows of **orange:** the common frog (*Rana temporaria*; N=150) and **blue:** the African clawed frog (*Xenopus laevis*; N=45) at the onset of metamorphosis (Gosner stage 42; Gosner 1960). Thermal tolerance polygons were generated from the critical thermal limits (CT_{min} and CT_{max}) at five constant developmental temperatures. Lower circles= CT_{min}. Upper circles= CT_{max}. Line between CT_{min} and CT_{max} = thermal range of tolerance. Dotted lines = estimated trend for thermal limits at temperatures below/above the experimental temperatures.

Phenotypic plasticity as key to cope with climate change: A preliminary conclusion

The first four chapters of this dissertation illustrate that both species studied here but larval anurans in general are able to show temperature-induced phenotypic plasticity. Furthermore, my results emphasize that anuran larvae may differ in their capacity for developmental and physiological plasticity due to their thermal adaptation according to the hypothesis of Janzen (1967). Therefore, I suggest that thermal adaptation determines the species-specific vulnerability to the impacts of climate change to a great extent. However, the capacity for

temperature-induced developmental plasticity is known to be related to local adaptations in anuran larvae (Laugen et al. 2003; Muir et al. 2014; Drakulic et al. 2016) and thus, is population-specific. Usually, the capacity for temperature-induced plasticity is highest in populations from heterogeneous environments and/or high latitudes (Stählberg et al. 2001). In this study, I tested individuals of a single population in both species. Therefore, I would expect different capacities for temperature-induced developmental plasticity in different populations of *R. temporaria* and *X. laevis* related to local adaptations. In *R. temporaria*, numerous studies could demonstrate that the capacity for temperature-induced developmental plasticity is population-specific and arises from local adaptations due to geographic differences in mean temperature and temperature variation (Laugen et al. 2002, 2003; Drakulic et al. 2016; Grözinger et al. 2018). In contrast, there is no geographical gradient in metabolic rate in *R. temporaria* tadpoles (Lindgren and Laurila 2009). Therefore, I assume that the population-specific differences in the capacity for physiological plasticity in SMR of *R. temporaria* may be smaller than for growth and developmental rate. However, despite SMR, a possible energetic mechanism for the higher plasticity in high-latitude populations (Stählberg et al. 2001) could be a difference in maximum metabolic rates and metabolic scope (Lindgren and Laurila 2009). If so, (northern) populations with a high capacity for phenotypic plasticity may show increased metabolic maxima compared to less-plastic populations. Consequently, I suggest that those differences in metabolic maxima dictate the population-specific difference in the capacity for physiological plasticity and thus. Such differences might be especially important in the light of ongoing (and predicted) climate change (Drakulic et al. 2016). As plasticity may play a key role in the initial steps of the adaptation to rapid environmental change when genetic adaptation, a typically slower process that may span many generations, is unable to generate optimal phenotypes at required pace. Not accounting for variation in plasticity within a species can lead to inaccurate predictions about the vulnerability of populations to environmental change (Orizaola and Laurila 2016).

Global climate change affects ecosystems and ecological communities, leading to changes in the phenology, geographic ranges, or population abundance of several species. Thus, predicting the impacts of global climate change on the current and future distribution of invasive species is an important subject in macroecological studies (Ihlow et al. 2016). As shown in **Chapter 2**, selected experimental temperatures range in the developmental thermal window of both species studied and represent temperatures occurring in natural breeding of *R. temporaria* and *X. laevis* (Drakulic et al. 2016, 2017; Ihlow et al. 2016). According to this, tadpoles of both species are likely to experience temperatures as high as selected as

experimental rearing temperatures in this study in their natural larval habitat. Predicted effects of climate change suggest even higher temperatures as climate change will increase the frequency of extreme thermal events (Seebacher et al. 2015; Pachauri et al. 2014; Gutiérrez-Pesquera et al. 2016). Nevertheless, this study is based on constant experimental temperatures which do not correspond to natural conditions as most terrestrial and aquatic ectotherms experience daily and seasonal variation in temperature. Mean organismal performance can differ in fluctuating and constant thermal environments, an effect due to Jensen's inequality for non-linear functions (Ruel and Ayres 1999; Martin and Huey 2008; Kingsolver et al. 2015). Thus, models of ectotherm responses to climate change that are parameterized from data sets gathered under constant (or that assume no change in the variance of thermal regimes) may contain systematic errors when compared with the real world (Paaijmans et al. 2013). Future studies need to examine the difference between the effects of temperature fluctuation and different constant temperatures on phenotypic plasticity in the context of climate change. Even if a long-term increase in annual average temperature is the consequence of global climate change, environmental change also results in short-term, both stochastic and predictable, extreme thermal events, particularly in temperate freshwater habitats (Seebacher et al. 2015; Gutiérrez-Pesquera et al. 2016; Burggren 2018; Fig. 8.3). Therefore, laboratory studies on the capacity for physiological plasticity in thermal tolerance (especially in CT_{max}) are of special relevance for predictions on vulnerability of anuran larvae to those short-term thermal challenges.

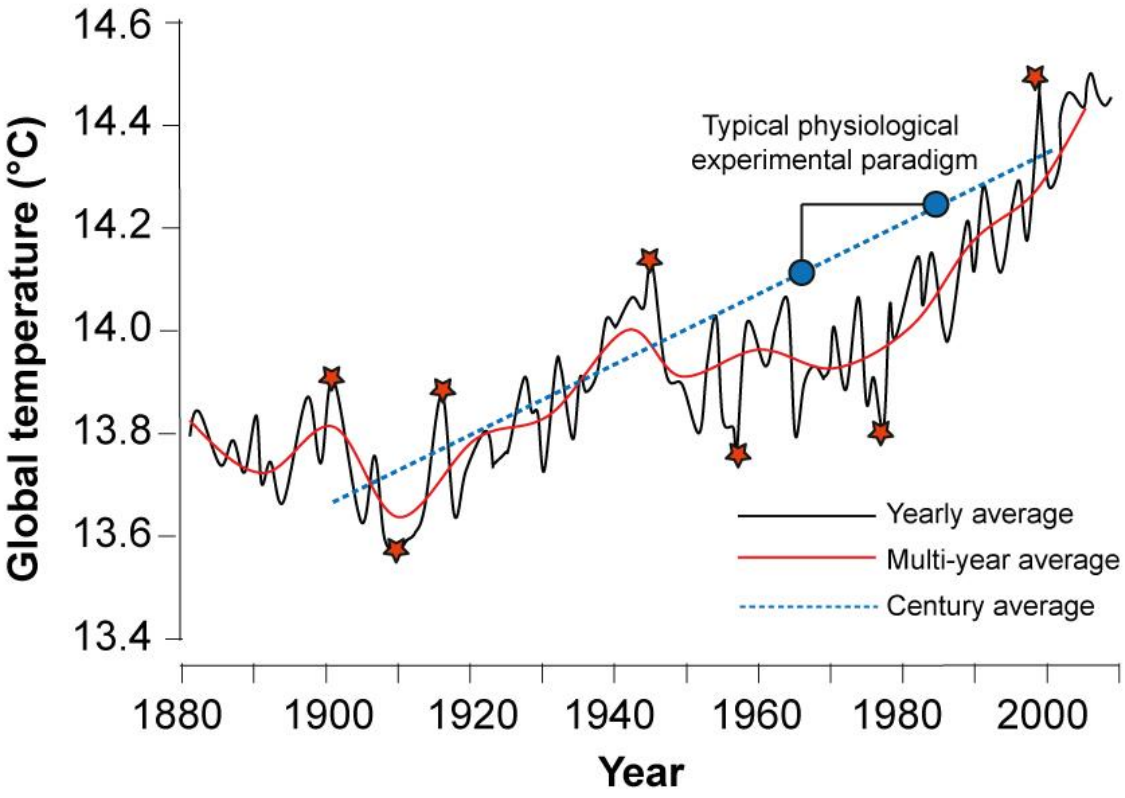

Fig. 8.3 Global temperature change (from 1880 to 2010) measured over different time frames and depicted in different ways, each leaving a different impression of the extent and rate of temperature change. Most physiological experiments exploring implications of global warming consider a change of a few degrees Celsius (taking the decades or century perspective), while fewer experiments consider the much larger, more stochastic temperature swings that actually represent what organisms experience over their individual life spans (modified from Burggren 2018; personal permission).

However, current IPCC models for global temperatures in the coming century (IPCC, 2014) do not suggest warming of the magnitude that would be likely to threaten temperate larvae such as *R. temporaria* with reaching their CT_{max} (Rowe and Crandall 2018). Contrary, positive effects of climate warming are expected in many organisms that currently occur below their thermal optima (Deutsch et al. 2008; Kingsolver et al. 2013; reviewed in Egea-Serrano and Van Buskirk 2016). This appears to be the case for *R. temporaria* in central and northern Europe (Duarte et al. 2012) but may not for populations in southern Europe as they are locally adapted to warmer temperatures (Lindgren and Laurila 2009). Climate change could therefore increase mean fitness of *R. temporaria*. Climatic anomalies as recorded in Europe for summer temperatures in 2003 (Neveu 2009) and recent spring and summer in 2018 (Deutscher Wetterdienst) representing the warmest seasonal temperatures since weather was recorded (Luterbacher et al. 2004; Neveu 2009; Deutscher Wetterdienst) may lead to sub-optimal temperatures accompanied by an increased desiccation risk and decreased food availability in larval habitats of *R. temporaria* (Fig. 8.4).

In contrast, previous studies demonstrated that tropical ectotherms have a limited acclimation ability (Janzen 1967, Ghalambor et al. 2006, Gunderson and Leal 2015, reviewed in Oyamaguchi et al. 2017) and low tolerance to extreme fluctuations in temperature (Janzen 1967, Addo-Bediako et al. 2000, Ghalambor et al. 2006, Deutsch et al. 2008, Tewksbury et al. 2008) compared with temperate species. My results are in line with those previous and generalized findings. As a consequence, the negative impacts of climate change should be greatest on tropical species such as *X. laevis* (Tewksbury et al. 2008; Oyamaguchi et al. 2017). Whereas range shifts have been observed in a wide range of (tropical) species with low thermal tolerance (Parmesan and Yohe 2003), individuals of *X. laevis* may have a lower capacity for dispersal as they remain aquatic after metamorphosis. Therefore, aquatic species such as *X. laevis* may be forced to persist in situ and compensate for environmental changes through phenotypic plasticity or genetic adaptation (Urban et al. 2014; Berg 2017). In contrast, numerous studies characterized *X. laevis* as a species capable of enduring extreme conditions and that both native and invasive populations of *X. laevis* move overland (reviewed in Measey et al. 2012). However, Ihlow et al. (2016) found that the future potential distributions in the species' native range in South Africa, as well as in the invaded areas in North and South America are predicted to decrease due to the increased frequency of heat waves (Fig. 8.5). In contrast, the potential range size in Europe is predicted to expand due to rising average temperatures and reduced precipitation across all climate change scenarios (Tinsley et al. 2015; Fig. 8.4, 8.5). If new thermal challenges outstrip the low capacity for phenotypic plasticity in *X. laevis*, prognoses may go from bad to worse. So far, it is challenging to project winners and losers in terms of climate change in light of the fact that multiple factors, beyond mere warming or increased variance in temperature, may simultaneously occur.

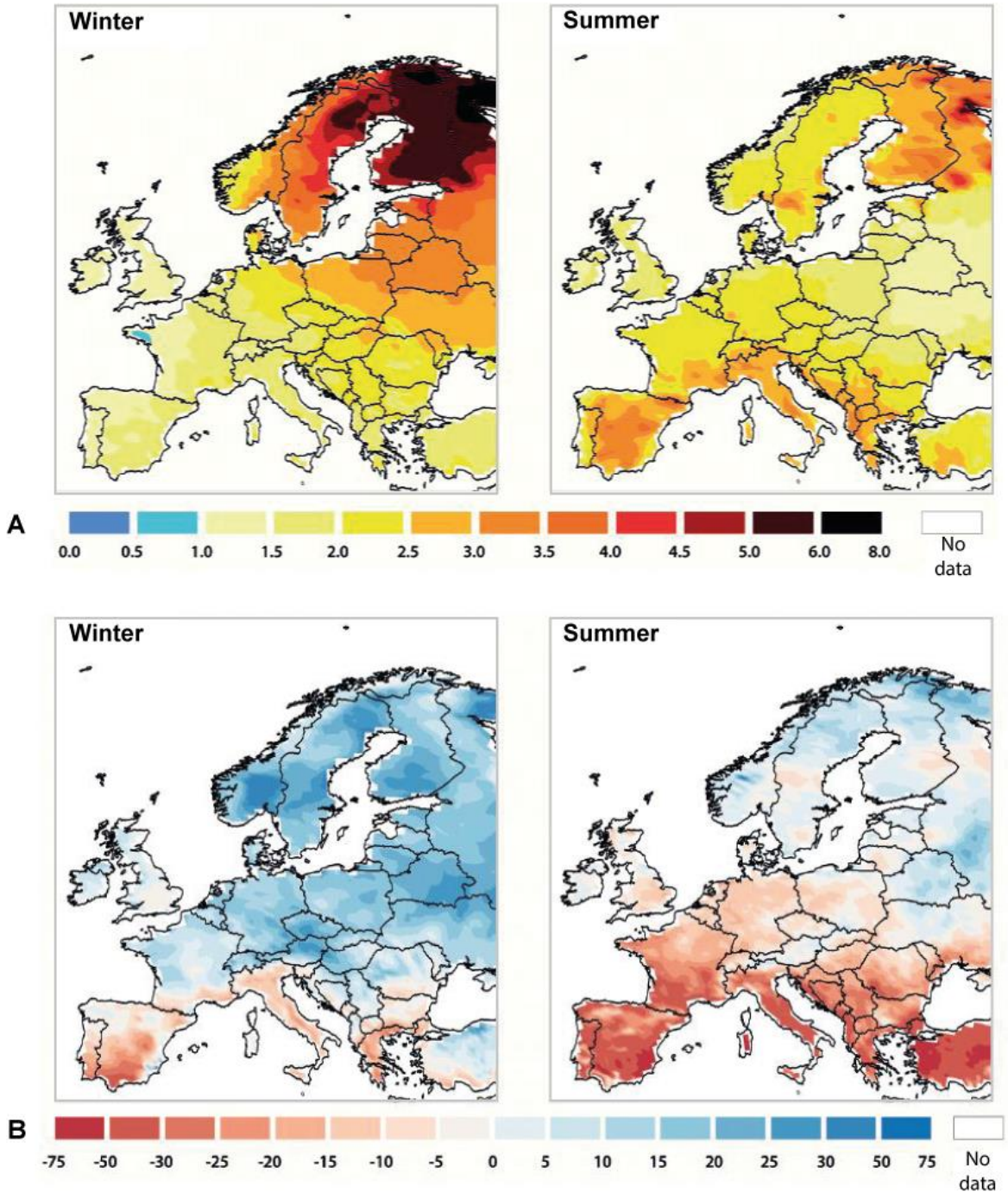

Fig. 8.4 Changes in annual temperature (in °C) and precipitation (in %) fluctuations for the timescale from 2071 to 2100 in comparison with the timescale from 1961 to 1990 predicted under climate change scenarious of a global temperature increase of 2°C. The scenario of a global temperature increase of 2 ° C represents a global average: even if it is realized, the temperatures in certain regions will rise by much more than 2 °C. In the winter months, temperatures in some parts of Scandinavia could rise by an average of 5 to 8 °C. In summer, temperatures could rise by an average of 3 to 4 °C in Spain and northern Scandinavia. The precipitation patterns for rain and snow may also change significantly. Winter precipitation is predicted to increase by more than 25% in some parts of Central Europe and Scandinavia. Summer rainfall is predicted to be reduced by more than 50% along the Mediterranean coast (modified from Ciscar et al. 2014).

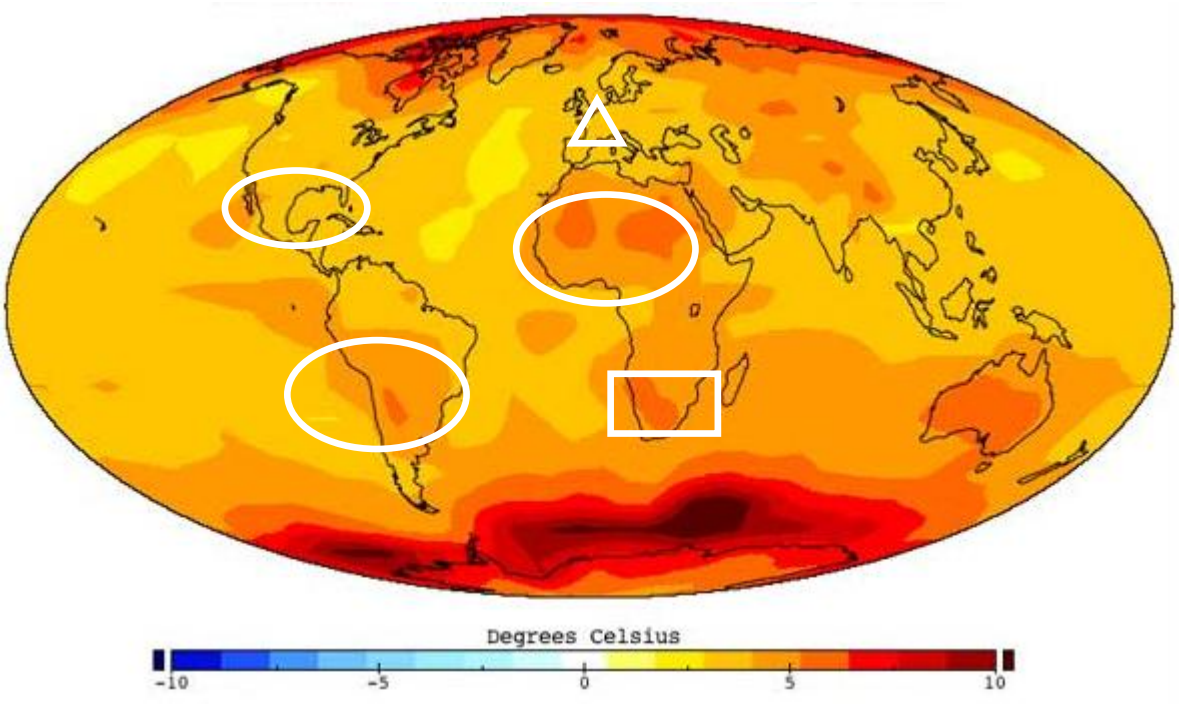

Fig. 8.5 Global temperature change from 1960 to 2060 (modified from NASA 2005). Square= native range of the African clawed frog (*X. laevis*) with predicted decreasing future distributions. Triangle= range of invasive *X. laevis* populations in Europe with predicted increasing future distributions. Ellipses= range of invasive *X. laevis* populations in North and South America and northern Africa with predicted decreasing future distributions (see Ihlow et al. 2016).

Environmental stress impairs the capacity for phenotypic plasticity

The capacity for developmental and physiological plasticity is not only related to the thermal adaptation but also to phylogenetic constraints (Somero 2005; Seebacher et al. 2012), biochemical limitations (reviewed in Little and Seebacher 2016), habitat structures (Angilletta et al. 2002), and behavioral ecology (Theisinger et al. 2017). In the course of ongoing anthropogenic global change, recent studies investigated abiotic and biotic factors which impair or disturb natural ecosystems, accordingly described as environmental stressors (Williams and Jackson 2007; Narayan 2016; Niinemets et al. 2017). The role of environmental stress in amphibians and amphibian life-history has recently received attention (Räsänen et al. 2003; Gabor et al. 2013, 2017; Kaiser et al. 2015; Egea-Serrano and Van Buskirk 2016; Freitas et al. 2017) but yet little is known about the interactive effects of different environmental stressors which occur simultaneously or sequentially in natural environments. As environmental stressors may proximately influence amphibian metamorphosis through their endocrine-disruptive effect on the TH system, **Chapter 2**, **3**,

and **4** investigated how proximate effects of environmental stressors affect the capacity for (temperature induced) phenotypic plasticity. In both species studied here, altered TH levels modified the capacity for developmental (**Chapter 2**) and physiological plasticity (**Chapter 3** and **4**) to a temperature-given change independent from their thermal adaptation. Consequently, stressed larvae may be more vulnerable to the impacts of climate change in terms of lacking the capacity for phenotypic plasticity.

Proximate effects of environmental stress modify the thermal reaction norm of metamorphic traits

Our results are in accordance with previous studies investigating the effect of altered TH levels on growth and developmental rate indicating an accelerating effect of increased TH levels on developmental rate whereas low TH levels decrease developmental rate (**Chapter 2**, **5**, and **6**). Therefore, endocrine disruption of the TH system may result in either large but old or young but small metamorphs which are both known to be disadvantaged in comparison with conspecifics developing in undisturbed larval habitats. Effects of altered TH levels were more pronounced at extreme temperatures in both species studied (**Chapter 2**). However, knowledge of how these proximate effects of environmental stressors affect the thermal reaction norm of metamorphic traits and thus, may impair the capacity for temperature-induced developmental plasticity is rare.

I demonstrated that altered TH levels alter the capacity for the two species studied here to express temperature-induced developmental plasticity but to different extent (**Chapter 2**). If environmental stressors inhibit or delay TH production during metamorphosis, the capacity for temperature-induced developmental plasticity can increase as demonstrated here in tadpoles exposed to the environmental relevant toxin SP. Inhibition of TH pathways is usually caused by environmental pollution and, therefore, chemical contamination of the larval habitat (Mann et al. 2009). Consequently, tadpoles in habitats polluted by agricultural fertilizers and pesticides (Mikó et al. 2017), industrial chemicals (Lefcort et al. 1998, Ossana et al. 2017) or fireworks (Sparling and Harvey 2006; Bulaeva et al. 2015), may have a slower rate of development, are larger at the onset of metamorphosis but maintain a higher developmental plasticity. Therefore, those tadpoles may be less vulnerable to the impacts of climate change. However, the majority of environmental factors activates stress-hormones and thus, causes high TH levels in anuran larvae (Dantzer et al. 2014; Denver 1997; Bonett et al. 2010). Tadpoles of both species studied exposed to high levels of TH were less able to adjust their rates of growth and development at warmer temperatures indicating that high levels of TH

impair the ability to display temperature-induced developmental plasticity (**Chapter 2**). Therefore, environmental stressors, which increase TH levels via the activation of stress-hormones, are likely to make tadpoles in general more vulnerable to the impact of warmer temperatures. However, the capacity for temperature-induced developmental plasticity is known to be population-specific due to local adapatations, I assume different impacts of altered TH levels on this capacity in different populations of *R. temporaria* and *X. laevis*. Those impacts might be worst in populations with a low baseline capacity for phenotypic plasticity.

Physiological plasticity under environmental stress

My results demonstrate that physiological traits in larvae of *R. temporaria* and *X. laevis* are affected by altered TH levels but to different extent (**Chapter 3** and **4**). Whereas SMR was increased by high TH levels (i.e. at exposure to T4) in both species studied here, low TH levels lead to different effects on SMR. In larvae of *X. laevis*, SMR was decreased when larvae were exposed to SP. In contrast, larvae of *R. temporaria* revealed an increased SMR at exposure to SP. My results demonstrate that high levels of TH have a distinct increasing effect on metabolic rate confirming that high levels of TH (i.e. by exposure to T4) determine the metabolic rate in ectotherms as shown for lizards (*Dipsosaurus dorsalis* and *Sceloporus occidentalis*; John-Alder 1983, 1990), snakes (*Thamnophis sirtalis*; Etheridge 1993), and the leopard frog (*Rana pipiens*; Steyermark et al. 2005). Thus, environmental stressors which increase TH levels (e.g. increased desiccation risk, crowding, presence of predators) may result in slightly increased SMRs. In amphibians, elevated SMR due to increased TH level manifests in increased activities of enzymes and densities of mitochondria in metabolic relevant tissues such as liver and red skeletal muscle (Chiu and Woo 1988; Rowe et al. 1998; Steyermark et al. 2005) with possible ramification for energy budgets and allocation (Orlofske and Hopkins 2009).

In contrast, low levels of TH (i.e. by exposure to SP or other endocrine disruptors) lead to contrary results in larvae of *R. temporaria* and *X. laevis* leading to different explanatory approaches: Data for *X. laevis* are in line with the above conclusion that SMR increases with TH level as THs are critical for regulating energy metabolism in vertebrates (Sheridan 1994; Rowe et al. 1998; Choi et al. 2017) even if the effect of THs on metabolic heat production in ectotherms such as amphibians is negligible (John-Alder 1983). Low levels of TH thus, result in low SMRs. As SMR was slightly increased at high TH levels as well as through exposure to SP in *R. temporaria*, I suggest that aquatic contaminants alone may also result in increased

metabolism due their toxicity, possibly independent from the thyroid metabolism, as shown in fish (Beyers et al. 1999), larval amphibians (McDaniel et al. 2004), and bivalves (Lannig et al. 2008; reviewed in Hallman and Brooks 2015). Therefore, larvae of *R. temporaria* may be more sensitive to aquatic contaminants compared with larvae of *X. laevis* at equal doses of T4 and SP indicating a generally higher vulnerability to chemical stressors in addition to possible endocrine-disruptive effects of those. Although THs have recently been shown to play a key regulatory role in thermal acclimation in fish (Little and Seebacher 2014; 2016) and several early studies provide an indication on thyroid-regulated acclimation in amphibians and reptiles (Locker and Weish 1966; Packard and Packard 1975; Little and Seebacher 2016), my results approve this merely obviously in larvae of *X. laevis* as altered TH levels impaired the thermal acclimation. In the present study, the effect of altered TH levels on SMR was intensified at warmer temperatures in larvae of *X. laevis* whereas SMR was decreased by the interactive effect of altered TH levels and warmer temperatures in larvae of *R. temporaria* indicating an overcompensation. Therefore, larvae of *X. laevis* exposed to warmer temperatures are likely to be more affected by environmental stressors than larvae at colder temperatures. Moreover, high TH levels reduced the generally low acclimation capacity in thermal tolerance in *X. laevis*. As global warming impacts wildlife in complex ways through synergistic interactions with other extreme environmental stressors, stressed larvae which cannot compensate for temperature variation such as *X. laevis* in the present study will be most affected.

Stressed larvae of R. temporaria and X. laevis in a warming world

Climate change is altering thermal patterns with potentially important repercussions for larval amphibians simultaneously stressed by contaminants or other environmental stressors (Hallman and Brooks 2015). The present findings demonstrate that tadpoles of *X. laevis* may suffer from this interactive effect of warmer temperatures and a disrupted TH system by several other environmental stressors in the course of global change as altered TH levels impair the ability for temperature-induced developmental plasticity. Furthermore, larvae of *X. laevis*, contrary to larvae of *R. temporaria*, were affected by the interactive effect of increased TH levels and warm temperature resulting in a more distinct effect on SMR with possible ramifications for energy stores and therefore a successful completion of metamorphosis (**Chapter 3**, **4**, and **5**). Moreover, owing to the generally lower capacity for phenotypic plasticity in warm-adapted species such as *X. laevis* in the present study (**Chapter 1-4**; Janzen 1967), effects of altered TH levels (especially of increased TH levels) on the

capacity for developmental and physiological plasticity may be more pronounced in larvae of *X. laevis* compared to larvae of *R. temporaria*. Consequently, environmental stress may face tropical species with a higher increase vulnerability to climate change effects than temperate species. Nevertheless, larvae of *R. temporaria* are known to respond more plastically to environmental variation than other species (Laurila and Kujasalo 1999) especially in terms of growth and developmental rate. Therefore, temperate species in general but especially larvae of *R. temporaria* may compensate better for direct (i.e. temperature increase) and indirect effects (i.e. shortening of hydroperiod, increasing tadpole density, and the concentration of contaminants as the pond desiccates; Egea-Serrano and Van Buskirk 2016) of global warming through temperature-induced developmental and physiological plasticity than tropical species. However, different populations of *R. temporaria* differ in their capacity for phenotypic plasticity due to local adaptations (Stählberg et al. 2001; Hjernquist et al. 2012; Muir et al. 2014; Orizaola and Laurila 2016; Drakulic et al. 2016; 2017). I suggest that other temperate species but also other populations of *R. temporaria* may be stronger affected by environmental stressors than the present population but also own a higher capacity for phenotypic plasticity and thus, making them less vulnerable to effects of global and climate change than tropical species. Since *R. temporaria* is the most widespread anuran species in Europe occurring in a variety of habitats it is a suitable model organism for temperate generalistic species which are expected to own a comparable capacity of phenotypic plasticity such as the Common toad (*Bufo bufo*) or species belonging to the water frog complex (*Pelophylax esculentus complex*) in Europe or the wood frog (*Rana sylvatica*) in Northern America. Temperate anuran specialists such as the European fire-bellied toad (*Bombina bombina*) or the yellow-bellied toad (*Bombina variegate*) may be more vulnerable to drivers of global change than larvae of *R. temporaria*.

My results emphasize that the capacity for temperature-induced phenotypic plasticity is not only determined by population-specific thermal adaptation but also by the multiple, covarying effects of natural and anthropogenic environmental stressors in a larval habitat. Even if this study presents *R. temporaria* and *X. laevis* as model organisms for temperate und tropical species, respectively, predictions of vulnerability to climate change effects should always consider and evaluate the complexity of factors which determine the population- or species-specific capacity for developmental and physiological plasticity.

Getting off to a bad start

The results of this dissertation highlight that the capacity for temperature-induced developmental and physiological plasticity is determined by a complexity of factors such as thermal adaptation but also environmental stress due to the drivers of global change. Consequently, tadpoles may differ in age, size, and energetics (i.e. SMR and body condition) at the onset of metamorphosis as a result of this population- or species-specific capacity for phenotypic plasticity with consequences for energy budget and allocation during metamorphic climax. Therefore, **Chapter 3-6** investigates whether environmental stressors impair the ability to accumulate energy in internal stores which are used to fuel the complex reorganization of larval to juvenile tissue during metamorphic climax. I found that warmer temperatures, altered TH levels and the interactive effect of both strongly affect the size of energy stores and thus, body condition at the onset of metamorphosis in both species studied here. Moreover, differences in age, size, and energetics may impair the energetic efficiency of metamorphic climax resulting in different total energetic costs and energy allocation to maintenance and developmental costs. Hence, the **final Chapter** of this dissertation investigates if different starting conditions in age, size, and energetics at the onset of metamorphosis as a result of environmental stressors experienced during larval stage impact energetic costs and energy allocation during metamorphic climax in *R. temporaria*. My results demonstrate that environmental stress during larval stage results in serious differences in terms of energetic efficiency of metamorphosis and thus, direct and indirect costs of metamorphosis.

Environmental stress impairs the starting conditions into metamorphic climax

Metamorphic climax is a period of profound changes in the morphology and physiology of anuran larvae and thus, a highly energy-consuming stage that is entirely supported by stored energy (Crump 1981, Beck and Congdon 2003; Orlofske and Hopkins 2009; Kirschman et al. 2017). Larvae, which experienced stressful conditions until the onset of metamorphosis, may differ in age, size, metabolic rate and body condition due to stressor-induced changes of TH level. In their complexity, these starting conditions determine efficiency and success of metamorphic climax with persisting effects on juvenile froglets. My data illustrate the following starting conditions into metamorphic climax after a stressful larval stage (**Chapter 6**): Larvae exposed to TH level decreasing environmental stressors (i.e. most aquatic contaminants) are 27% heavier, 28% larger, and 24% older compared with non-stressed larvae but reveal a 29% lower body condition. In contrast, larvae exposed to TH level

stimulating stressors (e.g. extreme temperature variation, predation pressure, and crowding) are 47% lighter, 46% smaller, and 23% younger compared with non-stressed larvae and reveal a 12% lower body condition. I suggest that exposure to environmental stressors which cause an alteration in TH status can alter the allocation of this stored energy to development during metamorphic climax and thus, may alter energetic costs of entire climax.

Environmental stress impacts energetic efficiency of metamorphosis

Tadpoles which experienced an alteration of TH status through environmental stress until the onset of metamorphosis started into metamorphic climax at different ages and sizes due to differences in growth and developmental rate (**Chapter 2, 5,** and **6**). Since developmental rate determines the total energy used for physiological and morphological reorganization and basal energy expenditure during metamorphic climax, tadpoles with high TH levels need absolutely the smallest amount of energy whereas tadpoles with low TH status claim the largest amount. However, depending on the TH status the percentage of this total amount of energy used which is allocated to development differed: Tadpoles with low TH status allocated about 60% more energy to development than those with high TH status and one third more than tadpoles with unaffected TH status. Further, tadpoles with high TH levels needed more energy to cover their maintenance costs although as much energy as possible should be allocated to development during this stage of profound physiological and morphological changes. Accordingly, metamorphic climax is energetically costlier when TH status is high as usually induced by environmental stressors such as predation pressure, increasing desiccation risk, or temperature variation. Environmental stress therefore results not only in different starting conditions into metamorphic climax through impacts on developmental and growth rate, but also alters energy allocation for development and maintenance costs during metamorphic climax.

Indeed, these differences in energetic costs may be related to a strong size-effect since TH level stimulating or decreasing environmental stressors resulted in different sizes at the onset of metamorphosis. Beck and Congdon (2003) and Orlofske and Hopkins (2009) found similar negative relationships between percent developmental costs and size at the initiation of metamorphic climax in the southern toad (*Bufo terrestris)* and in the pickerl frog (*Lithobathes palustris*). Therefore, a large body size at the onset of metamorphosis as found in tadpoles exposed to the environmental relevant endocrine disruptor SP has a significant physiological advantage over smaller tadpoles during the metamorphic climax because they complete metamorphosis more efficiently (i.e. used proportionally less total energy for metamorphic

climax) than their smaller counterparts (Pandian and Marian 1985, Orlofske and Hopkins 2009). However, I assume that energetic efficiency may be also affected by a size-independent effect of achieved TH status such as the anabolism and catabolism of macronutrients (Wright et al. 2011; Kirschman et al. 2017). This effect could compound the strong size effect on energetic efficiency. Nevertheless, the large size differences impede a more precise statement about this effect.

Costs of metamorphosis

Ongoing climate change and anthropogenic disturbances of larval habitats will result in altered phenotypes at the onset of metamorphosis (**Chapter 1-6**) and relative higher energetic costs during climax resulting in energetically inefficient completion of metamorphosis (**Chapter 6**) since most environmental stressors lead to an increase in TH level in anuran larvae (Dantzer et al. 2014; Denver 1997; Bonett et al. 2010). As in larvae with high TH levels less energy can be allocated to development, I suggest that this energy default during the complex reorganization of larval to juvenile structures may lead to malformations or morphological defects impairing fitness in later life stages. If environmental stressors inhibit or delay TH production during metamorphosis as caused by most aquatic contaminants (reviewed in Mann et al. 2009), the prolonged remaining in larval development is concomitant with increased pressures by predators in the aquatic habitat and desiccation risk (Lefcort et al. 1998; Kloas and Lutz 2006). Although climax is energetically more efficient at low TH levels, mortality risk increases as emerging from the larval pond is delayed. Consequently, environmental stress increases costs of metamorphosis in either cases of TH level alteration directly (i.e. through an increase of total energetic costs at low TH levels or energetic inefficiency at high TH levels) or indirectly (i.e. through a prolonged remaining in larval stage at low TH levels or developmental defects at high TH levels). Therefore, environmental stress may largely alter the direct or indirect costs of metamorphosis with possible ramifications of development resulting in carry-over effects in later life stages. Although larvae of *R. temporaria* own a high capacity for developmental plasticity and the ability to acclimate their SMR, the altered growth and developmental rates due to environmental stress may incur additional costs and an increased mortality risk. I suggest that environmental stress of any kind disrupts the natural balance of energy allocation to growth, development, and metabolism (i.e. maintenance costs) independent from the capacity for developmental and physiological plasticity resulting in a less successful and efficient metamorphosis.

Metamorphosis is not a new beginning

In organisms with complex life cycles such as anurans, factors in the larval environment have strong carry-over effects on juveniles and adults (Smith 1987; Berven 1990; Goater 1994; Pechenik 2006; Altwegg and Reyer 2003; Scott et al. 2007, Morey and Reznick 2001) and thus, important fitness consequences and persistent population-level impacts (Bouchard et al., 2016). Therefore, Pechenik (2006) emphasized that metamorphosis is not "a new beginning". This dissertation focusses on the capacity for temperature-induced phenotypic plasticity in anuran larvae and how environmental stressors as caused by drivers of natural and anthropogenic global change affect this capacity. As most studies only assume that age and size at the onset of metamorphosis is related to fitness in later life and very few studies really tested for complex carry-over effects including all metamorphic and physiological traits investigated in the present study, I examined whether environmental stress experienced during larval stage is really related to carry-over affects in juvenile stage. **Chapter 4** and **6** therefore investigate whether possible differences of body condition at the onset of metamorphosis due to environmental stress persist after completion of metamorphosis (**Chapter 4**) and in juvenile frogs (**Chapter 6**) of *R. temporaria*. I found that negative effects of warmer temperature, altered TH levels, and the interactive effect of both on body condition persist in froglets after completion of metamorphic climax (**Chapter 4**). Seven days after the completion of metamorphosis, juvenile froglets which experienced altered TH levels during larval stage compensated for these differences (**Chapter 6**). My results demonstrate that effects of altered TH levels can carry across metamorphosis and affect juvenile performance and thus, may decrease survival until maturity.

Environmental stress impairs post-metamorphic performance

I found that froglets which experienced a decrease in TH levels during larval stage reveal the best juvenile performance whereas froglets exposed to high TH levels reveal the poorest performance. As a consequence of the alteration in TH levels during larval stage, these froglets obviously differed in body size. Therefore, carry-over effects on juvenile performance were mainly mediated by variation in metamorphic body size due to the alteration of TH levels during larval stage (**Chapter 6**) which is in accordance with findings of previous studies. Beck and Congdon (1999) demonstrated a positive relationship of size and sprint speed, and endurance in the southern toad *(Bufo terrestris)* and John-Alder and Morin (1990) found that size at metamorphosis was positively related with jumping ability in the Fowler's toad *(Anaxyrus woodhousii,* formerly *Bufo woodhousii*). Furthermore, Goater et al. (1993)

found a positive relationship of size and burst speed in the Common toad *(Bufo bufo).* Nevertheless, I assume that juvenile performance may be also affected by a size-independent effect of achieved TH status since THs also control limb development during metamorphosis. If energy allocation to development during metamorphic climax is impaired due to altered TH levels (**Chapter 5**) limb development could be negatively affected as well. This effect could compound the strong size effect on juvenile performance especially in froglets formally exposed to high TH levels as metamorphosis is energetically inefficient. However, the large size differences impede a more precise statement about this effect.

Locomotor performance can have a positive influence on dispersal from natal ponds, food acquisition and predator avoidance (Beck and Congdon 2000; reviewed in Alvarez and Nicieza 2002). Therefore, tadpoles exposed to environmental stressors which increase TH levels may suffer from difficulties in food acquisition and predator avoidance associated with their poor locomotor performance. Those difficulties may inhibit compensation of growth and storage of energy reserves. Furthermore, Morey and Reznick (2001) found that smaller metamorphs sustained higher mortality rates due their increased foraging activity to compensate for growth differences and thus, a reduction of poor larval environment as in froglets exposed to exogenous T4 during larval stage. In contrast, froglets which experienced low TH levels during larval stage due to aquatic contamination may indeed reveal a strong juvenile performance and thus, may display an advantage in food acquisition and predator avoidance. Nevertheless, the number of froglets which emerge from the natal pond may be extremely reduced under natural conditions due to their substantially longer duration of metamorphosis. Consequently, environmental stress leads to size-dependent carry-over effects on juvenile performance with possible ramifications through impairing the natural balance of energy allocation to growth, development, and metabolism during larval stage independent from the capacity for phenotypic plasticity.

In temperate hibernating anurans such as the common frog (*R. temporaria*) a strong locomotor performance is also extremely beneficial for accumulating reserves of energy which are used during hibernation for survival and gonad development (Reading and Clarke, 1995) and thus, directly linked to juvenile and adult survival. Energy reserves are built up after emergence before the onset of the next winter (reviewed in Reading and Clarke 1995; Chen et al. 2011). Therefore, froglets which experienced environmental stress during larval stage may suffer from stressful larval conditions in two different ways: A small body size reduces locomotor performance which in turn impedes foraging and thus, storage of energy

reserves essential for successful hibernation. Werner (1986) found such a positive correlation of size at metamorphosis and adult survival in ranids and thus, a stronger performance in larger individuals. However, Boone (2005) could not find a strong relationship between size at metamorphosis associated with environmental stressors (i. e. crowding and chemical exposure) and probability of surviving the winter in ranids and bufonids. Therefore, Boone (2005) suggested that species may differ in the relationship between metamorphic size and terrestrial survival, and therefore, how metamorphic size interdepends with performance and terrestrial survival. Consequently, environmental stress may affect other species' performance less than *R. temporaria* in terms of a size-dependent carry-over effect.

Environmental stress and amphibian decline

It has been suggested that climate change itself may not affect larval amphibians, but rather will act in combination with abiotic and biotic factors increasing their effects (Lopez-Alcaide and Macip-Rios 2011; Baier et al. 2016). These possible interactions of different stressors and warmer temperatures are not well known (Boone et al. 2007; Polo-Cavia et al. 2017) and knowledge on relationships between amphibian declines and the interactions of impacts due to global change and other environmental stressors are still rare (Blaustein et al. 2011). Nevertheless, altered environmental scenarios may act directly and simultaneously at various levels or organization, including individuals, populations, and even communities; and likely affect fauna complex manners that include bottom-up and top-down interactions (Navas et al. 2016).

In this dissertation, I emphasize bottom-up effects perceivable at an individual level which influence the ecological performance of individuals and, in turn affect population dynamics and demography. Specifically, this work connects ecophysiological studies with anuran conservation since it contributes to a better understanding of the complex and various effects of global change on amphibian metamorphosis and possible impacts in later life stages and thus, long-lasting effects on amphibian populations. Although investigating the species-specific capacity for phenotypic plasticity is of key importance for predicting the vulnerability to global change, knowledge on how environmental stressors may affect the survival rate in tadpoles is likewise essential. Therefore, **Chapter 2-6** investigate how warmer temperatures, altered TH levels, and the interactive effect of both impairs survival until the onset of metamorphosis (**Chapter 2, 3, 5, 6**) and to juvenile stage (**Chapter 6**).

Environmental stress impairs survival to juvenile stage in amphibian larvae

My results demonstrate that survival until the onset of metamorphosis was reduced at warmer developmental temperatures in both species studied here (**Chapter 2, 3, 5**) but only *X. laevis* revealed an increased mortality due to the interactive effect of warmer developmental temperatures and altered TH levels as caused by environmental stressors (**Chapter 2** and **3**). **Chapter 2, 3,** and **5** demonstrate that survival was reduced at altered TH levels in *X. laevis* but only high TH levels reduced survival in larvae of *R. temporaria* (**Chapter 6**). Survival to juvenile stage was reduced in froglets of *R. temporaria* which were exposed to high TH levels during larval stage (**Chapter 6).** As survival of *X. laevis* was reduced by warmer temperatures, altered TH levels, and the interactive effect of both until the onset of metamorphosis (**Chapter 2, 3,** and **5**) and *X. laevis* generally reveal a higher sensitivity to environmental stressors (**Chapter 2** and **3**), I assume that survival to juvenile stage may also be detrimentally impacted in *X. laevis*. However, in order to be able to make exact statements for a species, it is indispensable to investigate the effects of environmental stress also regarding to this species. The negative impacts of warmer temperatures and the endocrine-disruptive effect of environmental stressors on survival seem to be independent from the capacity for phenotypic plasticity, since a species with a high ability for plastic responses such as *R. temporaria* in the present study revealed lower survival rates. Therefore, survival of larval amphibians in general may be reduced by environmental stressors. Since I could demonstrate that most environmental stressors may not only reduce survival to juvenile stage but may also reduce body size and impair the locomotor performance, I assume that these impacts may also impair survival until maturity and thus, could have important impacts on population persistence (James and Semlitsch 2011) which in turn may also affect metapopulation dynamics (Chelgren et al. 2006). Accordingly, the effects of warmer temperatures and environmental stressors due to global change on individual and population level investigated in the present study are definitely among the discussed causes of worldwide amphibian declines since aquatic and terrestrial habitat quality determines the reproductive success and persistence of amphibian populations (James and Semlitsch 2011).

General Conclusion

Environmental change due to global warming and other environmental stressors is complex and often unpredictable and anuran larvae will therefore be exposed to multiple environmental stressors. Climate change might increase the impact of other threatening factors, such as pathogens, land-use change, UV radiation, pollution or invasive alien species which in turn will impact populations (Ficetola and Maiorano 2016). Unfortunately, studies on amphibian conservation are increasingly focused on one single stressor. This ecophysiological study combines the proximate effects of the multiple stressors accompanying direct effects of climate change in a multifactorial design. Consequently, my results contribute to a better understanding of the complex and various effects of global change on amphibian metamorphosis and possible impacts in later life stages and thus, long-lasting effects on amphibian populations.

My results emphasize that in both studied species altered TH levels as caused by several environmental stressors modified the capacity for developmental plasticity to a temperature-given change independent of their thermal adaptation. Proximate effects of several environmental stressors due to global change may therefore result in ramifications for survival and fitness in later lifer stages. In contrast to larvae of the tropical *X. laevis,* larvae of the temperate *R. temporaria* were able to exhibit physiological plasticity in both metabolism and thermal tolerance. Altered TH levels affected this capacity for physiological plasticity in both species. Consequently, warm-acclimated and/or stressed larvae may be more vulnerable to the impacts of climate change in terms of lacking or losing the capacity for phenotypic plasticity.

The capacity for temperature-induced (developmental) plasticity is known to be related to local adaptations in anuran larvae and thus, is not only species-specific but also population-specific. Therefore, different populations may be differently affected by environmental stressors in their capacity for phenotypic plasticity. For general projections, investigations of further populations and/or species are indispensable. I therefore call for more studies on geographic variation in capacity for temperature-induced plasticity and how environmental stressors affect this capacity in different populations to make more precise predictions in the light of global (climate) change in both species studied here and other anuran species.

Laboratory studies like this in hand give detailed information about the influence of proximate factors such as temperature and other environmental stressors. However, there is little information on whether these factors act together in nature. To evaluate the factors that actually impact metamorphosis in natural amphibian populations, field studies are necessary (Loman 2004). In contrast, a field study cannot investigate whether or not a particular factor

actually affects tadpole performance since the effect of a factor may be offset by another factor having the opposite effect (Loman 2002). Therefore, research on how environmental stressors affect the capacity for developmental and physiological plasticity should be fortified with both laboratory and field experiments. Both methods combined will give a more precise overview how single and multiple stressors affect this capacity and thus, determine the vulnerability to global change.

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Contribution to thesis chapters

The following table summarizes my contributions to published articles and submitted manuscripts included in this thesis:

- Chapter 1 - **Patterns of temperature induced developmental plasticity in anuran larvae**
- Chapter 2 - **Developmental plasticity in amphibian larvae as a key to coping with the proximate impacts of global change**
- Chapter 3 - **Thyroid hormone levels and temperature during development alter thermal tolerance and energetics of *Xenopus laevis* larvae**
- Chapter 4 - **Multiple environmental stressors reduce physiological plasticity and body condition during and after metamorphosis in the common frog (*Rana temporaria*)**
- Chapter 5 - **Altered thyroid hormone levels affect body condition at metamorphosis in larvae of *Xenopus laevis***
- Chapter 6 - **Environmental stress alters costs of amphibian metamorphosis in the common frog (*Rana temporaria*)**

Contribution to	**Chapter 1**	**Chapter 2**	**Chapter 3**	**Chapter 4**	**Chapter 5**	**Chapter 6**
Study design	lead	lead	lead	lead	lead	lead
Data collection	lead	lead*,**	lead*	lead**	lead*	lead**
Data analysis	lead	contributed	contributed	lead	contributed	lead
Writing the manuscript	lead	lead	lead	lead	lead	lead

* Data collection was conducted equally by Janica Reese, Lisa Hartmann, Laura I. Becker and me.

** Data collection was conducted equally by Tom Robinson, Steffen Reinhardt and me.

Declaration of Oath

I hereby declare on oath that the work in this dissertation is my own and that I have not used other than the acknowledged resources and aids.

Katharina Ruthsatz

Hamburg, 18.10.2018

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