Spicule skeleton formation in the freshwater sponge *Ephydatia fluviatilis* under hypergravity conditions

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Successful dispersal of freshwater sponges depends on the formation of degenerative sponge bodies (gemmules) under adverse conditions. Gemmule formulation allows the sponge to overcome critical environmental conditions, for example desiccation or freezing, and to re-establish as a fully developed sponge when conditions are more favorable. A key process in sponge development from hatched gemmules is the construction of its silica skeleton. Silica spicules form the structural support for the three-dimensional filtration system the sponge uses to filter food particles from ambient water. We studied how sponges fed with dissolved ^{13}C- and ^{15}N-labelled amino acids and non-fed sponges develop their spicule skeleton under environmental stress, using different hypergravity forces (1, 2.5, 5, 10, and 20 x *g* for 48 h) as stressors. The results show that freshwater sponges can withstand prolonged periods of hypergravity exposure and successfully construct their skeleton, even under 20 x *g*. Developing sponges were found to take up and assimilate dissolved food before forming a functional filtering system. However, fed and non-fed sponges showed no differences in skeleton formation and relative surface area growth, suggesting that the gemmules' intrinsic energy fulfills the processes of skeleton construction. Additionally, non-fed sponges formed oscula significantly more often than fed sponges, especially under higher *g*-forces. This suggests that the eventual formation of a filtration system might be stimulated by food deprivation and environmentally stressful conditions. These findings indicate that the process of spicule skeleton formation is energy-efficient and highly flexible, and plays an important role in how sponges can adapt their size and shape required for indeterminate growth. The uptake of dissolved food

substances by freshwater sponges may contribute to the cycling of dissolved organic matter (DOM) in freshwater ecosystems where sponges are abundant.

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Abstract

Successful dispersal of freshwater sponges depends on the formation of degenerative sponge bodies (gemmules) under adverse conditions. Gemmule formulation allows the sponge to overcome critical environmental conditions, for example desiccation or freezing, and to re-establish as a fully developed sponge when conditions are more favorable. A key process in sponge development from hatched gemmules is the construction of its silica skeleton. Silica spicules form the structural support for the three-dimensional filtration system the sponge uses to filter food particles from ambient water. We studied how sponges fed with dissolved ^{13}C- and ^{15}N-labelled amino acids and non-fed sponges develop their spicule skeleton under environmental stress, using different hypergravity forces (1, 2.5, 5, 10, and 20 x *g* for 48 h) as stressors. The results show that freshwater sponges can withstand prolonged periods of hypergravity exposure and successfully construct their skeleton, even under 20 x *g*. Developing sponges were found to take up and assimilate dissolved food before forming a functional filtering system. However, fed and non-fed sponges showed no differences in skeleton formation and relative surface area growth, suggesting that the gemmules' intrinsic energy fulfills the processes of skeleton construction. Additionally, non-fed sponges formed oscula significantly more often than fed sponges, especially under higher *g*-forces. This suggests that the eventual formation of a filtration system might be stimulated by food deprivation and environmentally stressful conditions. These findings indicate that the process of spicule skeleton formation is energy-efficient and highly flexible, and plays an important role in how sponges can adapt their size and shape required for indeterminate growth. The uptake of

47 dissolved food substances by freshwater sponges may contribute to the cycling of dissolved
48 organic matter (DOM) in freshwater ecosystems where sponges are abundant.

Introduction

Sponges are among the oldest — approximately 700 million years old — still existing metazoans on Earth (Müller, 1998; Love *et al*., 2009; Ludeman *et al*., 2014). Due to its ancient heritage, the sponge body plan is traditionally seen as the original blueprint for multi-cellularity (Müller, 2004;Nosenko *et al*., 2013). Although sponges lack organs, they possess a high level of functional complexity (Leys *et al*., 2009; Srivastava *et al*., 2010). They have a well-developed food uptake and waste disposal system constructed of numerous small inflow openings (ostia) and chambers containing filter cells (choanocytes) with actively beating flagella to create an internal water flow. Sponges can contract their ostia and water channels to modulate this water flow (Elliot & Leys, 2007; Ludeman *et al*., 2014). After the indrawn water has passed the choanocyte chambers, waste products are discarded through excurrent canals (oscula). Sensory cilia inside the osculum use calcium channels to adapt the sponge' water filtering capacity, for example in response to temperature changes or increased suspended sediment (Ludeman *et al*., 2014; Cavalier-Smith, 2017).

The freshwater sponge *Ephydatia fluviatilis (*Porifera, Demospongia, Spongillidae), the species used in this study, is cosmopolitan and found throughout Earth's entire northern hemisphere (Van Soest *et al.* 2017). This shows the flexibility of this sponge to endure a wide range of environmental conditions. The colonization capacity of inland waters by freshwater sponges largely depends on the formation of small (~300 μm diameter) degenerative sponge bodies (gemmules) under adverse conditions (Manconi & Pronzato, 2008; Funayama, 2013). This form of asexual reproduction allows the sponges to overcome critical environmental conditions (e.g., low water temperatures during wintertime or desiccation during hot summers) (Manconi & Pronzato, 2016). When conditions are more favorable, a fully-developed miniature sponge can be re-established from a gemmule in approximately 1 week, depending on environmental conditions

(Höhr, 1977; Ilan *et al*., 1996; Funayama *et al*., 2005) (Fig 1A). First, during gemmule germination, totipotent stem cells contained within the gemmule differentiate in different cell types (Wierzejski 1915, 1935; Höhr, 1977). Secondly, during a process called hatching, the cells within the gemmule migrate outwards and attach the sponge to its substrate (Rozenfeld, 1970; Höhr, 1977; Harrison *et al*., 1981). Eventually, the migrated cells proliferate and differentiate into all types of cell to form a fully functional sponge (Höhr 1977; Funayama *et al*., 2005).

A key process in freshwater sponge development from hatched gemmules is the formation and construction of its skeleton. The sponge skeleton forms the structural support for the three-dimensional filtering system of the sponge (reviewed by Uriz *et* al., 2003). In demosponges, the skeleton consists of individualized elements (silica spicules), embedded in a fibrous organic matrix made from chitin and spongin (Larsen & Riisgård, 1994; Uriz *et al*., 2003; Ehrlich *et al*., 2013). After setting the spicules in an upright position, the developing sponge construct its filter systems and starts to obtain nutrients by active filter-feeding (Funayama *et al*. 2005).

Recently, it was shown for *E. fluviatilis* that the silica spicules are not randomly distributed throughout the developing sponge body, but are deliberately set-up in a highly-organized manner (Nakayama *et al*. 2010; 2015), similar to pitching a tent. Spicules are produced at different locations within the hatching gemmule and transported by so-called transport cells to their final position, where they are erected and cemented by a third type of specialized cells (Nakayama *et al*. 2015). This division of labor between various cell types within the sponge has revealed a fundamentally new mechanism of constructing the three-dimensional body shape of animals (Nakayama *et al.*, 2015). As stated by Nakayama (2015), the spicule skeleton construction process of *E. fluviatilis* is the only known biological mechanism in which a sequence of cooperative behaviors of individual cells leads to the active construction of a self-organized biological structure

using non-cellular materials. Moreover, the mediation of the different steps of spicule skeleton construction by specialized cells allows for high plasticity and helps to generate the morphological diversity of fully-grown *E. fluviatilis* specimens.

Gemmule formation and hatching are seasonally dependent and it appears that external factors such as water temperature, water turbidity and illumination are responsible for these processes (Harsha *et al.*, 1983; Ilan *et al*., 1996). However, at present it is unknown how most of these external factors affect the formation and hatching of gemmules. In addition, gemmule hatching and spicule body construction up to the moment of a fully-developed filter system is presumed to be mediated by internally-stored energy and therefore independent of externally available food sources. Germination studies are therefore usually performed in medium without added food sources (Funayama *et al.*, 2005;Elliott & Leys, 2007). This hypothesis is, however, never tested and therefore it is unknown whether newly hatched sponge gemmules can obtain external energy sources before they have developed the capacity to actively filter-feed, and if this influences their development.

Access to the large diameter centrifuge (LDC) of the European space agency (ESA) in Noordwijk, the Netherlands, enabled us to test the influence of increased mechanical stress (i.e. increased hypergravity (*g*) forces) on the construction of the siliceous skeleton of developing sponges. Hypergravity is an artificially created condition in which the acceleration exceeds the common terrestrial gravitational acceleration of 9.81 m s^{-2} (1 x *g*) and this creates a force working directly against the hatching sponge trying to erect its spicules. Furthermore, hypergravity has been shown to alter the intracellular transport and delivery of cell wall material in plants (Chebli *et al.*, 2012), polyp growth in stony corals (Meroz *et al.*, 2002) and effects skeletal architecture and bone-repair in mammals (Prodanov *et al.*, 2013; Canciani *et al*., 2015). Exposure to hypergravity acts

on the whole cell mass, and cells exposed to several *g*'s can adapt by decreasing the height of their microtubule network, but increasing the thickness of their actin fibers without affecting cell viability (Kacena *et al.,* 2004; Searby *et al.,* 2005; van Loon *et al*., 2009). We hypothesize that 1) increased *g*-forces decrease the ability of the sponge to transport their spicules to their correct orientation and to erect them due to the higher energy costs involved, preventing the formation of a fully-developed filter system (e.g., no osculum formation) and 2) that food addition will partially relieve developing sponges from the expected energy shortage caused by hypergravity (Fig 1B).

To study the effects of external stress on skeleton formation in developing freshwater sponges, we tested how prolonged exposure (48 h) to different hypergravity forces (1, 2.5, 5, 10, and 20 x *g*) influenced 1) relative surface area increase (as measure of growth) 2) the presence of a set-up skeleton and 3) the presence or absence of an osculum in hatched gemmules of *E. fluviatilis*. In addition, we tested the ability of the developing sponges under above-mentioned conditions of hypergravity exposure to take up dissolved food (i.e. ^{13}C- and ^{15}N-enriched amino acids) and whether additional feeding affects skeleton and osculum formation.

Materials & Methods

Gemmule harvesting and preparation

Gemmules were collected in the winter months (November 2013 to March 2014), from specimens of *E. fluviatilis* kept in outdoor aquaria at the University of Amsterdam. Sponge tissue containing the gemmules was collected and cleaned by rubbing the sponge tissue between two pieces of corduroy to free the gemmules from the sponge tissue scaffold that can inhibit germination. Detached gemmules were sterilized in 1% H_2O_2 for 5 min on a shaker at 4°C to remove bacterial and fungal contaminants included in the coat (Funayama *et al.*, 2005).

Gemmule hatching

A total of 420 gemmules from one individual sponge were plated on sterile 12-well culture plates in sterile M-Medium (Rasmont, 1961; 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.5 mM $NaHCO_3$, 0.05 mM KCl, 0.25 mM Na_2SiO_3) under ambient conditions. The plates were sealed by parafilm. Each well of the 12-well culture plates contained one gemmule in approximately 4 mL of M-medium. After four days, before placing the gemmules in the LDC, the individuals that did not hatch were discarded and of the remaining hatched specimens, only the sponges in stage II were selected (i.e. the sponges that had not yet erected any spicules nor formed an osculum, see Fig. 1 for a description of developmental stages). 72% of the plated gemmules hatched (302 out of 420), of which $n = 277$ sponges developed to stage II that were subsequently used for the experiment in the LDC (Table 1). Before placing the selected gemmules in the LDC, the medium was refreshed to prevent accumulation of waste products in the well plates.

157

158 **Sponge feeding with ^{13}C- and ^{15}N-enriched amino acids**

159 For each gravitational condition (see below), one group (i.e. approximately half) of the gemmules
160 was fed with isotopically-enriched ($^{13}C$and ^{15}N) dissolved amino acids in order to study nutrient
161 uptake, whereas the other group remained unfed (Table 1). The fed groups were randomly selected
162 and received M-Medium with 390 µg L^{-1} tracer ^{13}C- (11 µmol L^{-1}) and ^{15}N- (2.2 µmol L^{-1}) enriched
163 dissolved amino-acids added prior to the LDC runs.

164

165 **Hypergravity experiment in large diameter centrifuge**

166 Hypergravity experiments were performed using the large diameter centrifuge (LDC) of the
167 European Space Agency (ESA, Noordwijk, The Netherlands) following a predetermined protocol
168 (Fig. 2A). The LDC has a diameter of 8 m and comprehends four large arms fitted with outward
169 swinging gondolas. The rotational movement of the arms creates an artificial acceleration field at
170 the well plates positioned inside the gondolas, simulating different *g*-forces depending on gondola
171 placement on the arms and rotational speed (Fig. 2B). Experiments where performed in two 48-h
172 runs. In the first 48-h run developing sponges were exposed to 1 (i.e. as hypergravity control;
173 placed at the center of the rotating LDC), 2.5 and 5 x *g* and in the second run to 10 and 20 x *g* (Fig.
174 2B). Per hypergravity level, both fed and non-fed sponges were tested simultaneously. To assess
175 the effect of rotation on the development of the gemmules, a control experiment without feeding
176 was performed where gemmules were hatched on a non-rotating lab bench, in addition to the
177 rotating control at 1 x *g*. As conditions did not vary in the LDC room between the first (2.5 and 5
178 x *g*) and the second run (10 and 20 x *g*) no additional hypergravity control at 1 x *g* was performed
179 during the second run.

180

181 **Sponge surface area measurements**

182 Before and after placement in the LDC, the surface area of the substrate covered by the sponges
183 (in mm^2) was determined by light microscopy. We are aware that sponge volume is a better
184 metricfor growth of sponges building a three-dimensional structure (i.e. the skeleton). However,
185 we were unable to accurately measure volume and therefore used substrate area covered by the
186 sponge as a proxy for sponge size/growth. All sponges were photographed and visually checked
187 to assess substrate area cover, the set-up/no set-up of the sponge skeleton, and the presence/absence
188 of an osculum under a stereoscopic microscope (*Olympus SZH-ILLD* with *infinity1 microscopy*
189 *camera*). Surface area measurements were performed using *Image J* software
190 (*https://imagej.nih.gov/ij/*).

191

192 **Stable isotope analysis of sponges**

193 After LDC exposure and microscopy imaging, the labeled M-medium was replaced with non-
194 labeled M-medium in which the sponges were placed for 30 mins to remove any residual label.
195 Sponges were then taken out of the well-plates, rinsed with M-medium and pooled per treatment,
196 freeze-dried, homogenized, and stored at -20°C in silver boats. Pooling was necessary to ensure
197 that sufficient carbon and nitrogen was available for the stable isotope analysis. Silver boats were
198 acidified with 5% HCl to ensure removal of inorganic carbon, oven-dried at 60°C, pinched closed
199 and stored frozen before analysis on an Elemental Analyser *(EA, Firma Thermo Electron, Flash*
200 *EA 1112 analyzer*) that was coupled to a Delta V isotope ratio mass spectrometer (IRMS) for
201 simultaneous measurement of ^{13}C:^{12}C and ^{15}N:^{14}N ratios. Reproducibility for the EA-IRMS
202 analysis was 0.25‰ for ^{15}N and 0.2‰ for ^{13}C.

The uptake of dissolved amino acid C or N was expressed as uptake of µmol isotopically-enriched C or N mmol sponge^{-1} d^{-1}. Rates are calculated from the delta notations obtained from the IRMS as δX (‰) = (R_{sample}/R_{ref} -1) · 1000, in which X is the element (C or N), R_{sample} is the heavy : light isotope ratio in the sample and R_{ref} is the heavy : light isotope ratio in the reference material (R_{ref} = 0.0111797 for C and R_{ref} = 0.0036765 for N). The atomic fraction of the heavy isotope (F) in a sample is calculated as $F = R_{sample}/(R_{sample}+1)$. The excess (above background) atomic fraction is the difference between the F in an experimental sample and the atomic fraction in a control (i.e. non-enriched) sample: $E = F_{sample} - F_{control}$. The excess incorporation of ^{13}C and ^{15}N was multiplied by 1,000 to express rates in µmol tracer C or N per mmol sponge C or N and divided by the incubation time to convert to daily rates.

Statistical analysis

To analyze the effect of rotation without hypergravity exposure on the sponges, a two sample t-test was performed to compare the means of the two (rotating/non-rotating)1 x *g*groups. A chi-square test was used to compare osculum development between the two groups. Both tests were performed in *SPSS version 25* (*IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.).*

Surface area increase is expressed as a percentage relative to the original surface of each hatched gemmule in stage II at the start of the LDC experiment. The effect of hypergravity on percentage surface increase was investigated by a linear model, adopting a 0.05 significance level. Besides evaluation of the overall model significance, also the pairwise differences among the treatment-levels were made through a Tukey HSD test with a 95% family-wise confidence level.

Osculum formation was analyzed with a generalized linear model using a binomial error function. To address the effect of osculum formation and *g*-force on the mean dissolved organic matter uptake (measured by ^{13}C and ^{15}N enrichment), an additive linear model was evaluated, using a 0.05 significance level.

In the results, means and proportions are reported in combination with the standard deviation between brackets. All analyses were carried out in *Rstatistics programming environmentR 3.3.2*(*The R Foundation for Statistical Computing, 2004-2013*).

233 **Results**

234

235 **Sponge development under hypergravity exposure**

236 The non-rotating and rotating controls at 1 x *g* did not show a significant difference in sponge
237 growth (two-sample *t*-test, $t(31) = -0.341$, $p > 0.1$), skeleton formation (in both groups 100% op
238 sponges set up a skeleton) or osculum development (chi square test, χ^2 (2, $n = 33$) = 1.8397, $p >$
239 0.1). Over all levels of (hyper)gravity exposure, 96% (± 1%) of stage II hatched gemmules
240 developed to stage III sponges (i.e. with erected skeleton), 66% (± 7%) of all sponges reached
241 stage IV, (i.e. build an osculum) and formed a fully functional sponge during their 48-h treatment
242 (Table 1). Although all sponges showed a relative increase in surface area that they covered of on
243 average 196% (± 70%), the net surface area decreased slightly, but significantly, with increasing
244 hypergravity exposure ($R2 = 0.07$, $n = 227$, $p < 0.01$) (Figs. 3, 4). Comparing the different treatment
245 levels (using a Tukey HSD test), only the relative surface area change between 1 versus 20 x *g* (-
246 47%, $p<0.01$) and 5 versus 20 x *g* (-45%, $p<0.01$) were significant. No effect of feeding on surface
247 area cover increase was found (Fig. 4). A significant interaction effect of *g*-force and feeding on
248 mean osculum formation per group was observed, where fed sponges formed oscula less frequently
249 than non-fed sponges, especially at higher *g*-force (logit(osculum formation) = 0.71 – 0.13 ·
250 feeding + 0.06 · *g*-force – 0.10 · feeding · *g*-force; Nagelkerke-$R^2 = 0.08$, $n = 227$, $p < 0.05$).

251

252 **Tracer isotope incorporation**

253 Amino acid assimilation was confirmed by significant enrichment in both $\delta^{13}C$ (42.5 ± 11.1‰)
254 and $\delta^{15}N$ (1202.8 ± 280.2‰) in the tissues of all fed sponges versus background, non-fed, sponge
255 tissue (-30.5‰ $\delta^{13}C$ and 18.2‰ $\delta^{15}N$). On average, hatching *E. fluviatilis* assimilated 0.4 ± 0.1
256 µmol $C_{amino\ acids}$ mmol C_{sponge}^{-1} d^{-1} and 4.3 ± 1.0 µmol $N_{amino\ acids}$ mmol N_{sponge}^{-1} d^{-1} (Fig. 5). In

257 total, 9.1 ± 3.7% and 41.2 ± 18.5% of the amino acid carbon and nitrogen, respectively, that was
258 added was processed by the sponges during the 48-h incubations. No significant effect of *g*-force
259 was found for ^{13}C-uptake, while the overall effect for ^{15}N incorporation rates was significant (C~g-
260 force, $n = 10$, $p = 0.165$; N~g-force, $n = 10$, $p<0.05$) (Fig. 5). When comparing the effects on ^{15}N
261 for different treatment levels (using a Tukey HSD test), only the difference between 5 x *g* versus
262 20 x *g* (-2.7, $p< 0.01$) appeared significant.

Discussion

Spicule skeleton construction and osculum formation under hypergravity

Sponges are known for their enormous plasticity and opportunistic nature when it comes to food selection (e.g., Ribes *et al.* 1999; McMurray *et al.* 2016) and regeneration after environmental stress (e.g., Ayling *et al.* 1983; Wulff 2010; Alexander *et al.* 2015). This is particularly important in ecosystems with large seasonal fluctuations in water temperature and food availability, where the formation of gemmules by freshwater sponges is a very successful strategy to survive these adverse and possibly lethal conditions. When conditions are more favorable, gemmules hatch and develop into fully grown sponges in order to enter a successful sexual reproductive stage and obtain sufficient biomass to gemmulate (Funayama *et al*., 2005; Manconi & Pronzato, 2016). The formation of the spicule skeleton is a crucial feature of the morphogenesis of freshwater sponges, resulting eventually in the formation of an active filter-feeding system (Rozenfeld, 1980; Funayama *et al*., 2005), enabling the sponge to grow and reproduce. Albeit the hypergravity forces used in our experiment are not naturally occurring, this is the first *in vivo* study of freshwater sponges to show that a pivotal step in the seasonal life-cycle of *E. Fluviatilis* is highly resilient under extreme levels of mechanical stress. Even after the hatched gemmules were exposed to hypergravity forces up to 20 x *g* for 48 h, they managed to survive, grow, create and organize their spicules, and develop a functional food-uptake system. In comparison, a well-trained human astronaut has a *g*-force tolerance of approximately 9 x *g* for only short periods of seconds to minutes (Wu *et al.*, 2012). Sponges showed a significant decrease in surface area under increased *g*-force exposure, but almost all specimens (96% ± 1.2%) were able to set-up their spicule skeleton.

It is noteworthy that, although this study focused on the siliceous spicules of the *E.fluviatilis* skeleton, the fibrous matrix of sponging and chitin in which the spicules are embedded,

286 may also play a role in withstanding environmental stress (Ehrlich *et al.,* 2010, 2013). The presence
287 of chitin is known to be responsible for the rigidification or skeletal structures in invertebrates and
288 marine demosponges (Brunner *et al*., 2009; Ehrlich *et al*., 2010) and has since long been known
289 to be an important component of *E. fluviatilis* gemmules (Zykoff, 1892). However, it is at present
290 unknown whether or how the fibrous matrix of the skeleton changes in response to environmental
291 conditions.
292
293 **Fed versus non-fed sponges under hypergravity**
294 We also show that hatching *E. fluviatilis* can take up dissolved nutrients (i.e. amino acids).
295 Previous evidence corroborates that freshwater sponges can feed on dissolved organic food
296 sources, such as dissolved proteins (Weissenfels, 1976; Manconi & Pronzato, 2008). However,
297 this study shows, for the first time, that newly hatched gemmules take up and incorporate dissolved
298 food sources in their tissue *before* arranging their filter system and start active pumping. The
299 dissolved ^{13}C- and ^{15}N-labeled amino acids were most likely acquired either via passive diffusion
300 or through phagocytosis by choanocytes or surface pinacocytes (Willenz & van de Vyver, 1982;
301 Smith & Tiffon, 2013). However, the uptake of dissolved amino acids did not result in a significant
302 difference in the sponges' ability to erect their skeleton nor in their surface area growth throughout
303 the exposure to increasing levels of hypergravity, compared with non-fed sponges. This suggests
304 that the process of erecting the spicule sponge skeleton relies mainly on intrinsic energy. However,
305 fed sponges developed oscula significantly less frequently compared with non-fed sponges,
306 especially at exposure to the highest (10 and 20 x) *g*-forces. We speculate that under extremely
307 stressful conditions, when energy costs are highest, sponges invest in the formation of an active
308 filtration system to acquire new energy. Additional food can help prologue the process of hatching

and skeleton development, which might increase their chances of survival throughout severe stress conditions.

The role of dissolved food in the diet of freshwater sponges

The capacity of *E. fluviatilis* to readily take up dissolved food sources could be a valuable addition to their daily natural diet. Evidence is accumulating that many sponge species utilize dissolved organic matter (DOM) as major part (71–94 %) of their daily carbon intake (Yahel *et al*. 2003; De Goeij *et al*. 2008; Mueller *et al*. 2014; McMurray 2016), however, these studies were all done on marine sponge species. It has also been shown that mainly sponges with high numbers of associated bacteria (high microbial abundance (HMA) sponges) utilize DOM as food source, as opposed to the low microbial abundance (LMA) sponges (Hoer *et al.* 2018), unless sponges have an encrusting growth form (De Goeij *et al.* 2017). The freshwater sponge *E. fluviatilis* is a LMA species, but the algal symbionts of *E.fluviatilis* are not an obligate requirement for survival of the host (Wilkinson, 1980). Moreover, *E.fluviatilis* can be both encrusting and massive, depending on its stage of development. Therefore, it would be interesting to assess whether *E. fluviatilis* adjusts its diet depending on its algal symbionts or at various phases of its yearly life-cycle, and if it is able to process DOM continuously.

In tropical coral reefs, sponges play a key role in the cycling of nutrients by converting predominantly DOM into particulate organic matter (POM) through high cell turnover and detritus production, which is subsequently shunted to higher trophic levels – a process termed the sponge loop (de Goeij *et al.*, 2013). Since detritus production and rapid division of choanocytes by mitosis have been reported for *E. fluviatilis* (Weissenfels, 1976; Tanaka & Watanabe, 1984), it is likely

that similar sponge-loop processes also occur in freshwater systems. Additionally, the C:N ratio of the amino acids (5:1) in the medium was significantly lower than the assimilated C:N ratio by *E. fluviatilis* (1:5), and the sponge assimilated up to 4 times more nitrogen from the amino acids (41%) as compared with amino acid carbon (9%). Carbon could have been partly lost to respiration, but *E. fluviatilis* most likely assimilated nitrogen selectively from the amino acid source. As sponge detritus in eutrophic tropical ecosystems was found relatively enriched in nitrogen compared to sponge tissue (de Goeij *et al*., 2013), and the current view is that limitation of nitrogen is also common in eutrophic freshwaters (Downing & McCauley, 1992), freshwater sponges might fertilize their surrounding ecosystems in the same way as their marine counterparts.

Conclusions

Our results demonstrate that the process of skeleton formation in freshwater sponges plays an important role in their development towards a fully grown, actively pumping, and filter-feeding sponge. The finding that sponges are able to cope with extreme *g*-forces of up to 20 x *g* for as long as two days has to our knowledge never been found in a multicellular invertebrate aquatic organism. These results also support the ideas of Nakayama *et al*. (2015) that the mechanisms of self-construction shown by *E. fluviatilis* are adjusted by its (micro)environment and are potentially useful in other fields of research such as (bio)engineering. The underlying cellular and molecular mechanisms of processes such as spicule displacement, arrangement and cementing as well as their adaptive potential are at present unknown. In addition, this study supports the findings of Skelton & Strand (2013) that freshwater sponges may serve as an important energetic link between pelagic and benthic food webs in systems were sponges are abundant. The uptake of dissolved food may

be an important factor in the daily diet of freshwater sponges and they may contribute significantly to the cycling of dissolved nutrients in freshwater ecosystems.

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366 **References**

367 Alexander, B. E., Achlatis, M., Osinga, R., van der Geest, H. G., Cleutjens, J. P., Schutte, B., &
368 de Goeij, J. M. (2015). Cell kinetics during regeneration in the sponge *Halisarca caerulea*:
369 how local is the response to tissue damage?. *PeerJ*, *3*, e820.

370 Ayling, A. L. (1983). Growth and regeneration rates in thinly encrusting demospongiae from
371 temperate waters. *The Biological Bulletin*, *165*(2), 343-352.

372 Brunner, E., Ehrlich, H., Schupp, P., Hedrich, R., Hunoldt, S., Kammer, M., ... & Arnold, T.
373 (2009). Chitin-based scaffolds are an integral part of the skeleton of the marine
374 demosponge *Ianthella basta*. *Journal of structural biology*, 168(3), 539-547.

375 Canciani, B., Ruggiu, A., Giuliani, A., Panetta, D., Marozzi, K., Tripodi, M., ... & Tavella, S.
376 (2015). Effects of long time exposure to simulated micro-and hypergravity on skeletal
377 architecture. *Journal of the mechanical behavior of biomedical materials*, *51*, 1-12.

378 Cavalier-Smith, T. (2017). Origin of animal multicellularity: precursors, causes, consequences—
379 the choanoflagellate/sponge transition, neurogenesis and the Cambrian explosion.
380 *Philosophical Transaction of the Royal Society of Biological Sciences*, 372(1713),
381 20150476.

382 Chebli, Y., Van Loon, J., & Geitmann, A. N. J. A. (2012). Live cell imaging under hyper-gravity
383 conditions. *Bulletin microscopical society canada*, *40*(3), 8-12.

384 De Goeij, J. M., van den Berg, H., van Oostveen, M. M., Epping, E. H., & Van Duyl, F. C. (2008).
385 Major bulk dissolved organic carbon (DOC) removal by encrusting coral reef cavity
386 sponges. *Marine Ecology Progress Series*, *357*, 139-151.

387 De Goeij, J. M., Van Oevelen, D., Vermeij, M. J., Osinga, R., Middelburg, J. J., de Goeij, A. F.,
388 & Admiraal, W. (2013). Surviving in a marine desert: the sponge loop retains resources
389 within coral reefs. *Science*, *342*(6154), 108-110.
390 De Goeij, J. M., Lesser, M. P., & Pawlik, J. R. (2017). Nutrient Fluxes and Ecological Functions
391 of Coral Reef Sponges in a Changing Ocean. In *Climate Change, Ocean Acidification and*
392 *Sponges* (pp. 373-410). Springer, Cham.
393 Downing, J. A., & McCauley, E. (1992). The nitrogen: phosphorus relationship in
394 lakes. *Limnology and Oceanography*, *37*(5), 936-945.
395 Ehrlich, H., Ilan, M., Maldonado, M., Muricy, G., Bavestrello, G., Kljajic, Z., ... & Born, R. (2010).
396 Three-dimensional chitin-based scaffolds from Verongida sponges (Demospongiae:
397 Porifera). Part I. Isolation and identification of chitin. *International Journal of Biological*
398 *Macromolecules*, 47(2), 132-140.
399 Ehrlich, H., Kaluzhnaya, O. V., Brunner, E., Tsurkan, M. V., Ereskovsky, A., Ilan, M., ... & Born,
400 R. (2013). Identification and first insights into the structure and biosynthesis of chitin from
401 the freshwater sponge *Spongilla lacustris*. *Journal of Structural Biology*, 183(3), 474-483.
402 Elliott, G. R., & Leys, S. P. (2007). Coordinated contractions effectively expel water from the
403 aquiferous system of a freshwater sponge. *Journal of Experimental Biology*, *210*(21),
404 3736-3748.
405 Funayama, N., Nakatsukasa, M., Hayashi, T., & Agata, K. (2005). Isolation of the choanocyte in
406 the fresh water sponge, *Ephydatia fluviatilis* and its lineage marker, Ef
407 annexin. *Development, growth & differentiation*, *47*(4), 243-253.

408 Funayama, N. (2013). The stem cell system in demosponges: suggested involvement of two types
409 of cells: archeocytes (active stem cells) and choanocytes (food-entrapping flagellated
410 cells). *Development genes and evolution*, *223*(1-2), 23-38.
411 Harrison, F. W., Rosenberg, E. M., Davis, D. A., & Simpson, T. L. (1981). Correlation of cyclic
412 GMP and cyclic AMP immunofluorescence with cytochemical patterns during dormancy
413 release and development from gemmules in *Spongilla lacustris* L.(Porifera: Spongillidae).
414 *Journal of Morphology*, 167(1), 53-63.
415 Harsha, R. E., Francis, J. C., & Poirrier, M. A. (1983). Water temperature: a factor in the
416 seasonality of two freshwater sponge species, *Ephydatia fluviatilis* and *Spongilla*
417 *alba*. *Hydrobiologia*, *102*(3), 145-150.
418 Hoer, D. R., Gibson, P. J., Tommerdahl, J. P., Lindquist, N. L., & Martens, C. S. (2018).
419 Consumption of dissolved organic carbon by Caribbean reef sponges. *Limnology and*
420 *Oceanography*.
421 Höhr, D. (1977). Differenzierungsvorgänge in der keimenden Gemmula vonEphydatia fluviatilis.
422 *Wilhelm Roux's archives of developmental biology*, 182(4), 329-346.
423 Kacena, M. A., Todd, P., Gerstenfeld, L. C., & Landis, W. J. (2004). Experiments with osteoblasts
424 cultured under hypergravity conditions. *Microgravity-Science and Technology*, *15*(1), 28.
425 Larsen, P. S., & Riisgård, H. U. (1994). The sponge pump. *Journal of Theoretical Biology*, 168(1),
426 53-63.
427 Leys, S. P., Nichols, S. A., & Adams, E. D. (2009). Epithelia and integration in
428 sponges. *Integrative and Comparative Biology*, *49*(2), 167-177.

429 Love, G. D., Grosjean, E., Stalvies, C., Fike, D. A., Grotzinger, J. P., Bradley, A. S., ... & Bowring,
430 S. A. (2009). Fossil steroids record the appearance of Demospongiae during the
431 Cryogenian period. *Nature*, *457*(7230), 718-721.
432 Ludeman, D. A., Farrar, N., Riesgo, A., Paps, J., & Leys, S. P. (2014). Evolutionary origins of
433 sensation in metazoans: functional evidence for a new sensory organ in sponges. *BMC*
434 *evolutionary biology*, *14*(1), 3.
435 Manconi, R., & Pronzato, R. (2008). Global diversity of sponges (Porifera: Spongillina) in
436 freshwater. *Hydrobiologia*, *595*(1), 27-33.
437 Manconi, R., & Pronzato, R. (2016). How to survive and persist in temporary freshwater? Adaptive
438 traits of sponges (Porifera: Spongillida): A review. *Hydrobiologia*, *782*(1), 11-22.
439 McMurray, S. E., Johnson, Z. I., Hunt, D. E., Pawlik, J. R., & Finelli, C. M. (2016). Selective
440 feeding by the giant barrel sponge enhances foraging efficiency. *Limnology and*
441 *Oceanography*, *61*(4), 1271-1286.
442 Meroz, E., Brickner, I., Loya, Y., Peretzman-Shemer, A., & Ilan, M. (2002). The effect of gravity
443 on coral morphology. *Proceedings of the Royal Society of London B: Biological Sciences*,
444 269(1492), 717-720.
445 Mohri, K., Nakatsukasa, M., Masuda, Y., Agata, K., & Funayama, N. (2008). Toward
446 understanding the morphogenesis of siliceous spicules in freshwater sponge: Differential
447 mRNA expression of spicule-type-specific silicatein genes in *Ephydatia*
448 *fluviatilis*. *Developmental Dynamics*, *237*(10), 3024-3039.
449 Mueller, B., de Goeij, J. M., Vermeij, M. J., Mulders, Y., van der Ent, E., Ribes, M., & van Duyl,
450 F. C. (2014). Natural diet of coral-excavating sponges consists mainly of dissolved organic
451 carbon (DOC). *PloS one*, *9*(2), e90152.

452 Müller, W.E.G. (1998). Origin of Metazoa: sponges as living fossils. *Naturwissenschaften*, *85*(1),
453 11-25.
454 Müller, W. E., Wiens, M., Adell, T., Gamulin, V., Schröder, H. C., & Müller, I. M. (2004). Bauplan
455 of urmetazoa: basis for genetic complexity of metazoa. *International review of*
456 *cytology*, *235*, 53-92.
457 Nakayama S, Arima, K., Mohri,K., & Funayama, N. (2010). Spicule skeleton formation as a new
458 model to clarify pattern formation in demosponges: The roughly spaced spicule holding up
459 (SHU) points and the identification of spicule carrying cells. *Proceedings of 8th World*
460 *Sponge Symposium, Girona, Spain,* 86.
461 Nakayama, S., Arima, K., Kawai, K., Mohri, K., Inui, C., Sugano, W., ... & Arai-Shindo, M.
462 (2015). Dynamic transport and cementation of skeletal elements build up the pole-and-
463 beam structured skeleton of sponges. *Current Biology*, *25*(19), 2549-2554.
464 Nosenko, T., Schreiber, F., Adamska, M., Adamski, M., Eitel, M., Hammel, J., ... & Vacelet, J.
465 (2013). Deep metazoan phylogeny: when different genes tell different stories. *Molecular*
466 *phylogenetics and evolution*, *67*(1), 223-233.
467 Prodanov, L., Semeins, C. M., van Loon, J. J. W. A., Te Riet, J., Jansen, J. A., Klein-Nulend, J.,
468 & Walboomers, X. F. (2013). Influence of nanostructural environment and fluid flow on
469 osteoblast-like cell behavior: a model for cell-mechanics studies. *Acta biomaterialia*, *9*(5),
470 6653-6662.
471 Rasmont, R. (1961). Une technique de culture des éponges d'eau douce en milieu contrôlé. Ann.
472 Soc. R. Zool. Belg, 91, 147-155.

473 Ribes, M., Coma, R., & Gili, J. M. (1999). Natural diet and grazing rate of the temperate sponge
474 *Dysidea avara* (Demospongiae, Dendroceratida) throughout an annual cycle. *Marine*
475 *Ecology Progress Series*, 179-190.
476 Rozenfeld, F. (1970). Inhibition du développement des gemmules de spongillides: spécificité et
477 moment d'action de la gemmulostasine. *Archives de Biologie Liege*, 81, 193-214.
478 Rozenfeld, F. (1980). Effects of puromycin on the differentiation of the freshwater sponge:
479 *Ephydatia fluviatilis*. *Differentiation*, *17*(1-3), 193-198.
480 Searby, N. D., Steele, C. R., & Globus, R. K. (2005). Influence of increased mechanical loading
481 by hypergravity on the microtubule cytoskeleton and prostaglandin E2 release in primary
482 osteoblasts. *American Journal of Physiology-Cell Physiology*, *289*(1), C148-C158.
483 Skelton, J., & Strand, M. (2013). Trophic ecology of a freshwater sponge (*Spongilla lacustris*)
484 revealed by stable isotope analysis. *Hydrobiologia*, *709*(1), 227-235.
485 Smith, D. C., & Tiffon, Y. (Eds.). (2013). *Nutrition in the Lower Metazoa: Proceedings of a*
486 *Meeting Held at the University of Caen, France, 11-13 September 1979*. Elsevier.
487 Srivastava, M., Simakov, O., Chapman, J., Fahey, B., Gauthier, M. E., Mitros, T., ... & Larroux,
488 C. (2010). The *Amphimedon queenslandica* genome and the evolution of animal
489 complexity. *Nature*, *466*(7307), 720-726.
490 Tanaka, K., & Watanabe, Y. (1984). Choanocyte differentiation and morphogenesis of choanocyte
491 chambers in the fresh-water sponge, *Ephydatia fluviatilis*, after reversal of developmental
492 arrest caused by hydroxyurea. *Zoological science*, *1*(4), p561-570.
493 Uriz, M. J., Turon, X., Becerro, M. A., & Agell, G. (2003). Siliceous spicules and skeleton
494 frameworks in sponges: origin, diversity, ultrastructural patterns, and biological
495 functions. *Microscopy research and technique*, *62*(4), 279-299.

Van Loon, J. J. W. A., Van Laar, M. C., Korterik, J. P., Segerink, F. B., Wubbels, R. J., De Jong, H. A. A., & Van Hulst, N. F. (2009). An atomic force microscope operating at hypergravity for in situ measurement of cellular mechano-response. *Journal of microscopy*, *233*(2), 234-243.

Van Soest, R.W.M., Boury-Esnault, N., Hooper, J.N.A., Rützler, K., de Voogd, N.J., Alvarez de Glasby, B., Hajdu, E., Pisera, A.B., Manconi, R., Schoenberg, C., Klautau, M., Picton, B., Kelly, M., Vacelet, J., Dohrmann, M., Díaz, M.-C., Cárdenas, P., Carballo, J. L., & Rios Lopez, P. (2017). World Porifera database. Accessed at http://www.marinespecies.org/porifera on 2017-07-18

Weissenfels, N. (1976). Bau und Function des Susswasser-schwamms *Ephydatia fluviatilis* L.(Porifera) III. Nahrung-saufnahme, Verdauung und Defakation. *Zoomorphologie*, 85, 73-88.

Wierzejski, A. (1915). Beobachtungen über die Entwicklung der Gemmulae der Spongilliden und des Schwammes aus den Gemmulis. *Bulletin International De l'Academie Polonaise Des Sciences Et Des Lettre* (B), 2, 45-79.

Wierzejski, A. (1935). Süsswasserspongilliden: Monographische Bearbeitung. *Mémoires Academy Polony* (B) 9, 1-242

Wilkinson, C. R. (1980). Nutrient translocation from green algal symbionts to the freshwater sponge *Ephydatia fluviatilis. Hydrobiologia,* 75(3), 241-250.

Willenz, P., & Van De Vyver, G. (1982). Endocytosis of latex beads by the exopinacoderm in the fresh water sponge *Ephydatia fluviatilis*: an in vitro and in situ study in SEM and TEM. *Journal of ultrastructure research*, *79*(3), 294-306.

Wu, B., Xue, Y., Wu, P., Gu, Z., Wang, Y., & Jing, X. (2012). Physiological responses of astronaut candidates to simulated+ Gx orbital emergency re-entry. *Aviation, space, and environmental medicine*, *83*(8), 758-763.

Wulff, J. (2010). Regeneration of sponges in ecological context: is regeneration an integral part of life history and morphological strategies?. *Integrative and Comparative Biology*, *50*(4), 494-505.

Yahel, G., Sharp, J.H., Marie, D., Hase, C., & Genin, A. (2003). In situ feeding and element removal in the symbiont-bearing sponge *Theonella swinhoei*: Bulk DOC is the major source for carbon. *Limnology and Oceanography* 48:141-149

Zykoff, W. (1892). The development of the gemmules of *Ephydatia fluviatilis*, Auct. *Journal of Natural History*, 10(59), 413-415.

Figure 1

Developmental stages of germination in the freshwater sponge *Ephydatia fluviatilis*.

A: modified from Funayama *et al*. (2005). Stage 0: resting gemmule. Stage I: (approximately 2-d post-hatching): cells migrate outwards from the gemmule and differentiate into epithelial cells. Stage II: (3–4 d): cells start to proliferate and begin to differentiate into different cell types. This stage includes the development of the spicule-making sclerocytes. Spicule production generally starts around stage II and continues thereafter (Funayama *et al*., 2005; Mohri *et al*., 2008). Stage III: (4–5 d post-hatching): the canal system and choanocyte chambers are starting to form. Stage IV: (6–7 d post-hatching): The osculum is created approximately one week after hatching, creating a fully functional sponge. B: Hypothesized response of the influence of mechanical stress on developing sponges in combination with feeding. We hypothesize sponges will not be able to set up their spicule skeleton after prolonged exposure to increasing levels of hypergravity, but counteract this inability after additional feeding on dissolved food sources before forming a fully functional active filter-feeding system.

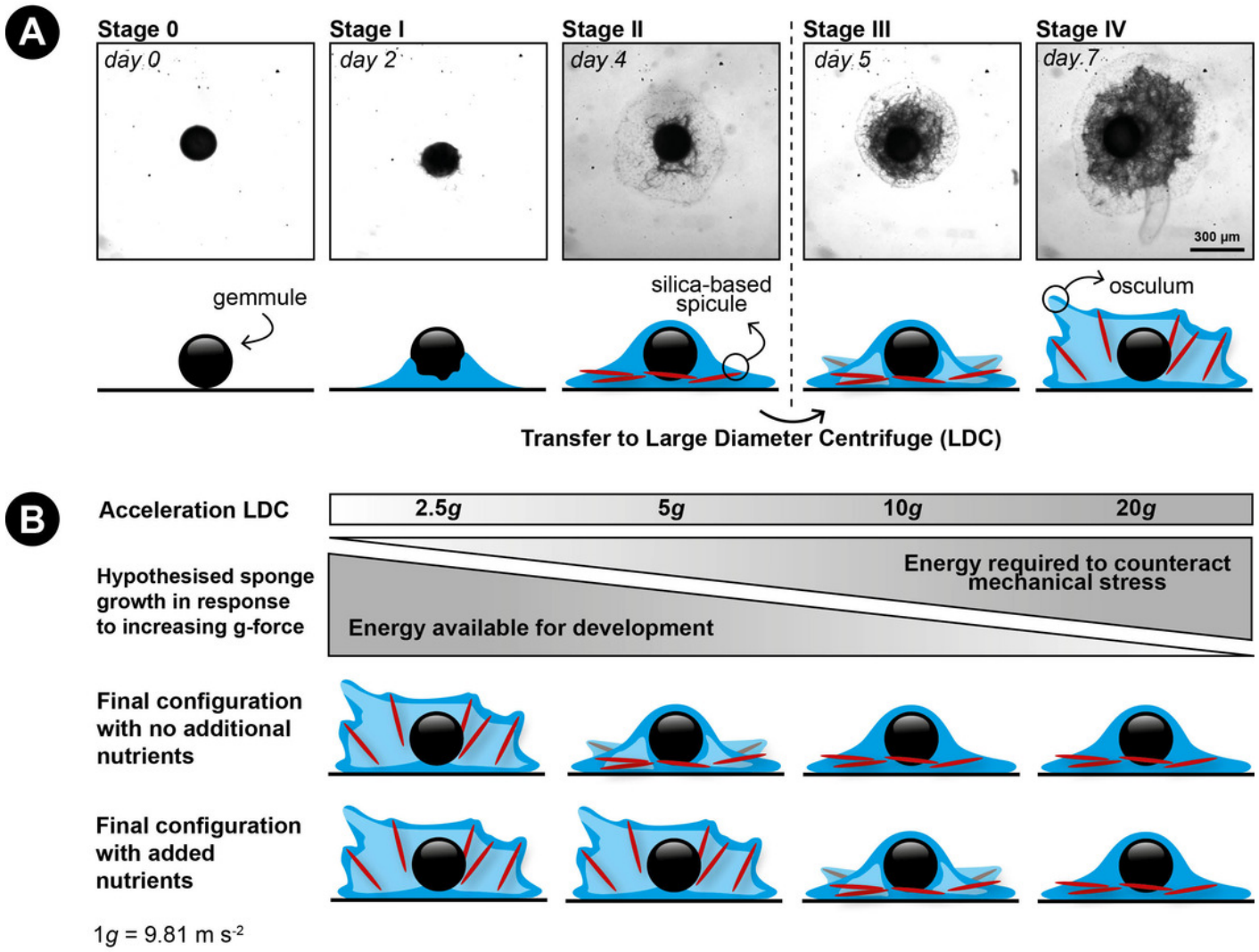
A
Stage 0
day 0
Stage I
day 2
Stage II
day 4
Stage III
day 5
Stage IV
day 7
300 µm
gemmule
silica-based spicule
osculum
Transfer to Large Diameter Centrifuge (LDC)
B
Acceleration LDC
2.5g
5g
10g
20g
Hypothesised sponge growth in response to increasing g-force
Energy required to counteract mechanical stress
Energy available for development
Final configuration with no additional nutrients
Final configuration with added nutrients
1g = 9.81 m s-2

Figure 2(on next page)

Schematic set-up of the experimental procedure using the large diameter centrifuge (LDC) at the European space agency (ESA), Noordwijk, the Netherlands.

A: Timeline of the experiment. B: Configuration of the LDC gondolas (top-view). The LDC has four arms each of which can accommodate two gondolas carrying the well plates with the gemmules. When the centrifuge is spun, the gondolas swing out at an angle (θ) and a hypergravity field (g_{eff}) inside the gondolas is created by the centripetal forces due to the rotation.

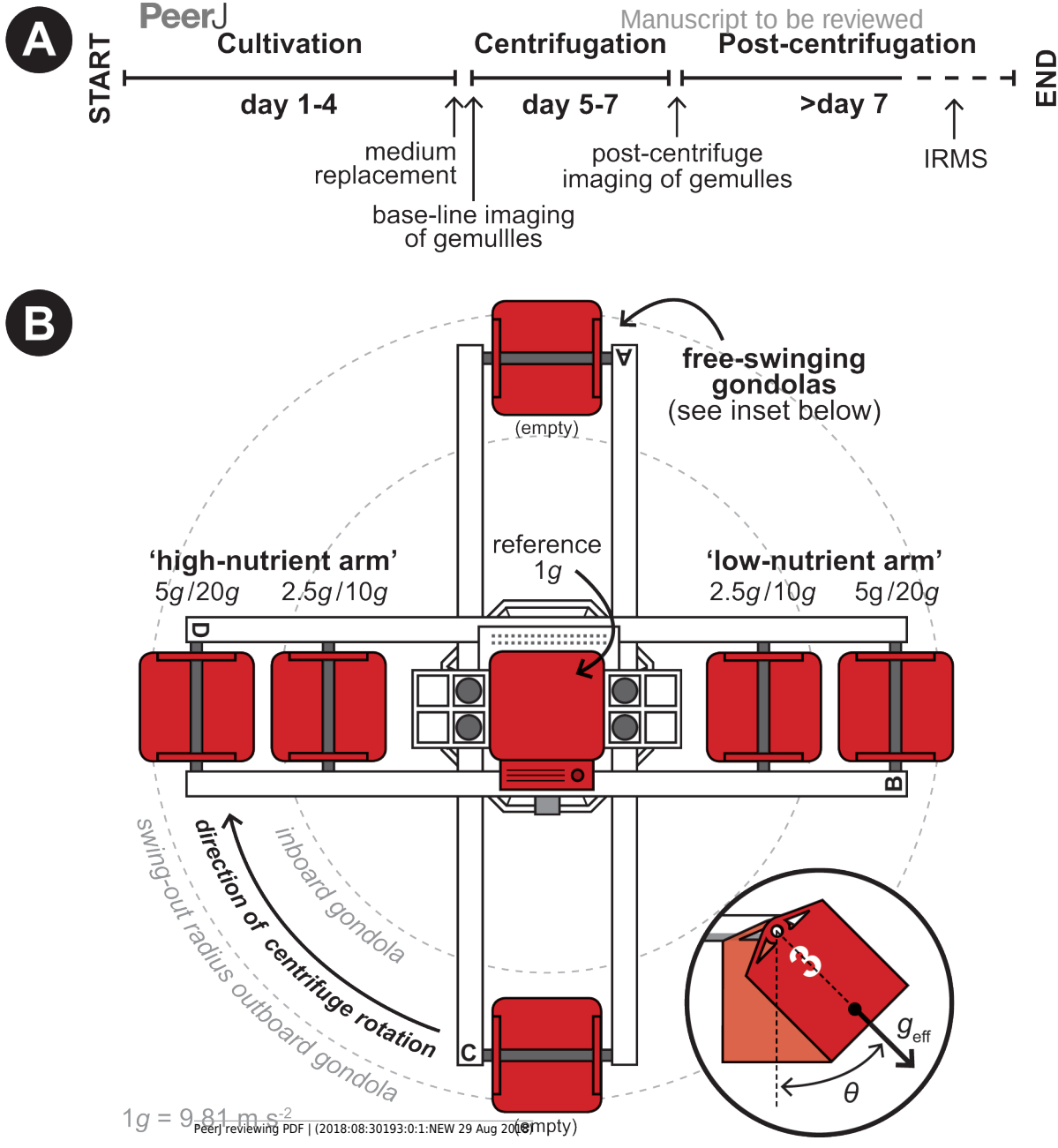
A
START
Cultivation
day 1-4
Centrifugation
day 5-7
Post-centrifugation
>day 7
END
medium replacement
base-line imaging of gemullles
post-centrifuge imaging of gemulles
IRMS
B
free-swinging gondolas
(see inset below)
(empty)
reference 1g
'high-nutrient arm'
5g/20g 2.5g/10g
'low-nutrient arm'
2.5g/10g 5g/20g
A
B
C
D
direction of centrifuge rotation
inboard gondola
swing-out radius outboard gondola
(empty)
g_{eff}
θ
1g = 9.81 m s-2

Figure 3(on next page)

Spicule skeleton in developing *E. fluviatilis* gemmules under different hypergravity forces.

A: Sponges photographed after the LDC centrifugation. Note that although surface area decreases with gravity level, under all hypergravity forces the sponges managed to produce spicules, set-up their three-dimensional skeleton and form an osculum. B: Zoomed-in photographs of osculum formation and spicules after 48 h exposure to 20 x *g*.

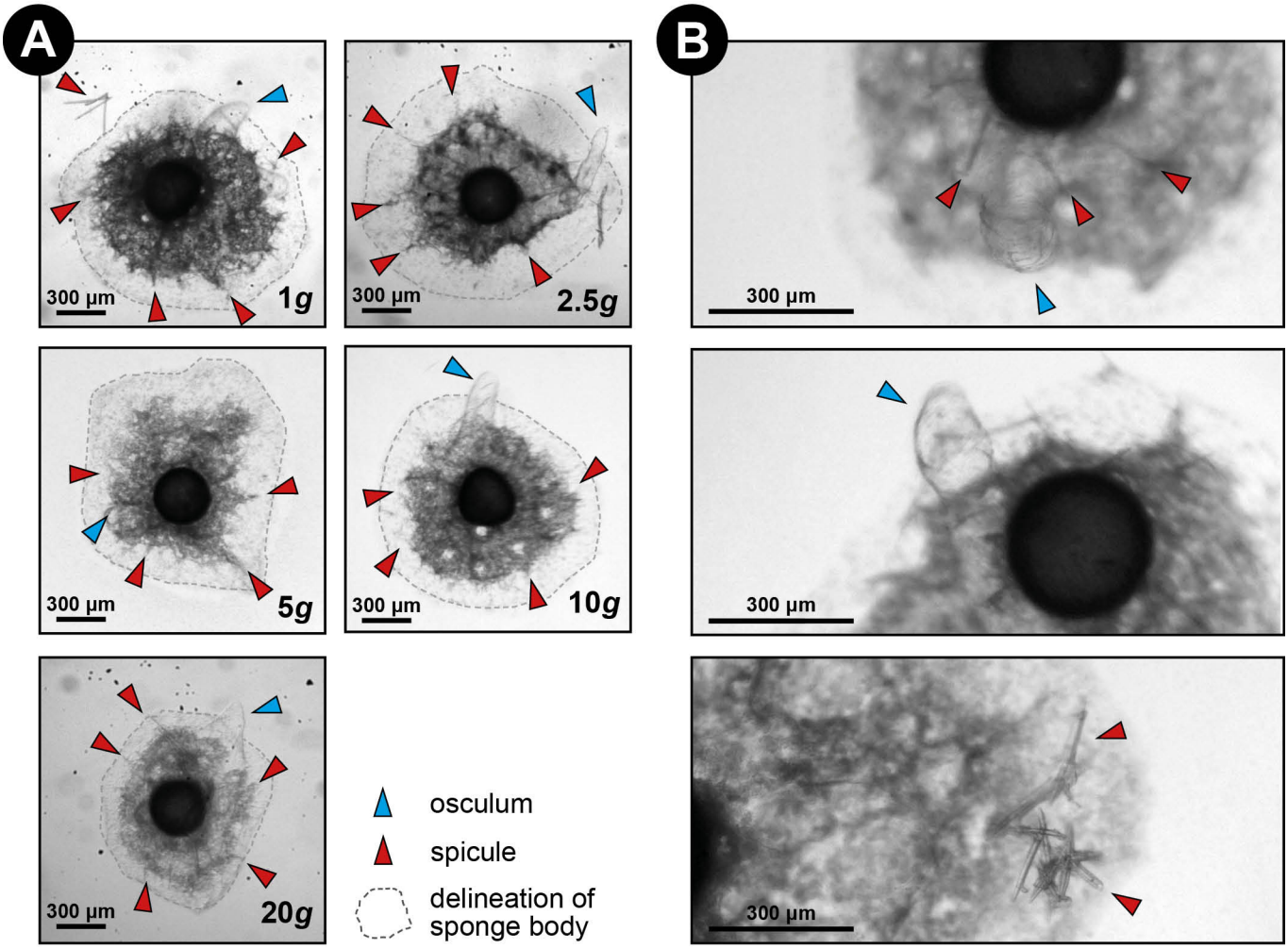
A
B
300 µm
1g
300 µm
2.5g
300 µm
5g
300 µm
10g
300 µm
20g
300 µm
300 µm
300 µm
osculum
spicule
delineation of sponge body

Figure 4(on next page)

Substrate area cover increase in fed and non-fed *E. fluviatilis* gemmules under different hypergravity conditions.

The average surface area cover increase (% of initial size) of fed and non-fed sponges under the different hypergravity forces. Error bars represent standard errors of the mean.

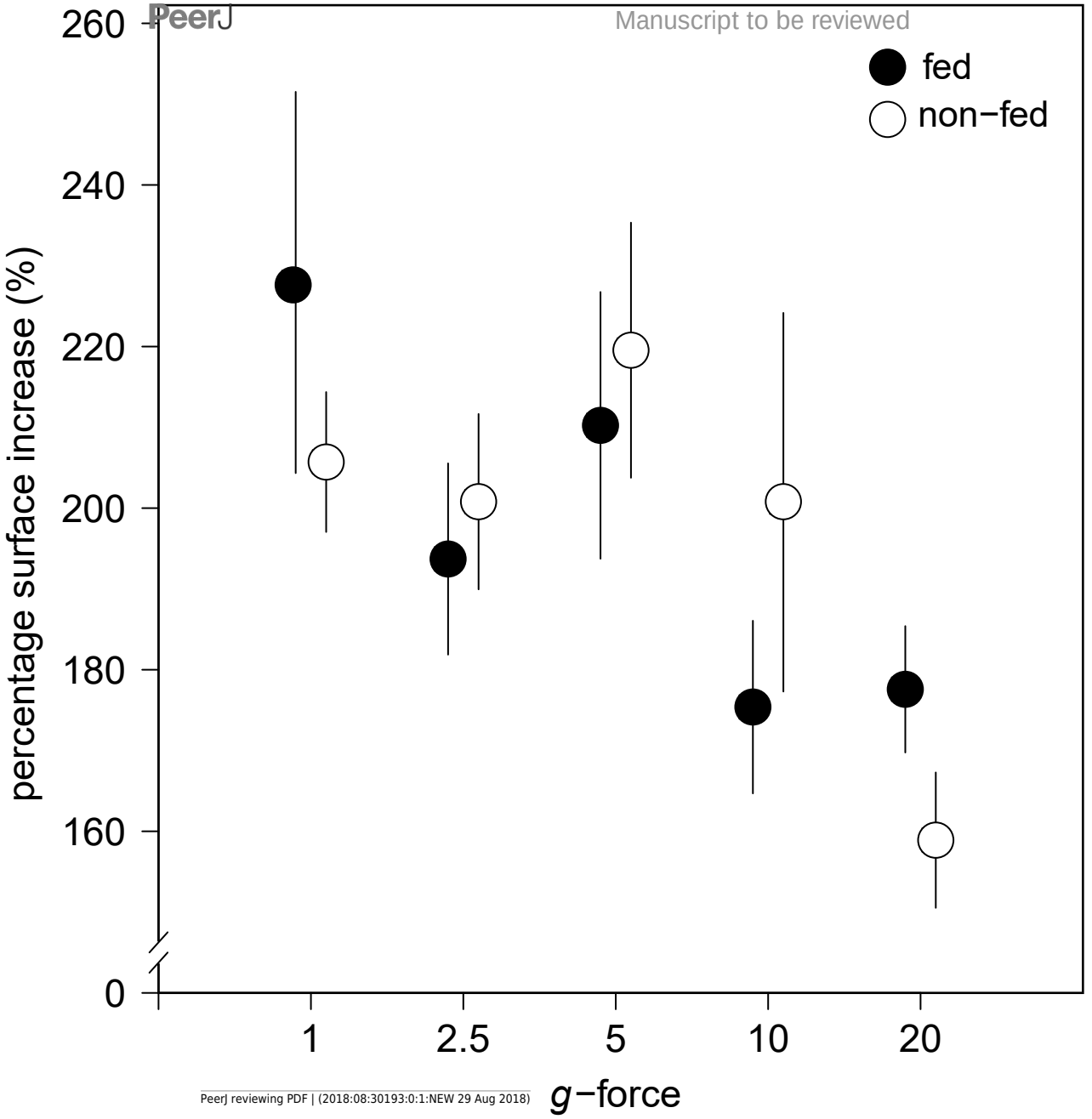
fed
non−fed
percentage surface increase (%)
260
240
220
200
180
160
0
1
2.5
5
10
20
g−force

Figure 5(on next page)

Incorporation of isotopically-enriched (^{13}Cand ^{15}N) amino acids in developing *E. fluviatilis*.

Average incorporation rates are expressed as µmol C or N_{tracer} $mmol_{sponge}^{-1}$ d^{-1}. The dotted line represents average uptake across all *g* force levels. Error bars represent standard errors of the mean. Asterisk indicates significant difference compared to uptake at 1 x *g*.

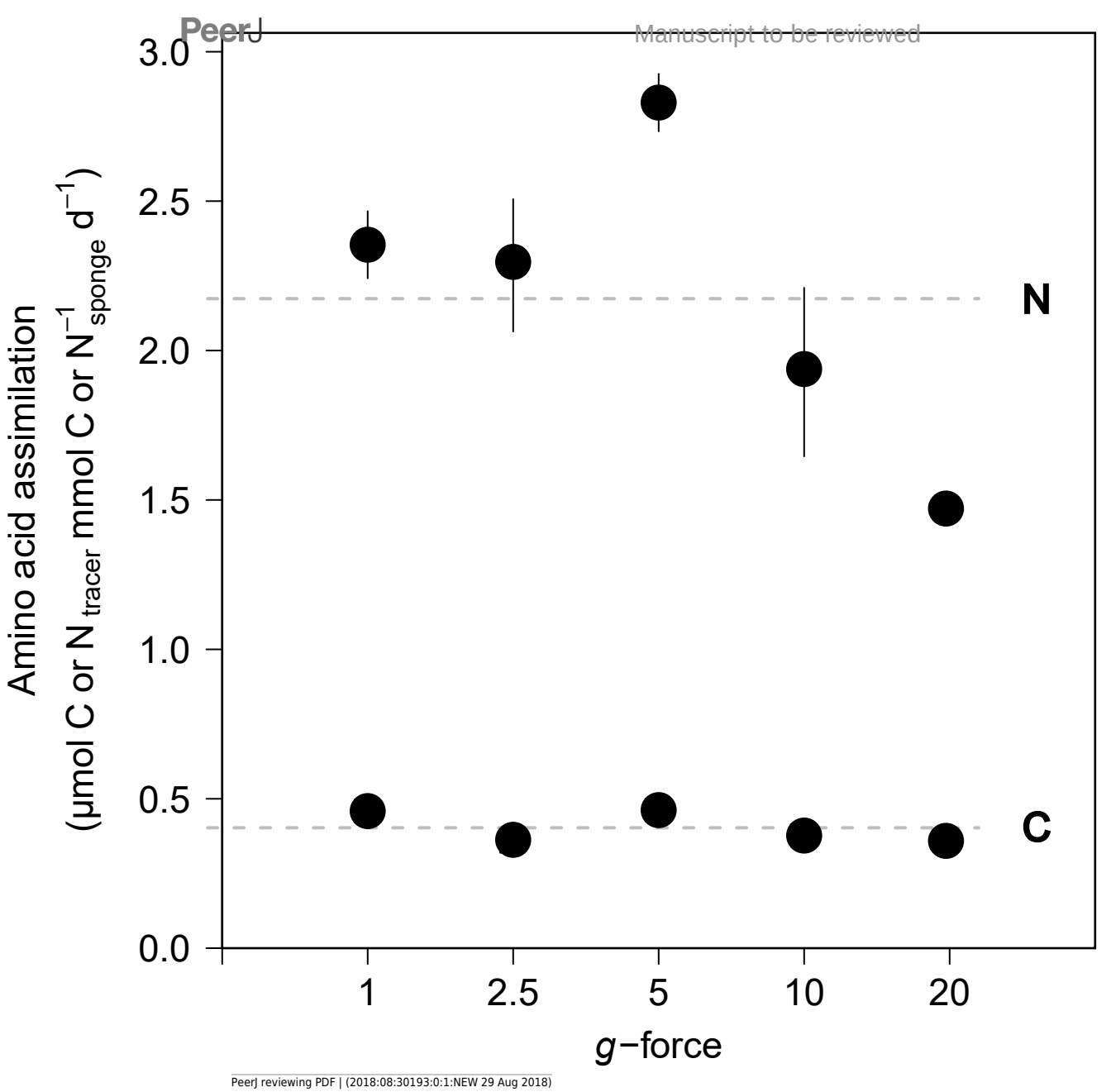
Amino acid assimilation
(µmol C or N tracer mmol C or N sponge−1 d−1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
N
C
1
2.5
5
10
20
g−force

Table 1(on next page)

Development of *E. fluviatilis* gemmules under hypergravity exposure (1 x g = 9.81 m s^{-2})

Stage III sponges have erected their silica spicules. Stage IV sponges have developed an osculum.

***g*-force**	**1**		**2.5**		**5**		**10**		**20**		**Total**	non-fed	fed
Fed	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	227	122	105
n	24	22	21	20	21	25	19	29	20	26			
Stage III reached (%)	100	95	100	90	95	92	100	93	100	96	**96%** (± 1%)	**99%** (± 1%)	**93%** (± 1%)
Stage IV reached (%)	79	73	95	60	29	48	79	59	95	46	**66%** (± 7%)	**75%** (± 13%)	**57%** (± 5%)