Emerging Model Organism

The Emergence of the Tardigrade *Hypsibius exemplaris* as a Model System

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The success of scientists in revealing biological mechanisms has depended in large part on choosing tractable model systems. In 1997, molecular phylogenetics revealed that two of biology's most tractable models—*Caenorhabditis elegans* and *Drosophila*—are much more closely related to each other than had been thought previously. I began to explore whether any of the little-studied members of this branch of the tree of life might serve as a new model for comparative biology that could make use of the rich and ongoing sources of information flowing from *C. elegans* and *Drosophila* research. Tardigrades, also known as water bears, make up a phylum of microscopic animals. The tardigrade *Hypsibius exemplaris* (recently disambiguated from a closely related species, *Hypsibius dujardini*) can be maintained in laboratories and has a generation time of <2 wk at room temperature. Stocks of animals can be stored frozen and revived. The animals and their embryos are optically clear, and embryos are laid in groups, with each synchronous clutch of embryos laid in a clear molt. We have developed techniques for laboratory study of this system, including methods for microinjection of animals, immunolocalization, in situ hybridization, RNA interference, transcriptomics, and methods for identifying proteins that mediate tolerance to extreme environments. Here, I review the development of this animal as an emerging model system, as well as recent molecular studies aimed at understanding the evolution of developmental mechanisms that underpin the evolution of animal form and at understanding how biological materials can survive extreme environments.

BACKGROUND INFORMATION

In 1997, a new molecular phylogeny revealed that *Caenorhabditis elegans* and *Drosophila* are much more closely related to each other than had been thought previously (Aguinaldo et al. 1997). Before then, *Drosophila* was thought to be more closely related even to humans than to *C. elegans* (Sidow and Thomas 1994). The 1997 molecular phylogeny placed the nematodes (which include *C. elegans*) and arthropods (which include *Drosophila*) together in a clade that includes six other animal phyla (Aguinaldo et al. 1997; Park et al. 2006). These eight phyla together are named the Ecdysozoa, or molting animals. I imagined that other ecdysozoan phyla closely related to the nematodes and arthropods might serve as valuable models for evo–devo biology (Goodman and Coughlin 2000) and for modern comparative biology (Hall 1994) more generally, if it were possible to develop a practical laboratory model from among these animals. In particular, animals related to but not within the nematodes and arthropods might maximally take advantage of having two reference model systems in *C. elegans* and *Drosophila*. In the long term, the use of a relative of *C. elegans* and *Drosophila* might dramatically expand the sets of genes and mechanisms of interest for comparative studies, beyond the

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narrower sets of genes and mechanisms known to have conserved functions across a much greater breadth of animal diversity (Erwin and Davidson 2002; Richter and King 2013). But little modern molecular work had been done in any of the phyla closely related to nematodes and arthropods, suggesting that it might be necessary to develop a new model.

Starting in 1999, I began an effort toward developing such a model by collecting species of one ecdysozoan phylum, the tardigrades, from multiple sources. The tardigrades, also known as water bears, are a phylum of eight-legged, microscopic animals estimated to include thousands of species, over a thousand of which have been described to date (Bartels et al. 2016). I contacted tardigrade zoologists Harold Heatwole, Diane Nelson, and Jette Eibye-Jacobsen, who kindly advised me on how to find and keep tardigrades. I sought a species with several key characteristics: with small, clear embryos so that development could be observed directly; that lacked the ornamented envelopes that surround the embryos of some tardigrade species (Nelson et al. 2016), which are beautiful but can obscure cell boundaries in DIC microscopy; and with small cells and rapid embryonic cell cycles, because these characteristics correlate at least very roughly with small genome size—a feature that was facilitating research in established model systems (Gregory and Hebert 1999; Gregory 2001). A culture from amateur scientist Bob McNuff, who runs the small biological supply company Sciento, appeared suitable. Embryos were about 60 µm long with a smooth outer envelope, and early cell divisions occurred every 50–55 min. Mark Blaxter's laboratory had some unpublished sequence data from them and from related species (Goldstein and Blaxter 2002; Blaxter et al. 2004). I began work on this species, and I sent some animals to tardigrade expert Roberto Bertolani, who identified the species as resembling most closely the identified species *Hypsibius dujardini* (Gabriel et al. 2007). In 2018, the animals we study were identified instead as a new species, a close relative of *H. dujardini* that was dubbed *Hypsibius exemplaris*—a Latin form of "exemplar" marking the by-then "wide use of the species as a laboratory model for various types of scientific studies" (Gąsiorek et al. 2018). The animals are about 250 µm long and typify tardigrades in having two eyespots and eight legs (Fig. 1).

Historically, most tardigrade zoologists collected their animals outdoors repeatedly as needed instead of maintaining cultures long-term in laboratories, and tardigrades had only rarely been reared continually in laboratories because success with long-term culture had remained elusive in many tardigrade species (Altiero and Rebecchi 2001; Suzuki 2003; Horikawa et al. 2008). So I was

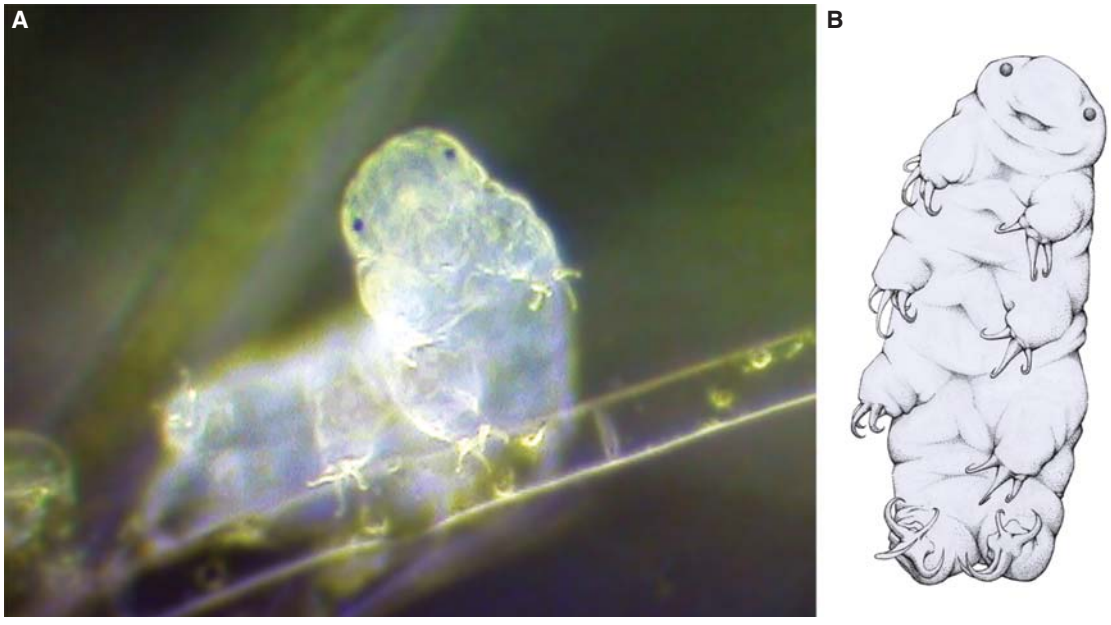

FIGURE 1. Adult *H. exemplaris*. (*A*) Photo by Sinclair Stammers, used with permission. (*B*) Illustration by Anya Brover-man-Wray based on a scanning electron micrograph.

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surprised when Bob McNuff first told me by phone in 2001 that he had been raising his cultures continuously, in a shed behind his home, for more than a decade—since 1987. Bob generously shared his culture methods, which enabled us to establish our own robust, continuous laboratory cultures, and then to begin to build a description of embryonic development and to work toward developing a battery of tools for laboratory study.

In the accompanying protocols, Bob McNuff shares his culture methods (see Protocol: **Laboratory Culture of *Hypsibius exemplaris*** [McNuff 2018]), and current and former members of my laboratory describe in detail techniques that we developed (see Protocol: **Desiccation of *Hypsibius exemplaris*** [Boothby 2018a], Protocol: **Total RNA Extraction from Tardigrades** [Boothby 2018b]; Protocol: **Live Imaging of Tardigrade Embryonic Development by Differential Interference Contrast Microscopy** [Heikes and Goldstein 2018], Protocol: **Embryonic In Situ Hybridization for the Tardigrade *Hypsibius exemplaris*** [Smith 2018], Protocol: **Embryonic Immunostaining for the Tardigrade *Hypsibius exemplaris*** [Smith and Gabriel 2018], and Protocol: **Microinjection of dsRNA in Tardigrades** [Tenlen 2018]). Most of the techniques that we developed had not been developed in the phylum previously, for example microinjection of animals and RNA interference (Tenlen et al. 2013), in situ hybridization (Smith et al. 2016), immunolocalization (Gabriel and Goldstein 2007), and methods for identifying specific, functionally validated mediators of desiccation tolerance (Boothby et al. 2017). We also share our newly developed methods for introducing live-cell fluorescent markers into *H. exemplaris* embryos by electroporation or soaking (see Protocol: **Fluorescent Cell Staining Methods for Living *Hypsibius exemplaris* Embryos** [McGreevy et al. 2018]).

SOURCES AND HUSBANDRY

Bob McNuff collected the standard *H. exemplaris* strain from a pond in Lancashire, England. This strain is designated Z151, a strain designation that originated as the Sciento catalog number that Bob had applied to the cultures deriving from the tardigrades he collected in 1987 (using Z for all zoological organisms he sold, with sets of numbers reserved for different types of organisms). To grow this strain continuously in the laboratory, animals are maintained in shallow liquid in Erlenmeyer flasks or Petri dishes and are fed a unicellular alga. Water and algae are changed every 10 d to 6 wk, depending on choice of culture methods (see Protocol: **Laboratory Culture of *Hypsibius exemplaris*** [McNuff 2018]). For scientists seeking to start their own cultures, *H. exemplaris* and their algal food can be purchased from Sciento and are distributed by mail. Animals can also be stored frozen (Gabriel et al. 2007). I receive frequent requests from other scientists for cultures, and to satisfy these without disrupting my laboratory's work, I have used Sciento as a stock center, placing orders to fulfill the requests.

RELATED SPECIES

Recent molecular studies on tardigrades have used mostly *H. exemplaris* and two other tardigrade species for which laboratory culture methods have been developed—*Milnesium tardigradum* and *Ramazzottius varieornatus* (Suzuki 2003; Horikawa et al. 2008)—as well as *Paramacrobiotus richtersi* recovered repeatedly from leaf litter (see, for example, Kondo et al. 2015; Smith et al. 2016; Hashimoto et al. 2016; Boothby et al. 2017; Yoshida et al. 2017). High quality genome sequences exist for *H. exemplaris* and *R. varieornatus* (Hashimoto et al. 2016; Yoshida et al. 2017), and a draft genome exists for *M. tardigradum* (Bemm et al. 2017). All four of the above species and the *H. exemplaris* close relative *H. dujardini* can survive extreme environmental conditions including desiccation (Wright 1989; Horikawa et al. 2008; Boothby et al. 2017). The proceedings of a recent international tardigrade symposium, introduced by Rebecchi and Nelson (2016), describe recent work on a broader array of tardigrade species.

We continue to use *H. exemplaris* more often than the other two laboratory-cultured species mentioned above because under laboratory conditions, *H. exemplaris* embryos consistently develop successfully (~100% in *H. exemplaris*, 77.2% to 90% in *M. tardigradum*, and 82.5% in *R. varieornatus*); embryonic development occurs in a consistent and shorter duration (4–4.5 d in *H. exemplaris*, 5–16 d in *M. tardigradum*, and 4–8 d in *R. varieornatus*); embryos are more transparent than the embryos of *M. tardigradum* and lack the ornamented eggshell that surrounds embryos of *R. varieornatus*; generation time is short (13–14 d in *H. exemplaris*, 15 d in *R. varieornatus*, and about 27 d in *M. tardigradum*) (Suzuki 2003; Gabriel et al. 2007; Horikawa et al. 2008); and because *H. exemplaris* can be grown on an algal source that is readily available (Gabriel et al. 2007). *H. exemplaris* and its close relative *H. dujardini* require more preconditioning to survive desiccation than do the other three species (Wright 1989; Horikawa et al. 2008; Boothby et al. 2017), suggesting that both *Hypsibius* species may activate a program to resist desiccation rather than being constitutively resistant. Consistent with this, *H. exemplaris* has the largest transcriptional responses to desiccation among these species (Boothby et al. 2017; Yoshida et al. 2017), which has facilitated finding genes that mediate desiccation tolerance. Some of these genes are induced during desiccation in *H. exemplaris* but constitutively expressed in a constitutively desiccation-tolerant species (Boothby et al. 2017). Although *H. exemplaris* has advantages as a laboratory model, each of the tardigrade species above also has distinct advantages. For example, males have been reported to arise rarely in otherwise parthenogenetic *M. tardigradum* and *H. dujardini* cultures (Ramazzotti and Maucci 1995; Suzuki 2008), which may prove valuable for genetic studies, and males have not yet been reported from the otherwise parthenogenetic *H. exemplaris*. *R. varieornatus* and *M. tardigradum* have even more compact genomes than does *H. exemplaris* (104 megabase pairs for *H. exemplaris*, 75 megabase pairs for *M. tardigradum*, and 56 megabase pairs for *R. varieornatus*) (Yoshida et al. 2017; Bemm et al. 2017).

Most of the recent developmental studies on tardigrades have used *H. exemplaris* strain Z151 from Sciento (Gabriel et al. 2007; Gabriel and Goldstein 2007; Gross and Mayer 2015; Hyra et al. 2016a,b; Smith et al. 2016; Smith and Goldstein 2017; Gross et al. 2017). Studies that used *H. exemplaris* strain Z151 prior to the 2018 species disambiguation from *H. dujardini* (Gąsiorek et al. 2018) generally referred to the species as *H. dujardini*, or as *Hypsibius* cf. *dujardini*, and in many of these studies the strain was indicated as Z151 and/or sourced from Sciento (Gabriel and Goldstein 2007; Gabriel et al. 2007; Bavan et al. 2009; Mali et al. 2010; Cesari et al. 2012; Beltrán-Pardo et al. 2013; Horikawa et al. 2013; Tenlen et al. 2013; Smith and Jockusch 2014; Boothby et al. 2015, 2017; Gross and Mayer 2015; Kondo et al. 2015; Arakawa et al. 2016; Bemm et al. 2016; Fernandez et al. 2016; Hering et al. 2016; Hyra et al. 2016a,b; Kosztyła et al. 2016; Koutsovoulos et al. 2016; Levin et al. 2016; Smith et al. 2016, 2017; Stec et al. 2016; Erdmann et al. 2017; Gross et al. 2017, 2018; Vasanthan et al. 2017; Yoshida et al. 2017, 2018; Fadero et al. 2018; Nelson 2018; Rost-Roszkowska et al. 2018). One study using *Thulinia stephaniae* (Hejnol and Schnabel 2005) indicates some contrasting developmental features in a second tardigrade species.

USES OF THE *H. exemplaris* MODEL SYSTEM

Studies using this model and the other tardigrade species mentioned above have focused mostly on survival in extreme conditions in the laboratory (Guidetti et al. 2011; Horikawa et al. 2013; Beltrán-Pardo et al. 2015; Fernandez et al. 2016; Vasanthan et al. 2017), identifying extremotolerance mechanisms (Kondo et al. 2015; Hashimoto et al. 2016; Boothby et al. 2017), development and anatomy and the evolution of development and anatomy (Gabriel and Goldstein 2007; Gabriel et al. 2007; Mayer et al. 2013; Smith and Jockusch 2014; Gross and Mayer 2015; Hyra et al. 2016a,b; Smith et al. 2016; Smith and Goldstein 2017; Gross et al. 2017), taxonomic methods and relationships (Rota-Stabelli et al. 2010; Stec et al. 2016), sequence comparisons with other species (Förster et al. 2009; Mali et al. 2010; Christie et al. 2011; D'Haese et al. 2011; Beltrán-Pardo et al. 2013; Hering and Mayer 2014; Mayer et al. 2015; Szitenberg et al. 2016; Thiruketheeswaran et al. 2017; Nelson 2018), and analyses of specific proteins (Bavan et al. 2009; Hering et al. 2016). Genomic studies are discussed in a section below.

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Here, I review briefly selected recent studies that highlight the use of *H. exemplaris* as a model to understand how animal body plans can evolve, and how biological materials can survive extreme environments.

Smith et al. (2016) sought to understand how new animal body plans can evolve using tardigrades as a model in which segment identities might be identified by Hox gene expression patterns (Hughes and Kaufman, 2002), but where body plan appears significantly different from that of *Drosophila* and its arthropod relatives. All known tardigrades have a compact body plan, with four apparent body segments plus a head. Multiple species of tardigrades lack apparent homologs of certain Hox genes, for example homologs of the *Drosophila* trunk-segment Hox genes *Antennapedia*, *Ultrabithorax* and *abdominal-A*, suggesting that these genes were lost early in the evolution of the tardigrades, perhaps as tardigrades first evolved (Smith et al. 2016; Yoshida et al. 2017). Expression patterns of the Hox genes that remain were found to be in a similar anterior-to-posterior register as in many other animals, but with the anterior boundaries of each of the head-identity Hox genes (as defined by arthropod homolog expression patterns) located not just in the head but instead through most of the tardigrade's body. The gene expression patterns suggested that nearly the entire body of tardigrades is homologous to just the head of *Drosophila*, and that an ancient ancestor to tardigrades must have lost a significant part of the body—corresponding to the entire thorax and nearly the entire abdomen of *Drosophila*—perhaps through loss of a posterior elongation zone (Smith et al. 2016). These results implied that an animal body plan can originate through loss of a greater part of an ancestor's body than had been recognized previously.

Tardigrades are a valuable model system for investigating longstanding questions about how animals, and biological materials more generally, can survive some remarkably extreme conditions (Keilin 1959; Crowe 1971). Recent work on identifying mechanisms by which tardigrades can survive extremes of desiccation and radiation has begun to identify proteins of special interest. Boothby et al. (2017) used transcriptomics to identify genes whose expression changed in response to desiccation or freezing, and then used RNAi targeting such genes to identify functional mediators of desiccation tolerance. The genes identified encode intrinsically disordered proteins, expression of which in bacteria or yeast can increase desiccation tolerance in these systems. This work, together with work that identified a DNA-associated protein from *R. varieornatus* that can suppress DNA damage in human cultured cells (Hashimoto et al. 2016) and mitochondrial proteins that modestly improved osmotic tolerance (Tanaka et al. 2015), suggest the promise of tardigrades as a continued source of proteins that can mediate tolerance to extremes.

GENOMICS AND ASSOCIATED RESOURCES

H. exemplaris has five pairs of chromosomes (Gabriel et al. 2007). A recent paper presents a high-quality genome assembly using animal collection and analysis methods that are significant improvements over previous methods (Yoshida et al. 2017). In my own laboratory's earlier draft genome publication, we had concluded that extensive horizontal gene transfer had occurred since the time when *H. exemplaris* ancestors had split from nontardigrade phyla, but several studies showed that this was an artifact of contamination and of an elevation of apparent levels of horizontal gene transfer that can occur when uncurated gene predictions are used; instead, current evidence is consistent with horizontal gene transfer having occurred at a rate that is typical for animals (Boothby et al. 2015; Arakawa 2016; Bemm et al. 2016; Delmont and Eren 2016; Koutsovoulos et al. 2016; Yoshida et al. 2017). A developmental transcriptome time series is available (Levin et al. 2016). This time series was produced from RNA-seq of 62 individual embryos spanning over a little more than the first 48 h of development. Currently the primary source for scientists seeking *H. exemplaris* RNA and DNA is directly from animal cultures obtained commercially from Sciento.

A genome database established and maintained by the Blaxter and Arakawa laboratories is available (http://ensembl.tardigrades.org/Hypsibius_dujardini_nhd315/Info/Index), and a physical map

of the genome can be accessed via NCBI (https://www.ncbi.nlm.nih.gov/genome/768?genome_assembly_id=313403).

TECHNICAL APPROACHES

The accompanying protocols describe methods for animal culture (Protocol: **Laboratory Culture of *Hypsibius exemplaris*** [McNuff 2018]), live filming of embryos by DIC microscopy (Protocol: **Live Imaging of Tardigrade Embryonic Development by Differential Interference Contrast Microscopy** [Heikes and Goldstein 2018]), in vivo fluorescent staining of embryos (Protocol: **Fluorescent Cell Staining Methods for Living *Hypsibius exemplaris* Embryos** [McGreevy et al. 2018]), immunolocalization (Protocol: **Embryonic Immunostaining for the Tardigrade *Hypsibius exemplaris*** [Smith and Gabriel 2018]), in situ hybridization (Protocol: **Embryonic In Situ Hybridization for the Tardigrade *Hypsibius exemplaris*** [Smith 2018]), desiccation (Protocol: **Desiccation of *Hypsibius exemplaris*** [Boothby 2018a]), RNA extraction (Protocol: **Total RNA Extraction from Tardigrades** [Boothby 2018b]), and microinjection and RNA interference (Protocol: **Microinjection of dsRNA in Tardigrades** [Tenlen 2018]). As with other emerging model organisms, the suite of technical approaches available to date makes it possible to address diverse questions of interest to scientists (Goldstein and King 2016). Use of this system would benefit at this stage from the development of additional tools, prominent among these being tools for transgenesis and CRISPR-based gene editing tools.

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REFERENCES

Aguinaldo AM, Turbeville JM, Linford LS, Rivera MC, Garey JR, Raff RA, Lake JA. 1997. Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature* **387:** 489–493.

Altiero T, Rebecchi L. 2001. Rearing tardigrades: Results and problems. *Zool Anz* **240:** 217–221.

Arakawa K. 2016. No evidence for extensive horizontal gene transfer from the draft genome of a tardigrade. *Proc Natl Acad Sci* **113:** E3057.

Arakawa K, Yoshida Y, Tomita M. 2016. Genome sequencing of a single tardigrade *Hypsibius dujardini* individual. *Sci Data* **3:** 160063.

Bartels PJ, Apodaca JJ, Mora C, Nelson DR. 2016. A global biodiversity estimate of a poorly known taxon: Phylum Tardigrada. *Zool J Linn Soc* **178:** 730–736.

Bavan S, Straub VA, Blaxter ML, Ennion SJ. 2009. A P2X receptor from the tardigrade species *Hypsibius dujardini* with fast kinetics and sensitivity to zinc and copper. *BMC Evol Biol* **9:** 17.

Beltrán-Pardo EA, Jönsson I, Wojcik A, Haghdoost S, Cruz RMB, Villegas JEB. 2013. Sequence analysis of the DNA-repair gene *rad51* in the tardigrades *Milnesium* cf. *tardigradum*, *Hypsibius dujardini* and *Macrobiotus* cf. *harmsworthi*. *J Limnol* **72:** 10.

Beltrán-Pardo E, Jönsson KI, Harms-Ringdahl M, Haghdoost S, Wojcik A. 2015. Tolerance to gamma radiation in the tardigrade *Hypsibius dujardini* from embryo to adult correlate inversely with cellular proliferation. *PLoS One* **10:** e0133658.

Bemm F, Weiß CL, Schultz J, Förster F. 2016. Genome of a tardigrade: Horizontal gene transfer or bacterial contamination? *Proc Natl Aacd Sci* **113:** E3054–E3056.

Bemm FM, Burleigh L, Förster F, Schmucki R, Ebeling M, Janzen C, Dandekar T, Schill R, Certa U, Schultz J. 2017. Draft genome of the Eutardigrade *Milnesium tardigradum* sheds light on ecdysozoan evolution. *bioRxiv* 122309. doi: 10.1101/122309.

Blaxter M, Elsworth B, Daub J. 2004. DNA taxonomy of a neglected animal phylum: An unexpected diversity of tardigrades. *Proc Biol Sci* **271(Suppl 4):** S189–S192.

Boothby TC. 2018a. Desiccation of *Hypsibius exemplaris*. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot102327.

Boothby TC. 2018b. Total RNA extraction from tardigrades. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot102376.

Boothby TC, Tenlen JR, Smith FW, Wang JR, Patanella KA, Nishimura EO, Tintori SC, Li Q, Jones CD, Yandell M, et al. 2015. Evidence for extensive horizontal gene transfer from the draft genome of a tardigrade. *Proc Natl Acad Sci* **112:** 15976–15981.

Boothby TC, Tapia H, Brozena AH, Piszkiewicz S, Smith AE, Giovannini I, Rebecchi L, Pielak GJ, Koshland D, Goldstein B. 2017. Tardigrades use intrinsically disordered proteins to survive desiccation. *Mol Cell* **65:** 975–984.e5.

Cesari M, Altiero T, Rebecchi L. 2012. Identification of the trehalose-6-phosphate synthase (*tps*) gene in desiccation tolerant and intolerant tardigrades. *Ital J Zool* **79:** 530–540.

Christie AE, Nolan DH, Garcia ZA, McCoole MD, Harmon SM, Congdon-Jones B, Ohno P, Hartline N, Congdon CB, Baer KN, et al. 2011. Bioinformatic prediction of arthropod/nematode-like peptides in non-arthropod, non-nematode members of the Ecdysozoa. *Gen Comp Endocrinol* **170:** 480–486.

Crowe JH. 1971. Anhydrobiosis: An unsolved problem. *Amer Naturalist* **105:** 563–573.

Delmont TO, Eren AM. 2016. Identifying contamination with advanced visualization and analysis practices: Metagenomic approaches for eukaryotic genome assemblies. *PeerJ* **4:** e1839.

D'Haese J, Traore-Freitag A, Kiehl E, Prasath T, Greven H. 2011. The actin gene of *Hypsibius klebelsbergi* (Eutardigrada)—Complete sequence and comparison with actin from related and non-related taxa. *J Zool Syst Evol Res* **49:** 84–89.

Erdmann W, Idzikowski B, Kowalski W, Szymański B, Kosicki JZ, Kaczmarek Ł. 2017. Can the tardigrade *Hypsibius dujardini* survive in the absence of the geomagnetic field? *PLoS One* **12:** e0183380.

Erwin DH, Davidson EH. 2002. The last common bilaterian ancestor. *Development* **129:** 3021–3032.

Fadero TC, Gerbich TM, Rana K, Suzuki A, DiSalvo M, Schaefer KN, Heppert JK, Boothby TC, Goldstein B, Peifer M, et al. 2018. LITE microscopy: Tilted light-sheet excitation of model organisms offers high resolution and low photobleaching. *J Cell Biol* **217:** 1869–1882.

Fernandez C, Vasanthan T, Kissoon N, Karam G, Duquette N, Seymour C, Stone JR. 2016. Radiation tolerance and bystander effects in the eutardigrade species *Hypsibius dujardini* (Parachaela: Hypsibiidae). *Zool J Linn Soc* **178:** 919–923.

Förster F, Liang C, Shkumatov A, Beisser D, Engelmann JC, Schnölzer M, Frohme M, Müller T, Schill RO, Dandekar T. 2009. Tardigrade workbench: Comparing stress-related proteins, sequence-similar and functional protein clusters as well as RNA elements in tardigrades. *BMC Genomics* **10:** 469.

Gabriel WN, Goldstein B. 2007. Segmental expression of Pax3/7 and engrailed homologs in tardigrade development. *Dev Genes Evol* **217:** 421–433.

Gabriel WN, McNuff R, Patel SK, Gregory TR, Jeck WR, Jones CD, Goldstein B. 2007. The tardigrade *Hypsibius dujardini*, a new model for studying the evolution of development. *Dev Biol* **312:** 545–559.

Gąsiorek P, Stec D, Morek W, Michalczyk Ł. 2018. An integrative redescription of *Hypsibius dujardini* (Doyère, 1840), the nominal taxon for Hypsibioidea (Tardigrada: Eutardigrada). *Zootaxa* **4415:** 45–75.

Goldstein B, Blaxter M. 2002. Tardigrades. *Curr Biol* **12:** R475.

Goldstein B, King N. 2016. The future of cell biology: Emerging model organisms. *Trends Cell Biol* **26:** 818–824.

Goodman CS, Coughlin BC. 2000. Introduction. The evolution of evo-devo biology. *Proc Natl Acad Sci* **97:** 4424–4425.

Gregory TR. 2001. Coincidence, coevolution, or causation? DNA content, cell size, and the C-value enigma. *Biol Rev Camb Philos Soc* **76:** 65–101.

Gregory TR, Hebert PD. 1999. The modulation of DNA content: Proximate causes and ultimate consequences. *Genome Res* **9:** 317–324.

Gross V, Mayer G. 2015. Neural development in the tardigrade *Hypsibius dujardini* based on anti-acetylated α-tubulin immunolabeling. *Evodevo* **6:** 12.

Gross V, Minich I, Mayer G. 2017. External morphogenesis of the tardigrade *Hypsibius dujardini* as revealed by scanning electron microscopy. *J Morphol* **278:** 563–573.

Gross V, Bährle R, Mayer G. 2018. Detection of cell proliferation in adults of the water bear *Hypsibius dujardini* (Tardigrada) via incorporation of a thymidine analog. *Tissue Cell* **51:** 77–83.

Guidetti R, Altiero T, Bertolani R, Grazioso P, Rebecchi L. 2011. Survival of freezing by hydrated tardigrades inhabiting terrestrial and freshwater habitats. *Zoology (Jena)* **114:** 123–128.

Hall BK. 1994. *Homology: The hierarchical basis of comparative biology*. Academic Press, San Diego.

Hashimoto T, Horikawa DD, Saito Y, Kuwahara H, Kozuka-Hata H, Shin-I T, Minakuchi Y, Ohishi K, Motoyama A, Aizu T, et al. 2016. Extremotolerant tardigrade genome and improved radiotolerance of human cultured cells by tardigrade-unique protein. *Nat Commun* **7:** 12808.

Heikes KL, Goldstein B. 2018. Live imaging of tardigrade embryonic development by differential interference contrast microscopy. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot102335.

Hejnol A, Schnabel R. 2005. The eutardigrade *Thulinia stephaniae* has an indeterminate development and the potential to regulate early blastomere ablations. *Development* **132:** 1349–1361.

Hering L, Mayer G. 2014. Analysis of the opsin repertoire in the tardigrade *Hypsibius dujardini* provides insights into the evolution of opsin genes in Panarthropoda. *Genome Biol Evol* **6:** 2380–2391.

Hering L, Bouameur J-E, Reichelt J, Magin TM, Mayer G. 2016. Novel origin of lamin-derived cytoplasmic intermediate filaments in tardigrades. *Elife* **5:** e11117.

Horikawa DD, Kunieda T, Abe W, Watanabe M, Nakahara Y, Yukuhiro F, Sakashita T, Hamada N, Wada S, Funayama T, et al. 2008. Establishment of a rearing system of the extremotolerant tardigrade *Ramazzottius varieornatus*: A new model animal for astrobiology. *Astrobiology* **8:** 549–556.

Horikawa DD, Cumbers J, Sakakibara I, Rogoff D, Leuko S, Harnoto R, Arakawa K, Katayama T, Kunieda T, Toyoda A, et al. 2013. Analysis of DNA repair and protection in the tardigrade *Ramazzottius varieornatus* and *Hypsibius dujardini* after exposure to UVC radiation. *PLoS One* **8:** e64793.

Hughes CL, Kaufman TC. 2002. Hox genes and the evolution of the arthropod body plan. *Evol Dev* **4:** 459–499.

Hyra M, Poprawa I, Włodarczyk A, Student S, Sonakowska L, Kszuk-Jendrysik M, Rost-Roszkowska MM. 2016a. Ultrastructural changes in the midgut epithelium of *Hypsibius dujardini* (Doyère, 1840) (Tardigrada, Eutardigrada, Hypsibiidae) in relation to oogenesis. *Zool J Linn Soc* **178:** 897–906.

Hyra M, Rost-Roszkowska MM, Student S, Włodarczyk A, Deperas M, Janelt K, Poprawa I. 2016b. Body cavity cells of Parachela during their active life. *Zool J Linn Soc* **178:** 878–887.

Keilin D. 1959. The problem of anabiosis or latent life: History and current concept. *Proc R Soc Lond B Biol Sci* **150:** 149–191.

Kondo K, Kubo T, Kunieda T. 2015. Suggested Involvement of PP1/PP2A Activity and de novo gene expression in anhydrobiotic survival in a tardigrade, *Hypsibius dujardini*, by chemical genetic approach. *PLoS One* **10:** e0144803.

Kosztyła P, Stec D, Morek W, Gąsiorek P, Zawierucha K, Michno K, Ufir K, Małek D, Hlebowicz K, Laska A, et al. 2016. Experimental taxonomy confirms the environmental stability of morphometric traits in a taxonomically challenging group of microinvertebrates. *Zool J Linn Soc* **178:** 765–775.

Koutsovoulos G, Kumar S, Laetsch DR, Stevens L, Daub J, Conlon C, Maroon H, Thomas F, Aboobaker AA, Blaxter M. 2016. No evidence for extensive horizontal gene transfer in the genome of the tardigrade *Hypsibius dujardini*. *Proc Natl Acad Sci* **113:** 5053–5058.

Levin M, Anavy L, Cole AG, Winter E, Mostov N, Khair S, Senderovich N, Kovalev E, Silver DH, Feder M, et al. 2016. The mid-developmental transition and the evolution of animal body plans. *Nature* **531:** 637–641.

Mali B, Grohme MA, Förster F, Dandekar T, Schnölzer M, Reuter D, Wełnicz W, Schill RO, Frohme M. 2010. Transcriptome survey of the anhydrobiotic tardigrade *Milnesium tardigradum* in comparison with *Hypsibius dujardini* and *Richtersius coronifer*. *BMC Genomics* **11:** 168.

Mayer G, Kauschke S, Rüdiger J, Stevenson PA. 2013. Neural markers reveal a one-segmented head in tardigrades (water bears). *PLoS One* **8:** e59090.

Mayer G, Hering L, Stosch JM, Stevenson PA, Dircksen H. 2015. Evolution of pigment-dispersing factor neuropeptides in Panarthropoda: Insights from Onychophora (velvet worms) and Tardigrada (water bears). *J Comp Neurol* **523:** 1865–1885.

McGreevy KM, Heikes KL, Kult S, Tharp ME, Goldstein B. 2018. Fluorescent cell staining methods for living *Hypsibius exemplaris* embryos. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot106021.

McNuff R. 2018. Laboratory culture of *Hypsibius exemplaris*. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot102319.

Nelson DR. 2018. Cytochrome P450 diversity in the tree of life. *Biochim Biophys Acta* **1866:** 141–154.

Nelson DR, Guidetti R, Rebecchi L. 2016. Chapter 15—Phylum Tardigrada. In *Thorp and Covich's freshwater invertebrates*, 4th ed. (ed. Thorp JH, Rogers DC), pp. 277–290. Academic Press, Boston.

Park J-K, Rho HS, Kristensen RM, Kim W, Giribet G. 2006. First molecular data on the phylum Loricifera: An investigation into the phylogeny of ecdysozoa with emphasis on the positions of Loricifera and Priapulida. *Zool Sci* **23:** 943–954.

Ramazzotti G, Maucci W. 1995. *The phylum Tardigrada*. Beasley CW (translator), McMurry University, Abilene, Texas.

Rebecchi L, Nelson DR. 2016. Introductory Text—Preface. *Zool J Linn Soc* **178:** 726–729.

Richter DJ, King N. 2013. The genomic and cellular foundations of animal origins. *Annu Rev Genet* **47:** 509–537.

Rost-Roszkowska MM, Janelt K, Poprawa I. 2018. The role of autophagy in the midgut epithelium of Parachela (Tardigrada). *Zoomorphology* doi:10.1007/s00435-018-0407-x.

Rota-Stabelli O, Kayal E, Gleeson D, Daub J, Boore JL, Telford MJ, Pisani D, Blaxter M, Lavrov DV. 2010. Ecdysozoan mitogenomics: Evidence for a common origin of the legged invertebrates, the Panarthropoda. *Genome Biol Evol* **2:** 425–440.

Sidow A, Thomas WK. 1994. A molecular evolutionary framework for eukaryotic model organisms. *Curr Biol* **4:** 596–603.

Smith FW. 2018. Embryonic in-situ hybridization for the tardigrade *Hypsibius exemplaris*. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot102350.

Smith FW, Gabriel WN. 2018. Embryonic immunostaining for the tardigrade *Hypsibius exemplaris*. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot102343.

Smith FW, Goldstein B. 2017. Segmentation in Tardigrada and diversification of segmental patterns in Panarthropoda. *Arthropod Struct Dev* **46:** 328–340.

Smith FW, Jockusch EL. 2014. The metameric pattern of *Hypsibius dujardini* (Eutardigrada) and its relationship to that of other panarthropods. *Front Zool* **11:** 66.

Smith FW, Boothby TC, Giovannini I, Rebecchi L, Jockusch EL, Goldstein B. 2016. The compact body plan of tardigrades evolved by the loss of a large body region. *Curr Biol* **26:** 224–229.

Smith FW, Bartels PJ, Goldstein B. 2017. A hypothesis for the composition of the tardigrade brain and its implications for panarthropod brain evolution. *Integr Comp Biol* **57:** 546–559.

Stec D, Gąsiorek P, Morek W, Kosztyła P, Zawierucha K, Michno K, Kaczmarek Ł, Prokop ZM, Michalczyk Ł. 2016. Estimating optimal sample size for tardigrade morphometry. *Zool J Linn Soc* **178:** 776–784.

Suzuki AC. 2003. Life history of *Milnesium tardigradum* Doyère (tardigrada) under a rearing environment. *Zool Sci* **20:** 49–57.

Suzuki AC. 2008. Appearance of males in a thelytokous strain of *Milnesium* cf. *tardigradum* (Tardigrada). *Zool Sci* **25:** 849–853.

Szitenberg A, Cha S, Opperman CH, Bird DM, Blaxter ML, Lunt DH. 2016. Genetic drift, not life history or RNAi, determine long-term evolution of transposable elements. *Genome Biol Evol* **8:** 2964–2978.

Tanaka S, Tanaka J, Miwa Y, Horikawa DD, Katayama T, Arakawa K, Toyoda A, Kubo T, Kunieda T. 2015. Novel mitochondria-targeted heat-soluble proteins identified in the anhydrobiotic Tardigrade improve osmotic tolerance of human cells. *PLoS One* **10:** e0118272.

Tenlen JR. 2018. Microinjection of dsRNA in tardigrades. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot102368.

Tenlen JR, McCaskill S, Goldstein B. 2013. RNA interference can be used to disrupt gene function in tardigrades. *Dev Genes Evol* **223:** 171–181.

Thiruketheeswaran P, Greven H, D'Haese J. 2017. Gelsolin in Onychophora and Tardigrada with notes on its variability in the Ecdysozoa. *Comp Biochem Physiol B Biochem Mol Biol* **203:** 47–52.

Vasanthan T, Alejaldre L, Hider J, Patel S, Husain N, Umapathisivam B, Stone J. 2017. G-equivalent acceleration tolerance in the eutardigrade species *Hypsibius dujardini*. *Astrobiology* **17:** 55–60.

Wright JC. 1989. Desiccation tolerance and water-retentive mechanisms in tardigrades. *J Exp Biol* **142:** 267–292.

Yoshida Y, Koutsovoulos G, Laetsch DR, Stevens L, Kumar S, Horikawa DD, Ishino K, Komine S, Kunieda T, Tomita M, et al. 2017. Comparative genomics of the tardigrades *Hypsibius dujardini* and *Ramazzottius varieornatus*. *PLoS Biol* **15:** e2002266.

Yoshida Y, Konno S, Nishino R, Murai Y, Tomita M, Arakawa K. 2018. Ultralow input genome sequencing library preparation from a single tardigrade specimen. *J Vis Exp* doi: 10.3791/57615.

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