Multi-level Analysis of Compensatory Growth

Katie Leigh Hector

A thesis

submitted to the University of Otago

in partial fulfilment of the requirements for the degree of

Master of Science

in Zoology

University of Otago, Dunedin,

New Zealand.

2011

Abstract

Compensatory growth is faster than optimal growth, and commonly occurs following dietary restriction in early development. This alternative growth strategy allows an animal to reach a "normal" adult size, influencing reproductive fitness. However, the rapid growth required to reach a large size after growth restriction comes at a cost of accumulated cellular damage. Compensatory growth has evolved as an alternative growth strategy because these costs are often incurred late in life, after the reproductive period. The aim of this thesis was to address the issue of compensatory growth on two levels: first, in an empirical study of one species, and second, in a quantitative review of numerous taxa.

I investigated the effects of early dietary restriction on the growth, development and morphology of *Litoria ewingii* tadpoles, as well as on three fitness-related behavioural traits. This is the first known study to follow the effects of compensatory growth in an amphibian beyond metamorphosis, and to simultaneously explore the effects of immune activation. Restricted tadpoles were fed half-rations for two weeks in early development, and tadpoles in half of each feeding treatment received an injection of phytohemagglutinin, PHA, a known immune-activating lectin. Dietary restriction prolonged the larval period of the tadpoles but resulted in larger, heavier frogs which were faster to capture prey and had increased survival. In contrast, immune activation caused high initial mortality but showed weak long-term effects. Whole-body corticosterone levels, as analysed by radioimmunoassay, were not affected by the dietary treatment. These results are unique for showing the rare effect of "over-compensation" and suggest dietary restriction is a stronger developmental influence than immune activation. The impact of compensatory growth on the post-metamorphic fitness

of *L. ewingii* was contrary to theoretical expectations and may possess some value as an alternative conservation strategy for amphibians.

The quantitative review, the first in this field, clarified the terminology of compensatory growth and catch-up growth (achieving the same final size as controls) and was able to confirm both growth patterns as reliable, wide-spread responses to dietary restriction. Meta-analysis and meta-regression analysis techniques were used to conduct eight analyses of the size, growth slopes and fitness outcomes related to compensatory growth, based on data collected from 88 studies, spanning 11 taxonomic classes. Overall, animals experienced higher mortality and reduced reproductive output as a result of the dietary treatment. Taken together, the results of the quantitative review verified the basic assumptions of compensatory growth but also highlighted a number of aspects which could guide future research, such as the significance of diet protocols, appropriate fitness correlates and possible effect of age-dependent growth.

Compensatory growth is a broad field of research, ranging from the small-scale physiological mechanisms to the vast evolutionary perspective. This research has far-reaching implications, from human health to agriculture to evolutionary theory. In addressing two levels of this field, this thesis provides answers for previous gaps in knowledge. In addition, these results open up further avenues of research, which not only extend the field of compensatory growth, but also have real-world medical and economic applications.

Acknowledgements

This project was approved by the University of Otago Animal Ethics Committee and financially supported by the Department of Zoology. I am exceedingly grateful for the financial support provided by the University of Otago Post-graduate Award. There are many people who deserve to be acknowledged for their contribution to this thesis. I owe a great debt to their kindness and generosity.

Firstly, my primary supervisor, Shinichi Nakagawa. Without the weekly dread of having to come up with something to "wow" him, this research would not have left the ground. I could not have asked for a more knowledgeable, humorous and inspiring supervisor and I feel very privileged to have been the first of the many future Masters students in his career. His hard-work and dedication has been a great gift.

Thanks also to Phil Bishop, for sharing his endless knowledge of all things amphibian and taking the time to help solve my many tadpole-related technical problems, including a crash-course in their internal anatomy. I owe many thanks to the University of Otago Frog Group, for sharing their expertise in both frogs and scientific writing: Helen White, Jen Germano, Kalinka Rexer-Huber, Sabine Melzer, Michel Ohmer, Bastian Egeter, Emma Shaw and Morgan Mclean.

I must also thank the SN Behavioural Ecology Group for broadening my mind with their varied research interests and providing (informative) light relief over discussions of recent publications: Morgan Mclean (again, and with a special mention for baby-sitting tadpoles

over Christmas), James Coats, Ed & Luana Santos, Jeff Vanderpham, Alistair Senior, Karin Ludwig, Losia Lagisz and Jaime McQuillan.

This research would not have been possible without the very generous donations of Brian Lowe and Sandra Aitken at Tekpak of 500 containers, which, otherwise, would have broken my budget. Thanks also to Warren Agnew at Gotcha Traps Ltd., Warkworth, for kindly providing tracking ink free of charge. Thank you to Murray McKenzie for craftily devising a "tadpole run" just as I had imagined and perfectly suited to the task. I greatly appreciate the generosity of Mary-Jane Sneyd for repeatedly allowing me access to her property to raid her pond of frog spawn.

I owe much of what I managed to achieve in this thesis to the sage advice of my superiors. Thank you to Mark Lokman & Alison Cree (who also donated expensive chemicals), Erica Crespi, Cristina Ledon-Rettig and Chelsea Ward for graciously answering my multitude of questions about radioimmunoassays and immune activators and encouraging me to persevere. Thanks also for the technical assistance of Matthew Downes, Karen Judge and Nicky McHugh, who were all great fonts of wisdom when I was lost in the laboratory.

Thank you to my wonderful, loving family for supporting and encouraging me in this endeavour (even though I was starving frogs more than saving them). Finally, I would like to thank my partner, Tom Holmes, for assisting with fieldwork, weathering the highs and lows of scientific enquiry, and providing unswerving moral support. I owe him my sanity. I am truly grateful.

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Chapter I: General Introduction

1.1 Phenotypic Plasticity

Phenotypic plasticity can be defined as one genotype giving rise to alternative phenotypes depending on the environmental conditions an organism experiences (West-Eberhard, 1989). Therefore, phenotypically plastic organisms have an important adaptive advantage, maintaining high fitness across a range of environments (Nylin & Gotthard, 1998). A subclass of phenotypic plasticity, developmental plasticity, is particularly crucial during early development. Life-history trade-offs to accommodate an adverse early environment may be permanent and can result in decreased fitness in a more conventional adult environment (Monaghan, 2008). For example, in mammals, adaptations to adverse foetal conditions can lead to a number of adult health concerns, such as asthma, anaemia, steroid insensitivity and behavioural deficits (Coe & Lubach, 2008). There are several important environmental factors governing the development of organisms and, thus, subsequent phenotypes. The primary focus of this thesis is the phenotypic outcome of inadequate nutrition in the early developmental environment. More specifically, I investigated compensatory growth at two levels: both in an introduced amphibian species and across a range of species in a quantitative review.

1.2 Compensatory Growth

When animals endure a period of under-nutrition, either *in utero* or in early postnatal development, the low energetic resources limit growth and/or maturation(Bauer *et al.*, 2009). If a subsequent change in the environment allows ample nutrition, many animals will enter a period of compensatory growth (Metcalfe & Monaghan, 2001). Compensatory growth is

defined as growth which is faster than the evolved "optimal" growth rate of the species (Nylin & Gotthard, 1998). Normal (optimal) growth rates are constrained by the numerous adverse consequences of rapid growth, which decrease cell functioning efficiency, immune function and resistance to physiological stressors (Mangel & Stamps, 2001). However, there remain many incentives to grow rapidly, such as environmental time constraints, predation risk and the fitness cost of delaying reproduction (Dmitriew, 2011). Most importantly, large adult size is a major contributor to sexual selection and fecundity and can only be achieved by considerable growth during the limited developmental period (Blanckenhorn, 2000). The necessity of rapid growth is greatly exacerbated by early growth restriction.

In response to early growth restriction, a physiological trade-off can be adopted as an alternative growth strategy, whereby the benefits of large size are traded against the costs incurred by rapid growth (Metcalfe & Monaghan, 2003). This seemingly detrimental trade-off, however, is mitigated by the time in which the accumulated negative costs become apparent (Yearsley *et al.*, 2004). Cell damage from faster than optimal growth tends to be expressed as heart disease, diabetes and liver failure, because the quality of organ tissues is jeopardized by rapid cell division (Barker *et al.*, 2002). Since these are later life diseases, an animal which has experienced compensatory growth can still increase its fitness during its reproductive phase because of its increased size, compared to the minimal reproductive success of small animals (Arendt, 1997). This ability to overcome early developmental setbacks in terms of fitness is believed to be how compensatory growth has evolved.

As well as food deprivation, the effects of immune activation, temperature and seasonal cues can lead to compensatory growth (Metcalfe & Monaghan, 2001). Compensatory growth can also be a consequence of the prenatal environment. Fetal programming in response to malnourishment *in utero* is now considered a key factor in explaining wide-spread metabolic syndrome in humans (James, 2002). Although the evolution of compensatory growth can be

explained in terms of delayed trade-offs, there are a number of detrimental effects which are manifested earlier and may affect fitness in the wild. Compensatory growth has been linked to incomplete bone ossification (Arendt & Wilson, 2000), prolonged fledging times (Bize *et al.*, 2006), altered body resource allocation (Stevens *et al.*, 2000), limited overwintering energy reserves (Morgan & Metcalfe, 2001), subordinance to size-matched controls (Royle *et al.*, 2005), poor cognitive performance (Fisher *et al.*, 2006) and behavioural suites which differ from those of consistently well-fed conspecifics (Stamps, 2007). Compensatory growth is also known to alter the regulation of stress hormones, specifically glucocorticosteroids, which in turn can have serious consequences for fitness (Kitaysky *et al.*, 1999). The effect of dietary restriction and rapid growth on stress hormones is discussed in greater detail in Chapter III. Both the stress response and restricted growth due to energy deficits are related to the secondary interest of this thesis: immune activation.

1.3 Immune Activation

Animals require an immune system to fight off invading foreign organisms including viruses, bacteria and fungi. Yet, it is essential that this defence is not always activated and only used when absolutely necessary (Sadd & Schmid-Hempel, 2009).Mounting an immune response is extremely energetically costly, not only because of the metabolic requirements but also by diverting resources from other functions such as reproduction or food-gathering, especially if resting is part of the "sickness behaviour" repertoire (Bonneaud *et al.*, 2003). Damage to cells resulting from autoreactivity can be severe, and even fatal, if additional stress is placed on the animal (Moret & Schmid-Hempel, 2000). Long and Nanthakumar (2004) stress the importance of distinguishing between T-helper lymphocyte populations when looking at immune responses in vertebrates. T-helper 1 (Th1) cells defend against intracellular infections like bacteria and viruses, while T-helper 2 (Th2) cells defend against non-invasive infections, such as metazoan parasites. Increasing one population of T-helper cells decreases

the other, so animals are faced with a constant battle trying to keep infections like viruses and flukes simultaneously at bay. Lymphocyte counts are also inversely related to testosterone and glucocorticoid levels (Rollins-Smith *et al.*, 1997; Boonekamp *et al.*, 2008).

Mounting an immune response occurs at the expense of many other costly functions, particularly reproductive success. Following successive immune challenges, house sparrows, *Passer domesticus*, were less active, feeding young less in fewer visits to the nest, and were more likely to desert small broods (Bonneaud *et al.*, 2003). Immune activated crickets, *Cyphoderris strepitans*, spent less time calling and had a significantly lower chance of securing a mating, with over 80% remaining virgins in one year (Leman *et al.*, 2009). Female Mallee dragons, *Ctenophorus fordi*, induced to mount an immune response also had lower reproductive output by producing smaller eggs (Uller *et al.*, 2006). Immune activation of collared dove nestlings, *Streptopelia decaocto*, had no effect on size or development period, yet survival was significantly lower than in controls within the first week after fledging (Eraud *et al.*, 2009). Although the nestlings were not observably different from the controls, immune activation impaired the birds' ability to avoid predators. The potential for immune activation to have long-lasting consequences for survival and reproduction make it a valuable developmental stressor for investigations of phenotypic plasticity.

1.4 Study Species: *Litoria ewingii*

L. ewingii was selected as the preferred study species for this investigation of compensatory growth and immune activation because amphibians have been largely overlooked in both these fields. The relatively short larval period and availability in the Dunedin area made this species convenient for study purposes, while its life-history is ecologically relevant to the questions being asked (Fig. 1.1). *L. ewingii* belongs to the Hylidae family of anurans and was introduced to New Zealand from Australia over 100 years ago (McCann, 1961). This species

Figure 1.1 Development of *Litoria ewingii*.

From top to bottom: *L. ewingii* egg clutches are a cluster of small eggs in a jelly-like mass attached to vegetation. The same tadpole, as photographed at age 10 days and at 38 days after hatching (1 cm scale bars shown, note the development of the hind-legs and forelimb development just visible under the transparent skin). Lastly, a juvenile frog, aged approximately four months. *L. ewingii* is identified by its distinctive dark brown "eye-mask" with a pale stripe beneath.

was originally introduced in to the Greymouth area, and has since spread the length of the South Island, extending to the lower North Island and south to Stewart Island (Lock *et al.*, 2005). As adults, *L. ewingii* are medium-sized frogs, with a snout-vent length up to 45mm (Robinson, 1996, Fig. 1.1). Adults breed throughout the year and may lay up to 500 eggs per clutch (Cree, 1984; Lauck *et al.*, 2005). Tadpoles are active swimmers and under controlled laboratory conditions at a temperature of 23°C most will complete metamorphosis 50-64 days after hatching (Cree, 1984). *L. ewingii* has been shown to be susceptible to outbreaks of Gram-negative bacterial species which cause fatal dermatosepticemia and infect the heart, liver and spleen of the frogs (Schadich & Cole, 2010). They are also vulnerable to infection by *Batrachochytrium dendrobatidis* (Bd), commonly known as chytrid fungus, which is believed to responsible for a proportion of amphibian population declines worldwide (Shaw *et al.*, 2010).

In the wild, *L. ewingii* is most commonly the prey of insect predators such as dragonflies, which show a preference for the smaller size classes of tadpoles (Richards & Bull, 1990). They have been known to exploit a variety of pond habitats in their native Australia, with the greatest reproductive success found in high elevation ponds with steep slopes, reduced shading and closeproximity to other ponds (Lauck *et al.*, 2005). In New Zealand, thesefrogs inhabitcool damp areas with daytime shelter and are often found in monocotyledonous vegetationsuch as flax and rushes, as well as under logs and rocks (Gill, 1973). Most notably, *L. ewingii*stand out as a particularly hardy species, capable of surviving up to 47.5% of their body water being frozen (Bazin *et al.*, 2007).

1.5 Statistical Tool: Meta-analysis

Meta-analysis is a powerful analytical technique which has been rapidly gaining popularity in the ecological and evolutionary sciences over the past 15 years, although it has been in the spotlight of medical, psychological and humanities research for some time (Arnqvist &

Wooster, 1995). In contrast with the misleading "vote-counting" method typical of literature reviews, meta-analysis allows us to quantify the direction and magnitude of the effect sizes across the literature (Cooper *et al.*, 2009). Although meta-analysis is commonly used in human sciences, it allows biologists to combine the results of numerous studies from diverse taxa (for example, vertebrates, invertebrates and fungi, Nakagawa *et al.*, submitted), with the relative influence of each study weighted by the variance of the effect size estimate. The variance is largely dependent on the sample size of the study, meaning that larger studies have a greater influence on the outcome of the analysis. Intuitively, this method suggests that we have a greater chance of detecting a real effect. Furthermore, a meta-regression technique can be used, which allows for various moderators to be included in order to ask relatively more sophisticated questions about the data (Thompson & Higgins, 2002).

Meta-analysis is used in Chapter IV to gain a broader perspective than is possible from the empirical study of compensatory growth with *L. ewingii* in Chapters II and III. There are two benefits to using a multi-level approach to compensatory growth research. Firstly, the *L. ewingii* study can answer specific questions about the effects of compensatory growth in a uniquely interesting taxon (as yet, no study has followed the effects of early dietary restriction beyond the point of metamorphosis in an amphibian). These data then contribute to the existing body of literature collected for the meta-analysis. Secondly, the overall outcome of the meta-analysis gives a broader biological perspective to tackle such questions as how compensatory growth has evolved and whether the conventions of this alternative growth strategy apply to all taxa. The results of the meta-analysis can then be compared against the data for *L. ewingii* to identify the ways in which this species is exceptional and postulate why it does not follow the general trends of compensatory growth across the animal kingdom. Overall, the two methods are highly complementary and provide a greater scope for understanding compensatory growth.

1.6 Aims

In summary, compensatory growth is a widespread and ecologically-relevant phenomenon which can result in a range of phenotypes depending on the plasticity of the growth strategies involved. However, there has yet to be a comprehensive analysis which can outline the prevalence and reliability of compensatory growth, as well as confirming the fitness consequences which explain the evolution of this alternative growth strategy. Amphibians have been a sorely neglected taxon for compensatory growth research, with only one strong paper supporting its existence in this taxonomic class (Capellan & Nicieza, 2007). Despite the speculation that metamorphosis could have a profound effect on the consequences of rapid growth, possibly allowing for the redistribution of resources in the adult form, evidence beyond metamorphosis is lacking (Metcalfe & Monaghan, 2001). Furthermore, the interaction of compensatory growth with other phenotypically plastic developmental stressors has also been overlooked, despite the potential of this type of research to shed light on the relative importance and potential interactions of multiple stressors.

In order to address these gaps in the literature, the aim of this study was to investigate the effect of dietary restriction and immune activation on the brown tree frog, *L. ewingii* (Chapter II). This research would be unique, firstly for exploring the two stressors in a factorial design, and secondly for attempting to observe the effects of compensatory growth beyond metamorphosis in an amphibian. It was hoped that this scope would allow for better interpretation of how the fitness consequences would manifest themselves in this taxa. In addition, the effect of the dietary restriction on the whole-body corticosterone levels of the tadpoles was also tested (Chapter III). To place these data into context, a meta-analysis was performed to quantify the impact of early dietary restriction on the subsequent growth and fitness characteristics of a diverse array of taxa (Chapter IV). This analysis would not only serve as a guideline for technical considerations of future compensatory growth research, it

would also address the serious concerns voiced about the interpretation of these data, which suggest that compensatory growth could be a mere statistical artefact (Nicieza & Alvarez, 2009). Overall, the aim of this thesis was to shed new light on this broad and diverse field by probing a few areas that had previously been overlooked.

1.7 Presentation of the Thesis

Each chapter in this thesis was written with the intention of presenting a stand-alone paper. Thus, each is presented with an abstract, references and appendices specific to the individual chapter. Although this practice engenders some redundancy of explanations, I believe that treating each chapter as an independent piece of scientific research is both in keeping with common publication guidelines of biological research and offers the most appropriate layout for the content of this thesis.

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Chapter II: Consequences of compensatory growth and immune activation in an amphibian.

ABSTRACT

Compensatory growth occurs when an organism grows faster than the optimal rate after a period of growth restriction. Immune activation restricts growth because energy resources are diverted to the immune system. Amphibians have been neglected in these fields despite their flexible life history and size-dependent fitness. I investigated the effects of early nutritional and immunological stress on the growth, morphology and three fitness-related behavioural traits of brown tree frog tadpoles, *Litoria ewingii,* before and after metamorphosis. I restricted the early nutrition of tadpoles and caused an immune response by injection of phytohemagglutinin, using a two-way factorial design (i.e. four different groups). Dietary restriction resulted in faster weight gain upon realimentation, larger final size and faster prey capture. Immune activation resulted in smaller-sized frogs and both treatments affected survival. Unlike previous work, nutritional restriction affected the developmental rate and resulted in "over-compensation" of growth. This experiment provides a unique comparison of two developmental stressors in amphibians adapted to ephemeral environments.

A version of this chapter is currently under revision for re-submission to the *Journal of Zoology.*

2.1 INTRODUCTION

Life-history trade-offs to accommodate an adverse early environment may be permanent and can result in decreased fitness in a more conventional adult environment (Monaghan, 2008). There are several important environmental factors governing the development of organisms and, thus, subsequent phenotypes. Two of these factors are the environmental availability of nutrition and pathogenic environments. Inadequate nutrition during development can have significant negative impacts on survival, developmental time, size at maturity and reproduction in diverse taxa (for example, mice, *Mus musculus*: Wu *et al.*, 2002; Nile tilapia, *Oreochromis niloticus*: Bhujel *et al.*, 2007; cockroach, *Nauphoeta cinerea*: Barrett *et al.*, 2009; ladybird beetle, *Harmonia axyridis*: Dmitriew *et al.*, 2009). These negative effects, however, may be mitigated by compensatory growth, also known as catch-up growth.

Compensatory growth is accelerated growth following a period of growth inhibition once environmental conditions improve. The accelerated growth is at a higher rate than expected in the absence of growth inhibition (Nicieza & Alvarez, 2009). Accumulated cellular damage from this rapid growth will often have negative effects later in life (Mangel & Munch, 2005). For example, rapid catch-up growth in human babies, born small for gestational age, has long been known to increase the risk of adult obesity and high blood pressure (meta-analytically reviewed in Huxley *et al.*, 2000; Ong & Loos, 2006). Despite these detrimental health consequences, compensatory growth may still be adaptive if the negative effects are delayed until after the reproductive phase, because increased size and earlier maturity commonly increase reproductive fitness (Metcalfe & Monaghan, 2001).

Immune activation, normally triggered by encountering pathogens and parasites, relates to compensatory growth in that the effects of an immune challenge are often similar to dietary restriction and can be compensated for; likewise, compensatory growth can have an impact on the immune function of an animal (Butz *et al.*, 2004; De Block & Stoks, 2008b). Immune

responses can be energetically costly, both in terms of metabolic requirements and in diverting resources from other functions such as reproduction and food-gathering (Bonneaud *et al.*, 2003). The long-term effects of having to mount an immune response during development seem to vary between species. For example, immune-activated lizards, *Ctenophorus fordi*, showed decreased reproductive investment and slower-growing offspring (Uller *et al.*, 2006), while immune-activated sagebrush crickets, *Cyphoderris strepitans*, spent less time calling and were significantly less likely to secure a mating in their first year (Leman *et al.*, 2009). Furthermore, red flour beetles, *Tribolium castaneum*, which mounted an immune defence, pupated earlier in order to avoid the energy expenditure altogether (Roth & Kurtz, 2008).

Amphibians are very sensitive to environmental factors during development, such as pond drying, food abundance and the densities of conspecifics and predators (Wilbur & Collins, 1973; Sokol, 1984). Metamorphosing amphibians show great plasticity in developmental rate, morphology and behaviour in response to these environmental factors (Newman, 1992; Schoeppner & Relyea, 2009). Previous work suggests that tadpoles are capable of rapid compensatory growth after early food restriction (Capellan & Nicieza, 2007). This pattern was also observed in an ecological context as a result of intraspecific competition (Travis, 1984). The potential for recovery by rapid growth declines with advancing developmental stage in tadpoles (Jasienski, 2008); however, the long-term fitness effects of catch-up growth have yet to be studied in amphibians. The flexibility that metamorphosis allows amphibians may play a major role in how tadpoles contend with both compensatory growth and immune activation by allowing resources to be redistributed in the adult body (Metcalfe & Monaghan, 2001). The tadpole immune system is known to decline approaching metamorphosis in order to prepare the body for a new adult immune pattern and prevent autoimmune complications (Rollins-Smith *et al.*, 1997).

The aim of this experiment was to investigate the effects of both compensatory growth and immune activation, and their possible interactions, in an amphibian. Based on previous work by Travis (1984), Capellan & Nicieza (2007) and Roth & Kurtz (2008), it was predicted that these stressors would affect the developmental rate, morphology and locomotive ability of the metamorphosed frogs. These phenotypic measures have been shown to contribute to lifelong fitness (Glennemeier & Denver, 2002; Arendt, 2009; Hunt *et al.*, 2009). I hypothesised that tadpoles exposed to a restricted diet early in development would show a subsequent accelerated growth rate to reach the same size as controls upon metamorphosis and that this rapid growth would be traded-off against post-metamorphic fitness traits. I also hypothesised that immune activation would stunt the growth of tadpoles and the compensatory growth of immune-activated tadpoles would be less complete than those restricted by diet due to premature development and metamorphosis, as shown in previous work on insects (Roth & Kurtz, 2008). Notably, my study is the first to explore whether the two variables (i.e. compensatory growth and immune activation) act additively or interactively on the survival and fitness-related traits of tadpoles.

2.2 METHODS

2.2.1 Study animals

Nineteen egg masses were collected from a pond in a residential area of Dunedin, New Zealand (45.8°S, 170.5°E). Clutches were relocated to a temperature-controlled animal husbandry room at the Department of Zoology, University of Otago, where they remained until the end of the experiment. The room had a constant photoperiod of 14:10 L:D and was maintained at a temperature of 23°C to maximise the growth potential of tadpoles (Cree, 1984). Each clutch (containing between 6 and 75 eggs, mean ± 1 SE, 21.5 ± 3.7) was placed in a separate 200 ml container (9 cm diameter, 4.8 cm height, Tekpak Ltd) with conditioned

water (AquaSafe water conditioner, TetraAqua) and a small amount of weed collected from the pond to provide refuge. Bubblers were used to gently aerate the water. Eggs were monitored daily and the tadpoles' development was recorded. The official hatching day of each clutch was considered as the day on which over half of the tadpoles were free swimming (Gosner stage 25; Gosner, 1960). All subsequent measurements and treatments took place in reference to this date as day one, so that all clutches were the same age for comparison.

On post-hatching day 4, tadpoles were individually housed in 200 ml containers with conditioned water, which was kept at room temperature at least 12 hours before use. A small amount of food was provided although the majority of tadpoles were sedentary and not yet eating. Weight and stage were not measured as tadpoles were too delicate for handling. Water was changed every second day and a small amount of food provided until day 10 when all tadpoles were active and had begun eating. Tadpoles were then identified by an ID number etched onto the lid which remained with them when they were transferred to clean containers. Two holes in the lid with approximately 1 cm diameters allowed oxygen transfer into the containers and prevented condensation obscuring viewing of the tadpoles.

Tadpole containers were arranged randomly within clutches every second day after water changes, preventing visual cues of neighbour size from affecting growth (Sutherland *et al.*, 2009). Adjacent clutches and position on shelf (either top or bottom shelf on two different shelving units) were randomly changed approximately weekly to prevent any spatial effects. After feeding, tadpole containers were partially covered with shredded bin-liners to allow lighting differences to simulate refugia.

Once tadpoles reached Gosner stage 42 (forelimb emergence), they were placed in clean 200ml containers with crumpled damp paper and approximately 5 mm of water in the bottom. This substrate prevented the majority of tadpoles from drowning yet allowed them mobility in the wet environment. Upon completing metamorphosis (Gosner stage 46, complete tail re-

absorption), froglets were re-housed individually in 500 ml plastic containers (10 cm diameter) with a damp paper substrate and plastic aquarium plants to provide climbing apparatus. Frogs were then fed three times weekly on wingless *Drosophila melanogaster*, juvenile crickets (*Teleogryllus commodus*) and houseflies (*Musca domestica*) depending on prey availability and the age of the frogs. All frogs were provided with the same amount of food at each feeding. Containers were cleaned, pellets removed and fresh substrate added once a week.

2.2.2 Food preparation and feeding treatment

Fresh lettuce leaves, excluding larger veins, were finely diced and boiled, before being diced again to prevent clogging the syringes used to distribute food. Boiled lettuce was mixed in a 7:2:1:1 ratio with Tetra goldfish flakes, Tetra spirulina flakes and gelatine which had been dissolved in boiled water at twice the volume of lettuce. Ingredients were thoroughly mixed and refrigerated until set. Food was prepared once a week and refrigerated between uses.

From nine clutches, 118 tadpoles successfully hatched (clutches containing from 1 to 63 tadpoles, mean ± SE, 13.1 ± 7). From day 10 onwards tadpoles were evenly divided within clutches between restricted and control treatments. Tadpoles were weighed, as described below, on day 10 and then weekly. The average weight for each treatment within each clutch was calculated and then used to establish daily feeding amounts (Fig. 2.1). Control tadpoles were fed their control clutch's mean weight daily for the duration of the larval period. For example, if the mean weight of control tadpoles in one clutch was 0.06 g, they received 0.06 g of food daily until the next weighing. This amount approximated *ad libitum* feeding as established by an earlier pilot test. The reasons for providing a finite amount as opposed to true *ad libitum* feeding were 1) to allow direct comparisons between the control and restricted

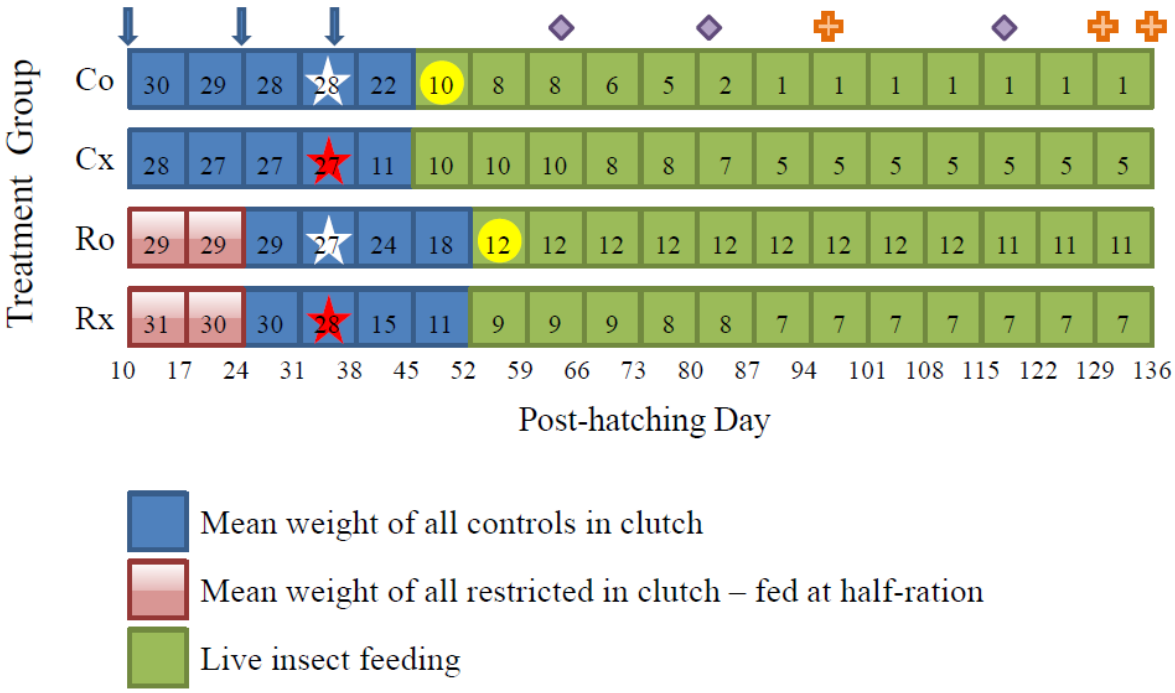

Figure 2.1. Diagram representing experimental timeline.

Daily food amounts for each feeding treatment within clutches were the mean weight at the previous weekly weighing. C = control-fed tadpoles; R = food-restricted tadpoles; o = PBS-injected (☆); x = PHA-injected (★). At forelimb emergence 12 metamorphs each from the control and restricted PBS-injected treatments were killed for use in another study (●). Individual frogs were shifted to live insect feeding upon completing metamorphosis. Arrows (⬇) represent swimming tests, diamonds (◆) represent feeding latency tests and crosses (✚) represent hopping tests. Numbers inside squares show the numbers of tadpoles in each treatment at the previous weighing.

groups to be made based on known food intake and 2) to prevent degradation of water quality which occurred rapidly if excess amounts of uneaten food remained in individual containers. In contrast, restricted tadpoles were fed only half of their restricted clutch's mean weight for the first two weeks of the experiment (Fig. 2.1). For example, if the mean weight of restricted tadpoles in one clutch was 0.06 g, they received 0.03 g of food daily until the next weighing. After this restriction period was over these tadpoles were then fed the full amount provided to the control tadpoles in their clutch (Fig. 2.1). In this way, restricted tadpoles were provided the same amount as what a tadpole of their age would be expected to eat under normal conditions. Food was provided by weighing out the full amount required by the treatment clutch then evenly dividing it among the tadpole containers via a syringe.

2.2.3 Immune activation treatment

On day 33, tadpoles within each treatment clutch were weighed and alternately assorted by weight into two injection treatments: a phosphate-buffered saline (PBS) treatment and a phytohemagglutinin (PHA) treatment. PHA (L8754, Sigma-Aldrich) is a lectin extracted from red kidney beans, *Phaseolus vulgaris*, which induces a T-lymphocyte response and is a known immune activator in amphibians (Gervasi & Foufopoulos, 2008). PHA was prepared at 4mg/ml PBS and was injected at 37.5 µg per gram body weight. Tadpoles were individually anaesthetised by being placed in a water bath containing 10 ml of 50 µL/L AquiS (AquiS New Zealand Ltd, Lower Hutt) in treated water for 6-10 minutes until loss of righting response. Tadpoles were then placed on their right side on a moist paper towel on the stage of a dissecting microscope. Injections (ranging from 5 to 19 µL volume, according to tadpole weight) were administered with a 24 gauge needle and 0.3 ml syringe subcutaneously near the tail junction on the left dorsal side. The needle tip was inserted vertically to pierce the skin then tilted horizontally to be inserted parallel with the tail at a depth which would prevent the injection fluid spilling out of the wound. Great care was taken to avoid the major

tail artery and other large vessels. Tadpoles were then placed in clean treated water with fresh food and were monitored closely for signs of revival. It is noted that this study had a balanced two-way factorial design with the diet treatments and immune activation treatments, at least, at the beginning of the study.

2.2.4 Morphometric measurements

Tadpoles were weighed, staged and had morphometric measurements taken weekly from day 10 until the completion of metamorphosis. Tadpoles were lightly dried on a paper towel before being placed in a half-filled 200 ml water container which had been tared on a set of electronic scales. Weight measurements were taken to the nearest 0.01 g. Staging was achieved by placing the tadpoles in a narrow test tube filled with treated water and comparing limb development through an eye glass (4x magnification) against the Gosner staging chart. Morphometric measurements were taken from digital photographs of tadpoles. Tadpoles were placed in a small glass chamber (72 x 22 x 23 mm) partially filled with treated water and were gently pressed against the side with a flat glass insert to allow a lateral view with minimal parallax error. The camera (Canon Powershot A2000 IS) was placed at uniform distance from the subject, under constant lighting conditions throughout the experiment, with macro focus (1-50 cm), no flash and automatic exposure. Photographs were only used for measurement when the subject was in focus, had a straight tail and was perpendicular to the camera. Measurements were taken from the photographs using ImageJ, with the lower edge of the container as a scale reference and at least a 75% zoom for straight line measurements. The four measurements taken were body length, tail length, tail muscle height and maximum tail height, as described by Altig and McDiarmid (1999; Fig. 2.2). In a pilot study, the validity of the digital photo measurement protocol was assessed by comparing digital measurements with those taken from the anaesthetised tadpole using callipers (see Appendix 2.6.1).

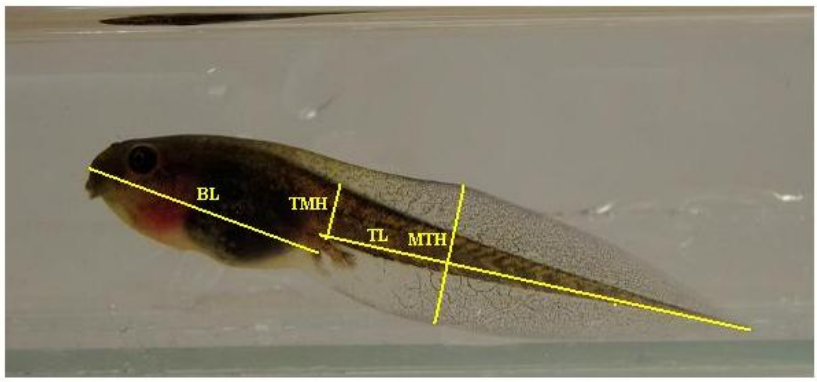

Figure 2.2 Tadpole measurements.

Morphometric measurements taken from tadpoles, as described by Altig & McDiarmid (1999). BL = body length; TL = tail length; TMH = tail muscle height; MTH = maximum tail height.

After metamorphosis only weight and snout-vent length were recorded. Frogs were placed on a small hand-held scale (accurate to 0.01 g) for weight measurement. They were then placed in a clear plastic zip lock bag and held restrained on the bench top. A finger was placed 1 cm either side of the frog to provide consistent levels of compression from the plastic bag across all frogs. Plastic callipers were then used to measure the snout-vent length. Frogs were not handled during the measuring process but were prompted to jump between the required vessels using a plastic spoon.

2.2.5 Fitness-related behavioural tests

Prior to restriction (day 10), at the end of restriction (day 24) and a day after the injection treatment (day 34), tadpoles were put through a swimming test. A specialised swim run was constructed from a thick plastic pipe with a length of 1 m and diameter of 7 cm. The upper portion of the pipe was removed and the ends were sealed to provide a long watertight enclosure. The run was divided into three 30 cm trials with 5 cm start and finish areas and

was filled to a depth of 2 cm with treated water. Individual tadpoles were held at the start area for one minute to adjust before the trial began. The tadpole was then prompted by a plastic spoon to begin swimming and again whenever movement ceased for more than 5 seconds or if a tadpole attempted to swim in the wrong direction. A lap timer was used to record the time the tadpole spent in each trial area and the number of prompts required was also recorded.

On days 62, 81 and 118, all frogs (*n* = 36, 30 and 24, respectively) were subjected to a feeding latency test. Plastic plants were removed from the container and fresh damp paper towels were added. Frogs had previously been fed at least two days prior to testing. The frog was prompted to sit facing towards the centre of the container on the substrate. One wingless *Drosophila melanogaster* was added to the middle of the container and the time taken for the frog to ingest the fly was recorded. If the *Drosophila* reached the top of the container unharmed it was flicked back to the bottom. Wingless *Drosophila* were selected as ideal prey for this test because of their constant movement, inability to escape the containers and small size which required greater accuracy in the frog's attack. This was repeated three times for each frog.

On days 97, 131 and 138 all surviving frogs (*n* = 25, 24 and 24 respectively) completed a hopping test. Each frog was placed on a 1 m^2 piece of paper and the largest hop as well as the number of hops achieved in 30 s was recorded. Initially each frog was placed on an ink pad soaked in green tracking ink (Gotcha Traps Ltd, Warkworth; for safety validation with *L. ewingii*,see Frost, 2007). This procedure made it possible for the location of the frog's hops to be clearly marked. After being placed on the paper, the frog was gently prompted by touching a pen to the caudal area if spontaneous hopping did not occur. The trial and cumulative number of each hop was recorded beside each print. Each frog completed three 30 s trials per test. Each test lasted less than five minutes before the frog was returned to its container to minimise the stress of testing. After testing, the paper was analysed by recording the

maximum number of hops in each trial and measuring the greatest distance between hops per trial from the markings made by the frog's vent in each successive hop, using a ruler.

2.2.6 Statistical analysis

The R 2.9.2 statistical package (R Development Core Team, 2009) was used for all statistical analyses. Survival was analysed using the R package, survival (Therneau, 2009) with a Cox proportional hazards regression model accounting for censored data and clustered by clutch. Accounting for clutch identity contributed no qualitative difference to the results so clutch was excluded from subsequent analyses because of the large variance in clutch size (range: 1 to 63). Specific comparisons made between groups for survival were analysed using a bias-reduced generalised linear model (BRGLM) with a binomial distribution (Kosmidis, 2007). Due to the irregular nature of the growth curve throughout metamorphosis, it was not practical to analyse growth in one statistical model. Instead, each morphometric measurement was analysed by day when measurements were taken, using a simple linear model (GLM) with food treatments as the only factor prior to injection (immune activation) treatments, then injection treatment and a food:injection treatment interaction were added. Notably, from day 80 onwards the food:injection interaction term was not considered due to unbalanced mortality among the treatment groups rendering the interaction effect incalculable.

For the analysis of the fitness-related behavioural tests (swimming, feeding and hopping), linear mixed effects models (LMM) and generalised linear mixed models (GLMM) with individual identity as a random effect were used accordingly (package: lme4; Bates & Maechler, 2009). The output variables of the continuous data were first multilinearised by a Box-Cox transform to account for the non-normal skew frequently found in timed data (package: car; Fox, 2009). Full models were fitted with a three-way interaction of food, injection and day (with the quasi-Poisson error for count data), then the best models were selected by sequentially eliminating non-significant interaction terms, then least significant

terms, to lower the AIC value of the model. The food:injection interaction term was not included for feeding and hopping data due to the unbalanced mortality mentioned above. Results from the analyses of the growth slope and fitness-related behavioural tests are reported without degrees of freedom due to the use of Markov chain Monte Carlo simulations to estimate *p*-values from the linear mixed effects models (package: languageR; Baayen, 2009; see Bolker *et al.*, 2009).

To visualise the impacts of the feeding and injection treatments in a more holistic way, the complete morphometric dataset (including stage) for post-hatching days 10 to 45 was analysed by feeding day using maximum-likelihood factor analysis. This analysis provided six variables per individual per day and each day was analysed by two factors for ease of interpretation. The sample size for each day was considered sufficient for a two-factor analysis (Budaev, 2010). This factor analysis was conducted to show the relative distinctions between feeding and immune activation treatments on the shape and development of the tadpoles as a whole across time. Likewise, a linear discriminant analysis was run to test how these morphological characters combined to provide distinctions between treatment groups (package: MASS; Venables & Ripley, 2002). Feeding treatments and immune activation treatments were analysed separately. All morphometric data including stage were included for each individual and the prior probabilities for the groups were 0.5 each. Following the linear discriminant analysis, the 'predict' function using the 'plug-in' method was used to sort the individuals based on the coefficients of the analysis and the accuracy of these predictions was recorded. Both the factor analysis and linear discriminant analysis were conducted following guidelines from Everitt (2005).

To place the effects of the feeding treatments into a broader context, the results were compared with published literature of experiments using *L. ewingii*. All available literature was searched for information on metamorphic characteristics and was accepted when no

extraneous stressors (e.g. predators, salinity) were placed on the animals and results were reported with standard errors (SE). Only data for tadpoles raised at the lowest density were included from Sokol (1984). To compare linear growth slopes, the slope from Chinathamby *et al*. (2006) was digitally calculated using ImageJ. These data were then analysed in two-sided *t*-tests, not assuming homogeneity of variance, in comparison to the corresponding data from the control and experimental groups of this study.

2.3 RESULTS

2.3.1 Survival analysis

Prior to the injection procedure, only six tadpoles died, three from each feeding treatment, indicating that the restricted feeding ration was not a significant health risk for young tadpoles. In the three days following the injection treatment, there was a considerable increase in mortality, presumably attributable to the effects of the injection. Mortality was significantly higher for those injected with PHA (49% mortality vs. 6% PBS-injected; BRGLM: *z*-value = 2.865, $p = 0.004$; Fig. 2.3) but there was no difference between feeding treatments (BRGLM: *z*-value = -0.014, $p = 0.989$).

Survival from completion of metamorphosis to the end of the experiment (136 days post-hatching) was significantly higher for the restricted group than the controls (BRGLM: *z*-value = 2.946, $p = 0.003$). In addition, heavier weight upon completion of metamorphosis significantly increased the likelihood of survival to the end of the experiment (BRGLM: *z*-value = 2.809, $p = 0.0018$).There was no significant effect of injection treatment on post-metamorphic survival (BRGLM: *z*-value = 1.538, $p = 0.124$). There was a significant interaction between the food and injection treatments (CPRH: *z*-value = 3.58, $p = 0.0003$). Survival was highest for the restricted PBS- injected tadpoles and lowest for the control PBS-

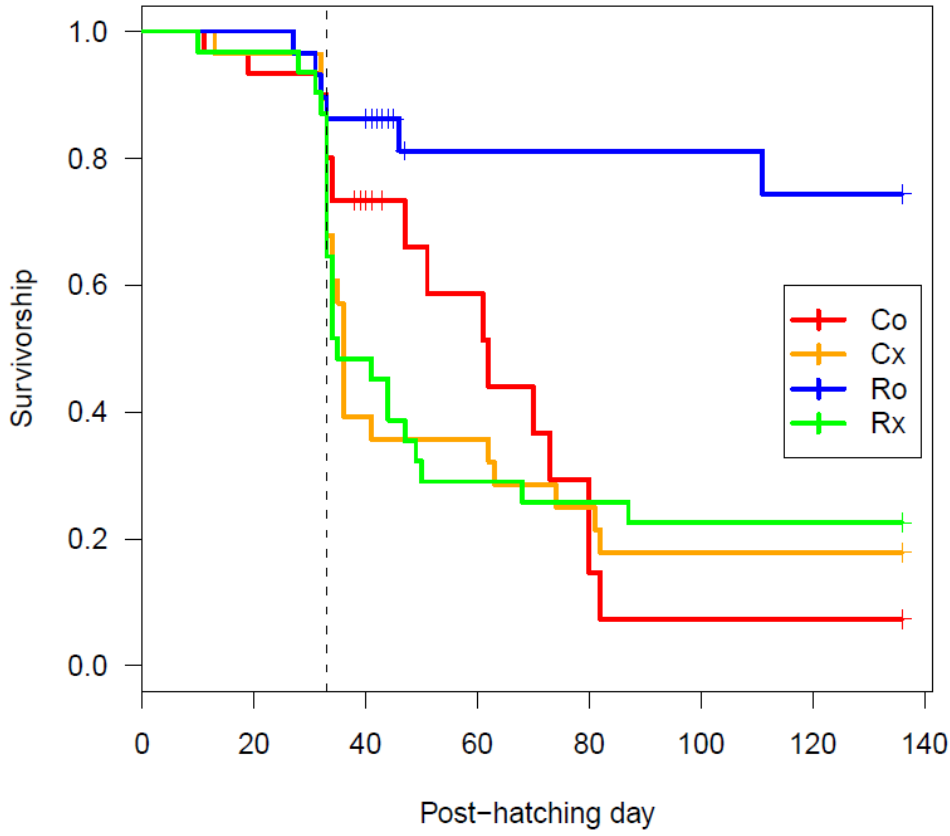

Figure 2.3 Survival across the entire experiment.

Survival throughout the experiment by treatment group (C = control-fed tadpoles; R = food-restricted tadpoles; o = PBS-injected; x = PHA-injected). The vertical dashed line represents the day of the injection procedure. Small vertical bars on the Co and Ro lines indicate tadpoles sacrificed at forelimb emergence (n = 12 per group).

injected tadpoles (Fig. 2.3). In contrast, both PHA-injected groups showed a similar survival trend with a steep decline as a result of the injection.

2.3.2 Pre-metamorphosis: the tadpole stages

Prior to metamorphosis, food restriction had a major impact on growth and development (Fig. 2.4). Control tadpoles were significantly heavier, had longer bodies and larger tail measurements between days 17 and 31 (Fig. 2.4; Tables A2.2-A2.6). After this period, all control measurements began to decline as a result of their natural advance towards metamorphosis. Control tadpoles were consistently at a more advanced stage of development than restricted tadpoles (Fig. 2.4b, Table A2.7). This pattern explains why they were significantly heavier and longer prior to the peak size and then significantly lighter, smaller and had greater tail re-absorption than restricted tadpoles after this peak had been reached. This advance in development resulted in a significantly earlier age at forelimb emergence for control tadpoles (mean days ± SE, control: 39.9 ± 0.4 *vs.* restricted: 44.7 ± 0.3; GLM, *t*-value = 7.43, *df* = 70, $p < 0.0001$). There was a small but significant difference in time between forelimb emergence and completion of metamorphosis between feeding treatments (mean days ± SE, control: 2.84 ± 0.16 *vs*. restricted 2.68 ± 0.1; GLM, *t*-value = -2.057, *df* = 40, $p = 0.047$). Notably, forelimb emergence was still considered a more practical and reliable estimate of metamorphosis compared to observing tail re-absorption (Sokol, 1984).

Immune activation had a significant effect only on the restricted tadpoles immediately after injection. PHA-injected tadpoles were significantly lighter than PBS-injected tadpoles on day 38 (mean weight (g) ± SE, restricted-PBS: 0.418 ± 0.013 *vs*. restricted-PHA: 0.366 ± 0.016; GLM, *t*-value = -2.461, *df* = 37, $p = 0.019$; Fig. 2.4a). Tail growth was also affected with immune activated tadpoles having smaller maximum tail heights (mean maximum tail height (mm) ± SE, restricted-PBS: 7.646 ± 0.124 *vs*. restricted-PHA: 7.2 ± 0.161; GLM, *t*-value =

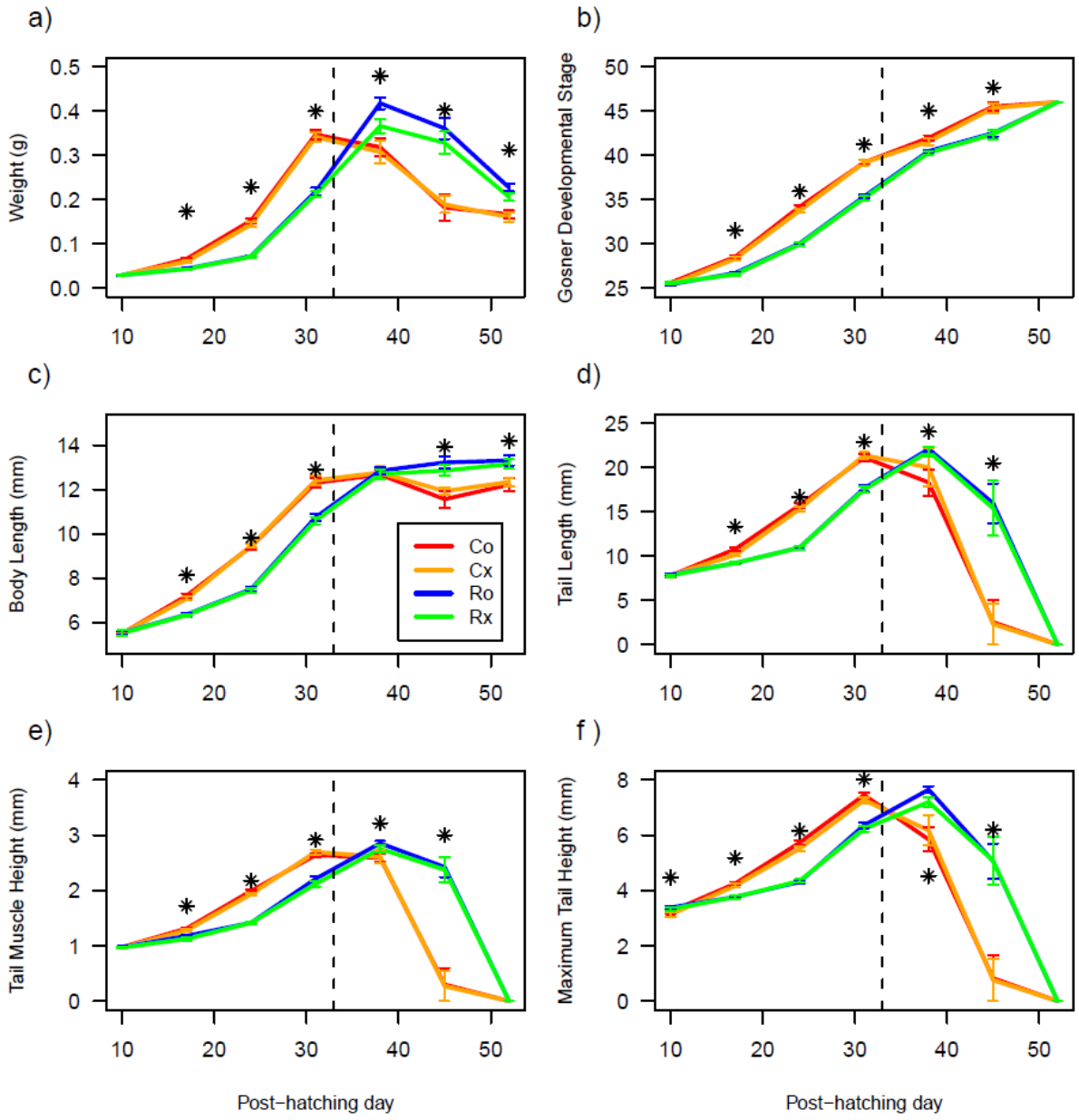

Figure 2.4 Pre-metamorphic morphometric measurements.

Mean (± SE) measurements for a) weight, b) Gosner development stage, c) body length, d) tail length, e) tail muscle height and f) maximum tail height by treatment group (C = control-fed tadpoles; R = food-restricted tadpoles; o = PBS-injected; x = PHA-injected). Vertical dashed line represents time of injection procedure. Asterisks indicate significant differences between feeding treatments where $p<0.05$, see text and Tables A2.2-A2.7 for details. See Fig. 2.1 for *n*.

- 0.446, *df* = 37, *p* = 0.034; Fig. 2.4f). Otherwise, immune activation had no significant effects on controls or other morphometric measurements prior to metamorphosis and the observed differences in restricted tadpoles had disappeared by the next measuring time (Tables A2.2-A2.7).

Alternatively, the impact of two-weeks of feed restriction on growth and development as a whole can be better visualised by factor analysis, which included all six measures and two factors (Fig. 2.5, Table A2.8). This pattern indicates a morphological distinction between feeding treatments increasing on day 17 to a peak on day 24, when food restriction ended. After this period, the separation of the groups decreases. This evidence is supported by a linear discriminant analysis (Table 2.1). The discrimination accuracy between control and restricted tadpoles peaks at the end of the restriction period then begins to decline. Notably the discrimination accuracy between PBS- and PHA-injected increases sharply following the injection then again declines.

Table 2.1 Linear discriminant analysis

Accuracy of the linear discrimination analysis between feeding treatments and between immune activation treatments. Morphological variables of the treatment groups were analysed by linear discriminant analysis and the resulting parameters which defined the groups were then used to predict which treatment group an individual belonged to and scored as correct or not.

Day	Feeding	Immune Activation
10	61.20%	58.47%
17	91.30%	59.13%
24	100%	58.77%
31	93.64%	59.09%
38	85.92%	77.46%
45	95.92%	69.39%

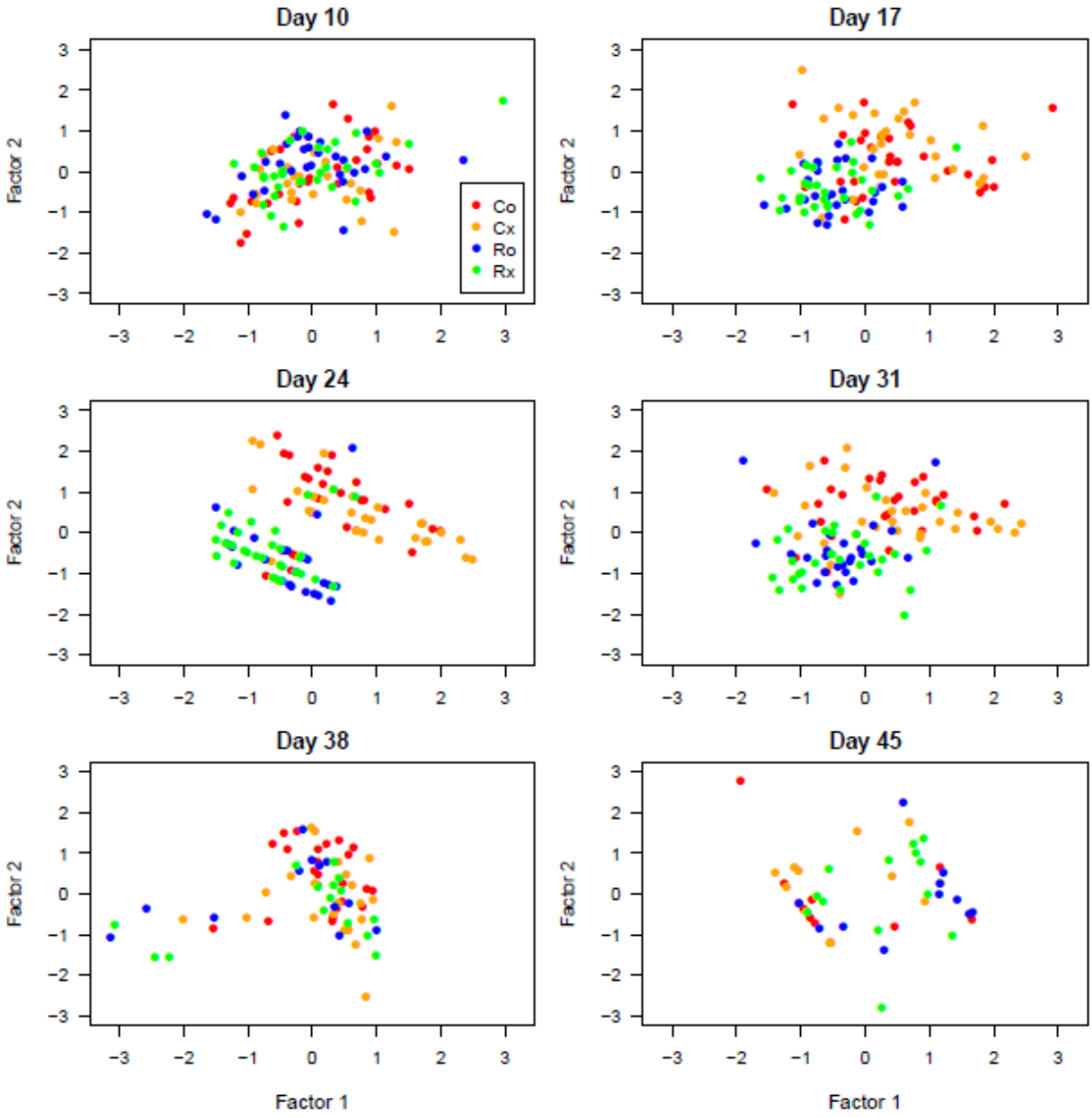

Figure 2.5 Factor analysis of pre-metamorphic morphology.

Plots of two first axes from the maximum-likelihood factor analysis of all morphometric variables for individuals prior to metamorphosis colour-coded by treatment group (C = control-fed tadpoles; R = food-restricted tadpoles; o = PBS-injected; x = PHA-injected). This analysis provided six variables per individual per day and each day was analysed by two factors for ease of interpretation. See Fig. 2.1 for *n* and Table A2.8 for loadings.

To ascertain that compensatory growth had occurred, the growth rates of all morphometric measures between day 10 and 31 for controls and days 17 and 38 for restricted (re-labelled for ease of comparison as 10 to 31) were compared. These periods constituted the most linear growth for each group and the seven day time-shift of the restricted data was comparable to the actual developmental difference as given by time of forelimb emergence. The growth rates of each group were extracted from a linear mixed model with random effects of the interaction of food and day over the entire period, accounting for individual identity as a random factor. The slopes of stage, body length, tail length and tail muscle height were not significantly different, indicating that for these traits, feed restriction only delayed growth but did not change the growth rate once food was equally available (Fig 2.6.; Table A2.9). In contrast, the slope of weight for the restricted tadpoles was significantly steeper than that of the controls (slope ± SE, control: 0.0146 ± 0.0005 *vs.* restricted: 0.0166 ± 0.0006 restricted; LMM, *t*-value = 2.95, $p = 0.004$; Fig. 2.6a). The slope of maximum tail height growth was steeper for the controls than restricted tadpoles (slope ± SE, control: 0.199 ± 0.004 *vs*. restricted: 0.186 ± 0.006; LMM, *t*-value = -2.13, $p = 0.043$; Fig. 2.6f).

The results of the comparisons with other papers (Table 2.2) showed that although both control and restricted tadpoles showed earlier forelimb emergence than has previously been published, their weights at forelimb emergence and the growth slopes were not significantly different than what is to be expected from *L. ewingii* tadpoles raised in a laboratory environment.

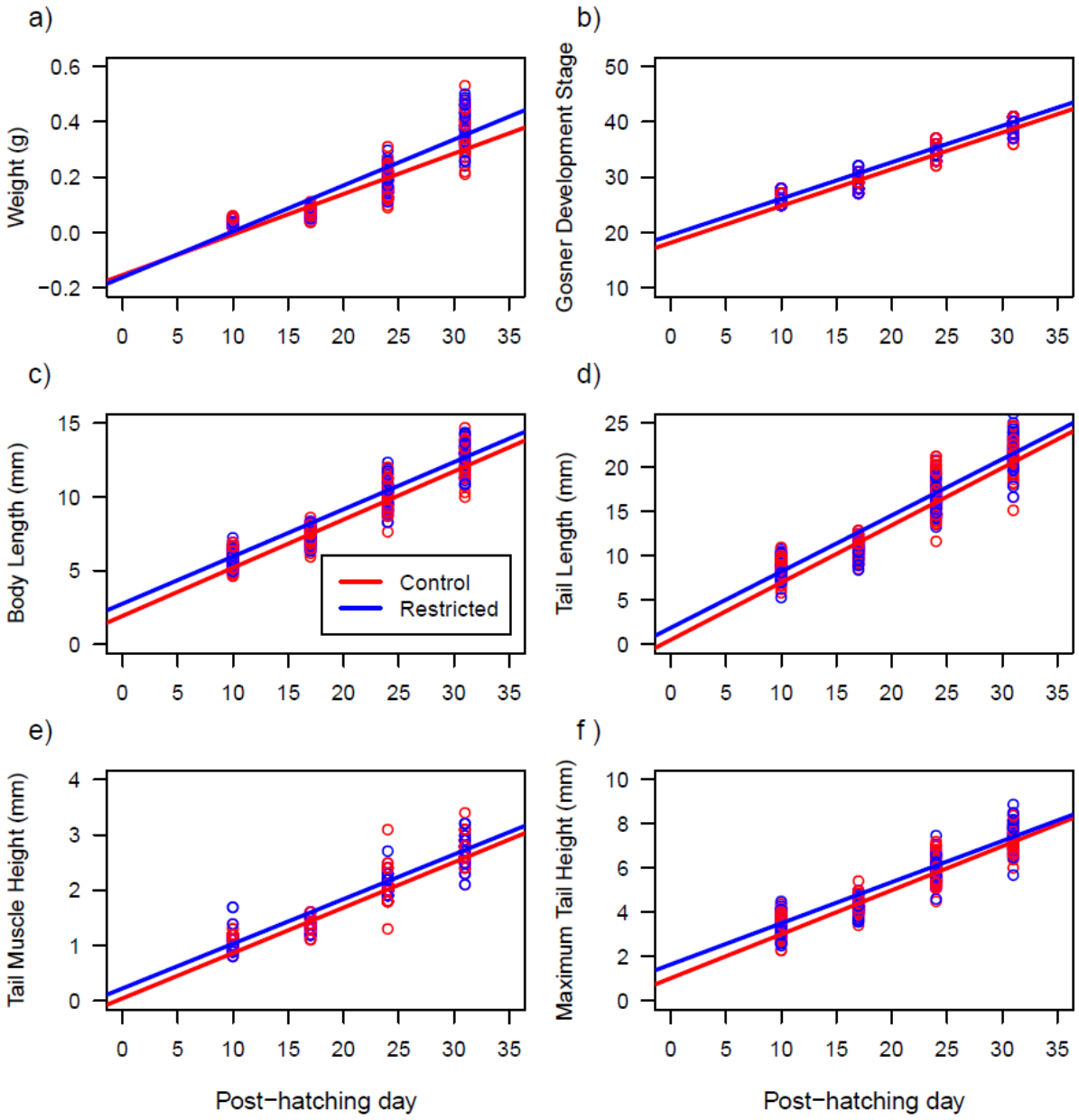

Figure 2.6 Pre-metamorphic linear growth slopes.

Linear regression slopes for a) weight, b) Gosner development stage, c) body length, d) tail length, e) tail muscle height and f) maximum tail height by feeding treatments prior to injection. Restricted tadpole data were shifted back by one week for ease of comparison. Original data measurements are included as circles. See Fig. 2.1 for *n*.

Table 2.2 Comparison with published literature

Statistical results of the comparisons of weight at forelimb emergence, larval period and linear growth slope prior to peak growth between published literature on *L. ewingii*, and the control and restricted treatment groups. The results are reported from multiple two-sided *t*-tests with unequal variances (note statistically significant results are in bold).

	Treatment Group	Paper	*t*	*df*	*p*
Weight	Control	Chinathamby *et al.*(2006)	-0.267	54	0.7903
	Restricted		0.331	57	0.7418
	Control	Squires *et al.* (2010)	-0.106	28	0.9161
	Restricted		0.200	31	0.8429
Larval Period	**Control**	**Sokol (1984)**	**-5.545**	**39**	**<0.0001**
	Restricted		**-2.043**	**42**	**0.0473**
	Control	**Chinathamby *et al.* (2006)**	**-13.560**	**54**	**<0.0001**
	Restricted		**-9.647**	**57**	**<0.0001**
	Control	**Squires *et al.* (2010)**	**-8.656**	**28**	**<0.0001**
	Restricted		**-4.515**	**31**	**0.0001**
Growth Slope	Control	Chinathamby *et al.* (2006)	-0.044	54	0.9654
	Restricted		0.016	57	0.9873

Tadpole swimming speed was significantly affected by feeding treatment (LMM, *t*-value = 3.67, *p*=0.0003; Fig. 2.7a, Table A2.10). Day also had a significant effect (LMM, *t*-value = -26.14, *p* <0.0001; Fig. 2.7a, Table A2.10). Initially there was little difference between the groups. However, after two weeks of food restriction the restricted tadpoles were considerably slower than the controls. Following only 10 days of realimentation both groups were equally fast. The number of prompts required to get the tadpoles to swim 30cm was affected by a significant interaction between day and feeding treatment (GLMM, *z*-value = 3.91, $p < 0.0001$; Fig. 2.7b, Table A2.11). The prompts restricted tadpoles required after the period of food restriction increased from baseline levels yet were no different than controls by the final swimming test.

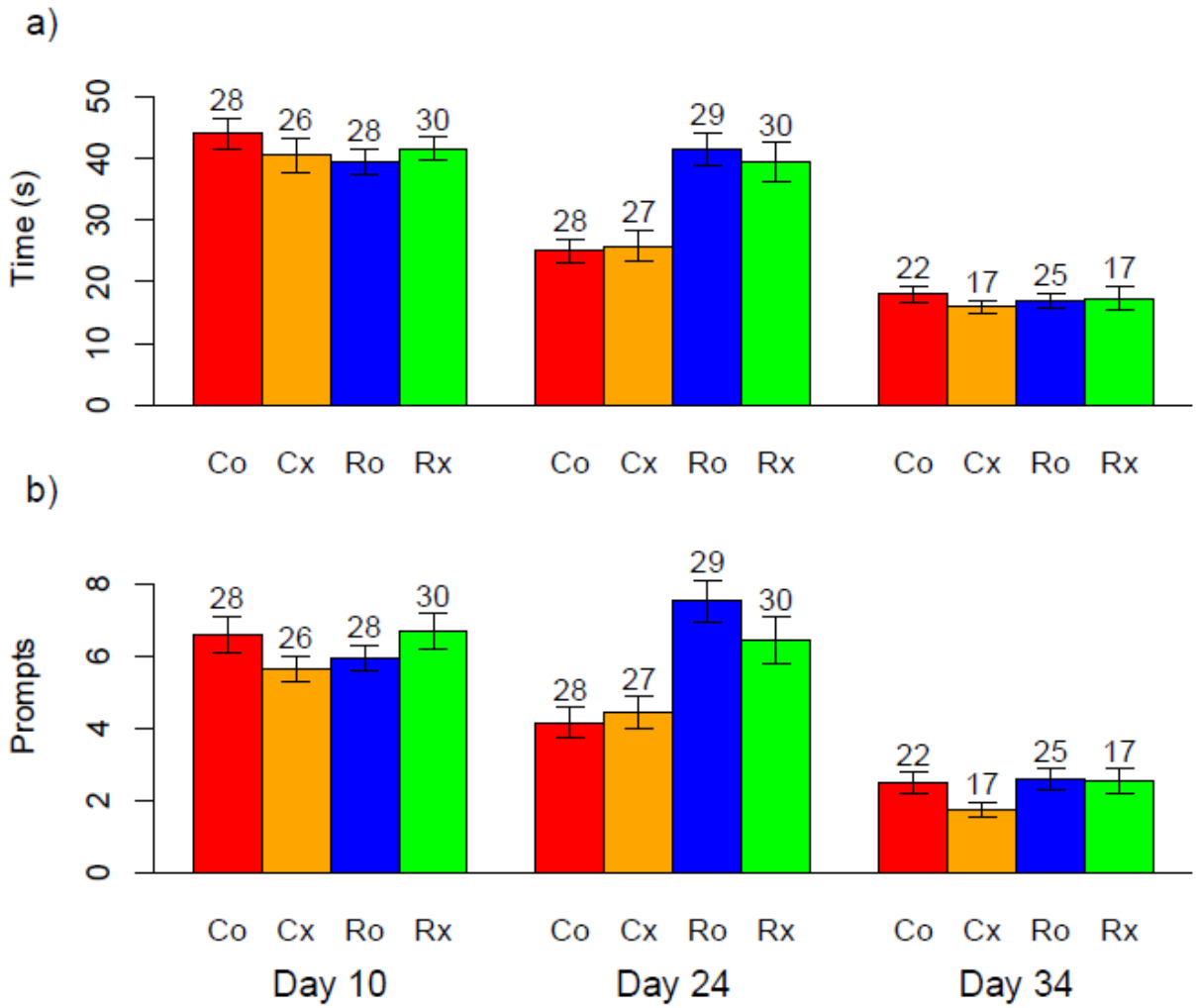

Figure 2.7 Swimming speed and prompts of tadpoles.

Mean (± SE) time taken (a) and the number of prompts required (b) for tadpoles to swim 30cm on three different testing days prior to metamorphosis (*n* given above bars). Restricted groups were fed a reduced diet from days 10 to 24. Treatment groups labelled as in Fig. 2.3.

2.3.3 Post-metamorphosis: the frog stages

After metamorphosis, the frogs from previously restricted tadpoles showed a distinct size advantage over the controls in both weight and snout-vent length (SVL; Fig. 2.8, Tables A2.2& A2.3). However, the difference in SVL appeared to be decreasing towards the end of the experiment, with the final measurement showing no significant difference (mean SVL (mm) ± SE, control: 16.12 ± 0.34 *vs*. restricted: 16.69 ± 0.16; GLM, *t*-value = 1.236, *df* = 21, $p = 0.23$; Fig. 2.8c). In contrast, the general trend of PHA injection being detrimental to weight appeared to be increasing towards the end of the experiment, with PBS-injected frogs weighing significantly more than the PHA-injected frogs on the second to last measurement (mean weight (g) ± SE on day 129, PBS: 0.447 ± 0.013 *vs*. PHA: 0.388 ± 0.016; GLM, *t*-value = -2.11, *df* = 21, $p = 0.047$; Fig. 2.8b). PHA-injection showed a lesser effect on SVL and was only significant at one measurement (Table A2.3, Fig. 2.8d).

Neither the feeding nor the injection treatments had a significant effect on the hopping ability of the frogs. Time did have an effect, with the hopping distance decreasing and the number of hops increasing over successive testing days (distance: LMM, *t*-value = -2.304, $p = 0.022$, Table A2.12; number: GLMM, *z*-value = 7.268, $p < 0.0001$, Table A2.13). The feeding latency of the frogs was significantly affected by their earlier feeding treatment. Previously restricted tadpoles were significantly faster to capture prey (wingless *Drosophila*) as frogs than the controls (feeding latency (s), control: 52.0 ± 9.7 *vs*. restricted: 20.7 ± 12.5; LMM, *t*-value = 3, $p = 0.003$, Table A2.14).

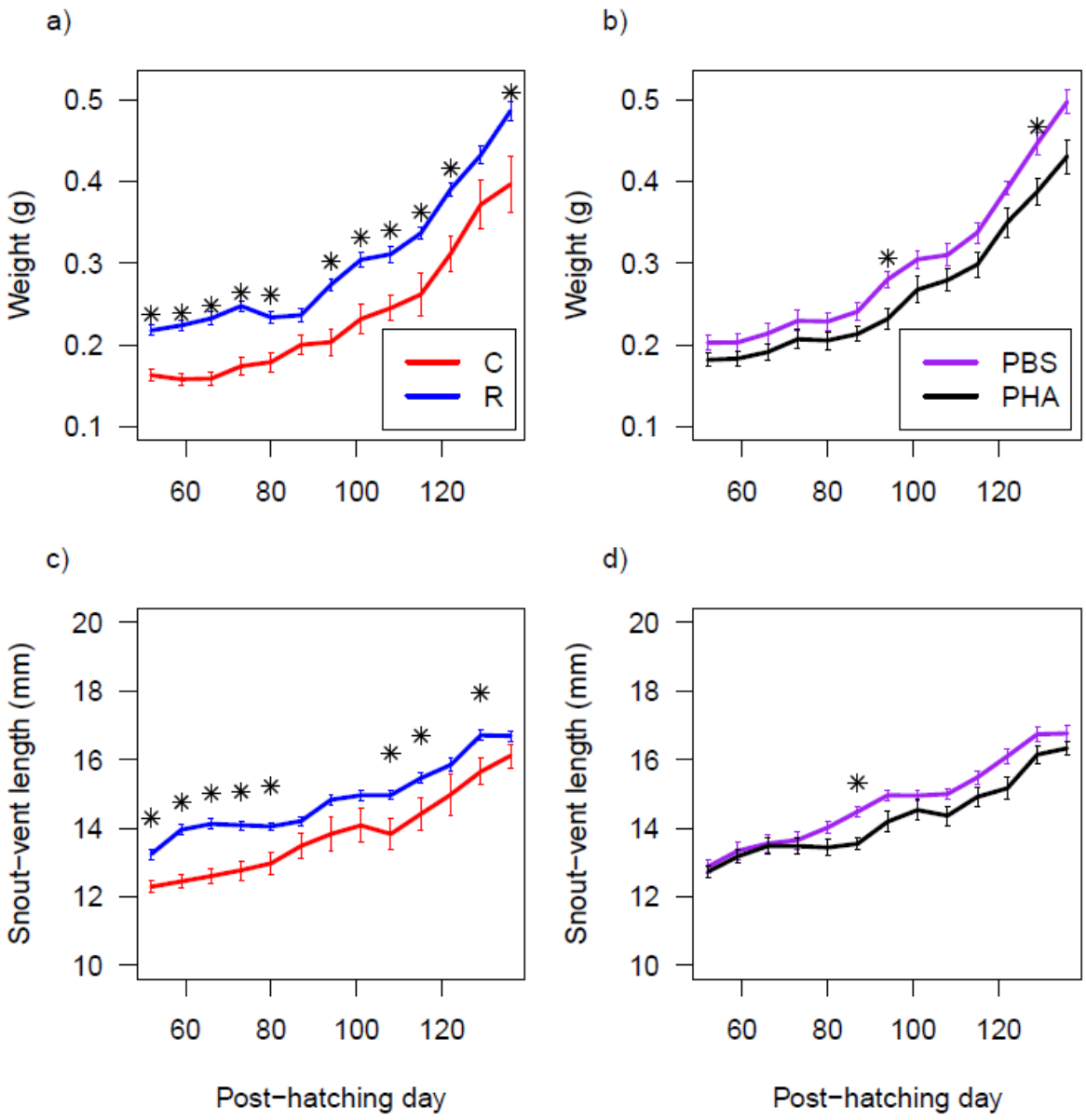

Figure 2.8 Post-metamorphic morphometric measurements

Mean (± SE) measurements for weight (a & b) and snout-vent length (c & d) by feeding treatment (a & c) and immune activation treatment (b & d). See text for explanation of inappropriateness of interaction, and thus the pooling of feeding treatment and injection group data in this graph. Asterisks indicate significant differences where $p<0.05$, see text and Table A2.2 & A2.3 for details. See Fig. 2.1 for *n*.

2.4 DISCUSSION

The two main findings of this study are that: 1) the feeding treatment caused compensatory growth in weight, delayed development and resulted in larger frogs with higher survival and 2) the immune activation caused 49% tadpole mortality following the injection and showed a trend of decreased size in PHA-injected frogs. Compensatory growth and delayed development are common responses to a restricted feeding treatment (Nylin & Gotthard, 1998; Metcalfe & Monaghan, 2001). However, few studies have observed the restricted group exceeding the final size of controls, sometimes referred to as "over-compensation" (Ali *et al.*, 2003). Likewise, increased survival due to restricted feeding is usually only observed in farmed poultry, as it has been shown to reduce the incidence of ascites-related mortalities (Baghbanzadeh & Decuypere, 2008). Compensatory growth was observed only in weight (Fig. 2.4a). There was a lower growth trajectory in maximum tail height, suggesting that tadpoles may have traded-off investment in tail growth in order to commit more resources to weight gain (Fig. 2.4f). Tail height may be expendable in a laboratory situation since it is usually associated with escaping predators (Kraft *et al.*, 2006).

Restricted feeding caused a significant delay in the development of the tadpoles, as has been recorded in a number of metamorphosing species as a result of early nutritional deprivation, from sea stars and clams to wax moths and damselflies (Allison, 1994; Bogus & Szolajska, 1995; De Block & Stoks, 2008a; Yan *et al.*, 2009). Larval fire salamanders, *Salamandra salamandra*, which had been maintained on a constant poor diet, took almost 30% longer than well-fed conspecifics to reach metamorphosis (Krause *et al.*, 2011). In a broad context, estimated larval periods for *L. ewingii* tadpoles in natural environments range from less than one month (Lauck *et al.*, 2005) to up to eight months (Alderton, 1985) and even successive years during wet summers (Gill, 1978). In a similar experimental design, wood frogs, *Rana temporaria*, showed compensatory growth but there was no difference in larval period

(Capellan & Nicieza, 2007). The *R. temporaria* tadpoles were able to fully compensate by the time of peak size prior to metamorphosis. Either their developmental rate was not hindered by food restriction or *R. temporaria* also showed compensatory development. Growth slope analysis shows that in the present study, developmental stage increased at the same rate as controls (Fig. 2.4), suggesting that these tadpoles did not attempt to compensate for a prolonged developmental period.

Since the weight of restricted tadpoles showed compensatory growth but their body length grew along the same trajectory as controls, it can be assumed that these tadpoles compensated by investing in fat stores rather than skeletal growth. The necessity of weight gain in place of skeletal growth is not obvious considering that both sexes prefer larger mates (Hunt et al., 2009). Instead, fat stores may be an investment in short-term survival rather than long-term reproductive fitness. The lighter weight, and presumably smaller fat stores, of the controls at metamorphosis may have reduced their chances of surviving. Their smaller size at completion of metamorphosis may in part be a result of the shorter larval period of the controls compared to restricted tadpoles. Analysis of the existing literature showed that mean larval periods for both feeding treatments were significantly shorter than all of these studies (Table 2.2). However, importantly, the linear growth trajectories of tadpoles in both groups were not significantly different from the existing literature.

One unusual finding in this study was the high mortality rate of the control animals (Fig. 2.2). Since PHA-injection caused a large number of deaths, further discussion of survival is limited to PBS-injected tadpoles to obtain a more biologically relevant perspective. The survival rate up to forelimb emergence (when 24 were sacrificed for a different experiment) was 79.7% for the 59 PBS-injected tadpoles. This rate falls within normal expectations of tadpole survival in laboratory studies which only follow tadpoles to metamorphosis (Cree, 1984: 100% survival; Sokol, 1984: 45% - 84% survival; Chinathamby *et al.*, 2006: 92% survival).

I was unable to locate studies with which to compare long-term post-metamorphic survival since all of the published laboratory studies on *L. ewingii* tadpoles use completion of metamorphosis as the experimental end point. Differential mortality between diet treatments after metamorphosis indicates that early larval diet had an important impact on later survival. Of the PBS-injected tadpoles that survived metamorphosis and were not sacrificed, 11 of the 13 previously food-restricted metamorphs survived until the end of the experiment, compared with only one of the eleven control metamorphs. Analysis of post-metamorphic survival showed a significant positive relationship between metamorphic weight and survival.

In comparison with other studies on *L. ewingii*, the larval periods of both feeding treatment groups were significantly shorter. One potential cause of the rapid developmental rate of both treatments is a pond drying effect, where tadpoles detect a decreasing water level and accelerate development in order to reach metamorphosis before a pond completely dries. Amphibians are known to be very sensitive to pond drying and can rapidly react by increasing their development rate (Newman, 1992). In the present experiment, individual tadpoles were raised in 200ml containers with a 4.8cm water column. Housing in these relatively shallow containers may have simulated a pond drying effect by exciting the proximate mechanisms of temperature or touch-pressure cues which are translated into phenotypic changes in development rate (Denver, 1995). All individuals were exposed to identical housing conditions; however, the restricted tadpoles may have been more successful at avoiding the costs of premature development due to their accelerated weight gain which increased their probability of post-metamorphic survival.

Compensatory growth does not come freely, as Metcalfe and Monaghan (2001) outline, but this cost was not detected by my fitness-related behaviour tests. The swimming test showed that following food restriction, tadpoles were much slower than controls and required more prompts (Fig. 2.7). This finding is likely due to small size, which for the restricted tadpoles

was still similar to initial size measurements, since the post-restriction speed and prompt measures were also similar to those recorded at the start of the experiment. At the time of the final swim trial, both treatment groups were of a similar size because the control tadpoles had begun their re-absorption following peak size and both groups showed a similar swimming speed. Interestingly, immune activation did not impede swimming ability, despite the high mortality for PHA-injected tadpoles and the fact that the final swim test was conducted one day after the injection was administered. This observation suggests that for the tadpoles which survived the immune activation, there was little immediate fitness cost or lethargy related to fighting the immune challenge.

The frogs from previously food-restricted tadpoles were much faster to capture prey than control frogs. The decreased feeding latency could have resulted from the previously restricted tadpoles being in better condition as frogs, with heavier weights and longer snout-vent lengths than control frogs (Fig. 2.8). In contrast, feeding and immune activation treatments had little effect on the hopping ability of the frogs. Many studies have failed to find a significant treatment effect on hopping despite other evidence of treatment effects (Van Buskirk & Saxer, 2001; Stamper *et al.*, 2008).

Although the mortality rate for PHA-injected frogs was relatively high and their growth seems to be stunted compared to the control group (PBS-injected), there was no differential survival between PHA-injected dietary treatments. There was also no effect of immune activation on time to metamorphosis. Both of these outcomes have been observed in wood frog tadpoles, *Rana sylvaticus*, immune activated with ranavirus (Warne *et al.*, 2010). Warne *et al.*(2010) found that mortality caused by the immune response to ranavirus was greater at later developmental stages, yet in this study there was no difference between feeding treatments despite the more advanced development of the control tadpoles (Fig. 2.3b).

Decreased investment in growth following an immune activation during development is a trade-off that has been shown in a number of species (reviewed in Sadd & Schmid-Hempel, 2009). Restricted tadpoles showed a temporary reduction in weight gain and maximum tail height growth immediately following immune activation, indicating that they had fewer energy reserves to invest in fighting off the challenge and therefore had to reduce their growth to compensate, unlike the controls which showed no difference in growth following the challenge (Fig. 2.3). It is unfortunate that due to unbalanced group sizes (because of unpredictable differential mortality), this interaction was not possible to investigate after metamorphosis. However, the trend towards smaller sized frogs after larval immune activation does suggest there are long-term effects of immune activation which may affect the stronger consequences of early dietary restriction.

Contrary to my initial hypotheses, the food-restricted tadpoles in this study actually showed increased survival and fitness traits, except for a prolonged developmental period. In particular, restricted tadpoles reached a larger size as frogs, which has strong implications for future reproductive fitness (Hunt et al., 2009), although this result could partially be due to my experimental setting. Compensatory growth was observed in the weight of restricted tadpoles, confirming that this species is capable of plastic development in order to overcome developmental nutritional setbacks. *L. ewingii* has evolved to survive in both permanent and ephemeral ponds with a wide variety of habitat characteristics, including pond productivity (Lauck *et al.*, 2005). Therefore, it is likely that variable levels of available nutrition are experienced by tadpoles in the wild. In a natural environment smaller *L. ewingii* tadpoles are more likely to be predated by dragonfly larvae; therefore the benefits of compensatory growth are obvious: grow fast or risk being eaten (Richards & Bull, 1990). On the whole, the results of this experiment provide additional support for the prevalence of compensatory growth across the animal kingdom and, in particular, emphasise the vast capacity for

phenotypic plasticity exhibited by amphibians in response to their developmental environment.

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2. 6 APPENDIX

2.6.1 Photo Validation

Tadpole morphology is sensitive to a number of environmental factors. Tadpoles showed an interaction effect between temperature and predator cues on morphology, with predator cues resulting in increased tail size and musculature only in cold water (Touchon & Warkentin, 2011). In nature, the overall shape of tadpoles from a number of species has been shown to vary depending on habitat characteristics such as pond permanence, canopy cover and conspecific density (Van Buskirk, 2009). To make accurate comparisons of morphological differences between the treatment groups in this experiment, it was first necessary to verify that the morphological information was correct. I validated the use of digitally measured photographs in an earlier pilot test, which also allowed me to test the efficacy of the anaesthetic and the ability of the tadpoles to recover from anaesthesia.

Fifteen tadpoles were initially photographed using the protocol described in Chapter II. However, in this instance three photographs were taken instead of one. Following this, the tadpoles were placed in 50 μL/L Aqui-S until spontaneous movement ceased. Each tadpole was placed laterally on a moist paper towel on the platform of a dissecting microscope and callipers were used to measure the four morphological measurements of interest (body length, tail length, tail muscle height and maximum tail depth). Measurements of each feature were taken three times for each tadpole. Tadpoles were doused with water between each series of measurements and were not out of water for longer than three minutes. After measurement, the tadpoles were returned to their containers. All tadpoles recovered from anaesthesia within 15 minutes and showed no adverse effects of the measuring procedure within the next few days.

To analyse whether the photographic measurements were accurate, the R statistical program (R Development Core Team, 2009) was used to measure the repeatability of each method for each morphological measurement (Schielzeth & Nakagawa, 2008). The results are presented below (Table A2.1). Linear mixed-effect models were also run for each morphological feature with tadpole identity as a random factor. There was no detectable difference between measurement techniques for body length (mean ± SE, calliper = 10.94 ± 0.63, photo = 10.93 ± 0.63; t = -0.12, df = 72, p = 0.905). However, both tail length and tail muscle height were recorded as being larger when measured by photograph (tail length: mean ± SE, calliper = 17.88 ± 1.27, photo = 18.23 ± 1.27; t = 3.59, df = 72, p = 0.0006; tail muscle height: mean ± SE, calliper = 2.37 ± 0.17, photo = 2.45 ± 0.17; t = 2.74, df = 72, p = 0.0078). In contrast, the measurements of maximum tail height were higher when taken from callipers (mean ± SE, calliper = 6.44 ± 0.33, photo = 6.33 ± 0.33; t = -2.39, df = 72, p = 0.019).

Table A2.1 Repeatability of photo measurements

Estimates and measures of variation for repeatability (R) of the measurement type for each morphological feature. (SE: standard error, 95% CI: 95% confidence interval)

	Photographic measurement			Direct measurement		
Morphological feature	*R*	SE	95% CI	*R*	SE	95% CI
Body length	0.997	0.002	(0.991 ,0.999)	0.995	0.004	(0.984, 0.998)
Tail length	0.999	0	(0.998, 1)	0.998	0.002	(0.993, 0.999)
Tail muscle height	0.984	0.01	(0.958, 0.993)	0.973	0.021	(0.915, 0.989)
Maximum tail height	0.991	0.005	(0.981, 0.998)	0.98	0.012	(0.952, 0.993)

Overall, these results indicate that there are consistent differences between the two measurement techniques. From the higher repeatability of the photographic techniques, it is likely that the differences stem from technical difficulties arising from measuring a tadpole by hand. This is consistent with the evidence that tail length and tail muscle height are higher when photographed, while maximum tail height is lower. Both tail length and tail muscle height require measurements to start at the tail base which is at least partially obstructed by the body when measuring with callipers. This means part of the length would have been missing from these calliper measurements, giving smaller results. Maximum tail height is more likely to be overestimated when measured by hand because of the difficulty in distinguishing where the edges of the transparent fin lie and the care required to prevent damaging the fragile tissue.

Overall, this validation provides confidence in the photographic measuring technique as being not only more consistent, but also more accurate than the traditional calliper technique (Altig & McDiarmid, 1999). Of course, the greatest advantage of the photographic technique is that it is relatively quick, non-invasive and much less stressful to the tadpole. Photographic measurement is unlikely to cause confounding effects on the experiment and allows for multiple measurements of numerous subjects throughout development. An additional advantage is that it also provides concrete and lasting evidence of the tadpoles which may be of some use to future research.

2.6.2 References

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Table A2.2 Weight statistics

Statistical table of the results of multiple linear models (GLMs) conducted separately for each day of measurement, both pre- and post-metamorphosis, for the weights (g) of tadpoles/frogs in the control or restricted feeding treatments and injected with immune-activator (PHA) or control injected (PBS). See statistical details in methods for rationale of use of interaction terms. "foodR" refers to the restricted treatment in comparison with the control feeding treatment and "injectx" refers to immune activated tadpoles in comparison with PBS-injected tadpoles, with "foodR:injectx" indicating an interaction term.

Day	Term	Estimate	SE	t-value	df	p-value
10	Intercept	0.0290	0.0008	36.677	116	<0.0001
	foodR	0.0002	0.0011	0.182	116	0.856
17	Intercept	0.0629	0.0014	45.910	113	<0.0001
	foodR	**-0.0195**	**0.0019**	**-10.180**	**113**	**<0.0001**
24	Intercept	0.1482	0.0028	53.400	112	<0.0001
	foodR	**-0.0770**	**0.0039**	**-19.960**	**112**	**<0.0001**
31	Intercept	0.3440	0.0067	51.530	108	<0.0001
	foodR	**-0.1293**	**0.0095**	**-13.640**	**108**	**<0.0001**
38	Intercept	0.3182	0.0169	18.817	68	<0.0001
	foodR	**0.0993**	**0.0234**	**4.243**	**68**	**<0.0001**
	injectx	-0.0109	0.0293	-0.372	68	0.711
	foodR:injectx	-0.0406	0.0392	-1.035	68	0.305
45	Intercept	0.1820	0.0280	6.495	45	<0.0001
	foodR	**0.1786**	**0.0350**	**5.109**	**45**	**<0.0001**
	injectx	0.0070	0.0396	0.177	45	0.861
	foodR:injectx	-0.0394	0.0522	-0.755	45	0.454
52	Intercept	0.1663	0.0099	16.722	35	<0.0001
	foodR	**0.0604**	**0.0128**	**4.707**	**35**	**<0.0001**
	injectx	-0.0063	0.0133	-0.469	35	0.642
	foodR:injectx	-0.0149	0.0182	-0.816	35	0.420
59	Intercept	0.1564	0.0101	15.523	35	<0.0001
	foodR	**0.0779**	**0.0130**	**5.996**	**35**	**<0.0001**
	injectx	0.0028	0.0135	0.204	35	0.840
	foodR:injectx	-0.0269	0.0184	-1.460	35	0.153

Table A2.2 (continued)

Day	Term	Estimate	SE	t-value	df	p-value
66	Intercept	0.1500	0.0131	11.451	31	<0.0001
	foodR	**0.0958**	**0.0160**	**5.974**	**31**	**<0.0001**
	injectx	0.0150	0.0173	0.866	31	0.393
	foodR:injectx	**-0.0464**	**0.0224**	**-2.074**	31	**0.047**
73	Intercept	0.1640	0.0150	10.942	29	<0.0001
	foodR	**0.0927**	**0.0178**	**5.194**	**29**	**<0.0001**
	injectx	0.0160	0.0191	0.837	29	0.409
	foodR:injectx	-0.0389	0.0245	-1.590	29	0.123
80	Intercept	0.1838	0.0163	11.310	26	<0.0001
	foodR	**0.0522**	**0.0153**	**3.406**	**26**	**0.002**
	injectx	-0.0063	0.0142	-0.446	26	0.659
87	Intercept	0.2149	0.0176	12.226	22	<0.0001
	foodR	0.0280	0.0164	1.705	22	0.102
	injectx	-0.0179	0.0140	-1.278	22	0.215
94	Intercept	0.2275	0.0173	13.176	22	<0.0001
	foodR	**0.0569**	**0.0161**	**3.527**	**22**	**0.002**
	injectx	**-0.0290**	**0.0138**	**-2.106**	22	**0.047**
101	Intercept	0.2440	0.0230	10.602	22	<0.0001
	foodR	**0.0657**	**0.0215**	**3.056**	**22**	**0.006**
	injectx	-0.0148	0.0184	-0.806	22	0.429
108	Intercept	0.2533	0.0227	11.148	22	<0.0001
	foodR	**0.0614**	**0.0212**	**2.896**	**22**	**0.008**
	injectx	-0.0100	0.0181	-0.550	22	0.588
115	Intercept	0.2755	0.0228	12.087	21	<0.0001
	foodR	**0.0676**	**0.0212**	**3.194**	**21**	**0.004**
	injectx	-0.0166	0.0183	-0.907	21	0.375
122	Intercept	0.3267	0.0211	15.481	21	<0.0001
	foodR	**0.0709**	**0.0196**	**3.616**	**21**	**0.002**
	injectx	-0.0180	0.0170	-1.063	21	0.300
129	Intercept	0.4098	0.0270	15.194	21	<0.0001
	foodR	0.0402	0.0250	1.606	21	0.123
	injectx	**-0.0458**	**0.0217**	**-2.110**	21	**0.047**
136	Intercept	0.4325	0.0306	14.150	21	<0.0001
	foodR	**0.0709**	**0.0284**	**2.497**	**21**	**0.021**
	injectx	-0.0430	0.0246	-1.751	21	0.095

Table A2.3 Body length statistics

Statistical table of the results of multiple linear models (GLMs) conducted separately for each day of measurement, both pre- and post-metamorphosis, for the body length (or SVL; mm) of tadpoles/frogs in the control or restricted feeding treatments and injected with immune-activator (PHA) or control injected (PBS). See statistical details in methods for rationale of use of interaction terms. "foodR" refers to the restricted treatment in comparison with the control feeding treatment and "injectx" refers to immune activated tadpoles in comparison with PBS-injected tadpoles, with "foodR:injectx" indicating an interaction term.

Day	Term	Estimate	SE	t-value	df	p-value
10	Intercept	5.4966	0.0660	83.290	116	<0.0001
	foodR	0.0251	0.0926	0.271	116	0.787
17	Intercept	7.1446	0.0565	126.440	113	<0.0001
	foodR	**-0.7989**	**0.0789**	**-10.130**	**113**	**<0.0001**
24	Intercept	9.4509	0.0787	120.030	112	<0.0001
	foodR	**-1.9713**	**0.1095**	**-18.010**	**112**	**<0.0001**
31	Intercept	12.3655	0.1284	96.334	108	<0.0001
	foodR	**-1.6909**	**0.1815**	**-9.315**	**108**	**<0.0001**
38	Intercept	12.6773	0.1974	64.180	68	<0.0001
	foodR	0.1769	0.2735	0.647	68	0.520
	injectx	0.0955	0.3421	0.279	68	0.781
	foodR:injectx	-0.2430	0.4583	-0.530	68	0.598
45	Intercept	11.5700	0.3048	37.961	45	<0.0001
	foodR	**1.6578**	**0.3801**	**4.361**	**45**	**<0.0001**
	injectx	0.3600	0.4310	0.835	45	0.408
	foodR:injectx	-0.7241	0.5673	-1.276	45	0.208
52	Intercept	12.2250	0.2534	48.244	35	<0.0001
	foodR	**1.0917**	**0.3271**	**3.337**	**35**	**0.002**
	injectx	0.1250	0.3400	0.368	35	0.71533
	foodR:injectx	-0.2861	0.4642	-0.616	35	0.542
59	Intercept	12.2000	0.2428	50.249	35	<0.0001
	foodR	**1.9000**	**0.3134**	**6.062**	**35**	**<0.0001**
	injectx	0.4500	0.3257	1.381	35	0.176
	foodR:injectx	-0.7833	0.4447	-1.761	35	0.087

Table A2.3 (continued)

Day	Term	Estimate	SE	t-value	df	p-value
66	Intercept	12.3333	0.2968	41.555	31	<0.0001
	foodR	**1.8333**	**0.3635**	**5.044**	**31**	**<0.0001**
	injectx	0.4792	0.3926	1.220	31	0.232
	foodR:injectx	-0.5681	-1.1210	-1.121	31	0.271
73	Intercept	12.6800	0.3542	35.802	29	<0.0001
	foodR	**1.3867**	**0.4216**	**3.289**	**29**	**<0.0001**
	injectx	0.1575	0.4515	0.349	29	0.728
	foodR:injectx	-0.0992	0.5784	-0.171	29	0.865
80	Intercept	13.1839	0.3179	41.469	26	<0.0001
	foodR	**0.9771**	**0.2999**	**3.258**	**26**	**0.003**
	injectx	-0.2650	0.2777	-0.955	26	0.349
87	Intercept	14.1638	0.3201	44.249	22	<0.0001
	foodR	0.3476	0.2988	1.163	22	0.257
	injectx	**-0.8165**	**0.2554**	**-3.196**	**22**	**0.004**
94	Intercept	14.2596	0.4389	32.492	22	<0.0001
	foodR	0.7604	0.4097	1.856	22	0.077
	injectx	-0.5115	0.3502	-1.461	22	0.158
101	Intercept	14.2037	0.4364	32.545	22	<0.0001
	foodR	0.8127	0.4074	1.995	22	0.059
	injectx	-0.1444	0.3483	-0.415	22	0.682
108	Intercept	14.0785	0.3660	38.470	22	<0.0001
	foodR	**0.9983**	**0.3416**	**2.922**	**22**	**0.008**
	injectx	-0.2942	0.2921	-0.415	22	0.325
115	Intercept	14.6304	0.4297	34.050	21	<0.0001
	foodR	**0.9304**	**0.3990**	**2.332**	**21**	**0.030**
	injectx	-0.2565	0.3455	-0.742	21	0.466
122	Intercept	15.6138	0.5172	30.189	21	<0.0001
	foodR	0.5304	0.4803	1.104	21	0.282
	injectx	-0.7565	0.4159	-1.819	21	0.083
129	Intercept	15.8828	0.4037	39.341	21	<0.0001
	foodR	**0.9370**	**0.3749**	**2.499**	**21**	**0.021**
	injectx	-0.2793	0.3247	-0.860	21	0.399
136	Intercept	16.3522	0.3939	41.514	21	<0.0001
	foodR	0.4522	0.3658	1.236	21	0.230
	injectx	-0.2826	-0.8920	-0.892	21	0.382

Table A2.4 Tail length statistics

Statistical table of the results of multiple linear models (GLMs) conducted separately for each day of measurement, up to complete tail re-absorption on completion of metamorphosis, for the tail length (mm) of tadpoles in the control or restricted feeding treatments and injected with immune-activator (PHA) or control injected (PBS). "foodR" refers to the restricted treatment in comparison with the control feeding treatment and "injectx" refers to immune activated tadpoles in comparison with PBS-injected tadpoles, with "foodR:injectx" indicating an interaction term.

Day	Term	Estimate	SE	*t*-value	*df*	*p*-value
10	Intercept	7.8035	0.1044	74.730	116	<0.0001
	foodR	0.0349	0.1464	0.238	116	0.812
17	Intercept	10.4643	0.1216	86.061	113	<0.0001
	foodR	**-1.2592**	**0.1698**	**-7.418**	**113**	**<0.0001**
24	Intercept	15.5309	0.1648	94.250	112	<0.0001
	foodR	**-4.5801**	**0.2291**	**-20.000**	**112**	**<0.0001**
31	Intercept	21.2582	0.2605	81.611	108	<0.0001
	foodR	**-3.6818**	**0.3684**	**-9.995**	**108**	**<0.0001**
38	Intercept	18.3180	1.0360	17.678	68	<0.0001
	foodR	**3.7650**	**1.4350**	**2.625**	**68**	**0.011**
	injectx	1.7090	1.7950	0.952	68	0.344
	foodR:injectx	-1.9920	2.4040	-0.829	68	0.410
45	Intercept	2.5300	2.8536	0.887	45	0.380
	foodR	**13.4311**	**3.5591**	**3.774**	**45**	**0.0005**
	injectx	-0.2200	4.0356	-0.055	45	0.957
	foodR:injectx	-0.3593	5.3116	-0.068	45	0.946

Table A2.5 Tail muscle height statistics

Statistical table of the results of multiple linear models (GLMs) conducted separately for each day of measurement, up to complete tail re-absorption on completion of metamorphosis, for the tail muscle height (mm) of tadpoles in the control or restricted feeding treatments and injected with immune-activator (PHA) or control injected (PBS). "foodR" refers to the restricted treatment in comparison with the control feeding treatment and "injectx" refers to immune activated tadpoles in comparison with PBS-injected tadpoles, with "foodR:injectx" indicating an interaction term.

Day	Term	Estimate	SE	*t*-value	*df*	*p*-value
10	Intercept	0.9707	0.0121	80.176	116	<0.0001
	foodR	-0.0007	0.0170	-0.041	116	0.968
17	Intercept	1.2964	0.0161	80.365	113	<0.0001
	foodR	**-0.1456**	**0.0225**	**-6.464**	**113**	**<0.0001**
24	Intercept	1.9636	0.0144	136.030	112	<0.0001
	foodR	**-0.5484**	**0.0201**	**-27.330**	**112**	**<0.0001**
31	Intercept	2.6673	0.0319	83.590	108	<0.0001
	foodR	**-0.5036**	**0.0451**	**-11.160**	**108**	**<0.0001**
38	Intercept	2.5909	0.0616	42.032	68	<0.0001
	foodR	**0.2633**	**0.0853**	**3.085**	**68**	**0.003**
	injectx	0.0182	0.1068	0.170	68	0.865
	foodR:injectx	-0.1190	0.1430	-0.832	68	0.408
45	Intercept	0.3000	0.2591	1.158	45	0.253
	foodR	**2.1222**	**0.3232**	**6.567**	**45**	**<0.0001**
	injectx	-0.0300	0.3665	-0.082	45	0.935
	foodR:injectx	-0.0195	0.4823	-0.040	45	0.968

Table A2.6 Maximum tail height statistics

Statistical table of the results of multiple linear models (GLMs) conducted separately for each day of measurement, up to complete tail re-absorption on completion of metamorphosis, for the maximum tail height (mm) of tadpoles in the control or restricted feeding treatments and injected with immune-activator (PHA) or control injected (PBS). "foodR" refers to the restricted treatment in comparison with the control feeding treatment and "injectx" refers to immune activated tadpoles in comparison with PBS-injected tadpoles, with "foodR:injectx" indicating an interaction term.

Day	Term	Estimate	SE	*t*-value	*df*	*p*-value
10	Intercept	3.1845	0.0523	60.950	116	<0.0001
	foodR	**0.1655**	**0.0733**	**2.259**	**116**	**0.026**
17	Intercept	4.2089	0.0422	99.664	113	<0.0001
	foodR	**-0.4479**	**0.0590**	**-7.597**	**113**	**<0.0001**
24	Intercept	5.6218	0.0514	109.370	112	<0.0001
	foodR	**-1.2930**	**0.0715**	**-18.100**	**112**	**<0.0001**
31	Intercept	7.3600	0.0719	102.380	108	<0.0001
	foodR	**-1.0691**	**0.1017**	**-10.520**	**108**	**<0.0001**
38	Intercept	5.8455	0.2993	19.532	68	<0.0001
	foodR	**1.8004**	**0.4143**	**4.345**	**68**	**<0.0001**
	injectx	0.3273	0.5184	0.631	68	0.530
	foodR:injectx	-0.7731	0.6944	-1.113	68	0.269
45	Intercept	0.8300	0.8405	0.988	45	0.329
	foodR	**4.2311**	**1.0482**	**4.036**	**45**	**0.0002**
	injectx	-0.0700	1.1886	-0.059	45	0.953
	foodR:injectx	0.0816	1.5644	0.052	45	0.959

Table A2.7 Developmental stage statistics

Statistical table of the results of multiple linear models (GLMs) conducted separately for each day of measurement, pre-metamorphosis, for the Gosner development stage (Gosner, 1960) of tadpoles in the control or restricted feeding treatments and injected with immune-activator (PHA) or control injected (PBS). Note that stage was measured between stage 25 and stage 46, completion of metamorphosis. "foodR" refers to the restricted treatment in comparison with the control feeding treatment and "injectx" refers to immune activated tadpoles in comparison with PBS-injected tadpoles, with "foodR:injectx" indicating an interaction term.

Day	Term	Estimate	SE	*t*-value	*df*	*p*-value
10	Intercept	25.4800	0.0875	291.359	116	<0.0001
	foodR	0.0005	0.1227	0.005	116	0.996
17	Intercept	28.4107	0.1267	224.230	113	<0.0001
	foodR	**-1.8005**	**0.1769**	**-10.180**	**113**	**<0.0001**
24	Intercept	33.9636	0.1354	250.860	112	<0.0001
	foodR	**-3.9806**	**0.1882**	**-21.150**	**112**	**<0.0001**
31	Intercept	39.1636	0.1748	224.070	108	<0.0001
	foodR	**-3.9273**	**0.2472**	**-15.890**	**108**	**<0.0001**
38	Intercept	41.9545	0.2233	187.911	68	<0.0001
	foodR	**-1.4962**	**0.3091**	**-4.841**	**68**	**<0.0001**
	injectx	-0.4091	0.3867	-1.058	68	0.294
	foodR:injectx	0.2174	0.5180	0.420	68	0.676
45	Intercept	45.5000	0.5199	87.512	45	<0.0001
	foodR	**-3.0000**	**0.6485**	**-4.626**	**45**	**<0.0001**
	injectx	-0.2000	0.7353	-0.272	45	0.787
	foodR:injectx	0.0636	0.9678	0.066	45	0.948

Table A2.8 Factor analysis loadings

Factor loadings from two-factor analysis of the six morphological variables for each post-hatching day of the pre-metamorphic period. Each day includes the uniqueness and the loadings for factors 1 and 2 of each variable, as well as the sum of square loadings (SS), proportional variance (PV) and cumulative variance (CV) for each factor. BL – body length, TL – tail length, TMH – tail muscle height, MTH – maximum tail height.

Day		Weight	BL	Stage	TL	TMH	MTH	SS	PV	CV
	Uniqueness	0.765	0.436	0.805	0.603	0.534	0.609			
10	Factor 1	0.423	0.652	0.438	0.375	0.641	0.116	1.36	0.226	0.227
	Factor 2	0.237	0.373	<0.001	0.506	0.236	0.614	0.888	0.148	0.375
	Uniqueness	0.129	0.178	0.41	0.379	0.578	0.401			
17	Factor 1	0.868	0.773	0.348	0.657	0.269	0.452	2.179	0.363	0.363
	Factor 2	0.342	0.474	0.685	0.436	0.592	0.629	1.746	0.291	0.654
	Uniqueness	0.07	0.093	0.193	0.134	0.005	0.147			
24	Factor 1	0.8	0.782	0.614	0.7	0.539	0.727	2.938	0.49	0.49
	Factor 2	0.539	0.544	0.656	0.613	0.839	0.569	2.421	0.404	0.893
	Uniqueness	0.017	0.168	0.247	0.201	0.183	0.241			
31	Factor 1	0.822	0.743	0.538	0.537	0.432	0.744	2.547	0.424	0.424
	Factor 2	0.681	0.553	0.453	0.529	0.715	0.794	2.396	0.399	0.824
	Uniqueness	0.17	0.353	0.186	0.168	0.273	0.021			
38	Factor 1	0.541	0.177	-0.878	0.809	0.53	0.849	2.752	0.459	0.459
	Factor 2	0.733	0.785	-0.206	0.421	0.668	0.508	2.076	0.346	0.805
	Uniqueness	0.083	0.005	0.025	0.029	0.095	0.009			
45	Factor 1	0.775	0.432	-0.898	0.878	0.823	0.887	3.83	0.638	0.638
	Factor 2	0.563	0.899	-0.412	0.446	0.477	0.451	1.925	0.321	0.959

Table A2.9 Growth slopes statistics

Statistical output from separate linear mixed model (LMM) analyses of the growth slopes of the feeding treatments during the linear growth phase with individual identity as a random factor. Results are reported from the interaction between feeding treatments and day for each morphological variable with the *p*-value estimated from MCMC simulation, including the lower and upper bounds of the 95% highest posterior density (95% HPD) as a credible interval. BL – body length, TL – tail length, TMH – tail muscle height, MTH – maximum tail height.

Trait	Estimate	SE	*t*-value	95% HPD lower	95% HPD upper	*p*MCMC
Weight	**0.0020**	**0.0007**	**2.95**	**0.0007**	**0.0033**	**0.004**
BL	-0.0075	0.0089	-0.84	-0.0259	0.0113	0.483
Stage	-0.0053	0.0147	-0.36	-0.0337	0.0231	0.715
TL	-0.0127	0.0193	-0.66	-0.0495	0.0299	0.557
TMH	-0.0013	0.0025	-0.53	-0.0061	0.0035	0.601
MTH	**-0.0129**	**0.0061**	**-2.13**	**-0.0252**	**-0.0005**	**0.043**

Table A2.10 Swimming speed statistics

Statistical table of the results of a linear mixed effect model (LMM) for the time taken (s) to swim 30 cm for three trials per tadpole per day on three days throughout development, with individual identity as a random factor. Results were Box-Cox transformed and the results shown are the optimal model according to the AIC values obtained by sequentially eliminating non-significant interaction terms then least significant terms to lower the AIC value of the model. See statistical analysis details in methods for reasoning why degrees of freedom are unable to be presented. "foodR" refers to the restricted treatment in comparison with the control feeding treatment and "day" refers to the three testing days in chronological order.

Term	Estimate	SE	*t*-value	*p*-value
Intercept	3.0314	0.0313	96.86	<0.0001
foodR	**0.1170**	**0.0319**	**3.67**	**0.0003**
day	**-0.2991**	**0.0114**	**-26.14**	**<0.0001**

Table A2.11 Swimming prompts statistics

Statistical table of the results of a generalised linear mixed effect model (GLMM) for the number of prompts required for tadpoles to swim 30 cm for three trials per tadpole per day on three days throughout development, with individual identity as a random factor. Results were analysed with a quasi-Poisson distribution and the results shown are the optimal model according to the AIC values obtained by sequentially eliminating non-significant interaction terms then least significant terms to lower the AIC value of the model. "foodR" refers to the restricted treatment in comparison with the control feeding treatment, "day" refers to the three testing days in chronological order and "foodR:day" indicates the interaction term.

Term	Estimate	SE	*z*-value	*p*-value
Intercept	2.2824	0.0680	33.58	<0.0001
foodR	-0.0325	0.0914	-0.36	0.722
day	**-0.4774**	**0.0322**	**-14.82**	**<0.0001**
foodR:day	**0.1631**	**0.0417**	**3.91**	**<0.0001**

Table A2.12 Hopping distance statistics

Statistical table of the results of a linear mixed effect model (LMM) for the longest hop (mm) in 30 seconds for three trials per frog per day on three days throughout development, with individual identity as a random factor. Results were Box-Cox transformed and the results shown are the optimal model according to the AIC values obtained by sequentially eliminating non-significant interaction terms then least significant terms to lower the AIC value of the model. See statistical details in methods for reasoning why degrees of freedom are unable to be presented. "foodR" refers to the restricted treatment in comparison with the control feeding treatment, "injectx" refers to immune activated tadpoles in comparison with PBS-injected tadpoles and "day" refers to the three testing days in chronological order. A colon indicates the interaction between two terms.

Term	Estimate	SE	*t*-value	*p*-value
Intercept	131.665	20.264	6.50	<0.0001
foodR	24.760	18.903	1.31	0.192
injectx	-27.309	16.190	-1.69	0.093
day	**-15.916**	**6.907**	**-2.30**	**0.022**
foodR:day	-8.928	6.420	-1.39	0.166
injectx:day	8.666	5.548	1.56	0.120

Table A2.13 Number of hops statistics

Statistical table of the results of a generalised linear mixed effect model (GLMM) for the number of hops achieved in 30 seconds for three trials per tadpole per day on three days throughout development, with individual identity as a random factor. Results were analysed with a quasi-Poisson distribution and the results shown are the optimal model according to the AIC values obtained by sequentially eliminating non-significant interaction terms then least significant terms to lower the AIC value of the model. "day" refers to the three testing days in chronological order.

Term	Estimate	SE	*z*-value	*p*-value
Intercept	1.7766	0.0645	27.527	<0.0001
day	**0.2021**	**0.0278**	**7.268**	**<0.0001**

Table A2.14 Feeding latency statistics

Statistical table of the results of a linear mixed effect model (LMM) for the time taken (s) to catch and consume a single wingless *Drosophila melanogaster* for three trials per frog per day on three days throughout development, with individual identity as a random factor. Results were Box-Cox transformed and the results shown are the optimal model according to the AIC values obtained by sequentially eliminating non-significant interaction terms then least significant terms to lower the AIC value of the model. See statistical details in methods for reasoning why degrees of freedom are unable to be presented. "foodR" refers to the restricted treatment in comparison with the control feeding treatment.

Term	Estimate	SE	*t*-value	*p*-value
Intercept	0.9815	0.0010	989.00	<0.0001
foodR	**0.0037**	**0.0012**	**3.00**	**0.003**

Chapter III: Effect of compensatory growth on whole-body corticosterone levels of tadpoles.

ABSTRACT

The stress response is necessary for the survival of animals, although its effects can be detrimental in the case of chronic stress. Corticosterone is a stress hormone most abundant in birds, reptiles and amphibians and acts to aid the body's recovery from an acute stress response by down-regulating the immune system and reproductive function. Dietary restriction has been shown to cause a stress response in various taxa, as measured by analysing glucocorticoid levels. I aimed to investigate the effects of early dietary restriction, and subsequent compensatory growth, on the whole-body corticosterone levels of *Litoria ewingii* tadpoles. Tadpoles were sacrificed at forelimb emergence and corticosterone levels were analysed by a single-antibody radioimmunoassay (RIA). No differences in baseline corticosterone levels were detected between groups; however, a number of impediments in the RIA procedure mean that this result is inconclusive. It remains unknown if there are any long-term consequences of early dietary restriction or compensatory growth on tadpole stress hormones.

3.1 INTRODUCTION

A stress response involves two phases: the immediate release of catecholamines (eg. adrenaline and noradrenalin) to stimulate a "fight or flight" response and the slower release of glucocorticoids (eg. cortisol and corticosterone) to aid in recovery (Romero & Butler, 2007). A stress response can also be classified as acute or chronic stress. Acute stress is a necessary survival function when an animal is confronted with immediate danger, such as predators, storms or rivals challenging for dominance (Romero, 2004). Glucocorticoids divert all possible resources to immediate survival at the cost of less immediate needs, such as digestion, immune function and reproduction (Creel, 2001). While this process is very useful in an emergency situation, heightened glucocorticoid levels for long periods, i.e. chronic stress, can have severely detrimental effects on bodily functions. Selye (1951) included diseases such as heart disease, diabetes, stomach ulcers and kidney failure as consequences of the exhaustion phase when he first defined stress as the general adaptation syndrome.

Long-term exposure to glucocorticoids can also result in less sensitivity to a repeated stressor because the animal becomes acclimated to the stressor (Romero, 2004). For example, European starlings, *Sturnus vulgaris*, exposed to repeated loud noises, restraint and cage-rolling, showed strongly suppressed baseline and restraint-induced corticosterone levels after 12 days (Cyr *et al.*, 2007). Suppression of corticosterone after prolonged stress allowed the birds to maintain a normal immune response to a T-cell mitogen. In contrast, juvenile alligators implanted with high-dose corticosterone-releasing implants showed a depletion of lymphoid cells, decreased percentages of lymphocytes, eosinophils and basophils, and 40% mortality within four weeks (Morici *et al.*, 1997). Acclimating to chronic stress can also cause animals to become more sensitive to new stressors. Consistently high glucocorticoid levels damage the hippocampal cells, limiting the ability to down-regulate glucocorticoid expression after a novel stressor (Black & Garbutt, 2002). With long-term stress, immune

functioning can become dysregulated to a degree, which has negative health implications (Padgett & Glaser, 2003). Mice, *Mus musculus*, stressed by restraint showed greatly elevated corticosterone levels and, following infection with Theiler's murine encephalomyelitis virus (TMEV), were five times more likely to succumb to this disease than unstressed infected mice (Young *et al.*, 2008). Likewise, reproduction is sacrificed during chronic stress, with female meerkats, *Suricata suricatta*, showing increased glucocorticoid levels, reduced conception rates and increased abortion rates when stressed by dominant female aggression (Young *et al.*, 2006). Minimising stress is therefore very important for an animal to live a healthy and fecund life, thereby maximising fitness.

There are a number of potential long-term stressors, which can cause chronic stress in animals, such as injury, intense predation, habitat loss and long-term subordinance (Romero, 2004). The stress response places an additional burden on an animal's energy resources since glucocorticoids convert protein to glycogen (in preparation for conversion to glucose) and reduce glucose re-uptake in target tissues, causing an overall increase in blood glucose levels (Romero & Butler, 2007). Severe nutritional restriction limits energy intake and further depletes an animal's energy reserves by also causing a stress response. Kittiwake chicks, *Rissa* sp., on various restricted diets maintained baseline levels of corticosterone two to four times higher than chicks on the most nutritious diet (Kitaysky *et al.*, 1999). Diet-restriction can also have contrasting effects to other stressors. Mice were exposed to two regimes which resulted in similar weight loss: 60% dietary restriction and separation from conspecifics (Avraham *et al.*, 2002). Dietary restriction caused a large increase in baseline glucocorticoid levels, whereas separation almost halved the baseline glucocorticoid levels compared to controls.

In amphibians, corticosterone is especially important during the tadpole phase. As the tadpole approaches metamorphosis, the corticosterone levels become elevated and act in concert with

thyroid hormones to promote metamorphosis (Wada, 2008). This peak in corticosterone levels is believed to serve the purpose of inhibiting the larval immune system as adult tissues are differentiating, in order to make way for a new adult immune system (Rollins-Smith *et al.*, 1997). A number of stressors have been shown to cause significantly elevated corticosterone levels in amphibians: restraint stress (Belden *et al.*, 2005), intra-specific competition (Cooperman *et al.*, 2004), pond drying (Denver, 1995), novel diet (Ledon-Rettig *et al.*, 2009) and chorusing in males (Burmeister & Wilczynski, 2000). Exposure to tadpole alarm pheromone actually decreased corticosterone levels in a time- and dose-dependent manner, which functioned to activate anti-predatory "freezing" behaviour in green frog, *Rana clamitans*, tadpoles (Fraker *et al.*, 2009). Species also has an effect on the typical stress response, with spadefoot toads, *Scaphiopus holbrooki*, failing to show the usual increase in corticosterone with handling, unlike wood frogs, *R. sylvatica*, and Jefferson salamanders, *Ambystoma jeffersonianum* (Belden *et al.*, 2010). The consequences of elevated corticosterone levels, as demonstrated by application of exogenous corticosterone to American leopard frog tadpoles, *R. pipiens*, are growth suppression, delayed development and a weaker response to adrenocorticotropic hormone (Glennemeier & Denver, 2002b). These effects are likely to have negative effects on future fitness, reducing the likelihood of breeding and increasing the chance of failing to respond to an immediate lethal stressor (Hunt *et al.*, 2009).

The aim of this experiment was to compare the corticosterone levels of two groups of tadpoles, a control group and a group that had experienced dietary restriction early in development, as they reached the peak of metamorphosis. The effects of this treatment on morphology, development and fitness-related behaviours have been described in Chapter II. I wished to observe whether this period of dietary restriction had had long-term effects on the corticosterone levels of the tadpoles. Glennemeier and Denver (2002a) found that limited

food did indeed cause a large increase in corticosterone levels of *R. pipiens* tadpoles when raised in low densities. However, the intervening period of realimentation in my study, in addition to the effects of approaching metamorphosis, made it difficult to predict whether early dietary restriction would have long-term effects in tadpoles.

3.2 METHODS

3.2.1 Dietary treatments

The tadpoles were raised according to the protocol described in Chapter II. Briefly, tadpoles were individually housed from day 10 post-hatching and fed according to two different feeding regimes. Control-fed tadpoles were provided with food at a daily amount equal to the mean weight of their clutch. Restricted tadpoles were provided with half the amount of their group's mean weight daily. This treatment was continued for two weeks. From post-hatching day 24 until metamorphosis, the restricted tadpoles were fed the same amount as the control-fed tadpoles from their clutch. Tadpoles were also injected with phosphate buffered saline on day 33 and completed three swimming speed trials on days 10, 24 and 34. Upon reaching forelimb emergence, Gosner stage 42 (Gosner, 1960), the tadpoles used in this experiment were killed by anaesthetic overdose (Aqui-S New Zealand Ltd, Lower Hutt). They were gently dried with paper towels and stored in Eppendorf tubes in a -20°C freezer until the time of analysis.

3.2.2 Radioimmunoassay

To assess the whole-body corticosterone levels of the tadpoles, a single antibody radioimmunoassay (RIA) was conducted. The validation protocol described by Brown *et al.* (2003) was primarily used to assess the reliability and accuracy of the RIA, although several other sources were consulted to clarify technical details. Most notably, C. Ledon-Rettig, R. Denver & E. Crespi very generously provided information on their tadpole corticosterone

RIA protocol, which was modified from that of K. Glennemeier based on the work of P. Licht (see Crespi & Denver, 2005; Ledon-Rettig *et al.*, 2009). Initially, a working concentration of [3H]-corticosterone was established, with a concentration of 3 nl/ml phosphate-buffered saline (PBS) equating to approximately 4000 cpm per well, an average of the 3000-5000 cpm recommended by Silvestre *et al.*(1998) and Collins *et al.*(1969). An appropriate dilution of the corticosterone antibody (C8784, Sigma-Aldrich) was then estimated by serial dilution and the optimal concentration at 30% binding of labelled steroid was selected. According to the product certificate of analysis, this antibody has the following cross-reactivities with other steroids: progesterone 15.7%, testosterone 7.9%, dehydroandosterone 0.1%, desoxicorticosterone 20%, androstenedione 2.6%, androsterone 0.1%, estrone 0.1%, estradiol 0.1%, estriol 0.1%, aldosterone 4.4%, 5-α dehydrotestosterone 1.4%, 17-hydroxy progesterone 1.8%, 20-α OH progesterone 8.8%, 20-β OH progesterone 5.2%, cortisone 3.2% and cortisol 4.5%.

Originally, the extraction process included a chromatographic separation (Wingfield & Farner, 1975; Kreutzmann *et al.*, 1982; see Appendix 3.6). Due to time and resource constraints, however, following the initial low extraction efficiency of this step, chromatography was removed and the whole-body corticosterone was analysed without purification. Tadpoles were thawed, weighed and homogenised in a volume of ethyl acetate equivalent to their body weight. Tadpoles were then homogenised at a slow speed in an UltraTurrax homogeniser. After homogenisation, 50 µl of [3H]-corticosterone at 4000 cpm was added to estimate recoveries. The homogenate was then extracted twice in ethyl acetate, including drying in a vacuum oven at 37°C, before being reconstituted in PBS to 100 µl. Extracts were incubated with antibody overnight at 4°C and dextran coated charcoal was used to separate the bound and unbound phases with centrifugation. The NSB was less than 2% in all assays, indicating that the charcoal performed effectively.

Standards and validation steps were run in duplicate, however in accordance with an ideal sample dilution of 50% binding in the validation for parallelism (Brown *et al.*, 2003; Fig. 3.1), samples could only be analysed by RIA once at full concentration. The intra-assay CV was 17.3% and the inter-assay CV was 19.4%. The extraction efficiency was 31.5% (± 7.7 SE). Recovery analysis gave an R^2 value of 0.9366 (F = 88.71, df = 6, p = <0.0001) indicating that hormone mass was being correctly estimated. The parallelism validation demonstrated that standards and samples showed limited parallelism (Fig 3.1.). This indicates that the immunoactivity of the corticosterone in the tadpole samples may not have been as similar to that of the standards as in an ideal RIA. The minimum detectable limit (as given by the mean of the maximum binding blanks plus two standard deviations) was 0.49ng/ml corticosterone in PBS, which is only slightly less sensitive than the 0.37ng/ml detected by another amphibian corticosterone RIA (Burmeister & Wilczynski, 2000).

3.2.3 Statistical analysis

Due to the failure of the standard curves to meet the template criteria of the RIA-smart program, it was necessary to manually calculate the sample concentrations of corticosterone. Healy (1972) provided the necessary procedure and equations which were then converted to an R script (R Development Core Team, 2009). To estimate each point of the curve, the following equation was used:

$$\hat{y} = a + b[\exp(c - d \cdot \ln x)/(1 + \exp(c - d \cdot \ln x))] \qquad (1)$$

where x is the known concentration of the standard, $\hat{y}$ is the estimated count, a is the meanblank counts and b is the mean maximum binding counts from the RIA, and c and d are twounknown parameters which describe the curve. To estimate the most likely values of c and d, I ran a script which calculated the residuals of the fitted values from given c and d valuesagainst the actual values provided by the mean standards from the RIA, excluding the

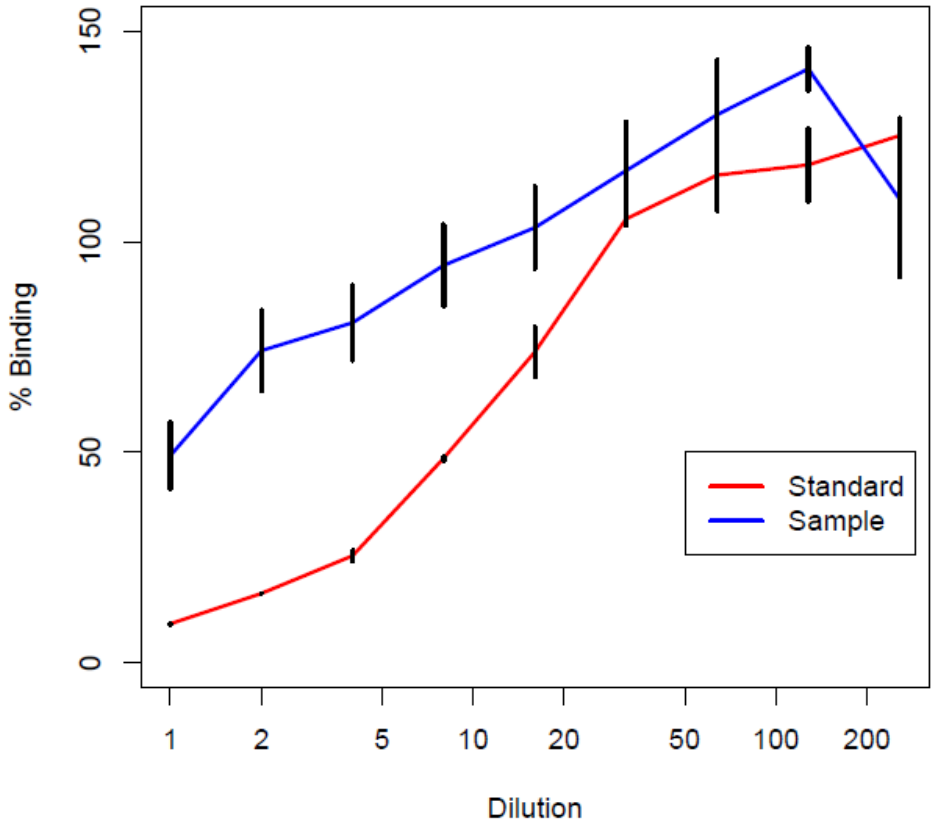

Figure 3.1 Parallelism of sample and standard.

Failure of serial dilution of [3H]-corticosterone-spiked sample to show desired parallelism with RIA standards (n = 2 per dilution). Note also that the sample dilution which shows the 50% binding recommended for use in analysis is not diluted (Brown *et al.*, 2003).

obvious outliers. The *c* and *d* values which together provided the lowest residual was therefore the best fit curve to the actual data. Once the equation of the curve was described, the following equation was used to calculate the actual concentration of corticosterone given by the samples:

$$x = \exp[(c - p)/d] \qquad (2)$$

where *x* is the concentration of corticosterone in the sample and *p* is the proportion of the zero-concentration rate as calculated by:

$$p = \ln\left[\frac{y - a}{b - (y - a)}\right] (3)$$

The given concentration of the sample was then divided by the original body weight of the tadpole to give a uniform concentration of corticosterone per gram of body weight. A simple linear model (LM) was then used to analyse the corticosterone levels of the two treatment groups, as well as possible confounding effects such as tadpole weight and analysis run.

3.3 RESULTS

There was no significant difference in whole body corticosterone levels detected between the two feeding treatments (mean corticosterone (ng/g) ± SE, control: 6.20 ± 0.78, restricted: 5.60 ± 0.63; LM, *t*-value = -0.62, *df* = 22, *p* = 0.541, Fig. 3.2). There was also no significant effect of tadpole weight (LM, *t*-value = -0.80, *df*= 22, *p* = 0.431) or analysis run (LM, *t*-value = -1.63, *df* = 22, *p* = 0.118).

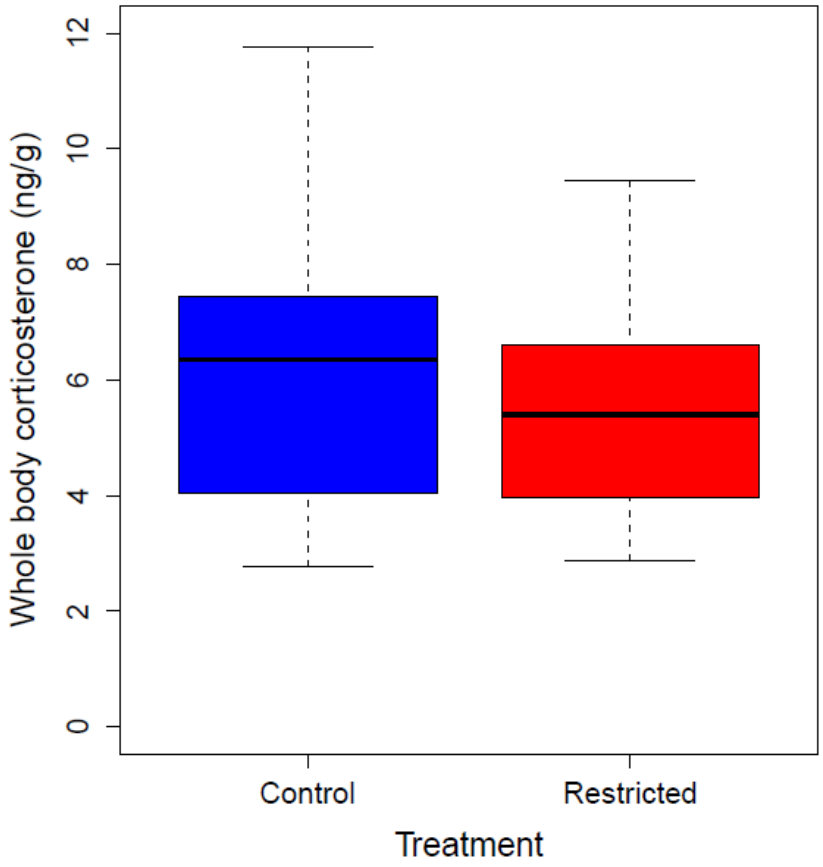

Figure 3.2 Corticosterone levels by dietary treatment.

Whole-body corticosterone levels of *L.ewingii* tadpoles at forelimb emergence (Gosner stage 42) as analysed by radioimmunoassay. Restricted tadpoles (n = 12) were diet-restricted for 14 days early in development before being placed on the *ad libitum* diet provided to the controls (n = 12). Dark line indicates the mean of each group, with inter-quartile range given by boxes and range by whiskers.

3.4 DISCUSSION

The results show that there were no statistically significant differences between the whole-body corticosterone levels of control-fed and food-restricted tadpoles at the time of forelimb emergence (Fig. 3.2). There are a number of reasons why the corticosterone levels of the tadpoles may not have been affected by their earlier exposure to a stressor. Firstly, they may not have been stressed by the experience. Restricted tadpoles were placed on a limited diet from day 10 post-hatching, approximately the same time that they started eating (Gosner stage 25). In Wistar rats, *Rattus norvegicus*, an equivalent degree of restriction (alternate day feeding) did not have an effect on corticosterone levels between weaning and 28 months of age (Dellwo & Beauchene, 1990). Thus, a moderately restricted diet in these taxa may not be sufficient to cause a stress response if the animals are accustomed to a limited amount of food from very early in life. This reasoning is in keeping with the finding that there were no detectable differences in mortality between dietary groups exposed to an immune activation (Chapter II). If the restricted tadpoleshad elevated corticosterone levels, their immune systems might be suppressed, so mounting an immune response to an apparent pathogen would be more taxing and likely to be fatal (Padgett & Glaser, 2003). Alternatively, the corticosterone levels of the restricted tadpoles may have initially been heightened but after chronic exposure the levels decreased and so were indistinguishable at the time of both immune activation and forelimb emergence (Cyr *et al.*, 2007).

A second possibility is that the tadpoles may have been stressed by the food-restriction but recovered without any long-term consequences in the intervening 3 weeks. In rats, it has been found that one week is sufficient time to recover from a week of chronic stress, returning corticosterone receptor levels to those of control animals (Sapolsky *et al.*, 1984). Finally, the experimental conditions by themselves may have been more stressful than the feedingtreatment intervention. Tadpoles were weighed, staged and photographed weekly, had

water changes every second day and were kept in small containers in isolation from other tadpoles. Frequent handling is a common stress-inducing procedure and although high density is known to increase corticosterone levels, isolation stress has yet to be tested in tadpoles (Belden *et al.*, 2007). Although great care was taken to minimise stress to the animals (minimal handling, clean water, visual cues from neighbours), it is possible that these processes had a greater effect on the corticosterone levels of the tadpoles than the feeding treatment so that no detectable difference was observed between the groups.

Although the methods used to measure the corticosterone levels of the tadpoles lacked the precision desirable of an RIA, the priority was on detecting a difference between the two groups rather than establishing a true indication of the corticosterone levels in the tadpoles. However, if we assume the results are correct, the whole-body corticosterone levels of both groups (~ 6 ng/g) are higher than expected for baseline whole-body corticosterone levels. Belden *et al.* (2005) provide a brief summary of corticosterone levels reported for amphibians in the published literature. The baseline levels range from 0.2 – 1.5 ng/g, while the peak levels obtained from a stress response range from 0.5 – 9.4 ng/g. Exposure to exogenous corticosterone produced even higher levels of 25 – 55 ng/g. This collective summary of data suggests that the results provided from this RIA were either incorrectly calibrated with true levels of corticosterone, a strong possibility given the limited parallelism (Fig. 3.1), or the tadpoles experienced a peak stress response during euthanasia. This response is unlikely given that tadpoles were euthanized by anaesthetic overdose. Alternatively, the high corticosterone levels of both groups could be a genuine reflection of the peak of baseline corticosterone levels at the climax of metamorphosis (Wada, 2008).

Because of the role corticosterone has to play in the progression of metamorphosis, it was considered that stress levels may have had some effect on the difference in developmental period between the two groups (mean days ± SE, control: 39.9 ± 0.4; restricted: 44.7 ±

0.3).Elevated corticosterone during food restriction may have slowed the progression of metamorphosis. Glennemeier and Denver (2002b) found that development could be restricted by exogenous corticosterone application. However, without an assessment of baseline corticosterone levels at the end of food restriction, it is impossible to know if stress was a major contributor to the delayed development of food-restricted tadpoles.

Overall, these results leave a number of conflicting and inconsistent avenues of interpretation. This conflict is in part due to the unreliability of the RIA according to the validation protocols but also owes some degree of confusion to the contradictory nature of glucocorticoids. Much of their biological interpretation is dependent on the specific context in which they are observed, especially the difference between acute and chronic stressors and whether these effects enhance or suppress other physiological functions (Dhabhar & McEwen, 1999). To obtain a better insight into the effects of dietary restriction on corticosterone levels in tadpoles, an experiment would need to be designed which not only had more precision in the RIA analysis, but could capture snapshots of the average corticosterone levels of both groups across time. Such a procedure was not possible for this experiment since the primary goal was to observe the morphological and fitness effects beyond metamorphosis. However, for the purpose of observing corticosterone levels at this one significant point in development, this study suggests that early dietary restriction has no observable long-term effect on later stress levels at metamorphosis.

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3.6 APPENDIX

3.6.1 Column chromatography

The original experimental design of the tadpole corticosterone analysis involved column chromatography as a purification step between homogenisation of the tadpoles and analysis by radioimmunoassay (RIA). While many researchers use thin-layer chromatography for this purpose (Glennemeier & Denver, 2002; Warne *et al.*, 2010), resource availability made column chromatography a better choice in this instance. Column chromatography is most commonly used to separate small amounts of plasma into fractions containing different steroids of interest so as to reduce the amount of blood which is required from small animals (Wingfield & Farner, 1975). However, for this study, it was intended to separate the corticosterone-containing fraction from a whole-body homogenate, since the tadpoles were too small to provide an adequate amount of blood for analysis. It was hoped that column chromatography would prevent interference from other steroids and proteins included in the homogenate (Abraham, 1975). This appendix outlines the preliminary experiment testing the effectiveness of column chromatography and is included to clarify why the final protocol did not include a purification step.

3.6.2 Method

Celite 545 (419931, Sigma-Aldrich), diatomaceous earth commonly used as an analytical filter aid, was first cooked overnight at 400°C to ensure maximum dehydration (Kreutzmann *et al.*, 1982). Columns were built in sterile disposable 5ml glass pipettes. Celite was mixed in a 2:1:1 ratio with ethylene glycol and propylene glycol and left for 10 minutes before packing into the columns. A small glass bead was placed into the pipette, followed by 0.3g of a 3:1 Celite:distilled water mixture to form a "glycol trap" (Wingfield & Farner, 1975). This was packed down with a thin rod and followed by 1g of the Celite:glycols mixture. Two aliquots

of 3.5ml isooctane (also known as 2,2,4-trimethylpentane) were run through the column before use.

Prior to preparing the column, two tadpoles had been homogenised and 50µl of ~9000cpm [3H]-corticosterone was added to each to determine which fraction contained corticosterone (Lokman *et al.*, 2002). The homogenate was extracted in ethyl acetate, centrifuged and the liquid layer dried in a vacuum oven (Crespi & Denver, 2005). Dried extract was then resuspended in 100µl isooctane saturated with ethylene glycol (Tousignant *et al.*, 1995). The 100µl of sample was then added to the top of the column and 50% ethyl acetate in isooctane was allowed to flow through at a rate no faster than one drop per seven seconds (Wingfield & Farner, 1975). Sample tubes were then used to collect each 250µl of eluate. For one tadpole, this allowed a range of 6ml of eluate, while the other had only 3ml due to restricted flow. The fractions were dried in the vacuum oven then resuspended in 50µl PBS before being analysed by scintillation. Two wells with 50µl of ~9000cpm [3H]-corticosterone and two wells with no [3H]-corticosterone were also run as references.

3.6.3 Results

The elution pattern of sample 1 showed two sharp peaks: one at 0.75ml and one at 2ml (Fig. A3.1). However, the presence of a large outlier at 2.5ml makes it difficult to determine whether collecting between 2ml and 3ml would have provided a purified corticosterone-containing fraction. The irregular pattern of sample 1 hindered the selection of a preferred fraction and, therefore, was not analysed for the extraction efficiency of one fraction. The overall extraction efficiency of sample 1, excluding the outlier, was 60.4%. With sample 2 the pattern is more obvious, with the most obvious eluate collection lying between 1.5ml and 2.75ml. The overall extraction efficiency was 17.4%, with 16.3% of the [3H]-corticosterone recovered from the preferred fraction. The same preferred fraction from sample 1, excluding

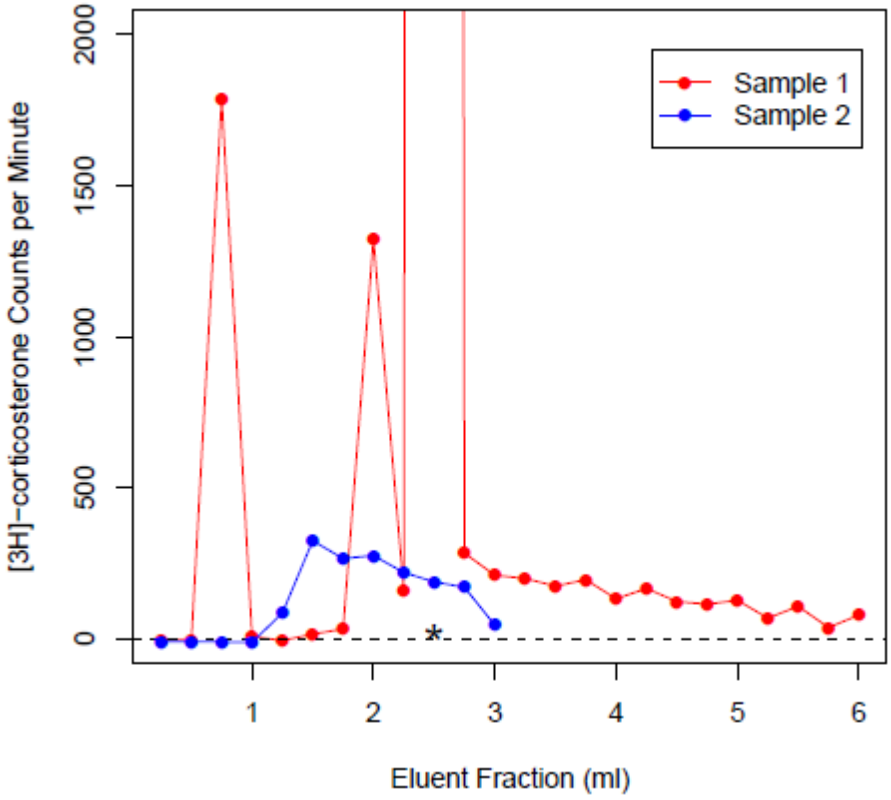

Figure A3.1 Column chromatography elution profile.

Elution profile from column chromatography for two tadpole samples containing [3H]-corticosterone. The dashed line indicates the background (mean of two blank wells) as the background was subtracted before plotting. The asterisk indicates an outlier of 60,674cpm.

the outlier, gives an extraction efficiency of 20.5%.

3.6.4 Discussion

Clearly, the column chromatography protocol was not a success, given the large variation between the two elution patterns and the overall disappointing extraction efficiency (compared to most published studies which achieve an extraction efficiency of at least 90%). There were a number of areas which could have been improved in the protocol: sample size, finding the correct ethyl acetate:isooctane ratio used for elution for this particular system (see Wingfield & Farner, 1975), variation in how tightly Celite was packed, variation in the concentration of sample and more stringent anti-contamination measures. Unfortunately, due to limited time, restricted access to laboratory resources and, most importantly, a rapidly dwindling supply of non-experimental tadpoles, I decided that further exploration into this technique was simply not possible.

Although the double extraction technique used in the final protocol lacks the precision of the purification step, the advantage was that it provided fewer opportunities for corticosterone to be removed from the final sample used in the RIA analysis. The main priority of this experiment was to detect whether a difference in corticosterone levels existed between the two treatment groups. Therefore, steps which were more likely to have differential effects between samples (such as the variation in patterns seen in this experiment) had to be minimised, even at the cost of reporting inaccurate whole-body corticosterone levels. It was more important that all samples were exposed to the same protocol. With a larger pool of tadpoles for use in validation techniques, more time and access to resources such as the nitrogengas which many researchers use to speed up the repeated drying processes, I believe could have successfully refined this column chromatography protocol for inclusion in an improved tadpole corticosterone RIA. Unfortunately, I was not able to do so for this thesis.

3.6.5 References

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Chapter IV: Quantitative analysis of compensatory and catch-up growth in diverse taxa

ABSTRACT

"Compensatory growth" and "catch-up growth" are often used interchangeably to describe the faster than optimal growth which occurs following a period of dietary restriction in the development of many animals. This study distinguishes the two terms to clarify the fitness consequences of rapid growth. Eight meta-analyses and meta-regression analyses were conducted on data extracted from 88 papers, including 11 taxonomic classes. The results confirmed that both growth strategies occur across a wide range of taxa and result in decreased direct fitness. Importantly, the meta-analytic techniques made it possible to identify specific experimental techniques which most successfully promoted rapid growth after restriction. The results also address recent concerns regarding high rates of false detection of compensatory growth. As the first quantitative analysis to be conducted in this field, this study provides not only essential support for the premises of compensatory growth, but also future guidelines and new possibilities for relevant research.

A version of this chapter will be submitted to *Functional Ecology*.

4.1 INTRODUCTION

Developmental plasticity is an important advantage for growing organisms, because it allows them to be better suited to their environment (Pigliucci, 2001). Making phenotypic adjustments to suit a sub-optimal developmental environment enables an organism to make the best of a bad situation (Metcalfe & Monaghan, 2001). Growth is a crucial component of life history, not only for its relationship with maturation time and subsequent body size, but also for the impact of these collective life-history traits on fitness (Nylin & Gotthard, 1998). Under ideal circumstances, animals grow at an optimal rate, limited more by quality control of differentiating tissues than by a lack of resources (Metcalfe & Monaghan, 2003). Faster than optimal growth (maximal growth) can decrease cell functioning efficiency, immune function and resistance to physiological stressors (Mangel & Stamps, 2001). Optimal growth reduces the negative physiological costs of the accumulated cellular damage observed when maximal growth occurs. However, growth rates closer to maximal are commonly observed when an organism has previously experienced a period of limited growth during development, known as compensatory growth or catch-up growth.

Compensatory growth has long been of interest to scientists (Jackson, 1937), primarily because it begs the question: if some animals are willing to grow at a maximal rate, why don't they all? The answer seems to lie in the altered cost-benefit equation of an animal with a poor start in life. An animal's adult size is often a major factor in fitness; it is known to affect mate selection, fecundity and offspring survival (Blanckenhorn, 2005). Therefore, animals with slow growth during development are at a distinct disadvantage if they reach a small adult size. If the environmental cause of restricted growth passes, the opportunity may present itself for an animal to increase growth before reaching a small final size. In this case, it is more beneficial for the growth-restricted animal to risk the negative consequences of maximal growth. In many circumstances, the cost of maximal growth, in the form of

accumulated cellular damage, is not paid until after the reproductive phase. For example, in humans, rapid growth in childhood after a "small-for-gestational-age" birth weight is associated with increased risk of late-onset adult diseases such as heart disease, diabetes and obesity (Cottrell & Ozanne, 2008). Similar findings have been reported, for example, from the intensive studies of metabolic syndrome in rats (Bol *et al.*, 2009; Porrello *et al.*, 2009). Compensatory growth is known to decrease the maximum lifespan of a number of species (Metcalfe & Monaghan, 2003). Thus, compensatory growth allows animals to reach a larger size for increased reproductive fitness before the consequences of rapid growth negatively affect them.

The theory of compensatory growth has been reviewed by a number of researchers, with a variety of perspectives. Metcalfe and Monaghan (2001) provide a broad outline of the forms and consequences of growth compensation across many taxa. Compensatory growth has also been reviewed with specific taxa in mind (domestic fowl: Nir *et al.*, 1996; fish: Ali *et al.*, 2003)and with life-history model simulations to support theoretical assumptions (Mangel & Munch, 2005). Collectively, these reviews voice strong support for compensatory growth as a measurable, repeatable and taxonomically diverse real-world phenomenon. However, the review by Nicieza & Alvarez (2009) casts doubt on the collated evidence from decades of research into compensatory growth by criticising the statistical methods used to analyse this type of data. They claim that many analyses do not take into account the size-dependence of growth rates, meaning that growth slows as an animal gets larger. West *et al.*(2001) propose a universal growth curve, since the relationship between resource allocation to maintenance and to growth changes as an animal grows larger. Clearly, ignoring the size-dependence of growth is a major oversight. Another confounding factor is an animal's allocation of resources to storage as opposed to structural growth. Preferential recovery of fat stores as

opposed to skeletal growth may be leading to a bias in the reporting of detectable compensatory growth (Nicieza & Alvarez, 2009).

The terminology used in this area of research could be considered another impediment to the accurate reporting of compensatory growth. In many cases, compensatory growth and catch-up growth are used as interchangeable terms, both meaning that the growth rate of a previously restricted group is significantly higher than a control. This is the explanation which has the most relevance to a trade-off for fitness, owing to the relationship between maximal growth and cellular damage (Mangel & Stamps, 2001). However, in other studies, the term catch-up growth is defined more by the actual "catching-up"of adult size rather than the growth. Reaching the same final size as the controls is important in many species where size-dependant fitness traits occur, such as predation, mate choice, social dominance and fecundity (Blanckenhorn, 2005). Catching up to the weight of normal conspecifics can be achieved by extending the developmental period while continuing at an optimal growth rate (Arendt, 1997). While there may some cost to life-time reproductive success due to the extra time spent in a non-reproductive phase (Oli *et al.*, 2002), this strategy can often be favourable in variable environments and comes at minimal physiological cost (Wilbur & Rudolf, 2006). Thus, not only are the fitness consequences confused by the lack of clarity in terminology, but it is also unclear what the authors really mean in reporting results as "compensatory" or "catch-up" growth. For the sake of clarity in this study, "catch-up growth" will refer to the attainment of a non-significant difference in size between the control and previously restricted animals (Fig. 4.1b & 4.1d). This can be achieved at a normal growth rate (Fig. 4.1b). In contrast, "compensatory growth" refers to a significantly steeper growth rate of the previously restricted animals, which may or may not result in catching up to the same weight of control-fed animals (Fig 4.1c-d).

The experimental design, in particular, the duration of the relevant periods of restriction and

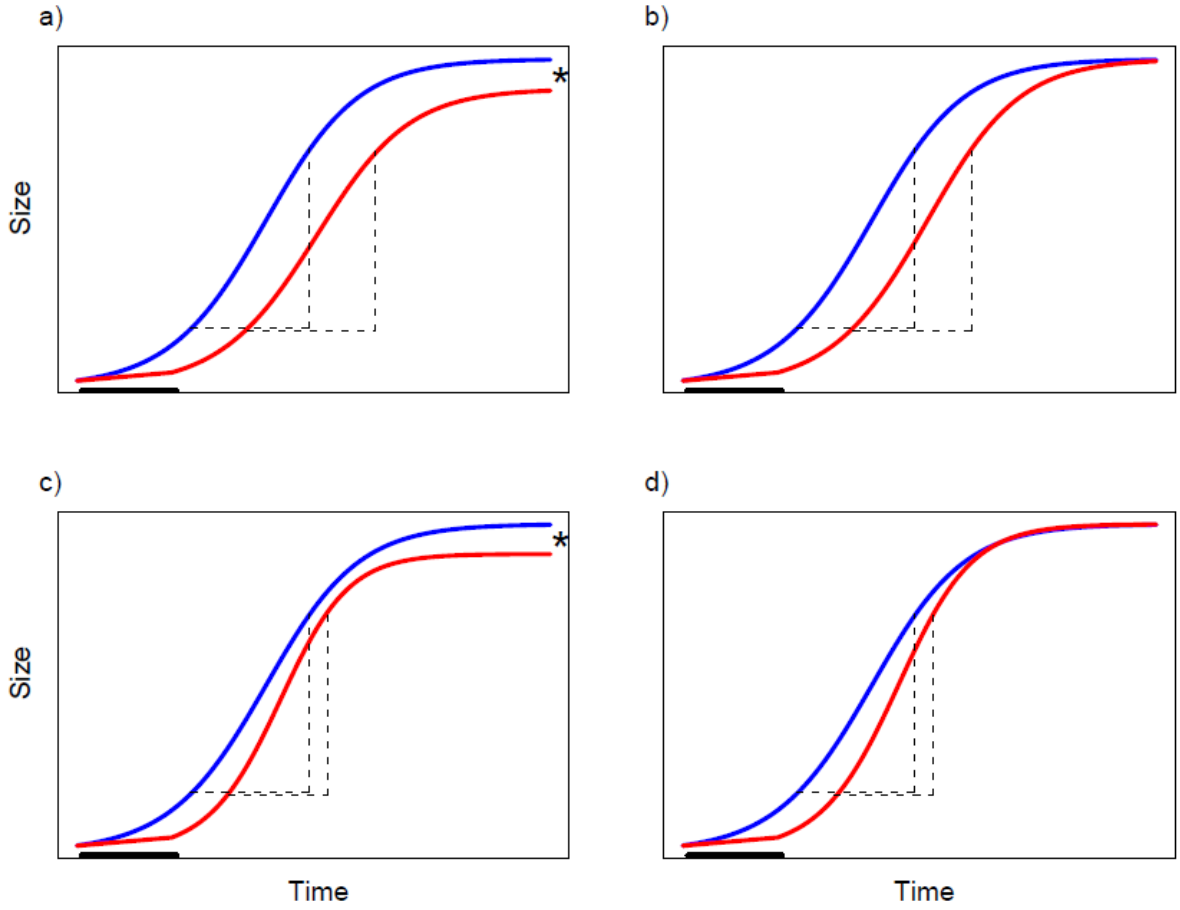


Figure 4.1. Diagram of compensatory and catch-up growth patterns.

Theoretical representation of four growth patterns following restricted growth: a) no compensation, b) catch-up growth only, c) compensatory growth only and d) both catch-up and compensatory growth. The dark line on the time axis indicates the period of growth restriction for the restricted group (red). The control group is in blue. The asterisk indicates a significantly different final size between the groups, while the dashed lines outline the time taken between 20% and 80% of the final size for ease of comparison between growth slopes.

realimentation, has a large effect on the conclusions drawn from each experiment, although the length of each period of the experiment is often a seemingly arbitrary decision. The approximate time during the growth phase that restriction takes place is of particular significance to growth outcomes (Mangel & Munch, 2005).Furthermore, the degree to which one group is restricted is also widely varied across the body of literature. It is surprising that few studies are able to report direct fitness outcomes, given the effects of compensatory growth on fitness seem to be of primary interest. In part, this trend may be due to the limitations of working with relatively long-lived species. Agricultural studies also make up a large proportion of the literature on compensatory growth and, therefore, report effects in terms of economic value rather than effects of relevance to life history. Due to the limited nature of fitness consequences reported, combining the results of a number of studies should give a more holisitic view of the impacts of compensatory growth on fitness. Such an approach also requires comparing a variety of taxa.

Compensatory growth is hypothesised to have evolved because the fitness consequences are delayed until after reproduction (Metcalfe & Monaghan, 2001). Therefore, selection favours those individuals capable of compensatory growth because of their increased size-dependent fitness compared to individuals with limited developmental plasticity in growth (Yearsley *et al.*, 2004). This selection is dependent on the delay before health consequences occur being sufficiently long to allow reproduction. Another factor worth consideration is the life history flexibility of the species involved. Many endotherms have a fairly rigid developmental pathway compared to ectotherms, because of their decreased sensitivity to the environment and the potential for endothermy to indirectly increase intrinsic growth rates (Arendt, 1997). Taken together, it is imperative that we attempt to gain a better perspective on the universality of compensatory growth and its associated effects on fitness. While there are a

number of reviews on the topic, there has yet to be a quantitative assessment of compensatory growth.

The aim of this paper was to use meta-analytical techniques to explore a number of assumptions about compensatory growth. I tested whether: 1) nutritional restriction affects size across a variety of taxa; 2) food-restricted animals are able to reach the same final size as controls ("catch-up") after a period of nutritional restriction and a subsequent return to normal feeding amounts; 3) food-restricted animals show faster than normal growth ("compensatory growth") after nutritional restriction; 4) early restriction has an effect on later fitness traits. By analysing each of these assumptions with a number of moderators included, such as the degree to which an animal was restricted and the method used for restriction, I aimed to gain a better understanding of the variation in the literature and compensatory growth as a whole.

4.2 METHODS

4.2.1 Data collection

Data were collected from papers published in peer-reviewed journals, but also included the results of an experiment in this thesis. Primarily, papers were sourced from searches of ISI Web of Science, the most recent of which was conducted on 5th August, 2010. Only compensatory growth as a result of dietary restriction, as opposed to temperature or seasonal effects, was considered to be of interest for this study. The study was also limited to post-natal effects of restriction, although there is a vast literature on the consequences of inadequate nutrition in prenatal development. The search terms for titles, keywords and abstracts were: "catch-up growth", "compensatory growth", realiment* (to include variations on "realimentation"), refeed* (to include "refeeding"), refed, "early undernutrition", "early under nutrition", "early nutritional defici*" (to include variations on "deficiency"), "early

diet* restrict*" (to include variations on "dietary restriction"), "early food restrict*" (to include variations on "restriction"), "early feed* restrict*" (to include variations on "feeding restriction"), "early calori* restrict*" (to include variations on "caloric restriction"), "early nutri* restrict*" (to include variations on "nutritional restriction") and "growth compensation". This provided over 8,000 papers. These papers were scanned for relevance to the meta-analysis by title and many were instantly rejected because they related to human disorders, proximal effects of feeding on tissue or prenatal nutritional deficits. To ensure that few relevant papers were missed by this collection method, I also back-referenced from all of the available reviews of the topic and forward-referenced from Metcalfe and Monaghan (2001), which is the most cited review of the topic and relates specifically to the area of interest in this analysis. This procedure yielded only six additional papers which were of use in analysis, indicating that the initial search was sufficiently thorough.

The remaining papers were then filtered by adherence to the following rules. For inclusion in this analysis, a paper must: 1) have a control group, 2) report body weight before and after restriction and after realimentation along with estimates of uncertainty (e.g. standard deviation and/or standard error), 3) be an empirical study (i.e. no computational simulations), 4) not include animals which have been genetically modified or have a known disorder, 5) have quantifiable degrees of feed restriction in a controlled environment (thus excluding correlational data from wild populations in different areas of food abundance) and 6) report the sample size. To be included studies also had to provide both treatment groups the same diet but in a smaller quantity for restricted animals, as opposed to studies of qualitative dietary restriction which limit the amounts of specific nutrients, such as protein. I felt that this was the most ecologically relevant way to determine the effects of compensatory growth in relation to a period of poor nutrition in the natural environment. Some allowances were made for papers that restricted animals by manipulating clutch size, diluting food with indigestible

material or feeding animals less frequently rather than a smaller amount. However, I noted these exceptions in order to control for the method of restriction in later analysis.

Length, an alternative size measurement to weight, was also recorded where available and ranged from total body length to tarsus length. This size estimate would allow me to compare compensatory growth in weight with compensatory growth in length. Studies which used farmed or model laboratory species were also required to have some fitness estimate included. I decided this criterion was the best way to optimise the variety of species included (one of the main aims of this study), while ensuring that fitness data was important, since some studies of taxonomically diverse wild-caught animals did not include fitness estimates. This criterion also reduced the representation of agricultural studies (approximately 300 agricultural studies with no fitness estimate) which would have overwhelmed the other taxa in the analysis. I noted that agricultural papers were found to rarely report fitness data, more commonly reporting meat quality and characteristics instead.

In summary, I collected data from 88 papers (Table A4.2), from which I extracted 226 comparisons of the size of restricted animals with their reported control groups. These data included 58 species, spanning eight classes within the phyla Chordata, Arthropoda and Mollusca. Since some papers reported multiple fitness measures, I collected a total of 207 fitness comparisons between restricted and control animals, which I categorised into the six broad classes of fitness traits (see Appendix 4.6.1 for analysis of indirect fitness traits). For use in the main study, only those fitness traits which were considered direct fitness estimates (survival, number of offspring, lifetime reproductive output, fertility and hatchability) were included. This limited the fitness analysis to 94 comparisons.

4.2.2 Effect size extraction

I recorded the publication details of each paper, the study species, the number of animals in each treatment group, their sex, age at initiation of the treatment, duration of the restriction

treatment, duration of the realimentation period (i.e. end of restriction to end of experiment) and the degree of restriction (percentage comparison to the amount the controls were fed). In addition, it was recorded whether or not the controls were fed *ad libitum* and if the restricted animals were refed *ad libitum*, if the animals were farmed (including laboratory colonies) or not, and which type of study design was used among the three methods of food restriction, mentioned earlier (clutch size manipulation, food dilution or intermittent feeding). Estimates of the mean weight at the three crucial time points (prior to restriction, after the restriction period and after realimentation) were derived either from direct reporting or from graphs. In the case of taking estimates from graphs, the image was enlarged to screen size and analysed using ImageJ. The last reported weight was accepted as the end of realimentation. Estimates of variation were recorded as standard errors as this error statistic was the most common format. Conversions from standard deviation or 95% confidence intervals were performed in some cases. In many papers, there were multiple treatment groups reported, in some cases all compared to one control group and in other studies each treatment group had its own control.

Additional data on the taxonomic ranking of each species studied was sought from the NCBI Entrez Taxonomy website (http://www.ncbi.nlm.nih.gov/taxonomy; Table A4.3). The taxonomic information allowed higher level analysis instead of grouping each species individually. Along with the taxonomic data, the average longevity for each species was also researched. The Animal Aging and Longevity Database website (http://genomics.senescence.info/species) provided the expected longevity of many species using data collected from a number of published sources (Table A4.4). Where the longevity data was not available or the legitimacy of the source was doubted, independent searches were conducted among the published literature on the species in question (Table A4.4). The longevity data was required in order to standardise data on the duration of restriction and realimentation. Even though these standardised durations were somewhat imprecise

measurements, the estimates of longevity would, nonetheless, allow a better comparison of species with lifespans at the more extreme ends of the scale. For example, compare *Daphnia magna*, with a recorded average lifespan of 80 days, and the green turtle, *Chelonia mydas*, which is estimated to live for 75 years.

4.2.3 Statistical analysis

Data were analysed using R 2.9.0 (R Development Core Team, 2009) and S-PLUS 8.0.4 (TIBCO; http://www.tibco.com/). Estimates of variance for weight and fitness data were first converted from standard errors to standard deviations for use in calculating Hedges' *d*. Hedges' *d* was considered the most appropriate measure of effect size because it compares the difference between two groups. Using this measure, as opposed to Cohen's *d*, controls for an upward bias caused by small sample size (Nakagawa & Cuthill, 2007).

Hedges' *d* was calculated as:

$$\text{Hedges}'\, d = \text{Cohen}'\text{s}\, d \left[1 - \frac{3}{4(n_1 + n_2 - 2) - 1}\right] \qquad (1)$$

where n_1 and n_2 are the sample size of each treatment group and Cohen's *d* was calculated from:

$$\text{Cohen}'\text{s}\, d = \frac{m_2 - m_1}{s_{\text{pooled}}} \qquad (2)$$

where $m_1$and m_2 are the means of each treatment group and s_{pooled} was calculated from:

$$s_{\text{pooled}} = \sqrt{\frac{(n_2 - 1)s_2^2 + (n_1 - 1)s_1^2}{n_1 + n_2 - 2}} \qquad (3)$$

where $s_1^2$and s_2^2 are the standard deviations from each treatment group. The associated estimate of standard error with each *d* estimate was calculated as:

$$se_d = \sqrt{\frac{n_1 + n_2}{n_1 n_2} + \frac{d^2}{2(n_1 + n_2 - 2)}} \qquad (4)$$

The standard error of *d* was then squared to represent the variance of *d*. The variance of *d* was used as the weighting value for statistical analysis of the overall effects, such that estimates with lower variance and therefore more reliability (either from more consistent results or a greater sample size) contributed more to the model (i.e. meta-analytical models).

The fitness data I collected were broadly of two categories: continuous estimates and percentage data. For the continuous data, a separate sample size was recorded in addition to the mean estimate and error because in many cases only a subset of the experimental subjects was used. Continuous data was able to be calculated as an effect size in the same manner as weight data. However, the percentage data first had to be logit transformed with $\pi^2/3$ as the variance estimate, assuming the logistic distribution of the transformed percentage data (Borenstein *et al.*, 2009).

Estimating growth slopes from the two periods observed (A: from the beginning to the end of restriction; and B: from the end of restriction to the end of the experiment; Fig. 4.2) required additional analysis. Slopes were straightforward to calculate, for example:

$$\text{Growth slope}_A = \frac{m_{A2} - m_{A1}}{t} \qquad (5)$$

where m_{A1} is the mean size of a treatment group at the start of period A, while m_{A2} is mean size of the group at the end of period A and *t* is the duration of period A. However, it was not possible to calculate the estimate of error analytically, as far as I know, since m_{A1} and m_{A2} each had their own standard error. I, therefore, used a simulation to calculate the standard error of the slope. For each slope, the number of samples in the original experiment was drawn from a normal distribution of m_{A1} and m_{A2} using their known standard deviations. These values were then used in a linear regression model which calculated the slope and its

associated measure of error. This procedure was repeated 1000 time for each slope and the means of the final values were taken as the growth slope and standard error for each treatment group. To verify that this procedure was accurate, regression analysis of the original estimates of growth and those calculated in the model returned an R^2 value of 1 (slope ± SE: 1 ± 0.00006, t = 16570, df = 418, p< .0001). The simulated slope and standard deviation values could then be used to calculate d between the two slopes, as described above.

In order to answer the questions which I had initially posed, several sets of meta-analyses were required: 1) models investigating size differences at each point of interest (before restriction, after restriction and after realimentation at the end of the experiment); 2) the effect the dietary treatment had on fitness; 3) the effect on growth rates as measured by the growth slopes at the different periods. This last point was difficult because of the size-dependent growth Nicieza and Alvarez (2009) addressed as a prominent confounding variable in analysis of compensatory growth (i.e. control animals are larger and therefore grow at a slower rate, so comparison with post-restriction growth of small restricted animals is biased). As such, I compared the "compensatory growth" (period B) of the previously restricted animals with both period A and period B of the control animals, and also compared differences when the studies were limited to those which had controls showing only "linear" growth (Fig. 4.2). By only comparing studies where controls showed linear growth over the entire experiment, I could be certain that the size-dependence of growth would not be a concern. To identify linear growth, a visual inspection of the control slopes was made and those that appeared to be of a linear nature were selected. This method was then verified by selecting only control data where the larger, steeper slope was within 40% of the value of the smaller slope and this method was found to have 93% agreement with the visual inspection (see Appendix 4.6.2 for method rationale). This method reduced the number of data points

for inclusion in the linear slopes analyses to 87, down from the original 226 included in the "all slopes" analyses. There were also 226 values in the final size meta-analysis, but only 212 in the pre-restriction and 218 in the post-restriction because some experiments which gave information on length did not provide measurements at these points. The fitness meta-analysis included 94 comparisons.

Each meta-analysis was analysed as a linear mixed-effects model, using a modified version of the method described by Nakagawa *et al.* (2007). In all analyses, I controlled for experimental design effects by including both paper identity and taxonomic class as random factors (the former nested within the later). Both null models (model with the intercept; classically considered and referred to as meta-analysis) and scaled best models with moderators (often referred to as meta-regression) were used to interpret my results. For scaling, all continuous moderators had the mean subtracted from each value and were divided by two times the standard deviation. This method of scaling and centering allows the outputs of the model to be more fairly interpreted (Gelman, 2008; Schielzeth, 2010). Binary variables were left unscaled, and sex (which had the values both, male and female) was analysed with "both" as the reference variable so that the male and female output values could be interpreted as the effect of looking at only one sex as opposed to mixed-sex experiments (see Table A4.5 for the complete list of moderators). Best models were then selected by running the full model using the maximum likelihood method, as opposed to restricted maximum likelihood (REML), because the changes in the AIC values are more relevant under maximum likelihood. Least significant moderators were then sequentially removed until the AIC value was no longer lowered and the best model was then reverted to REML so that the effect size estimates could be used in interpreting the data. Full models are reported including all the moderators initially included in the Appendix. Moderators were first tested for collinearity among one another and were only included if correlated by less than 0.5 (Table

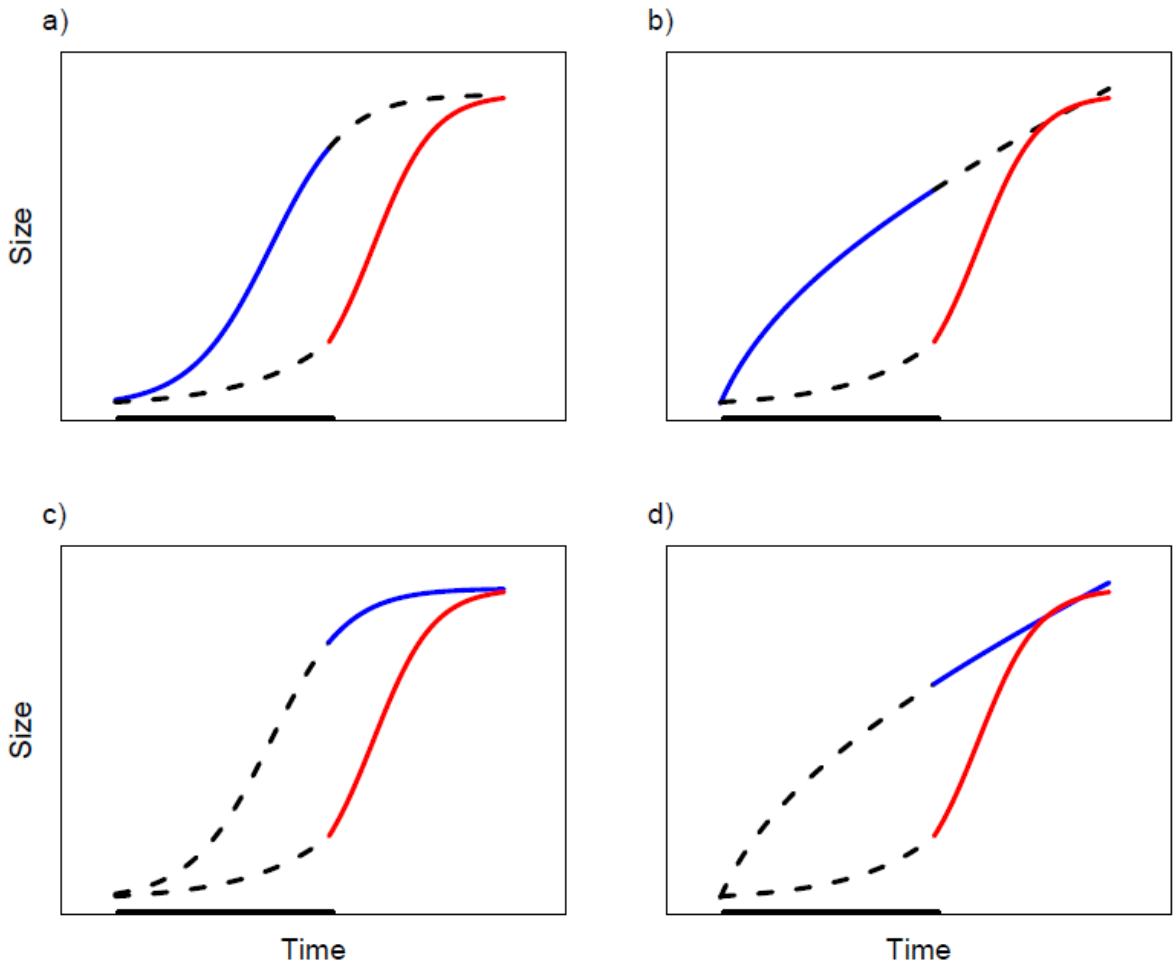


Figure 4.2 Diagram of non-linear and linear growth slopes.

Representation of the four analyses performed on the growth slopes. The blue line is the period of growth calculated for the control group, which is then compared to the growth rate of the restricted animal during realimentation (red line). The dark line of the time axis indicates the period of growth restriction (Period A), while the time after this is realimentation (Period B). The four analyses are: a) A vs. B (all), b) A vs. B (linear), c) B vs. B (all) and d) B vs. B (linear).

A4.6). Two pairs (*ad libitum* feeding of control and of restricted for realimentation, and relative duration of realimentation and proportion of realimentation compared to restriction) failed to meet this criterion so only one moderator per pair could be used. I selected *ad libitum* feeding of restricted animals for its possible relation to hyperphagia following restriction, and the relative proportion of realimentation, because it is probably a better reflection of the experimental methods. Only moderators which could reasonably have affected the outcome, and were biologically meaningful, were included in each model. For example, the duration of realimentation had no bearing on the pre-restriction size and therefore was not included. Some of the moderators were not directly given from the original papers and were instead calculated by combining data. Namely, the relative age, duration of restriction and duration of realimentation were all calculated as a percentage of the reported maximum longevity of the species. The proportion of the duration of realimentation to restriction was calculated by dividing the length of the former by the latter.

4.2.4 Validation of meta-analytical techniques

To ensure that the analyses were not affected by publication bias, funnel plots of the datasets used in each analysis were visually inspected (Fig. 4.3, see Fig. A4.4 for fine scale). No obvious asymmetry was detected in the plots, other than that which was expected to reflect true biological heterogeneity in the post-restriction and post-realimentation datasets (Egger *et al.*, 1997, Fig. 4.3b-c). Heterogeneity was assessed by calculation of the I^2 statistic, using the following equation (adapted from Higgins & Thompson, 2002):

$$I^2 = 100\frac{\sigma_B^2}{\sigma_T^2} \qquad (6)$$

where σ^2_B is the between study variance and the total variance, σ^2_T, is given by:

$$\sigma_B^2 = \sigma_C^2 + \sigma_S^2 \qquad (7)$$

$$\sigma_T^2 = \sigma_B^2 + \sigma_M^2 \qquad (8)$$

where σ^2_C is the class (-specific) variance, σ^2_S is the study (-specific) variance and σ^2_M is the measurement variance. The measurement variance was calculated as follows:

$$\sigma_M^2 = \frac{\sum_{j-1}^{k} w_j(k-1)}{(\sum_{j-1}^{p} w_j)^2 - \sum_{j-1}^{j} w^2} \qquad (9)$$

where w is the inverse variance of d_j (i = 1,..., k; see earlier number of studies per model for k). The high I^2 values reported reflect the high degree of inconsistency across studies (Higgins *et al.*, 2003; Table 4.1). The large contribution of between-study differences (as opposed to between-class differences) to the overall heterogeneity reflects the importance of moderators in controlling for differing experimental techniques (Table 4.1). In most cases, the best model actually increased the heterogeneity compared to the null model, although the AIC value of all best models was lower than the null models.

Figure 4.3 Funnel plots for each analysis.

Funnel plots showing the distribution of the effect size (*d*) extracted from each study plotted against the precision of the study (*w* = 1/SE). Asymmetry about the null intercept (red line) can indicate either publication bias or true biological heterogeneity. Period A (restriction) and B (realimentation) are defined in text. For a-h, *n* = 212, 218, 226, 94, 226, 87, 226 and 87 respectively.

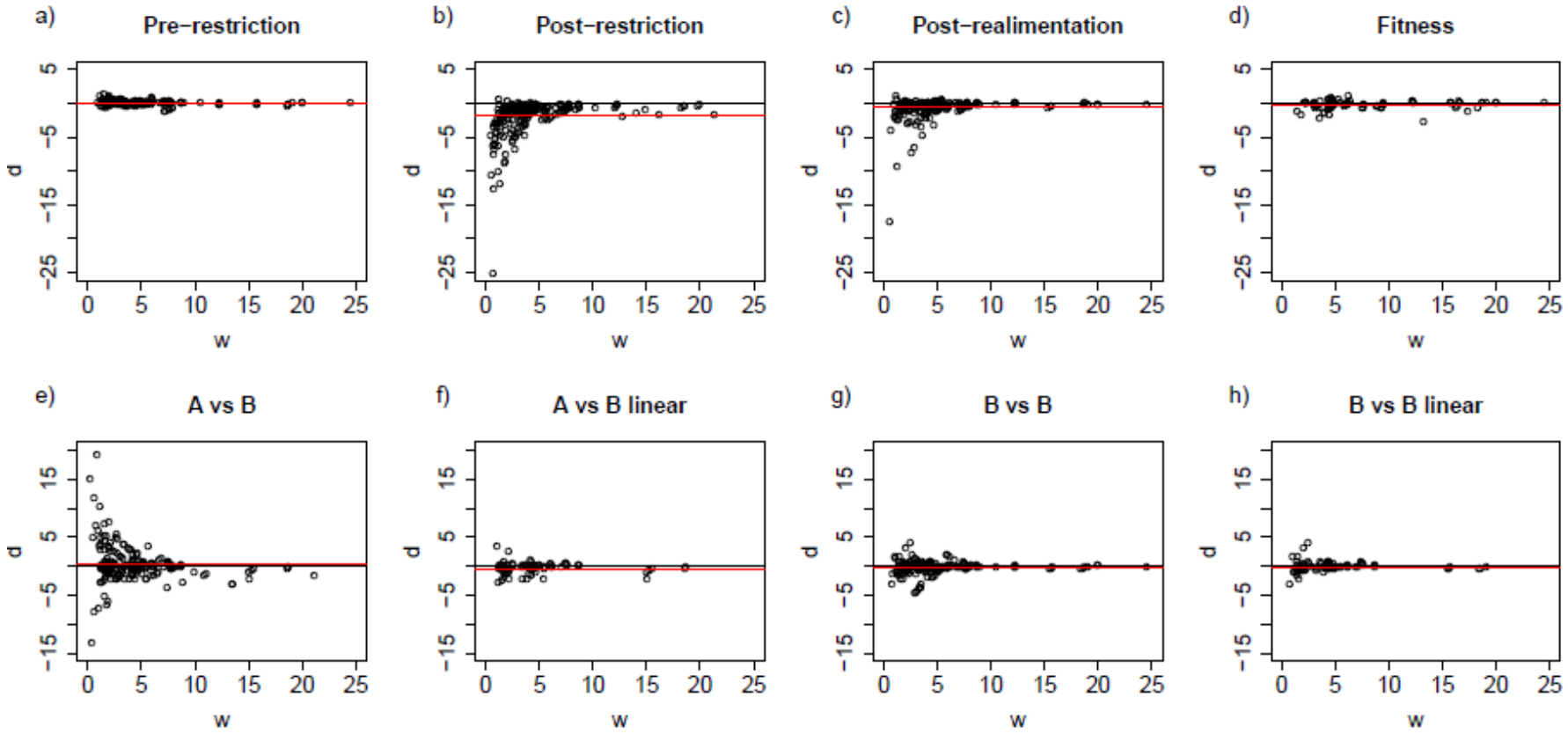


Table 4.1 I^2 statistics for each analysis.

Values of the I^2 statistic reflecting the heterogeneity of each null and scaled best model. The relative contribution of class and paper to model heterogeneity is also reported as well as the AIC value calculated by maximum likelihood for each model.

	Model	I^2	Class	Paper	AIC
Pre-restriction	null	56.57	0.00	56.57	32
	best	54.89	0.00	54.89	31
Post-restriction	null	98.83	0.64	98.19	1628
	best	99.05	0.07	98.97	1484
Post-realimentation	null	95.81	1.80	94.02	909
	best	96.71	0.00	96.71	797
Fitness	null	95.63	0.00	95.63	836
	best	95.21	2.22	92.99	724
A v B	null	99.52	0.00	99.52	1744
	best	99.57	0.00	99.57	1694
A v B linear	null	96.14	27.03	69.12	301
	best	95.84	21.65	74.19	292
B v B	null	95.93	8.07	87.86	665
	best	95.99	15.45	80.54	646
B v B linear	null	93.24	5.22	88.01	196
	best	93.72	46.34	47.38	189

4.3 RESULTS

4.3.1 Meta-analysis: null models

The null models reflect the overall effect size of *d* without considering any moderators but still accounting for paper identity nested within taxonomic class. The pre-restriction intercept (i.e. meta-analytic mean) shows that there was no difference between treatment groups prior to restriction (intercept ± SE: 0.02 ± 0.03, $t = 0.642$, $df = 124$, $p = 0.522$; Fig. 4.4a). By the end of restriction, the restricted group was significantly smaller than controls (intercept ± SE: -1.78 ± 0.02, $t = -9.57$, $df = 130$, $p < 0.0001$; Fig. 4.4a). By the end of the experiment, the

restricted groups had failed to "catch-up" to the size of controls and were still significantly smaller (intercept ± SE: -0.54 ± 0.10, t = -5.416, df = 138, p<0.0001; Fig. 4.4a). Fitness was significantly affected by the treatment and the effect was in the expected negative direction (intercept ± SE: -0.25± 0.09, t = -2.824, df = 57, p = 0.0065; Fig. 4.4a).

The slopes showed more ambiguous results than the absolute models (Fig. 4.4b). Intercept for A vs. B (see Fig. 4.2a for explanation) shows that there was no detectable difference between the initial growth of the controls and the growth rate of the restricted animals during realimentation (intercept ± SE: 0.25 ± 0.27, t = 0.894, df = 124, p = 0.373; Fig. 4.4b). However, when only experiments during the linear growth phase of the controls were considered (A vs. B linear, Fig. 4.2b), the restricted animals appeared to show "compensatory growth" at a faster rate than the early growth of controls (intercept ± SE: -0.45 ± 0.13, t = -2.090, df = 49, p = 0.042; Fig. 4.4b). This effect was also found to be true when comparing the period B growth of all the control animals (B vs. B, Fig. 4.2c), although this result would be expected if size-dependent growth is causing over-inflated estimations of compensatory growth, as Nicieza and Alvarez (2009) suggest (intercept ± SE: -0.30 ± 0.13, t = -2.379, df = 124, p = 0.019; Fig. 4.4b). Their claim of false detection is supported by the finding that the difference for B vs. B is eliminated when only linear control growth is considered (B vs. B linear, Fig. 4.2d; intercept ± SE: -0.15 ± 0.13, t = -1.172, df = 49, p = 0.247; Fig. 4.4b).

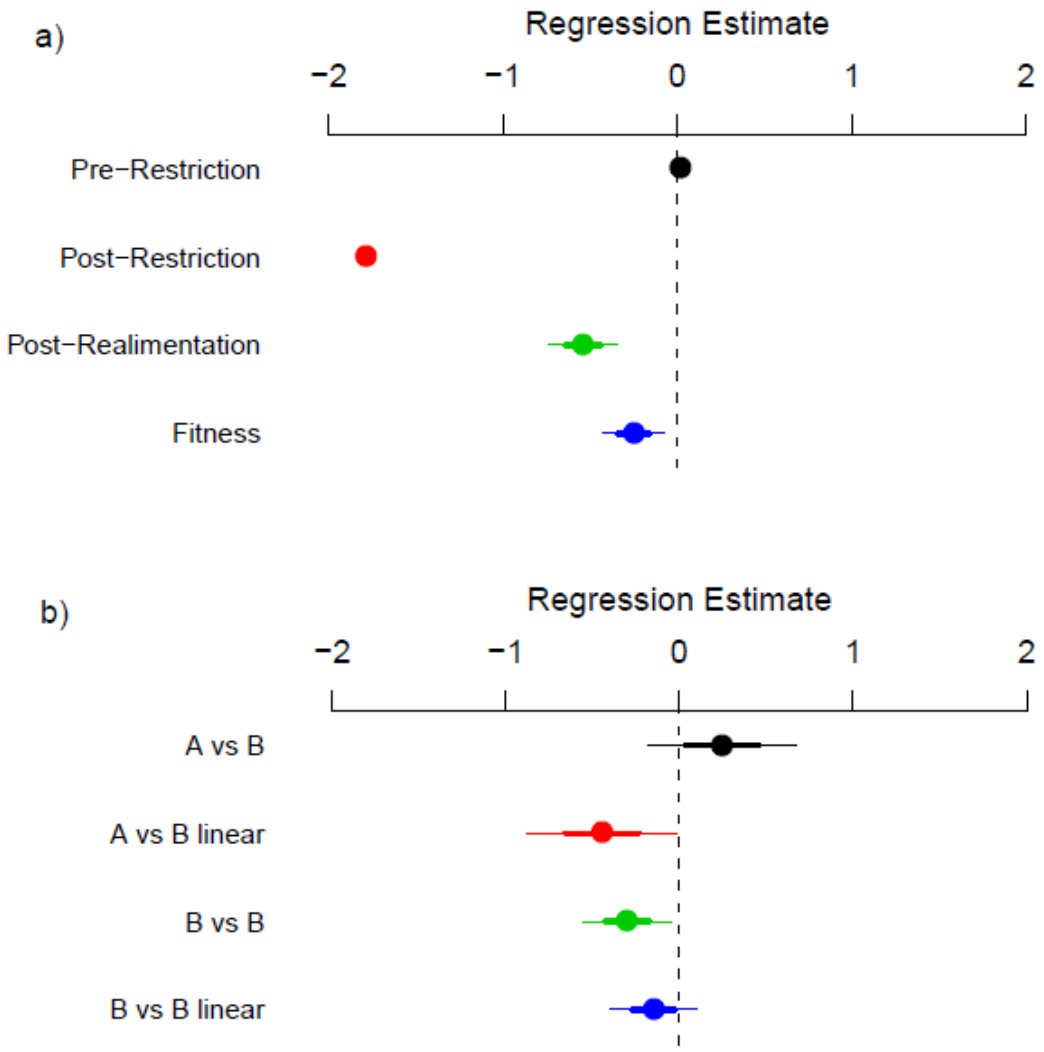


Figure 4.4 Meta-analysis null intercepts.

The intercepts of the null models for each of the eight meta-analyses, grouped as a) standardized difference between point estimates in size and fitness and b) standardized difference between growth slopes. For the former, a negative value of *d* indicates smaller size or decreased fitness in the restricted group compared to the control group. For the latter, a negative value indicates that the restricted group grew faster than the control group during the period of interest (see Fig. 4.2). Confidence intervals which span zero indicate no significant effect at $\alpha = 0.05$.

4.3.2 Meta-regression: scaled best models

These models take into account the effect of various moderators, as selected by the methods described earlier. To interpret the output of these models, it is necessary to remember that the models are scaled. The intercepts reflect the overall effect size based on the mean values of continuous variables and the default value of binary variables. For example, an intercept which had dietary restriction and farming as moderators would reflect the overall effect based on a moderate degree of restriction and non-farmed animals. The moderator values provided could then be interpreted as the slope of the degree of restriction centred on this intercept and the effect when farmed animals are considered. Inclusion in the best model did not necessarily produce statistical significance in all moderators. For ease of reading, statistical values are only provided for intercepts, but full statistical information for moderators is available in Tables A4.7-14.

There were no statistically significant moderators for the pre-restriction analysis, suggesting experiments were indeed fair and both treatment groups started at the same size (intercept ± SE: -0.04 ± 0.04, t = -1.006, df = 124, p = 0.316; Fig. 4.5a). Following restriction, the restricted animals were again found to be significantly smaller than controls (intercept ± SE: -1.51 ± 0.31, t = -4.946, df = 126, $p < 0.0001$; Fig. 4.5b). The size difference between the control and the treatment groups was heavily dependent on how severe the restriction was and how long the diet lasted. Restriction also showed a greater effect on weight than on length. Intermittent feeding appeared to be less effective than other diet methods and farmed animals were more likely to be significantly smaller.

Contrary to the results of the null model, animals did achieve “catch-up” growth when taking into account a number of variables, most significantly the duration and severity of the diet (intercept ± SE: 0.05 ± 0.24, t = 0.196, df = 133, p = 0.845; Fig. 4.5c). Length was more

likely to attain the same size as controls than weight, though this could be because length was not as severely affected by restriction. When the realimentation period was longer than the restriction period, the restricted group was more likely to catch up. This result suggests that either restricted animals eventually catch up by prolonging the growth period or that experiments which fail to achieve "catch up growth" do not allow adequate time for compensatory growth.

In support of the null model, fitness was found to be negatively affected by the diet treatment overall (intercept ± SE: -.597, t = -3.005, df = 52, p = 0.0041; Fig. 4.5d). The effect size of the impact on fitness was even larger when accounting for moderators. Mortality was more negatively affected by treatment than reproduction and there was less of an impact on fitness if the period of realimentation was longer than restriction. Unlike males, for which there was no detectable difference, females in single-sex experiments had higher fitness than mixed sex experiments. These results were extracted from direct fitness measurements only. The analysis of fitness with indirect fitness measurements included came to a different conclusion (Appendix 4.6.1).

With experimental methods taken into account, both A vs. B and the linear selection of A vs. B were found to show no significant difference in growth rate between period A of the controls and realimentation of the restricted group (A vs. B intercept ± SE: 0.28 ± 0.31, t = 0.920, df = 119, p = 0.359; Fig. 4.6a; A vs. B linear intercept ± SE: -0.20 ± 0.21, t = -0.936, df = 46, p = 0.354; Fig. 4.6b). Both analyses also agreed that weight was more likely to show compensatory growth than length. For all studies, a longer duration of restriction was found to increase the likelihood of compensatory growth, while the linear studies showed that a longer proportion of realimentation increased the chance of compensatory growth. Intermittent feeding and clutch size manipulation were found to cause slower growth of the restricted group compared to initial growth of controls.

Figure 4.5 Meta-regression of size and fitness.

Coefficient plots from meta-regression analyses showing the effects of the treatment on the restricted group in comparison to the control group a) before the experiment, b) after restriction, c) after realimentation and d) on fitness. The intercept shows the overall model outcome and the other points describe the contribution of each moderator to the intercept. Confidence intervals which span zero indicate no significant effect at $\alpha = 0.05$. For graphs a-d, n = 212, 218, 226 and 94 respectively. For binary variables, the coefficient shows the impact of the named effect in contrast with the default value, upon which the intercept depends. For farmed, clutch size, intermittent feeding (IF), dilution and *ad libitum* realimentation (AdLibReal), T is shown for true, as opposed to the default (F - false) when experiments did not include these methods. For Type, the effect of weight compared to length is shown. For Fitness Trait, the effect of mortality compared to reproduction is shown. Male and Female indicate their respective effects as single-sex studies when compared to the mixed-sex studies, upon which the intercept was based. The continuous variables of degree of dietary restriction (Degree DR), duration of restriction (DurRes), proportion of realimentation: restriction (PropReal) and relative age (Age) indicate the slopes of these variables against the effect of *d*. The intercept is based on intermediate values of these variables. For example, degree of restriction tends to have a positive slope in relation to *d*, indicating that more food for the restricted group means a smaller difference in the typically negative relationship between control and restricted size (*d*). See also Table A4.5 for information on moderators and A4.7-10 for relevant statistics to these analyses.

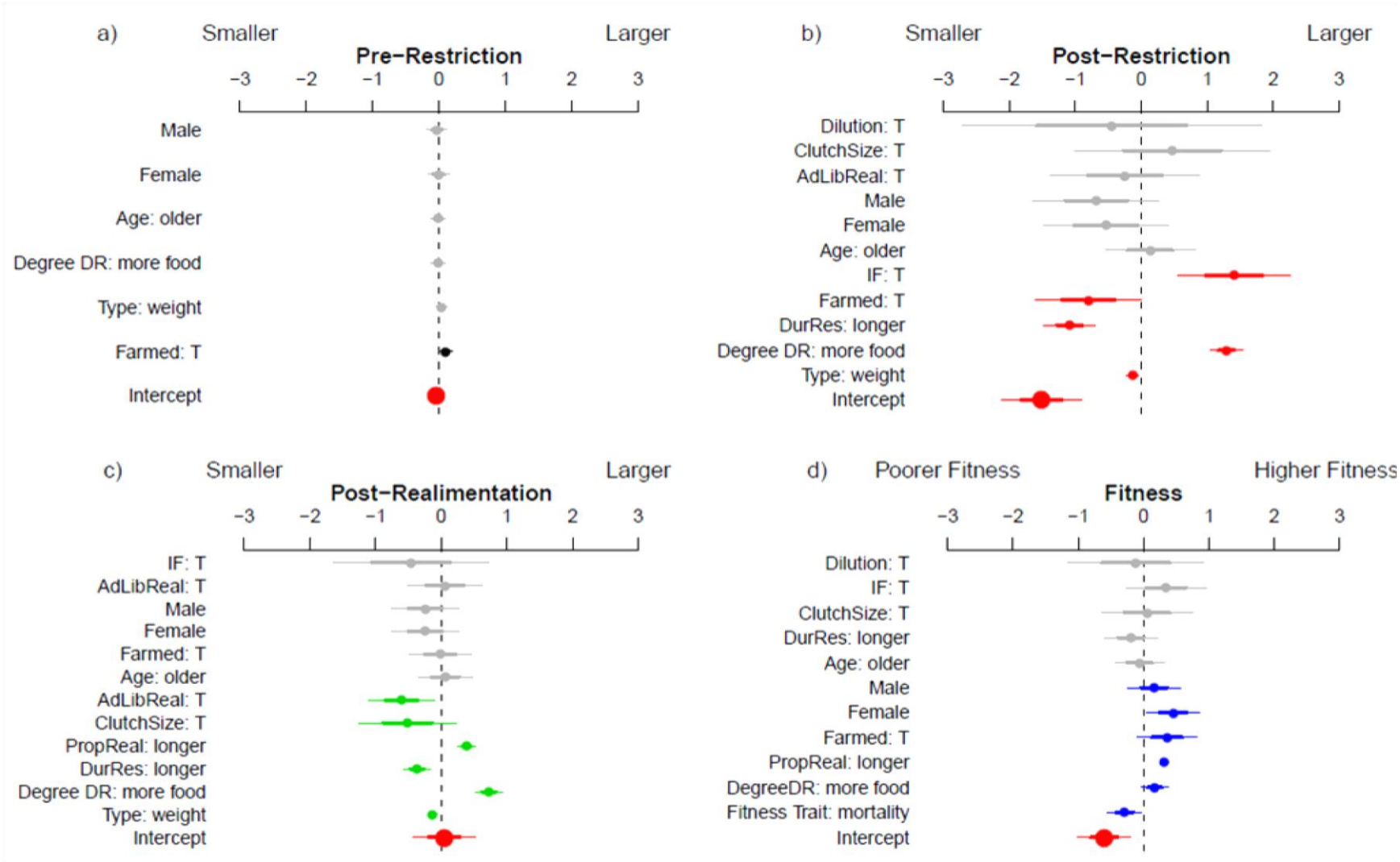
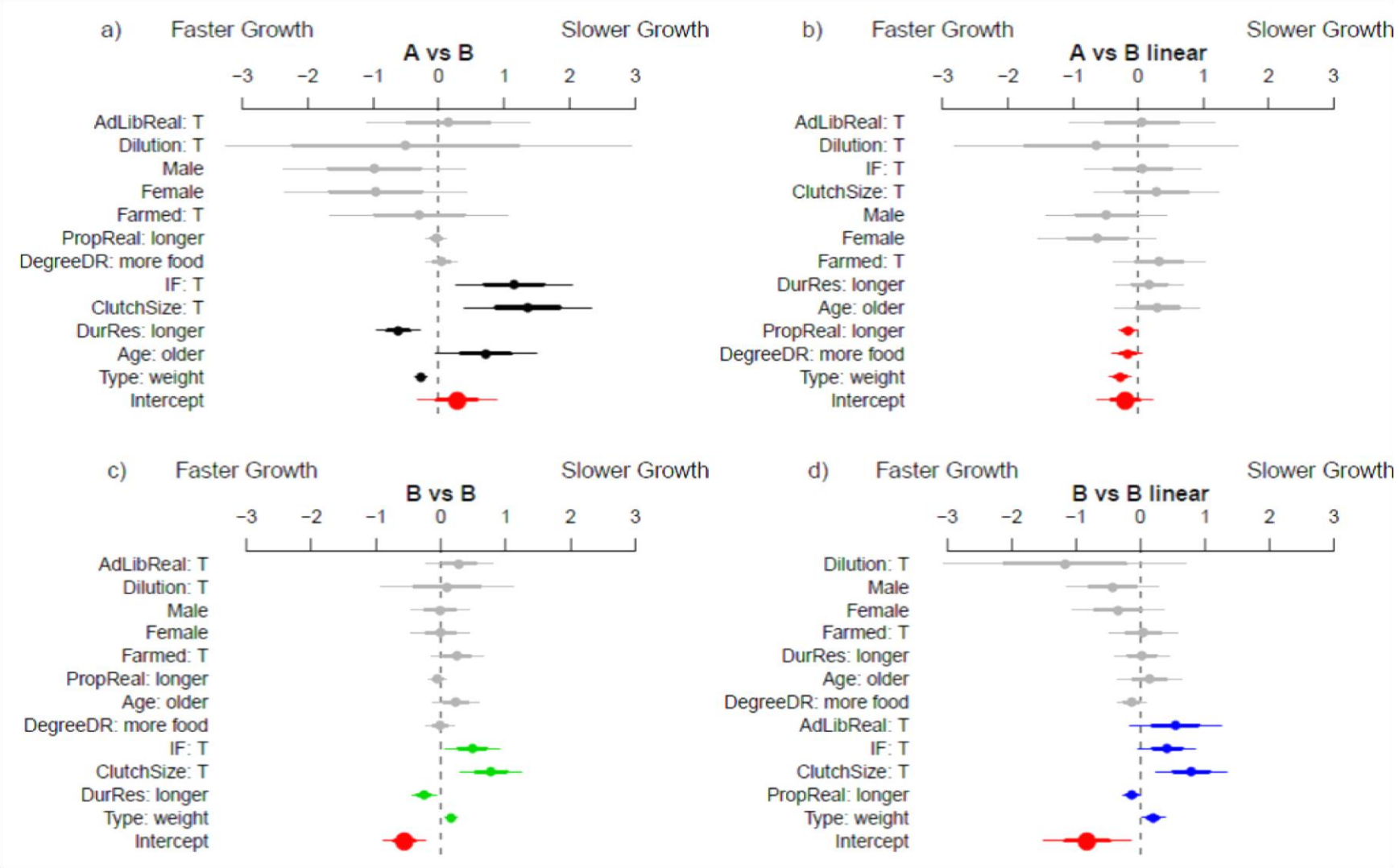

a)
Smaller
Larger
Pre−Restriction
−3
−2
−1
0
1
2
3
Male
Female
Age: older
Degree DR: more food
Type: weight
Farmed: T
Intercept
b)
Smaller
Larger
Post−Restriction
Dilution: T
ClutchSize: T
AdLibReal: T
Male
Female
Age: older
IF: T
Farmed: T
DurRes: longer
Degree DR: more food
Type: weight
Intercept
c)
Smaller
Larger
Post−Realimentation
IF: T
AdLibReal: T
Male
Female
Farmed: T
Age: older
AdLibReal: T
ClutchSize: T
PropReal: longer
DurRes: longer
Degree DR: more food
Type: weight
Intercept
d)
Poorer Fitness
Higher Fitness
Fitness
Dilution: T
IF: T
ClutchSize: T
DurRes: longer
Age: older
Male
Female
Farmed: T
PropReal: longer
DegreeDR: more food
Fitness Trait: mortality
Intercept

Figure 4.6 Meta-regression of growth slopes.

Coefficient plots showing the treatment effects on the growth rate of the restricted group after realimentation ("compensatory growth") compared to a) the control group during the restriction period (Period A) from all studies, b) the control group during period A where only control groups in the linear growth phase were included, c) the control group during the realimentation period (Period B) from all studies and d) the linear control groups during Period B (see Fig. 4.2). The intercept shows the overall model outcome and the other points describe the contribution of each moderator to the intercept. Confidence intervals which span zero indicate no significant effect at $\alpha = 0.05$. For graphs a-d, n = 226, 87, 226 and 87 respectively. For further explanation on interpreting moderator values, see Fig. 4.5 and Table A4.5. Full statistical information for each meta-regression analysis is reported in Tables A4.11-14.


a)
Faster Growth
Slower Growth
A vs B
−3 −2 −1 0 1 2 3
AdLibReal: T
Dilution: T
Male
Female
Farmed: T
PropReal: longer
DegreeDR: more food
IF: T
ClutchSize: T
DurRes: longer
Age: older
Type: weight
Intercept
b)
Faster Growth
Slower Growth
A vs B linear
−3 −2 −1 0 1 2 3
AdLibReal: T
Dilution: T
IF: T
ClutchSize: T
Male
Female
Farmed: T
DurRes: longer
Age: older
PropReal: longer
DegreeDR: more food
Type: weight
Intercept
c)
Faster Growth
Slower Growth
B vs B
−3 −2 −1 0 1 2 3
AdLibReal: T
Dilution: T
Male
Female
Farmed: T
PropReal: longer
Age: older
DegreeDR: more food
IF: T
ClutchSize: T
DurRes: longer
Type: weight
Intercept
d)
Faster Growth
Slower Growth
B vs B linear
−3 −2 −1 0 1 2 3
Dilution: T
Male
Female
Farmed: T
DurRes: longer
Age: older
DegreeDR: more food
AdLibReal: T
IF: T
ClutchSize: T
PropReal: longer
Type: weight
Intercept

Controlling for experimental methods shows that compensatory growth is achieved by the restricted group when compared with the post-restriction growth of the controls (intercept ± SE: -0.57 ± 0.16, t = -3.516, df = 120, p = 0.0006; Fig. 6c). This finding was moderated by the type of measurement, with compensatory growth in weight being weaker, and the duration of restriction, indicating longer restriction periods leads to swifter compensatory growth at realimentation. The evidence for compensatory growth could be interpreted as false detection, since faster period B growth is predicted given size-dependent growth of the controls. However, analysis of linear control growth shows that compensatory growth is a robust effect (intercept ± SE: -0.83 ± 0.34, t = 45, df = -2.421, p = 0.020; Fig. 6d). The linear analysis again demonstrates that weight has weaker compensatory growth than length and that, like the full analysis, clutch size and intermittent feeding are unlikely to lead to compensatory growth.

4.4 DISCUSSION

These meta-analyses and meta-regression analyses support the basic assumptions of altered growth patterns in response to early dietary restrictions: animals may reach the same size as controls after a period of restriction (catch-up growth) and can grow at a faster than optimal rate to achieve this end (compensatory growth; Metcalfe & Monaghan, 2001; Fig. 4.1). In reality, the realization of both growth patterns is largely dependent on the experimental methods used, as indicated by the large effects of a number of the moderators included and the high I^2 values (reflecting heterogeneity among studies; Table. 4.1). In particular, the inclusion of such important moderators as the degree and duration of restriction changed the net result of the post-realimentation analysis, which previously indicated that animals do not "catch-up" to the size of controls after restriction (Fig. 4.4a). Likewise, the meta-analyses for growth slopes showed equivocal results prior to the inclusion of the preeminent moderators "measurement type" and the duration or proportion of restriction (Fig. 4.4b). Biologically,

these experimental factors have an obvious influence on the growth of animals. In meta-regression analyses I was able to account for these inter-study differences by scaling and centering data on intermediate values, in order to observe the true effects of "catch-up growth" and "compensatory growth".

These results also support the claim that compensatory growth comes at a cost to fitness, at least in direct measurements of survival and reproductive output (Mangel & Munch, 2005; Fig. 4.5d, see Appendix 4.6.1 for indirect measurements). The greater impact on mortality than reproduction likely has some basis in the different protocols used between classes. Extreme dietary restriction (i.e. total starvation for long periods) is commonly used on fish and invertebrates and often uses mortality as a measure of effect (for example, Vidal *et al.*, 2006; Wang *et al.*, 2009). In contrast, much of the reproductive data comes from birds and mammals where possibly more consideration is given to the welfare of the animal, keeping restriction levels to a minimum (for example, Bernardis *et al.*, 1989; Hassan *et al.*, 2003). In this study, almost all classes (nine of eleven) were represented in the mortality data, while only birds, fish and mammals provided direct reproduction data. Taxonomic class and mortality biases could also be accountable for the difference between female-only and mixed-sex fitness effects. Female-only studies were mostly investigating reproductive effort, so females were in a reasonably healthy state, while many invertebrate studies did not sex the subjects and included large mortality statistics (for example, Zhang *et al.*, 2009). However, the overall negative effect of the treatment on fitness is indisputable.

Compensatory growth is believed to have evolved as an alternative growth strategy because it improves reproductive success, compared to under-size animals, and the fitness consequences are not paid until later in life (Yearsley *et al.*, 2004). Although it is tempting to accept the fitness results of this analysis as confirmation of the evolutionary foundation of compensatory growth, these results are largely artificial. All studies selected for these meta-analyses were

conducted in controlled environments in order to ensure that feeding amounts could be manipulated and monitored. Therefore, the subjects were spared the real life trials of predator avoidance, mate selection and pathogen exposure, which all clearly have great bearing on the fitness outcomes of wild animals. In order to truly understand whether it was the compensatory growth which caused the fitness deficits and not simply the early-life experience of dietary restriction, it is necessary to compare fitness with permanently restricted animals (a treatment group which is almost always missing in empirical work).

Research suggests that long-term dietary restriction increases longevity but decreases reproductive capacity (Shanley & Kirkwood, 2000). However, many of these claims are also laboratory-based and therefore do little to enlighten us on the evolutionary benefits of compensatory growth. Theoretically, the ideal proof of compensatory growth as an evolved alternative strategy would show that animals that grew faster than optimal would have worse fitness than well-fed animals but better fitness than animals that did not compensate for small size early in life, all in a real-world environment. However, since I have only the collated evidence of hundreds of laboratory experiments, the most I can safely conclude is that the treatment of dietary restriction and realimentation is sufficient to cause reduced direct fitness measurements in some classes of animals. The evidence of measurable compensatory growth does hint at the evolutionary theory on optimal growth being correct, though poor early life conditions alone could also explain lower survival and reproduction.

Overall, the analyses show that intermittent feeding and clutch size manipulation are poor ways of conducting compensatory research. Manipulating clutch size seems to cause little restrictive effect on offspring and, therefore, does little to display compensatory growth (Alonso-Alvarez *et al.*, 2006). It is possible that intermittent feeding is ineffective because the animals simply gorge on feeding days, known as "hyperphagia" (Ali & Wootton, 2000). Recent research on the effect of dietary restriction on longevity found that whether animals

were multi-generation laboratory dwellers or not had a significant impact on their response to the diet treatment (S. Nakagawa, *pers. comm.*). It is, therefore, surprising that whether animals were farmed or not had little impact on their potential for catch-up or compensatory growth. The only significant difference between farmed and non-farmed animals was detected after restriction (Fig. 4.5b), though this may have been biased by the number of fish studies which showed extremely severe restriction for long periods (for example, Santiago *et al.*, 2004). The heavy dependence of results on the experimental protocol used, as discussed earlier, suggests that a call be made for unified restriction protocols, at least for similar species. Such protocols would allow the evolutionary context of "compensatory growth" to be much more easily interpreted. Sogard & Olla (2002) give a good example of this strategy in their comparison of compensatory growth between sablefish, *Anoplopoma fimbria*, and walleye pollock, *Theragra chalcogramma*. Despite the similar environmental and ecological niches of these two pelagic fish species, the researchers were able to detect a marked difference in the mechanisms of compensatory growth and evolved baseline growth rates by exposing the fish to identical dietary regimes.

It is interesting that, on the whole, the linear analyses (Fig. 4.6b & 4.6d) are actually in agreement with the results derived from the complete slopes analyses (Fig. 4.6a & 4.6c). These results suggest that the impact of researchers not taking size-dependent growth into account when analysing compensatory growth is fortunately minimal and not as problematic as Nicieza and Alvarez (2009) would have us believe. However, the conflict between results derived by comparison of the restricted group's realimentation with part A and part B of the controls relates to this concern (Fig. 4.2). Compensatory growth after restriction was faster than the rate of controls at the same time, but, importantly, it was no different than the rate observed in the controls during period A. Although this difference could be explained by

size-dependent growth, the linear comparison makes the growth curve of the controls an unlikely explanation. Instead, it is possible that time is the important factor.

The rapid growth after restriction is faster than optimal growth for the age of the animals, but not faster than early on in life. The importance of age-dependent growth has been shown in response to the natural dietary restriction of alpine swift nestlings, *Apus melba* (Bize *et al.*, 2006). Chicks which were older during the poor weather delayed fledging in order to compensate for wing growth which had been sacrificed in place of mass during restriction. In contrast, younger chicks lost mass during undernutrition, but were able to reach the same size as controls at fledging by rapid weight gain. Bize *et al.* (2006) explained the difference in strategies on the varying "developmental windows" of tissues, organs and morphological traits, which in turn alter the stress resistance and tissue preservation hierarchy at different ages. The negative consequences of compensatory growth on fitness are, therefore, due to rapid growth at an age when physiological restrictions inhibit growth. For examples, one proposed mechanism for the later life health consequences of catch-up growth in humans, is that the critical period for muscle growth is around 30 weeks *in utero*(Robinson & Barker, 2002). As there is little cell replication after birth, any gain in mass is likely to give a disproportionately high body fat ratio, which leads to similar symptoms as in obesity, although an individual may not be obese. Surprisingly, the age (or timing) that restriction was initiated had little effect on the size and growth rate outcomes.

The other major concern of Nicieza and Alvarez (2009), was that preferential recovery of fat stores as opposed to skeletal growth may cause an overestimation of compensatory growth. This is a difficult issue to argue in terms of fitness; fat stores undeniably aid survival for the many animals that experience nutritional stress (such as hibernation, migration, nest-sitting or seasonal prey abundance; Morrison *et al.*, 2007), yet in size-dependent mate-selection body length is more important than mass, although the two are correlated (McElligott *et al.*, 2001).

The meta-analytic results indicate that weight was more severely affected by food restriction than length and was, therefore, less likely to catch-up to the same weight as controls. Compensatory weight gain was faster than the initial weight gain of the controls, but length had a stronger impact on compensatory growth when compared to the growth of controls after restriction. To a degree, these results support the concerns of Nicieza and Alvarez (2009), as it appears that weight is more easily gained and lost. Size-dependent growth explains why length shows greater compensation when compared to the post-restriction growth of controls, since the controls have already completed the majority of their skeletal growth by the end of restriction. This argument is supported by the stronger effect when non-linear studies are included (Fig. 4.6c). However, since the linear analyses came to the same conclusions as the complete analyses and measurement type had only small effect sizes, it appears that controlling for size-dependent growth and preferential fat storage is a matter of thorough investigating practice. In practice, failure to keep to these ideals may not be as misleading and counterfactual as originally feared. Nevertheless, controlling for size-dependent growth is encouraged as a matter of good science.

In conclusion, these analyses provide strong support for the theoretical concept of compensatory growth as an evolved type of phenotypic plasticity common to many disparate animal taxa. Dependent on the degree of restriction and the period within which animals were restricted, both catch-up growth and compensatory growth are feasible outcomes following realimentation (Fig. 4.5-6). As predicted, there is a cost to fitness as a trade-off for the larger size, which is manifested as higher mortality and lower reproductive output. Further work is needed to disentangle how much of this cost is generated purely by faster than optimal growth and not restriction. The major contribution of the present study has been to clarify the terminology (Fig. 4.1), such that the causes and consequences of “catch-up growth” and “compensatory growth” can be compared and contrasted without confusion. Given more

clear-cut definitions of key vocabulary, the field of compensatory growth research may use the findings of this quantitative review as a guideline for planning future research. With the implementation of more standardised restriction protocols, there can be greater emphasis on uncovering the proximate mechanisms, as well as unravelling the ecological and evolutionary consequences, of compensatory growth across taxa.

4.5 REFERENCES

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4.6 APPENDIX

4.6.1 INDIRECT FITNESS MEASUREMENTS

In addition to the required weight and length measures, any traits potentially relating to fitness were recorded. Broadly, these were categorised as traits relating to mortality, reproduction, development, physiology, sociality and activity (Table A4.1). Each sub-category within these groups was selected for its effect on the potential fitness on the animals. For example, swimming speed was included in the activity group, because it is known that faster swimming increases likelihood of avoiding predators and, therefore, increases survival. In comparison, fat content was not included as a physiological measure because it was unknown whether additional fat stores would have a positive or negative effect on the fitness of wild animals. For example, excess fat in humans is known to have negative consequences (Solomon & Manson, 1997), however fat stores in wild Atlantic salmon are essential for surviving winter (Morgan & Metcalfe, 2001).

4.6.1.1 Statistical Analysis

Analysis of the complete dataset of fitness-related traits (n = 207) was performed as described in the main text with a few differences. The effect of each trait was recorded as either positive or negative for use in later analysis, meaning that a high positive score and a low negative score both increase fitness. For example, data on survival was recorded as a percentage and was considered positive, while number of mating attempts to achieve conception was considered a negative, since fewer attempts indicate increased fitness. The effect sizes of all the traits which had been coded as negative (meaning a lower number is better for fitness) were multiplied by negative one for inclusion with the rest of the data. Analysis of this dataset indicated that a high percentage of the heterogeneity originated from the effect of taxonomic class as a random factor. Repeating the analysis without the inclusion of

taxonomic class caused no qualitative differences in the results, so class was included for consistency with the analyses in the main text.

TableA4.1 Fitness traits.

Lists of fitness traits for which data was collected, as grouped by the six major fitness categories. Percentage (%) indicates the estimates were recorded as a percentage of the experimental group to achieve the trait and an asterisk (*) indicates this value was calculated as a negative, see text for further explanation.

Activity	Development	Mortality
activity	eclosion time*	recapture (%)
endurance	puberty onset*	risky feeder (%)*
spatial learning	sexual maturity (%)	survival post-fledging (%)
speed*		survival pre-fledging (%)

Physiology	Reproduction	Social
cortisol/corticosterone*	age at first reproduction*	aggression
free radical resistance	estrus interval*	dominance
heterocyte:lymphocyte ratio	fertility (%)	
	hatchability (%)	
immune response	lifetime reproduction	
plasma carotenoids	mating attempts to conception*	
righting response*	number of offspring	
	offspring weight	
	receptive to mating (%)	
	reproductive investment	
	seed output	
	sexual maturity	

4.6.1.2 Results

Fitness was not significantly affected by the treatment (intercept ± SE: -0.20 ± 0.17, t = -1.223, df = 141, p = 0.223). The best model did not detect any significant effect of treatment on the fitness of restricted animals (intercept ± SE: -0.30 ± 0.20, t = -1.534, df = 132, p = 0.127; Fig. A4.1). In comparison with "activity" as a reference variable, all fitness traits showed the same non-significant negative bias, with the exception of "social" which showed a non-significant positive trend. The fitness measures of the restricted animals in comparison to controls were likely to be improved if there was a short restriction and a long realimentation. Females alone, and, to a lesser degree, males alone, were less likely to bear the fitness costs of treatment than males and females together. The number of classes represented in each fitness analysis (direct fitness or inclusive fitness ie. this analysis) were observed as percentage contributions (Fig. A4.2). The relative proportions of the three major contributing classes (Aves, Actinopterygii and Mammalia) show more indirect fitness data from fish and mammals and a decreasing contribution of the direct data from birds. More indirect data also comes from the class Insecta; however, the contribution of data from other invertebrates becomes less substantial when all fitness data is included.

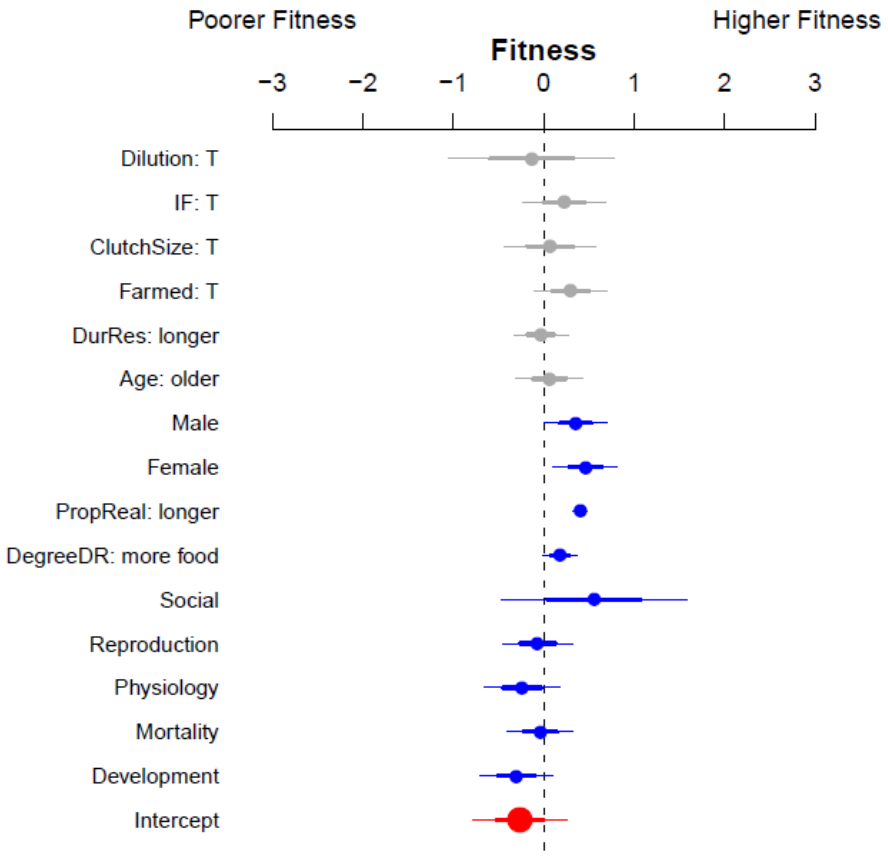

Figure A4.1 Fitness coefficient plot.

Coefficient plot of the inclusive fitness analysis showing the effect of treatment on the fitness of the restricted group compared to the control group. The intercept includes zero in the confidence interval indicating there is no overall significant effect. As a multiple factor comparison, the fitness traits were set against "Activity" as a reference variable, though no traits were found to be significantly different. Degree of restriction (DegreeDR) and proportion realimentation:restriction (PropReal) have better fitness prospects when given more food or longer realimentation. Male and Female indicate their respective effects as single-sex studies when compared to mixed-sex studies. For other moderators, see Fig. 4.5 or Table A4.5.

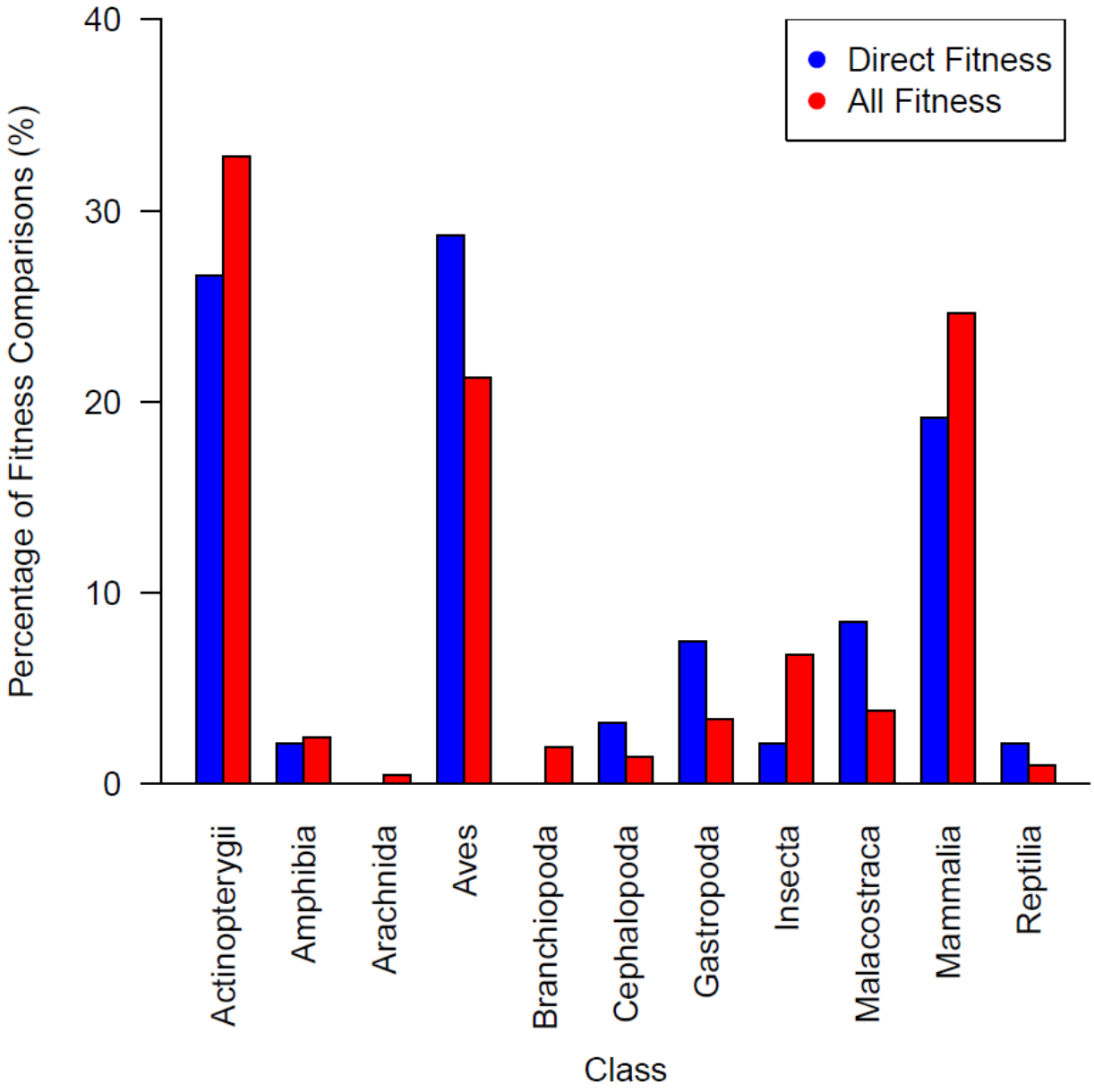

Figure A4.2 Taxonomic class contributions to direct and inclusive fitness analyses.

The percentage of fitness comparisons derived from each taxonomic class for the direct fitness analysis (main text, *n* = 94) and the inclusive fitness analysis, "all fitness" (presented in this appendix, *n* = 207).

4.6.1.3 Discussion

In contrast with the analysis of the direct fitness traits reported in the main data, this inclusive analysis shows that there is no net effect of compensatory growth on fitness. Given the high level of heterogeneity derived from class, the results suggest that not all taxa are required to trade-off fitness for rapid growth. While the absence of a trade-off could be true, considering the varying importance of size in relation to fitness across taxa, this conclusion does not satisfactorily explain the overall confirmation of compensatory growth across taxa. If there is no negative trade-off, there is no reason to limit growth to an optimal rate. Therefore, compensatory growth would not be detected. However, this conundrum could be explained by the types of fitness measures recorded in many studies.

The analysis of direct fitness effects was limited to two incontrovertible measures: mortality and reproduction. In contrast, the indirect measures of fitness included activity, development, physiology and social interactions (Table A4.1). Each of these groups, and the sub-groups within them, has a debateable influence on actual fitness. For example, earlier development was included as a positive factor because it results in earlier reproduction. However, there is much debate over the effects of early development on lifetime reproductive output, since early sexual maturity is correlated with reduced longevity (Partridge, 1987). Likewise, the interpretation of physiological measures such as corticosterone levels is highly context dependent and therefore may not be a good indicator of fitness (see Chapter III for further discussion).

The proportionally much greater contribution of indirect fitness data from fish, mammals and insects (and considering this analysis had over twice the sample size) suggests that many of the indirect fitness measures reported from these taxa are misleading. To negate the overall effect of negative fitness consequences observed from the direct study, the indirect evidence must have overwhelmingly found no effect or, more likely, found evidence in the opposite

direction. Measures of indirect fitness ultimately aspire to reaching the same conclusions as direct fitness measures but without the inconvenience of long-term studies. This analysis suggests that researchers take greater care in making assumptions about fitness from indirect measures and also highlights the need for more research on invertebrate compensatory growth to gain a greater understanding of its global fitness consequences.

4.6.1.4 References

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4.6.2 LINEAR SLOPES SELECTION

Initially, periods A and B of the control animals (Fig. 4.2) were analysed together and those that had a *d*-value that was not significantly different from zero with a 95% confidence interval were included. However, this method was found to be unsatisfactory, since data with large standard deviations could then be included despite having vastly different A and B slopes. While the dependence of *d* on standard deviations is very useful in making conservative estimates of true differences between groups, the linear selection process required bold estimates of differences between slopes so that they could be excluded as non-linear. The different effects of the two selection methods is presented below, and gives a clear indication of why the less statistically-based method was preferred (Fig. A4.3). Ideally with two linear slopes, slope A divided by slope B should be 1. The preferred method is less likely to include statistically non-significant results which actually have quite different slopes (up to four times larger). Consequently this method was chosen in order to select control growth slopes which were linear throughout the entire experiment.

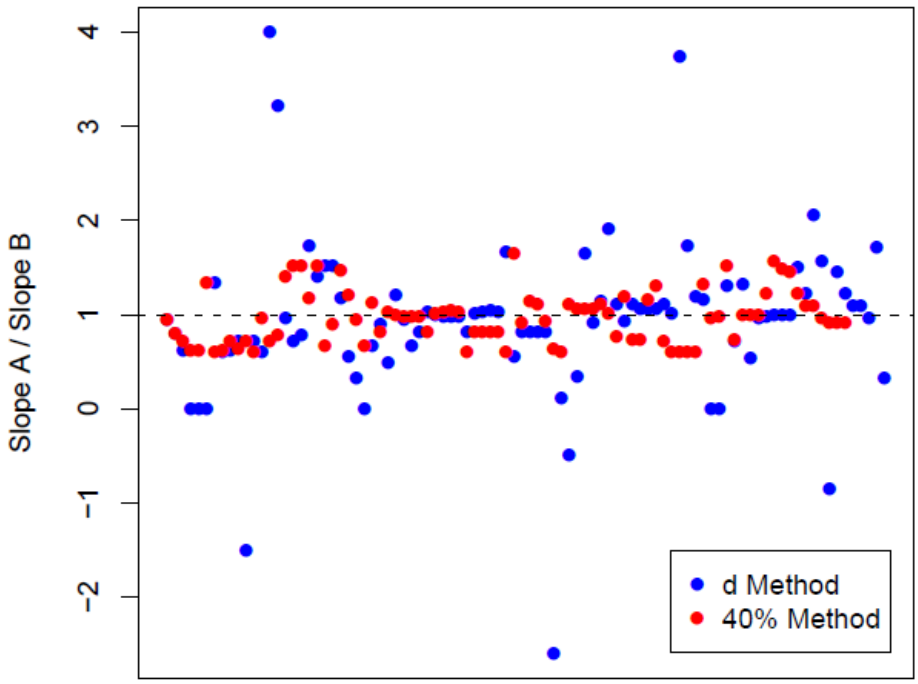

Figure A4.3 Comparison of linear slope selection methods.

Difference between slopes for each data point included by the "statistical *d*"method and by the "40% of the smaller slope" method (which approximates the visual inspection method). While many of the data points are likely to be included by both methods, it is clear that the *d* method is much more inclusive of extreme values (slope A up to four times larger than slope B) because of its dependence on standard deviations to detect differences.

Table A4.2 Study details.

Publication details of the studies included in the meta-analyses (n = 88).

Author	Year	Title	Journal	Volume	Pages
Ali, M., Cui, Y.B., Zhu, X.M. & Wootton, R.J.	2001	Dynamics of appetite in three fish species (*Gasterosteus aculeatus*, *Phoxinus phoxinus* and *Carassius auratus gibelio*) after feed deprivation	*Aquaculture Research*	32	443-450
Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B., Chastel, O. & Sorci, G.	2006	An experimental manipulation of life-history trajectories and resistance to oxidative stress	*Evolution*	60	1913-1924
Alvarez, D. & Metcalfe, N.B.	2005	Catch-up growth and swimming performance in threespine sticklebacks (*Gasterosteus aculeatus*): seasonal changes in the cost of compensation	*Canadian Journal of Fisheries and Aquatic Sciences*	62	2169-2176
Alvarez, D. & Nicieza, A.G.	2005	Compensatory response 'defends' energy levels but not growth trajectories in brown trout, *Salmo trutta* L.	*Proceedings of the Royal Society B-Biological Sciences*	272	601-607
Auer, S.K., Arendt, J.D., Chandramouli, R. & Reznick, D.N.	2010	Juvenile compensatory growth has negative consequences for reproduction in Trinidadian guppies (*Poecilia reticulata*)	*Ecology Letters*	13	998-1007
Benyi, K., Acheampong-Boateng, O., Norris, D. & Mikasi, M.S.	2008	Effects of early and late skip-a-day feeding on the growth performance of male Hybro broiler chickens	*Asian Journal of Animal and Veterinary Advances*	3	244-253
Bernardis, L.L., Bellinger, L.L., Kodis, M. & Feldman, M.J.	1989	Normal catch-up growth in rats severely food-restricted prior to lesions of the dorsomedial hypothalamic nucleus: the 1st 48 hours	*Pharmacology Biochemistry and Behavior*	32	957-960
Bernardis, L.L., Bellinger, L.L., McEwen, G., Kodis, M. & Feldman, M.J.	1988	Further evidence for the existence of an organismic set point in rats with dorsomedial hypothalamic nucleus lesions (Dmnl rats) - normal catch-up growth	*Physiology & Behavior*	44	561-568

Bhujel, R.C., Little, D.C. & Hossain, A.	2007	Reproductive performance and the growth of pre-stunted and normal Nile tilapia (*Oreochromis niloticus*) broodfish at varying feeding rates	*Aquaculture*	273	71-79
Bradley, M.C., Perrin, N. & Calow, P.	1991	Energy allocation in the cladoceran *Daphnia magna* Straus, under starvation and refeeding	*Oecologia*	86	414-418
Brumm, H., Zollinger, S.A. & Slater, P.J.B.	2009	Developmental stress affects song learning but not song complexity and vocal amplitude in zebra finches	*Behavioral Ecology and Sociobiology*	63	1387-1395
Capellan, E. & Gill, A.G.	2007	Non-equivalence of growth arrest induced by predation risk or food limitation: context-dependent compensatory growth in anuran tadpoles	*Journal of Animal Ecology*	76	1026-1035
Cho, S.H.	2005	Compensatory growth of juvenile flounder *Paralichthys olivaceus* L. and changes in biochemical composition and body condition indices during starvation and after refeeding in winter season	*Journal of the World Aquaculture Society*	36	508-514
Cho, S.H., Cho, Y.J. & Yoo, J.H.	2009	Compensatory growth of juvenile abalone *Haliotis discus hannai* with different feeding regime	*Aquaculture Research*	40	984-987
Cho, S.H., Lee, S.M., Park, B.H., Ji, S.C., Lee, J., Bae, J. & Oh, S.Y.	2006	Compensatory growth of juvenile olive flounder, *Paralichthys olivaceus* L., and changes in proximate composition and body condition indexes during fasting and after refeeding in summer season	*Journal of the World Aquaculture Society*	37	168-174
Damsgard, B. & Dill, L.M.	1998	Risk-taking behavior in weight-compensating coho salmon, *Oncorhynchus kisutch*	*Behavioural Ecology*	9	26-32
De Block, M., McPeek, M.A. & Stoks, R.	2008	Stronger compensatory growth in a permanent-pond *Lestes* damselfly relative to temporary-pond *Lestes*	*Oikos*	117	245-254
De Block, M., Slos, S., Johansson, F. & Stoks, R.	2008	Integrating life history and physiology to understand latitudinal size variation in a damselfly	*Ecography*	31	115-123
DeKogel, C.H.	1997	Long-term effects of brood size maniplation on morphological development and sex-specific mortality of offspring	*Journal of Animal Ecology*	66	167-178

Desai, M., Gayle, D.A., Casillas, E., Boles, J. & Ross, M.G.	2009	Early undernutrition attenuates the inflammatory response in adult rat offspring	*Journal of Maternal-Fetal & Neonatal Medicine*	22	571-575
Dmitriew, C., Carroll, J. & Rowe, L.	2009	Effects of early growth conditions on body composition, allometry, and survival in the ladybird beetle *Harmonia axyridis*	*Canadian Journal of Zoology*	87	175-182
Dmitriew, C., Cooray, M. & Rowe, L.	2007	Effects of early resource-limiting conditions on patterns of growth, growth efficiency, and immune function at emergence in a damselfly (Odonata : Coenagrionidae)	*Canadian Journal of Zoology*	85	310-318
Engelbregt, M.J.T., van Weissenbruch, M.M., Lips, P., van Lingen, A., Roos, J.C. & Delemarre-van de Waal, H.A.	2004	Body composition and bone measurements in intra-uterine growth retarded and early postnatally undernourished male and female rats at the age of 6 months: comparison with puberty	*Bone*	34	180-186
Fermin, A.C.	2002	Effects of alternate starvation and refeeding cycles on food consumption and compensatory growth of abalone, *Haliotis asinina* (Linnaeus)	*Aquaculture Research*	33	197-202
Fink, R., Tauson, A.H. & Forsburg, M.	1998	Influence of different planes of energy supply prior to the breeding season on blood metabolites in female mink (*Mustela vison*)	*Reproduction Nutrition Development*	38	107-116
Fraser, D.J., Weir, L.K., Darwish, T.L., Eddington, J.D. & Hutchings, J.A.	2007	Divergent compensatory growth responses within species: linked to contrasting migrations in salmon?	*Oecologia*	153	543-553
Freetly, H.C., Ferrell, C.L. & Jenkins, T.G.	2000	Timing of realimentation of mature cows that were feed-restricted during pregnancy influences calf birth weights and growth rates	*Journal of Animal Science*	78	2790-2796
Gidenne, T., Combes, S., Feugier, A., Jehl, N., Arveaux, P., Boisot, P., Briens, C., Corrent, E., Fortune, H., Montessuy, S. & Verdelhan, S.	2009	Feed restriction strategy in the growing rabbit. 2. Impact on digestive health, growth and carcass characteristics	*Animal*	3	509-515

Gilbert, M.E., MacPhail, R., Baldwin, J., Moser, V.C. & Chernoff, N.	2010	Moderate developmental undernutrition: impact on growth and cognitive function in youth and old age	*Neurotoxicology and Teratology*	32	362-372
Gill, C.J. & Rissman, E.F.	1997	Female sexual behavior is inhibited by short- and long-term food restriction	*Physiology & Behavior*	61	387-394
Gonzales, E., Buyse, J., Loddi, M.M., Takita, T.S., Buys, N. & Decuypere, E.	1998	Performance, incidence of metabolic disturbances and endocrine variables of food-restricted male broiler chickens	*British Poultry Science*	39	671-678
Hassan, S.M., Mady, M.E., Cartwright, A.L., Sabri, H.M. & Mobarak, M.S.	2003	Effect of early feed restriction on reproductive performance in Japanese quail (*Coturnix coturnix japonica*)	*Poultry Science*	82	1163-1169
Hector, K.L., Bishop, P.J. & Nakagawa, S.	2010	Compensatory growth and immune activation in the brown tree frog, *Litoria ewingii*	*Unpublished*		
Hegyi, G. & Torok, J.	2007	Developmental plasticity in a passerine bird: an experiment with collared flycatchers *Ficedula albicollis*	*Journal of Avian Biology*	38	327-334
Heide, A., Foss, A., Stefansson, S.O., Mayer, I., Norberg, B., Roth, B., Jenssen, M.D., Nortvedt, R. & Irmsland, A.K.	2006	Compensatory growth and fillet crude composition in juvenile Atlantic halibut: effects of short term starvation periods and subsequent feeding	*Aquaculture*	261	109-117
Hervant, F., Mathieu, J. & Durand, J.	2001	Behavioural, physiological and metabolic responses to long-term starvation and refeeding in a blind cave-dwelling (*Proteus anguinus*) and a surface-dwelling (*Euproctus asper*) salamander	*Journal of Experimental Biology*	204	269-281
Isaacs, K.L., Owens, J.L., Littlejohn, R.P., Johnstone, P.D. & Fennessy, P.F.	1991	Influence of maternal liveweight on reproductive-performance and wool production of heterozygous Booroola Merino x Coopworth (Fecb Fec+) and Merino x Coopworth ewes	*New Zealand Journal of Agricultural Research*	34	55-67
Jackson, C.M.	1937	Recovery of rats upon refeeding after prolonged suppression of growth by underfeeding	*Anatomical Record*	68	371-381

Jang, I.S., Kang, S.Y., Ko, Y.H., Moon, Y.S. & Sohn, S.H.	2009	Effect of qualitative and quantitative feed restriction on growth performance and immune function in broiler chickens	*Asian-Australasian Journal of Animal Sciences*	22	388-395
Johnsson, J.I. & Bohlin, T.	2005	Compensatory growth for free? A field experiment on brown trout, *Salmo trutta*	*Oikos*	111	31-38
Johnsson, J.I. & Bohlin, T.	2006	The cost of catching up: increased winter mortality following structural growth compensation in the wild	*Proceedings of the Royal Society B-Biological Sciences*	273	1281-1286
Kerr, G.R., Lozy, M.E. & Scheffler, G.	1975	Malnutrition studies in *Macaca mulatta* .4. Energy and protein consumption during growth failure and catch-up growth	*American Journal of Clinical Nutrition*	28	1364-1376
Konarzewski, M., Kowalczyk, J., Swierubska, T. & Lewonczuk, B.	1996	Effect of short-term feed restriction, realimentation and overfeeding on growth of song thrush (*Turdus philomelos*) nestlings	*Functional Ecology*	10	97-105
Le Cozler, Y., Peccatte, J.R. & Delaby, L.	2010	A comparative study of three growth profiles during rearing in dairy heifers: effect of feeding intensity during two successive winters on performances and longevity	*Livestock Science*	127	238-247
Lepczyk, C.A. & Karasov, W.H.	2000	Effect of ephemeral food restriction on growth of house sparrows	*Auk*	117	164-174
Li, Z.H., Xie, S., Wang, J.X., Sales, J., Li, P. & Chen, D.Q.	2009	Effect of intermittent starvation on growth and some antioxidant indexes of *Macrobrachium nipponense* (De Haan)	*Aquaculture Research*	40	526-532
Lippens, M., Room, G., De Groote, G. & Decuypere, E.	2000	Early and temporary quantitative food restriction of broiler chickens. 1. Effects on performance characteristics, mortality and meat quality	*British Poultry Science*	41	343-354
Luna-Pinto, G. & Cronje, P.B.	2000	The roles of the insulin-like growth factor system and leptin as possible mediators of the effects of nutritional restriction on age at puberty and compensatory growth in dairy heifers	*South African Journal of Animal Science*	30	155-163
Mantysaari, P., Ingvartsen, K.L. & Toivonen, V.	1999	Feeding intensity of pregnant heifers: effect of feeding intensity during gestation on performance and plasma parameters of primiparous Ayrshire cows	*Livestock Production Science*	62	29-41

Marczak, L.B. & Richardson, J.S.	2008	Growth and development rates in a riparian spider are altered by asynchrony between the timing and amount of a resource subsidy	*Oecologia*	156	249-258
Maurya, V.P., Naqvi, S.M.K. & Mittal, J.P.	2004	Effect of dietary energy level on physiological responses and reproductive performance of Malpura sheep in the hot semi-arid regions of India	*Small Ruminant Research*	55	117-122
Mazzuco, H., Guidoni, A.L. & Jaenisch, F.R.	2000	Effects of qualitative feed restriction on compensatory growth in the broiler chicken	*Pesquisa Agropecuaria Brasileira*	35	543-549
Morgan, I.J. & Metcalfe, N.B.	2001	Deferred costs of catch-up growth after autumnal food shortage in juvenile salmon	*Proceedings of the Royal Society B - Biological Sciences*	268	295-301
Myszkowski, L., Kaminski, R. & Kamler, E.	2006	Compensatory growth and matter or energy deposition in *Vimba vimba* juveniles fed natural food or a formulated diet	*Folia Zoologica*	55	211-222
Nicieza, A.G. & Metcalfe, N.B.	1997	Growth compensation in juvenile Atlantic salmon: responses to depressed temperature and food availability	*Ecology*	78	2385-2400
Onbasilar, E.E., Yalcin, S., Torlak, E. & Ozdemir, P.	2009	Effects of early feed restriction on live performance, carcass characteristics, meat and liver composition, some blood parameters, heterophil-lymphocyte ratio, antibody production and tonic immobility duration	*Tropical Animal Health and Production*	41	1513-1519
Pan, J.Q., Tan, X., Li, J.C., Sun, W.D. & Wang, X.L.	2005	Effects of early feed restriction and cold temperature on lipid peroxidation, pulmonary vascular remodelling and ascites morbidity in broilers under normal and cold temperature	*British Poultry Science*	46	374-381
Partadiredja, G. & Bedi, K.S.	2010	Undernutrition during either the pre- or immediate post-weaning period does not affect longevity in Quackenbush mice	*Nutritional Neuroscience*	13	33-42
Peterson, B.C. & Small, B.C.	2004	Effects of fasting on circulating IGF-binding proteins, glucose, and cortisol in channel catfish (*Ictalurus punctatus*)	*Domestic Animal Endocrinology*	26	231-240

Pierce, B.J. & McWilliams, S.R.	2004	Diet quality and food limitation affect the dynamics of body composition and digestive organs in a migratory songbird (*Zonotrichia albicollis*)	*Physiological and Biochemical Zoology*	77	471-483
Poore, K.R., Cleal, J.K., Newman, J.P., Boullin, J.P., Noakes, D.E., Hanson, M.A. & Green, L.R.	2007	Nutritional challenges during development induce sex-specific changes in glucose homeostasis in the adult sheep	*American Journal of Physiology-Endocrinology and Metabolism*	292	E32-E39
Qian, X., Cui, Y., Xiong, B. & Yang, Y.	2000	Compensatory growth, feed utilization and activity in gibel carp, following feed deprivation	*Journal of Fish Biology*	56	228-232
Radder, R.S., Warner, D.A. & Shine, R.	2007	Compensating for a bad start: catch-up growth in juvenile lizards (*Amphibolurus muricatus*, Agamidae)	*Journal of Experimental Zoology Part A - Ecological Genetics and Physiology*	307	500-508
Reimers, E., Kjorrefjord, A.G. & Stavostrand, S.M.	1993	Compensatory growth and reduced maturation in 2nd sea winter farmed Atlantic salmon following starvation in February and March	*Journal of Fish Biology*	43	805-810
Richner, H., Schneiter, P. & Stirnimann, H.	1989	Life-history consequences of growth rate depression: an experimental study on carrion crows (*Corvus corone corone* L)	*Functional Ecology*	3	617-624
Roark, A.M., Bjorndal, K.A. & Bolten, A.B.	2009	Compensatory responses to food restriction in juvenile green turtles (*Chelonia mydas*)	*Ecology*	90	2524-2534
Rommers, J.M., Kemp, B., Meijerhof, R. & Noordhuizens, J.P.T.M.	2001	The effect of litter size before weaning on subsequent body development, feed intake, and reproductive performance of young rabbit does	*Journal of Animal Science*	79	1973-1982
Rueda, F.M., Martinez, F.J., Zamora, S., Kentouri, M. & Divanach, P.	1998	Effect of fasting and refeeding on growth and body composition of red porgy, *Pagrus pagrus* L.	*Aquaculture Research*	29	447-452
Saleh, E.A., Watkins, S.E., Waldroup, A.L. & Waldroup, P.W.	2005	Effects of early quantitative feed restriction on live performance and carcass composition of male broilers grown for further processing	*Journal of Applied Poultry Research*	14	87-93

Santiago, C.B., Gonzal, A.C., Aralar, E.V. & Arcilla, R.P.	2004	Effect of stunting of juvenile bighead carp *Aristichthys nobilis* (Richardson) on compensatory growth and reproduction	*Aquaculture Research*	35	836-841
Sears, J. & Hatch, S.A.	2008	Rhinoceros auklet developmental responses to food limitation: an experimental study	*Condor*	110	709-717
Sogard, S.M. & Olla, B.L.	2002	Contrasts in the capacity and underlying mechanisms for compensatory growth in two pelagic marine fishes	*Marine Ecology-Progress Series*	243	165-177
Stefansson, S.O., Imsland, A.K. & Handeland, S.O.	2009	Food-deprivation, compensatory growth and hydro-mineral balance in Atlantic salmon (*Salmo salar*) post-smolts in sea water	*Aquaculture*	290	243-249
Stoks, R., De Block, M. & McPeek, M.A.	2006	Physiological costs of compensatory growth in a damselfly	*Ecology*	87	1566-1574
Sun, L., Akha, A.A.S., Miller, R.A. & Harper, J.M.	2009	Life-span extension in mice by preweaning food restriction and by methionine restriction in middle age	*Journals of Gerontology Series A-Biological Sciences and Medical Sciences*	64	711-722
Velkoska, E., Cole, T.J., Dean, R.G., Burrell, L.M. & Morris, M.J.	2008	Early undernutrition leads to long-lasting reductions in body weight and adiposity whereas increased intake increases cardiac fibrosis in male rats	*Journal of Nutrition*	138	1622-1627
Vidal, E.A.G., DiMarco, P. & Lee, P.	2006	Effects of starvation and recovery on the survival, growth and RNA/DNA ratio in loliginid squid paralarvae	*Aquaculture*	260	94-105
Wang, Y., Li, C., Qin, J.G. & Han, H.	2009	Cyclical feed deprivation and refeeding fails to enhance compensatory growth in Nile tilapia, *Oreochromis niloticus* L.	*Aquaculture Research*	40	204-210
Weatherly, A.H. & Gill, H.S.	1981	Recovery growth following periods of restricted rations and starvation in rainbow trout, *Salmo gairdneri* Richardson	*Journal of Fish Biology*	18	195-207
Wieser, W., Krumschnabel, G. & Ojwangokwor, J.P.	1992	The energetics of starvation and growth after refeeding in juveniles of 3 cyprinid species	*Environmental Biology of Fishes*	33	63-71
Wiggins, D.A.	1990	Food availability, growth, and heritability of body size in nestling tree swallows (*Tachycineta bicolor*)	*Canadian Journal of Zoology*	68	1292-1296

Wright, H.A., Wootton, R.J. & Barber, I.	2007	Compensatory growth in threespine sticklebacks (*Gasterosteus aculeatus*) inhibited by experimental *Schistocephalus* infections	*Canadian Journal of Fisheries and Aquatic Sciences*	64	819-826
Wu, L.X. & Dong, S.L.	2002	Compensatory growth responses in juvenile Chinese shrimp, *Fenneropenaeus chinensis*, at different temperatures	*Journal of Crustacean Biology*	22	511-520
Zak, L.J., Cosgrove, J.R., Aherne, F.X. & Foxcroft, G.R.	1997	Pattern of feed intake and associated metabolic and endocrine changes differentially affect postweaning fertility in primiparous lactating sows	*Journal of Animal Science*	75	208-216
Zhang, P.D., Zhang, X.M., Li, J. & Gao, T.X.	2009	Starvation resistance and metabolic response to food deprivation and recovery feeding in *Fenneropenaeus chinensis* juveniles	*Aquaculture International*	17	159-172
Zhao, Z.J. & Cao, J.	2009	Plasticity in energy budget and behavior in Swiss mice and striped hamsters under stochastic food deprivation and refeeding	*Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology*	154	84-91
Zhao, Y., Chen, Y. & Brown, J.A.	2001	Impacts of egg and larval size on survival and growth of Atlantic cod under different feeding conditions	*Journal of Fish Biology*	59	569-581
Zhu, X., Wu, L., Cui, Y., Yang, Y. & Wootton, R.J.	2003	Compensatory growth response in three-spined stickleback in relation to feed-deprivation protocols	*Journal of Fish Biology*	62	195-205

Table A4.3 Taxonomic information.

Taxonomic information for data species, with number of data points per species for post-realimentation and slopes analyses.

Common Name	Phylum	Class	Order	Family	Genus	Species	*n*
Amber-winged damselfly	Arthropoda	Insecta	Odonata	Lestidae	*Lestes*	*eurinus*	4
Ass's ear abalone	Mollusca	Gastropoda	Orthogastropoda	Haliotidae	*Haliotis*	*asinina*	4
Atlantic cod	Chordata	Actinopterygii	Gadiformes	Gadidae	*Gadus*	*morhua*	4
Atlantic halibut	Chordata	Actinopterygii	Pleuronectiformes	Pleuronectidae	*Hippoglossus*	*hippoglossus*	1
Atlantic salmon	Chordata	Actinopterygii	Salmoniformes	Salmonidae	*Salmo*	*salar*	23
Bighead carp	Chordata	Actinopterygii	Cypriniformes	Cyprinidae	*Aristichthys*	*nobilis*	8
Brown tree frog	Chordata	Amphibia	Anura	Hylidae	*Litoria*	*ewingii*	2
Brown trout	Chordata	Actinopterygii	Salmoniformes	Salmonidae	*Salmo*	*trutta*	12
Carrion crow	Chordata	Aves	Passeriformes	Corvidae	*Corvus*	*corone*	2
Cave salamander	Chordata	Amphibia	Caudata	Proteidae	*Proteus*	*anguinus*	1
Channel catfish	Chordata	Actinopterygii	Siluriformes	Ictaluridae	*Ictalurus*	*punctatus*	1
Chicken	Chordata	Aves	Galliformes	Phasianidae	*Gallus*	*gallus*	15
Chinese shrimp	Arthropoda	Malacostraca	Decapoda	Penaeidae	*Fenneropenaeus*	*chinensis*	8
Coho Salmon	Chordata	Actinopterygii	Salmoniformes	Salmonidae	*Oncorhynchus*	*kisutch*	1
Collared flycatcher	Chordata	Aves	Passeriformes	Muscicapidae	*Ficedula*	*albicollis*	2
Common blue damselfly	Arthropoda	Insecta	Odonata	Coenagrionidae	*Enallagma*	*cyathigerum*	3
Cow	Chordata	Mammalia	Cetartiodactyla	Bovidae	*Bos*	*taurus*	8
Danubian bleak	Chordata	Actinopterygii	Cypriniformes	Cyprinidae	*Chalcalburnus*	*chalcoides mento*	2
Daphnia	Arthropoda	Branchiopoda	Diplostraca	Daphniidae	*Daphnia*	*magna*	4
Eastern forktail damselfly	Arthropoda	Insecta	Odonata	Coenagrionidae	*Ischnura*	*verticalis*	2
European common frog	Chordata	Amphibia	Anura	Ranidae	*Rana*	*temporaria*	1

Gibel carp	Chordata	Actinopterygii	Cypriniformes	Cyprinidae	*Carassius*	*auratus gibelio*	3
Green emerald damselfly	Arthropoda	Insecta	Odonata	Lestidae	*Lestes*	*viridis*	2
Green turtle	Chordata	Reptilia	Testudines	Cheloniidae	*Chelonia*	*mydas*	2
Guppy	Chordata	Actinopterygii	Cyprinodontiformes	Poeciliidae	*Poecilia*	*reticulata*	2
House sparrow	Chordata	Aves	Passeriformes	Passeridae	*Passer*	*domesticus*	1
Jacky dragon	Chordata	Reptilia	Squamata	Agamidae	*Amphibolurus*	*muricatus*	4
Japanese quail	Chordata	Aves	Galliformes	Perdicinae	*Coturnix*	*coturnix japonica*	4
Ladybird beetle	Arthropoda	Insecta	Coleoptera	Coccinellidae	*Harmonia*	*axyridis*	2
Long-armed shrimp	Arthropoda	Malacostraca	Decapoda	Palaemonidae	*Macrobrachium*	*nipponense*	3
Mink	Chordata	Mammalia	Carnivora	Mustelidae	*Mustela*	*vison*	1
Minnow	Chordata	Actinopterygii	Cypriniformes	Cyprinidae	*Phoxinus*	*phoxinus*	2
Mouse	Chordata	Mammalia	Rodentia	Muridae	*Mus*	*musculus*	4
Musk shrew	Chordata	Mammalia	Insectivora	Soricidae	*Suncus*	*murinus*	1
Nile tilapia	Chordata	Actinopterygii	Perciformes	Cichlidae	*Oreochromis*	*niloticus*	3
Olive flounder	Chordata	Actinopterygii	Pleuronectiformes	Paralichthyidae	*Paralichthys*	*olivaceus*	8
Pacific abalone	Mollusca	Gastropoda	Orthogastropoda	Haliotidae	*Haliotis*	*discus hannai*	10
Pig	Chordata	Mammalia	Cetartiodactyla	Suidae	*Sus*	*scrofa domestica*	1
Pyrenean salamander	Chordata	Amphibia	Caudata	Salamandridae	*Euproctus*	*asper*	1
Rabbit	Chordata	Mammalia	Lagomorpha	Leporidae	*Oryctolagus*	*cuniculas*	5
Rainbow trout	Chordata	Actinopterygii	Salmoniformes	Salmonidae	*Oncorhynchus*	*mykiss*	1
Rat	Chordata	Mammalia	Rodentia	Muridae	*Rattus*	*norvegicus*	11
Red porgy	Chordata	Actinopterygii	Perciformes	Sparidae	*Pagrus*	*pagrus*	3
Rhesus monkey	Chordata	Mammalia	Primates	Cercopithecidae	*Macaca*	*mulatta*	2
Rhinocerous auklet	Chordata	Aves	Charadriiformes	Alcidae	*Cerorhinca*	*monocerata*	2
Rudd	Chordata	Actinopterygii	Cypriniformes	Cyprinidae	*Scardinius*	*erythropthalmus*	2

Sablefish	Chordata	Actinopterygii	Scorpaeniformes	Anoplopomatidae	*Anoplopoma*	*fimbria*	2
Sheep	Chordata	Mammalia	Cetartiodactyla	Bovidae	*Ovis*	*aries*	4
Song thrush	Chordata	Aves	Passeriformes	Turdidae	*Turdus*	*philomelos*	1
Spider (Long-jawed orb weaver)	Arthropoda	Arachnida	Araneae	Tetragnathidae	*Tetragnatha*	*versicolor*	2
Squid	Mollusca	Cephalopoda	Teuthida	Loliginidae	*Loligo*	*opalescens*	3
Striped hamster	Chordata	Mammalia	Rodentia	Cricetidae	*Cricetulus*	*barabensis*	1
Three-spined stickleback	Chordata	Actinopterygii	Gasterosteiformes	Gasterosteidae	*Gasterosteus*	*aculeatus*	10
Tree swallow	Chordata	Aves	Passeriformes	Hirundinidae	*Tachycineta*	*bicolor*	2
Vimba	Chordata	Actinopterygii	Cypriniformes	Cyprinidae	*Vimba*	*vimba*	1
Wall-eyed pollock	Chordata	Actinopterygii	Gadiformes	Gadidae	*Theragra*	*chalcogramma*	2
White-throated sparrow	Chordata	Aves	Passeriformes	Fringillidae	*Zonotrichia*	*albicollis*	2
Zebra finch	Chordata	Aves	Passeriformes	Estrildidae	*Taeniopygia*	*guttata*	8

Table A4.4 Longevity data.

Longevity data by species and the origin of longevity estimates. “AA” stands for AnAge.com, followed by the preferred source within AnAge.

Common Name	Longevity (days)	Source
Amber-winged damselfly	365	Lutz (1968) Life-history studies on *Lestes eurinus* say (Odonata). *Ecology* **49**, 576-579
Ass's ear abalone	2920	Poore, Gary C. B.(1972) 'Ecology of New Zealand abalones, Haliotis species (Mollusca: Gastropoda)', *NZJ Marine and Freshwater Research*, 6: 4, 534 — 559
Atlantic cod	9125	AA - fishbase
Atlantic halibut	7300	AA - Roff (2007) Contributions of genomics to life-history theory. *Nat Rev Genet* **8** 116-125
Atlantic salmon	4745	AA - Longevity Records, Carey & Judge
Bighead carp	3285	Jennings (1988) Bighead Carp (*Hypophthalichthys nobilis*): biological synopsis. U.S. Fish Wild. Serv., Bio. Rep.**88**35pp
Brown tree frog	1825	Secretary, NZ Herpetological Society. On www.pondturtle.com/lfrog.html#Litoria
Brown trout	2190	AA -fishbase
Carrion crow	6935	AA - Monaghan and Metcalfe (2000) Genome size and longevity
Cave salamander	21170	AA - Snider & Bowler (1992) Longevity of Reptiles and Amphibians in North American Collections, Second Edition
Channel catfish	5840	AA - fishbase
Chicken	1825	Saunders Comprehensive Veterinary Dictionary 3rd Ed., Blood, Studdert & Gay. Elsevier
Chinese shrimp	730	Korea-US Aquaculture - www.lib.noaa.gov/retiredsites/korea/main_species/chinesefleshy.htm
Coho Salmon	1825	AA - fishbase
Collared flycatcher	2884	AA - Brommer et al. (2007), Exploring the genetics of aging in a wild passerine bird
Common blue damselfly	365	Cordero (1993) The effect of sex and age on survviorshi[of adult damselflies in the laboratory (Zygoptera: Coenagrioidae). *Odonatologica***23**, 1-12; Bots et al. (2010) Exposure to PFOS adversely affects the life-cycle of the damselfly, *Enallagma cyathigerum*. *Environmental Pollution* **158**, 901-905.

Cow	3650	Saunders Comprehensive Veterinary Dictionary 3rd Ed., Blood, Studdert & Gay. Elsevier
Danubian bleak	1825	Freshwater Fishes of Iran - Species accounts - Cyprinidae - Alburnus. Www.briancoad.com/species accounts/Alburnus.htm
Daphnia	80	Pietrzak et al (2010) Longevity of *Daphnia magna* males and females. *Hydrobiologia***643** 71-75
Eastern forktail damselfly	365	Dmitriew & Rowe (2005) Resource limitation, predation risj and compensatory growth in a damselfly. *Oecologia* 142 **150-154**
European common frog	5110	AA - Smirina (1994), Age determination and longevity in amphibians
Gibel carp	10950	AA - Longevity Records, Carey & Judge - Moyle, P.B., 1976. Inland Fishes of California. Berkeley, CA: University California Press.
Green emerald damselfly	365	De Block et al (2008) - Integrating life history and physiology to understand latitudinal size variation in a damselfly. *Ecography* **31** 115-123
Green turtle	27375	AA - Roger Conant et al. (1998), A Field Guide to Reptiles and Amphibians: Eastern and Central North America
Guppy	1825	AA - fishbase
House sparrow	8395	AA - Moller (2006), Sociality, age at first reproduction and senescence: comparative analyses of birds,
Jacky dragon	1460	Harlow & Taylor (2000) Reproductive ecology of the jacky dragon (*Amphibolurus muricatus*): an agamid lizard with temperature-dependent sex determination. *Austral Ecology***25** 640-652
Japanese quail	2190	AA - Ottinger (2001), Quail and other short-lived birds
Ladybird beetle	84	Agarwala et al (2008) Life history response of a predatory ladybird, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), to food stress. *Appl. Entomol. Zool.* **43** 183-189
Long-armed shrimp	1460	Mantel & Dudgeon (2005) Reproduction and sexual dimorphism of the palaemonid shrimp *Macrobrachium hainanese* in Hong Kong streams. *Journal of Crustacean Biology***25** 450-459.
Mink	3650	AA - Grizmek's Encyclopaedia of Mammals (1990)
Minnow	4745	AA - fishbase
Mouse	900	Saunders Comprehensive Veterinary Dictionary 3rd Ed., Blood, Studdert & Gay. Elsevier
Musk shrew	1095	AA - Longevity of Mammals in Captivity, from the living world collection. Weigl (2005)
Nile tilapia	3285	AA - fishbase
Olive flounder	4015	Yoneda et al (2007) Age validation and growth variability of Japanese flounder *Paralichthys*

		olivaceus off the Pacific coast of northern Japan. *Fisheries Science* **73** 585-592
Pacific abalone	2920	Poore, Gary C. B.(1972) 'Ecology of New Zealand abalones, Haliotis species (Mollusca: Gastropoda)', *NZJ Marine and Freshwater Research*, 6: 4, 534-559
Pig	3285	Saunders Comprehensive Veterinary Dictionary 3rd Ed., Blood, Studdert & Gay. Elsevier
Pyrenean salamander	9490	AA - Smirina (1994), Age determination and longevity in amphibians
Rabbit	1825	Saunders Comprehensive Veterinary Dictionary 3rd Ed., Blood, Studdert & Gay. Elsevier
Rainbow trout	4015	AA - fishbase
Rat	1460	Saunders Comprehensive Veterinary Dictionary 3rd Ed., Blood, Studdert & Gay. Elsevier
Red porgy	5475	Vaughan et al (1992) population characteristics of the red porgy, *Pagrus pagrus*, stock off the Carolinas. *Bulletin of Marine Science*,**50,** 1-20
Rhesus monkey	14600	AA - Bodkin et al (2003) Mortality and morbidity in laboratory-maintained Rhesus monkeys and effects of long-term dietary restriction. *J Gerontol A Biol Sci Med Sci*, **58**, 212-219
Rhinocerous auklet	3650	AA - BBL - Longevity Records of North American Birds
Rudd	6935	AA - fishbase
Sablefish	14600	AA - Cailliet et al. (2001) Age determination and validation studies of marine fishes: do deep-dwellers live longer?
Sheep	2190	Saunders Comprehensive Veterinary Dictionary 3rd Ed., Blood, Studdert & Gay. Elsevier
Song thrush	6460	AA - EURING list of longevity records for European birds
Spider (Long-jawed orb weaver)	365	Marczak & Richardson (2008) Growth and development rates in a riparian spider are altered by asynchrony between the timing and amount of a resource subsidy. *Oecologia* **156** 249-258
Squid	730	Recksiek & Frey (1978) Biological, oceanographic and acoustic aspects of the market squid *Loligo opalescens* Berry. Dept Fish and Game. *Fish Bulletin* **169** 185pp
Striped hamster	1935	Saunders Comprehensive Veterinary Dictionary 3rd Ed., Blood, Studdert & Gay. Elsevier
Three-spined stickleback	820	Inness & Metcalfe (2008) The impact of dietary restriction, intermittent feeding and compensatory growth on reproductive investment and lifespan in a short-lived fish. *Proc Royal Soc B* **275** 1703-1708
Tree swallow	4380	AA - BBL - Longevity Records of North American Birds
Vimba	1533	Ermolin and Shashulovskii (2006) On the results of *Vimba vimba* (Cypriniformes, Cyprinidae) introduction in the Volgograd Reservoir. *Voprosy Ikhtiologii* **46** 569-571
Wall-eyed pollock	5475	AA - fishbase
White-throated sparrow	3541	AA - Klimkiewicz & Futcher (1987) Longevity records of North American Birds: Coerbinae

		to Estrildidae
Zebra finch	5292	AA - Wang et al (2002) Vocal control neuron incorporation decreases with age in the adult zebra finch. *J Neurosci,* **22,** 10864-10870

Table A4.5 Moderators.

Descriptions of the moderators incorporated in the models. "Relative" means calculated as a percentage of the average longevity for each species (see Table A4.4).

Moderator	Meaning	Type	Scale
Intercept	Overall effect of *d*	Continuous	Absolute measures: + large restricted group - small restricted group Fitness: + higher fitness for restricted - lower fitness for restricted Slopes: + slower growth for restricted - faster growth for restricted
Type	Measurement type	Binary	+ weight - length
Degree DR	Degree of restriction	Continuous	+ more food - less food
Age	Relative age of restriction onset	Continuous	+ older - younger
Duration Restriction	Relative duration of restriction	Continuous	+longer - shorter
Proportion Realimentation	Proportion of duration of realimentation:restriction	Continuous	+longer - shorter
Farmed	Farmed or laboratory colony	Binary	+ True - False
Female	Female compared to mixed sex	Binary	+ True - False
Male	Male compared to mixed sex	Binary	+ True - False
Clutch Size	Restricted by clutch size manipulation	Binary	+ True - False
Intermittent Feeding	Restricted by intermittent feeding	Binary	+ True - False
Dilution	Restricted by dilution of diet	Binary	+ True - False
Ad libitum Realimentation	Restricted group fed *ad libitum* for realimentation	Binary	+True - False
Development	Compared to Activity	Binary	+True - False
Mortality	Compared to Activity	Binary	+True - False
Physiology	Compared to Activity	Binary	+True - False
Reproduction	Compared to Activity	Binary	+True - False
Social	Compared to Activity	Binary	+True - False

Table A4.6 Collinearity of moderators.

Results of correlation tests between moderators prior to modelling. Values reported are the Pearson's product-moment correlation. Only values of less than 0.5 were included in the full model to prevent the negative effects of collinearity (red values were excluded). DurRes = duration restriction, DurReal = duration realimentation, PropReal = proportion realimentation:restriction, AdLibCon = *ad libitum* control, IF = intermittent feeding, AdLibReal = *ad libitum* realimentation. For further explanation of moderators, see Table A4.5.

	Type	DegreeDR	Age	DurRes	DurReal	PropReal	Farmed	Sex	AdLibCon	ClutchSize	IF	Dilution
Type	1	-	-	-	-	-	-	-	-	-	-	-
DegreeDR	0.087	1	-	-	-	-	-	-	-	-	-	-
Age	0.053	-0.131	1	-	-	-	-	-	-	-	-	-
DurRes	-0.016	0.024	-0.045	1	-	-	-	-	-	-	-	-
DurReal	-0.048	0.105	-0.069	0.357	1	-	-	-	-	-	-	-
PropReal	-0.061	0.082	-0.038	-0.132	**0.646**	1	-	-	-	-	-	-
Farmed	0.152	0.306	-0.234	0.155	0.116	-0.08	1	-	-	-	-	-
Sex	NA	0.443	0.057	0.053	0.077	-0.026	0.223	1	-	-	-	-
AdLibCon	-0.114	-0.073	-0.427	-0.22	0.008	0.125	0.253	-0.029	1	-	-	-
ClutchSize	0.017	0.109	-0.144	-0.047	0.286	0.318	0.149	0.185	0.102	1	-	-
IF	-0.078	0.151	0.134	0.157	-0.051	-0.196	0.032	0.217	-0.139	-0.108	1	-
Dilution	0.006	0.173	0.059	-0.051	-0.069	-0.017	-0.152	0.162	0.068	-0.044	-0.072	1
AdLibReal	-0.128	-0.031	-0.484	-0.19	-0.058	0.153	0.161	-0.078	**0.854**	0.091	-0.099	0.061

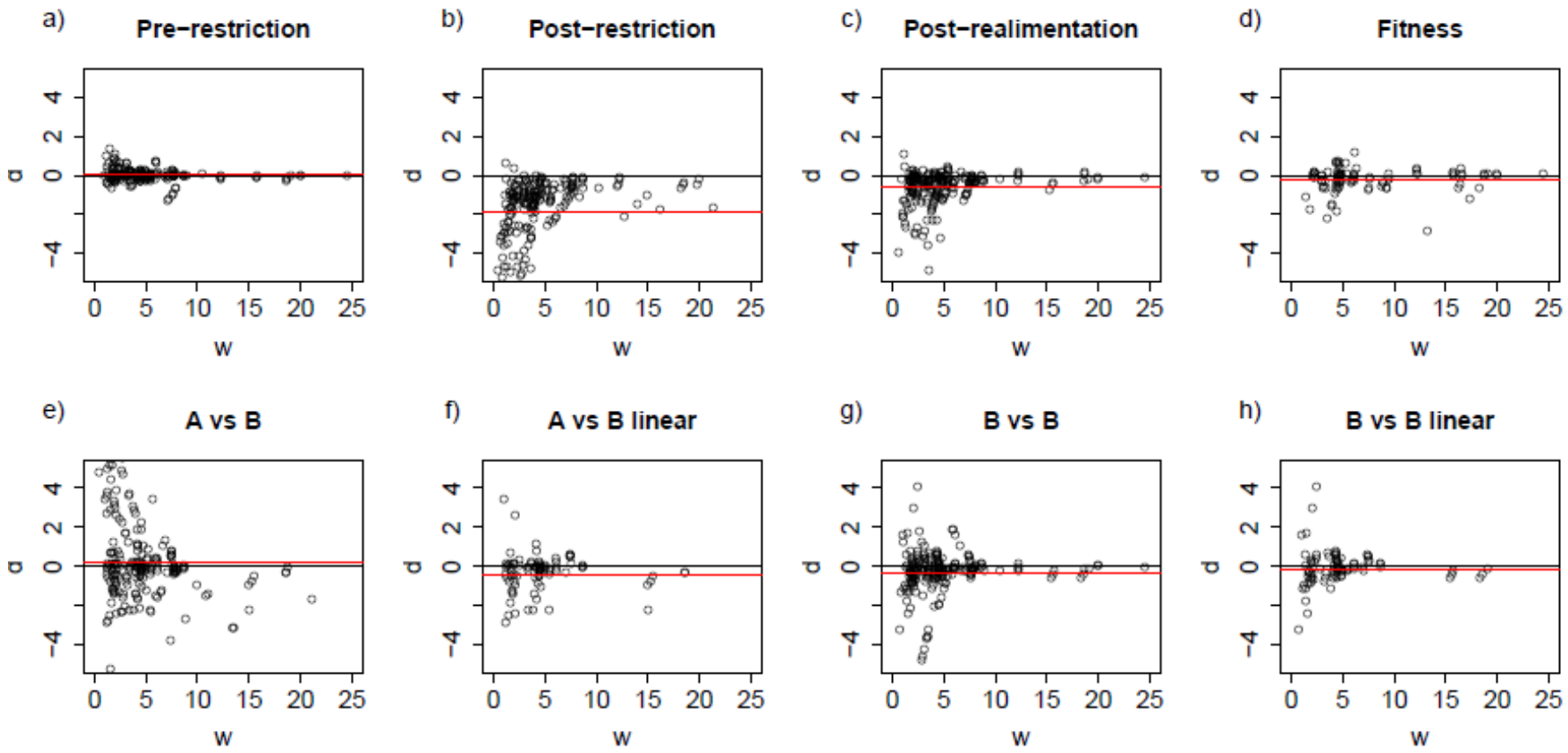

Figure A4.4 Fine-scale funnel plots.

Funnel plots showing the distribution of the effect size (*d*) extracted from each study plotted against the precision of the study (1/SE). This version includes only effect sizes between 5 and -5in order to allow easier viewing of symmetry. See Fig. 4.3 for further explanation.

Table A4.7 Pre-restriction size.

Statistical table of the LMM model used to analyse pre-restriction size. Data in bold are reported from the scaled best model, while other data are from the full model (see Methods). See Table A4.5 for description of moderators. *n* = 212.

	Estimate	SE	*df*	*t*-value	*p*
Intercept	**-0.042**	**0.041**	**124**	**-1.006**	**0.3164**
Farmed	**0.102**	**0.055**	**76**	**1.860**	**0.0667**
Type	0.039	0.041	119	0.954	0.3418
Degree DR	-0.011	0.056	119	-0.203	0.8392
Age	-0.009	0.058	119	-0.150	0.8806
Female	-0.003	0.082	119	-0.033	0.9735
Male	-0.028	0.079	119	-0.362	0.7183

Table A4.8 Post-restriction size.

Statistical table of the LMM model used to analyse post-restriction size. Data in bold are reported from the scaled best model, while other data are from the full model (see Methods). Asterisks denote moderators which reached statistical significance (α = 0.05). See Table A4.5 for description of moderators. *n* = 218.

	Estimate	SE	*df*	*t*-value	*p*	
Intercept	**-1.514**	**0.306**	**126**	**-4.946**	**<0.0001**	*****
Type	**-0.129**	**0.045**	**126**	**-2.853**	**0.0051**	*****
Degree DR	**1.293**	**0.119**	**126**	**10.819**	**<0.0001**	*****
Restriction Duration	**-1.087**	**0.192**	**126**	**-5.650**	**<0.0001**	*****
Farmed	**-0.800**	**0.398**	**76**	**-2.010**	**0.0480**	*****
Intermittent Feeding	**1.411**	**0.429**	**126**	**3.291**	**0.0013**	*****
Age	0.180	0.325	123	0.553	0.5812	
Female	-0.505	0.465	123	-1.086	0.2795	
Male	-0.658	0.465	123	-1.413	0.1601	
Clutch Size	0.446	0.730	74	0.611	0.5431	
Dilution	-0.485	1.116	74	-0.434	0.6653	

Table A4.9 Post-realimentation size.

Statistical table of the LMM model used to analyse post-realimentation size. Data in bold are reported from the scaled best model, while other data are from the full model (see Methods). Asterisks denote moderators which reached statistical significance (α = 0.05). See Table A4.5 for description of moderators. *n* = 226.

	Estimate	SE	*df*	t-*value*	*p*	
Intercept	**0.046**	**0.236**	**133**	**0.196**	**0.8450**	
Type	**-0.135**	**0.037**	**133**	**-3.654**	**0.0004**	*
Degree DR	**0.726**	**0.105**	**133**	**6.925**	**<0.0001**	*
Restriction Duration	**-0.368**	**0.102**	**133**	**-3.598**	**0.0005**	*
Proportion Realimentation	**0.387**	**0.064**	**133**	**6.043**	**<0.0001**	*
Clutch Size	**-0.510**	**0.367**	**76**	**-1.389**	**0.1688**	
***Ad libitum* Realimentation**	**-0.602**	**0.250**	**133**	**-2.408**	**0.0174**	*
Age	0.065	0.207	129	0.314	0.7542	
Farmed	-0.008	0.237	74	-0.034	0.9731	
Female	-0.244	0.254	129	-0.959	0.3391	
Male	-0.240	0.254	129	-0.943	0.3475	
Intermittent Feeding	0.059	0.284	129	0.208	0.8353	
Dilution	-0.457	0.597	74	-0.765	0.4469	

Table A4.10 Fitness.

Statistical table of the LMM model used to analyse fitness. Note that only direct fitness measurements were included, thus fitness trait reflects the effect of mortality in contrast to reproduction. Data in bold are reported from the scaled best model, while other data are from the full model (see Methods). Asterisks denote moderators which reached statistical significance ($\alpha = 0.05$). See Table A4.5 for description of moderators. $n = 94$.

	Estimate	SE	*df*	*t*-value	*p*	
Intercept	**-0.597**	**0.199**	**52**	**-3.005**	**0.0041**	*
Fitness Trait	**-0.291**	**0.130**	**52**	**-2.230**	**0.0301**	*
Degree DR	**0.172**	**0.106**	**52**	**1.626**	**0.1099**	
Proportion Realimentation	**0.316**	**0.030**	**52**	**10.421**	**<0.0001**	*
Farmed	**0.361**	**0.224**	**27**	**1.611**	**0.1189**	
Female	**0.452**	**0.202**	**52**	**2.237**	**0.0296**	*
Male	**0.158**	**0.198**	**52**	**0.799**	**0.4277**	
Age	-0.065	0.186	23	-0.350	0.7294	
Duration Realimentation	-0.185	0.198	51	-0.934	0.3548	
Clutch Size	0.051	0.344	23	0.148	0.8837	
Intermittent Feeding	0.332	0.302	23	1.099	0.2830	
Dilution	-0.082	0.510	23	-0.160	0.8742	

Table A4.11 Slopes A vs. B.

Statistical table of the LMM model used to analyse the slopes of the controls in period A against the slopes of the restricted group in period B (Fig. 4.2a). Data in bold are reported from the scaled best model, while other data are from the full model (see Methods). Asterisks denote moderators which reached statistical significance (α = 0.05). See Table A4.5 for description of moderators. *n* = 226.

	Estimate	SE	*df*	*t*-value	*p*	
Intercept	**0.282**	**0.307**	**119**	**0.920**	**0.3592**	
Type	**-0.268**	**0.047**	**119**	**-5.728**	**<0.0001**	*
Age	**0.722**	**0.384**	**119**	**1.880**	**0.0626**	
Duration Realimentation	**-0.616**	**0.169**	**119**	**-3.641**	**0.0004**	*
Clutch Size	**1.362**	**0.486**	**119**	**2.802**	**0.0059**	*
Intermittent Feeding	**1.152**	**0.443**	**119**	**2.598**	**0.0106**	*
Degree DR	0.043	0.124	114	0.352	0.7257	
Proportion Realimentation	-0.033	0.079	114	-0.420	0.6751	
Farmed	-0.295	0.678	75	-0.435	0.6651	
Female	-0.958	0.698	114	-1.372	0.1727	
Male	-0.983	0.699	114	-1.405	0.1627	
Dilution	-0.503	1.722	75	-0.292	0.7709	
Ad libitum Realimentation	0.147	0.625	114	0.235	0.8145	

Table A4.12 Slopes A vs. B linear.

Statistical table of the LMM model used to analyse the slopes of the controls in period A against the slopes of the restricted group in period B, where the controls showed linear growth throughout the experiment (Fig. 4.2b). Data in bold are reported from the scaled best model, while other data are from the full model (see Methods). Asterisks denote moderators which reached statistical significance ($\alpha = 0.05$). See Table A4.5 for description of moderators. $n = 87$.

	Estimate	SE	*df*	*t*-value	*p*	
Intercept	**-0.200**	**0.214**	**46**	**-0.936**	**0.3540**	
Type	**-0.275**	**0.088**	**46**	**-3.119**	**0.0031**	*
Degree DR	**-0.162**	**0.116**	**46**	**-1.400**	**0.1682**	
Proportion Realimentation	**-0.155**	**0.074**	**46**	**-2.089**	**0.0423**	*
Age	0.294	0.325	40	0.906	0.3704	
Restriction Duration	0.170	0.259	40	0.658	0.5140	
Farmed	0.322	0.357	25	0.904	0.3745	
Female	-0.628	0.451	40	-1.391	0.1719	
Male	-0.487	0.461	40	-1.058	0.2965	
Clutch Size	0.283	0.477	40	0.593	0.5566	
Intermittent Feeding	0.067	0.443	40	0.151	0.8807	
Dilution	-0.644	1.093	25	-0.589	0.5610	
Ad libitum Realimentation	0.057	0.557	25	0.102	0.9192	

Table A4.13 Slopes B vs. B.

Statistical table of the LMM model used to analyse the slopes of the controls in period B against the slopes of the restricted group in period B (Fig. 4.2c). Data in bold are reported from the scaled best model, while other data are from the full model (see Methods). Asterisks denote moderators which reached statistical significance (α = 0.05). See Table A4.5 for description of moderators. *n* = 226.

	Estimate	SE	*df*	*t*-value	*p*	
Intercept	**-0.565**	**0.161**	**120**	**-3.516**	**0.0006**	*
Type	**0.155**	**0.045**	**120**	**3.455**	**0.0008**	*
Duration Restriction	**-0.260**	**0.096**	**120**	**-2.704**	**0.0078**	*
Clutch Size	**0.773**	**0.237**	**120**	**3.264**	**0.0014**	*
Intermittent Feeding	**0.487**	**0.211**	**120**	**2.307**	**0.0228**	*
Degree DR	-0.011	0.107	114	-0.105	0.9163	
Age	0.231	0.182	114	1.265	0.2084	
Proportion Realimentation	-0.051	0.070	114	-0.718	0.4741	
Farmed	0.251	0.204	75	1.227	0.2237	
Female	-0.004	0.227	114	-0.018	0.9856	
Male	-0.132	0.226	114	-0.582	0.5619	
Dilution	0.095	0.510	75	0.186	0.8530	
Ad libitum Realimentation	0.282	0.255	114	1.105	0.2714	

Table A4.14 Slopes B vs. B linear.

Statistical table of the LMM model used to analyse the slopes of the controls in period B against the slopes of the restricted group in period B, where the controls showed linear growth throughout the experiment (Fig. 4.2d). Data in bold are reported from the scaled best model, while other data are from the full model (see Methods). Asterisks denote moderators which reached statistical significance ($\alpha = 0.05$). See Table A4.5 for description of moderators. $n = 87$.

	Estimate	SE	*df*	*t*-value	*p*	
Intercept	**-0.830**	**0.343**	**45**	**-2.421**	**0.0196**	*****
Type	**0.201**	**0.088**	**45**	**2.268**	**0.0282**	*****
Proportion Realimentation	**-0.131**	**0.072**	**45**	**-1.826**	**0.0746**	
Clutch Size	**0.790**	**0.273**	**45**	**2.893**	**0.0059**	*****
Intermittent Feeding	**0.413**	**0.223**	**45**	**1.851**	**0.0707**	
***Ad libitum* Realimentation**	**0.544**	**0.354**	**27**	**1.538**	**0.1356**	
Degree DR	-0.133	0.114	40	-1.171	0.2485	
Age	0.143	0.249	40	0.575	0.5686	
Restriction Duration	0.026	0.212	40	0.124	0.9017	
Farmed	0.042	0.264	25	0.160	0.8745	
Female	-0.342	0.356	40	-0.961	0.3423	
Male	-0.427	0.359	40	-1.190	0.2409	
Dilution	-1.168	0.939	25	-1.244	0.2250	

Chapter V: General Discussion

5.1 Summary of the major findings

Dietary restriction of *Litoria ewingii* tadpoles during early development resulted in compensatory growth in weight after realimentation (Chapter II). The final size of the food-restricted metamorphs actually exceeded that of the well-fed controls and the food-restricted group had a much higher rate of survival. Dietary restriction caused a delay in metamorphosis of approximately six days, which can be considered a negative fitness trait given the importance of minimising the larval period due to the cumulative risk of predation or pond drying (Wilbur, 1980). However, the rapid metamorphosis of the control group may have been at least partially due to the experimental apparatus, which may have simulated a pond-drying effect. Nevertheless, the restricted group was able to compensate in swimming speed after realimentation and were also faster to capture prey as frogs, indicating a superior survival advantage. No difference in hopping ability was detected between the groups, adding support to the growing evidence that hopping is a poor proxy for fitness. The radioimmunoassay conducted was unable to detect a difference between the corticosterone levels of the dietary treatment groups, although this result was most likely due to a lack of accuracy and reliability in the RIA procedure (Chapter III).

Immune activation with phytohemagglutinin (PHA) was found to be frequently lethal, although there was no indication of why some tadpoles survived and others did not (Chapter II). Mortality was not correlated with weight, stage, clutch or, most importantly, dietary treatment. Immune activation had little effect on the tadpoles which survived the period immediately following the injection, although there was a slight decline in the growth rate of

restricted tadpoles directly after immune activation. The larval period and fitness-related behavioural traits were not affected by immune activation, but there was a slight trend towards immune-activated frogs being smaller and lighter by the end of the experiment. This trend was, however, far surpassed by the difference between dietary treatment groups.

The meta-analyses were in agreement with the results of the *L. ewingii* experiment, concluding that compensatory growth is a widespread alternative growth strategy across a range of taxa (Chapter IV). Restricted animals were able to catch up to a size statistically indistinguishable from the controls, which in this thesis is defined as catch-up growth. This contrasted with the restricted group which was significantly larger in the frog experiment, also known as "over-compensation" (Chapter II). Meta-regression analysis was also able to confirm that the fitness of the previously restricted animals was negatively affected by the dietary treatment, with mortality being more severely affected than reproduction (Chapter IV). Unfortunately it is not possible to discern whether decreased fitness is due solely to the accumulated cellular damage of faster than optimal growth or whether this cost is simply a consequence of the period of restriction. A number of experimental methods were implicated in the strength of both the catch-up and compensatory growth. Unsurprisingly, longer periods of restriction and more severe diets (i.e. starvation) caused a greater difference between groups after restriction and were less likely to result in complete catch-up to the size as controls. Long periods of restriction did, however, make the occurrence of compensatory growth more likely.

Analysis of only the studies which were conducted during the linear growth phase of the control animals reached the same overall conclusions as the analyses which included all available data, regardless of control growth patterns. In a large-scale meta-analysis, size-dependent growth (i.e. slower growth as animals get larger) is therefore not as strong a confounding factor as previously alleged (Nicieza & Alvarez, 2009). However, future

compensatory growth studies should take care when analysing results because the reduced power of a single study may make the analysis of results more susceptible to the influence of size-dependent growth. In contrast, it appears that age-dependent growth may explain the negative impacts of compensatory growth. The rapid growth of the restricted groups upon refeeding was actually no different to the rate at which the controls had grown at a younger age. By growing at this rate at an older age, animals may be causing physiological damage because of the different developmental windows and changing tissue preservation hierarchy as animals age (Bize *et al.*, 2006). The measurement type can also result in significantly different conclusions, given that weight was more negatively influenced by restriction and length was more likely to reach the same final size as controls. This suggests that animals are more willing to sacrifice fat and muscle mass in place of skeletal growth. It is more difficult to compensate for skeletal losses due to finite developmental periods and body length is often of greater consequence than mass for sexual selection (McElligott *et al.*, 2001).

5.2 Limitations of the study

There were a number of aspects of each section of this thesis which could have been improved with the benefit of hindsight. In the *L. ewingii* experiment, the use of containers which may have simulated a pond-drying effect was a major confounding factor, both because it may have altered the growth trajectories of the control tadpoles and also because their apparent premature metamorphosis (compared to existing *L. ewingii* literature) may have been the cause of their high post-metamorphic mortality rate. Not only was the high control mortality a confusing result, it also meant that the unbalanced group sizes after metamorphosis prevented thorough investigation of the interactive effects of the diet and immune-activation treatments after metamorphosis. While the fitness-related behavioural traits and the overall size of the frogs were reasonable proxies for future fitness according to the literature (Van Buskirk & Saxer, 2001), monitoring the on-going growth patterns and

later reproductive success of these individuals would have been a very valuable contribution to the field. Not only could a long-term experiment confirm or deny the legitimacy of the fitness substitutes, on-going research would have been of great worth to the field of catch-up growth, since these frogs achieved "over-compensation" which is very rare (Ali *et al.*, 2003).

The immune activation treatment was also severely affected by the high mortality of PHA-injected tadpoles. The resulting sample sizes also prevented the corticosterone analysis of immune-activated tadpoles since there were insufficient numbers to allow sacrifice. I suggest that an alternative to PHA be used in future immune activation experiments in this species, because despite the high mortality, there were few long-term consequences from PHA-injection in the tadpoles which survived. It is likely that given a weaker dose of the immune activator, these trends would disappear altogether. The dosage used in the experiment was selected because a pilot study with a weaker dose of PHA did not even cause the swelling response which this lectin is typically used for (Gervasi & Foufopoulos, 2008). Likewise, lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria and another common immune-activator (Bonneaud *et al.*, 2003; Uller *et al.*, 2006), did not affect the heterocyte:lymphocyte ratio of *L. ewingii* tadpoles in an early pilot test. One potential alternative is real infection with either *Batrochochytrium dendrobatidis* or *Klebsiella pneumonia* to which this species is known to be susceptible (Schadich & Cole, 2010; Shaw *et al.*, 2010). However, this would involve the confounding factor of fighting an actual disease rather than merely activating the immune system, which was the primary goal of this experiment.

Without a doubt, the RIA is the section of this thesis which could have been most improved. Numerous failures to achieve desirable validation results meant that the overall result was not trustworthy, ultimately making the analysis redundant. However, as well as gaining a working experience of the technique, I was able to identify a number of areas which could

have been improved given sufficient time and a surplus of sample tadpoles. First amongst these was the column chromatography, which required a number of fine-tuning adjustments (described in the Chapter III Appendix). Inclusion of a functional purification step would hopefully have allowed for more clear-cut results without the interference of other proteins. This additional step may have been sufficient to increase the extraction efficiency to a more desirable 90% or higher. Better extraction would, in turn, allow for sample dilution prior to the RIA which means that the samples could have been run in triplicate, as was initially intended. Aside from technical accuracy, this experiment could have been improved by the inclusion of not only the immune-activated groups (as discussed earlier) but also an outside control group of "wild-caught" tadpoles to observe the effect of laboratory living on baseline corticosterone levels.

In contrast with the RIA, there are fewer improvements which are recommended for the meta-analysis section. Firstly, the study may have been improved by more intensive data collection methods, for example, contacting overseas archives or original authors for information. However, publication bias was not an obvious concern in the dataset and all available texts in New Zealand were consulted thanks to the Borrow Direct service offered by the Library Consortium of New Zealand through the University of Otago. Many papers were excluded for not including the required weight measures because these were essential to the basic analysis. Broadening the requirements to any measures (including fitness estimates alone) would have provided a larger dataset, although the interpretation of fitness data without confirmation of compensatory growth is even more difficult. Analysis of the actual protein content of the diets may have provided a more in-depth analysis of compensatory growth, given that protein-content rather than mere caloric restriction is believed to be primarily responsible for the life-extending qualities of prolonged dietary restriction

(Nakagawa *et al.*, submitted). Unfortunately there was insufficient time to undertake such a time-consuming task on top of the regular data collection and analysis.

5.3 Future directions for research

Based on the findings of this thesis, there are a number of potential avenues of new research which are worthy of further exploration. To begin with, the *L. ewingii* experiment ought to be repeated to verify that the results were not entirely dependent on the influence of a pond-drying effect caused by the small containers. With this in mind, further research could be conducted to verify the possible existence of a threshold weight for survival after metamorphosis. The implications of this research could be of use to conservationists. If such a threshold exists, it would be beneficial to the survival of metamorphs for external manipulation of environmental cues in order to prolong metamorphic climax. Delayed metamorphosis could be achieved by increasing the depth of natural ponds or by restricting the food supply to tadpoles early in the breeding season as a surrogate for the individual dietary restriction performed in this thesis. Since the restricted tadpoles outperformed the control animals in this study, it is possible that this intervention could be of benefit to the many campaigns trying to prevent the rapid decline of amphibian populations worldwide (Wright *et al.*, 2001).

The meta-analysis opens up a wide range of new angles from which to consider compensatory growth. When indirect fitness measures were considered alongside direct measurements (Chapter IV Appendix), the overall conclusion was that compensatory growth had little effect on fitness (at least, no consistent effects on fitness). Not only does this indicate that more research needs to be done on which traits are really appropriate fitness substitutes, it suggest that compensatory growth does not affect (or possibly even enhances) traits in the areas of activity, development, physiology and social behaviour. Thus, there could be even greater benefits to compensatory growth than attaining the same adult size as

controls. A further meta-analysis could be conducted using the same criteria as this thesis but limited to pre-natal growth. Although there are a number of human medical meta-analyses of prenatal dietary restriction (for example, Huxley *et al.*, 2000), a pre-natal study which broadened its perspective to include as many taxa as possible and a wide-range of fitness outcomes (not just coronary disease) would be very interesting to compare with the post-natal effects catalogued here.

In terms of commercial benefits, compensatory growth has already been used to produce exceptionally tender pork due to the protein degradation that occurs following accelerated growth (Therkildsen *et al.*, 2002). Compensatory growth also causes Indian major carp, *Labeo rohita*, to invest in muscle mass by greater protein production than fat deposition, which is preferred by aquaculturists (Sardari *et al.*, 2008).Dietary restriction has been used to cut feeding costs for pregnant cows during the second trimester because the same final weight can be attained by parturition without affecting the weight of calves (Freetly *et al.*, 2000). Dietary restriction also reduces the cost of employing farm workers to feed animals. For example, whitefish, *Coregonus lavaretus*, farmers were able to forego expensive weekend feedings without loss of saleable fish mass (Kankanen & Pirhonen, 2009). Based on the evidence of the meta-analyses, it appears that compensatory growth is a widely applicable and very useful agricultural technique, since it does not appear to affect minor fitness traits. However, if animals are destined for breeding or if a long working life is desirable, the results of this meta-analysis suggest that paying the extra feed costs are worthwhile to prevent negative costs to mortality or reproductive output. A meta-analysis devoted to purely agricultural outcomes would be of great consequence to future agricultural practices.

5.4 Overall contribution of the study

In summary, this thesis has had a number of outcomes, which could be considered contributions to the scientific field. The *L. ewingii* experiment clarified that the effects of

early dietary restriction do extend beyond metamorphosis in an amphibian. The superior performance of the restricted frogs also suggests that the adult body re-organisation at metamorphosis did potentially aid the restricted group in overcoming their early setback, as speculated by Metcalfe & Monaghan (2001). The extreme flexibility of amphibian development is the most likely reason that these tadpoles were able to show results contrary to the expectations of compensatory growth. As such, this study promotes further amphibian research in this field, in order to discover the extremes of compensatory growth. The consequences of dietary restriction seemed to far outweigh the long-term costs of immune activation, except for the tadpoles which died immediately from PHA injection. The minor differences between immune treatments in frogs which survived the injections suggest that a weaker dose of PHA to promote immediate survival would have led to even less distinction between immune treatment groups.

Furthermore, this experiment generated a novel fitness-related behavioural test in the form of the feeding latency protocol. Since the common test of hopping ability is becoming more and more dubious as a fitness predictor (Stamper *et al.,* 2008), the feeding latency method could be of use to herpetologists in the future. As well as confirming the fitness expectations of the frogs based on size, a personal observation of this technique is that capturing *Drosophila* was an excellent test of the co-ordination skills of the frogs. Weak performers with higher latencies often missed prey due to misjudged jumping distance or timing. I hypothesise that this test is likely to correlate with actual prey-capture performance in the wild, and potentially also predator avoidance.

The contribution of this thesis to the field of compensatory growth is not only additional support from a neglected taxonomic class (Chapter II), but the first quantitative analysis of compensatory growth in a wide range of taxa (Chapter IV). Using a meta-regression analysis, I was able to not only confirm the primary expectations of compensatory and catch-up growth

(faster than optimal growth to attain same size as controls at a cost to fitness), but was also able to identify a number of experimental methods which influence this outcome. These confirmatory results were based on intermediate levels of all variables included, however severe values for the degree of restriction and the duration of restriction are more likely to result in extreme differences between treatment groups and an inability to catch-up. These moderators also made it possible to identify that intermittent feeding and clutch size manipulation experiments are the techniques least consistent with the compensatory growth paradigm and, therefore, should perhaps be reconsidered for future compensatory growth research.

Nicieza and Alvarez (2009) sought to reduce the rate of false detection in compensatory growth studies by drawing attention to a number of statistical and biological impediments to data analysis. They argue that an animal's preferential allocation of energy to immediately restoring depleted fat reserves at realimentation may cause over-estimation of compensatory growth when size is measured as mass rather than skeletal growth. My meta-analysis did indeed detect a difference between weight and length responses to dietary treatment; however, the effect size between the two units of measurement was very small. Measurement type did not have a strong effect on the overall outcome, which is reassuring for researchers working with animals which are not easily measured in both dimensions, for example, weights of flying insects.

The other concern of Nicieza & Alvarez (2009) was in regards to size-dependent growth, where as controls get larger, their growth slows and, therefore, the growth comparison with the smaller, restricted group is unfair. This issue was also found to be a minor concern, given that both linear growth slope analyses (i.e. no size-dependent growth in controls) drew the same conclusions as their complete (non-linear included) counterparts. Instead it seems that age-dependent growth may be a stronger influence. The potential influence of post-natal

developmental windows in compensatory growth further unites this field with genetic and human health research (Gardner *et al.*, 2009). It is recommended that future researchers do take fat allocation and size-dependent growth into consideration when analysing the results of their research (using the recommended statistical techniques encouraged by Nicieza & Alvarez, 2009); however, with a sample size as large as this meta-analysis, these confounding factors were insufficient to change the overall outcome.

Importantly, this thesis was able to clarify the distinction between catch-up growth and compensatory growth, which are often used interchangeably in the literature. Although both are possible responses to the same dietary treatment and are not mutually exclusive, the fitness outcomes expected with respect to each outcome are quite different. Fitness consequences of compensatory growth are the result of accumulated cellular damage from faster than optimal growth. It is this type of cost which is involved in the trade-off which makes compensatory growth an alternative growth strategy (as opposed to "optimal" growth becoming faster and faster). In contrast, the consequences of catch-up growth are much more benign, because animals which achieve the same size as well-fed conspecifics are likely to have increased fitness, due to size-related mortality and sexual selection. In a sense, compensatory growth is the price food-restricted animals have to pay to enjoy the benefits of catch-up growth.

By examining compensatory growth in a neglected taxonomic class, as well as performing an all-encompassing quantitative analysis, this thesis provides a multi-layered perspective of a popular, contemporary topic in life-history theory. The applications of this field of research are many and varied: from human pre-natal epidemiology to economic gains in agriculture to evolutionary theory on body size. The novel and thought-provoking results of this thesis demonstrate how much still remains to be learned about compensatory growth and have the potential to open a wide variety of avenues for future research.

5.5 REFERENCES

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