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Lack of metabolic ageing in the long-lived flatworm *Schmidtea polychroa*.

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Abstract

Freshwater planarians have a large totipotent stem cell population allowing high rates of cell renewal and morphological plasticity. It is often suggested that they are able to rejuvenate during fission, regeneration and starvation. These features, together with the rapidly expanding molecular toolset, make planarians such as *Schmidtea polychroa* and *S. mediterranea* interesting for ageing research. Yet, the basic demographic and physiological data is lacking or still based on fragmentary observations of one century ago.

Here, we present the first longitudinal physiological study of the species *Schmidtea polychroa*. Survival, size and metabolic rate, measured by microcalorimetry, of a cohort of 28 individuals were followed over a period of three years. Sexual maturity was reached during the second month after which the worms continued growing up to 5 months. This initial growth phase was followed by alternating periods of synchronized growth and degrowth. Although mass-specific metabolic rates declined during the initial growth phase, no changes were found later in life. The absence of metabolic ageing may be explained by the very high rate of cell renewal during homeostasis and alternating phases of degrowth and growth during which tissues are renewed.

Surprisingly, all deaths occurred in pairs of worms that were housed in the same culture recipient, suggesting that worms did not die from ageing. Taking into account the metabolic and demographic data, we suggest that *S. polychroa* shows negligible ageing.

Detailed analyses of size and metabolic rate revealed a remarkable biphasic allometric scaling relation. During the initial growth phase (month 1-5) the allometric scaling exponent b was 0.86 while later in life, it increased to an unusually large value of 1.17, indicating that mass-specific metabolic rate increases with size in adult *S. polychroa*.

Keywords: Planarian, Ageing, Metabolism, Allometry, Stem cells

1. Introduction

Due to their high regenerative capacity and active stem cell population, freshwater planarians have drawn attention of biogerontologists. These worms maintain a stable population of totipotent stem cells, which are the only proliferating cells in the adult body (Baguña and Slack, 1981; Newmark and Alvarado, 2002). These cells, called neoblasts, are responsible for the very high rate of somatic cell renewal during normal tissue homeostasis (Pellettieri and Alvarado, 2007). They also allow an impressive morphological plasticity of the planarian during fission (asexual reproduction), regeneration (ability to regenerate a complete organism from any tiny body fragment), and starvation (controlled shrinkage during prolonged starvation) (Salo, 2006).

Interestingly, these three processes are often claimed to induce rejuvenation in these worms. This is based on the observations of an everlasting clonal lifespan in asexuals, a lifespan extension after inducing regeneration or starvation and the classical metabolic experiments of Child and Hyman (Child, 1911; Child, 1915; Egger et al., 2006; Haranghy and Balázs, 1964; Hyman, 1919a; Hyman, 1919b; Hyman, 1919c). Child and Hyman indirectly measured oxygen consumption by means of the susceptibility and Winkler methods and found that planarian metabolic rate appeared to decrease with age. After fission, regeneration and starvation, they noticed that metabolic rates were restored to juvenile levels, leading to the conclusion that these are rejuvenating transformations (Child, 1911; Child, 1915; Hyman, 1919a; Hyman, 1919b; Hyman, 1919c). These metabolic data should be interpreted with caution. Hyman studied metabolic changes only during development and not during the ageing process (Hyman, 1919a). Child measured metabolic rates during both development and adulthood, but the adults were selected based on their size, which was used as a proxy of physiological age (Child, 1911; Child, 1915). Because adult planarians may repeatedly grow and shrink during adulthood, this study is severely flawed and little can be derived concerning

metabolic patterns in ageing planarians. In addition, the rejuvenation hypothesis became equivocal as later metabolic studies of fission, regeneration and starvation reported contradictory results (Allen, 1919; Brøndsted, 1969; Pedersen, 1956). Surprisingly, no genuine longitudinal metabolic studies of ageing flatworm cohorts have been performed and it is yet unknown whether these animals show a clear metabolic decline at advanced age or whether metabolic rejuvenation occurs after regeneration or starvation.

In the three-year longitudinal study presented here, we analyse the correlation among size, age and metabolic rate during both development and adulthood of the planarian *Schmidtea polychroa*. Our data provide evidence that mass-specific metabolic rate decreases during the initial growth period, but remains constant during adulthood. Our demographic data suggests that this planarian species may not age significantly after all. We further show a remarkable morphological plasticity in our flatworm cohorts and suggest that there is an age-related biphasic allometric scaling relation.

2. Material and methods

2.1. Species, culture and design of the longitudinal experiment

Schmidtea polychroa is a free-living freshwater planarian (Platyhelminthes). Because it is a cocoon-laying species, the lifespan can be simply defined as the time from hatching till death. Standard lab cultures were maintained in square plastic containers with a surface of about 200 cm², filled with about 300 ml 1:1 tap water:distilled water and incubated at 20°C in the dark. The worms were fed weekly with raw veal liver. Small pieces of liver, purchased locally every few months, were kept at -20°C and before feeding, several of these pieces were chopped into thin slices while they thawed. After worms stopped showing feeding behaviour (usually about 4 hours) cocoons were collected, containers were cleaned and the medium was renewed. The collected cocoons were kept in a Petri dish with fresh medium.

For the longitudinal study, individuals of the same age were obtained by collecting juveniles that hatched within a 24-hour time frame from several cocoons produced by a batch of about 30 parthenogenic adults. The experiment started with 14 replicates. Each replicate contained 2 animals as sufficient biomass was necessary for accurate microcalorimetry. The experimental animals were maintained, per replicate, in a Petri dish under the same conditions as the standard cultures. Survival was scored weekly, after feeding. If one of the two individuals died, the replicate was excluded from further metabolic measurements. Metabolic rate was measured monthly, three days after feeding to control for the influence of food intake and digestion on metabolic rate. One day after microcalorimetry, pictures were taken for automated analysis of body surface. As accurate life tables of *Schmidtea polychroa* do not exist, we ran the study until the median lifespan of the cohort was reached.

2.2. Measuring metabolic rate and normalization to body surface

During the longitudinal study, metabolic rate and a normalization parameter were measured in a non-destructive manner. Metabolic rate was determined by means of microcalorimetry, a very accurate technique that directly measures metabolic heat which reflects total catabolism, including fermentation (Braeckman et al., 2002a; Braeckman et al., 2002b). The metabolic heat produced by each replicate was quantified with the thermal activity monitor from Thermometric (Järfälla, Sweden). Before measurement, the two worms of a replicate were transferred to a 20-ml glass ampoule containing 2 ml culture medium. After a 1–2 h equilibration period, stable power (μW) recordings were obtained for at least 3 hours at 24°C. Mass-specific metabolic rate was obtained by dividing the total heat production rate of a replicate by the summed surface area of the two worms. Worm surface area was measured by transferring the slow moving animals to a moist rolling paper. At each time point, five replicate photographs of each individual were taken with an Olympus C-5050 Zoom camera

mounted on an Olympus SZX12 stereo microscope. Body surfaces were quantified by semi-automated image analysis (KS400 software, ZEISS).

To verify whether body surface area is an appropriate normalization parameter, body surface and the commonly used biomass proxies protein content and wet weight were measured in 48 individuals of different sizes. We found a highly significant correlation between body surface and both protein content ($R^2 = 0.97$; $P < 0.0001$) and wet weight ($R^2 = 0.97$; $P < 0.0001$) (Figure 1).

2.3. Statistical analyses

The accuracy of body surface as normalisation parameter was verified by performing Pearson's product – momentum correlation tests between body surface, wet weight and protein content.

Because of the limited population size, mortality rates were calculated over 5-month intervals. To test if the log mortality rate significantly increases with age, a linear regression analysis was performed.

To investigate whether age and/or size had a significant effect on mass-specific metabolic rate, a linear mixed model was fitted to the data (Proc mixed SAS 9.2). Here, we related the effect of 'age', 'body surface' and their interaction 'age*body surface' to the mass-specific metabolic rate. Both independent variables were treated as continuous variables. As the same replicates were repeatedly measured over time, replicate and its interaction with age were included as random effects. Detailed analyses were performed on the following data subsets: development (month 1-3), the initial growth phase (month 1-5) and adulthood (≥ 3 months). For the determination of the scaling exponent b during the complete lifespan, we log-transformed all body surface and metabolic rate data. Data from worm replicates undergoing the typical severe degrowth ($< 35\text{mm}^2$) which is preceding death were censored. Next, linear

regression was performed and the parameter values were determined. This function can be written as the log form of the allometric scaling law, $\log Y = \log Y_0 + b \cdot \log M$, in which b represents the slope. Y represents the metabolic heat production rate, M is the total body surface and Y_0 is a constant characteristic to the species (West et al., 1997). To test whether the b -value differed significantly from 0.75 and 1, one sample median tests were performed. To study the allometric scaling relation in several data subsets in detail, linear regression analyses of the log-transformed body surface and mass-specific metabolic rate data of the complete lifespan, initial growth phase (month 1-5), adulthood (≥ 3 months), adult growth (17-22, 25-27 months), and adult degrowth (20-23, 27-29 months) were carried out. Phases of adult growth and degrowth during these two periods were determined per replicate. Furthermore, Mann-Whitney U Tests were performed to compare young growing adults (3-5 months old) with all older adults showing morphological plasticity on the one hand and to compare adult growth with degrowth during above mentioned periods on the other hand. To compare the mass-specific metabolic rate of consecutive growth phases (9-11, 17-20, 25-27 months), a linear regression analysis was performed.

All analyses were performed with Statistica 7, except the linear mixed model for which SAS 9.2 was used. In all statistical analyses, we considered effects significant at a P level < 0.05 .

3. Results and discussion

3.1. *Schmidtea polychroa* shows high morphological plasticity during its long lifespan

During the three-year period of the experiment, we observed an impressive morphological plasticity with advancing age. After sexual maturation during the second month, the initial growth phase continued up to the fifth month in life. From then onward, the flatworms undergo alternating phases of degrowth and growth which occur rather synchronically between (Figure 2a) and within (Figure 2b) all replicates. This remarkable phenomenon is

hard to explain. A seasonal clock could underlie physiological fluctuations in animals kept under laboratory conditions (Pengelley and Asmundson, 1969), but the body size fluctuations we observed did not show a clear-cut circannual rhythm. Abiotic culture conditions (temperature, medium type, continuous darkness, constant feeding schedule) were kept constant during the complete experiment. However, small variations in the composition of the medium or phytochemical contaminants in the liver batches may have influenced the dramatic periods of growth or degrowth.

In contrast to the classic developmental models, in which development takes place only once and the pattern becomes fixed until the next generation, planarians seem to regulate their morphogenetic process continuously (Salo, 2006). During adulthood, the total body surface per replicate (two worms) varies between 35 and 100 mm². However, death is usually preceded by a period of intense degrowth, resulting in small worms of juvenile-like size ($\leq 35\text{mm}^2$ per two worms), some of which showed some deformations. It would be interesting to find out whether this is a process of controlled degrowth or an uncontrolled shrinkage because of compromised cellular homeostasis. To our surprise, this phenomenon seemed to be contagious as it was always observed in both individuals of the same replicate (Figure 2c).

We cannot exclude pathological effects although we found no clear signs of bacterial or fungal infection, which are known problems in planarian cultures (Haranghy and Balázs, 1964; Oviedo et al., 2008). The typical symptoms such as a bad medium odour, the presence of lesions and tissue-loss at the anterior end were not present.

The replicate worm pairs also died together in a narrow time interval of 2 to 12 weeks (Table 1). The median lifespan of the experimental cohort was reached at the age of 34 months (Figure 3a), when both individuals of seven replicates died. In each of the remaining replicates, both individuals were still alive. A median lifespan of 34 months is much larger than expected based on the existing literature. Detailed mortality tables of planarians are not

available, but there are some reports on old age and maximum lifespan. For *S. polychroa*, Voigt (1928) observed a maximum lifespan of 9.5 months at room temperature and 43.5 months for individuals kept on a meagre diet in the cold, two conditions that are known to extend the lifespan. Lindh (1957) described two-year old individuals as being “very old” (see references in Haranghy and Balázs, 1964).

Demographically, ageing is demonstrated by the exponential increase of the age-specific mortality rate and a resulting sigmoid shape of the survival curve (Arking, 1998). In non-ageing animals, where death is caused by random external influences, the survival curve is L-shaped (Arking, 1998). Here, the mortality rate did not seem to increase significantly ($R^2 = 0.51$; $P = 0.17$) with age and the obtained survival curve is rather linear (Figure 3b). Due to the small initial population size ($n=28$) it is impossible to obtain high resolution demographic data and draw any conclusions about ageing rates. However, the combined observation that the median lifespan of these small animals was almost three years and that most animals died in pairs, suggests that ageing rates may be negligible.

3.2. Do young flatworms have an increased mass-specific metabolic rate?

The highest peak of mass-specific metabolic rate was observed in one-month old individuals ($1.05\mu\text{W}/\text{mm}^2$, Figure 4), hinting at a decreasing metabolic rate during development. Indeed, during the initial growth phase (month 1-5), both age ($F_{1, 13} = 5.85$; $P = 0.03$) and size ($F_{1, 13} = 6.94$; $P < 0.01$) were found to have a significant negative effect on mass-specific metabolic rate. Furthermore, their effect is synergetic ($F_{1, 27} = 10.87$; $P = 0.003$). In other words, the mass-specific metabolic rate decreases as animals are growing during the first months of their life. This observation seems to confirm the conclusion of Child and Hyman, postulating that small (juvenile) planarians maintain higher mass-specific metabolic rates than large (mature) individuals (Child, 1915; Hyman, 1919a).

3.3. Mass-specific metabolic rate does not decline during adulthood

We anticipated a decline of mass-specific metabolic rate during the ageing process of *S. polychroa*. This would have been an interesting starting phenotype to test potential metabolic rejuvenation after starvation or after inducing regeneration of specific body parts. To our surprise, we could not detect a significant decline in mass-specific metabolic rate over time in adults ($F_{1, 13} = 0.05$; $P = 0.82$) (Figure 4). The absence of metabolic ageing can be explained by high rates of cell renewal during homeostasis (Pellettieri and Alvarado, 2007). As old and damaged cells are continuously replaced by new cells, produced by the neoblasts, the body may maintain a metabolically young status. One should keep in mind that each tissue type may have a unique cellular turnover rate and may age at different pace (Rando, 2006). Yet, nothing is known about tissue-specific turnover rates in planarians and replicative ageing of the neoblasts. Additionally, the maintenance of a youthful status during adulthood may also be promoted by alternating phases of degrowth (cell removal) and growth (tissue formation). Considering the high mass-specific metabolic rates found in juveniles, it would be reasonable to expect temporary increases of mass-specific metabolic rate during adult growth phases and corresponding declines during periods of degrowth. This assumption is not supported by our data; no significant difference ($P = 0.62$) could be found in mass-specific metabolic rate during growth and degrowth. Thus, mass-specific metabolic rate appears constant over adult age. We also asked whether mass-specific metabolic rates decreased over consecutive cycles of regrowth, which may possibly represent an estimate of stem cell ageing. We compared the mass-specific metabolic rate of replicates exhibiting growth during the periods 9-11 months (0.67 ± 0.08 standard deviation (s.d.) $\mu\text{W}/\text{mm}^2$), 17-20 months (0.79 ± 0.12 s.d. $\mu\text{W}/\text{mm}^2$) and 25-27 months (0.68 ± 0.09 s.d. $\mu\text{W}/\text{mm}^2$). Linear regression demonstrated that there is no decreasing trend ($R^2 = 0.01$; $P = 0.32$) which is not indicative for stem cell ageing.

In other vertebrate and invertebrate species, both metabolic constancy and decline have been observed during ageing (Chappell et al., 2003; Galbraith et al., 1999; Greenberg, 1999; Keys et al., 1973; Klausen et al., 1997; Lecomte et al., 2010; Moe et al., 2007; Moe et al., 2009; O'Connor et al., 2002; Raman et al., 2007; Ruggiero et al., 2008; Speakman et al., 2003; Sukhotin et al., 2002; Vanfleteren and DeVreese, 1996). The underlying biological reason for this discrepancy is not clear but it may reflect the relative importance of somatic maintenance in the animal. It would be interesting to study age-dependent mass-specific metabolic rate in more flatworm species, and especially those reproducing through fission and those with limited morphological plasticity, regeneration capacity and cell renewal.

In contrast to the initial growth phase, there is an age-independent, significant positive effect of size on mass-specific metabolic rate in *S. polychroa* (size effect: $F_{1, 13} = 6.42$; $P = 0.02$; age*body surface: $F_{1, 304} = 0.22$; $P = 0.64$). This positive effect is rather exceptional and therefore, we analysed the relation between metabolic rate and size in more detail.

3.4. The allometric scaling relation has an age-related biphasic pattern

The significant positive correlation between size and mass-specific metabolic rate in adult *S. polychroa* is in contrast with the general negative allometric scaling relation, predicting that metabolism per unit body mass is decreasing with size according to the Brody-Kleiber equation (Finch, 1990; West *et al.*, 1997). To study the allometric scaling relation in more detail, we calculated the scaling component b of specific data subsets. A negative allometric scaling relation is reflected by a b -value smaller than 1, while a positive relation is indicated by a b -value larger than 1.

When b was determined over the whole lifespan, including the juvenile phase (but censoring data of premortal degrowth as pathological effects could not be excluded), we obtained a value of 0.90 (± 0.03 standard error (s.e.)) while Y_0 was 0.04 (± 0.05 s.e.) ([Figures 5 and 6](#)).

In contrast to most other species, the metabolic rate of *S. polychroa* does not obey the three quarters power scaling law ($b = 0.75$) (West *et al.*, 1997) ($P < 0.0001$). This confirms West's (1997) hypothesis that the three in the $\frac{3}{4}$ power law is explained by the three-dimensionality of the body and that nearly two-dimensional organisms such as flatworms may not obey this law. As the body of triclads is just a rough approximation of two-dimensionality, the metabolic rate of *S. polychroa* does also differ significantly from the corresponding theoretical b -value of 1 ($P = 0.0002$). In contrast to the metabolic rate, the changing cell number itself was suggested to follow three quarters scaling during growth and degrowth in planarians (Oviedo *et al.*, 2003).

When we considered adult stage only, the b value was $1.17 (\pm 0.04 \text{ s.e.})$. Excluding the juveniles from the analysis thus results in a shift from a negative to a positive effect of size on mass-specific metabolism, confirming the observations discussed above. We could not detect large variations in b values during periods of adult growth ($b = 1.17 \pm 0.11 \text{ s.e.}$) and degrowth ($b = 1.13 \pm 0.07 \text{ s.e.}$), indicating that these values are consistent over the whole adult range. The shift in size-effect on metabolism, between juveniles and adults, suggests that there is an age-related biphasic allometric scaling relation. As expected from the analyses mentioned above, we observed that the mass-specific metabolic rates of young adults in the initial growth phase (month 3-5) are significantly higher than those of older adults ($P < 0.0001$). This prompted us to calculate the allometric scaling factor during initial growth (month 1-5), which was found to be $0.86 (\pm 0.03 \text{ s.e.})$. Thus, it appears that the allometric scaling factor differs between young, growing worms and adults that undergo repeated cycles of growth and degrowth.

The causes of this shift and the molecular mechanisms underlying the positive allometric effect during adulthood are not yet understood. It may suggest that growth during

development is physiologically different from the growth and degrowth cycles that are observed during adulthood.

4. Conclusions

Schmidtea polychroa is an invertebrate species showing negligible senescence, a lifespan of at least several years, and it maintains a high morphological plasticity during adulthood.

Metabolic ageing could not be observed, which may be explained by a very high rate of cell renewal during homeostasis (Pellettieri and Alvarado, 2007) and consecutive phases of degrowth and growth during which tissues are renewed. Metabolism of *S. polychroa* shows an age-related biphasic allometric scaling relation. Analysis of the complete lifespan resulted in an allometric scaling exponent of 0.90, while during adulthood a *b*-value of 1.17 was found as size had a significant positive effect on mass-specific metabolism.

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Figures and tables

Figure 1:

(a) Protein density as a function of body surface. (b) Wet weight as a function of body surface.

Figure 2:

(a) Total body surface per replicate (two worms) as a function of age. Size alternates synchronously in all replicates in consecutive phases of growth and degrowth. Replicate body surface decreases to juvenile dimension immediately before death. (b) Individual body surfaces of four replicate pairs showing intense growth and degrowth in the period between 15 and 25 months. Phases of growth and degrowth occur rather simultaneously in both worms of each replicate. (c) Individual body surfaces of replicate pairs during the last 5 months of their life. Degrowth consistently occurs in both worms of each replicate.

Figure 3:

(a) Survival curve of the experimental cohort. (b) The mortality rate of this cohort as a function of age. Data points represent the logarithm of the age-specific mortality rate per five months.

Figure 4:

Mass-specific metabolic rate, measured directly as heat production rate by microcalorimetry, as a function of age. During initial growth a decrease in mass-specific metabolic rate can be observed after which it remains stable during adulthood.

Figure 5:

The allometric relation between body size and metabolic rate of *S. polychroa*. All replicates and ages of the longitudinal experiment are included except worms undergoing dramatic degrowth preceding death (because pathologies could not be excluded). The scaling exponent b (0.90) is the slope of the linear regression.

Figure 6:

The allometric relation between body size and mass-specific metabolic rate. The highest values of mass-specific metabolic rate were observed in 1-month-old individuals. Mass-specific metabolic rates of young adults in their initial growth phase (month 3 – 5) are significantly higher than those of older adults (> 5 months). The allometric scaling exponent b shifts from 0.86 during the initial growth phase to 1.17 during the rest of the adult life. Data from adults < 35 mm² due to extreme degrowth before death were censored.

Table 1:

The occurrence of paired deaths in the replicates.

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Table 1

Replicate	Age of first death (months)	Age of second death (months)	Time between deaths (weeks)
3	9	9	2
5	19	20	5
6	29	31	10
7	25	27	12
9	33	34	5
13	16	16	2
14	23	24	5

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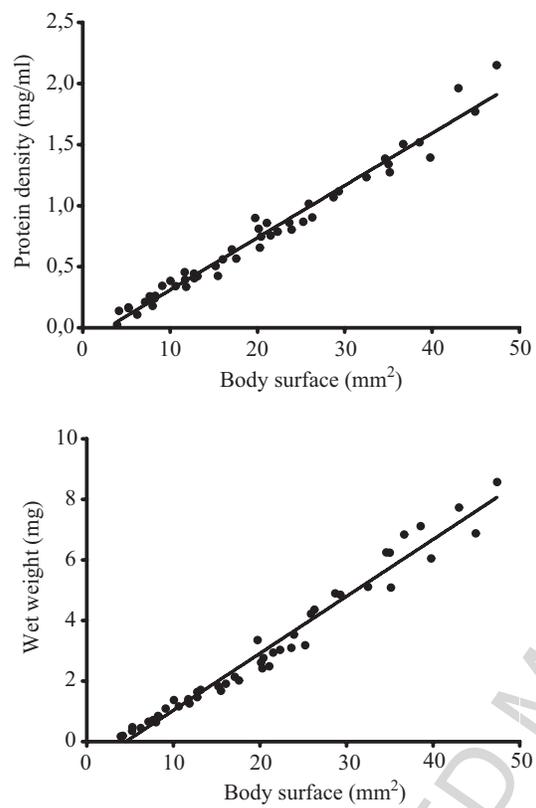


Figure 1

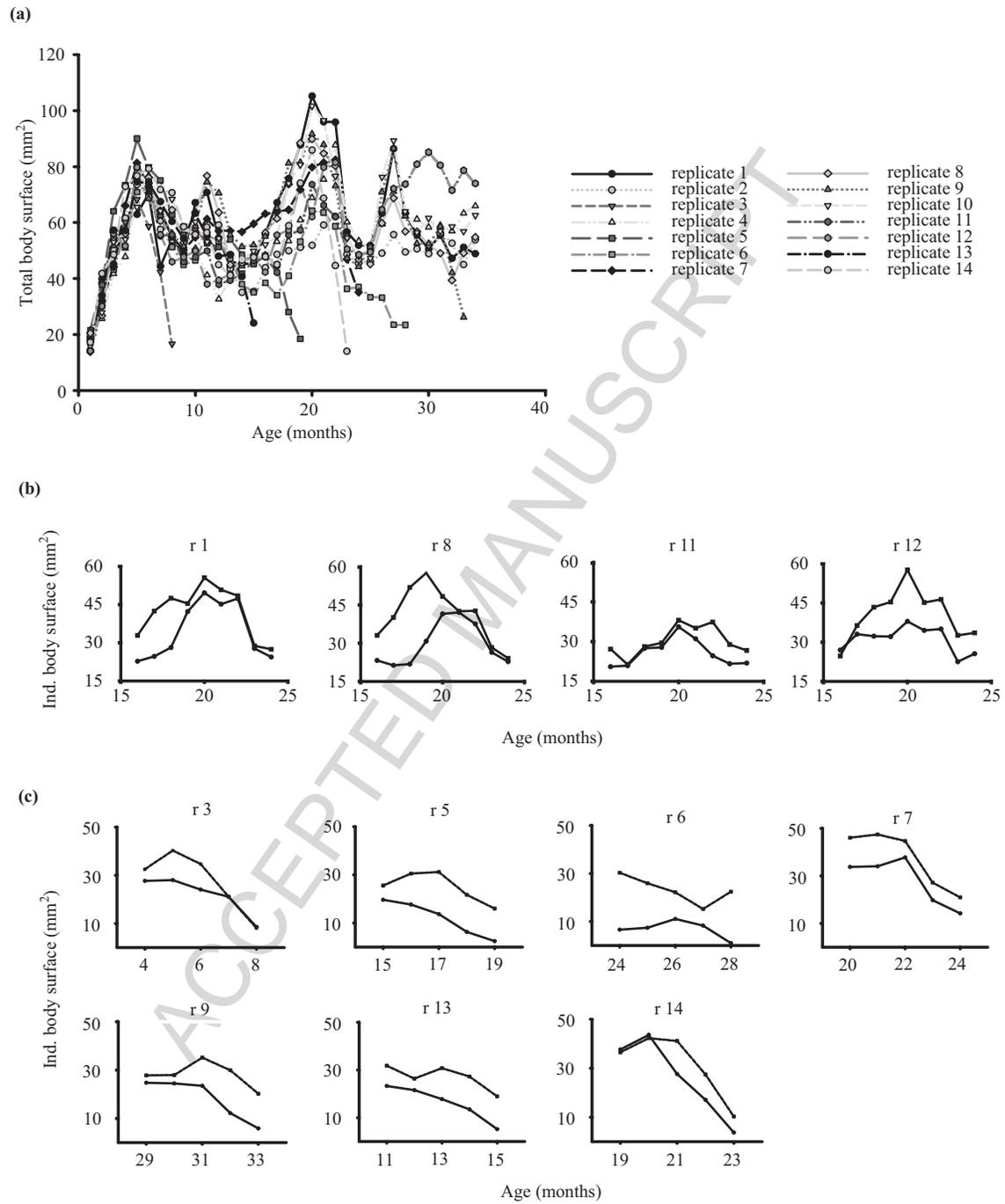


Figure 2

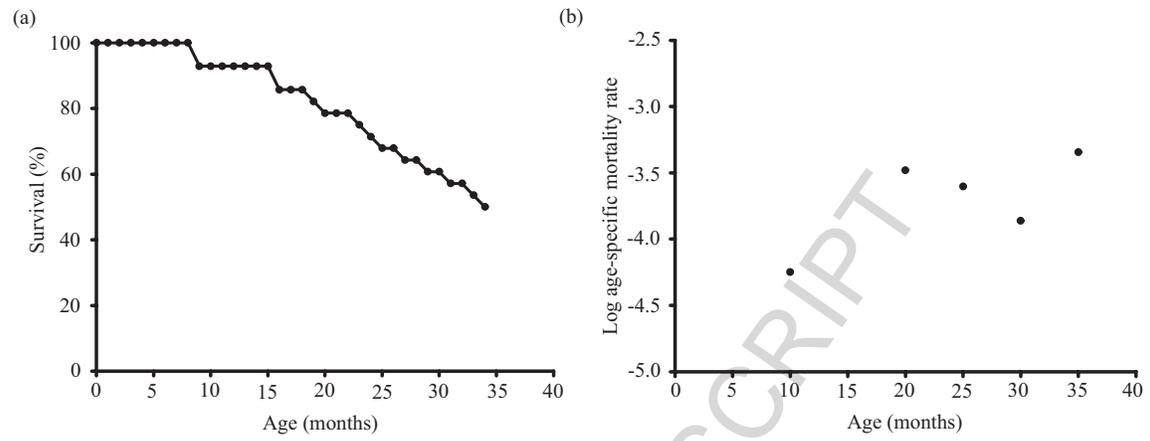


Figure 3

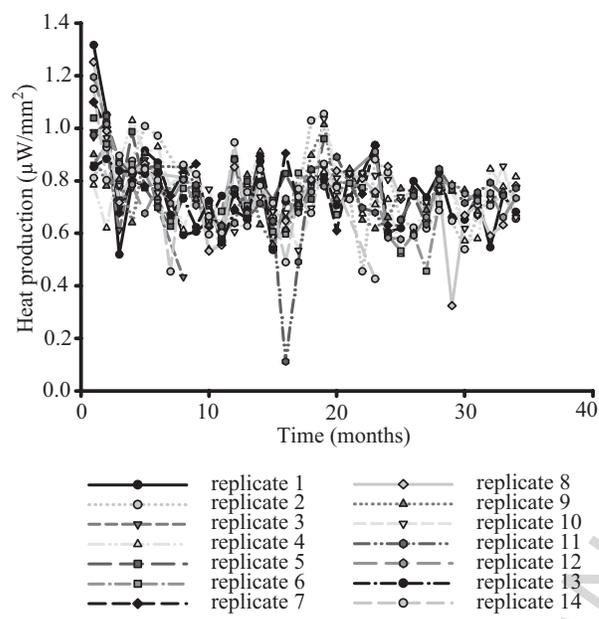


Figure 4

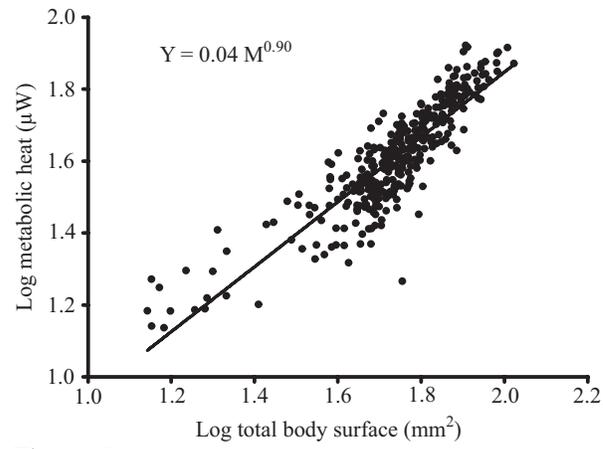


Figure 5

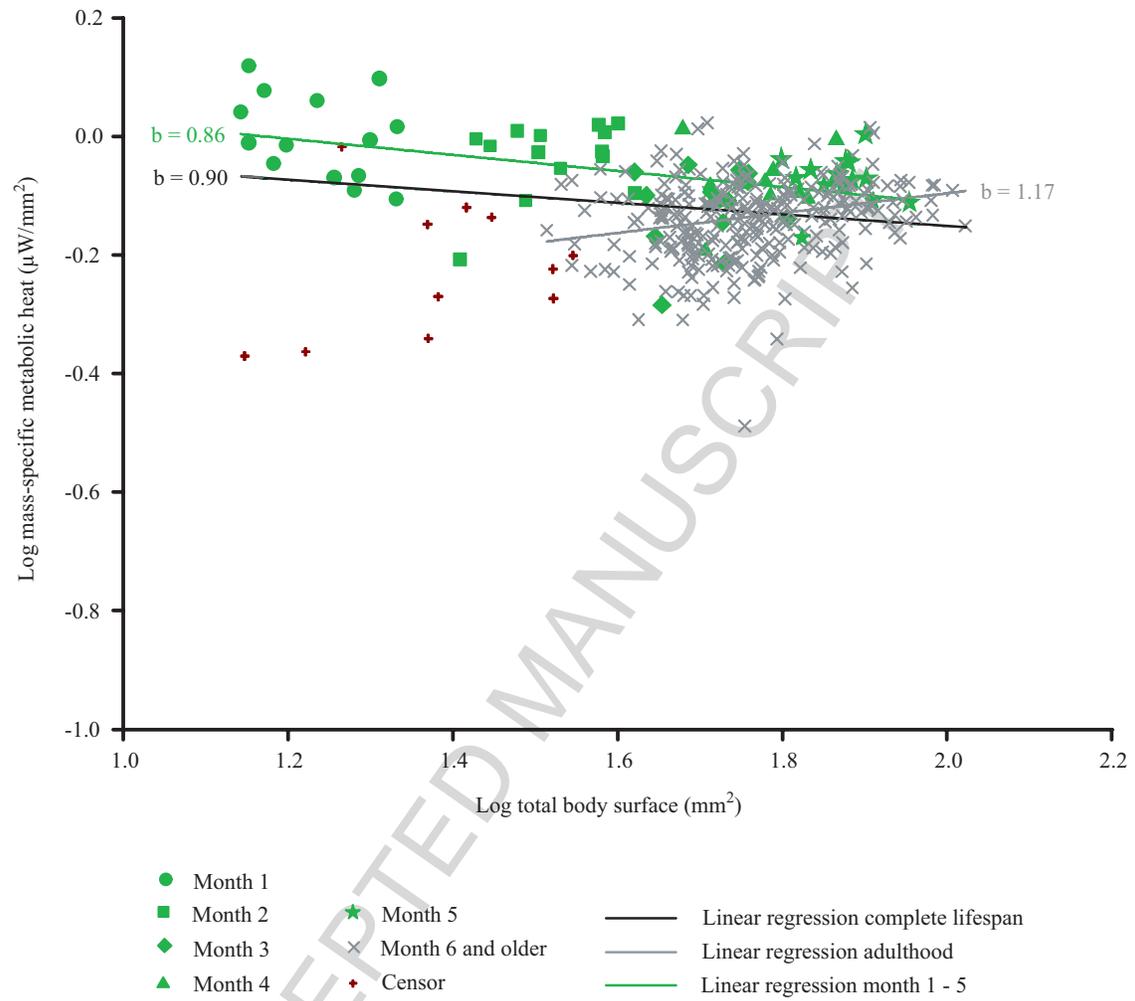


Figure 6

Research Highlights

- We present the first longitudinal flatworm aging study with emphasis on metabolism.
- *Schmidtea polychroa* shows negligible ageing.
- Mass-specific metabolic rate declines during initial growth, but not later in life.
- *S. polychroa* shows an unusual biphasic allometric scaling relation during its life.

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