Sex-dependent plasticity of reproductive traits in relation to body size and environmental seasonality in *Hydra oligactis*

Thesis for the Degree of Doctor of Philosophy (PhD)

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Debrecen, 2026

Hereby I declare that I prepared this thesis within the Doctoral Council for Natural Sciences and Engineering, Doctoral School of **Juhász-Nagy Pál**, University of Debrecen in order to obtain a PhD Degree in Natural Sciences at Debrecen University.
The results published in the thesis are not reported in any other PhD theses.

Debrecen, 2026. . . . . .

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Réka Gergely

Hereby I confirm that **Réka Gergely** candidate conducted her studies with my supervision within the **Evolutionary and Biodiversity** Doctoral Program of the Doctoral School of **Juhász-Nagy Pál** between 2022 and 2026. The independent studies and research work of the candidate significantly contributed to the results published in the thesis.

I also declare that the results published in the thesis are not reported in any other theses.

I support the acceptance of the thesis.

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Dr. Jácint Tökölyi

SEX-DEPENDENT PLASTICITY OF REPRODUCTIVE TRAITS IN RELATION TO BODY SIZE AND ENVIRONMENTAL SEASONALITY IN HYDRA OLIGACTIS

Dissertation submitted in partial fulfilment of the requirements for the doctoral (PhD) degree in Biology

Written by **Réka Gergely** certified Biologist

Prepared in the framework of the **Juhász-Nagy Pál** doctoral school of the University of Debrecen
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I. General introduction and research question

In the following sections, I will outline the principal research questions of this dissertation and their theoretical background, organized into two major parts. Accordingly, Part One concentrates on sex differences in the effects of body size on life-history traits, emphasizing how sex-specific allocation patterns generate pronounced differences in reproductive output and survival. Part Two addresses differential responses to seasonal temperature change, focusing primarily on variation in sexual modes and the occurrence of sex change, again in the context of the focal study organism.

Life-history theory aims to explain how organisms allocate limited resources among growth, reproduction, and survival, and how these allocation patterns vary across individuals, environments, and evolutionary contexts (Roff, 1993; Stearns, 1989). A central element of life-history evolution is phenotypic plasticity, defined as the capacity of traits such as body size, reproductive investment, and survival probability to vary in response to internal physiological state and external environmental conditions (Kaliszewicz & Lipińska, 2013; Kirkwood & Rose, 1991; West-Eberhard, 2003). Through such plasticity, organisms can modify their life-history strategies over the course of their lifespan, for example by adjusting somatic growth, the timing and magnitude of sexual reproduction, or allocation to maintenance and/or survival.

Sex differences constitute another fundamental axis of life-history variation. Sex-specific selection pressures have generated widespread divergence between males and females in morphology, behaviour, lifespan, and reproductive strategies across taxa (Fairbairn et al., 2007; Maklakov et al., 2008; Tessier & Cáceres, 2004). These differences frequently manifest as sex-

specific life-history trajectories, arising from contrasting reproductive roles, differential costs of reproduction, and distinct allocation trade-offs (Kaliszewicz, 2018; Kraus et al., 2013; Stearns, 1989). Consequently, a comprehensive understanding of life-history plasticity requires the explicit integration of sex as a biological variable, as males and females may differ not only in trait values but also in the magnitude and direction of their plastic responses to environmental variation.

To investigate sex-specific life-history plasticity, we employed a freshwater cnidarian model organism belonging to the genus *Hydra*, occurring in Hungary. *Hydra* and our model species from the genus, *Hydra oligactis* represents a well-established experimental system in evolutionary and ecological research owing to its small body size, simple tissue organization, dioecious reproduction, and ease of laboratory maintenance (Bosch & David, 1986; Galliot, 2012; Tomczyk et al., 2015). Importantly, this genus exhibits pronounced variation in body size, reproductive mode (asexual-, sexual male- or sexual female- polyp), and survival, rendering it particularly suitable for examining life-history trade-offs and plasticity (Harvell & Grosberg, 1988; Martínez & Bridge, 2012). *H. oligactis* has a relatively simple life cycle connected to environmental seasonality. Such as, the polyps reproduce asexually through budding in the warmer seasons and start to reproduce sexually when it's getting colder. If the individuals survive post-reproduction, then they start to reproduce asexually again (Burnett & Reisa, 1973; Tökölyi et al., 2021). Previous studies have documented sex-specific differences in reproductive strategies and ageing patterns in *Hydra*, underscoring its value for analysing how sex shapes life-history variation even in relatively simple metazoans (Schaible et al., 2015; Sebestyén et al., 2018, 2020; Tökölyi et al., 2021). Collectively, these characteristics establish *Hydra* as an appropriate

model system for disentangling the interaction between phenotypic plasticity and sex-specific life-history strategies.

Hence, in the first part of this thesis I address how sex, as a biological variable, influences life-history trajectories, specifically examining how effect of body size depends on the animals' sex. In this context, I investigated how variation in body size affects reproductive investments in both male and female individuals. The second part focuses on environmental influences on phenotypic plasticity and sex change, examining how environmental variation shapes sexual modes and plastic responses in the studied organism.

I.I - Part One - Sex-dependent body size effects on reproduction

Variation in life history traits across mammals, reptiles, birds, and invertebrates is to a large extent explained by body size (Lindstedt & Boyce, 1985). As one of the most fundamental quantitative traits in evolutionary ecology, body size is strongly associated with both physiological functions and fitness outcomes (Blanckenhorn, 2000; Reiss M.J., 1989; Roff, 1993). Considerable variation exists both within and among species, and because body size influences multiple components of fitness in different ways, it is subject to opposing selective pressures. If the variation in body size among species within taxa is shaped by selection, then it must result from differences in the balance between selective pressures favoring large body size and those favoring early maturation, which indirectly promotes smaller size (Amarillo-Suárez et al., 2011). In some cases, body size is linked to the acceleration of certain aspects of life history and the slowing of others. For example, small dogs generally reach sexual maturity earlier than large dogs, while also remaining healthy and living significantly longer (Austad, 2010).

Furthermore, sexual size dimorphism (SSD) is common in animals, and evidence suggest that it reflects the adaptation of the two sexes for their differing reproductive roles. Hence it is important to examine both sexes separately when examining life-history traits. Many functional hypotheses have been proposed to explain the evolution of SSD. In the end, it is shaped by processes that create variations - such as reproductive selection - both within each sex and between males and females (Blanckenhorn, 2005; Fairbairn, 1997). For example, larger individuals may be favoured because they tend to be more fecund, whereas smaller body size may be advantageous when early maturation is selected for (Amarillo-Suárez et al., 2011; Blanckenhorn, 2000; Harvey et al., 2006).

From an energetic standpoint, several mechanisms may underlie the link between body size and fitness. For example, in our "energy reserve" hypothesis posits that larger organisms have greater stored energy, which can be allocated among competing processes such as survival, reproduction, and maintenance (Gergely & Tökölyi, 2023; Lindsey, 1966; Millar & Hickling, 1990; Roff, 1993; Stearns S.C., 1992). This framework predicts that individuals of larger size can initiate reproduction earlier and at higher intensity, thereby attaining greater reproductive success even under resource-limited conditions due to their energy reserves (Lindstedt & Boyce, 1985; Reim et al., 2006a).

A positive relationship between body size and fecundity has been observed across diverse taxa, including sponges (e.g., *Rhopaloeides odorabile*) (Whalan et al., 2007), cnidarians (e.g., *H. oligactis* and *Tripalea clavaria*) (Excoffon et al., 2011; Ngo et al., 2021), and numerous insect orders such as Coleoptera, Diptera, Heteroptera, Lepidoptera, Odonata, Orthoptera etc. (Honěk, 1993). For instance, in Odonata, larger body size enhances mating success, fecundity, longevity, and survival, conferring an overall fitness advantage (Sokolovska et

al., 2000). Similarly, in *Drosophila pseudoobscura*, fecundity, viability, and lifespan increase with size, and grasshoppers such as *Romalea microptera* also show a positive relationship between size and reproductive output (Akman & Whitman, 2008; Tantawy & Vetukhiv, 1960).

By contrast, our "energy demand" hypothesis suggests that larger size may carry disadvantages under restricted conditions, as increased body mass requires more energy for tissue maintenance (Gergely & Tökölyi, 2023; Reim et al., 2006). Under this scenario, smaller individuals may gain a reproductive advantage by needing less energy for self-maintenance and consequently reproducing earlier (Blanckenhorn, 2000). For example, in water striders, smaller males can still achieve copulation at lower food availability thresholds compared to larger males (Blanckenhorn, 2005).

Disentangling the relative contribution of the "energy reserve" and "energy demand" hypotheses to correlations between body size and fitness traits is challenging in most cases (e.g., across taxa or sexes), as both traits may be shaped by shared underlying factors. For instance, food availability influences both growth and condition, meaning that the observed positive association between body size and fitness could primarily reflect variation in resource access rather than direct effects of large size or rapid growth itself (Gori et al., 2013; Reznick et al., 2000; van Noordwijk & de Jong, 1986; Yom-Tov et al., 2006). In addition, investigating the impact of body size in species with larger size and extended lifespan is not readily feasible under laboratory conditions. Body size also exerts strong pleiotropic effects on other life-history traits; for example, while larger individuals often achieve greater fecundity, they may also experience increased mortality later in life due to reproduction–survival trade-offs (Kirkwood & Rose, 1991; Sebestyén et al., 2020).

The energy costs and benefits caused by body size can differ depending on the animals' sex, resulting in differing body size – life-history relations in males and females. From a life-history perspective, body size is a central trait influencing energy acquisition, allocation, and reproductive output, and its fitness consequences often differ between the sexes. Because males and females typically experience different reproductive constraints and selection pressures, the energetic costs and benefits associated with body size are sex-specific. As a result, relationships between body size and key life-history traits - such as age at maturation, reproductive investment, and survival - frequently diverge between males and females (Blanckenhorn et al., 2007; Shine, 1989).

In many invertebrates, including spiders, males are under selection to mature earlier in order to maximize mating opportunities. For earlier maturation development time shortens and somatic growth can get constrained, resulting in smaller male body size. In contrast, female fitness is often more directly linked to fecundity, which typically scales positively with body size due to increased capacity for egg production. Consequently, selection favours greater somatic investment and delayed maturation in females when larger size enhances reproductive output (Head, 1995; Vollrath & Parker, 1992). These patterns reflect classic life-history trade-offs between growth, timing of reproduction, and fecundity.

Importantly, the optimal body size for each sex depends on the mating system and ecological context. In species where male reproductive success depends strongly on direct competition or monopolization of mates, selection may favour increased male body size despite the associated developmental and maintenance costs (Blanckenhorn et al., 2007; Fairbairn & Preziosi, 1996). Thus, sex-specific selection on body size emerges from differences in how

growth, survival, and reproduction contribute to lifetime fitness in males versus females.

These divergent selective pressures are underpinned by differences in energy allocation strategies. Males often allocate proportionally more energy toward behaviours and traits that enhance mating success - such as mate searching, courtship, or competitive structures - whereas females allocate a larger share of resources to gamete production and offspring provisioning (Shine, 1989; Stillwell et al., 2010). Because body size mediates both maintenance costs and reproductive capacity, it becomes integrated into sex-specific life-history strategies, generating distinct size - life-history relationships in males and females.

In our first study, we employed the freshwater cnidarian *H. oligactis* (common name: brown hydra) as a model system to investigate the direct effects of body size on fitness-related traits. We used male and female individuals so we could examine the differing effects of body size on traits between the sexes. Owing to their exceptional regenerative capacity, hydra permit precise tissue excision and grafting between individuals, enabling experimental enlargement or reduction of the body column in individual polyps. Together with resource manipulation we examined the effects of these factors and their interaction on the reproduction of male and female polyps (Gergely & Tökölyi, 2023).

I.II - Part Two - Effects of seasonality and temperature fluctuations on reproduction and sexual readiness

Facultatively sexual species combine clonal (asexual) reproduction with occasional sexual reproduction and exhibit remarkably diverse life-history

strategies. These range from short clonal cycles with frequent sexual events to predominantly asexual lineages capable of extensive geographic spread and potentially millennia-long persistence (Arnaud-Haond et al., 2012; Kokko, 2020; Stelzer & Lehtonen, 2016). The transition from asexual to sexual reproduction in such organisms is highly variable, both among and within species (Franch-Gras et al., 2017; Ryan & Miller, 2019; Sánchez Navarro et al., 2013; Tessier & Cáceres, 2004). For example, in freshwater invertebrates such as monogonont rotifers, water fleas and hydras, clonal lineages often vary in their tendency toward sex: some genotypes exhibit no sexual investment at all, whereas others undergo full sexual induction when exposed to particular environmental cues (Gilbert, 2020; Tessier & Cáceres, 2004; Tökölyi et al., 2017).

A central objective in the study of facultatively sexual organisms is to uncover the factors underlying this variation. Many such species inhabit ephemeral or strongly seasonal habitats where periods of favourable conditions alternate with less favourable ones. During favourable periods, clonal propagation dominates, facilitating rapid colonization and efficient exploitation of abundant resources (Hadany & Otto, 2009; Stelzer, 2012; Stelzer & Lehtonen, 2016). Conversely, the onset of unfavourable conditions (when drought imposes high mortality or when freezing becomes a major stressor) often triggers sexual reproduction, leading to the production of dormant or diapausing stages - just like the resting eggs characteristic of aphids, rotifers, water fleas, and hydras (Schröder, 2005; Simon et al., 2002; Steele et al., 2019; Tessier & Cáceres, 2004). In such seasonal habitats, these recurring environmental shifts act as reliable cues that organisms use to detect habitat deterioration and to initiate entry into a diapausing stage. Because of the tight ecological link between sexuality and diapause in these taxa, variation in reproductive investment must be interpreted within the broader framework of

environmental variability and selection for diapause (Stelzer & Lehtonen, 2016; Tessier & Cáceres, 2004).

In hydras, temperature fluctuations linked to seasonality - either increases or decreases - can signal the onset of gametogenesis, depending on the species. Warm-crisis hydras (*H. vulgaris*, *H. circumcincta*, *H. viridissima*) initiate sexual reproduction through summer when rising temperatures increase the likelihood that their aquatic habitats will desiccate (Burnett & Reisa, 1973; Kaliszewicz & Lipińska, 2013; Schuchert, 2010). In contrast, cold-crisis species such as *H. oligactis* and *H. oxycnida* respond to sustained decline in temperature that forecast the approach of winter and the possible freezing of their habitats (Burnett & Reisa, 1973; Kaliszewicz, 2015; Schuchert, 2010).

Although seasonally fluctuating environmental cues often serve as reliable indicators of environmental change, no habitat is entirely predictable. Consequently, the correlation between such cues and the actual environmental shift is imperfect. Under these conditions, organisms experience a trade-off - responding too early may result in a premature switch to sexual reproduction and the loss of potential asexual reproductive opportunities if conditions remain favourable, whereas responding too late increases the risk of mortality should the environment deteriorate unexpectedly. To balance these opposing risks, an adaptive strategy in seasonal yet unpredictable environments may involve gradually increasing sensitivity to sex-inducing cues as the season progresses.

Empirical evidence supports this idea. For example in *Daphnia* species, maternal experiences of environmental factors such as food availability and photoperiod can be transmitted to offspring, thereby promoting appropriately timed production of resting eggs (Alekseev & Lampert, 2001). Comparable patterns occur in hydras maintained under laboratory conditions, where sexual

propensity is low immediately after a strain is established from a newly hatched polyp but increases over years of continuous asexual propagation (Noda, 1982). Collectively, these cases indicate that the initiation of sexual reproduction in facultatively sexual species represents a complex, dynamic process rather than a simple response to an environmental stimulus. Nevertheless, little is currently known about how sensitivity to environmental cues shifts over the life cycle in facultatively clonal species or what mechanisms govern these changes.

Given the substantial costs of sexual reproduction, it is unsurprising that not all *H. oligactis* individuals reproduce sexually even when exposed to identical environmental triggers. In natural populations, only a fraction of individuals engage in sexual reproduction during autumn (Ribi et al., 1985; Sebestyén et al., 2018; Welch & Loomis, 1924). Laboratory studies further reveal that strains originating from the same population and sustained under uniform conditions differ in their sexual propensity (Tökölyi et al., 2017). Moreover, even within a single strain clonally derived from one polyp, individuals vary in their tendency to initiate sexual reproduction when exposed to cooling (Sebestyén et al., 2020). Autumn-collected polyps and their asexual offsprings will start their sexual reproduction faster and much more likely than strains collected in spring and their asexual clones. Furthermore, cold- adapted strains exposed to differing length of warm periods are influencing the sexual propensity, in a way that its increasing with the warm exposures length. This indicate that reciprocal cold and warm periods with a certain length are required for inducing sexual reproduction in *H. oligactis*, thus ensuring the accurate timing. Thus, heightened sensitivity to environmental deterioration may enhance fitness in habitats characterized by both predictable (seasonal) and unpredictable environmental variation (Tökölyi et al., 2021).

Based on the above, earlier study about sexual readiness gave the spark for our next questions on seasonalit, sexual reproduction and sex change. What happens when the animals survive their sexual reproduction, start to reproduce asexually, go through a sufficiently long warm period and with the next sexual season (cooling with winter) they start to reproduce again?

I.III - Part Two - Sexual modes and sex change

The majority of animal species are gonochoristic, meaning that individuals develop as one of two distinct sexual phenotypes, and male and female gametes are produced by separate organisms (Sasson & Ryan, 2017). Nevertheless, some gonochoristic taxa possess the remarkable capacity to switch their phenotypic sex during their lifetime—a phenomenon termed sex change or sequential hermaphroditism (Jarne & Auld, 2006; Pla et al., 2022; Sasson & Ryan, 2017). In such cases, an adult's sexual phenotype (e.g., possessing testes and male secondary sexual traits) is transformed into the opposite phenotype (e.g., ovaries and female secondary traits), allowing the same individual to reproduce first as one sex and later as the other. This process, observed for instance in the clownfish *Amphiprion percula* (Munday et al., 2006), has sometimes been referred to as "sex reversal." However, this term is increasingly reserved for a distinct phenomenon in which the phenotypic sex expressed during development diverges from the genetically determined sex. Such sex reversal can only occur in species with genotypic sex determination (GSD; e.g., those possessing sex chromosomes), and the affected individual reproduces exclusively as the resulting phenotypic sex throughout life, as seen in several ectothermic vertebrates (Baroiller & D'Cotta, 2016; Holleley et al., 2016; Nemesházi & Bókony, 2022). Sequential hermaphroditism, by contrast,

is independent of GSD and has been documented across numerous animal phyla, for example Cnidaria, Porifera, Annelida, Mollusca, Platyhelminthes, Arthropoda, Echinodermata, and Chordata (Vega-Frutis et al., 2014).

Sex change is widely considered an adaptive mechanism enabling individuals to maximize reproductive success under changing internal or external conditions (Munday et al., 2006). In sequential hermaphrodites, sex change is most commonly triggered by alterations in social status (e.g., body size, age) or in the social environment (e.g., population density, sex ratio), which shift the relative fitness benefits of reproducing as male *versus* female. For example, in the small reef fish *Trimma okinawae*, females transition to males when they become the biggest individual within their social group, but can revert to the female phenotype if the social structure changes again (Munday et al., 2006).

Abiotic factors may also influence sex-specific fitness and thus play a role in shaping sexual phenotype development. This principle is best understood in species with environmental sex determination (ESD), in which phenotypic sex is primarily governed by environmental conditions during early ontogeny (Bock et al., 2023; Schwanz & Georges, 2021). Comparable sex-dependent fitness effects may also underlie environmentally induced sex reversal in taxa with GSD (Geffroy & Douhard, 2019). Among abiotic influences, temperature stands out as a major determinant of both ESD and GSD-related sex reversal (Nemesházi & Bókony, 2022; Schwanz & Georges, 2021). Because temperature profoundly affects physiological performance and ecological interactions, it is reasonable to hypothesize that local thermal conditions could also influence sex-change decisions in sequential hermaphrodites (Pankhurst & Munday, 2011; Pla et al., 2021; Straková et al., 2020). However, although temperature's role in sex determination is well established in ESD species, its influence on sex switching in sequential hermaphrodites remains poorly

understood. Only a few invertebrate case studies have investigated this relationship (Carré & Carré, 2000; Parker et al., 2018; Santerre et al., 2013). To clarify the thermal mechanisms - such as environmental seasonality - underlying sex change is therefore crucial not only for advancing evolutionary and ecological theory but also for assessing the potential impacts of climate change. Climate-driven sex ratio distortions are known to elevate extinction risk in species with ESD and environmentally induced sex reversal (Mitchell & Janzen, 2010; Nemesházi et al., 2021), yet comparable risks in sequentially hermaphroditic species remain largely unquantified due to insufficient empirical evidence.

Cnidarians represent an excellent taxonomic group for exploring how ecological factors shape sex determination, as they display remarkable diversity in sexual strategies and a high degree of sexual plasticity. Within this phylum, hydrozoans—such as *Hydractinia*, *Clytia*, and *Hydra*—are among the best-studied taxa in terms of the mechanisms underlying sex determination and maintenance (Siebert & Juliano, 2017). This extensive understanding largely stems from the discovery of interstitial stem cells (ISCs), which are crucial for the formation of both germline and multiple somatic cell types, including nerve, gland, and nematocyte cells, and in some species, even epithelial cells (Nishimiya-Fujisawa & Kobayashi, 2012; Siebert & Juliano, 2017). The developmental potential of ISCs varies among hydrozoan species, ranging from multipotent to totipotent capacities.

In *Hydra*, the multipotent interstitial stem cells (MPSC) retain the ability to create new germline stem cells (GSCs) when required. Once established, however, unipotent GSCs become primarily responsible for gamete production (Nishimiya-Fujisawa & Kobayashi, 2012; Siebert & Juliano, 2017). Downstream along the differentiation pathway, GSCs produce either sperm-

restricted or egg-restricted stem cells, and the presence of these sex-specific precursors determines the expressed sexual phenotype (Nishimiya-Fujisawa & Kobayashi, 2012). In some hydrozoans, both male and female GSCs differentiate simultaneously, rendering these species *simultaneous hermaphrodites*. In others, the production of male and female gametes occurs sequentially within the same individual but not concurrently, which characterizes *sequential hermaphroditism* (Bosch & David, 1986). Conversely, certain *Hydra* species are strictly gonochoristic (e.g., *H. oligactis*), with individuals typically producing only one type of gamete throughout life. In such species, sex-specific stem cells undergo self-renewal within the polyp (the individual hydra) and are typically transferred to offspring during asexual budding, resulting in lineages with stable, inherited sex (Bosch & David, 1986; Nishimiya-Fujisawa & Kobayashi, 2012; Sugiyama & Sugimoto, 1985).

Despite this stability, rare cases of sex change have been documented in laboratory conditions (Bosch & David, 1986; Littlefield, 1986). For example, male-to-female sex change has been observed in the gonochoristic Brown hydra when strains were maintained at elevated temperatures compared with standard culture conditions (22 *vs*. 18 °C) (Littlefield, 1986). Interestingly, a recent population genetic study on the same species inferred relatively high rates of sex change in natural populations (Miklós et al., 2021), suggesting a discrepancy between field and laboratory observations, the underlying causes remain unclear.

From a proximal perspective, sex change can be viewed as the outcome of environmental influences acting on the organism's internal physiological and cellular state. During sexual reproduction, GSCs differentiate into gametes, and when the population of resident GSCs is abundant, the existing sexual phenotype is maintained—likely through inhibitory signalling from the

prevailing germline that suppresses the differentiation of opposite-type GSCs (Dranow et al., 2013). Conversely, depletion of the resident GSC pool may enable the emergence of GSCs of the opposite sex, leading to the development of a different sexual phenotype. Experimental evidence supports this hypothesis too: in *H. vulgaris*, polyps regenerated from tissue fragments devoid of GSCs (such as the basal foot region) frequently develop the opposite sexual phenotype compared to their parent (Nishimiya-Fujisawa & Kobayashi, 2012, 2018).

I.IV - Part Two - Study species – *Hydra oligactis*

H. oligactis is a small (1-2 cm in lenght) gonochoristic, facultatively sexual cnidarian species living in freshwaters around the world (Fig. 1.). In strongly seasonal environments, reproduction occurs predominantly through budding (asexually) for most of the year. With the onset of late autumn and the associated decline in temperature, however, hydra polyps switch to sexual reproduction. Exposure to cold induces the development of gonads: male individuals form testes, while female individuals develop ovaries distributed along their body column. These sexual structures are externally visible and individual gonads can be counted using a stereomicroscope. Notably, males commence gonad development approximately two weeks earlier than females, and sperm formation and release continue for several weeks, whereas females produce fertilizable eggs only for a shorter interval. This seasonal reproductive phase is the only stage in their life cycle during which males and females can be morphologically distinguished (Burnett & Reisa, 1973; Quinn et al., 2012; Schuchert, 2010; Tökölyi et al., 2021).

Sexual reproduction results in the production of gametes, with sperm released into the surrounding water to fertilize eggs that remain attached to females. Fertilization gives rise to resting eggs capable of enduring adverse conditions, such as freezing. Following sexual reproduction, most polyps exhibit a decline in somatic function in the following months, resembling senescence-like deterioration (Tökölyi et al., 2017; Yoshida et al., 2006). In *H. oligactis*, this post-reproductive decline involves depletion of interstitial stem cells (ISCs), impaired prey capture, reduced tactile responsiveness, smaller body size, and elevated mortality (Martínez & Bridge, 2012; Sebestyén et al., 2018; Tökölyi et al., 2017). Nevertheless, if individuals are able to restore their feeding capacity, they may recover and possibly engage in another cycle of sexual reproduction during the next breeding season.

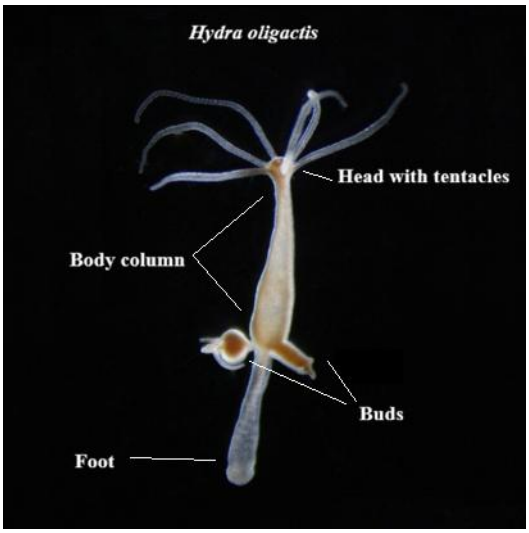

Figure 1. Hydra oligactis, *an asexual polyp with two buds. (Picture by Hanna Révész)*

II. Objectives and results

In this section of the thesis, I briefly present the aims, methods and major results of the two studies that form the basis of this dissertation. The description of the detailed methodology and results can be found in the original articles in the section IX. and X.

II.I – Part One - Study I

Resource availability modulates the effect of body size on reproductive development. (Gergely, R., & Tökölyi, J. 2023)

Study I - Objectives

Our primary objective was to investigate, in both males and females, how body size influences key life-history traits, including development time, fecundity, and survival. Specifically, we examined the energetic role of body size in each sex. Does larger body size function as an *"energy reserve"*, supplying additional resources for processes such as reproduction? Alternatively, does it represent an *"energetic demand"*, imposing metabolic costs that may reduce physiological performance? Furthermore, we considered whether individuals of smaller body size - characterized by more limited intrinsic energy reserves - can compensate for this constraint through access to additional external resources. We also assessed the reproductive consequences of small body size under conditions of food limitation. Do small, starved individuals exhibit delayed reproduction, reduced reproductive output, or complete reproductive suppression? Finally, we evaluated whether these patterns differ between males and females.

Study I - Methods

To decipher which energy/resource focused hypothesis stands its ground, we experimentally manipulated body size in male and female *H. oligactis* and its resource (food) availability for two weeks in parallel to inducing sexual reproduction, thus aiming to distinguish direct effects of sex-dependent body size from those attributable to resource conditions on sexual development time, fecundity, and survival.

We used 3 female and 3 male strains (X11/14; T3/1; M26/9/10 and C2/7; T3/2; M83/4) (a strain is a clonal line obtained from a single individual through asexual propagation; a female or a male strain is a clonal line that has the capacity to produce female or male individuals, respectively, in response to experimental triggering of gametogenesis) to decipher sex-dependent body size effects.

For the body size manipulation, we applied a tissue-grafting procedure to generate enlarged, control, and reduced individuals using randomly selected animals from the six strains. Two polyps from the same strain were chosen and paired at random, after which a ring-shaped section was excised from the body column of each individual. In the "enlarged - reduced" pairing, the excised rings differed in size and were then exchanged among the two animals: the individual designated to be enlarged got a larger ring in place of its smaller original ring, whereas the reduced individual received a smaller ring in exchange for the larger one removed from its body. This procedure yielded polyps with increased and decreased body size, respectively. In the "control - control" pairs, rings of nearly identical size were swapped between partners to maintain unaltered body size. The head, grafted ring, and foot regions were reassembled in the appropriate order and mounted on a glass microcapillary needle until the tissues adhered. The polyps were then removed from the needle

and allowed to heal overnight before inducing sexual reproduction with cooling in the next day.

To assess how food availability interacts with the experimentally altered body sizes, we established three feeding treatments using the size-manipulated individuals. Food manipulation began directly after cooling, at the onset of gonadogenesis. The first group was deprived of food for two weeks (low food, 0x). The second group was provided with food twice per week for two weeks (normal or medium food, 2x), and the third group obtained food four times, weekly for the same period (high food, 4x). All animals were fed individually - thus excluding any possibility of food competition - using 20 µl of fresh *Artemia nauplii* suspension. After the two-week feeding treatment, all groups were subsequently maintained on a twice-weekly feeding schedule as far as the end of the experiment, which concluded 22 weeks after cooling (Fig. 2.).

We aimed to have 16 animals in each strain and group (e.g., strain: C2/7/food treatment: starved/size manipulation: reduced). For the experiment, 864 individuals (12 sets/strain = 144 polyps/strain) were used in total, and 820 remained after failed, sex-changed, and hermaphrodite animals were taken out of the analysis. The exact number of polyps in each strain and experimental groups are shown in Table 1.

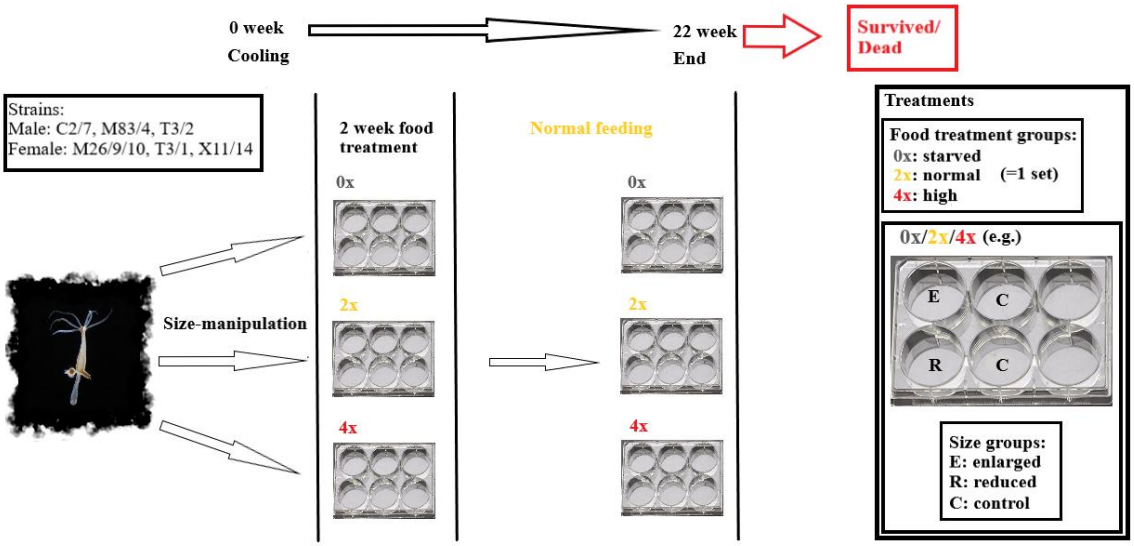

Figure 2. From the six strains (C2/7, M83/4, T3/2, M26/9/10, T3/1 and X11/14), individuals were randomly selected and paired, following the size manipulation treatment (E- enlarged, R- reduced, C- control), polyps were assigned to one of the three food treatment groups (0×- starved, 2×- normal, 4×- high). In the first 2 weeks, individuals were fed according to their feeding group, after 2 weeks of food treatment, all polyps were fed 2 times per week until the end of the study. In the end, the animals' final status was defined as survived or dead.

Male strains									
Strain	C2/7 N=142			M83/4 N=131			T3/2 N=140		
Food treatment	0x N=48	2x N=48	4x N=46	0x N=43	2x N=16	4x N=42	0x N=48	2x N=46	4x N=46
Size manipulation	E=16 R=16 C=16	E=16 R=16 C=16	E=14 R=16 C=16	E=15 R=13 C=15	E=15 R=16 C=15	E=14 R=14 C=14	E=16 R=16 C=16	E=15 R=15 C=16	E=14 R=16 C=16
Female strains									
Strain	M26/9/10 N=139			T3/1 N=134			X11/14 N=134		
Food treatment	0x N=47	2x N=46	4x N=46	0x N=45	2x N=47	4x N=42	0x N=47	2x N=43	4x N=44
Size manipulation	E=16 R=15 C=16	E=15 R=15 C=16	E=15 R=15 C=16	E=15 R=16 C=14	E=16 R=15 C=16	E=14 R=14 C=14	E=15 R=16 C=16	E=16 R=13 C=14	E=15 R=14 C=15

Table 1. Exact sample size in each strain (divided into male (C2/7, M83/, T3/2) and female M26/9/10, T3/1, X11/14 strains) and experimental group (divided into food treatment and size manipulation). In the food treatment "0x" is starved, "2x" is normally and "4x" is a highly fed group, next to it, the number of individuals in each group. In the size manipulation experimental group, "E" is enlarged, "R" is reduced and "C" is control animals' number.

Through the 22 weeks we collected data on the polyps four times per week. We recorded the timing of sexual reproduction - defined as the appearance of the first mature egg in females and the first mature testis in males - along with gonad number (total egg count in females and the maximum number of testes in males) using an Euromex StereoBlue binocular stereomicroscope.

Photographs were taken at the end of the size-manipulation procedure but prior to cooling to quantify the magnitude of the induced size change, and again following the two-week food treatment to assess its impact (Fig. 3.). Animals were imaged under a stereomicroscope using a CMEX microscope camera. Each individual was photographed on 1-mm grid paper, and body size was quantified with ImageJ (Schneider et al., 2012) by calculating the area of the

body column. Body size was expressed as the measured polyp area in pixels divided by the squared pixel length corresponding to one millimetre.

Finally, the animals' condition at the end of the experiment (22 weeks after cooling) was recorded. Individuals were classified as regenerated/survived if they appeared healthy - characterized by intact tentacles, feeding activity, tactile responsiveness, and an orange coloration - and/or if they produced bud after completing sexual reproduction, or if they remained alive without engaging in sexual reproduction (i.e., remained asexual) until the final day. Individuals were classified as dead if, by the end of the experiment, they had disintegrated or consisted solely of necrotic tissue.

Study I - Results

Size-manipulation

The size-manipulation procedure effectively altered body size in the experimental animals, producing enlarged and reduced individuals relative to controls (Gaussian GLMM, $\chi^2 = 949.790$, $p < 0.001$). Reduced animals were roughly 50% smaller than control polyps, whereas enlarged animals exhibited an approximate 50% increase in size (Fig. 3.).

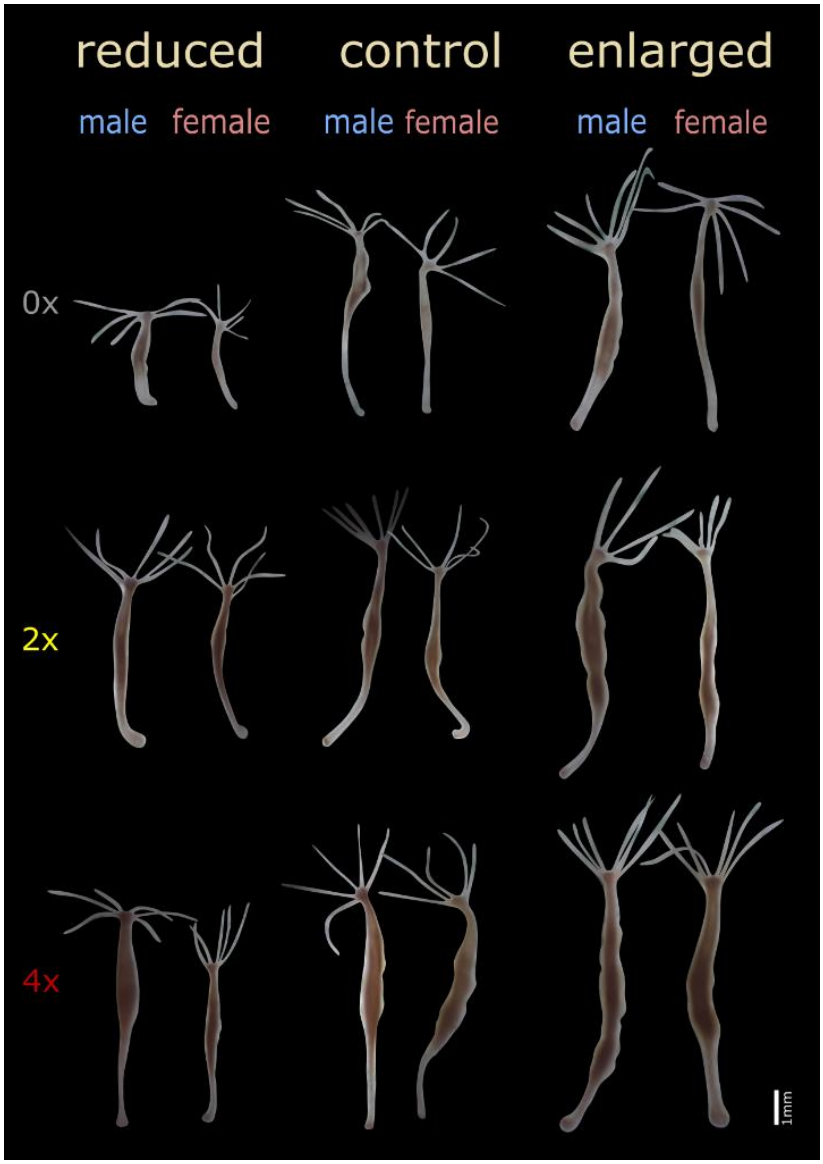

Figure 3. Male and female Hydra *polyps after two weeks of food availability treatment in the reduced, control and enlarged size-manipulated experimental groups. First row is the starved (0x), second row is the group which was fed two times per week (2x) and the last row is the highly fed (4x) group. In the three columns (reduced, control, enlarged) the first animal depicted is always male and the second one is always female.*

Sexual development time

In female strains, the time required to produce the first gonads decreased with increasing body size as well as with higher resource availability. However, the influence of body size was strongly contingent on food levels during gonad development: enlarged individuals receiving the 4x feeding regime initiated sexual reproduction earliest, whereas reduced individuals showed pronounced delays, particularly when food availability was also low (significant interaction between body size and food availability on sexual development time; Gaussian GLMM, $\chi^2 = 21.154$, $p < .001$). Across the three strains, the first eggs were generally produced 20-28 days after cooling, but reduced and starved individuals required, on average, 29-43 days to produce their first eggs, with strain T3/1 exhibiting the greatest delay (Fig. 4.).

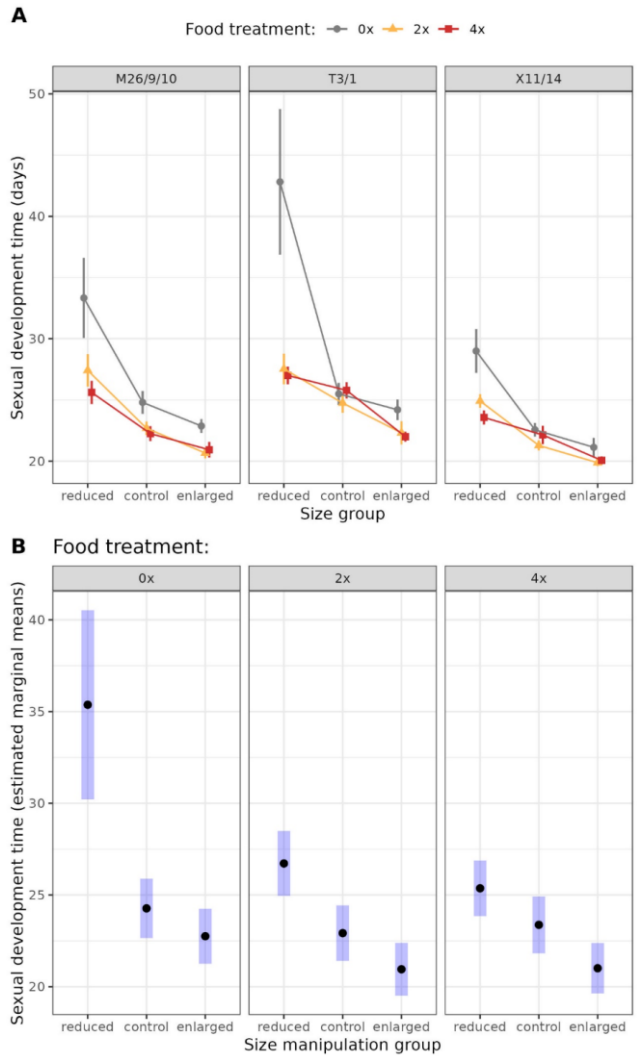

Figure 4. Female hydra polyps with experimentally reduced body size have a longer sexual development time than individuals with an enlarged or control size. The effect of body size depends dramatically on resource availability during gonad development, such that starved small polyps produce eggs latest. (A) raw data for the three female strains (M26/9/10, T3/1, X11/14) and (B) estimated marginal means for parameter estimates from a Gaussian GLMM containing size manipulation, food manipulation and their interaction as fixed effects, as well as strain ID and size manipulation pair ID as random effects.

In male animals, enlarged individuals and those subjected to starvation initiated sexual reproduction the earliest, although the effect of size manipulation was amplified in polyps that received higher food levels (significant interaction between body size and food availability on sexual development time; Gaussian GLMM, $\chi^2 = 13.051$, $p = 0.011$). The three strains differed in the timing of sexual development: strain C2/7 began reproducing the earliest (approximately 10-14 days after the cold stimulus), whereas strain T3/2 exhibited the latest onset (approximately 13-18 days after cooling), with strain M83/4 in the middle. Despite these strain-specific differences, reduced animals in all three strains advanced sexual reproduction to a greater extent when they were also starved (Fig. 5.).

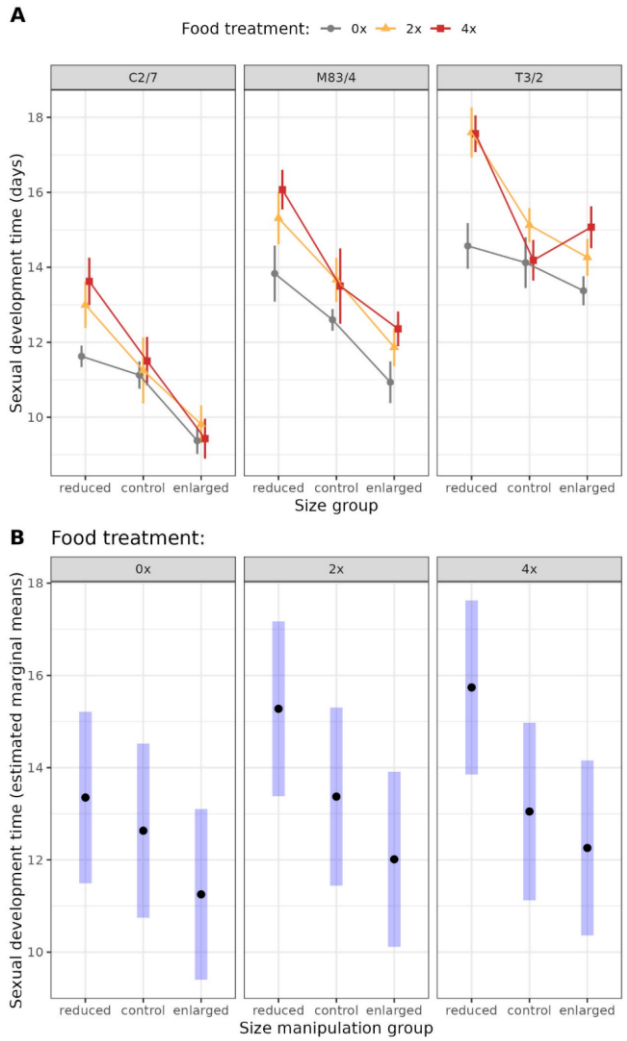

Figure 5. Male hydra polyps with experimentally reduced body size have a longer sexual development time than individuals with an enlarged or control size. However, the effect of body size depends on resource availability during gonad development, such that the effect of size manipulation is higher in individuals that received more food. (A) raw data for the three male strains (C2/7, M83/4, T3/2) and (B) estimated marginal means for parameter estimates from a Gaussian GLMM containing size manipulation, food manipulation and their interaction as fixed effects, as well as strain ID and size manipulation pair ID as random effects.

Fecundity

In females, enlarged and well-fed polyps produced the highest numbers of gonads. No significant interaction was detected between body size and food treatments (Negative Binomial GLMM, $\chi^2 = 4.771$, $p = .312$); however, both factors independently exerted strong effects on egg production (size treatment: $\chi^2 = 57.855$, $p < .001$; food treatment: $\chi^2 = 110.260$, $p < .001$). The three strains differed in their overall levels of egg production, ranging from approximately 2-15 eggs per animal in strain M26/9/10, 5-20 in T3/1, and 6-32 in X11/14, but their responses to the experimental treatments were qualitatively alike (Fig. 6.).

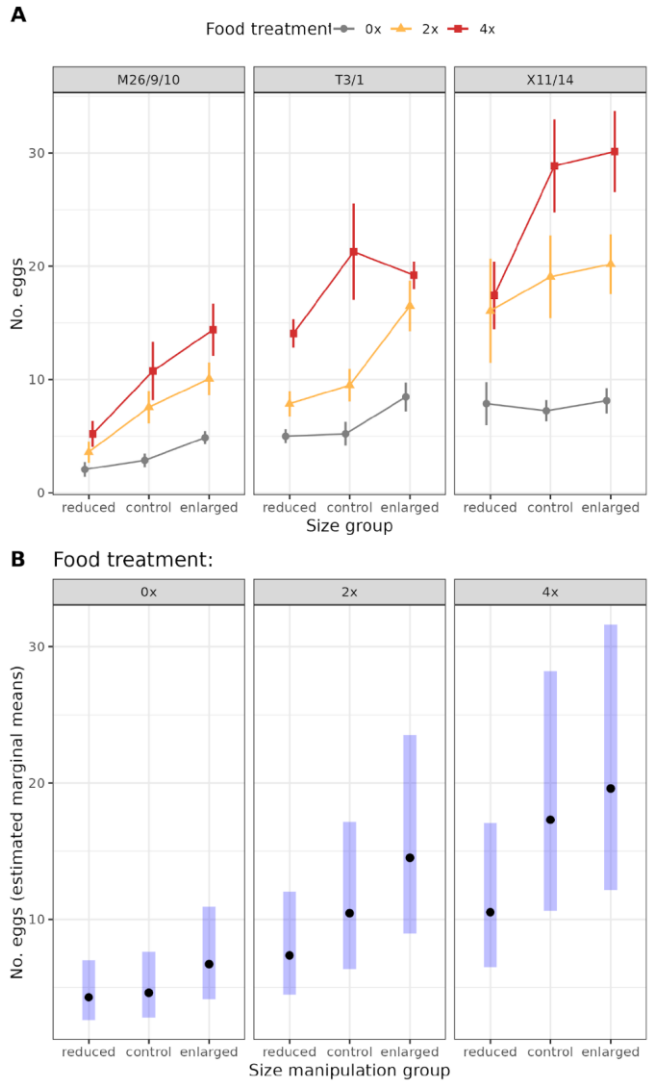

Figure 6. Female hydra polyps with enlarged body size and receiving more food produce a higher number of eggs, but there is no interaction between food and size treatments. (A) raw data for the three female strains (M26/9/10, T3/1, X11/14) and (B) estimated marginal means for parameter estimates from a Gaussian GLMM containing size manipulation, food manipulation and their interaction as fixed effects, as well as strain ID and size manipulation pair ID as random effects.

In males, enlarged and fed individuals likewise produced the greatest number of gonads. As in females, the interaction between size and food treatments was not statistically significant (Gaussian GLMM, $\chi^2 = 8.334$, $p = .080$). Nevertheless, both body size and food availability had strong independent effects on the number of testes produced (size treatment: $\chi^2 = 242.310$, $p < .001$; food treatment: $\chi^2 = 243.370$, $p < .001$). Although the three strains differed in overall testes production - averaging 4-16 testes per individual in C2/7, 5-18 in M83/4, and 2-26 in T3/2 depending on manipulation - the direction and magnitude of treatment responses were consistent across strains (Fig. 7.).

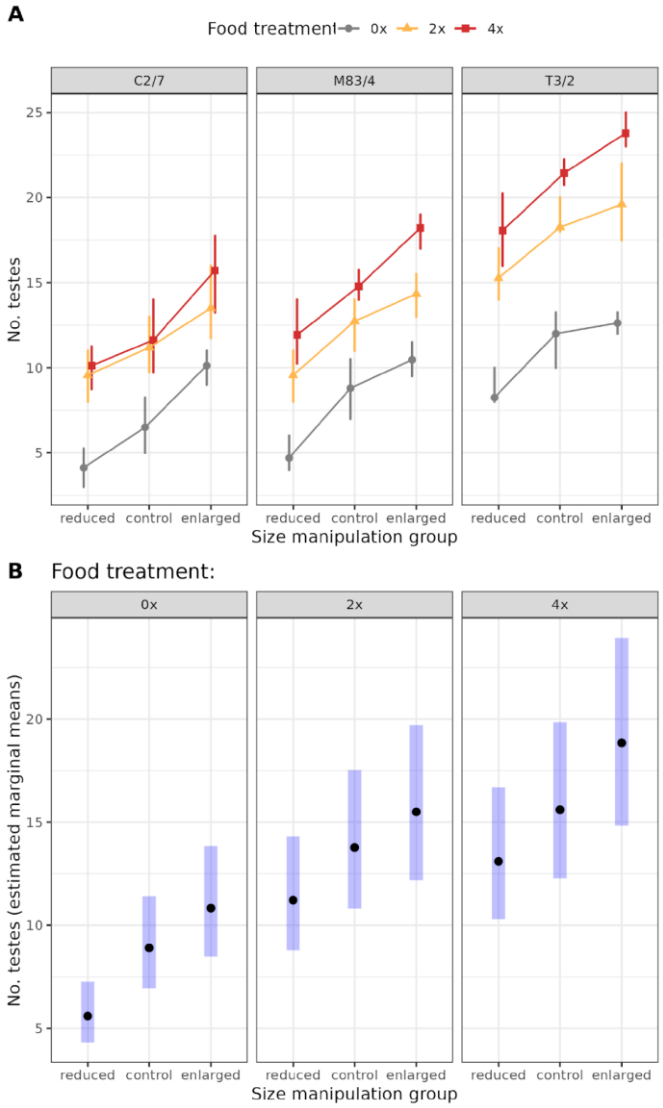

Figure 7. Male hydra polyps with enlarged body size and those receiving more food produce a higher number of testes, but there is no interaction between food and size treatments. (A) raw data for the three male strains (C2/7, M83/4, T3/2) and (B) estimated marginal means for parameter estimates from a Gaussian GLMM containing size manipulation, food manipulation and their interaction as fixed effects, as well as strain ID and size manipulation pair ID as random effects.

Survival

No significant interaction between size manipulation and food availability was detected for survival probability (Binomial GLMM, $\chi^2 = 7.532$, $p = .110$). However, both experimental factors independently influenced survival, with the highest survival rates observed in reduced and starved individuals (Binomial GLMMs: size manipulation $\chi^2 = 64.364$, $p < .001$; food treatment $\chi^2 = 44.219$, $p < .001$). Overall survival varied markedly among the six strains (female strains: M26/9/10 = 64%, T3/1 = 8.2%, X11/14 = 8.9%; male strains: C2/7 = 12.6%, M83/4 = 63.3%, T3/2 = 100%). Notably, all individuals in strain T3/2 survived irrespective of body size or food treatment. In contrast, the female strain T3/1 exhibited the largest post-reproductive mortality, with only 11 out of 134 individuals (8.2%) surviving to the end of the experiment, all of which were reduced polyps (Fig. 8.).

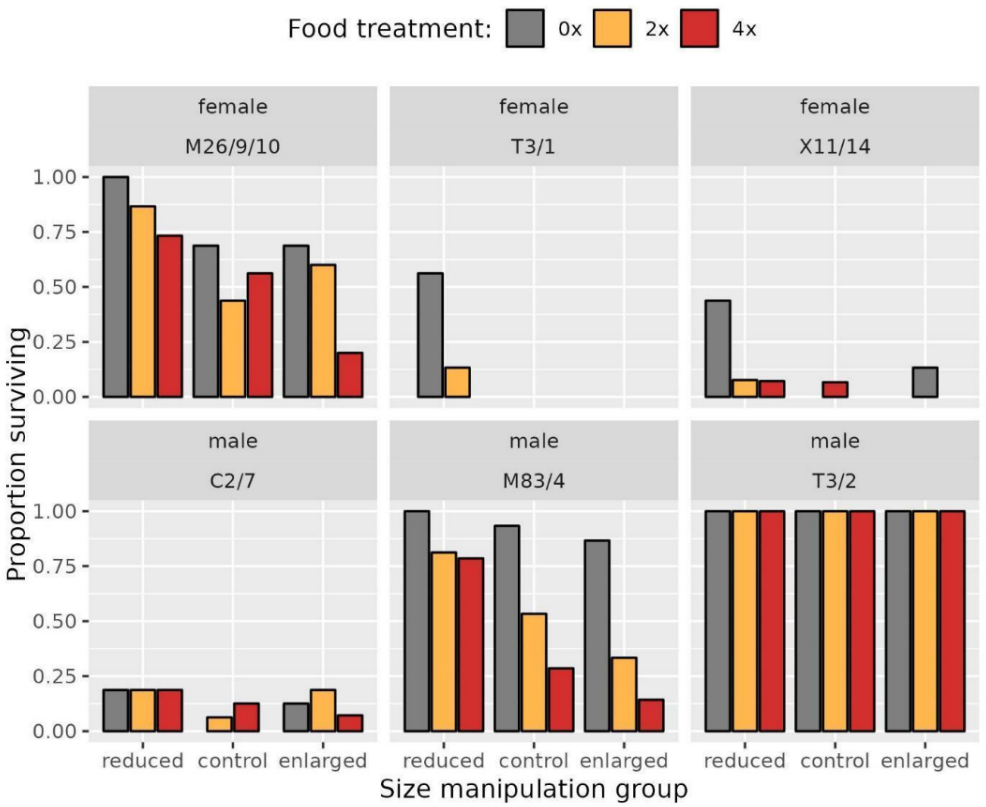

Figure 8. Both size manipulation and resource availability had a significant effect on survival, such as the reduced and starved animals' survival rate was the highest, but there was no interaction between the two treatments in affecting survival rate. Moreover, there are exceptions in male strains C2/7, T3/2 and female strain X11/14, where the highest survival rate is not in the starved and reduced groups. Furthermore, male polyps (C2/7, M83/4, T3/2) had higher survival rate, than the females (M26/9/10, T3/1, X11/14).

II.II – Part Two - Study II

Seasonal environmental change and sex change in a cnidarian. (Gergely, R., et al. 2025)

Study II - Objectives

Temperature fluctuation study

Based on our prior observations that sexual reproduction does not occur under constant cold conditions, we sought to identify the environmental cues responsible for its induction. Specifically, we investigated whether a change in temperature, rather than low temperature alone, serves as the critical trigger. Additionally, we evaluated whether the duration of warm periods influences the initiation and extent of sexual reproduction, and, if so, in what manner. This study's observations inspired us to examine further the influence of temperature fluctuations, because among others we observed sex change too.

Sex change study

In this experiment, following up on the temperature fluctuation study, we aimed to examine the role of seasonal environmental variation in inducing plasticity in sex determination in *H. oligactis*, specifically by exploring the role of temperature fluctuations in driving sequential hermaphroditism. We hypothesized that seasonal changes in temperature and photoperiod characteristic of natural habitats may be at least partly responsible for the elevated frequency of sex change observed in field populations. To test this hypothesis, we experimentally simulated seasonal fluctuations in temperature and photoperiod under laboratory conditions and assessed the sex of clonal lineages exposed to these environmental regimes.

Study II - Methods

Temperature fluctuation study

Accordingly, we investigated the sex ratio of *Hydra* polyps under fluctuating temperature regimes in both male and female strains (C2/7 and X11/14). For each sex, four control groups were established. Three warm control groups were maintained at 18 °C ("warm strain", propagated from 1 polyp on 18 °C) and exposed to warm conditions for 1, 4, or 8 weeks prior to cooling (18 °C "warm strain" - 18 °C for 1/4/8 weeks – 7 °C for 8 weeks). In addition, one cold control group was maintained continuously at 7 °C without any prior exposure to 18 °C ("cold strain", propagated from 1 polyp on 7 °C). Furthermore, three experimental - fluctuating temperature -groups were set up for each sex. These groups were exposed to 1, 4, or 8 weeks of warm conditions between two cold periods (7 °C "cold strain" – 18 °C for 1/4/8 weeks –7 °C for 8 weeks).

The experimental groups and sample sizes as follows: Control groups: Three warm (18 °C) controls, which received 1 (N=170), 4 (N=169) and 8 (N=174) weeks of warm treatment before cooling. One cold (7 °C) control (N=113). Thus, 4-4 groups of males and females were formed. Fluctuating temperature groups: Based on the same scheme, 3-3 groups were formed to study fluctuating temperature (7 - 18 - 7 °C). 1 week (N=94), 4 weeks (N=95) and 8 weeks (N=95) of warm treatment. Total 910 animals.

Polyps were maintained individually in six-well cell culture plates, each well containing 5 ml of standard hydra medium. Individuals were fed separately with 20 µl of fresh *Artemia nauplii* suspension four times per week using an automatic pipette. Approximately two hours after feeding, each animal was transferred to a new six-well plate containing fresh hydra medium. Throughout the experiment, we recorded data on the time to sexual development and, at the

conclusion of the study (8 weeks after treatment), the final status of each individual (asexual, sexual, or dead) (Gergely, R. 2020).

Sex change study

To assess sex ratios and the probability of sex change in hydra following a complete seasonal cycle, we used individuals that had undergone sexual reproduction and survived the subsequent post-reproductive senescence phase (Fig. 9.B). The polyps were maintained under the same experimental conditions as in the temperature fluctuation study.

We conducted the experiments using three male strains (C2/7, M83/4, T3/2) and three female strains (X11/14, M26/9/10, T3/1). A strain represents a clonal lineage derived from a single individual through asexual propagation; male and female strains are defined as clonal lines capable of producing male or female individuals, respectively, in response to experimental induction of gametogenesis. Initially, all animals were maintained at 18 °C under a 12:12 h light-dark cycle to simulate warm spring/summer conditions. Subsequently, randomly selected individuals (approximately 36 polyps per strain) were moved to a cooled incubator set to 8 °C with an 8:16 h light-dark cycle to mimic winter conditions and induce sexual reproduction *via* cold exposure.

After the first round of sexual reproduction, surviving individuals were collected to establish new lines, each initiated from a single regenerated polyp and its descendants. This procedure was carried out for all six strains maintained at 8 °C, yielding approximately 24 new lines per strain. Through asexual budding, each line was expanded from one individual to six polyps. Once all wells of a six-well plate were occupied, the animals were kept for an additional two weeks at 8 °C, counted from the birth of the youngest polyp.

Thereafter, plates were transferred first to 12 °C (8:16 h light-dark cycle) and subsequently to 18 °C to re-establish summer conditions. This intermediate temperature step was included to minimize stress associated with abrupt warming. To simulate a second summer period, animals were maintained at 18 °C for three weeks, after which they were returned to 8 °C to induce sexual reproduction again. At 8 °C, sex was determined once gonads redeveloped. Individuals were classified as sex-changed if they produced gonads atypical for their original strain - namely, testes in a female strain or ovaries in a male strain (Fig. 9.C).

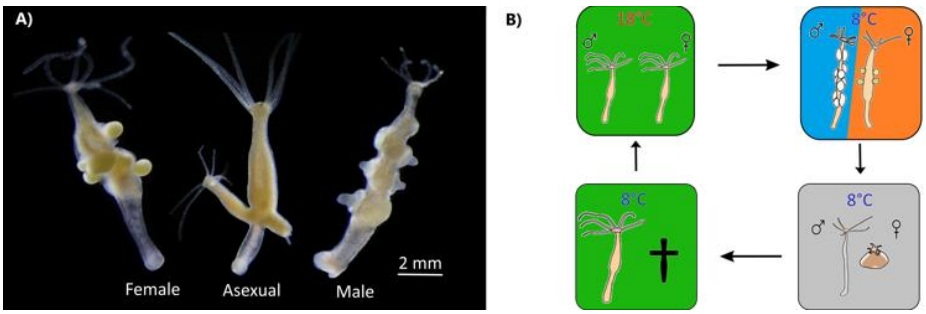

Figure 9. A)B) Brown hydra reproductive phenotypes (A), reproductive cycle (B)

In panel A) *the three polyps are, from left to right, a female with ovaries, an asexual individual with two buds, and a male with testes. In panel* B) *seasonal reproduction in the laboratory is illustrated, starting with asexual reproduction under summer conditions (with asexual male and female polyp) (top left) through sexual reproduction under winter conditions (with a male polyp with testes and a female polyp with eggs along their body column) (top right) and post-reproductive senescence (with senescent male and female polyps) (bottom right) to either death ("dagger" symbol) or regeneration (asexual polyp) (bottom left).*

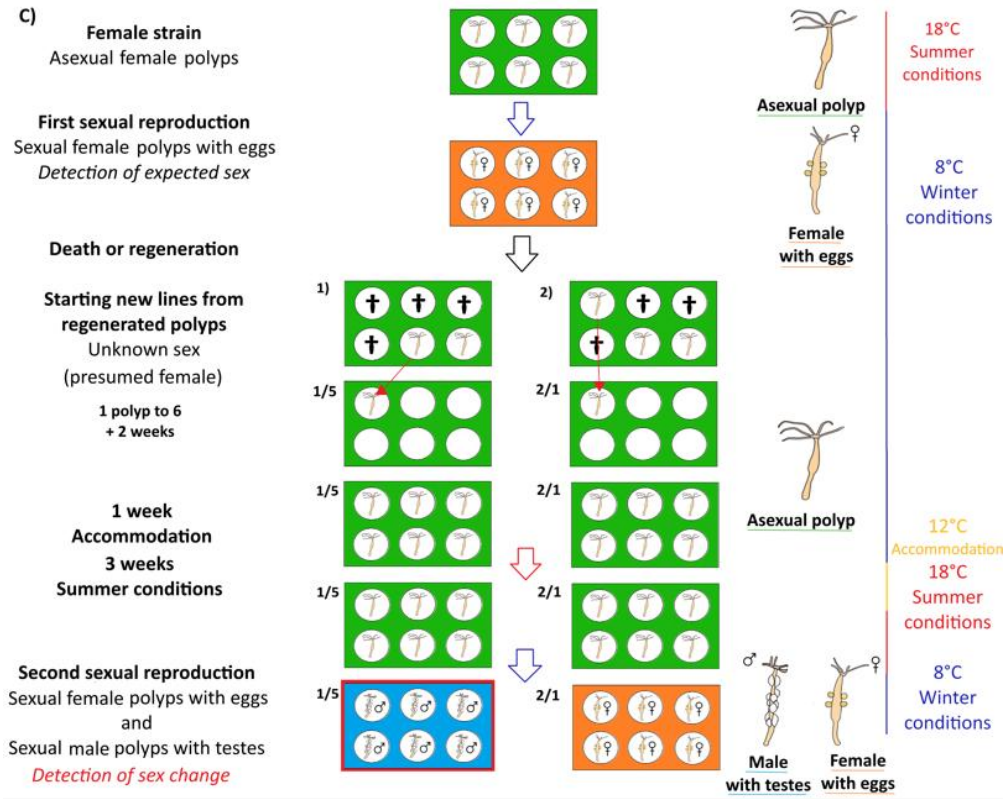

Figure 9.C), Flowchart of the experimental protocol.

The panel depicts our experimental design through an example of a female strain where in one line there is a sex change to male phenotype (line 1/5 marked with red box) while in the other line the sex was stable (line 2/1).

Study II - Results

Temperature fluctuation study

In the fluctuating temperature experiment, three treatments with differing durations of warm exposure (7–18–7 °C: 1, 4, or 8 weeks at 18 °C) and four control treatments (18 °C for 1, 4, or 8 weeks prior to cooling, and constant 7 °C) were evaluated.

In the male strain, polyps transferred from warm to cold conditions initiated sexual reproduction. In the 4 and 8 week warm exposure control groups, all individuals began sexual reproduction, whereas in the 1 week group nearly all individuals reproduced, with only a few exceptions. In the cold control group, individuals remained asexual, except for a small number that initiated reproduction at a markedly later time point.

In the fluctuating temperature treatments, a 1 week warm period did not induce sexual reproduction, whereas the 4 and 8 week treatments resulted in a mixture of sexual and asexual individuals. A statistically significant difference was detected among these groups (Fig. 10).

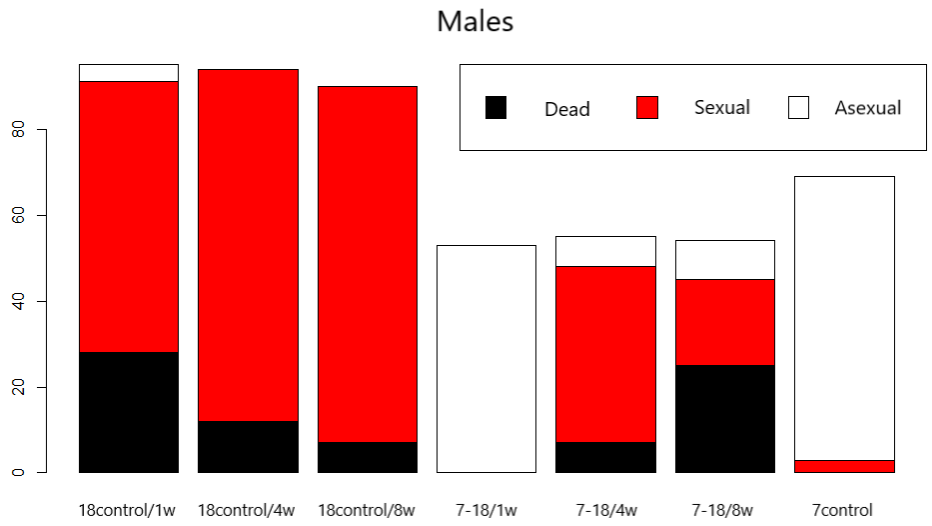

Figure 10. Sex ratio in the male control (18control/1w: 1 week 18 °C before 7 °C; 18control/4w: 4 week 18 °C before 7 °C; 18control/8w: 8 week 18 °C before 7 °C; 7control: constant 7 °C) and experimental groups (7-18/1w: 7 °C-18°C for 1 week-7 °C; 7-18/4w: 7 °C-18 °C for 4 week-7 °C; 7-18/8w: 7 °C-18 °C for 8 week-7 °C).

The duration of the warm period influenced the timing of sexual development: longer warm exposure resulted in earlier onset of testis development following the transition to cold conditions. Although no significant difference in the timing of sexual initiation was observed between the 4 and 8 week control treatments, the onset of the sexual phase occurred later in the fluctuating temperature treatments compared to the corresponding control groups (Fig. 11).

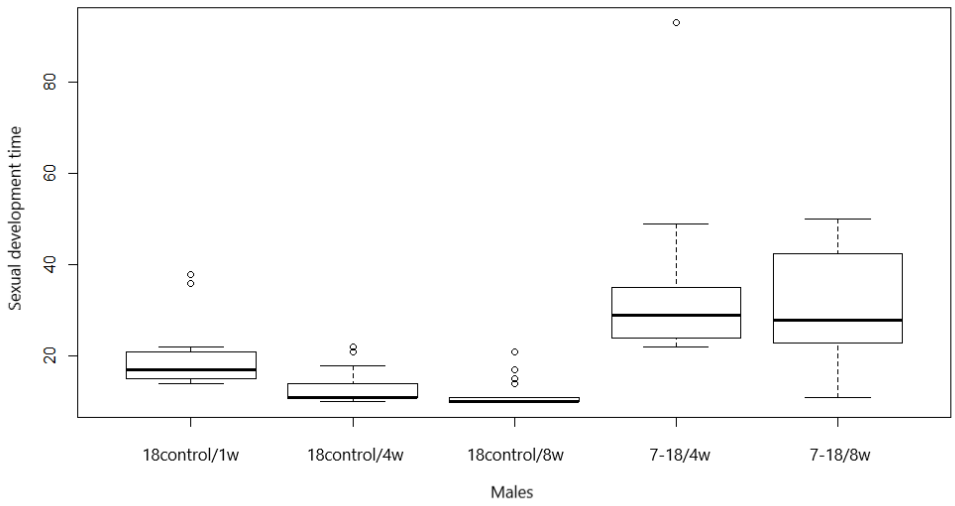

Figure 11. Sexual development time in males in control (18control/1w: 1 week 18 °C before 7 °C; 18control/4w: 4 week 18 °C before 7 °C; 18control/8w: 8 week 18 °C before 7 °C) and experimental groups (7-18/4w: 7 °C-18 °C for 4 week-7 °C; 7-18/8w: 7 °C-18 °C for 8 week-7 °C).

In the female strain egg production was observed in a high proportion in the warm control groups and in the cold control there was no sexual reproduction. In the fluctuating experimental groups, where the warm period lasted for one week, ovaries were formed in a quarter of the female individuals. After four and eight weeks of treatment, this form of reproduction was observed in a similar proportion to males. And it can be observed that the longer the female animals received heat treatment, the more of them reproduced sexually. To exclude chance, we also calculated a P-value using Fisher's exact test and got a significant difference.

(Fig.12.).

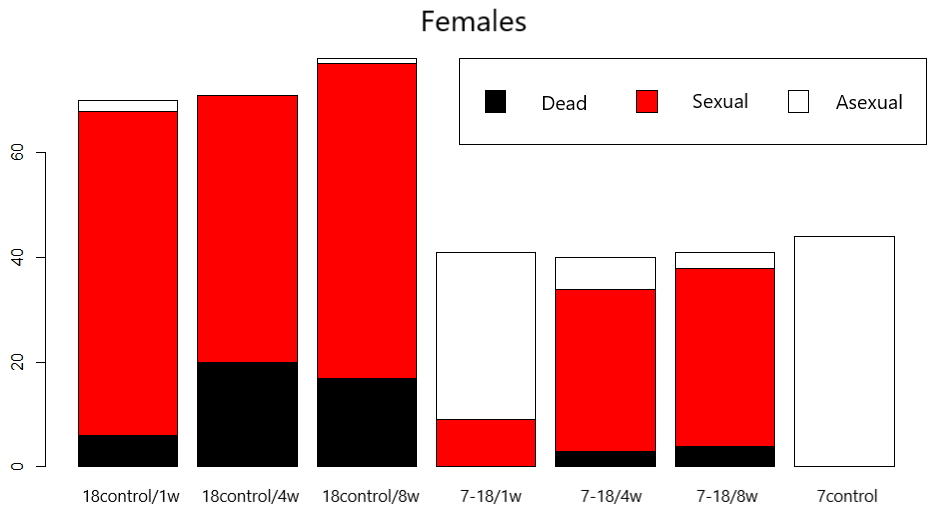

Figure 12. Sex ratio in the female control (18control/1w: 1 week 18°C before 7°C; 18control/4w: 4 week 18°C before 7°C; 18control/8w: 8 week 18°C before 7°C; 7control: constant 7°C) and experimental groups (7-18/1w: 7°C-18°C for 1 week-7°C; 7-18/4w: 7°C-18°C for 4 week-7°C; 7-18/8w: 7°C-18°C for 8 week-7°C).

Here too, as in the male groups, the length of the warm period affected the onset of sexual reproduction, and a significant difference was observed between the two sexes. Ovaries were observed much later in the female animals compared to the testes in males. The temperature fluctuating treatment did not reveal a significant difference between the 4 week and 8 week groups regarding the onset of sexual reproduction. The low sexual reproduction rate following the 1 week treatment started egg production significantly later than the other groups (Fig.13.) (Gergely, R. 2020).

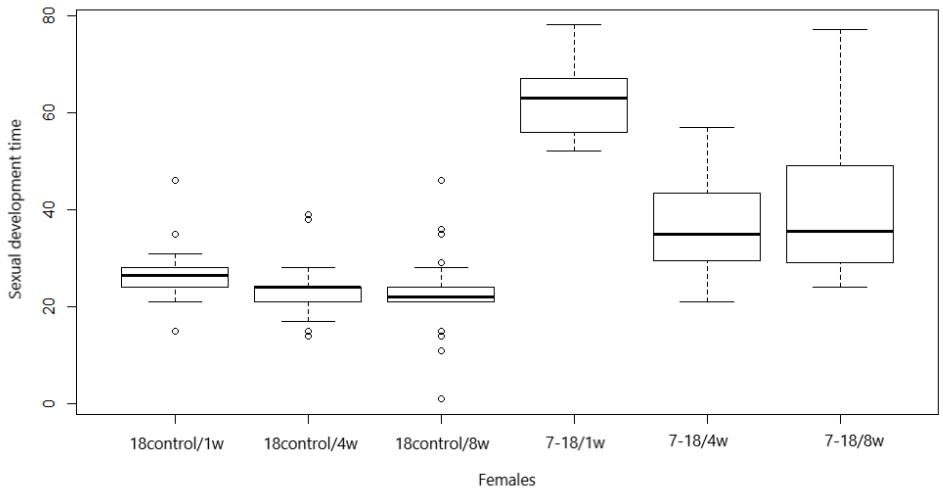

Figure 13. Sexual development time in females in control (18control/1w: 1 week 18 °C before 7 °C; 18control/4w: 4 week 18 °C before 7 °C; 18control/8w: 8 week 18 °C before 7 °C) and experimental groups (7-18/1w: 7 °C-18 °C for 1 week-7 °C; 7-18/4w: 7 °C-18 °C for 4 week-7 °C; 7-18/8w: 7 °C-18 °C for 8 week-7 °C).

Sex change study

During the second reproductive cycle, sex change was observed exclusively in strains that were initially female. Among these strains, the proportion of individuals transitioning to a male phenotype ranged from 36.5% to 60%, whereas the remaining animals either retained the female phenotype or remained asexual. In contrast, in strains that were originally male, 71–94.2% of polyps maintained the male phenotype, with the remaining individuals persisting in an asexual state (Fig. 14.).

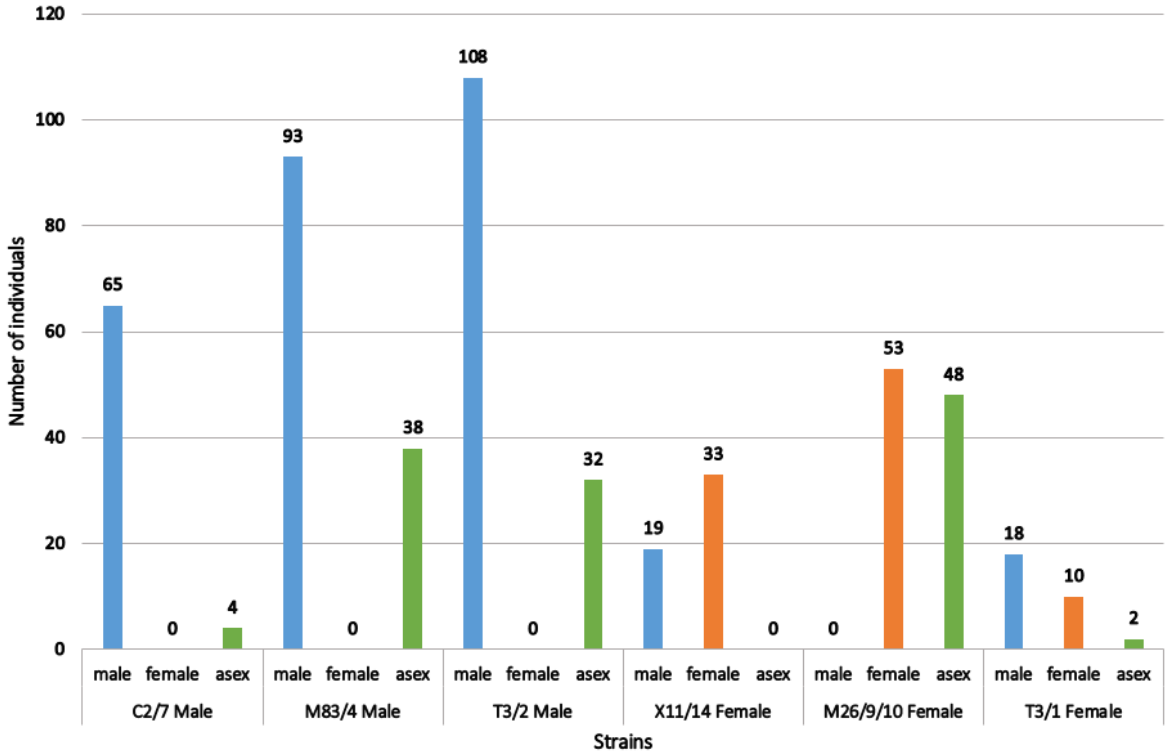

Figure 14. Number of male and sex-changed male (blue), female and sex-changed female (orange), and asexual (green) individuals in the second reproductive cycle of the experiment in the 3 male and 3 female strains.

III. General discussion and conclusions

Study I.

Body size is expected to exert complex influences on an organism's energetic balance, with large size potentially functioning either as an energy reserve or as an additional energetic cost that must be sustained (Blanckenhorn, 2000; Blanckenhorn et al., 1995). In the first study, we examined these alternative energy-balance mechanisms by experimentally manipulating male and female body size in freshwater *Hydra*, thereby generating artificial size variation that enabled us to assess the direct effects of body size in a sex-dependent way, independently of prior environmental conditions and life-history trajectories. Size manipulation was combined with three levels of resource availability manipulation in both sexes during the period of sexual development. Under both hypotheses, we predicted interactive effects of body size and resource availability on reproductive traits and/or survival.

Specifically, under the "energy reserve" hypothesis, we expected larger individuals to be less sensitive to reduced resource availability, as increased body size could provide greater energy reserves that may be allotted to multiple life-history functions even under limiting conditions (Lindstedt & Boyce, 1985; Roff, 1993; Stearns S.C., 1992). In contrast, under the "energy demand" hypothesis, we anticipated that larger individuals would be more strongly influenced by variation in resource supply, because their greater maintenance requirements would impose higher energetic costs that could be met under high, but not low, resource conditions (Blanckenhorn, 2000, 2005; Reim et al., 2006). Although we treated these hypotheses as conceptually distinct, it is important to note that the energetic costs and benefits associated with large body size may represent complementary aspects of a single underlying energy-

balance mechanism, within which resource availability contributes more transient inputs. Consequently, a strict separation of body-size and resource-availability effects may not be fully achievable.

With this caveat in mind, our results indicate that sexual development time was more strongly influenced by food availability in smaller than in larger animals, consistent with an "energy reserve" effect of body size. By contrast, we detected no food-dependent modulation of body size effects on fecundity or survival.

Sexual development time

Larger females initiated gonad production earlier, in agreement with our previous findings (Ngo et al., 2021). The amplifying effect of increased body size was strongly contingent on food availability: enlarged, well-fed polyps produced eggs earliest, whereas reduced and starved females exhibited the greatest delay, requiring approximately one additional week to produce their first gonads. Larger males (both enlarged and control individuals) also commenced sexual reproduction earlier, with the effect of size manipulation being most pronounced under high food conditions. In contrast to females, however, food deprivation advanced the onset of reproduction in male polyps irrespective of body size, with this pattern being particularly evident under low food availability, such that starved, reduced males initiated reproduction first.

These results are consistent with an "energy reserve" effect of large body size, as sexual development time was influenced by both body size and resource availability, and the influence of food limitation was weakest in enlarged individuals. Notably, however, the direction of the food effect differed between the sexes: food scarcity delayed reproduction in females but accelerated it in males. A number of mechanisms may underlie this sex-specific response. Sexual reproduction is likely more energetically demanding for females, given

that egg production is generally more costly than sperm production (Hayward & Gillooly, 2011). Consequently, females are expected to be most strongly affected in the reduced-starved treatment, where both internal (body size) and external (food availability) energy reserves are limited. While this explanation accounts for the stronger response seen in females, it does not explain why small males responded in the opposite manner to resource limitation.

One possible explanation is that, in addition to their lower energetic requirements for gamete production (Hayward & Gillooly, 2011), males may interpret low food availability as an additional environmental cue triggering sexual reproduction (Burnett & Reisa, 1973; Tökölyi et al., 2021). Under this scenario, increasing intensity of cues signalling the onset of winter—such as reduced food availability and lower temperatures—could induce earlier reproduction regardless of body size. Males may therefore respond differently to the interaction between body size and resource availability because delayed sperm production could place them at a competitive disadvantage relative to other males, particularly given that *Hydra* eggs don't remain fertilizable for extended periods (Kaliszewicz & Lipińska, 2012; Littlefield et al., 1991; Tökölyi et al., 2021).

Taken together, large body size likely functions as a net long-term physiological energy reserve in both male and female *Hydra*, facilitating faster gonad development and earlier reproduction. Conversely, short-term food availability (i.e., immediate energy supply) further accelerates reproduction in females, exerting an effect similar to that of long-term energy reserves, but has the opposite effect in males. In males, increased food availability may be preferentially allocated not to additional sperm (testis) production but to other, more long-term life-history characteristics, such as growth or longevity, given

the relatively low energetic costs of sperm production (Blanckenhorn, 2005; Reim et al., 2006).

Fecundity

Gonad number increased additively with body size - reflecting the bigger energy reserves of enlarged individuals - and with food availability, indicating higher energetic input, in both sexes. This pattern is widely documented across diverse taxa, including insects, birds, mammals, and reptiles (Allainé et al., 1987; Ford & Seigel, 1989; Honěk, 1993). We had anticipated interactive effects of body size and resource availability on gonad production, such that, under the "energy reserve" hypothesis, larger individuals would exhibit smaller differences in fecundity between low and high food conditions, whereas under the "energy demand" hypothesis, fecundity differences would be amplified in larger individuals. However, no such interaction was detected.

One possible explanation for the absence of an interaction is the shift in reproductive timing observed in polyps with reduced body size. Reduced and starved females produced their first eggs approximately one week later than all other experimental groups, which may have allowed them to accumulate additional resources from feeding prior to reproduction, thereby increasing fecundity and effectively masking any interaction effect. A longer period of starvation, or reduced feeding following the treatment phase, might have produced a more pronounced effect, although such conditions could also have further delayed reproduction in reduced and starved polyps. Thus, whether energetic constraints were imposed through long-term limitation of energy reserves *via* body size or through short-term restriction of food availability, individuals appeared to compensate by postponing reproduction rather than by creating fewer eggs earlier.

Adjustment in reproductive timing, nonetheless, does not fully account for the lack of interaction between resource and size treatments in males. Reduced and starved males initiated reproduction earlier, yet their fertility was not more strongly reduced. Two factors may contribute to this unexpected result. First, the advancement of reproduction in smaller, reduced males was modest—approximately 3.5 days under 4x feeding and about 2 days under 0x feeding—which may have been insufficient to generate detectable differences in fertility. Second, our assessment of male fertility may have lacked the resolution required to capture subtle effects, as we quantified only the number of gonads visible to the naked eye, despite potential variation among gonads in size and sperm production.

Survival

Consistent with earlier work in *Hydra* (Ngo et al., 2021), reduced animals exhibited higher survival than larger individuals. By including a greater number of strains in the present study, we were able to generalize the negative relationship between body size and longevity in *Hydra*. Comparable inverse associations between body size and lifespan have been documented across breeds of several domesticated animals, such as dogs and horses (Austad, 2010; Bartke, 2017; Kraus et al., 2013; Rollo, 2002). We propose that the enhanced survival of smaller individuals reflects their lower reproductive investment, allowing a greater proportion of resources to be allocated to somatic maintenance and survival (Kirkwood & Rose, 1991; Sebestyén et al., 2020).

As in the fecundity analyses, nevertheless, we did not detect an interaction between body size and food availability in their effects on survival. One contributing factor may be the pronounced variation in survival among strains, with some exhibiting complete survival and others experiencing nearly total

mortality. Such large variation among strains likely reduced statistical power and obscured potential treatment effects. These strain-specific survival patterns are difficult to predict. Genetic divergence among strains is thought to be low (Miklós et al., 2021), and all animals were maintained under identical laboratory conditions. Nevertheless, strains may differ in the composition of their associated microbiota - an aspect not assessed here - which is known to strongly influence *Hydra* physiology (reviewed in (Taubenheim et al., 2022)). This possibility is further supported by the markedly reduced survival of strain C2/7 in the present study compared with our previous work (Ngo et al., 2021). As no other experimental parameters were altered, changes in host-associated microbial communities represent a plausible explanation, although this remains speculative. Future studies should therefore explicitly consider the role of specific host-associated microbes.

We also observed higher survival in starved individuals compared with those receiving high food levels. This pattern closely resembles the effects of dietary restriction reported across a wide range of animal taxa (Magwere et al., 2004; Moatt et al., 2016) and may likewise be explained by shifts in the trade-off between reproduction and survival in both sexes (Adler et al., 2016; Kirkwood & Rose, 1991; Sebestyén et al., 2020). Interestingly, such a dietary restriction effect was not detected in a former study on the same *Hydra* species (Tökölyi et al., 2017). Several differences between the studies may account for this discrepancy. First, the present experiment involved a two-week period of complete starvation, whereas the prior study applied a continuous but low food supply. Second, and perhaps more importantly, the strains used previously differed and exhibited uniformly high survival rates, comparable to strain T3/2 in the current study. Such high baseline survival may have limited the ability to detect additional survival benefits under reduced food conditions. Taken together, these observations suggest that dietary restriction can enhance

survival in *Hydra*, but its effects may be attenuated in strains with inherently high survival.

In summary, our results support the hypothesis that body size functions as an energetic safeguard in *Hydra*, enabling larger polyps to initiate sexual development at a relatively constant rate largely independent of present environmental conditions. In contrast, small animals with long-term energy reserves presumably below a critical threshold plastically adjusted their reproductive timing in response to resource limitation. As a result, the effects of body size on fecundity and survival were largely independent of food availability. We therefore found little evidence for an energetic cost of large body size, as enlarged individuals did not suffer disproportionately reduced fitness under resource scarcity relative to smaller polyps. This apparent absence of an "energy demand" effect may be characteristic of ectothermic organisms, which require substantially less energy for tissue maintenance than endotherms (Gillooly et al., 2001). More generally, experimentally calibrating short-term (food supply) and long-term (body size) energy reserves around the physiological thresholds governing reproductive life-history responses remains challenging (Blanckenhorn et al., 1995). Future experiments combining body-size manipulation with increased tissue maintenance costs - for example, through elevated temperatures - may provide a promising avenue to address this issue.

Study II.

Although substantial evidence demonstrates that environmental temperature plays a key role in early-life sex determination in species with environmental sex determination (ESD) and in genetically sex-determined (GSD) species exhibiting sex reversal, considerably less is known about how abiotic environmental variation affects the sexual phenotype of adults in species with sequential hermaphroditism. In the second study, we exposed *Hydra* polyps to alternating high and low temperatures accompanied by corresponding changes in photoperiod, thereby simulating natural seasonal variation. Under these conditions, clonal lineages underwent gonadogenesis and in a subsequent reproductive cycle, occasionally exhibited female-to-male sex change. Thus, sex change in response to seasonal temperature variation can indicate protogyny in Brown hydra.

Although Brown hydra is generally regarded as gonochoristic - where individuals typically express a single sexual phenotype that is clonally inherited - sporadic cases of sex change have previously been reported, such as male-to-female transitions in strains maintained at high temperature (Littlefield, 1986). However, attempts to replicate male-to-female sex change in our laboratory have so far been unsuccessful (unpublished observations of J. Tökölyi and R. Gergely). By contrast, the present study demonstrates that experimentally simulated seasonal fluctuations in temperature and photoperiod can induce occasional female-to-male transitions in strains designated as female, while no male-to-female sex change was detected across multiple strains. These observations resemble sequential hermaphroditism, although classification is less straightforward in modular organisms such as hydra.

According to the framework proposed by Wasson & Newberry (1997), sexual systems can be characterized at multiple organizational levels: the individual

(e.g. polyp, ramet, or clonemate), the genotype (genet or clonal lineage), and the colony (for species in which individuals remain physically connected). At the individual level, *H. oligactis* can be considered gonochoristic, as single polyps develop only one type of gonad. At the genotype level, however, our results indicate that *H. oligactis* is effectively hermaphroditic, because a single genotype is capable of producing both sexes. Thus, *H. oligactis* corresponds to the SG category of Wasson and Newberry's (1997) classification: genets that are sequentially hermaphroditic while modules are gonochoristic.

The potential adaptive value of sex change in this system remains an open question. Given hydra's clonal mode of reproduction, strict gonochorism may entail fitness costs. Extensive clonal propagation can reduce clonal diversity, potentially resulting in local populations composed predominantly of a single sex during the reproductive phase, thereby limiting sexual fitness. Although hydra polyps are capable of dispersal, for example by floating on the water surface, it is unclear whether such mechanisms reliably ensure access to the opposite sex in natural settings. Furthermore, clonal lineages can persist in populations for multiple years (Miklós et al., 2022), providing ample opportunity for clonal expansion and increasing the likelihood of stochastic loss of one sex, particularly in small or fragmented populations. If sex change were primarily driven by local sex ratios, however, transitions should occur with similar frequency in both directions, which was not observed here, as individuals were housed separately. An alternative explanation is that males and females interpret social or environmental cues differently. For males, the absence of competitors might signal favourable conditions for maximizing reproductive success through increased sperm production. For females, by contrast, the absence of males could indicate reduced fertilization prospects, potentially altering the relative payoff of remaining female and triggering

female-to-male transition. This hypothesis could be tested in the future experimentally by manipulating population density and sex ratio.

These patterns are consistent with the idea that germline stem cells may be depleted during sexual reproduction, potentially through differentiation into gametes. Polyps surviving sexual reproduction may therefore lack residual GSCs and require redevelopment of the germline from multipotent somatic stem cells. The exclusive observation of female-to-male transitions in our experiments may indicate that female GSCs are more costly to regenerate under the conditions prevailing during post-reproductive redevelopment, although direct evidence for sex-specific reproductive costs in this system is currently lacking.

In a previous study about seasonal variation and sexual readiness made by our lab ("Temperature fluctuation study") (Gergely, R. 2020; Tökölyi et al., 2021), we investigated whether sexual readiness in *H. oligactis* varies seasonally when individuals are exposed to identical environmental conditions. In this species, sexual reproduction and the production of resting eggs are restricted to the period preceding winter (Burnett & Reisa, 1973). Consequently, sustaining a high level of reproductive preparedness year-round may be suboptimal, particularly if such preparedness incurs physiological costs (Tökölyi et al., 2012). We therefore hypothesized that *Hydra* polyps would exhibit increasing levels of reproductive readiness as winter approaches.

To determine whether seasonal differences in sexual readiness arise from phenotypic plasticity, as predicted by our hypothesis, we conducted warm-exposure experiments using two laboratory strains, one male: C2/7 and one female: X11/14 - so we could take sex as a variable into consideration - and assessed changes in sexual readiness following exposure to elevated temperature.

Hydra oligactis polyps typically initiate sexual reproduction in response to a drop in temperature. However, this response varies between sexes, such as males start their reproduction faster and their reproductive period takes longer than in females (Burnett & Reisa, 1973). It varies among individuals, younger polyps show reduced sexual propensity, lower fecundity, and higher post-reproductive survival (Sebestyén et al., 2020). Furthermore, we formally demonstrated that sexual reproduction does not occur in animals propagated asexually at 8 °C for extended periods (up to several months) (Tökölyi et al., 2021). This prolonged asexual phase likely reflects a natural component of the species' life cycle following winter, when asexual polyps persist after sexual reproduction and may reach high population densities (Welch & Loomis, 1924; J. Tökölyi personal observation). Taken together, these findings suggest that cold exposure alone is insufficient to induce sexual reproduction in this system.

Because a temperature drop - rather than continuous cold exposure - appears to be the critical cue for inducing sex in *H. oligactis*, we conducted the study where we tested how reciprocal cold and warm periods influence sexual readiness in a male and a female strain. To simulate seasonal temperature fluctuations, we first lowered the temperature to 7 °C and then increased it to 18 °C for either 1, 4 or 8 weeks in the treatment groups, while control animals remained continuously at 18°C and on 7 °C. After the warm-exposure phase, treated polyps were returned to 7 °C to attempt induction of sexual reproduction.

Sexual reproduction was rare in the group exposed to warm conditions for only 1 week (none of the males, but 1/5 of the females reproduced sexually) but substantially higher in the groups that experienced a 4 and 8 week warm period. These results indicate that warm exposure enhances sexual readiness and that reciprocal transitions between warm and cold periods are necessary to reliably

trigger sexual reproduction in this species (Gergely, R. 2020; Tökölyi et al., 2021).

Furthermore, the repeated, cyclic seasonal shifts may reflect reduced GSC abundance in early spring, which could create conditions favourable for the emergence and proliferation of GSCs of the opposite sex, thereby facilitating sex change. Such a process could be interpreted as a form of random sex determination, in which sexual phenotype is established through stochastic developmental processes rather than fixed genetic or environmental cues (Perrin, 2016). Definitive evaluation of this hypothesis will require future studies that directly identify and track GSCs across developmental stages.

In conclusion, in Study II. we demonstrated that environmental cues characteristic of seasonal change, together with the alternation between sexual and asexual life stages, are associated with sex change in an invertebrate species. This phenomenon likely arises through altered differentiation dynamics of the cell lineages underlying sexual phenotype determination. Whether sex change in Brown hydra represents an adaptive strategy analogous to sequential hermaphroditism in other taxa - serving to maximize fitness by adjusting sexual phenotype to mate availability or individual condition such as age - remains to be established. Our results suggest that such adjustment is contingent upon the induction of sexual reproduction by declining temperatures following regeneration. Consequently, ongoing climate change and increasingly mild winters may disrupt sex ratios in hydra populations by reducing opportunities for adaptive sex change, with potential downstream effects on population demography and ecosystem function. Further research will be necessary to elucidate the cellular mechanisms underlying sex change in hydra, the basis of its sex-biased expression, and its implications for population dynamics and life-history evolution.

IV. Key findings - Summary

- Resource availability modulates the relationship between body size and sexual development, with sex-specific differences in this effect
- Fecundity is enhanced by increases in body size as well as by greater food availability
- Body size may function as an energy reserve
- Survival is higher when fecundity is lower - reduced body size and lower resource availability are both associated with improved survival
- Seasonal environmental changes and repeated life cycles can lead to sex change - seasonal variation may induce protogyny in *Hydra oligactis*
- *Hydra oligactis* exhibits gonochorism at the level of the individual while being sequentially hermaphroditic at the genetic level

V. Összefoglalás – Jelentősebb eredmények

Az életmenet-elmélet azt vizsgálja, miként osztják el az élőlények korlátozott erőforrásaikat a növekedés, a szaporodás és a túlélés között, valamint hogy e forrásallokációs mintázatok miként különböznek az egyedek között, a környezeti feltételek változásának függvényében, illetve eltérő evolúciós kontextusokban (Roff, 1993; Stearns, 1989). Az életmenet-evolúció egyik központi fogalma a fenotípusos plaszticitás, amelynek során olyan tulajdonságok, mint a testméret, a reprodukciós befektetés vagy a túlélési valószínűség, egyaránt módosulhatnak a belső állapot és a külső környezeti tényezők hatására (Kaliszewicz & Lipińska, 2013; Kirkwood & Rose, 1991; West-Eberhard, 2003). A plaszticitás lehetővé teszi, hogy az élőlények életük során adaptív módon módosítsák életmenet-stratégiáikat, például a szomatikus növekedésbe történő befektetés, az ivaros szaporodás időzítése és intenzitása, valamint az általános fenntartásra és túlélésre fordított erőforrások arányának változtatásával.

A ivarok közötti különbségek az életmenet-variációk egy további alapvető tengelyét alkotják. Az ivarspecifikus szelekciós nyomások széles körben dokumentált különbségekhez vezetnek a hímek és nőstények között a morfológia, a viselkedés, az élettartam és a szaporodási stratégiák tekintetében különböző taxonokban (Fairbairn et al., 2007; Maklakov et al., 2008; Tessier & Cáceres, 2004). Ezek az eltérések gyakran ivarspecifikus életmenet-pályákban öltenek testet, amelyeket az eltérő reprodukciós szerepek, a szaporodás különböző költségei és az erőforrás-allokációs kompromisszumok formálnak (Kaliszewicz, 2018; Kraus et al., 2013; Stearns, 1989). Ennek megfelelően az életmenetbeli plaszticitás vizsgálata megköveteli az ivar, mint biológiai változó explicit figyelembevételét, mivel a hímek és

nőstények nem csupán a tulajdonságértékekben térhetnek el, hanem a környezeti feltételekre adott plasztikus válaszok mértékében és irányában is.

Az ivarspecifikus életmenetbeli plaszticitás vizsgálatához egy csalánozó modellállatot, a Magyarországon is előforduló édesvízi *Hydra* nem egyik faját használtuk a *Hydra oligactist*, vagy Magyar nevén a Nyeles hidrát. A *Hydra* régóta alkalmazott kísérleti modell az evolúciós és ökológiai kutatásokban, kis testmérete, egyszerű szöveti szerveződése, váltivarú szaporodása és könnyű laboratóriumi fenntarthatósága miatt (Bosch & David, 1986; Galliot, 2012; Tomczyk et al., 2015). Emellett a *Hydra* fajok jelentős variációt mutatnak a testméret, a szaporodási módok és a túlélés tekintetében, ami különösen alkalmassá teszi őket az életmenet-variációk és a plaszticitás tanulmányozására (Harvell & Grosberg, 1988; Martínez & Bridge, 2012). Korábbi vizsgálatok ivarspecifikus különbségeket fedeztek fel a szaporodási stratégiákban és az öregedési mintázatokban egyaránt, kiemelve e modellrendszer jelentőségét annak megértésében, hogy az ivar miként formálja az életmenetbeli variációkat még viszonylag egyszerű szerveződésű metazoák esetében is (Schaible et al., 2015; Sebestyén et al., 2018, 2020; Tökölyi et al., 2021). E tulajdonságok együttesen a hidrát ideális modellé teszik a fenotípusos plaszticitás és a nemspecifikus életmenet-stratégiák közötti kölcsönhatások feltárására. Ennek megfelelően e modellrendszer alkalmazásával végeztük el az alábbi két vizsgálatot.

1. Vizsgálat Gergely, R., és Tökölyi, J. (2023) A forráselérhetőség modulálja a testméret hatását a szaporodási fejlődésre

A fajon belüli testméret-variáció ismerten jelentős különbségeket jelez előre az életmenet-jellegekben, beleértve az ivaréretté válás időzítését, a fekunditást és az élettartamot. Energetikai megközelítésben a nagyobb egyedek nagyobb energiatartalékokkal rendelkezhetnek, amelyek különböző funkciókat – például a szaporodást és a túlélést – szolgálhatnak („energiatartalék" hipotézis). Ugyanakkor a nagyobb testméret az általános fenntartási költségek növekedésével is járhat, ami erőforrás-szegény környezetben csökkent szaporodási teljesítményt és túlélést eredményezhet a nagyobb egyedek esetében („energiaigény" hipotézis). Elsődleges célunk annak vizsgálata volt, *H. oligactis* hímekben és nőstényekben a testméret miként befolyásolja a kulcsfontosságú életmenet-jellegeket, beleértve a fejlődésiidőt, a fekunditást és a túlélést. Különösen a testméret energetikai szerepét elemeztük mindkét nemben.

Ennek érdekében három hím és három nőstény hidra-törzs egyedeinek testméretét, valamint táplálékelérhetőségét kísérletesen manipuláltuk. Három testméret-kategóriát (kicsinyített, nagyított, kontroll) és három etetési csoportot alakítottunk ki (a lehűtéstől számított két héten át: éheztetett (0x), normál (2x) és magas (4x) táplálékellátás). A testméret-manipulációt és a táplálékcsoportba sorolást követően a szexuális szaporodást lehűtéssel indukáltuk. Az egyedeket összesen 22 héten keresztül követtük (az első két hét a táplálék-manipuláció időszaka volt), rögzítve az ivaros szaporodás kezdetének időpontját, a fekunditást (hímek esetében a herék maximális száma, nőstények esetében az összesített pete-szám), valamint a végső státuszt (túlélt/regenerálódott; elpusztult/nekrotikus).

Eredményeink szerint a nőstény törzsekben mind a testméret, mind a táplálékmennyiség növekedése csökkentette az ivaros szaporodás megkezdéséhez szükséges időt. Ugyanakkor a testméret hatása szignifikánsan függött a táplálékelérhetőségtől: a nagyított egyedek a magas táplálékszinten kezdték meg legkorábban az ivaros szaporodást, míg a kicsinyített, éheztetett egyedek pedig a legkésőbb. A hím törzsekben a nagyított, de éheztetett egyedek kezdték meg legkorábban az ivaros szaporodást, ugyanakkor a testméret-manipuláció hatása a táplálékelérhetőség növekedésével erősödött.

Mind a hím, mind a nőstény egyedek esetében a testméret és a táplálékelérhetőség növekedésével párhuzamosan nőtt a fekunditás, azonban a két tényező között nem találtunk interakciót.

A túlélés tekintetében szintén nem detektáltunk kölcsönhatást a két faktor között, ugyanakkor mindkettő önállóan befolyásolta a túlélést. A legmagasabb túlélési arányt a kicsinyített és éheztetett egyedeknél figyeltük meg, továbbá a hímek általánosságban magasabb túlélést mutattak, mint a nőstények.

Összességében megállapítható, hogy az ivaros szaporodás megkezdésének időzítésére a két vizsgált tényező együttesen hatott, és a táplálékelérhetőség hatása kifejezettebb volt a kisebb egyedek esetében, ami az „energiatartalék” hipotézissel áll összhangban. Ugyanakkor a fekunditás és a túlélés vonatkozásában nem találtunk táplálékfüggő moduláló hatást.

2. Vizsgálat Gergely, R. és mtsai. (2025) Szezonális környezetváltozás és ivarváltás csalánozókban

A második vizsgálatban arra törekedtünk, hogy feltárjuk a szezonális környezeti változások szerepét az ivarmeghatározás plaszticitásának kiváltásában a *Hydra oligactis* esetében, különös tekintettel arra, hogy a hőmérsékleti ingadozások miként járulhatnak hozzá a szekvenciális hermafroditizmus kialakulásához. Feltételezésünk szerint a természetes élőhelyekre jellemző, szezonális hőmérsékleti és fotoperiódus-változások legalább részben felelősek lehetnek a terepi populációkban megfigyelt, emelkedett gyakoriságú ivarváltásért. Hipotézisünk tesztelésére laboratóriumi körülmények között kísérletesen szimuláltuk a szezonális hőmérsékleti és fotoperiódus-fluktuációkat, majd meghatároztuk az ilyen környezeti kezelés kombinációknak kitett klonális vonalak nemét.

A nemarány és az ivarváltás valószínűségének vizsgálatához olyan egyedeket használtunk, amelyek már átestek egy teljes szaporodási cikluson (ivartalan szaporodás melegben, ivaros szaporodás hidegben, túlélés az ivaros szaporodást követően, majd ismét ivartalan szaporodás hidegben és melegben). Három hím és három nőstény törzset alkalmaztunk, amelyek klonális vonalaknak tekinthetők, mivel egyetlen egyed aszexuális bimbózásával felszaporított, genetikailag azonos, a hím vagy nőstény nem irányába elkötelezett egyedekből álltak.

A törzseket kezdetben nyári körülményeket szimuláló (18 °C, 12–12 órás fény–sötét ciklus) környezetben tartottuk. Az innen véletlenszerűen kiválasztott egyedeket egy telet szimuláló (8 °C, 8–16 órás fény–sötét ciklus) inkubátorba helyeztük át, amellyel indukáltuk az ivaros szaporodást. Az első ivaros szaporodást követően a túlélő egyedekből – egy regenerálódott polip és

annak bimbózással létrejött klónjai felhasználásával – új vonalakat hoztunk létre azonos körülmények között. A felszaporított vonalakat egy hétig 12 °C-on tartottuk, majd ismét 18 °C-ra helyeztük a nyári körülmények szimulálására, ahol három hétig maradtak, mielőtt újból 8 °C-ra kerültek volna a második ivaros szaporodás indukálására. Ekkor ismét meghatároztuk az egyedek nemét, és az állatokat ivarváltott (az eredeti törzs nemétől eltérő gonád kialakulása: here nőstény törzsben vagy pete hím törzsben) illetve nem ivarváltott csoportba soroltuk.

A második szaporodási ciklust követően ivarváltást kizárólag az eredetileg nőstény törzsekben figyeltünk meg, és az ivarváltás aránya e törzsek között változónak bizonyult. Ezzel szemben a hím törzsekben egyetlen ivarváltási eseményt sem detektáltunk. Eredményeink alapján a szezonális hőmérséklet-ingadozás és a fotoperiódus fluktuáció protogíniát indukálhat a Nyeles hidrában.

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VII. List of publications serving as the basis for the thesis

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VIII. Personal contributions to the publications

	Study 1	Study 2
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Investigation and data curation	lead	lead
Data analysis	supporting	supporting
Manuscript preparation	equal	lead

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Resource availability modulates the effect of body size on reproductive development

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Abstract

Within-species variation in animal body size predicts major differences in life history, for example, in reproductive development, fecundity, and even longevity. Purely from an energetic perspective, large size could entail larger energy reserves, fuelling different life functions, such as reproduction and survival (the "energy reserve" hypothesis). Conversely, larger body size could demand more energy for maintenance, and larger individuals might do worse in reproduction and survival under resource shortage (the "energy demand" hypothesis). Disentangling these alternative hypotheses is difficult because large size often correlates with better resource availability during growth, which could mask direct effects of body size on fitness traits. Here, we used experimental body size manipulation in the freshwater cnidarian *Hydra oligactis*, coupled with manipulation of resource (food) availability to separate direct effects of body size from resource availability on fitness traits (sexual development time, fecundity, and survival). We found significant interaction between body size and food availability in sexual development time in both males and females, such that large individuals responded less strongly to variation in resource availability. These results are consistent with an energy reserve effect of large size in *Hydra*. Surprisingly, the response was different in males and females: small and starved females delayed their reproduction, while small and starved males developed reproductive organs faster. In case of fecundity and survival, both size and food availability had significant effects, but we detected no interaction between them. Our observations suggest that in *Hydra*, small individuals are sensitive to fluctuations in resource availability, but these small individuals are able to adjust their reproductive development to maintain fitness.

KEYWORDS
body size, food availability, reproduction, size manipulation, survival

TAXONOMY CLASSIFICATION
Behavioural ecology, Evolutionary ecology, Zoology

1 | INTRODUCTION

A large proportion of life history traits' variation can be attributed to body size in mammals, reptiles, birds, and invertebrates (Lindstedt & Boyce, 1985). Body size is one of the most important quantitative traits in evolutionary ecology research because of its strong correlation with physiological and fitness characters (Blanckenhorn, 2000; Reiss, 1989; Roff, 1993). Body size varies considerably both within and among species and is affected by contrasting selective forces due to its complex effects on fitness components. For instance, large size can be under positive selection due to its association with higher fecundity, but selection could also favor early maturation, thus small size (Amarillo-Suárez et al., 2011; Blanckenhorn, 2000; Harvey et al., 2006).

Purely from an energetic perspective, the correlation between body size and fitness traits could stem from several distinct mechanisms. First, large body size entails larger energy reserves, which can be allocated between different tasks (e.g., self-maintenance, reproduction, survival; the "energy reserve" hypothesis) (Lindsey, 1966; Millar & Hickling, 1990; Roff, 1993; Stearns, 1992). This hypothesis predicts that larger animals will start to reproduce earlier with greater effort, resulting in higher reproduction, even in limited environments, because of the higher amount of accumulated energy (Lindstedt & Boyce, 1985; Reim et al., 2006). Body size positively affects fecundity in taxa as diverse as sponges (e.g., *Rhopaloeides odorabile*) (Whalan et al., 2007), cnidarians (e.g., *H. oligactis* and *Tripalea clavaria*) (Excoffon et al., 2011; Ngo et al., 2021), or insects (e.g., Coleoptera, Diptera, Heteroptera, Hymenoptera, and Lepidoptera) (Honěk, 1993), to name a few. For example, in Odonates, large body size has positive effect on mating rate, fecundity, longevity, and survivorship; hence, there is a general fitness benefit to large size for this order of insects (Sokolovska et al., 2000). In *Drosophila pseudoobscura*, longevity and viability as well as fecundity are positively affected by large body size, whereas in grasshoppers too (e.g., *Romalea microptera*), large size has positive effect on fecundity (Akman & Whitman, 2008; Tantawy & Vetukhiv, 1960).

By contrast, larger body size might also demand more energy for maintenance simply because of the extra tissue that needs to be maintained (the "energy demand" hypothesis) and larger individuals might actually do worse in limited conditions (Reim et al., 2006). According to this hypothesis, smaller individuals might need less energy for self-support and can thus reproduce sooner, causing advantage over bigger size (Blanckenhorn, 2000). For instance, in water striders, the food availability threshold over which small males are still able to copulate is more permissive than in larger males (Blanckenhorn, 2005).

Untangling which of these two alternative hypotheses (the "energy reserve" or "energy demand" hypothesis) contributes to the body size—fitness trait correlations are difficult in most occurrences (e.g., taxa and sex) because both of these traits might be affected by common causes. For example, food availability affects both growth and condition; hence, the positive relationship between size and fitness might in fact represent variation in resource availability (Gori et al., 2013; Reznick et al., 2000; van Noordwijk & de Jong, 1986; Yom-Tov et al., 2006) and not direct consequences of large size or high growth per se. Moreover, in animals with larger size and longer life span, the study of body size effects in a laboratory is unfeasible. Furthermore, body size shows substantial pleiotropic effects on other traits. For instance, large size entails better fecundity, but may cause higher mortality later, because of the reproduction/survival trade-off (Kirkwood & Rose, 1991; Sebestyén et al., 2020).

Here, we used the freshwater cnidarian *Hydra oligactis* (Pallas, 1766) as a model system to understand direct effects of body size on fitness traits. *Hydra* have remarkable regeneration abilities, which allow tissue excision and grafting between individuals, such that the body column of individual polyps can be experimentally increased or reduced. Previously, we have shown that these size changes are associated with altered sexual development time, fecundity, and postreproductive survival (Ngo et al., 2021). However, the reasons for these effects are still to be explained. To address this gap, we surgically manipulated *Hydra* body size, and the size-manipulated individuals were subjected to different food availability environments during their sexual development. We hypothesized that the effect of body size should depend on the availability of food in a way that would reveal whether the "energy reserve" or the "energy demand" hypotheses are involved in explaining body size effects in *Hydra*. Specifically, if the "energy reserve" hypothesis is true, then larger animals will have better reproduction, such as earlier start and higher fecundity and/or postreproductive survival, because of the accumulated energy reserves, even in limited conditions. Conversely, if the "energy demand" hypothesis is correct, then large animals will do worse in reproduction and/or survival, because of the higher maintenance costs especially under limiting conditions.

2 | MATERIALS AND METHODS

2.1 | Study system

Hydra oligactis, commonly known as Brown Hydra, is a freshwater, gonochoristic invertebrate species, which is capable of asexual and sexual reproduction. It lives in highly seasonal habitats and reproduces by budding (asexually) in the major part of the year and starts to reproduce sexually at the onset of winter, when temperature is getting lower. After the cold stimulus, male polyps produce testes, while female polyps produce ovaries (about 2 weeks of difference between sexes) (Burnett & Reisa, 1973; Schuchert, 2010; Tökölyi et al., 2021; Quinn et al., 2012). Sexual reproduction results in the production of resting eggs that can survive harsh conditions, such as freezing. After sexual reproduction, a substantial proportion of polyps die in the next few months, when they go through a senescence-like degradation (Tökölyi et al., 2017; Yoshida et al., 2006). This postreproductive senescence in *H. oligactis* is associated with reduction in the number of interstitial stem cells, a decreased ability to catch food, decline in tactile movements, decline in body size, and increase in mortality rate (Martínez & Bridge, 2012; Sebestyén et al., 2018; Tökölyi et al., 2017).

2.2 | Hydra strains

All strains used for the experiment originate from one animal each from East Hungary. Strain X11/14 (female) and C2/7 (male) were established from two polyps collected from Tiszadorogma (47.6712°N, 020.8641°E) in September 2016, from a floodplain lake of Tisza river. Strain T3/1 (female) and T3/2 (male) originate from Tiszalúc (48.03420°N, 02.07894°N) an oxbow lake of Tisza river, in summer 2020. M26/9/10 (female) strain derives from river Hortobágy (47.57805°N, 021.14587°E), collected in summer 2020. Strain M83/4 (male) is from Gávavencsellő (48.17484°N, 021.61385°E); it was collected from an oxbow lake linked to Tisza river, in spring 2020. In all cases, *Hydra* polyps were collected from free-floating and submerged macrophytes (e.g., *Ceratophyllum demersum*, *Ceratophyllum submersum*, *Myriophyllum spicatum*, *Stratiotes aloides*, *and Nuphar lutea*), and all of the collected polyps were multiplied to strains by asexual budding and were maintained asexually under standard conditions in the laboratory since their collection (standard feeding, temperature, and light–dark cycle, see below).

2.3 | Experimental maintenance

Animals were kept individually in six-well cell culture plates with 5-ml standard *Hydra* medium per well (*Hydra* medium composition: 1 mM Tris, 1 mM NaCl, 1 mM $CaCl_2$, 0.1 mM KCl, 0.1 mM $MgSO_4$; pH: 7.6 (Tökölyi et al., 2017)). They were fed individually with 20 μl fresh *Artemia nauplii* suspension using an automatic pipette (FinnPipette). During feeding, the number of gonads (testes and eggs) and the number of detached buds were recorded, and about 2 h after feeding, the animals were moved to clean *Hydra* medium. For the experiment, 3-week-old animals cultured on 18°C degrees with 12/12 h' light/dark cycle (simulating summer conditions) were randomly selected and paired with similar individuals from the three female and three male strains before the experimental treatments.

2.4 | Size manipulation treatment

For the size manipulation treatment, tissue grafting was used to produce individuals with enlarged, control, and reduced body size with randomly picked individuals from the six strains. Two individuals were selected from the same strain and paired randomly, then a ring shaped tissue was cut from both *Hydra* polyps' body column. For the "enlarged-reduced" pair, we cut rings differing in size and then switched these rings between the animals (the enlarged animal received a big ring in return for the small one that was cut out; the reduced animal received a small ring in return for the big one that was cut out). As a result, we obtained animals with enlarged and reduced body size. For the "control–control" pair, rings approximately identical in size were switched between the paired polyps. The head, the ring, and the foot region were put together in the correct order and strung up on a glass microcapillary needle until the pieces stuck together (which took about 1–2 h). Then, we removed the polyps from the needle and left them to heal until the next day (see the procedure in: Ngo et al., 2021).

2.5 | Food availability manipulation

The day after size manipulation, experimental animals were moved to a cooled incubator (Pol-Eko ST2) set to 8°C degrees with 8/16 h' dark/light cycle (simulating winter conditions) to induce sexual reproduction by cold stimulus (first day of experiment). To examine the effect of food availability on the different body sizes, three feeding groups were made with the size-manipulated animals. Food manipulation was performed immediately after cooling, when animals started gonadogenesis. The first group was starved for 2 weeks (low food, 0×). The second group received food two times per week for 2 weeks (normal or medium food, 2×), and the third group received food four times per week (high food, 4×) for 2 weeks. All experimental animals were fed individually (hence, no food competition was involved) with 20 μl fresh *Artemia nauplii* suspension, as detailed in the section *Experimental maintenance*. After the 2-week food treatment, all groups were fed two times per week until the end of the experiment, which was 22 weeks after cooling.

Throughout the study, animals were maintained in sets, which contained one plate/feeding group (0×, 2× or 4×) with four size-manipulated animals (one "enlarged-reduced" pair and a "control–control" pair" or two "enlarged-reduced" pairs; Figure 1). The individuals' location in the plate was randomly chosen, and the three feeding treatment plates' sequence in the stock was randomized too. We aimed to have 16 animals in each strain and group (e.g., strain: C2/7/food treatment: starved/size manipulation: reduced).

For the experiment, 864 individuals (12 sets/strain = 144 polyps/strain) were used in total, and 820 remained after failed (e.g., the body parts did not attach after size manipulation), sex-changed, and hermaphrodite animals were taken out of the analysis. The exact number of polyps in each strain and experimental groups are shown in Table 1.

2.6 | Data recording

Timing of sexual reproduction (first mature egg on females and first mature testis on males), and the number of gonads (total number of eggs in females and maximum number of testes in males) were recorded four times per week under a binocular stereo microscope (Euromex StereoBlue). Sexual development time is defined as the number of days elapsed after lowering temperature until gonads were clearly visible on polyps for the first time. In order to record the number of eggs on female individuals, we counted the eggs on them, as well as the detached eggs in the well. After gametogenesis, the number of detached eggs was summed up (unfertilized eggs detach from the polyp after some time), to obtain total egg production.

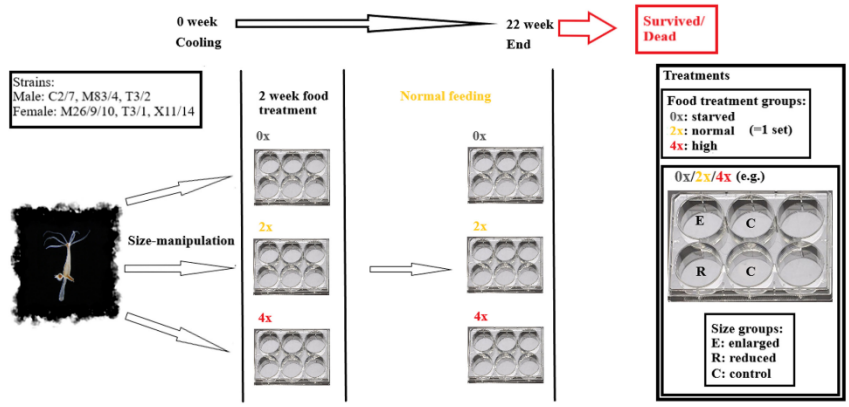

FIGURE 1 From the six strains (C2/7, M83/4, T3/2, M26/9/10, T3/1, and X11/14), individuals were randomly selected/paired and after the size manipulation treatment (E-enlarged, R-reduced, C-control), polyps were assigned to one of the three food treatment groups (0x-starved, 2x-normal, 4x-high). In the first 2 weeks, polyps were fed according to their feeding group, after 2 weeks of food treatment, all animals were fed normally (2x) until the end of the study. Finally, the animals' final status was defined as survived or dead.

TABLE 1 Exact sample size in each strain (divided into male (C2/7, M83/, T3/2) and female M26/9/10, T3/1, X11/14 strains) and experimental group (divided into food treatment and size manipulation).

Male strains									
Strain	**C2/7 *N* = 142**			**M83/4 *N* = 131**			**T3/2 *N* = 140**		
Food treatment	0× *N* = 48	2× *N* = 48	4× *N* = 46	0× *N* = 43	2× *N* = 16	4× *N* = 42	0× *N* = 48	2× *N* = 46	4× *N* = 46
Size manipulation	E = 16	E = 16	E = 14	E = 15	E = 15	E = 14	E = 16	E = 15	E = 14
	R = 16	R = 16	R = 16	R = 13	R = 16	R = 14	R = 16	R = 15	R = 16
	C = 16	C = 16	C = 16	C = 15	C = 15	C = 14	C = 16	C = 16	C = 16
Female strains									
Strain	**M26/9/10 *N* = 139**			**T3/1 *N* = 134**			**X11/14 *N* = 134**		
Food treatment	0× *N* = 47	2× *N* = 46	4× *N* = 46	0× *N* = 45	2× *N* = 47	4× *N* = 42	0× *N* = 47	2× *N* = 43	4× *N* = 44
Size manipulation	E = 16	E = 15	E = 15	E = 15	E = 16	E = 14	E = 15	E = 16	E = 15
	R = 15	R = 15	R = 15	R = 16	R = 15	R = 14	R = 16	R = 13	R = 14
	C = 16	C = 16	C = 16	C = 14	C = 16	C = 14	C = 16	C = 14	C = 15

Note: In the food treatment "0x" is starved, "2x" is normally and "4x" is a highly fed group, next to it, the number of individuals in each group. In the size manipulation experimental group, "E" is enlarged, "R" is reduced and "C" is control animals' number.

To record the number of testes on a male individual, we laid it on its side and counted the clearly visible testes. In males, the maximum number of testes was used as a proxy for fertility, because the number of testes are changing from the first day of gametogenesis to the last day (there is an increase in the early stages until they reach a maximum, thereafter, when the individual start to go through senescence, there is a decrease in the number of gonads). Testes number was used as a proxy for fertility in males, because sperm counting is difficult. We expect higher sperm number, consequently higher fertility from a polyp with more testes.

Photographs of the animals were taken after size manipulation, but before cooling to quantify size change as a result of size manipulation and after the 2 weeks of food treatment to see its effect (Figure 2). We used a CMEX microscope camera to photograph animals under a stereo microscope. Photographs of the individuals were taken on 1 mm grid paper, and the program ImageJ (Schneider et al., 2012) was used for measuring the area of the body column from which body size was calculated (expressed as the area of the polyps in pixels, divided by the square length of standard millimeter in pixels).

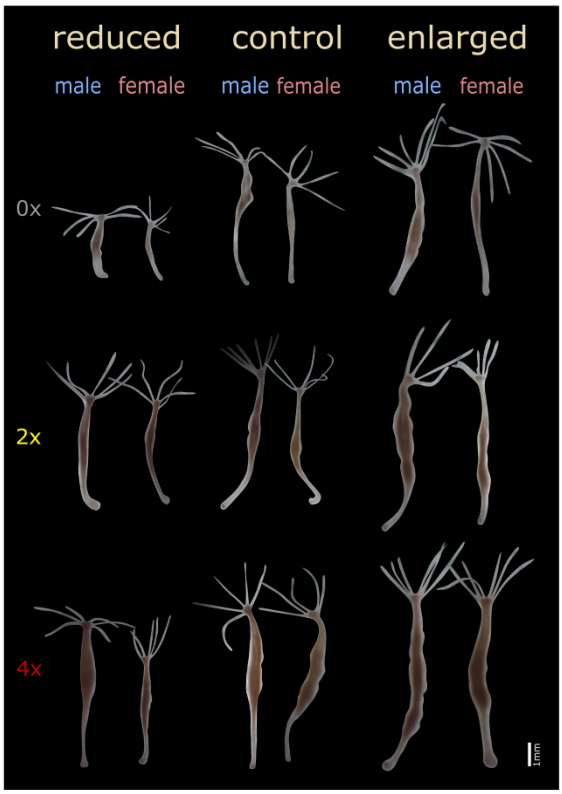

FIGURE 2 Male and female hydra polyps after 2 weeks of food treatment in the reduced, control and enlarged size-manipulated groups. First row is the starved (0×), second is the group which was fed two times per week (2×), and the last is the highly fed (4×) group. In the three columns (reduced, control, and enlarged), the first animal depicted is always male and the second one is always female.

Finally, the animals' final status was recorded on the last day of experiment (22 weeks after cooling). The individual was scored as regenerated/survived if the polyp looked healthy (intact tentacles/eating, tactile movements, and orange color), and/or it made a new bud after sexual reproduction and if it did not reproduce sexually (asexual), but stayed alive until the last day. The animals were scored as dead if they disintegrated or consisted only of necrotic tissue on the last day of experiment.

2.7 | Statistical analysis

To test the interactive effects of body size and food availability on life history traits, we used Generalized Linear Mixed Models (GLMMs), as implemented in the glmmTMB v1.1.4 package in R v4.2.1 (Brooks et al., 2017; R Core Team, 2022). As dependent variables, we used sexual development time (after experimentally lowering the temperature), fecundity (total no. detached eggs in females, and maximum no. testes for males), and survival. Models were fitted separately for female and male strains with strain ID as a random effect. Size manipulation pair ID was included as an additional random effect to take into account the fact that individuals exchanging body parts might be more similar to each other than expected by chance.

We considered GLMMs with Gaussian, Poisson, or Negative Binomial distribution (with quadratic or linear parametrizations; Brooks et al., 2017) to analyze sexual development time and fecundity. The type of distribution was selected based on model comparisons with Akaike's information criteria corrected for small sample size (AICc). Based on this, sexual development time and the number of testes produced in males were analyzed with Gaussian

GLMMs, while a Negative Binomial model with quadratic parametrization ranked best to analyze the number of eggs produced by females. Where needed, we explicitly modeled heterogeneity of variance using the *dispformula* argument in glmmTMB. Finally, survival was analyzed with GLMMs with binomial distribution. Model diagnostics were performed with the DHARMa package v0.4.5 (Hartig, 2022), checking for approximate normality of residuals, homogeneity of variance, and the presence of outliers. For each model, we fitted a full model with size manipulation group, food treatment group, and their interaction as fixed effects (in addition to strain and size manipulation pair ID as random effects). From these full models, we performed stepwise model simplification followed by Likelihood Ratio Tests (LRTs) to remove nonsignificant predictors. Graphs were produced with the *ggplot2* package v3.3.6 (Wickham, 2016).

3 | RESULTS

3.1 | Size manipulation

Size manipulation successfully increased or decreased the size of experimental polyps in enlarged and reduced animals relative to controls (Gaussian GLMM, $\chi^2 = 949.790$, $p < .001$). Reduced animals were approximately 50% smaller than controls, while enlarged animals were about 50% larger (Figure A1).

3.2 | Sexual development time

In female strains, the time required to produce the first gonads decreased with increasing body size and with the amount of food received (Figure 3a). However, the effect of body size depended dramatically on resource availability during gonad development, such that enlarged and 4× fed individuals started sexual reproduction the earliest, while reduced polyps delayed their reproduction substantially more, especially if they also received less food (significant interaction between body size and food availability on sexual development time; Gaussian GLMM, $\chi^2 = 21.154$, $p < .001$; Figure 3a). In all three strains, the first eggs were produced on average 20–28 days after lowering the temperature, but reduced and starved polyps needed 29–43 days on average to produce the first eggs, with strain T3/1 delaying reproduction most (Figure 3a).

In male animals, enlarged and starved individuals started sexual reproduction earliest, but the effect of size manipulation was higher in individuals that received more food (significant interaction between body size and food availability on sexual development time; Gaussian GLMM, $\chi^2 = 13.051$, $p = .011$; Figure 3b). The three strains differed in their sexual development time: polyps in strain C2/7 started sexual reproduction earliest (about 10–14 days after the cold stimulus), while those in strain T3/2 developed gonads the latest (about 13–18 days after the cold stimulus), with strain M83/4 being intermediate between the two (Figure 3b). However, despite the differences in timing, reduced animals advanced their reproduction more when they were also starved in all three strains (Figure 3b).

3.3 | Fecundity

In females, enlarged and fed individuals produced the most gonads. There was no significant interaction between size and food treatments (Negative Binomial GLMM, $\chi^2 = 4.771$, $p = .312$). However, both the size and the food treatments significantly affected the number of eggs produced (size treatment $\chi^2 = 57.855$, $p < .001$; food treatment $\chi^2 = 110.260$, $p < .001$; Figure 4a). The three strains differed in overall egg production (about 2–15 eggs produced/polyp in strain M26/9/10; 5–20 in T3/1 and 6–32 in X11/14), but the response to treatments was similar across strains.

In males, enlarged and fed individuals produced the most gonads, but the interaction between size and food treatments was not significant just like in the female strains (Gaussian GLMM, $\chi^2 = 8.334$, $p = .080$). However, separately, both the size and the food treatments significantly affected the number of testes produced (size treatment $\chi^2 = 242.310$, $p < .001$; food treatment $\chi^2 = 243.370$, $p < .001$; Figure 4b). The three strains differed in overall testes production (on average, 4–16 testes/polyp in C2/7, 5–18 in M83/4 and 2–26 in T3/2, depending on treatment), but all strains responded similarly to treatments.

3.4 | Survival

There was no interaction between the two treatments in affecting survival rate (Binomial GLMM, $\chi^2 = 7.532$, $p = .110$), but both size manipulation and resource availability had a significant effect on survival, such that survival rate was highest in reduced and starved animals (Binomial GLMMs, size manipulation: $\chi^2 = 64.364$, $p < .001$; food treatment, $\chi^2 = 44.219$, $p < .001$; Figure 5). Total survival rate of the six strains was highly variable (female strains: M26/9/10 = 64%; T3/1 = 8.2%; X11/14 = 8.9%; male strains: C2/7 = 12.6%; M83/4 = 63.3%; T3/2 = 100%). All of the polyps survived in strain T3/2 regardless of their size and food treatment. By contrast, in the female strain T3/1, which had the highest postreproduction mortality rate, just 11 polyps survived out of 134 (8.2% survival rate), and all of them were reduced animals.

4 | DISCUSSION

Body size should have complex effects on the energetic balance of an organism, with large size either acting as a reserve or as an extra cost to be expended (Blanckenhorn, 2000; Blanckenhorn et al., 1995). Here, we investigated this energy balance mechanism by applying experimental body size manipulation in freshwater *Hydra* to obtain artificially created size variation, allowing us to investigate the direct effects of body size, independent of the past environmental

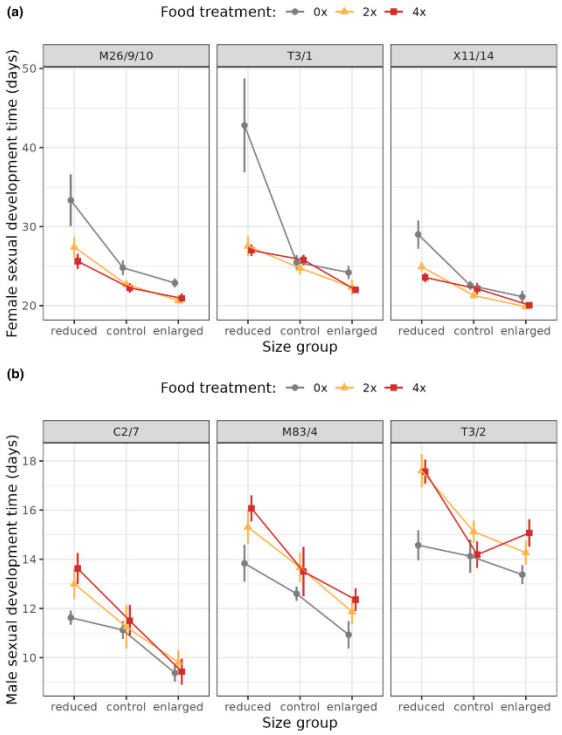

FIGURE 3 Time required to develop the first gonads after lowering the temperature in three female strains (M26/9/10, T3/1, X11/14; a) and three male strains (C2/7, M83/4, T3/2; b) of *H. oligactis* exposed to body size manipulation and kept under three different feeding regimes (0×, 2×, 4×; see Figure 1 for experimental design). Female hydra polyps with experimentally reduced body size have a longer sexual development time than individuals with an enlarged or control size. The effect of body size depends dramatically on resource availability during gonad development, such that starved small polyps produce eggs latest. Male hydra polyps with experimentally reduced body size have a longer sexual development time than individuals with an enlarged or control size. However, the effect of body size depends on resource availability during gonad development, such that the effect of size manipulation is higher in individuals that received more food.

conditions and life history experienced by these animals. We combined size treatment with three distinct resource levels during the sexual development period. Based on both of our hypotheses, we expected interactions between body size and food availability in their effect on reproduction and/or survival. Specifically, we expected that large animals will be less affected by reduced resources if the "energy reserve" hypothesis is true, because larger body size could entail more energy reserves that can be allocated to different life history components even under limiting conditions (Lindstedt & Boyce, 1985; Roff, 1993; Stearns, 1992). By contrast, we expected large animals to be more affected by variation in resource availability if the "energy demand" hypothesis is true, because large size would demand more energy for maintenance, and this extra energy demand could be more easily satisfied under high but not low resource conditions (Blanckenhorn, 2000, 2005; Reim et al., 2006). Although we treated the two hypotheses as separate mechanisms, it has to be emphasized that energetic costs and benefits of large size might be just two facets of the same mechanism (i.e., energy balance) to which resource availability also integrates, albeit providing shorter term contributions. Hence, a clear separation of size- and resource availability effects might not be entirely possible.

With this limitation in mind, we found that sexual development time depended on food availability more strongly in small than in large individuals, consistent with an "energy reserve" effect of body size. On the contrary, we did not find any food-dependent effects of body size on fecundity or survival. We discuss these findings in turn below.

4.1 | Sexual development time

Larger females produced gonads earlier, consistent with our previous observations (Ngo et al., 2021). The accelerating effect of body size dramatically depended on food treatment, as enlarged, highly fed individuals produced eggs earliest and diminished, starved ones latest, requiring about one more week to produce their first eggs.

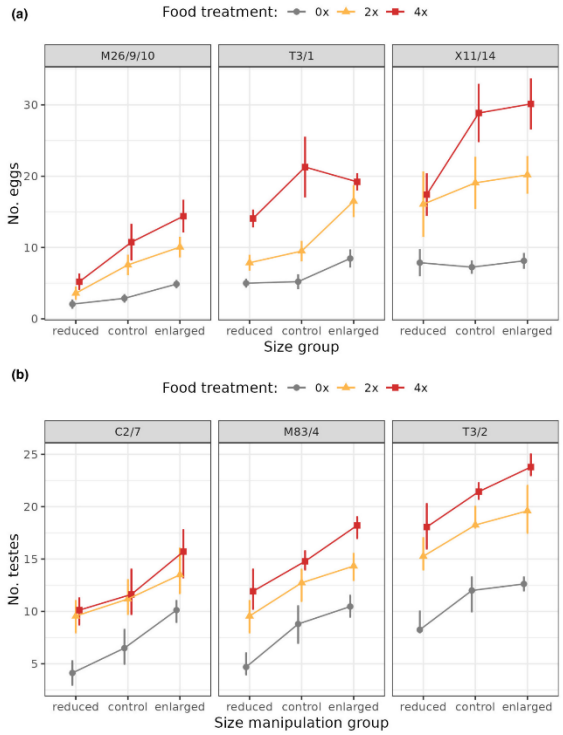

FIGURE 4 Number of eggs (a) and number of testes (b) produced by hydra polyps exposed to body size manipulation and kept under three different feeding regimes (0×, 2×, 4×; see Figure 1 for experimental design). Female hydra polyps with enlarged body size and receiving more food produce a higher number of eggs, but there is no interaction between food and size treatments. Likewise, male hydra polyps with enlarged body size and those receiving more food produce a higher number of testes, but there is no interaction between food and size treatments.

Larger (enlarged and control) males also started to reproduce earlier, with the size manipulation effect amplified at high food availability. However, in contrast to females, starved male polyps started reproduction earlier than well-fed ones, regardless of their body size, this effect was most pronounced on low food (i.e., starved, reduced polyps started reproduction first).

These results support the "energy reserve" effect of large size, since sexual development time was affected by both body size and resource availability, and the effect of resource availability was lowest in enlarged individuals. Surprisingly, however, the effect of food availability differed in the two sexes: lack of food delayed reproduction in females, but accelerated reproduction in males. Several explanations could lie behind these sex differences. Most importantly, sexual reproduction might be more expensive for females, since eggs are thought to be more costly to produce than sperm (Hayward & Gillooly, 2011). As a result, females should be impacted most in the reduced-starved group because they lack both internal (body size) and external (food) reserves. However, while this hypothesis can explain the stronger effect observed in females, it cannot account for small males responding the opposite way to resource shortage. A potential explanation could be that, besides their lower energy need for gamete production (Hayward & Gillooly, 2011), males may interpret low resource availability as an additional cue for starting sexual reproduction (Burnett & Reisa, 1973; Tökölyi et al., 2021). Hence, the stronger the intensity of cues signaling the approach of winter (low food and reduced temperature) may cause earlier start of reproduction regardless of their size. Thus, males might be differently affected by the interaction between body size and resource availability, because they must not fall behind in sperm production to other competing males, since *Hydra* eggs are not fertilizable for a long time (Kaliszewicz & Lipińska, 2012; Littlefield et al., 1991; Tökölyi et al., 2021). Taken together, large body size may well physiologically constitute a net long-term energy reserve for both male and female *Hydra* to be invested in reproduction, thus speeding up their gonad development. By contrast, short-term food (i.e., energy) supply further accelerates reproduction (i.e., has the same effect as

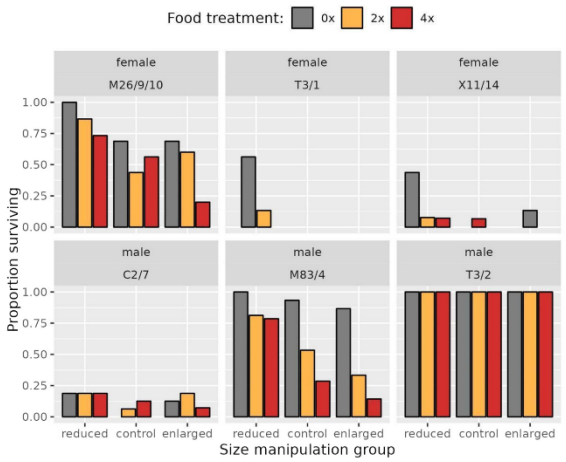

FIGURE 5 Both size manipulation and resource availability had a significant effect on survival, such as the reduced and starved animals' survival rate was the highest, but there was no interaction between the two treatments in affecting survival rate. There are exceptions in male strains C2/7, T3/2 and female strain X11/14, where the highest survival rate is not in the starved and reduced groups. Male polyps (C2/7, M83/4, T3/2) had higher survival rate, than the females (M26/9/10, T3/1, X11/14).

long-term energy reserves) in females but has the opposite effect in males, likely because males invest not in more sperm (testes) reproduction but into other, more long-term life history traits such as, for example, growth or longevity (because they already have enough cheap sperm) (Blanckenhorn, 2005; Reim et al., 2006).

4.2 | Fecundity

The number of gonads increased additively with body size (reflecting the larger energy reserve of enlarged animals) and with food (reflecting the larger energy influx), in both sexes. This pattern can be observed in most insects, birds, mammals, or reptiles too (Allainé et al., 1987; Ford & Seigel, 1989; Honěk, 1993). We had expected interactive effects between body size and resource availability on the number of gonads, such that larger individuals would show a smaller fecundity difference on low vs. high food if the "energy reserve" hypothesis is true and a larger fecundity difference according to the "energy demand" hypothesis. However, we did not find such an interaction.

One potential explanation behind this lacking interaction could be the altered reproductive timing of individuals with reduced body size. Reduced and starved females produced their first egg about 1 week later than all other experimental groups. This apparently allowed them to accumulate additional reserves from their food to increase their fecundity, effectively canceling any interaction effect. A longer starvation period (or reduced feeding after the treatments) might have elicited a stronger effect, although it is possible that the reduced and starved polyps would have delayed their reproduction even further. Thus, regardless of whether energy reserves were limited via body size (long-term) or directly via food supply (short-term), a presumed fecundity target was adjusted by delaying reproduction rather than by producing fewer eggs earlier.

Adjustment in reproductive timing, however, cannot fully explain a lack of interaction between food and size treatments on fertility in males. Reduced and starved males advanced their reproduction, yet their fertility was not more negatively affected. Two factors might explain this surprising finding. First, the reproductive advancement in the smaller, reduced males was merely 3.5 days at 4× feeding, while it was 2 days at 0× feeding conditions. This small difference might not be sufficient to generate an observable effect on fertility. Second, our measurement of male fertility might not be precise enough to detect such small effects, since we counted gonads visible to the naked eye, whereas these gonads might differ in size and/or sperm production. Future studies will require assessing male fertility more precisely, for example, via reproductive cell counts or reproductive gene expression assays.

4.3 | Survival

Consistent with a previous study in *Hydra* (Ngo et al., 2021), reduced polyps showed enhanced survival. The present study tested a larger number of *Hydra* strains so we could generalize the body size–longevity relationship in *Hydra*. Similar negative relationship between body size and longevity is seen across breeds of a number of domesticated animals (e.g., dogs and horses; Austad, 2010; Bartke, 2017; Kraus et al., 2013; Rollo, 2002). We hypothesize that the higher survival of small polyps is due to their lower investment in reproduction, so more resources could be shunted into survival (Kirkwood & Rose, 1991; Sebestyén et al., 2020). However, we again did not observe an interaction between body size and food

availability on survival, which could be explained as the lacking of interaction in the fecundity analysis. Moreover, there was a big difference between the strains in the number of survived animals, some of them showing 100% survival, and others almost complete mortality. These large deviations certainly reduced the power of our analysis by obscuring any treatments effects. These different survival rates of the strains are currently hard to predict. Genetic differences among our strains are likely to be low (Miklós et al., 2021), and the laboratory conditions under which they were maintained were identical. However, they might differ in the composition of their associated microbiota (something that we currently do not know), and microbiota composition has strong effects on *Hydra* physiology (reviewed in: Taubenheim et al., 2022). This is all the more likely because we here detected a substantial drop in the survival rate of strain C2/7 relative to our previous study (Ngo et al., 2021). Since nothing else changed in our experimental conditions, we suspect that these animals might have experienced a change in the composition of host-associated microbes, although we cannot currently verify this assumption. Future studies should take into account the presence of specific host-associated microbes as well.

We also found that starved individuals had a higher survival rate than highly fed ones. This pattern is very similar to the dietary restriction effects observed in a number of different animal species (Magwere et al., 2004; Moatt et al., 2016), and it could be also explained by an altered reproduction/survival trade-off in both sexes (Adler et al., 2016; Kirkwood & Rose, 1991; Sebestyén et al., 2020). Interestingly, we did not find such a dietary restriction effect in a previous study performed on the same *Hydra* species (Tökölyi et al., 2017). However, there are several differences among this and our previous study. First, in the current experiment, we applied a 2-week-long intense starvation, whereas animals in our previous study were exposed to a continuous low food supply. Second, and perhaps more importantly, the strains in the previous study were different and they had a very high overall survival rate, similar to strain T3/2 presented here. This high survival rate might have precluded detecting any increase in survival in response to food reduction. We therefore conclude that there appears to be a dietary restriction effect on survival in *Hydra*, with strains with a high overall survival rate merely less affected.

To conclude, we found support for the hypothesis that body size acts as an energetic buffer in *Hydra*, enabling large individuals to achieve sexual development at the same speed largely independent of the current environmental conditions. Small polyps with long-term energy reserves under a presumed threshold, by contrast, plastically adjusted their reproductive timing to food shortage. Likely as a consequence, any effects of body size on fecundity and survival did not depend on food availability. We thus did not find strong support for an energetic cost of large body size, since animals with an enlarged body size did not experience a stronger reduction in fitness traits under resource shortage than small individuals. This lacking "energy demand" effect might be specific to ectothermic animals that require much less energy for tissue maintenance than endothermic species (Gillooly et al., 2001). In general, it is difficult to experimentally calibrate short-term (i.e., food supply) and long-term (i.e., body size) energy reserves supply around the putative individual physiological energetic thresholds mediating their reproductive life history responses (Blanckenhorn et al., 1995). Experimental size manipulation might address that question in the future by exposing size-manipulated *Hydra* to increased tissue maintenance costs (e.g., by experimentally increasing temperature).

AUTHOR CONTRIBUTIONS

Réka Gergely: Conceptualization (supporting); data curation (lead); funding acquisition (equal); investigation (lead); methodology (equal); project administration (lead); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Jácint Tökölyi:** Conceptualization (lead); formal analysis (lead); funding acquisition (equal); methodology (equal); project administration (supporting); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

All authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

Data: Gergely, Reka; Tokolyi, Jacint (2022): data_resource_availability_modulates_the_effect_of_body_size_on_reproductive_development.xlsx. figshare. Dataset. https://doi.org/10.6084/m9.figshare.21428130.v1. Code: https://github.com/jtokolyi/Hydra-SizeFoodInteraction.

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APPENDIX

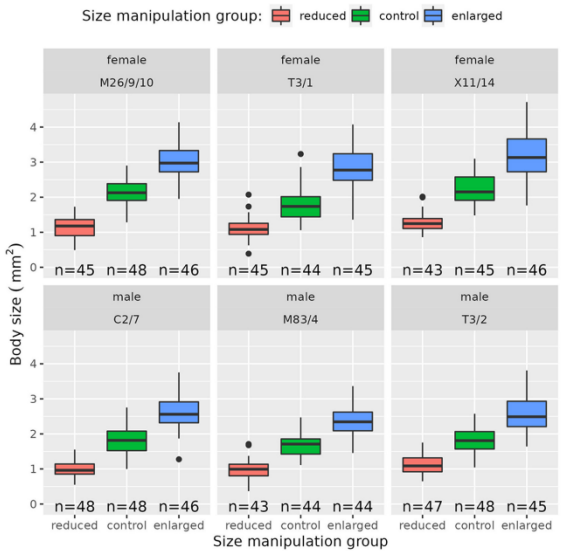

FIGURE A1 Body sizes after size manipulation in the three female (M26/9/10, T3/1, X11/14) and three male (C2/7, M83/4, T3/2) strains. Reduced (red box), control (green box) and enlarged (blue box) polyps' sizes on the *y*-axis in mm^2.

X. Study 2 (original article)

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Seasonal environmental change and sex change in a cnidarian

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In various animal species with distinct sexes, sequential hermaphroditism occurs whereby adults change their sex. The role of environmental factors in triggering sex change events is still relatively poorly understood, despite that climate-driven sex-ratio distortion may increase extinction risk. Here, we investigated the effect of seasonal temperature fluctuations on the expression of sex-related genes and sex change in the gonochoristic freshwater cnidarian, brown hydra (*Hydra oligactis*). Individuals of this species reproduce clonally throughout much of the year but switch to sexual reproduction when temperature decreases in autumn. After sexual reproduction, some individuals can revert back to clonality again. We found that clonal offspring derived from these individuals had a relatively high rate of female-to-male transition. The sexual stage was most differentiated in terms of gene expression, with a large number of genes involved in cellular differentiation and gametogenesis differentially expressed between males and females undergoing gamete production, while asexual individuals belonging to the male and female strain were transcriptomically nearly identical following sexual reproduction. These results show that seasonal environmental changes and associated life stage transitions 'homogenize' the gene expression profiles of males and females, ultimately enabling the emergence of alternative sexual phenotypes within strains with an otherwise stable sex.

1. Introduction

The vast majority of animals exhibit gonochorism, where distinct sexual phenotypes exist and male and female gametes are produced by separate individuals [1]. However, certain gonochoristic organisms possess the ability to switch their phenotypic sex, a phenomenon known as sex change or sequential hermaphroditism [1–3]. This means that an adult individual's sexual phenotype (e.g. testes and male secondary sexual characteristics) changes to the other phenotype (e.g. ovaria and female secondary sexual traits), and throughout its life the individual can reproduce first as male and later as female or vice versa. This type of sex change, as seen, for example in anemonefish, *Amphiprion percula* [4] is sometimes also called sex reversal, although recently the latter term is increasingly reserved for another phenomenon, whereby the phenotypic sex forming at the beginning of ontogeny deviates from genotypic sex. This latter sex reversal can only happen in organisms with genotypic sex determination (GSD; e.g. sex chromosomes), and the individual can only reproduce as one (phenotypic) sex during its life, as seen in various ectothermic vertebrates [5–7]. Sequential hermaphroditism, by contrast, does not rely on GSD and has

been reported for a wide range of animal phyla, such as Cnidaria, Porifera, Annelida, Entoprocta, Gnathostomulida, Mollusca, Platyhelminthes, Arthropoda, Echinodermata and Chordata [8].

Sex change is considered an adaptive strategy by which individuals can maximize their reproductive success under the prevailing internal and external conditions [4]. For sequential hermaphrodites, the well-known triggers of sex change are changes in the individual's social status (e.g. size, age) and social environment (e.g. population density, sex ratio) which create different fitness prospects for reproducing as a male versus female. For example, in the small reef fish *Trimma okinawae* female-to-male sex change occurs when the female becomes the largest individual in a social group, and it can switch back to female phenotype if the social environment changes again [4].

Abiotic environmental conditions may also have sex-dependent effects on fitness, and such effects may also influence individual decisions on developing one sexual phenotype or the other. This theory is best described, and supported by empirical data, for gonochoristic animals with environmental sex determination (ESD) where phenotypic sex develops predominantly as a result of environmental experiences during early life [9,10], but similar sex-dependent fitness effects may explain environmentally induced sex reversal in GSD [11]. Among the abiotic factors influencing ESD and sex reversal in GSD, temperature is the most prominent [7,10]. Temperature has key roles in mediating animal fitness and has important consequences for a species' ecology. Therefore, we might expect that the decision to change sex may depend on local thermal conditions in sequential hermaphrodites, too [12–14]. However, while the role of temperature in sex determination is well studied in species with ESD, its influence on decisions to switch sex in sequential hermaphrodites is much less well known, with only a few case studies in invertebrates [15–17]. Understanding the thermal drivers of sex change is important not only for advancing the field of evolutionary ecology but also for predicting the consequences of ongoing climatic changes. Climate-driven sex-ratio distortions increase extinction risk for species with ESD and environmental sex reversal [18,19], but we currently lack the knowledge necessary for evaluating such risk in species with sequential hermaphroditism.

Cnidarians are an intriguing taxon for studying the role of ecological factors in sex determination as they exhibit a wide range of sexual strategies and sexual plasticity. Among Cnidaria, hydrozoans, such as *Hydractinia*, *Clytia* and *Hydra* have garnered the most comprehensive understanding of sex determination and maintenance mechanisms [20]. This deeper understanding is primarily attributed to the discovery of interstitial stem cells (ISCs). ISCs play a role in the formation of the germline and various somatic cells, including nerve cells, gland cells, nematocytes, and, in some cases, epithelial cells [20,21]. Their somatic potential varies across taxa, with ISCs exhibiting multipotent or totipotent characteristics, depending on the species. In *Hydra*, the multipotent ISC retains the capacity to generate new germline stem cells (GSCs) when needed, but once created, the unipotent GSCs take charge of the majority of gamete production [20,21]. Downstream in the differentiation pathway, GSCs give rise to sperm-restricted stem cells or egg-restricted stem cells and the presence of these sex-specific stem cells is responsible for generating the sexual phenotype [21]. For instance, in some species male and female GSCs can differentiate at the same time and these species are considered 'simultaneous hermaphrodites'. In others, simultaneous male and female gamete production does not occur, but both gamete types can be generated within the same individual at distinct time points (i.e. these are sequential hermaphrodites) [22]. Finally, some hydra species are considered gonochoristic, such that an individual produces the same gamete types throughout its life. In these species, sex-specific stem cells undergo self-renewal within polyps (i.e. a hydra individual) and are typically passed on to a new bud during asexual reproduction from a parental polyp, giving rise to asexual lineages with stable sex [21–23]. However, rare sex change has been observed in this system in the laboratory [22,24]. For instance, male-to-female sex change was observed in the gonochoristic brown hydra (*Hydra oligactis*) when strains were cultured at higher temperature (22°C compared to the typical culture conditions of 18°C) in the laboratory [24]. Intriguingly, in a recent population-genetic study of the same species, relatively high rates of sex change have been inferred in natural populations [25], but the reasons for the discrepancy in sex-change frequency between laboratory and field populations are unclear.

Here, we aimed at investigating the role of seasonal environmental changes in sequential hermaphroditism in brown hydra. We hypothesized that seasonal variation in temperature and photoperiod observed in the natural environment could be at least partly responsible for the higher rates of sex change observed in the field. Therefore, we simulated seasonal temperature and photoperiod variation in the laboratory and determined sex of clonal lineages that experienced these temperature fluctuations.

From a proximal perspective, sex change could be considered a consequence of environmental conditions exerting their effects on the internal physiological and cellular state of animals. During sexual reproduction, GSCs undergo differentiation into gametes. When GSCs are abundant, the sexual phenotype remains stable because any appearance of GSCs of the opposite sex is suppressed by the existing ones (e.g. by signals produced by the residing germ cells; [26]). However, a decrease in the abundance of resident GSCs could lead to an increase in another type, potentially resulting in the emergence of an opposite sexual phenotype. This phenomenon has been observed in *Hydra vulgaris* under experimental conditions. When *H. vulgaris* polyps are induced to regenerate from a small piece of tissue lacking GSCs (specifically, the foot region), they frequently exhibit a sexual phenotype distinct from that of their parent [21,27]. Therefore, to gain additional insight into the sexual capacity of brown hydra individuals varying in their exposure to different environmental conditions, we performed a transcriptomic study in a male and a female strain along the asexual–sexual life cycle. While this method cannot directly track individual GSCs during development (a challenging task with current methodology), it nonetheless gives an overview of the regulation of sex-specific genes. We hypothesized that a greater disparity in gene expression would indicate a higher abundance of either GSCs or gametes at a specific life stage, thereby reflecting the differentiation level of the sexual phenotypes.

2. Methods

(a) Study system

Brown hydra is a facultatively sexual cnidarian, capable of both asexual and sexual reproduction. Typically, in highly seasonal habitats, this species primarily reproduces through budding (asexual reproduction) for most of the year. However, it shifts to sexual reproduction during late autumn/early winter, when temperature drops. In response to this cold stimulus, male polyps develop testes, while female polyps develop ovaries along their body column, which can be differentiated by the naked eye, and individual gonads can be counted with a stereomicroscope (figure 1A). Males initiate gonadogenesis about two weeks earlier than females and the formation and release of sperm in males takes place continuously for several weeks, while females form fertile eggs for a shorter time. This is the only period during their life cycle when the two phenotypic sexes can be visually separated [28–31].

Sexual reproduction results in gamete production. Male gametes are released into the water and fertilize eggs attached to females. Fertilization leads to the formation of resting eggs that can withstand harsh environmental conditions, such as freezing temperature. Following sexual reproduction, the majority of polyps experience a decline in somatic performance in the subsequent months, resembling a senescence-like degradation [32,33], a phenotype regulated by canonical Wnt/β-catenin signalling [34]. This post-reproductive senescence in *H. oligactis* is characterized by a reduction in the number of ISCs in the animal, a decreased ability to capture food, a decline in tactile movements, decrease in body size and an increase in mortality rate [32,35,36]. However, if the animals regain their feeding abilities, they have a chance at regeneration and can potentially undergo another round of sexual reproduction in the following breeding season (figure 1B).

(b) Sex change experiment

To investigate the sex ratio and the likelihood of sex change in *Hydra* after a full seasonal cycle, we used animals that had undergone sexual reproduction and survived the subsequent post-reproductive senescence phase, as follows (figure 1C). Individual animals were housed in six-well cell culture plates, with each well containing 5 ml of standard hydra medium. The hydra medium consisted of 1 mM Tris, 1 mM NaCl, 1 mM $CaCl_2$, 0.1 mM KCl and 0.1 mM $MgSO_4$, with a pH of 7.8 [32]. The animals were fed individually, receiving 20 µl of fresh *Artemia nauplii* suspension four times a week using an automatic pipette (FinnPipette). Approximately 2 h after being fed, the animals were transferred individually to a new six-well plate with fresh hydra medium.

We used three male strains (C2/7, M83/4, T3/2) and three female strains (X11/14, M26/9/10, T3/1) for the experiments reported here (a strain is a clonal line obtained from a single individual through asexual propagation; a female or a male strain is a clonal line that has the capacity to produce female or male individuals, respectively, in response to experimental triggering of gametogenesis). Animals were initially cultured at 18°C with a 12 L : 12 D cycle to simulate summer conditions (figure 1C '18°C, summer conditions, female strain, asexual female polyps') [37]. Subsequently, randomly chosen animals (approximately 36 polyps per strain) were transferred to a cooled incubator (Pol-Eko ST2) set to 8°C with an 8 L : 16 D cycle to simulate winter conditions, inducing sexual reproduction via cold stimulus. Following sexual reproduction (figure 1C '8°C, first sexual reproduction'), we collected the surviving animals to establish new lines, each consisting of one regenerated polyp and its clones (figure 1C '8°C, death or regeneration, starting new lines from regenerated polyps'). This was done with all the six strains in the 8°C incubator (approximately 24 new lines per strain). Through asexual budding, we expanded each line from one polyp to six. Once the six-well cell-culture plate was full, the animals were maintained for an additional two weeks (from the birth of the youngest polyp) at 8°C (figure 1C '+2 weeks'). Subsequently, the plate was transferred first to 12°C (8 L : 16 D cycle) then to 18°C to mimic summer conditions. This intermediate step was included because sudden warming is stressful for this species. To simulate summer conditions for the second time, the animals were kept at 18°C for three weeks (figure 1C '18°C, 1 week, accommodation (12°C), 3 weeks, summer conditions'), then we transferred them back to 8°C to induce sexual reproduction once more (figure 1C '8°C, second sexual reproduction, detection of sex change'). At 8°C, we determined the sex of the polyps when they developed gonads again. If an animal developed gonads not characteristic for the strain, i.e. testes in a female strain (figure 1C '1/5 line, marked with red box', male with testes) or ovaries in a male strain, then we considered those individuals sex-changed (figure 1A–C).

(c) Transcriptomic study

To investigate gene expression changes associated with seasonal changes of the reproductive cycle in male and female hydra polyps, RNA was isolated from one male strain (C2/7) and one female strain (X11/14) at distinct life stages. These life stages were selected to reflect both the asexual and sexual parts of the life cycle. Hence, the first two groups consisted of individuals maintained asexually at 18°C and were either freshly detached buds (1–3 days after detachment from an asexual parent; henceforth called 'buds 18°C') or three weeks old adult asexual polyps (asexual 18°C). The third group included polyps that were initially cultured at 18°C for three weeks and were subsequently transferred to 8°C for 2 days (2 days 8°C). This group served to reflect gene expression changes associated with sudden temperature reduction. The fourth group consisted of individuals that were first cultured asexually for three weeks at 18°C, then transferred to 8°C for two weeks (sexual 8°C). These individuals were undergoing gametogenesis and served to reflect gene expression patterns in sexual individuals. Finally, the last group consisted of asexual individuals that had reproduced sexually, survived post-reproductive senescence and reverted

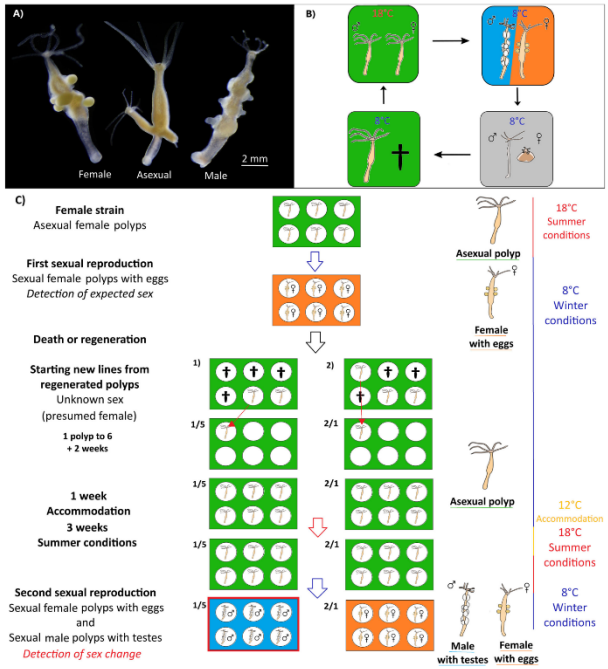

Figure 1. Brown hydra reproductive phenotypes (A), reproductive cycle (B), and flowchart of the experimental protocol (C). In (A), the three polyps are, from left to right, a female with ovaries, an asexual individual with two buds, and a male with testes. In (B), seasonal reproduction in the laboratory is illustrated, starting with asexual reproduction under summer conditions (with asexual male and female polyps; top left) through sexual reproduction under winter conditions (with a male polyp with testes and a female polyp with eggs along their body column; top right) and post-reproductive senescence (with senescent male and female polyps; bottom right) to either death ('dagger' symbol) or regeneration (asexual polyp), bottom left. (C) depicts our experimental design through an example of a female strain where in one line there is a sex change to male phenotype (line 1/5 marked with red box) while in the other line the sex was stable (line 2/1).

to asexual reproduction at 8°C (asexual 8°C). The animals selected for RNA isolation were different from those involved in the estimation of sex change frequency but were maintained under the same conditions.

For each group, three biological replicates were used per strain. Each replicate was obtained by pooling 2–3 polyps for RNA extraction, with the exception of the buds, which were smaller in size and 5–10 had to be pooled to obtain the required RNA amount. RNA was extracted using the protocol described in Tökölyi [38] and subsequently shipped to Novogene, Beijing for library preparation and sequencing. Library preparation involved preliminary quantification of RNA concentration in samples with Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA), checks for degradation and potential contamination with agarose gel electrophoresis, and checks for RNA integrity with BioAnalyzer 2100 (Agilent, Santa Clara, CA, USA). Following quality control, messenger RNA (mRNA) samples were enriched with oligo(dT) beads, then mRNA was fragmented randomly by adding fragmentation buffer, then the complementary DNA (cDNA) was synthesized by using mRNA template and random hexamers primer, after which a custom second-strand synthesis buffer (Illumina), dNTPs, RNase H and DNA polymerase I were added to initiate the second-strand synthesis. Second, after a series of terminal repair, a ligation and sequencing adaptor ligation, the double-stranded cDNA library was completed through size selection and polymerase chain reaction (PCR) enrichment. Library preparation was followed by quantification of concentration with Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA, USA), testing the insert size with BioAnalyzer 2100 and library quantification with quantitative PCR (qPCR). Finally, sequencing was performed on an Illumina Novaseq 6000 platform (paired-end 150 nt).

Sequencing reads underwent processing with fastp v0.23.4 [39] for the detection of adaptor contamination and elimination of low-quality reads. The parameters used were a quantified quality score of 30, a minimum required length of 50 bp, a sliding window from front to tail and tail to front with a window size of 4 bp, and a required mean quality of 30, with adaptor detection and base correction enabled. Subsequently, SortMeRNA v4.3.6 [40] was used to identify and remove ribosomal RNA contamination, employing the smr_v4.3_sensitive RNA reference database. To eliminate contamination from other organisms potentially present in the hydra or introduced before sequencing, a two-step decontamination process was conducted. In

the first step, reads were mapped against the *H. oligactis* high-quality draft genome [41] using HISAT2 v2.2.1 [42], and only concordantly aligned reads were retained. In the second step, non-aligning reads were subjected to mapping with Kraken2 v2.1.3 [43] against the prebuilt Kraken2 *nt* database (5/2/2023) to identify missing hydra sequences in the draft genome. Following Kraken2 taxonomy assignments, reads mapping to cnidarian sequences were preserved, and all other reads were discarded. The remaining reads, mapping to the *H. oligactis* genome and cnidarian sequences, were concatenated individually for each sample.

The *de novo* transcriptome assembly was conducted using Trinity v2.15.1 [44], employing the concatenated set of forward and reverse reads. Subsequent assessment of transcriptome quality involved the re-mapping of reads from individual samples to the assembly using Bowtie2 v2.5.1 [45] and BUSCO v.5.4.7 [46] completeness analysis, with the Metazoa_odb10 database as a reference.

Trinotate pipeline v4.0.1 [47] was used for transcript annotation. Initially, coding regions within transcripts were identified with TransDecoder v3.7.0 (https://github.com/TransDecoder/TransDecoder), followed by homology searches against the Swissprot database using blastx and blastp (ncbi blast v1.14.0, [48]) and the Pfam database with hmmscan using HMMER v3.3.2 [49].

Differential expression analysis was conducted using the DESeq2 v.1.40.2 package [50] in R v.4.3.1 [51]. Initially, transcripts with low and sporadic expression were filtered out. To do so, we required 10 counts or greater in at least three samples for transcripts to be retained in the analysis. Subsequently, DESeq2 analysis was performed with experimental groups (i.e. life stages, strain and their interaction) as predictors.

Principal components analysis (PCA) was performed to obtain an overview of gene expression differences between male and female strains at different life stages. To reduce the variance of the log-transformed read counts with low mean, variance-stabilizing transformation of the read counts was performed with the *vst* function [50].

Gene regulation differences between males and females were estimated by performing differential expression analysis between the two strains at each life stage separately in DESeq2. Log_2-fold change values were adjusted using the *apeglm* shrinkage estimator [52] to reduce overestimation of effect sizes owing to small sample size and high variability in transcriptomic data. Transcripts with an adjusted *p*-value <0.01 and $|log_2\text{-fold change}|>1$ were selected. Subsequently, these transcripts underwent gene ontology (GO) gene enrichment analysis with length bias correction using the GOseq v1.52.0 R package [53]. GO terms significantly enriched with an adjusted p <0.05 were selected. The resulting list of GO terms was clustered based on semantic similarity with the *binary_cut* function from the *simplifyEnrichment* package [54] to obtain groups of functionally similar transcripts that were differentially expressed.

Transcripts differentially expressed between sexes (adjusted *p*-value <0.01 and $|log_2\text{-fold change}|$) were compared across life stages separately for males and females. Co-expressing transcripts were identified through hierarchical clustering, and cluster functions were inferred by generating word clouds from the GO terms associated with the transcripts.

We also looked specifically for the expression of transcripts purportedly involved in sex determination in hydra. A recent study performed in *H. vulgaris* [55] uncovered that hydra GSCs have a substantial overlap in their genetic repertoire with mammalian primordial germ cells. In particular, hydra orthologues to bilaterian "Doublesex and Male abnormal-3" (DM) domain genes of *Doublesex (Dsx*) in insects and *Dmrt1* in vertebrates were inferred to have male-determining functions in hydra, while transcripts in the Boule/Boule-like (BOLL) RNA binding protein family were found to be expressed exclusively in GSCs and required for the meiotic progression of germ cells. In addition, the "Positive Regulatory Domain I-Binding Factor 1 and Retinoblastoma protein-Interacting Zinc finger protein 1" (PR) domain zinc finger proteins (*Prdms*) were inferred to have a putative role in germline determination. To understand the potential role of these genetic factors in *H. oligactis*, we plotted the expression pattern of *Dmrt*, *Boule/Boll* and *Prdm* transcripts from the *H. oligactis* transcriptome across the five life cycle categories and looked for increases/decreases in the expression of these transcripts in sexual relative to asexual individuals.

3. Results

(a) Sex change in response to seasonal temperature change reveals protogyny in brown hydra

In the second reproductive cycle, sex change occurred exclusively in strains that were initially female. Specifically, the proportion of animals that transitioned to the male phenotype varied between 36.5% and 60%, while the rest either retained the female phenotype or remained asexual (figure 2). In the originally male strains, 71%–94.2% of the polyps retained the male phenotype while the rest remained asexual (figure 2).

(b) Male and female differentiation in gene expression is reduced in asexual individuals

We obtained an average of 19.8 million paired-end reads per sample (range: 16.8–24.3 million; see the electronic supplementary material, table S1 for detailed information on sequencing read statistics) after performing quality control and decontamination. The PCA revealed the greatest differences in gene expression between sexually reproducing males and females, relative to each other and to the other life stages (figure 3A). Based on the first two principal components that collectively explained 51% of total variance, individuals from male and female strains were relatively similar to each other in the bud stage, and adult females cultured on 18°C were similar to this group (figure 3A). The lowest differentiation between gene expression of males and females was observed in asexuals cultured at cold temperature (Asex. 8°C, figure 3A). Supporting these observations, the number of differentially expressed transcripts was highest in the sexually reproducing individuals and lowest in buds and asexuals cultured at low temperature (figure 3B–F).

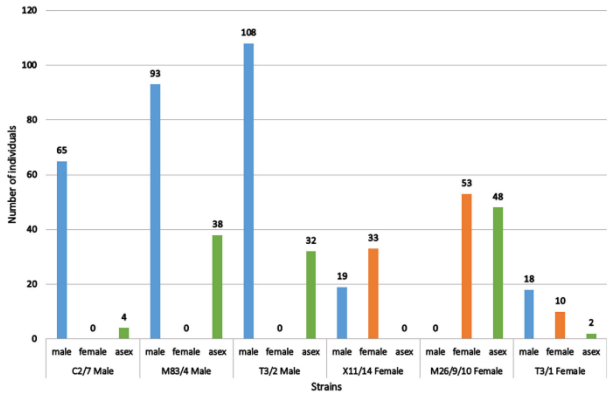

Figure 2. Number of male and sex-changed male (blue), female and sex-changed female (orange), and asexual (green) individuals in the second reproductive cycle of the experiment in the three male and three female strains.

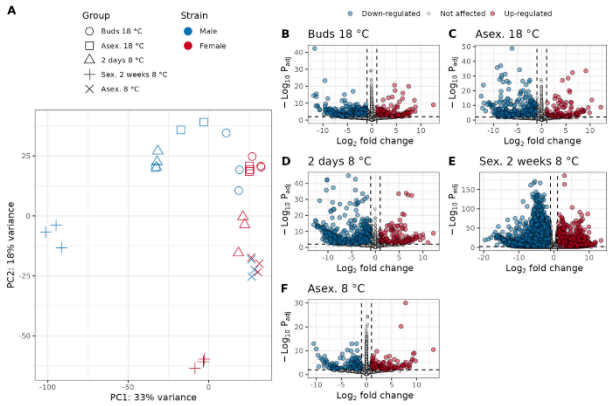

Figure 3. (A) Principal components analysis comparing gene expression of a male and female strain of *H. oligactis* from five distinct stages of the asexual–sexual life cycle: asexual buds at 18°C, asexual adults (three weeks after detachment from their asexual parent) at 18°C, asexual adults after 2 days at 8°C, sexually reproducing polyps (after two weeks at 8°C) and asexual polyps cultured at 8°C. (B–F) Volcano plots showing gene expression differences between males and females at each distinct life stage. Genes that are overexpressed (positive $\log_2$-fold change; shown in red) are female-specific genes, while those underexpressed (negative $\log_2$-fold change; shown in blue) are male-specific genes.

To find out the functional roles of differentially expressed transcripts, we performed GO over-representation analysis and clustered over-represented GO terms based on semantic similarity. Most over-represented GO terms were found within the sexual individuals (figure 4). Several GO terms over-represented in males clustered into functional groups involved in spermatozoa synthesis (e.g. meiosis, cilium assembly, nucleotide synthesis). Most of the GO terms within these clusters were over-represented in sexual individuals, but a few were also over-represented in the buds 18°C, asexual 18°C and the 2 days 8°C life stages preceding the sexual stage (figure 4A). However, there were no male-specific over-represented GO terms in asexual individuals cultured at cold temperature (figure 4A). Female-specific over-represented GO terms were mostly found in the sexual stage and a few in the bud stage, while other stages did not show female-specific GO terms (figure 4B). Most female-specific over-represented GO terms were associated with cell differentiation and not specifically with female gamete generation.

Next, we looked at patterns of differential expression across the life stages separately for the two strains. In males (figure 5), many transcripts were strongly upregulated in the sexual individuals. These transcripts were otherwise expressed at low

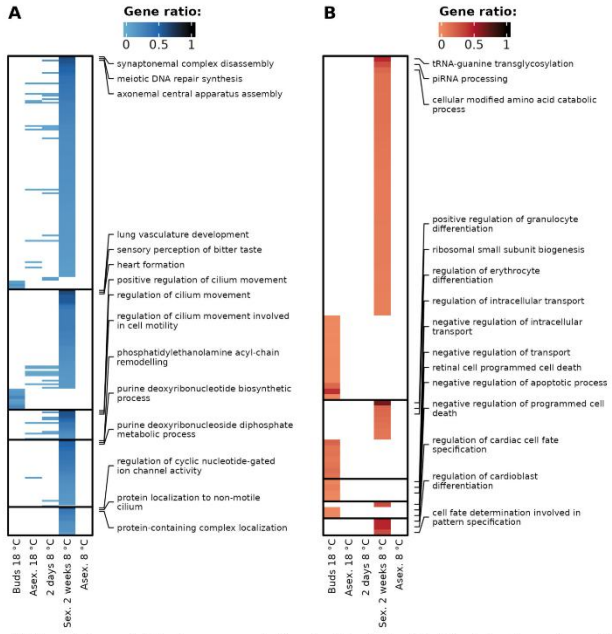

Figure 4. Gene ontology (GO) terms that were significantly over-represented in males (A) or females (B) relative to the other sex in one of the five life stages. Gene ratio is the number of differentially expressed transcripts associated with a GO term, relative to the total number of transcripts associated with that term. Darker colours indicate GO terms that were more strongly over-represented among the differentially expressed transcripts. GO terms within each cluster were ranked based on mean gene ratio and the name of the GO terms with the highest mean gene ratio are shown on the plot for each cluster.

levels and had functions in cell cycle and cell division (both mitotic and meiotic), probably reflecting the advanced stages of spermatogenesis happening in these individuals. Buds and asexual individuals at both temperatures showed overexpression of transcripts that were associated with metabolism, biosynthesis and catabolism, but interestingly, two different sets of these transcripts were upregulated at low versus high temperature (see clusters 5, 9 and 11 versus clusters 3, 6 and 12 in figure 5). A small group of transcripts associated with autophagy was specifically upregulated 2 days after lowering the temperature (cluster 4 in figure 5).

In contrast to males, there were fewer transcripts upregulated in the sexual individuals among females (figure 6). A large cluster was specifically associated with females undergoing gonadogenesis (the sexual 2 weeks 8°C group), and this included transcripts associated with metabolism, development and differentiation (cluster 4 in figure 6). Another cluster included transcripts downregulated in females undergoing gonadogenesis (cluster 9), and this cluster included genes associated with metabolism and biosynthesis. Several clusters were associated with buds, as well as asexual individuals at low or high temperature and these included transcripts involved in metabolism (biosynthesis or catabolism), mRNA alternative splicing and transmembrane transport processes (figure 6). In addition, a cluster involving transcripts with roles in autophagy was upregulated after lowering temperature, just as in males (cluster 10 in figure 6).

We found four differentially regulated *Dmrt* transcripts in our transcriptome database, which belonged to two genes, *Dmrt2* and *DmrtA* (figure 7A). Three of these transcripts showed an expression pattern suggesting an involvement in sex determination (i.e. they were differentially expressed in sexual individuals, relative to asexuals at both temperatures). Two *Dmrt2* transcripts were upregulated in male sexual individuals, while a third was downregulated (figure 7A; electronic supplementary material, figure S1). In *Boule/Boll* transcripts, there were in total 17 differentially regulated transcripts, most of which were substantially upregulated either in male or female sexual individuals (figure 7B; electronic supplementary material, figure S2). Finally, among 18 differentially regulated *Prdm* transcripts, four were upregulated in sexual versus asexual individuals in the female strain (*Prdm5*: DN19508_c0_g1_i4, *Prdm6*: DN1311_c0_g1_i2, *Prdm9*: DN6269_c0_g1_i2 and i12), while five were upregulated in sexual versus asexual individuals of the male strain (*Prdm9*: DN644_c4_g1_i1 and i2, DN9823_c1_g1_i1, i3 and i5; figure 7C; electronic supplementary material, figure S3). Importantly, for all these transcripts, asexual individuals cultured at 8°C showed very low expression, being most similar to buds cultured at 18°C, suggesting that these life stages had small amounts of the sex-determining factors or a reduced number of cell populations expressing these sex-determining genes.

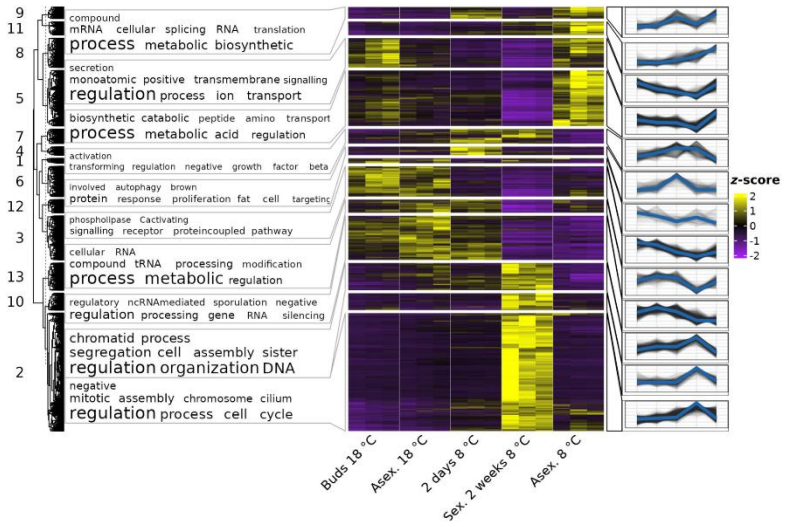

Figure 5. Patterns of differential expression in the five life stages of the male strain. The heatmap shows differentially expressed transcripts with $|\log_2\text{-fold change}|>1$ and adjusted p-value <0.01. Transcripts were clustered based on their expression patterns using hierarchical clustering and subsequently grouped into clusters by cutting the tree at a constant height. Numbers on the left denote cluster IDs. The mean expression within clusters is shown on the right side. Word clouds constructed from GO terms are presented on the left, with word sizes proportional to their frequency among the GO terms associated with their cluster.

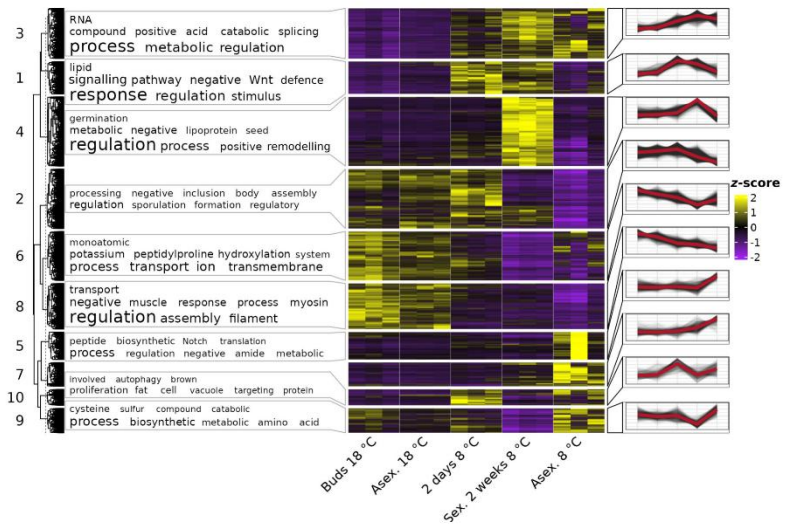

Figure 6. Patterns of differential expression in the five life stages of the female strain. The interpretation of the figure elements is the same as in figure 5.

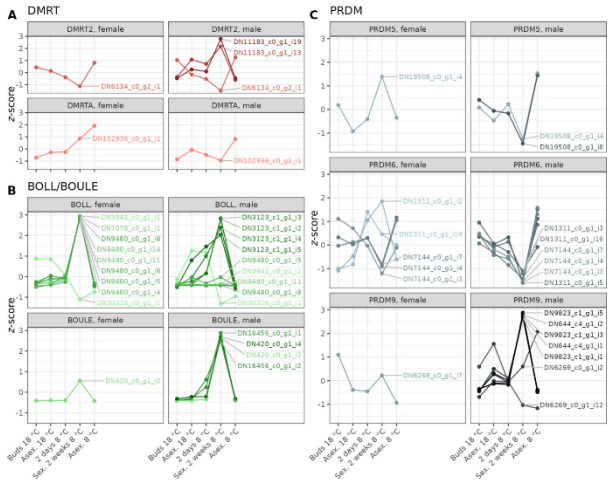

Figure 7. Expression of genes supposedly involved in sex determination in brown hydra across the life cycle: (A) two *Dmrt* genes, (B) two Boule-like (*Boll*)/*Boule* genes and (C) three *Prdm* genes. Each line in each panel corresponds to an isoform transcribed from the same gene (e.g. owing to alternative splicing). Transcript IDs generated by the Trinity software (starting with 'DN') are shown on the graph along each transcript. Each isoform is shown by a different colour.

4. Discussion

While ample evidence shows that environmental temperature plays a crucial role in early life sex determination in species with ESD and in species with GSD with sex reversal, much less information is available about the effects of abiotic environmental changes on the sexual phenotype of adults in species with sequential hermaphroditism. In this study, we exposed hydra polyps to alternating high and low temperatures, mimicking seasonal temperature variation with accompanying photoperiod changes. We found that clonal lineages subjected to these conditions underwent gonadogenesis and showed occasional female-to-male sex change in a subsequent round of gonadogenesis. Although the brown hydra is classified as a gonochoristic species, where individuals typically express a single sexual phenotype that is asexually transmitted to clonal offspring, sporadic occurrences of sex change have been noted in this species, e.g. male-to-female transition in strains cultured at high temperature [24]. However, attempts to replicate male-to-female sex change observations have been so far unsuccessful in our laboratory (J. Tökölyi and R. Gergely, 2023, unpublished data). By contrast, our present study demonstrated that simulated seasonal changes in temperature and photoperiod were followed by occasional female-to-male transitions in the strains designated as female, whereas no male-to-female sex change occurred across multiple strains. These instances of sex change resemble sequential hermaphroditism, although the terminology is more complicated for modular animals such as hydra. The classification scheme introduced by Wasson & Newberry [56] considers sexual mode at various levels: the individual level (e.g. polyps, ramets, or clonemates), the genotype level (genet or clonal lineage) and the colony level (relevant for species where individuals remain connected). At the individual level, *H. oligactis* can be considered gonochoristic, as individual polyps develop a single gonad type. However, our findings suggest that at the genotype level, *H. oligactis* can be considered hermaphroditic, because a single genotype can produce both sexes. Thus, *H. oligactis* aligns with the SG category in Wasson & Newberry's [56] classification (genet sequentially hermaphroditic, module gonochoristic).

What potential adaptive advantages might sex change confer in this system? In the context of hydra's clonality, maintaining a strictly gonochoristic reproductive strategy could pose fitness challenges. The proliferation of clones and subsequent reduction in clonal diversity could lead to a scenario where only one sex is present during the reproductive phase, effectively compromising sexual fitness. While hydra polyps can disperse longer distances via floating on the surface of water, it is unclear whether this dispersal potential is able to maintain access to the opposite sex under natural conditions. Additionally, given that clonal lineages in this species can persist in a population for multiple years [57], there exists a prolonged window for clonal expansion, which could result in the stochastic loss of either sex, especially in small and fragmented populations. Nevertheless, if sex change in hydra would be a response to local sex ratio, we should have observed it with similar frequency in both directions, as both male and female strains were housed individually in our experiment. Alternatively, males and females might perceive these conditions differently: for males, lack of other males could signal an ideal condition where they can increase their reproductive success by producing lots of sperm that fertilize a large number of eggs. For females, the lack of males could signal low expected fertilization success, which could shift the perceived reproductive pay-off of being a female and provide a cue

for female-to-male transition. Future experiments could test this hypothesis by investigating sex change in hydra at different population densities and sex ratios.

Upon comparing the transcriptomic profiles of animals subjected to different temperatures, we observed variations in gene expression differences between males and females across life stages. Not surprisingly, significant differences were evident in individuals undergoing gametogenesis. However, some disparities were also observed in stages preceding gametogenesis, suggesting that asexual individuals cultured under summer conditions already exhibit, at least partially, the expression of sex-related genes, potentially accounting for the stability of sex in these strains at constant high temperature. Conversely, in post-reproductive individuals, these differences diminished, with individuals from both male and female strains exhibiting strikingly similar transcriptomic profiles, including at the level of putative sex-determining genes. In that life stage, few differentially expressed genes were identified comparing the male and the female strain, and these did not cluster into any GO categories. Consequently, it appears that sex-related genes are expressed at low levels in asexual individuals cultured at low temperature. These findings suggest that GSCs may become depleted during sexual reproduction, possibly through differentiation into gametes. Polyps surviving sexual reproduction may lack residual GSCs and consequently must undergo redevelopment from somatic multipotent stem cells. The observation that only female-to-male transition has been observed in our experiment could indicate that female GSCs are costlier to produce under the particular conditions when GSCs are re-developed, although currently we have no evidence for a sex-dependent cost of reproduction in this system.

Overall, our findings are consistent with earlier observations in this species. In a prior investigation, asexual *H. oligactis* polyps exhibited low sexual propensity (probability of initiating gametogenesis in response to cold stimulus) in early spring but demonstrated a heightened sexual propensity by late summer, ensuring the appropriate timing of sexual reproduction and production of resting eggs before the onset of winter freezing [31]. On the proximal level, this shift could potentially be attributed to the diminished abundance of GSCs in early spring. Such reduced abundance may create a developmental environment conducive to the emergence and proliferation of GSCs of the opposite sex, thereby facilitating the occurrence of sex-changed individuals. This process might be classified as random sex determination, whereby the sexual phenotype is set by stochastic processes rather than genotypic or environmental factors [58]. Further research would be necessary to identify and track these GSCs throughout hydra development, thereby conclusively testing this hypothesis.

In conclusion, we have shown here that environmental cues characteristic of seasonal environmental change, and the associated alternation of sexual and asexual life stages, are associated with sex change in an invertebrate, most likely through altering the differentiation dynamics of the cell lineages responsible for generating the sexual phenotype. It remains to be tested if sex change in brown hydra is an adaptive strategy, similarly to other species with sequential hermaphroditism, serving to maximize fitness by adjusting the sexual phenotype to the local availability of mates or to individual state such as ageing. Our findings suggest that such adjustments can happen only when dropping temperatures trigger sexual reproduction after regeneration. Therefore, our results raise the possibility that rising global temperatures and ensuing mild winters might disrupt sex ratios in hydra populations, by reducing the chances of adaptive sex adjustment by sex change events, with downstream consequences for the demography of these populations and ultimately, the ecosystems they inhabit. Further studies will be required to understand the cellular mechanism behind sex change in hydra, the sex-biased expression of sex change and the implications of this phenomenon for the species' population dynamics and life-history evolution.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. Code availability [59]. Dataset uploaded to Dryad [60]. It includes the phenotype data from the sex change experiment, plus the de novo transcriptome and read counts from the RNA-Seq experiment. Raw sequencing data https://www.ncbi.nlm.nih.gov/bioproject/PRJNA937400.

Supplementary material is available online [61].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. R.G.: data curation, funding acquisition, investigation, methodology, visualization, writing—original draft; V.B.: writing—review and editing; Z.B.: writing—review and editing; J.T.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, validation, visualization, writing—original draft.

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