CHARLES UNIVERSITY
FIRST FACULTY OF MEDICINE
Institute of Immunology and Microbiology

Ph.D. study program: Parasitology

# Encystace a životní cyklus volně žijících améb rodu *Acanthamoeba* spp.

# Encystation and life cycle of free living amoebae of the genus *Acanthamoeba* spp.

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Summary of the Ph.D. Thesis

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

CONTENTS:

# 1 Abstrakt

Améby rodu *Acanthamoeba* jsou celosvětově rozšířená, volně žijící, fakultativně patogenní jednobuněčná eukaryota. Jejich nebezpečnost pro člověka spočívá ve schopnosti pronikat do organismu, odolat obranným mechanismům, množit se, poškozovat napadené tkáně a tím vyvolat onemocnění, proti kterým chybí kauzální léčba a která nejčastěji postihují oko a centrální nervovou soustavu (CNS). Jedním z hlavních důvodů často neúspěšné terapie je schopnost akantaméb vytvářet v postižených tkáních cysty, vysoce rezistentní klidová stádia. Kromě cyst, které akantaméby tvoří jak v přírodě, tak v infikovaných tkáních pod vlivem dlouhodobého stresu, se tyto organismy vlivem akutního ohrožení rychle transformují v méně odolná klidová stádia, pseudocysty. Předkládaná práce se zaměřuje na dosud neznámé aspekty odolnosti obou rezistentních stádií akantaméb, cyst a pseudocyst, a současně si klade za cíl popsat další charakteristiky odlišující cysty a pseudocysty a procesy spojené s jejich tvorbou a rezistencí.

Jedním ze studovaných aspektů odolnosti klidových stádií akantaméb byla přítomnost cukerného alkoholu manitolu a neredukujícího cukru trehalózy, sacharidů, které se v buňkách mnoha organismů účastní obranných reakcí vůči abiotickému stresu. Ačkoli v genomu *A. castellanii* jsou enzymy pro syntézu obou cukrů popsány, v buňkách akantaméb jsme prokázali pouze přítomnost disacharidu trehalózy a to jak v klidových stádiích, cystách a pseudocystách, tak v aktivně se množících trofozoitech. Manitol se nám nepodařilo prokázat u žádné ze studovaných životních forem. Podrobnější analýza sekvencí genů kódujících enzymy podílející se na syntéze trehalózy, trehalóza fosfát syntázy (TPS), trehalóza fosfát fosfatázy (TPP) a trehalóza syntázy (TS), odhalila, že v genomu se nachází enzymy dvou syntetických drah, z nichž jedna je prokaryontního původu. qRT-PCR pak prokázala rozdíly v míře exprese těchto genů v závislosti na dané životní formě, cysty vs. pseudocysty. V průběhu tvorby pseudocyst se na syntéze trehalózy podílí geny obou syntetických drah a v mnohem větší míře, než je tomu v průběhu encystace. K nárůstu množství trehalózy však dochází v průběhu obou ochranných reakcí. Trehalóza přetrvává, i když v menší míře, i v buňkách zralých cyst a pseudocyst, jak bylo doloženo pomocí hmotnostní spektrometrie.

Studovali jsme také vztah mezi encystací resp. tvorbou pseudocyst a buněčným cyklem resp. množstvím DNA. Pomocí průtokové cytometrie jsme prokázali, že u akantaméb lze jasně rozlišit dvě populace buněk s odlišným množstvím DNA, populaci v G1 fázi buněčného cyklu a populaci s již nově nasyntetizovanou DNA v G2 fázi. Zjistili jsme, že zatímco encystaci vždy předchází syntéza DNA, probíhá tedy z G2 fáze buněčného cyklu, tvorba pseudocyst je na pozici v buněčném cyklu nezávislá a může k ní dojít kdykoli během buněčného cyklu. Co je však pro obě rezistentní stádia totožné, je obsah DNA u zralých cyst a pseudocyst. Obě rezistentní stádia přečkávají dobu nepříznivých vnějších podmínek s G2 fázovým, obsahem DNA. Popsali jsme také účinek inhibitorů afidikolinu a hydroxyurey na růst a buněčný cyklus akantaméb. Zjistili jsme, že ani jedna ze studovaných látek nesynchronizuje akantaméby a nezastavuje je v množení na rozhraní G1/S fáze, jak je to popsáno u jiných protozoií a savčích buněk. Současně jsme pozorovali koncentračně závislý vliv hydroxyurey na míru množení trofozoitů akantaméb. Klasifikace a následná fylogenetická analýza hlavních regulátorů buněčného cyklu odhalila přítomnost celkem 14 genů 9 typů cyklinů a 6 genů 3 typů cyklin-dependentních kináz.

## 2 Abstract

Amoebae of the genus *Acanthamoeba* spp. are free-living unicellular organisms found in disparate ecosystems all over the world. Due to their ability to invade human body, evade its defensive mechanisms and cause extensive tissue damage, *Acanthamoeba* infection can lead to serious, if rare, diseases, affecting most commonly the eye and the central nervous system. Specific therapy for *Acanthamoeba* infections is not available.

A major reason for therapeutic failure in ameobiasis is the ability of the protist to differentiate into resistant stages. These are *cysts*, known to be formed under prolonged unfavorable conditions, both in the environment and the infected tissues, and the *pseudocysts*, less durable but rapidly formed under acute stress. The present thesis focuses on as yet unexplored mechanisms of resistance of cysts and pseudocysts. Moreover, further characteristics distinguishing cysts and pseudocysts as well as the processes involved in their formation are investigated.

One of the issues addressed is a presence of protective carbohydrate compounds mannitol and trehalose that participate in defensive reactions against abiotic stress in many organisms. Although putative genes for enzymes of the trehalose and mannitol synthetic pathways are present in the genome of *Acanthamoeba*, only one of the two compounds, disaccharide trehalose, was found. Trehalose was identified not only in cysts and pseudocysts but also in growing trophozoites. In contrast, none of the life stages were shown to contain mannitol. Detailed analysis of the sequences of the enzymes of the trehalose synthetic pathways, trehalose phosphate synthase (TPS), trehalose phosphate phosphatase (TPP) and trehalose synthase (TS), revealed that the genome contains enzymes belonging to two distinct enzymatic pathways, one being of a prokaryotic origin. Quantitative RT-PCR demonstrated a significant difference in expression profiles of the synthetic pathway genes in cyst and pseudocyst. Genes of both synthetic pathways are involved in trehalose synthesis during pseudocyst formation and at higher level than during encystation. Amounts of trehalose are nevertheless increased during both stress defense reactions. The presence of trehalose in mature cysts and pseudocysts was also demonstrated using mass spectrometry.

Furthermore, the relationship between encystation and pseudocyst formation and the progress of the cell cycle was also studied. Phylogenetic analysis and classification of the main cell cycle regulators in the *Acanthamoeba* genome revealed presence of 14 genes of 9 types of cyclins and 6 genes of 3 types of cyclin-dependent kinases.

By using flow cytometry analysis we clearly distinguished cell populations with distinct DNA content, G1 cell cycle population and population with newly synthetized DNA, G2 population. Our results strongly indicate that *A. castellanii* enters encystation from the G2 phase of the cell cycle. In contrast, initiation of differentiation into pseudocysts is independent of the progression of the cell cycle. Nevertheless, DNA content in mature cysts and pseudocysts is the same, both resistant stages survive harsh environmental conditions with G2 phase DNA content. We also described the effect of DNA synthesis inhibitors aphidicolin and hydroxyurea on *Acanthamoeba* growth and cell cycle. Our data revealed that neither of the studied compounds synchronized *Acanthamoeba* cell populations on the G1/S boundary, in contrast to what was described in other protists as well as mammalian cell lines. Along with this, we did observe concentration dependent impact of hydroxyurea on *Acanthamoeba* trophozoites growth rate.

## 3 Introduction

Free-living amoebae of the genus *Acanthamoeba* represent one of the most prevalent protists found in the environment. They are also causative agents of rare but serious

human diseases, granulomatous amoebic encephalitis and amoebic keratitis (Marciano-Cabral and Cabral 2003; Schuster and Visvesvara 2004). The life cycle of *Acanthamoeba* consists of two mononuclear stages: an active amoeba (a trophozoite) and a dormant cyst. *Acanthamoeba* cysts are formed upon exposure of trophozoites to long-lasting unfavorable environmental conditions, such as drought or starvation (Neff et al. 1964). As a cyst, *Acanthamoeba* cells can remain viable for years (Aksozek et al. 2002; Mazur et al. 1995). Kliescikova et al. (2011) found that under acute stress (induced in vitro by exposure of cells to organic solvents), trophozoites rapidly differentiate into pseudocysts, enabling acanthamoebae to survive life- threatening conditions. The most evident differences between *Acanthamoeba* cysts and pseudocysts are the rate of the cell response to stress and the structure and composition of the envelopes formed on the surface of these resting stages (a double-layered wall on the cyst or a single-layered fibrillar coat on the pseudocyst). So far very little is known about other differences between both dormant stages, especially on the molecular level, as well as mechanisms of their resistance.

An analysis of the genome of *A. castellanii* revealed many proteins that are putatively involved in the modulation of the cellular response to external cues (Clarke et al. 2013). In many eukaryotes, stress resistance is accompanied by the synthesis of protective compounds to alleviate the effects of anhydrobiosis, freezing, and osmotic pressure on macromolecular assemblies such as membranes. This role is often played by carbohydrates, trehalose or the sugar alcohol mannitol (Lourenço et al. 2016). Genes for mannitol-metabolizing enzymes, mannitol phosphate dehydrogenase and mannitol dehydrogenase as well as trehalose synthetic pathway, trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, identified in the genome of *A. castellanii* (Anderson et al. 2005; Clarke et al. 2013) led authors to suggestion that mannitol and/or trehalose may participate in stress tolerance also in acanthamoebae.

Cell differentiation is believed to be closely associated with cell cycle regulation. Before the onset of differentiation, cells usually exit the cell cycle from a specific point during the gap (G) phase. In mammalian cells, the exit point for differentiation usually occurs in the G1 phase, which is the longest phase of the cell cycle (Cooper, 2001). Cyst-forming protozoan parasites, e.g., the intestinal flagellate *Giardia intestinalis*, differentiate into cysts from the G2 phase, which is also the dominant and longest phase in the *Giardia* cell cycle (Reiner et al., 2008). Likewise, the sporulation, i.e., adaptive response of vegetative cells to unfavorable environmental conditions that allows the organism to survive, of the free-living amoebozoan protist *Dictyostelium* occurs from the G2 phase (Muramoto and Chubb, 2008). In the genus *Acanthamoeba*, which is a relative of *Dictyostelium*, the relationship between encystation and the cell cycle has not yet been fully determined. In some earlier works, encystation initiation was thought to occur from the G1 phase (Byers et al., 1969; Rudick, 1971), but later works suggested that it occurred from the G2 phase (Stöhr et al., 1987). Regarding the formation of pseudocysts, Kliescikova et al. (2011) proposed that this process is probably not connected to any specific phase of the *Acanthamoeba* cell cycle. This hypothesis is based on the observation that pseudocyst formation is a rapid response to immediately life-threatening conditions that require immediate action to protect the inner cell environment. In contrast, it can be expected that encystation, which is a slow response to long-term stressful environmental conditions that allow enough time for DNA synthesis, is initiated from a specific point in the cell cycle. However, neither data about DNA content during the formation of pseudocysts nor satisfactory evidence concerning the phase from which *Acanthamoeba* encysts have been presented so far.

## 4 Aims of the thesis

The main objectives were:

**I.** To identify genes of metabolic pathways involved in synthesis of protective sugars, trehalose and mannitol in *Acanthamoeba* genome and to perform the phylogenetic and functional analysis of the protein sequences.

**II.** To determine the role of trehalose and mannitol in *Acanthamoeba* protection, especially during encystation and pseudocyst formation and in the dormant stages cyst and pseudocyst.

**III.** To identify the point within the cell cycle from which *Acanthamoeba* enters encystation and pseudocyst formation and what is the DNA content of the mature cysts and the mature pseudocysts.

## 5 Summary and conclusions

This thesis summarizes results of three publications in peer-reviewed journals. These are focused on the role of mannitol in *Acantamoeba* resistant stages (I.) the role of trehalose in protection of encysting and pseudocysting *Acanthamoeba* cells as well as in cysts and pseudocysts (II.) and the cell cycle positions from which *Acanthamoeba* enters encystation and pseudocyst formation (III.)

I. In the first study we discussed the potential role of the sugar alcohol mannitol in *Acanthamoeba* protection. By using qRT-PCR, comparison has been made of mRNA levels of the enzymes for mannitol metabolism identified in *Acanthamoeba* genome, at various time intervals during encystation and pseudocyst formation. We observed gradual decrease of both enzymes during encystation and slight increases at the beginning of pseudocyst formation. By method of Mass spectrometry we detected no mannitol in any *Acanthamoeba* stage (trophozoite, pseudocyst, cyst). Detailed analysis of mRNA sequences of the two genes revealed similarities with various alcohol dehydrogenases rather than mannitol dehydrogenases. An explanation of this finding is a possible misinterpretation of previously published sequences. Our results simultaneously indicate that there is probably no protective role for mannitol in *Acanthamoeba* as no mannitol was detected in any *Acanthamoeba* life form.

II. The second article focused on the possible role of trehalose in *Acanthamoeba* protection. Our detailed phylogenetic analysis of the sequences for the enzymes of trehalose synthetic pathways revealed that *A. castellanii* contains genes for enzymes from two different pathways for trehalose synthesis, the prokaryotic trehalose synthase (TreS) and the widespread trehalose phosphate synthase – trehalose phosphate phosphatase (TPS-TPP)-based pathways and that, according to the bioinformatics data, these pathways are functional. By quantitative RT-PCR we demonstrated variable expression levels of the genes depending on the life stage, cyst vs. pseudocyst. We found out that genes of both synthetic pathways are most likely involved in trehalose synthesis during pseudocyst formation and at higher level, than during encystation. By using HILIC ESI Mass spectrometry we detected trehalose in all known forms of *Acanthamoeba,* i.e., the vegetative trophozoites and two stress-induced stages, pseudocysts and cysts and described the quantitative changes in the amount of trehalose during both stress defence reactions. Based on the results, we propose the presence of two functional pathways of trehalose synthesis in *Acanthamoeba* which could mean that

trehalose should be used by *Acanthamoeba* as a cell protectant especially in the presence of stress environmental conditions. Moreover, our results clearly support the view that encystation and pseudocyst formation, represent fundamentally different processes.

III. In the third paper we analysed by using flow cytometry DNA content of exponentially growing *Acanthamoeba* cell population as well as the changes in DNA content during *Acanthamoeba* encystation and pseudocyst formation. Our data conclusively showed a bimodal DNA distribution in asynchronous *Acanthamoeba* populations and revealed that *Acanthamoeba* enters encystation from the G2 phase of its cell cycle, whereas it differentiates into pseudocysts from the G1 and G2 phases of the cell cycle. The DNA content of both mature cysts and pseudocysts is, however, the same, indicating that the cells survive unfavourable environmental conditions with replicated genomes. We described the effect of DNA synthesis inhibitors aphidicolin and hydroxyurea on *Acanthamoeba* growth and its cell cycle. Our data revealed that no one of the studied compounds synchronized *Acanthamoeba* cell populations on the G1/S boundary, as it is described in mammalian cell lines and other protists. Together with this we observed concentration dependent impact of hydroxyurea on *Acanthamoeba* trophozoites growth rate. We also performed a phylogenetic analysis and classification of the main cell cycle regulators, namely, cyclin-dependent kinases and cyclins that are found in the genome of *A. castellanii*. The analysis revealed presence of 14 genes of 9 types of cyclins and 6 genes of 3 types of cyclin-dependent kinases. By using the modern methods of cell sorting and analysis as well as phylogenetic data we partially fill the knowledge gap regarding *Acanthamoeba* cell cycle progression and differentiation.

## 6 List of publications (including abstracts)

**I. Bínová E**, Klieščiková **J**, Ashford D, Thomas-Oates J, Nohýnková E (2012). Mannitol is not involved in protective reactions of *Acanthamoeba*. Molecular and Biochemical Parasitology 184:118-121.

Impact factor: 2.158 (2018)

**Abstract.** Genes for mannitol-metabolizing enzymes, mannitol phosphate dehydrogenase (MPDH) and mannitol dehydrogenase (MDH), have been recently identified in the genome of *Acanthamoeba castellanii* and their potential role in stress tolerance was proposed. Using qRT-PCR, comparison has been made of mRNA levels of the enzymes for mannitol metabolism at various time intervals during the stress defense reactions of encystation and pseudocyst formation. Gradual decrease of both enzymes during encystation and slight increases at the beginning of pseudocyst formation were observed. Detailed analysis of mRNA sequences of the two genes revealed similarities with various alcohol dehydrogenases rather than mannitol dehydrogenases. Our results indicate there is probably no protective role for mannitol in *Acanthamoeba* as no mannitol was detected using HILIC ESI MS, in any *Acanthamoeba* life cycle stage. Possible misinterpretation of previously published sequences as encoding enzymes of the mannitol metabolic pathway is discussed.

**II. Bínová E**, Bína D, Ashford DA, Thomas-Oates J, Nohýnková E (2017). Trehalose during two stress responses in *Acanthamoeba*: Differentiation between encystation and pseudocyst formation. Protist 168:649-662.

Impact factor: 3.000 (2018)

**Abstract.** The non-reducing disaccharide trehalose can serve as a protectant against a range of environmental stressors, such as heat, cold, or dehydration, in both prokaryotes and eukaryotes, with the exception of vertebrates. Here, we analyzed trehalose metabolism in the facultatively parasitic organism *Acanthamoeba castellanii*, known to respond to unfavorable external conditions by forming two resistant stages: a cyst, produced in the case of chronic stress, and a pseudocyst, formed in reaction to acute stress. The possible role of trehalose in the resistant stages was investigated using a combination of bioinformatic, molecular biological and biochemical approaches. Genes for enzymes from a widespread trehalose-6-synthase-trehalose-6-phosphate phosphatase (TPS-TPP) pathway and a prokaryotic trehalose synthase (TreS) pathway were identified. The expression patterns of the genes during encystation and pseudocyst formation were analyzed and correlated with the time course of cellular trehalose content determined mass spectrometrically. The data clearly demonstrate fundamental differences between encystation and pseudocyst formation at the level of cellular metabolism.

**III. Bínová E**, Bína D, Nohýnková E (2020). DNA content in *Acanthamoeba* during two stress defense reactions: Encystation, pseudocyst formation and cell cycle. European Journal of Protistology doi: 10.1016/j.ejop.2020.125745.

Impact factor: 2.395 (2019)

**Abstract.** During environmental stress, the vegetative cells of the facultative pathogenic amoeba *Acanthamoeba castellanii* reversibly differentiate into resistant dormant stages, namely, cysts or pseudocysts. The type of resistant stage depends on the nature and duration of the stressor. Cell differentiation is accompanied by changes in morphology and cellular metabolism. Moreover, cell differentiation is also expected to be closely linked to the regulation of the cell cycle and, thus, to cellular DNA content. While the existence of the resistant stages in *A. castellanii* is well known, there is no consensus regarding the relationship between differentiation and cell cycle progression. In the present work, we used flow cytometry analysis to explore the changes in the DNA content during *Acanthamoeba* encystation and pseudocyst formation. Our results strongly indicate that *A. castellanii* enters encystation from the G2 phase of the cell cycle. In contrast, differentiation into pseudocysts can begin in the G1 and G2 phases. In addition, we present a phylogenetic analysis and classification of the main cell cycle regulators, namely, cyclin-dependent kinases and cyclins that are found in the genome of *A. castellanii*.

## 7 Grant support

- Grant Agency of the Czech Republic, grant no. 310/09/1120 (principal investigator: Eva Nohýnková)

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- Support of the Charles University in Prague, institutional projects PROGRES, number Q26/LF1 and grants No. SVV 260 369, SVV-2012-264506

## 8 Curriculum vitae

**Education**

2016 - present Ph.D. study in Parasitology
The First Faculty of Medicine, Institute of Immunology and Microbiology, Charles University, Prague
Ph.D. Thesis: Encystation and life cycle of free living amoebae of the genus *Acanthamoeba* spp.

2008 - 2016 Ph.D. study in Parasitology
Department of Infectious and Tropical Diseases, The First Faculty of Medicine, Charles University and Na Bulovce Hospital, Prague
Ph.D. Thesis: Encystation and life cycle of free living amoebae of the genus *Acanthamoeba* spp.

2004 - 2007 MSc. in Parasitology
Department of Parasitology, Faculty of Science, University of South Bohemia in České Budějovice
Supervisor: Prof. RNDr. Tomáš Scholz, CSc.
Diploma Thesis: Biologie a výskyt larválních stádií motolice obrovské (*Fascioloides magna*) v České Republice

**Stays abroad**

2011 Department of Molecular Microbiology, School of Medicine, Washington University in St. Louis, USA
Laboratory of Professor Jennifer Lodge
Duration: 3 months

2010 Department of Chemistry, University of York, York, UK
Laboratory of Professor Jane Thomas-Oates
Duration: 2 weeks

**Conference presentations**

2012 **X. české a slovenské parazitologické dny**, Brno, 28. 5. – 1.6. 2012
oral presentation: Úloha trehalózy v ochraně vnitřního prostředí akantaméby během její diferenciace (E. Bínová, J. Klieščiková, D. Ashford, J. Thomas-Oates, E. Nohýnková)

2011 **14.th International Meeting on The Biology and Pathogenicity of Free-Living Amoebae**, Montego Bay, Jamaica, 10. – 15.10. 2011
oral presentation: Role of Trehalose in *Acanthamoeba* protection (E. Bínová, J. Klieščiková, D. Ashford, J. Thomas-Oates, E. Nohýnková

2011 **12. Studentská vědecká konference**, Praha, 24.5. 2011
oral presentation: Changes in DNA content during *Acantamoeba* stress defence reactions (E. Bínová, E. Nohýnková)

2010 **11th International Workshops on Opportunistic Protists (IWOP-11)**, Hilo, Hawaii, USA, 1.-5.8. 2010
oral presentation: Propylene glycol induces pseudocyst formation in *Acanthamoeba* spp. (J. Klieščiková, E. Horáčková, E. Nohýnková)

2010 **40. Jírovcovy protozoologické dny**, Ledeč nad Sázavou-Kouty, 3.-7.5. 2010
oral presentation: Buněčný cyklus a encystace akantaméb (E. Horáčková, E. Nohýnková)

2009 **10. Studentská vědecká konference**, Praha, 27.5. 2009
oral presentation: Trehalóza a manitol jako možné protektanty akantaméb (E. Horáčková, E. Nohýnková)

2009 **13th International Meeting of Free-Living Amoebae**, Puerto de la Cruz, Tenerife, Spain, 17.-21.5. 2009
poster: Tracing possible involvement of trehalose and mannitol in protection of *Acanthamoeba* (E. Horáčková, J. Klieščiková, E. Nohýnková)

2009 **39. Jírovcovy protozoologické dny**, Hradec nad Moravicí, 4.- 8. 5.2009
poster: Tracing possible involvement of trehalose and mannitol in protection of *Acanthamoeba* (E. Horáčková, J. Klieščiková, E. Nohýnková)

**Work experience**

2020 – present University of South Bohemia, Faculty of Science
researcher

2019 – present Waldorfská škola České Budějovice o. p. s.
biology teacher in high school

2018 – present Centrum ekologické a globální výchovy Cassiopeia, České Budějovice
lecturer, regional coordinator of the global project Ekoškola and ŠUŽ

2014 – 2017 maternity leave

## 9 Full list of publications

**Bínová E,** Bína D, Nohýnková E (2020). DNA content in *Acanthamoeba* during two stress defense reactions: Encystation, pseudocyst formation and cell cycle. European Journal of Protistology doi: 10.1016/j.ejop.2020.125745.

**Bínová E**, Bína D, Ashford DA, Thomas-Oates J, Nohýnková E (2017). Trehalose during two stress responses in *Acanthamoeba*: Differentiation between encystation and pseudocyst formation. Protist 168:649-662

**Bínová E**, Klieščiková J, Ashford D, Thomas-Oates J, Nohýnková E (2012). Mannitol is not involved in protective reactions of *Acanthamoeba*. Molecular and Biochemical Parasitology 184:118-121

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**Kliescikova J, Kulda J, Nohynkova E** Stress-induced pseudocyst formationnewly identified mechanism of protection against organic solvents in acanthamoebae of the T4 genotype. Protist 2011 162:58-69

**Lourenço TF, Barros PM, Saibo NJM, Abreu IA, Santos AP, António C, Pereira JS, Oliveira MM** Genomics of Drought. In: Edwards D., Batley J. (eds) Plant Genomics and Climate Change. Springer, New York, NY. 2016 Pp85-135

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