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The influence of simulated microgravity on the proteome of *Daphnia magna*

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BACKGROUND: The waterflea *Daphnia* is an interesting candidate for bioregenerative life support systems (BLSS). These animals are particularly promising because of their central role in the limnic food web and its mode of reproduction. However, the response of *Daphnia* to altered gravity conditions has to be investigated, especially on the molecular level, to evaluate the suitability of *Daphnia* for BLSS in space.

METHODS: In this study, we applied a proteomic approach to identify key proteins and pathways involved in the response of *Daphnia* to simulated microgravity generated by a two-dimensional (2D) clinostat. We analyzed five biological replicates using 2D-difference gel electrophoresis proteomic analysis.

RESULTS: We identified 109 protein spots differing in intensity ($P < 0.05$). Substantial fractions of these proteins are involved in actin microfilament organization, indicating the disruption of cytoskeletal structures during clinorotation. Furthermore, proteins involved in protein folding were identified, suggesting altered gravity induced breakdown of protein structures in general. In addition, simulated microgravity increased the abundance of energy metabolism-related proteins, indicating an enhanced energy demand of *Daphnia*.

CONCLUSIONS: The affected biological processes were also described in other studies using different organisms and systems either aiming to simulate microgravity conditions or providing real microgravity conditions. Moreover, most of the *Daphnia* protein sequences are well-conserved throughout taxa, indicating that the response to altered gravity conditions in *Daphnia* follows a general concept. Data are available via ProteomeXchange with identifier PXD002096.

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INTRODUCTION

Since the first moon landing in 1969, technologies have advanced and the urge to further explore space has not diminished. At the moment, the ultimate goal of manned space missions is the exploration of Mars. Yet manned missions face several key issues that have to be solved, not only concerning human health,[1] but also the provision with essential supplies, e.g., food, water, oxygen.

A solution for this supply problem, especially for long duration missions, is the development of a bioregenerative life support system (BLSS), which minimizes reliability on delivered supplies and enhances autochtonous production. Up to now, such systems have only been installed as modules on the Mir and ISS space stations.[2] Since then, new components were added to the life support system, e.g., waste water recovery, forming a so-called ecological control and life support system.[3] The functions that have to be fulfilled by this system are the regeneration of atmosphere, purification of water, waste processing, food production, and food processing. The organisms involved in these systems include bacteria and fungi for the decomposition of organic waste and excrements, as well as unicellular microalgae, which produce the oxygen for astronauts, but also comprise higher organisms such as vegetables or fish to provide the astronauts with food.[4]

The waterflea *Daphnia* (Crustacea) might be a candidate in aquatic modules of such a BLSS for several reasons: (i) *Daphnia* occupies a central role in limnic food webs by being a primary consumer, hence serving as a link between oxygen producing, autotrophic producers such as algae and secondary consumers, such as planktivorous fish.[5] Fish, in respect, serve as an animal protein source for the human crew. Positive side effects of this constellation are that no additional fish food has to be transported, as well as that the growth of algae populations is controlled. (ii) *Daphnia* reproduces by the mode of cyclic parthenogenesis, thus enabling *Daphnia* to reproduce asexually in favorable environmental conditions and sexually in unfavorable ones, which leads to the formation of dormant eggs that are encased in a protective structure, the so called ephippium. Those resting eggs could be used to restart the BLSS in case of a system collapse and it was already shown that dormant eggs of *Daphnia* are able to hatch living neonates after long-term exposure to the space environment on the ISS.[6] Combining these modes of reproduction with the short generation time and the high number of offspring per clutch, a high bio mass production can be guaranteed.[7]

Daphnia does not only serve as a model organism in the fields of ecology, evolution, and ecotoxicology,[8] but also in ecological genomics.[9] Here, especially the improved availability of genomic resources[10] facilitates untargeted holistic approaches, such as transcriptomics[11] or proteomics,[12,13] which may reveal unpredicted key players underlying biological traits.

Long-term spaceflight is known to affect human physiology leading to bone demineralization, skeletal muscle atrophy, and

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Figure 1. Cuvette clinostat provided by the German Aerospace Center (DLR).

immune system suppression.[1] Furthermore, microgravity-induced responses were described in a variety of biological systems reaching from alterations of cytoskeletal formation in cells[14] to altered plant forms in *Arabidopsis*.[15] *Daphnia* has already been part of missions to space stations, demonstrating that resting eggs were viable even after exposure to outer space for >1 year.[6] Some animals survived up to 4 months in space but showed alterations in swimming behavior with an unusual high proportion of looping movements.[16] However, more information on the influence of microgravity on *Daphnia* is needed to decide on the suitability of these animals for BLSS in long-duration missions. Studies at the molecular level are especially interesting, as they may elucidate additional biological processes not detectable at the morphological or physiological level.

To determine the effect of long-term exposure to weightlessness, high technical complexity and financial investment is needed. Most facilities providing free fall conditions, like parabolic flights or drop-tower experiments can only deliver short duration of weightlessness. Yet a cost-effective ground-based method is the use of a two-dimensional (2D)-clinostat,[17] however, carefully considering the operational mode and limitation of the simulation.

In this study, we investigated the effect of simulated microgravity on *Daphnia* at the protein level, as proteins are the main effectors of biological functions in an organism. We exposed *Daphnia* to simulated microgravity using a 2D-clinostat and subsequently performed a proteomic approach to study quantitative changes in the proteome of animals exposed to altered gravity conditions compared with a control group.

MATERIALS AND METHODS

Animal husbandry

To investigate the effect of simulated microgravity on the proteome of *Daphnia magna* the laboratory cultivated genotype K34J was used, which originated from a fishpond near Munich, Germany. The animals were kept in a density of 5–12 adult animals per 1.5-l jar filled with semi-artificial medium[18] in an illuminated climate chamber (Binder KBWF 240, Binder GmbH, Tuttlingen, Germany) at 21 ± 1 °C and a photoperiod of 12 h (L18W 865 Cool Daylight, Osram, Munich, Germany). Animals were fed every second day with the unicellular algae *Scenedesmus obliquus*, thereby reaching a carbon concentration of 1.5 mg/l.

Simulated microgravity—clinostat experiment

To simulate microgravity, we used a cuvette 2D-clinostat, designed and provided by the German Aerospace Center (Deutsches Zentrum für Luft- und Raumfahrt, DLR) in Cologne, Germany (Figure 1).

The clinorotation principle is based on the fast rotation around a small diameter thereby preventing physical sedimentation.[19] As a consequence of clinorotation, the composition of the *D. magna* proteome was investigated in comparison to the proteome under normal gravity conditions, which means in a static 1 g control. For the experiment, *D. magna* of 2 ± 0.2 mm size were used. Body size was measured with a Leica MS5 stereomicroscope (Leica Microsystems, Wetzlar, Germany). The clinostat was loaded with 10 1-ml serological BD-Falcon pipettes (BD Biosciences, Heidelberg, Germany). Each pipette contained 1 ml of medium enriched with algae (carbon concentration: 1.5 mg/l) and five randomly selected *D. magna*. Animals were able to move freely in the cuvette. Alternately one pipette was mounted on the clinostat and the next one was put next to the clinostat as a control, therefore the control pipettes were exposed to the same vibrations as the clinorotated pipettes. This process was repeated until 10 pipettes of each treatment were arranged within the experimental setup.

The rotation speed of the clinostat was set at 60 r.p.m., with a residual gravity of ~ 0.008*g*.[20] The duration of the experiment was set to 60 min at a room temperature of 20 °C. Longer exposure times of several days, as implemented for plants,[17] would not be possible without creating food limitations for *Daphnia*, especially as a higher starting amount of algae may have harmful effects on the animals and adding of additional algae is not feasible during clinorotation because of turbulence generation. However, as an exposure time of 60 min was sufficient to show effects in other studies, e.g., study by Eiermann *et al*.,[21] and the algae concentration was sufficient for this amount of time, we chose this duration for our clinorotation experiment. After 60 min, the pipettes were emptied during rotation by tilting the device into cryo tubes and excessive water was immediately removed and cryo tubes were snap frozen in liquid nitrogen. The process of water removal and freezing was performed in less than 5 s. A total number of 10 runs was performed, each consisting of 10 serological cuvettes and therefore 50 animals of each treatment.

Sample preparation

To generate samples for proteomic analysis, the frozen biological samples were pulverized in a mortar containing liquid nitrogen to prevent thawing. The resulting powder was solubilised in 330 µl lysis buffer (2 mol/l Thiourea, 6 mol/l Urea, 4% CHAPS, 1 cOmplete ULTRA Tablets Mini (Roche, Penzberg, Germany) per 5 ml buffer). Afterwards, each sample was centrifuged using a QIA Shredder Mini Spin Column (Qiagen, Hilden, Germany) for 3 min at 16,100 r.c.f. to get rid of debris. Proteins were precipitated using 30% trichloroacetic acid for 20 min on ice to inhibit proteolytic activity.[22] Subsequently, samples were centrifuged for 10 min at 16,100 r.c.f., the supernatant was discarded and the protein pellet was washed three times with cold acetone. The pellet was dried and resolved in lysis buffer. The pH of the solution was adjusted to 8 by adding 50 mM NaOH. Protein concentration was determined by Bradford Assay (Coomassie Plus (Bradford) Assay Reagent, Thermo Scientific, Braunschweig, Germany) according to the manufacturer's instructions. To reach sufficient protein concentrations for 2D-difference gel electrophoresis (DIGE), two clinorotation runs per group were pooled leading to five biological replicates.

2D-DIGE

2D-DIGE method was conducted following the general procedure described in the study by Otte *et al*.[13] Briefly, 50 µg protein per biological replicate was labeled with 2D-DIGE Cy3 Dye for control and Cy5 Dye for the clinorotated group following the protocol of the manufacturer (GE Healthcare Life Sciences, Munich, Germany). An internal pooled standard was prepared by pooling 25 µg protein of all biological replicates and labeling of 300 µg with 2D-DIGE Cy2 Dye. For each 2D-DIGE gel, 50 µg of one Cy3-labeled control replicate, 50 µg of one Cy5-labeled clinorotated replicate, and 50 µg of Cy2-labeled internal pooled standard were combined.

For first dimension separation, 24-cm gel strips (DryStrips pH 4–7, GE Healthcare) and an IPGPhore (Pharmacia Biotech, Uppsala, Sweden) were used. For second dimension separation, the gel strips were fixed on top of

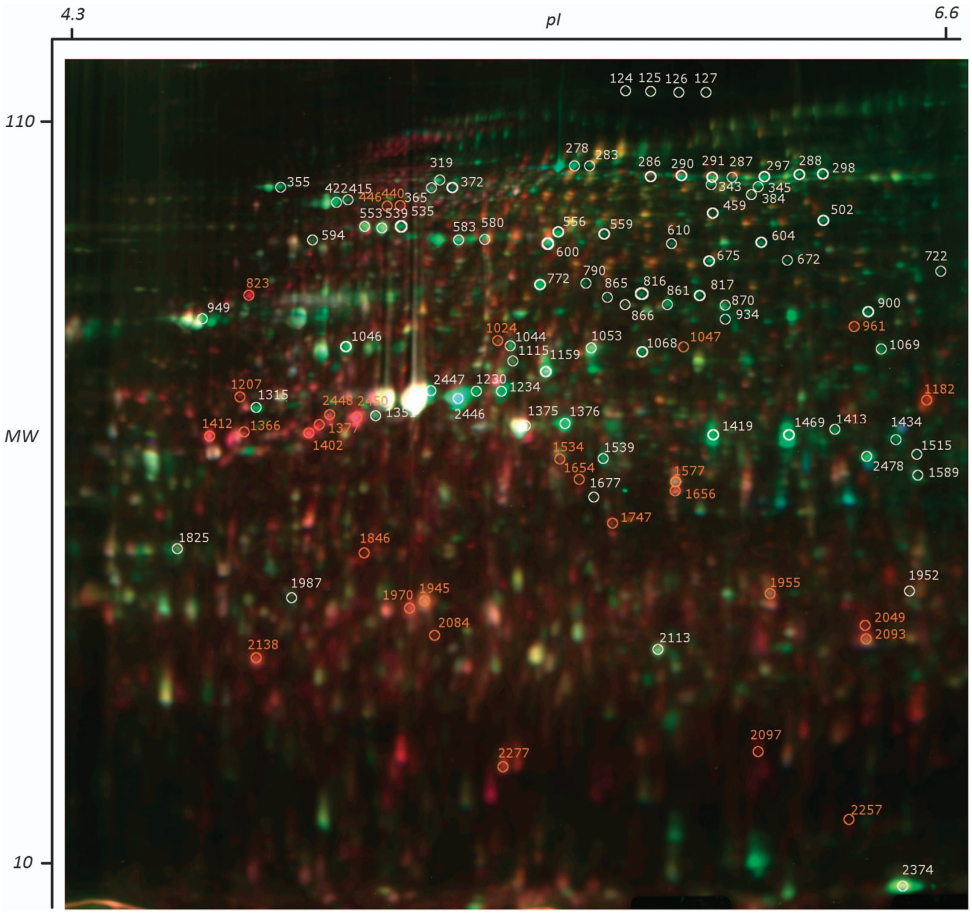

Figure 2. Example 2D-DIGE gel image. Here, protein spots which were significantly different between treatments and were identified using LC-MS/MS were marked. Red labeled spots were less abundant in the clinorotated treatment, whereas white labeled spots were more abundant in the clinorotated treatment. LC-MS/MS, liquid chromatography-tandem mass spectrometry; 2D-DIGE, two-dimensional difference gel electrophoresis.

lab cast gels and electrophoresis was performed using an ETTANDaltsix electrophoresis unit (GE Healthcare Life Sciences). During the whole 2D-DIGE procedure, all five biological replicates were processed in parallel.

Imaging and quantitative analysis

Immediately after electrophoresis, gels were scanned using a Typhoon 9400 fluorescence scanner (GE Healthcare Life Sciences) with parameter settings recommended by the manufacturers for 2D-DIGE experiments. Image analysis and relative quantification were performed with DeCyder 2D Software version v7.0 (GE Healthcare Life Sciences). Coordinates of corresponding spots differing significantly in their intensity ($P \leqslant 0.05$ after false discovery rate correction, ratio $\geqslant |2|$) were used to generate a pick list for further processing.

Excision of spots and tryptic hydrolysis

Gels were stained overnight with colloidal Coomassie staining solution (Carl Roth GmbH, Karlsruhe, Germany) and then destained using 25% methanol. Further processing of proteins spots was performed according to in the study by Otte *et al*.[13] In summary, spots of interest were cut out of the gel and digested with trypsin (Sequencing Grade–Modified Trypsin, Promega, Mannheim, Germany) to generate peptides for protein identification.

LC-MS/MS analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed on a multi-dimensional LC system (Ettan MDLC, GE Healthcare Life Sciences) coupled to a LTQ mass spectrometer (Thermo Scientific, Braunschweig, Germany). Further settings were the same as described in the study by Otte *et al*.[13]

Bioinformatic processing

For protein identification, MS/MS data were searched with Mascot Version: 2.3.00 (Matrix Science, London, United Kingdom). As database, pre-released gene-predictions of *D. magna* (V2.4 effective May 2012) were used. These sequence data were produced by The Center for Genomics and Bioinformatics at Indiana University and distributed via wFleaBase in collaboration with the Daphnia Genomics Consortium (http://daphnia.cgb.indiana.edu). Further data processing was done as described in the study by Otte *et al*.[13] Protein spots having multiple protein identifications were not included in the final data set. To get further information on similar proteins, all significant protein sequences were blastp searched against NCBI non-redundant (nr) and Swiss-Prot databases using local standalone blast.[23] The NCBI nr database combines non-redundant protein sequences of several sources, including translations from annotated coding regions in GenBank, RefSeq, and TPA, as well as records from PIR, PRF, and PDB, whereas the Swiss-Prot database is manually annotated and therefore contains less but more reliable data. Thus, recent data of genomic studies on several organisms are found in nr database, whereas Swiss-Prot consist mainly of well-annotated protein information of a few well-studied model organisms.

Preliminary annotation data were received from http://server7.wfleabase.org/genome/Daphnia_magna/ (V2.4 effective May 2012). Protein-associated gene ontology (GO) terms were tested for enrichment using customized standalone EASE[24] (Benjamini-corrected P value < 0.05).

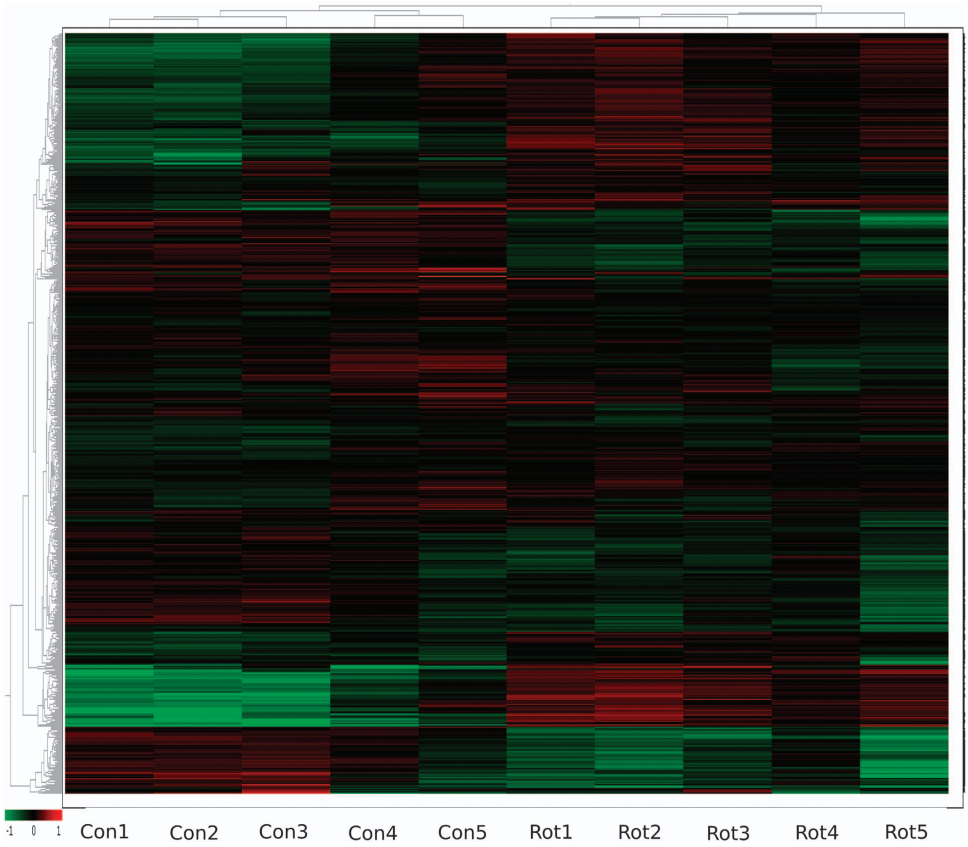

Figure 3. Heatmap and hierarchical cluster of all protein spot intensity data present in all biological replicates. Con refers to the control replicates, whereas Rot refers to the clinorotated replicates.

Enriched terms were tested for redundant terms and semantic similarities using the online-tool REViGO[25] and visualized as treemap graphs using R.[26]

In addition, protein sequences were also processed using the software Blast2go,[27] which uses results of NCBI blast search to map sequences directly to GO terms. These GO terms were used as additional information.

RESULTS

We analyzed the effects of altered gravity conditions on *D. magna* at the protein level by exposing animals for 1 h to clinorotation followed by a proteomic 2D-DIGE approach. We studied five clinorotated and five control replicates. We generated 5 2D-DIGE gels, which showed reproducible spot map patterns (Supplementary Data S2), and were able to match and quantify 1,211 protein spots in at least 4 of these 5 spot maps. Unsupervised hierarchical clustering of spot intensity data present in all spot maps showed a good clustering according to treatment (Figure 2).

About 109 of these protein spots showed significantly different intensity signals between the control and the clinorotated treatment ($P \leqslant 0.05$, ratio $\geqslant |2|$) and were identified using LC-MS/MS (Figure 3 and Table 1). Of these protein spots, 30 were less abundant, whereas 79 protein spots were more abundant in the clinorotated treatment.

As information on protein function of *D. magna* proteins is scarce so far, we performed a blastp search against NCBI nr and Swiss-Prot database to look for similar and possibly better characterized proteins in other species. Here, we used a strict BLAST bit score threshold of $\geqslant 244$, which ensures accurate prediction of protein function similarity.[28] Interestingly, nearly all hits in the nr database refer to proteins of *D. pulex*. In contrast to *D. magna*, the *D. pulex* genome is published[10] and therefore completely represented in this database. Furthermore, a majority of proteins had a similar blast hit in the Swiss-Prot database, indicating the presence of similar proteins in well-studied model organisms and therefore a good conservation of these proteins within organismal taxa (Supplementary Data S1).

Enrichment analysis of GO terms yielded five overrepresented terms in biological process and molecular function database, namely protein folding, unfolded protein binding, actin binding, ATP binding, and glycolytic process (Figure 4 and Table 2). Furthermore, nine protein spots were identified as *Daphnia* hemoglobins.

About 27 protein spots were identified as proteins connected to actin binding and they were either involved in muscular structures or the cytoskeleton. Beyond actin itself, we identified myosin, α-actinin, filamin-A, gelsolin, and advillin. Some of these proteins are present in multiple spots with isoelectric point and molecular weight shifts, and varying abundances, indicating post-translational modifications (PTMs). Most spots identified as actin, myosin, α-actinin, and filamin-a were less abundant, whereas advillin and gelsolin were identified in spots to be more abundant after clinorotation.

Furthermore, 20 protein spots were identified as proteins involved in protein folding. Here, heat shock proteins and other chaperones like endoplasmin, a protein disulfide-isomerase, and different subunits of T-complex protein 1 were found. All spots except one had a higher abundance in the animals exposed to clinorotation.

In addition, 17 protein spots were connected to different metabolic pathways involved in energy generation. Proteins were involved in glycolysis, a GO term which was also found to be

Table 1. Significantly altered proteins involved in the response of *Daphnia* to altered gravity

Spot. no.	*2D-DIGE_results*				*First_Blast_hit*		*Enriched_GO_terms*
	*ID_*D. magna*_database*	*T-test*	*Av. ratio*	*Acc*	*Name*	*Org*	
288	daphmag3mtv3l5529t1	0.001	25.87	EFX88163	Hypothetical protein DAPPUDRAFT_311761	*Daphnia pulex*	F: actin binding
556	daphmag3mtv3l7809t1	0.004	11.43	EFX86275	Hypothetical protein DAPPUDRAFT_313359	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, F: ATP binding
1469	daphmag3mtv3l6920t1	0.007	9.87	BAJ72724	2-domain hemoglobin	*Daphnia magna*	—
1068	daphmag3mtv3l11111t1	0.003	9.35	EFX83276	Enolase	*Daphnia pulex*	P: glycolysis
297	daphmag3mtv3l5529t1	0.007	8.62	EFX88163	Hypothetical protein DAPPUDRAFT_311761	*Daphnia pulex*	F: actin binding
559	daphmag3mtv3l7809t1	0.006	7.67	EFX86275	Hypothetical protein DAPPUDRAFT_313359	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, F: ATP binding
604	daphmag3mtv3l6730t1	0.003	7.42	EFX71530	Hypothetical protein DAPPUDRAFT_308853	*Daphnia pulex*	—
1419	daphmag3mtv3l6920t1	0.007	7.01	BAJ72724	2-domain hemoglobin	*Daphnia magna*	—
772	daphmag3mtv3l9572t1	0.008	6.94	EFX84424	Hypothetical protein DAPPUDRAFT_301074	*Daphnia pulex*	C: cytoplasm, F: ATP binding
1376	daphmag3mtv3l8231t1	0.001	6.91	EFX89163	Hypothetical protein DAPPUDRAFT_220693	*Daphnia pulex*	F: ATP binding
900	daphmag3mtv3l9835t1	0.012	6.9	EFX70620	Hypothetical protein DAPPUDRAFT_202253	*Daphnia pulex*	—
1234	daphmag3mtv3l7094t1	0.003	6.78	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
372	daphmag3mtv3l2246t2	0.013	6.13	EFX89391	Hypothetical protein DAPPUDRAFT_303199	*Daphnia pulex*	F: actin binding
817	daphmag3mtv3l10162t1	0.013	6.05	EFX77428	Hypothetical protein DAPPUDRAFT_213377	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
290	daphmag3mtv3l5529t1	0.011	5.82	EFX88163	Hypothetical protein DAPPUDRAFT_311761	*Daphnia pulex*	F: actin binding
1230	daphmag3mtv3l7094t1	0.011	5.6	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
1589	daphmag3mtv3l15212t1	0.013	5.44	EFX80600	Hypothetical protein DAPPUDRAFT_196566	*Daphnia pulex*	—
343	daphmag3mtv3l4901t1	0.009	5.31	EFX86312	Hypothetical protein DAPPUDRAFT_308519	*Daphnia pulex*	—
2446	daphmag3mtv3l7094t1	0.005	5.24	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
502	daphmag3mtv3l592t1	0.013	5.17	EFX87607	Hypothetical protein DAPPUDRAFT_192333	*Daphnia pulex*	—
2478	daphmag3mtv3l16955t1	0.013	5.1	BAA76873	Hemoglobin	*Daphnia magna*	—
298	daphmag3mtv3l11111t1	0.029	5.01	EFX83276	Enolase	*Daphnia pulex*	P: glycolysis
1046	daphmag3mtv3l10909t1	0.008	4.87	EFX90019	Hypothetical protein DAPPUDRAFT_309746	*Daphnia pulex*	F: ATP binding
675	daphmag3mtv3l10134t1	0.007	4.78	EFX75422	Hypothetical protein DAPPUDRAFT_306806	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
459	daphmag3mtv3l6920t1	0.003	4.61	BAJ72724	2-domain hemoglobin	*Daphnia magna*	—
1315	daphmag3mtv3l17606t1	0.015	4.25	EFX78249	Hypothetical protein DAPPUDRAFT_320706	*Daphnia pulex*	—
1159	daphmag3mtv3l13427t1	0.011	4.08	EFX90443	Hypothetical protein DAPPUDRAFT_299795	*Daphnia pulex*	P: glycolysis
286	daphmag3mtv3l5529t1	0.023	4.07	EFX88163	Hypothetical protein DAPPUDRAFT_311761	*Daphnia pulex*	F: actin binding
816	daphmag3mtv3l10162t1	0.013	3.82	EFX77428	Hypothetical protein DAPPUDRAFT_213377	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
1413	daphmag3mtv3l16955t1	0.026	3.82	BAA76873	Hemoglobin	*Daphnia magna*	—
535	daphmag3mtv3l7770t1	0.004	3.81	ADA79522	Heat shock protein 70	*Daphniopsis tibetana*	F:ATP binding
345	daphmag3mtv3l4901t1	0.012	3.77	EFX86312	Hypothetical protein DAPPUDRAFT_308519	*Daphnia pulex*	—
1987	daphmag3mtv3l6078t1	0.003	3.71	EFX87538	Hypothetical protein DAPPUDRAFT_192225	*Daphnia pulex*	C: cytoplasm, F: ATP binding
291	daphmag3mtv3l7067t1	0.041	3.65	EFX71215	Hypothetical protein DAPPUDRAFT_309186	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, F: ATP binding
2374	daphmag3mtv3l21839t1	0.012	3.63	NA	NA	NA	—
355	daphmag3mtv3l7067t1	0.013	3.57	EFX71215	Hypothetical protein DAPPUDRAFT_309186	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, F: ATP binding
287	daphmag3mtv3l5529t1	0.021	3.48	EFX88163	Hypothetical protein DAPPUDRAFT_311761	*Daphnia pulex*	F: actin binding
1515	daphmag3mtv3l15212t1	0.044	3.37	EFX80600	Hypothetical protein DAPPUDRAFT_196566	*Daphnia pulex*	—
422	daphmag3mtv3l4176t1	0.02	3.36	EFX66769	Hypothetical protein DAPPUDRAFT_302452	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, F: ATP binding
861	daphmag3mtv3l11254t1	0.031	3.32	EFX74207	Cct5-prov protein	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
934	daphmag3mtv3l9835t1	0.012	3.17	EFX70620	Hypothetical protein DAPPUDRAFT_202253	*Daphnia pulex*	—
1539	daphmag3mtv3l13753t1	0.017	3.16	EFX71334	Cytosolic malate dehydrogenase	*Daphnia pulex*	—
790	daphmag3mtv3l9572t1	0.011	3.14	EFX84424	Hypothetical protein DAPPUDRAFT_301074	*Daphnia pulex*	C: cytoplasm, F: ATP binding
278	daphmag3mtv3l6051t1	0.012	3.1	EFX71787	Hypothetical protein DAPPUDRAFT_326816	*Daphnia pulex*	—
365	daphmag3mtv3l2246t2	0.027	3.09	EFX89391	Hypothetical protein DAPPUDRAFT_303199	*Daphnia pulex*	F: actin binding
865	daphmag3mtv3l11254t1	0.011	3.08	EFX74207	Cct5-prov protein	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
1069	daphmag3mtv3l5529t1	0.026	3	EFX88163	Hypothetical protein DAPPUDRAFT_311761	*Daphnia pulex*	F: actin binding
722	daphmag3mtv3l4092t1	0.013	2.97	EFX87506	Hypothetical protein DAPPUDRAFT_306375	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
415	daphmag3mtv3l4176t1	0.028	2.97	EFX66769	Hypothetical protein DAPPUDRAFT_302452	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, F: ATP binding
672	daphmag3mtv3l9343t1	0.012	2.94	XP_003700942	PREDICTED: coatomer subunit delta like	*Megachile rotundata*	—
600	daphmag3mtv3l2675t1	0.023	2.84	EFX81902	Hypothetical protein DAPPUDRAFT_302856	*Daphnia pulex*	F: ATP binding
126	daphmag3mtv3l1194t1	0.013	2.81	EFX79782	Hypothetical protein DAPPUDRAFT_304363	*Daphnia pulex*	—
870	daphmag3mtv3l9792t1	0.025	2.71	EFX87987	Hypothetical protein DAPPUDRAFT_127024	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
319	daphmag3mtv3l4116t1	0.026	2.69	EFX72171	Hypothetical protein DAPPUDRAFT_308570	*Daphnia pulex*	F: ATP binding
283	daphmag3mtv3l6051t1	0.02	2.66	EFX71787	Hypothetical protein DAPPUDRAFT_326816	*Daphnia pulex*	—
580	daphmag3mtv3l5322t1	0.012	2.63	EFX90349	Hypothetical protein DAPPUDRAFT_300069	*Daphnia pulex*	F: ATP binding
1044	daphmag3mtv3l9038t1	0.023	2.62	EFX87450	Hypothetical protein DAPPUDRAFT_207615	*Daphnia pulex*	—
1115	daphmag3mtv3l12548t1	0.012	2.6	EFX81896	Hypothetical protein DAPPUDRAFT_302792	*Daphnia pulex*	—
1434	daphmag3mtv3l8815t1	0.023	2.57	EFX88463	Hypothetical protein DAPPUDRAFT_305568	*Daphnia pulex*	—
124	daphmag3mtv3l1194t1	0.019	2.56	EFX79782	Hypothetical protein DAPPUDRAFT_304363	*Daphnia pulex*	—
127	daphmag3mtv3l1194t1	0.018	2.56	EFX79782	Hypothetical protein DAPPUDRAFT_304363	*Daphnia pulex*	—
125	daphmag3mtv3l1194t1	0.016	2.54	EFX79782	Hypothetical protein DAPPUDRAFT_304363	*Daphnia pulex*	—
1053	daphmag3mtv3l11111t1	0.02	2.51	EFX83276	Enolase	*Daphnia pulex*	P: glycolysis
594	daphmag3mtv3l13753t1	0.001	2.44	EFX71334	Cytosolic malate dehydrogenase	*Daphnia pulex*	—
949	daphmag3mtv3l11112t1	0.034	2.43	EFX88851	Hypothetical protein DAPPUDRAFT_234212	*Daphnia pulex*	—
1952	daphmag3mtv3l6078t1	0.043	2.36	EFX87538	Hypothetical protein DAPPUDRAFT_192225	*Daphnia pulex*	C: cytoplasm, F: ATP binding

Table 1. (Continued)

Spot. no.	2D-DIGE_results				First_Blast_hit		Enriched_GO_terms
	ID_D. magna_database	T-test	Av. ratio	Acc	Name	Org	
539	daphmag3mtv3l7770t1	0.021	2.35	ADA79522	Heat shock protein 70	*Daphniopsis tibetana*	F: ATP binding
2113	daphmag3mtv3l14633t1	0.011	2.34	EFX88365	Hypothetical protein DAPPUDRAFT_230303	*Daphnia pulex*	—
977	daphmag3mtv3l2256t2	0.017	2.3	EFX74558	Hypothetical protein DAPPUDRAFT_307231	*Daphnia pulex*	—
583	daphmag3mtv3l5322t1	0.036	2.27	EFX90349	Hypothetical protein DAPPUDRAFT_300069	*Daphnia pulex*	F: ATP binding
866	daphmag3mtv3l4092t1	0.025	2.24	EFX87506	Hypothetical protein DAPPUDRAFT_306375	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
610	daphmag3mtv3l2732t1	0.05	2.23	EFX80327	Hypothetical protein DAPPUDRAFT_304064	*Daphnia pulex*	—
1825	daphmag3mtv3l10239t1	0.008	2.2	EFX70674	Hypothetical protein DAPPUDRAFT_256736	*Daphnia pulex*	F: ATP binding
384	daphmag3mtv3l4116t1	0.025	2.18	EFX72171	Hypothetical protein DAPPUDRAFT_308570	*Daphnia pulex*	F: ATP binding
1677	daphmag3mtv3l8231t1	0.008	2.16	EFX89163	Hypothetical protein DAPPUDRAFT_220693	*Daphnia pulex*	F: ATP binding
1375	daphmag3mtv3l8231t1	0.003	2.13	EFX89163	Hypothetical protein DAPPUDRAFT_220693	*Daphnia pulex*	F: ATP binding
553	daphmag3mtv3l7770t1	0.039	2.08	ADA79522	Heat shock protein 70	*Daphniopsis tibetana*	F: ATP binding
1351	daphmag3mtv3l12256t1	0.033	2.07	EFX68536	Hypothetical protein DAPPUDRAFT_189444	*Daphnia pulex*	—
2447	daphmag3mtv3l7094t1	0.017	2.02	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
1654	daphmag3mtv3l8855t1	0.008	−2.02	EFX80562	Hypothetical protein DAPPUDRAFT_188180	*Daphnia pulex*	P: glycolysis
1047	daphmag3mtv3l5529t1	0.026	−2.03	EFX88163	Hypothetical protein DAPPUDRAFT_311761	*Daphnia pulex*	F: actin binding
1747	daphmag3mtv3l8231t1	0.033	−2.06	EFX89163	Hypothetical protein DAPPUDRAFT_220693	*Daphnia pulex*	F: ATP binding
1534	daphmag3mtv3l11651t1	0.018	−2.09	EFX82035	Hypothetical protein DAPPUDRAFT_302845	*Daphnia pulex*	C: cytoplasm
1945	daphmag3mtv3l7094t1	0.043	−2.28	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
961	daphmag3mtv3l1503t1	0.008	−2.46	EFX86436	Hypothetical protein DAPPUDRAFT_208250	*Daphnia pulex*	F: actin binding
1412	daphmag3mtv3l7094t1	0.043	−2.46	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
1577	daphmag3mtv3l8855t1	0.017	−2.52	EFX80562	Hypothetical protein DAPPUDRAFT_188180	*Daphnia pulex*	P: glycolysis
2093	daphmag3mtv3l6920t1	0.04	−2.55	BAJ72724	2-domain hemoglobin	*Daphnia magna*	—
440	daphmag3mtv3l733t1	0.012	−2.73	EFX87106	Myosin heavy chain isoform 3	*Daphnia pulex*	F: ATP binding, F: actin binding
1024	daphmag3mtv3l5322t1	0.004	−2.87	EFX90349	Hypothetical protein DAPPUDRAFT_300069	*Daphnia pulex*	F: ATP binding
1656	daphmag3mtv3l8855t1	0.015	−2.89	EFX80562	Hypothetical protein DAPPUDRAFT_188180	*Daphnia pulex*	P: glycolysis
1955	daphmag3mtv3l6920t1	0.008	−2.89	BAJ72724	2-domain hemoglobin	*Daphnia magna*	—
1207	daphmag3mtv3l7770t1	0.012	−2.93	ADA79522	Heat shock protein 70	*Daphniopsis tibetana*	F: ATP binding
2257	daphmag3mtv3l9572t1	0.031	−2.95	EFX84424	Hypothetical protein DAPPUDRAFT_301074	*Daphnia pulex*	C: cytoplasm, F: ATP binding
2084	daphmag3mtv3l9835t1	0.012	−3.06	EFX70620	Hypothetical protein DAPPUDRAFT_202253	*Daphnia pulex*	—
1402	daphmag3mtv3l7094t1	0.027	−3.12	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
446	daphmag3mtv3l733t1	0.031	−3.13	EFX87106	Myosin heavy chain isoform 3	*Daphnia pulex*	F: ATP binding, F: actin binding
1970	daphmag3mtv3l7094t1	0.032	−3.13	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
2097	daphmag3mtv3l6920t1	0.01	−3.17	BAJ72724	2-domain hemoglobin	*Daphnia magna*	—
1366	daphmag3mtv3l7094t1	0.019	−3.2	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
2448	daphmag3mtv3l7094t1	0.012	−3.26	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
1182	daphmag3mtv3l1503t1	0.031	−3.4	EFX86436	Hypothetical protein DAPPUDRAFT_208250	*Daphnia pulex*	F: actin binding
2450	daphmag3mtv3l7094t1	0.012	−3.42	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
1846	daphmag3mtv3l8815t1	0.028	−3.49	EFX88463	Hypothetical protein DAPPUDRAFT_305568	*Daphnia pulex*	—
2049	daphmag3mtv3l6920t1	0.013	−3.69	BAJ72724	2-domain hemoglobin	*Daphnia magna*	—
1377	daphmag3mtv3l7094t1	0.019	−3.99	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
2138	daphmag3mtv3l8231t1	0.013	−4.01	EFX89163	Hypothetical protein DAPPUDRAFT_220693	*Daphnia pulex*	F: ATP binding
2277	daphmag3mtv3l733t1	0.025	−4.05	EFX87106	Myosin heavy chain isoform 3	*Daphnia pulex*	F: ATP binding, F: actin binding
823	daphmag3mtv3l2501t1	0.004	−4.6	EFX84778	Hypothetical protein DAPPUDRAFT_99081	*Daphnia pulex*	F: actin binding

Abbreviations: 2D-DIGE, two-dimensional difference gel electrophoresis; GO, gene ontology; NA, not applicable; nr, non-redundant.
2D-DIGE results, first nr Blast Hit and enriched GO terms are displayed.

overrepresented in our data set, in the tricarboxylic acid cycle, the respiratory chain, or pentose phosphate pathway. All proteins except fructose-bisphosphate-aldolase were more abundant in *Daphnia* exposed to simulated microgravity.

For a summary of identified proteins see Table 3, for details on protein spots see Supplementary Data S1.

DISCUSSION

To analyze the effects of altered gravity conditions on the waterflea *D. magna*, we exposed animals to fast clinorotation, an established method to simulate microgravity conditions on ground. Nevertheless one has to keep in mind, that this kind of simulation approach has to be verified under real microgravity conditions.[17] The clinorotated animals were compared with a control group using a proteomic 2D-DIGE approach (five biological replicates). As a general result of this study, proteins involved in actin microfilament organization were less abundant in clinorotated animals, whereas proteins connected to protein folding and energy metabolism were more abundant.

Exposure to simulated microgravity may disrupt actin microfilament organization in *Daphnia*

Around 25% of the significantly altered and identified protein spots in our data set were related to muscular structures or the cytoskeleton. All of these proteins were annotated with the GO term actin binding, which was also found to be significantly overrepresented (Figure 4).

We identified the structural protein actin in 12 spots, showing isoelectric point and molecular weight shifts on the 2D-gel. Two different kinds of spots were observed, indicating two groups of PTMs. The first group consists of four spots which were more

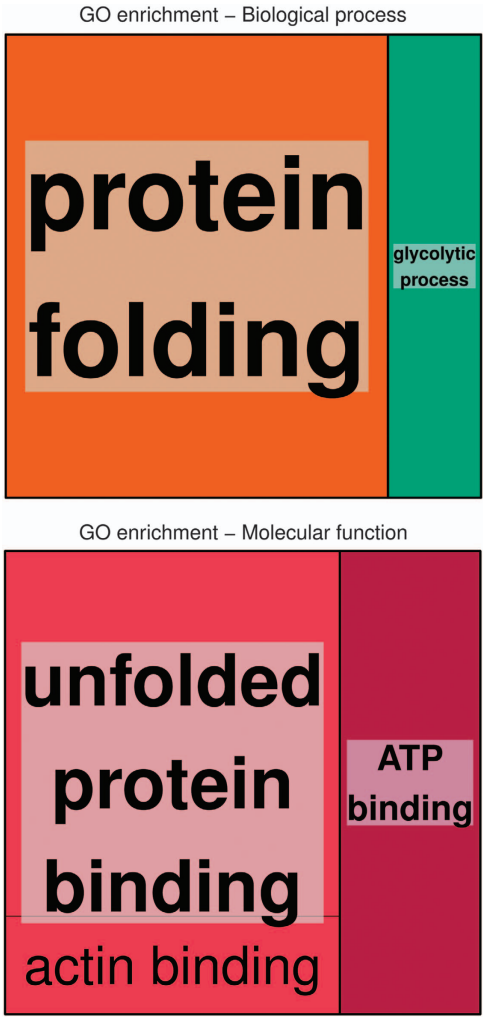

Figure 4. Results of REViGO semantic analysis of EASE results for enrichment of GO biological process terms and molecular function terms (p.adjust < 0.05). Colors mark semantic similarity, whereas the size of the corresponding area reflects the *P* value. GO, gene ontology.

abundant in the clinorotated treatment and have a pI near the theoretical value but an increased molecular mass. The second group of eight spots is less abundant in the clinorotated treatment and has a decreased pI but the molecular weight is near the theoretical value or below (Supplementary Data S1). These spots, which were located differently on the gel showing different abundance ratios, may indicate changes of actin PTMs. These changes may be related to alterations of actin microfilament organization, as PTMs are known to modulate structure and function of actin.[29]

The effect of clinorotation on actin filaments in our study is further emphasized by abundance alterations of actin-related proteins. Here, not only the actin-filament-associated motor protein myosin but also the actin-binding proteins α-actinin and filamin-A were less abundant in animals exposed to simulated microgravity. Filamin is known to be involved in the recruitment of actin filaments in *Drosophila*[30] and its decreased abundance may therefore indicate cytoskeletal disorganization. α-actinin is a cross-linker of actin filaments in muscle and non-muscle cells. *Drosophila* knock-down mutants are known to suffer from muscle weakness and atrophy,[31] which may also be the case in *D. magna* exposed to microgravity. Furthermore, α-actinin was also found to be less abundant in human neuroblastoma cells exposed to simulated microgravity.[32] In contrast to these proteins, the actin-binding proteins advillin and gelsolin were more abundant in clinorotated *Daphnia*. Both proteins are members of the gelsolin/villin family, which are involved in the regulation of actin polymer organization[33] and are also able to sever actin filaments. Therefore, it can be stated that our findings on actin-related proteins provide strong evidence for a disruption of actin microfilament organization in *D. magna* exposed to simulated microgravity, at least in the time frame of 60-min exposure.

Alterations of the cytoskeleton as a result of different gravity conditions are a phenomenon described in many studies analyzing various mammalian cell lines either exposed to real or simulated microgravity.[14] In several studies, the disorganization of the actin cytoskeleton was observed in human monocytes[34] and human neuroblastoma cells[32] using immunohistochemistry and studying cell morphology. Furthermore, changes of cytoskeletal element gene expression and morphology in human cells were detected on a short time-scale after only 2 s in real microgravity during parabolic flights.[35,36] In addition, actin protein abundance decreased after 12 days of spaceflight in *Arabidopsis*.[37]

Table 2. EASE results for enrichment of GO biological process terms and molecular function terms (p.adjust < 0.05, *P* value was Benjamini corrected)

	GO term	*p.adjust*	*Ids* D. magna *database*
GO:0051082	F: unfolded protein binding	0.000000114	DAPHMAG3MTV3L10134T1; DAPHMAG3MTV3L10162T1; DAPHMAG3MTV3L11254T1; DAPHMAG3MTV3L4092T1; DAPHMAG3MTV3L4176T1; DAPHMAG3MTV3L7067T1; DAPHMAG3MTV3L7809T1; DAPHMAG3MTV3L9792T1
GO:0006457	P: protein folding	0.00000375	DAPHMAG3MTV3L10134T1; DAPHMAG3MTV3L10162T1; DAPHMAG3MTV3L11254T1; DAPHMAG3MTV3L4092T1; DAPHMAG3MTV3L4176T1; DAPHMAG3MTV3L7067T1; DAPHMAG3MTV3L7809T1; DAPHMAG3MTV3L9792T1
GO:0005737	C: cytoplasm	0.0000684	DAPHMAG3MTV3L10134T1; DAPHMAG3MTV3L10162T1; DAPHMAG3MTV3L11254T1; DAPHMAG3MTV3L11651T1; DAPHMAG3MTV3L4092T1; DAPHMAG3MTV3L6078T1; DAPHMAG3MTV3L7094T1; DAPHMAG3MTV3L9572T1; DAPHMAG3MTV3L9792T1
GO:0005524	F: ATP binding	0.0002	DAPHMAG3MTV3L10134T1; DAPHMAG3MTV3L10162T1; DAPHMAG3MTV3L10239T1; DAPHMAG3MTV3L10909T1; DAPHMAG3MTV3L11254T1; DAPHMAG3MTV3L2675T1; DAPHMAG3MTV3L4092T1; DAPHMAG3MTV3L4116T1; DAPHMAG3MTV3L4176T1; DAPHMAG3MTV3L5322T1; DAPHMAG3MTV3L6078T1; DAPHMAG3MTV3L7067T1; DAPHMAG3MTV3L7094T1; DAPHMAG3MTV3L733T1; DAPHMAG3MTV3L7770T1; DAPHMAG3MTV3L7809T1; DAPHMAG3MTV3L8231T1; DAPHMAG3MTV3L9572T1; DAPHMAG3MTV3L9792T1
GO:0003779	F: actin binding	0.0136	DAPHMAG3MTV3L1503T1; DAPHMAG3MTV3L2246T2; DAPHMAG3MTV3L2501T1; DAPHMAG3MTV3L5529T1; DAPHMAG3MTV3L733T1
GO:0006096	P: glycolysis	0.0477	DAPHMAG3MTV3L11111T1; DAPHMAG3MTV3L13427T1; DAPHMAG3MTV3L8855T1

Abbreviation: GO, gene ontology.

Table 3. Summary of significantly altered proteins involved in the response of *Daphnia* to altered gravity

Dmag*ID*	*Swissprot accession*	*Protein name*	*No. spots*	*Average ratio*
Actin binding				
daphmag3mtv3l7094t1	P07837	Actin	12	−0.44
daphmag3mtv3l5529t1	O75366	Advillin	7	6.98
daphmag3mtv3l2501t1	P18091	α-actinin	1	−4.6
daphmag3mtv3l1503t1	Q9VEN1	Filamin-A	2	−2.93
daphmag3mtv3l2246t2	Q27319	Gelsolin	2	4.61
daphmag3mtv3l733t1	P05661	Myosin	3	−3.3
Protein binding				
daphmag3mtv3l7770t1	P29844	Heat shock protein 70	4	1.33
daphmag3mtv3l7067t1	Q66HD0	Endoplasmin	2	3.61
daphmag3mtv3l4176t1	P02828	Heat shock protein 83	2	3.17
daphmag3mtv3l7809t1	Q5R511	Stress-70 protein	2	9.55
daphmag3mtv3l2732t1	Q12931	Heat shock protein 75	1	2.23
daphmag3mtv3l10134t1	Q6P502	T-complex protein 1	8	3.62
daphmag3mtv3l9835t1	P38657	Protein disulfide-isomerase	1	6.9
Energy metabolism				
daphmag3mtv3l11111t1	P15007	Enolase	3	5.62
daphmag3mtv3l13427t1	P91427	Phosphoglycerate kinase	1	4.08
daphmag3mtv3l8855t1	P07764	Fructose-bisphosphate aldolase	3	−2.48
daphmag3mtv3l4901t1	Q9XTL9	Glycogen phosphorylase	2	4.54
daphmag3mtv3l13753t1	Q5ZME2	Malate dehydrogenase	2	2.8
daphmag3mtv3l6730t1	P50137	Transketolase	1	7.42
daphmag3mtv3l592t1	Q66HF1	NADH-ubiquinone oxidoreductase	1	5.17
daphmag3mtv3l10909t1	Q05825	ATP synthase subunit β	1	4.87
Hemoglobin				
daphmag3mtv3l6920t1	BAJ72724	2-domain hemoglobin	7	1.31
daphmag3mtv3l16955t1	BAA76873	Hemoglobin	2	4.46

Sequence ID from *D. magna* protein database and corresponding blast hit in Swiss-Prot database are displayed in addition to total number of identified protein spots and average ratio of spot intensity (clinorotated/control).

Large-scale effects of microgravity on the muscular system of humans, mice, and rats are also well-studied,[38] leading to atrophy and reduced functional capacity of the muscles. However, the source and function of actin found in our study, either cytoplasmic or muscular, is hard to predict without further experiments. Röper *et al.*[39] showed that muscle-specific actin is incorporated into cytoplasmic structures, and cytoskeletal actin is incorporated into muscles for all actin paralogues of *Drosophila Melanogaster*. Therefore, it is not possible to deduce actin function solely from its protein sequence.

Chaperones are involved in the stress response of *Daphnia* to simulated microgravity

Another substantial fraction of protein spots were associated with protein binding, a GO term found overrepresented in the enrichment analysis (Figure 4). In this group, several molecular chaperones were identified with nearly all proteins being more abundant in the clinorotated treatment.

Heat shock proteins and other chaperones facilitate protein folding, unfolding, and transportation[40] playing an important role in both normal cellular homeostasis and stress response.[41] In *Daphnia*, they were found to be involved in responses to several stressors, e.g., temperature changes,[11] presence of a predator,[13,42] exposure to the drug diclofenac,[43] and exposure to copper.[44]

Activation of the heat shock system was also observed as response to microgravity in other systems, leading to increased gene expression or higher abundance of heat shock proteins. This was reported for plant cells exposed to simulated microgravity and microgravity during spaceflight,[45] for animal cells exposed to simulated microgravity[32] and also for *Drosophila* during spaceflight.[46] Our data clearly indicate that in *Daphnia*, the heat shock system possibly reacts to stress-dependent changes in cell, tissue, or organ structures caused by altered mechanical (gravitational) forces.[47] Therefore, proteins related to protein folding seem to be involved in the response to microgravity.[46]

In addition to heat shock proteins, we identified subunits of the chaperonin containing T-complex protein, also known as CCT, in several spots, which were all more abundant in the clinorotated treatment. CCT is known to contribute to the folding of a distinct subset of cellular proteins including cytoskeletal proteins like actin and myosin.[48] Moreover, protein disulfide-isomerase was more abundant in the clinorotated animals. This protein is an essential folding catalyst and chaperone located in the endoplasmatic reticulum, which introduces disulfide bonds into proteins and catalyses the rearrangement of incorrect disulfides.[49]

Therefore, the high abundance of proteins related to protein folding in *Daphnia* exposed to clinorotation is a strong indicator for a microgravity-induced breakdown of protein structures in general.

Clinorotation leads to an increased energy demand in *Daphnia*

Further molecular consequences of simulated microgravity on *Daphnia* is seen in the field of energy related proteins. Here, proteins associated with various energy pathways were altered in their abundance, most of them more abundant in the clinorotated treatment.

We found proteins involved in glycolysis (enolase, phosphoglycerate kinase, and fructose-bisphosphate aldolase), the TCA cycle (malate dehydrogenase), and in the respiratory chain (NADH-ubiquinone oxidoreductase and ATP synthase subunit β). Most of

these proteins were more abundant in the clinorotated treatment, indicating an enhanced energy metabolism in simulated microgravity. Furthermore, the enhanced abundance of glycogen phosphorylase is also an indicator of increased energy consumption in clinorotated animals, as it catalyses the degradation of glycogen to provide an increased amount of glucose.

In contrast to the other glycolytic proteins, fructose-bisphosphate aldolase was less abundant in clinorotated animals. However, this protein is also involved in processes apart from glycolysis, e.g., pentose phosphate pathway, and fructose and mannose metabolism, which may also be affected by the stressful condition of altered gravity. Similarly, transketolase, is also involved in the pentose phosphate pathway and showed only a slightly higher abundance in clinorotated *Daphnia*.

Interestingly, proteins related to energy metabolism were also found to be affected by microgravity in other systems. In *Arabidopsis thaliana* grown for 12 days on the ISS, a lower abundance of these proteins was observed.[37] In contrast, exposure to simulated microgravity for several hours using a clinostat led to an increased abundance of carbohydrate metabolism proteins in *Arabidopsis* callus[50] and root cells.[51] The differences that were found in the abovementioned studies might be based on different exposure times (short-term versus long term) or different exposure methods (simulated versus real microgravity), which may influence the response to microgravity.[17] However, indications for an increased energy metabolism found in our study correlated well with the similar clinorotation experiments in *A. thaliana*.[50,51] Changes in protein abundance related to energy metabolism were also observed in *Pseudomonas aeruginosa*, an opportunistic pathogen, when exposed to microgravity. Here, proteins related to glycolysis were of higher abundance in ground control treatments when compared with *P. aeruginosa* exposed to microgravity.[52] Furthermore, alterations in the expression of proteins related to energy metabolism were also reported in the bacterial pathogen *Salomonella* in spaceflight environment.[53,54]

Another indicator for an increased energy demand in *Daphnia* in our study is the altered abundance of hemoglobin. *Daphnia*, is known to increase its hemoglobin concentration in response to environmental hypoxia as well as to temperature increase.[55] Here, elevated water temperature has two effects, a decrease of oxygen partial pressure due to decreased oxygen solubility and an increase of metabolic rate in the ectotherm animal.

Daphnia hemoglobin genes are located in a tandem-duplicated gene cluster, which contributes to the varying composition of the protein. Both, the abundance of different Hb subunits, as well as their post translational modifications change in an oxygen-dependant manner, most probably optimizing oxygen affinity of hemoglobin according to oxygen concentration.[56] The same may be true for our study, explaining the occurrence of both, more and less abundant hemoglobin spots in the clinorotated treatment with pI and mass shifts.

As average hemoglobin abundance is higher in clinorotated animals, oxygen consumption may be increased. Therefore, hemoglobin abundance is most probably not primarily affected by microgravity, but clinorotation may lead to a stress response in *Daphnia*, resulting in higher energy demand and therefore increased oxygen consumption. As the amount of available oxygen in the cuvette is limited due to the experimental device, this increased oxygen consumption may lead to hypoxic conditions induced by the experimental device rather than microgravity. Animals in the control cuvette did not show an increase in hemoglobin abundance although oxygen concentration in the cuvette was similar, which indicates higher oxygen consumption of clinorotated animals. However, it cannot be excluded that the increased energy demand found in our study is related to altered swimming behavior of clinorotated *Daphnia*, as it is known that *Daphnia* in space shows an increased amount of looping/summersaulting movements.[16]

CONCLUSION

Using a proteomic approach, we were able to identify molecular key processes affected by clinorotation. Assuming that this simulation approach is suitable for *Daphnia*, which has to be verified in space, our results indicate impacts of microgravity on the parameters of investigations. We found strong indicators of actin cytoskeleton disruption and breakdown of protein structures in general and an increase of energy demands. These results are in agreement with results obtained from other organisms and microgravity systems. Interestingly, most of the proteins found to be affected are well-conserved throughout taxa.

Our proteomic approach led to interesting insights into the responses to altered gravity conditions. These results not only resemble important starting points for further *Daphnia* space research concerning life support systems but also increase the knowledge on the influence of gravity on biochemical processes. In addition, our data strongly suggest that a lack of gravity affects similar molecular processes in a variety of organisms.

ACKNOWLEDGMENTS

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CONTRIBUTIONS

CL, RH, and KS designed the study. BT performed clinorotation and proteomic experiments. BT and KAO analyzed proteomic data. KAO conducted further bioinformatical analysis. TF supervised mass spectrometry analysis. KAO and BT wrote the first draft of the manuscript, and CL, TF, RH, KS, and GJA contributed substantially to revisions. All authors read and approved the final manuscript.

DATA DEPOSITION

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium[57] via the PRIDE partner repository with the data set identifier PXD002096.

COMPETING INTERESTS

The authors declare no conflict of interest.

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