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Research Article

Life history traits for the freshwater Tardigrade Species *Hypsibius exemplaris* reared under laboratory conditions

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Abstract

Known tardigrade species have increased in number over the years, but individual life history traits for most are unknown. The present study documents life history traits such as growth, reproductive activity, and lifespan for the parthenogenetic Eutardigrada species *Hypsibius exemplaris* when reared under laboratory conditions. Embryos were characterized by 100% hatching success, and hatchlings were characterized by approximately 90% survival to sexual maturity and 80% healthy growth to adult size, suggesting that mortality and growth defects occur early in life history. Egg production increased with age, eggs per exuvium maximizing on days 18, 21, and 24 post-hatching. The mean lifespan was 61.9 ± 9.9 days, with the maximum, 75 days, achieved by four among the 70 individuals whose life histories were documented. Given its short generation time, amenability to laboratory conditions, stereotypical embryonic stages, recently sequenced genome, and now chronicled life history, *H. exemplaris* rapidly is becoming a popular candidate in the phylum Tardigrada for advancing scientific inquiry.

Keywords: Hatching success, *Hypsibius dujardini*, Lifespan, Reproduction

Introduction

The phylum Tardigrada contains over 1200 known species (Degma *et al*. 2019). Cosmopolitan in distribution, tardigrades can be found in marine, freshwater and terrestrial environments (Devasurmutt and Arpitha 2016). While recognized species have been increasing in numbers over the years, scant data are available for individual life history traits for most species. And while tardigrade tolerance has been investigated for a variety of factors, fundamental biological phenomena like embryonic development, reproduction and lifespan have been described for relatively few species (i.e., < 5%; Schill *et al*. 2013).

We examined life history traits for the freshwater eutardigrade species *Hypsibius exemplaris* sp. nov. (recently redescribed from a strain in *H. dujardini* (Doyère, 1840); Gąsiorek *et al*. 2018). The species is characterized by traits shared among tardigrades – bilaterally symmetric body, comprising a head and four segments, each bearing paired lobopod legs that terminate in digits or claws; as a eutardigrade, it lacks cuticular dorsal plates, so internal anatomy can be observed; and populations are found in freshwater ecosystems and predominantly comprise parthenogenetic females that reproduce by depositing relatively large eggs into exuvia during molting (Fig. 1). This species was chosen because it is a suitable model organism for evolutionary-developmental studies (Gabriel *et al*. 2007), characterized by a short generation times, stereotypical in its embryonic stages and cultured easily and continuously under laboratory conditions. Discrepancies among results in recent *H. exemplaris* genome sequencing projects (the first tardigrade genome to have been published) have received much attention in the scientific community (e.g., Boothby *et al*. 2015, Koutsovoulos *et al*. 2016, Yoshida *et al*. 2017). Researchers additionally have made significant advances in exploring responses elicited by exposure to environmental extremes such as ionizing radiation and associated radiation-induced bystander effects, desiccation, cold temperature and hypergravity on *H. exemplaris* (respectively, Beltrán-Pardo *et al*. 2015 and Fernandez *et al*. 2016, Boothby *et al*. 2015, Guidetti *et al*. 2011, Vasanthan *et al*. 2017). Whereas *H. exemplaris* research has been concentrated on extreme tolerance, life history traits like hatching success, fecundity rates and maximum lifespan are unavailable (Schill 2013). The present study was implemented predominantly to provide those data (some morphological data also were recorded and are reported herein).

Figure 1. A *Hypsibius exemplaris* specimen (left) depositing two eggs (right) while emerging from a shed exuvium during molting.

Material and methods

Tardigrade Sampling

To characterize life history traits in *H. exemplaris* (Z151, Sciento, Whitefield, Manchester), approximately 100 egg-laying mothers were isolated. Individuals were monitored under light microscopy (Nikon SMZ1000) until eggs had been deposited, and mothers had exited their exuvia. Eggs were isolated and housed in 1.5 mL microtubes containing 15 µL spring water (Aberfoyle, Puslinch Ontario; Nestlé Pure Life). Exuvia typically contained a single embryo, but some containing 2 and 3 embryos also were used. To maintain synchronous development among individuals, only eggs collected from mothers within 1-hour post laying were used. Seventy eggs were sampled. Eggs were monitored for approximately 2.5 hours daily under light microscopy until hatching. Hatchlings were stored individually in 1.5 mL microtubes and monitored daily until day 6, when individuals began ovipositing. Adults were monitored every three days until death. To score growth, egg production and survivorship, individuals were transferred from microtubes onto a 35-mm Petri dish (Falcon) and observed by light microscopy. Survivorship data were recorded every three days when water (100 µL) and algal food (10 µL; Chlorococcum sp.; A68, Sciento, Whitefield, Manchester) were replenished and used microtubes were replaced with new microtubes. Eggs produced by individuals were recorded and removed subsequently.

Body Length Measurements

An additional 12 eggs were collected to measure individual body lengths for hatchlings (before first feeding) and adults (days 6 and 8 post-hatching). Measurements were taken via stereomicroscope eyepiece reticule (Wild Heerburg, M3C) and calibrated with a stage micrometer. Body lengths were measured during walking when specimens were extended maximally. As described by Suzuki (2003), body length measurements were taken as the distance from head to juncture on the posterior-most segment with legs.

Tardigrade Life History Traits

The following life history traits were documented in the 70 individuals: gestational period (days), growth, age at fist oviposition (days), oviposition per lifespan, eggs laid per molt, maximum clutch size, eggs laid per lifetime, average lifespan (days) and maximum lifespan (days). Where applicable, values were reported with means and ranges.

Results

Hatching Success and Growth

All eggs (N=70) hatched on day four post laying, confirming previous reports that *H. exemplaris* embryos take four days to complete embryogenesis (Gabriel *et al*. 2007). Whereas hatching always was synchronous, hatchlings were asynchronous in growth, with 21.43% (N=15/70) and 11.43% (N=8/70), respectively exhibiting delayed or no growth by day 6 (Fig. 2). The individuals that failed to grow were removed from the study. From a qualitative perspective and analogous to embryonic development in *Milnesium tardigradum*, where eggs become more transparent with development, *H. exemplaris* embryos transitioned from dark brown to a more-transparent hue as development ensued (Suzuki 2003).

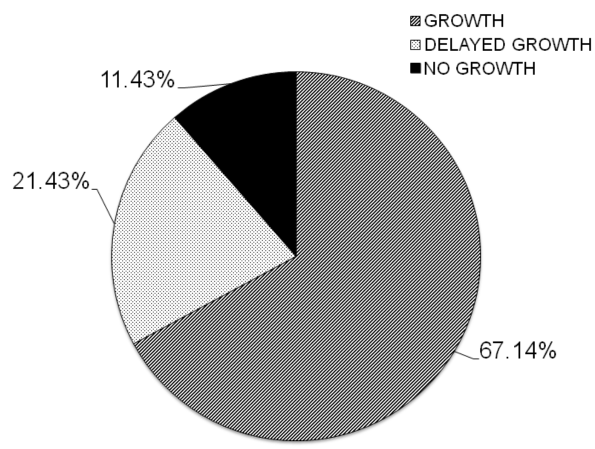

Figure 2. The proportion among individuals (N=70) that exhibited growth (increase in body length by day 3 of life; 67.1%), delayed growth (increase in body size by day 9 of life; 21.4%) or no growth (11.4%; individuals that did not grow by day nine were removed from the study).

Body Measurements

Day 1 hatchling (unfed; N=12 individuals) body lengths ranged from 119.0 µm (minimum) to 142.8 µm (maximum), averaging 138.0 µm. Day 6 and day 8 post-hatching adult (fed; N=12) body lengths measured on average 220.15 µm (minimum = 190.4 µm; maximum = 238.0 µm) and 263.8 µm (minimum = 238.0; maximum = 285.6), respectively. Gravid females were observed to be slightly longer than were non-egg-carrying females; this difference, however, was only transitory, as pregnant mothers returned to their original sizes once eggs had been deposited. *H. exemplaris* adults are small relative to adults in most tardigrade species (Table 1).

Oviposition, Clutch Size and Molting

By day 6, almost two-thirds (65.7%) among the specimens had deposited eggs into shed exuvia for the first time. First egg production generally yielded between one and three eggs; one individual produced 6 eggs in its first round.

On average, individuals molted approximately 11 times throughout their lives (Table 2). Lifetime ova production ranged from 15 to 59 eggs, individuals laying on average 42 eggs. Eggs laid per exuvium steadily increased with time, with egg production peaking on days 18, 21, and 24, after which it declined gradually (Fig. 3).

Longevity
Lifespan averaged 61.9 ± 9.9 days; the maximum age achieved, attained by 5 individuals, was 75 days (Table 1; Figure 4).

Table 1. Body measurements for adult tardigrade species, including data from the present study

Species	Habitation	Mean (µm)	Range (µm)	Gender	Reference
Hypsibius exemplaris	Freshwater	263.8	238- 285.6	Females	Present Study
Dactylobiotus vulcanus	Freshwater	343	235 -450	n.d.	Kaczmarek *et al.* 2012
Parastygarctus renaudue	Marine	153	112 -168	Females	Grimaldi de Zio *et al.* 2009
Stygarctus lambertii	Marine	93	69-106	Females	Grimaldi de Zio *et al.* 2009
Stygarctus lambertii	Marine	93	76 -112	Males	Grimaldi de Zio *et al.* 2009
Ramazzottius oberhaeuseri	Limno-terrestrial	228.7	107.3-375.4	Females	Rebecchi *et al.* 2006
Paramacrobiotus tonal/ii	Limno-terrestrial	654 b	n.d.	Females and Males	Lemloh *et al.* 2011
Macrobiotus sapiens	Limno-terrestrial	518 b	n.d.	Females and Males	Lemloh *et al.* 2011

[a] Values obtained from mounted spec ies
[b] Maximum body size

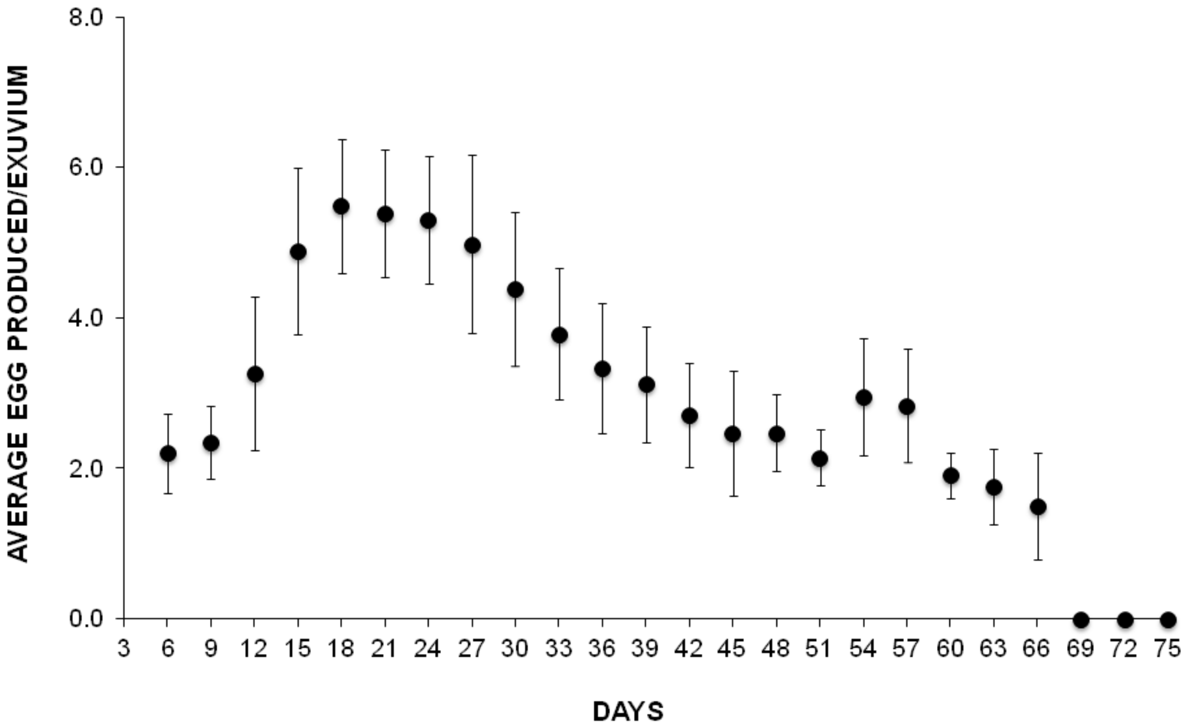

Figure 3. Eggs laid per exuvium (Mean± SD) every three days in *H. exemplaris* (N=59)

Table 2. Life history traits of tardigrade species, including data from the present study

Species	Reproduction	Lifespan (days)	Max Lifespan (days)	Age at Oviposition (days)	Hatching Success	Max Eggs/ clutch	Oviposition / Lifespan	Reference
Hypsibius exemplaris	Parthenogenesis	61.9 ± 9.9	75	8.0 ± 3.1	90-100 %	8	10.5 ± 2.2	Present Study
Acutuncus antarticus	Parthenogenesis	69.2 ± 36.4	162	9.3 ± 1.1	97.6 %	10	7.5	Tsujmoto *et al.* 2015
Paramacrobiotus kenianus (I)	Parthenogenesis	125 ± 35	204	10 a	33 %	n.d.	n.d.	Schill 2013
Paramacrobiotus kenianus (II)	Parthenogenesis	141 ± 54	212	10 a	51 %	n.d.	n.d.	Schill 2013
Paramacrobiotus palaui	Parthenogenesis	97 ± 31	187	10 a	54 %	n.d.	n.d.	Schill 2013
Paramacrobiotus tonollii	Bisexual	69.0 ± 45.1	237	24.4 ± 4.4 b	82.2 %	19	n.d.	Lemich *et al*. 2011
Macrobiotus sapiens	Bisexual	83.0 ± 33.5	145	16.5 ± 3.8 b	78 %	16	n.d.	Lemich *et al*. 2011
Macrobiotus richtersi (I)	Parthenogenesis	194.9 ± 164.4	518	76.9 ± 16.4	83.1 ± 12.7	39	3.4 ± 2.1	Altiero *et al*. 2006
Macrobiotus richtersi (II)	Parthenogenesis	137.3 ± 136.4	457	70.7 ± 19.4	51.0 ± 36.0	30	2.0 ± 1.2	Altiero *et al*. 2006
Milnesium tardigradum	Parthenogenesis	42.7 ± 11.8	58	15.3 ± 1.6 c	77.2 %	12	3.7 ± 1.3	Suzuki *et al*. 2003

a Median
b Eggs observed in ovary
c As reported by Tsujmoto *et al.* 2015

Discussion

Hatching Success and Growth

Whereas 100% hatching success was observed in this study, previous pilot projects indicated that successful hatching rates typically are lower (approximately 90%). The impaired (21.43%) or lacking (11.43%) growth that was observed might be explained by malnutrition. Given their semi-transparent bodies, guts in *H. exemplaris* are visible when magnified. Unfilled guts observed among individuals that exhibited delayed or no growth suggests that they either began feeding at later stages or had failed to feed entirely. Similar observations have been documented for the tardigrade species *Hypsibius convergens* (Bauman 1961) and *Paramacrobiotus tonollii* (Lemloh *et al*. 2011), where survival in hatchlings was attributed to adequate food intake during the first day (i.e., 24 hours).

Body Measurements

The body length measurements obtained (average 138.0 μm for hatchlings, 220.15 μm for adults) are comparable to those described previously (McInnes 1995), which ranged from 145 μm to 280 μm. While the correlation between growth and molting was evident initially (i.e., days 1 through 8 post hatching) – adults were approximately double hatchling size, no correlation was observed once individuals began ovipositing.

Oviposition, Clutch Size and Molting

Most (65.7%) specimens started ovipositing by day 6 post-hatching, laying between one and three eggs. While sexual maturity in tardigrades often is described as the time point at which first eggs are laid (i.e., oviposition), this constitutes a slight overestimation (Schill 2013). In *H. exemplaris*, individuals typically can be observed carrying eggs approximately one day before eggs are released and deposited into exuvia; thus, a more accurate designation for achieving sexual maturity in *H. exemplaris* would be between days 5 and 6 post-hatching. Whereas clutch size in *H. exemplaris* has been reported previously to range between 1 (minimum) and 10 (maximum) eggs (Gabriel *et al*. 2007), the maximum output observed in a single molt by any individual in the present study was 8 eggs (Table 2). This number is small compared to values associated with other tardigrades species, which can lay as many as 30 to 40 eggs; thus, exuvia size may dictate egg production in *H. exemplaris* (Glime 2013).

While egg laying and molting almost always occurred synchronously, molting also occurred without egg production during the earlier and later stages. The average molts per lifecycle thus is estimated to be slightly greater than 11. Molting without eggs during the early stages may be attributed to growth. Like many tardigrade species, *H. exemplaris* is eutelic (Bertolani 1970). Growth occurs through cell enlargement, where individuals increase body size by increasing cell sizes. Molting without eggs during later stages may result from senescence, where the ability to generate eggs decreases with increased biological age.

Longevity

Individuals typically lived approximately 62 days, with 5.7% achieving the maximum, 75 days. Although a slight decrease in survivorship was observed immediately after hatching, survival rates were high during early and middle life history stages. Ecdysozoans typically follow survivorship curves with mortality (and fertility) greatest during early life history stages. The survivorship curve for *H. exemplaris*, however, was characterized by least mortality during the early and middle stages with a steep increase at later stages. Explanations for this pattern include species-specific characteristics like egg provisioning to enable rapid development and population-specific characteristics attributable to maintenance under constant laboratory conditions. The protection provided by exuvia particularly might explain high hatching rates (i.e., 90-100 %). High survival rates in freely laid eggs in other tardigrade species (e.g., *Paramacrobiotus richters*i) can be attributed to ornamentation, which includes pores and reticulation patterns (Schill 2013, Thorp and Covich 2009).

Conclusion

The parthogenetic tardigrade species *Hysibius exemplaris* is characterized by a life history in which individuals hatch from eggs in 4 days, grow rapidly for and start producing eggs in approximately 8 days; and growth gradually and deposit eggs into exuvia during molting for another approximately 50 days. Mortality is very low at hatching, increases slightly during early and middle growth stages, and increases greatly at later stages. Fecundity initiates with approximately 2 eggs (day 8), maximizes at approximately 5 eggs (day 18-24), and declines steadily back to 2 eggs (until death). Body length is approximately 138 µm at hatching, 220 µm at first egg production, and thence increases to approximately 264 µm.

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