

## ARTICLE



# Trophic flexibility of marine diplomonads - switching from osmotrophy to bacterivory

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Diplomonads are one of the most abundant groups of heterotrophic planktonic microeukaryotes in the world ocean and, thus, are likely to play an essential role in marine ecosystems. So far, only few species have been introduced into a culture, allowing basic studies of diplomonad genetics, morphology, ultrastructure, metabolism, as well as endosymbionts. However, it remains unclear whether these heterotrophic flagellates are parasitic or free-living and what are their predominant dietary patterns and preferred food items. Here we show that cultured diplomonads, maintained in an organic-rich medium as osmotrophs, can gradually switch to bacterivory as a sole food resource, supporting positive growth of their population, even when fed with a low biovolume of bacteria. We further observed remarkable differences in species-specific feeding patterns, size-selective grazing preferences, and distinct feeding strategies. Diplomonads can discriminate between low-quality food items and inedible particles, such as latex beads, even after their ingestion, by discharging them in the form of large waste vacuoles. We also detected digestion-related endogenous autofluorescence emitted by lysosomes and the activity of a melanin-like material. We present the first evidence that these omnipresent protists possess an opportunistic lifestyle that provides a considerable advantage in the generally food resource-limited marine environments.

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## INTRODUCTION

As part of the largest food web on the planet, marine microbial eukaryotes lay a foundation for global biogeochemical cycles, ultimately sustaining life in the ocean and controlling the atmosphere's composition [1]. Together with prokaryotes, they account for about 70% of the total biomass in the marine environment [2] and, through a multitude of interactions with other microeukaryotes, bacteria and viruses, form highly complex microbial food webs [3]. Consequently, they play a pivotal role in driving primary and secondary production and degradation of organic material and nutrient recycling [4]. Despite their importance, the remarkable diversity of protist-dominated marine microeukaryotes, particularly their most abundant heterotrophic components, has been uncovered only recently [5–7].

Although previously overlooked, recent environmental sequencing studies identified diplomonads (Diplonemea) as one of the major groups of protists populating various habitats of the world ocean [6–10], while they seem to be rarer in freshwater bodies [11]. They inhabit virtually every niche in the water column, including anoxic regions, yet predominate in the mesopelagic and bathypelagic zones [12], as well as in the sediment surfaces [13]. First discovered in 1913 by Griessmann [14], fewer than two dozen diplomonads have been cultivated since then, allowing detailed studies of their morphology and ultrastructure [15–19]. Their

uniquely inflated mitochondrial genome [20] and massively edited mitochondrial transcripts have been described to considerable detail [21], although complete nuclear genomes remain unavailable, largely because of their repetitive nature. The genomic complexity of many protists significantly limits our attempts to infer their ecological functions from environmental sequence data alone [5]. To obtain better insights into the biology and ecophysiological traits of protists, environmental sequencing has to be complemented by cultivation efforts, as well as metabolic and biochemical studies, allowing the deciphering of their trophic interactions and modes of behavior.

As heterotrophs, diplomonads depend on the availability of preformed organic compounds in their environment. However, since they dwell preferentially in deep waters and sediments lacking primary photosynthetic production [22], the dominant feeding modes providing a sufficient amount of organic carbon supporting the growth of these relatively large flagellates remain enigmatic. Previously, diplomonads have been associated with an array of organisms, including diatoms, copepods, haptophytes, dinoflagellates, and plants [15, 16, 23], as well as clams, crabs, and lobsters [24, 25]. Some researchers have credited them with a parasitic lifestyle [8, 13], yet an *in silico* global planktonic interactome [26], as well as genomic features [9] do not support this. Several reports have hinted that diplomonads may possibly

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ingest bacteria [9, 11, 27, 28], but convincing evidence that these protists might be efficient bacterivores were not provided. A successful cultivation of some diplomonid isolates on carbon-rich artificial media revealed their ability of osmotrophic nutrition [15, 16], however, this also influenced further research. While the use of osmotrophically maintained cultures has greatly contributed to the study of diplomonid cells structure and biology, the understanding of their *in situ* ecological role as part of marine food webs has been neglected.

One can assume that diplomonids may exploit inherent patchiness of aquatic environments with hotspots of enhanced concentrations of organic particles and slowly sedimenting decaying organisms, which also attract abundant prokaryote populations, forming a rapidly growing biomass of heterotrophic bacteria. These considerations led us to hypothesize that diplomonids can grow on a concentrated bacterial biomass. Here, we explored this hypothesis and aimed to fill the gap in our knowledge on the behavior and feeding modes of these flourishing flagellates.

## MATERIALS AND METHODS

Eight diplomonid species (*Diplonema aggregatum* YPF1606, *D. papillatum* ATCC 50162, *D. japonicum* YPF1604, *Rhynchopus humris* YPF1608, *Lacrimia lanifica* YPF1601, *Sulcionema specki* YPF1618, *Namystynia karyoxenos* YPF1621, and *Hemistasia phaeocysticola* YPF1303), initially maintained axenically as osmotrophs in the nutrient-rich Hemi medium at 15 °C, were tested for their ability to feed on bacteria. The heat-killed fluorescently labeled bacteria (FLB) were prepared from a mixed bacterial inoculum collected from the Baltic Sea, using the 5-(4,6-dichlorotriazin-2-yl)aminofluorescein [29], and together with unstained stocks of the same bacteria were stored frozen at -20 °C until use in grazing experiments. The bacterial community was characterized *via* 16S rRNA gene sequences, and its morphometric structure was characterized by sizing of >300 cells in 4',6'-diamidino-2-phenylindole (DAPI)-stained preparations.

Diplomonids were adapted to graze on bacteria through weekly sub-culturing in gradually diluted (50%, 25%, 10%, 5%, 1%, 0%) media by nutrient-free artificial seawater (ASW), with simultaneous additions of heat-killed bacterial mixture (from  $5 \times 10^6$  progressing to  $10^7$  cells/ml). All incubations were performed at 15 °C in 25 cm<sup>2</sup> tissue culture flasks kept in a horizontal position. For grazing experiments, diplomonids were pre-starved in ASW and subsequently dispensed into ASW containing either  $7-9 \times 10^6$  cells/ml or  $1.5 \times 10^6$  cells/ml of FLB that supposedly correspond to the typical densities of prokaryotes in the benthic and pelagic marine habitats, respectively. The growth of diplomonids and the depletion of FLB were assessed by flow cytometry, and the diplomonids' morphology, ultrastructure, and morphometry were examined using light, fluorescence, and transmission electron microscopies. Transcriptomic analysis of osmotrophic, phagotrophic and starved diplomonids was done on guanidine extracted RNA using NovaSeq 6000 (Illumina) paired-end 150 bp technology.

Grazing rate was calculated as the ratio between the amount of ingested bacteria, based on their decay rate/ml in the culture medium, and the diplomonids' abundance achieved during this decay phase. The doubling time was calculated using ln-transformed data on diplomonids abundance changing with time. The digestion rates of the bacterial prey were assessed using heat-killed bacteria labeled with amine-reactive dye pHrodo Green. To examine size-dependent species-specific uptake rates as well as the consumption of indigestible particles, diplomonids were fed fluorescent-labeled latex beads (1 µm diameter) resuspended in either ASW or Hemi medium.

The unexpected appearance of red autofluorescence in grazing and starved diplomonids (see Results) was verified by their resuspension into fresh Hemi medium or ASW. The detection of reactive oxygen species in these cultures was performed with dihydrorhodamine 123. To attest the lysosomal origin of autofluorescence, cells were stained with LysoTracker Green, immobilized by low-melting agarose, and observed *via* confocal laser scanning microscopy. To confirm the presence of melanin, Western blot and immunofluorescence assays were performed, using anti-melanin monoclonal antibody 6D2. The isolation of the melanin-containing fraction was conducted by solubilizing the cells in 1 M NaOH.

Statistical analysis of diplomonid growth and morphometry was performed using SAS software, with a set at 0.05.

For detailed protocols see Supplemental material.

## RESULTS

### Cell growth

Direct transfer of the osmotrophically grown diplomonids to a particulate bacterial diet resulted in the inability of cells to sustain their growth. Taking into account the high nutritional value of the Hemi medium (total organic carbon - 0.3 mg/ml and nitrogen - 0.08 mg/ml), we applied gradual pre-adaptation of diplomonid cultures by exposing them to a cross-gradient of increasing bacterial densities (mean cell volume  $0.37 \pm 0.30 \mu\text{m}^3$ , corresponding to 54.9 fg carbon per bacterial cell) and decreasing concentrations of the Hemi medium, inducing bacterivory. Species heterogeneity of the bacterial culture was reflected by pairwise identities of obtained 16S rRNA genes, ranging from 91.8% to 93.7%, with most sequences falling into the genus *Pseudomonas*.

Although most tested species could ingest bacteria, only *D. japonicum* and *R. humris* grew well solely on this food source (Fig. 1A). Both species proliferated on the bacterial diet even when fed 5 to 6 times lower concentrations of FLB (Supplementary Fig. S1A, B). While *S. specki* endured the course of adaptation, as well as prolonged cultivation with bacteria, always showing a high bacterial decay rate (Supplementary Fig. S1C, inset), it remained in the permanent stationary phase irrespective of bacterial concentration used (Supplementary Fig. S1C, data not shown). The dynamics of bacterial uptake significantly differed (*T*-test,  $P < 0.05$ ) between *D. japonicum* and *R. humris*, consuming 2–31 ( $17.7 \pm 8.1$ ; average  $\pm$  SD) and 1–24 ( $13.0 \pm 8.2$ ; average  $\pm$  SD) FLB flagellate<sup>-1</sup> h<sup>-1</sup>, respectively, reflecting their growth dynamics (Fig. 1A, B). Indeed, *D. japonicum* started to proliferate almost immediately (Fig. 1A, B) with a doubling time of ~14 h (T<sub>0</sub>–T<sub>48</sub>), while *R. humris* displayed growth after a 48 h lag phase (Fig. 1B), yielding the doubling time of ~21 h. The growth of both species was exponential for ~2 days (Fig. 1B). After 96 h of active ingestion of bacteria by *R. humris*, the number of prey cells remained relatively stable ( $\sim 2 \times 10^6$  cells/ml) for another 144 h (Fig. 1A, inset).

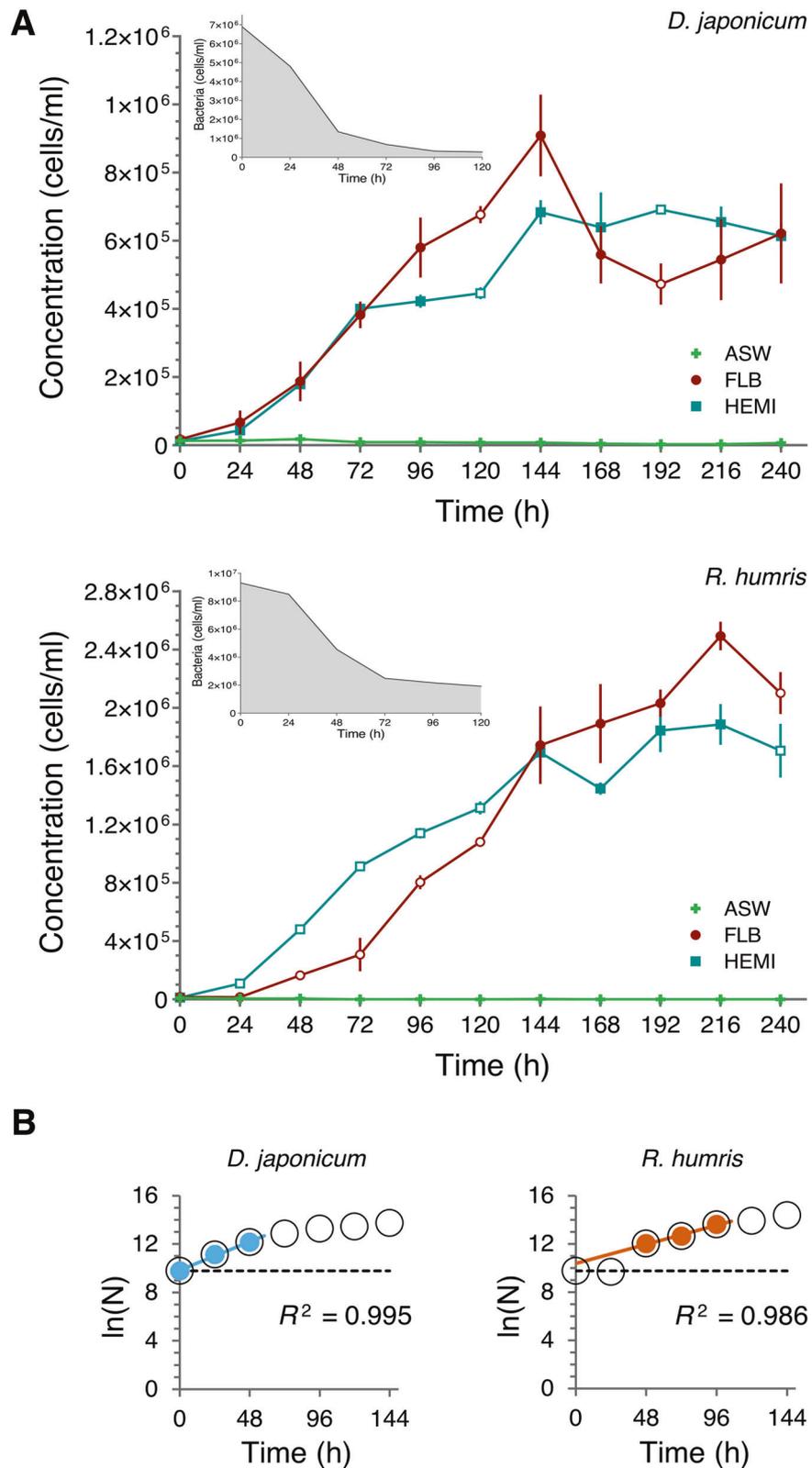
Except for two time points (120 h and 192 h), the cell counts of *D. japonicum* fed on bacteria and cultured in the Hemi medium were similar (Fig. 1A), while the density of *R. humris* reached the level of osmotrophically maintained cells only by 144 h (Fig. 1A).

### Kinetics of consumption and digestion of bacteria

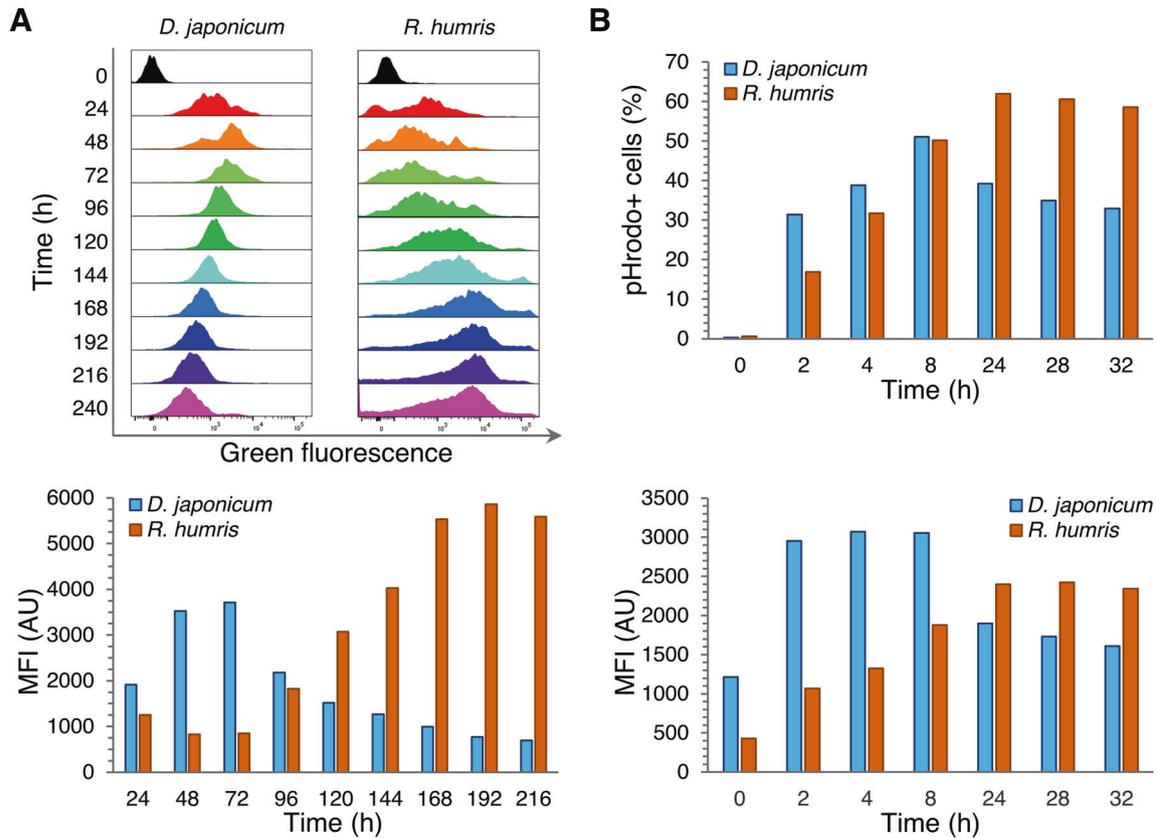
The species-specific uptake rates of FLB were also reflected in variable trends of the green fluorescent signal emitted from food vacuoles (Figs. 2A and 3B, E), suggesting different metabolic and digestion rates. To estimate the bacterial digestion rate, diplomonids were fed a mixture of heat-killed bacteria prelabeled by the pHrodo dye, a fluorescence signal of which gradually increases in the acidic environment. Upon uptake, *D. japonicum* immediately began to digest the prokaryotes, whereas bacterial consumption and digestion rates were somewhat lower in *R. humris* (Fig. 2B). Both flagellates rapidly consumed the small amount of bacteria used in this assay, and the emitted fluorescence signal started to decline after active digestion within ~8 h and ~32 h in *D. japonicum* and *R. humris*, respectively (Fig. 2B). However, the signal remained relatively stable after 76 h and did not completely disappear even after a week, reflecting the long-term presence of remnants of undigested bacteria in the food vacuoles.

### Morphology of diplomonids maintained on bacterial diet

General appearances and morphometrics of both species grown with and without FLB revealed marginal changes in shape but significant differences in cell volume (Fig. 3). While bacteria-fed *D. japonicum* and *R. humris* contained several prominent food vacuoles filled with FLB at different degrees of digestion, numerous storage vacuoles of homogeneous consistency were scattered throughout their cytoplasm when maintained on the Hemi medium (Figs. 3A–F and 4A–G). The bacteria-fed *D. japonicum* were significantly larger than their osmotrophically maintained counterparts at 72 h, while 120-h-old *R. humris* revealed an opposite trend (Fig. 3).



**Fig. 1** Dynamics of bacterial uptake and growth of diplonemids. **A** Growth curves of *Diplonema japonicum* and *Rhynchopus humris* cultured in either the nutrient-rich Hemi medium (turquoise), artificial seawater (ASW) supplemented with FLB [ $7\text{--}9 \times 10^6$  cells/ml] (maroon), or nutrient-free ASW (bright green). Values are means for triplicates, and the error bars show SDs. Open symbols in FLB and Hemi curves indicate significantly different values (*T*-test,  $P < 0.05$ ). The insets show the exponential decay of FLB. **B** Growth rates of *D. japonicum* (blue) and *R. humris* (orange) represented as the best-fit lines.



**Fig. 2 Kinetics of consumption and digestion of bacteria.** **A** Time-course changes in green fluorescence signal emitted by FLB in food vacuoles of *D. japonicum* (blue) and *R. humris* (orange). Upper panel: each individual histogram represents the intensity of the green fluorescence (corresponding to the amount of ingested FLB) on the x-axis and the number of events on the y-axis. The composite of histograms shows the extent of changes in the relative fluorescence signal over time intervals of 24 h, which are color-coded. Lower panel: column chart showing comparison of mean fluorescence intensity (MFI) of both diplomonids. **B** Changes in abundance of *D. japonicum* (blue) and *R. humris* (orange) with ingested pHrodo Green dye-labeled bacteria over time and MFI emitted by these bacteria inside the food vacuoles of the respective diplomonids.

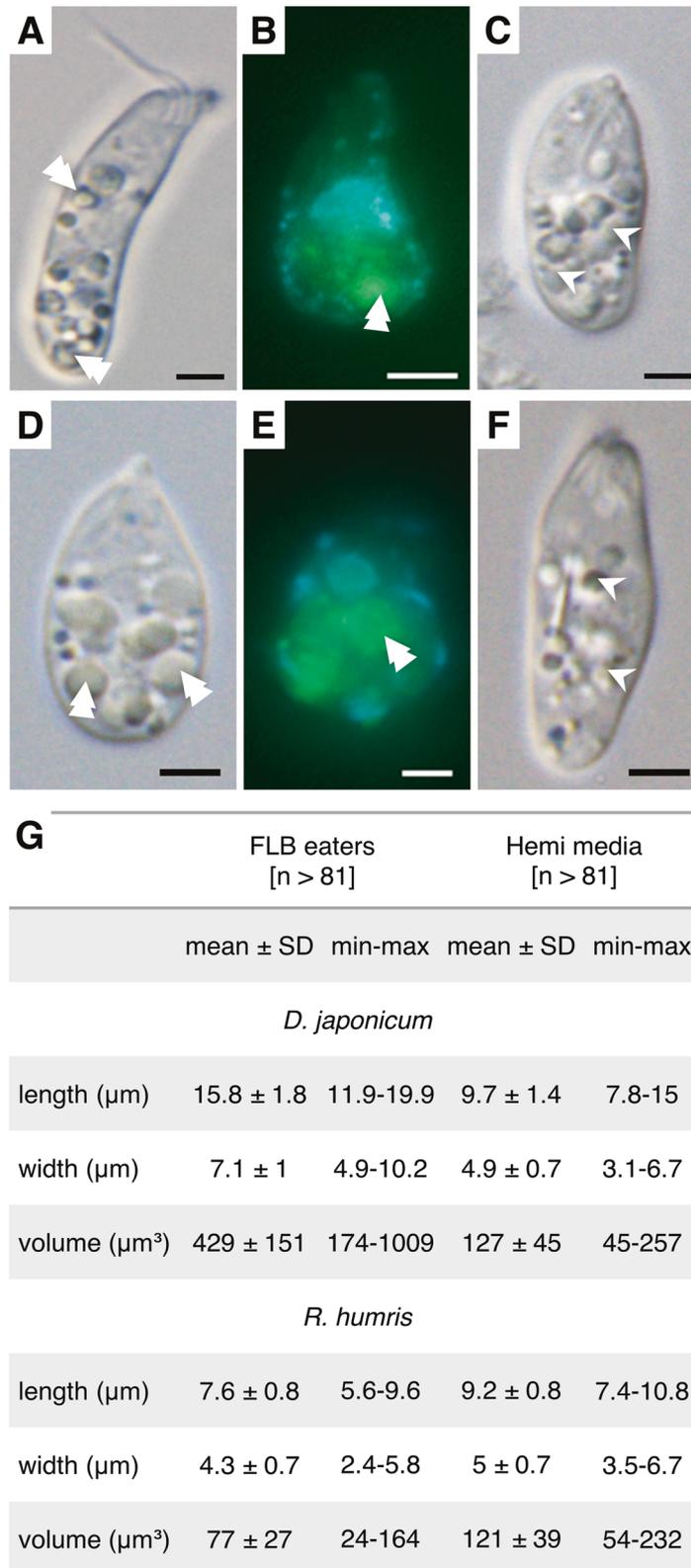
A close inspection of food vacuoles revealed species-specific differences in their content (Fig. 4A–G). While *D. japonicum* was stuffed with larger bacteria, *R. humris* ingested smaller prey (Fig. 4A–G). This preference was confirmed by the average volumes  $0.36 \pm 0.44 \mu\text{m}^3$  and  $0.50 \pm 0.61 \mu\text{m}^3$  of bacteria suspended in the cultures at 72 h and 120 h for *D. japonicum* and *R. humris*, respectively. The size-selective variability in grazing rates among the species was further demonstrated using fluorescent  $1 \mu\text{m}$  ( $0.52 \mu\text{m}^3$ ) polystyrene latex beads (Supplementary Fig. S2). While *D. japonicum* showed a rapid consumption of FLB, *R. humris* preferentially ingested beads over bacteria, indicating their distinct food selectivity patterns (Supplementary Fig. S2D). Nevertheless, with the proportion of large-sized bacteria gradually increasing in time, *R. humris* was able to efficiently graze on them as well, as reflected by an increasing proportion of larger bacteria in its food vacuoles (Fig. 4E–G) and cell sizes during the treatment period (Fig. 4H). As for *D. japonicum*, their food vacuoles content progressively declined over time (Fig. 4A–D), thus reflecting the rapid FLB decay and digestion rates. In one-week-old cultures, almost no ingested prey was found, and only membranous residues of digested bacteria could be distinguished (Fig. 4C). Moreover, as a consequence of the starvation, *D. japonicum* entered the sessile life stage (Fig. 4D). Notably, in addition to a regular cell coat that *D. japonicum* produces during this phase for aggregation, the cells become enveloped by another coat, sometimes enclosing several cells (Fig. 4D). By this barrier, sessile *D. japonicum* creates an electron-transparent void in which no bacteria can be seen. However, a structure reminiscent of a defecation vacuole was

occasionally noticed within the layers of the coating (Fig. 4D). Indeed, when most of the cellular body of diplomonids was filled with large vacuoles containing undigested particles, their excretion from the cell's posterior occurred (Fig. 4I–K). These events lasted  $\sim 90$  s, during which the cell contracted, and released one to several vacuoles consecutively, usually accompanied by some filaments. It is not yet clear what happens to the plasma membrane and the peripheral microtubular corset at this moment; nevertheless, the cell readily continues its locomotion (Fig. 4I–K). Examination of the excreted vacuoles by transmission electron microscopy revealed that they are filled with aggregates of bacterial residues at different stages of digestion (Fig. 4K).

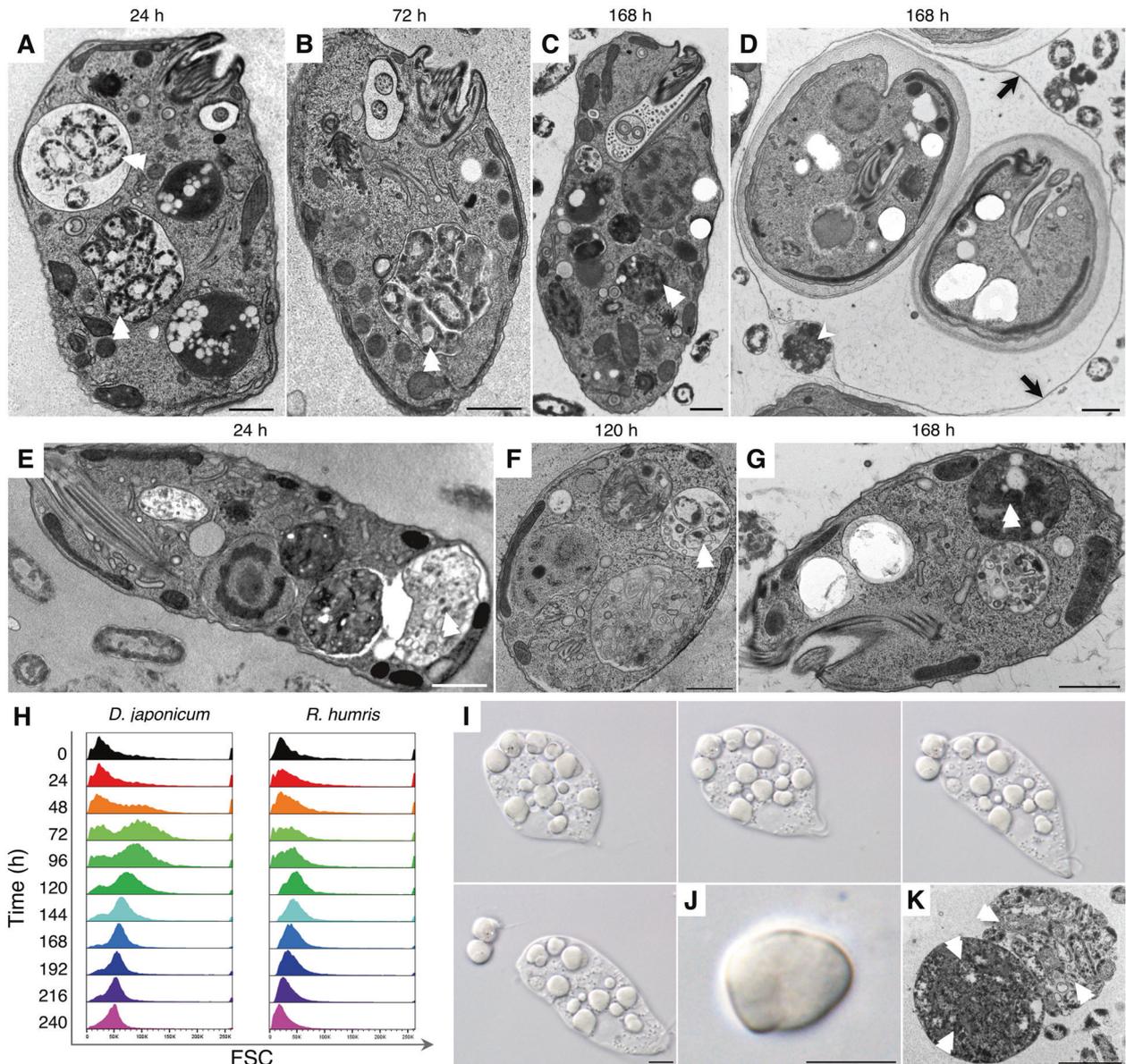
#### Autofluorescence of diplomonids

Apart from the green fluorescence signal emitted by the intravacuolar FLB, we have also detected a signal in the red fluorescent channel with an emission maximum of 560 nm. Curiously, this signal appeared only upon feeding (Fig. 5). In *D. japonicum*, a weak signal can be distinguished after 24 h of feeding on the FLB, with its intensity gradually increasing till 72 h (Fig. 5A). In *R. humris*, fluorescence at this emission appeared at 48 h, which corresponds to the latency phase, reaching its maximum at 144 h (Fig. 5A). In both cases, the emission maxima correlated with the maxima of cell abundance and ingested bacteria (green fluorescence signal), with the signal progressively declining with the decrease of both parameters (Figs. 2A, 4H, and 5A).

The nature of autofluorescence was further evaluated in diplomonids either fed with unlabeled bacteria or grown on the Hemi medium. The red fluorescence signal was emitted in both



**Fig. 3 Characteristics of diplomonads under different conditions.** General morphology of *D. japonicum* (A, C) and *R. humris* (D, F) at 72 h and 120 h time points, respectively, by differential interference contrast (DIC) microscopy. Double arrowheads point to numerous food vacuoles filled with FLB (A, D), and arrowheads indicate storage vacuoles in the Hemi medium-grown cells (C, F). Epifluorescence microscopy of *D. japonicum* (B) and *R. humris* (E) showing DAPI-stained nucleus (blue) and food vacuoles (double arrowheads), containing FLB (green) at different stages of digestion. Scale bar: 2.5 µm. **G** A comparison of major morphometric characteristics (shown as the mean value ± SD and range of cell length, width, and cell volume) of the *D. japonicum* and *R. humris* from both culture conditions.



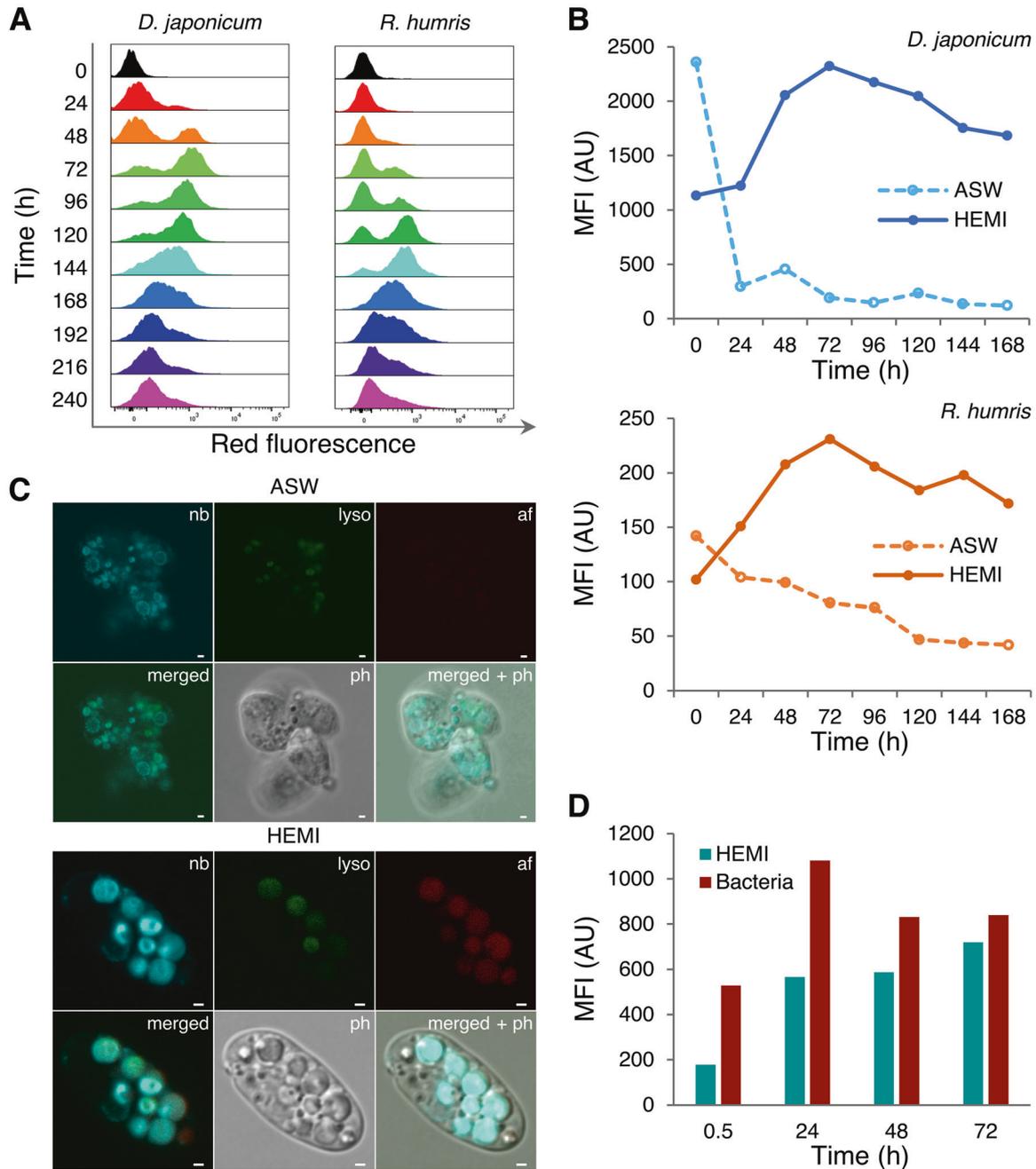
**Fig. 4** Characteristics of FLB-fed diplomonads. Transmission electron micrographs (TEM) of *D. japonicum* (A–D) and *R. humris* (E–G) revealing bacterial prey (double arrowheads) inside the food vacuoles at indicated time points. D Arrows point to the coat enveloping two sessile *D. japonicum* cells, and the arrowhead shows an excreted vacuole with bacterial residues. H Time-course changes in sizes of *D. japonicum* and *R. humris* fed with FLB, represented by forward side scatter (FSC). Time intervals as in Fig. 2A. I DIC micrographs of *Sulcionema specki* showing successive events of excretion of undigested bacterial residues and released vacuole (J). K TEM shows the content of vacuoles filled with bacteria (double arrowheads) at different stages of digestion. Scale bars: 1  $\mu\text{m}$  (A–G), 5  $\mu\text{m}$  (I–J) and 2  $\mu\text{m}$  (K).

cases yet ceased upon starvation or transfer into the nutrient-free environment (Fig. 5B). Notably, the autofluorescence did not appear when cells were fed solely with latex beads (Supplementary Fig. S2E). The emergence of the red signal with digestible food uptake implied its association with the lysosomal activity. Indeed, colocalization by confocal microscopy of fluorescently labeled lysosomes and the autofluorescence signal in living cells showed positive spatial overlap (Fig. 5C). Moreover, the appearance of the red signal correlated with the production of reactive oxygen species, suggesting that autofluorescence may be associated with acidification (Fig. 5D).

#### Transcriptomic analysis

We next sought to identify the molecular mechanisms underlying the response to the different food sources and the red

autofluorescence using transcriptomic data. In the absence of a model diplomonid, and due to the large evolutionary distance of diplomonids from well-annotated eukaryotes, we were able to reliably annotate only 28% transcripts in *D. japonicum* and 40% in *R. humris*. In both species, most of the highly expressed transcripts (TPM > 400) were related to translation (187 in *D. japonicum*/175 in *R. humris*), followed by chromatin structure (25/21), protein modification (17/21), and cytoskeleton (14/13) (Fig. 6). In *D. japonicum*, the samples cluster according to the respective condition (Fig. 6A). The Hemi medium-fed cells were most distinctive by the increased expression of genes related to the chromatin structure and dynamics, coenzyme transport and metabolism, transcription, and cytoskeleton. Bacteria-fed cultures showed an increased transcription of genes related to RNA processing and modification, while starved *D. japonicum* invested in translation, energy conversion, metabolism,

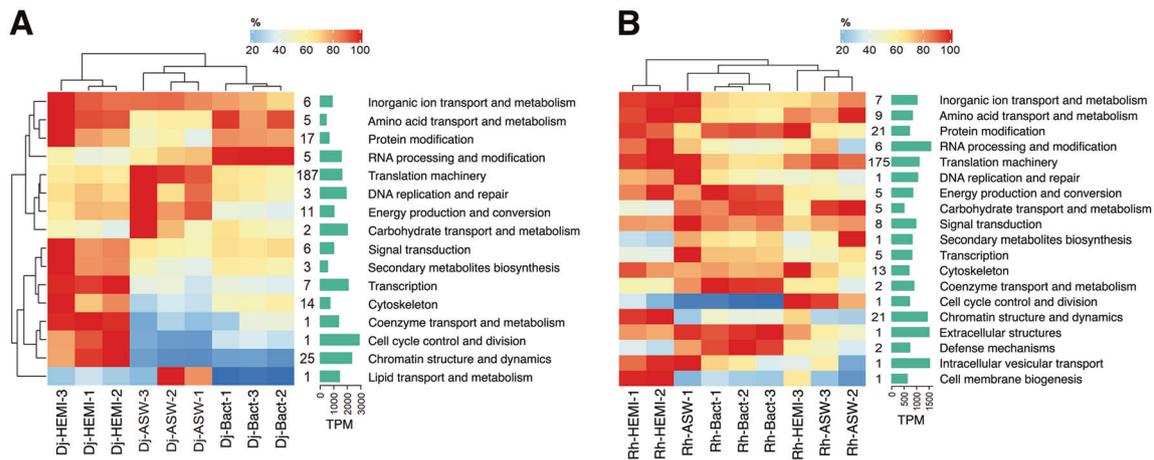


**Fig. 5** Characterization of red autofluorescence of diplomonads. **A** Panels showing emergence and dynamics of red fluorescence signal emitted by *D. japonicum* and *R. humris*. Time intervals as in Fig. 2A. **B** Time-course changes in mean red autofluorescence intensity of *D. japonicum* and *R. humris* freshly subjected to either the Hemi medium (HEMI; solid line) or artificial seawater (ASW; dashed line). **C** Confocal microscopy images of live *D. japonicum* showing the presence of red autofluorescence (af) and its colocalization with lysosomes stained with green LysoTracker (lyso); the signal is absent in the ASW-starved cells. Nucleus was stained with NucBlue (nb). Ph, phase contrast. Scale bar: 1  $\mu$ m. **D** Time-course changes in abundance of reactive oxygen species produced by *D. japonicum* grown on bacteria or medium.

and transport of lipids. Despite the lack of clustering according to respective cultivation conditions in *R. humris* (Fig. 6B), in the bacteria-fed cells, we were able to observe some emerging patterns, such as investment in protein synthesis and protection from reactive oxygen species.

Using a conservative filtering approach (passing both edgeR and DESeq2 tests [FDR 0.001, 4-fold change), 3 402 and 2 854 differentially expressed transcripts in *D. japonicum* and *R. humris*, respectively, were found. Considerable differences in expression were observed between the Hemi-medium vs. bacteria-fed cells, while only small differences occurred between the medium-fed vs.

starved cells. The medium-fed *D. japonicum* invested more in systemic processes, such as DNA replication, protein assembly, and folding, while the bacteria-fed diplomonads up-regulated genes involved in phagocytosis and oxidation (vacuolar ATP synthase, proton pumps), vesicular transport (serine-threonine kinases), peptidases, cell defense, and synthesis of melanin (Supplementary Fig. S3 and Supplementary Table S1). Starved *D. japonicum* increasingly invested in ribosome assembly, DNA replication, and translation. The Hemi-medium fed *R. humris* increased its ribosome assembly, translation, and nutrition metabolism, especially nitrogen. Bacterial prey enhanced vesicular



**Fig. 6 Differentially expressed genes of osmotrophic, phagotrophic, and starved diplomonids.** Overall relative levels of the gene expression for the most expressed transcripts (TPM > 400) in *D. japonicum* (A) and *R. humris* (B) maintained on a bacterial diet (Bact) and medium (HEMI) or starved in ASW broken down to the respective functional categories (COG). For each COG category, the sample with the highest expression (TPM) was assigned 100% (red), and the expression of the remaining samples was related to this value. The horizontal bars show the cumulative TPM values of all abundant transcripts belonging to the respective COG category. Numbers count the most expressed transcripts included in a given COG.

transport, as well as the (endo)membrane system organization and remodeling, whereas starved *R. humris* up-regulated mainly the expression of genes responsible for cell movement, (endo) membrane organization, and the phosphotransferase system, used in bacteria for import of sugars from the environment (Supplementary Fig. S4 and Supplementary Table S2).

#### Melanin as possible source of autofluorescence

Based on the upregulation of tyrosine transporters and tyrosinase – a key enzyme of melanin synthesis, the enrichment of melanin synthesis and metabolism (GO:0006582, GO:00482438) as revealed by the Fisher exact test, and also the emission spectrum, we hypothesized that the autofluorescence might be linked to melanin-like pigments. Western blotting with melanin-specific monoclonal antibody 6D2 revealed multiple bands at various molecular weights, consistent with synthetic melanin, which served as a positive control (Supplementary Fig. S5); however, the strongest signal remained in the stacking gel, suggesting that most of the polymer failed to enter the resolving gel (Fig. 7A). As also confirmed by immunofluorescence, melanin was depleted yet still detectable in diplomonids maintained in ASW and did not disappear even after 72 h of starvation (Fig. 7A, B, Supplementary Fig. S5). Instead of being located in the lysosomes, melanin was dispersed throughout the cytoplasm, seemingly in the vicinity of the endoplasmic reticulum (Fig. 7B).

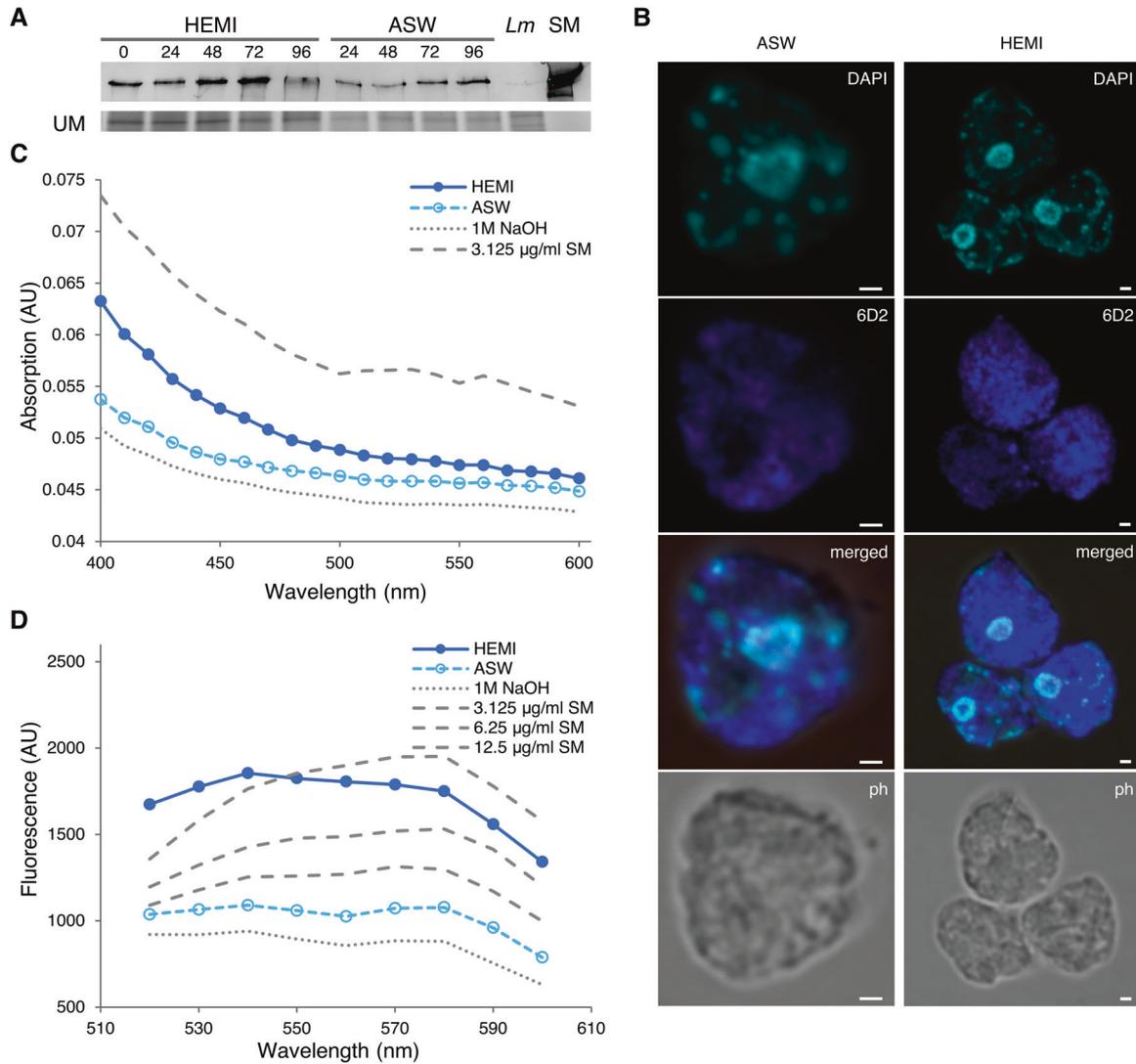
To further validate that the signal indeed represents melanin, we performed the isolation of pigment-containing fractions from both trophic and starved *D. japonicum* and characterized their optical properties. The absorption and the emission spectra of the extracted fractions closely resembled the exponential absorption pattern and the profiles, respectively, of synthetic melanin (Fig. 7C, D).

#### DISCUSSION

Based on extensive data from environmental sequencing [6–10, 12], diplomonids seem to be the most abundant eukaryotic component of the oceanic ecosystem for which any ecological function remains largely speculative. Extensive co-occurrence studies did not reveal any convincing associations between them and other eukaryotes [26], and their size distribution only hinted that some of them might be parasitic [8, 13]. Here we focused on nutritional modes of three marine members of the family Diplonemidae, and demonstrate their capability to switch from

osmotrophy to phagotrophy under cultivation preconditioning. In the absence of cultured representatives of Eupelagonemidae, which constitute 98% of diplomonid diversity in the oceans [8], our findings are of ecological relevance. While members of both families inhabit a range of habitats and may or may not (significantly) differ in their biology, they seem to share a lot of similarities in general morphology [30] and feeding behavior [9]. We posit that feeding flexibility of diplomonids is highly advantageous and provides strong clues to the successful life strategy of these understudied yet omnipresent protists.

Oceanic biodiversity is numerically dominated by prokaryotes, recognized to modulate the protistan communities and vice versa. Indeed, protistan bacterivores largely impact the structure and dynamics of bacterial assemblages in the aquatic environment [31, 32]. The ability to grow on bacteria as a sole food source shown here suggests that the abundant diplomonids may considerably contribute to the aggregated predator grazing pressure on prokaryotes. While nothing is known about grazing preferences of diplomonids under natural conditions, our experimental setup indicated substantial differences in their species-specific feeding patterns. Indeed, the growth dynamics of studied diplomonids were impacted by bacterial size, biotic state, composition, and total biomass available. *R. humris* selectively grazed on smaller prey and only with its depletion switched to larger bacteria, whereas *D. japonicum* appears to be more flexible in its prey selection and feeds rather erratically, yet this could have been tested only under the constraints inherent to our experimental setup. One possible explanation may be the lineage-specific preferences of flagellates for certain bacterial taxa, although the phenomenon of such grazing specificity is more profound in freshwater habitats [33–37]. As supporting evidence, we have also observed that phagotrophic *D. japonicum* grazed on living cyanobacteria belonging to the *Synechococcus* group (Supplementary Fig. S6A), while *R. humris* ignored this prey. Notably, both species did not grow when the heat-killed bacteria were replaced with their live cultures as prey. Though this may deviate from the general paradigm [34], it is worth noting that some protists have been also reported to prefer dead bacteria to living ones [34]. On the other hand, the well-known virulence factor of *Pseudomonas* endotoxin, inhibiting the growth of other organisms in the culture, may be an alternative explanation for the limited growth [38]. Another factor leading to moderate consumption of bacteria is a suboptimal prey-size ratio and, as a



**Fig. 7** Characterization of melanin-like pigment in trophic and starved *D. japonicum*. **A** Western blot analysis showing the time course (0–96 h) of melanin expression using anti-melanin antibody 6D2 in the diploemids freshly inoculated into either the Hemi medium (HEMI) or artificial seawater (ASW). *Leishmania mexicana* (*Lm*) and synthetic melanin (SM) served as negative and positive controls, respectively. UM – stain-free membrane. **B** Confocal microscopy images of melanin detected in 72-h-old cultures by immunofluorescence using 6D2 and Alexa Fluor 647 goat anti-mouse antibody. DAPI stained the nucleus. Ph, phase contrast. Scale bar: 1 μm. Optical absorption spectra (**C**) and fluorescence (**D**) emission spectra of pigment-containing fraction extracted from 72-h-old cultures and compared with synthetic melanin (SM).

consequence, the potentially longer food particle processing time and adaptation of a predator to prey [37, 39, 40]. In addition, even closely related prokaryotes may have a distinct nutritional impact on flagellate bacterivores, thereby affecting their community composition and ecophysiological characteristics, such as the growth rate and population biomass [41]. It appears to be the case of *S. specki*, which exhibits a high rate of total bacterial uptake, but at the same time excretes them almost undigested in numerous waste vacuoles, indicating a low nutritional value of the consumed bacteria. The slower growth and small cell volumes of *R. humris* fed on FLB may be associated with a deficiency of nutrients in the preferentially ingested small-sized prokaryotes [42], or simply that bacteria alone are not their preferred food items. Apart from the requirement of different types of nutrients, the studied species revealed strikingly distinct feeding strategies. While *D. japonicum* stuffed itself with the prey and quickly digested it, *R. humris* accumulated and digested prokaryotes at a much slower rate. These fundamental differences in trophic speciation are likely associated with food preferences and food particle processing.

The feeding strategy of *R. humris*, which may potentially be a lobster parasite [17, 25], may reflect its life strategy accustomed to high food availability not requiring its storage.

The ability to switch between different nutritional modes provides cells with great flexibility and may be critical for their survival in the broad habitat range they occupy. While in our experimental setup diploemids had to undergo preadaptation, possibly due to their long-term cultivation as osmotrophs, we speculate that the transition would likely be instant under natural conditions. Indeed, the presence of a fully developed cytopharynx [17–19, 43] in the osmotrophic cells indicates their phagotrophic capacity [44]. The species studied here were originally isolated from an aquarium and coastal waters, and utilization of bacterivory is likely beneficial during coastal bacterial blooms or any kind of accumulation of organic matter driven by inherent oceanic processes. The trophic stages of *D. japonicum* and *R. humris* are characterized by gliding cells [17, 18], suggesting their colonization of surfaces, which are typically nutrient-rich. At the same time, the starved cells are free swimmers, like most other

diplonemids, and while the expression patterns show that they are adapted to utilize bacteria as a food source, bacterivory may not be their main carbon source. Indeed, the generally low C-content of oceanic bacterioplankton compared to cultured bacteria [45] is unlikely to be sufficient to meet metabolic requirements of large flagellates, such as diplonemids. In addition, since in the seawater column the bacterial abundance is decreasing with depth, diplonemids are unlikely to feast on them in the deep sea where any nutritional particle is of great importance [46]. Instead, they likely use other carbon sources, such as sinking marine snow particles, rich in associated bacteria, decaying algae, and other planktonic organisms, representing hotspots of microbial biomass and degradation in the highly diluted, organic particle-poor environments. Near such lavish hotspots (Supplementary Video S1), the opportunistic lifestyle combined with the flexible feeding mode of diplonemids might add a considerable competitive advantage in cropping of food resources compared to suspension-feeding protists [39].

Species-specific feeding responses to a similar prey may indicate a variety of specialized feeding modes of diplonemids, reflecting difference in their thus far neglected ecological roles. The ubiquitous occurrence of diplonemids in different oceanic depths together with the assumed broad array of survival strategies might indicate that this highly diverse group involves many opportunistic strategists with high growth potential once the food resource or decaying biotic particles become abundant. The case of *S. specki* shows that even when food is depleted, decreased metabolic rates allow survival of dormant stages in adverse conditions, a behavior recently exemplified by flexible metabolic adaptations of diplonemids [47], which suggest an opportunistic lifestyle. Furthermore, the transcriptome-based study revealed an extremely high metabolic versatility of diplonemids [48]. Accordingly, we assume that they are omnivorous or opportunistic generalist predators and scavengers. This assumption is supported by the clearance rates of artificial latex beads as well as grazing on live and dead microalgae (Supplementary Figs. S6B–D). Diplonemids seem to adapt to food dynamics in their habitat, and, at the same time, can discriminate the low-quality or non-digestible food particles, such as latex beads, even after their ingestion, a feature known also for other protists [49]. To a large extent, such selectivity can be attributed to the biotic and physiological state, structural features, particle- or cell-surface properties, or chemical composition of predation-resistant bacteria [33, 40, 50], as well as repulsive chemicals in the environment [49]. In this context, we hypothesize that the appearance of red autofluorescence in cells during food digestion is associated with a self-defense mechanism. It appears that the process of food digestion is accompanied by the production of reactive oxygen species, the excess of which leads to the degradation of the lysosomal membrane [51]. To prevent the damage, diplonemids seem to trigger melanin synthesis, which is known to neutralize free radical toxicity and has other protective functions [52]. While melanin is widely distributed in animals, plants, fungi, and bacteria, it is less common in protists [53], although its presence under specific cultivation conditions was suggested in *Leishmania* [54], a parasitic relative of diplonemids. We further hypothesize that being produced in the endoplasmic reticulum [55], melanin then enters lysosomes, where its oxidative degradation causes fluorescence [56]. Ultimately, this leads to quenching of the vacuole with the sequestration of waste products and subsequent excretion from the cell. The expulsion of large waste vacuoles, in the absence of any recognizable dedicated cell structures, such as cytopycge or cytoproct, has been observed in cultured cells (this work) as well as freshly captured eupelagonemids (Okamoto N and Keeling PJ, pers. commun.). Since this prominent excretion happens at the posterior end of the cell, it may occur at the convergence of the peripheral microtubular corset or even require disassembly of microtubules,

followed by membrane healing [57]. In addition, we revealed another putatively protective extracellular feature of *D. japonicum*, the electron-transparent coat, which was not observed in cells cultured in the absence of bacteria [18]. These findings imply that in addition to great flexibility in feeding modes, diplonemids also have a broad array of defense and survival strategies as part of their successful lifestyle in permanently changing environments.

## CONCLUSIONS

We present the first detailed observation of the feeding behavior of diplonemids, one of the understudied yet highly abundant marine protistan groups. We demonstrate that diplonemids possess extensive flexibility in feeding modes, being able to switch from osmotrophy to phagotrophy. Such a broad scale of trophic adaptations can explain their ubiquity in different marine habitats assumed to be largely limited by particulate food sources. In order to unveil all the fascinating adaptations of diplonemids and their fundamental influence on the marine food web, further research on their cell biology, ecology and interaction with other planktonic organisms is needed. In addition, cultured diplonemids can serve as model organisms for studying other possible feeding modes.

## DATA AVAILABILITY

The raw RNA-Seq reads and transcriptome assemblies used in this study are available at NCBI under the BioProject PRJNA746563.

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## AUTHOR CONTRIBUTIONS

GP, TK, JL, and KŠ designed the study. GP and TK carried out the experiments and analyzed the data. VJ and AH performed the transcriptomic analysis. JM carried out Western blotting. GP, KŠ, and JL wrote the manuscript with input from all authors. All authors discussed the results and commented on the manuscript.

## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

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