Aging in Basal Metazoans – A Biodemographic Approach

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ZUSAMMENFASSUNG

Basale Metazoa besitzen eine große Anzahl vielfältiger Lebenszyklen, inklusive klonaler Fortpflanzung, woraus sich eine einzigartige Möglichkeit ergibt, die Evolution der Alterung von einem biodemografischen Standpunkt aus zu untersuchen. Dieses Dissertationsprojekt zielt darauf, die Diversität der Alterungsmuster zwischen und innerhalb Spezies auf dem Genet- und Ramet-Level durch Vergleiche alterungsspezifischer Merkmale wie Überlebenszeit und Reproduktion in experimentellen Laborstudien und durch die Analyse demografischer Daten vielerlei basaler Metazoen zu beleuchten. Zum ersten Mal überhaupt bei dieser Art konnten wir alterungsspezifisches Überleben, Größe und Reproduktion in einem Langzeit-Laborexperiment mit *Eleutheria dichotoma* (Cnidaria: Hydrozoa), einem metagenetischen marinen Hydrozoon mit einer halbsessilen Meduse, unter konstanten Bedingungen für die Polypen und Medusenstadien eines Klons (Genets) messen. Etablierte Polypenkolonien zeigten fast keine Mortalität innerhalb von mehr als 3.5 Beobachtungsjahren. Dagegen war die Larven- und Primärpolypenmortalität relativ hoch, was ein negatives Seneszenz-Muster auf dem Polypenkolonie-Ramet-Level andeutet. Die demografischen Merkmale der isogenen Medusen-Ramets unterschieden sich substantiell dazu, indem sie buckelartige Kurven („Hump shape senescence“) hinsichtlich des Überlebens und beider Reproduktionsmodi zeigten. Medusengröße war nicht mit dem Überleben korreliert und wir konnten keine Heritabilität der Lebensspanne oder des Reproduktionsoutputs feststellen. Hervorzuheben war ein signifikanter Trend zu einem qualitativen Abfall des Überlebens und beider Reproduktionsmodi mit der Aufeinanderfolge vegetativer Medusengenerationen. Wir schlussfolgern, dass *E. dichotoma*-Genets negative Seneszenz aufweisen. In weiteren Laborexperimenten testeten wir die Ressourcenverteilungsflexibilität eines anderen Hydrozoons, eines nicht-alternden und rein vegetativ reproduzierenden Süßwasserpolypen-Stamms von *Hydra magnipapillata*. Wir untersuchten die individuelle phänotypische Variation der isogenen *Hydra* Polypen unter konstanten Bedingungen und *Hydra*‘s phänotypische Plastizität in Bezug auf verschiedene Umwelteinflüsse, so wie Temperaturgradienten, Hunger oder Bisektion. *Hydra* Polypen zeigten hochvariable und nicht vererbliche Knospungsphänotypen unter konstanten Bedingungen, was auf einen zufälligen Phänotypen-Generierungs-Prozess hindeutet. Umweltstress kann hormetische Reaktionen in *Hydra* auslösen, ohne dass direkte Kosten dafür gefunden werden konnten. Dies zeigt, dass variable und fluktuierende Umwelteinflüsse sogar von Vorteil für *Hydra* sein können.

Schlagworte: Basale Metazoa, Alterung, Biodemografie

ABSTRACT

Basal metazoans show large variations in life-cycle patterns, including clonal propagation modes, offering unique opportunities to study the evolution of aging from a biodemographic point of view. Comparing age-specific life history traits such as survival and reproduction in experimental laboratory studies and analyzing demographic data across various basal metazoans at both genet and ramet levels this dissertation project aims to shed light on the different resource allocation strategies and diversity of aging patterns across and within species. For the first time ever in this species, we measured age-specific survival, size and reproduction in a longitudinal laboratory experiment with *Eleutheria dichotoma* (Cnidaria: Hydrozoa), a metagenetic marine hydrozoan with a crawling medusa, under constant conditions for both polyp and medusa stages of one clone (i.e. genet). Established polyp colonies suffered almost no mortality at all within more than 3.5 years of observation whereas larva and primary polyp mortality was rather high, pointing towards a negative senescence pattern at the polyp colony ramet level. Demographic traits of isogenic medusae differed substantially from polyp colonies, exhibiting hump shaped trajectories in survival and both reproduction modes, suggesting a "hump shape senescence" on the medusa ramet level. Medusa size was not correlated with survival and no heritability of lifespan or reproductive output could be found, indicating a stochastic origin of generally high trait variability in medusae. Remarkable was a significant trend towards a qualitative decline in survival and both reproduction outputs with succession of vegetative medusa generations. We reason that the overall aging pattern of *E. dichotoma* genets is of a negative senescent type. In further sets of laboratory experiments, we tested the resource allocation flexibility of another hydrozoan, a non-senescing and purely asexually reproducing freshwater *Hydra* strain (*Hydra magnipapillata).* We examined individual phenotypic variation of isogenic *Hydra* polyps under constant conditions and *Hydra*'s phenotypic plasticity in response to various environmental challenges such as temperature gradients, hunger and bisection. We recorded budding rates, size and starvation survival as indicators to changes in the allocation of resources to asexual reproduction and maintenance. *Hydra* polyps showed highly variable, non-heritable budding phenotypes under constant conditions, hinting towards a random phenotype generation process. Environmental stresses triggered hormetic responses in *Hydra* without any detectable trade-off costs, showing that variable stressful and fluctuating environments can be salutary for *Hydra*.

Key words: basal Metazoa, aging, biodemography

CONTENTS

GENERAL INTRODUCTION

AGING AND BIODEMOGRAPHY

What is aging and how can it be measured?

Aging is intrinsically tied to life and questioned as a mysterious process since time immemorial. But what exactly do we mean by ‘aging’? Across the tree of life (ToL, fig. 1) organisms are not only highly diverse in terms of their body structures, bauplan, behaviour, sizes and genes, but also in terms of their aging patterns (Baudisch and Vaupel 2012; Baudisch et al. 2013; Jones et al. 2014). There are extremely long lived and short lived species, semelparous species which die soon after reproduction and species which do not seem to age, or more specifically senesce, at all, like the hydrozoan freshwater polyp *Hydra* (Martinez 1998; Jones et al. 2014; Schaible et al. in preparation for submission). The definitions of aging and senescence are often confused. Aging is more than just the familiar decline in physiological functioning with age that negatively affects the ability to survive and/or to reproduce, which is more precisely termed senescence (*derived from the Latin word senex, meaning "old man" or "old age" or "advanced in age“). Aging is most clearly defined and measurable using a demographic approach, whereby aging shall be a broader generic term for: “variation in functioning with age, for the better or worse” (Baudisch 2008; Baudisch and Vaupel 2012). Thus aging, in its most basic and abstract sense, is merely “change over time”. Furthermore, in contradiction to the common dogma in gerontology and life-history science that mortality starts to rise after the age of reproductive maturity, there are, next to senescence, even aging patterns which can be named negligible, non- and negative senescence (Vaupel et al. 2004). Following and modified after Vaupel et al., aging

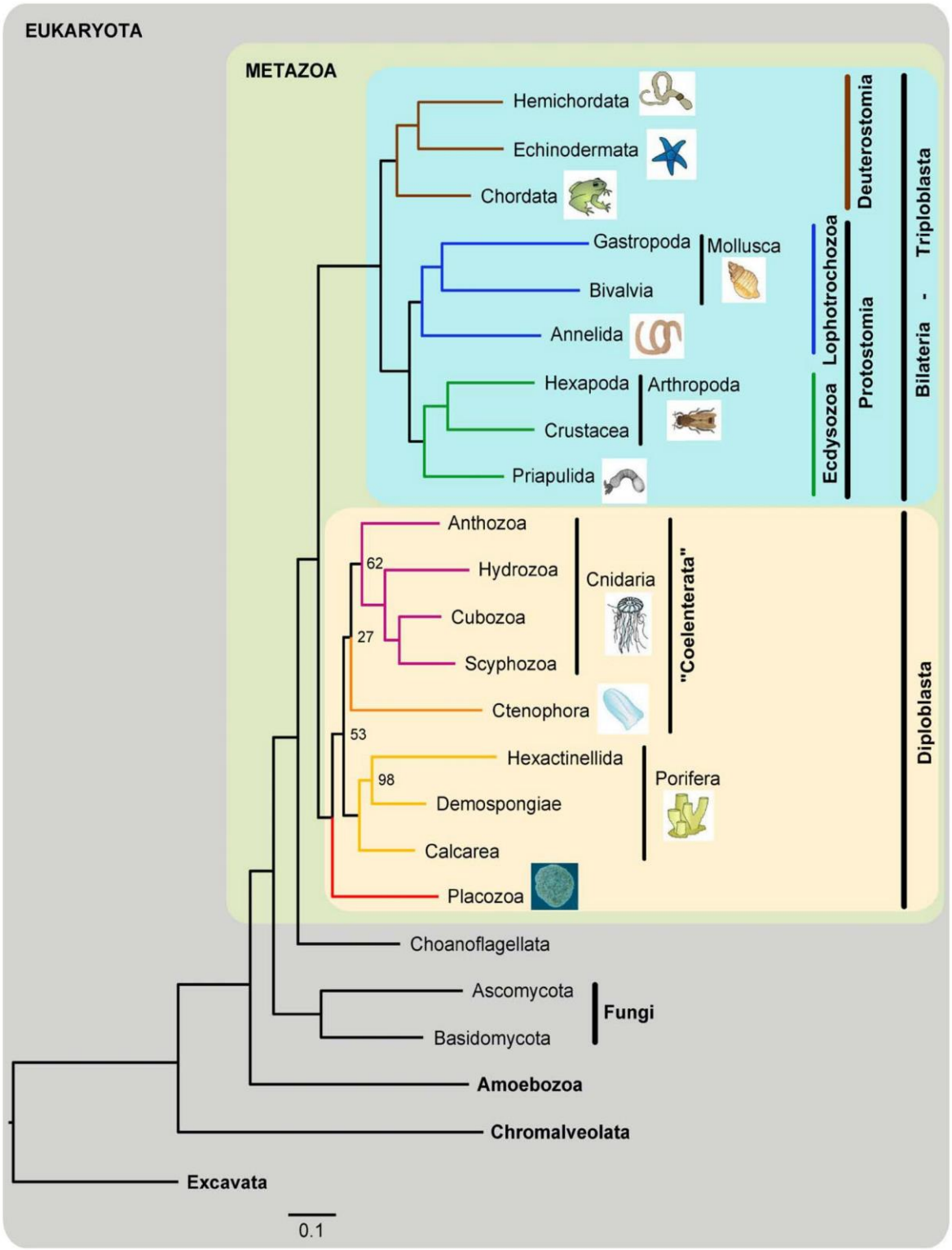

Figure 1. Maximum Likelihood Phylogenetic Tree of Metazoan Relationships (Schierwater et al. 2009)

can thus be fragmented into three different patterns, described in a demographic understanding (Vaupel et al. 2004; Baudisch 2008; Baudisch 2011):

- **Senescence:** *Age-related changes in an organism that adversely affect its vitality and functions, but most importantly increase its mortality rate and/or decrease its fertility as a function of time*

- **Non-senescence (a.k.a. negligible senescence):** *Death rates stay rather constant with age, no measurable reductions in reproductive capability with age, or measurable functional decline with age*

- **Negative senescence:** *a decline in mortality with age after reproductive maturity, generally accompanied by an increase in fertility*

It is important to note, that negative or non-senescence do not imply that some species have acquired individual immortality - every known life form can still die from intrinsic factors or be killed by extrinsic (environmental) influences, such as heat or predation. It is meant in an actuarial sense that the risk of dying, however high it may be for a specific species, does not increase with age.

I believe that the biodemographic approach is most promising to understand more about the evolutionary pathways of aging across the tree of life. Biodemography combines methods used in demography, which studies population dynamics and structure, with evolutionary biology, which is concerned with the origin, descent, multiplication and diversity of species over time, coming up with a research path on how evolution shapes age-specific trajectories of life-history parameters such as mortality, fertility, and growth (Metcalf and Pavard 2007). Biodemographic methods include gathering and analysing age- or size-specific survival,

growth and ideally also reproductive data of individuals from populations of various species in longitudinal laboratory or field studies, supplemented by additional measurements of size and other physiological traits, if possible. However, there is no generally agreed upon measure of senescence and the most frequently used demographic approach is to look at the change in mortality with age. This is a simple and widely accepted working definition (Finch 1990). But since both mortality and fertility are closely linked and crucial for the fitness and natural selection of each species, an ultimate measure of senescence should include both survival and reproduction (Baudisch 2008).

Evolutionary theories of aging

The evolutionary origin of senescence remains a fundamental unsolved problem in biology. At first glance evolution should tend to eradicate senescence because senescence reduces survival and reproduction. Several evolutionary theories of aging have been developed aiming to explain why senescence nevertheless evolved (Weismann 1891; Medawar 1952; Williams 1957; Hamilton 1966; Kirkwood 1977), but none of them can explain the diversity of aging patterns, including non- and negative senescence, that we find across the tree of life (Martinez 2002; Vaupel et al. 2004; Baudisch and Vaupel 2012; Jones et al. 2014).

August Weismann (1891) was the first biologist of the evolutionary era to advance a theory of senescence in his broad, but also contradictory and disputed "Essays upon Heredity". He believed that "death is not a primary necessity, but that it has been secondarily acquired as an adaptation…that life is endowed with a fixed duration, not because it is contrary to its nature to be unlimited, but because the unlimited existence of individuals would be a luxury without any corresponding advantage." But he also stated, that death is by no means an attribute of all organisms and elaborated on the potentiality of unending life in 'low organisms' like Amoebae. Weismann thought that such animals are too simply

constructed for any deterioration to take place within them. Later on, his explanations were heavily criticized as being incomplete and circular (Medawar 1952; Comfort 1954; Comfort 1956; Kirkwood 1977). The main critique concerned Weismann's statements, that death and "'ageing' is a mechanism for ridding a population of old and worn-out individuals, who would otherwise compete for resources with younger and fitter ones" (Kirkwood 1977). This was indeed regarded as circular since it assumes initially what it purports to explain (wear-out and 'ageing'), without explaining the reason for the wear-out with age. On the other hand, Weismann also concluded, following his own remarks on on the origin and necessity of death, "that the organism did not finally cease to renew the worn-out cell material because the nature of the cells did not permit them to multiply indefinitely, but because the power of multiplying indefinitely was lost when it ceased to be of use." Although Weisman did not state it particularly, these thoughts, to my understanding, already imply a natural selection of death and senescence in conjunction with a declining selection pressure on longevity with age due to the declining fertility output with age in a theoretical population of 'potentially immortal organisms' under a constant (external) mortality regime, which was well explained by Sir Peter B. Medawar (1952) in his test tube example to criticize Weismann's ideas. In conclusion, it is tempting to say, as Medawar commented on Weismann: "This is all a great muddle, but there is certainly some truth in it…." - a statement which might apply to all aging theories to date, as we shall see.

Apart from the inconsistent wear-out assumption, Weismann's theory is subject to a number of other criticisms (Williams 1957), namely: 1) the problematic nature of identifying senescence with mechanical wear 2) the extreme rarity of finding decrepit individuals in the wild in natural populations of any species and 3) the failure to uncover any death-mechanism or evidence for an adaptive and naturally selected senescence programme during

many decades of gerontological research and 4) the difficulties in understanding how such a programme could be produced by natural selection.

Further theories followed in an attempt to solve the inconsistencies. In 1952, Medawar proposed that aging is basically a matter of neglect. His theory, which is referred to as *Mutation Accumulation Theory*, states that senescence results from a decline in selection pressure with age for traits that maintain viability. Older ages matter less to life-time reproductive success because only a small proportion of all individuals reaches old ages due to external hazards such as diseases, accidents or predation. Thus detrimental mutations showing an effect only late in life accumulate via natural selection and cause physiological decline and damage with increasing age, which is usually associated with senescence. There is no selection pressure for genes which would promote increased longevities or immortality of individuals.

George C. Williams proposed another theory in 1957, called *Antagonistic Pleiotropy Hypothesis*. Pleiotropy means basically that one gene can have two or more effects on the phenotype. Antagonistic pleiotropy imposes that one of these effects is beneficial and another is detrimental to an organism. Williams referred this mechanism to genes that offer benefits in early life, but exact costs later in life. He assumed that enhanced early fertility can be selected for even if it includes a price tag in form of an earlier death or physiological decline with age. Senescence, accordingly, evolved basically as a side effect of beneficial fitness traits during younger ages. Williams also stated, that his theory predicted senescence as an evolved characteristic of the soma – just as Weismann did already in 1891 and Kirkwood later in 1977, too - and that senescence should not be present in organisms without a clear germ-soma segregation. However, Williams differentiated, that while clones, i.e. genets, should be non-senescent, asexually reproducing individuals of a clone, i.e. modules/ramets, should show senescence because they could be regarded as soma.

In 1966 William D. Hamilton claimed that senescence is an inevitable outcome of evolution because the force of selection declines with age, implying that later ages become unimportant to evolution. By combining insights from Medawar and Williams with concepts and models of population dynamics (Lotka 1924) he showed theoretically by mathematical modeling that ‘‘senescence is an inevitable outcome of evolution’’ and ‘‘cannot be avoided by any conceivable organism’’ - survival and fertility have to decline with age. This dogma became established among gerontologists until Vaupel et al. introduced the concept of negative senescence in 2004 and Annette Baudisch disproved Hamilton’s claim in her article on “Hamilton’s indicators of the force of selection” (Baudisch 2005). Baudisch concludes that “life histories are likely to be shaped largely by optimization rather than by a burden of deleterious mutations, at least over ages where the bulk of life-time reproduction is realized” (Baudisch 2008). Baudisch’s models show that, theoretically, senescence is not an inherent part of life and that optimal life histories can cover a broad range of senescent and non-/negative senescent strategies, just as we can truly observe them in real life scenarios (Jones et al. 2014).

Another prominent mainstream theory of aging is the *Disposable soma theory*, proposed in 1977 by Thomas B. L. Kirkwood. The term disposable soma came in analogy with disposable products. Why spend energy in making something durable, if it will only be used and needed for a limited amount of time? Each organism must budget the amount of energy available to its body. Energy resources must be allocated for metabolism, reproduction, growth, repair and maintenance. With a finite supply of food or energy, compromises and trade-offs occur within the resource allocation system, which finally result in physiological deterioration with age. Kirkwood conjectured that the critical part of an organism that must survive is the genetic code, which contains all information necessary to ensure the persistence of a lineage. The germ line acts, therefore, as the keeper of the genetic code of each respective species and

thus has to be protected from any damage and remains basically immortal, whereas the rest of the body cells, the soma, can be replaced. The soma can be understood as the vehicle for carrying and transporting the genetic code over generations, which is consistent with the gene-centered view of evolution proposed by Dawkins in "The Selfish Gene" (Dawkins 1976). In his theory, Kirkwood assumes that the costs required for the continuous repair of the soma are too high in perpetuity; therefore evolution transposes the protection of the germ line against senescence of the soma, "the evolutionary optimum leads directly to senescence" (Kirkwood and Rose 1991). According to Kirkwood's theory, somatic senescence evolved because of the accumulation of unrepaired somatic defects with age. The problem with the *Disposable soma theory* is, though, that it only applies to organisms with a sequestered germ-line, like most bilaterians, but not to clonal organisms without a germ-soma distinction, where the concept becomes blurry, as Kirkwood admitted himself (Kirkwood and Rose 1991).

To date, as noted above, none of the evolutionary theories of aging can describe the diversity of aging patterns found across the tree of life (Jones et al. 2014) and further theoretical research is deeply in need. The current published literature is full of empirical results that challenge and contradict the established theories. For example, it has been suggested for asexual metazoans via experimental data on a marine oligochaete and a rhabdocoel, that the evolution of somatic differentiation, preceding germ-line sequestration, is the necessary condition for the evolution of senescence (Martinez and Levinton 1992; Martinez 2002). Other studies suggest that senescence exists also in unicellular organisms like bacteria, contrasting previous aging concepts even more (Stewart et al. 2005; Wang et al. 2010). And then there is the case of the non-senescent *Hydra* polyp (fig. 2), a multicellular and mostly vegetatively reproducing basal metazoan without germ-line sequestration, exhibiting constant low mortality rates on its polyp-ramet level with only one exception in *Hydra oligactis*,

which relates to an environmentally triggered semelparous like mortality response when temperature drops (Martinez 1998; Yoshida et al. 2006; Jones et al. 2014; Schaible et al. in preparation for submission). In light of the vast diversity and complexity of life cycles and life stages across basal metazoans, plants and fungi, and their potential for clonal reproduction, theoretical modeling of all possible ways of their aging pattern evolution becomes seriously complicated, if not impossible - not to mention the huge gaps in knowledge regarding the diversity of living beings still existing today. Helpful are more empirical demographic studies of such organisms, including bacteria, which could serve as a cornerstone to modified theories on aging. In this thesis, I chose to focus on one of the least studied groups regarding their aging patterns: the basal metazoans.

BASAL METAZOANS

As the term "basal metazoans" is not very well-defined in general (Collins et al. 2005), I refer it from here on to animals from clades at the base of the metazoan (multicellular animal) evolutionary tree (fig. 1), i.e. Placozoa (*Trichoplax spp.*), Porifera (sponges), Cnidaria (jellyfish and polyps) and Ctenophora (comb jellies). Excluded from the diploblastic basal metazoans, having only two germ layers (ecto- and endoderm), are by this definition all higher metazoans with three germ layers (ecto-, meso- and endoderm), namely the triploblastic Bilateria, and the enigmatical Myxozoa, since their phylogenetic position and relationship, and whether they have diploblastic or triploblastic ancestors, are not so clear to date (Petralia et al. 2014). Hence, according to the definition I apply, all basal metazoans can also be called diploblasts (Schierwater et al. 2009), which does not have the potentially misleading term ‘basal’ in it, since species of basal metazoans living today can be as much derived as today’s species of bilaterians (Collins et al. 2005).

Most basal metazoans have complex life cycles with various life stages and are capable of both sexual and asexual reproduction (we use the terms asexual and vegetative as synonyms, see (Schierwater and Hauenschild 1990) for a precise definition of asexual versus sexual reproduction which I apply throughout this thesis). Hence, they do not exhibit a clear germ-line sequestration in a classical sense of the term in contrast to most higher metazoans (see (Weismann 1891; Buss 1987; Finch 1990; Martinez and Levinton 1992; Martinez 2002) for more on the concept of germ-soma segregation). When thinking about aging in basal metazoans, it is crucial to consider the organizational level of the individual one is looking at. Since asexual propagation modes are prevalent and commonly distributed across the lower tree of life, it is important to always distinguish between the genet (i.e. clone) and the ramet (i.e. module) level in clonal populations (Harper 1981; Silander 1985; Karlson 1988; Karlson 1991; Martinez 2002). How does the additional dimension of asexual reproduction affect the aging patterns of both ramets and genets? What are the differences and variations between these levels, and what kind of aging diversity can we expect? These are core issues I want to raise and tackle with this thesis.

Information on this subject is very scant throughout the literature and the genet/ramet distinction is most often completely neglected in aging studies about clonal animals, which complicates correct understanding and interpretations even further. Just few thorough reviews elaborating on the genet/clone/colony versus ramet/module senescence stand out (Orive 1995; Gardner and Mangel 1997; Tanner 2001; Martinez 2002; Skold and Obst 2011; Arnaud-Haond et al. 2012), though all tend to remain tangled and blurry in their conclusions about the potentially distinct demographic aging patterns of genets and ramet stages.

GOALS OF THIS THESIS

Since the established theories and concepts of aging become very unclear when it comes to clonal organisms, one of the main goals of this thesis is to raise this awareness and describe the possibilities and potential differences between the demographic aging patterns of the genet and ramet levels. The diversity of aging across the tree of life has just begun to be described and undermined by quantitative demographic data (Jones et al. 2014). Large gaps need to be filled both theoretically and experimentally and it is time to fit the clonal aging puzzle piece into the aging diversity assemblage. Empirical demographic aging data are especially scant at the root of the metazoan tree of life, where we find extremely plastic animals regarding their life cycles and regeneration abilities. Metagenetic basal metazoans like most Medusozoa (Kayal et al. 2013), including one of our experimental organisms: *Eleutheria dichotoma* (fig.3), possess parallel living life stages which can be simultaneously potent of reproducing either sexually or asexually or both. *Hydra*, on the other hand, is a Medusozoan which lost the medusa stage (Kayal et al. 2013) and was left with the polyp stage alone (fig. 2). Hence, when thinking about aging on the genet versus the ramet level, each species has to be evaluated differently and potentially parallel ramet life stages have to be considered as well.

Figure 2. *Hydra magnipapillata* polyp

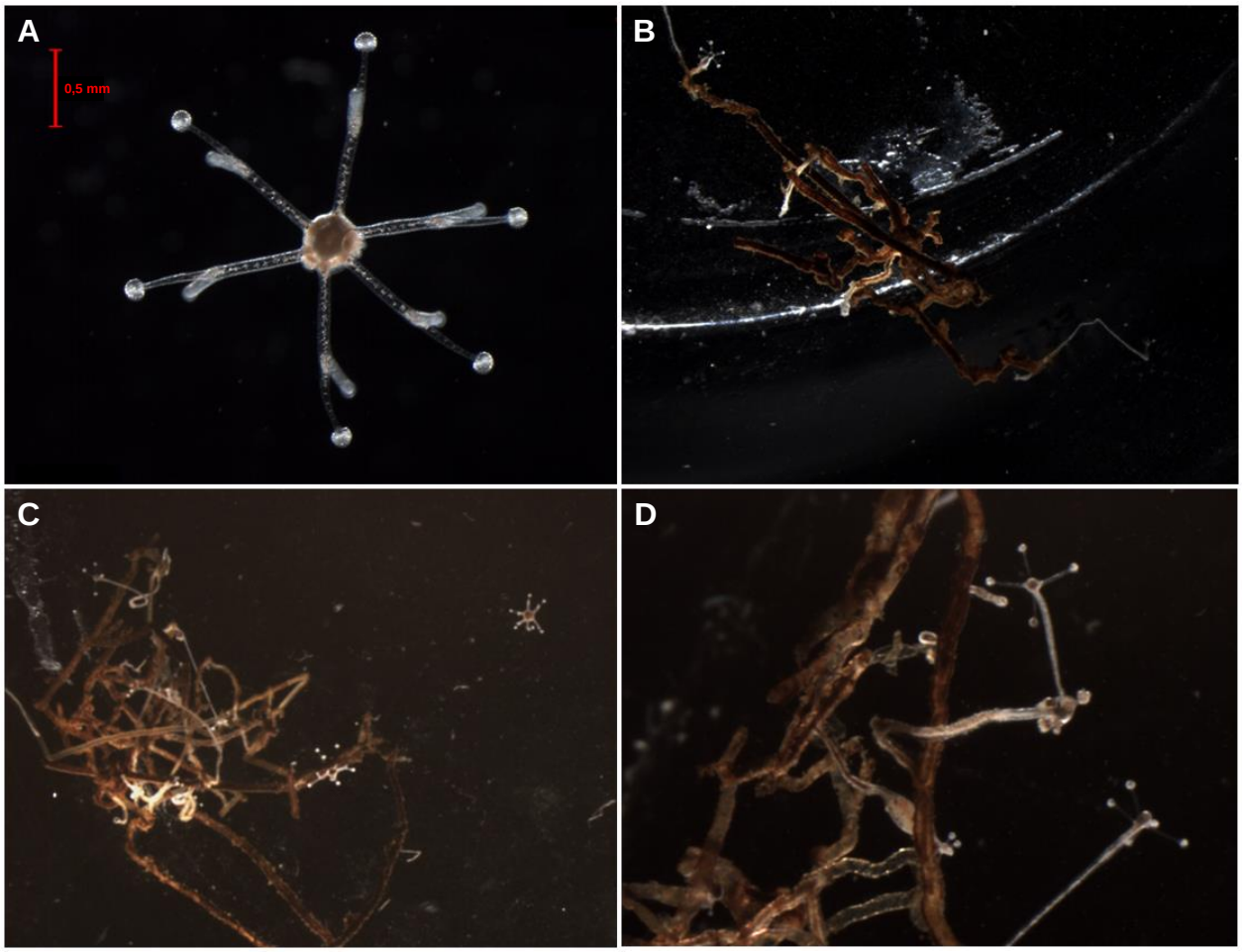

Figure 3. *Eleutheria dichotoma***. A** Medusa (aboral view) with two growing buds on the umbrella. **B** Stolonal colony with polyps. **C** Stolonal colony with polyps and medusae. **D** Section of a stolonal colony showing polyps close-up. Growing primary medusae can be seen at the hydrocaulus of the polyp in front and a the polyp in the back ingested an *Artemia salina* nauplius. Scale bar on the upper right (**A**) applies only to **A**.

Throughout my thesis I will discuss aging in basal metazoans from a biodemographic point of view. A major core question in evolutionary demography is: Why evolution favoured a certain path of aging in a certain species? To look into this question, I chose to examine aging in two extremely interesting basal metazoans belonging to the Hydrozoans: *Hydra magnipapillata* and *Eleutheria dichotoma*.

The freshwater polyp *Hydra* (Hydrozoa, Athecata, Aplanuata) is a well-studied model-organism across various biological disciplines (Campbell 1967; Gierer et al. 1972; Martinez 1998; Bosch et al. 2010; Bosch 2012; Chapter III). The cosmopolite genus (except

Antarctica) comprises four morphologically distinct and recognizable species clusters with various species (12-15 in total according to recent estimates) and more than a hundred species strains (Jankowski et al. 2008; Martinez et al. 2010). Populations usually undergo frequent seasonal fluctuations in temperate zones regarding population size and switches in reproduction patterns, changing from purely vegetative to sexual or simultaneous reproduction in summer times to a more dormant and unproductive living style during winter (Ribi et al. 1985)(own observations). However, in comparison with asexual reproduction, sexual reproduction seems to play only a marginal role for the proliferation and population growth in the *Hydra* (Bosch 2009). Connected to the mode of vegetative proliferation is the high stem cell potential, or "stem cellness" (Bosch 2007), and the continuous proliferative cell renewal and cell turnover of *Hydra* which equips the polyp also with its remarkable regeneration capabilities (Bosch 2007; Bosch et al. 2010). These features of Hydra are more than likely playing an important role in its exceptional non-senescent aging pattern and flexible life history responses towards changing environmental conditions, including hormetic reactions or phenotypic heterogeneities and random phenotype allocations (Hydra chapters). I chose to examine *Hydra magnipapillata* strain 105, a *brown Hydra* belonging to the *Hydra vulgaris* cluster, which was isolated in 1973 in Japanese wetlands near Mishima on Honshu (Sugiyama and Fujisawa 1977; Sugiyama and Fujisawa 1977; Sugiyama and Fujisawa 1978). This strain reproduces solely vegetatively in our lab cultures since more than 10 years, although older ramets were reported to produce gametes as well (Sugiyama and Fujisawa 1977; Sugiyama and Fujisawa 1977).

In contrast, *Eleutheria dichotoma* (Hydrozoa, Athecata, Cladonematidae), nowadays a cosmopolitan marine Cnidarian inhabiting the littoral zone (Hauenschild 1956; Fraser et al. 2006), is a metagenetic organisms with a planula larva, polyp colony and medusa life stage. Under moderate conditions, a live larva is released from a medusa to search actively for a

suitable settlement spot for a few days to metamorphose then into a primary polyp, which develops into a stolonal polyp colony. These polyps proliferate only vegetatively by either growing as an intact colony network with increasing numbers of polyps, whereby colonies may break apart and live on as disjoined parts, or by budding primary medusae which detach from the parent colony. In contrast to most other hydrozoan medusa forms which are incapable of vegetative reproduction, the crawling and non-swimming medusa of *E. dichotoma* can proliferate both sexually and asexually. Sexually by bisexual self-fertilization, whereby sperm and eggs form embryos within brood chambers below the umbrella of a medusa which are released as hatched swimming planula larvae to settle and form new polyp colonies (Schuchert 2006). The asexual reproduction, which is the prior mode of reproduction in *E. dichotoma* medusae, consists of budding secondary medusae which have the same potential reproduction modes as the primary medusae (Hauenschild 1956).

On the basis of the experimental laboratory studies me and my colleagues conducted with these two model organisms and the few data which are available throughout the literature on aging in basal metazoans I aim to tackle and discuss further key aging research questions:

- Why evolution favoured a certain path of aging in a certain species?
- Where on the tree of life is the evolutionary origin of senescence and why did senescence evolve?
- Did senescence evolve convergently or as a single event?
- Which basal metazoans show patterns of non- or negative senescence?
- Under which circumstances can negative and non-senescence evolve?
- How can we incorporate the ramet and genet concept into the concept of aging?
- What are the differences between aging on the genet and ramet levels.
- Does senescence on the ramet level necessarily lead to senescence on the genet level?

- How are the different aging patterns maintained by evolution and which biological, physiological, ecological or life history factors are important to understand the evolution of non-senescent mortality trajectories?
- How flexible are resource allocation strategies regarding aging in basal metazoans?

Via the basal metazoan examples, this thesis aims to offer more insights into the concept of aging and into the evolutionary origin of senescence, why senescence evolved and how the evolution of non- and negative senescence patterns can be explained. To understand the remarkable diversity of aging is the key to our understanding of the process and evolution of aging in general.

CHAPTERS

This thesis is based on the following five research articles

(presented and referred to as chapters I to V):

I) **Ringelhan, F**.; Schierwater B.; Campos Rodigues, I. R.; Schaible, R.: Aging in a metagenetic basal metazoan I – The biodemography of *Eleutheria dichotoma* (Cnidaria: Hydrozoa)

For the first time ever in a longitudinal experiment, the survival, size, and reproduction of isogenic *Eleutheria* were measured at two different feeding regimes under constant conditions for both polyp and medusa stages. Three successive vegetative medusa generations were studied, whereby the first was constituted from primary polyps of one chosen parent stem polyp colony and the two successive ones were descendants of the primary and secondary medusae, respectively. The stem colony was continuously observed

while larvae and polyps were collected and raised from the primary medusae. In this chapter we display and discuss the demographic survival and reproduction patterns and the differences between the feeding levels, vegetetative medusa generations and the polyp/medusa ramets. Additionally, we reflect on aging in genet versus ramet levels.

II) **Ringelhan, F**.; Schierwater B.; Schaible, R.: Aging in a metagenetic basal metazoan II – demographic trade-offs in *Eleutheria dichotoma* (Cnidaria: Hydrozoa)

Following on chapter I, we examined the correlations and trade-offs between the measured traits in the experiment, including size. Furthermore we elaborate on the phenotypic diversity between the isogenic medusa ramets and relate the results to the known ecological factors influencing *E. dichotoma* in their natural environments.

III) Schaible, R., **Ringelhan, F.**, Kramer, B.H., Miethe, T. (2011): Environmental challenges improve resource utilization for asexual reproduction and maintenance in hydra *Experimental Gerontology* 46 (10): 794-802.

Variation in life history traits can reflect (epi-)genetic differences, and may be caused by environmental effects on phenotypes. To gain a deeper understanding of *Hydra*'s exceptional aging patterns we examined *Hydra*'s phenotypic plasticity in response to various environmental challenges. In a set of laboratory experiments, we studied the variation in the allocation of resources to vegetative reproduction and to somatic maintenance of isogenic and purely asexually reproducing *Hydra* polyps of a *H. magnipapillata* strain in relation to differences in temperature and food availability. We recorded budding rates and starvation survival as indicators to changes in the allocation of resources to asexual reproduction and maintenance. Finally, we discuss our findings regarding resource allocation trade-offs, hormetic reactions and ecological interactions of *H. magnipapillata*.

IV) Schaible, R.; Danko, M. J.; **Ringelhan, F.**; Wagner, P.; Kramer, B. H.: Variation in individual fitness of Hydra polyps and the importance of stochasticity

In this experiment, we explicitly examined the individual variation in life-history traits of isogenic, purely asexual *H. magnipapillata*. To further look into the phenotypic heterogeneity within a *Hydra* clone, we analysed patterns of vegetative reproduction and age at first reproduction of more than 1118 isogenic polyps, subdivided into six cohorts of different ramet ages (2-5 years old). Environmental conditions were kept constant throughout the study. We discuss the effects of stochasticity and random phenotype allocation in clonal organisms.

V) **Ringelhan, F**. and Schaible, R: To cut or not to cut - Biscetion trade-offs in the polyp *Hydra magnipapillata* (Cnidaria: Hydrozoa)

Following on chapter III&IV and in light of the remarkable plasticity, regeneration abilities and non-senescent aging pattern of *Hydra* polyps, this study examines its resource allocation strategy in response to strong environmental stressors. We reared isogenic, purely asexually reproducing *Hydra* polyps of a *H. magnipapillata* strain at five different feeding regimes and bisected them horizontally to simulate predation stress. We observed reproduction, size, and starvation survival after regeneration and compared these traits between groups to reveal eventual allocation trade-offs and hormesis effects. We also looked into trait heterogeneity within and between polyps and checked for heritability as we monitored fed and unfed budded offspring generations at the two highest feeding levels.

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CHAPTER I

Aging in a metagenetic basal metazoan I –

The biodemography of *Eleutheria dichotoma* (Cnidaria: Hydrozoa)

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ABSTRACT

Basal metazoans show large variations in life-cycle patterns, offering unique opportunities to study the evolution of aging from a biodemographic point of view. Laboratory experiments were conducted on *Eleutheria dichotoma* (Cnidaria: Hydrozoa: Cladonematidae), a metagenetic hydrozoan with a crawling medusa, to test the resource allocation flexibility of a remarkably plastic and variable marine organism. For the first time ever in a longitudinal experiment, the survival, size and reproduction of isogenic *Eleutheria* were measured at different feeding regimes under constant conditions for both polyp and medusa stages. *E. dichotoma* medusae have the ability to reproduce both via asexual budding and bisexual self-fertilization. The polyps stay asexual, growing as stolonal polyp colonies with the ability to form medusa as buds. Established polyp colonies suffered almost no mortality at all within more than three years of observation while larva and primary polyp mortality was rather high. This points towards a negative senescence pattern at the polyp colony ramet level. Medusa demography differed substantially, exhibiting hump shaped trajectories in survival and both reproduction modes, suggesting a "hump shape senescence" at the medusa ramet level. The maximum medusa lifespan was 359 days and low feeding regimes had a positive effect on survival at generally lower reproduction rates. Striking was a significant trend towards a qualitative decline in successive vegetative medusa generations in survival and both reproduction outputs. In context with the reported natural seasonal occurrence of medusae these results broaden our understanding of the genet-ramet complex and its evolutionary demographic implications for the diversity of aging patterns in basal metazoans. We reason that the overall aging pattern of *E. dichotoma* genets is of a negative senescent type.

INTRODUCTION

Aging and the demography of basal metazoans (i.e. Diploblasta: Placozoa, Porifera, Cnidaria and Ctenophora) are still poorly understood. Aging is most clearly defined and measurable using a demographic approach, with aging itself being a summary term, broadly meaning 'change over time', comprising the three following main aging types: 1) senescence - described as increasing mortality and/or declining fertility with age, i.e. a decline in individual fitness with age, 2) non-senescence - with both traits staying constant over age and 3) negative senescence - with trait directions opposite to the senescent types, respectively (Vaupel, Baudisch et al. 2004; Baudisch 2008; Baudisch 2011; Baudisch and Vaupel 2012). Evolutionary theory proposes that the decline in the force of natural selection with age is the fundamental cause of senescence (Medawar 1952; Hamilton 1966). As follows, senescence should commence inevitably from reproductive maturity onwards for every living being – but this is not the case. Especially recent literature on the diversity of aging patterns across the tree of life suggest a far more diverse nature of aging than proposed by previous theories (Martinez 1998; Martinez 2002; Vaupel, Baudisch et al. 2004; Baudisch and Vaupel 2012; Baudisch, Salguero-Gómez et al. 2013; Jones, Scheuerlein et al. 2014). Yet, the diversity of aging across the tree of life has just begun to be described and undermined by quantitative demographic data. Large gaps need to be filled both theoretically and experimentally. The data are especially scant at the root of the metazoan tree of life, where we find the most plastic animals regarding their life cycles and regeneration abilities. Most basal metazoans are capable of both sexual and asexual reproduction (see Schierwater and Hauenschild 1990 for a precise definition of asexual versus sexual reproduction which we apply consistently). Consequently, they do not exhibit a clear germ-line sequestration in the classical Weismannian sense of the term compared to higher metazoans (Weismann 1891; Buss 1987;

Finch 1990; Martinez and Levinton 1992; Martinez 2002). August Weismann proposed more than a century ago that “natural death”, meaning senescence, appeared for the first time in multicellular organisms with a natural germ-line (non-senescent) - soma (senescent) distinction (Weismann 1891). Accordingly, organisms that sequester the germ-line should senesce (at the soma level) and those without a clear distinction between the germ-line and the soma, generally capable of both sexual and asexual reproduction (Martinez and Levinton 1992, see above), should not. Complicating this even further, metagenetic basal metazoans, like most Medusozoa, possess parallel life stages which can be simultaneously potent of reproducing either sexually or asexually or both (Kayal, Roure et al. 2013). From these considerations follows that we have to distinguish in such non-germ-line-sequestering organisms between aging on the ramet level (individual organism unit) and aging on the genet level (the entire clone), the latter being the evolutionary actor sensu stricto (Harper 1981; Silander 1985; Karlson 1991; Martinez 2002).

Till today, contradicting results have been found and it has been suggested that the evolution of somatic differentiation, preceding germ-line sequestration, is the necessary condition for the evolution of senescence (Martinez and Levinton 1992; Martinez 2002). Other studies suggest that senescence exists also in unicellular organisms like bacteria, contrasting previous aging concepts even stronger (Stewart, Madden et al. 2005; Wang, Robert et al. 2010). And then there is the case of the non-senescent *Hydra*, a multicellular basal metazoan without germ-line sequestration, exhibiting a constant low mortality rate with only one exception in *Hydra oligactis*, which relates to an environmentally triggered semelparous like mortality response (Martinez 1998; Yoshida, Fujisawa et al. 2006; Jones, Scheuerlein et al. 2014; Schaible, Scheuerlein et al. in preparation for submission). However, the obligatory distinction between senescence on the ramet/genet level has been left out and unmentioned in most of these works, which complicated proper understanding of the aging concept strongly.

This study aims to help disentangling this evident aging mess by contributing new quantitative demographic data of a yet undescribed basal metazoan regarding aging, while emphasizing the genet/ramet complex and its distinct aging deductions. The extents of the variety of aging patterns might become apparent by considering metagenetic species expressing different aging phenotypes/life stages within the same genome. The two distinct generations of most Medusozoa are, apart from a usually short (~ hours to days) planula larva stage after hatching, typically a polyp form as the sessile, vegetative state and the jellyfish/medusa form as dispersal unit capable of sexual reproduction. Interestingly, both of these exemplary cnidarian life cycle stages seem to show different aging patterns – generally it is assumed for most metagenetic cnidarians that the (possibly colonial) polyp stage represents a longer-lived, perennial life stage surviving through winter periods in temperate regions and that the medusa is a shorter-lived, only seasonal and ephemeral stage (Mills 2001; Ojimi, Isomura et al. 2009; Lucas, Graham et al. 2012). Generally, a typical lifespan assumption for "a jellyfish" (Cnidaria: Medusozoa) is about 6-9 months, although many exceptions in various taxa exist (Lucas, Graham et al. 2012). Individual *Aurelia* polyps (scyphistomae) have been maintained in the laboratory under controlled conditions with artificial seawater without signs of deterioration or individual mortality for three years, while the medusae reached maximally six months (Spangenberg 1965).

Much less is known about our study object, the hydrozoan *Eleutheria dichotoma*. *E. dichotoma's* distinctively complex life-cycle makes it a perfect study organism to gain deeper insights into the demographic aging patterns of a metagenetic basal metazoan at both the genet and ramet level. The genus *Eleutheria* belongs to the family Cladonematidae, of which the medusae are adapted to a benthic life style with crawling or swimming medusa, while the polyps grow as colonies (hydroid colonies), usually attached to a substrate, whereby polyps

are connected with root like hydrocauli and stolons typical for many hydrozoans (Schuchert 2006). An additionally most interesting and unusual feature for Medusozoa the *E. dichotoma* medusa shares with some of its family sister species is the ability to reproduce both vegetatively by budding plus sexually by bisexual self-fertilization (Hauenschild 1956; Schuchert 2006). *E. dichotoma* enables us hereby to study not only the aging differences between parallel life-cycle stages (medusa vs. polyp) and aging at the genet vs. ramet level, but also the effects and trade-offs of the varying reproductive modes on its aging patterns.

Only few examples of detailed demographic studies on basal metazoans exist and these are most often very limited in their representative relevance for general patterns since individual numbers are usually low in ecological field studies but also in most laboratory studies (Babcock 1991; Martinez 1998; Garrabou and Harmelin 2002; Martinez 2002; Vaupel, Baudisch et al. 2004). The diversity of demographic aging patterns across the basal metazoans is supposedly very high considering the enormous plasticity in life cycle variations and the abundant potential of asexual reproduction coupled to high regeneration capabilities. A full spectrum of patterns, including senescent, non-senescent and even negatively senescent species and species stages, with declining mortality risks and/or rising fertility with age, might be expected (Baudisch 2008; Baudisch 2011; Baudisch and Vaupel 2012; Baudisch, Salguero-Gómez et al. 2013; Jones, Scheuerlein et al. 2014). More demographic data are needed to study these patterns, whereby the differences between genet and ramet aging patterns are most interesting and consequential to examine considering the specific life cycle stages at which the respective ramet units occur (Karlson 1991; Martinez 2002).

Here we report the first extensive demographic laboratory study of the life of a metagenetic hydrozoan, including the measurement of the survival, size, and reproduction measured at different feeding regimes for both polyp and medusa stages, including released planula larva

survival. We chose two different feeding regimes to observe potential shifts and trade-offs in resource allocation patterns regarding aging.

MATERIAL AND METHODS

Study Organism

The Hydrozoan *Eleutheria dichotoma* Quatrefages, 1842 (Hydrozoa, Athecata, Cladonematidae) is a metagenetic, nowadays cosmopolitan marine Cnidarian inhabiting the littoral zone (Hauenschild 1956; Fraser, Capa et al. 2006). The polyps proliferate only asexually by either growing as an intact colony network with increasing numbers of polyps whereby colonies may break apart and live on as disjoined parts, or by budding primary medusae which detach from the parent colony. The crawling medusa can proliferate both sexually and asexually. Sexually by bisexual self-fertilization, whereby sperm and eggs form embryos within brood chambers below the umbrella of a medusa which are released as hatched swimming planula larvae to settle and form new polyp colonies (Schierwater and Hadrys 1998; Schuchert 2006). The asexual reproduction, which is the prior mode of reproduction in medusae, consists of budding secondary medusae which have the same potential reproduction modes as the primary medusae (Hauenschild 1956).

The genetic consequences of the vegetative and the bisexual reproduction via self-fertilization are presumably similar for *Eleutheria*, both modes lead to genetically identical offspring generations through time (Williams 1975; Schierwater and Hauenschild 1991; Ender 1997). *E. dichotoma* shows evidence of being one of the remarkable clonal/inbred species without any signs of genetic exhaustion. The often-quoted Muller's ratchet dead end scenario (Muller 1964) for clonal/inbred species may not apply for *Eleutheria* although there is no evidence of cross fertilization at all in the wild (Ender 1997).

E. dichotoma medusae are unable to swim and are adapted to a benthic and crawling lifestyle which is specific for and widespread among Cladonematidae medusae (Schuchert 2006). *E. dichotoma* medusae have been found in shallow depths (< 20 m) and tide pools on algae such as *Ulva, Cystoseira* and *Gelidium*, sessile polyps have rarely been found in the wild so far but seem to prefer lithoidal and hard substrate (Hauenschild 1956; Brinckmann-Voss 1970; Ender 1997; Fraser, Capa et al. 2006; Schuchert 2006). The seasonal pattern of medusa and polyp occurrence and their corresponding reproduction modes is not yet clear and fully understood. *E. dichotoma* medusae may not sustain winter conditions since medusa were frequently found in all seasons except in winter (Riedl 1983; Schierwater 1989a), so the polyp colony stage may serve as the reservoir and backup stage of *Eleutheria* in harsher winter conditions. Surprisingly, reproductive patterns are not synchronized with seasonal external environmental factors such as temperature, photoperiod and population density (Schierwater 1989a). Still, differences in food abundance between winter and the other seasons could be a relevant factor for the direction of the reproductive mode, although evidence is lacking that such food abundance differences between seasons really exist for *Eleutheria* (Schierwater 1989a) and are likely not true as Calbet et al found out in 2001 for a bay not far away from the original sampling spots of the *Eleutheria* we used (Calbet, Garrido et al. 2001; García-Comas, Stemmann et al. 2011). Both reproductive modes can be observed in medusae from spring throughout autumn (Schierwater 1989a) which leads to the assumption that no optimization in terms of an allocation of seasonal reproductive modes (Giese 1959) is necessary for *Eleutheria* in the field and that rather stochastic heterogeneity or endogenous processes are underlying the reproductive patterns of this species.

Culturing conditions

We used individuals of a single clone (Ω) of *E. dichotoma*, derived from the laboratory of

the ITZ Ecology and Evolution, Tieraerztliche Hochschule Hannover, Germany (ITZ). The original medusa specimen for this clonal line was collected in 1984-1986 from the Mediterranean shores of Banyuls-sur-Mer, France (Schierwater 1989a; Ender 1997) and cultured in the laboratory since then. Culturing conditions applied for this study were modified after Hauenschild (1956) and Schierwater (1989). Both polyp and medusa stages were cultured in the laboratory in artificial seawater of 35 ‰ ("Reef Crystals" by Aquarium Systems mixed with Milli Q filtered water) in either glass dishes of about 50-100 ml or plastic six-well microwell plates with about 9 ml saltwater per well. Constant temperature (23°C) and light conditions (18/6-light/dark diurnal rhythm) were provided in BINDER incubators. Light was hereby offered by Osram Lumilux Cool Daylight lamps (L18W/865) at PFD's (Photosynthetically Active Photon Flux Density) around 7-20 $\mu mol/m^2/s$, constituting a low light environment resembling natural sublittoral illumination conditions. Both polyps and medusae were constantly fed a mono-diet of *Artemia salina* nauplii (2 days post hatching), water was exchanged at least once a week (for more one the feeding behaviour of E. dichotoma, see (Hadrys, Schierwater et al. 1990).

Experimental Design

The isogenic medusa cohorts for the biodemographic monitoring study were built up from a single and separated polyp colony of the original clone Ω (minimum age > 6 months, minimum of 6 live polyps during primary medusa isolation). This stem parent colony was continuously well maintained in a separate glass dish and fed with a gush or *Artemia* three times a week. Three cohorts were generated with 60 medusae per each cohort, whereby half of each cohort were fed with 6 *Artemia* per week (= High feeding regime (HFR), fed Mo, Wed, Fr) and the other half with 2 *Artemia* per week (= Low feeding regime (LFR), fed Mo & Fr). Feeding rates were chosen according to a pilot experiment and experiences in previous

culturing and experiments (Hauenschild 1956; Schierwater 1989a; Raudonat 1995). The first medusa cohort , so called primary medusae, was constituted by the first medusae budded off by the parent polyp colony (PH = primary medusa at HFR, PL = primary medusa at LFR). The first medusa bud of each isolated primary medusa was kept to generate the second cohort, so called secondary medusae (SH = secondary medusa at HFR, SL = secondary medusa at LFR). To prevent any direct inter-generational transmission of signals, we made sure that only these medusae were taken for the secondary medusa cohort, which have not been already in development when the primary medusae were still attached to their parent polyps. The same procedure was applied to the third cohort, only that these tertiary medusae were now isolated from their respective parent secondary medusa individuals (TH = tertiary medusa at HFR, TL = tertiary medusa at LFR).

Each isolated medusa was kept and traced individually in a well of a plastic six-well microwell plate with about 9 ml saltwater per well. Wells were checked for uneaten *Artemia* before feeding to compensate for uneaten food in the subsequent feeding round. At least twice per week medusae were checked for detached medusa buds, which were discarded, to record individual budding rates. The sexual state of a medusa as well as the number of released and free swimming planula larvae were recorded also at least twice per week during the experiment under a binocular. From each medusa of the primary cohort the first five released planulae were isolated into separate glass dishes. Feeding started here as soon as the first tentacles were visible after settlement and metamorphosis into a polyp. The same feeding schedule was applied to the primary polyps as to the respective parent primary medusa, whereby each additionally growing polyp on the developing colony got additionally always the same amount of *Artemia* as the first polyp. With the successful emergence of the first polyp with tentacles the remaining planulae and smaller polyps were discarded to maintain and monitor only one polyp (-colony) offspring per parent primary medusa individual for

practical and laboratory space reasons.

This experimental design allowed us to analyse for the first time completely the demographic survival and both sexual and asexual reproductive patterns of the parent stem polyp colony, three successive medusa generations and one polyp offspring generation of the primary medusae at two different feeding levels, respectively.

Medusa size was also monitored by photographing all living cohort medusa individuals four times during the experiment under the microscope. Two- and three dimensional surface areas were calculated (Schierwater 1989b) and used to compare medusa size and growth (see the follow-up chapter II for the anaylsis of size patterns).

Analyses

SPSS and R software were used for the statistical analysis of the obtained data. We tested for cohort and feeding level differences regarding survival and reproduction (vegetative and sexual output). We applied non-parametric Mann-Whitney U, Jonckheere-Terpstra and Kendall's tau b tests in compliance with the respective statistical requirements of the data. Furthermore, we used graphical data representation to assess demographic patterns where appropriate. We applied a smooth spline fit to mortality data via a Generalized Additive Model (GAM).

For the survival curve analysis, graphical methods were used in order to assess the assumption of proportional hazards between the generations, namely: (1) the log of the cumulative hazard functions (using the – *log* transformation of the Kaplan-Meier estimates) against time were plotted and checked for parallelism and (2) the differences in the log cumulative hazard for each pair of functions against time were plotted and checked for constancy. Based on the obtained plots (not shown), the assumption of proportional hazards was dismissed both between the high feeding as among the low feeding groups. Accordingly,

we used the distribution-free Gehan-Breslow (generalized Wilcoxon) test (Gehan 1965; Breslow 1970) to test the null hypothesis that the survival functions of the three generations are the same in each feeding regime, versus the global alternative hypothesis that, at least, one of the survival functions is different. The trend version of the Gehan-Breslow test (Moeschberger and Klein 2003) was used to test the same null hypothesis against the ordered alternative hypothesis that $S_P(t) \geq S_S(t) \geq S_T(t)$, with $S_P(t)$, $S_S(t)$and $S_T(t)$ being the survival functions of the primary, secondary and tertiary generations, respectively. As in all other tests in this study, a p-value of 0.05 or less was considered to be statistically significant.

RESULTS

In *Eleutheria* we see a difference of life history patterns between the polyp and medusa stages. While the stem polyp colony survived well throughout the whole experiment until today (minimum age > 3.5 years), the longest observed medusa lifespan was almost exactly 1 year (359 days). The polyp colonies produce continuously new stolonal branches, polyps and medusa buds if fed and maintained under constant environmental conditions. Medusae start off with early medusa bud production, switch quickly to often simultaneous sexual self-fertilization parallel to ongoing bud production until both reproductive modes cease and medusae stop feeding and finally die.

We found a significant overall trend towards a qualitative decline in all measured traits with asexually progressing medusa generations.

Survival

Medusa

Cohort and feeding level comparisons

Based on the survival information from table 1 and figure 1, it is considered that there are no substantial differences in the censoring pattern between the generations in both feeding regimes. We found significant differences in the survival functions of the three generations in the HFR, both when considering the global alternative hypothesis (χ^2=9.67, d.f.=2, p=0.0079) and the ordered alternative (Z=3.074, p=0.00106, see fig. 1 and table 1). On the other hand, the results concerning the medusa generations in the LFR showed significant differences only when considering the global alternative, at which at least one of the survival functions is different (χ^2=6.25, d.f.=2, p=0.044), but not when testing against the ordered alternative (Z=0.788, p=0.215, see fig. 1 and table 1).

Comparing all LFR with HFR medusae, medusae in the LFR lived significantly longer than in HFR (Mann–Whitney U test, p<0.001, fig. 2). Separate cohort comparison reveals significantly longer survival for SL and TL medusae compared to SH and TH, respectively (Mann–Whitney U tests, p<0.001, fig. 3.), while P medusa cohorts were not different in survival.

Mortality trajectories

Most interesting regarding *Eleutheria*'s aging pattern are the survival and mortality trajectories for medusae (figs. 1 & 4-7). We calculated and compared mortality (qx) on a monthly scale - in our opinion a reasonable interval choice regarding the death distributions (to reduce interval 'gaps' with no death occurrences) and the medusas' lifespan of ≤ 1 year. Medusa mortality follows a striking hump-shape senescence pattern with falling mortality after an earlier mortality rise for all cohorts separated (fig. 4), HFR and LFR combined (fig.

5) and all cohorts combined together again (fig. 6). An exception are the tertiary medusa cohorts at both feeding levels, which displayed a steady monthly mortality increase (fig. 4). Additionally, the hump shape could be confirmed by a smooth spline fit, fitting a Generalized Additive Model (GAM) to the data (see fig 7, last death interval excluded for the shape fit). If the last interval, including the last three medusa deaths out of 162 individuals at the start of the experiment, is not excluded from the data, the smoothed GAM trajectory increases again at the end due to the nature of mortality calculation. In conclusion, the shape captured by the raw monthly mortality plus the GAM fit (while excluding the last interval) is a strong statement for falling mortality through most of a long-lived medusa's lifetime.

Remarkably, freshly budded medusa offspring does not have any mortality risk in the first weeks of its lifetime in neither cohort (fig. 4). Generally, medusa lifespan is very heterogeneous between and within isogenic cohorts, with only less than 50% of a cohort surviving to half of the maximum observed lifespan of each cohort, respectively (figs. 1 & 7).

Polyps.

LpH – Larva isolated from medusa of the high feeding regime

Most of the isolated larvae (88) did not develop into polyps and did not make it to an observed attachment to the ground including a metamorphosis into a primary polyp. Furthermore, most of the metamorphosed primary polyps (33) did not develop further to grow into a colony with stolon formation and died/starved to death within seven weeks after original larva isolation. Only four out of the 88 isolated larvae developed further into a colony, which was defined as a polyp(s) with stolon formation. None of the four primary polyp colonies is still alive today, though colony mortality was very low and flat, considering that only 4 colonies were present. Colonies contained always varying numbers of polyps, just like in the stem polyp colony, and deaths occurred after 38, 221, 579 and 977 days of

lifetime. Colony deaths were assigned to colonies, which had no living polyp and no polyp formation out of resting stolon fragments for at least one month.

LpL – Larva isolated from medusa of the high feeding regime

The pattern for LpL larvae was similar to the ones from LpH. Most of the isolated larvae (74) did not become polyps and did not metamorphose into primary polyps. In contrast to LpH, none of the metamorphosed primary polyps (18) developed further to grow into a colony with stolon formation. The last and "oldest" of the LpL polyps died after about seven weeks post-larva isolation.

Stem Parent Polyp Colony

The parent polyp colony survived throughout the full experiment until today (minimum age > 3.5 years). Still, the degree of stolon partition (5 - 26 disconnected stolon parts), alive polyp number of the colony (1 - 21), individual polyp lifetime at a stolon (not observed) and the biofilm environment varies considerably through time, confirming previous observations (Hauenschild 1956; Schierwater 1989a; Raudonat 1995).

Reproduction

Medusa Reproduction

Asexual

Cohort and feeding level comparisons

Comparing medusa total bud release (TBR) and budding rates per day alive (BRR) between cohorts (figs. 8-9), significant trends towards a decline in both measurements with succeeding generations could be observed in the HFR (Jonckheere-Terpstra-Tests, $p<0.001$, Kendall's tau b = -0.446 & -0.312, $p<0.001$), in the LFR regime (Jonckheere-Terpstra-Tests, $p<0.001$,

Kendall's tau b = -0.535 & -0.499, $p<0.001$) as well as in both regimes analysed together (Jonckheere-Terpstra-Tests, $p<0.001$, Kendall's tau b = -0.358 & -0.236, $p<0.001$).

Budding trajectories

The overall pattern of the budding behavior of medusae again was a hump shaped trajectory (fig. 10). Young medusae begin very quickly to produce their first buds themselves, often already while still being attached to their parent polyp or medusa. Hence, first medusa bud detachment can happen very early in a medusa life, sometimes even in not yet detached medusa buds. Age specific budding rates peak around the second to fourth week (after detachment from parent) throughout cohorts and decrease from then on with possible minor peaks afterwards (fig. 10). TBR and BRR are, in accordance with food intake, much higher in HFR than in LFR (Mann–Whitney U tests, $p<0.001$, see figs. 8-11). Almost all medusa produced detached bud offspring, only in the SL and TL cohorts were medusae with no bud production at all (fig. 11).

Budding behavior was very heterogeneous between and within isogenic cohorts, with minimum 0 and maximum 15 buds released in the LFR and minimum 3 and maximum 32 buds released per medusa in the HFR (fig. 11).

Sexual

Cohort and feeding level comparisons

Medusa total planula larva release (TLR) and larva release rates per day (LRR) compared between cohorts showed similar patterns as in medusa buds (figs. 12-13). Significant trends towards a decline in both measurements with succeeding generations could be observed only in the LFR (Jonckheere-Terpstra-Tests, $p<0.05$, Kendall's tau b = -0.202 & -0.207, $p<0.05$). In the HFR and both regimes analysed together a trend was only visible for TLR and not for

LRR (Jonckheere-Terpstra-Tests, $p<0.05$ & < 0.01, Kendall's tau b = -0.212 & -0.163, $p<0.05$ & < 0.01).

Larva Release trajectories

The overall pattern of the larva release of medusae followed as well a hump shaped trajectory (fig. 14). Young medusa buds can rarely begin very early to produce their first embryos while still being attached to their parent medusa (at parent polyps it has not been observed but also not so frequently checked). Hence, first larva release can happen very early in a medusa life as well (e.g. while still being connected to parent) and even larva sharing between connected parent and offspring medusa has been observed. Age specific larva release rates peak around the fourth to sixth week throughout cohorts, which is later than the budding release rates, and decrease from then on with possible minor peaks afterwards (fig. 14). TLR and LRR are, in accordance with food intake, much higher in HFR than in LFR (Mann–Whitney U tests, $p<0.001$, see figs. 12-15). Almost all medusae produced embryos and released larva offspring, only few medusae stayed completely asexual without any observed embryo development at all (in the SL and TL cohorts were medusae with no larva production at all (one in SL, one in TH & five in TL). Not all medusa with observed embryogenesis released functional and live planula larvae as well.

The sexual larva release behavior was very heterogeneous between and within isogenic cohorts, with minimum 0 and maximum 20 larvae released in LFR and minimum 0 and maximum 31 larvae released per medusa in HFR (fig. 15).

The onset and offset of sexual reproduction differed between HFR and LFR with LFR medusae needing a longer time to produce “first seen larva in medusa” and “first released larva” and also a longer time (counted from medusa birth onwards) to go back to the (degenerative) vegetative state whereafter no sexual reproduction occurred (Mann–Whitney

U tests, $p<0.001$, see cumulative plots (fig. 15) regarding larva release patterns). The "days from last released larva until death" did not differ between HFR (median = 26 days) and LFR (median = 28 days) with observations exhibiting a broad scale ranging from -3 to 110 days. Although HFR seemed to show a slight trend towards longer total sexual reproductive phases, this trait was not compared directly because of the huge heterogeneity of medusas' sexual behavior. Many medusae had phases without larva releases and larva observations within medusae in between and hence could have switched completely to a vegetative state in between sexual phases as well (varying also in their possibly simultaneous bud production and larva release all the time), which would confound a direct comparison of this trait.

Polyp Reproduction (only Asexual)

From the four (all LpH) of totally 162 isolated embryos which developed further into a polyp with stolons, three made it to a multipolyp- and medusae-releasing colony stage, the other one died already after 38 days. The colony deaths occurred since then after 221, 579 and 977days of living with average medusa bud release rates of 1.05, 2.06 and 2.17 per week. The longest living colony thus produced 303 medusa buds in about 140 weeks of lifetime at the HFR of 6 *Artemia* per week per polyp at the colony.

DISCUSSION

Demographic Trajectories – *Eleutheria* medusae are 'hump shaped'

In all measured medusa traits an overall occurring pattern was the hump shape. The hump in mortality, expressed as age-specific mortality (qx) and/or death rate (mx), can be seen in all cohorts separated, HFR and LFR combined and all cohorts combined, except in the tertiary cohorts both in HFR and LFR. Regarding both sexual and vegetative age-specific fertility,

measured as mean weekly larva/bud release per medusa, a hump can be seen in every comparison (all cohorts separate, HFR and LFR combined and all combined). The hump shaped medusa traits can be distinguished into three phases, with the first phase showing absolutely no mortality and reproduction yet. The second phase is determined by increasing mortality risk and increasing budding- and larva release rates. Finally, in the third phase all traits start to decrease again on the population level after having reached a hump characteristic climax before. In all traits, the climax tends to be relatively early, at a young medusa age regarding maximum lifespan, in contrast to the observed humps in other species, e.g. in medflies (Carey, Liedo et al. 1992; Vaupel, Carey et al. 1998). Remarkably, the age-specific fertility trajectories run though similar absolute values between sexual and asexual reproduction but in slightly shifted phases, with the budding trajectory preceding in its phase the larva release curves around one to three weeks.

Several conclusions can be drawn from this observation, namely that the phase of vegetative, sexual and simultaneous reproduction including tissue bursts by larva releases constitute the most risky medusa life phase, whatever vegetative generation. The declining mortality risk phase is accompanied by lower larva and budding release rates (down to zero in both traits, or just shutting sexual reproduction), less food consumption and starvation. Two explanations for this pattern seem most probable to us, both not mutually exclusive: heterogeneity and medusa physiology. Heterogeneity between isogenic medusae could be an important driver for this phenomenon, potentially caused by epigenetic differences between medusa tissues and cell lines and distributed differently across the isogenic medusae in the cohort. Similar phenotypic variability observations due to random phenotype allocation to ramets regarding budding rate and starvation survival have been made by us with the freshwater polyp *Hydra*, for example (Chapter IV; Chapter V). Our second hypothesis is that the early risky life phase associated with the high mortality hump is an inherent physiological feature of all medusae.

High reproduction rates in both asexual and especially sexual reproduction seem to pose a high risk to the medusa survival. Not all larvae are released through the sexual channels without damage to the parent medusa (Hauenschild 1956, own observations), larva release can be associated with tissue bursts in the umbrella, sometimes larvae even hatch within the brood pouches of the medusa and may cause serious damage to the parent. As a result of this highly reproductive phase a high mortality hump phase is following, with deaths occurring randomly among otherwise isogenic and similar medusae. The relatively few long lived medusa survivors of the critical phase expressed rather low age-specific weekly budding and larva release rates later on after the peak, pointing to a generally different resource allocation in them emphasizing more investment in maintenance which is strengthened by the overall low BRR and LRR values of the long lived medusa (without that lifetime had an effect on TLR in contrast to TBR, which was generally positively correlated with lifetime (see chapter II)), or even to a post-peak trade-off shift in some to more maintenance and less reproduction relative to their total energy investment. Generally, longer lived medusae had also longer absolute sexual phases and some parent medusae actually merged and fused with their still attached buds, prolonging their 'life' in this way. Most medusae displayed phases of simultaneous sexual and asexual reproduction and purely sexual or asexual individuals were extremely rare. This leaves the question if all these observations are due to heterogeneity from medusa birth on or linked to random survival and late life resource allocation patterns. Most likely, both heterogeneity and medusa physiology are interactively at work. Heterogeneity between isogenic medusae could as well be related to differences and variations among their proposed associated bacterial epicommunity (i.e. metaorganismic/holobiontic variations), which turns recently more and more into focus of research, especially in the freshwater polyp *Hydra* (Bosch and McFall-Ngai 2011; Bosch 2012; Bosch 2012; McFall-Ngai, Hadfield et al. 2013). The trade-off features of our

experiment will be further elaborated in a separate follow-up article on *Eleutheria* trade-offs (Chapter II).

The risky mortality hump of *Eleutheria* medusae might be abstractly compared to the "accident hump" we observe in human mortality at young ages around 15-30 years of age, whereby after this hump decline mortality keeps on rising exponentially with age according to the Gompertz curve with late deceleration after age 80 till reaching a mortality plateau at very high ages for supercentenarians (Heligman and Pollard 1980; Vaupel, Carey et al. 1998; Remund 2012). Additionally, no ontogenescence, i.e. ontogenetic decline of death rate between conception and maturity (Levitis 2011), is observed in the vegetatively produced medusa cohorts, which do not show any early ontogenetic death at all in the first month after being budded off.

Polyp vs. Medusa Aging

The finding that the stolonal polyp stage (colony) lives longer than the medusa stage in *Eleutheria* is not a complete new discovery of this study but it could be confirmed here with the most extensive demographic study on *Eleutheria* so far. Previous studies on *Eleutheria* touched this feature along the way as well (Hauenschild 1956; Schierwater 1989a; Schierwater and Hauenschild 1990; Schierwater and Hauenschild 1991; Raudonat 1995; Ender 1997; Schierwater and Hadrys 1998) but never studied aging patterns of both polyps and medusa on a demographic basis, except Raudonat 1995, where 15 medusae (5 parental and 2 x 5 horizontal offspring medusae) were observed over their complete lifespan (max. ca. 22 weeks at 18°C and low feeding level) plus additionally the development and survival of 10-15 planula larvae for four months. The sexual conception, brooding and spawning stages and the following settling and establishment stages of the embryos, planula larvae and metamorphosing primary polyps we observed in our experiment were all very risky phases at

which we found high mortality levels compared to low mortality levels of older and established stolonal colonies, speaking for sexual ontogenescence in *Eleutheria*. Once a primary polyp is established, producing its first stolonal protrusions and starting to grow new secondary polyps, mortality starts to decline sharply and seems to sink into a plateau shaped non-senescence, with sporadic colony deaths in between but without signs of an increased mortality due to age. Individual polyps on a colony may be absorbed and/or degenerate through time, but can reappear at the same position, somewhere else on the stolon or on a new stolonal protrusion. Additionally, colonies may break apart and split to continue life as several colony ramets. These patterns are in stark contrast to the complete absence of ontogenescence and early-life mortality in medusae. Considering the different developmental steps involved in the budding process compared with the bisexual self-fertilization including the following hatching, settlement and metamorphosis steps, it is not surprising to find this result. Sexual reproduction is the much riskier way of propagation for *Eleutheria*, but it opens up a different route of dispersal completing the full life cycle including a possible epigenetic reset for the clonal unit which might not be accomplished by pure asexual vegetative reproduction via budding. Surely, more detailed demographic ontogenescence data including embryo, larva and primary polyp mortality and following stolonal colony data need to be collected over longer time spans to confirm this finding and our rather low feeding rate for the primary polyps could be adjusted to higher levels to compare the outcomes.

Considering the presumed seasonal occurrence of *Eleutheria* medusa, medusae were not found during winter periods in European temperate zones, i.e. along Atlantic and Mediterranean shores (Hincks 1868; Brinckmann-Voss 1970; Riedl 1983; Schierwater 1989a) - not including occurrences in the aquarium or in the laboratory - but it is still not understood what causes their winterly absence and why only the polyps shall survive this period as a reservoir stage. Winterly medusa absence could be due to substrate (*Ulva* algae)

loss or declines at very cold water temperatures (<5°C, see (Schierwater and Hauenschild 1990)) or due to somehow low food abundance (zooplankton), although evidence of this strict seasonal zooplankton pattern is still lacking and likely not true as discussed before (Calbet, Garrido et al. 2001; García-Comas, Stemmann et al. 2011). Low water temperatures in winter, with minimum surface sea water temperatures seldom short below 10°C in the Northwest Mediterranean, (Schierwater 1989a; Calbet, Garrido et al. 2001) are most likely not directly affecting *Eleutheria* medusa in a negative sense according to Schierwater 1989, who showed that medusae and polyps have a similar temperature tolerance regime (5°C lethal for polyps, 10-25°C tolerable for both polyps and medusa, 29°C also tolerable for polyps but 30°C deadly for medusa). Generally, seasonal synchronization patterns of jellyfish appearances and abundances coupled with food availability and other environmental factors are very unclear and understudied for jellyfish in the field (Lucas 2001; Lucas, Graham et al. 2012). Just like for most Cnidarians with a metagenetic life-cycle, a deep understanding of the ecology and especially the (natural) biodemography of all life stages is still lacking, as Mills pointed out in 2001 regarding jellyfish bloom occurrences '*Knowledge about the ecology of both the medusa and the polyp phases of each life cycle is necessary if we are to understand the true causes of these increases and decreases, but in most cases where changes in medusa populations have been recognized, we know nothing about the field ecology of the polyps.*' Lucas, Graham et al. 2012 carried this on and mentioned that this gap has started to be addressed over the past decade and summarized recent and related studies in their comprehensive review on '*Jellyfish life histories: role of polyps in forming and maintaining scyphomedusa populations*', but definitely more detailed observational field monitoring and laboratory studies are needed for the extremely diverse and complex Cnidarian taxa to gain a glimpse of the true ecological and demographic diversity across all relevant life stages of each species.

Our finding of the different demography patterns across the three life stages of *Eleutheria* (larva – stolonal polyp form – medusa) can be linked to the qualitative decline we observed with successive vertical vegetative medusa generations. This decline in medusa survival, asexual and sexual reproduction may be explained by an evolved endogenous natural seasonal rhythm/adaptation of the examined *Eleutheria* genet, determining that medusa are only 'made for one season' (< one year) since in winter, likely unfavourable for medusae, only the polyp stage is needed as a reservoir and survivor anyway, thus it does not matter if asexual secondary medusa generations loose quality in their demographic traits. Assuming this purely seasonal occurrence of medusae, the selection pressure for high maintenance and reproduction levels should decline with time during a medusa season for successive medusa generations, pushing the earliest medusae of each season (especially the primary medusae released by the polyps) to highest fitness levels maximizing maintenance and reproduction. Mechanistically, this quality decline could possibly be shown by a reduced amount of stem cells within medusae of successive vegetative vertical (and possibly horizontal) generations or differential gene expressions between generations. To prove this theory, more genets from various geographical zones differing in climate need to be checked for the presence of this qualitative decline. Additionally, more laboratory studies and field observations and monitorings are needed during all seasons to understand the probable winterly medusa absence in the Mediterranean.

Alternatively and not mutually exclusive, the qualitative decline could be evidence that the mode of bisexual self-fertilization via forming a zygote to restart the life cycle is an inherent and necessary feature for *Eleutheria* to survive, because by only reproducing themselves via medusa vegetative budding without the polyp stage the stem cell potential may be somehow lost through time resulting in a dead end scenario for pure medusa propagation. This proposed process still needs to be clarified, since previous results already showed successive

propagation of vegetative secondary medusae for more than 40 generations (Hauenschild 1956; Hauenschild 1957). Hauenschild thus proposed a theoretical vegetative medusa reproduction *ad infinitum*. Strikingly, several of these medusa generation lines lost their sexuality through time which might hint to a loss or decline in stem cell potential, although further on a recovery of this lost sexuality was reported and discussed as well (Hauenschild 1957). This loss of sexuality in many vertical survivor generations through repeated vegetative propagation could hint to a connection to an increased demand of sexuality on the stem cell potential of each medusa – contrasted to the stem cell potential of the stolonal polyp stage, which does not induce the gene expression cascade and cell differentiation for sexual reproduction. The polyp stage remains overall 'simpler' compared to the more complex medusa stage, serving as a reservoir stage for the genet with a possible complete cell replacement and continuous turnover of all cells similar as reported for the freshwater hydrozoan polyp *Hydra* (Campbell 1967; Campbell 1974; Bosch 2007; Bosch 2009; Bosch, Anton-Erxleben et al. 2010; Galliot and Ghila 2010; Chapter III), enabling them complete regeneration and long-term maintenance and survival in contrast to the medusa. Complexity differences in various aspects between less complex planula and polyp stages to the more complex medusa stage have been described and confirmed for many Medusozoa in several previous studies (Piraino, Boero et al. 1996; Boero, Gravili et al. 1998; Piraino, De Vito et al. 2004; Seipel and Schmid 2005; Boero, Schierwater et al. 2007). The induction of sexual reproduction might itself constitute a much higher demand on the medusa impeding complete long term cell maintenance and replacement, leading mechanistically to a lower threshold for a system failure and a higher probability of an earlier death of the individual medusa. However, the few purely asexual medusae we observed in our experiments (only in successive SL, TH and TL cohorts) did not live longer than the sexual ones, although this pure asexuality does not imply that no sexual potential or pathways had been present or

(unsuccessfully) induced. *Eleutheria* medusae show a remarkable plasticity regarding their activated or suppressed sexuality, which could most likely be a stochastic result of vegetative allocation and proliferation of competing interstitial cell (I-cell) lines of different (sexual/asexual) expressions within each medusa, as discussed already in Hauenschild 1956 & 1957. *Eleutheria* does not seem to have a fixed "Keimbahn" and a strict germ/soma segregation (Hauenschild 1957). The complexity difference between the stolonal polyp life stage and the medusa might therefore promote the evolved life-stage biodemography we have observed in our experiment. The mode of bisexual self-fertilization could constitute a 'reset' of the stem cell potential within the genet by forming new solitary zygotes of the clone, with an (nearly) identical genotype (Williams 1975; Schierwater and Hauenschild 1991; Ender 1997), enabling the genet to assemble a new independent ramet line untouched by effects of possibly previously accumulated damages or stem cell losses within the medusa. Via this proposed mode, the genet would maintain its survival through time with varying, possibly even increased fitness over time (by increasing its ramet population with time), overcoming the separate dead end scenarios for each of the ramet lines which may by itself not necessarily be mechanistically or physiologically inevitable but which evolved as optimized adaptation to the specific seasonal and organismic features. Additionally, by sexual reproduction the genet always gets the bonus of distributing its presence on two (three including the short term planula) life stages with different characteristics and requirements, extending the stolonal polyp stage as a perennial backup stage with dispersing medusa as 'bonus' units not totally necessary for the survival of the genet but offering the advantage to spread the presence of the genet.

Still unclear is why it seems to be an advantage for *Eleutheria* not to cross fertilize and only shows signs of self-fertilization as studied and discussed by Schierwater and Hauenschild 1991 and Ender 1997.

This molecularly confirmed finding concurs at least with the biological features of *Eleutheria* with its sessile mode of living of both medusa (semi-sessile) and polyp stages and a limited dispersal ability via the short-termed (several days according to own observations) planulae (Jackson 1986). Recently it has been shown that even with only mitotic recombination extensive chromosomal reshuffling can drive the evolution of virulence in a fungal, strictly asexual plant pathogen (de Jonge, Bolton et al. 2013), opening up ways of understanding how clonal lines, if meiotic/mitotic or both through several stages, can persist and react to selection forces over long evolutionary time scales. Indeed, the age of the collected clone lines of *E. dichotoma* has been estimated to be .2 – 2.4 Million years, according to 16S-mtDNA analyses (Ender 1997). One haplotype-line, collected on Mallorca, was even estimated to be 5 – 10 Million years old. This renders clonal lines of *E. dichotoma* among the "oldest" organisms ever measured.

Moreover, it has been found in plants that inbreeding and selfing is accompanied by major changes in the offspring's transcriptome by epigenetic modifications, i.e. gene silencing/activation by methylations and demethlyations of the genome, affecting inbreeding depression effects directly (Vergeer, Wagemaker et al. 2012; Cheptou and Donohue 2013). This opens up new ways of thinking about inbreeding depression, seeing it not anymore as an unavoidable evolutionary consequence and constraint for every (inbred/selfed) organism imposed by the accumulation of recessive homozygous deleterious mutations. The epigenetic pathway, influenceable by both environmental and genetic factors (Cheptou and Donohue 2013), may have a much more concise role which even allows negative inbreeding effects to be altered and avoided epigenetically in clonal lines (genets) of e.g. *Eleutheria* over evolutionary long time scales. Additionally, inbreeding leading to more homozygous and less variable genets may have more chances to survive selection in less complex organism where a relatively low number of processes and interactions have to be maintained. Optimally

inbred genets may be achieved and sustained when all processes are maintained in an optimal state suited for the ecological niche the organism conquered – non beneficially mutated ramets of the genet get selected out, beneficially mutated ramets will spread and add more variability and plasticity to the dynamic clone - the same is true for clonal vegetative propagation. In more complex organisms more processes are prone to failure and thus they are more vulnerable to negative inbreeding effects. We hypothesize an organism dependent complexity threshold for inbreeding depression - a threshold from which inbreeding, leading to increased homozygosity, is not successful anymore due to an increased failure vulnerability with increasing organism complexity. Ultimately, with increasing organism complexity, sex via cross fertilization becomes necessary to recombine and refresh genomes to overcome inbreeding depression effects.

There is still the possibility that the qualitative decline with successive medusa generations is just an experimental artifact and a matter of random heterogeneity between cohorts, since it could not be clearly confirmed in the LFR survival data, 'only' three generations were tested, a huge heterogeneity within cohorts for all measured traits was observed and, of course, more individuals per cohort would give an even clearer picture – however, this is rather unlikely. The LFR treatment may have somehow masked and prevented the qualitative decline in survival – in contrast to reproduction - since this low food stress, i.e. caloric restriction, could have induced hormetic counter responses (Calabrese and Baldwin 2003; Stebbing 2003; Parsons 2005; Mangel 2008; Rattan 2008) in all LFR medusae, especially in successive generations. We observed similar hormetic responses to low food stress and bisection before in *Hydra*, for example (Chapter III; Chapter V). Indicators for this kind of hormetic reaction are, besides the absent decline in mean survival time with successive generation, the relatively long average survival of SL medusae compared to the other LFR and even HFR groups and that both SL and TL medusae have a higher average survival than their

counterparts SH and TH, respectively. Alternatively, or additionally, a trade-off could be seen here, with proportionally more resources allocated to maintenance than to reproduction in the LFR medusa compared to the HFR, especially in the two succeeding LFR generations. If both effects are at work simultaneously, happen to be coupled and the relative share of each to the found patterns in these cases remain unclear – to us, the hormesis effect seems to have a far larger impact considering the exceptional survival times of SL and TL, the low energy availability to produce offspring in the LFR anyway and the similar reproductive declines with succeeding generations in both feeding levels. However, we can conclude that the particular response to low food stress in *E. dichotoma* medusae is not a pure hormesis effect without any costs, but instead coupled to a reduction in offspring output and an eventual resource allocation trade-off between maintenance and reproduction. The parental exposure to the low food conditions additionally seems to play a crucial role for the hormesis/trade-off response since the primary medusa of the LFR did not show longer average survival compared to their counterparts in the HFR.

CONCLUSIONS

Aging patterns in *E. dichotoma* are multisided. We found the aging patterns between the polyp and medusa ramet life stages to be highly different, with indices for non- and negative senescence in polyp colonies, displaying a flat and low mortality, accompanied by a rather continuous, feeding dependent asexual reproduction output, producing polyps and medusae. In contrast, medusa ramets displayed a distinct hump shape regarding mortality and both vegetative and sexual reproduction output, suggesting a "hump shape senescence" pattern. Furthermore, a vast heterogeneity of both survival and reproduction seems to be extant within the isogenic ramet life stages, especially within the medusae.

Strikingly, we observed a trend towards a quality decline with successive medusa generation, which was more emphasized in the high feeding regime. We suggest that hormetic responses towards the low feeding stress, coupled to a maintenance-reproduction trade-off in favour of maintenance, may have masked the qualitative decline in successive medusa generations in the low feeding regime. The quality decline may be an indicator of adaptation to the reported seasonal occurrence of *E. dichotoma* in the Mediterranean.

The aging pattern of *E. dichotoma* genets still remains unclear, also in light of missing cross-fertilization in *E. dichotoma.* All indices we found point towards a negative senescent pattern at the *E. dichotoma* genet level (i.e. all polyp colonies of a genet combined with their medusa offspring and again their self-fertilized inbred offspring), i.e. the clone or haplotype level, similar to stony corals (Babcock 1991) and most likely many more basal metazoans and other organisms capable of asexual reproduction (see Jones et al. 2014 for more negative senescence examples). The extremely old age estimates of *E. dichotoma* haplotypes, ranging from .2 to 10 Million years of age (Ender 1997), render *E. dichotoma* among the "oldest" organisms ever measured and strengthen our assumptions.

The case of *E. dichotoma* displays beautifully the wide range and diversity of aging patterns not only between, but also within species. More demographic field and laboratory studies of basal metazoans with various ramet life stages are needed to verify our conclusions. A further very promising target of research will be to find the (epi-) genetic pathways controlling the diverse aging patterns.

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FIGURES AND TABLES

Table 1. Censored observations and median survival time for each generation, by feeding regime

Feeding regime	Generation	Censored n (%)	Median survival (days)
High	Primary (n=30)	2 (6,7%)	82
	Secondary (n=30)	4 (13,3%)	73
	Tertiary (n=30)	0 (0,0%)	66
Low	Primary (n=30)	3 (10,0%)	93
	Secondary (n=28)	2 (7,14%)	125
	Tertiary (n=26)	2 (7,7%)	91

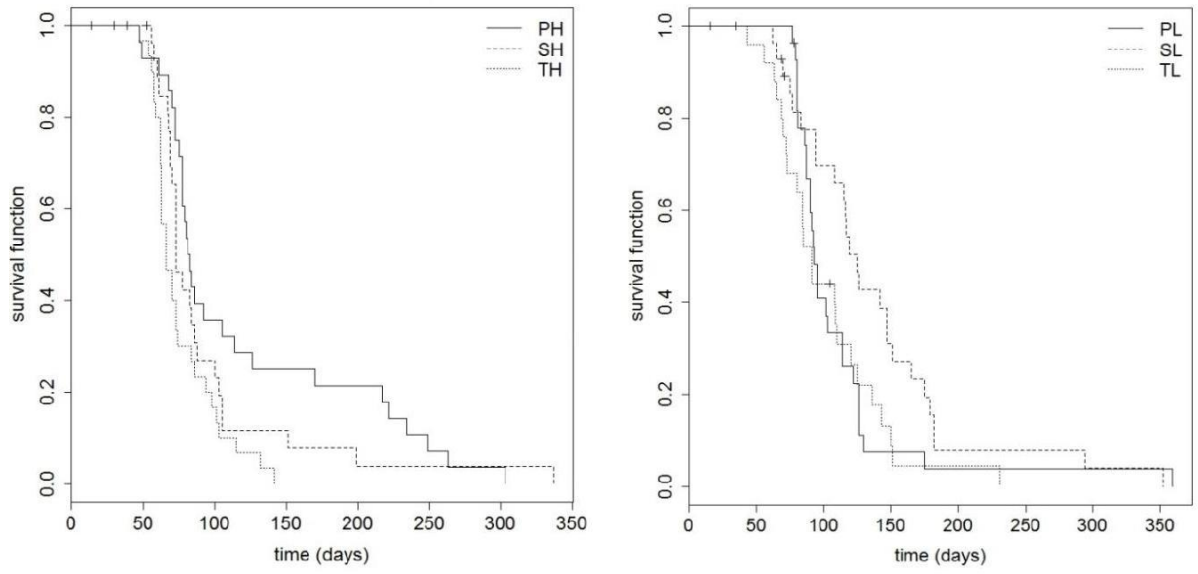

Figure 1. Kaplan-Meier estimates of the survival functions for each generation (P=Primary; S=Secondary; T=Tertiary), high feeding regime (left) and low feeding regime (right).

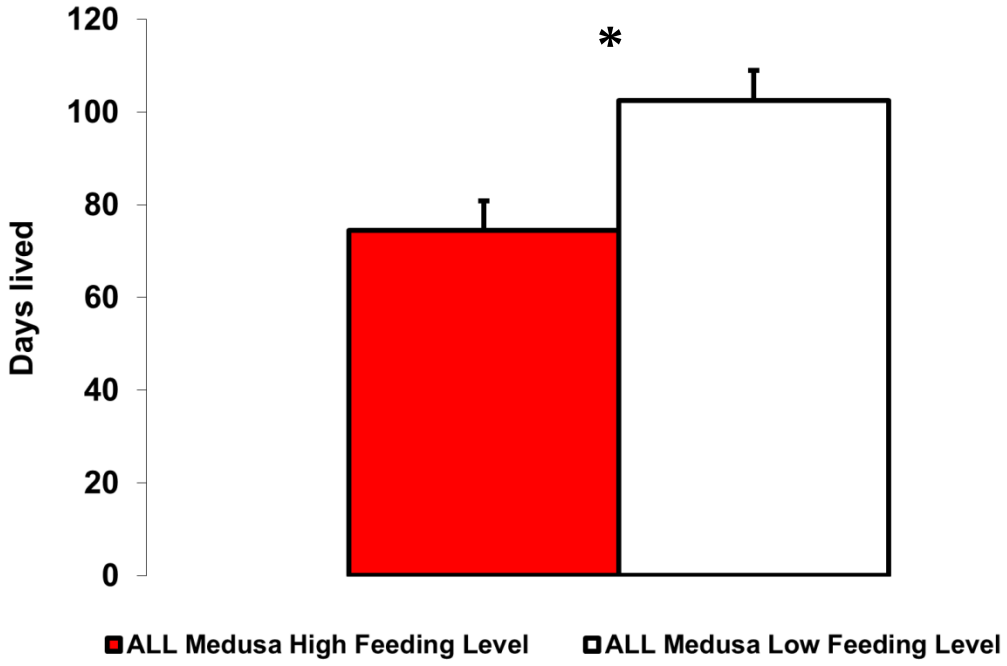

Figure 2. Median medusa survival. High- versus low feeding, * indicates a significant difference (Mann–Whitney U test, $p<0.001$). Error bars represent standard errors.

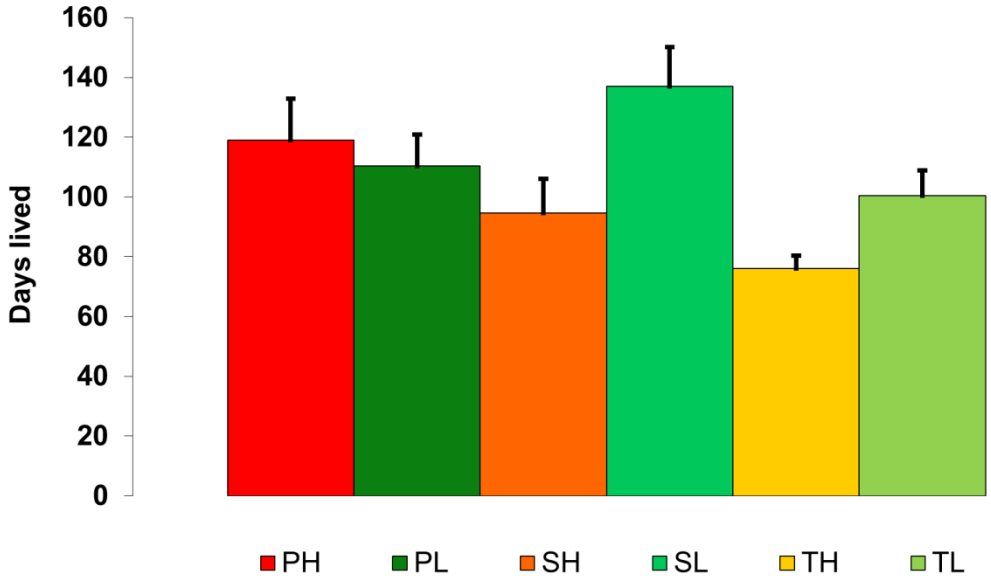

Figure 3. Mean medusa survival. All cohorts separated. P, S and T stand for Primary, Secondary and Tertiary medusa cohort, H and L represent High and Low feeding regime. Error bars are standard errors.

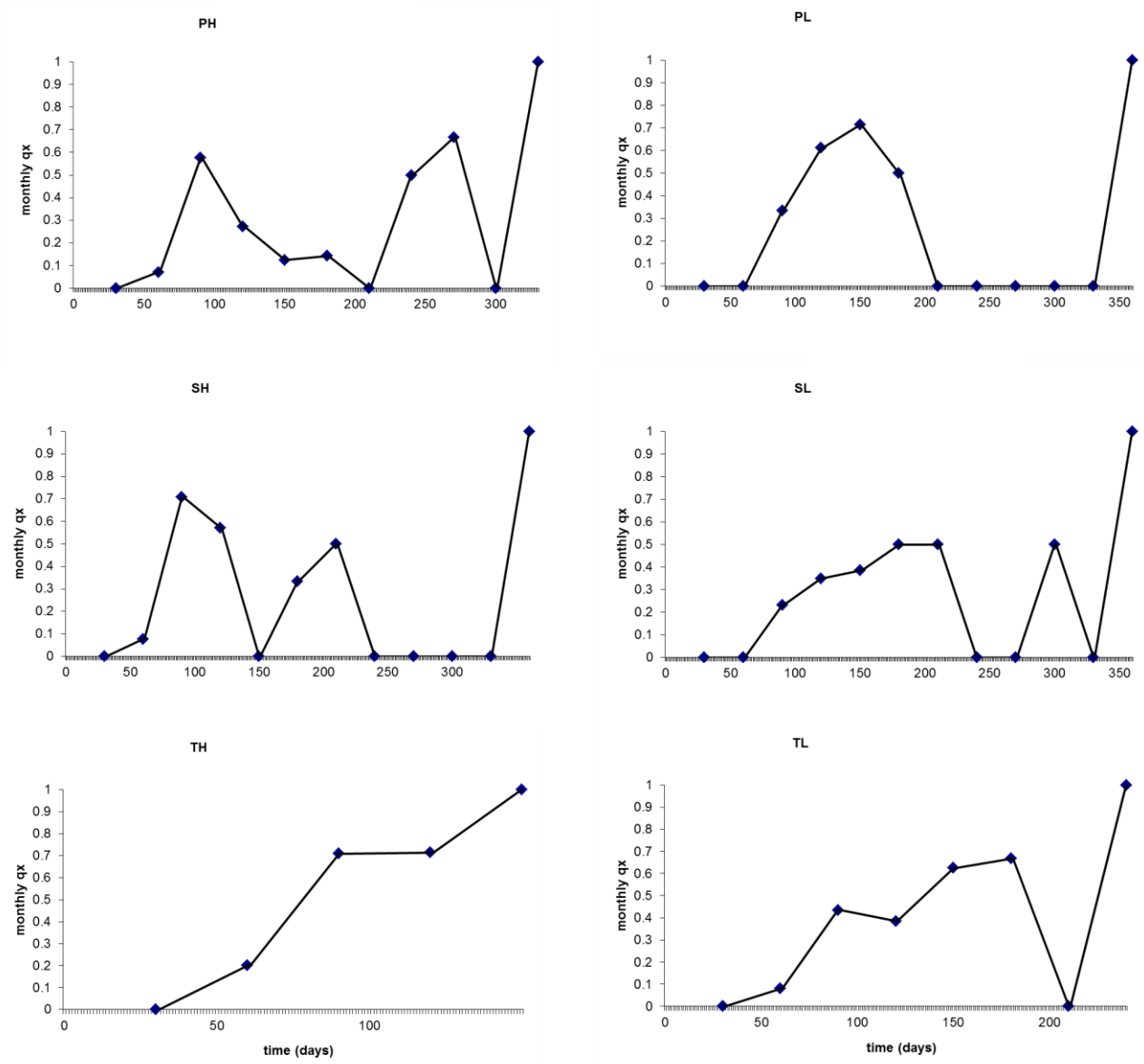

Figure 4. Raw monthly medusa mortality (qx). All cohorts separated. P, S and T stand for Primary, Secondary and Tertiary medusa cohort, H and L represent High and Low feeding.

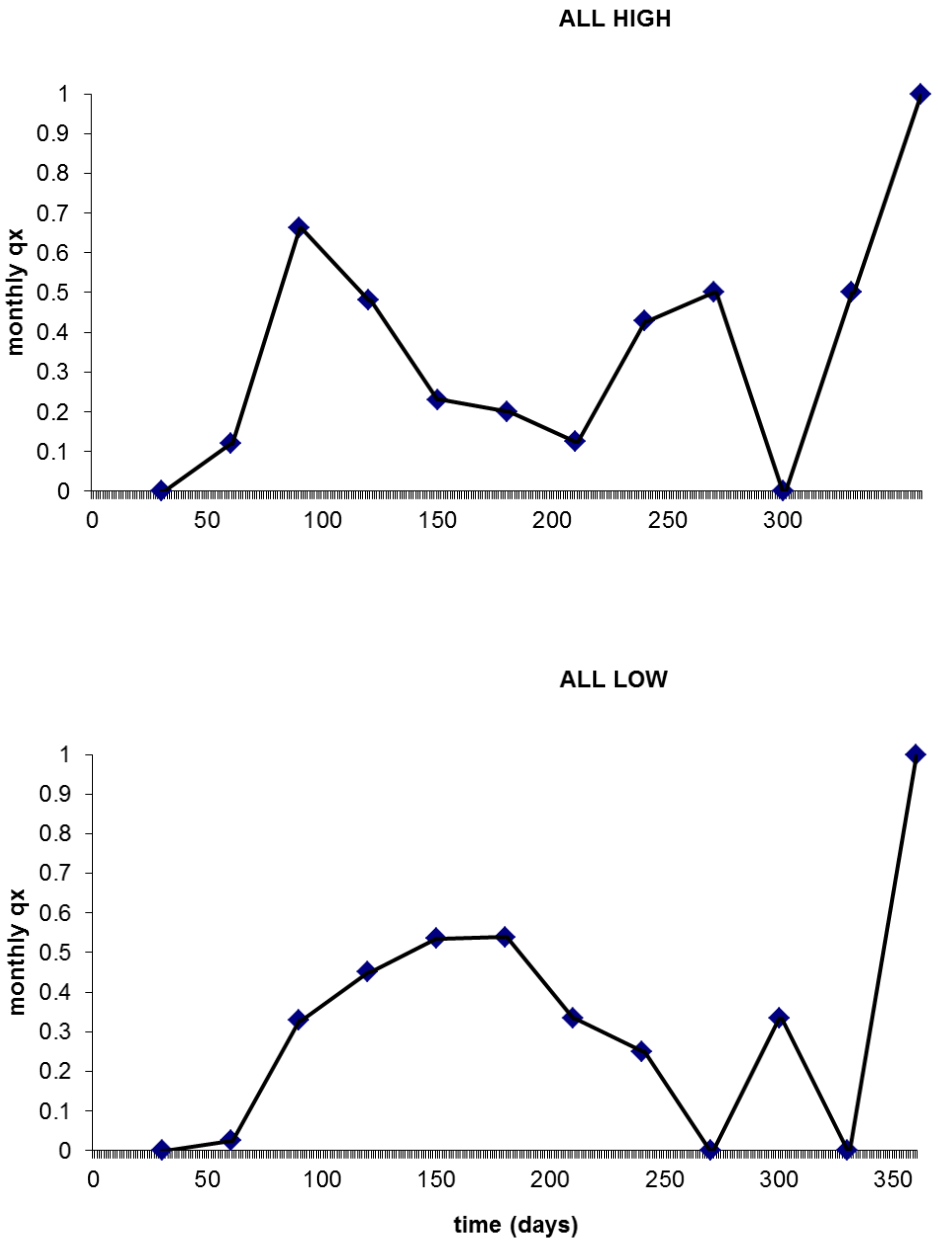

Figure 5. Raw monthly medusa mortality (qx). High- and low feeding regimes separated.

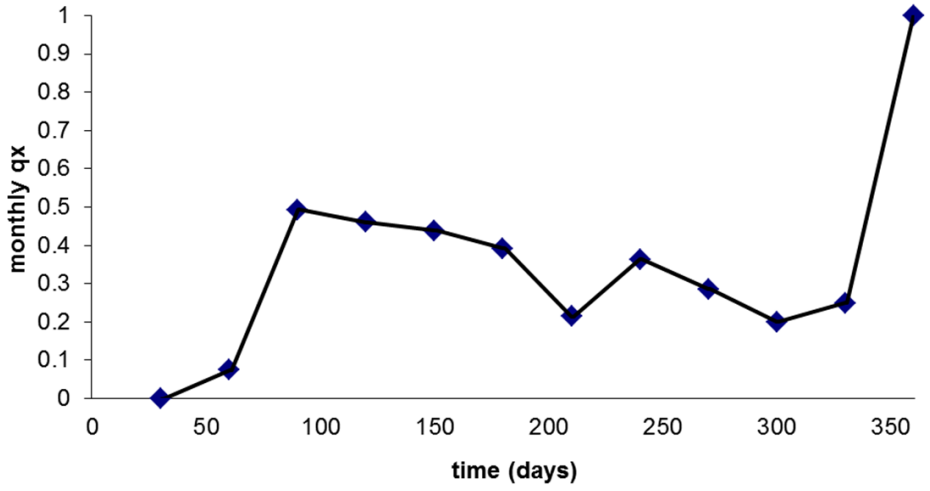

Figure 6. Raw monthly medusa mortality (qx). All cohorts combined.

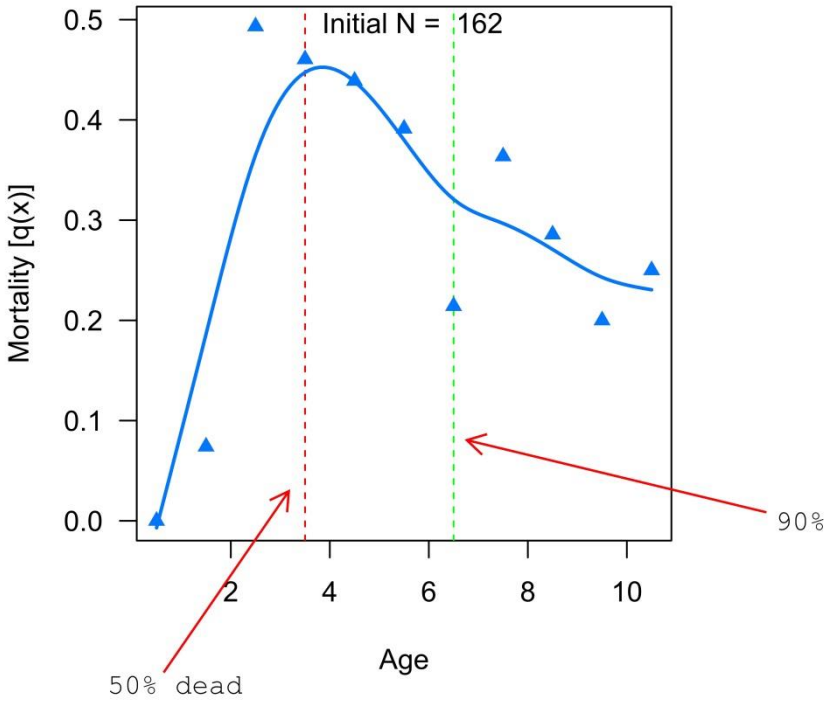

Figure 7. Smoothed monthly medusa mortality (qx) using a Generalized Additive Model (GAM). All cohorts combined. The last month interval with the last three medusa deaths is excluded for the shape fit.

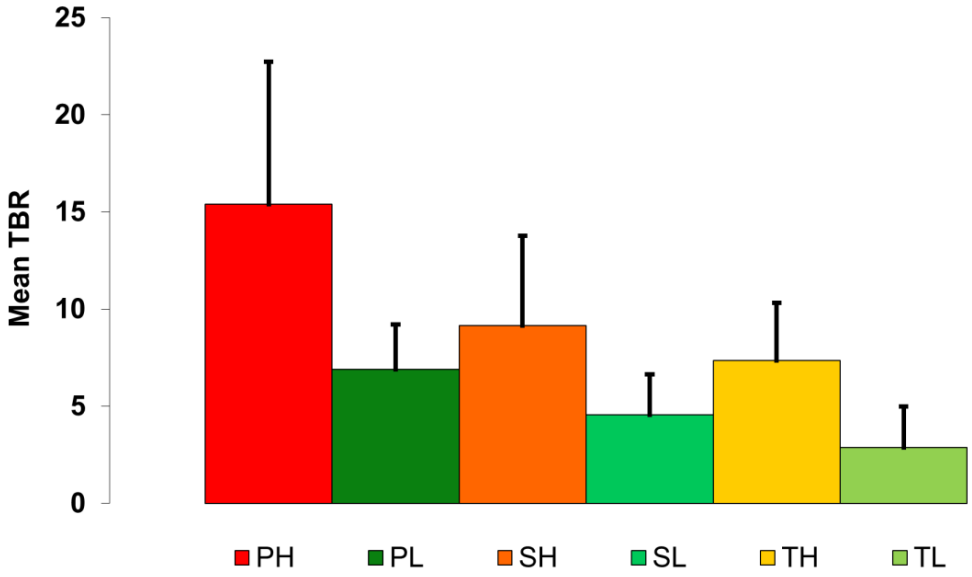

Figure 8. Mean Total Bud Release per medusa (TBR). P, S and T stand for Primary, Secondary and Tertiary medusa cohort, H and L represent High and Low feeding regime Error bars are standard deviations.

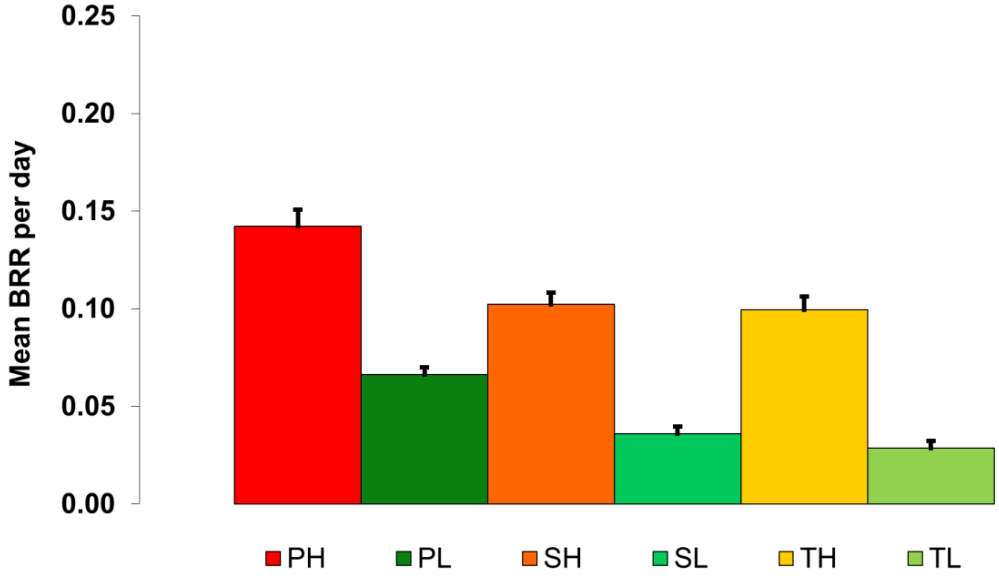

Figure 9. Mean Bud Release Rate per medusa per day (BRR). P, S and T stand for Primary, Secondary and Tertiary medusa cohort, H and L represent High and Low feeding regime Error bars are standard errors.

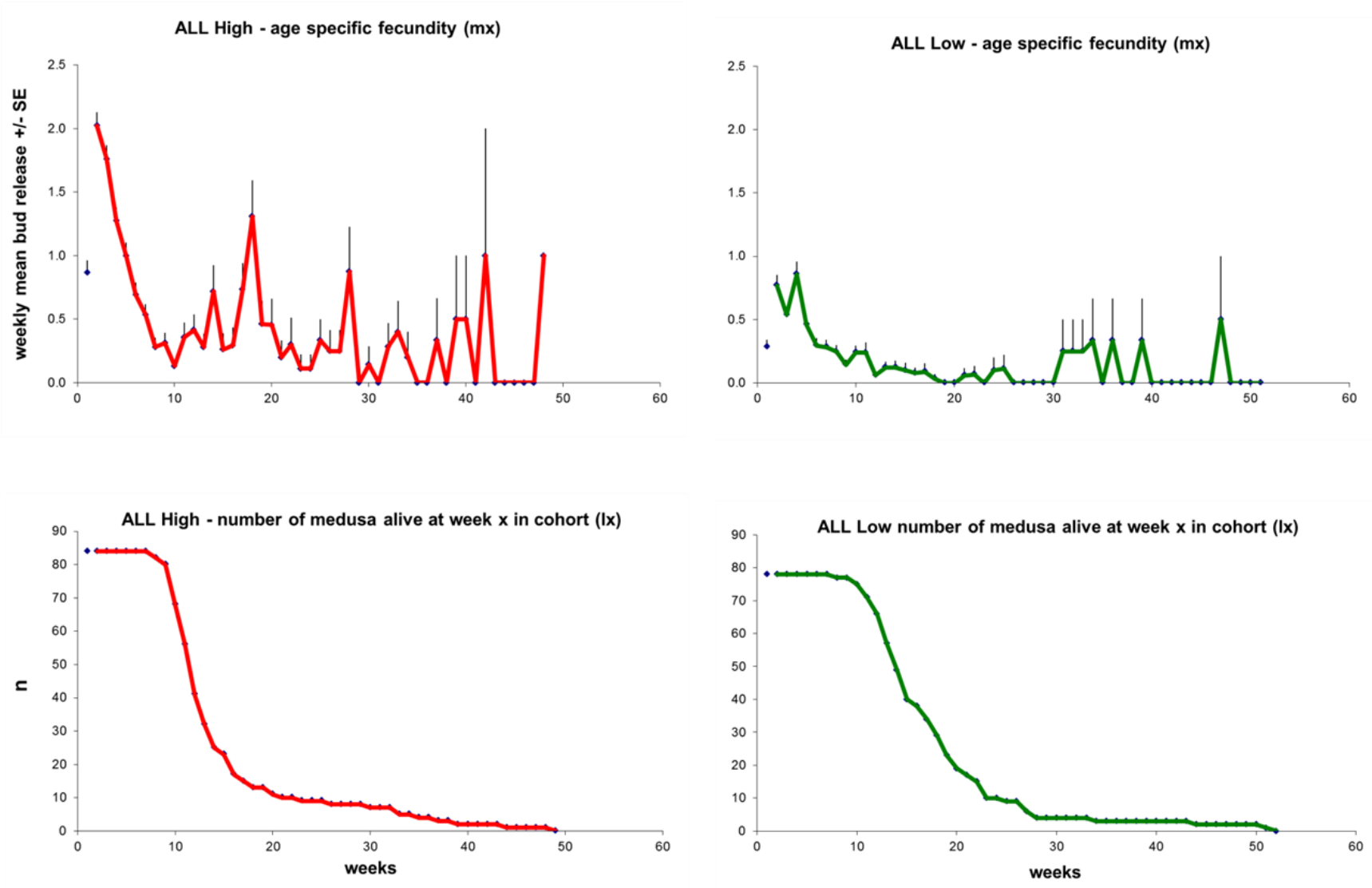

Figure 10. Age-specific asexual fertility. Mean weekly bud release per medusa (upper graphs) and parallel medusa survival (lower graphs). Left (red) graphs represent all HFR cohorts combined, right (green) graphs represent all LFR cohorts combined. Error bars are standard errors.

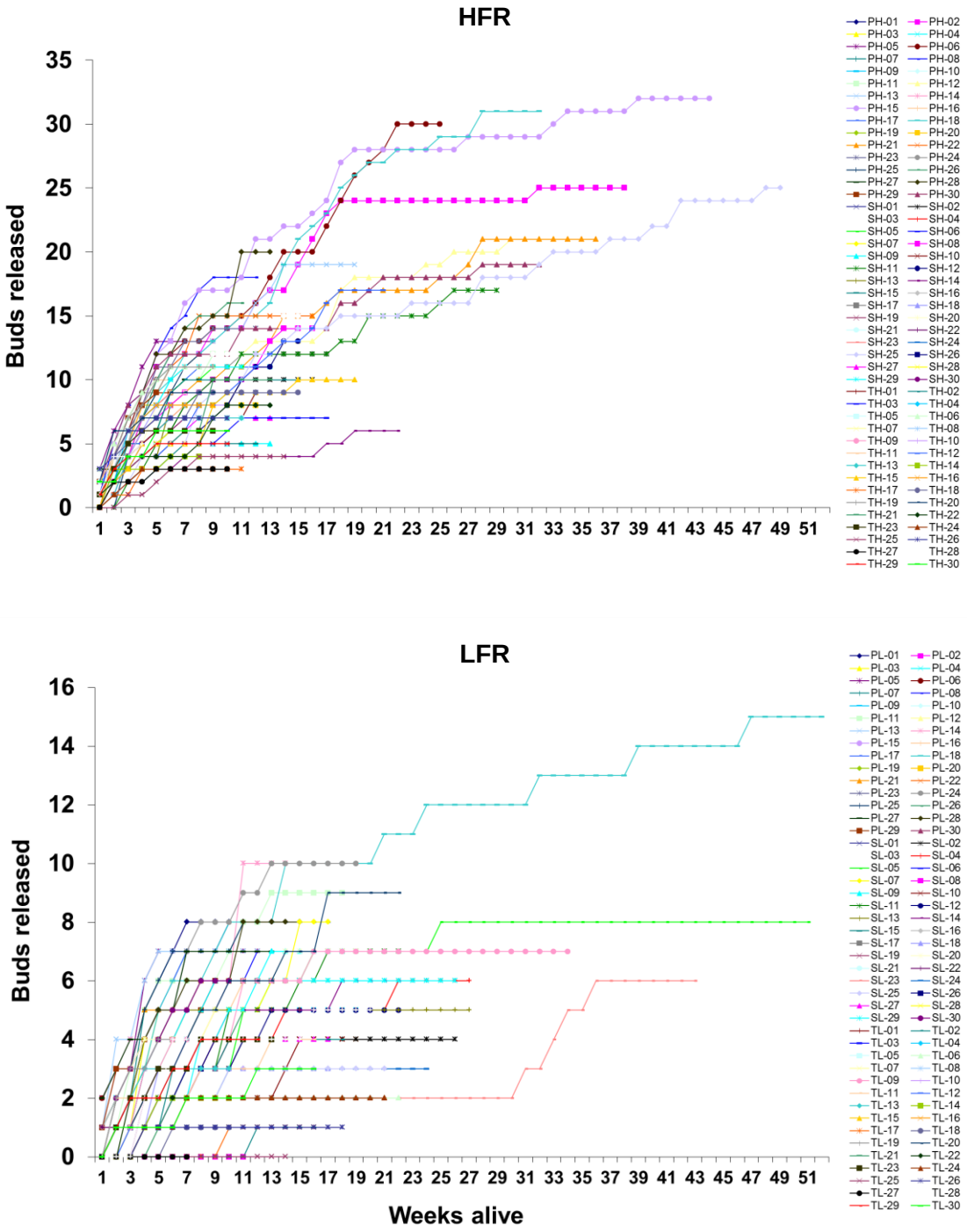

Figure 11. Cumulative bud release. Upper graph represents all HFR cohorts combined, lower graph represents all LFR cohorts combined.

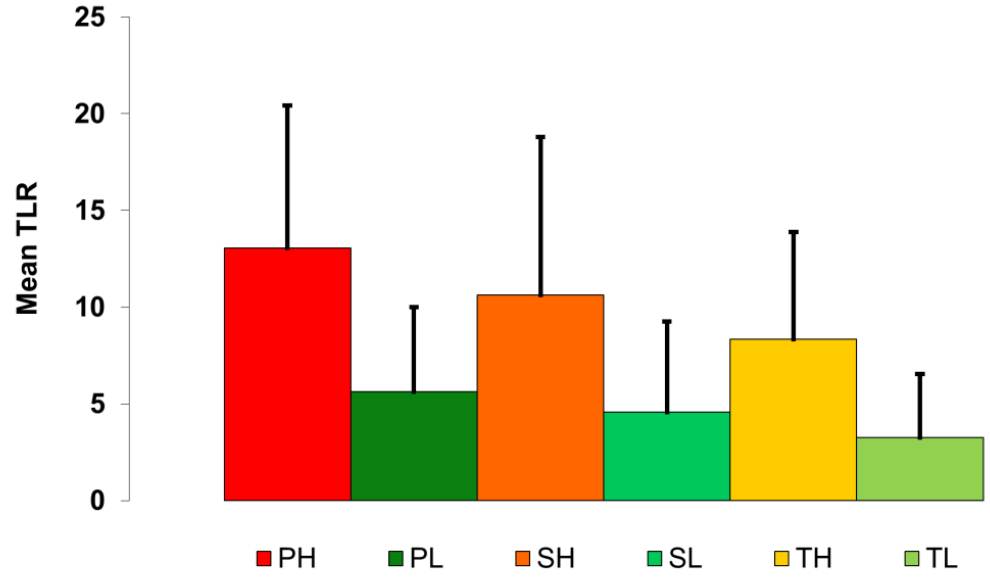

Figure 12. Mean Total Larva Release per medusa (TLR). P, S and T stand for Primary, Secondary and Tertiary medusa cohort, H and L represent High and Low feeding regime Error bars are standard deviations.

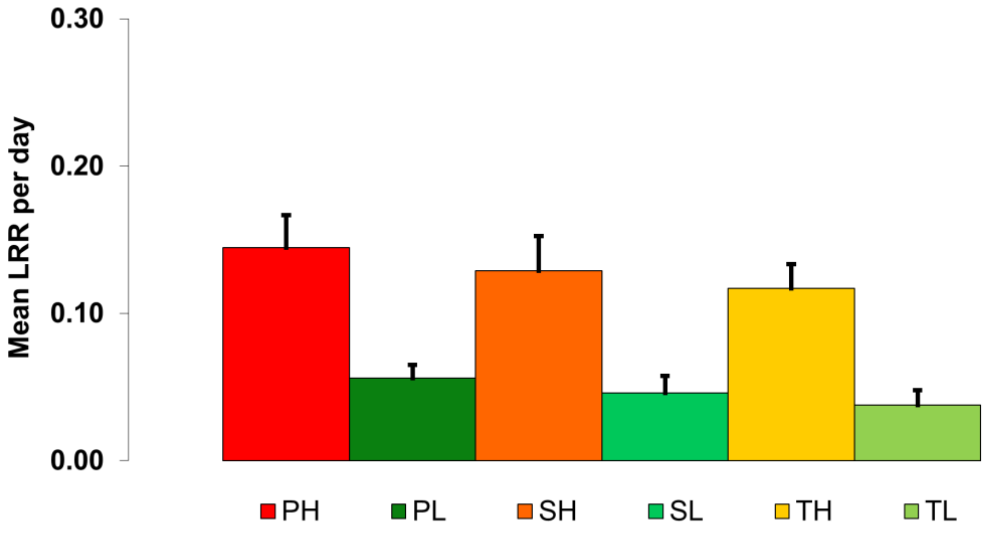

Figure 13. Mean Larva Release Rate per medusa per day (LRR). P, S and T stand for Primary, Secondary and Tertiary medusa cohort, H and L represent High and Low feeding regime Error bars are standard errors.

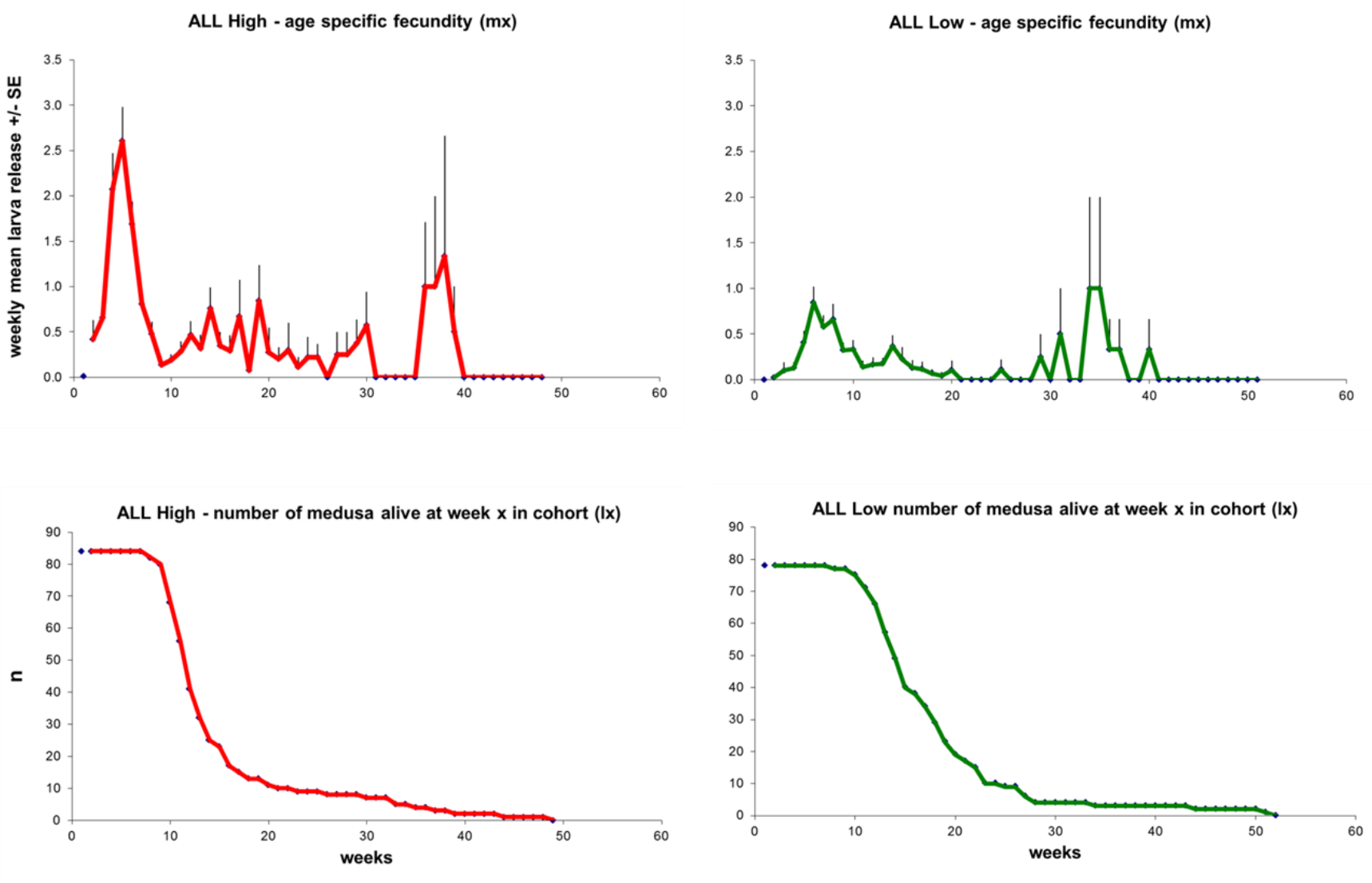

Figure 14. Age-specific sexual fertility. Mean weekly larva release per medusa (upper graphs) and parallel medusa survival (lower graphs). Left (red) graphs represent all HFR cohorts combined, right (green) graphs represent all LFR cohorts combined. Error bars are standard errors.

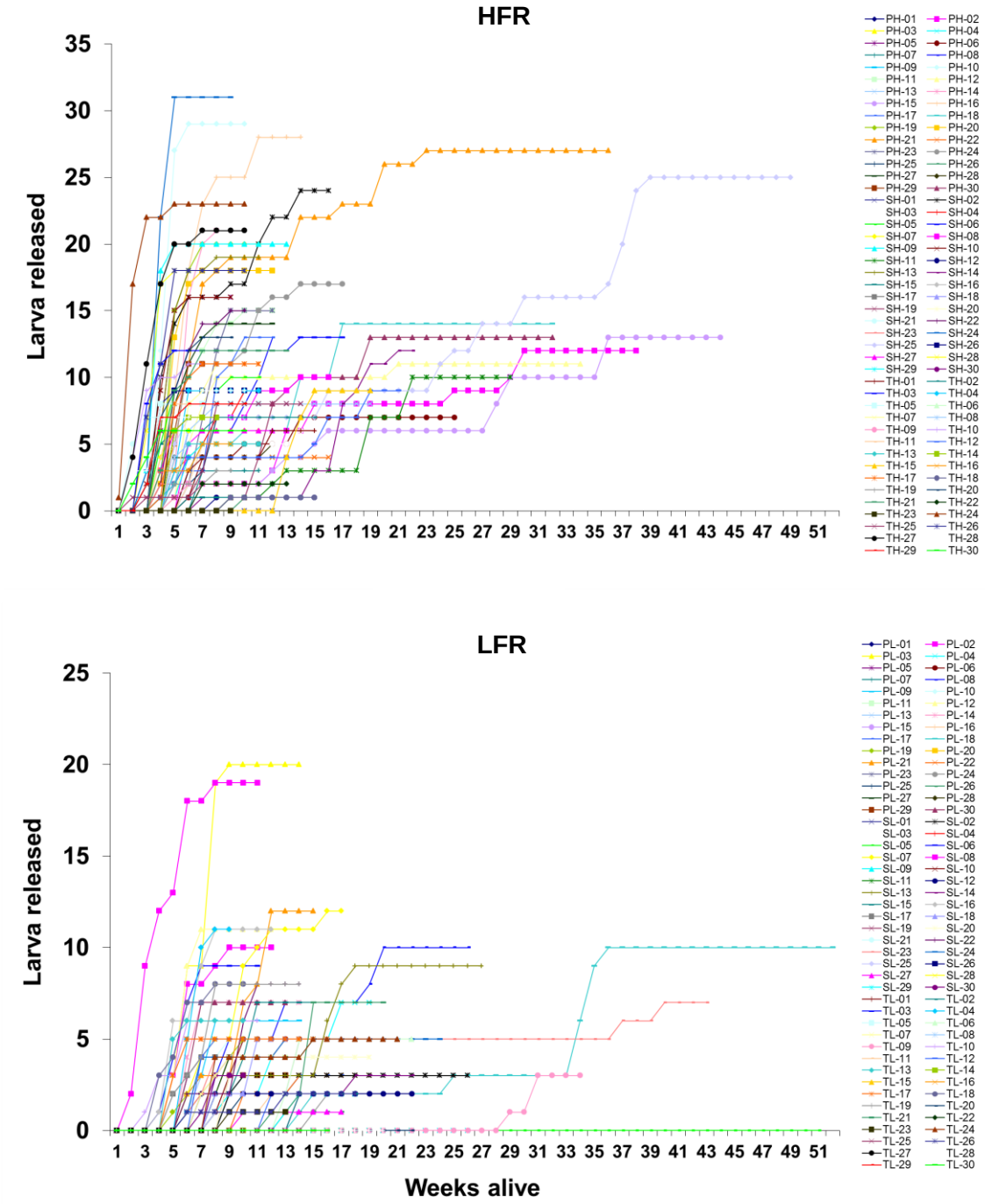

Figure 15. Cumulative larva release. Upper graph represents all HFR cohorts combined, lower graph represents all LFR cohorts combined.

CHAPTER II

Aging in a metagenetic basal metazoan II –

Demographic trade-offs in *Eleutheria dichotoma* (Cnidaria: Hydrozoa)

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ABSTRACT

Basal metazoans show large variations in life-cycle patterns, offering great opportunities to study the evolution of aging from a biodemographic point of view. Laboratory experiments were conducted with *Eleutheria dichotoma* (Cnidaria: Hydrozoa: Cladonematidae), a metagenetic hydrozoan with a crawling medusa, to test the resource allocation flexibility of a remarkably plastic and variable marine organism. For the first time ever in a longitudinal experiment, the survival, size and reproduction of isogenic *Eleutheria* were measured at different feeding regimes under constant conditions for both polyp and medusa stages. *E. dichotoma* medusae have the ability to reproduce both via asexual budding and bisexual self-fertilization. The polyps stay asexual, growing as stolonal polyp colonies with the ability to form medusae as buds. Our results suggest a huge phenotypic diversity within the studied clonal line (Omega). Huge heterogeneity could be observed in the survival and in the vegetative medusa bud production as well as in the bisexual self-fertilization and the resulting released planula larvae of isogenic medusae within the cohorts. We propose that the nutritional level affects the resource allocation trade-off in *E. dichotoma,* acting like a lifetime pacesetter for a medusa - the more food is available, the faster and compressed it lives. More specifically, the feeding level seems to influence the fine tuning of the adjusting screw of the trade-off between reproduction and maintenance. The missing heritability of the measured traits and the huge variability within all traits between medusa individuals and cohorts suggest a random phenotype generating process in *E. dichotoma.* However, another trade-off was observable between successive medusa generations, expressed by a quality decline with consecutive generations. This trade-off might have evolved in the light of the seasonal appearance of *E. dichotoma* medusae, whereby the force of selection pushed emphasis on the primary medusae. Our findings strikingly show and confirm that senescence is not inevitable. Instead, aging patterns can vary greatly between genet and ramet level and ramet life stages as well. Our results support the idea that the evolution of different aging paths and various life history strategies substantially depend on the type of the underlying trade-offs between survival and reproduction.

INTRODUCTION

Demographic aging patterns of basal organisms capable of both sexual and asexual reproduction are still poorly known and understood until today. Understanding aging from a demographic perspective still remains a major challenge in evolutionary biology. A vast variety of different aging patterns seems to be extant and spread across the tree of life (Baudisch 2008; Baudisch 2011; Jones, Scheuerlein et al. 2014; Chapter I). Species with senescent patterns, i.e. increasing mortality&/decreasing fertility with age, non-senescent patterns, i.e. non-increasing mortality&/fertility with age and even negative senescent patterns, i.e. declining mortality/increasing fertility with age, have just been started to be described and gathered in various databases (Martinez 1998; Martinez 2002; Vaupel, Baudisch et al. 2004; Jones, Scheuerlein et al. 2014; Chapter I). This immense variation in aging should depart one from the generally negative connotation with the term aging, and more precise terms such as non-/negative senescence should be used instead. In this paper we follow up on chapter I, in which mortality and fertility patterns of the metagenetic hydrozoan *Eleutheria dichotoma* have been in focus, discussing here the plasticity of the described aging patterns in *E. dichotoma* and the demographic trade-offs we observed during the first extensive biodemographic study on this basal metazoan.

S. C. Stearns stated very profoundly, that *"Trade-offs represent the costs paid in the currency of fitness when a beneficial change in one trait is linked to a detrimental change in another"* (Stearns 1989). And Kirkwood and Rose claimed that, according to the disposable soma theory, which assumes a trade-off of resource allocation between maintenance and reproduction, the evolutionary optimum would lead directly to senescence (Kirkwood 1977; Kirkwood and Holliday 1979; Kirkwood and Rose 1991). But considering the vast amount of positive and negative aging patterns being discovered now, also within species between

different life stages, this general claim needs to be revised. More precisely, the evolution of different aging paths and various life history strategies substantially depend on the type of the underlying trade-offs between survival and reproduction (Baudisch 2009). These trade-offs need to be studied to fully comprehend ageing.

E. dichotoma is an ideal organism to study the demographic trade-offs affecting aging. *E. dichotoma* develops from a live released, free-swimming planula larva into a polyp colony, which can propagate asexually by growing more polyps formed in a stolonal network and additionally medusae buds at the polyps bases. The medusa can propagate both vegetatively and sexually by budding secondary medusae and self-fertilize itself to brood and release planula larvae (Hauenschild 1956; Hauenschild 1957; Schierwater 1989a; Schierwater and Hauenschild 1990). Both sexual and vegetative offspring of a clone can hence be allocated to that same clone since no indications of any cross-fertilization has been found in *E. dichotoma* to date (Schierwater and Hauenschild 1991; Ender 1997). Interestingly, both of the exemplary Cnidarian life cycle stages seem to show different aging patterns, as shown in chapter I – polyp colonies show signs of non- or even negative senescence when including the high recruitment mortality, whereas medusae showed an explicit hump shaped mortality pattern, with the highest death risks occurring usually after initial sexual and asexual reproduction bursts. In this paper we want to further discuss our findings on the fine tuning of *E. dichotoma's* aging patterns, how the different traits we measured affected each other in respect of the different feeding levels we chose and the successive medusa cohorts we investigated. How heterogeneous and how heritable are reproductive patterns, size and lifespan? Here, we pursue these questions with measurements of the survival, size, and reproduction measured at different feeding regimes for both polyp and medusa stages kept under controlled laboratory conditions to gain more insights into the plasticity of the trade-off system of *E. dichotoma.*

MATERIAL AND METHODS

Study Organism

The Hydrozoan *Eleutheria dichotoma* Quatrefages, 1842 (Hydrozoa, Athecata, Cladonematidae) is a metagenetic, nowadays cosmopolitan marine Cnidarian inhabiting the littoral zone (Hauenschild 1956; Fraser, Capa et al. 2006). A brief description about the biology and ecology of *E. dichotoma* can be found in chapter I at this point.

Culturing conditions

We used individuals of a single clone (Ω) of *E. dichotoma*, derived from the laboratory of the ITZ Ecology and Evolution, Tieraerztliche Hochschule Hannover, Germany (ITZ). The original medusa specimen for this clonal line was collected in 1984-1986 from the Mediterranean shores of Banyuls-sur-Mer, France (Schierwater 1989a; Ender 1997). Culturing conditions were modified after Hauenschild (1956) and Schierwater (1989). Both polyp and medusa stages were cultured in the laboratory in artificial seawater of 35 ‰ ("Reef Crystals" by Aquarium Systems mixed with Milli Q filtered water) in either glass dishes of about 50-100 ml or plastic six-well microwell plates with about 9 ml saltwater per well. Further culturing details are described in chapter I.

Experimental Design

The isogenic medusa cohorts for the biodemographic monitoring study were built up from a single and separated polyp colony of the original clone Ω (minimum age > 6 months, minimum of 6 live polyps during primary medusa isolation). This stem parent colony was continuously well maintained in a separate glass dish and fed with a gush or *Artemia* three

times a week. Three cohorts were generated with 60 medusae per each cohort, whereby half of each cohort were fed with 6 *Artemia* per week (= High feeding regime (HFR), fed Mo, Wed, Fr) and the other half with 2 *Artemia* per week (= Low feeding regime (LFR), fed Mo & Fr). Feeding rates were chosen according to a pilot experiment and experiences in previous culturing and experiments (Hauenschild 1956; Schierwater 1989a; Raudonat 1995). The first medusa cohort , so called primary medusae, was constituted by the first medusae budded by the parent polyp colony (PH = primary medusa at HFR, PL = primary medusa at LFR). The first medusa bud of each isolated primary medusa was kept again to generate the second cohort, so called secondary medusae (SH = secondary medusa at HFR, SL = secondary medusa at LFR). To prevent any direct inter-generational transmission of signals, we made sure that only these medusae were taken for the secondary medusa cohort, which have not been already in development when the primary medusae were still attached to their parent polyps. The same procedure has been applied to the third cohort, only that these tertiary medusae were now isolated from their respective parent secondary medusa individuals (TH = tertiary medusa at HFR, TL = tertiary medusa at LFR). Further details on the experimental design are described in chapter I.

Our experimental design allowed us to analyse for the first time the complete demographic survival and both sexual and asexual reproductive patterns of the parent stem polyp colony, three successive medusa generations and one polyp offspring generation of the primary medusae at two different feeding levels, respectively.

Medusa size was additionally monitored and controlled for by taking successive comparable pictures of all living cohort medusa individuals four times during the experiment under the microscope. Two- and three dimensional surface areas were calculated (Schierwater 1989b) and used to compare medusa size and growth.

Analyses

SPSS software were used for the statistical analysis of the obtained data. We tested for cohort and feeding level differences regarding size (Mann-Whitney U tests) and checked for heritability of survival, asexual- and sexual reproduction (linear regressions). Trade-offs were investigated via linear regressions between measured traits and several quotients: budding rate per day (BRR)/survival, larva release rate per day (LRR)/survival, BRR/size and LRR/size. The quotients were applied as a kind of trade-off measure between maintenance (survival), growth (size) and reproduction (BRR and LRR). In the example of BRR/survival, a low value close to zero implies no asexual reproduction at all (all resources are indicated to be focused on individual maintenance), and an increased value implies a higher asexual reproduction-resource allocation contrasted with survival. The quotients regarding size are just rough measures, because we took size, in this case, as average individual total surface area constituted by maximum four successive photo-size measurements taken within 3 months (see above in experimental design). All tests were performed in compliance with the respective statistical requirements of the data. Furthermore, we used graphical data representation to assess demographic patterns, where appropriate.

RESULTS

The demographic experiment with *E. dichotoma* suggests a huge phenotypic diversity within and between cohorts and life stages within the studied clonal line (Omega). Large variances can be observed in the survival and in the vegetative medusa bud production as well as in the bisexual self-fertilization and the resulting released planula larvae of isogenic medusae within the cohorts (see also chapter I).

Medusa Size

Cohort and feeding level comparisons

Medusae of the HFR were significantly larger than in LFR when comparing all cohorts together or each cohort separately, either in two dimensional or three dimensional total surface area extrapolations (Mann–Whitney U tests, $p<0.001$, fig. 1). Analysing and comparing size trajectories and trends throughout cohorts was left out because of only four conducted size measurements within three months of the experiments resulting in a resolution too coarse to capture accurate size trajectories with age and the possible changes and differences between cohorts through time, considering as well the strong influence of the varying reproductive states on size. Still, with the coarse data, general trends go towards smaller sizes in successive generations (at least in LFR and both feeding regimes together) and hump shaped size trajectories when plotted against relative individual medusa lifetime (in basically all groups).

Heritability of traits

Medusa Survival

No clear heritability patterns regarding survival could be found between generations in either HFR and LFR. Offspring lifespan is independent of parent lifespan in both feeding regimes comparing primary parent and secondary offspring medusae and secondary parent and tertiary offspring medusae (linear regressions, $p>0.05$). Also when comparing all primary parents with secondary offspring medusae (feeding regimes merged) and all secondary parents with tertiary offspring medusae no significant correlation could be found, although significant survival differences existed between HFR and LFR (cohorts combined) with longer survival times in LFR (Mann–Whitney U test, $p<0.001$, chapter I). Only a slight correlation could be found when comparing PH with TH (linear regression, $p<0.05$, $y =$

0.2107x + 50.135, R^2 = 0.468), indicating a low heritability of .21 as the slope of the regression, illustrating the much narrower distribution of lifespans in TH. Still, the coefficient of determination (r^2) is rather low due to the heterogeneity of lifespans. PL and all primary parents lifespans were not correlated with those of TL or all tertiary medusae, respectively.

Budding

No clear budding heritability patterns could be found between generations in either HFR and LFR. Linear regressions for total bud release (TBR) were not significant in HFR comparing PH with SH and SH with TH, in LFR comparing PL with SL and PL with TL. In contrast, PH and SL parent TBR was significantly correlated with TH and TL offspring TBR, respectively (linear regressions, $p<0.05$, $y = 0.1774x + 4.6624$, $R^2 = 0.1781$; $p < 0.01$, $y = 0.6356x - 0.3241$ $R^2 = 0.2805$). Merging feeding regimes and comparing generations, all three comparisons yielded significantly positive but weak regressions (P vs. S $p<0.05$, $R^2 = 0.106$; S vs. T $p<0.01$, $R^2 = 0.158$; P vs. T $p<0.001$ $R^2 = 0.368$;), which is mostly attributed to the significant TBR difference between HFR and LFR with HFR having generally a much higher TBR (Mann–Whitney U test, $p<0.001$, see chapter I). Maximum R^2 values were not higher than .368 in all significant correlations indicating the high variation of TBR in each of the cohorts and low linear relationship even between significantly correlated groups. The maximum slope, i.e. heritability was here between SL and TL with .6356, indicating the general decline of TBR in successive medusa generations.

Looking at BRR, none of the separate cohort comparisons showed significant heritability correlations. Merged feeding regimes resulted in highly significant ($p<0.001$) correlations throughout all three comparisons again, but as in TBR this is mainly attributable to the generally higher BRR in HFR compared to LFR (Mann–Whitney U test, $p < 0.001$, see chapter I). R^2 values were never higher than .504 in all significant correlations; the maximum

slope was between S and T with .683, indicating also the general decline of BRR in successive medusa generations.

Larva Release

Larva release was not a heritable trait. All comparisons between parent and offspring TLR and LRR in either HFR or LFR showed no significant correlations, except for comparing PL with TL TLR, which was significant (linear regression, $p<0.05$, $y = 0.4243x + 1.2633$ $R^2 = 0.1863$). Still, the R^2 value was very low again indicating the weak linear relationship (i.e. heritability) between the compared cohorts. Merging feeding regimes and comparing generations, two comparisons yielded significant correlations for TLR and LRR (S vs. T, P vs. T; P vs. S non-significant), but this is mostly attributed to the significant TLR and LRR difference between HFR and LFR with HFR having generally a much higher TLR and LRR (Mann–Whitney U tests, $p<0.001$, see chapter I). Additionally, R^2 values were never higher than .232 in all significant correlations indicating the high variation of TLR in each of the cohorts and low linear relationship even between significantly correlated groups. The maximum slope, i.e. heritability was here between PL and TL TLR with .4243, indicating the general decline of LRR and TLR in successive medusa generations.

Trade-offs

Survival vs. Size vs. Feeding Level

No correlation could be found between survival and individual average size over all four size measurement times (linear regressions - all cohorts combined and HFR and LFR separate, size both in 2D and 3D total surface area extrapolations, see fig. 2). However, medusae in the HFR were generally larger but lived shorter than in LFR when compared separately (see size results section, fig. 1 and chapter I regarding survival).

Survival vs. Reproduction

Survival vs. Sex

Sexual reproduction is not directly coupled with survival. Neither total larva output nor larva release rate were clearly correlated with survival (tested in all optional groups, r^2 always below 0.3). Still, the few long lived individuals had mostly rather low or medium LRR values in respect of their feeding level, without that TLR showed such a trend.

The onset of sexual reproduction, either measured as "days from birth to first seen embryo in medusa" or "days from birth to first released larva of medusa" was only well positively correlated, with survival in TL (linear regressions, $p<0.001$, $y = 1{,}4811x + 46{,}878$, $R^2 = 0{,}6027$ & $y = 0.865x + 56.319$, $R^2 = 0.769$, see figs. 3-4). All other cohorts were much more heterogeneous and without a clear linear trend when comparing these traits, with R^2 values ranging from 0.02 (PL) to 0.31 (PL). Still, the longest lived individuals tended to have a medium to late onset of sexual reproduction in respect of their feeding level (see *cumulative larva release*, chapter I). Lifespan correlated positively with "days from birth to last released larva" throughout all cohorts with R^2 values ranging from 0.73 to 0.94 (linear regressions, $p<0.001$). The "days lived after last released larva till death of medusa" showed only a correlation with total individual lifespan in both primary medusa cohorts (linear regressions, $p<0.001$, R^2 from 0.61 to 0.69), whereas in all others these two traits remained uncorrelated (R^2 0.03 to 0.21).

Survival vs. Asex

Longer survival increased the total bud output, as TBR was significantly positively correlated with survival in all cases exhibiting mostly convincing linear trends with variable data spreads (linear regressions, $p<0.05$, 0.2 (ALL LFR) as lowest and 0.68 (PH) as highest r^2, see fig. 5). BRR showed a different picture without any good correlation with survival (highest r^2

= 0.23 (PH)). The long lived individuals tended again to have BRR values at the lower end of the scale in respect to their feeding level, just as with LRR.

BRR/Survival Quotient – a trade-off measure

BRR/S is much higher in HFR than in LFR in accordance with food intake and TBR and BRR (Mann–Whitney U tests, $p<0.001$). Despite a strong spread, survival tended to be weakly negatively correlated with BRR/S in all comparisons (linear regressions, $p<0.05$, all cohorts separate, HFR and LFR separate and all combined, with r^2 from 0.19 to 0.57). Distinctively, all longer lived individuals tended to have very low quotients.

LRR/Survival Quotient – a trade-off measure

LRR/S is much higher in HFR than in LFR in accordance with food intake and TLR and LRR (Mann–Whitney U tests, $p<0.001$). Similar as in BRR/S, survival tended to be weakly negatively correlated with LRR/S in all comparisons, despite an even stronger spread (linear regressions $p<0.05$, all cohorts separate, HFR and LFR separate and all combined, with r^2 from 0.11 to 0.31). Again, all longer lived individuals tended to have very low quotients.

Sex vs. Asex

No directional trend of a trade-off was observable between sexual and asexual reproduction when comparing either total budding with larva output or budding- with larva release rate (linear regressions for all cohorts separate or together or HFR vs. LFR, with r^2 from = 0.0002 to 0.22.

BRR/Survival vs. LRR/Survival Quotients

Both quotients were quite independent of each other and showed no correlation in any

comparison (linear regressions for all cohorts separate, HFR and LFR separate and all combined, with r^2 from 0.0001 to 0.27).

Size vs. Reproduction

Size vs. Sex

Size was not correlated with sexual reproduction in neither comparison (linear regressions - TLR, LRR & LRR/S for all cohorts combined and HFR and LFR separate in 2D and 3D total surface area extrapolations, r^2 from 5E-05 to 0.14).

Size vs. Asex

Size was also uncorrelated with asexual reproduction in all comparisons (linear regressions - TBR, BRR & BRR/S for all cohorts combined and HFR and LFR separate in 2D and 3D total surface area extrapolations, r^2 from 0.0035 to 0.21).

BRR/ Size and LRR/Size Quotients – further trade-off measures

BRR/Size and LRR/Size quotients are much higher in HFR than in LFR in accordance with food intake and TBR, TLR & BRR and LRR (Mann–Whitney U tests, $p<0.001$). Despite a strong spread, size tended to be weakly negatively correlated with BRR/S in all comparisons (linear regressions $p<0.05$, all cohorts separate, HFR and LFR separate and all combined, with r^2 from 0.11 to 0.51). Distinctively, all larger individuals tended to have very low BRR/Size quotients. LRR/Size quotients revealed a different picture without a clear trend towards a correlation (linear regressions, r^2 from 0.01 to 0.32).

Survival was also independent of both trade-off measures in all comparisons (linear regressions, r^2 from 0.01 to 0.23). Strikingly, the longest lived individuals tended again to have quotients at the lower end of the scale.

BRR/ Size vs. LRR/Size Quotients

We found mixed results for these correlations. PH, SH, All HFR and all cohorts combined showed a trend towards positive correlations (linear regressions $p<0.05$, with r^2 from 0.22 (PH) to 0.83 (SH)). In all other cohorts and ALL LFR no correlation trend could be found (r^2 from 0.0004 to 0.08).

DISCUSSION

Trade-Offs

Not surprisingly, food levels had a significant impact on the growth and size of medusae. However, size is very plastic and flexible for *E. dichotoma* medusae. By trend, a freshly detached medusa bud starts off growing in size, producing buds vegetatively and larvae sexually to finally stop eating and shrinking again down to a completely disintegrating tissue clump. Sometimes, this decomposition process seems to happen rather fast, in an 'explosive manner', when intact, but tiny medusae, with already reduced tentacles, dissociate from one day to the next into many tiny tissue fragments. In addition to the impact of nutrition level on the size of a medusa found here, especially aging and reproductive state influence medusa size as well (own observations and Schierwater 1989b; Hadrys, Schierwater et al. 1990) - two traits which vary a lot within and between cohorts in our experiment.

Although we found no correlation between survival and individual average size of all four size measurement times, medusae in the HFR were generally larger but lived shorter than in LFR. The difference between HFR and LFR in size was consistent for cohorts compared separately or for all medusae of the respective feeding regime combined, but non consistent for survival because of one exception, the absence of a difference in primary medusa between HFR and LFR (see chapter I). The feeding regime shows hereby overall to have a significant

influence on the survival and size of medusae, but size itself is too variable within and between treatments, at least in the way the averaged size was measured here, to be a good predictor of the survival time of a medusa, or vice versa. Concordantly, reproductive output, either sexual or asexual, was not correlated with average medusa size. However, these are very rough comparisons and one needs to be aware of the heterogeneity within the feeding regimes and cohorts, especially as seen in the survival trend analysis (chapter I). Finer temporal resolution of size measurements will be needed to offer deeper insights into the age and cohort effects on size.

Surprisingly, in contrast to hypothesized, survival turned out not to be directly linked to sexual reproduction. Long-lived medusae tended to have low LRR (but not TLR) values and a medium to late onset of larva release (yet following longer sexual phases), but sexual onset and output of the majority of the medusae was rather heterogeneous and no reliable predictor of the survival time. After initial budding and larva release phases, medusae were either stopping both or showing long-term simultaneous reproduction, some switched back to pure vegetative reproduction before dying while others could have more (eventually simultaneous to bud production) larva release bursts or periods again as well. However, the long-lived individuals could be regarded as reproducing sexually at a generally rather slow pace in respect of their released larvae per lifetime, which might explain at least partially their longevity. Sexual maturity has been shown to state the onset of senescence in many cases for various species across the tree of live, since selection pressure usually declines with age after first reproduction (Hamilton 1966; Cui, Chen et al. 2000; Baudisch and Vaupel 2012). Additionally, sexual reproduction uses resources otherwise potentially allocated for somatic maintenance and adds more complexity to an organism comparing it to a ‘less spectacular’ vegetative mode of reproduction as present in *E. dichotoma*. The negligible influence of sexual reproduction on medusa survival is remarkable in the light of a declining selection

pressure with age, which should be present for *E. dichotoma* on the ramet level as well, in contrast to the genet level. The potential for vegetative reproduction plus the bisexual self-fertilization mode of *E. dichotoma* should both neutralize the decline of selection pressure with age for a clone. The population of ramets can grow with genet age by either vegetative polyp- or medusa reproduction and/or by inbreeding sexually, providing that a certain level of ramet sustenance is met. These characteristics lead to the potential of a greater fitness at older ages for a genet resulting in an increasing selection pressure with age for the genet, a selection directed towards maximizing genet lifespan leading to a pattern of non- or even negative senescence for the genet. However, the selection pressure on the ramet level is declining with age after first reproduction. Ramets could be exchanged and need not necessarily be sustained for a long time, at least not all, but the selection pressure on the genet would push towards larger and/or sustainable ramet populations, met either by 'fast pace' ramets (fast reproduction and death), or 'slow pace' ramets (slow reproduction and death), depending on the respective environmental conditions and hazards.

Considering this and the observed phenotypic variability between isogenic medusa ramets, stochastic phenotype allocation leading to vitality differences between medusae and random survival through physiologically demanding phases like the larva breeding and -release are presumably more dominant influences for lifespan outcome than individual sexual output itself. The mortality rise and peak at the hump-shaped mortality we observed throughout medusa cohorts in chapter I indicates the declining selection pressure for medusa ramets following their first vegetative and sexual offspring outputs. The following mortality decline might be explained by the stochastic phenotype variability, whereby the most robust individuals persist much longer than average and drag population mortality with age down again. The hump-shaped trajectories observed for vegetative and sexual reproduction (Chapter I) speak additionally for the declining selection pressure with age for medusa

ramets. Concluding, the crucial points about the negligible influence of sexual reproduction on medusa survival may not be the absence of a trade-off on the ramet-level between sexual output and survival, but the phenotypic heterogeneity of medusae, with medusae mixed along the 'fast' and 'slow' paced continuum, which obscure a clear trade-off between (sexual) output and survival. Both the low LRR and BRR levels of the long-lived individuals support this "vitality heterogeneity hypothesis".

The observation of positive correlations between medusa lifespan and TBR shows that vegetative reproduction is not averting but fostering medusa longevity, although BRR values tended again to be at the mid to lower end of the scale. Cell growth and division, which are coupled to vegetative budding, can also facilitate somatic maintenance, offering an easy 'by-the-way' mode to an individual medusa to sustain itself while allocating resources to 'growth' and supporting hereby its vegetative proliferation plus individual maintenance. The same should apply to the supposedly non-senescent perennial polyp colony (Chapter I), where cell proliferation can lead to colony growth by adding more polyps or stolonal tissue to the colony, to medusa production or to pure maintenance by replacing degenerated polyps, cells and tissues. Mechanistically, all these processes are very closely related, that no real trade-off between reproduction and maintenance or growth must exist. Similar observations have been made with isogenic *Hydra* polyps, which show no senescence on the ramet level (Martinez 1998; Schaible, Scheuerlein et al. in preparation for submission), whereby the question arises, if non-senescence for ramets is just a by-product of this special trade-off paradox. As discussed before, ramets need not necessarily be non-senescent to preserve the non-senescence of the genet, but they still are so in the case of *Hydra* and most likely *E. dichotoma* polyp colonies as well, in contrast to the *E. dichotoma* medusa (Chapter I). More demographic long-term studies comparing genet with ramet aging trajectories are needed to reveal these proposed patterns in many more organisms capable of vegetative reproduction.

Medusae might show the different ‘hump’ pattern due to their increased complexity in connection to their sexual reproduction which adds another resource allocation cost not ending up in maintenance. An additional important medusa death factor could be (still undetermined) seasonal environmental condition changes (see chapter I), posing higher mortality risks at winter times specifically for medusae, in contrast to presumably lower risks for the more sheltered, robust and less demanding polyp colonies, explaining further the differently emerged aging patterns between these two ramet life stages.

Expectedly, all longer lived medusae had low sexual and asexual trade-off quotients regarding size and survival in respect of the applied feeding level, indicating a resource allocation shift towards maintenance in these specimen. Generally higher quotients for both reproductive modes at higher feeding levels are simply explainable with more energy availability for reproduction. Considering the overall shorter lifespan of medusae at HFR compared to LFR, a higher food abundance seems to trigger a resource allocation shift towards more reproduction, including a size increase, affecting maintenance negatively hereby as well, although more total energy is available at HFR. Consequently, the idea of a positive low food stress effect on survival, i.e. calorie restriction (CR) effect (Heilbronn and Ravussin 2003; Chung, Kim et al. 2013), at the cost of reduced reproduction (and in our case smaller body sizes), seems to apply also for *E. dichotoma* medusae. Another interesting finding was that no direct trade-off between sexual and asexual reproduction could be found. Both traits apparently function independently of each other.

Heritability & Stochasticity

Remarkably, none of the measured traits was heritable. Neither the lifespan, nor the asexual or sexual reproductive output of the medusa parents, measured as total offspring count or offspring release rate, determined the respective patterns in their vegetative medusa offspring.

These results are a strong argument for a stochastic allocation mechanism being at work for the phenotypic outcome of each individual. We found similar patterns in the freshwater polyp *Hydra*, where we suggest them to be an evolutionary selected feature of the species enabling it to cope quickly to changing conditions in a frequently fluctuating environment (Chapter IV). The vegetative mode of reproduction might play a crucial role for this potential, including its associated differential tissue shift into the offspring buds. Each vegetative bud might get different cell lines and signals from the parent resulting in a completely individualized transcriptome and phenotype plasticity, always dependent on the current state of the parent, affected by the present environmental and individual conditions during which the bud is growing at the parent (see chapter I).

CONCLUSIONS

The patterns found in our demographic experiment with *E. dichotoma* suggest a huge phenotypic diversity within the studied clonal line (Omega). Huge variances can be observed in the survival and in the vegetative medusa bud production as well as in the bisexual self-fertilization and the resulting released planula larvae of isogenic medusae within the cohorts. Nevertheless, several demographic trends can be observed between different feeding levels and between different vegetative medusa generations. Although medusa survival seems not directly linked to both sexual- and asexual reproduction output (in the case of TLR, LRR and BRR, just TBR tended to be positively correlated with survival), the overall results indicate that the nutritional level affects the resource allocation trade-off in *E. dichotoma* by setting a metabolic pace level for a medusa - the more food is available, the faster and compressed it lives. The feeding level influences the fine tuning of the adjusting screw of the trade-off between reproduction and maintenance. The missing heritability of the measured traits and

the huge variability within all traits between individuals and cohorts suggest a random phenotype generating process in *E. dichotoma.* However, as discussed in chapter I, a trade-off seems to be extant between successive medusa generations with an observable quality decline with consecutive generations. This trade-off might be beneficial for the genet in the light of the seasonal appearance of *E. dichotoma* medusae (Chapter I).

Our findings, together with chapter I, support a revision of the general claim by Kirkwood and Rose, that the evolutionary optimum leads directly to senescence. Instead, senescence seems not inevitable, but multifaceted in pace and shapes (Baudisch 2011), when considering the "hump shape senescence" of *E. dichotoma* medusa ramets, the indications of negative- and non-senescence of polyp colony ramets and *E. dichotoma* genets and the diverse patterns found for many more species across the tree of life (Martinez 1998; Martinez 2002; Vaupel, Baudisch et al. 2004; Jones, Scheuerlein et al. 2014; Schaible, Scheuerlein et al. in preparation for submission). Our results support the idea that the evolution of different aging paths and various life history strategies substantially depend on the type of the underlying trade-offs between maintenance and reproduction.

It is next to study *E. dichotoma* under further environmental challenges and to take a closer look at early-life mortality of embryos and developing polyp colonies. Furthermore, studying the proximate mechanisms for the fitness difference between *E. dichotoma* polyp colonies and medusae and between the vegetatively produced medusa generations, e.g. if it is the result of measurable changes and differences in the transcriptome and/or the stem cell distribution between them, will also help to gain a deeper understanding of the ultimate causes of the vast diversity of aging patterns in basal metazoans, with *E. dichotoma* being one among them.

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FIGURES AND TABLES

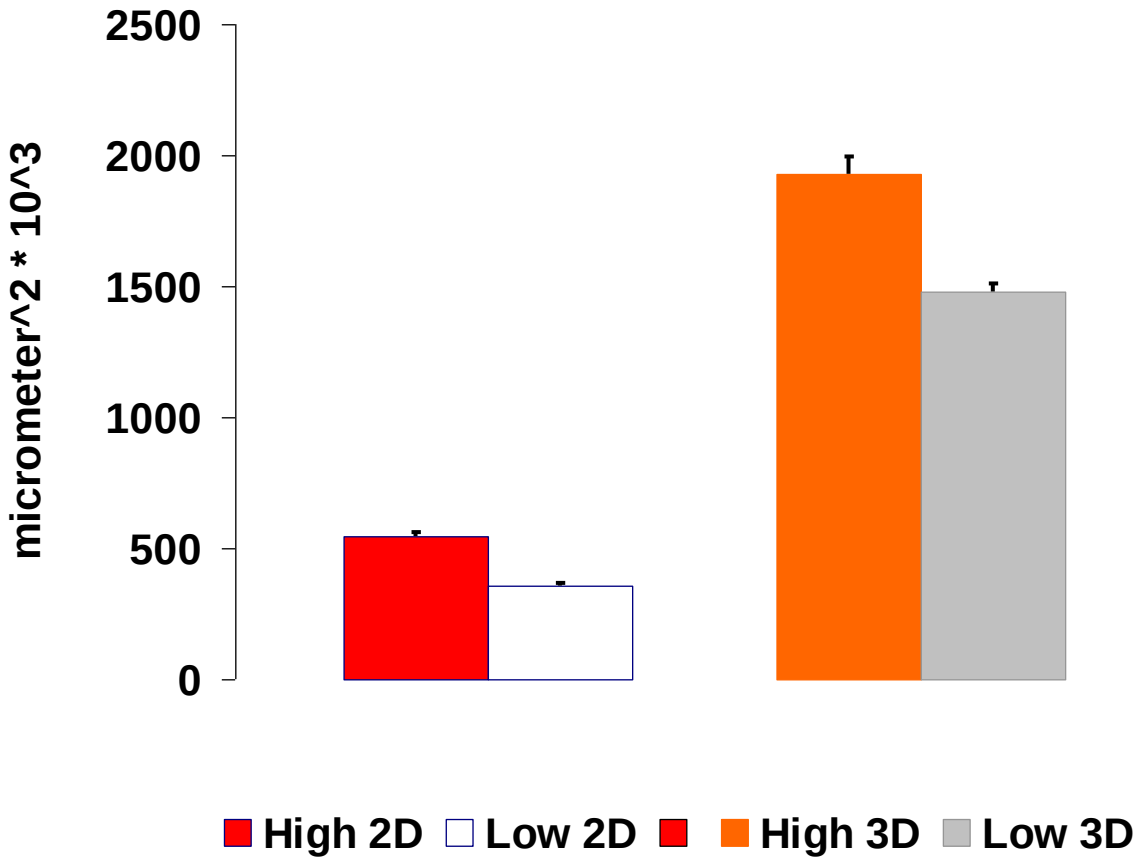

Figure 1. Size comparison – mean HFR- versus mean LFR sizes. Mean total surface areas of medusae averaged over all measurement time points are shown. Error bars represent standard errors. Medusae of the HFR were significantly larger than in LFR when comparing all cohorts of a feeding regime together (shown here) or each generation separately (not shown), either in two dimensional or three dimensional total surface area extrapolations (Mann–Whitney U tests, $p<0.001$).

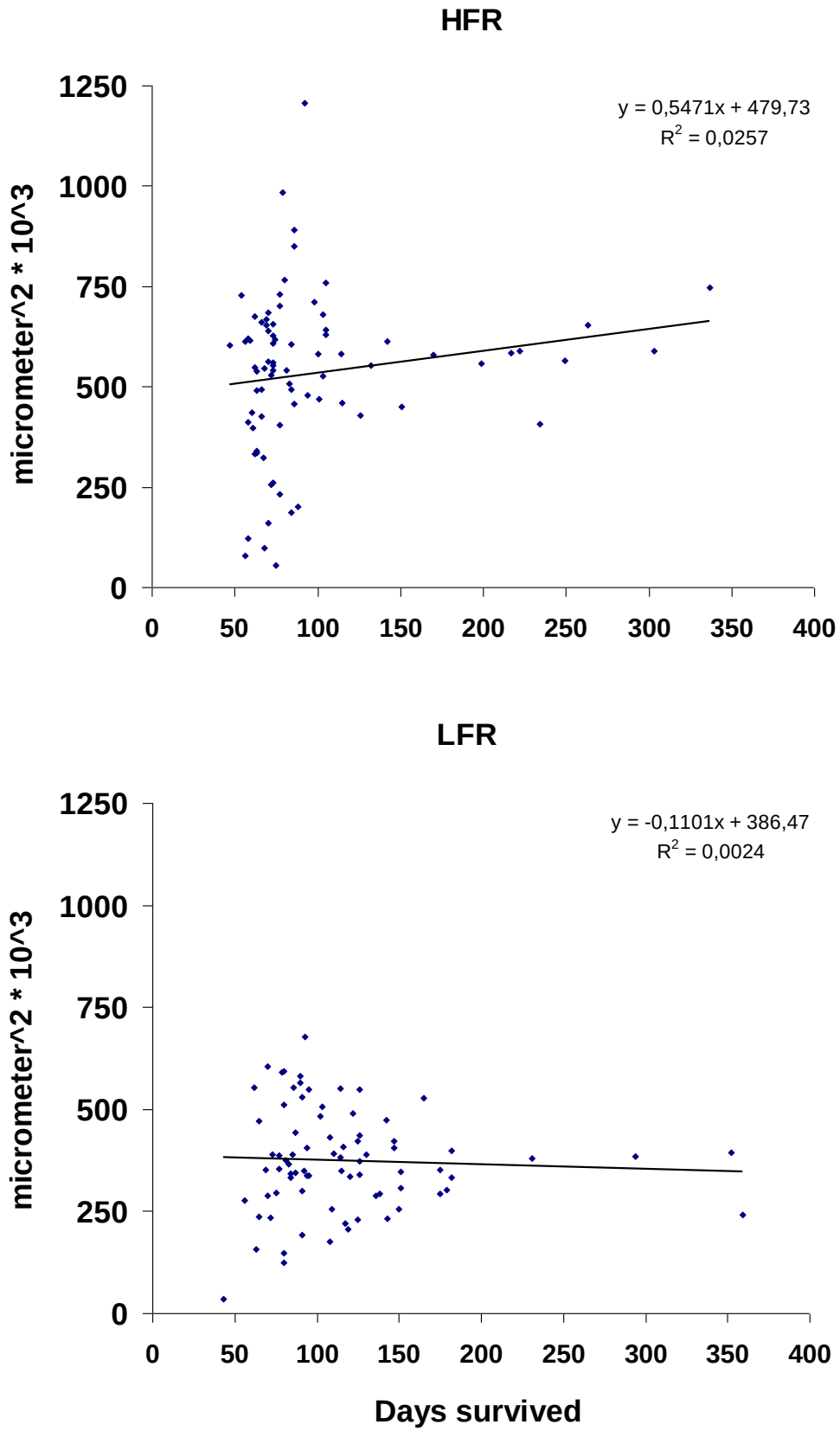

Figure 2. Medusa Size versus Survival. Individual average 2-dimensional total surface areas of medusae were taken. Upper graph shows all cohorts of the HFR combined, lower graph shows LFR, respectively. Linear regressions were non-significant in both cases ($p>0.05$, equations given in graphs).

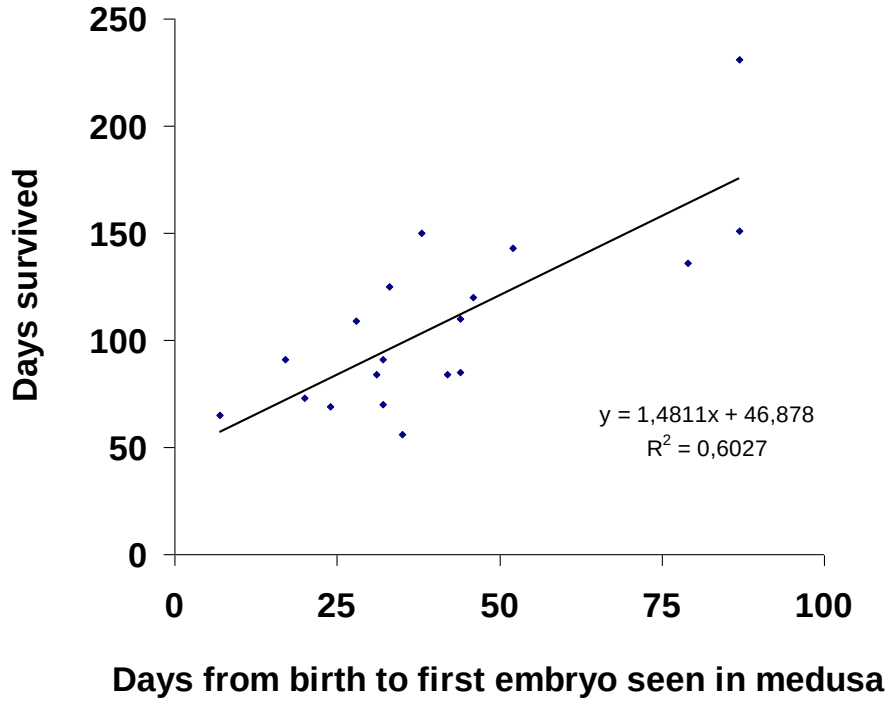

Figure 3. Medusa survival versus onset of sexual reproduction. TL cohort. Onset of sexual reproduction is here shown as days from birth to first seen embryo in medusa. The strongest correlation is shown here with an r^2 of .6 (linear regression, $p<0.001$). All other cohorts had much lower r^2 values, were much more heterogeneous and without a clear linear trend when comparing these traits.

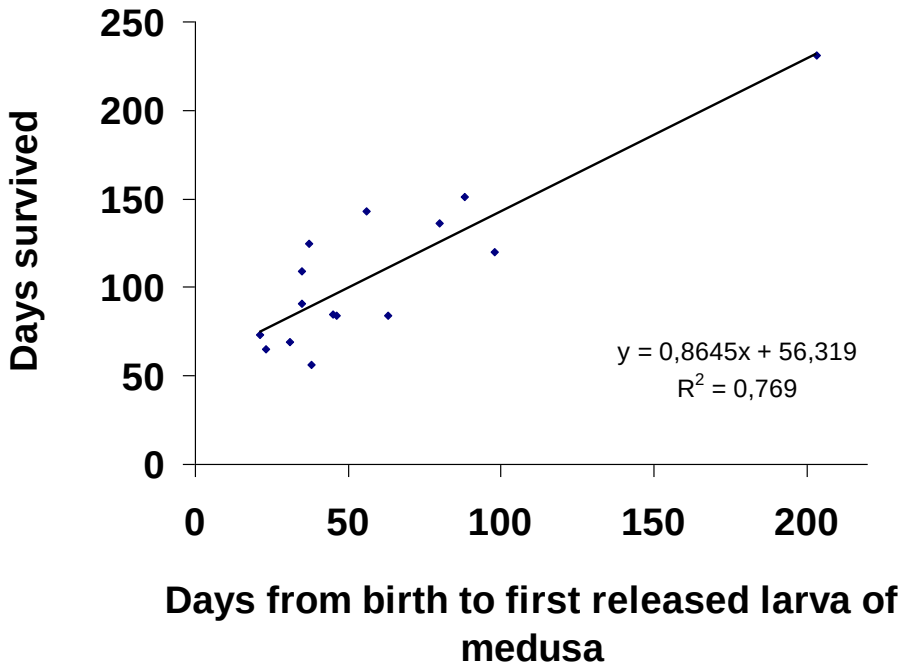

Figure 4. Medusa survival versus onset of sexual reproduction. TL cohort. Onset of sexual reproduction is here shown as days from birth to first released larva of medusa. The strongest correlation is shown here with an r^2 of .77 (linear regression, $p<0.001$). All other cohorts had much lower r^2 values, were much more heterogeneous and without a clear linear trend when comparing these traits.

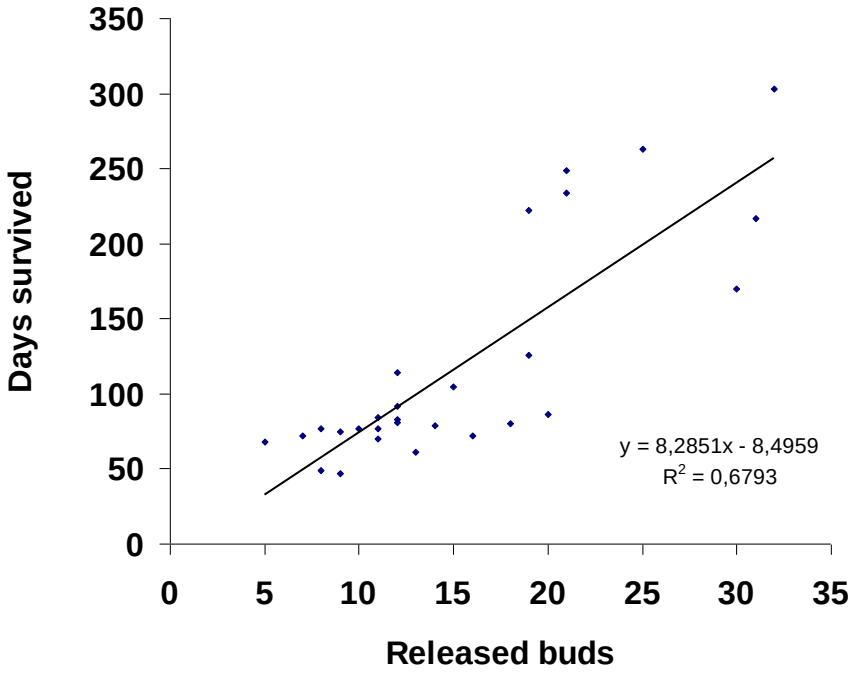

Figure 5. Medusa survival versus total bud release (TBR). PH cohort. The strongest correlation is shown here with an r^2 of .68 (linear regression, $p<0.001$). All other cohorts showed similar significant trends, but with lower r^2 values.

CHAPTER III

Environmental challenges improve resource utilization for asexual reproduction and maintenance in hydra

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ABSTRACT

Variation in life history can reflect genetic differences, and may be caused by environmental effects on phenotypes. Understanding how these two sources of life history variation interact to express an optimal allocation of resources in a changing environment is central to life history theory. This study addresses variation in the allocation of resources to asexual reproduction and to maintenance of *Hydra magnipapillata* in relation to differences in temperature and food availability. Hydra is a non-senescent, persistent species with primarily clonal reproduction. We recorded changes in budding rate and mean survival under starvation, which indicate changes in the allocation of resources to asexual reproduction and maintenance. In constant conditions we observed a clear trade-off between asexual reproduction and maintenance, where budding increased linearly with food intake while starvation survival stayed rather constant. In contrast, an environment with fluctuations in temperature or food availability promotes maintenance and increases the survival chances of hydra under starvation. Surprisingly, asexual reproduction also tends to be positively affected by fluctuating environmental conditions, which suggests that in this case there is no clear trade-off between asexual reproduction and maintenance in hydra. Environmental stresses have a beneficial impact on the fitness-related phenotypical traits of the basal metazoan hydra. The results indicate that, if the stress occurs in hormetic doses, variable stressful and fluctuating environments can be salutary for hydra. A closer examination of this dynamic can therefore enable us to develop a deeper understanding of the evolution of aging and longevity.

1. Introduction

Organisms differ in their growth and breeding schedules, the extent of parental investment, the number of offspring, and the investments made in body maintenance and survival. A perfect match between these life history traits of an organism can never be attained because organisms must cope with constraints, changing environments and trade-offs (Roff, 1992). Thus, the question of how resources can be efficiently allocated among growth, reproduction and survival is of particular interest.

As anticipated by life history theory, a change in one phenotypic trait may occur together with a change in another; these are referred to as trade-offs (Roff, 1992): i.e., energy that is allocated to maintenance, which in turn increases the chances of survival, cannot be used for other processes, such as reproduction (Hercus et al., 2003; Le Bourg, 2009; Boggs, 2009). The presence of trade-offs between reproduction and survival is a central feature in life history theory, and affects the evolution of longevity (Le Bourg, 2009; Stearns, 1992). Trade-offs may change in response to environmental stresses (Parsons, 2005; Rattan, 2008), and this response may differ when stresses are applied as constant or fluctuating regimes (Rattan, 2008; Parsons, 2007; Gomez et al., 2009; Marshall and Sinclair, 2010). Thermal stresses (low or high temperature) and resource scarcity induce stress responses in organisms that can lead to increased longevity, but not necessarily at the expense of their reproductive output. The improvement in the survival of an organism following exposure to mild stresses is a response known as hormesis (Parsons, 2005; Calabrese and Baldwin, 2003), and has been observed in various species across the tree of life, e.g., in yeast (Minois, 2000), *Drosophila* (Semenchenko et al., 2004; Le Bourg and Minois, 1999) and nematode worms (Yashin et al., 2001; Lee et al., 2006).

Hydra (Cnidaria, Hydrozoa) deviates from typical life histories, and offers researchers the opportunity to gain insight into the role of trade-offs in shaping age-specific life histories. Hydra reproduces mostly clonally, through budding from the body tissues by a process of mitosis and cell migration. Asexual reproduction is the main reproductive mode in hydra, while sexual reproduction occurs less frequently. Therefore, hydra is ideal for studying neglected variations in life history traits based on environmental effects. The fact that the offspring are genetically identical facilitates the observation of phenotypic variation in the allocation of resources that depend exclusively on environmental conditions.

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In hydra, three distinct stem cell lineages have been found: the epithelial stem cells (endoderm and ectoderm) provide epidermal and digestive cells, while interstitial cells provide all of the remaining cell types. The epithelial cells have been shown to have a cell cycle time of approximately three days, and to be continuously cycling (Bosch and David, 1984), so that the epithelial tissue mass of the animal doubles every three to four days (David and Campbell, 1972). Thus, the pattern of cell turnover is highly dynamic, with differentiated cells persisting for only a short time before they are lost or destroyed through apoptosis and autophagy (Chera et al., 2009). Rather than investing energy into the repair of damaged cells, hydra instead replaces whole cells, presumably because doing so is more cost-effective. These cell dynamics are of great importance for the life history of hydra, and for decisions regarding resource allocation. Furthermore, because of this dynamic balance of cell loss and cell gain, hydra is a model of continuous regeneration, and is considered to be a non-senescent invertebrate (Martinez, 1998).

Another advantage of using hydra in our experiments is that individuals kept at a constant food level attain a relatively stable maximum size (which may be species-specific). At low food levels, the size of a polyp shrinks, and the use of dead cells from apoptosis as a food source increases, as does autophagy (Bosch and David, 1984; Chera et al., 2009). With these mechanisms, the polyps can maintain their body in perpetuity, and therefore survive very low food levels or starvation for several days. Once a polyp has grown to full size (for which a constant food level is necessary), most of the newly generated cells will be channeled into budding. At this stage, resources can be allocated to asexual reproduction as well as to maintenance, and maintenance will in turn influence survival. The rate of bud production is mainly governed by temperature and food intake.

The apparent lack of senescence, the efficient asexual reproduction mechanism and the regeneration potential of hydra suggest that the response of maintenance efficiency to changing environmental conditions may be crucial to understanding the evolution of longevity and non-senescence. In many organisms, mild environmental stresses have been shown to induce a higher degree of resistance against further or new stresses, and stressed organisms have been found to have better maintenance or longer life spans than unstressed organisms. A negative effect for maintenance could be observed in overfeeding experiments conducted by Bode et al. (1977): the polyps in the experiments died, suggesting a strong decline in somatic strength. However, all of the previous experiments in which food level change and starvation were combined with an analysis of budding rate and survival lasted fewer than 30 days (Chera et al., 2009; Bode et al., 1977; Otto and Campbell, 1977). Therefore, no clear pattern with respect to resource allocation and the trade-off between maintenance and asexual reproduction could be observed.

It is the aim of our study to examine the allocation of resources to asexual reproduction and maintenance in genetically identical individuals under both controlled and stressful environmental conditions, with respect to food availability and temperature. We explore the question of whether allocation strategies are phenotypically plastic within a lifetime of a polyp, and whether they vary in response to environmental conditions. We attempt to identify the mechanism that could play a crucial role in the optimal resource allocation between survival and asexual reproduction in hydra. We compare the nutritional costs of maintenance with the nutritional costs of asexual reproduction to examine whether either one is large enough to compete for a significant fraction of the resources allocated to the other.

2. Materials and methods

2.1. Species

There are similarities in life histories across the genus *Hydra*. In this study, we use the well-studied strain *Hydra magnipapillata* 105, as this line has been kept successfully in the laboratory for over 30 years where it reproduced exclusively by clonal budding. The strain 105 does not show any signs of sexual reproduction including production of gametes. We were able to follow standard proven laboratory procedures, which includes feeding a mono-diet of *Artemia salina* nauplii (1 day post hatching). We thus benefited from the long history and experience of researchers who have worked with this model system e.g. (Martinez, 1998).

2.2. Definitions

2.2.1. Asexual reproduction = budding

We measured budding (asexual reproduction) as a reproductive rate, expressed as the average number of buds produced per hydra per unit of time. We defined asexual reproduction as a developmental process with two states based on Sanyal (1966). 1. The development of a bud starts with the tissue recruitment from the parent polyp to the bud; both are genetically identical and all three independent stem cell lineages are involved in this process (Otto and Campbell, 1977; Sanyal, 1966). 2. This process ends at the time when the bud builds its first tentacle rudiments (bud hydranth morphogenesis) and the bud separates from the parent polyp. Asexual reproduction is strongly correlated with food concentration. Furthermore, the individual size (number of cells per hydra) and the individual budding rate depend on the food intake (Otto and Campbell, 1977). Therefore, it is very important to separate budding from polyp growth, which describes the change in the size or the cell number of a polyp. We predicted that, after a long period of constant food intake, the size of the polyps would stay constant over time and within treatment groups (Otto and Campbell, 1977). Furthermore, we predicted that the size could vary among groups with different food intakes. Under constant feeding regimes with a constant number of food items per day per hydra, individuals could reach a steady state in size (number of epithelial cells), which is proportional to the food intake (Bode et al., 1977; Otto and Campbell, 1977). The respective food concentration was held constant for more than three months to allow each individual to attain this stable maximum size. We thereby ensured that 1) at the beginning of each experiment, size effects between the polyps could be nearly excluded; 2) all polyps would have acclimatized to the respective condition; and 3) newly produced buds would have nearly the same size in each treatment.

2.2.2. Food utilization

We analyzed the efficiency of energy utilization. We quantified the number of *Artemia* needed to produce one bud by calculating the number of *Artemia* per bud per time for each individual. To do this, we integrated all of the *Artemia* fed, up to the day when the last bud separated from the mother. This is an indirect measure for detecting a change in resource allocation due to environmental stresses.

2.2.3. Survival under starvation

Another way to quantify changes in the pattern of resource allocation in hydra under environmental stresses is to assess the energy needed for maintenance; i.e., survival. In hydra, the identification of a relationship between environmental stresses and survival expansion, as observed in other species, is not directly possible due to its unknown and extraordinary lifespan. Thus, the indirect measurement of life expectancy under starvation in days, as it is used in our experiments, serves as a comparable approach. Significant differences in such an artificial mortality curve can then be explained as differences in the allocation of resources to maintenance. To avoid effects of cell number or size of polyps on this parameter (because survival can be positively correlated with the number of cells per polyp, as mentioned above) we started with an experiment in which we fed groups of polyps at different constant food levels (between 0.2

and 20 *Artemia* per day and polyp). The feeding period was followed by a final starvation time to test the influence of the food level on the survival under starvation (= maintenance). We predicted that, because the size of a polyp depends on food level, starvation survival would be higher in polyps fed with more *Artemia* prior to starvation. This is because these polyps contain more cells and thus more substance (Otto and Campbell, 1977) undergoing apoptosis and autophagy (Bosch and David, 1984; Chera et al., 2009), which should result in a longer life span without food.

2.3. Experimental design

2.3.1. Culture conditions

We cultured individuals in plastic multi-well culture plates with a medium containing 0.05 mM $NaHCO_3$, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.001 mM $MgSO_4$, 0.003 mM KNO_3 in deionised water, and maintained them in incubators at 18 °C with a 12 h-light, 12 h-dark regime (= control environment). For the experiments, each polyp was kept separately in a single container containing 8 ml of hydra medium on plastic 6-well plates. Hydra polyps were fed with freshly hatched nauplii of *Artemia salina*. Exact numbers of these *Artemia* can be fed directly to the hydra, offering a simple and practical way of manipulating the resources given to individuals. In all of the experiments, all of the polyps were checked for complete food intake after feeding. We are aware that the exclusive use of *Artemia* is not natural (mono-diet) as compared with multiple food sources in the wild. But using *Artemia* as food source offers the opportunity to compare the levels of food intake between individuals since the *Artemia nauplii* we used were nearly constant in size and energy level (1 day post hatching).

To reduce age effects within/between the experimental groups, we used polyps of a similar age in all experiments. We collected newly released buds from an existing adult polyp culture in our lab within 3–5 months. All individuals used for the experiments in this study were taken out of this pool.

2.3.2. Experiments

The exact details of the individual experiments and their simulated environmental stresses (like feeding regime, hunger periods, temperature) are depicted in Table 1. Treatments with lower food levels lasted longer to ensure that the polyps did not die due to the low food level itself. The results of the following experiments, in which we treated groups of hydra with varying temperatures and starvation regimes, are compared and discussed with respect to the possible size effect on life expectancy under starvation.

(1) Constant food levels
We studied the effect of different food levels on asexual reproduction and survival under laboratory conditions (18 °C and 12 h dark: 12 h light conditions). Food level responses are measured as the reproductive rate, food utilization per bud and the survival time (mean life expectancy under final starvation). Groups of hydra with constant but differing mean food levels between 0.2 and 20 *Artemia* per day per hydra were compared (Table 1).
(2) Variation in mean food level
Here we compared both the budding rate and the life expectancy under starvation in two scenarios: low food (1.3 *Artemia* per day per hydra) after 119 days of high food (3 *Artemia* per day per hydra), and high food after 119 days of low food. Both of these outcomes are compared to the outcomes of control groups (Table 1).
(3) Food scarcity in-between
Two additional experiments were run to analyze the effect of a limited starvation time: the low-food group was fed with 1.3 and the high-food group with 3 *Artemia* per day per hydra. We ceased feeding after 101 days in both groups for 61 days. Thereafter, the original feeding schedule was resumed for an additional 61 days (Table 1), after which life expectancy under final starvation was measured.
(4) Temperature and food concentration
To test the influences of temperature on hydra longevity, asexual reproduction and food utilization, we conducted two different experiments. In the first experiment, hydra were kept under a constant temperature regime (10 °C or 18 °C) and were fed either 1 or 4 *Artemia* per day per hydra (Table 1).

In the second experiment, we exposed hydra polyps to a temperature cycle (18–14–10–6–10–14-18 °C; each temperature for four weeks). One group was fed 1.3 *Artemia* per day per hydra, while a second group was fed 3 *Artemia* per day per hydra. (Table 1).

2.3.3. Statistics

SPSS software was used for the statistical analysis of the obtained data. We tested the hypothesis that mild stresses (temperature and food variation or food scarcity) would have an effect on lifetime asexual reproduction, food utilization and starvation survival, when compared to constant conditions without stresses using an analysis of variance (one-way ANOVA). The analysis of variance was followed by Tukey *post-hoc* comparisons to test for differences between the treatments with and without mild stresses. To test the effects of different environmental conditions and their interactions on the survival and reproduction of hydra, we compromised on the number of individuals in each treatment and thus reduced the statistical strength of the analysis for feasibility reasons.

3. Results

3.1. Constant food

Fig. 1 shows how asexual reproduction and survival were affected by the overall mean daily food intake. Increasing resource levels caused a linear increase in budding rates (linear model: $F_{1,287} = 3317$; $p < 0.001$; $r^2 = 0.92$ and ANOVA, $F_{10,287} = 383$, $p < 0.001$). At the food level of 0.21 *Artemia* per day, no buds developed (Fig. 1). The food utilization per bud per hydra was nearly constant across all food levels (with the exception of food treatment < 1 *Artemia* per day), with an average requirement of 16 *Artemia* per bud ($F_{8,233} = 1.9$; $p = 0.069$; data not shown).

With increasing food intake, starvation survival was at a fairly constant level across all food treatments, with more than 0.6 *Artemia* per day (linear model: $F_{1,256} = 3.1$; $p = 0.075$; $r^2 = 0.012$), but there were significant differences between single groups (Fig. 1; ANOVA, $F_{10,287} = 47$, $p < 0.001$). At higher food levels (<3 *Artemia*), the mean starvation survival was nearly 80 days. At 0.21 *Artemia* per day per hydra, the mean starvation survival of 48 days was significantly lower (Fig. 1; Tukey *post-hoc*: $p < 0.05$). The highest values of starvation survival could be observed at one and 1.3 *Artemia* per day per hydra treatments (Fig. 1). Surprisingly, these levels showed a higher starvation survival than groups with higher food intake. The nearly constant level of life expectancy under starvation over all higher food concentrations showed that, in our experiments, starvation survival (= maintenance) seemed to be nearly independent of food intake (except the minimum food levels). It thus appears that starvation survival behaves independently of size and the number of cells per polyp, respectively. At a very low food level (<1 *Artemia* per day per polyp), we cannot exclude an effect of size or cell number.

Table 1
Information about experiments.

	Number of Individuals	First feeding regime					Second feeding regime			
		Temperature °C	*Artemia* per day per Hydra	Feeding frequency: *Artemia* per day	Feeding days	Changing treatments	*Artemia* per day per Hydra	Feeding frequency: *Artemia* per day		Feeding days
Constant food	30	18	0.21	1–0–0–0–0–1–0–0–0–0	112	– –	–	–	–	–
	24	18	0.57	2–0–0–0–2–0–0	112	– –	–	–	–	–
	24	18	0.79	3–0–0–2–0–0–2–0–0	95	– –	–	–	–	–
	30	18	1.00	2–0–2–0–3–0–0	95	– –	–	–	–	–
	24	18	1.29	3–0–3–0–3–0–0	95	– –	–	–	–	–
	24	18	2.43	3–2–3–2–3–2–2	95	– –	–	–	–	–
	24	18	3.00	3–3–3–3–3–3–3	95					
	30	18	4.00	4–4–4–4–4–4–4	77	– –	–	–	–	–
	24	18	6.00	6–6–6–6–6–6–6	77	– –	–	–	–	–
	24	18	10.00	10–10–10–10–10–10–10	77	– –	–	–	–	–
	24	18	15.00	15–15–15–15–15–15–15	77	– –	–	–	–	–
	24	18	20.00	20–20–20–20–20–20–20	77	– –	–	–	–	–
Different temperatures	30	10	1.00	1–1–1–1–1–1–1	65	– –	–	–	–	–
	30	10	4.00	4–4–4–4–4–4–4	65	– –	–	–	–	–
Changing food level	24	18	1.29	3–0–3–0–3–0–0	119	– –	3.00	3–3–3–3–3–3–3		91
	24	18	3.00	3–3–3–3–3–3–3	119	– –	1.29	3–0–3–0–3–0–0–		91
Scarcity in–between	24	18	3.00	3–3–3–3–3–3–3	102	Scarcity of 61 days	3.00	3–3–3–3–3–3–3		63
	24	18	1.29	3–0–3–0–3–0–0	102		1.29	3–0–3–0–3–0–0–		63
Temperature-gradient	24	18–14–10–6–10–14–18	1.29	3–0–3–0–3–0–0	252	Weeks per temperature: 8–4–4–4–4–4–8	–	–	–	–
	24		3.00	3–3–3–3–3–3–3	252		–	–	–	–

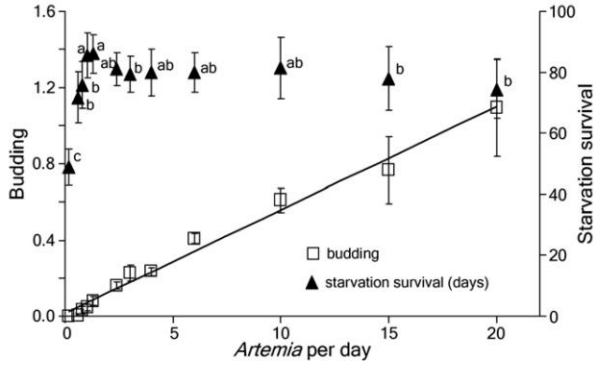

Fig. 1. Relationship between daily budding rate and remaining life expectancy under starvation after various food regimes. Different lower case letters indicate significant differences in life expectancy (one-way ANOVA, Tukey *post-hoc* test, $p<0.05$).

3.2. Variation in mean food level

To simulate the varying levels of food abundance in the natural environment, we performed two different experiments: by changing food levels from high to low, and from low to high. We then compared these groups to a control group in which food levels remained constant. In general, the reproductive effort was affected by the direction of the changing food concentration. The mean budding rate in the 1.3 *Artemia* per day per hydra treatment was significantly higher in the high-low group than in the low-high food and in the control group (Fig. 2A; ANOVA, $F_{2,71}=130$, $p<0.001$; Tukey *post-hoc*: $p<0.05$).

The efficiency of food utilization was positively affected by the direction of the changing food concentration. When the food intake was reduced from 3 to 1.3, hydra individuals used a lower number of *Artemia* to produce a bud (Fig. 2B; ANOVA, $F_{2,71}=47$, $p<0.001$). Under constant food conditions, hydra needed an average of 13 *Artemia* per bud, but after food levels were lowered, this fell to nearly 10 *Artemia* per bud (Tukey *post-hoc*: $p<0.05$). At low food levels, the budding rate was reduced as well, while food utilization increased, such that relatively more buds were produced per amount of food.

In addition, the starvation survival was significantly affected by the direction of the changing food concentration (ANOVA, $F_{3,95}=70$, $p<0.001$). A strong increase in mean starvation survival (91 ± 9 and 101 ± 8 days) for changing food treatments from 1.3 to 3 and 3 to 1.3

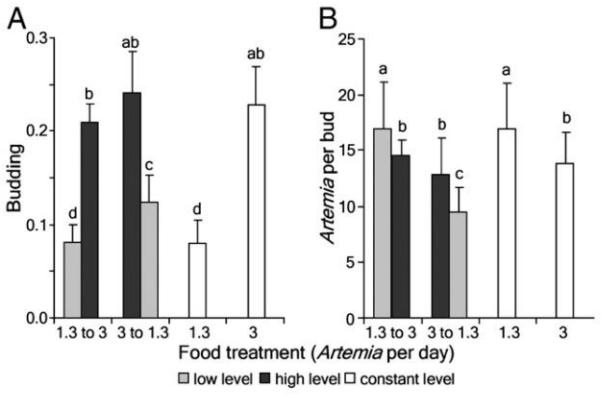

Fig. 2. Relationship of budding rate (A) and food utilization (*Artemia* per bud) (B) of one treatment with change mean in food concentration from 1.3 to 3 *Artemia* per day and the second treatment with change in mean food concentration from 3 to 1.3 *Artemia* per day as well as two control treatments with constant food levels (1.3 and 3 *Artemia* per day). Different lower case letters indicate significant differences in budding rate or in food utilization between the various food treatments (one-way ANOVA, Tukey *post-hoc* test, $p<0.05$).

Artemia per day (Fig. 3A) was observed when compared to the constant food concentration at 1.3 (86 ± 6 days) and 3 *Artemia* per day per hydra (79 ± 6 days) (Tukey *post-hoc*: $p<0.05$). Again, the individuals in the group changing from higher to lower food intake show longer starvation survival than the individuals in the group with higher food intake as the final food level (3 *Artemia* per day). In the constant controls, the polyps fed 1.3 *Artemia* per day per hydra were living longer under starvation than their control counterparts, which were fed 3 *Artemia* per day (Fig. 1).

3.3. Food scarcity in-between

To further test the influence of hunger time and food level on life history traits, we conducted a series of experiments with a longer hunger period between two feeding regimes with a constant food level. The groups were subject to a high and a low food regime, with 3 and 1.3 *Artemia* fed per day per hydra, respectively. Both groups were exposed to a fasting period of 61 days before re-feeding. In the 1.3 *Artemia* treatment, no change in asexual reproduction between before and after the fasting time and the control group could be observed (Fig. 4A; ANOVA, $F_{2,71}=2.4$, $p=0.098$). In the 3 *Artemia* treatments, the reproductive success after the fasting time was significantly lower (Fig. 4A; ANOVA, $F_{2,71}=4.1$, $p=0.028$). The food utilization was not affected by the starvation period (data not shown; ANOVA, $p>0.13$ for 1.3 *Artemia* treatment; $p>0.43$ for 3 *Artemia* treatment).

The long fasting period strongly affected the starvation survival in both treatment groups (Fig. 3B). Compared to the control group (Fig. 1 and Fig. 3B), without a hunger period, the mean starvation survival increased by 29 days and 17 days for the 3 and 1.3 *Artemia* treatments, respectively and were significantly higher (ANOVA, $F_{3,95}=136$, $p<0.001$). But again, we did not detect higher rates of survival under starvation for individuals with higher food intake (3 *Artemia*) than for those with lower food intake (1.3 *Artemia*) in the treatment groups. Instead, the trend was found to be opposite in the controls.

3.4. Temperature

3.4.1. Constant temperature

To test the consequences of a constant low temperature on life expectancy and asexual reproduction, we compared two food treatments with one and four *Artemia* per day per hydra at two different temperatures: 10 °C and 18 °C.

The results of these experiments suggest that temperature has a strong influence on starvation survival (Fig. 3C) and asexual reproduction (Fig. 4B). The asexual reproduction at 10 °C decreased dramatically relative to the 18 °C treatments in both food treatments (Fig. 4B; ANOVA, $F_{3,119}=498$, $p<0.001$). Moreover, low temperature increased mean starvation survival significantly in both food treatments (Fig. 5; ANOVA, $F_{3,119}=172$, $p<0.001$), by 57 days for the four *Artemia* treatments, and by 46 days for the one *Artemia* per day per hydra treatments (Fig. 3C). Within a temperature treatment, mean starvation survival was independent of food concentration (Fig. 3C; Tukey *post-hoc*: $p>0.05$).

3.4.2. Temperature cycle

In this experiment, the effects of mild temperature stress, simulating winter conditions, were tested: survival after starvation (Fig. 3D) was measured following the temperature cycle, and the budding rate (Fig. 5A) was measured and compared at the beginning and at the end of the temperature cycle at 18 °C. In both food treatments (3 and 1.3 *Artemia* per day and per hydra), the budding rate increased significantly relative to the results at 18 °C before (first 30 days) and after (last 30 days) the temperature cycle, and also relative to the control groups at constant 18 °C (Fig. 1; ANOVA, $F_{2,71}=11$, $p<0.001$ for 1.3 *Artemia* treatment; $F_{2,71}=88$, $p<0.001$ for 3 *Artemia* treatment).

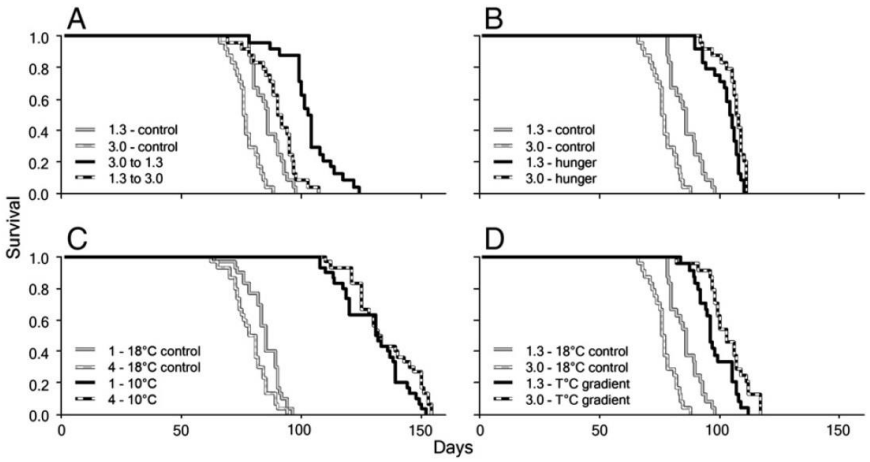

Fig. 3. Survival curves starting at the onset of starvation for different treatments: A, survival curves for change in mean food concentration from 3 to 1.3 *Artemia* per day and from 1.3 to 3 *Artemia* per day compared to the control group (constant 1.3 and 3 *Artemia* per day); B, survival curves for treatments with intermediate hunger period of 61 days and 1.3 and 3 *Artemia* per day feeding regimes. Control group without hunger but same feeding regime; C, survival curves for two different food regimes (4 and 1 *Artemia* per day) and two temperature treatments (10 and 18 °C); D, survival curves for the temperature gradient treatment (1.3 and 3 *Artemia* per day) compared to the control group (constant temperature; 1.3 and 3 *Artemia* per day).

The efficiency of food utilization measured as *Artemia* per bud per hydra improved significantly after the temperature cycle in both food treatment groups, compared to the control group with a constant temperature of 18 °C (Fig. 5B; ANOVA, $F_{2,71}=17$, $p<0.001$ for 1.3 *Artemia* treatment; $F_{2,71}=38$, $p<0.001$ for 3 *Artemia* treatment). The changes in food utilization after the temperature cycle were independent of the food concentration (Fig. 5B; Tukey *post-hoc*: $p>0.05$).

The individual survival after final starvation was significantly affected by the temperature cycle (Fig. 3D; ANOVA, $F_{3,95}=77$, $p<0.001$). The mean starvation survival of these individuals was 29 (3.0 *Artemia*) and 20 (1.3 *Artemia*) days longer than the mean survival starvation of individuals under a constant temperature of 18 °C. Interestingly, the individuals that underwent the 3 *Artemia* treatment had a significantly longer mean starvation survival (105 ± 4 days) than those that underwent the 1.3 *Artemia* treatments (99 ± 7 days) following the temperature cycle (Tukey *post-hoc*: $p<0.05$). In contrast, under a constant temperature of 18 °C, the differences between food treatments were reversed (Tukey *post-hoc*: $p<0.05$).

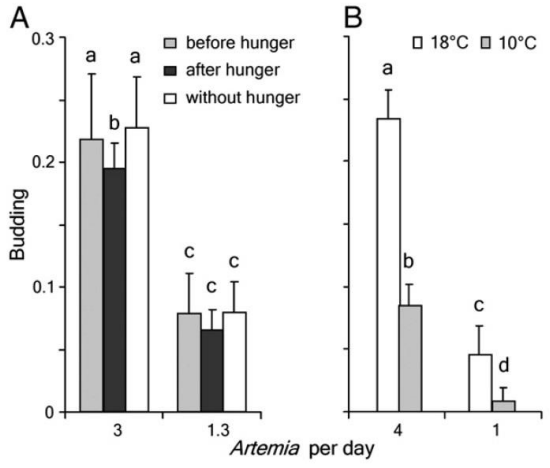

Fig. 4. The budding behavior of two different treatments: A, mean budding rate for 1.3 and 3 *Artemia* per day with intermediate hunger period of 61 days in both treatments. Control groups without hunger (1.3 and 3 *Artemia* per day); B, mean budding rate under different but constant food regimes (4 and 1 *Artemia* per day) and constant temperature (10 and 18 °C). Different lower case letters indicate significant treatment effect (one-way ANOVA, Tukey *post-hoc* test, $p<0.05$).

4. Discussion

4.1. Environment with constant food and temperature levels

A polyp's size, the size of its buds and its budding rate depends on food intake (Otto and Campbell, 1977). Once a polyp has grown to full size, most of the newly generated cells will be channeled into budding. At this stage, resource allocation to growth is negligible. Resources can be allocated to budding as well as to maintenance, which in turn influences survival.

At constant temperatures and at constant food services, budding rates showed a linear increase with increasing food levels (Fig. 1), thus confirming previous studies (Otto and Campbell, 1977). In contrast, starvation survival was not positively affected by different constant food levels. Survival thus appears to increase with food intake only up to a critical level (one *Artemia* per day). Once this critical food level is reached, the variation in starvation survival rates remains very small, with no trend towards an increase in survival for higher feeding regimes. We anticipated that higher food levels would

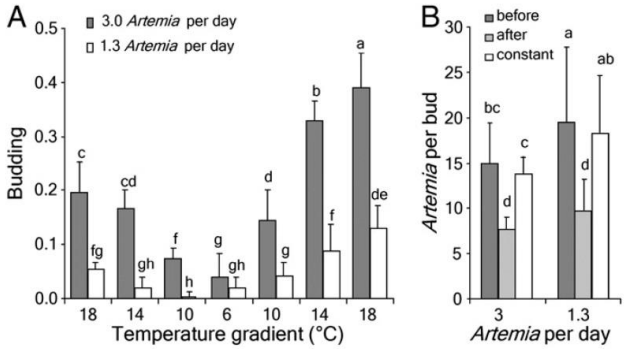

Fig. 5. Budding rate (A) of individuals exposed to different temperatures in a temperature cycle for 1.3 and 3 Artemia per day and energy utilization (*Artemia* per bud; B) at 18 °C before and after the temperature cycles compared with the control treatment at constant 18 °C.

increase the cell number per polyp (Otto and Campbell, 1977), and would therefore increase survival during starvation. Instead, we observed that larger polyps did not increase their starvation survival. Moreover, it seems that being kept at very high food levels for a long time may even be disadvantageous for hydra's survival probability under starvation.

It appears that larger polyp size comes at a cost, which indicates that a trade-off exists between growth, asexual reproduction and survival. More food increases the budding rate linearly, and enough food (>3 *Artemia*) increases the cell number per polyp, which results in a lower surface area to volume ratio than for polyps with a lower number of cells. But it seems that larger polyps lack an advantage by having a lower surface area to volume ratio with respect to energy-use efficiency, as predicted by the Bergmann's rule, at least with measured survival during starvation. By contrast, the similar survival for survival for polyps under starvation that were previously exposed to lower food levels indicates that a shift occurs in resource allocation to growth and budding, while maintenance allocation is kept at a constant or even lower level, which is indicated by the reduced starvation survival among previously highly fed groups. Consequently, smaller and less fed polyps seem to have a more balanced resource allocation, and they proportionally invest more energy in maintenance than the highly fed polyps.

Another explanation for the observed survival pattern could be that constant excessive food supply leads to more inefficient energy consumption, affecting basically all physiological functions in hydra. Such a wasteful handling of energy could lead to a lowered allocation to maintenance or a proportional increase prior to starvation, thus resulting in a similar or slightly shorter survival during starvation compared to less fed hydra. After a sudden cessation of food supply, the lower energy efficiency results in a quicker decline in cell numbers per polyp, because proportionally more cells are used as food and energy in autophagy and apoptosis to cover the cost of starvation.

A third explanation would be that, at high food levels, a decline in the somatic strength (i.e., maintenance efficiency) of the polyp results in increasing costs per polyp for maintenance, and leads to a lower life expectancy under starvation. This decline in polyp health could be explained by increasing maintenance costs due to the need among larger polyps to allocate more energy to repairing and building defense mechanisms to avoid the accumulation of permanent deleterious damages in cells. These higher costs are a consequence of the persistence of high metabolic rates per cell that were established during times when enough or too much food was available. Similar effects were observed by Bode et al. (1977) and Galliot and Ghila (2010), as their overfeeding experiments resulted in the natural death of the polyps.

Constant lower temperature treatments at 10 °C force also a trade-off in both life history traits: maintenance and asexual reproduction. The remarkable increase in starvation survival and the decreased budding rate at a constant temperature of 10 °C, compared to 18 °C, demonstrates that, at low temperature, the allocation to asexual reproduction is lowered, while the allocation to maintenance increases. These results are similar to those seen previously in hydra (Schroeder and Callaghan, 1981; Schroeder and Callaghan, 1982; Park and Ortmeyer, 1972). At low temperature, a reduced asexual reproduction could be observed, while polyp size was sometimes found to increase. The finding of an increase in life span may not be too surprising considering the reduction in the metabolic rate and the potential for a greater investment in body size under constant lowered temperature (McCabe and Partridge, 1997; Bochdanovits and De Jong, 2003; Castilho et al., 2007). Another possible explanation could be that there is no change in the allocation resources; instead, the observed patterns could be simply the result of the reduction in the total metabolic rate, which lowers the budding and cell division rates, and thereby promotes longer starvation survival under low temperatures.

4.2. Environment with variable food and temperature levels

In these experiments, we tested the abilities of hydra to withstand variable environments. After hunger periods between two constant feeding times, starvation under final starvation was largely extended (Fig. 2B). Imposing a short period of food deprivation is one of the tools for manipulating the maintenance investments of various organisms that has been most frequently investigated in studies on aging (Hulbert et al., 2007), and the effects of this technique have been observed in *C. elegans* (Lee et al., 2006; Lakowski and Hekimi, 1998), in some fruit fly species (Partridge et al., 2005; Carey et al., 2008), rodents (Masoro, 1988) and rotifers (Verdonesmith and Enesco, 1982; Ozdemir, 2009).

Not only the absolute abundance of food, but also the direction of change in food abundance is important for hydra. In our study, a change from an abundant food supply to a scarce or moderate food supply was found to result in higher asexual reproduction compared to the moderate food supply of the opposite direction treatment. Secondly, we observed an increase in the energy utilization ratio relative to a constant food supply (i.e., the number of *Artemia* per bud) (Fig. 2B). In the high to low food treatment, food utilization—i.e., food conversion efficiency—increased in hydra. This may seem surprising, but can be explained by the fact that food limitation is mostly associated with harsher conditions, and, consequently, with high extrinsic mortality in the field (predation and eutrophication). A relatively high budding rate allows the population to survive such conditions for a while. Consequently, there should be consistently strong directional selection to enhance food utilization whenever food is scarce. In contrast, increasing food supply leads to reduced food utilization. Starvation survival increased in both food treatments, irrespective of the direction of change and of asexual reproduction, though the survival increase was even more pronounced in the high to low feeding treatments (Fig. 3A). The increase in life expectancy indicates an increase in the maintenance performance and an increase in the maintenance efficiency of the polyps as a result of the changing food regimes prior to starvation. This interesting response could be related to a hormetic effect, i.e., a beneficial effect on the starvation survival or on the polyp maintenance of hydra under hormetic doses of increased environmental stress (Calabrese and Baldwin, 2003).

Most interestingly, fluctuation in temperature was found to have a beneficial effect on both asexual reproduction and maintenance in hydra. In a temperature cycle that simulates the seasonal variability in temperature in the field at low extrinsic mortality and high food abundance, hydra polyps were found to survive at a temperature of 6 °C. At low temperatures, asexual reproduction was shown to decline, but after the temperature was raised again to 18 °C, asexual reproduction was found to increase substantially at each food level compared to the same levels before reaching the lowest temperature (Fig. 5A). After temperature cycles were imposed, starvation survival was found to increase significantly compared to polyps in constant 18 °C regime (Fig. 3D). In this case, the short period of lower temperature was shown to induce an increased allocation of resources into both asexual reproduction and maintenance after the temperatures returned to higher levels. We suggest that the resources that were allocated to maintenance during lowered temperatures were reinvested into an efficient asexual reproduction and maintenance. These positive processes were sustained for several weeks at a constant 18 °C, even during starvation. Consequently, habitats with lower temperatures appear to support strategies in which individuals allocate more resources to asexual reproduction and an individual's maintenance following these lower temperatures, thereby increasing survival during starvation as well.

4.3. Causes and consequences

We had anticipated that *Hydra* would have evolved mechanisms by which the energy requirements for reproduction and survival can

be met to optimize fitness in a given environment, such that energy saved by a reduction in asexual reproduction can be used to increase maintenance, i.e., survivorship. The results of our study provide direct evidence that both asexual reproduction and maintenance in particular benefit from environmental stresses.

A very interesting observation in our study is that polyps in a steady-state condition (concerning all morphogenetic processes like homeostasis in cell production and loss, animal size and budding), have a lower starvation survival than polyps in more stressful conditions, in which homeostasis was interrupted. A higher resistance to stress, which leads to increases in maintenance and reproduction efficiency, could be attributed to the beneficial effects that may result from exposure to low doses of mild stress, a response known as hormesis (Rattan, 2008; Calabrese and Baldwin, 2003; Mangel, 2008). But the mechanisms of such benefits are multi-faceted and difficult to assess. In general, hormesis induces stimulation of protective cell mechanisms, leading to an improvement in overall cellular functions and performance (Rattan, 2008). Stress triggers a signal transduction network, which can result in an enhancement of metabolic efficiency, thus allowing the body to maintain, survive and reproduce with a lower supply of energy. These processes, which can play a decisive role in the development of higher resistance to environmental stress—like cell proliferation (with constant cell cycling lengths), autophagy and apoptosis—are responsible for the morphogenesis and the survival of the polyps in general (Galliot and Ghila, 2010). Constant conditions with sufficient food and low extrinsic mortality hazard lead to homeostasis or steady-state conditions in organisms: when cell turnover, cell production and cell loss, as well as the polyp size (cell number) and asexual reproduction, are stable, levels of autophagy and phagocytosis of apoptotic cell bodies are low or negligible. For polyps that have acclimated to constant environmental conditions over a period of several months, a sudden change in environmental conditions creates a dramatic break in their homeostasis. Such breaks can influence the morphogenetic processes, resulting in rapid activation of apoptosis and autophagy, and in a decrease in the epithelial cell cycle length (Bosch and David, 1984; Bode et al., 1977). Thus, stress conditions can favor the metabolic efficiency and "wake up the polyps from their dormancy"; the cellular and developmental reactions of the polyp to stress enhance simultaneously metabolic and utilization rates of resources. This adjustment response to new environmental conditions should lead to an improvement in metabolic and physiological functions, and result in both greater maintenance efficiency and a higher survival under starvation conditions. Given that hydra has a plastic resource allocation system and can increase its maintenance efficiency through environmental stresses, the question of whether this also produces long-term benefits arises, as hormetic effects were also reported to be potentially transient effects (Stebbing, 1982). To answer this question, it is necessary to know the memory retention time of previous specific stressors for the physiological processes in a single hydra polyp or a population. Although we did not analyze the memory retention time of previous stressors to hydra in this study, there are two facts that lend support to the possibility that the patterns we observed are transient effects (Stebbing, 1982). First, the dynamic equilibrium of cell gain and cell loss leads to a continuous renewing of all cells in a polyp body within a few weeks (Campbell, 1967). Consequently, there are supposed to be no long-lived cells in the hydra body, making it difficult for the organism to maintain a long-term memory (Terman and Brunk, 2005). Second, hydra is exposed to dramatic environmental changes in its natural environment, where temperature and the availability of resources (in addition to other factors) vary greatly in time and space. Hence, we suggest that a long-term memory retention time may not be necessary, as fluctuations in the environment occur frequently and allow for recurrent and continuous improvement of somatic maintenance efficiency via hormetic-dose stress responses. Stress itself does not need to induce a lasting adaptive and heritable response in hydra.

Our results are obtained from a laboratory strain of hydra. Our finding of improved reproduction and maintenance after mild stresses found under laboratory conditions cannot directly be linked to responses in the wild. Because, in the wild we can assume that irregular and unexpected multiple changes of environmental conditions occur, which can lead to interaction effects of environmental conditions. Additional experiments in consideration of the possible interaction effects of the natural environmental have to be done in future.

5. Conclusion

Hydra presents an interesting deviation from typical life-histories: It appears to show no senescence, providing an outstanding opportunity to gain insights into the role that trade-offs may play in shaping age-specific life-histories. Therefore, a set of laboratory experiments investigated how trade-offs between reproduction and maintenance (here measured as survival under starvation) are modified under environmental stresses. For our isogenic hydra strain, lacking both genetic variation and generalist traits, we observed a clear trade-off between asexual reproduction and maintenance under constant conditions with maintenance staying rather constant while budding increased linearly with food intake. Moreover, under fluctuating environmental conditions both reproduction and maintenance can benefit from these stresses, i.e. producing a hormetic stress response. Under recurrent hormetic stresses polyps may emerge stronger in terms of health and reproductive activity with time. Such recurrent environmental stresses may have contributed in the evolution of the extraordinary aging pattern without senescence in hydra including its hormetic stress response ability. Hydra's constant cell turnover and the resulting regenerative capacity may play a crucial role in its remarkable plasticity of resource allocation responses including its hormetic abilities.

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CHAPTER IV

Variation in individual fitness of Hydra polyps and the importance of stochasticity

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ABSTRACT

It is widely recognized that the fitness of individuals varies substantially within populations. It is difficult to allocate the different sources of variation as there are genetic, environmental and residual stochastic variabilities. While it is widely assumed that genetic and environmental variations are major components of fitness differences, there have been few attempts to examine whether stochasticity alone could be sufficient to account for fitness differences. The asexually reproducing freshwater polyp *Hydra* represents an ideal study organism to examine the features of realized inter-individual variation in a clonal animal under constant environmental conditions.

In this study, we examined the individual variation in life-history traits, using an isogenic laboratory population of *Hydra magnipapillata*. We analysed the traits "asexual reproduction" and "age at first reproduction" of more than 1118 genetically identical polyps, subdivided into six cohorts of different ages (2-5 years). The individuals were kept rigorously under constant and equal environmental conditions.

We detected a highly uneven distribution of phenotypes of both traits. The analysis of the budding behaviour reveals that half of the offspring was produced by about a quarter of parent individuals (17-25%) and about 25% of the individuals changed their budding rate more than once throughout their lifetime. The high variation in budding behaviour is also supported by a high coefficient of variation of 70% and more per cohort. Comparisons of the budding behaviour between generations reveal that budding potential is not a heritable trait for *Hydra*.

The high variation of budding phenotypes in isogenic *Hydra* individuals provide clear evidence for a random phenotype generation process leading to stochasticity in phenotype plasticity in a basal multi-cellular organism. We assume that this phenotype stochasticity is an adaptive bet-hedging strategy ensuring survival for some individuals in case of sudden und unpredictable environmental changes.

INTRODUCTION

Individuals within a wild population can differ substantially in their fitness. It is well established that survival and lifetime reproductive success are far from homogeneous across individuals within populations. Such differences within populations are usually driven by genetic differences between individuals, providing the basis for the populations' ability to adaptively respond to natural selection caused by environmental changes (Weismann 1889; Burt 2000).

Alternatively, individual phenotypic variations determine the ability of an individual to respond to environmental changes. Such individual plasticity can be defined as a reaction to changing environmental conditions by altering the individual's morphological, physiological or developmental traits during its lifetime. Therefore, different environments may result in different phenotypes even if the genotypes are the same (Stearns 1989).

Even if both genetic and environmental causes of phenotypic variation have been controlled for, individuality still exists. For instance, under constant laboratory conditions isogenetic populations of nematodes (Finch and Kirkwood 2000); (Rea et al. 2005) and marble crayfishes (Vogt 2008), inbred lines of Drosophila (Gartner 1990) and genetically homogeneous bacteria cultures (Spudich and Koshland 1976; Avery 2006) show substantial variation in various life history traits such as survival, lifetime reproduction and other measures of individual performance. However, this individuality in isogenic populations should not necessarily be interpreted as evidence for quality differences between individuals. Stochastic variation could also be important. Stochasticity is a biologically important variable for the fitness of organisms (Kussell and Leibler 2005; Steiner and Tuljapurkar 2011) that is subject to natural selection (Beaumont et al. 2009; Lenormand et al. 2009).

Here, we aimed to assess if phenotypic variation can be observed among individuals within an isogenetic population of the freshwater polyp *Hydra* kept at controlled and constant laboratory conditions. Such a variation would be generated solely by stochastic phenotype expression. *Hydra*'s body plan is well defined. It contains stem cells, its nerve system is simple and the tissue consists of only two differentiated tissue layers. *Hydra* species reproduce mostly clonally by budding. The cells from the mother individual migrate into the bud without meiosis or the reduction to a single cell. This excludes phenotypic variation due to instability in developmental processes (see literature in (Vogt 2008)). Thus, *Hydra* offers an ideal opportunity to analyse the influence of stochastic mechanisms on individual phenotypes in contrast to recent research that mostly focuses on the importance of stochasticity for phenotypic variation due to instabilities in developmental processes (Gartner 1990; Lajus and Alekseev 2004; Vogt 2008). If high phenotypic variation in the life history traits of isogenetic *Hydra* individuals kept under uniform environmental conditions were detected, this would be the first evidence for phenotype plasticity due to stochastic variability in this genus.

In order to identify the importance of stochasticity for variation in *Hydra* life history, we analysed the traits “asexual reproduction” and “age at first reproduction” of 1118 genetically identical polyp individuals that were kept rigorously under constant and equal environmental conditions. We tested the hypothesis that these isogenetic individuals have a high variation in the measured traits as can express statistically by mean value with high variance. Such variance would indicate the expression of multiple phenotypes by one genotype as caused by stochastic processes, implying a high degree of genome plasticity and differential gene expression of one clone. Also, if the assumed process was at work those traits should not be heritable.

MATERIAL AND METHODS

Study Organism

Hydra is a derived member of the basal metazoan phylum Cnidaria (Hydrozoa), belonging to the Diploblasta, the sister group of the Bilateria. *Hydra* has a defined body plan, stem cells, a simple nerve system and two differentiated tissue layers. (Martinez et al. 2010) estimate the origin of *Hydra* about 60 Ma ago. The experiment was performed using a laboratory population of *Hydra magnipapillata* strain 105 under conditions described in chapter III. Strain 105 was established as lab culture from a single polyp (= single clone) collected from a freshwater pond in Japan in 1973 (Toshitaka Fujisawa, personal communication, (Sugiyama and Fujisawa 1977)). The polyps are well adapted to artificial environmental conditions. No sexual reproduction has ever been observed within our cultures. For further culturing details see chapter III.

Our lab stock culture was founded in March 2005 by budded offspring from a single polyp (= one member of the clone strain 105), which originated from a sub lab culture of the Irvine University (U.S.A.). On 1 March 2006 individuals for the first cohort A derived by budding from the stock culture. This process of building a new cohort of 204 polyp individuals was finished after 55 days. About six months later (10 December 2006) cohort B (all subsequent cohorts consist of 204 individuals) was established by budding by the individuals of cohort A. Three months later (16 March 2007) cohort C was established from cohorts A and B. The following cohorts were all derived from cohorts A, B and C and were separated on 10 January 2008 (cohort D), on 9 September 2008 (cohort E), on 1 April 2009 (cohort F), on 18 March 2010 (cohort G) and on 1 November 2010 (cohort H). Exceptionally, 19 buds of cohort E were produced by cohort D.

Experimental setup

In order to minimize sources for variation in the life history of single polyps we followed standard proven laboratory procedures (Martinez 1998; Chapter III). All individuals were cultured individually in plastic multi-well culture plates using a medium containing 0.05 mM NaHCO3, 1 mM CaCl2, 0.1 mM MgCl2, 0.001 mM MgSO4 and 0.003 mM KNO3 in deionised water. The medium (~ 9 ml per well and per polyp) was exchanged once in a week. The culture plates with the polyps were reared in an incubator at a constant temperature of 18°C under a constant 12/12 light/dark cycle.

Following rules for the experimental set-up were applied: The polyps were cultured under identical and constant laboratory conditions in order to standardize macro-environmental parameters and minimize micro-environmental conditions; we used only genetically identical polyps that were derived from asexual reproduction (budding) of originally one polyp; (3) all individuals were set on a controlled mono-diet with the same quality and quantity of food (*Artemia salina* nauplii, 1 day post hatching; (4) each polyp was reared in a single well on a 6-well micro-plate and could thus be individually recognized and fed; (5) polyps were regularly inspected (4 days per week) and checked for new buds or non-consumed *Artemia.*

Feeding regime

Hydra in general shows deterministic growth, but size can vary as a function of food and environments (Otto and Campbell 1977). *Hydra* can shrink or grow depending on the food supply (Chera et al. 2009). The rate of asexual reproduction (budding) also depends on the supply of food, but additionally on the ability of the parents to utilize it, as well as on how resources are allocated within the organism (Chapter III). In order to minimize phenotypic changes in reproductive traits as a response to differing feeding regimes within and between individuals the feeding regime was highly controlled. Throughout the whole experiment,

every *Hydra* was fed 3 *Artemia* every Monday, Wednesday and Friday resulting in 9 *Artemia* per week per *Hydra*. These *Artemia* were fed directly to the *Hydra* by placing each of the three nauplii with a pipette onto the *Hydra* tentacles. All polyps were checked for complete food intake after feeding on the subsequent day or on the medium exchange day.

Data analyses

Data

Each newly detached bud was counted. "Budding rate" (buds produced per day per *Hydra*) as well as "age at first reproduction" (when a polyp produced its first bud) are used as a measure of fitness of each polyp. We assume that fitness is a direct measure of the individuals' efficiency to allocate energy into reproduction. Healthy, fit and well adapted polyps have enough energy to reproduce by budding. We here assume that differences in reproduction of equal polyps in a constant environment are evidence for phenotypic differentiation. Important, 106 individuals died by accidents during the experiments and were not included into the calculations.

Concentration curve

An approach to find inequalities within our experimental cohorts are to use concentration or Lorenz curves (Lorenz 1905) and the Gini coefficient (G, (Gini 1912)) which summarizes the total amount of inequality within a cohort. "Budding" was used to evaluate the degree of inequality among all isogenetic polyps within each cohort. We compared the concentration of reproduction across six *Hydra* cohorts. In a concentration curve individuals are ranked by their reproduction, and the cumulative proportion of polyps (x-axis) is plotted against the corresponding cumulative proportion of their total reproduction on the y-axis. These concentration measures are estimated from distributions of all adult *Hydra* individuals within

a cohort according to the number of buds they had throughout the study. If the *Hydra* share is equal to the share of buds, then perfect homogeneity of reproduction exists and the concentration curve is simply diagonal (G=0). Greater differences in individual budding rates lead to greater deviation from the diagonal concentration curve (concave shape; G>0). The *Havehalf* and *Halfhave* are two measures of reproductive concentration used in prior studies (Shkolnikov et al. 2007). In this study *Havehalf* was defined as a minimum proportion of a cohort of parent *Hydra* producing 50% (half) of all buds. *Halfhave* denotes a maximum proportion of buds produced by 50% (half) of all parent *Hydra*s. The value of *Havehalf* decreases whereas the value of *Halfhave* increases when phenotypic variation is high for the reproductive trait (curve becomes concave).

Budding behaviour within a polyp's lifetime

We aimed to analyse the budding pattern of polyps under constant environmental conditions. We used linear regressions on the cumulative buds throughout the experimental time, the life span of an individual. The obtained slope (buds/days), from now on called b-value, was used to describe the budding rate of an individual. This measure is only appropriate if the budding rate of an individual is constant over time. To check if budding rate was constant over time and to verify our approach we tested if the b-value displaying the mean budding rate also described the budding behaviour on subsets of individuals' lifetime.

Since we observed a slight delay for most individuals before they reached a constant production of buds, we tested if either birth, the day of first reproduction (first bud produced) or the day the second bud was produced is best to be used for the fit of the linear regression. Additionally, we tested the possibility of changing reproductive output during a polyp's lifetime, which would reject the use of the b-values. For that purpose we divided the lifetime interval of each individual into ten disjoint test intervals of equal length (number of days).

Assuming constant reproduction mean via probability model in each of the ten small intervals, the expected number of expected buds equals the budding rate times the interval length. Thus we could check whether the counted number of buds per day lied within a 90% confidence interval around the calculated expected number of buds (b-value). If we could not find derivations from the calculated b-value a score of 0 was given to this interval. A significant deviation from the 90% CI resulted in the allocation of the score 1 for that interval. Also, we chose a minimum length of 50 days for each small interval in order to be able to make sensible use of estimations involving the Central Limit Theorem. In this way, the theoretical cumulative score over all ten intervals is binomially distributed for each polyp ($n=10$, $p=0.1$). The more the distribution of the real cumulative score deviates from the theoretical binomial distribution, the less support exists that our b-value model describes the budding behaviour, and the more a polyp's budding pattern is governed by changes in its budding rate. To realize this comparison some specific prerequisites must be fulfilled to draw reasonable conclusions: Only individuals with $\geq$12 buds and $\geq$500 days between the second and the last bud could be used, reducing our sample of 1118 polyps to 519. Our findings are that the distribution of the real cumulative score obtained by considering the period from the day of the second bud to the day of the last bud resembles the theoretical binomial distribution extremely well, providing strong support for our b-value model.

Heritability

To test the heritability of the measured traits (budding rate, age at first bud) we compared parents and their offspring as well as parents and their grand offspring and parents and their grand-grand offspring. If the traits are inherited by the next generation we should expect minor differences in the measured traits between generations. Additionally, traits could be constant over two or more generations, which we tested here. Heritability of budding rates

between isogenetic individuals of various generations were obtained directly from the slope of the linear regressions between generations. If the slope of the regression is 1, the traits are inherited between generations. If the offspring performed worse the slope of the regression would be smaller than one. If the measured trait improved in the following generations the slope would be greater than one. Since we study a clonal organism without a mother and father in the traditional sense we used 1 as the coefficient of relationship between the generations for the heritability estimation. Therefore, the heritability is equal to the slope of the regression between parents and offspring.

RESULTS

Reproduction

The concentration curves of reproduction reveal a strongly unequal concentration of reproduction among individuals in all six cohorts (fig. 1). Minorities of highly reproductive individuals in all cohorts lead to these inequalities. The Have-half statistic of the concentration curves indicate that, depending on the cohort, 17-26% of the polyps provided about 50% of all reproduction, while 50% of the polyps are responsible for 78-88% of the reproduction (fig. 1; Table 1). The concentration curves of reproduction are nearly similar between the cohorts except cohort D and E. The Gini coefficient reflects these results. A lower inequality in budding in the cohorts A, B, C, and F (G=0.38-0.42; Table 1) is followed by a higher inequality in budding in the cohort D and E (G=0.53 and G=0.54; Table 1).

The distribution of the polyps' reproduction in the cohorts A, B and C were right skewed (fig. 2) caused by a high intra-cohort variation in reproduction, ranging from 0 to 105 buds per polyp per measured lifetime. For the cohorts E and D the distribution of reproduction were weakly skewed right and showed a high number of individuals with only low or no budding

rate. Cohort F is, as the youngest cohort, tending to be right skewed as well. The coefficients of variation for reproduction vary between 0.79 and 1.12 (Table 1) and indicating a high individuality in reproduction of *Hydra* under constant conditions.

Age at first reproduction

All cohorts showed an unequal distribution (fig. 3) of age at first reproduction. Individuals needed between 20 and 1635 days to produce their first bud, 63 of 1118 individuals never produced a bud. Between 78-91% of the individuals produced their first bud until about 300 days after their birth.

In the cohorts D and E 50 (~26%) and 43 (~22%) individuals, respectively, have not started reproduction after 600 days of observation (fig. 3). Moreover, 22 individuals of cohort D and 27 of cohort E never produced a bud even after 1026 and 783 maximal observation days (data not shown).

Variation in reproduction during polyp lifetime

Our data consist of 1118 polyps with their daily budding information, the oldest cohort observed for up to five years. A total of 1055 polyps budded at least once during the observation time, and 963 polyps budded at least twice. Due to our b-value model specifications we could use 519 polyps (see methods). Fig. 4 describes the lifetime budding behaviour of four example individuals and the deviations from the b-value. In two of the shown example individuals the observed interval budding rates differed in five and six time intervals significantly from the expected overall budding rate (b-value). About 25% of all individuals analysed showed two or more deviations from the expected overall budding rates, suggesting that the budding pattern of those polyps is governed by frequent changes in their budding rate during lifetime (fig. 5). In contrast, 384 out of 519 tested individuals (around 75%; fig. 5), do not or only at most once change their budding behaviour during their

lifetime. Two of such individuals are depicted in figure 4 (bottom), they showed a constant budding rate over their lifetime.

Heritability estimates

The heritability analysis was done independently for budding rate and age at first reproduction between one, two and three generations. All linear regressions between consecutive generations showed no significant relationship between related individuals (all linear regressions; $p>0.05$) and low r^2 values, which shows that the linear regressions cannot describe the data appropriately (fig. 6). Therefore heritability estimates for budding rates and ages at first reproduction from all generation regressions were close to zero, indicating no heritable reproductive traits between generations. Consequently, the measured trait of the offspring does not depend on the trait of the parents. This means that an efficiently reproducing parent polyp does not necessarily produce similar efficiently reproducing buds.

DISCUSSION

In our study, we observed considerable variation in two reproductive traits between hundreds of isogenetic *Hydra* individuals reared under constant conditions over four generations. The analysis of the lifetime reproductive success of each single individual revealed that the most productive individuals (17-25%) produced half of the offspring for the next generation. Such an uneven distribution of budding rate in an isogenetic organism is surprising and it is, however, supported by a high coefficient of variation of 70% and more per cohort. A high variation in budding behaviour could mostly be observed between overall lifetime budding rates (b-value), and just weakly during a polyp's lifetime. Only about 25% of the individuals changed their reproduction rate throughout the experiment. Most individuals (~75%) expressed a constant budding rate throughout their lifetime, once it was established, which usually was the case after the production of the first bud. This indicates clearly that the laboratory conditions were constant and equal over the course of the experiment. However, several questions arise from our observations: what triggers (i) the huge variation of budding rate between individuals and (ii) the changing budding behaviour per lifetime of the remaining 25%.

Such great phenotypic variation as shown by the uneven distribution of reproductive success is clearly not derived from genetic or environmental differences, as these have been experimentally excluded, and should therefore not be viewed as evidence for differences in quality between individuals. It might be argued that individuals can never be optimally adapted to an environment and hence heterogeneity in a cohort or population is just a natural occurrence. But it is also very unlikely that such a huge phenotypic variation as we observed, is a consequence of small genetic or microenvironmental disparity. Rather, the results of this study point to the importance of stochasticity in producing phenotypic variation as supported

by a growing number of publications (Rea et al. 2005; Sanchez-Blanco and Kim 2011; Steiner and Tuljapurkar 2011).

Several studies show that individual fates may be shaped by a sequence of stochastic events that generate significant variations in different life history traits e.g. lifespan, reproduction or germination ability (Gartner 1990; Finch and Kirkwood 2000; Rea et al. 2005; Vogt 2008; Sanchez-Blanco and Kim 2011). Johnson (Johnson 1990) describes considerable variations of lifespan between genetically identical individuals of highly inbred populations of the nematode *C. elegans*. Also, the production of many different phenotypes measured as reproductive success and lifespan was observed in isogenetic populations of *Daphnia* (Lajus and Alekseev 2004) and in marble crayfish (Vogt 2008). Other intriguing examples are unicellular organisms where individuality in growth rate and reproduction are of particular significance. Here, individual cells may randomly switch among a number of different inheritable phenotypes (Kussell and Leibler 2005; Davidson and Surette 2008). Even in out-bred populations, similar non-genetic and non-environmental variations seem to be a major component for life history traits such as lifetime reproductive success or lifespan of species (kittiwakes: (Steiner and Tuljapurkar 2011), in *C. elegans* and others: (Finch and Kirkwood 2000). Another example is annual plants and insects where stochastic mechanisms are often related to great variations in the duration of diapause and dormancy (Cohen 1966; Childs et al. 2008). Taken together, these examples and the results of this study support the view that the intrinsic individual variability of *Hydra* is of stochastic nature.

Stochastic mechanisms introducing high phenotypic variability

Which mechanisms can create variations in genetically identical *Hydra* reared under constant conditions? Despite the possible importance of stochasticity in the life history of *Hydra*,

nothing is known about the interaction between stochastic events and biological processes. There are some interesting aspects, which might be worth considering in the future:

Stochastic processes have been described in a variety of species from unicellular organisms to plants and animals. For example, stochastic fluctuations occur in gene expression (Fraser et al. 2004; Rea et al. 2005), or in epigenetic changes (Henderson and Jacobsen 2007; Levy et al. 2012; Pujadas and Feinberg 2012). This can result in multiple states of phenotypes varying strongly in their activity, which in turn affects growth or metabolic rates of the entire organism (Raser and O'Shea 2004; Davidson and Surette 2008; Levy et al. 2012). In a laboratory study of the worm *C. elegans*, Rea et al. (Rea et al. 2005) showed that a significant amount of phenotypic variation could be related to the stochastic variation in the expression of a single gene involved in thermal stress resistance.

Most likely, such molecular processes within single cells also cause stochastic variation in *Hydra*, resulting in variations of basic cellular processes such as cell division, migration and differentiation. Such cell-to-cell variability may influence the individuality of a single polyp. If stochasticity was also responsible for the selection of cells that migrate during budding between parental polyp and offspring, the different distribution of cell phenotypes between different offspring lines would trigger the polyp-to-polyp differences in reproduction. A remarkable phenotypic plasticity of epithelial cells with diverse architectural design and physiology in *Hydra* was described in the study of Anton-Erxleben et al. (Anton-Erxleben et al. 2009).

Another source for stochastic variation, although not generated by the polyp itself, is the interplay between epithelial cells and microbial communities living on those cells (Fraune et al. 2009; Fraune and Bosch 2010). Already in 1982, (Rahat and Dimentman 1982) showed that bacteria might be important for tissue proliferation and successful budding in *Hydra*. But whether stochastic processes contribute to the variation in budding rate in *Hydra* depends on

whether the interaction between polyp and microbial community enhances or suppresses the effect of stochasticity. Furthermore, stochastic phenotypic switching often observed in bacterial cells (see (Kussell and Leibler 2005; Davidson and Surette 2008)) has been proposed to facilitate immune changes within and colonization of new niches by the host (Salathe et al. 2009). This might also influence the reproduction rate of polyps in a stochastic way.

However, the mechanisms underlying stochastic changes in *Hydra* are yet completely unknown and it is uncertain, whether stochasticity is of significance for the survival of *Hydra* under natural conditions.

Benefits for *Hydra*'s life history

A major question that arises from the findings in our study is: Why does *Hydra* display such high phenotypic variability without genetic variability? Most likely, this results from a risk spreading strategy in uncertain environments that are called bet-hedging (Stumpf et al. 2002; Thattai and van Oudenaarden 2004). This strategy could enhance long-term fitness by increasing the likelihood that a subset of individuals expresses a phenotype that allows the genet to sustain well through time in different and fluctuating environments (Cohen 1966; Kussell and Leibler 2005). Understanding the role of individuality in *Hydra* requires a greater appreciation of the ecological context of *Hydra*'s natural environment – freshwater rivers, lakes or ponds (Holstein and Emschermann 1995). Besides seasonal variation in ambient temperatures, photoperiod or food density, unpredictable fluctuations of *Hydra*'s environment could be water-level fluctuations, freezing, sudden spatial or temporal changes of the biotic or abiotic substrate niches or abrupt inter-species competition for resources or predation. All those environmental cues influence and affect the life history of individual *Hydra* polyps on different levels in several ways. On the basis of our experimental design,

that ensured constant environmental conditions, we can only speculate whether stochasticity significantly affects the fitness in the wild. In order to test whether the high variation of phenotypes is an evidence for an adaptive strategy to survive in fluctuating environmental conditions we should transfer individuals in an environment that fluctuates in time among a finite number of different environmental types (Kussell and Leibler 2005; Acar et al. 2008; Beaumont et al. 2009). Observing various subgroups of phenotypes within these environments would support the hypothesis that stochasticity and randomness are biologically important for *Hydra*'s life history and beneficial for its fitness and adaption.

Evolution of phenotypic variability

A high number of phenotypic variations in a population can reduce the mean fitness of the organism in a constant environment. Thus, from an evolutionary point of view, low phenotypic fluctuation around the fittest state is beneficial for maintaining optimal and maximal function over time (Landry et al. 2007; Lehner 2008). If genetic variability governed phenotypic adaptation and plasticity, the influence of stochasticity on phenotypic traits should be minimized through natural selection thus reducing any deleterious effects. In contrast, a faster adaptation to fluctuating environments by stochastically induced phenotypic variation of *Hydra* buds could be a selective advantage if we assume that genetic variation plays a minor role in the wild. One way this could have evolved in *Hydra* is by exhibition to multiple environmental cues that vary over small spatio-temporal scales. Such conditions may lead to differences in fitness between individual phenotypes but should not affect the distribution of phenotypic variation within a genet in general. In that case, selection pressures vary (with temporal environmental fluctuation) in such a way that in a given environment only a fraction of phenotypes is exposed to selection. A subsequent, sudden environmental change may affect another fraction. Such a fluctuating selection could favour the evolution of

phenotypic stochasticity within a population (Slatkin 1974; Meyers and Bull 2002; Kussell and Leibler 2005) and can therefore enhance long-term fitness of the *Hydra* genet, by increasing the chance that a subgroup of individuals exhibits a phenotype that will be well adapted to future environments (Slatkin 1974; Acar et al. 2008; Beaumont et al. 2009). Selection would favour the genotype which generates the stochastic variability of individual offspring phenotypes in an optimal phenotypic variability range of the affected traits, according to their living environment, to increase the genotype's lifetime reproductive success through time. In millions of year's evolution, *Hydra* experienced various environmental conditions, which should allow the development of genotypes with a high potential and ability for phenotypic plasticity.

CONCLUSIONS

The findings of our study provide the first evidence that stochasticity might be very important for the adaption ability of mainly clonally reproducing *Hydra* populations. Stochasticity can be an important component for *Hydra* life history by modifying individual fitness and population dynamics. Therefore, this study on *Hydra* supports the suggestion by (Beaumont et al. 2009) that stochastic processes may have been among the earliest evolutionary solutions to survival in variable environments.

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FIGURES AND TABELS

Table 1. The Number of samples, the age range of individuals per cohort, the "Have-half", "Half-have" statistic, Gini coefficient for the concentration curve of reproduction and the coefficient of variations (SD / mean) for budding rate and age at first reproduction were presented.

Cohort	N	age range (days)	Have-half (%)	Half-have (%)	Gini-coefficient	%CV Budding	%CV Age at first reproduction
A	164	1593-1706	25	78	0.39	70.5	99.7
B	185	1419-1513	26	78	0.38	68.4	92.6
C	184	1216-1326	25	79	0.40	71.8	79.7
D	192	861-1026	18	87	0.53	105.1	112.9
E	198	627-783	17	88	0.54	106.1	101.9
F	195	430-579	25	79	0.42	80.9	95.9

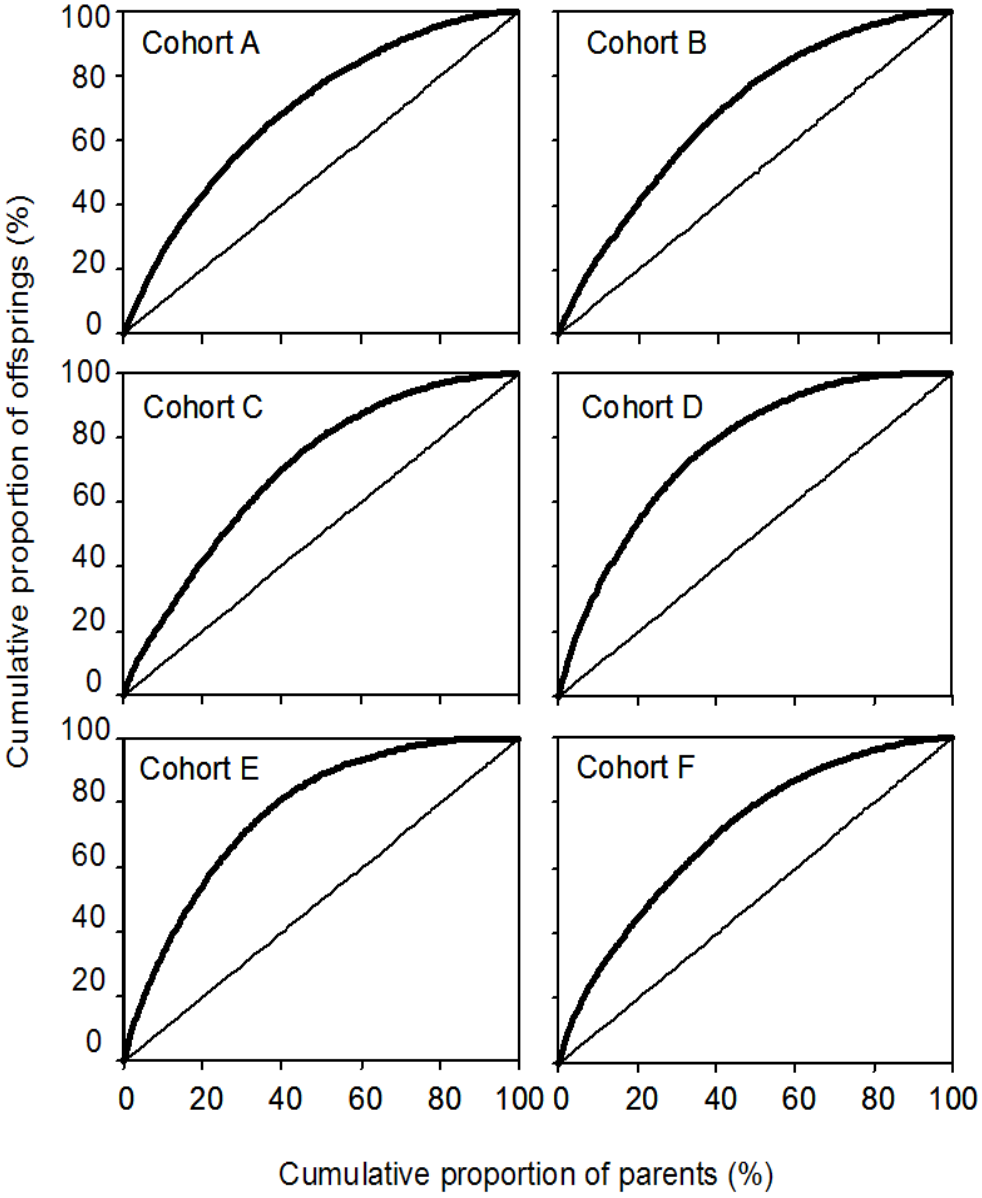

Figure 1. Concentrations of reproduction among genetically identical *Hydra magnipapillata* individuals of six cohorts with different ages at similar and constant environmental conditions are shown. The diagonal line is identical with the line of equality of reproduction within each cohort.

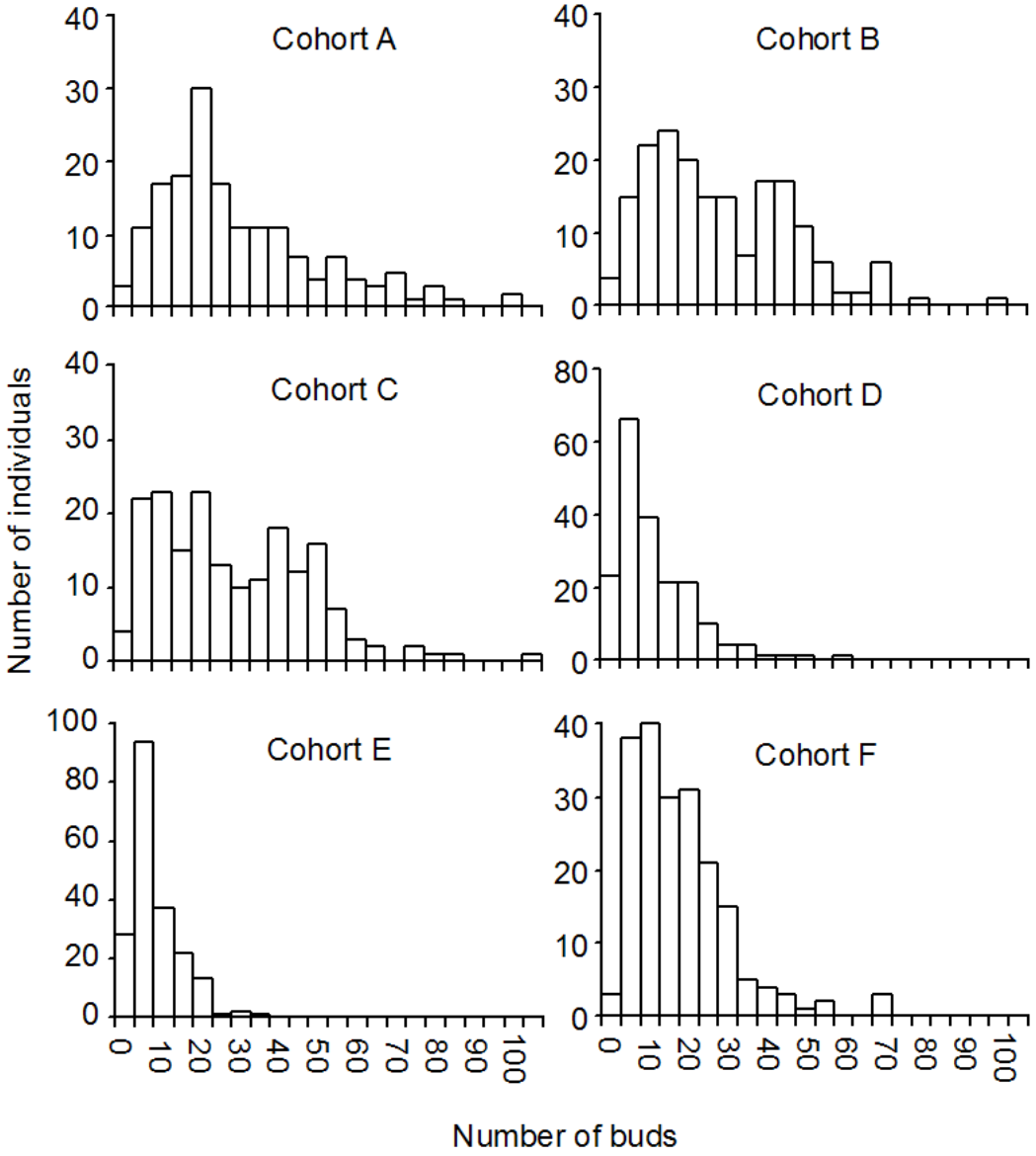

Figure 2. Frequency distribution of the differences in "budding rate" between genetically identical *Hydra magnipapillata* individuals of six cohorts with different ages at similar and constant environmental conditions (Table 1).

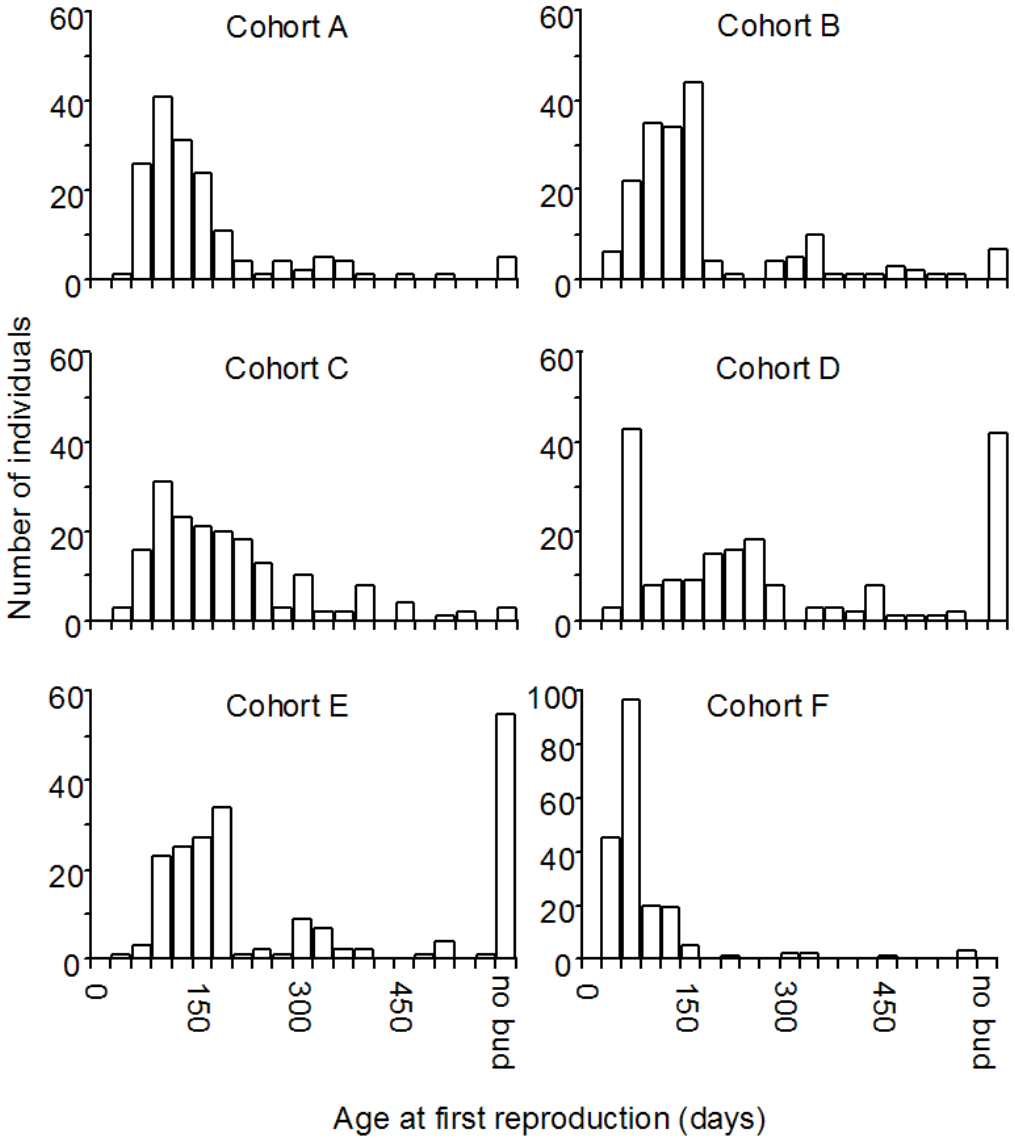

Figure 3. Frequency distribution of the differences in "age at first reproduction" between genetically identical *Hydra magnipapillata* individuals of six cohorts with different ages at similar and constant environmental conditions (Table 1).

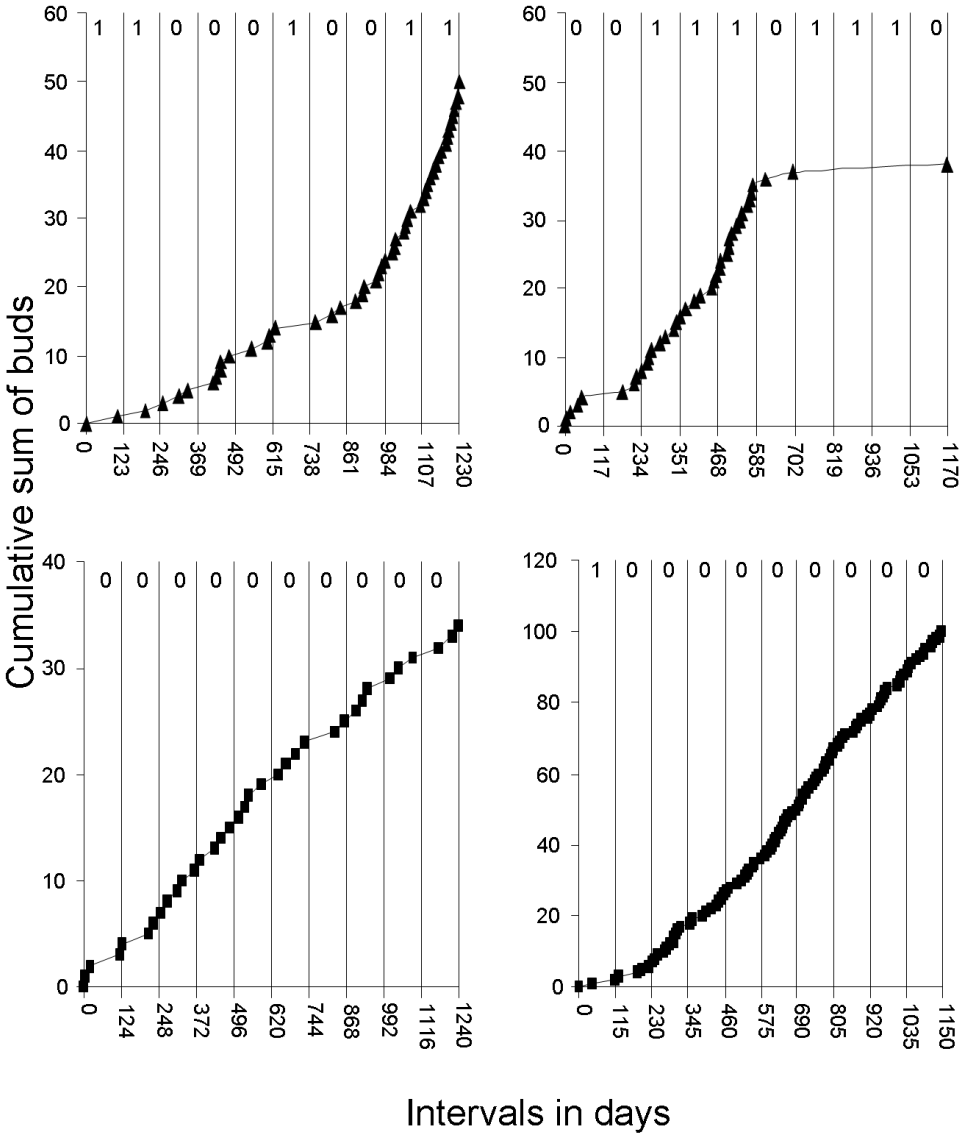

Figure 4. Budding behaviour during lifetime of four example individuals. Two polyps (above) have 5 and 6 changes, respectively, here scored as 1 in their observed budding behaviour as compared to the expected overall budding rate (b-value). Two other examples (bottom) showed no or only one deviation from the expected overall budding rate value (b-value).

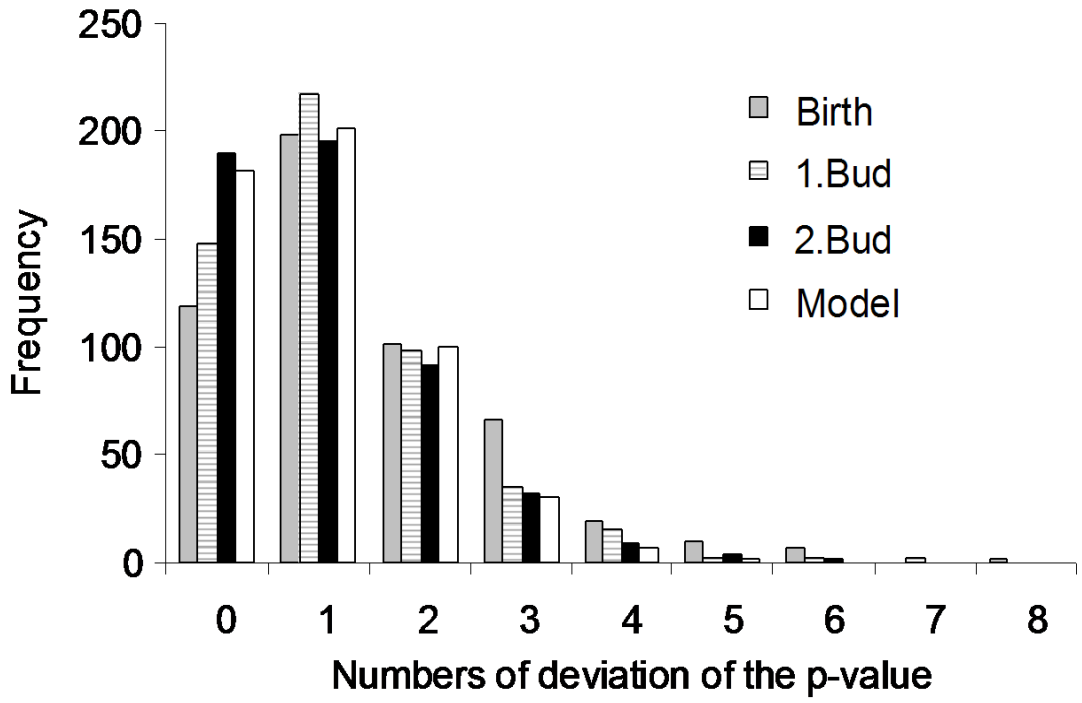

Figure 5. The distribution of the number of interval budding rate deviations from the expected overall budding rate value (b-value). Presented here are the results of the theoretical expected distribution of the deviations (model) and the distribution of the observed deviations in budding rates after the times of starting from birth, production of the first and of the second bud.

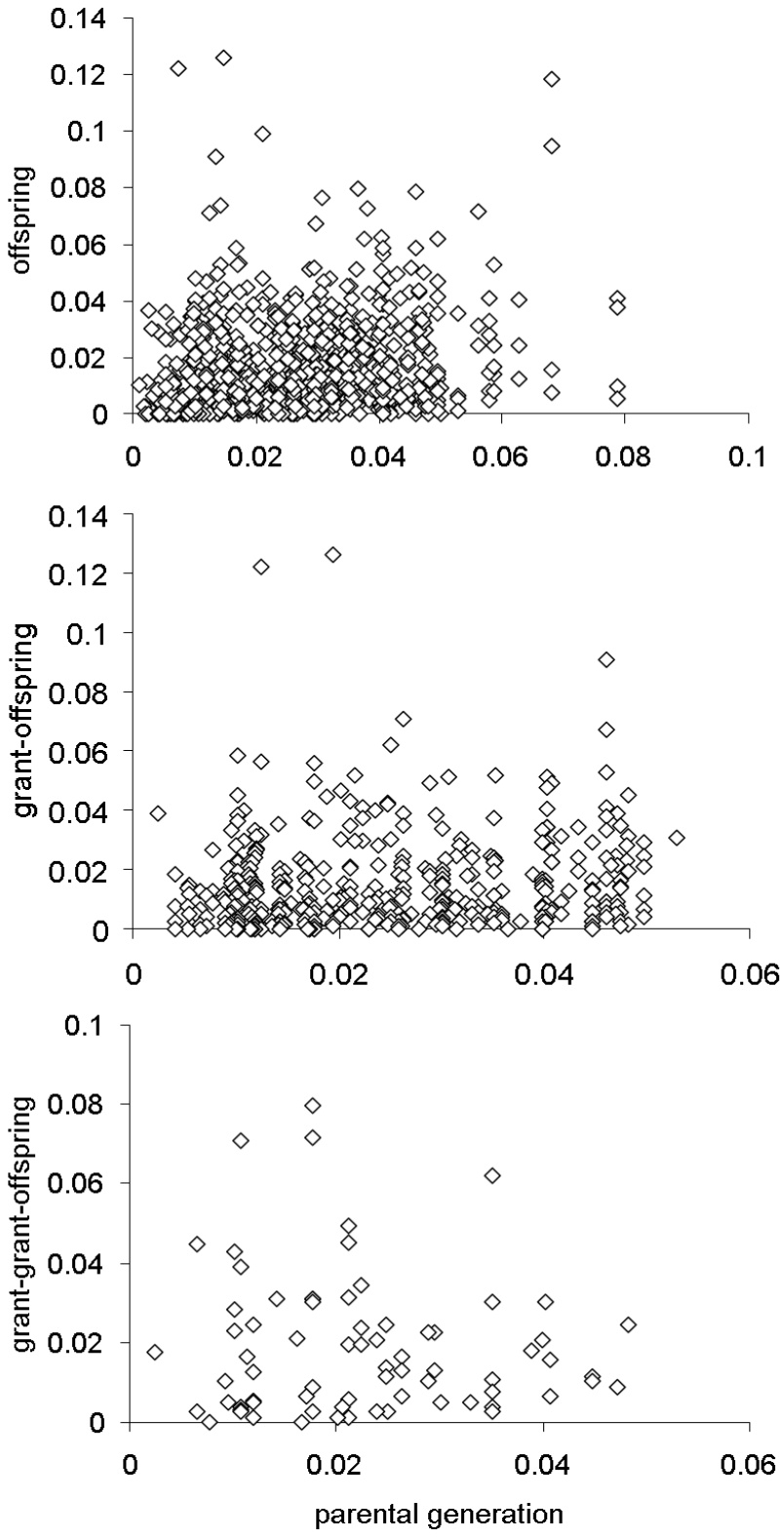

Figure 6. The relationship as measure of heritability of the expected budding value (b-value) between different generations; parents polyps are compared with offspring lines of different generations (children; grand-children; grand-grand-children) of *Hydra magnipapillata*. The linear regression model for parental generation *vs*. offspring: $y_{1,853}$ = 0.23x + 0.011, r^2 = 0.038, SE = 0.001, F = 33.2, p < 0.001; for parental generation *vs*. grant-offspring: $y_{1,398}$ = 0.15x + 0.012, r^2 = 0.016, SE = 0.002, F = 6.4, p = 0.012; for parental generation *vs*. grant-grant-offspring: $y_{1,72}$ = -0.14x + 0.02, r^2 = 0.005, SE = 0.001, F = 0.54, p = 0.46.

CHAPTER V

To cut or not to cut - Bisection trade-offs in the polyp *Hydra magnipapillata* (Cnidaria: Hydrozoa)

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ABSTRACT

Basal metazoans have large variations in life-cycle patterns, offering great opportunities to study the evolution and demographic patterns of aging. In light of the remarkable regeneration abilities and non-senescent aging pattern of the freshwater polyp *Hydra*, this study aims to examine its resource allocation strategy in response to environmental stressors. In a set of laboratory experiments, we tested the resource allocation flexibility of a purely asexually reproducing *Hydra* strain (*Hydra magnipapillata*). Isogenic *Hydra* polyps were reared at different feeding regimes and bisected to simulate predation stress while reproduction, size, and starvation survival after regeneration were compared between groups to reveal eventual allocation trade-offs and hormesis effects. Surprisingly, bisection effects were similar in both halves. Both head and foot regenerates showed similar if not slightly enhanced trait merits in comparison to the uncut controls, from which we conclude a hormetic efficiency increase response. Short feeding regime interruptions accompanying the bisection treatment reinforced this hormetic response. Feeding level prior to starvation had a substantial influence. More food resulted in higher budding rates, but not necessarily longer starvation survival. Instead, a clear trade-off could be shown between the intermediate and second-highest food levels, with the highest and lowest average starvation survival, respectively. A shift of resource allocation focus towards reproduction is a reasonable explanation for the low survival group, since all groups with lower food levels had none or negligible offspring output. This effect is neutralized with increasing food availability, at which size, budding rate and starvation survival all increase again. However, all three key life history traits were uncoupled from each other - size, survival or budding showed no correlation. No heritability of any trait pattern from parent to bud could be detected. We conclude that *Hydra* is a dynamic organism with heterogeneous phenotypic states between and within polyps, a feature which is most likely linked to its constitutive proliferative cell renewal machinery. This vitality is particularly emphasized in its hormetic responses without that any costs of it were detectable in our experiment.

INTRODUCTION

Hydra (Cnidaria, Hydrozoa) is truly an enigmatic organism concerning its aging and life-history patterns. From a demographic point of view, *Hydra* polyps show no senescence, meaning that neither their mortality nor fertility schedules change with age, for the better or worse (Martinez 1998; Jones et al. 2014; Schaible et al. in preparation for submission). To understand this extraordinary evolutionary outcome it is helpful to study the flexibility of *Hydra*'s resource allocation and the trade-offs between maintenance, reproduction and growth which may be involved in shaping this pattern. According to present theories, organisms allocate resources either to 1) *repair, regeneration and somatic maintenance,* 2) *growth and/or* 3) *reproduction;* and this allocation is hypothesized to be optimized according to the selected course of life (Kirkwood 1977; Kirkwood and Holliday 1979; Stearns 1989; Kirkwood and Rose 1991; Vaupel et al. 2004; Baudisch 2007; Baudisch 2009; Flatt 2011). Hence, resource allocation can be thought of as a flexible system affecting an organism's aging pattern directly, contributing to the evolution of the various and diverse aging patterns found across the tree of life (Jones et al. 2014), which make it hard to claim a general exclusion of potentially lifelong growth and reproduction as well as continuous maintenance.

The freshwater polyp *Hydra* is a well-studied organism across various biological disciplines (Campbell 1967; Gierer et al. 1972; Martinez 1998; Bosch et al. 2010; Bosch 2012; Chapter III). The worldwide spread genus (except Antarctica) comprises four morphologically distinct and recognizable species clusters with various species (12-15 in total according to recent estimates) and more than a hundred species strains (Jankowski et al. 2008; Martinez et al. 2010). Populations undergo frequent seasonal fluctuations in temperate zones regarding population size and switches in reproduction patterns, changing from purely asexual to sexual or simultaneous reproduction in summer times to a more dormant and unproductive living

style during winter (Ribi et al. 1985)(own observations). However, sexual reproduction appears to play only a marginal role for proliferation and population growth in comparison with asexual reproduction in the genus *Hydra* (Bosch 2009). Asexual reproduction, which in the case of *Hydra* means vegetative proliferation via budding, can be viewed as extended growth, at which continuous individual growth results simply in more isogenic individuals ("ramets") after some time, providing a Hydra clone with an enormous proliferation power. Connected to this mode of vegetative proliferation, and most likely evolutionary selected by it, is the continuous proliferative cell renewal and cell turnover of *Hydra*. This machinery equips the polyp also with its remarkable regeneration capabilities (Bosch 2007; Bosch et al. 2010), and is thereby likely to play an important role in *Hydra's* exceptional maintenance and non-senescent aging pattern as well as in its flexible life-history responses, including hormetic reactions, towards changing environmental conditions (Chapter III; Schaible et al. in preparation for submission).

In chapter III we conducted a series of laboratory experiments by exposing a purely asexually reproducing brown *Hydra* strain, *Hydra magnipapillata* strain 105, to challenging environmental conditions to investigate the trade-off between reproduction and somatic maintenance. Selected stressors were shifts in temperature and variation of food level, including a starvation period, while budding rates during feeding periods were recorded continuously. Instead of the normal survival observation, survival in a final starvation period was taken as a feasible measurement for maintenance allocation in *Hydra* since one had to wait for a long time for the last polyp to die under constant feeding conditions due to the low level of their flat mortality rates (Martinez 1998; Schaible et al. in preparation for submission). The trade-off found in the study was that budding increased linearly with food intake while survival under starvation stayed rather constant from normal and non-starving

feeding levels on. Under fluctuating environmental influence by temperature or food limitations, both maintenance and budding tended to show enhanced levels, pointing to a hormetic type of response lacking a trade-off. Such an effect could increase maintenance and reproduction efficiency, though possibly only temporarily, resulting from exposure to low and specific doses of stress, a phenomenon known as hormesis (Calabrese and Baldwin 2003; Stebbing 2003; Parsons 2005; Mangel 2008; Rattan 2008). How exactly this effect is achieved, though, is still uncertain and several different mechanisms have been proposed, including physiological counteractions to stress with overcorrections to retain homeostasis, leading to a hormetic response pattern known as homeostatic hypothesis for hormesis (Stebbing 2003). In chapter III we proposed that "environmental stresses could have a beneficial impact on the fitness-related phenotypical traits of the basal metazoan *Hydra*", and that hormetic stress doses posed by variable and fluctuating environments could be salutary for the persistence of *Hydra*.

To follow up on these findings and examine *Hydra*'s resource allocation closer we conducted our study by applying a more direct type of stress. We simulated a predation injury by bisecting the polyps into two equally sized halves and combined this treatment with varying feeding levels. We measured sizes and budding rates before and after bisection and measured survival under starvation after the feeding period as maintenance measurement. We hypothesized that both bisected halves would show different trait values than uncut controls and that the foot regenerates would survive the longest under starvation whereas the head parts would have most buds compared to the respective counterparts. We supposed that the increased regeneration demand for the foot halve would trigger an increased general maintenance allocation within the polyp. Whereat the foot halves had to regenerate the complete head region including a new hypostome with the tentacle crown, the head halves had only to regenerate the adhesive basal foot region, which involves much less growth and

cell re- and transformation of *Hydra*'s dynamic cell column (Bosch 2007; Galliot and Chera 2010; Galliot and Ghila 2010; Galliot 2013). Five different feeding regimes were chosen to gain more insights into the required energy for complete regeneration after bisection and their effect on reproduction, growth and maintenance, measured as starvation survival, with respect to the bisection effect. Following up on our previous findings (Chapter III), we hypothesized that low food levels could increase allocation to maintenance and enhance starvation survival rates in comparison with the higher ones. Furthermore, we tested for heritability and persistence of any of the trait patterns in fed and unfed offspring generations. We suspected no heritability in any trait as no indications for inheritable reproductive traits have been found previously regarding budding rates and ages at first reproduction (Chapter IV).

MATERIAL AND METHODS

Study Organism

In our study we used the *Hydra magnipapillata* strain 105, a *brown Hydra* belonging to the *Hydra vulgaris* cluster, which was isolated in 1973 in Japanese wetlands near Mishima on Honshu (Sugiyama and Fujisawa 1977; Sugiyama and Fujisawa 1977; Sugiyama and Fujisawa 1978). The isogenic strain has been kept successfully in the laboratory for over 40 years with reproducing exclusively by clonal budding. No successful sexual reproduction nor clear production of either male or female gametes has been observed in the lab for this strain so far. Throughout the experiment we followed standard proven laboratory procedures with controlled environmental conditions benefiting from the long history of *Hydra* research.

Culturing conditions

All experimental animals were cultured in the laboratory under controlled and constant environmental conditions in plastic multi-well culture plates with a '*Hydra* medium' (detailed culturing conditions in chapter III). *Artemia salina* nauplii (instar II to instar IV stages, 1.5 dph (days post-hatching, Schaible & Houliston, personal communication)) were used as constant food source for all polyps.

Experimental Design

To test and compare bisection effects on *Hydra* we chose five different feeding regimes, from low (1 / 3 / 5 *Artemia* per week) to high (7 / 21 *Artemia* per week). The *Artemia* amount per feeding level was evenly distributed throughout a week with regularly three feeding days per week. Bisection was performed horizontally via scalpel in the middle of the body column (see fig. 1). Control groups without bisecting polyps were kept for both higher feeding regimes.

Following measurements were taken:

budding rate (daily)
survival (daily)
tentacle number (weekly)
volume/size via photometric area analysis with Adobe Photoshop© (weekly)
n = 18 in low feeding regimes n = 30 in high feeding regimes

All polyps were allocated individually to specified wells filled with 9ml culture medium on six-well-plates. Wells & culture medium were changed weakly to maintain favourable

conditions and feeding was conducted individually via pipettes, placing alive *Artemia* directly onto the tentacles of the polyps to ensure the correct amount of food per individual.

All investigated *Hydra* individuals were isogenic and originated from a long-term mixed stem population established in our lab. First measurements in all groups were taken in an initial phase before bisection. After 25 days, each polyp was horizontally bisected via scalpel, except the polyps in the control groups. Individual foot and head pieces (see fig. 1) were followed up and observed regarding their regeneration in terms of survival, tentacle number, size, feeding ability and budding rates. To synchronize growth and individual food intake between foot and head halves and controls, each polyp got the same amount of *Artemia* in respect of its feeding regime throughout the experiment. Thus, each bisected head half and its respective control partner was only fed when the corresponding foot half was able to ingest *Artemia* again after hypostome regeneration. Regeneration and feeding ability for the foot halves was tested daily after bisection until all foot halves were able to feed again. Original feeding regimes were gradually installed again individually within two to three weeks and maintained for another four to five weeks. Finally, feeding was ceased and polyps were starved to measure starvation survival as a maintenance measurement (Chapter III).

The budded offspring polyp generation after bisection was additionally followed up in parallel to check for the heredity of possible bisection effects. The first bud was taken and monitored from all polyps fed with 7 and 21 *Artemia* per week, the second bud was only isolated from each polyp in the highest feeding regime. All first buds of the highest feeding level were fed in the identical feeding regime as their parent counterparts before the start of the final starvation phase whereas the first buds of the 2nd highest feeding regime were starved immediately after isolation from their parents. The second buds of the parental polyps

in highest feeding regime were starved immediately as well. The experiment ended with the last polyp's death.

Size, as a measure for individual polyp growth, was measured by taking standardized pictures of elongated *Hydra* polyps free of ingested food (Levitis and Goldstein 2013). The same procedure as Levitis and Goldstein 2013 described it for measuring size in *Hydra vulgaris strain AEP* was applied to our measurements. Since we standardized our photo samples and photographed only elongated polyps we decided to use polyp the intuitive body volume as a size indicator (without the hypostome and tentacle crown for simplicity), which was calculated from the respective measured body area and length according the formulas given in Levitis and Goldstein 2013, who assumed a cylindrical polyp body shape for their calculations and showed that either volume, cylindrical body-column surface area or their proposed corrected version of the surface area of an individual give reliable size estimates in this case.

Analyses

SPSS software was used for the statistical analysis of the obtained data. We tested for bisection and feeding treatment effects on starvation survival, budding rate and polyp size (volume). One- and two-way ANOVAs were applied and followed by Tukey or Bonferroni post-hoc comparisons in compliance with the respective statistical requirements of the data. Trade-offs between the response variables and heritability of traits were checked with linear regressions, where appropriate. We compromised on the number of individuals in each treatment to maximize the treatment resolution, thus the statistical strength of the analysis might have got reduced for feasibility reasons.

RESULTS

A clear trade-off between resource allocation to maintenance versus reproduction could be shown for *Hydra* in our experiments. No polyps, neither parent or offspring, died naturally during the feeding phases.

Starvation Survival

Comparing all parental cohorts, feeding level had a pronounced and significant influence on starvation survival while bisection tended to have a slight but minor and statistically non-significant effect, no interaction effect was found (two-way ANOVA and Tukey post-hoc tests, $p<0.05$, see fig. 2 and supplementary tables S1-3). After a stepwise increase from the lowest feeding level up to 5 *Artemia* survival dropped sharply for polyps fed with 7 *Artemia* per week, increasing again to long survival at the highest feeding level. The shortest average survival was at the 7 *Artemia* feeding regime, while the cohort with 5 *Artemia* survived the longest. Bisection had basically no effect when comparing survival of head versus foot halves, while a slight trend towards longer survival in bisected polyps compared to uncut controls could be observed (only conducted at 7 and 21 *Artemia* per week). This positive survival effect was slightly more pronounced in foot halves compared to head halves.

Never fed offspring generations survived much shorter in starvation than previously fed parent and offspring generations (see figs. 3 & 4). Still, never fed offspring from parents of the highest feeding regime survived longer than their counterparts from parents of the *7 Artemia* regime, while no bisection or interaction effects existed (two-way ANOVA and Tukey post-hoc test, $p<0.05$, see fig. 4 and supplementary tables S8-9).

The fed first offspring generation from parent polyps of the highest feeding regime survived slightly, but significantly longer in starvation than their parents, with a trend towards a

bisection effect not within, but between generations, with survival lightly increased in the offspring of bisected polyps compared to unbisected control parents and offspring foot halves slightly increased compared to parent head halves, with the same, though here not significant, trend towards the parent foot halves as well (one- and two-way ANOVA with Tukey post-hoc tests, $p<0.05$, see fig. 3 and supplementary tables S4-7).

Comparing all groups separated, including fed and unfed offspring cohorts, significant survival differences were never observable between head, foot or control polyps within any feeding regime per generation (Bonferroni post-hoc test after a significant ($p<0.05$) one-way ANOVA, $p>0.05$, fig. 2-4, test results not shown due to length). Clear survival differences existed here only between specific feeding levels, most pronounced between the short starvation survival of all unfed groups and groups fed with 7 *Artemia* per week compared to the relatively long survival of polyps fed with 5 and 21 *Artemia* per week.

Budding behaviour

Budding rates of parent polyps differed considerably between feeding levels but the comparison between before and after bisection budding rates per respective feeding level showed only differences at the highest feeding level (two-way ANOVA with Tukey post-hoc tests, $p<0.05$, see fig. 5 and supplementary tables S10-12). Most strikingly, a huge difference between the highest and second highest feeding level could be observed, while budding ceased almost completely at the three lowest feeding regimes (fig. 5). Among the low regimes, only the budding rates at 5 *Artemia* prior bisection stood out and were minimally increased compared to all low feeding groups, though just above the significance level (Tukey post-hoc, $p>0.05$, fig. 5 and supplementary table S11). At 7 *Artemia* per week a low budding output could be observed throughout groups, a weak, but non-significant trend towards an increased budding after vs. before bisection could be seen, whereby this effect

was strongly significant at 21 *Artemia* per week. Interestingly, budding rates prior bisection were similar at 5 and 7 *Artemia* per week.

Irrespective of the significant group differences at the highest feeding regime between budding rates before vs. after bisection, we could not find clear correlations between individual budding rates before versus after bisection within treatments and feeding levels as well (linear regressions, maximum r^2=0.145, see fig. 11 and supplementary tables S22), indicating independence of budding rates before versus after bisection.

Bisection itself had no overall effect on the budding output (two-way ANOVA with Tukey post-hoc test, $p>0.05$, fig. 5 and supplementary table S12). Treatment budding rates before bisection were not different from head or foot halves in combined low feeding regimes while heads did not differ from feet as well. For the two combined high feeding levels, though, both control and treatment budding rates prior bisection were significantly lower than the respective controls, heads and feet after bisection and higher than all respective combined low feeding groups, without that they were different from each other themselves. No differences between heads, feet and controls after bisection were found. No overall interaction could be found in the two-way ANOVA.

Within the highest feeding regime, parent versus first generation fed offspring buds yielded no significant differences between any group (one-way ANOVA and Tukey post-hoc test, $p>0.05$, see fig. 6 and supplementary tables S13-14). Combining groups and comparing generations, we found no difference between budding rates (two-way ANOVA and Tukey post-hoc test, $p>0.05$, see fig. 6 and supplementary tables S15-16). But when comparing bisection treatments of combined generations, we found a weak bisection effect, with foot regenerates having lower budding rates than heads, without that any of the treatments were different to the controls (Tukey post-hoc, $p<0.05$, fig. 6 and supplementary table S16). No buds were produced in both unfed offspring cohorts.

Size

We recorded huge variations of polyp volumes within and between feeding levels as shown in fig. 7. Qualitatively, as visible in the graph, bisection and the associated feeding restriction had the effect of reducing every polyps' size in the first days after the bisection treatment in all groups. After reinstalling feeding regimes, polyps grew back to previous sizes at feeding levels of 5 and 7 *Artemia* and tended to grow even larger in the highest feeding regime. In the two lowest regimes polyps did not regain original sizes, no growth at all could be seen at 1 *Artemia* per week and slight growth was visible at 3 *Artemia*. Head, foot or control polyps did not differ substantially in volumes within their respective feeding levels while size heterogeneity was highest during the feeding phases in all groups. Interestingly, size started to decrease already in the last two weeks of the feeding phase for the two highest feeding levels.

Budding versus Starvation Survival

We found no clear correlation between starvation survival and budding rate within the groups at the two highest feeding levels, starvation survival appeared to be independent of budding rate throughout all groups (linear regressions, maximum r^2=0.132, see fig. 12 and supplementary table S23). The same pattern was evident in the fed offspring polyps and in parents combined with fed offspring polyps, no strong correlation could be found within or across bisection groups (linear regressions, maximum r^2=0.22, see fig. 13 & 14 and supplementary tables S24-25).

Size versus Starvation Survival

Starvation survival did not depend on the polyp size (its volume) at the initiation of the starvation phase (linear regressions, all insignificant, $p>0.05$, see fig. 15 and supplementary

table S26). Polyps at all feeding levels have been analysed (within feeding levels) at separated or combined bisection treatments.

Heritability

Budding rates (after bisection) and starvation survival were not clearly correlated between parents and their first buds of the 21 *Artemia* feeding regime (linear regressions, maximum r^2=0.23, see fig. 8-9 and supplementary tables S17-18). The only significant regression could be seen at budding rates between parent and offspring heads, though very weakly pronounced due to the high variance (linear regression, $p<0.05$, slope=0.707, r^2=0.23, see fig. 9 and supplementary table S18).

Unfed buds had also no direct heritability to their parents in starvation survival, budding was non-existent in unfed polyps anyway. When comparing parents with unfed offspring within their respective feeding regime, ignoring bisection treatments, no heritability of starvation survival was found. Taking both bisection and feeding into account, we found a slight trace of inherited starvation survival patterns just in one group, the heads of the 7 *Artemia* feeding regime (linear regression, $p<0.01$, slope=0.356, r^2=0.521, see fig. 10 and supplementary table S19). All other groups had even weaker, i.e. none, linear relationships regarding survival (supplemental material, fig. S1-2 and tables S20-21).

DISCUSSION

To study and understand the plasticity of aging patterns in *Hydra* the concept of life-history trade-offs proved to be useful (Baudisch 2012; Chapter III). Assuming that resource gains of an organism are limited and also costly, allocation patterns of these to individual maintenance and reproduction are the crucial point to determine life-history patterns. If one of these

processes increases, there must be a cost for the other one, thus higher reproduction generally comes at the cost of lower survival chances. Extensive overviews of trade-off concepts and how trade-offs are involved in current evolution and aging theories are given in recent reviews (Parsons 2005; Flatt 2011; Baudisch 2012).

Our finding of an increased starvation survival combined with a cease of budding at low feeding rates, especially for the groups at 5 *Artemia* per week, in contrast to the lowest survival in combination with starting reproduction at 7 *Artemia* per week, without that size patterns were different between these groups over time, represents a clear resource allocation trade-off between maintenance and reproduction similar to the observed trade-offs in one of our previous studies on *Hydra* (Chapter III). At the highest feeding regime, abundant resources allowed the polyps to have much higher budding rates and larger sizes than at all other lower feeding regimes, combined with a long starvation survival similar to the one at 5 *Artemia*. Thus, the trade-off switch in resource allocation appears most clearly between feeding levels of 5 & 7 *Artemia* per week. Other than expected and proposed in chapter III, growth seems to be rather unaffected at this most pronounced trade-off shift as size patterns were very similar between these two feeding levels across all treatments and controls over time. In chapter III the results were different for their observed trade-off. For the same strain of *Hydra*, highest starvation survival was found to be at 7 & 9 *Artemia* per week, whereby not the same signs for a trade-off such as in our experiments could be observed due to the steadily increasing budding rates with increasing food intake. Still, from their next higher feeding level onwards (17 *Artemia* per week), survival declined slightly to a lower level while budding rates kept on increasing linearly, thus indicating the same maintenance-reproduction trade-off we found in another way.

These trade-off pattern differences between the studies could be either due to the bisection and the coupled food shortage days after bisection, or temporal size variations within the

feeding levels or the different amount of feeding lengths applied. Measured sizes varied considerably in our study not only between feeding levels but also within, as indicated in the huge standard deviations over time in fig. 7, whereas no size measurements were undertaken in chapter III for comparison. Furthermore, Levitis and Goldstein 2013 showed for *Hydra vulgaris strain AEP* that individual polyp size can vary considerably over time (two- to threefold changes in three-dimensional surface area over the course of a few days) within constant feeding regimes, without any obvious relationship to the timing of budding. To our surprise, we found that the actual polyp size at the point when feeding was stopped and the starvation phase began did not have any impact on the outcome of the final individual starvation survival, though. Within all groups and independent of the feeding level or bisection, no relationship existed between these two variables, implying that large or small body sizes did not have any cost for the maintenance allocation, other than suggested in our previous study (Chapter III). This leaves either effects due to bisection, accompanied by the food shortage, or the different feeding durations as a more probable explanation for the observed differences. In this experiment, polyps were fed for about one month before and one month after bisection on their respective feeding levels, while in chapter III feeding durations ranged from about two to eight months.

Our hypothesis that bisection, as a simulated predation stress, could alter Hydra's trade-off allocation directly by triggering a higher maintenance at the cost of reproduction due to the high regeneration activities could not be confirmed. Bisection did not cause significant differences overall, both in starvation survival and daily budding rates between head and foot halves, neither were the bisected ones different to the controls. This leads to the conclusion that our bisection treatment had not changed the general trade-off allocation in *Hydra*. Either the difference between head and foot regeneration patterns and their pattern difference to the controls was not strong enough to invoke the hypothesized trade-off shift or bisection simply

cannot lead to such a shift. However, that the controls behaved similarly to bisected polyps could also be related to the feeding treatment we applied to all of our groups. Controls were always getting the same amount of *Artemia* as both treated halves, in the same order, thus, similar starvation survival and budding rates between all groups could potentially also be due to the similar feeding regime. These effects could have overruled potential differences between bisected and unbisected polyps. That a general plasticity on *Hydra*'s trade-off allocation system exists, though, is evident based on the shift effect we found in response to different feeding levels and based on what our previous experiments showed in response to changed environmental conditions like feeding regimes, temperature and hunger treatments (Chapter III).

Intriguing are the offspring generations results. At the highest feeding regime, we observed a trend towards increased performance in the first offspring generation, which was left unbisected and fed with the same feeding regime as their parents before their starvation period. Overall, starvation survival of offspring polyps was slightly higher than that of their parents, while no significant generation difference could be found at budding rates. Within treatment groups, generation did not have any effects for both traits. Bisection had, if at all, only a weak impact. When comparing generations, we found slight, but significant survival increases in the offspring of bisected polyps compared to unbisected control parents, as well as in offspring foot halves compared to parent head halves. In reproduction, just a slight bisection effect occurred at budding rates when generations were combined and foot halves showed slightly lower budding rates than heads, without that both bisected groups were different to the controls nor general interaction effects were evident. Overall, these results indicate a transmitted and even potentially enforced hormesis effect from the parent to the offspring generation. The higher performance enhancement of the treatment offspring compared to the control might be explained by the doubled hormesis trigger, namely both

parent bisection and the following food shortage during regeneration, while the control parents' only hormesis trigger could have been the foot shortage due to the treatments' bisection.

Unfed offspring generations, the first buds of parents at 7 *Artemia* per week and the second buds of parents at the highest feeding level, did not produce any buds at all and lived much shorter, about a quarter less starvation survival, than their respective parents and their fed generation siblings in case of the highest feeding regime. This result is not surprising at all since buds are typically smaller and provided with less tissue, although not in all cases and depending on the feeding rates, than their parents when detaching and budding off (Otto and Campbell 1977; Slobodkin et al. 1991; Levitis and Goldstein 2013). In contrast, our results that size at the start of starvation of previously fed polyps does not influence the survival outcome in this period are relativizing the importance of size for maintenance and survival.

Remarkably, we could not find traces for a direct trade-off between starvation survival and asexual reproduction within feeding levels (with bisection treatments separated and combined). Neither was size at the initiation of the starvation phase correlated with the following starvation survival time. From this we can conclude that no real trade-off between size, reproduction and maintenance seems to exist in *Hydra*, they seem to be uncoupled from each other although all three traits are basically run by the same system. A recent review on the transcription factor FOXO, an important key regulator for many target genes and molecular pathways in a cell determining resource allocation in metazoans, suggests that all of the three traits - maintenance, asexual reproduction and growth/size - are regulated via FOXO in *Hydra* (Schaible and Sussman 2013). *Hydra*'s constitutive proliferative cell renewal machinery seems to offer a certain plasticity which is responsible for the observed patterns of a missing direct trade-off in our experiment, since all three key traits are regulated

by that same mechanism, which itself is regulated via FOXO activity. The only level where we could detect a trade-off was between the feeding regimes of 5 and 7 *Artemia* per week, where spare resources at the lower regime were limiting *Hydra* to use its cell proliferating renewal system just to maintain a stable body size and keep up maintenance without proliferating sufficient cells to allow budding, whereby at the higher regime budding could be established but at the cost of reduced maintenance capabilities resulting in much shorter starvation survival times. This might contradict somehow the suggested uncoupled nature of the three traits, but it does not necessarily oppose it. This trade-off rather reveals the interaction of key functions within *Hydra* at the threshold where just enough resources are provided that a polyp starts to propagate buds, which evidentially resulted in a weakened maintenance of such a parent polyp in our experiment. At this threshold, and only there, a cost of reproduction becomes evident with less resources available for maintenance, resulting in a coupled pattern. Increased cell proliferation accompanied with the loss of a substantial amount of cells due to the budding process could be an explanation for the reduced maintenance potential at this threshold level. The proportion of resources taken away from a polyp via budding at this food level seems to be too large to be compensated for by the constitutive proliferative cell renewal to sustain as much maintenance as on respective lower or higher feeding levels. With increasing resources provided at increasing feeding levels the budding cost vanishes and the imbalance gets resolved within the polyp, maintenance is rising.

The uncoupled nature of the three traits displays itself also in the vast heterogeneity we find in all traits within and across treatments and controls. Interestingly and throughout treatments of the two higher feeding levels at which budding occurred regularly, polyps tended not to maintain an individual-specific averaged daily budding rate during our experiments since rates before and after bisection were uncorrelated, even in the controls. This contrasts our

previous findings for *Hydra*'s budding behaviour (see chapter IV), where we observed rather stable budding rates per polyps and suggested a specific individual character stability and homeostasis over time in the light of the huge heterogeneity found between polyps, even when all controllable environmental factors are kept on a constant level. Possibly, our feeding phases here were too short to measure stabilized budding rates since we fed polyps only for four to five weeks before and after bisection compared to much longer durations in the experiments of chapter IV. In light of the slight budding increase we found in both treatments and controls after bisection in both higher feeding regimes compared to before, this non-stability in budding may be affected by a hormetic stress effect. Since not only the bisected polyps were affected, but also the controls, the short starvation period after bisection, where feeding was lowered in all groups due to the applied treatment, rather than the bisection itself seems to have triggered that effect. Our findings underline hereby the plasticity of *Hydra*'s efficiency, we found similar hormetic effects before in *Hydra* in response to temperature and starvation stresses (Chapter III). Another striking feature and indication of a hormetic response we found in our study is that both head and foot regenerates had similar, if not even slightly enhanced budding rates and starvation survival compared to the controls, without that a clear trade-off regarding size patterns was obvious between the groups either. This is surprising since these two groups consisted just of half of the tissue after bisection as their uncut counter controls and all individuals got the same amount of food after on. Here, the bisection itself seems to have caused that additional hormetic response on top of the effect of the interim starvation. *Hydra* shows evidence of being able to increase its metabolic efficiency as a stress response. It is still puzzling why the optimal metabolic efficiency is not reached during rather stable, optimal and non-stressful conditions and unclear what remains the trade-off to this effect and how long such an increased performance can be maintained, as hormetic effects appear to be mostly of a transient nature (Stebbing 1982), but not by all

means (Rattan 2008; Calabrese et al. 2012). Previously, we hypothesized that a constant excessive food supply may lead to more inefficiency and a certain slackness on energy utilization (Chapter III), but further experiments are needed to clear these propositions.

A so far unmentioned but potentially very influential factor regarding phenotypic heterogeneity within and between ramets is the epibiome of polyps, consisting of various usually single celled organisms. These "holobiontic" or "metaorganismic" relationships and variations turn recently more and more into focus of research, especially in *Hydra* (Bosch and McFall-Ngai 2011; Bosch 2012; Bosch 2012; McFall-Ngai et al. 2013), and constitute a promising concept to reveal new insights into microecosystems of clonal organisms.

Heritability

Budding behaviour and starvation survival are not directly inherited in *Hydra* from parent to bud as our results show in the comparison of the offspring generations to their parents at the two highest feeding levels. Bisection and feeding level did not alter this pattern at all. From these results it seems that each bud is attaining its specific individual phenotypic character rather independently of its parent, if we can speak of a phenotypic character at all. Such a character would determine the resource allocation pattern of an individual onto a certain level and express itself in an individual specific, rather stable budding rate and starvation survival. Regarding the budding rates we could not find a stable character when comparing before versus after budding rates, as mentioned above. Plus, the hormetic responses we measured hint to a specific character plasticity over time with dynamic resource allocations within a polyp responding to actual environmental stressors. This expands the findings on *Hydra*'s phenotypic plasticity and its random phenotype allocation of chapter IV, specifically regarding *Hydra*'s budding behaviour, where we found rather stable budding rates over time, but measured under constant conditions (Chapter IV).

It is to be considered, though, that we did not have completely identical situations between parents and offspring generations in our experiments. In the fed buds at the highest feeding level, polyps were treated in the same way as their parents after bisection, but without the bisection itself. The two other offspring generations we surveyed at 21 and 7 *Artemia* feeding levels were both kept unfed and unbisected. Thus, when checking for heritability here we did not have a parent-offspring comparison under exactly the same (pre-)conditions. Still, since we could not determine clear trait inheritance patterns in any of the groups, these considerations might be overcautious in the end and we can conclude that *Hydra* shows no indication of inheriting any individual-specific phenotypic trait merits from parent to offspring via budding.

CONCLUSIONS

Simulated predation stress in the form of bisection triggers a hormetic response in *Hydra*, whereby head and foot regenerates do not display significant differences from each other. An increased maintenance and reproduction efficiency could be detected after bisection, both budding rates and starvation survival reached similar, if not slightly increased levels compared to uncut controls. Considering that only half of the original body mass was available to both bisected groups, a clear hormetic response can be inferred from these results. Additionally to bisection, temporal food shortage alone seems to trigger hormetic reactions as well, as both control and bisected groups had enhanced budding rates after the shortened food supply period accompanying bisection.

A trade-off could be determined only at the threshold feeding level of 7 *Artemia* per week per polyp. With resources deviated to starting reproduction, less were available for maintenance, starvation survival decreased sharply. At lower or higher feeding regimes, none such an

effect was visible. Apart from this trade-off, all three key life history traits were uncoupled from each other - size, survival or budding showed no correlation. No signs of inheritance of trait merits from parent to bud could be detected, except of an overall hormetic effect transmission shown in the offspring buds at the highest feeding regime. The constitutive proliferative cell renewal machinery in *Hydra* renders the polyp into a dynamic organism with heterogeneous phenotypic states between and within polyps, respondent to environmental stress triggers. Furthermore, the overall organismal efficiency can be enhanced in a hormetic response without that any costs were detectable in our experiment.

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FIGURES & TABLES

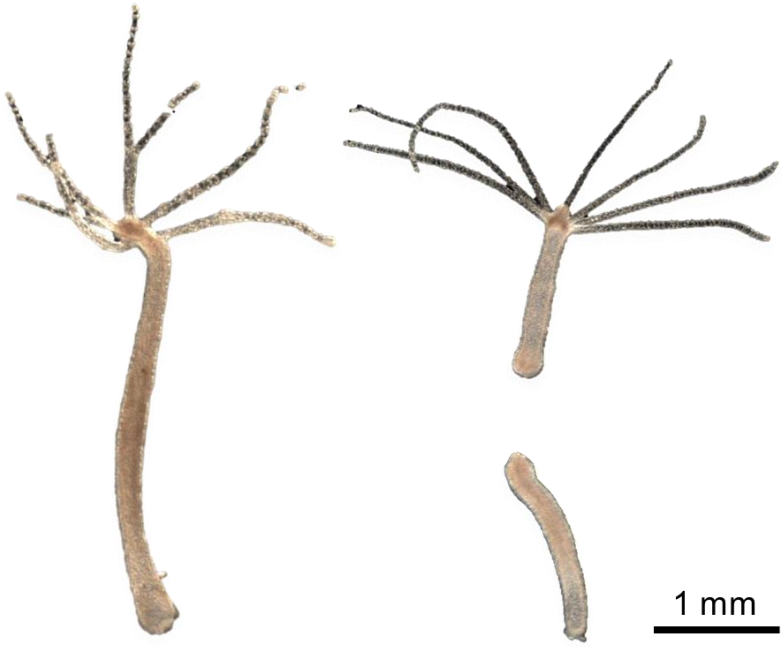

Figure 1. Normal (left) and bisected (right) polyp. *Hydra magnipapillata*

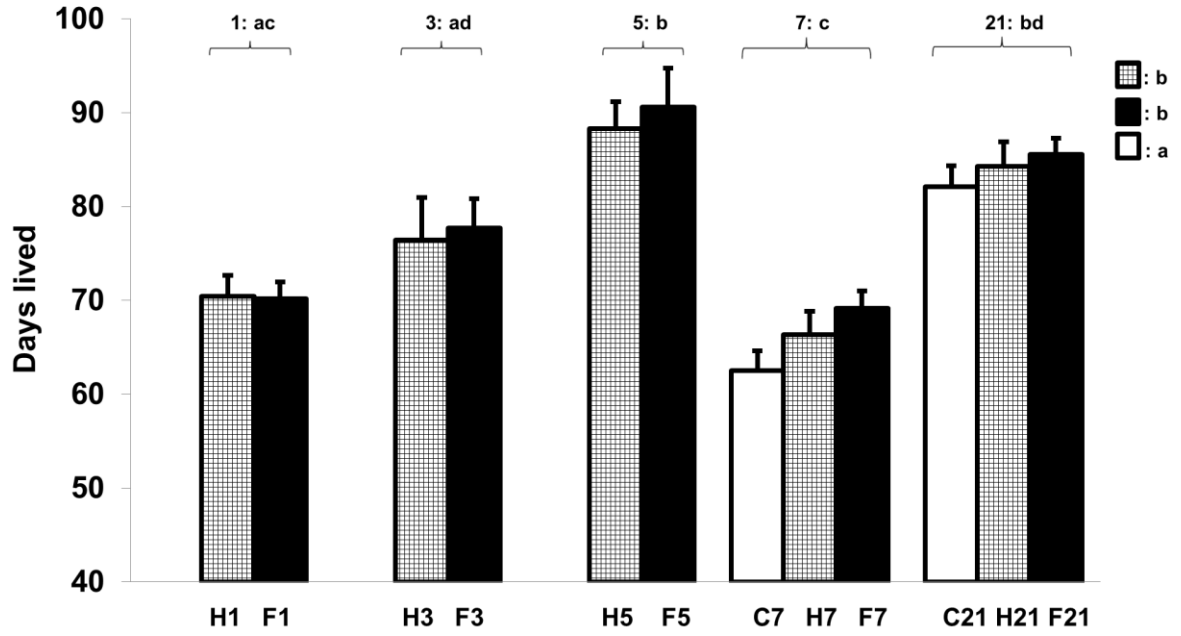

Figure 2. Average starvation survival of *Hydra* polyps across all feeding regimes and bisection treatments. H, F and C represent polyp head/foot halves and controls. The adjoint numbers stand for the respective feeding levels. Different lower case letters indicate significant differences between feeding levels or bisection treatments in the post-hoc comparisons (two-way ANOVA, Tukey post-hoc tests, $p<0.05$, see tables S1-3 in the supplemental material). Error bars represent standard errors.

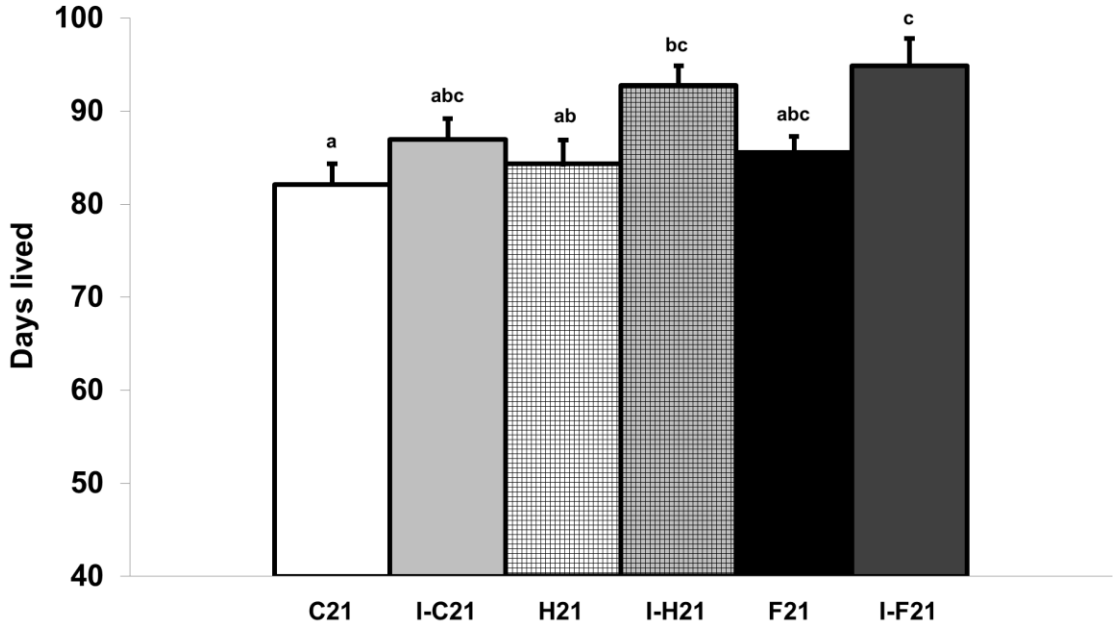

Figure 3. Average starvation survival of *Hydra* polyps. Parents versus fed offspring cohort in the highest feeding regime. H, F and C represent polyp head/foot halves and controls. The adjoint numbers stand for the respective feeding level, while I- indicates the offspring polyps. Error bars represent standard errors. A significant overall generation effect was found (two-way ANOVA, Tukey post-hoc test, $p<0.05$, see tables S4-5 in the supplemental material). Different lower case letters indicate significant differences between separated groups (one-way ANOVA, Tukey post-hoc test, $p<0.05$, see tables S6-7 in the supplemental material).

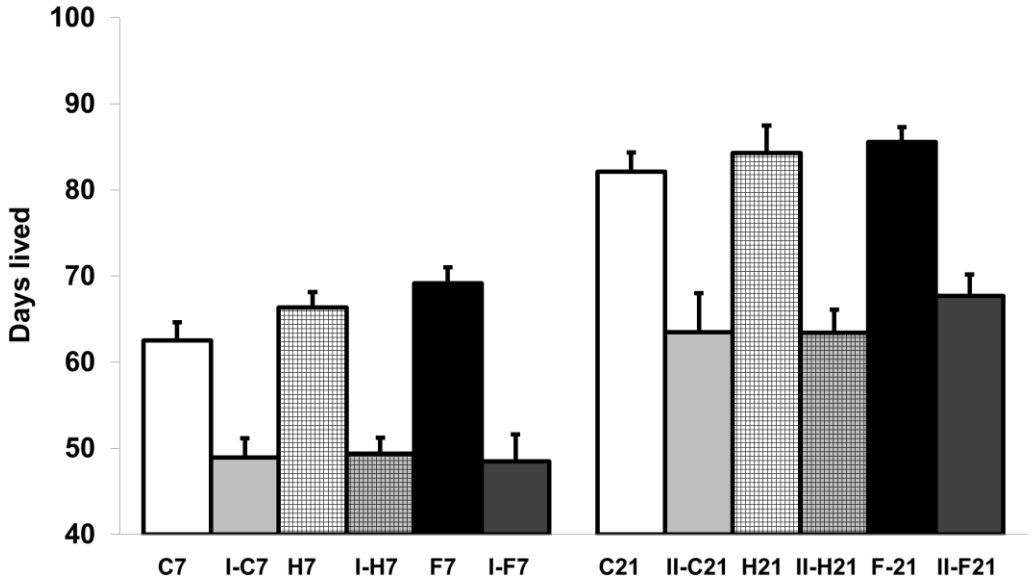

Figure 4. Average starvation survival of *Hydra* polyps. Unfed offspring cohorts in comparison to parents. H, F and C represent polyp head/foot halves and controls. The adjoint numbers stand for the respective feeding levels, while I- & II- indicate the offspring polyps. Error bars represent standard errors. Unfed polyps survived obviously much shorter than their parents during starvation. Feeding level of parents still had a significant effect on unfed offspring's starvation survival, no bisection effects were evident (two-way ANOVA, Tukey post-hoc test, $p<0.05$, see tables S8-9 in the supplemental material).

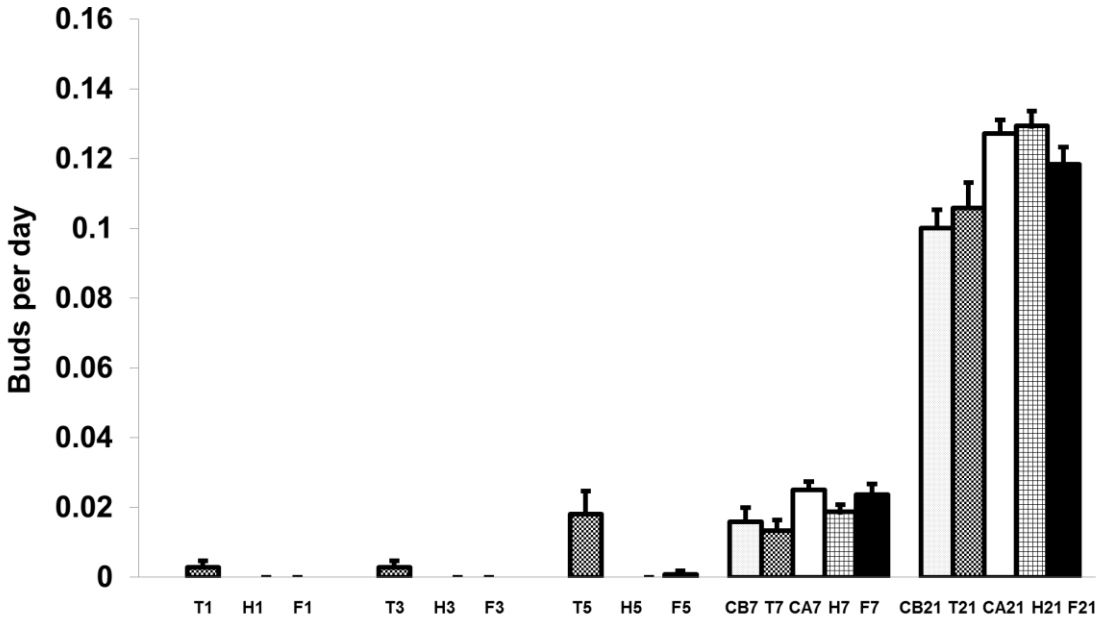

Figure 5. Average budding rate of *Hydra* polyps across all feeding regimes and bisection treatments. Budding before and after bisection. H, F and CA represent polyp head/foot halves and controls after bisection. T and CB represent treatment and control polyps before bisection. The adjoint numbers stand for the respective feeding levels. Error bars represent standard errors. See supplementary tables for distinct treatment effects (two-way ANOVA, Tukey post-hoc tests, $p<0.05$, see tables S10-12 in the supplemental material).

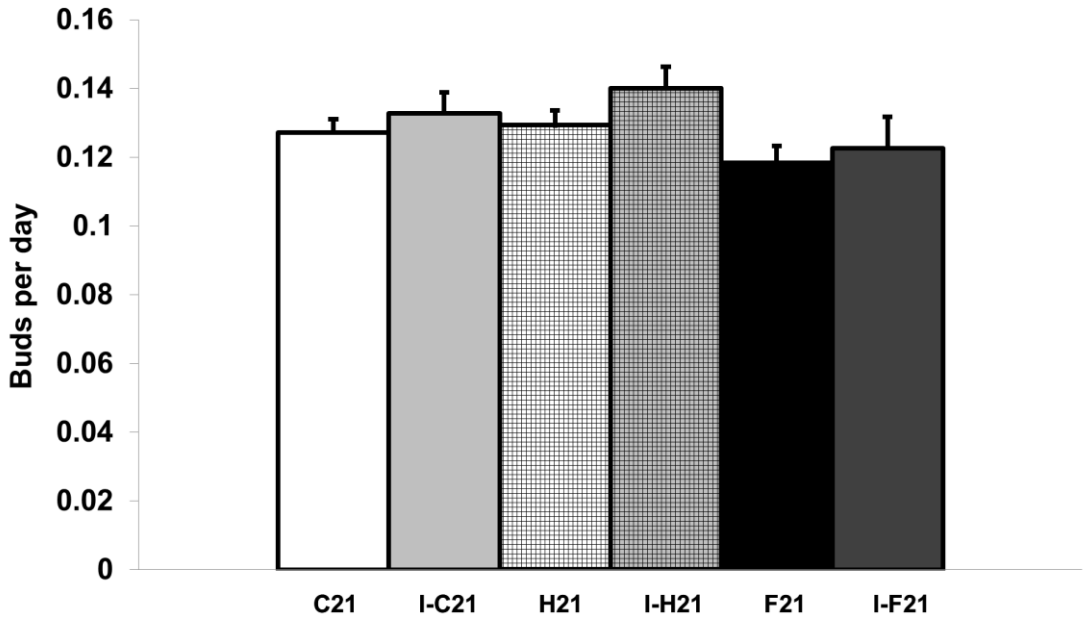

Figure 6. Average budding rate of *Hydra* polyps. Parents versus fed offspring budding in the highest feeding regime. H, F and C represent polyp head/foot halves and controls (after bisection for parents). The adjoint numbers stand for the respective feeding levels, while I- indicates the offspring polyps. Error bars represent standard errors. No differences between parents and offspring groups could be found and bisection had no general effect (one and two-way ANOVAs, Tukey post-hoc tests, $p>0.05$, see tables S13-16 in the supplemental material).

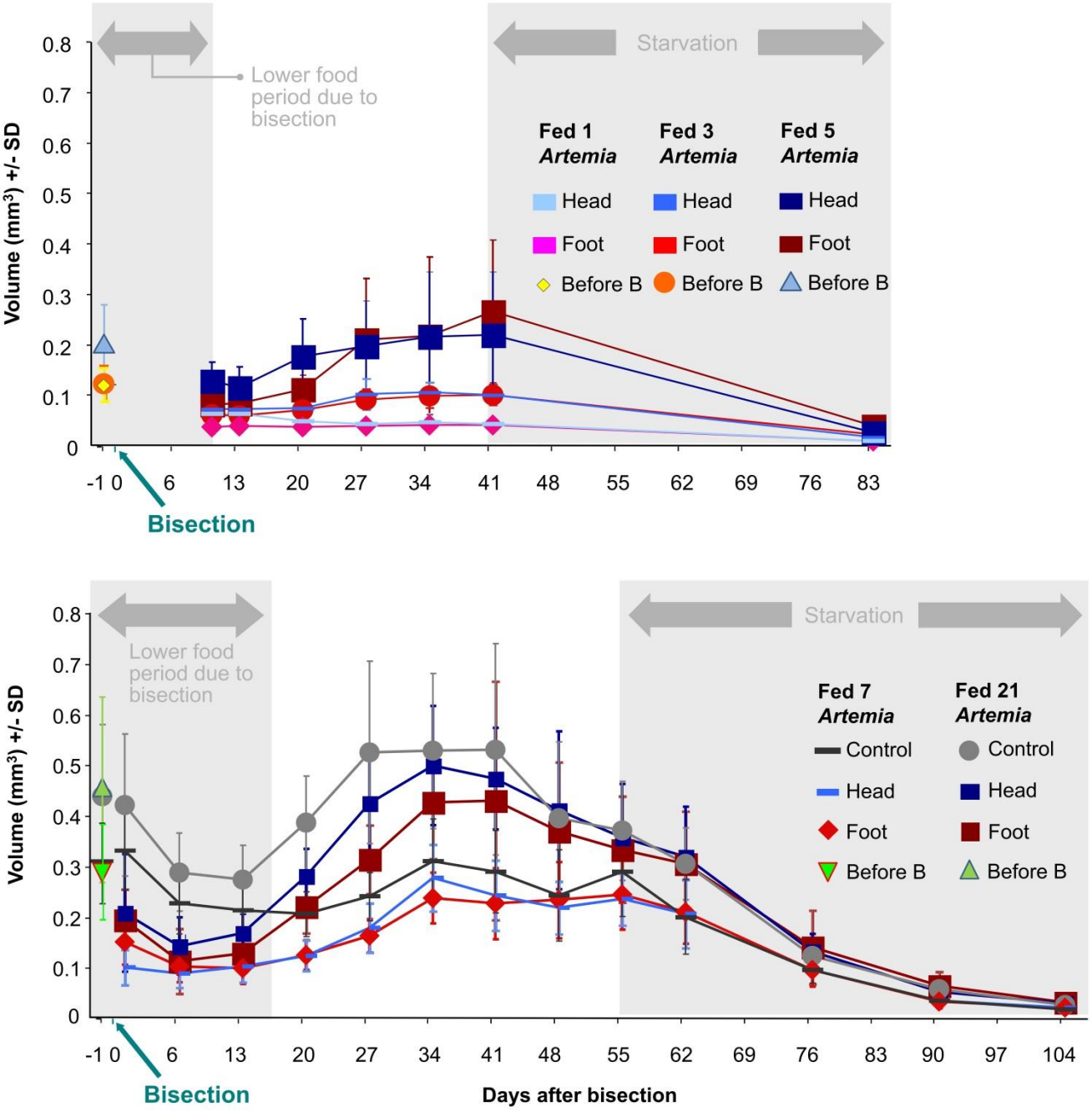

Figure 7. Average *Hydra* sizes throughout the experiment. Error bars represent standard deviations.

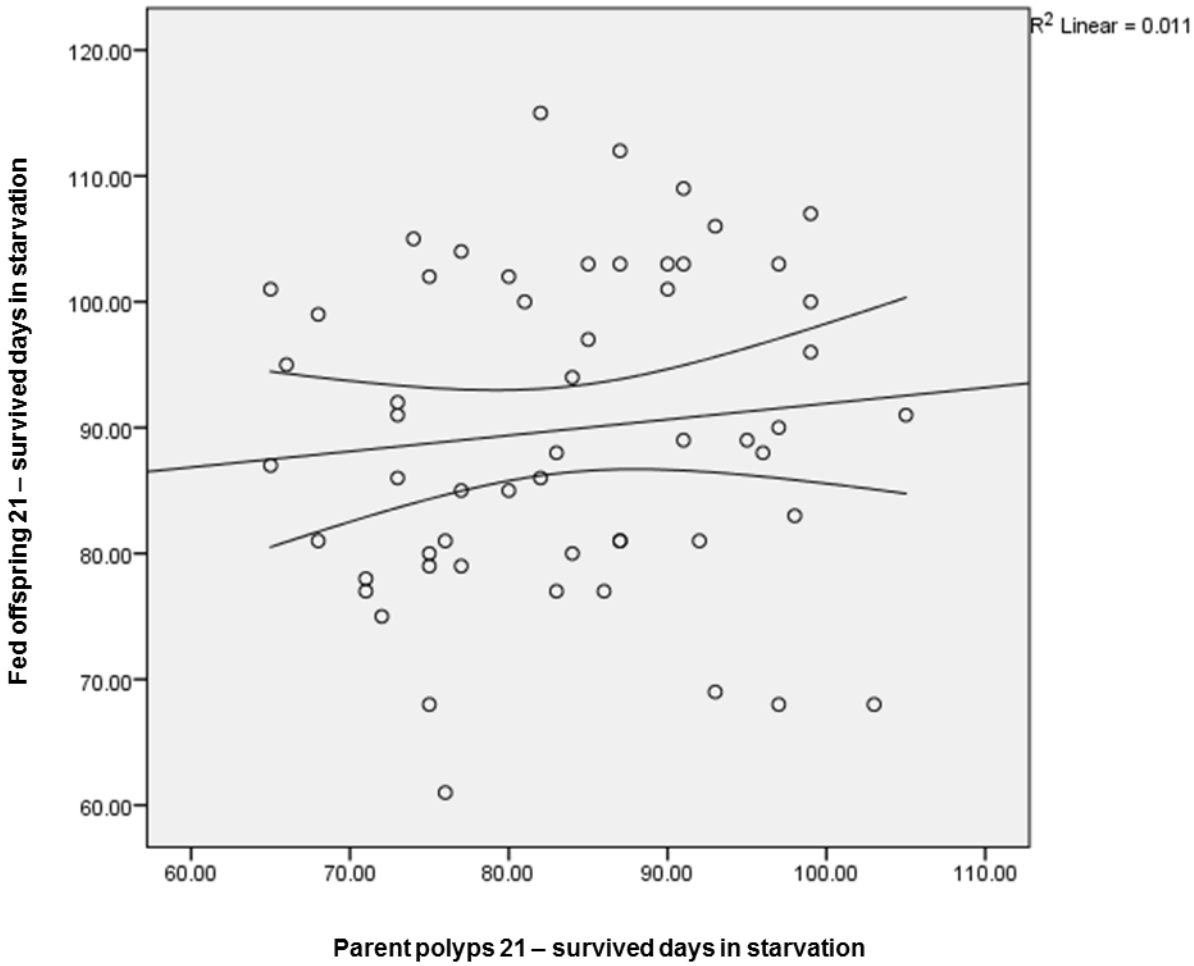

Figure 8. Survival heritability between parent versus fed offspring in the highest feeding regime. Error bars represent 95% confidence intervals. No significant heritability existed (linear regression, $p>0.05$, see table S17, supplemental material), separated bisection groups and controls showed also no significant heritability (data not shown).

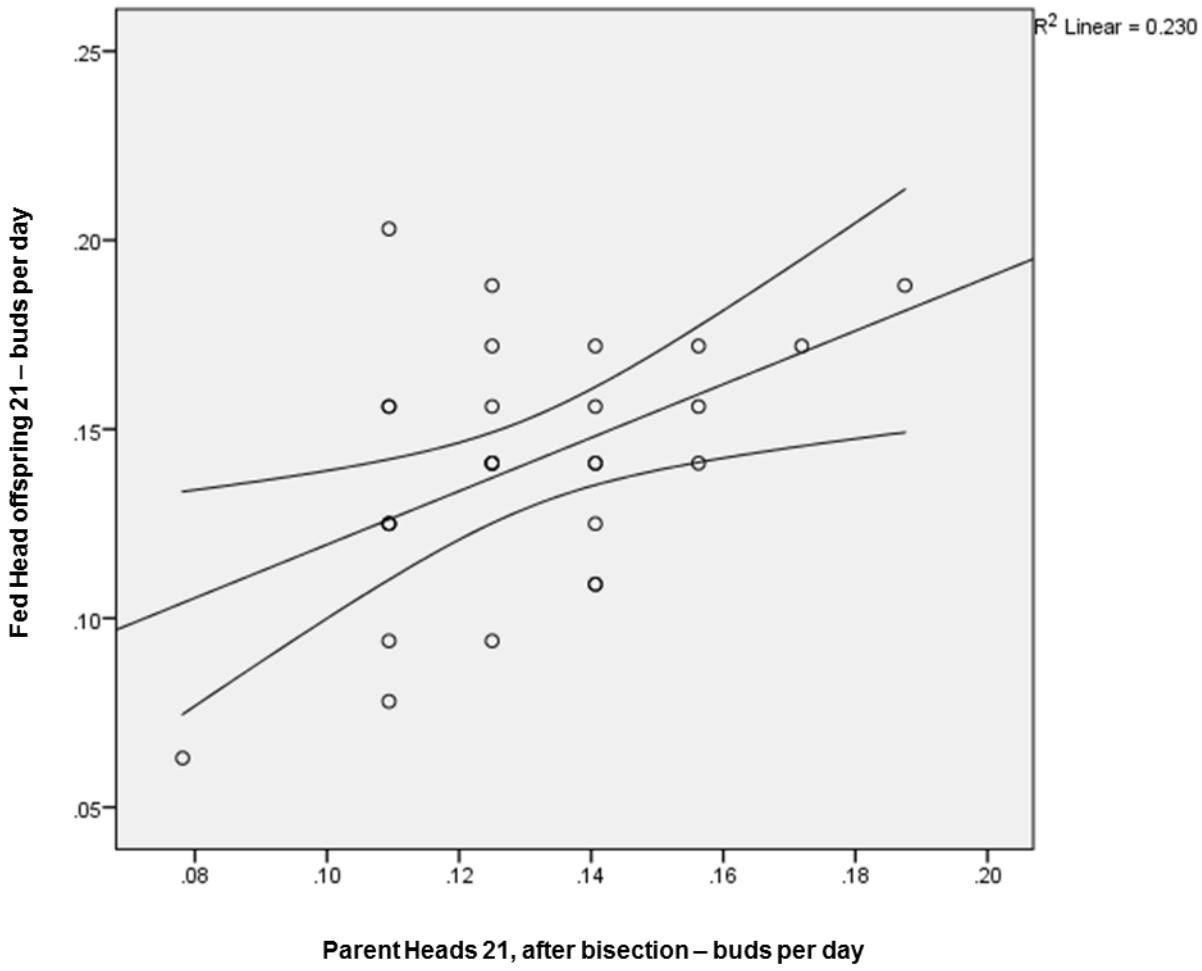

Figure 9. Budding heritability between parents versus fed offspring in the highest feeding regime. Error bars represent 95% confidence intervals. The only significant heritability is shown here with a positive slope of .707, but an r^2 of .23 (linear regression, $p<0.05$, see tables S18, supplemental material), no heritability was found in feet and controls and all three groups combined (data not shown).

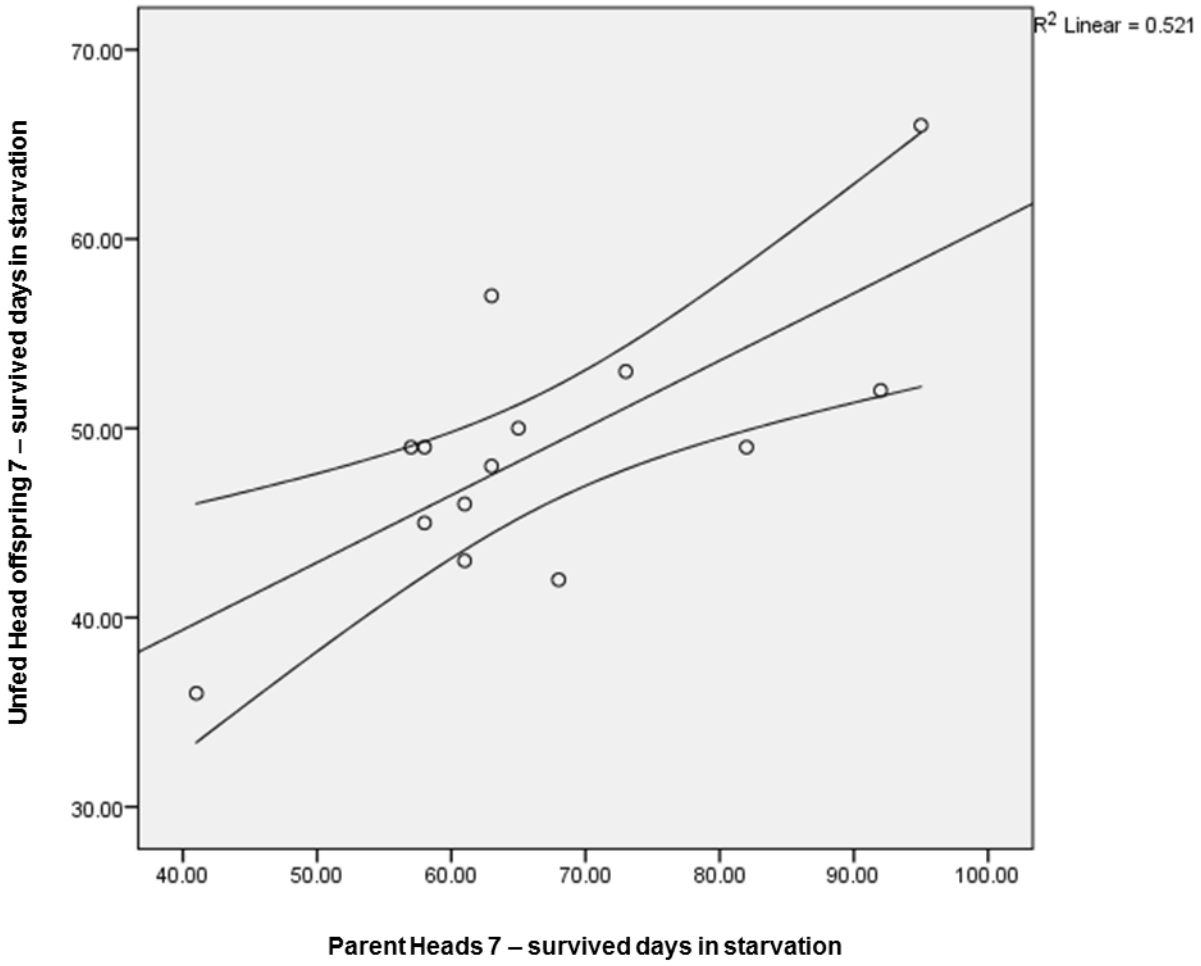

Figure 10. Survival heritability between parents versus their unfed offspring in the two highest (separated) feeding regimes. Error bars represent 95% confidence intervals. The strongest heritability is shown here with a positive slope of .356 and an r^2 of .521 (linear regression, $p<0.05$, see tables S19, supplemental material), all other separate and combined (within feeding levels) regressions had varying slopes (positive/negative) with much lower r^2 values and/or were insignificant (see figures S1-2 and tables S20-21 for combined regression results, supplemental material, further data not shown).

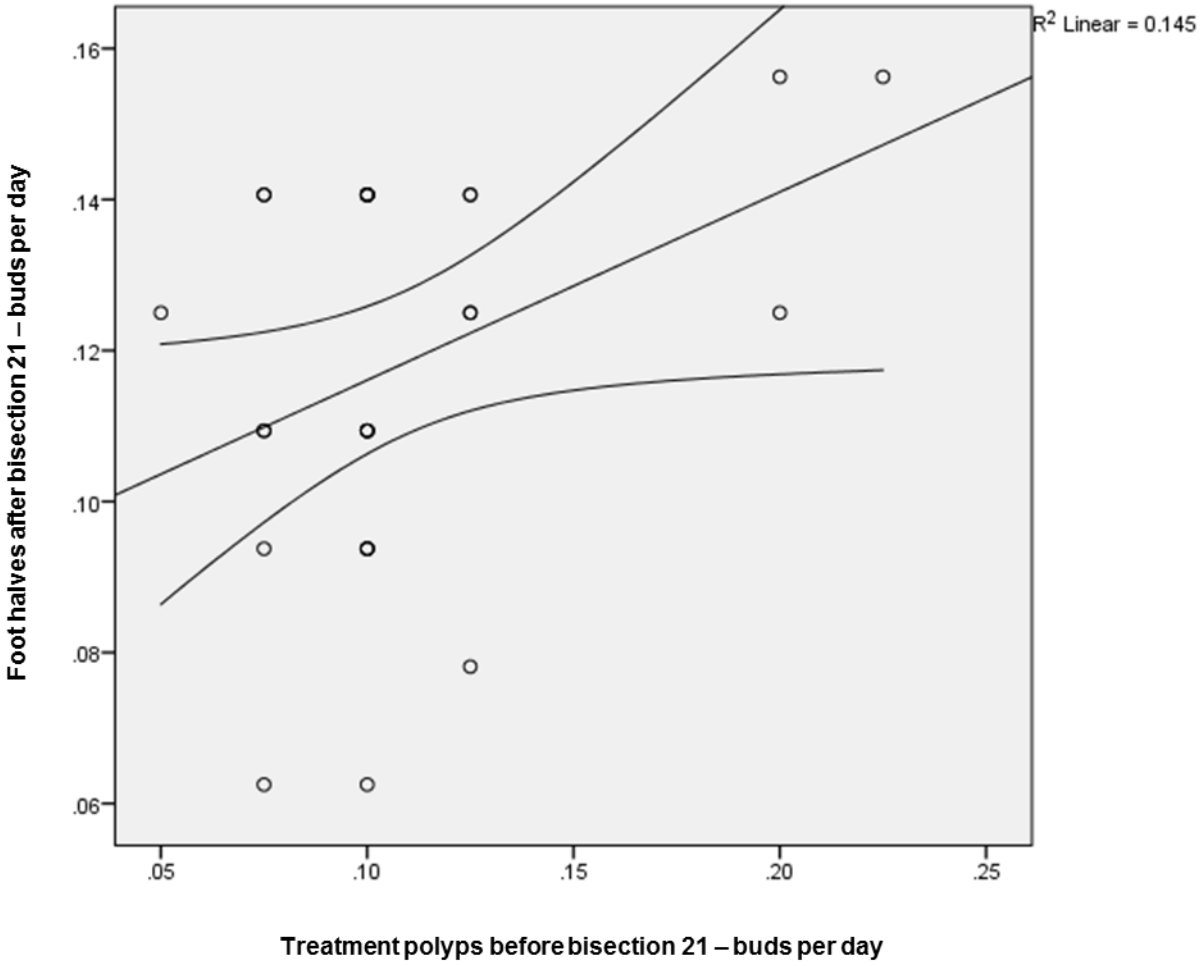

Figure 11. Budding rates before versus after bisection in the two highest (separated) feeding regimes. Error bars represent 95% confidence intervals. The strongest relationship is shown here with a positive slope of .249, but an r^2 of .145 (linear regression, $p<0.05$, see tables S22, supplemental material). All other separate and combined (within feeding regimes) regressions had varying slopes (positive/negative) with even lower r^2 values and/or were insignificant (data not shown).

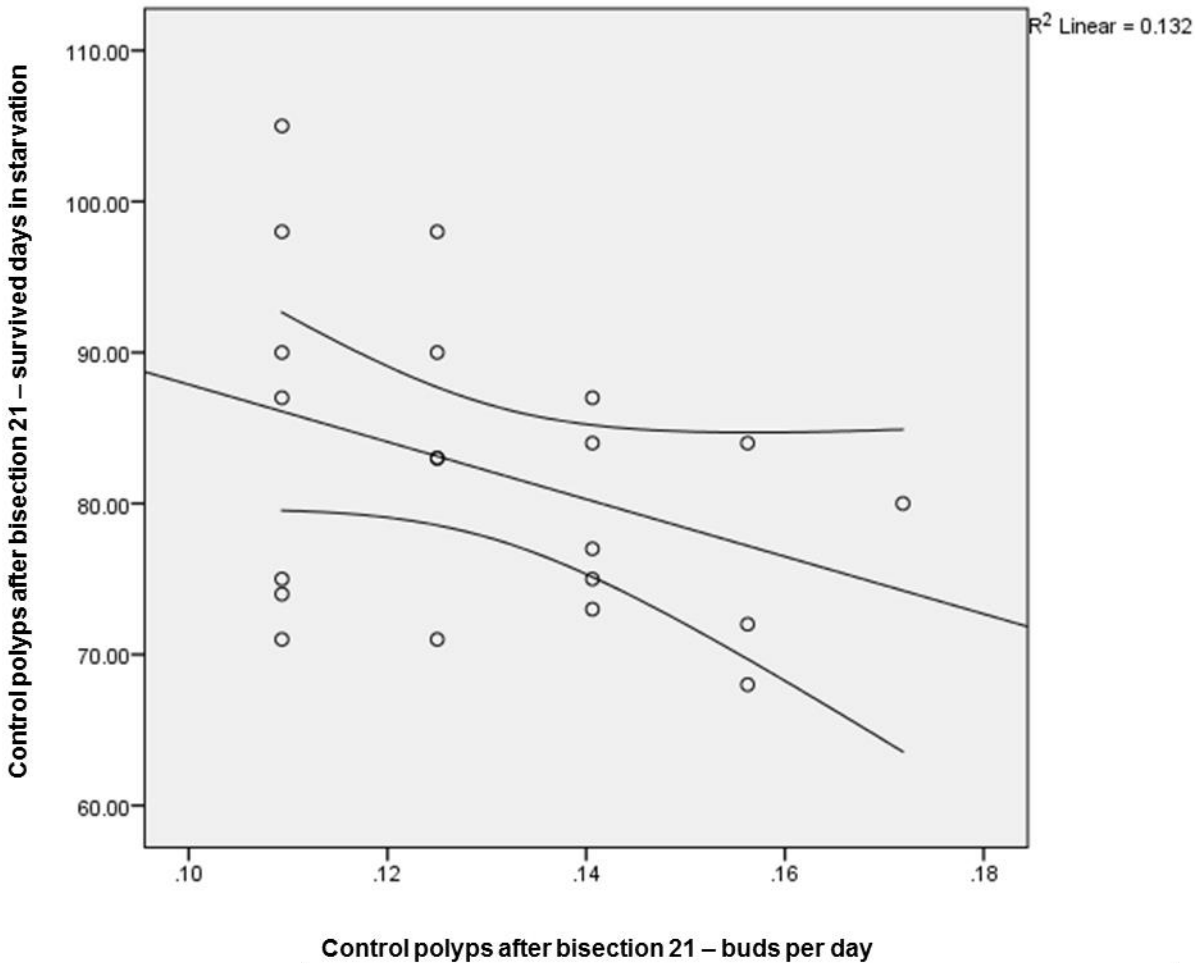

Figure 12. Budding versus starvation survival trade-off in the two highest (separated) feeding regimes. Error bars represent 95% confidence intervals. The non-significant regression with the highest r^2 is shown here (linear regressions, $p>0.05$, see table S23, supplemental material). All other separate and combined (within feeding regimes) regressions had varying slopes (positive/negative) with even lower r^2 values (data not shown).

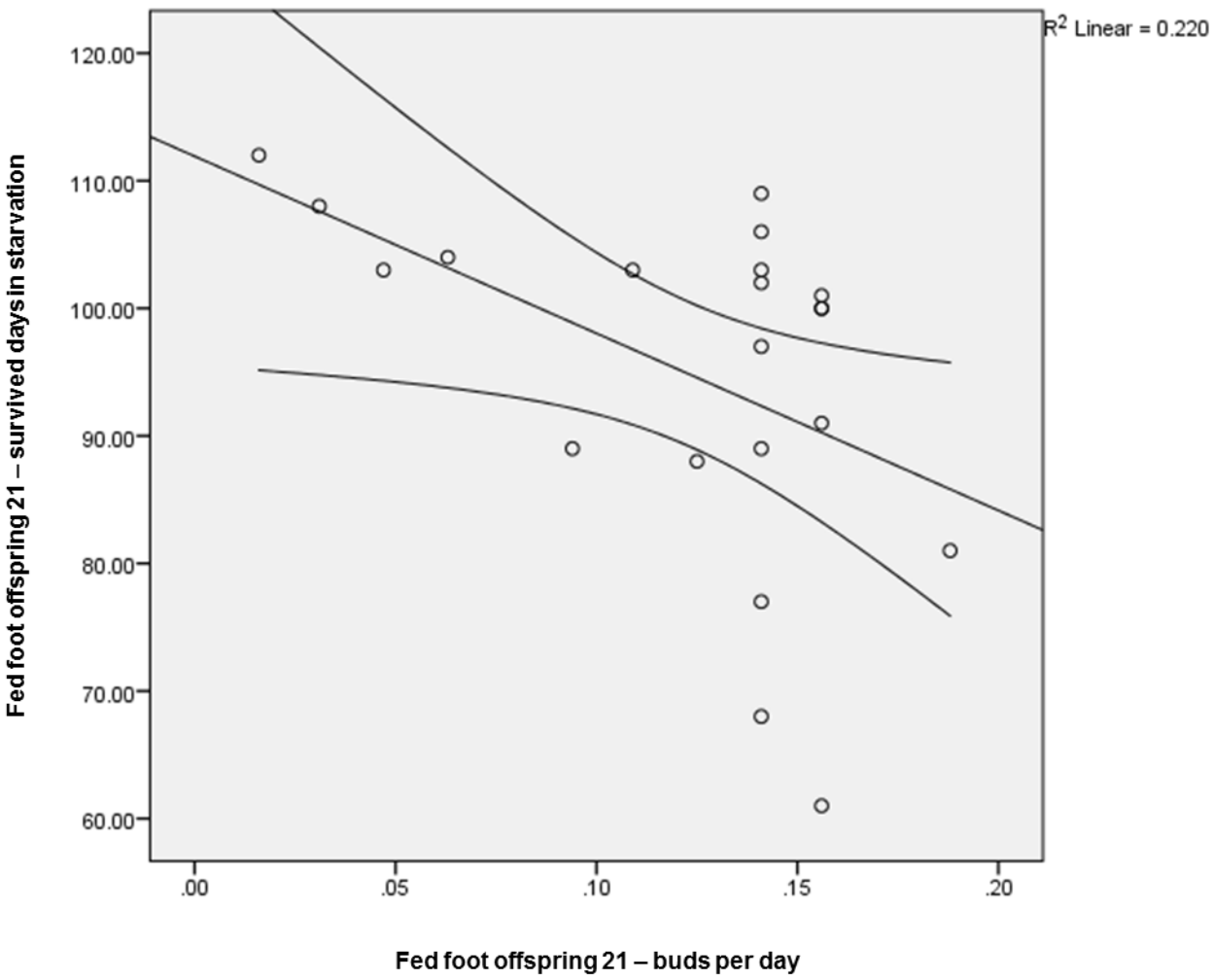

Figure 13. Budding versus starvation survival trade-off in fed offspring polyps of the highest feeding regime. Error bars represent 95% confidence intervals. The strongest correlation is shown here with an r^2 of .22 (linear regression, $p<0.05$, see tables S24, supplemental material). All other separate and combined regressions had lower r^2 values and/or were insignificant (data not shown).

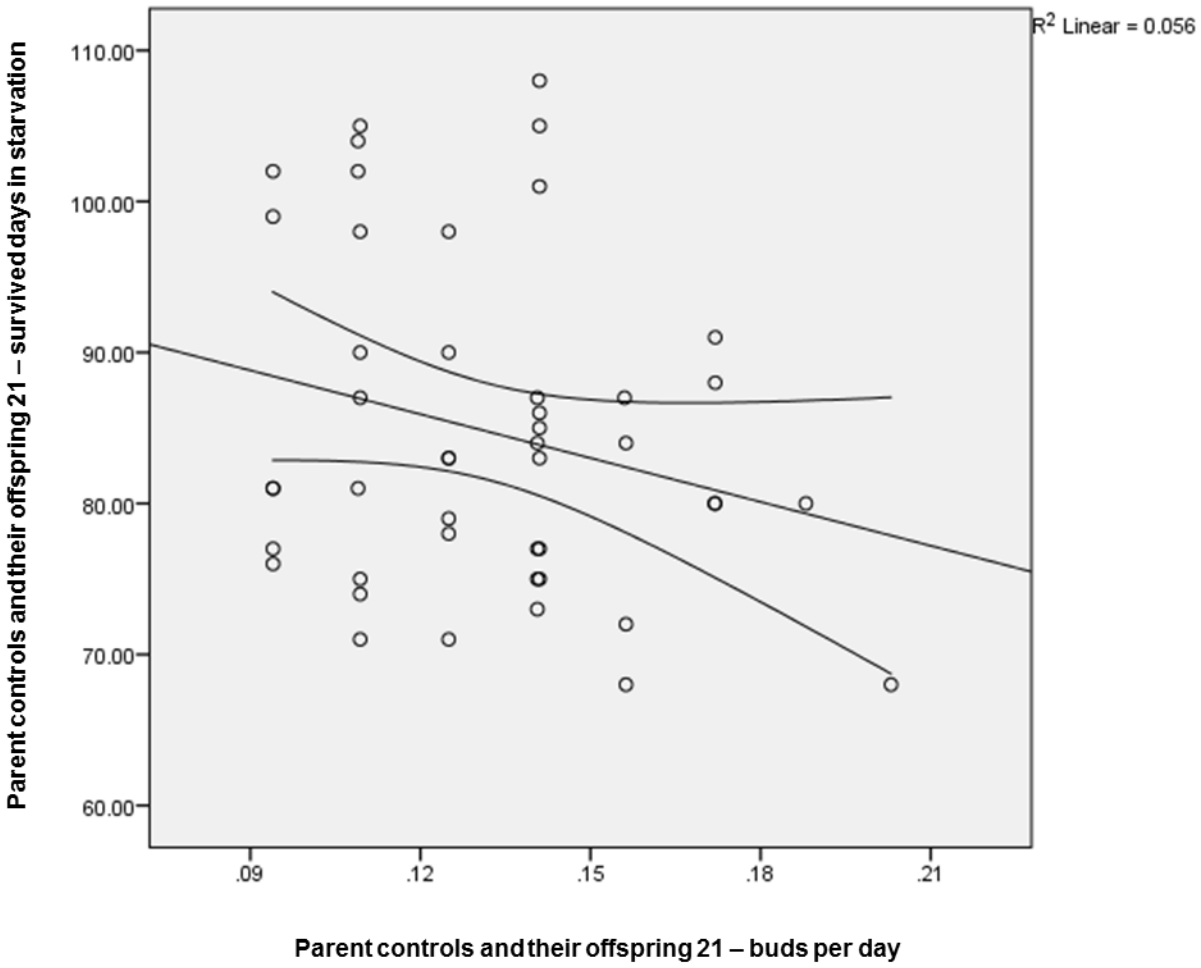

Figure 14. Budding versus starvation survival trade-off in parents and fed offspring polyps of the highest feeding regime combined. Error bars represent 95% confidence intervals. The non-significant regressions with the highest r^2 is shown here (linear regressions, $p>0.05$, see table S25, supplemental material). All other separate and combined regressions had even lower r^2 values and/or were insignificant as well (data not shown).

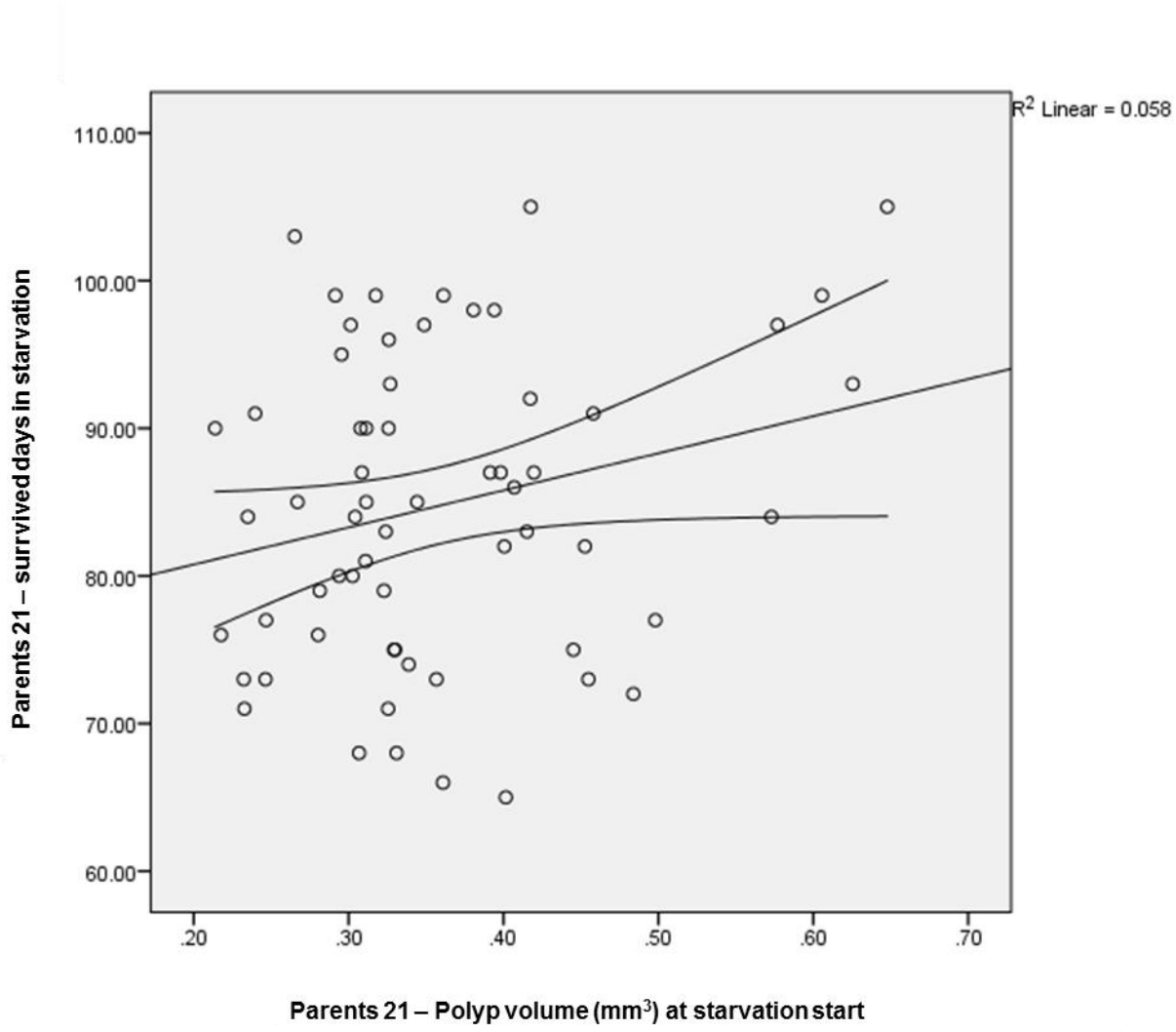

Figure 15. Size at starvation start versus starvation survival trade-off in all (separated) feeding regimes. Error bars represent 95% confidence intervals. No significant regression was found, parents 21 are shown as example (linear regression, $p>0.05$, see table S26, supplemental material). All other separate and combined (within feeding regimes) regressions were also insignificant (data not shown).

SUPPLEMENTAL MATERIAL

Table S1. Two-way ANOVA relating to **figure 2.**

Tests of Between-Subjects Effects

Dependent Variable: Survivalparents

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	17262.144[a]	11	1569.286	14.182	.000
Intercept	896535.591	1	896535.591	8102.304	.000
HeadFootorControl	578.379	2	289.189	2.614	.076
Feedingrate	16069.854	4	4017.464	36.307	.000
HeadFootorControl * Feedingrate	94.196	5	18.839	.170	.973
Error	22241.039	201	110.652		
Total	1262506.000	213			
Corrected Total	39503.183	212			

a. R Squared = .437 (Adjusted R Squared = .406)

Table S2. Tukey post-hoc test relating to **figure 2.** 6, 9 and 8 represent foot/head halves and controls.

Dependent Variable: Survivalparents

	(I) HeadFootorControl	(J) HeadFootorControl	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	6.00	8.00	5.7912*	1.94733	.009	1.1931	10.3892
		9.00	1.1519	1.62325	.758	-2.6809	4.9848
	8.00	6.00	-5.7912*	1.94733	.009	-10.3892	-1.1931
		9.00	-4.6392*	1.93926	.046	-9.2182	-.0602
	9.00	6.00	-1.1519	1.62325	.758	-4.9848	2.6809
		8.00	4.6392*	1.93926	.046	.0602	9.2182

Based on observed means.

The error term is Mean Square(Error) = 110.652.

*. The mean difference is significant at the .05 level.

Table S3. Tukey post-hoc test relating to **figure 2.**

Dependent Variable: Survivalparents

	(I) Feedingrate	(J) Feedingrate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	1.00	3.00	-6.8537	3.10336	.181	-15.3963	1.6889
		5.00	-18.8942*	3.06061	.000	-27.3191	-10.4692
		7.00	4.2042	2.35673	.386	-2.2832	10.6916
		21.00	-13.8052*	2.38786	.000	-20.3782	-7.2321
	3.00	1.00	6.8537	3.10336	.181	-1.6889	15.3963
		5.00	-12.0405*	3.28660	.003	-21.0875	-2.9935
		7.00	11.0579*	2.64359	.000	3.7809	18.3349
		21.00	-6.9514	2.67138	.074	-14.3049	.4020
	5.00	1.00	18.8942*	3.06061	.000	10.4692	27.3191
		3.00	12.0405*	3.28660	.003	2.9935	21.0875
		7.00	23.0984*	2.59328	.000	15.9599	30.2369
		21.00	5.0890	2.62160	.299	-2.1275	12.3055
	7.00	1.00	-4.2042	2.35673	.386	-10.6916	2.2832
		3.00	-11.0579*	2.64359	.000	-18.3349	-3.7809
		5.00	-23.0984*	2.59328	.000	-30.2369	-15.9599
		21.00	-18.0093*	1.74917	.000	-22.8243	-13.1944
	21.00	1.00	13.8052*	2.38786	.000	7.2321	20.3782
		3.00	6.9514	2.67138	.074	-.4020	14.3049
		5.00	-5.0890	2.62160	.299	-12.3055	2.1275
		7.00	18.0093*	1.74917	.000	13.1944	22.8243

Based on observed means.

The error term is Mean Square(Error) = 110.652.

*. The mean difference is significant at the .05 level.

Table S4. Two-way ANOVA relating to **figure 3.**

Tests of Between-Subjects Effects

Dependent Variable: Survival21parentsvs21offspringfed

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2764.365[a]	5	552.873	4.349	.001
Intercept	1077930.080	1	1077930.080	8479.888	.000
HeadFootorControl	770.292	2	385.146	3.030	.052
Generation	1968.875	1	1968.875	15.489	.000
HeadFootorControl * Generation	128.616	2	64.308	.506	.604
Error	17160.671	135	127.116		
Total	1106731.000	141			
Corrected Total	19925.035	140			

a. R Squared = .139 (Adjusted R Squared = .107)

Table S5. Tukey post-hoc test relating to **figure 3.** 6, 9 and 8 represent foot/head halves and controls.

Dependent Variable: Survival21parentsvs21offspringfed

	(I) HeadFootorControl	(J) HeadFootorControl	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	6.00	8.00	5.0435	2.35091	.085	-.5278	10.6148
		9.00	1.0492	2.31465	.893	-4.4361	6.5346
	8.00	6.00	-5.0435	2.35091	.085	-10.6148	.5278
		9.00	-3.9942	2.31465	.199	-9.4796	1.4911
	9.00	6.00	-1.0492	2.31465	.893	-6.5346	4.4361
		8.00	3.9942	2.31465	.199	-1.4911	9.4796

Based on observed means.

The error term is Mean Square(Error) = 127.116.

Table S6. One-way ANOVA relating to **figure 3.**

Tests of Between-Subjects Effects

Dependent Variable: Survival21parentsvs21offspringfed

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2764.365[a]	5	552.873	4.349	.001
Intercept	1077930.080	1	1077930.080	8479.888	.000
HeadFootControlparentvsoffspring	2764.365	5	552.873	4.349	.001
Error	17160.671	135	127.116		
Total	1106731.000	141			
Corrected Total	19925.035	140			

a. R Squared = .139 (Adjusted R Squared = .107)

Table S7. Tukey post-hoc test relating to **figure 3.** 6, 9 and 8 represent parent foot/head halves and controls. 60, 90 and 80 represent offspring foot/head halves and controls.

Dependent Variable: Survival21parentsvs21offspringfed

Tukey HSD

(I) HeadFootControl parentvsoffspring	(J) HeadFootControl parentvsoffspring	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	8.00	3.4171	3.33733	.909	-6.2313	13.0656
	9.00	1.2557	3.25752	.999	-8.1621	10.6734
6.00	60.00	-9.2971	3.33733	.066	-18.9456	.3513
	80.00	-1.4000	3.18893	.998	-10.6195	7.8195
	90.00	-7.1323	3.15812	.219	-16.2627	1.9981
	6.00	-3.4171	3.33733	.909	-13.0656	6.2313
	9.00	-2.1615	3.40293	.988	-11.9996	7.6766
8.00	60.00	-12.7143*	3.47941	.005	-22.7735	-2.6550
	80.00	-4.8171	3.33733	.700	-14.4656	4.8313
	90.00	-10.5495*	3.30790	.021	-20.1129	-.9860
	6.00	-1.2557	3.25752	.999	-10.6734	8.1621
	8.00	2.1615	3.40293	.988	-7.6766	11.9996
9.00	60.00	-10.5528*	3.40293	.028	-20.3909	-.7147
	80.00	-2.6557	3.25752	.964	-12.0734	6.7621
	90.00	-8.3880	3.22736	.105	-17.7185	.9426
	6.00	9.2971	3.33733	.066	-.3513	18.9456
	8.00	12.7143*	3.47941	.005	2.6550	22.7735
60.00	9.00	10.5528*	3.40293	.028	.7147	20.3909
	80.00	7.8971	3.33733	.176	-1.7513	17.5456
	90.00	2.1648	3.30790	.986	-7.3986	11.7282
	6.00	1.4000	3.18893	.998	-7.8195	10.6195
	8.00	4.8171	3.33733	.700	-4.8313	14.4656
80.00	9.00	2.6557	3.25752	.964	-6.7621	12.0734
	60.00	-7.8971	3.33733	.176	-17.5456	1.7513
	90.00	-5.7323	3.15812	.460	-14.8627	3.3981
	6.00	7.1323	3.15812	.219	-1.9981	16.2627
	8.00	10.5495*	3.30790	.021	.9860	20.1129
90.00	9.00	8.3880	3.22736	.105	-.9426	17.7185
	60.00	-2.1648	3.30790	.986	-11.7282	7.3986
	80.00	5.7323	3.15812	.460	-3.3981	14.8627

Based on observed means.

The error term is Mean Square(Error) = 127.116.

*. The mean difference is significant at the .05 level.

Table S8. Two-way ANOVA relating to **figure 4.**

Tests of Between-Subjects Effects

Dependent Variable: Survivalunfed

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6470.051[a]	5	1294.010	8.032	.000
Intercept	309137.872	1	309137.872	1918.791	.000
Feedingrate	6054.669	1	6054.669	37.581	.000
HeadFootControlunfed	66.071	2	33.035	.205	.815
Feedingrate * HeadFootControlunfed	131.784	2	65.892	.409	.666
Error	14983.302	93	161.111		
Total	356697.000	99			
Corrected Total	21453.354	98			

a. R Squared = .302 (Adjusted R Squared = .264)

Table S9. Tukey post-hoc test relating to **figure 4.** 6, 9 and 8 represent foot/head halves and controls of unfed offspring polyps.

Dependent Variable: Survivalunfed

Tukey HSD

(I) HeadFootControlunfed	(J) HeadFootControlunfed	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6.00	8.00	3.8753	3.20844	.452	-3.7667	11.5172
	9.00	2.7402	3.03543	.640	-4.4896	9.9700
8.00	6.00	-3.8753	3.20844	.452	-11.5172	3.7667
	9.00	-1.1351	3.16715	.932	-8.6786	6.4085
9.00	6.00	-2.7402	3.03543	.640	-9.9700	4.4896
	8.00	1.1351	3.16715	.932	-6.4085	8.6786

Based on observed means.

The error term is Mean Square(Error) = 161.111.

Table S10. Two-way ANOVA relating **to figure 5.**

Tests of Between-Subjects Effects

Dependent Variable: BuddingBeforeAndAfter

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.136[a]	18	.063	163.350	.000
Intercept	.577	1	.577	1493.388	.000
Beforevsafterinclfeedingdistinct	.670	6	.112	289.039	.000
BisectionHFCT	.001	4	.000	.535	.710
Beforevsafterinclfeedingdistinct * BisectionHFCT	.002	5	.000	1.192	.312
Error	.165	428	.000		
Total	2.182	447			
Corrected Total	1.301	446			

a. R Squared = .873 (Adjusted R Squared = .868)

Table S11. Tukey post-hoc test relating to **figure 5.** 1,3,5,7 and 21 represent polyps at respective feeding levels before bisetion, 10, 30, 50, 70 and 210 represent polyps after bisection.

Dependent Variable: BuddingBeforeAndAfter

	(I) Beforevsafterinclfeedingdistinct	(J) Beforevsafterinclfeedingdistinct	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	1.00	3.00	.0000	.00655	1.000	-.0208	.0208
		5.00	-.0153	.00655	.371	-.0361	.0056
		7.00	-.0118	.00528	.434	-.0286	.0050
		10.00	.0028	.00567	1.000	-.0153	.0208
		21.00	-.1002*	.00529	.000	-.1170	-.0834
		30.00	.0028	.00567	1.000	-.0153	.0208
		50.00	.0023	.00567	1.000	-.0157	.0204
		70.00	-.0198*	.00512	.005	-.0361	-.0035
		210.00	-.1222*	.00510	.000	-.1384	-.1060
	3.00	1.00	.0000	.00655	1.000	-.0208	.0208
		5.00	-.0153	.00655	.371	-.0361	.0056
		7.00	-.0118	.00528	.434	-.0286	.0050
		10.00	.0028	.00567	1.000	-.0153	.0208
		21.00	-.1002*	.00529	.000	-.1170	-.0834
		30.00	.0028	.00567	1.000	-.0153	.0208
		50.00	.0023	.00567	1.000	-.0157	.0204
		70.00	-.0198*	.00512	.005	-.0361	-.0035
		210.00	-.1222*	.00510	.000	-.1384	-.1060
	5.00	1.00	.0153	.00655	.371	-.0056	.0361

	3.00	.0153	.00655	.371	-.0056	.0361
	7.00	.0035	.00528	1.000	-.0133	.0203
	10.00	.0181*	.00567	.050	.0000	.0361
	21.00	-.0849*	.00529	.000	-.1017	-.0681
	30.00	.0181*	.00567	.050	.0000	.0361
	50.00	.0176	.00567	.062	-.0004	.0357
	70.00	-.0045	.00512	.997	-.0208	.0118
	210.00	-.1069*	.00510	.000	-.1232	-.0907
	1.00	.0118	.00528	.434	-.0050	.0286
	3.00	.0118	.00528	.434	-.0050	.0286
	5.00	-.0035	.00528	1.000	-.0203	.0133
	10.00	.0146*	.00414	.017	.0014	.0278
7.00	21.00	-.0884*	.00360	.000	-.0998	-.0769
	30.00	.0146*	.00414	.017	.0014	.0278
	50.00	.0141*	.00414	.024	.0010	.0273
	70.00	-.0080	.00335	.337	-.0186	.0027
	210.00	-.1104*	.00331	.000	-.1210	-.0999
	1.00	-.0028	.00567	1.000	-.0208	.0153
	3.00	-.0028	.00567	1.000	-.0208	.0153
	5.00	-.0181*	.00567	.050	-.0361	.0000
	7.00	-.0146*	.00414	.017	-.0278	-.0014
10.00	21.00	-.1030*	.00416	.000	-.1162	-.0897
	30.00	.0000	.00463	1.000	-.0147	.0147
	50.00	-.0004	.00463	1.000	-.0152	.0143
	70.00	-.0226*	.00394	.000	-.0351	-.0100
	210.00	-.1250*	.00391	.000	-.1374	-.1126
	1.00	.1002*	.00529	.000	.0834	.1170
	3.00	.1002*	.00529	.000	.0834	.1170
	5.00	.0849*	.00529	.000	.0681	.1017
	7.00	.0884*	.00360	.000	.0769	.0998
21.00	10.00	.1030*	.00416	.000	.0897	.1162
	30.00	.1030*	.00416	.000	.0897	.1162
	50.00	.1025*	.00416	.000	.0893	.1157
	70.00	.0804*	.00336	.000	.0697	.0911
	210.00	-.0220*	.00333	.000	-.0326	-.0114
	1.00	-.0028	.00567	1.000	-.0208	.0153
	3.00	-.0028	.00567	1.000	-.0208	.0153
	5.00	-.0181*	.00567	.050	-.0361	.0000
	7.00	-.0146*	.00414	.017	-.0278	-.0014
30.00	10.00	.0000	.00463	1.000	-.0147	.0147
	21.00	-.1030*	.00416	.000	-.1162	-.0897
	50.00	-.0004	.00463	1.000	-.0152	.0143
	70.00	-.0226*	.00394	.000	-.0351	-.0100
	210.00	-.1250*	.00391	.000	-.1374	-.1126
	1.00	-.0023	.00567	1.000	-.0204	.0157
50.00	3.00	-.0023	.00567	1.000	-.0204	.0157
	5.00	-.0176	.00567	.062	-.0357	.0004

	7.00	-.0141*	.00414	.024	-.0273	-.0010
	10.00	.0004	.00463	1.000	-.0143	.0152
	21.00	-.1025*	.00416	.000	-.1157	-.0893
	30.00	.0004	.00463	1.000	-.0143	.0152
	70.00	-.0221*	.00394	.000	-.0346	-.0096
	210.00	-.1246*	.00391	.000	-.1370	-.1121
	1.00	.0198*	.00512	.005	.0035	.0361
	3.00	.0198*	.00512	.005	.0035	.0361
	5.00	.0045	.00512	.997	-.0118	.0208
	7.00	.0080	.00335	.337	-.0027	.0186
70.00	10.00	.0226*	.00394	.000	.0100	.0351
	21.00	-.0804*	.00336	.000	-.0911	-.0697
	30.00	.0226*	.00394	.000	.0100	.0351
	50.00	.0221*	.00394	.000	.0096	.0346
	210.00	-.1024*	.00305	.000	-.1121	-.0927
	1.00	.1222*	.00510	.000	.1060	.1384
	3.00	.1222*	.00510	.000	.1060	.1384
	5.00	.1069*	.00510	.000	.0907	.1232
	7.00	.1104*	.00331	.000	.0999	.1210
210.00	10.00	.1250*	.00391	.000	.1126	.1374
	21.00	.0220*	.00333	.000	.0114	.0326
	30.00	.1250*	.00391	.000	.1126	.1374
	50.00	.1246*	.00391	.000	.1121	.1370
	70.00	.1024*	.00305	.000	.0927	.1121

Based on observed means.

The error term is Mean Square(Error) = .000.

*. The mean difference is significant at the .05 level.

Table S12. Tukey post-hoc test relating to **figure 5.** 4, 5 and 7 represent low/high treatment and control before bisection. 6, 9 and 8 represent foot/head halves and controls after bisection in the two highest feeding regimes. 60 and 90 stand for foot and head halves after bisection in the three lower feeding regimes.

	(I) BisectionHFCT	(J) BisectionHFCT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	4.00	5.00	-.0493*	.00370	.000	-.0606	-.0381
		6.00	-.0640*	.00377	.000	-.0755	-.0525
		7.00	-.0517*	.00369	.000	-.0629	-.0405
		8.00	-.0683*	.00375	.000	-.0797	-.0569
		9.00	-.0691*	.00377	.000	-.0806	-.0576
		60.00	.0076	.00378	.482	-.0039	.0191
		90.00	.0079	.00378	.429	-.0037	.0194
	5.00	4.00	.0493*	.00370	.000	.0381	.0606
		6.00	-.0147*	.00368	.002	-.0259	-.0034
		7.00	-.0024	.00360	.998	-.0134	.0086
		8.00	-.0190*	.00367	.000	-.0301	-.0078
		9.00	-.0198*	.00368	.000	-.0310	-.0086
		60.00	.0569*	.00370	.000	.0456	.0682
		90.00	.0572*	.00370	.000	.0459	.0685
	6.00	4.00	.0640*	.00377	.000	.0525	.0755
		5.00	.0147*	.00368	.002	.0034	.0259
		7.00	.0123*	.00367	.020	.0011	.0235
		8.00	-.0043	.00373	.945	-.0157	.0071
		9.00	-.0051	.00375	.873	-.0165	.0063
		60.00	.0716*	.00377	.000	.0601	.0830
		90.00	.0719*	.00377	.000	.0604	.0833
	7.00	4.00	.0517*	.00369	.000	.0405	.0629
		5.00	.0024	.00360	.998	-.0086	.0134
		6.00	-.0123*	.00367	.020	-.0235	-.0011
		8.00	-.0166*	.00365	.000	-.0277	-.0055
		9.00	-.0174*	.00367	.000	-.0286	-.0062
		60.00	.0593*	.00369	.000	.0481	.0705
		90.00	.0596*	.00369	.000	.0484	.0708
	8.00	4.00	.0683*	.00375	.000	.0569	.0797
		5.00	.0190*	.00367	.000	.0078	.0301
		6.00	.0043	.00373	.945	-.0071	.0157
		7.00	.0166*	.00365	.000	.0055	.0277
		9.00	-.0008	.00373	1.000	-.0122	.0105
		60.00	.0759*	.00375	.000	.0645	.0873

	90.00	.0762*	.00375	.000	.0648	.0876
	4.00	.0691*	.00377	.000	.0576	.0806
	5.00	.0198*	.00368	.000	.0086	.0310
	6.00	.0051	.00375	.873	-.0063	.0165
9.00	7.00	.0174*	.00367	.000	.0062	.0286
	8.00	.0008	.00373	1.000	-.0105	.0122
	60.00	.0767*	.00377	.000	.0652	.0882
	90.00	.0770*	.00377	.000	.0655	.0885
	4.00	-.0076	.00378	.482	-.0191	.0039
	5.00	-.0569*	.00370	.000	-.0682	-.0456
	6.00	-.0716*	.00377	.000	-.0830	-.0601
60.00	7.00	-.0593*	.00369	.000	-.0705	-.0481
	8.00	-.0759*	.00375	.000	-.0873	-.0645
	9.00	-.0767*	.00377	.000	-.0882	-.0652
	90.00	.0003	.00378	1.000	-.0112	.0118
	4.00	-.0079	.00378	.429	-.0194	.0037
	5.00	-.0572*	.00370	.000	-.0685	-.0459
	6.00	-.0719*	.00377	.000	-.0833	-.0604
90.00	7.00	-.0596*	.00369	.000	-.0708	-.0484
	8.00	-.0762*	.00375	.000	-.0876	-.0648
	9.00	-.0770*	.00377	.000	-.0885	-.0655
	60.00	-.0003	.00378	1.000	-.0118	.0112

Based on observed means.

The error term is Mean Square(Error) = .000.

*. The mean difference is significant at the .05 level.

Table S13. One-way ANOVA relating **to figure 6.**

Tests of Between-Subjects Effects

Dependent Variable: buds21parentsvsoffspringfed

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.008[a]	5	.002	1.712	.135
Intercept	2.752	1	2.752	2832.527	.000
HeadFootControlparentvsoffspring	.008	5	.002	1.712	.135
Error	.156	161	.001		
Total	2.923	167			
Corrected Total	.165	166			

a. R Squared = .050 (Adjusted R Squared = .021)

Table S14. Tukey post-hoc test relating to **figure 6.** 6, 9 and 8 represent parent foot/head halves and controls. 60, 90 and 80 represent offspring foot/head halves and controls.

Dependent Variable: buds21parentsvsoffspringfed

	(I) HeadFootControl parentvsoffspring	(J) HeadFootControl parentvsoffspring	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
		8.00	-.0089	.00833	.892	-.0330	.0151
		9.00	-.0110	.00826	.766	-.0348	.0128
	6.00	60.00	-.0045	.00841	.995	-.0288	.0197
		80.00	-.0147	.00849	.516	-.0391	.0098
		90.00	-.0219	.00826	.092	-.0457	.0020
		6.00	.0089	.00833	.892	-.0151	.0330
		9.00	-.0021	.00826	1.000	-.0259	.0217
	8.00	60.00	.0044	.00841	.995	-.0198	.0287
		80.00	-.0057	.00849	.984	-.0302	.0188
		90.00	-.0129	.00826	.621	-.0368	.0109
		6.00	.0110	.00826	.766	-.0128	.0348
		8.00	.0021	.00826	1.000	-.0217	.0259
	9.00	60.00	.0065	.00834	.971	-.0175	.0305
		80.00	-.0037	.00842	.998	-.0279	.0206
Tukey		90.00	-.0109	.00819	.770	-.0345	.0127
HSD		6.00	.0045	.00841	.995	-.0197	.0288
		8.00	-.0044	.00841	.995	-.0287	.0198
	60.00	9.00	-.0065	.00834	.971	-.0305	.0175
		80.00	-.0101	.00856	.844	-.0348	.0146
		90.00	-.0174	.00834	.302	-.0414	.0067
		6.00	.0147	.00849	.516	-.0098	.0391
		8.00	.0057	.00849	.984	-.0188	.0302
	80.00	9.00	.0037	.00842	.998	-.0206	.0279
		60.00	.0101	.00856	.844	-.0146	.0348
		90.00	-.0072	.00842	.956	-.0315	.0171
		6.00	.0219	.00826	.092	-.0020	.0457
		8.00	.0129	.00826	.621	-.0109	.0368
	90.00	9.00	.0109	.00819	.770	-.0127	.0345
		60.00	.0174	.00834	.302	-.0067	.0414
		80.00	.0072	.00842	.956	-.0171	.0315

Based on observed means.

The error term is Mean Square(Error) = .001.

Table S15. Two-way ANOVA relating **to figure 6.**

Tests of Between-Subjects Effects

Dependent Variable: buds21parentsvsoffspringfed

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.008[a]	5	.002	1.712	.135
Intercept	2.752	1	2.752	2832.527	.000
HeadFootorControl	.006	2	.003	3.017	.052
Generation	.002	1	.002	2.123	.147
HeadFootorControl * Generation	.000	2	.000	.166	.847
Error	.156	161	.001		
Total	2.923	167			
Corrected Total	.165	166			

a. R Squared = .050 (Adjusted R Squared = .021)

Table S16. Tukey post-hoc test relating to **figure 6.** 6, 9 and 8 represent combined foot/head halves and controls.

Dependent Variable: buds21parentsvsoffspringfed

	(I) HeadFootorControl	(J) HeadFootorControl	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	6.00	8.00	-.0095	.00597	.254	-.0236	.0047
		9.00	-.0142*	.00587	.043	-.0281	-.0003
	8.00	6.00	.0095	.00597	.254	-.0047	.0236
		9.00	-.0048	.00589	.700	-.0187	.0092
	9.00	6.00	.0142*	.00587	.043	.0003	.0281
		8.00	.0048	.00589	.700	-.0092	.0187

Based on observed means.

The error term is Mean Square(Error) = .001.

*. The mean difference is significant at the .05 level.

Table S17. Linear Regression output relating to **figure 8. Starvation survival heritability.** Parent polyps versus fed offspring in highest feeding regime.

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	97.411	1	97.411	.602	.441[b]
	Residual	8732.142	54	161.706		
	Total	8829.554	55			

Tables S18. Linear Regression output relating to **figure 9. Budding rate heritability.** Parent head halves versus their fed offspring cohort in highest feeding regime.

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.007	1	.007	7.775	.010[b]
	Residual	.024	26	.001		
	Total	.031	27			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	.049	.033		1.460	.156	-.020	.118
	BudsAfterBparents	.707	.253	.480	2.788	.010	.186	1.228

Tables S19. Linear Regression output relating to **figure 10. Starvation survival heritability.** Parent head halves versus their unfed offspring cohort in the second highest feeding regime.

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	343.409	1	343.409	13.061	.004[b]
	Residual	315.519	12	26.293		
	Total	658.929	13			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	25.134	6.725		3.737	.003
	SurvivalALL	.356	.098	.722	3.614	.004

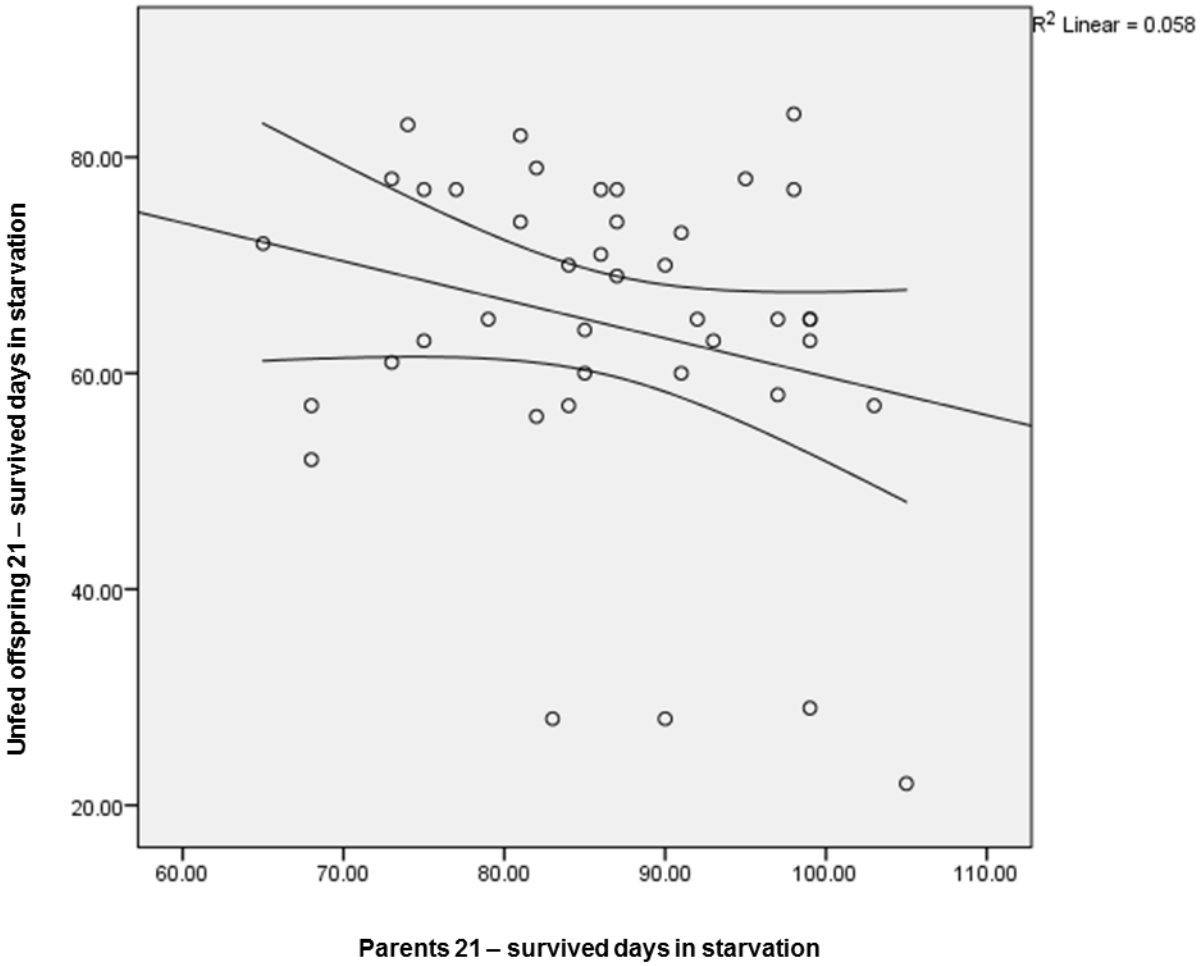

Figure S1. Extra to **figure 10. Survival heritability** between parents versus unfed offspring in the highest feeding regime. Error bars represent 95% confidence intervals. Linear regression was non-significant ($p>0.05$, see table S20).

Table S20. Extra to **figure 10. Linear Regression output** relating to **figure S1. Starvation survival heritability.** Parents versus their unfed offspring cohort in the highest feeding regime.

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	525.516	1	525.516	2.405	.129[b]
	Residual	8522.728	39	218.531		
	Total	9048.244	40			

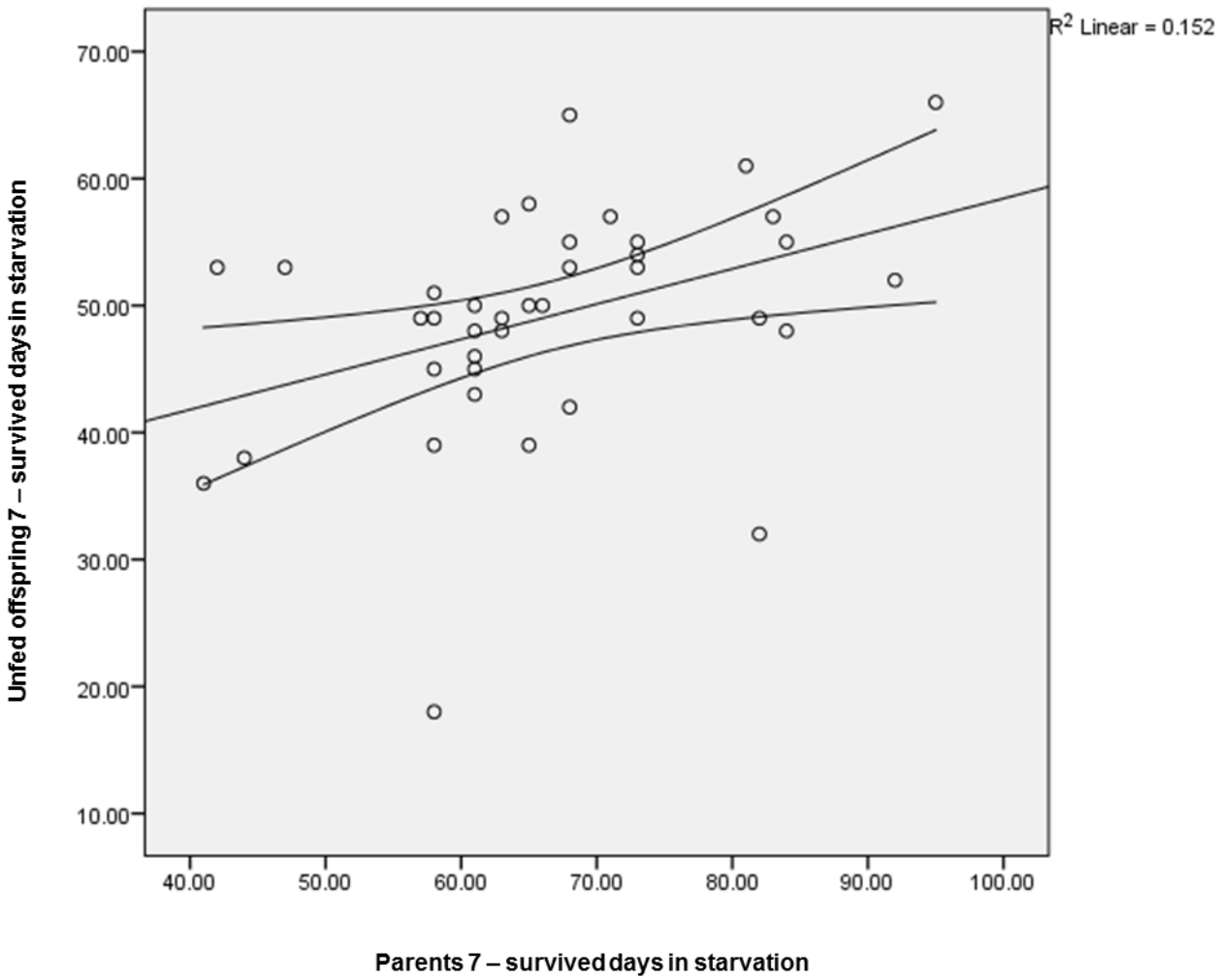

Figure S2. Extra to **figure 10. Survival heritability** between parents versus unfed offspring in the second highest feeding regime. Error bars represent 95% confidence intervals. Linear regression was significant ($p<0.05$, see table S21).

Tables S21. Extra to **figure 10. Linear Regression output** relating to **figure S2. Starvation survival heritability.** Parents versus their unfed offspring cohort in the second highest feeding regime.

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	462.141	1	462.141	6.625	.014[b]
	Residual	2580.936	37	69.755		
	Total	3043.077	38			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	30.725	7.284		4.218	.000
	SurvivalALL	.277	.108	.390	2.574	.014

Tables S22. Linear Regression output relating to **figure 11. Budding rates before versus after bisection** in the highest feeding regime. Treatment polyps before bisection versus foot halves after bisection.

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.003	1	.003	4.422	.045[b]
	Residual	.016	26	.001		
	Total	.018	27			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.091	.014		6.646	.000
	BeforeT21	.249	.119	.381	2.103	.045

Table S23. Linear Regression output relating to **figure 12. Budding versus starvation survival trade-off.** Control polyps in the highest feeding regime.

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	270.033	1	270.033	2.901	.105[b]
	Residual	1768.539	19	93.081		
	Total	2038.571	20			

Tables S24. Linear Regression output relating to **figure 13. Budding versus starvation survival trade-off.** Fed foot offspring in the highest feeding regime.

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	820.978	1	820.978	5.365	.032[b]
	Residual	2907.593	19	153.031		
	Total	3728.571	20			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	111.921	7.846		14.265	.000
	B21OfffedF	-138.836	59.941	-.469	-2.316	.032

Table S25. Linear Regression output relating to **figure 14. Budding versus starvation survival trade-off** in parent controls and their offspring in the highest feeding regime combined.

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	300.835	1	300.835	2.615	.113[b]
	Residual	5061.534	44	115.035		
	Total	5362.370	45			

Table S26. Linear Regression output relating to **figure 15. Size at starvation start versus starvation survival trade-off** in parents of the highest feeding regime.

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	384.128	1	384.128	3.699	.059[b]
	Residual	6231.243	60	103.854		
	Total	6615.371	61			

GENERAL DISCUSSION

AGING PATTERNS IN BASAL METAZOANS

It needs to be emphasized that it is absolutely essential to consider the organizational level of the individual one is looking at when it comes to aging in basal metazoans with their complex life cycles and –stages and their potential of clonal reproduction. A clonal organism could have disparate aging patterns between the ramet and genet level, comparable to differences in aging patterns within a non-clonal multicellular organism between its cells and the individual itself. Parallel different ramet life stages, like in many Medusozoans, need to be considered as well. In the clonal polyp *Hydra* for example, one can conclude that polyps on both ramet and genet levels show no senescence (Martinez 1998; Jones et al. 2014; Schaible et al. in preparation for submission), with a strong probability that *Hydra*'s senescence is even negative on the genet level. In general, sexual reproduction is a very risky way to propagate. It coincides with high mortality risks at the very early stages of e.g. (embryonic) development, metamorphosis and settlement - compared to the vegetative asexual reproduction mode in which all these difficult phases are lacking, as in the case of *Hydra*. Taking this increased early life mortality risk for newly created genets into account, a negative senescence pattern on the clone level can be expected even in *Hydra*. In fact, the prevalence of negative senescence on the genet level is very likely to be the dominant pattern for all clonal organisms with both sexual and asexual reproduction modes. Surely, more detailed demographic studies of ramet and genet populations of clonal basal metazoans are needed to reconfirm this hypothesis, since the empirical demographic data situation is not yet very comprehensive till this day. Therefore, it is one of the major aims of this dissertation to bring more light into this neglected area of aging research by adding more demographic

experimental data on basal metazoans and raise awareness about the diversity of aging patterns in general.

Most of the scant existent studies with life-history data on basal metazoans (Strehler 1961; Reiswig 1973; Brock 1974; Fell and Jacob 1979; Fell and Lewandrowski 1981; Brock 1984; Hughes 1987; Carre and Carre 1990; Babcock 1991; Carre and Carre 1991; Bavestrello et al. 1992; Piraino et al. 1996; Lirman and Fong 1997; Miyake et al. 1997; Martinez 1998; Lucas 2001; Bell and Barnes 2002; Garrabou and Harmelin 2002; Martinez 2002; Tanaka 2002; Wilson et al. 2002; Marschal et al. 2004; Albert 2005; Yoshida et al. 2006; Elahi and Edmunds 2007; Elahi and Edmunds 2007; Linares et al. 2007; McMurray et al. 2008; McMurray et al. 2010; Kubota 2011; Lucas et al. 2012; Jones et al. 2014; Schaible et al. in preparation for submission) tended to neglect the important distinction of genet versus ramet aging. One of the reasons for this neglect may be that most of the studies were conducted from an ecological point of view, without that aging or the demographic population patterns were on their focus. Therefore, the number of studied and sampled individuals per population also varied a lot and was usually rather low from a demographic perspective. Accordingly, relatively short time scales and low frequencies of observations were common. From those reviewed studies it is very hard to draw a conclusion about demographic aging in basal metazoans and the urgent need for more and thorough demographic aging studies on basal metazoans with complex life-cycles becomes apparent.

Throughout the literature and based on the studies of me and my colleagues presented in this dissertation, I could not find clear evidence of genet senescence in basal metazoans. Martinez (2002) showed in his revised version of Hughes' data (1987), that hydroid colonies of *Laomedea flexuosa*, monitored and grown on Plexiglas sheets in the field in the southern North Sea, had abruptly declining age-specific survival rates after 18 days of rather constant

survival. Even if those colonies died of ‘age’, and not by a disturbing natural force or environmental factor, which is always a potential uncontrollable factor in field studies, this would not necessarily imply genet senescence. Since hydroids of this species grow in huge stolonal networks, naturally on fronds of intertidal alga such as e.g. *Fucus* or *Ascophyllum* (Marfenin and Belorustseva 2008), and survive cutting experiments (Stebbing 2011), vegetative propagation by colony fission/breakoff is another plausible method of ramet multiplication. The genet itself may thus survive with continuously propagating ramet colonies of itself, as implied by previous studies (Marfenin and Belorustseva 2008; Stebbing 2011). Furthermore, it has been noted that a *L. flexuosa* colony clone has been successfully maintained in a laboratory for over a decade (Wermuth 1980), which suggests that the number of degeneration-regeneration cycles of polyps on these colonies and of the colonies itself ‘appear to be intrinsically limitless’ (Hughes 1989). The polyp recycling process, whereby polyps grow to a predetermined size, function for about a week in laboratory cultures of *L. flexuosa* (Strehler 1961), regress afterwards by autolysis (Brock 1970) and are replaced by new polyps developing from primordial cells (Crowell 1953), is the usual growth form of thecate hydroids (Hughes 1989). Hughes suggests that this cycle serves at least three main functions: 1) to replace ‘aging’ polyps; 2) to excrete waste products and 3) to shed fouling organisms. While the latter two points make intuitively immediate sense, the first point might need some more consideration. Hughes proposes that this ‘aging’ (meaning senescence strictly speaking) ‘seems to be an inevitable consequence of reduced mitotic activity associated with the cessation of growth and development’. Considering a hydroid stolonal polyp colony as a dynamic cell system with a continuous high cell turnover sustaining its vitality, as it has been shown and discussed for the solitary polyp *Hydra* (Bosch and David 1984; Bosch 2007; Bosch et al. 2010; Schaible et al. in preparation for submission), the proposed localized stop of mitotic activity in a polyp of a colony can be

understood as a mechanism to establish the successful degeneration-regeneration cycle to sustain the latter two mentioned points. The polyps of the colony are hereby analogous to leaves on a tree who are shed frequently for similar reasons. This is supported by data within the same study by Hughes (1987), revised by Martinez (2002), in which a decline of age-specific polyp survival within 16-24 days on the field colonies of *L. flexuosa* has been reported. However, Hughes found here in the field no evidence of the polyp regression-regeneration cycles observed for this species under laboratory conditions. Interestingly, hydranths on older, central sections of the colony lived longer than the ones generated in young, peripheral parts of the colony. The lab data, on the other hand, describe these cycles very clearly (Brock 1974; Wermuth 1980; Hughes 1989; Stebbing 2011), and Brock (1974) reports detailed successive hydranth survivorship data of *L. flexuosa* laboratory colonies maintained for over three years under constant environmental conditions. Nevertheless, despite the finding of endogenous circannual rhythms in growth, development and hydranth longevity in absence of periodic signals from the environment (Brock 1974), a clear trend towards a decline in age-specific hydranth survival rates could be seen also for the laboratory cultures over observation time-spans of 12-26 days (Martinez 2002). Longest hydranth life spans between growth periods reached here 12 - 42 days at 10°C, while the colonies itself persisted continuously (Brock 1974).

The aging patterns we see in *L. flexuosa* are, associated with the growth patterns, exemplary for thecate hydroids and many more Cnidarians. In athecate hydroids, like *Eleutheria*, the stolonal and polyp growth patterns are, in respect to the degeneration-regeneration cycles, principally very alike. The colonies persist over many years, whereby the polyps regress and regrow continuously (Schierwater 1989a)(own observations, see chapter I and II). Analogous patterns can be found in corals and colonial anemones.

Important to note is, wherever an organism has the potential to propagate clonally during one of its life stages, or grows in a colonial form, its genet distributes the chance of dying onto several units, i.e. the ramets, thereby lowering the mortality risk for itself in general. The typical and most expected genet aging pattern for such clonal organisms is therefore of a negative senescent type. An exemplary case of negative genet senescence has been described in the thorough study by Babcock on three scleractinian corals in Australia (Babcock 1991; Vaupel et al. 2004). The patterns of ramets of clonal organisms, on the other hand, vary enormously, without affecting the overall type of the aging pattern of the genet. We find senescent (e.g. *L. flexuosa* hydranths), non-senescent (e.g. *Hydra* polyps), hump-shaped (e.g. *Eleutheria* medusae) and even negative senescent (e.g. *G. aspera* coral ramets – Babcock and Rob revision, unpublished) patterns for ramets.

Demographic patterns between different ramet life stages of a species, as in the case of *E. dichotoma*, can vary as well. While polyps on the stolonal colony grow and regress in varying periods in the above discussed polyp cycling system, the fractional stolonal colony ramets of an *E. dichotoma* genet may persist through much longer times (own observations, chapter I & II). Still, the exact aging pattern of both of these ramet modules needs to be further examined and I can just speculate that no senescence occurs in both cases. On the other hand, we found a very specific hump shaped mortality for the medusa ramet stage, with humps in size and both sexual and asexual vegetative reproduction, too (Chapter I; Chapter II). Concluding, aging patterns might not only vary between different parallel living adult life stages of a species, instead, the humpy pattern of the medusae traits of *E. dichotoma* indicate a huge heterogeneity within this life stage as well. Not to forget the obviously existing mortality differences between very young sexually produced (larval) life stages (e.g. planula larvae) and (young, vegetatively produced) adult life stages (stolonal polyp colonies, medusae). All these examples illustrate the evident need to look deeply at the specific demography of each

life-stage of a metamorphic organism to draw a distinct conclusion about its aging patterns in relation to the eco-evolutionary factors involved in the specific life-cycle.

A general interesting observation is the higher mortality risk within sexual propagation compared to vegetative asexual propagation forms. Vegetatively produced offspring shows basically no early life mortality, whereas sexual reproduction poses usually much higher risks to the offspring (see *Hydra* polyps and *E. dichotoma* polyps & medusae, chapter I & II & V) (Martinez 1998; Levitis 2011; Levitis and Martinez 2013; Schaible et al. in preparation for submission). Several reasons may account for these risk differences and have been elaborated in previous works, such as the usually completely different development forms of sexual versus asexual offspring and the high regeneration potential of metazoans capable of asexual development (Martinez 2002). Vegetative asexual propagation, as seen in *Hydra* or *Eleutheria,* can be viewed as an extended form of individual growth, resulting in yet another module of the same genet. As in the exemplary case of *Hydra*, with vegetative reproduction as its main mode of reproduction (Bosch 2009), its exceptional proliferative stem cell renewal machinery seems to play a key role in the intriguing non-senescence of *Hydra* ramets from their birth onwards. The evolution of this machinery is likely to be strongly linked to the fact that vegetative reproduction is *Hydra*'s main mode of reproduction. From this point of view, non-senescence of *Hydra* polyps, i.e. on the ramet level, can be interpreted as a by-product of its fast, effective and overall successful asexual reproduction strategy. In accordance, its remarkable maintenance, including its regeneration abilities, would be a part of this by-product.

In contrast, sexual reproduction has, besides various extrinsic costs related to the mating system itself, the disadvantage of random genetic shuffling and thereby creating a huge share of failing or nonfunctional offspring units out of functional parents (Agrawal 2006), which leads to high early-life sexual offspring (i.e. genet) mortality. Additionally, organismal

development throughout the ontogenesis of sexually produced offspring may pose in general much higher mortality risks than in vegetative offspring, where typically fully functional adult ramets are 'born'. To complicate things further, clonal genet lines can also occur through sexual reproduction by continuous self-fertilization and inbreeding, as seen in *Eleutheria* (Schierwater and Hauenschild 1991; Ender 1997; Chapter I; Chapter II). In this case, the process of sexual reproduction, and not the fact of inbreeding or cloning a successful genet by this self-fertilization, dictates the demographic outcome regarding early-life mortality (e.g. chapter I & II, larva mortality). How obligate clonal reproducers, such as *Eleutheria* for example (Schierwater and Hauenschild 1991; Ender 1997), circumvent the proposed negative consequences of long-term cloning and inbreeding in absence of cross-fertilization, i.e. the accumulation of harmful mutations known as Muller's Ratchet (Muller 1964), is not clear, yet. That it seems possible, though, show not only the findings on *Eleutheria* (Schierwater and Hauenschild 1991; Ender 1997), but also studies on exclusively asexual metazoans like bdelloid rotifers (Wilson and Sherman 2010; Wilson and Sherman 2013). Our finding of a loss of medusa quality with successive vegetative medusa generation regarding survival and both sexual and asexual reproduction in *E. dichotoma* is not likely to contradict the obligate clonal reproducer hypothesis. The quality decline rather depicts the seemingly seasonal nature of medusa population occurrences and emphasizes the importance of continuous clonal line 'refreshment' by the means of sexual self-fertilization associated with the development of new polyp colonies and medusa cohorts through embryogenesis and metamorphosis steps. That *E. dichotoma* medusa generations can be potentially bred *ad infinitum* through successive vegetative generations showed Hauenschild 1956 already more than 50 years ago with the propagation of vegetative secondary medusae for more than 40 generations (Hauenschild 1956; Hauenschild 1957). Nevertheless, a possible overall quality decline with successive generations cannot be excluded here since it was not checked. But

strikingly, several of these medusa generation lines were reported to have lost their sexuality through time which might hint to a loss or decline in stem cell potential (Hauenschild 1956); but some regained sexuality (Hauenschild 1957), which contradicts an irrevocable quality decline with pure vegetative reproduction. Whether purely vegetatively or both by vegetative plus sexual self-fertilization, established *E. dichotoma* clones show us to date no evidence of the necessity to cross-fertilize to sustain the survival of the species. Indeed, the age of the collected clone lines of *E. dichotoma* has been estimated to be .2 – 2.4 Million years, according to 16S-mtDNA analyses (Ender 1997). One haplotype-line, collected on Mallorca, was even estimated to be 5 – 10 Million years old. This renders clonal lines of *E. dichotoma* among the "oldest" organisms ever measured.

MULTICELLULARITY AS A WAY TO OVERCOME RAMET-SENESCENCE

As mentioned in the thesis introduction, the traditional theories on the evolution of aging focus on animals with a clear germ-soma segregation (Medawar 1952; Williams 1957; Hamilton 1966; Kirkwood 1977; Kirkwood and Holliday 1979; Kirkwood 1991) and remain very blurry about aging and senescence in organisms without this clear distinction, such as Protozoans, basal metazoans, fungi and plants. Several studies suggest, that organisms without a clear germ/soma cell line segregation are not completely free from senescence. Senescence on the ramet level has already been found in bacteria (Stewart et al. 2005; Wang et al. 2010) and, entering the multicellular animal kingdom, in hydranths of hydrozoans (Brock 1974; Hughes 1987; Martinez 2002) and, entering higher metazoans and bilaterians, in oligochaetes and Platyhelminthes (Martinez and Levinton 1992). With senescence already found to be present in bacteria, the occurrence of senescence, if only at the ramet level, seems to be deeply rooted in life itself, starting with the first reproductive (cell) units, making the

‘parent’ (cell) units replaceable. It seems that senescence is an inseparable feature of life, where anything ‘old’ becomes replaceable by something new, whereby this ‘old’ and ‘new’ units can be represented by either parent and offspring cells or cell lines (in asymmetric division), or even genets/clones (in asymmetrically or symmetrically dividing ramet units when sexual reproduction - with a rejuvenating character for the sexual offspring - is additionally present). Thinking in an even more abstract way about reproduction enabling senescence, the replaceable unit does not have to be the “old” one – the replaceability can apply to any unit, as long as it is compensated for by multiplication and survival. Eventually, it seems not to be the case, as previously postulated, that “the evolution of somatic differentiation and hence of an integrated multicellular soma, and not of germ-line sequestration, was the necessary condition for the evolution of senescence” (Martinez and Levinton 1992).

So why do *Hydra* ramets NOT senesce? One simple, but intriguing explanation is, that multicellularity itself opens a way for *Hydra* polyps to overcome senescence. Each cell of a *Hydra* polyp, just as shown in the experiments with the bacteria, may still ‘wear out’ and senesce over time, as long as a kind of rejuvenation is ensured in the offspring cells, for example by asymmetrical division of stem cells. But the individual ramet unit, which is the polyp in *Hydra*, and not the single cell as in bacteria, can be sustained by the continuous cell proliferation and turnover machinery rooted in *Hydra*. *Hydra*’s stem cell community thus may act as an ever rejuvenating cell line, analogous to a ‘germ line’ in sexual organisms with a sequestered germ line, providing each *Hydra* ramet plus its genet with an unlimited source of cell proliferation potential - with the combined feature of eventual germ cell production in between. A *Hydra* polyp can be seen as a dynamic, continuously recycling active cell colony with constantly dividing, dying, renewing and proliferating cells in it, preventing senescence even on the ramet (polyp) level, additionally to the senescence prevention on the genet level.

It's body plan simplicity, low number of different cell types and low complexity in general have also an important share in this and allow *Hydra* to express this remarkable aging pattern. *Hydra* could be just an exemplary case for many more basal metazoans – a similar mechanism could be very likely at work for many more basal metazoans, especially hydranth (e.g. *E. dichotoma*) and coral colonies, for example. It is on future studies about the biology and demography of more basal metazoan cases to test this hypothesis.

HETEROGENEITY & RANDOMNESS OF AGING

The patterns we found in both *Hydra* (polyps) and *Eleutheria* (medusae) suggest a huge phenotypic diversity in the studied clonal lines. We observed huge variabilities between individuals in basically all measured traits, i.e. the survival, sexual (in the case of *Eleutheria*) and vegetative reproduction, as well as in individual size (all chapters). Furthermore, we did not find any traces for direct heritability of trait patterns between vegetative generations in both study organisms. These findings hint to an evolutionary successful randomized phenotype generation process during vegetative reproduction in both species in their different life stages. Our studies provide hereby first evidence that this random phenotype production might be favourable for the adaptation ability of the mainly asexually reproducing *Hydra*- and the metagenetic *Eleutheria* populations. Via this system, each genet automatically creates ramet populations consisting of multiple phenotypes. Each phenotype seems to have its own plasticity window, in which its trait pattern fluctuate according to the individual's dynamic state. This hypothesis is supported by our bipolar findings of trait stability over time. On the one hand, we found heterogeneous *H. magnipapillata* budding patterns to be stable over time within individuals under constant conditions (Chapter IV). On the other hand, we showed that budding rates after stress induction (hunger period in between/bisection) differ within

individuals (Chapter V). And we reported size fluctuations in both *H. magnipapillata* polyps and *E. dichotoma* medusae within constant regimes (Chapter II; Chapter V), as it has been reported for *Hydra vulgaris strain AEP* before (Levitis and Goldstein 2013). Both of these trait instabilities do not contradict our random phenotype generation hypothesis, instead, these cases display the plasticity window of each phenotype very nicely. While the budding rate change after stress indicates a hormetic response in *H. magnipapillata* polyps overriding the original phenotype setting, the size fluctuations reveal the dynamic states of each *Hydra* polyp or *Eleutheria* medusa. As both entities are involved in regular vegetative reproduction, fluctuating sizes are not really surprising. Additionally, as in the case shown for *Hydra*, high cell turnover rates may add to this phenotypic character plasticity (Bosch and David 1984; Bosch 2007; Bosch et al. 2010; Schaible et al. in preparation for submission). Through this high cell turnover and cell proliferation rates each polyp or medusa exists in a dynamic state with continuously changing cell proportions. It follows, that each bud is grown from a unique combination of parent cells leading to differing epigenetic profiles of each bud. Hence, from this mechanistical point of view the observed heterogeneity between, but also within polyps in the mentioned cases, is not so surprising anymore. Since vegetative reproduction is a very fast and effective way of producing high adult ramet numbers within a short time, circumventing thereby the risky development pathways annexed to sexual reproduction, this randomized phenotype generation process seems to be a most successful propagation strategy. Most likely, this has been selected due to its risk spreading feature, further described as bet-hedging in the literature (Cohen 1966; Stumpf et al. 2002; Thattai and van Oudenaarden 2004; Kussell and Leibler 2005; Beaumont et al. 2009; Chapter IV). This strategy could enhance long-term fitness by providing the genet continuously with many phenotype subsets of ramets which secure the survival of the genet in changing or fluctuating environments when one or several of these subsets are already optimized to the altered

conditions. Whether the natural conditions for *H. magnipapillata* in freshwaters of Japan (Sugiyama and Fujisawa 1977; Sugiyama and Fujisawa 1977; Sugiyama and Fujisawa 1978; Sugiyama and Fujisawa 1978; Sugiyama and Fujisawa 1979) or *E. dichotoma* in the Mediterranean (Schierwater 1989a; Ender 1997) are particularly fluctuating or challenging to support the bet-hedging hypothesis is relatively difficult to prove. Various unpredictable and sudden environmental changes could occur in both environments, such as temperature or salinity shifts (especially in rock pools for *E. dichotoma),* food shortages, inter-species competition for resources or predation. In all these cases, an array of already existent and differing ramet phenotypes could infer an advantage to the sustenance of the genet. *Sensu stricto,* this would not be bet-hedging under the previously proposed (ramet) phenotype switching strategy during the lifetime of (ramet) individuals (Thattai and van Oudenaarden 2004; Kussell and Leibler 2005; Beaumont et al. 2009), but via our proposed random phenotype generation process in *Hydra* polyps or *E. dichotoma* medusae. In both cases, though, subpopulations of differing phenotypes will exist in the clonal (genet) population of ramets, compared to a hypothetical genet which relies on responsive switching of ramets. Kussel and Leibler (2005) found in a modeled clonal population that stochastic phenotype switching - or referring to our case: the random generation of phenotypic diversity within the ramet population - is favored over responsive switching in environments with less frequent changes. The longer an environment remains constant, the less it pays to invest resources in a sensoring system for a responsive switch. Interestingly, bet-hedging strategies have already been found and discussed for bacteria and it has been concluded that these risk-spreading strategies may have been among the earliest evolutionary solutions to life in clonal populations in fluctuating environments, "perhaps even preceding the evolution of environmentally responsive mechanisms of gene regulation" (Beaumont et al. 2009). This

exemplifies how primordial the survival strategies of *Hydra*, *E. dichotoma* and many more basal metazoans may be.

Another, so far mostly neglected factor regarding phenotypic heterogeneity is the epibiome of organisms, consisting of various usually single celled species. Heterogeneity within and between isogenic ramets, as in the case of *Hydra* polyps or *Eleutheria* medusae, could possibly be related to differences and variations among their associated bacterial epicommunity. These metaorganismic or holobiontic relationships and variations turn recently more and more into focus of research, especially in the freshwater polyp *Hydra* (Bosch and McFall-Ngai 2011; Bosch 2012; Bosch 2012; McFall-Ngai et al. 2013), and pose a promising approach to gain new insights into the microecosystems of clonal organisms.

AGING TRADE-OFFS

Trade-offs between the allocation of limited resources to either maintenance or reproduction are of crucial importance when thinking about the evolution of aging patterns and are a centerpiece of the disposable soma theory of aging (Kirkwood 1977; Kirkwood and Holliday 1979; Kirkwood and Rose 1991) which brought trade-offs into the limelight of aging research in the end. We found several indices for important trade-offs in *Hydra* and *E. dichotoma* in our studies. In *E. dichotoma* medusae, our results indicated direct effects of the nutritional level on the pace of medusa lifetime. The more food is available, the faster and compressed it lives. Hence, the feeding level directly affects the trade-off setting between reproduction and maintenance in *E. dichotoma*. The other, different kind of trade-off we discovered in the medusae emerged between successive medusa generations. Medusa quality, in terms of all measured traits, declined with consecutive generations. I propose that this trade-off might be

advantageous for the genet regarding the proposed seasonal appearance of *E. dichotoma* (Chapter I; Chapter II).

In the *Hydra* bisection experiment, a clear trade-off between vegetative reproduction and maintenance did only occur at the threshold feeding level of 7 *Artemia* per week per polyp for both cut and uncut polyps (Chapter V). With resources deviated to starting reproduction at this feeding level, less seemed available for maintenance and starvation survival decreased sharply, independent of the previous bisection treatment. At lower or higher feeding regimes, none such an effect emerged in this experiment. Furthermore, as shown in chapter III & V, budding rates of polyps increased rather linearly with increasing food levels, while starvation survival stayed rather constant around 80 days from > 7 *Artemia* per week onwards, though slight variations occurred between the studies. Apart from these trade-off reactions to different feeding levels, all three key life history traits were rather uncoupled from each other in *Hydra* - size, survival or budding showed generally no correlations within treatment levels (Chapter V). This uncoupling of the three traits seems to be also the case for *E. dichotoma* medusae according to our findings. I can conclude that, next to the mentioned feeding level and generation trade-offs, no real trade-off in a traditional sense between size, reproduction and maintenance (Kirkwood 1977; Kirkwood and Holliday 1979; Stearns 1989; Kirkwood and Rose 1991; Vaupel et al. 2004; Baudisch 2007; Baudisch 2009; Flatt 2011) seems to exist in both *Hydra* and *E. dichotoma.*

HORMETIC AGING RESPONSES

Hormesis is a stress response phenomenon, where maintenance- and reproduction efficiency levels of an organism are found to be increased, though possibly only temporarily, resulting from exposure to low and specific doses of stress (Calabrese and Baldwin 2003; Stebbing

2003; Parsons 2005; Mangel 2008; Rattan 2008). The exact mechanisms at work are still unclear and several suggestions were made, including physiological counteractions to stress with overcorrections to retain homeostasis leading to a hormetic response pattern, known as homeostatic hypothesis for hormesis (Stebbing 2003).

We found clear indications for hormetic responses at work in both *Hydra* and *E. dichotoma.* In *Hydra* polyps, various stressors, such as bisection, hunger periods and minor starvation, temperature or food level changes caused hormetic responses in both starvation survival and budding rates, without any signs of a trade-off (Chapter III; Chapter V). In *E. dichotoma* medusae, constant low feeding levels caused a positive effect on survival (Chapter I; Chapter II), comparable to the caloric restriction effect reported for many species (Heilbronn and Ravussin 2003; Chung et al. 2013). I propose, that this type of positive low food stress response is a variant of a hormetic stress response, but, in contrast to the *Hydra* results, with the additional feature of a visible trade-off. Medusae in the low feeding level tended to live longer, but were generally smaller and had lower budding- and larva release rates than at the higher feeding level. This effect became more pronounced at successive generations. While our findings on *Hydra* suggest that it is able to increase its metabolic efficiency in response to stress, free from a trade-off, our results on *E. dichotoma* medusae are less clear regarding interpretation. Low food stress could have induced a pure resource allocation shift, pushing resource utilization towards maintenance and attenuating reproduction, speaking for a pure trade-off reaction. On the other hand, this response may have been additionally coupled with a hormetic counter response increasing metabolic efficiency, just as in *Hydra*. Indicators for both responses working together are the relatively long average survival of SL medusae compared to the other LFR and even HFR groups and that both SL and TL medusae had a higher average survival than their counterparts SH and TH, respectively.

As for all hormetic reactions, it is still puzzling why the optimal metabolic efficiency is not reached during rather stable, optimal and non-stressful conditions. The costs, or trade-off, to this effect remain still unclear, as well as how long such an increased performance can be maintained, as hormetic effects appear to be mostly of a transient nature (Stebbing 1982), but not by all means (Rattan 2008; Calabrese et al. 2012). We hypothesize that a constant excessive food supply may lead to more inefficiency and a certain slackness on energy utilization (Chapter III), but further experiments are needed to resolve these propositions. Important to keep in mind is, that environmental stresses can have a beneficial impact on fitness-related phenotypical traits and that hormetic stress doses posed by variable and fluctuating environments could be, in the end, salutary for the persistence of clonal lineages of *Hydra*, *Eleutheria* and presumably many more basal metazoans.

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Publications

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Schaible, R., **Ringelhan, F.**, Kramer, B.H., Miethe, T. (2011). Environmental challenges improve resource utilization for asexual reproduction and maintenance in hydra. Experimental Gerontology 46:794-802.

Presentations

Ringelhan, F.; Schierwater B.; Schaible, R. (2014). Diversity of Aging in Clonal Animals. Evolutionary Demography Society's 2nd Annual Meeting, 10–12 November, Stanford University, Palo Alto, California, USA. [Talk]

Danko, A.; **Ringelhan, F.**; Danko, M. J.; Schaible, R. (2014). Environmental influence on reproduction and aging of the medusa of *Eleutheria dichotoma*. European Meeting of PhD Students in Evolutionary Biology (EMPSEB), 1-6 September, La Roche-en-Ardenne, Belgium. [Talk]

Schaible, R.; Sussman, M.; Kramer, B. H.; Sugareva, V.; Danko, M. J.; **Ringelhan, F.**; Scheuerlein, A. (2014). Evolutionary Biodemography and the Age-Specific Mortality of Hydra. Scientific Advisory Board (Fachbeirat) Evaluation Meeting, 19–21 May 2014, Max Planck Institute for Demographic Research, Rostock, Germany. [Poster]

Danko, A.; Schaible, R.; **Ringelhan, F.** (2013). Influence of high temperature on life history traits of *Eleutheria dichotoma.* Evolutionary Demography Society's 1st Annual Meeting, 6–10 October, Max-Planck Odense Center on the Biodemography of Aging at the University of Southern Denmark (SDU), Odense, Denmark. [Poster]

Ringelhan, F.; Schaible, R.; Schierwater B. (2013). Aging in a metagenetic basal metazoan – the biodemography of *Eleutheria dichotoma* (Cnidaria: Hydrozoa). Fourth International Jellyfish Bloom Symposium, 5-7 June, International Conference Center Hiroshima, Japan. & Seto Marine Biological Laboratory, Field Science Education and Research Center, Kyoto University, Shirahama, Nishimuro, Wakayama, Japan. [Talks]

Baudisch, A.; Salguero-Gomez, R.; Colchero, F.; Jones, O.; Wrycza, T.; Burger, O.; Conde Ovando, D. A.; Kramer, B.; Rebke, M.; **Ringelhan, F.**; Schaible, R.; Scheuerlein, A. and Vaupel J. (2012). When and why senescence evolves. 97th meeting of the Ecological Society of America (ESA), 5-10 August, Oregon Convention Center, Portland, Oregon, USA. [Talk]

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Ringelhan, F. and Schaible, R. (2011 & 2012). Aging in basal metazoans – a biodemographic approach: Trade-offs in Hydra. International Workshop "Searching for Eve: basal metazoans and the evolution of multicellular complexity", 12–15 September 2011, Evangelische Akademie Tutzing, Germany. & Scientific Advisory Board (Fachbeirat) Evaluation Meeting, 22–24 May 2012, Max Planck Institute for Demographic Research, Rostock, Germany. & Evolutionary Demography Workshop, 6-9 June 2012, Max Planck Institute for Demographic Research, Rostock, Germany. [Poster]

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Ringelhan, F.; Schaible, R.; Vaupel J. W. (2010). Aging in basal metazoans - resource allocation flexibility in Hydra. MaxNetAging Conference VIII "Early life-late life: an interdisciplinary approach towards aging", 6–8 October, Parkhotel Schlangenbad, Germany. [Poster]

Ringelhan, F. and Cunningham, C. (2010). Aging in basal metazoans - a biodemographic approach. 7th Workshop of the Hydrozoan Society, 10–18 September, Porto Cesareo, Lecce, Italy. [Talk]

Milisenda, G.; Longo, C.; **Ringelhan, F.** (2010). Placozoa sp. H2 (The "Roscoff Clone") Endosymbionts and cell-cell communication at the base of the metazoan ToL. 3rd Volker Schmid Training Course on Experimental Developmental Biology of Marine Invertebrates, 4-14 May, Station Biologique de Roscoff, France. [Poster]

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Molis, M.; **Ringelhan, F.**; Rohnstock, L.; Lenz, M.; Wahl, M. (2009). Effects of grazing pressure on the induction of anti-herbivory defences in bladderwrack (Fucus vesiculosus) in different seasons. 8th International Temperate Reefs Symposium, 12–16 January, Adelaide, Australia. [Poster]