Vol. 26: 49–56, 2017
https://doi.org/10.3354/ab00673

AQUATIC BIOLOGY
Aquat Biol

Published May 15

Effects of rapamycin on life span and on expression of *TOR* and *S6K* in *Brachionus calyciflorus* (Rotifera)

Huanhuan Xu, Lulu Liu, Yuqi Su, Ye Liang, Jiaxin Yang*

School of Life Sciences, Nanjing Normal University, Nanjing, Jiangsu 210023, PR China

ABSTRACT: The mechanistic target of rapamycin (mTOR) coordinates a complex signal pathway from translation to autophagy that is a key regulator of not only growth and proliferation but also metabolism and aging. mTOR is sensitive to many environmental and endocrine stimuli. We investigated the influence of TOR signaling on aging and reproduction of the rotifer *Brachionus calyciflorus* using rapamycin as an exogenous inhibitor. We found that 2 and 4 µM rapamycin extended *B. calyciflorus* life span by 15 and 22%, respectively compared with controls ($p < 0.05$). The reproductive peak was significantly delayed by rapamycin at 2 and 4 µM ($p < 0.01$), but the pre- and post-reproduction periods were not significantly different from controls ($p > 0.05$). Partial cDNAs coding 375 bp for *TOR* and 951 bp for S6 kinase (*S6K*) were obtained from *B. calyciflorus* expressed sequence tags. The identities of the deduced amino acid sequences of *B. calyciflorus* cDNAs to their human orthologs were 58% for *TOR* and 68% for *S6K*. *TOR* and *S6K* mRNA expression were up- or down-regulated by different rapamycin concentrations (0.5, 1, 2, 4, 8, and 16 µM) and treatment intervals (control, 12, 24, 36, and 48 h). The results indicated that TOR inhibition acted additively to extend rotifer life span, with up- and down-regulation simultaneously impacting reproduction and gene expression.

KEY WORDS: Aging · Life span · mRNA expression levels · Rapamycin · Reproduction · Rotifer

INTRODUCTION

Rotifers have long been used as a model organism in the study of aging (Enesco 1993). Tractable model systems have been developed to investigate responses to genetic and environmental interventions relevant to human aging and longevity extension (de Magalhães et al. 2012). Aging is influenced by the expression of hundreds of genes, their interactions with environmental variables, and cellular regulation (Wilkinson et al. 2012). Understanding how the environment modulates aging gene expression, especially the effects of diet, dietary supplements, lifestyle, heavy metals, and drugs, is a major challenge (López-Otin et al. 2013). The intent of ongoing research is to extend healthy life by identifying key aging mechanisms that enable some organisms to resist a variety of environmental stresses, and selectively manipulate signaling pathways to slow aging.

Aging research seeks to understand how changes in gene expression that can extend life are likely to benefit from stress reduction (Snell et al. 2014). Comparing gene expression in closely related species with differences in aging will increase our understanding of molecular targets of intervention (Snell 2014). Nutrient sensing pathways, such as those involving *IGF*, *TOR*, *SIRT*, and *FOXO* genes, are involved in the regulation of aging (Haigis & Sinclair 2010, Katewa & Kapahi 2011).

Rapamycin was discovered because of its antifungal activity, but was soon found to have immunosuppressant activity as an inhibitor of T-cell proliferation

*Corresponding author: yangjx@njnu.edu.cn

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(Martel et al. 1977, Dumont et al. 1990). Since it was first shown to extend the longevity of wild-type mice in 2009, many studies have confirmed the antiaging effect of rapamycin (Arriola Apelo & Lamming 2016). The target of rapamycin (TOR) pathway responds to nutrient availability, energy status, and physiological stress and matches growth conditions to stimulation of anabolic and suppression of catabolic pathways in insects, flies, *Caenorhabditis elegans*, and mice (Thoreen et al. 2009, Bjedov et al. 2010, Gibbens et al. 2011, Gu et al. 2012, Mirth & Shingleton 2012). The kinase mTOR (mechanistic target of rapamycin) integrates growth-factor signaling with nutrient availability and directly regulates protein translation needed for cell growth and proliferation (Schmelzle & Hall 2000, Chiang & Abraham 2007). Rapamycin also induces G1/S cell cycle arrest, autophagy, and inhibits initiation of translation (Takeuchi et al. 2005).

mTOR is a serine/threonine protein kinase member of the PI3K-related kinase (PIKK) family (Laplante & Sabatini 2012), and it complexes with other proteins to form mTORC1 and mTORC2, which differ in structure and function. mTORC1 stimulates protein and lipid synthesis, ribosome biogenesis, energy metabolism, and lysosome biogenesis (Foster 2013), whereas the mTORC2 pathway in adipocytes for the body is very important for the energy balance (Kumar et al. 2010). mTORC1 is known to be strongly inhibited by rapamycin (Kim et al. 2002, Sarbassov et al. 2004), but the sensitivity of mTORC2 to rapamycin varies among cell lines and tissue types. In some tissues (e.g. thymus, kidney, and stomach) mTORC2 is known to be completely resistant to rapamycin (Sarbassov et al. 2006, Schreiber et al. 2015). The differential sensitivity of each mTOR complex to rapamycin is of great relevance for aging research (Arriola Apelo & Lamming 2016). mTORC1 targets p70 S6 kinase (S6K) and eIF4E-binding protein-1 (4E-BP1), which regulate translation of mRNA, and can be blocked using rapamycin as a pharmacological probe (Teleman 2010, Magnuson et al. 2012). Rapamycin acts through a gain-of-function mechanism in which it binds to intracellular protein to generate a drug-receptor complex that then binds and inhibits the kinase activity of mTORC1 (Jacinto et al. 2004).

Rapamycin robustly affects the average and maximum life-span of genetically heterogeneous mice (Harrison et al. 2009). The regulatory importance of TORC1 was indicated by a report that mutation of the *daf-15* gene coding for the regulatory associated protein of mTOR (RAPTOR) also extends life span (Jia et al. 2004), as does knockdown of an intestinal peptide transporter (*pep-2*) thought to act upstream of TORC1 in *C. elegans* (Meissner et al. 2004). It has been reported that dominant negative alleles of TOR or S6K, as homologs of TSC1 and TSC2, act as negative regulators of TORC1 and increase longevity in flies (Kapahi et al. 2004). The role of TOR as an important aging pathway has been firmly established in 3 invertebrate model systems: a budding yeast *Saccharomyces cerevisiae*, the nematode *C. elegans*, and the fruit fly *Drosophila melanogaster* (Arriola Apelo & Lamming 2016). In these organisms, a reduction in TORC1 activity can significantly increase longevity.

As the antiaging mechanisms of rapamycin have not been widely investigated in aquatic invertebrates, we evaluated this signaling pathway inhibitor for its ability to extend longevity in rotifers. A recent study by Snell et al. (2014) found that exposure of the marine rotifer *B. manjavacas* to a rapamycin inhibitor extended the mean life span by 35% and the maximum life span by 37% at a 1 µM concentration. RNAi knockdown of *TOR* gene expression resulted in a 29% extension of mean life span (Snell et al. 2014). Real-time RNA (RT-RNA) was used to determine the specific roles of the *TOR* and *S6K* genes in life-span regulation. Microscope observation was used to determine the life cycle. We also observed that rapamycin treatment altered rotifer reproductive patterns. The study objective was to increase our understanding of aging regulation by TOR signaling in a rotifer model system.

MATERIALS AND METHODS

Rotifers

Brachionus calyciflorus, the species of rotifer used in this study, was collected in Gainesville, FL, USA in 1983 (Snell et al. 1991) and has since been continuously cultured with periodic collection and storage of resting eggs. *B. calyciflorus* used in the experimental procedures were female neonates (0 to 2 h old) hatched from resting eggs in artificial freshwater (EPA medium, pH ~7.8) composed of 96 mg $NaHCO_3$, 60 mg $CaSO_4{\cdot}H_2O$, 123 mg $MgSO_4$, and 4 mg KCl in 1 l deionized water at 25°C (ASTM International 2001). A 16:8 h light:dark photoperiod and constant fluorescent illumination at 2000 lux were used during the experimental procedures. Females were fed the green alga *Chlorella pyrenoidosa* (~3.0×10^6 cells ml^{-1}). *C. pyrenoidosa* (Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan) was cultured in Bold's Basal Medium at 25°C

in a 5 l bag under constant fluorescent illumination at 2000 lux.

Experimental treatment protocol

Rapamycin (Sigma-Aldrich) was added at final concentrations of 0.5, 1, 2, 8, and 16 µM to 1 ml cultures of *B.calyciflorus* in 24 well plates. Controls were treated with 0.16% dimethyl sulfoxide (DMSO) in EPA medium. Six replicates were prepared for each life span treatment. The rotifer cultures were checked every 12 h to record the number of neonates and remove them until all the experimental animals had died. Cultures were monitored every 2 h to determine pre-reproduction (PR), reproduction (RP) and post-reproduction (PP) intervals (in h). Six replicates were prepared for each treatment to determine its life cycle. Rotifers were transferred to fresh culture medium containing feeding alga every 24 h. Rapamycin was stored in 10 mM stock solutions by dissolving in DMSO and was then diluted with EPA medium to the test concentrations. The percentage of DMSO in treatment solutions was 0.16%. The highest DMSO concentration without significant effect on rotifer reproduction was previously determined to be 0.3% (Snell et al. 2014). The pretest DMSO concentration (<0.3%) had no significant effect on *B. calyciflorus* reproduction (data not shown).

Rotifer culture for quantitative real-time PCR

The effects of rapamycin on the expression of *TOR* and *S6K* in *B. calyciflorus* were tested in triplicate in cultures of 100 rotifers in 50 ml EPA medium at concentrations of 0.5, 1, 2, 4, 8 and 16 µM. The controls were treated with DMSO only in EPA medium. Treatment effects were measured at 12, 24, 36, and 48 h (relative to controls at 0 h) in replicate groups of 100 individuals for each treatment. Rotifers were transferred to new medium every 24 h and checked every 12 h to record the number of neonates. Neonates were removed each day. Rotifers were collected and total RNA was extracted.

RT-RNA isolation

Total rotifer RNA was isolated using Trizol reagent (Invitrogen) following the manufacturer's instructions. RNA concentration was determined by measuring the absorbance at OD_{260} and RNA integrity was checked by electrophoresis. Aliquots of total RNA were reverse-transcribed into cDNA using an aMMLV RTase cDNA Synthesis Kit (TaKaRa).

Table 1. Specific primers used in the experiment

Primers	Sequence (5′–3′)
TOR-RT-F	CGAACCTGAAACTGAGAAGAAAGA
TOR-RT-R	CGTAATTGTAGGAGCACATTGTCT
S6K-RT-F	AGTTGATTGGTGGTCTTTTG
S6K-RT-R	ATCCTTAGCGTCGTTTGTT
β actin -RT-F	GAAATTGTGCGCGACATCAAGGA
β actin -RT-R	GCAATGCCCGGGTACATGGTGGT

RT-PCR assay of relative gene transcription

Total RNA was extracted from 100 rotifers as described above for each rapamycin concentration and treatment time. *TOR* and *S6K* mRNA expression was assayed by RT-PCR using Mastercycler ep realplex system (Eppendorf). After RT-PCR, we determined whether the dissolution curve was normal and whether there was a single peak. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). The TOR and S6K primers that were used are shown in Table 1. Primers that were designed for the gene fragments of the *B. calyciflorus* transcriptome are shown in Table 2. β-actin primers were used to amplify a 308 bp fragment of β actin (GenBank accession no. JX441322) as an internal control. All partial sequences from organisms were obtained using the NCBI-BLAST search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To verify that each primer hybridized specifically to the target sequence, PCR products were separated on 0.2% agarose gels and single bands was visualized by UV exposure. We used a CyClone cloning kit to construct pMD19-T vectors from the PCR products and sequenced them to determine the applicant identity.

Table 2. cDNA gene fragments from the *Brachionus calyciflorus* rotifer transcriptome. *TOR*: target of rapamycin; *S6K*: p70S6 kinase

Clone	Accession number	Completeness	Size (bp)	Identity (similarity) to human ortholog (%)
TOR	KX119430	Partial	375	58 (100)
S6K	KX119431	Partial	951	68 (84)

Statistical analysis

Statistical analyses were carried out using the SPSS 16.0 analytic package. Results of life span experiments were tested by survival analysis; effects of rapamycin on reproduction were tested by 1-way ANOVA and *t*-tests. Differences in mRNA expression were tested for significance by 1-way ANOVA. Differences were considered significant at $p < 0.05$. All figures were drawn using Sigma Plot version 11.0 (SYSTAT Software).

RESULTS

Effect of rapamycin on rotifer life span and reproduction

The average life spans of rotifers treated with 2 µM (180.01 ± 5.51 h) and 4 µM (190.11 ± 2.41 h) rapamycin were significantly longer than those of controls (156.01 ± 1.21 h; $p = 0.025$ and 0.017, respectively) (Table 3). However, the average life spans of rotifers treated with 8 and 16 µM rapamycin were significantly shorter ($p < 0.05$) than those of controls (Table 3). Means (±SE) of the PR, RP and PP in *Brachionus calyciflorus* exposed to different concentrations of rapamycin are shown in Table 3. PR of rotifers treated with 0.5 or 1 µM rapamycin were not significantly different from controls, but were significantly longer in those treated with 2 or 4 µM (both $p < 0.001$) than in controls. RP and PP were both significantly shorter in rotifers treated with 8 and 16 µM rapamycin than in controls ($p < 0.001$). DMSO alone had no obvious effect on life span and reproductive period in the control cultures (data not shown). Fig. 1 shows the fecundity of *B.calyciflorus* rotifers in controls and treatment groups. The average number of offspring produced by individual rotifers treated with 0.5, 1, 2, and 4 µM rapamycin was not significantly different from results for untreated controls. At 8 and 16 µM, rapamycin caused significant reductions in the number of offspring compared with controls (13.60 ± 3.50, $p < 0.001$).

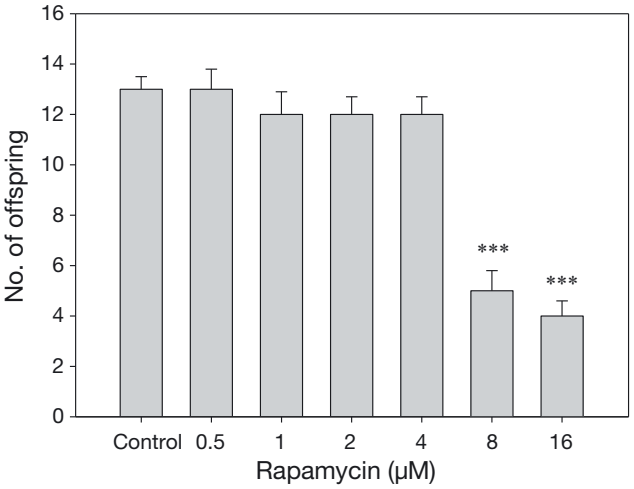

Fig. 1. Number of offspring (mean ± SE) produced by each *Brachionus calyciflorus* after rapamycin treatment compared with controls. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (1-way ANOVA with Dunnett's test).

Effect of rapamycin on *TOR* mRNA expression

The effect of rapamycin on *TOR* mRNA expression was assayed at 0.5, 1, 2, 4, 8, and 16 µM (relative to the controls) over 48 h. A low concentration (0.5 µM) of rapamycin induced a 1.7-fold ($p < 0.01$) increase in *TOR* gene expression, but expression was significantly suppressed at higher concentrations (2, 4, 8, and 16 µM) at 12 h (Fig. 2A). *TOR* expression was significantly increased at 24 h (Fig. 2B; $p < 0.05$) and 36 h (Fig. 2C; $p < 0.05$) exposure at each concentration, but expression levels were lower at 36 h than at 24 h. With 16 µM rapamycin, *TOR* expression increased rapidly and reached a maximum level (20-fold increase, $p < 0.001$) at 24 h exposure (Fig. 2B). Expression levels declined at 48 h exposure to rapamycin at 0.5, 1, 2, and 4 µM (Fig. 2D), but *TOR* expression continued to increase with 8 and 16 µM rapamycin (Fig. 2D; $p < 0.05$). DMSO had no obvious effect on *TOR* expression (data not shown).

Table 3. Duration (mean ± SE) of principal developmental stages and life span of *Brachionus calyciflorus* exposed to different concentrations of rapamycin. PR: pre-reproduction; RP: reproduction; PP: post-reproduction. Letters indicate significant differences from control (1-way ANOVA with *t*-test): *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Conc. (µM)	Life span (h)	PR (h)	RP (h)	PP (h)
0	156.01 ± 1.21	22.69 ± 0.51	104.24 ± 2.81	30.07 ± 1.11
0.5	155.21 ± 3.11	22.61 ± 0.53	103.98 ± 1.52	28.89 ± 2.12
1	156.81 ± 3.57	23.31 ± 0.52	103.84 ± 2.80	29.66 ± 1.83
2	180.01 ± 5.51***	46.75 ± 0.71***	104.87 ± 0.11	29.18 ± 2.03
4	190.11 ± 2.41***	56.85 ± 0.50***	104.21 ± 0.30	29.05 ± 1.10
8	103.12 ± 4.72***	20.08 ± 0.30***	67.65 ± 0.25***	20.02 ± 1.91***
16	67.23 ± 1.65***	18.23 ± 1.50***	29.01 ± 1.21***	20.16 ± 1.34***

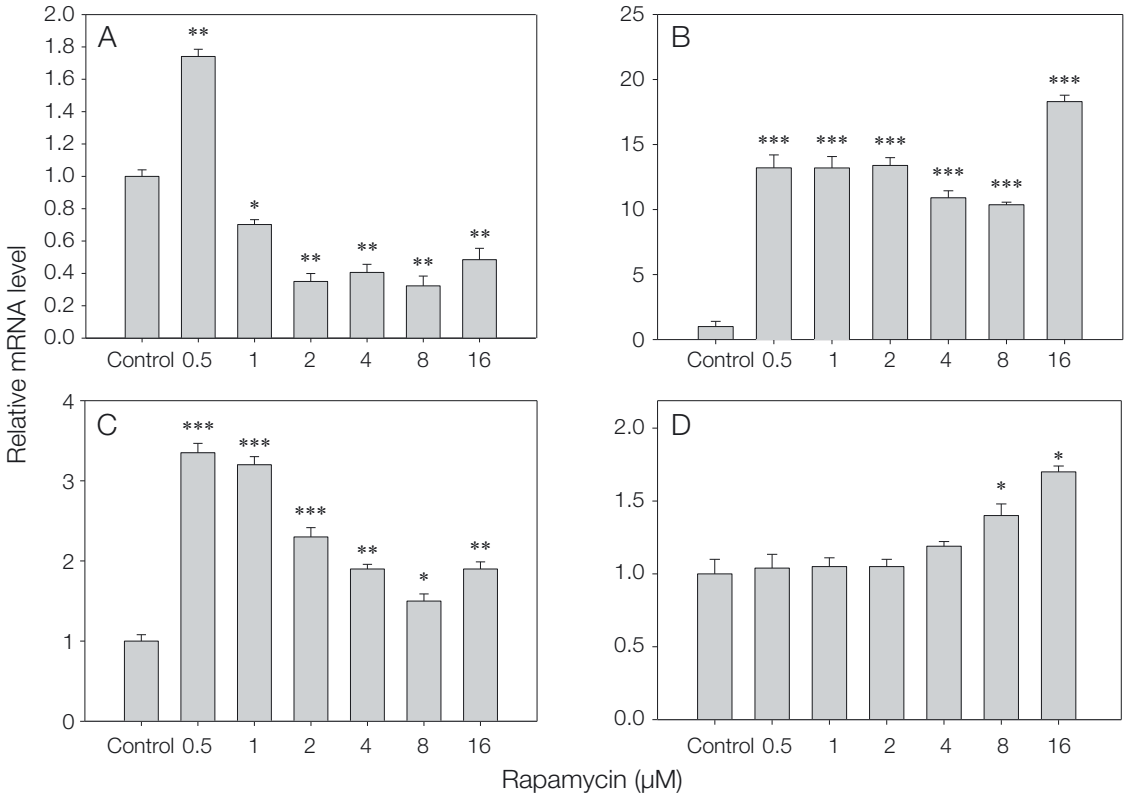

Fig. 2. Rapamycin effect on *TOR* mRNA expression levels (mean ± SE) in *Brachionus calyciflorus* after (A) 12 h, (B) 24 h, (C) 36 h, and (D) 48 h. *TOR* mRNA levels were assayed by RT-PCR. *p < 0.05, **p < 0.01, ***p < 0.001 (1-way ANOVA with Dunnett's test)

Effect of rapamycin on *S6K* mRNA expression

Fig. 3 shows the effect of rapamycin on *S6K* mRNA expression at the same rapamycin concentrations used for the TOR assays. At 12 h, 0.5, 4, and 8 μM rapamycin significantly increased *S6K* mRNA expression, but expression was significantly suppressed by 2 μM and 16 μM rapamycin (Fig. 3A; $p < 0.01$). RT-PCR demonstrated that rapamycin also increased *S6K* mRNA expression at 24 h (Fig. 3B; $p < 0.01$) and 36 h (Fig. 3C; $p < 0.001$), and the expression level returned to normal with exposure beyond 48 h (Fig. 3D; $p > 0.05$). DMSO treatment had no obvious effect on *S6K* expression (data not shown).

DISCUSSION

mTOR controls cellular growth and gene translation, and is upregulated in mammalian cancers (Zoncu et al. 2011, Laplante & Sabatini 2012, Alayev & Holz 2013, Cornu et al. 2013).

Brachionus calyciflorus has a small body size (0.3 mm) and short life span (5 to 7 d), making it suitable for ecotoxicological and aging studies (Snell & Janssen 1995, Snell & Joaquim-Justo 2007, Snell et al. 2014). *TOR* is linked to invertebrate aging, and rapamycin is a specific inhibitor of *TOR* expression (Sarbassov et al. 2006, Fontana et al. 2010). Rotifers treated with 2 and 4 μM rapamycin had 15 and 22% longer life span than controls, respectively, without significant effects on female reproduction (Table 3). This study is consistent with a previous report that rapamycin (1 μM) extended mean *B. manjavacas* life span by 35% (Snell et al. 2014). *B. calyciflorus* life span was shortened and PP and reproductive patterns were changed by treatment with 8 or 16 μM rapamycin, but the specific cause is not clear. Only the reproductive period timing changed, and PP was significantly delayed with 2 and 4 μM rapamycin (Table 3). Rapamycin thus resulted in a significant extension of the life span of *B. calyciflorus*. A similar study in *B. manjavacas* reported that rapamycin (1 μM) extended life span by 19% and shifted peak reproduction from Day 4 in controls to Day 6. Spindler et al. (2012) found that everolimus, a rapamycin derivative, increased the longevity of male *Drosophila* by 17%. Bjedov et al. (2010) also demonstrated that rapamycin treat-

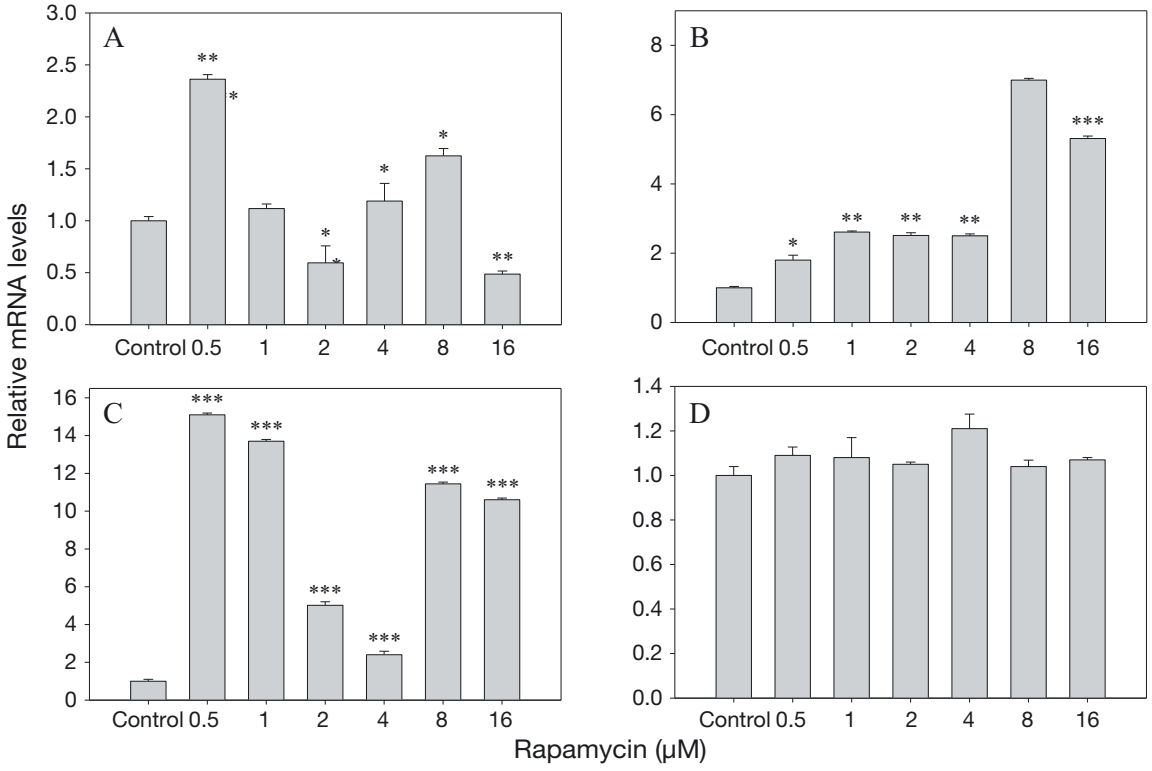

Fig. 3. Rapamycin effect on *S6K* mRNA expression levels (mean ± SE) in *Brachionus calyciflorus* after (A) 12 h, (B) 24 h, (C) 36 h, and (D) 48 h. mRNA levels were assayed by RT-PCR. Bars represent standard errors. *p < 0.05, **p < 0.01, ***p < 0.001 (1-way ANOVA with Dunnett's test)

ment mimicked the extension of longevity seen in some adult *Drosophila melanogaster TOR* mutants.

TOR (Fig. 2) and *S6K* (Fig. 3) mRNA expression were initially increased by rapamycin, declined, and then returned to normal levels with time. A surprising discovery was that the *TOR* expression levels reached a maximum 20-fold increase ($p < 0.001$) and *S6K* reached a 15-fold increase ($p < 0.001$). Rapamycin inhibition of p70S6K and cell cycle activity is closely associated with many different key cellular processes, but in a cell-free system, a rapamycin-PKBP complex did not inhibit p70S6K activity, which showed that there was no direct interaction between rapamycin and p70S6K (Hirose et al. 2014).

Perhaps relative mRNA expression levels reflect stress-induced adaptation mediated by rapamycin. We speculate that the corresponding changes in expression had an impact on the TORC1 targets, p70 S6 kinase (S6K) and eIF4E-binding protein-1 4E-BP1, which regulate translation of mRNA. Inhibition of TORC1 signaling from 24 to 72 h after amputation in a zebrafish fin regeneration model suppressed cell proliferation through the inhibition of S6K activation (Hirose et al. 2014). Thus, we speculate that it suppressed cell proliferation through the inhibition of S6K activation in *B. calyciflorus.*

Mutations that inhibit TOR function extend life span in various invertebrates including flies (Kapahi et al. 2004) and nematodes (Vellai et al. 2003, Jia et al. 2004). Inhibition of TOR activity also extends longevity in yeast (Kaeberlein et al. 2005, Powers et al. 2006). We propose that rapamycin extended *B. calyciflorus* longevity via a mechanism that affected TOR activity. Snell et al. (2014) reported a significant increase in the number of lysosomes after rapamycin treatment, suggesting an up-regulation of autophagy that potentially contributes to life extension. However, in *Carcinus maenas,* molting had no effect on *TOR* and *S6K* gene expression, suggesting that TOR signaling was not regulated by transcriptional mechanisms (Abuhagr et al. 2014). We also hypothesize that in *B. calyciflorus*, the mechanism of life prolongation involves regulation of autophagy.

Another environmental variable (as temperature, reactive oxygen species, pH) is known to influence rotifer reproduction and life span (Kauler & Enesco 2011). Expression of genes expressing CuZn SOD, Mn SOD, and CAT increases with temperature stress

(Yang et al. 2013). The resulting reduction of reactive oxygen species would otherwise result in the accumulation of irreversible cell damage and affect the parameters of rotifer life history (Houthoofd et al. 2004). Dietary supplement combinations, including antioxidants, are also able to extend rotifer life span (Snell et al. 2012), and dietary restriction can also extend life span and reduce fecundity of *B. calyciflorus* (Yang et al. 2014). Aging in rotifers is thus regulated by a variety of factors. As rapamycin (1 µM) can also modulate ecdysteroid secretion in *C. maenas* and *Gecarcinus lateralis* and in Y-organs *in vitro*, ecdysteroid activity may also require TOR-dependent protein synthesis (Abuhagr et al. 2014).

CONCLUSIONS

In this study, we investigated the impact of rapamycin on life span and molecular characteristics of rotifers. Rapamycin extended the average life-span and impacted reproductive patterns of *Brachionus calyciflorus*. It also had significant effects on *TOR* and *S6K* expression. Rapamycin intervention might impact aging by altering gene expression and reproduction. The TOR pathway might also interact with other pathways to influence reproduction and longevity. Determining the cause of rapamycin effects on TOR in rotifers requires additional study, as does an understanding of downstream signaling of the TOR pathway proteins. While several potential candidates (e.g. PP2A-related phosphatases and Tap42p) have been associated with the effects of rapamycin, the link between these proteins and downstream targets remains unclear. Some TOR proteins have been proposed to be involved in environmental adaption but few TOR binding partners have been identified, and their roles remain to be elucidated.

Acknowledgements. This work was supported by the National Natural Science Foundation of China (No. 31272388) to J.X.Y. and Three Term Project of Aquaculture of Jiangsu Province, China (No. Y2014-32).

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Editorial responsibility: Roderick Finn, Bergen, Norway

Submitted: January 12, 2017; Accepted: March 16, 2017
Proofs received from author(s): April 29, 2017