Using the Experimental Evolution of Long-Lived Yeast Species for Testing Evolutionary Theories of Aging

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ABSTRACT

Using the Experimental Evolution of Long-Lived Yeast Species for Testing Evolutionary Theories of Aging

Anastasia Glebov, M.Sc.

Aging of multicellular and unicellular eukaryotic organisms is a highly complex biological phenomenon that has various causes and affects numerous processes within cells. As a model organism for elucidating the basic biology and molecular mechanisms of cellular aging in multicellular eukaryotes, we use the baker's yeast *Saccharomyces cerevisiae*. The employment of this budding yeast as an advantageous model organism in aging research during the past decade has convincingly demonstrated that longevity signaling pathways and mechanisms of their modulation by dietary and pharmacological interventions are conserved across phyla.

Recently, we designed a chemical genetic screen for small molecules that increase the chronological lifespan (CLS) of yeast under caloric restriction (CR) conditions by targeting lipid metabolism and modulating housekeeping longevity pathways that regulate longevity irrespective of the number of available calories. Our high-throughput screen identified a bile acid called lithocholic acid (LCA) as one of such molecules. The results of our pharmacophore modeling of the anti-aging potential of various species of bile acids imply that the maintenance of the minimal polarity of both the hydrophilic (concave) and hydrophobic (convex) sides of the steroid nucleus - by avoiding the

presence of polar substituents at the positions 6, 7 and 12 - is mandatory for the extreme life-extending efficacy of LCA under CR conditions. Such stringent structural requirements are consistent with a target specificity of LCA action as an anti-aging small molecule. We found that the life-extending efficacy of LCA under CR exceeds that under non-CR conditions, being inversely proportional to the concentration of glucose in growth medium and thus in correlation with the extent of calorie supply limitation.

Yeast do not synthesize LCA or any other bile acids produced by mammals. Therefore, we propose that bile acids released into the environment by mammals may act as interspecies chemical signals providing longevity benefits to yeast and, perhaps, other species within an ecosystem. We hypothesize that, because bile acids are known to be mildly toxic compounds, they may create selective pressure for the evolution of yeast species that can respond to the bile acids-induced mild cellular damage by developing the most efficient stress protective mechanisms. It is likely that such mechanisms may provide effective protection of yeast against molecular and cellular damage accumulated with age. Thus, we propose that yeast species that have been selected for the most effective mechanisms providing protection against bile acids may evolve the most effective anti-aging mechanisms that are sensitive to regulation by bile acids. We analyzed how small anti-aging molecules other than LCA (including resveratrol, caffeine and rapamycin) synthesized and released into the environment by one species of the organisms composing an ecosystem extend longevity of many other species within this ecosystem. Based on such analysis, we extended our initial hypothesis on how bile acids govern longevity regulation and drive longevity evolution by suggesting a unified

hypothesis of the xenohormetic, hormetic and cytostatic selective forces that impel the evolution of longevity regulation mechanisms at the ecosystemic level.

To test the validity of our hypothesis, we carried out the LCA-driven experimental evolution of long-lived yeast species. Our serial-batch-transfer type of selection yielded three laboratory-evolved yeast strains with greatly extended lifespan. All these strains were able to maintain their prolonged lifespan following storage at -80^{o}C and multiple successive passages in medium without LCA. We demonstrated that in the absence of LCA a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species extend longevity and alter the age-dependent dynamics of mitochondrial respiration under both CR and non-CR conditions. We therefore concluded that, consistent with its sought-after effect on longevity regulation pathways, our LCA-driven multistep selection process under laboratory conditions yielded long-lived yeast species whose greatly delayed chronological aging was caused by the selection of a mutant allele or alleles that activate housekeeping longevity assurance pathways. As we recently demonstrated, these housekeeping longevity assurance pathways modulate longevity irrespective of the number of available calories and do not overlap with the adaptable longevity pathways that are under the stringent control of calorie availability [167].

We also revealed that under CR conditions in the absence of LCA a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species: 1) enhance the resistance of yeast to chronic oxidative, thermal and osmotic

stresses; 2) suppress mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide; and 3) attenuate lipid-induced necrotic cell death induced by exogenously added palmitoleic acid. All these processes are known to be governed by the housekeeping longevity assurance pathways that modulate longevity irrespective of the number of available calories [167].

Moreover, the experiments described in this Master's thesis revealed that the addition of LCA to the laboratory-selected long-lived yeast species cultured under CR conditions 1) further extends their CLS, although to a lesser degree than that of wild-type (WT) strain; 2) causes further changes in the age-dependent dynamics of mitochondrial respiration, although not as dramatic as those seen in WT; 3) further enhances the resistance of yeast to chronic oxidative, thermal and osmotic stresses, although to a lesser extent than those observed in WT; 4) further suppresses mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide, although to a lesser degree than those seen in WT; and 5) further attenuates lipid-induced necrotic cell death induced by exogenously added palmitoleic acid, although to a lesser extent than those observed in WT. These findings imply that, although a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species greatly impact a compendium of the LCA-sensing housekeeping longevity assurance processes, this allele or alleles do not activate such processes sufficiently enough to attain the maximal CLS achievable under life-extending CR conditions in the presence of LCA.

Altogether, these findings provide the comprehensive evidence of our hypothesis in which the evolution of longevity regulation mechanisms within an ecosystem can be driven by the xenohormetic, hormetic and cytostatic selective forces created by a lasting exposure of an organism to an anti-aging natural compound released by other organisms composing this ecosystem.

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a lesser extent than those observed in WT 99

List of Abbreviations

AMPK/TOR, AMP-activated protein kinase/target of rapamycin; cAMP/PKA, cAMP/protein kinase A; CFU, colony forming units; CLS, chronological lifespan; CR, caloric restriction; DHR, dihydrorhodamine 123; DR, dietary restriction; ER, endoplasmic reticulum; FA-CoA, CoA esters of fatty acids; FFA, free fatty acids; FoxO, Forkhead box type O; IGF-1, insulin/insulin-like growth factor 1; LBs, lipid bodies; LCA, lithocholic acid; ROS, reactive oxygen species; RLS, replicative lifespan; SD, standard deviation; TORC1, TOR complex 1; TAG, triacylglycerols; WAT, white adipose tissue.

1 Introduction

1.1 Longevity is controlled by a signaling network that integrates three signaling pathways governing numerous longevity-defining cellular processes

Aging of multicellular and unicellular eukaryotic organisms is a highly complex biological phenomenon, which affects numerous processes within cells. These cellular processes include cell cycle, cell growth, stress response, protein folding, apoptosis, autophagy, proteasomal protein degradation, actin organization, signal transduction, nuclear DNA replication, chromatin assembly and maintenance, ribosome biogenesis and translation, lipid and carbohydrate metabolism, oxidative metabolism in mitochondria, NAD^+ homeostasis, amino acid biosynthesis and degradation, and ammonium and amino acid uptake [1 - 36]. Only three nutrient- and energy-sensing signaling pathways have been shown to modulate the functional states of all these numerous longevity-defining processes and their spatiotemporal organization. These signaling pathways are conserved across phyla and include the AMP-activated protein kinase/target of rapamycin (AMPK/TOR), cAMP/protein kinase A (cAMP/PKA) and insulin/insulin-like growth factor 1 (IGF-1) pathways (Figure 1.1) [4, 10, 25 – 27, 30, 35].

By operating as an intracellular sensor for the nutrient and energy status, the AMPK/TOR pathway regulates longevity in yeast, worms, fruit flies and mammals (Figure 1.1) [37 - 42]. Being activated in response to the high AMP:ATP ratio characteristic of a low level of nutrients or energy within a cell, AMPK phosphorylates and inhibits TOR protein

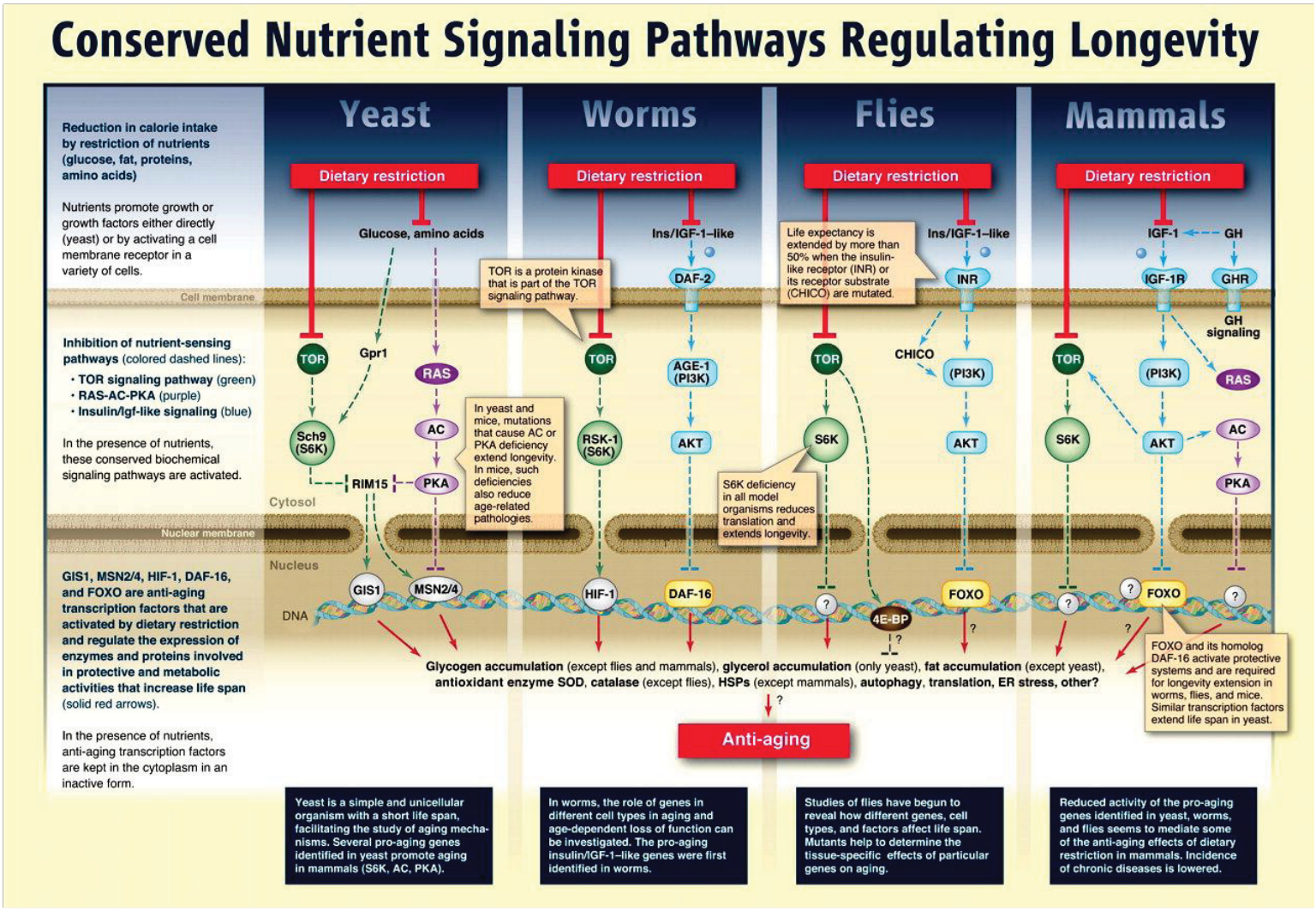

Figure 1.1. The functional states of numerous longevity-defining processes and their spatiotemporal organization are modulated by only a few nutrient- and energy-sensing signaling pathways that are conserved across phyla and include the AMP-activated protein kinase/target of rapamycin (AMPK/TOR), cAMP/protein kinase A (cAMP/PKA) and insulin/insulin-like growth factor 1 (IGF-1) pathways (see text in section 1.2 for details). Reproduced from: Fontana, L., Partridge, L. and Longo, V.D. (2010). Extending healthy lifespan - from yeast to humans. *Science* 328:321-326.

kinase associated with several other proteins into the TOR complex 1 (TORC1) [37 - 42]. The resulting inhibition of TORC1 triggers global changes in cell physiology by 1) slowing down the processes of cellular growth, ribosome biogenesis and protein translation, all of which are extremely energy-consuming; 2) causing a switch from cap-dependent to cap-independent translation, thereby initiating a distinct pro-longevity

translational program; 3) stimulating the removal of damaged macromolecules and organelles by activating nutrient- and energy-producing autophagy; 4) accelerating mitochondrial synthesis of proteins encoded by mitochondrial genes for core subunits of the oxidative phosphorylation protein machinery; and 5) causing global changes in mitochondrial gene expression pattern [37, 43 - 45]. Noteworthy, TORC1 can be also inhibited in an AMPK-independent manner, in response to low intracellular levels of amino acids [39, 41, 46 - 49]. Moreover, if AMPK is phosphorylated by the LKB1 protein kinase under low nutrient or energy conditions within a cell, it can extend the lifespan of worms in a TORC1-independent fashion by slowing down hydrolysis of the neutral lipids triacylglycerols (TAG) [50].

The cAMP/PKA pathway has been shown to regulate longevity in mice and yeast (Figure 1.1). In mice, this signaling pathway accelerates aging if activated by cAMP produced by AC5, an isoform of adenylate cyclase that is highly abundant in the heart and brain [51]. By attenuating AMP/PKA signaling in mice, lack of AC5 reduces the inhibitory effect of PKA on the Raf/MEK/ERK protein kinase cascade [51]. The resulting activation of the protein kinase ERK extends longevity and delays age-related degenerative processes in the heart and bone, likely by elevating the level of the reactive oxygen species (ROS) scavenging enzyme manganese superoxide dismutase, increasing the resistance of mice to oxidative stress and attenuating apoptotic cell death [51]. Recent findings that lack of either a catalytic subunit of PKA or its regulatory subunit in mice extends lifespan and improves health by attenuating age-related pathologies provided a convincing

confirmation for the essential role for cAMP/PKA in regulating longevity and modulating physiological processes underlying healthy aging [52 - 53].

In yeast, glucose deprivation attenuates the cAMP/PKA pathway by inhibiting such activators of adenylate cyclase Cyr1p as the G protein-coupled receptor Gpr1p, Gα protein Gpa2p and two small GTP-binding proteins, Ras1p and Ras2p (Figure 1.1) [54, 55]. The attenuation of cAMP/PKA signaling in glucose-deprived yeast cells impairs the ability of cytosolic PKA to phosphorylate the following three proteins in the cytosol: 1) the protein kinase Rim15p (thereby restoring the kinase activity of Rim15p inhibited by PKA-dependent phosphorylation); and 2) the stress response transcription factors Msn2p and Msn4p (thereby enabling the import of their unphosphorylated forms into the nucleus) [56 - 58]. Following their import into the nucleus, the unphosphorylated forms of Msn2p and Msn4p collaborate with a nuclear pool of unphosphorylated Rim15p in activating transcription of numerous pro-longevity genes that govern carbohydrate metabolism, the tricarboxylic acid cycle, trehalose biosynthesis, proteolysis, stress protection, ROS detoxification and cell growth regulation [59 - 62]. The resulting establishment of such global pro-longevity transcriptional pattern ultimately extends both the chronological and replicative lifespans of yeast [56, 63, 64]. Noteworthy, similar to the Raf/MEK/ERK signaling cascade in mice [51], its homologous Ste11p/Ste7p/Kss1p protein kinase cascade in yeast is attenuated by PKA [65]. Therefore, if the Ste11p/Ste7p/Kss1p protein kinase cascade is active due to reduced PKA signaling, it promotes longevity of chronologically aging yeast [65]. Such a pro-longevity effect of the Ste11p/Ste7p/Kss1p cascade in yeast under conditions of attenuated cAMP/PKA

signaling is synergistic with the pro-longevity action of the nuclear forms of Msn2p, Msn4p and Rim15p not subjected to phosphorylation by cytosolic PKA (see above) [65].

Unlike the AMPK/TOR and cAMP/PKA signaling pathways that regulate longevity by acting as sensors of the nutrient and energy status of an individual eukaryotic cell, the insulin/IGF-1 signaling pathway regulates longevity in worms, fruit flies and mice by responding to an endocrine signal that monitors the nutritional status of the whole organism (Figure 1.1) [66]. The insulin/IGF-1 pathway responds to an insulin-like ligand by stimulating a phosphatidylinositol-3-kinase, which then activates a cascade of protein kinases that ultimately cause phosphorylation of a Forkhead box type O (FoxO) family transcription factor and its sequestration in the cytosol [67, 68]. Reduced signaling through the insulin/IGF-1 pathway inhibits phosphorylation of a FoxO transcription factor (DAF-16 in worms, dFOXO in fruit flies and FoxO1 in mice), thereby permitting its import into the nucleus [69]. After being imported into the nucleus, the FoxO transcription factor orchestrates the establishment of a global life-extending transcriptional pattern by activating or repressing transcription of numerous longevity-defining genes involved in metabolism, detoxification, apoptosis, stress response, transcription, translation, signaling and development [70, 71].

By sharing several protein kinases and adaptor proteins, the AMPK/TOR, cAMP/PKA and insulin/IGF-1 signaling pathways converge into a network that regulates longevity in yeast, worms, fruit flies and mammals [4, 35 - 38]. In addition to the well-known individual protein components of the AMPK/TOR, cAMP/PKA and insulin/IGF-1

pathways, this network includes several proteins that currently are not viewed as being in any of these signaling pathways; among these proteins are 1) Rtg2p in yeast; 2) CLK-1, ISP-1, JNK, MST-1, PHA-4, Rictor/TORC2/SGK-1, SIR-2.1, SKN-1 and SMK-1 in worms; 3) dSir2 and JNK in fruit flies; and 4) MCLK, $P66^{Shc}$, SIRT1, SIRT6, and SIRT7 in mammals [4, 36, 72 - 74]. Importantly, this longevity regulation network responds to the age-related partial mitochondrial dysfunction and to fluctuations in the intracellular concentration of mitochondrially produced ROS [36, 37, 73, 75]. By sensing the nutritional status of the whole organism as well as the intracellular nutrient and energy status, functional state of mitochondria, and concentration of ROS produced in mitochondria, the longevity network regulates lifespan across species by coordinating information flow along its convergent, divergent and multiply branched signaling pathways.

1.2 Some dietary regimens can extend longevity and improve health

By altering the organismal and intracellular nutrient and energy status, nutrient intake plays an important role in defining both lifespan and healthspan [7, 76 - 78]. The dietary regimens known as caloric restriction (CR) and dietary restriction (DR) are known not only to exhibit a robust longevity-extending effect in evolutionarily distant organisms ranging from yeast to rhesus monkeys, but also to improve health by attenuating age-related pathologies and delaying the onset of age-related diseases across phyla (Table 1.1) [76 - 83]. A CR diet reduces the intake of calories without compromising the supply of amino acids, vitamins and other nutrients [77 - 80]. In contrast, a DR dietary regimen

reduces the intake of nutrients (but not necessarily of calories) by limiting food supply without causing malnutrition [76, 81 - 83].

Table 1.1. Caloric restriction (CR) and dietary restriction (DR) exhibit a robust longevity-extending effect in evolutionarily distant organisms ranging from yeast to rhesus monkeys and improve health by attenuating age-related pathologies and delaying the onset of age-related diseases across phyla. Adapted from Fontana, L., Partridge, L. and Longo, V.D. (2010). Extending healthy lifespan - from yeast to humans. *Science* 328:321-326 with modifications.

Organism	**Life-span increase by CR or DR**	**Health improvement by CR or DR**
Yeast	3-fold (chronological lifespan)	Extended reproductive period
Worms	2- to 3-fold	Resistance to misexpressed toxic proteins
Fruit flies	2-fold	None reported
Mice	30-50%	Protection against cancer, diabetes, atherosclerosis, cardiomyopathy, autoimmune, kidney and respiratory diseases; reduced neurodegeneration
Rhesus monkeys	Tend noted	Prevention of obesity; protection against cancer, diabetes and cardiovascular disease
Humans	Not tested	Prevention of obesity, diabetes, hypertension; reduced risk factors for cancer and cardiovascular disease

In a "TOR-centric" model of longevity regulation, AMPK/TOR is the only signaling pathway that underlies all life-extending and health-improving effects of CR and DR [84 - 89]. According to this model, TORC1 - a core protein complex governing the

AMPK/TOR pathway - performs three functions that are pivotal to the beneficial effects of CR and DR on longevity and healthspan. First, TORC1 integrates the flow of information on the organismal and intracellular nutrient and energy status from AMPK (a protein kinase in the AMPK/TOR pathway), PKA (a protein kinase in the cAMP/PKA pathway), PKB/AKT (a protein kinase in the insulin/IGF-1 pathway), ERK1/2 (a protein kinase in the Raf/MEK/ERK signaling cascade) and $P66^{Shc}$ (a mitochondria-confined sensor of the redox status within this organelle) [84 - 86]. Second, TORC1 operates (independently of AMPK) as a sensor of the intracellular levels of amino acids [84 - 86]. Third, by gathering and processing the information on the organismal and intracellular nutrient, energy and redox status and amino acid availability, TORC1 governs numerous longevity-related processes independently of sirtuins [84 - 86]. The validity of the TOR-centric model for TORC1-orchestrated modulation of the life-extending effects of CR and DR has been confirmed in worms and replicatively aging yeast. In fact, DR is unable to extend longevity of worms with reduced TOR signaling [88, 89]. Furthermore, lack of key components of the TOR signaling pathway eliminates the beneficial effect of CR on the replicative lifespan of yeast [87].

Although in replicatively aging yeast the TOR pathway alone mediates the longevity benefit associated with CR, the life-extending effect of this dietary regimen in chronologically aging yeast relies on a signaling network in which the protein kinase Rim15p operates as a nutritional integrator of the TOR and cAMP/PKA pathways; it seems that this network also includes some other, yet to be identified signaling pathways that are not converged on Rim15p [35]. Furthermore, because the AMPK/TOR,

cAMP/PKA and insulin/IGF-1 pathways in worms, fruit flies and mammals are known to converge into a complex network regulating their longevity (see above) [4, 7, 36 - 38], it is likely that these three divergent and multiply branched signaling pathways equally contribute to the life-extending effects of CR and DR in these organisms [4, 90 - 99]. The emerging concept of a signaling network (rather than the AMPK/TOR pathway alone) that mediates the longevity benefit associated with CR and DR by coordinating information flow along its convergent, divergent and multiply branched signaling pathways has been recently supported by studies in worms. Specifically, it has been demonstrated that the life-extending efficacies of different DR regimens in these organisms depend on both independent and overlapping signaling pathways [100].

1.3 Some pharmacological interventions can delay aging and attenuate age-related pathologies

CR and DR are not the only interventions known to extend longevity in evolutionarily distant organisms. Aging can be slowed down, health improved, age-related pathologies attenuated and the onset of age-related diseases delayed also by certain small molecules, many of which exhibit the beneficial effects on longevity and healthspan in organisms across phyla (Table 1.2). Many of these longevity- and healthspan-extending pharmacological interventions target the AMPK/TOR signaling pathway known to modulate longevity in response to the intracellular nutrient and energy status. Metformin, a type 2 diabetes therapeutics that activates AMPK, extends longevity in worms and mice by reducing TORC1 signaling (Table 1.2) [107, 108]. Methionine sulfoximine, a glutamine synthetase inhibitor that attenuates TORC1 signaling by decreasing the

intracellular concentration of the TORC1 activator glutamine, extends chronological lifespan in yeast (Table 1.2) [110, 111]. LY294002 and U0126, which lower TORC1 signaling by inhibiting upstream TORC1 activators PI3K and MEK (respectively), increase the replicative lifespan of cultured human fibrosarcoma cells (Table 1.2) [136]. By acting through FKBP12 to inhibit TORC1, the macrocyclic lactone rapamycin has been shown to 1) extend longevity in fruit flies and mice [113, 116]; 2) increase the replicative lifespans of cultured rodent fibroblasts, human epithelium cells and human fibrosarcoma cells [117]; and 3) prolong the replicative and chronological lifespans of yeast (Table 1.2) [56, 110, 114]. Moreover, the xanthine alkaloid caffeine has been demonstrated to increase chronological lifespan in yeast by reducing the catalytic activity of Tor1p (Table 1.2) [101].

Table 1.2. Known anti-aging compounds, their abilities to increase lifespan in different organisms (under caloric or dietary restriction [CR or DR, respectively], on a standard diet or fed a high-calorie diet), and the mechanisms of their anti-aging action.

Compound	**Increases lifespan***		**Mechanism**
	CR/DR	**Standard or high-calorie diet**	
Caffeine	Not tested (NT)	+ (yeast, CLS) [101]	By inhibiting TORC1, modulates unspecified longevity-related processes [101] governed by the TOR pathway [37, 102]
Li^+	+ (nematodes**) [103]	+ (nematodes) [103]	By altering transcription of genes involved in histone methylation, nucleosome composition and chromatin structure, modulates unspecified longevity-related

			processes [103] known to be influenced by age-related chromatin reorganization [104]
Lipoic acid, propyl gallate, trolox and taxifolin	NT	+ (nematodes [105] (all); fruit flies [106] (lipoic acid))	Antioxidants that may increase lifespan by detoxifying free radicals and/or enhancing resistance to age-related oxidative stress [105, 106]
Metformin, buformin and phenformin	- (nematodes[**]) [107]	+ (nematodes [107]; mice [108])	Type 2 diabetes therapeutics that - by activating LKB1/AMPK signaling and thereby inhibiting TORC1 [38, 109] - modulate unspecified longevity-related processes [107, 108] known to be governed by the TOR pathway [37, 38, 102]
Methionine sulfoximine	NT	+ (yeast, CLS) [110]	By inhibiting glutamine synthetase and reducing both intracellular glutamine level and TORC1-signaling [111], increases lifespan - perhaps by activating gluconeogenesis and enhancing stress resistance [110]
Mianserin	- (nematodes) [112]	+ (nematodes) [112]	A serotonin receptor antagonist used as an antidepressant in humans; may increase lifespan by inhibiting neurotransmission related to food sensing, thereby mimicking a DR-like physiological state [112][***]

Rapamycin	+ (fruit flies) [113]	+ (yeast, CLS [101, 110, 114] and RLS [115]; fruit flies [113]; mice [116]; rodent fibroblasts, human epithelium and fibrosarcoma cells - all RLS [110])	By inhibiting TORC1 (yeast and fruit flies) [101, 102, 113, 114] and mTORC1 (mammals) [38, 102, 116], increases lifespan by activating macroautophagy (yeast and fruit flies) [113, 118] and inhibiting cap-dependent protein translation (fruit flies and mice) [113, 116] as well as - perhaps - by promoting gluconeogenesis (yeast) [110], enhancing stress resistance (yeast) [110, 115], and increasing neutral lipid levels (fruit flies) [113]
Resveratrol	- (yeast, RLS [119]; fruit flies [120]; mice [121])	+ (yeast, RLS [119] but not CLS [119]; nematodes [120]; fruit flies [120]; fishes [122]; mice [121, 124]; human fibroblasts, RLS [123])****	Increases life span by modulating a number of longevity-related processes (*e.g.*, by altering transcription of numerous genes involved in key longevity pathways, stimulating p53 deacetylation, increasing insulin sensitivity and mitochondrial number, reducing IGF-1 levels, activating AMPK and PGC-1α, promoting ER stress response, repressing transcription of PPAR-γ, inhibiting adipocyte differentiation, accelerating storage fat mobilization,

			inhibiting mTORC1, and activating autophagy [119, 121, 123 - 131]; its life-extending ability in yeast, nematodes and fruit flies depends on Sir2p - a member of the conserved sirtuin family of NAD^+-dependent protein deacetylases/mono-ADP-ribosyltransferases [119, 120, 125]*****
SkQ1	NT	+ (fungi, daphnias, fruit flies, mice) [132]	By being specifically targeted to mitochondria, acts as an antioxidant that may increase lifespan by preventing oxidative damage to proteins and lipids (*i.e.*, cardiolipin), altering mitochondrial morphology, reducing hydrogen peroxide-induced apoptosis and necrosis, and/or slowing down the age-related phosphorylation of histone H2AX [132]
Sodium nitroprusside	NT	+ (human PBMC,RLS) [133]	By activating expression of the human sirtuin SIRT1 and thereby increasing the extent of SIRT1-dependent histone H4 lysine 16 deacetylation, may cause the development of an anti-aging pattern of transcription of numerous genes involved in longevity

			regulation [133]
Spermidine	NT	+ (yeast, CLS; nematodes; fruit flies; human PBMC, RLS) [134]	By inhibiting histone acetyltransferases and promoting histone H3 deacetylation, increases lifespan by activating transcription of numerous autophagy-related genes; the resulting induction of autophagy suppresses age-related necrotic cell death [134]
Valproic acid	NT	+ (nematodes) [135]	Is used as a mood stabilizer and an anticonvulsant in humans; may increase lifespan by promoting nuclear localization of the DAF-16 forkhead transcription factor, thereby reducing the pro-aging effect of the insulin/IGF-1 signaling pathway [135]
LY294002	NT	+ (human fibrosarcoma cells, RLS) [136]	An inhibitor of phosphatidylinositol-3-kinase that – by reducing mTORC1 signaling [37, 102] – modulates unspecified longevity-related processes [136] known to be governed by the TOR pathway [37, 38, 102]
U0126	NT	+ (human fibrosarcoma cells, RLS) [136]	An inhibitor of the protein kinase MEK that – by reducing mTORC1 signaling [37, 102] – modulates unspecified longevity-related processes [136] known to be governed

			by the TOR pathway [37, 38, 102]

* Mean, median and/or maximum lifespans.

** Nematodes carrying mutations that mimic DR under non-DR conditions [103, 105].

*** The ability of mianserin to increase nematode lifespan can only be seen in liquid media [112], whereas in solid media the compound reduces lifespan [137].

**** Increases the replicative lifespan of yeast grown under non-CR conditions [119] only in one out of four different yeast strain backgrounds [138]; one group has been unable to reproduce the lifespan extension by resveratrol in nematodes and fruit flies [139]; increases the life of mice only if fed a high-calorie diet, but not a standard diet [121, 124].

***** Although the life-extending ability of resveratrol in yeast, nematodes and fruit flies depends on Sir2p [119, 120, 125], it is currently debated whether this anti-aging compound binds to Sir2p (or SIRT1, a mammalian sirtuin) in vivo and/or activates Sir2p or SIRT1 in living cells [126, 138 - 142]; importantly, resveratrol has been shown to inhibit or activate many proteins other than sirtuins by interacting with them [143, 144].

Abbreviations: AMPK, the AMP-activated serine/threonine protein kinase; CLS, chronological lifespan; IGF-1, insulin-like growth factor 1; LKB1, a serine/threonine protein kinase that phosphorylates and activates AMPK; mTORC1, the mammalian target of rapamycin complex 1; NT, not tested; PBMC, peripheral blood mononuclear cells; PGC-1α, peroxisome proliferator-activated receptor-γ co-activator 1α; RLS, replicative lifespan; TORC1, the yeast target of rapamycin complex 1.

Some anti-aging compounds extend longevity by targeting the insulin/IGF-1 pathway, which monitors the nutritional status of the whole organism. By modulating an upstream step in this signaling pathway, the serotonin receptor antagonist mianserin (which is used as an antidepressant in humans) increases lifespan in worms, likely by inhibiting neurotransmission related to food sensing and mimicking a DR-like physiological state (Table 1.2) [112]. A downstream step in this signaling pathway is a target of valproic acid (a mood stabilizer and an anticonvulsant in humans), which extends longevity in

worms by promoting the translocation of the pro-longevity FoxO transcriptional factor DAF-16 into the nucleus (Table 1.2) [135].

Some anti-aging small molecules extend longevity, improve health and attenuate age-related pathologies by modulating longevity-defining processes controlled by proteins that are not viewed as components of the AMPK/TOR, cAMP/PKA and insulin/IGF-1 signaling pathways but are dynamically integrated into a longevity regulation network (see section 1.1 in this chapter of the thesis) governed by these pathways. One of such longevity-extending molecules is resveratrol, a small polyphenol produced by plants (Table 1.2). Although the demonstrated by *in vitro* studies ability of resveratrol to activate the protein deacetylase activities of the sirtuins Sir2p (yeast), SIR-2.1 (worms), dSir2 (fruit flies) and SIRT1 (mammals) [131, 145] is disputed [142, 146], it has been shown to modulate activities of many other longevity-related proteins by interacting with them [143, 144] – thereby influencing a number of longevity-defining processes [119, 121, 124 - 127, 129]. It is likely that the resveratrol-driven modulation of activities of numerous longevity-defining proteins and processes they govern underlies the observed ability of this small polyphenol molecule 1) to extend replicative (but not chronological) lifespan in yeast [119]; 2) to increase the replicative lifespan of cultured human fibroblasts [123]; and 3) to extend longevity of worms, fruit flies, fishes and mice [120 - 122, 124] (Table 1.2).

Similar to resveratrol, sodium nitroprusside exhibits a potent anti-aging effect by stimulating sirtuin-dependent protein deacetylation. Unlike resveratrol, sodium

nitroprusside activates transcription of the gene encoding sirtuin SIRT1 [133]. The resulting enhancement of SIRT1-driven deacetylation of lysine 16 in histone H4 may underlie the observed establishment of an anti-aging pattern of transcription and the concomitant replicative lifespan extension in cultured human peripheral blood mononuclear cells (PMBC) (Table 1.2) [133].

Reduced histone acetylation is a longevity-extending process that can be stimulated not only by sodium nitroprusside, but also by spermidine. This natural polyamine has been shown to inhibit histone acetyltransferases and activate histone H3 deacetylation, thereby activating transcription of numerous autophagy-related genes, suppressing age-related necrotic cell death and ultimately extending the chronological lifespan of yeast, the replicative lifespan of cultured human PMBC, and longevity of worms and fruit flies (Table 1.2) [131, 134]. Noteworthy, histone modification is also a target of another longevity-extending compound, Li^+. The ability of this alkali metal ion to cause the age-related chromatin reorganization by altering transcription of genes involved in histone methylation, nucleosome composition and chromatin structure may underlie its beneficial properties as a mood stabilizer in humans and an anti-aging compound in worms (Table 1.2) [103].

Mitochondria are known to play a pivotal role in longevity regulation across phyla [36, 73, 75, 147]. Therefore, it is not surprisingly that several anti-aging compounds extend longevity by influencing longevity-defining processes confined to or governed by these organelles. SkQ1, a plastoquinone derivate, is sorted to mitochondria and operates as an

antioxidant that attenuates oxidative damage to mitochondrial proteins and lipids, changes mitochondrial morphology, and impairs mitochondria-controlled forms of apoptotic and necrotic cell death caused by an excessive accumulation of mitochondria-produced hydrogen peroxide (Table 1.2) [132]. By influencing these longevity-defining processes in mitochondria, SkQ1 not only extends longevity in fungi, daphnias, fruit flies and mice, but also improves health and delays the onset of age-related diseases in mice [132]. Furthermore, longevity in worms and fruit flies can be extended by the thermal stress mimetics lipoic acid, propyl gallate, trolox and taxifolin; all these compounds may delay aging by detoxifying free radicals and enhancing resistance to age-related oxidative stress (Table 1.2) [106, 105].

It is important to emphasize that all anti-aging compounds known until recently have been called "CR mimetics" and "DR mimetics" because they: 1) similar to CR and DR dietary regimens, increase lifespan and/or healthspan under non-CR or non-DR conditions; 2) unlike CR and DR diets, do not restrict caloric and nutrient intake; and 3) mimic life-extending and health-improving effects of CR and DR on metabolic pathways, physiological processes, stress response and gene expression (Table 1.2) [148 - 150]. Furthermore, as detailed in Table 1.2, almost all longevity-extending compounds known prior to the study presented in this thesis target the AMPK/TOR and insulin/IGF-1 pathways and impact the sirtuin-governed protein deacetylation module of the longevity signaling network that integrates these pathways. As it has been mentioned in sections 1.1 and 1.2, this network defines longevity only in response to the organismal and intracellular nutrient and energy status. Moreover, such CR mimetics and DR mimetics as

resveratrol, metformin and mianserin beneficially influence lifespan and healthspan only under non-CR or non-DR conditions, but do not extend longevity or improve health if the supply of calories or nutrients is limited (Table 1.2) [107, 112, 119 - 121]. Therefore, we coined the term “adaptable” to define the AMPK/TOR, cAMP/PKA and insulin/IGF-1 signaling pathways and sirtuin-governed protein deacetylation module - because they all are integrated into the longevity regulation network targeted by the currently known anti-aging CR mimetics and DR mimetics. The term “adaptable” emphasizes the fact that all these pathways and the sirtuin-governed module exhibit the beneficial effects on longevity and attenuate age-related pathologies only in response to certain changes in the extracellular and intracellular nutrient and energy status of an organism. It is important to mention, however, that recent studies revealed that Li^+ in worms and rapamycin in fruit flies can extend lifespan even under DR conditions – although their longevity-extending efficacy under such nutrient-limited conditions is significantly lower than that under non-CR conditions (Table 1.2) [103, 113]. Based on these findings, we thought that some longevity assurance pathways could be “constitutive” or “housekeeping” by nature, assuming that they 1) modulate longevity irrespective of the extracellular and intracellular nutrient and energy status – in contrast to the adaptable AMPK/TOR, cAMP/PKA and insulin/IGF-1 signaling pathways and sirtuin-governed protein deacetylation module; and 2) do not overlap (or only partially overlap) with the adaptable pathways that are under the stringent control of calorie and nutrient availability.

1.4 Baker’s yeast as an advantageous model organism for elucidating mechanisms of cellular aging in multicellular eukaryotes

The budding yeast *Saccharomyces cerevisiae*, a unicellular eukaryote, is a valuable model for studying the basic biology of aging and revealing longevity regulation mechanisms in multicellular eukaryotes [6, 34, 152]. Two different paradigms of aging are known for this unicellular eukaryotic organism, which is amenable to comprehensive biochemical, genetic, cell biological, chemical biological and system biological analyses. In the replicative aging paradigm, yeast aging is defined by the maximum number of daughter cells (buds) that a mother cell can form before becoming senescent (Figure 1.2) [6, 31]. Replicative aging in yeast mimics aging of dividing ("mitotic") cells in a multicellular eukaryotic organism [6, 31]. In the chronological aging paradigm, yeast aging is defined by the length of time during which a cell remains viable following entry into a non-proliferative quiescent state (Figure 1.2) [6, 63, 152]. Chronological aging in yeast is an advantageous model for elucidating aging of non-dividing ("post-mitotic") cells in a multicellular eukaryotic organism [6, 63, 152]. Yeast chronological aging is assessed using a simple clonogenic assay, which measures the percentage of yeast cells that remain viable at different time points following the entry of a cell population into the non-proliferative stationary phase [63]. A CR diet, which in yeast can be imposed by reducing the initial glucose concentration in a growth medium from 2% to 0.5% or lower, decelerates both replicative and chronological aging of yeast [6, 35, 63, 151].

As it has been mentioned in sections 1.1, 1.2 and 1.3 of this Master's thesis, longevity signaling pathways and mechanisms of their modulation by dietary and pharmacological

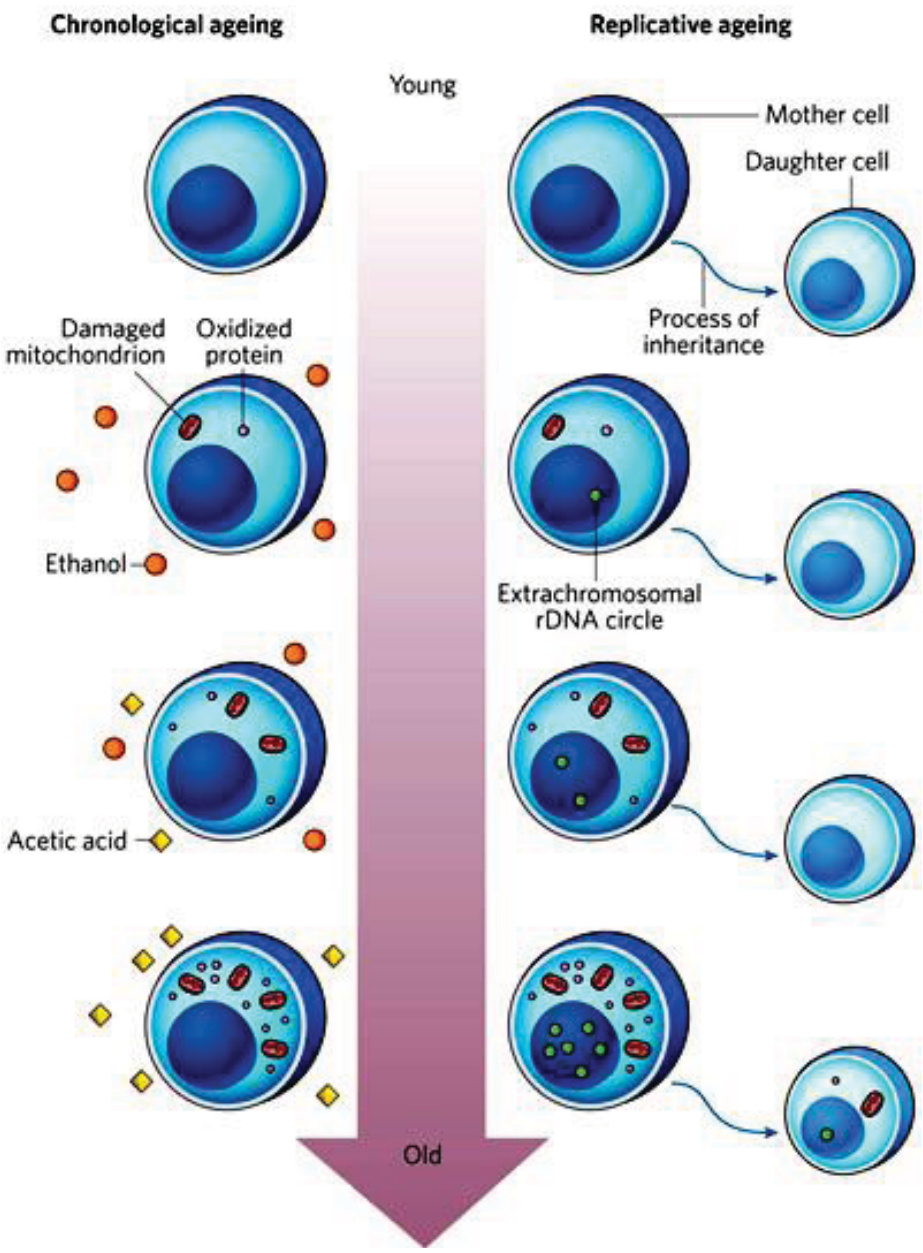

Figure 1.3. Two different paradigms of yeast aging. In the replicative aging paradigm, yeast aging is defined by the maximum number of daughter cells that a mother cell can produce before becoming senescent. Replicative aging in yeast mimics aging of dividing ("mitotic") cells in a multicellular eukaryotic organism. In the chronological aging paradigm, yeast aging is defined by the length of time during which a cell remains viable following entry into a non-proliferative state. Chronological aging in yeast is an advantageous model for studying aging of non-dividing ("post-mitotic") cells in a multicellular eukaryotic organism. Yeast chronological aging is assessed using a simple

clonogenic assay, which measures the percentage of yeast cells that remain viable at different time points following the entry of a cell population into the non-proliferative stationary phase. (See text in section 1.5 for details). Reproduced from [151].

interventions are conserved across phyla. It should be emphasized that the use of yeast as an advantageous model organism in aging research greatly contributed to the current understanding of the molecular and cellular mechanisms underlying longevity regulation in evolutionarily distant eukaryotic organisms. Due to the relatively short and easily monitored replicative and chronological lifespans of this genetically and biochemically manipulable unicellular eukaryote with annotated genome, it has been successfully used to 1) identify numerous novel longevity genes, all of which have been later implicated in regulating longevity of multicellular eukaryotic organisms; 2) establish the chemical nature of molecular damage that causes aging and accelerates the onset of age-related diseases across phyla; and 3) identify several longevity-extending small molecules, all of which have been later shown to slow down aging, improve health, attenuate age-related pathologies and delay the onset of age-related diseases in eukaryotic organisms across phyla [6, 7, 31, 79, 134, 151 - 153].

1.5 Objectives of this Master's thesis work

One of the objectives of this Master's thesis work was to use a chemical genetic high-throughput screen to identify small molecules that can extend longevity of chronologically aging yeast cultured under CR conditions. Under such conditions a pro-aging signaling network that integrates the TOR and cAMP/PKA pathways is greatly attenuated. We thought that the availability of such anti-aging compounds would provide

a potent chemical biological tool for defining of what we call the "housekeeping" longevity assurance pathways. We introduced the term "housekeeping longevity assurance pathways" to emphasize the following: 1) these pathways modulate longevity irrespective of the extracellular and intracellular nutrient and energy status – in contrast to TOR and cAMP/PKA, the two longevity signaling pathways that are "adaptable" by nature because they define yeast chronological lifespan only in response to certain changes in such status; and 2) these pathways do not overlap (or only partially overlap) with the adaptable TOR and cAMP/PKA pathways that in yeast are under the stringent control of calorie availability.

Our high-throughput screen identified several bile acids as anti-aging compounds targeting housekeeping longevity assurance pathways. One of the objectives of this Master's thesis work was to conduct pharmacophore modeling of the anti-aging potential of various species of bile acids under CR and non-CR conditions.

Our pharmacophore modeling of the anti-aging potential of various species of bile acids revealed that the most hydrophobic of them, lithocholic acid (LCA), provides yeast with the greatest longevity benefit when calorie supply was limited. Indeed, the life-extending efficacy of LCA under CR exceeded that under non-CR conditions, being inversely proportional to the concentration of glucose in growth medium and thus in correlation with the extent of calorie supply limitation.

Unlike mammals, yeast do not synthesize bile acids. We proposed that bile acids released into the environment by mammals may act as interspecies chemical signals extending longevity of yeast species and, perhaps, of other organisms that can: 1) sense these mildly toxic molecules with detergent-like properties; and 2) respond to the resulting mild cellular damage by developing the most efficient stress protective mechanisms. Therefore, one of the objectives of this Master's thesis work was 1) to analyze how LCA and other small anti-aging molecules (including resveratrol, caffeine and rapamycin) synthesized and released into the environment by one species of the organisms composing an ecosystem extend longevity of other species within this ecosystem; and then 2) to formulate a hypothesis on how these interspecies chemical signals could create xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms at the ecosystemic level.

One of the objectives of this Master's thesis work was to test the validity of the proposed here unified hypothesis of the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms within ecosystems by carrying out the LCA-driven experimental evolution of long-lived yeast species. Our serial-batch-transfer type of selection yielded three laboratory-evolved yeast strains with greatly extended lifespan. One of the objectives of this Master's thesis work was to use these extremely long-lived yeast species for establishing the molecular mechanisms underlying their greatly extended longevity.

1.6 Thesis outline and contributions of colleagues

Chapter 2 describes our pharmacophore modeling of the anti-aging potential of various species of bile acids. Our data imply that the maintenance of the minimal polarity of both the hydrophilic (concave) and hydrophobic (convex) sides of the steroid nucleus - by avoiding the presence of polar substituents at the positions 6, 7 and 12 - is mandatory for the extreme life-extending efficacy of LCA under CR conditions. Such stringent structural requirements are consistent with a target specificity of LCA action as an anti-aging small molecule. We also found that the life-extending efficacy of LCA under CR exceeds that under non-CR conditions, being inversely proportional to the concentration of glucose in growth medium and thus in correlation with the extent of calorie supply limitation. We therefore concluded that LCA delays aging of chronologically aging yeast by modulating housekeeping longevity assurance processes. Our other published data imply that these processes are not regulated by the adaptable TOR and cAMP/PKA signaling pathways. We expect that this knowledge will be instrumental for defining molecular mechanisms underlying the ability of LCA to extend longevity by impacting these various housekeeping longevity assurance processes and by modulating signaling pathways governing them.

Chapter 3 describes our hypothesis in which interspecies chemical signals released into the environment create xenohormetic, hormetic and cytostatic selective forces that drive the ecosystemic evolution of longevity regulation mechanisms. In our hypothesis, the ability of a species of the organisms composing an ecosystem to undergo life-extending metabolic or physiological changes in response to hormetic or cytostatic chemical

compounds released to the ecosystem by other species: 1) increases its chances of survival; 2) creates selective forces aimed at maintaining such ability; and 3) enables the evolution of longevity regulation mechanisms.

To verify empirically our hypothesis of the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms at the ecosystemic level, in experiments described in Chapter 4 we carried out the LCA-driven multistep selection of long-lived yeast species under laboratory conditions. We found that a lasting exposure of wild-type (WT) yeast to LCA results in selection of yeast species that live significantly longer in the absence of this bile acid than their ancestor. Our data imply that the evolved under laboratory conditions long-lived yeast species 3, 5 and 12 were able to maintain their greatly extended chronological life spans (CLS) following numerous successive passages in medium without LCA under CR conditions. Furthermore, we revealed that each of these long-lived yeast species also exhibited extended CLS when cultured in the absence of LCA under non-CR conditions (although to a lesser extent than that under CR conditions). We therefore concluded that, consistent with its sought-after effect on longevity regulation pathways, our LCA-driven multistep selection process under laboratory conditions yielded long-lived yeast species whose greatly delayed chronological aging was caused by the accumulation of mutant alleles that activate so-called housekeeping longevity assurance pathways. These housekeeping longevity assurance pathways modulate longevity irrespective of the number of available calories and do not overlap with the adaptable longevity pathways that are under the stringent control of calorie availability [167].

Chapter 5 describes the results of testing other aspects of our hypothesis on the ecosystemic evolution of longevity regulation mechanisms. Sprcifically, we assessed how a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 influence a compendium of the housekeeping longevity-related processes that, as we recently revealed, are modulated by LCA in chronologically aging yeast. We found that in the absence of LCA this allele or alleles alter the age-dependent dynamics of mitochondrial respiration under both CR and non-CR conditions. Thus, consistent with its sought-after effect on longevity regulation pathways, our LCA-driven multistep selection process under laboratory conditions yielded long-lived yeast species whose greatly delayed chronological aging was caused by the selection of a mutant allele or alleles that activate so-called housekeeping longevity assurance pathways.

Most of the findings described in Chapter 2 have been published in *Aging* [Goldberg, A.A., Richard, V.R., Kyryakov, P., Bourque, S.D., Beach, A., Burstein, M.T., Glebov, A., Koupaki, O., Boukh-Viner, T., Gregg, C., Juneau, M., English, A.M., Thomas, D.Y. and Titorenko, V.I. (2010). Chemical genetic screen identifies lithocholic acid as an anti-aging compound that extends yeast chronological lifespan in a TOR-independent manner, by modulating housekeeping longevity assurance processes. *Aging* 2:393-414]. I carried out the pharmacophore modeling of the anti-aging potential of various species of bile acids and prepared the first draft of two sections relevant to my work. Dr. V. Titorenko provided intellectual leadership of this project and edited the manuscript.

Findings described in Chapter 3 have been published in *Dose-Response* [Burstein, M.T., Beach, A., Richard, V.R., Koupaki, O., Gomez-Perez, A., Goldberg, A.A., Kyryakov, P., Bourque, S.D., Glebov, A. and Titorenko, V.I. (2011). Interspecies chemical signals released into the environment may create xenohormetic, hormetic and cytostatic selective forces that drive the ecosystemic evolution of longevity regulation mechanisms. *Dose-Response* 9: in press. I prepared the first draft of a section describing the ability of rapamycin released into environment to act as a cytostatic chemical signal that extends longevity of the other organisms within an ecosystem. Dr. V. Titorenko provided intellectual leadership of this project and edited the manuscript.

Findings described in Chapter 4 are presented in the manuscript of a paper [Glebov, A., Gomez-Perez, A., Kyryakov, P., Burstein, M.T., Beach, A., Richard, V.R. and Titorenko, V.I. Laboratory evolution of longevity regulation mechanisms by a lasting exposure of the yeast *Saccharomyces cerevisiae* to a bile acid] that is currently in preparation for submission to *Current Biology*. I expect this manuscript to be submitted for publication in the late spring or early summer of 2012. I carried out a 3-step process of the laboratory evolution of long-lived yeast species by a lasting exposure of wild-type yeast to lithocholic acid and prepared the first draft of four sections relevant to my work. Dr. V. Titorenko provided intellectual leadership of this project and edited the manuscript.

2 Pharmacophore modeling of the anti-aging potential of bile acids

2.1 Abstract

Longevity of chronologically aging yeast can be extended by administering a caloric restriction (CR) diet [6, 7, 10, 24, 35, 63] or some small molecules [101, 110, 114, 134]. These life-extending interventions target the adaptable target of rapamycin (TOR) and cAMP/protein kinase A (cAMP/PKA) signaling pathways that are under the stringent control of calorie availability [6, 7, 10, 35, 101, 110, 114, 134]. We designed a chemical genetic screen for small molecules that increase the chronological life span (CLS) of yeast under CR by targeting lipid metabolism and modulating housekeeping longevity pathways that regulate longevity irrespective of the number of available calories. By screening the total of approximately 19,000 representative compounds from seven commercial libraries, we identified 24 small molecules that greatly extend the CLS of a short-lived mutant *pex5Δ* deficient in peroxisomal fatty acid oxidation, which we used for our high-throughput screen. These anti-aging small molecules belong to 5 chemical groups. Group I consisted of 6 bile acids, including lithocholic acid (LCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), cholic acid (CA), dehydrocholic acid (DHCA) and hyodeoxycholic acid (HDCA). The results of our pharmacophore modeling of the anti-aging potential of various species of bile acids imply that the maintenance of the minimal polarity of both the hydrophilic (concave) and hydrophobic (convex) sides of the steroid nucleus - by avoiding the presence of polar substituents at the positions 6, 7 and 12 - is mandatory for the extreme life-extending efficacy of LCA under CR conditions. Such stringent structural requirements are consistent with a target specificity

of LCA action as an anti-aging small molecule. We found that the life-extending efficacy of LCA under CR exceeds that under non-CR conditions, being inversely proportional to the concentration of glucose in growth medium and thus in correlation with the extent of calorie supply limitation.

2.2 Introduction

Aging of multicellular and unicellular eukaryotic organisms is a multifactorial biological phenomenon that has various causes and affects a plethora of cellular activities [1 - 3, 5, 8, 10, 24]. These numerous activities are modulated by only a few nutrient- and energy-sensing signaling pathways that are conserved across phyla and include the insulin/insulin-like growth factor 1 (IGF-1), AMP-activated protein kinase/target of rapamycin (AMPK/TOR) and cAMP/protein kinase A (cAMP/PKA) pathways [4, 10, 36]. By sharing a compendium of protein kinases and adaptor proteins, the insulin/IGF-1, AMPK/TOR and cAMP/PKA pathways in yeast, worms, fruit flies and mammals converge into a network regulating longevity [4, 10, 36 - 38, 154]. This network may also include several proteins that currently are not viewed as being in any of these three pathways [4, 10, 36, 73, 74]. Moreover, this network responds to the age-related partial mitochondrial dysfunction and is modulated by mitochondrially produced reactive oxygen species (ROS) [36, 73, 75, 147]. By sensing the nutritional status of the whole organism as well as the intracellular nutrient and energy status, functional state of mitochondria, and concentration of ROS produced in mitochondria, the longevity network regulates life span across species by coordinating information flow along its convergent, divergent and multiply branched signaling pathways.

By defining the organismal and intracellular nutrient and energy status, nutrient intake plays an important role in modulating life span and influences age-related pathologies [7, 155]. Two dietary regimens are known to have the most profound life-extending effects across species and to improve overall health by delaying the onset of age-related diseases. They include: 1) caloric restriction (CR), a diet in which only calorie intake is reduced but the supply of amino acids, vitamins and other nutrients is not compromised [13, 76, 77, 80, 155]; and 2) dietary restriction (DR), in which the intake of nutrients (but not necessarily of calories) is reduced by limiting food supply without causing malnutrition [66, 76, 77, 91]. In a "TOR-centric" view of longevity regulation, TOR alone governs the life-extending and health-improving effects of CR/DR by: 1) integrating the flow of information on the organismal and intracellular nutrient and energy status from the protein kinases AMPK, PKA, PKB/AKT (the insulin/IGF-1 pathway) and ERK1/2 (the PKA-inhibited Raf/MEK/ERK protein kinase cascade) as well as from the mitochondrial redox protein $P66^{Shc}$; 2) sensing the intracellular levels of amino acids in an AMPK-independent manner; and 3) operating as a control center which, based on the information it has gathered and processed, modulates many longevity-related processes in a sirtuin-independent fashion [84 - 86]. The inability of CR to increase the replicative life span (RLS) of yeast mutants lacking components of the TOR pathway [87] and the lack of the beneficial effect of DR on life span in worms with reduced TOR signaling [88, 89] support the proposed central role for TOR in orchestrating the life-extending effect of CR/DR in these two longevity paradigms. Moreover, while the postulated by the TOR-centric model dispensability of sirtuins for the longevity benefit associated with DR has been confirmed in worms [89], the importance of the sirtuin Sir2p in mediating the life-

extending effect of CR in replicatively aging yeast is debated [56, 87, 145, 151]. Noteworthy, while TOR is a central regulator of the life-extending effect of CR in replicatively aging yeast, the longevity benefit associated with CR in chronologically aging yeast is mediated by a signaling network that includes: 1) the TOR and cAMP/PKA pathways converged on Rim15p, which therefore acts as a nutritional integrator; and 2) some other, currently unknown pathways that are not centered on Rim15p [154]. Considering the likely convergence of the insulin/IGF-1, AMPK/TOR and cAMP/PKA signaling pathways into a network regulating longevity in worms, fruit flies and mammals (see above), it is conceivable that - akin to TOR - the insulin/IGF-1 and cAMP/PKA pathways may contribute to the beneficial effect of CR/DR on their longevity. Although some findings in worms, fruit flies and mammals support the involvement of the insulin/IGF-1 pathway in the longevity benefit associated with CR/DR, other data imply that such benefit is independent of insulin/IGF-1 (reviewed by Narasimhan *et al*. [36]). The role of cAMP/PKA signaling in the life-extending effect of CR/DR in these multicellular eukaryotes remains to be tested. Importantly, the recently reported in worms involvement of both independent and overlapping pathways in life span extension by different DR regimens [100] supports the notion that the longevity benefit associated with nutrient limitation is mediated by a signaling network that integrates several pathways.

Akin to CR and DR regimens, certain pharmacological interventions can extend longevity across phyla and improve health by beneficially influencing age-related pathologies. Noteworthy, all of the currently known anti-aging compounds increase life

span under non-CR or non-DR conditions (see chapter 1 of my thesis for a detailed discussion of this topic). Under such conditions, these compounds have been shown to: 1) provide the longevity and health benefits associated with CR and DR, but without restricting caloric and nutrient intake; and 2) mimic numerous life-extending effects of CR and DR on gene expression pattern, metabolic and physiological processes, and stress response pathways. Therefore, the names "CR mimetics" and "DR mimetics" have been coined for them [149, 150]. Importantly, most CR mimetics and DR mimetics target signaling pathways that modulate longevity in response to the organismal and intracellular nutrient and energy status, including the insulin/IGF-1 and AMPK/TOR pathways as well as the sirtuin-governed protein deacetylation module of the longevity signaling network integrating these pathways (see chapter 1 of my thesis for a detailed discussion of this topic). Furthermore, such compounds as resveratrol, metformin and mianserin increase life span only under non-CR or non-DR conditions, but are unable to do so if the supply of calories or nutrients is limited [107, 112, 119 - 121]. Hence, one could envision that most, if not all, longevity pathways are "adaptable" by nature, *i.e.*, that they modulate longevity only in response to certain changes in the extracellular and intracellular nutrient and energy status of an organism. However, Li^+ in worms and rapamycin in fruits flies extend life span even under DR conditions [103, 113]. It is likely therefore that some longevity pathways could be "constitutive" or "housekeeping" by nature, *i.e.*, that they: 1) modulate longevity irrespective of the organismal and intracellular nutrient and energy status; and 2) do not overlap (or only partially overlap) with the adaptable longevity pathways that are under the stringent control of calorie and/or nutrient availability. The challenge is to identify such housekeeping longevity

pathways, perhaps by using a chemical screen for compounds that can extend longevity even under CR/DR conditions. Because under such conditions the adaptable pro-aging pathways are fully suppressed and the adaptable anti-aging pathways are fully activated, a compound that can increase life span is expected to target the housekeeping longevity pathways.

Noteworthy, two anti-aging compounds alter lipid levels in mammals and fruit flies under non-DR conditions. In fact, resveratrol treatment reduces the levels of the neutral lipids triacylglycerols (TAG) and increases free fatty acid (FFA) levels in mouse adipocytes [127]. Furthermore, feeding rapamycin to fruit flies results in elevated TAG levels [113]. Although it remains to be seen if such effects of resveratrol and rapamycin on lipid levels play a casual role in their anti-aging action under non-DR conditions, it should be stressed that lipid metabolism has been shown to be involved in longevity regulation in yeast [24, 156], worms [50, 74, 157, 158], fruit flies [157, 159] and mice [127, 157, 160 - 163]. Recently, our laboratory proposed a mechanism linking yeast longevity and lipid dynamics in the endoplasmic reticulum (ER), lipid bodies and peroxisomes. In this mechanism, a CR diet extends yeast CLS by activating FFA oxidation in peroxisomes [24, 156]. It is conceivable that the identification of small molecules targeting this mechanism could yield novel anti-aging compounds. Such compounds can be used as research tools for defining the roles for different longevity pathways in modulating lipid metabolism and in integrating lipid dynamics with other longevity-related processes. Furthermore, the availability of such compounds would enable a quest for housekeeping longevity assurance pathways that do not overlap (or only partially overlap) with the

adaptable TOR and cAMP/PKA pathways. Moreover, such compounds would have a potential to be used as pharmaceutical agents for increasing life span and promoting healthy aging by delaying the onset of age-related diseases, regardless of an organism's dietary regimen.

We sought to identify small molecules that increase the CLS of yeast under CR conditions by targeting lipid metabolism and modulating housekeeping longevity assurance pathways. Our chemical genetic screen identified bile acids as one of the 5 chemical groups of such anti-aging small molecules. Our pharmacophore modeling of the anti-aging potential of various species of bile acids under CR and non-CR conditions provided important new insights into mechanisms of longevity regulation, as outlined below.

2.3 Materials and Methods

Strains and media

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) was used in this study. Media components were as follows: 1) YEPD (0.2% Glucose), 1% yeast extract, 2% peptone, 0.2% glucose; 2) YEPD (0.5% Glucose), 1% yeast extract, 2% peptone, 0.5% glucose; 3) YEPD (1% Glucose), 1% yeast extract, 2% peptone, 1% glucose; and 4) YEPD (2% Glucose), 1% yeast extract, 2% peptone, 2% glucose.

A plating assay for the analysis of chronological life span (CLS)

Cells were grown in YEPD medium initially containing 0.2%, 0.5%, 1% or 2% glucose as carbon source at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a flask volume/medium volume ratio of 5:1. A sample of cells was removed from each culture at various time points. A fraction of the cell sample was diluted in order to determine the total number of cells per ml of culture using a hemacytometer. 10 μl of serial dilutions (1:10 to 1:10^{3}) of cells were applied to the hemacytometer, where each large square is calibrated to hold 0.1 μl. The number of cells in 4 large squares was then counted and an average was taken in order to ensure greater accuracy. The concentration of cells was calculated as follows: number of cells per large square x dilution factor × 10 × 1,000 = total number of cells per ml of culture. A second fraction of the cell sample was diluted and serial dilutions (1:10^{2} to 1:10^{5}) of cells were plated onto YEPD (2% Glucose) plates in triplicate in order to count the number of viable cells per ml of each culture. 100 μl of diluted culture was plated onto each plate. After a 48-h incubation at 30^{o}C, the number of colonies per plate was counted. The number of colony forming units (CFU) equals to the number of viable cells in a sample. Therefore, the number of viable cells was calculated as follows: number of colonies × dilution factor × 10 = number of viable cells per ml. For each culture assayed, % viability of the cells was calculated as follows: number of viable cells per ml / total number of cells per ml × 100%. The % viability of cells in mid-logarithmic phase was set at 100% viability for that particular culture.

Pharmacological manipulation of CLS

CLS analysis was performed as described above in this section. The chenodeoxycholic (C9377), cholic (C1129), dehydrocholic (D3750), deoxycholic (D2510), hyodeoxycholic (H3878), lithocholic (L6250) and ursodeoxycholic (U5127) bile acids were from Sigma. Their stock solutions in DMSO were made on the day of adding each of these compounds to cell cultures. Compounds were added to growth medium at the indicated concentration immediately following cell inoculation. The final concentration of DMSO in yeast cultures supplemented with a bile acid (and in the corresponding control cultures supplemented with drug vehicle) was 1% (v/v).

2.4 Results

2.4.1 Pharmacophore modeling of the anti-aging potential of bile acids

To perform a chemical genetic screen for small molecules that increase the CLS of yeast by targeting lipid metabolism, we chose the single-gene-deletion mutant strain *pex5Δ*. Because *pex5Δ* lacks a cytosolic shuttling receptor for peroxisomal import of Fox1p and Fox2p, these two enzymes of the β-oxidation of free fatty acids (FFA) reside in the cytosol of *pex5Δ* cells [164]. In contrast, the Pex5p-independent peroxisomal import of Fox3p, the third enzyme of the FFA β-oxidation pathway, sorts it to the peroxisome in *pex5Δ* cells [164]. By spatially separating Fox1p and Fox2p from Fox3p within a cell, the *pex5Δ* mutation impairs FFA oxidation. In chronologically aging yeast grown under CR conditions on 0.2% or 0.5% glucose, peroxisomal FFA oxidation regulates longevity by 1) efficiently generating acetyl-CoA to synthesize the bulk of ATP in mitochondria; and

2) acting as a rheostat that modulates the age-related dynamics of FFA and diacylglycerol (DAG), two regulatory lipids known to promote longevity-defining cell death [24, 156, 165, 166]. Unlike CR yeast, chronologically aging non-CR yeast grown on 1% or 2% glucose are unable to generate significant quantities of ATP by oxidizing peroxisome-derived acetyl-CoA in mitochondria and, instead, produce the bulk of ATP via glycolytic oxidation of glycogen- and trehalose-derived glucose [24, 156, 165]. Consistent with the essential role of peroxisomal FFA oxidation as a longevity assurance process only under CR, the *pex5Δ* mutation substantially shortened the CLS of CR yeast but caused a significantly lower reduction of longevity in non-CR yeast, especially in yeast grown on 2% glucose [167]. Our published data imply that, by impairing peroxisomal FFA oxidation and affecting lipid metabolism in the endoplasmic reticulum (ER) and lipid bodies, the *pex5Δ* mutation alters the levels of numerous pro- and anti-aging proteins and impacts many longevity-related processes, thereby shortening the CLS of yeast when calorie supply is limited [167]. We therefore chose the short-lived *pex5Δ* strain to carry out a chemical genetic screen for anti-aging compounds that target lipid metabolism to extend CLS in yeast placed on a CR diet. By screening the total of approximately 19,000 representative compounds from seven commercial libraries, we identified 24 small molecules that greatly extend the CLS of *pex5Δ* under CR and belong to 5 chemical groups. Group I consisted of 6 bile acids, including lithocholic acid (LCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), cholic acid (CA), dehydrocholic acid (DHCA) and hyodeoxycholic acid (HDCA).

Our pharmacophore modeling of the anti-aging potential of various species of bile acids revealed that some of them extended the CLS of wild-type (WT) strain under CR conditions. Specifically, we found that LCA and two other bile acids - DCA and CDCA - increased both the mean and maximum CLS of WT yeast grown under CR on 0.2% glucose (Figures 2.1A to 2.1D). Moreover, DHCA increased only the mean CLS of WT yeast under CR at 0.2% glucose, whereas HDCA increased only their maximum CLS (Figures 2.1A to 2.1D). The most hydrophobic bile acid, LCA [168], provided WT cells with the greatest longevity benefit when calorie supply was limited. In fact, we found that LCA increased the mean CLS of WT strain under CR at 0.2% glucose by almost 250% and its maximum CLS by more than 200% (Figures 2.1A - 2.1D). Our comparative analysis of the structural differences between various bile acids and their relative life-extending efficacies revealed that the positions 6, 7 and 12 in the six-member rings B and C of the steroid nucleus are important for the anti-aging potential of a bile acid. Indeed, the ability of LCA to extend both the mean and maximum CLS of WT yeast under CR can be: 1) eliminated (with respect to the mean CLS) or greatly reduced (with respect to the maximum CLS) by attaching an α-oriented hydroxyl group at the position 6 (as in HDCA); 2) greatly reduced (with respect to both the mean and maximum CLS) by attaching an α-oriented hydroxyl group at the position 7 (as in CDCA); and 3) greatly reduced (with respect to both the mean and maximum CLS) by attaching an α-oriented hydroxyl group at the position 12 (as in DCA) (Figures 2.1B to 2.1E). All these modifications to the structure of LCA increase polarity of the hydrophilic (concave) side [α-face] of the steroid nucleus by positioning a hydroxyl group below the nucleus and axially to its plane (Figure 2.1E). Furthermore, the anti-aging potential of LCA can be

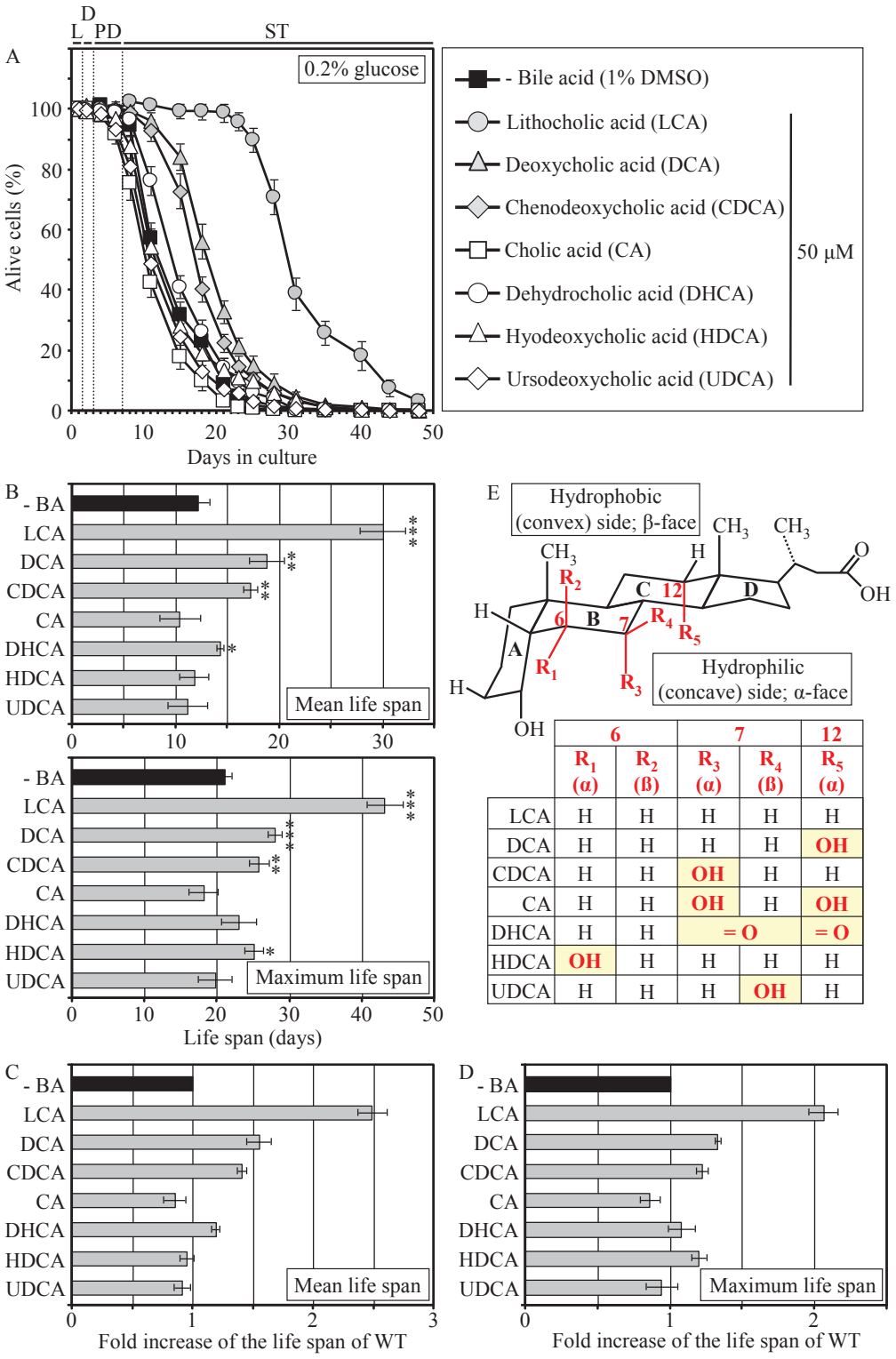

Figure 2.1. LCA and some other bile acids extend the CLS of WT strain under CR conditions. (A - D) Effect of various bile acids on survival (A) and on the mean and

maximum life spans (B - D) of chronologically aging WT strain grown under CR conditions on 0.2% glucose. Data are presented as means ± SEM (n = 3-28; ***p < 0.001; **p < 0.01; *p < 0.05). (E) Structure and hydrophilic/hydrophobic properties of bile acids. The R1 (α), R3 (α) and R5 (α) hydroxyl groups at the positions 6, 7 and 12 in the six-member rings B and C of the steroid nucleus increase polarity of the hydrophilic (concave) side [α-face] of the nucleus by being located below the nucleus and axially to its plane. The R4 (β) hydroxyl group at the position 7 in the six-member ring B of the steroid nucleus confers polarity of the hydrophobic (convex) side [β-face] of the nucleus by being located above the nucleus and equatorially to its plane.

abolished by attaching a β-oriented hydroxyl group at the position 7 (as in UDCA), thereby conferring polarity to the hydrophobic (convex) side [β-face] of the steroid nucleus by positioning a hydroxyl group above the nucleus and equatorially to its plane (Figures 2.1B to 2.1E). Moreover, the simultaneous attachments of two α-oriented hydroxyl groups (as in CA) or two keto groups (as in DHCA) at the positions 7 and 12 eliminated the ability of LCA to extend both the mean and maximum CLS of WT yeast under CR (Figures 2.1B to 2.1E).

Altogether, the results of our pharmacophore modeling of the anti-aging potential of bile acids imply that the maintenance of the minimal polarity of both the hydrophilic (concave) and hydrophobic (convex) sides of the steroid nucleus - by avoiding the presence of polar substituents at the positions 6, 7 and 12 - is mandatory for the extreme life-extending efficacy of LCA under CR conditions. Such stringent structural requirements are consistent with a target specificity of LCA action as an anti-aging small molecule.

2.4.2 LCA extends the CLS of WT yeast under both CR and non-CR conditions, although to a different extent

We found that, if added to growth medium at the time of cell inoculation, LCA increased both the mean and maximum CLS of WT strain not only under CR at 0.2% or 0.5% glucose (Figures 2.2A, 2.2B and 2.2G – 2.2I) but also under non-CR conditions administered by culturing yeast in medium initially containing 1% or 2% glucose (Figures 2.2C, 2.2D and 2.2G – 2.2I). At any tested concentration of glucose in growth medium, LCA displayed the greatest beneficial effect on both the mean and maximum CLS of WT strain if used at a final concentration of 50 μM (Figures 2.2E and 2.2F). It should be stressed that the life-extending efficacy of 50 μM LCA under CR exceeded that under non-CR conditions, being inversely proportional to the concentration of glucose in growth medium and thus in correlation with the extent of calorie supply limitation (Figures 2.2G to 2.2I). Importantly, although 50 μM LCA displayed a profound effect on CLS, it did not cause significant changes in growth of WT strain at any tested concentration of glucose in medium. In fact, we found that both growth rate in logarithmic phase and time prior to entry into stationary (ST) phase were similar for WT cells cultured in medium with or without LCA (Figure 2.3).

2.5 Discussion

In studies described in this chapter, we carried out a high-throughput chemical genetic screen for small molecules that increase the CLS of yeast under CR conditions by targeting lipid metabolism and modulating housekeeping longevity pathways that regulate longevity irrespective of the number of available calories. By screening the total

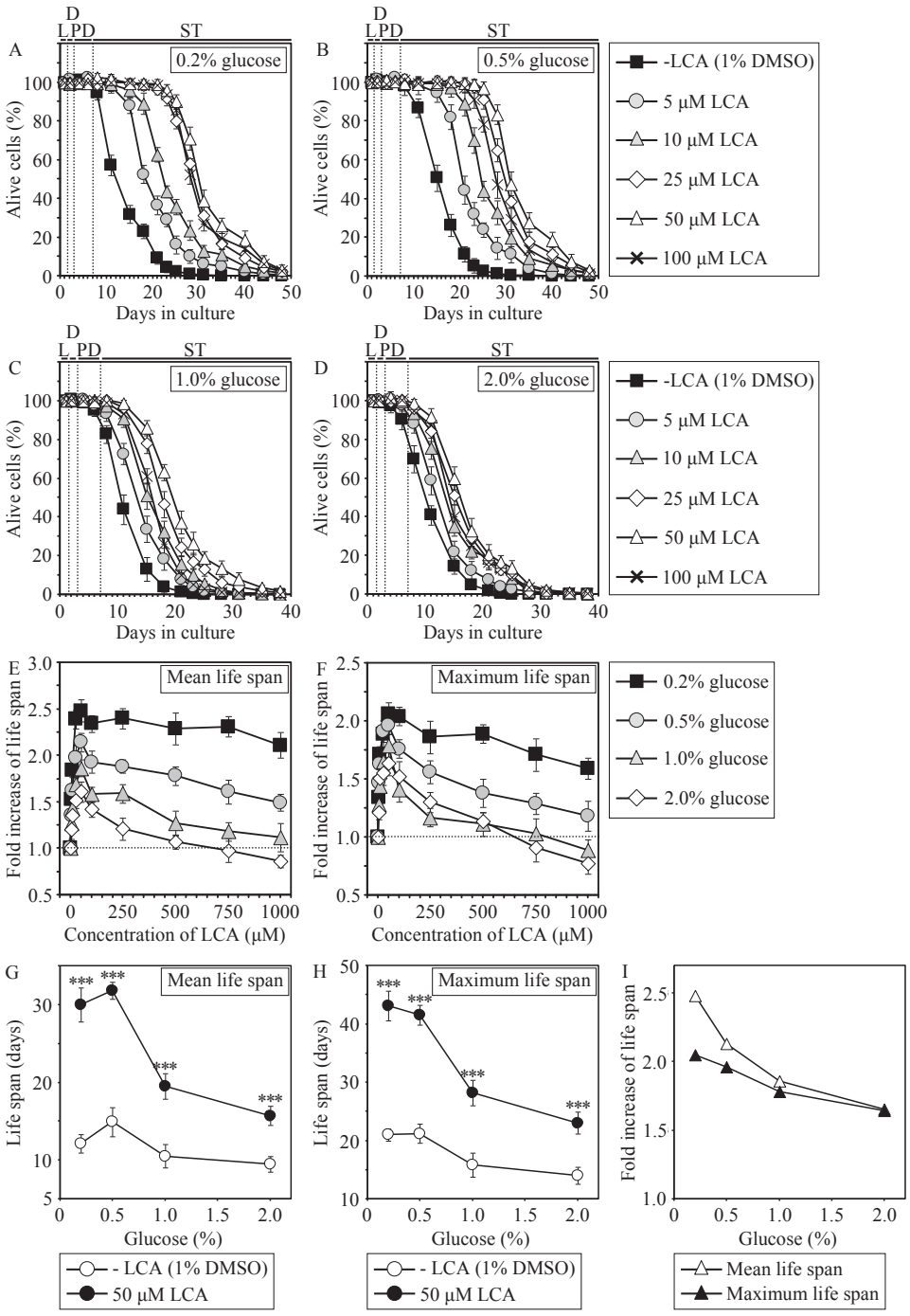

Figure 2.2. In chronologically aging WT yeast, the life-extending efficacy of LCA under CR exceeds that under non-CR conditions. (A - F) Effect of various concentrations of LCA on survival (A - D) and on the fold increase in the mean (E) or maximum (F) life span of chronologically aging WT strain cultured in medium initially containing 0.2%, 0.5%, 1% or 2% glucose. Data are presented as means ± SEM (n = 3-28). (G - I) Effect of

50 μM LCA on the mean or maximum CLS of WT yeast cultured in medium initially containing 0.2%, 0.5%, 1% or 2% glucose. Data are presented as means ± SEM (n = 12-28; ***p < 0.001).

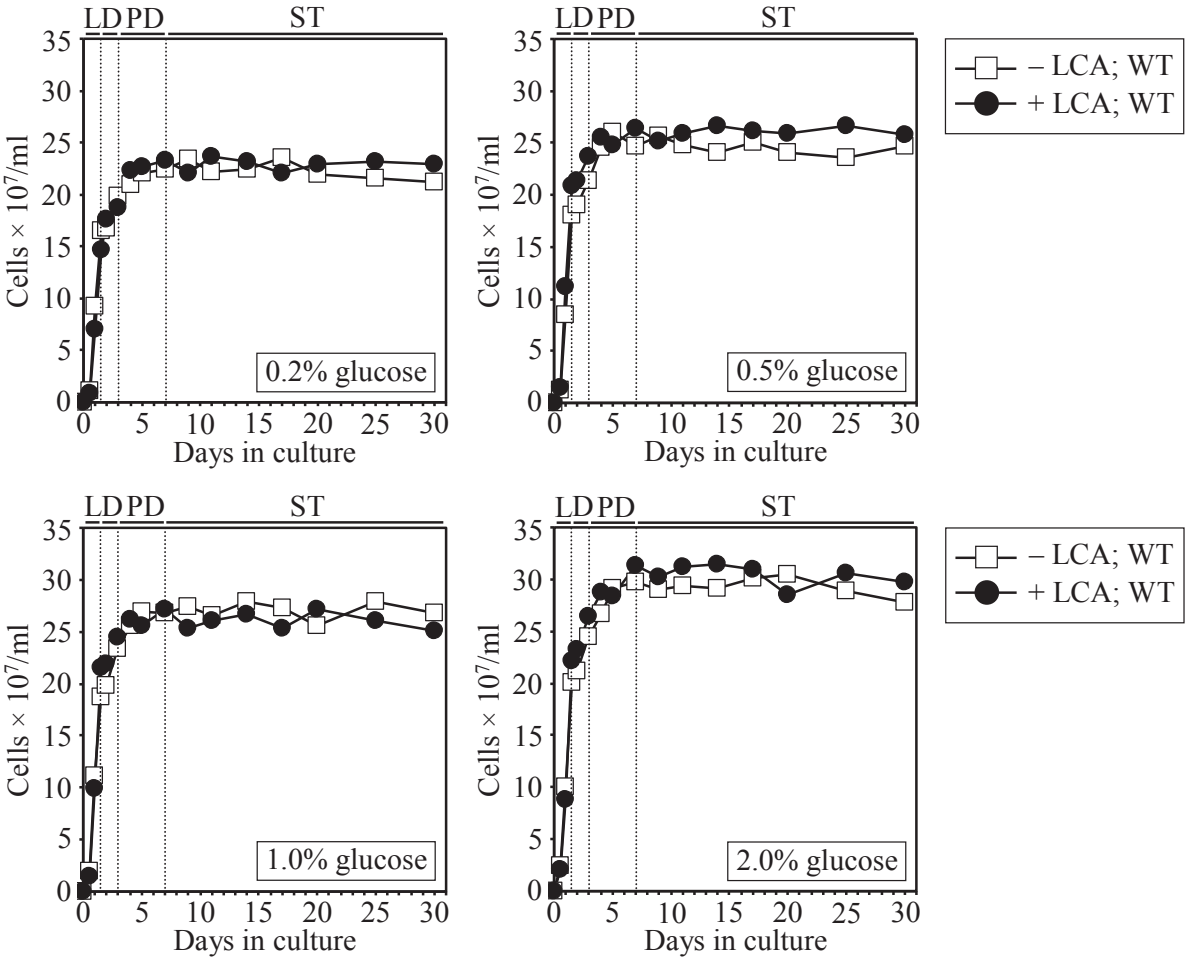

Figure 2.3. LCA does not cause significant changes in growth pattern of wild-type (WT) strain at any tested concentration of glucose in medium. Kinetics of growth for WT strain in medium initially containing 0.2%, 0.5%, 1.0% or 2.0% glucose in the presence of LCA (50 μM) or in its presence. Each plot shows a representative experiment repeated 4-7 times in triplicate with similar results. Abbreviations: D, diauxic growth phase; L, logarithmic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase.

of approximately 19,000 representative compounds from seven commercial libraries, we identified bile acids as one of the five chemical groups of such anti-aging small molecules. The results of our pharmacophore modeling of the anti-aging potential of various species of bile acids imply that the maintenance of the minimal polarity of both the hydrophilic (concave) and hydrophobic (convex) sides of the steroid nucleus - by avoiding the presence of polar substituents at the positions 6, 7 and 12 - is mandatory for the extreme life-extending efficacy of LCA under CR conditions. Such stringent structural requirements are consistent with a target specificity of LCA action as an anti-aging small molecule. We also found that the life-extending efficacy of LCA under CR exceeds that under non-CR conditions, being inversely proportional to the concentration of glucose in growth medium and thus in correlation with the extent of calorie supply limitation. We therefore concluded that LCA delays aging of chronologically aging yeast by modulating housekeeping longevity assurance processes. Our other published data imply that these processes are not regulated by the adaptable TOR and cAMP/PKA signaling pathways [167].

Noteworthy, although we found that LCA greatly extends yeast longevity, yeast do not synthesize this or any other bile acid found in mammals [168, 169]; a recent mass spectrometry-based analysis of the total yeast lipidome conducted by Simon Bourque and Adam Beach in Dr. Titorenko's laboratory has confirmed lack of endogenous bile acids. One could envision that during evolution yeast have lost the ability to synthesize bile acids but have maintained the life-extending response to these biologically active molecules by retaining certain longevity-related processes that are sensitive to regulation

by bile acids. Alternatively, one could think that during evolution yeast have developed the ability to sense bile acids produced by mammals (and/or bile acid-like lipids synthesized by worms), recognize these mildly toxic molecules as environmental stressors providing hormetic benefits and/or as indicators of the state of the environment or food supply, and then to respond by undergoing certain life-extending changes to their physiology that ultimately increase their chances of survival. It is conceivable therefore that the life-extending potential of LCA and other bile acids as well as, probably, the mechanisms underlying their anti-aging action are evolutionarily conserved.

In fact, following their synthesis from cholesterol in the intestine, hypodermis, spermatheca and sensory neurons of worms, bile acid-like dafachronic acids (including 3-keto-LCA) have been shown to be delivered to other tissues where they activate the DAF-12/DAF-16 signaling cascade that in turn orchestrates an anti-aging transcriptional program, thereby increasing the life span of the entire organism [157]. Bile acids also provide health benefits to mammals. Synthesized from cholesterol in hepatocytes of the liver, these amphipathic molecules have been for a long time considered to function only as trophic factors for the enteric epithelium and as detergents for the emulsification and absorption of dietary lipids and fat-soluble vitamins [168 - 170]. Recent years have been marked by a significant progress in our understanding of the essential role that bile acids play as signaling molecules regulating lipid, glucose and energy homeostasis and activating detoxification of xenobiotics [168, 170, 171]. By stimulating the G-protein-coupled receptor TGR5, bile acids activate the cAMP/PKA signaling pathway that 1) enhances energy expenditure in brown adipose tissue and muscle by stimulating

mitochondrial oxidative phosphorylation and uncoupling; 2) improves liver and pancreatic function by activating the endothelial nitric oxide synthase; and 3) enhances glucose tolerance in obese mice by inducing intestinal glucagon-like peptide-1 release [168, 170, 171]. Furthermore, by activating the farnesoid X receptor (FXR) and several other nuclear hormone receptors inside mammalian cells, bile acids 1) modulate the intracellular homeostasis of cholesterol, neutral lipids and fatty acids; 2) regulate glucose metabolism by enhancing glycogenesis and attenuating gluconeogenesis; and 3) stimulate clearance of xenobiotic and endobiotic toxins by activating transcription of numerous xenobiotic detoxification genes [168 - 172]. All these health-improving, beneficial metabolic effects of bile acids prevent the development of obesity following administration of high-fat diet [168 - 170]. Thus, bile acids have a great potential as pharmaceutical agents for the treatment of diabetes, obesity and various associated metabolic disorders, all of which are age-related [168, 169]. Moreover, bile acids have been shown to inhibit neuronal apoptosis in experimental rodent models of neurodegenerative disorders by promoting mitochondrial membrane stability, preventing the release of cytochrome c from mitochondria, reducing activities of various caspases, and activating the NF-κB, PI3K and MAPK survival pathways [172, 173].

It should be emphasized that many of the metabolic, stress response and apoptotic processes modulated by bile acids in mammals are essential for healthy aging and longevity regulation. Importantly, we found that, by modulating several of these health- and longevity-related processes in chronologically aging yeast, LCA increases their life span [167]. Moreover, the long-lived $Ghrhr^{lit/lit}$ mice displayed elevated levels of several

bile acids and exhibited increased FXR-dependent transcription of numerous xenobiotic detoxification genes; if administered to food consumed by wild-type mice, cholic acid - one of these bile acids - mimicked the FXR-governed gene expression pattern observed in $Ghrhr^{lit/lit}$ mice [174, 175]. It has been therefore proposed that, by promoting chemical hormesis in mammals, these mildly toxic molecules with detergent-like properties may extend their longevity by acting as endobiotic regulators of aging [175 - 177].

Altogether, these findings support the notion that bile acids act as endobiotic and xenobiotic regulators of aging that are beneficial to health and longevity across phyla. Our comparative analysis of the mechanisms underlying such health-improving and life-extending action of bile acids implies that these mechanisms are likely to be evolutionarily conserved.

2.6 Conclusions

Our high-throughput chemical genetic screen identified LCA, a bile acid, as a novel anti-aging compound that extends yeast chronological life span under CR conditions by targeting lipid metabolism and modulating housekeeping longevity assurance pathways. The results of our pharmacophore modeling of the anti-aging potential of various species of bile acids imply that the maintenance of the minimal polarity of both the hydrophilic (concave) and hydrophobic (convex) sides of the steroid nucleus is mandatory for the extreme life-extending efficacy of LCA under CR conditions. Such stringent structural requirements are consistent with a target specificity of LCA action as an anti-aging small molecule. Moreover, we found that the life-extending efficacy of LCA under CR exceeds

that under non-CR conditions, being inversely proportional to the concentration of glucose in growth medium and thus in correlation with the extent of calorie supply limitation. We therefore concluded that LCA delays aging of chronologically aging yeast by modulating housekeeping longevity assurance processes. Our other published data imply that these processes are not regulated by the adaptable TOR and cAMP/PKA signaling pathways. We expect that this knowledge will be instrumental for defining molecular mechanisms underlying the ability of LCA to extend longevity by impacting these various housekeeping longevity assurance processes and by modulating signaling pathways governing them.

3 Interspecies chemical signals released into the environment may create xenohormetic, hormetic and cytostatic selective forces that drive the evolution of longevity regulation mechanisms within ecosystems: A hypothesis

3.1 Abstract

In our recent chemical genetic screen, we identified lithocholic acid (LCA) as a novel anti-aging molecule that extends yeast longevity. Yeast do not synthesize this or any other bile acids produced by mammals [168, 169]; a recent mass spectrometry-based analysis of the total yeast lipidome conducted in Dr. Titorenko's laboratory has confirmed lack of endogenous bile acids. Noteworthy, various organisms (*i.e.*, bacteria, fungi, plants and animals) within an ecosystem can synthesize and release into the environment certain longevity-extending small molecules. In this chapter, we hypothesize that these interspecies chemical signals can create xenohormetic, hormetic and cytostatic selective forces driving the ecosystemic evolution of longevity regulation mechanisms. In our hypothesis, following their release into the environment by one species of the organisms composing an ecosystem, such small molecules can activate anti-aging processes and/or inhibit pro-aging processes in other species within the ecosystem. The organisms that possess the most effective (as compared to their counterparts of the same species) mechanisms for sensing the chemical signals produced and released by other species and for responding to such signals by undergoing certain hormetic and/or cytostatic life-extending changes to their metabolism and physiology are expected to live longer than their counterparts within the ecosystem. We therefore propose that the ability

of a species of the organisms composing an ecosystem to undergo life-extending metabolic or physiological changes in response to hormetic or cytostatic chemical compounds released to the ecosystem by other species: 1) increases its chances of survival; 2) creates selective forces aimed at maintaining such ability; and 3) enables the evolution of longevity regulation mechanisms.

3.2 Introduction

Aging of multicellular and unicellular eukaryotic organisms is a highly complex biological phenomenon, which affects numerous processes within cells [178 - 181]. These cellular processes include cell cycle, cell growth, stress response, protein folding, apoptosis, autophagy, proteasomal protein degradation, actin organization, signal transduction, nuclear DNA replication, chromatin assembly and maintenance, ribosome biogenesis and translation, lipid and carbohydrate metabolism, oxidative metabolism in mitochondria, NAD^+ homeostasis, amino acid biosynthesis and degradation, and ammonium and amino acid uptake [182 - 186]. Across phyla, such plethora of longevity-defining processes is governed by a nutrient signaling network integrating the AMP-activated protein kinase/target of rapamycin (AMPK/TOR), cAMP/protein kinase A (cAMP/PKA) and insulin/insulin-like growth factor 1 (IGF-1) pathways, as well as a sirtuin-governed protein deacetylation module [179, 182, 184, 187 – 189]. Because this evolutionarily conserved signaling network regulates longevity only in response to certain changes in the organismal and intracellular nutrient and energy status, it is "adaptable" by its nature [167]. By altering the organismal and intracellular nutrient and energy status, caloric restriction (CR) and dietary restriction (DR) extend longevity and

improve health across species by modulating the adaptable longevity network [179, 190 - 194]. Unlike signaling pathways and sirtuin-governed protein deacetylation module integrated into the adaptable longevity network, some longevity-defining pathways are "constitutive" or "housekeeping" by their nature as they regulate longevity irrespective of the organismal and intracellular nutrient status [167].

It should be stressed that both adaptable and housekeeping longevity pathways are the targets of longevity-extending and health-improving small molecules that are produced and then released into the environment by various organisms (*i.e.*, bacteria, fungi, plants and animals) within an ecosystem. We therefore propose a hypothesis in which interspecies chemical signals released into the environment create xenohormetic, hormetic and cytostatic selective forces that empower the ecosystemic evolution of longevity regulation mechanisms.

3.3 Hypothesis

3.3.1 Plants and other autotrophs release into the environment xenohormetic and cytostatic interspecies chemical signals that extend longevity of other organisms within an ecosystem

According to the "xenohormesis" hypothesis of Howitz and Sinclair, in response to various hormetic environmental stresses - such as UV light, dehydration, infection, predation, cellular damage and nutrient deprivation - plants and other autotrophic organisms synthesize a group of secondary metabolites called xenohormetic phytochemicals [195 - 197]. Prior to being released into the environment, these secondary

metabolites activate defense systems protecting the host autotrophic organisms against hormetic environmental stresses that caused their synthesis [196, 197]. After being released into the environment, these xenohormetic phytochemicals provide benefits to health and longevity of heterotrophic organisms within the ecosystem. It was proposed that xenohormetic phytochemicals cause such life-extending and health-improving effects not by operating as mildly toxic hormetic molecules, but by activating the key enzymes of stress-response, anti-aging pathways known to govern longevity-related processes in heterotrophic organisms [195 - 197]. Recent studies revealed that some xenohormetic phytochemicals, such as resveratrol and caffeine, extend longevity of heterotrophic organisms by attenuating the adaptable TOR signaling pathway known to accelerate their aging [198 - 202]. Because the TOR pathway also plays a pivotal role in promoting proliferative growth of all heterotrophic organisms, resveratrol and caffeine exhibit a cytostatic effect in these organisms [201 - 204].

By extending the xenohormesis hypothesis of Howitz and Sinclair, we propose that within each of the heterotrophic species composing an ecosystem there are organisms that 1) possess the most effective (as compared to their counterparts of the same species) mechanisms for sensing xenohormetic and cytostatic phytochemicals released into the environment by autotrophic species; and 2) can respond to these phytochemicals by activating the key enzymes of stress-response, anti-aging pathways and/or by attenuating the adaptable, pro-aging TOR signaling pathway - thereby undergoing life-extending changes to their metabolism and physiology. These heterotrophic organisms are expected to live longer than their counterparts within the same species. Thus, their ability to sense

the longevity-extending xenohormetic and cytostatic phytochemicals released into the environment by autotrophic species and to respond to these phytochemicals by undergoing certain life-extending metabolic and physiological changes will: 1) increase their chances of survival; 2) create selective forces aimed at maintaining such ability; and 3) power the evolution of their longevity regulation mechanisms.

3.3.2 Mammals release into the environment bile acids, hormetic interspecies chemical signals that extend longevity of yeast and perhaps of other organisms within an ecosystem

In mammals, bile acids operate not only as trophic factors for the enteric epithelium and detergents for the emulsification and absorption of dietary lipids, but also as signaling molecules regulating lipid, glucose and energy homeostasis and activating detoxification of xenobiotics [205 - 210]. Bile acids have been shown to cause numerous health-improving metabolic effects in mammals and to protect them from xenobiotic toxins [205 - 210]. Therefore, it was proposed that by promoting chemical hormesis in mammals, bile acids – mildly toxic molecules with detergent-like properties – may extend their longevity by acting as endobiotic regulators of aging [211 - 214].

Importantly, our recent study identified lithocholic acid, a bile acid, as an anti-aging compound that extends yeast longevity by activating a compendium of anti-aging processes and attenuating a distinct set of pro-aging processes [167]. Unlike mammals, yeast do not synthesize bile acids [167, 209, 215]. We therefore propose that bile acids released into the environment by mammals may act as interspecies chemical signals

extending longevity of yeast species and, perhaps, of other organisms that can: 1) sense these mildly toxic molecules with detergent-like properties; and 2) respond to the resulting mild cellular damage by developing the most efficient stress protective mechanisms. We hypothesize that such mechanisms may provide effective protection of yeast and other organisms not only against cellular damage caused by bile acids but also against molecular and cellular damage accumulated with age. Thus, those species of the organisms within an ecosystem that that have been selected for the most effective (as compared to their counterparts of the same species) mechanisms providing protection against bile acids are expected to 1) live longer than their counterparts within the same species; and 2) evolve the most effective anti-aging mechanisms that are sensitive to regulation by bile acids. Thus, the ability of certain non-mammalian species within an ecosystem to sense bile acids produced by mammals and then to respond by undergoing certain longevity-extending changes to their physiology will increase their chances of survival - thereby creating selective force aimed at maintaining such ability and driving the evolution of their longevity regulation mechanisms.

3.3.3 Soil bacteria release into the environment rapamycin, a cytostatic interspecies chemical signal that extends longevity of other organisms within an ecosystem

The adaptable TOR signaling pathway can be attenuated not only by resveratrol and spermidine - the two longevity-extending xenohormetic and cytostatic phytochemicals released into the environment by autotrophic species – but also by rapamycin [203, 216, 217]. Rapamycin - a macrocyclic lactone synthesized by soil bacteria to inhibit growth of

fungal competitors - extends longevity of yeast, fruit flies and mice by specifically inhibiting the nutrient-sensory protein kinase TOR, a master negative regulator of the pro-aging TOR signaling pathway [203, 217 - 220].

Rapamycin exhibits a potent cytostatic effect by causing G1 cell cycle arrest and greatly delaying proliferative growth of organisms across phyla [203, 216 - 218]. We therefore hypothesize that rapamycin released into the environment by soil bacteria not only suppresses growth of fungal competitors, but may also may create selective pressure for the evolution of yeast, fruit fly and mammalian species that can respond to rapamycin-induced growth retardation by developing certain mechanisms aimed at such remodeling of their anabolic and catabolic processes that would increase their chances of survival under conditions of slow growth. We propose that some of these mechanisms delay aging by optimizing essential longevity-related processes and remain sensitive to modulation by rapamycin. Therefore, the ability of yeast, fruit fly and mammalian species composing an ecosystem to undergo life-extending metabolic or physiological changes in response to rapamycin produced by soil bacteria will: 1) increase their chances of survival; 2) create selective forces aimed at maintaining such ability; and 3) empower the evolution of longevity regulation mechanisms.

3.3.4 A hypothesis of the xenohormetic, hormetic and cytostatic selective forces that drive the ecosystemic evolution of longevity regulation mechanisms

Our analysis of how several small molecules synthesized and released into the environment by one species of the organisms composing an ecosystem extend longevity

of other species within this ecosystem suggests a hypothesis in which these interspecies chemical signals create xenohormetic, hormetic and cytostatic selective forces that impel the ecosystemic evolution of longevity regulation mechanisms. In our hypothesis, after being released into the environment by one species of organisms capable of synthesizing such small molecules, they can activate anti-aging processes and/or inhibit pro-aging processes in other species within an ecosystem (Figure 3.1). Within each of these other species, there are organisms that possess the most effective (as compared to their counterparts of the same species) mechanisms for sensing the interspecies chemical signals and for responding to such signals by undergoing certain life-extending changes to their metabolism and physiology; such life-extending changes could be hormetic and/or cytostatic by their nature. These organisms therefore are expected to live longer than their counterparts of the same species within the ecosystem. Thus, the ability of a species of the organisms composing an ecosystem to sense the longevity-modulating interspecies chemical signals released into the environment by other species within the ecosystem and to respond to these signals by undergoing certain life-extending metabolic and physiological changes is expected to increase its chances to survive, thereby creating selective force aimed at maintaining such ability.

Our hypothesis implies that the evolution of longevity regulation mechanisms in each species of the organisms composing an ecosystem is driven by the ability of this species to undergo specific life-extending metabolic or physiological changes in response to hormetic or cytostatic chemical compounds that are released to the ecosystem by other species (Figure 3.1).

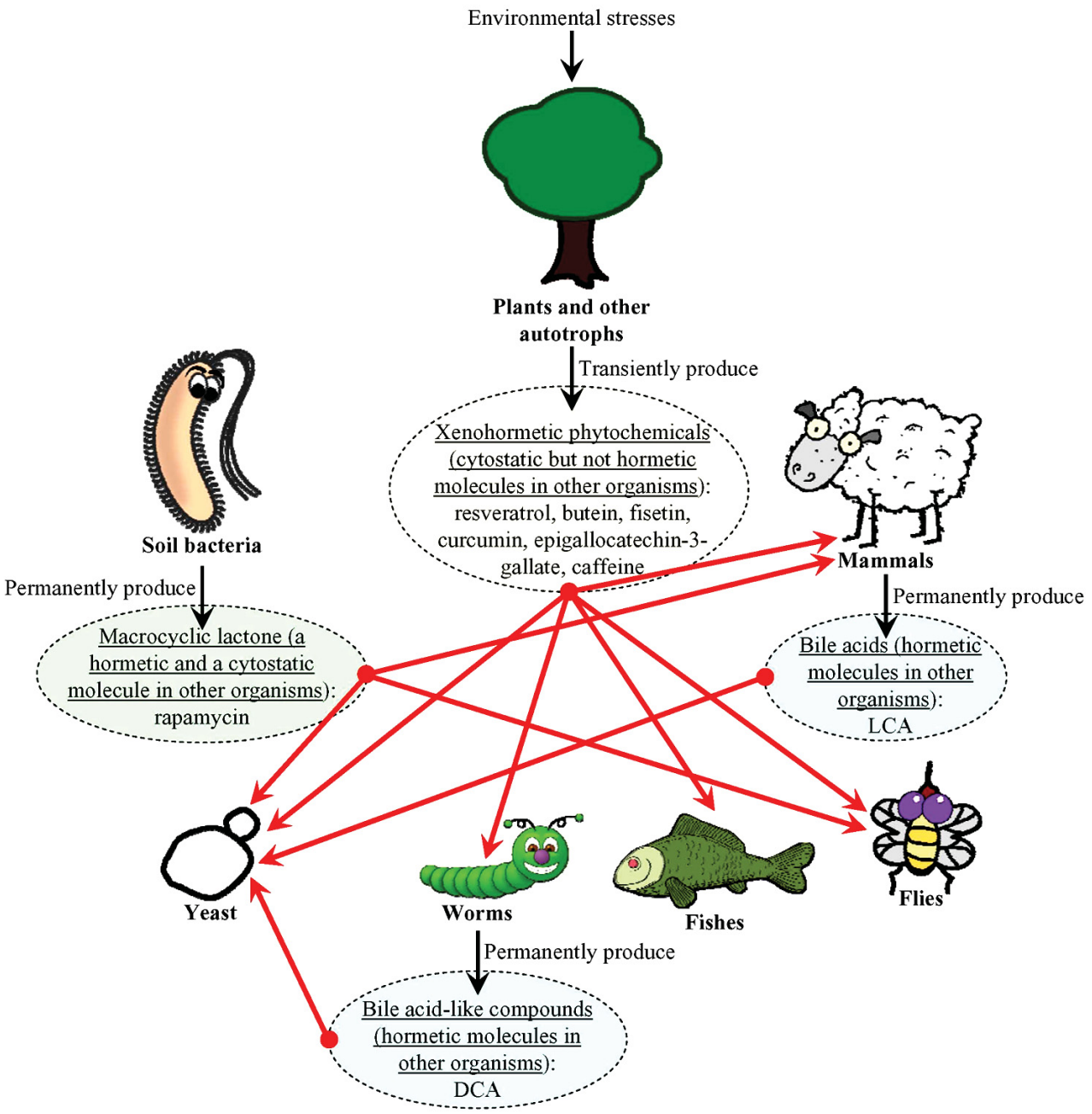

Figure 3.1. The xenohormetic, hormetic and cytostatic selective forces may drive the evolution of longevity regulation mechanisms within an ecosystem. We propose that organisms from all domains of life within an ecosystem synthesize chemical compounds that 1) are produced and then released into the environment permanently or only in response to deteriorating environmental conditions, increased population density of competitors and/or predators, or changes in food availability and its nutrient and/or

caloric content; 2) are mildly toxic compounds that trigger a hormetic response in an organism that senses them or, alternatively, are not toxic for any organism within the ecosystem and do not cause a hormetic response; 3) are cytostatic compounds that attenuate the TOR-governed signaling network or, alternatively, do not modulate this growth-promoting network; and 4) extend longevity of organisms that can sense these compounds (red arrows), thereby increasing their chances of survival and creating selective force aimed at maintaining the ability of organisms composing the ecosystem to respond to these compounds by undergoing specific life-extending changes to their physiology. In our hypothesis, the evolution of longevity regulation mechanisms in each group of the organisms composing an ecosystem is driven by the ability of this group of organisms to undergo specific life-extending changes to their physiology in response to a compendium of "critical" chemical compounds that are permanently or transiently released to the ecosystem by other groups of organisms. Abbreviations: LCA, lithocholic acid; DCA, bile acid-like dafachronic acids.

3.4 Conclusions

Our analysis of how LCA and other small anti-aging molecules (including resveratrol, caffeine and rapamycin) synthesized and released into the environment by one species of the organisms composing an ecosystem extend longevity of many other species within this ecosystem let us to propose a unified hypothesis of the xenohormetic, hormetic and cytostatic selective forces that drive the evolution of longevity regulation mechanisms at the ecosystemic level. In this hypothesis, the ability of a species of the organisms composing an ecosystem to undergo life-extending metabolic or physiological changes in response to hormetic or cytostatic chemical compounds released to the ecosystem by other species: 1) increases its chances of survival; 2) creates selective forces aimed at maintaining such ability; and 3) enables the evolution of longevity regulation mechanisms.

4 Using the experimental evolution of long-lived yeast species for testing our hypothesis of xenohormetic, hormetic and cytostatic forces driving the evolution of longevity regulation mechanisms within ecosystems

4.1 Abstract

In Chapter 3 of this Master's thesis, we proposed that bile acids released into the environment by mammals may act as interspecies chemical signals providing longevity benefits to yeast and, perhaps, other species within an ecosystem. We hypothesized that, because bile acids are known to be mildly toxic compounds, they may create selective pressure for the evolution of yeast species that can respond to the bile acids-induced mild cellular damage by developing the most efficient stress protective mechanisms. It is likely that such mechanisms may provide effective protection of yeast against molecular and cellular damage accumulated with age. Thus, we proposed that yeast species that have been selected for the most effective mechanisms providing protection against bile acids may evolve the most effective anti-aging mechanisms that are sensitive to regulation by bile acids. We extended our hypothesis on longevity regulation by bile acids by suggesting a hypothesis of the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms at the ecosystemic level. To verify our hypothesis empirically, we carried out the LCA-driven multistep selection of long-lived yeast species under laboratory conditions. We found that a lasting exposure of wild-type yeast to LCA results in selection of yeast species that live longer in the absence of this bile acid than their ancestor. Our data enabled to rank different concentrations of LCA with respect to the efficiency with which they cause the

appearance of long-lived yeast species. We revealed that, if used at the most efficient concentration of 5 μM, this bile acid induced life-extending mutations with the frequency of about 4×10^8/generation. At the concentration of 50 μM, LCA caused the appearance of long-lived species with the frequency of 1×10^8/generation, whereas a lasting exposure of yeast to 250 μM LCA did not result in selection of such species. Because the lowest used concentration of LCA resulted in the highest frequency of long-lived species appearance, we believe that it is unlikely that the life-extending mutations they carry are due to mutagenic action of this bile acid.

We found that the evolved under laboratory conditions long-lived yeast species 3, 5 and 12 were able to maintain their greatly extended CLS following numerous successive passages in medium without LCA under CR conditions. Our data imply that each of these long-lived yeast species also exhibited extended CLS when cultured in the absence of LCA under non-CR conditions (although to a lesser extent than that under CR conditions). Thus, consistent with its sought-after effect on longevity regulation pathways, our LCA-driven multistep selection process under laboratory conditions yielded long-lived yeast species whose greatly delayed chronological aging was caused by the selection of a mutant allele or alleles that activate so-called housekeeping longevity assurance pathways. These housekeeping longevity assurance pathways modulate longevity irrespective of the number of available calories and do not overlap with the adaptable longevity pathways that are under the stringent control of calorie availability [167].

4.2 Introduction

We recently found that LCA greatly (and some other bile acids to a lesser degree) increases the chronological life span of yeast under caloric restriction (CR) conditions [167]. Our findings provided evidence that LCA extends longevity of chronologically aging yeast through two different mechanisms (Figure 4.1). In one mechanism, this bile acid targets longevity pathways that control chronological aging irrespective of the number of calories available to yeast. Because these pathways modulate longevity regardless of calorie availability, we called them "constitutive" or "housekeeping" (see chapter 1 and 2) [167]. LCA modulates these housekeeping longevity assurance pathways by suppressing lipid-induced necrosis, attenuating mitochondrial fragmentation, altering oxidation-reduction processes in mitochondria, enhancing resistance to oxidative and thermal stresses, suppressing mitochondria-controlled apoptosis, and enhancing stability of nuclear and mitochondrial DNA ([167]; Figure 4.1C). The housekeeping longevity pathways do not overlap with the TOR (target of rapamycin) and cAMP/PKA (cAMP/protein kinase A) signaling pathways ([167]; Figure 4.1A), both of which are "adaptable" by nature because they are under the stringent control of calorie and/or nutrient availability ([4, 7, 10, 36, 221, 222]; Figure 4.1B). In the other mechanism, LCA targets the adaptable cAMP/PKA pathway by unmasking an anti-aging potential of PKA under non-CR conditions, perhaps by activating PKA-dependent phosphorylation of the cytosolic pool of the key nutrient-sensory protein kinase Rim15p (see chapters 1 and 2) [167]. The phosphorylation of Rim15p by PKA inactivates its protein kinase activity [58]. Hence, the LCA-driven inactivation of Rim15p may reduce the phosphorylation status of its known [223] target proteins in the cytosol, thereby lowering their pro-aging

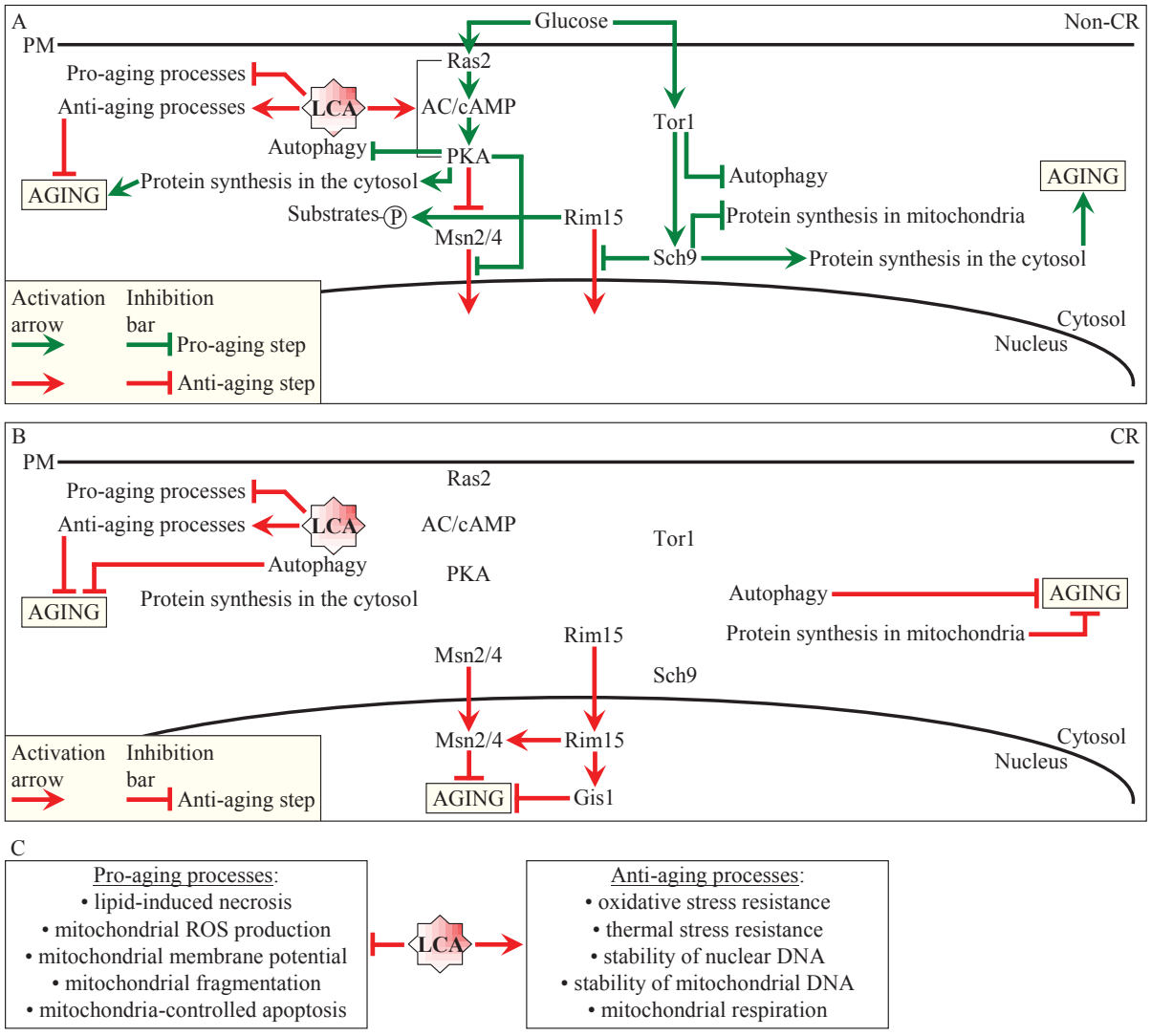

Figure 4.1. Lithocholic acid (LCA) extends longevity of chronologically aging yeast through two different mechanisms. (A and B) Outline of pro- and anti-aging processes that are controlled by the TOR and/or cAMP/PKA signaling pathways and are modulated by LCA in yeast cells grown under non-CR (A) or CR (B) conditions. Activation arrows and inhibition bars denote pro-aging (displayed in green color) or anti-aging (displayed in red color) processes. Under both non-CR and CR conditions, LCA targets housekeeping longevity assurance processes listed in (C). Under non-CR conditions only, LCA also targets the adaptable cAMP/PKA pathway. By activating PKA-dependent phosphorylation of the cytosolic pool of the key nutrient-sensory protein kinase Rim15p, LCA causes the inactivation of Rim15p. The resulting reduction of the phosphorylation

status of several Rim15p target proteins in the cytosol lowers their pro-aging efficacy. Abbreviations: CR, caloric restriction; PM, plasma membrane.

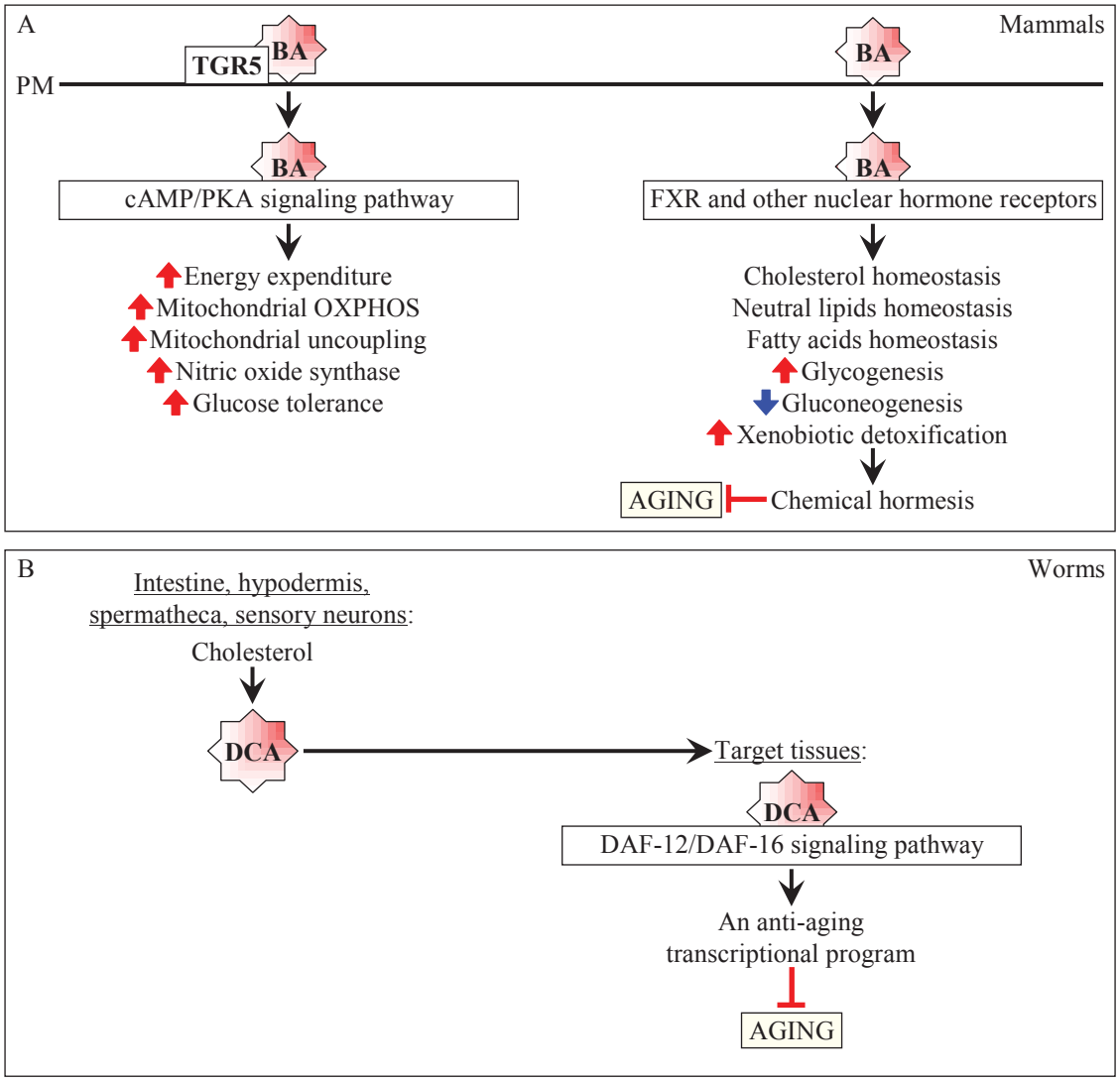

Figure 4.2. Bile acids are beneficial to health and longevity in animals. (A) In mammals, bile acids (BA) function not only as trophic factors for the enteric epithelium and detergents for the emulsification and absorption of dietary lipids, but also as signaling molecules that regulate lipid, glucose and energy homeostasis and activate detoxification of xenobiotics. By improving overall health, BA may delay the onset of age-related

diseases and have beneficial effect on longevity. By activating transcription of numerous xenobiotic detoxification genes and thus promoting chemical hormesis, BA may extend their longevity by acting as endobiotic regulators of aging. (B) In worms, following their synthesis from cholesterol in the intestine, hypodermis, spermatheca and sensory neurons, bile acid-like dafachronic acids (DCA) are delivered to other tissues where they activate the DAF-12/DAF-16 signaling cascade, thereby orchestrating an anti-aging transcriptional program and increasing the life span of the entire organism.

efficacy ([167]; Figure 4.1A). Although bile acids in mammals have been traditionally considered only as trophic factors for the enteric epithelium and detergents for the emulsification and absorption of dietary lipids [168 - 170], they are now also recognized for their essential role as signaling molecules regulating lipid, glucose and energy homeostasis and activating detoxification of xenobiotics ([168 - 173]; Figure 4.2A). Many of the numerous health-improving metabolic effects caused by bile acids and their demonstrated ability to protect mammals from xenobiotic toxins ([169 - 173]; Figure 4.2A) suggest that, by improving overall health, these amphipathic molecules may delay the onset of age-related diseases and have beneficial effect on longevity. Furthermore, because of the elevated levels of several bile acids in the long-lived $Ghrhr^{lit/lit}$ mice and due to the ability of cholic acid administered to food of wild-type mice to activate transcription of numerous xenobiotic detoxification genes, it has been proposed that, by promoting chemical hormesis in mammals, these mildly toxic molecules with detergent-like properties may extend their longevity by acting as endobiotic regulators of aging [174 - 177]. Moreover, bile acid-like dafachronic acids (including 3-keto-LCA) in worms function as endocrine regulators of aging by activating an anti-aging transcriptional program governed by the DAF-12/DAF-16 signaling cascade ([157, 224, 225]; Figure

4.2B). Altogether, these findings support the notion that bile acids are beneficial to health and longevity in animals because of their ability to operate as potent signaling molecules that modulate a compendium health- and longevity-related processes. Noteworthy, by modulating many of these processes also in yeast, LCA extends their longevity [167]. It is likely therefore that the life-extending capacity of LCA and other bile acids as well as, perhaps, the mechanisms underlying their anti-aging action are conserved across animal species and other phyla.

4.3 Materials and Methods

Strains and media

The wild type strain *Saccharomyces cerevisiae* BY4742 (*MATα, his3Δ leu2Δ0 lys2Δ ura3Δ0)* was cultured in liquid YP medium (1% yeast extract, 2% peptone) contained 0.2% glucose as a carbon source, with or without LCA, as detailed in the "Results" section. Cell cultures were incubated at 30°C with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 5:1. For a spot assay of cell viability, serial 10-fold dilutions of cell cultures were spotted onto plates with solid YP medium (1% yeast extract, 2% peptone) containing 2% glucose.

4.4 Results

4.4.1 The LCA-driven multistep selection of long-lived yeast species under laboratory conditions

To empirically verify our hypothesis of the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms at the

ecosystemic level, we carried out a 3-step selection of long-lived yeast species by a lasting exposure to LCA under laboratory conditions. For the first step of such selection, yeast cells from an overnight culture were inoculated into a fresh YP medium (with or without LCA) containing 0.2% glucose to the initial cell titre of 1×10^5 cells/ml (Figure 4.3). After one week of the incubation in this medium and following approximately 45 cell generations, yeast entered into a non-proliferative state by reaching stationary (ST) growth phase at the cell titre of 2×10^8 cells/ml (Figure 4.5). Following their entry into a non-proliferative state, yeast cells were cultured for additional 5 weeks. By the end of this cultivation, only ~ 0.02% of cells in the medium without LCA remained viable. In the cell cultures that were supplemented with LCA, ~ 1% of cells remained viable – due to the ability of LCA to increase the chronological life span (CLS) of non-dividing (senescent) yeast cells. Thus, the enrichment factor for long-lived mutants by the end of each selection step was 10^2 (Figure 4.5). Five cultures were used to carry out the selection of long-lived yeast species: the first culture lacked LCA; the second culture contained 5 μM LCA added at the moment of cell inoculation; the third culture contained 50 μM LCA added at the moment of cell inoculation; the fourth culture contained 250 μM LCA added at the moment of cell inoculation; and the fifth culture was supplemented with 10 doses of 5 μM LCA each by adding this bile acid every 3 or 4 days (Figure 4.6). At the end of the first selection step, aliquots of each of the five cultures were plated onto plates with solid YP medium containing 2% glucose. After 2 days, each of the 10 randomly chosen colonies was inoculated into a liquid medium without LCA to carry out a viability testing step (Figure 4.3). Every week, an aliquot of each culture was used for a spot assay of cell viability to identify long-lived mutants (if any) induced due to a lasting

exposure of yeast to LCA during the selection step (Figure 4.3). If such mutants were detected, an aliquot of their culture in medium lacking LCA was frozen at -80°C.

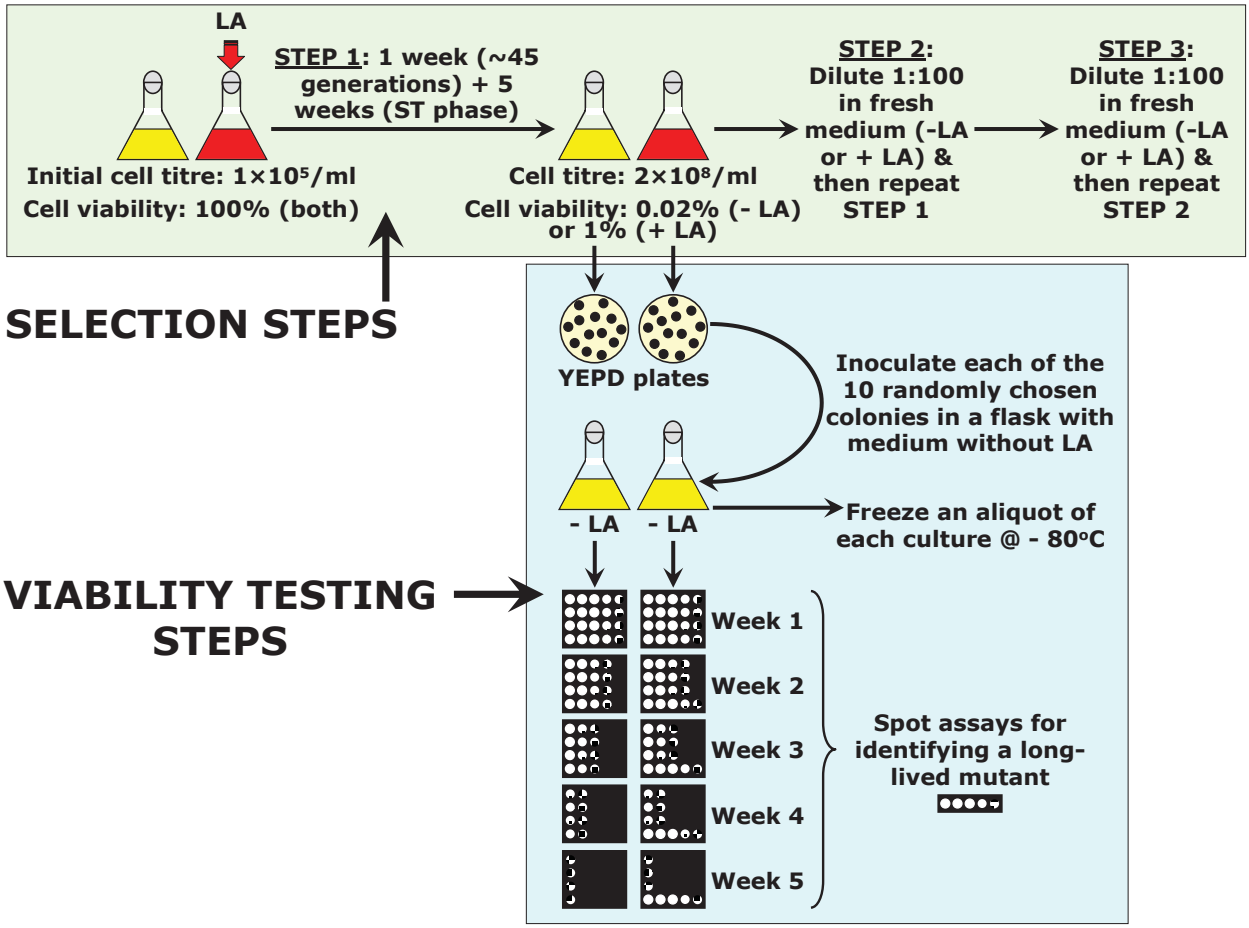

Figure 4.3. A 3-step process of the LCA-driven experimental evolution of longevity regulation mechanisms by conducting selection of long-lived yeast species through a lasting exposure to LCA. See text for details.

To conduct the second selection step, an aliquot of the culture recovered at the end of the first step of selection was diluted 100 folds in a fresh YP medium containing 0.2% glucose, with or without LCA; if added to a culture for the second selection step, LCA

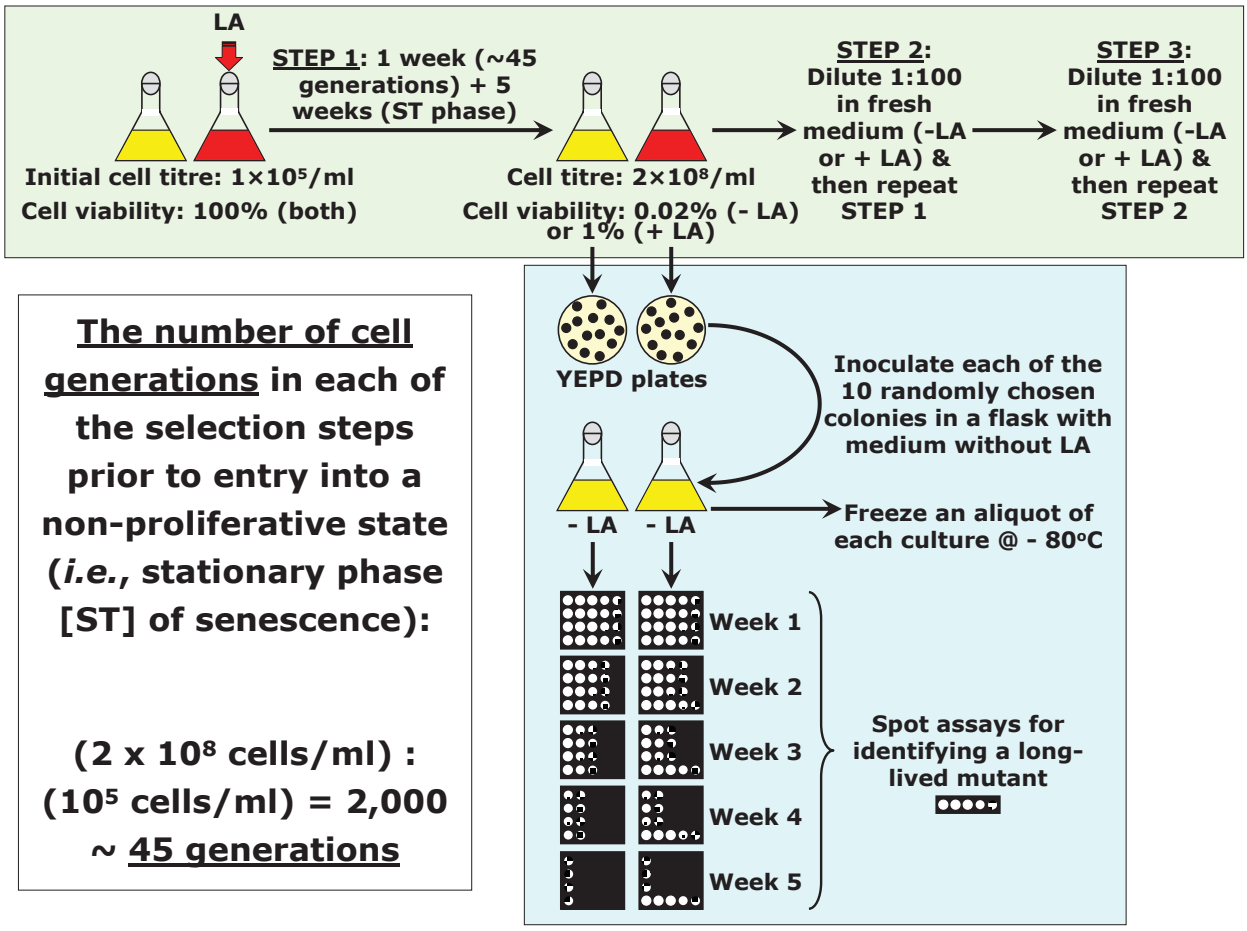

Figure 4.4. A 3-step process of the LCA-driven experimental evolution of longevity regulation mechanisms by conducting selection of long-lived yeast species through a lasting exposure to LCA. The number of cell generations in each of the selection steps prior to entry into a non-proliferative state is calculated. See text for details.

was present at the same final concentration as that in the medium used for the first step of selection (Figure 4.3). From that point, the second selection step was carried out exactly as the first one and was followed by a spot-assay viability step described above (Figure 4.3). The second selection step was followed by the third selection step and then by a spot-assay viability step, both conducted as described previously (Figure 4.3).

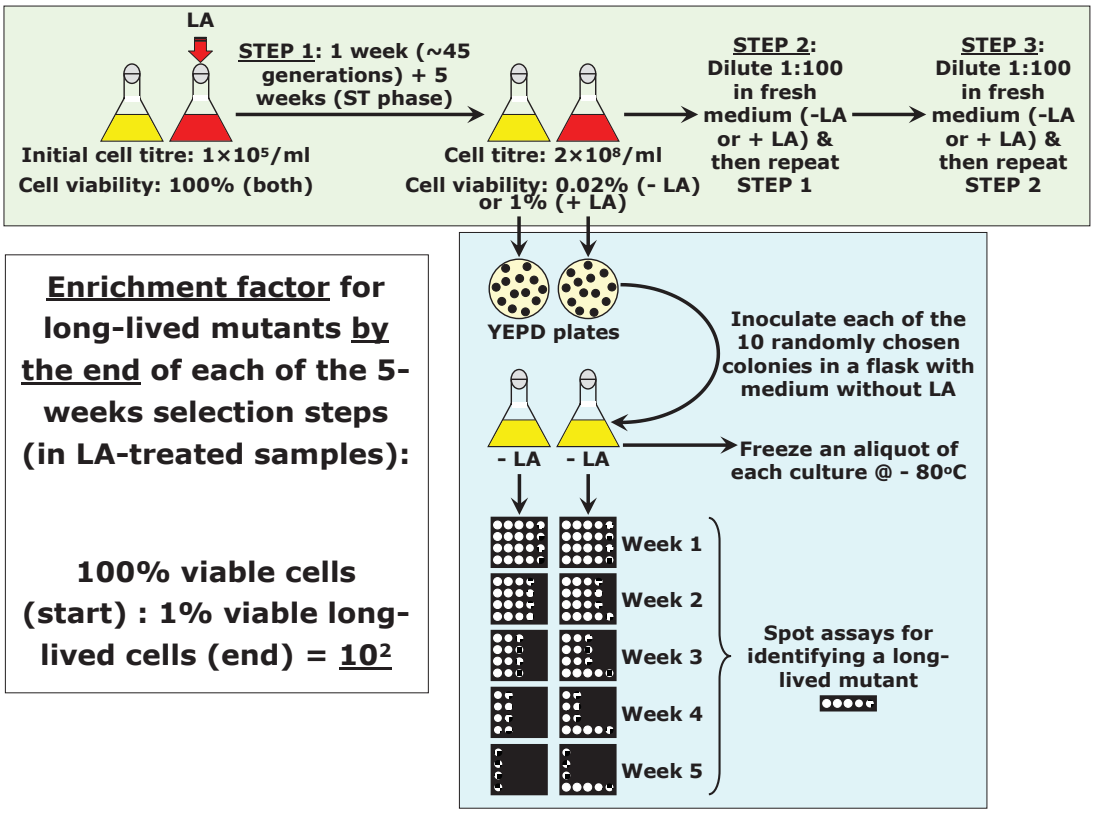

Figure 4.5. A 3-step process of the LCA-driven experimental evolution of longevity regulation mechanisms by conducting selection of long-lived yeast species through a lasting exposure to LCA. The enrichment factor for long-lived mutants by the end of each of the selection steps is calculated. See text for details.

The 3-step selection of long-lived yeast species depicted in Figures 4.3 to 4.6 was expected to result in the increased fraction of long-lived mutants in a population of yeast exposed to LCA (Figure 4.7). Moreover, the fraction of such long-lived mutants in a population was anticipated to progressively increase at each of the consecutive selection steps (Figure 4.7 and Table 4.1).

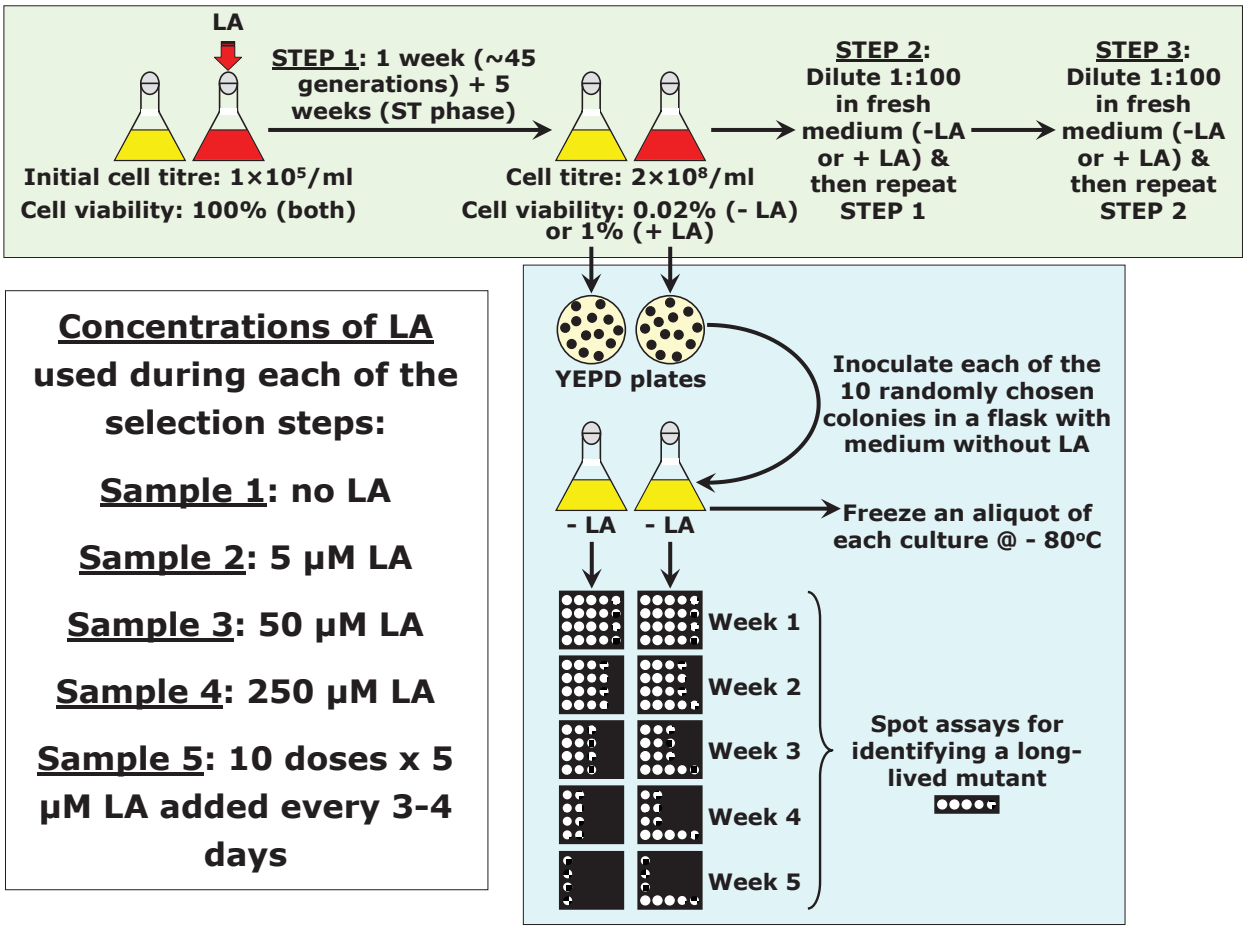

Figure 4.6. A 3-step process of the LCA-driven experimental evolution of longevity regulation mechanisms by conducting selection of long-lived yeast species through a lasting exposure to LCA. Concentrations of LCA used during each of the selection steps are shown. See text for details.

By carrying out this 3-step selection of long-lived yeast species under laboratory conditions, we found no such mutants at the end of the first selection step, 4 mutants at the end of the second selection step, and 16 mutants at the end of the third selection step (Figures 4.8, 4.90 and 4.10, respectively). We therefore concluded that a long-term exposure of wild-type yeast to LCA following the 3 selection steps did result in selection

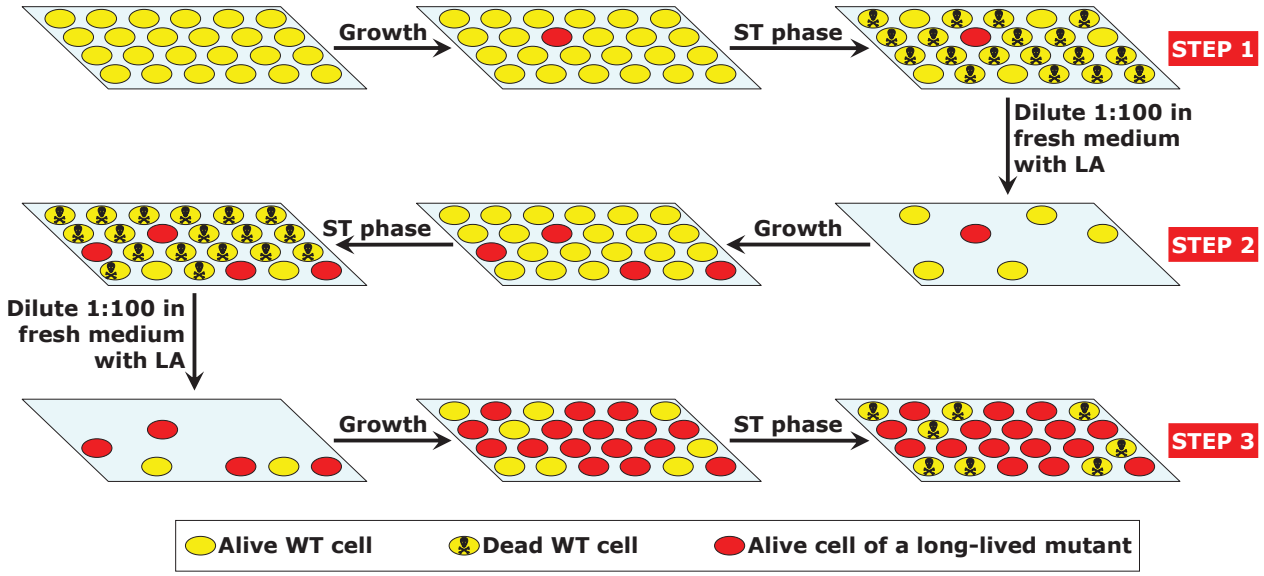

Figure 4.7. The fraction of long-lived mutants in a population of yeast is increased by the end of each of the 3 steps of the LCA-driven experimental evolution of longevity regulation mechanisms.

of yeast species that live longer in the absence of LCA than their ancestor. Our findings revealed the following order of different LCA concentrations ranked by the efficiency with which they cause the appearance of long-lived species (frequencies of such appearance are shown): 5 μM LCA (~4 × 10^{-8}/generation) > 10 doses x 5 μM LCA (~3 × 10^{-8}/generation) > 50 μM LCA (~1 × 10^{-8}/generation) > 250 μM LCA (no long-lived species found). It should be emphasized that, because the lowest used concentration of LCA resulted in the highest frequency of long-lived species appearance, it is unlikely that the life-extending mutations they carry are due to mutagenic action of LCA.

Table 4.1. The fraction of long-lived mutants in a population of yeast is increased by the end of each of the 3 steps of the LCA-driven experimental evolution of longevity regulation mechanisms.

If the frequency of mutations is:	Enrichment factor for long-lived mutants	The fraction of long-lived mutants & their number in a population of yeast by the end of each of the 3 steps of LA-driven experimental evolution	
		Fraction	Number
10^{-8}/ generation	10^2	STEP 1: $(10^{-8} \times 45 \text{ generations}) \times 10^2 = 4.5 \times 10^{-5}$	4-5 out of 100,000
		STEP 2: $[(4.5 \times 10^{-5}) + (10^{-8} \times 45 \text{ gen.})] \times 10^2 \sim 4.5 \times 10^{-3}$	4-5 out of 1,000
		STEP 3: $[(4.5 \times 10^{-3}) + (10^{-8} \times 45 \text{ gen.})] \times 10^2 \sim 4.5 \times 10^{-1}$	4-5 out of 10
10^{-7}/ generation	10^2	STEP 1: $(10^{-7} \times 45 \text{ generations}) \times 10^2 = 4.5 \times 10^{-4}$	4-5 out of 10,000
		STEP 2: $[(4.5 \times 10^{-4}) + (10^{-7} \times 45 \text{ gen.})] \times 10^2 \sim 4.5 \times 10^{-2}$	4-5 out of 100
		STEP 3: $[(4.5 \times 10^{-2}) + (10^{-7} \times 45 \text{ gen.})] \times 10^2 \sim 4.5 \times 10^{0}$	ALL
10^{-6}/ generation	10^2	STEP 1: $(10^{-6} \times 45 \text{ generations}) \times 10^2 = 4.5 \times 10^{-3}$	4-5 out of 1,000
		STEP 2: $[(4.5 \times 10^{-3}) + (10^{-6} \times 45 \text{ gen.})] \times 10^2 \sim 4.5 \times 10^{-1}$	4-5 out of 10
		STEP 3: $[(4.5 \times 10^{-1}) + (10^{-6} \times 30 \text{ gen.})] \times 10^2 \sim 4.5 \times 10^{1}$	ALL

4.4.2 Three long-lived yeast species evolved under laboratory conditions were able to maintain their greatly extended life spans following numerous passages in medium without LCA

The 3 steps of experimental evolution of long-lived yeast species by a prolonged exposure of wild-type yeast to LCA resulted in selection of 20 species that in a spot-assay lived longer than their ancestor in medium lacking LCA (see Figures 4.8 – 4.10). An aliquot of the culture of each of these long-lived yeast species was frozen at -80^{o}C immediately after being recovered during the second or third selection step. To test the

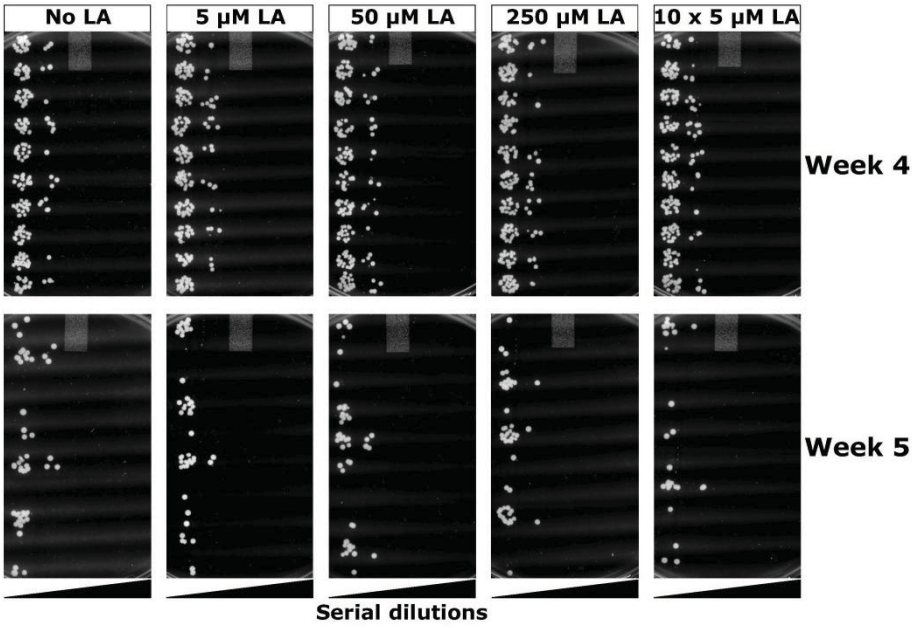

Figure 4.8. A spot-assay of cell survival for the 1st step of the LCA-driven experimental evolution of longevity regulation mechanisms.

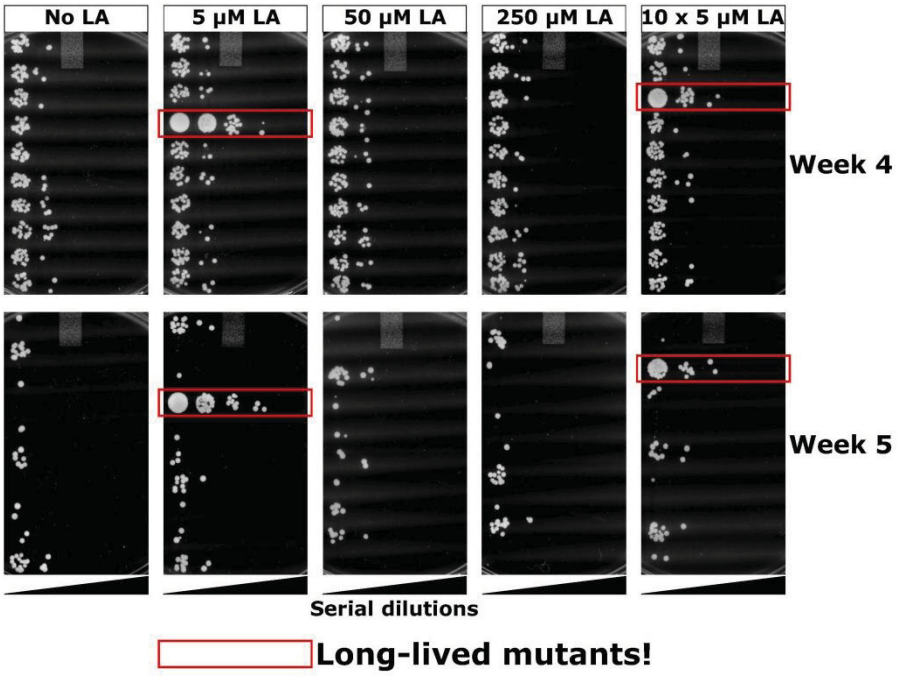

Figure 4.9. A spot-assay of cell survival for the 2nd step of the LCA-driven experimental evolution of longevity regulation mechanisms.

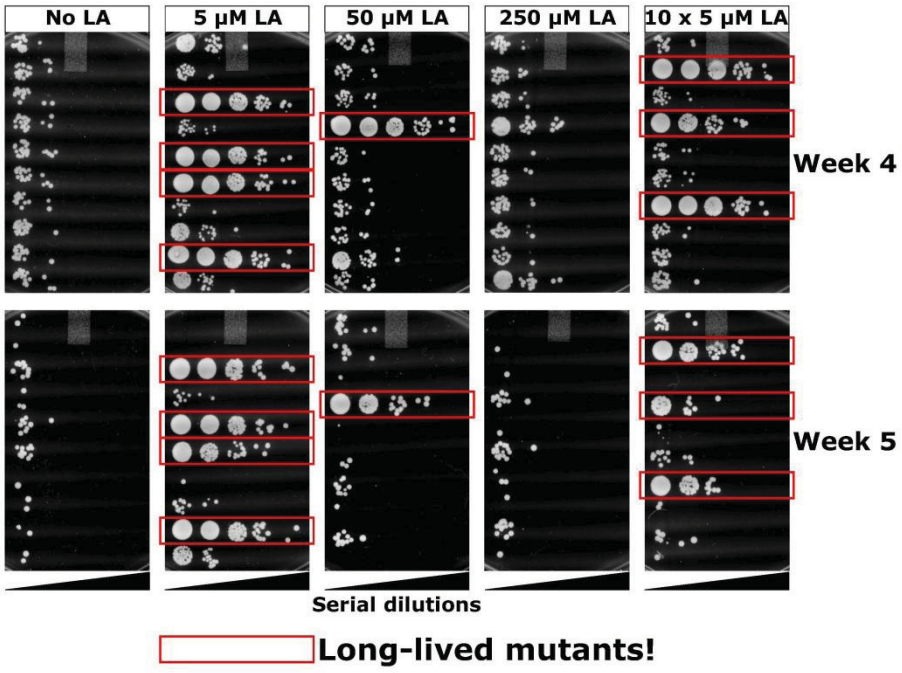

Figure 4.10. A spot-assay of cell survival for the 3rd step of the LCA-driven experimental evolution of longevity regulation mechanisms.

ability of these species to maintain their greatly extended life spans following the 1st passage in medium without LCA, each aliquot was thawed and then inoculated into liquid YP medium lacking this bile acid and containing 0.2% glucose. Our comparative analysis of the chronological life spans (CLS) of wild-type (WT) strain and all of these selected long-lived yeast species revealed that species 3, 5 and 12 maintained their ability to live much longer than WT following the 1st passage (Figure 4.11).

The selected long-lived yeast species 3, 5 and 12 underwent four more passages (each carried out as described for the 1st passage) in liquid medium without LCA and then tested again for their ability to maintain greatly extended life spans, now following five successive passages in medium without LCA. As we found, each of these three long-

lived yeast species evolved under laboratory conditions maintained its greatly extended life span following five passages in medium without LCA (Figure 4.12).

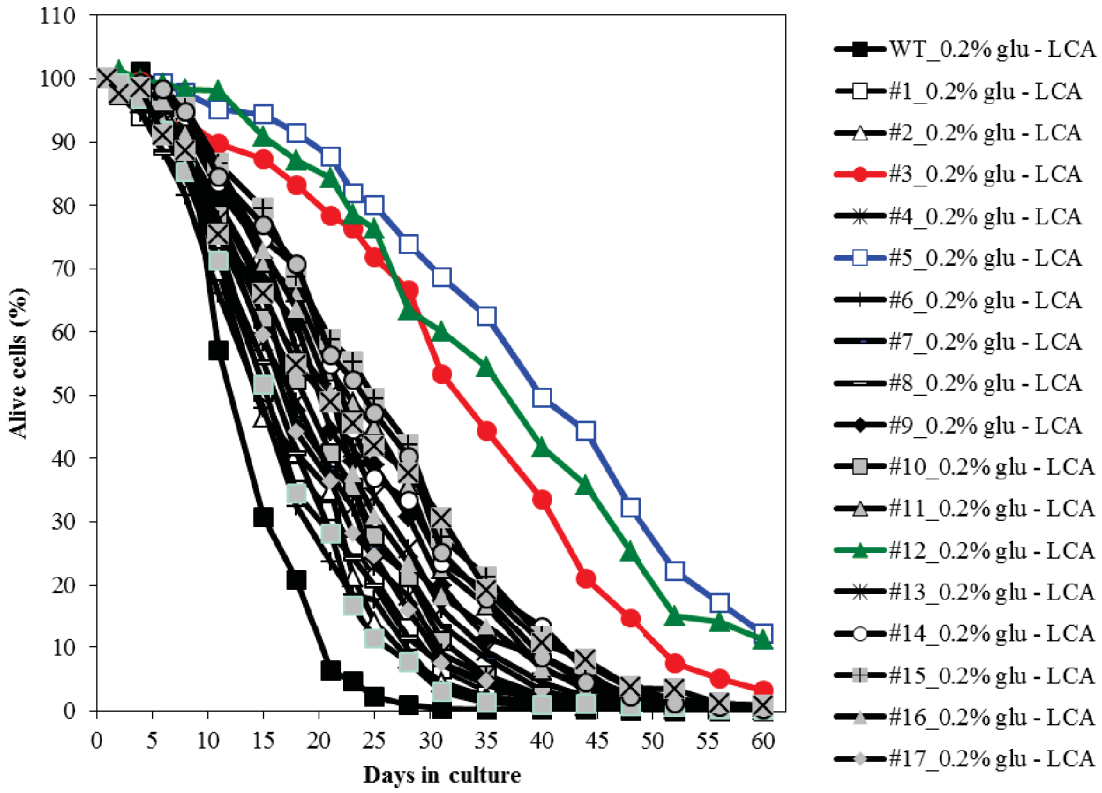

Figure 4.11. The selected long-lived yeast species 3, 5 and 12 maintain their ability to live much longer than WT following the 1st passage in medium lacking LCA. Survival of chronologically aging WT and of the yeast species 3, 5 and 12 cultured under CR conditions in liquid YP medium initially containing 0.2% glucose without LCA.

4.4.3 The selected under CR long-lived yeast species 3, 5 and 12 exhibit extended CLS when cultured in medium without LCA under non-CR conditions, although to a lesser extent than that under CR conditions

When the selected long-lived yeast species 3, 5 and 12 that underwent five successive

passages in medium without LCA under CR were cultured under non-CR conditions on 2% glucose in the absence of this bile acid, each of them exhibited extended CLS as compared to WT (Figure 4.13). For each of these three species, the efficacy of CLS extension under CR conditions exceeded that under non-CR conditions (Figure 4.13).

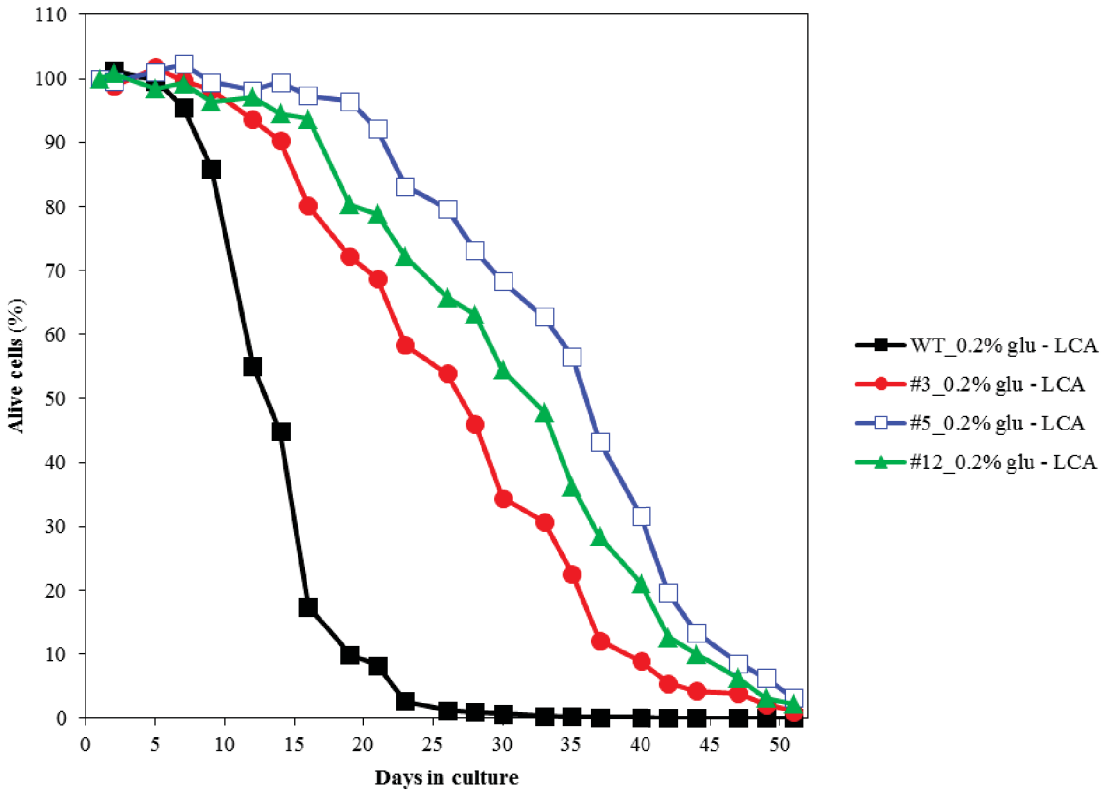

Figure 4.12. The selected long-lived yeast species 3, 5 and 12 maintain their ability to live much longer than WT following five successive passages in medium lacking LCA. Survival of chronologically aging WT and of the yeast species 3, 5 and 12 cultured under CR conditions in liquid YP medium initially containing 0.2% glucose without LCA.

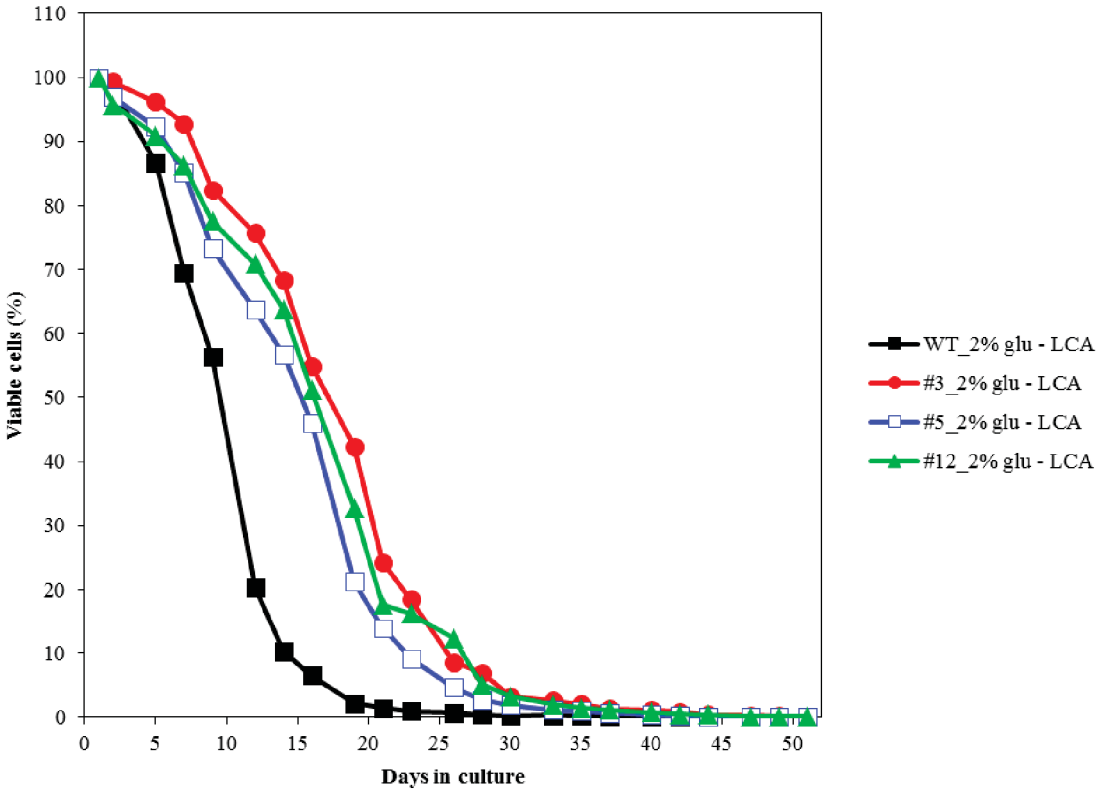

Figure 4.13. Following five successive passages in medium lacking LCA, the selected under CR long-lived yeast species 3, 5 and 12 exhibit extended CLS when cultured in medium without LCA under non-CR conditions - although to a lesser extent than that under CR conditions. Survival of chronologically aging WT and of the yeast species 3, 5 and 12 cultured under CR conditions in liquid YP medium initially containing 0.2% glucose without LCA.

4.5 Discussion

To verify empirically our hypothesis of the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms at the ecosystemic level, we carried out the LCA-driven multistep selection of long-lived yeast species under laboratory conditions. We found that a lasting exposure of WT yeast to LCA results in selection of yeast species that live significantly longer in the absence of this bile acid than their ancestor. Our data enabled to rank different concentrations of

LCA with respect to the efficiency with which they cause the appearance of long-lived yeast species. Because the lowest used concentration of LCA resulted in the highest frequency of long-lived species appearance, it seems unlikely that the life-extending mutations they carry are due to mutagenic action of this bile acid. The evolved under laboratory conditions long-lived yeast species 3, 5 and 12 were able to maintain their greatly extended CLS following numerous successive passages in medium without LCA under CR conditions. Importantly, each of these long-lived yeast species also exhibited extended CLS when cultured in the absence of LCA under non-CR conditions (although to a lesser extent than that under CR conditions). Thus, consistent with its sought-after effect on longevity regulation pathways, our LCA-driven multistep selection process under laboratory conditions yielded long-lived yeast species whose greatly delayed chronological aging was caused by the selection of a mutant allele or alleles that activate so-called housekeeping longevity assurance pathways. These housekeeping longevity assurance pathways modulate longevity irrespective of the number of available calories and do not overlap with the adaptable longevity pathways that are under the stringent control of calorie availability [167].

4.6 Conclusions

In experiments described in this Chapter, we verified empirically our hypothesis that the evolution of longevity regulation mechanisms within an ecosystem can be driven by the xenohormetic, hormetic and cytostatic selective forces created by a lasting exposure of an organism to an anti-aging natural compound released by other organisms composing this ecosystem. Indeed, a long-term exposure of wild-type yeast to LCA (a bile acid that

under field-like conditions is synthesized and released into the environment by mammals) results in selection of yeast species that live longer in the absence of this bile acid than their ancestor. To test the validity of other aspects of our hypothesis on the ecosystemic evolution of longevity regulation mechanisms, future studies need to address the following most critical questions: 1) what genes are affected by mutations responsible for the extended longevity of selected long-lived yeast species? 2) how these mutations influence a compendium of the housekeeping longevity-related processes modulated by LCA in chronologically aging yeast ([167]; Figure 4.1)?; 3) 3) will these mutations affect the growth rate of yeast in media with or without LCA? 4) will selected long-lived yeast species be able to maintain their ability to live longer than wild-type yeast if they undergo several successive passages in medium without LCA? - and, thus, is there selective pressure aimed at maintaining of an "optimal" rather than a "maximal" chronological life span of yeast (due to, *e.g.*, a proposed selective advantage of the envisioned "altruistic" program [226 - 231] of chronological aging in yeast)? and 5) if mixed with an equal number of wild-type yeast cells, will selected long-lived yeast species out-grow and/or out-live them in medium without LCA or the opposite will happen (due to selective pressure on yeast aimed at maintaining of the so-called "altruistic" program [226 - 231] of their chronological aging)?

5 A mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 affect a compendium of the housekeeping longevity-related processes modulated by LCA in chronologically aging yeast

5.1 Abstract

In experiments described in Chapter 4 of this Master's thesis, we verified empirically our hypothesis that the evolution of longevity regulation mechanisms within an ecosystem can be driven by the xenohormetic, hormetic and cytostatic selective forces created by a lasting exposure of an organism to an anti-aging natural compound released by other organisms composing this ecosystem. Specifically, we found that a long-term exposure of wild-type yeast to LCA (a bile acid that under field-like conditions is synthesized and released into the environment by mammals) results in selection of yeast species that live longer in the absence of this bile acid than their ancestor.

To test the validity of other aspects of our hypothesis on the ecosystemic evolution of longevity regulation mechanisms, we assessed how a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 influence a compendium of the housekeeping longevity-related processes that, as we revealed [167], are modulated by LCA in chronologically aging yeast. We found that in the absence of LCA this allele or alleles alter the age-dependent dynamics of mitochondrial respiration under both CR and non-CR conditions. This finding further confirms our conclusion (see Chapter 4 of this Master's thesis) that, consistent with its

sought-after effect on longevity regulation pathways, our LCA-driven multistep selection process under laboratory conditions yielded long-lived yeast species whose greatly delayed chronological aging was caused by the selection of a mutant allele or alleles that activate so-called housekeeping longevity assurance pathways. As we previously demonstrated, these housekeeping longevity assurance pathways modulate longevity irrespective of the number of available calories and do not overlap with the adaptable longevity pathways that are under the stringent control of calorie availability [167].

We also revealed that under CR conditions in the absence of LCA a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species: 1) enhance the resistance of yeast to chronic oxidative, thermal and osmotic stresses; 2) suppress mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide; and 3) attenuate lipid-induced necrotic cell death induced by exogenously added palmitoleic acid.

As we found, the addition of LCA to long-lived yeast species 3, 5 and 12 cultured under CR conditions 1) further extends their chronological life spans (CLS), although to a lesser degree than that of WT; 2) causes further changes in the age-dependent dynamics of mitochondrial respiration, although not as dramatic as those seen in WT; 3) further enhances the resistance of yeast to chronic oxidative, thermal and osmotic stresses, although to a lesser extent than those observed in WT; 4) further suppresses mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide, although to a lesser degree than those seen in WT; and 5) further attenuates

lipid-induced necrotic cell death induced by exogenously added palmitoleic acid, although to a lesser extent than those observed in WT. We therefore concluded that, although a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 greatly impact a compendium of the LCA-sensing housekeeping longevity assurance processes, this allele or alleles do not activate such processes sufficiently enough to attain the maximal CLS achievable under life-extending CR conditions in the presence of LCA.

Altogether, these findings further confirm the validity of our hypothesis in which the evolution of longevity regulation mechanisms within an ecosystem can be driven by the xenohormetic, hormetic and cytostatic selective forces created by a lasting exposure of an organism to an anti-aging natural compound released by other organisms composing this ecosystem.

5.2 Introduction

We recently found that under field-like CR conditions LCA extends longevity of chronologically aging yeast cultured under laboratory conditions by modulating the housekeeping longevity assurance pathways, and thereby 1) altering the age-dependent dynamics of mitochondrial respiration and of some other oxidation-reduction processes in mitochondria; 2) enhancing resistance to chronic oxidative, thermal and osmotic stresses; 3) suppressing mitochondria-controlled apoptotic cell death; and 4) attenuating lipid-induced necrotic cell death [167]. By conducting the LCA-driven multistep selection of long-lived yeast under laboratory conditions (which is described in Chapter 4 of this

Master's thesis), we selected the new yeast species 3, 5 and 12 that live significantly longer in the absence of LCA than their ancestor. Because the evolution of these long-lived yeast species under laboratory conditions was driven by LCA, one could assume that a mutant allele or alleles selected during such evolution affect the same set of the housekeeping longevity-related processes that are modulated by LCA in chronologically aging yeast. In a series of experiments described in this Chapter of the thesis, we tested the validity of our assumption.

5.3 Materials and Methods

Strains and media

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MATα, his3Δ leu2Δ0 lys2Δ ura3Δ0)* and long-lived mutant species 3, 5 and 12 derived from this strain were cultured in liquid YP medium (1% yeast extract, 2% peptone) contained 0.2% or 2% glucose as a carbon source, with or without LCA, as detailed in the "Results" section. Cell cultures were incubated at 30°C with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 5:1.

A plating assay for the analysis of chronological life span (CLS)

Cells were grown in YP medium initially containing 0.2% or 2% glucose as carbon source at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a flask volume/medium volume ratio of 5:1. A sample of cells was removed from each culture at various time points. A fraction of the cell sample was diluted in order to determine the total number of cells per ml of culture using a hemacytometer. 10 μl of serial dilutions

(1:10 to $1:10^3$) of cells were applied to the hemacytometer, where each large square is calibrated to hold 0.1 µl. The number of cells in 4 large squares was then counted and an average was taken in order to ensure greater accuracy. The concentration of cells was calculated as follows: number of cells per large square x dilution factor × 10 × 1,000 = total number of cells per ml of culture. A second fraction of the cell sample was diluted and serial dilutions ($1:10^2$ to $1:10^5$) of cells were plated onto YEPD (2% Glucose) plates in triplicate in order to count the number of viable cells per ml of each culture. 100 µl of diluted culture was plated onto each plate. After 48-h incubation at 30°C, the number of colonies per plate was counted. The number of colony forming units (CFU) equals to the number of viable cells in a sample. Therefore, the number of viable cells was calculated as follows: number of colonies × dilution factor × 10 = number of viable cells per ml. For each culture assayed, % viability of the cells was calculated as follows: number of viable cells per ml / total number of cells per ml × 100%. The % viability of cells in mid-logarithmic phase was set at 100% viability for that particular culture.

Oxygen consumption assay

The rate of oxygen consumption by yeast cells recovered at various time points was measured continuously in a 2-ml stirred chamber using a custom-designed biological oxygen monitor (Science Technical Center of Concordia University) equipped with a Clark-type oxygen electrode. 1 ml of YP medium supplemented with 0.2% glucose was added to the electrode for approximately 5 minutes to obtain a baseline. Cultured cells of a known titre were spun down at 3,000 × g for 5 minutes. The resulting pellet was resuspended in YP medium supplemented with 0.2% glucose and then added to the

electrode with the medium that was used to obtain a baseline. The resulting slope was used to calculate the rate of oxygen consumption in $O_2\% \times min^{-1} \times 10^9$ cells.

Plating assays for the analysis of resistance to various chronic stresses

For the analysis of oxidative stress resistance, serial dilutions (1:10 to $1:10^5$) of wild-type and mutant cells removed from various phases of growth YP medium initially containing 0.2% glucose were spotted onto two sets of plates. One set of plates contained YEPD (2% Glucose) medium alone, whereas the other set contained YEPD (2% Glucose) medium supplemented with 5 mM hydrogen peroxide. Pictures were taken after 3-day incubation at 30°C.

For the analysis of heat-shock resistance, serial dilutions (1:10 to $1:10^5$) of wild-type and mutant cells removed from various phases of growth YP medium initially containing 0.2% glucose were spotted onto two sets of YEPD (2% Glucose) plates. One set of plates was incubated at 30°C. The other set of plates was initially incubated at 55°C for 30 min, and was then transferred to 30°C. Pictures were taken after 3-day incubation at 30°C.

For the analysis of osmotic (salt stress) resistance, serial dilutions (1:10 to $1:10^5$) of wild-type and mutant cells removed from various phases of growth YP medium initially containing 0.2% glucose were spotted onto two sets of plates. One set of plates contained YEPD (2% Glucose) medium alone, whereas the other set contained YEPD (2% Glucose) medium supplemented with 1M NaCl. Pictures were taken after 3-day incubation at 30°C.

5.4 Results

5.4.1 A mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 alter the age-dependent dynamics of mitochondrial respiration under CR and non-CR conditions

We found that in wild-type (WT) yeast grown under CR conditions on 0.2% glucose without LCA, the rate of oxygen consumption by mitochondria was 1) greatly amplified when yeast entered diauxic (D) growth phase that begins on day 2 of culturing; and 2) sharply declined during the subsequent post-diauxic (PD; occurs between days 3 and 7 of culturing) phase of growth; and 3) gradually reduced during stationary (ST; begins after day 7 of culturing) growth phase that follows PD phase (Figure 5.1). In contrast, in long-lived yeast species 3, 5 and 12 grown under CR conditions on 0.2% glucose without LCA, the rate of oxygen consumption by mitochondria 1) was amplified to a significantly lesser extent during D phase than it was in WT yeast cultured on 0.2% glucose; 2) did not declined as sharply during the subsequent PD phase of growth as it was in WT yeast cultured under these conditions; and 3) was elevated again for a long period of time during ST phase (to the level similar to that seen in these yeast species during PD phase) and began to decline slowly only deep into ST phase – unlike the situation seen in WT yeast cultured under these conditions (Figure 5.1).

A similar effect of a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 on the age-dependent dynamics of mitochondrial respiration was observed in yeast grown under non-CR conditions on 2% glucose without LCA – although under non-CR conditions 1) the rates of oxygen

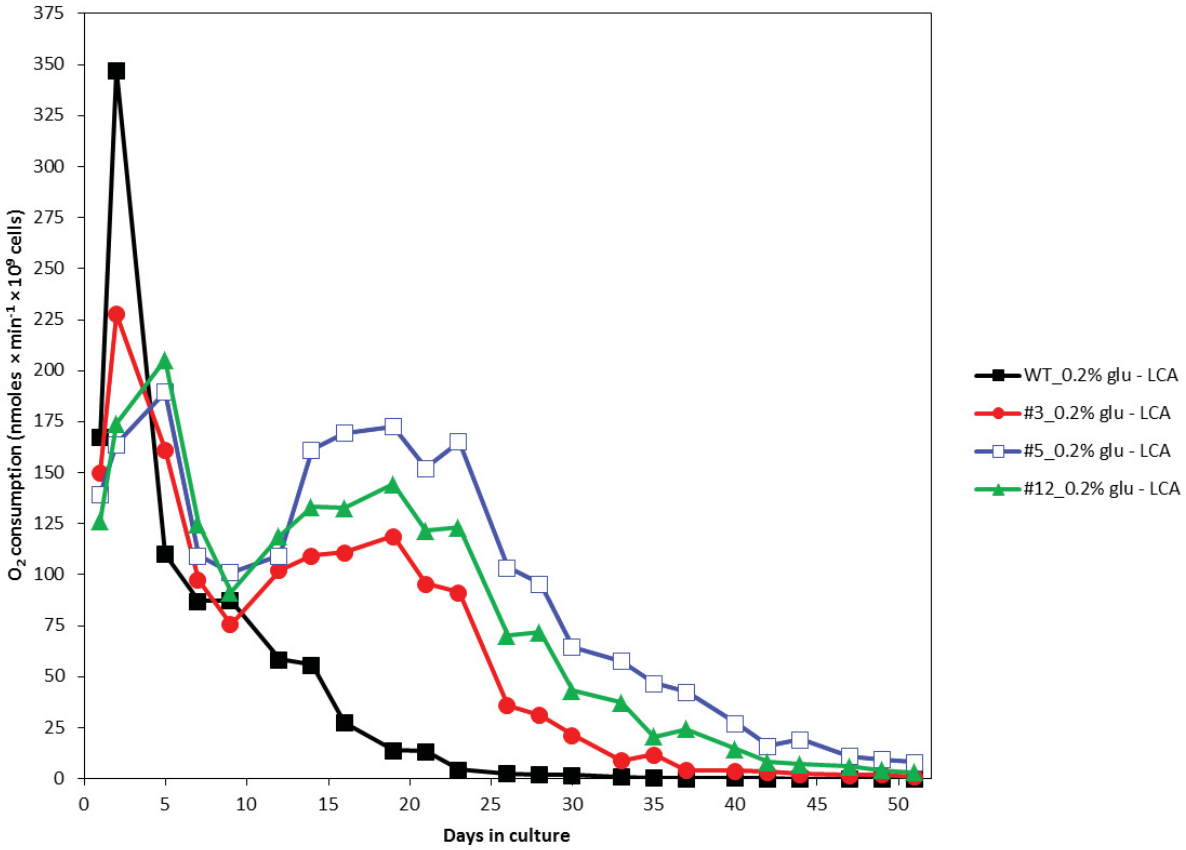

Figure 5.1. A mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 alter the age-dependent dynamics of mitochondrial respiration under CR conditions. The dynamics of age-dependent changes in the rate of oxygen consumption by cells of WT strain and yeast species 3, 5 and 12. Cells were cultured in YP medium without LCA initially containing 0.2% glucose.

consumption by cells of WT strain and yeast species 3, 5 and 12 were lower during D, PD and ST phases than those seen in their counterparts cultured under CR conditions; and 2) the differences between cells of WT and yeast species 3, 5 and 12 in amplitudes of the oxygen consumption amplification during D phase, its decline during the subsequent PD phase and its strain-characteristic changes during ST phase were less pronounced than those observed in their counterparts cultured under CR conditions (Figure 5.2).

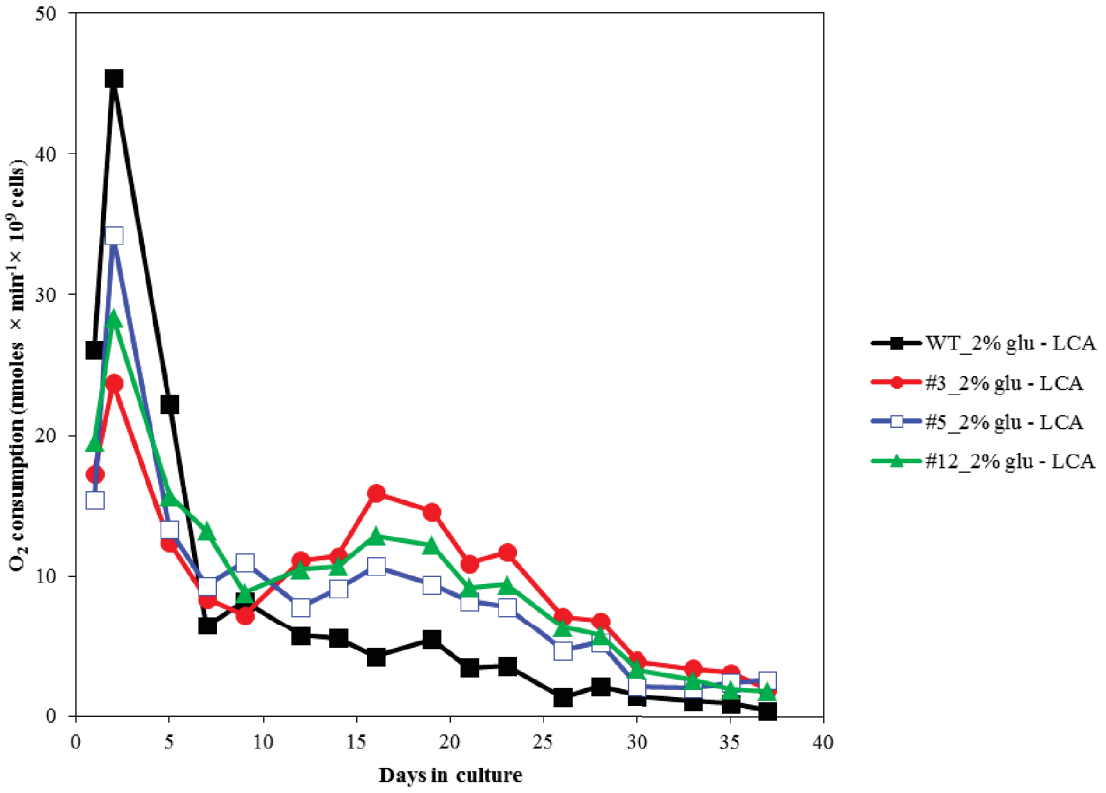

Figure 5.2. A mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 alter the age-dependent dynamics of mitochondrial respiration under non-CR conditions. The dynamics of age-dependent changes in the rate of oxygen consumption by cells of WT strain and yeast species 3, 5 and 12. Cells were cultured in YP medium without LCA initially containing 2% glucose.

5.4.2 A mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 enhance the resistance of yeast to chronic oxidative, thermal and osmotic stresses under CR conditions

We recently demonstrated that under CR conditions LCA extends longevity of chronologically aging yeast by activating a set of housekeeping longevity assurance processes leading, in part, to the enhanced resistance of yeast to chronic oxidative,

thermal and osmotic stresses [167]. Because the evolution of long-lived yeast species 3, 5 and 12 under laboratory conditions was driven by LCA, one could assume that a mutant allele or alleles selected during such evolution further activate the same set of the housekeeping assurance processes that are activated by LCA in chronologically aging WT yeast. In fact, we found that this mutant allele or alleles enhance the resistance of yeast cultured under CR conditions without LCA to chronic oxidative, thermal and osmotic stresses (Figure 5.3).

5.4.3 A mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 suppress mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide under CR conditions

As we recently showed, under CR conditions LCA extends longevity of chronologically aging yeast by activating a set of housekeeping longevity assurance processes leading, in part, to the reduced susceptibility of yeast to cell death triggered by a short-term exposure to exogenous hydrogen peroxide and caused by mitochondria-controlled apoptosis [167]. Because the evolution of long-lived yeast species 3, 5 and 12 under laboratory conditions was driven by LCA, it is conceivable that a mutant allele or alleles selected during such evolution further activate the same set of the housekeeping assurance processes that are activated by LCA in chronologically aging WT yeast. In fact, we found that in yeast cultured under CR conditions without LCA this mutant allele or alleles suppress mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide (Figure 5.4).

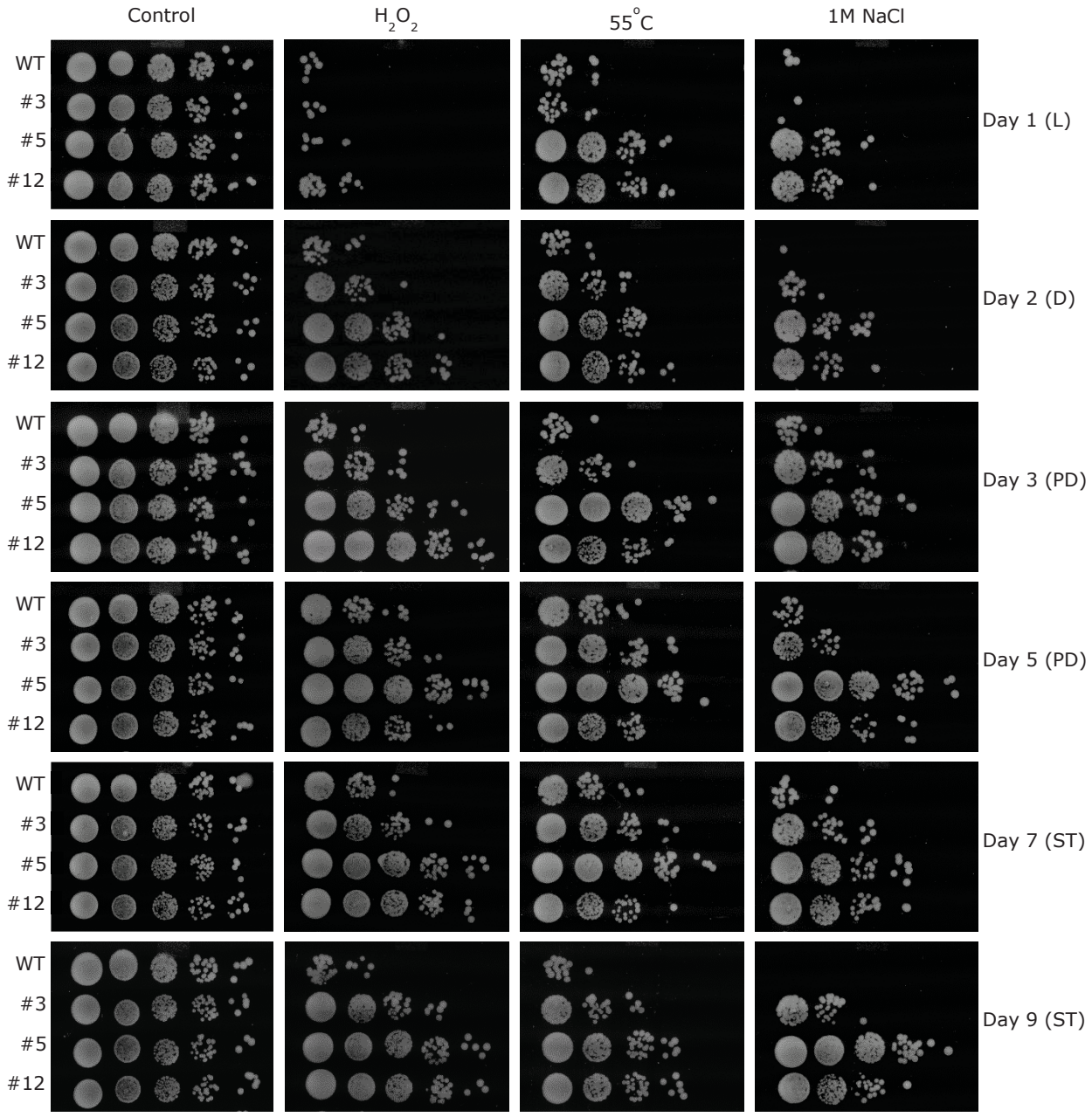

Figure 5.3. A mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 enhance the resistance of yeast cultured under CR conditions without LCA to chronic oxidative, thermal and osmotic stresses. Yeast cells were grown in YP medium without LCA initially containing 0.2% glucose. Cell aliquots were recovered from various growth phases. The resistance of yeast to chronic oxidative, thermal and osmotic stresses was monitored as described in the

Materials and Methods section. Abbreviations: D, diauxic growth phase; L, logarithmic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase.

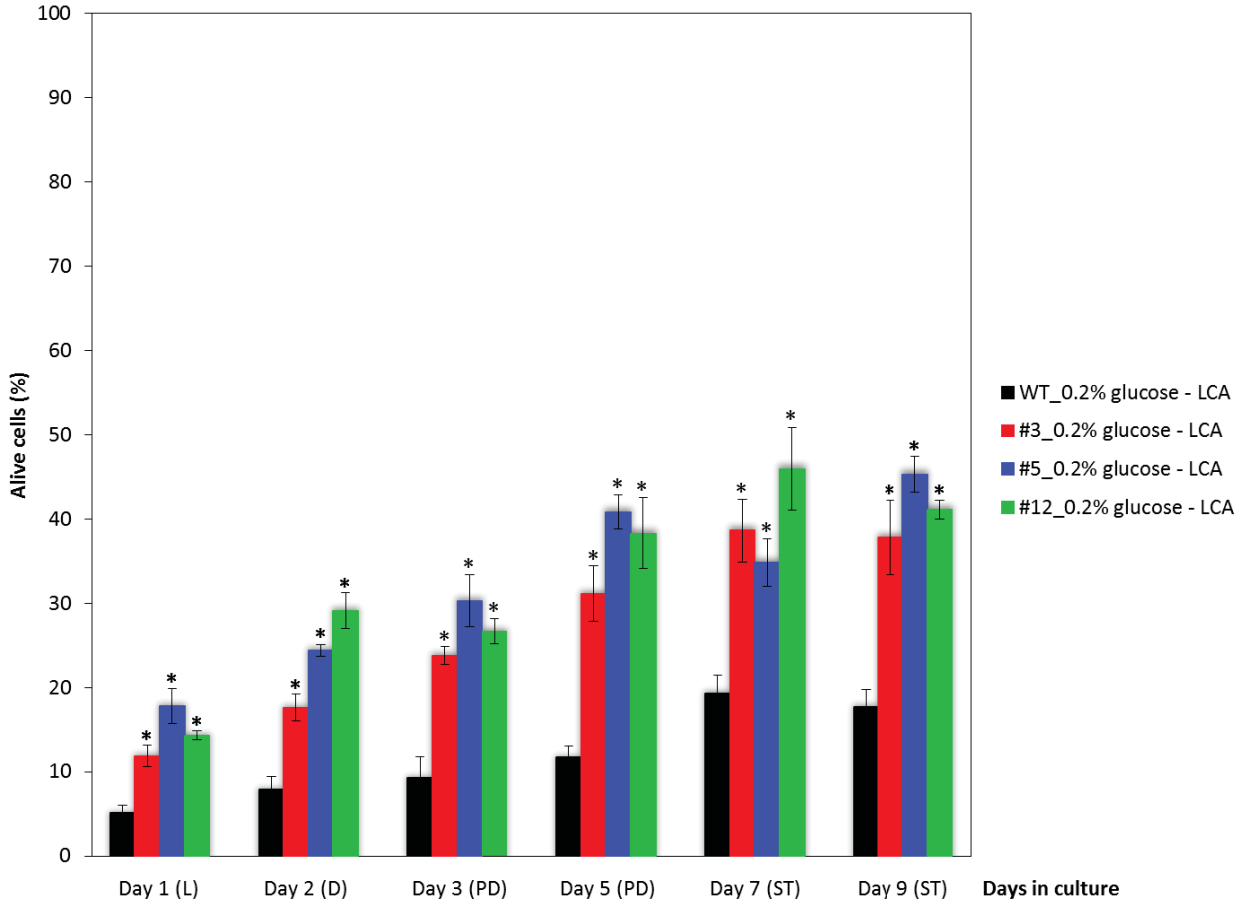

Figure 5.4. A mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 suppress mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide in yeast cultured under CR conditions without LCA. Yeast cells were pre-grown in YP medium without LCA initially containing 0.2% glucose. Cell aliquots were recovered from various growth phases and then treated for 2 h with 2.5 mM hydrogen peroxide to induce mitochondria-controlled apoptosis. Abbreviations: D, diauxic growth phase; L, logarithmic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase.

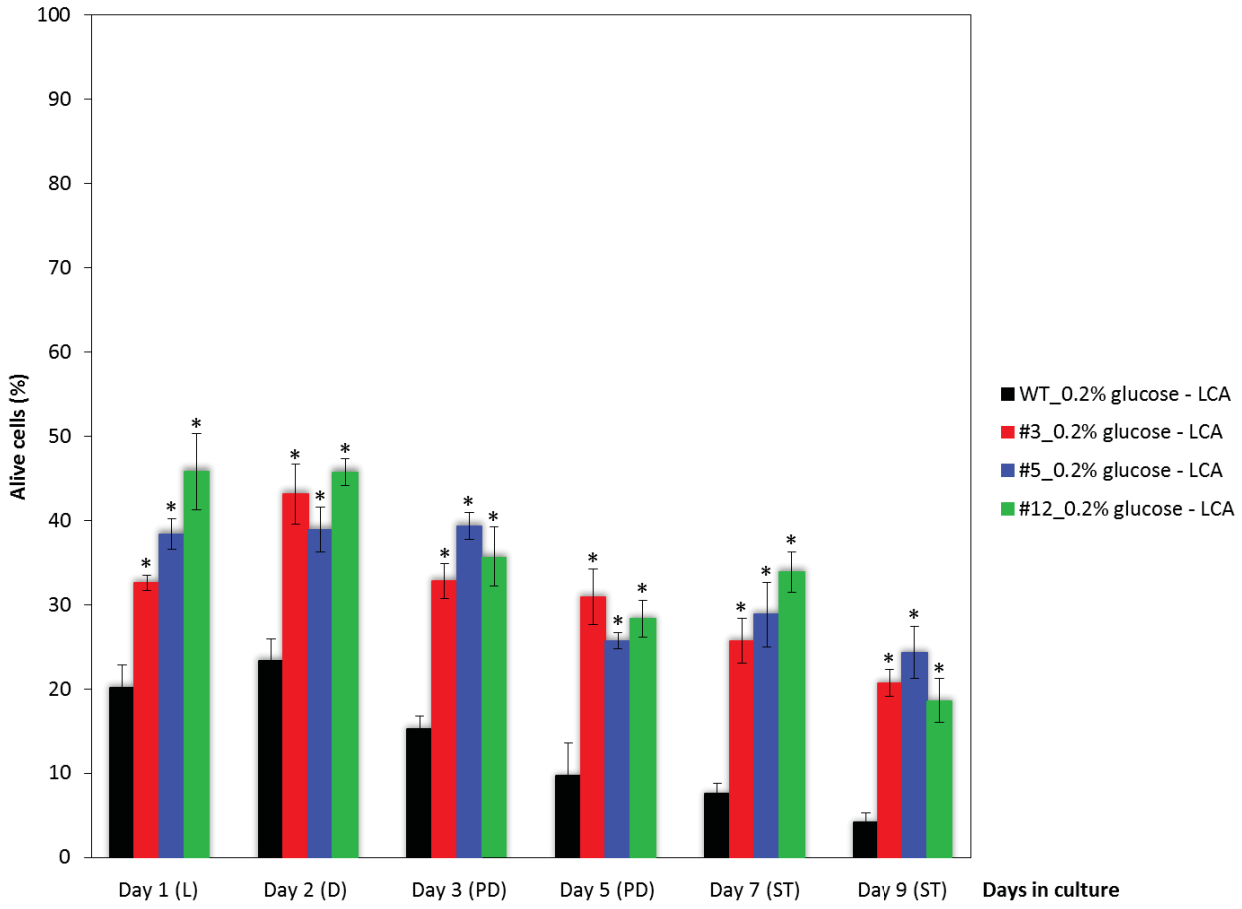

Figure 5.5. In yeast cultured under CR conditions without LCA, a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 attenuate lipid-induced necrotic cell death induced by exogenously added palmitoleic acid. Yeast cells were pre-grown in YP medium without LCA initially containing 0.2% glucose. Cell aliquots were recovered from various growth phases and then exposed for 2 h to 0.2 mM palmitoleic acid to induce a necrotic mode of cell death. Abbreviations: D, diauxic growth phase; L, logarithmic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase.

5.4.4 A mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 attenuate lipid-induced

necrotic cell death induced by exogenously added palmitoleic acid under CR conditions

A set of housekeeping longevity assurance processes whose activation by LCA under CR conditions partially underlies the anti-aging effect of LCA includes an attenuation of lipid-induced necrotic cell death induced by exogenously added palmitoleic acid [167]. One could expect that due to the fact that LCA has been a driving force for the evolution of long-lived yeast species 3, 5 and 12 under laboratory conditions, a mutant allele or alleles selected during such evolution would further reduce susceptibility of yeast to this mode of cell death. Indeed, in yeast cultured under CR conditions without LCA this mutant allele or alleles attenuated lipid-induced necrotic cell death triggered by a short-term exposure to exogenous palmitoleic acid (Figure 5.5).

5.4.5 A mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 do not activate the LCA-sensing housekeeping longevity assurance processes sufficiently enough to attain the maximal CLS achievable under CR conditions in the presence of LCA

We found that the addition of LCA to long-lived yeast species 3, 5 and 12 cultured under CR conditions further extends their CLS, although to a lesser degree than that of WT (Figure 5.6). This finding implies that, although a mutant allele or alleles selected during the LCA-driven multistep selection process of these yeast species greatly impact a compendium of the LCA-sensing housekeeping longevity assurance processes (see Figures 5.1 to 5.5), this allele or alleles do not activate such processes sufficiently enough

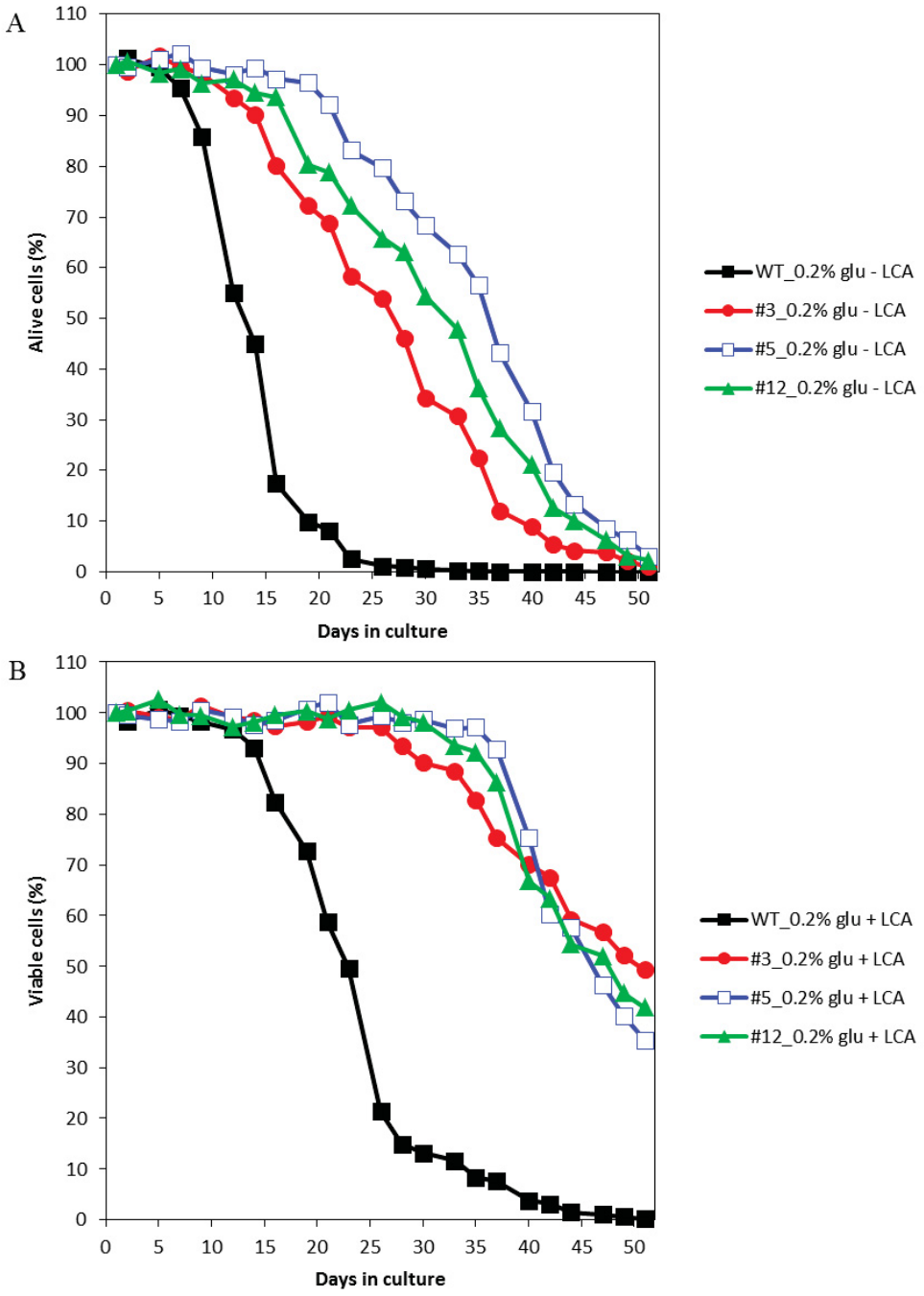

Figure 5.6. The addition of LCA to long-lived yeast species 3, 5 and 12 cultured under CR conditions further extends their CLS, although to a lesser degree than that of WT. Survival of chronologically aging WT and of the yeast species 3, 5 and 12 cultured under CR conditions in liquid YP medium initially containing 0.2% glucose without (A) or with (B) LCA.

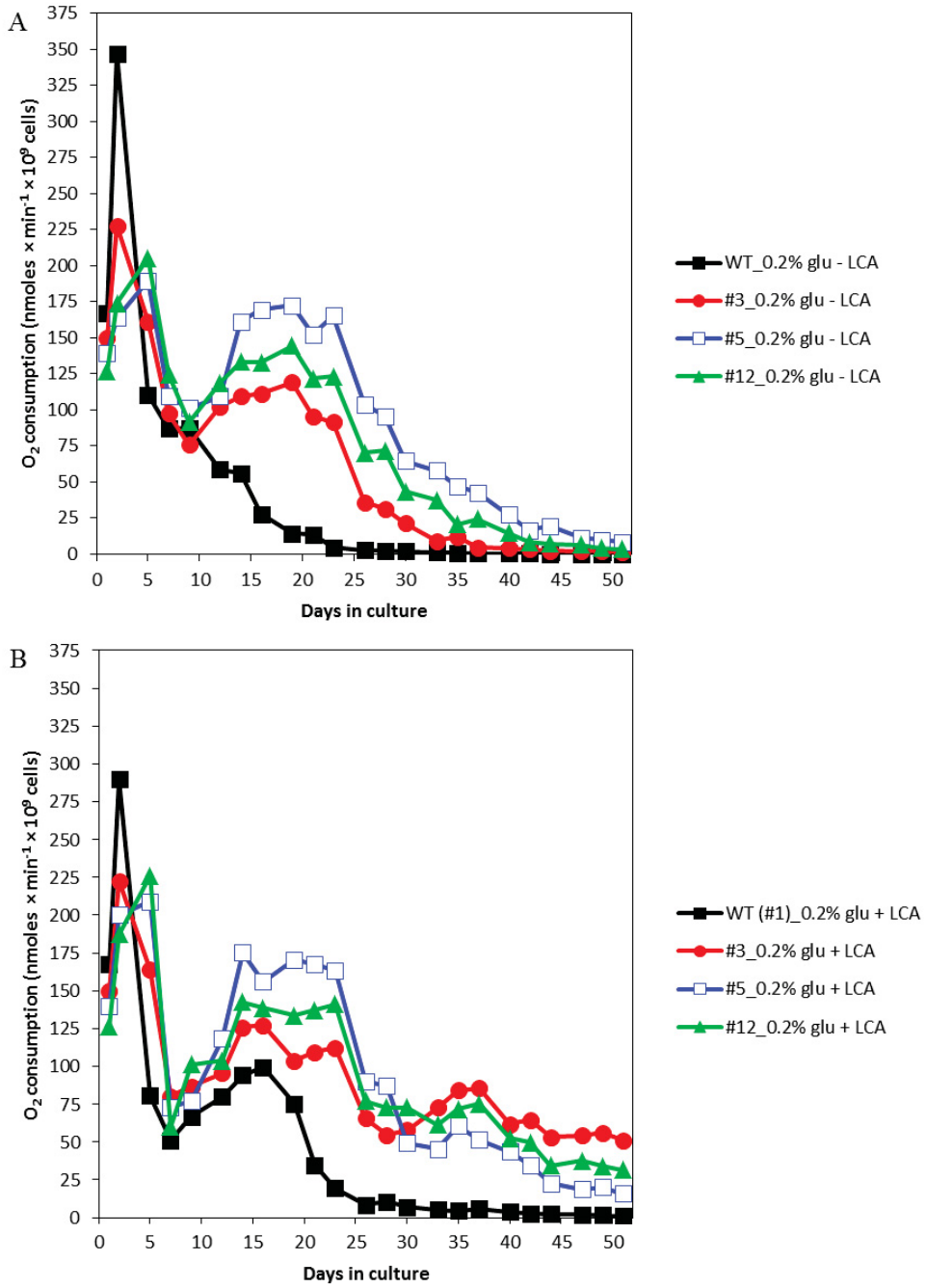

Figure 5.7. The addition of LCA to long-lived yeast species 3, 5 and 12 cultured under CR conditions causes further changes in the age-dependent dynamics of mitochondrial respiration, although not as dramatic as those seen in WT. The dynamics of age-dependent changes in the rate of oxygen consumption by cells of WT strain and yeast species 3, 5 and 12. Cells were under CR conditions in liquid YP medium initially containing 0.2% glucose without (A) or with (B) LCA.

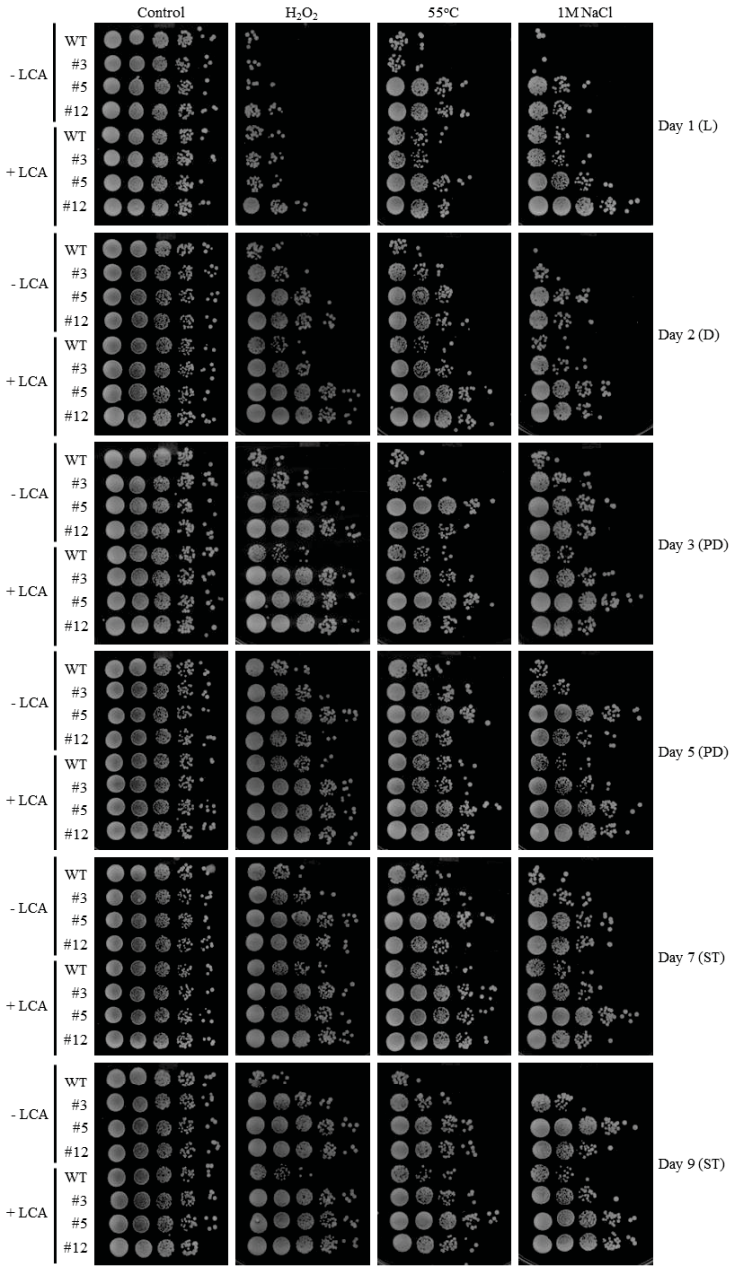

Figure 5.8. The addition of LCA to long-lived yeast species 3, 5 and 12 cultured under CR conditions further enhances the resistance of yeast to chronic oxidative, thermal and osmotic stresses, although to a lesser extent than those observed in WT. Yeast cells were grown in YP medium initially containing 0.2% glucose, with or without LCA. Cell aliquots were recovered from various growth phases. The resistance of yeast to chronic oxidative, thermal and osmotic stresses was monitored as described in the Materials and

Methods section. Abbreviations: D, diauxic growth phase; L, logarithmic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase.

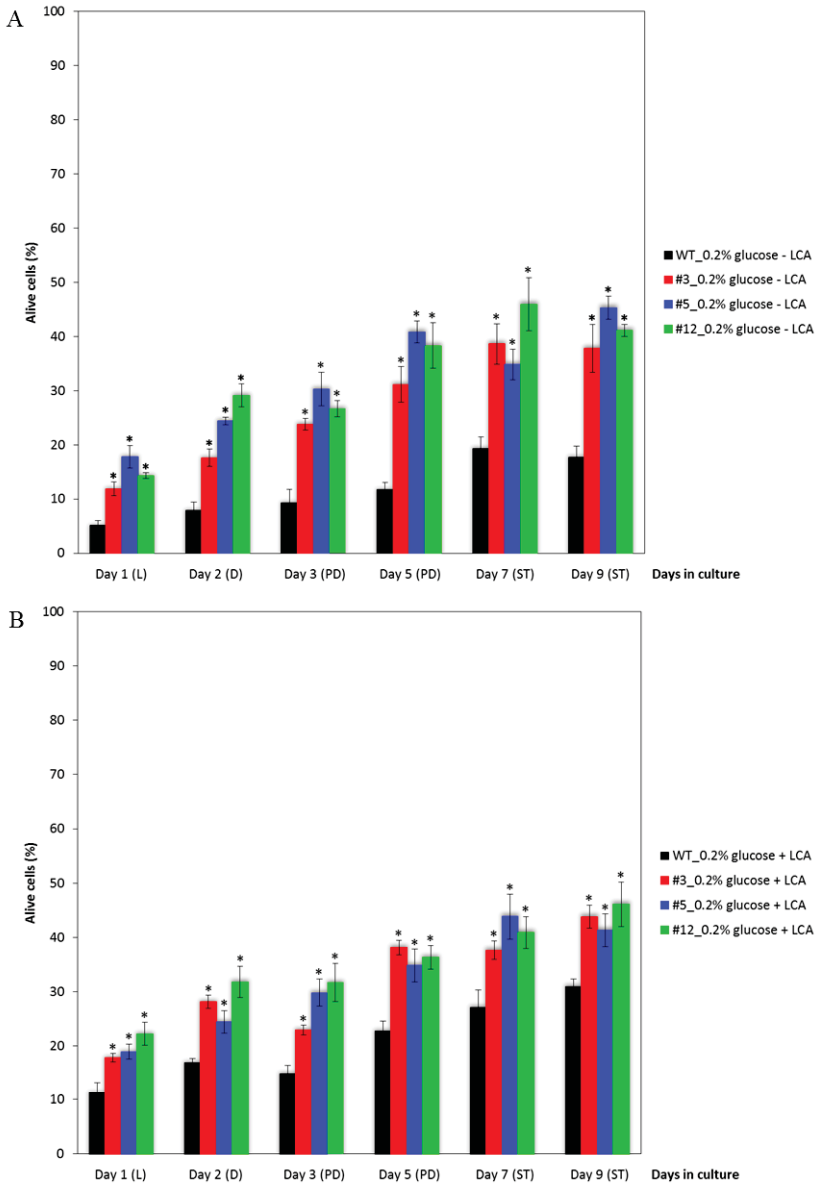

Figure 5.9. The addition of LCA to long-lived yeast species 3, 5 and 12 cultured under CR conditions further suppresses mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide, although to a lesser degree than those seen in WT. Yeast cells were pre-grown under CR conditions in liquid YP medium initially containing 0.2% glucose without (A) or with (B) LCA. Cell aliquots were recovered from various growth phases and then treated for 2 h with 2.5 mM hydrogen peroxide to induce

mitochondria-controlled apoptosis. Abbreviations: D, diauxic growth phase; L, logarithmic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase.

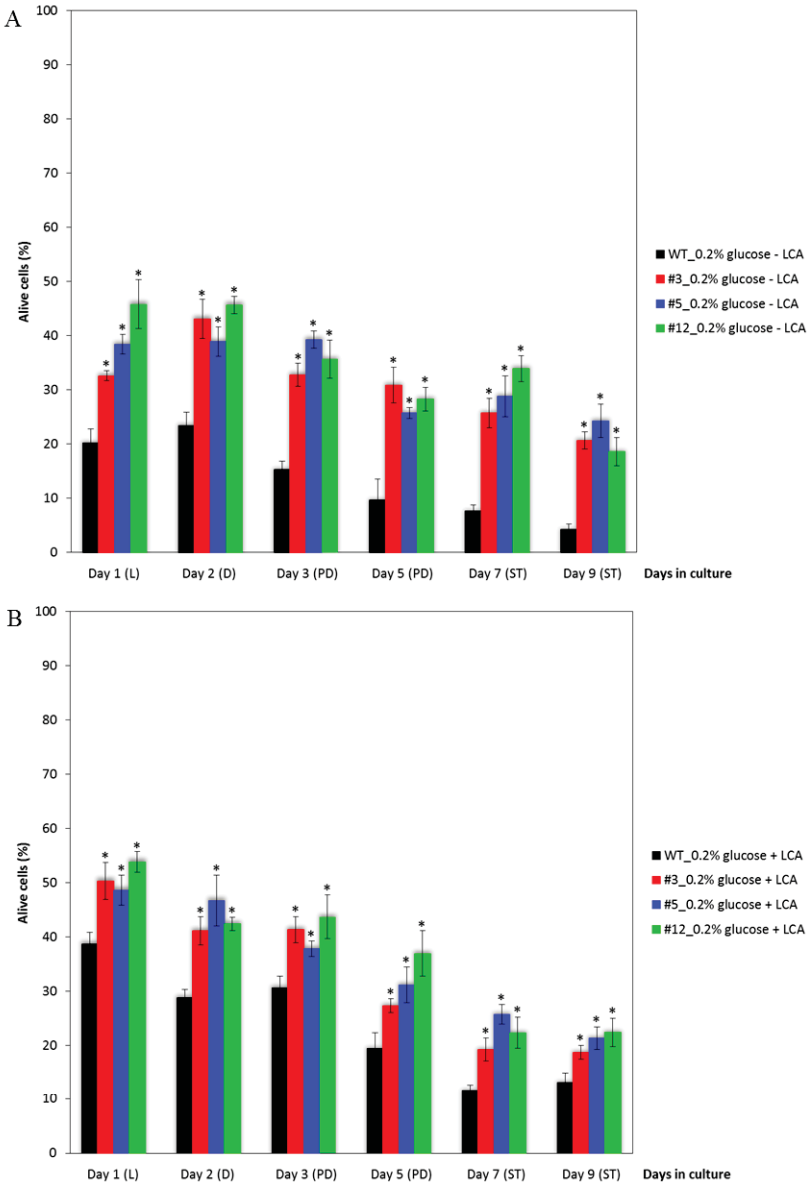

Figure 5.10. The addition of LCA to long-lived yeast species 3, 5 and 12 cultured under CR conditions further attenuates lipid-induced necrotic cell death induced by exogenously added palmitoleic acid, although to a lesser extent than those observed in WT. Yeast cells were pre-grown under CR conditions in liquid YP medium initially containing 0.2% glucose without (A) or with (B) LCA. Cell aliquots were recovered from various growth phases and then exposed for 2 h to 0.2 mM palmitoleic acid to induce a

necrotic mode of cell death. Abbreviations: D, diauxic growth phase; L, logarithmic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase.

to attain the maximal CLS achievable under life-extending CR conditions in the presence of LCA. In support of this notion, we revealed that the addition of LCA to long-lived yeast species 3, 5 and 12 cultured under CR conditions 1) causes further changes in the age-dependent dynamics of mitochondrial respiration, although not as dramatic as those seen in WT (Figure 5.7); 2) further enhances the resistance of yeast to chronic oxidative, thermal and osmotic stresses, although to a lesser extent than those observed in WT (Figure 5.8); 3) further suppresses mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide, although to a lesser degree than those seen in WT (Figure 5.9); and 4) further attenuates lipid-induced necrotic cell death induced by exogenously added palmitoleic acid, although to a lesser extent than those observed in WT (Figure 5.10).

5.5 Conclusions

A described in this Chapter observation that a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 alter the age-dependent dynamics of mitochondrial respiration under both CR and non-CR conditions further confirms our conclusion (see Chapter 4 of this Master's thesis) that, consistent with its sought-after effect on longevity regulation pathways, our LCA-driven multistep selection process under laboratory conditions yielded long-lived yeast species whose greatly delayed chronological aging was caused by the selection of a mutant allele or alleles that activate so-called housekeeping longevity assurance pathways.

Findings described in this Chapter also imply that under CR conditions in the absence of LCA a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species: 1) enhance the resistance of yeast to chronic oxidative, thermal and osmotic stresses; 2) suppress mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide; and 3) attenuate lipid-induced necrotic cell death induced by exogenously added palmitoleic acid. As we previously demonstrated, all these processes are governed by the housekeeping longevity assurance pathways that modulate longevity irrespective of the number of available calories [167].

Moreover, the experiments described in this Chapter revealed that the addition of LCA to long-lived yeast species 3, 5 and 12 cultured under CR conditions 1) further extends their CLS, although to a lesser degree than that of WT; 2) causes further changes in the age-dependent dynamics of mitochondrial respiration, although not as dramatic as those seen in WT; 3) further enhances the resistance of yeast to chronic oxidative, thermal and osmotic stresses, although to a lesser extent than those observed in WT; 4) further suppresses mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide, although to a lesser degree than those seen in WT; and 5) further attenuates lipid-induced necrotic cell death induced by exogenously added palmitoleic acid, although to a lesser extent than those observed in WT. Altogether, these findings imply that, although a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species greatly impact a compendium of the LCA-sensing housekeeping longevity assurance processes, this allele or alleles do not activate

such processes sufficiently enough to attain the maximal CLS achievable under life-extending CR conditions in the presence of LCA.

In sum, findings presented on this Chapter further confirm the validity of our hypothesis proposing that the evolution of longevity regulation mechanisms within an ecosystem can be driven by the xenohormetic, hormetic and cytostatic selective forces created by a lasting exposure of an organism to an anti-aging natural compound released by other organisms composing this ecosystem.

6 Conclusions and suggestions for future work

We designed a chemical genetic screen for small molecules that increase the chronological life span (CLS) of yeast under CR by targeting lipid metabolism and modulating housekeeping longevity pathways that regulate longevity irrespective of the number of available calories. By screening the total of approximately 19,000 representative compounds from seven commercial libraries, we identified 24 small molecules that greatly extend the CLS of a short-lived mutant *pex5Δ* deficient in peroxisomal fatty acid oxidation, which we used for our high-throughput screen. These anti-aging small molecules belong to 5 chemical groups. Group I consisted of 6 bile acids, including lithocholic acid (LCA), deoxycholic acid, chenodeoxycholic acid, cholic acid, dehydrocholic acid and hyodeoxycholic acid. The results of our pharmacophore modeling of the anti-aging potential of various species of bile acids imply that the maintenance of the minimal polarity of both the hydrophilic (concave) and hydrophobic (convex) sides of the steroid nucleus - by avoiding the presence of polar substituents at the positions 6, 7 and 12 - is mandatory for the extreme life-extending efficacy of LCA under CR conditions. Such stringent structural requirements are consistent with a target specificity of LCA action as an anti-aging small molecule. We found that the life-extending efficacy of LCA under CR exceeds that under non-CR conditions, being inversely proportional to the concentration of glucose in growth medium and thus in correlation with the extent of calorie supply limitation.

Yeast do not synthesize this or any other bile acids produced by mammals [168, 169]. We noticed that various organisms (*i.e.*, bacteria, fungi, plants and animals) within an

ecosystem can synthesize and release into the environment certain longevity-extending small molecules. We therefore hypothesized that these interspecies chemical signals can create xenohormetic, hormetic and cytostatic selective forces driving the ecosystemic evolution of longevity regulation mechanisms. In our hypothesis, following their release into the environment by one species of the organisms composing an ecosystem, such small molecules can activate anti-aging processes and/or inhibit pro-aging processes in other species within the ecosystem. The organisms that possess the most effective (as compared to their counterparts of the same species) mechanisms for sensing the chemical signals produced and released by other species and for responding to such signals by undergoing certain hormetic and/or cytostatic life-extending changes to their metabolism and physiology are expected to live longer than their counterparts within the ecosystem. We therefore proposed that the ability of a species of the organisms composing an ecosystem to undergo life-extending metabolic or physiological changes in response to hormetic or cytostatic chemical compounds released to the ecosystem by other species: 1) increases its chances of survival; 2) creates selective forces aimed at maintaining such ability; and 3) enables the evolution of longevity regulation mechanisms.

To verify our hypothesis empirically, we carried out the LCA-driven multistep selection of long-lived yeast species under laboratory conditions. We found that a lasting exposure of wild-type yeast to LCA results in selection of yeast species that live longer in the absence of this bile acid than their ancestor. Our data enabled to rank different concentrations of LCA with respect to the efficiency with which they cause the appearance of long-lived yeast species. We revealed that, if used at the most efficient

concentration of 5 μM, this bile acid induced life-extending mutations with the frequency of about 4×10^8/generation. At the concentration of 50 μM, LCA caused the appearance of long-lived species with the frequency of 1×10^8/generation, whereas a lasting exposure of yeast to 250 μM LCA did not result in selection of such species. Because the lowest used concentration of LCA resulted in the highest frequency of long-lived species appearance, we believe that it is unlikely that the life-extending mutations they carry are due to mutagenic action of this bile acid.

We found that the evolved under laboratory conditions long-lived yeast species 3, 5 and 12 were able to maintain their greatly extended CLS following numerous successive passages in medium without LCA under CR conditions. Our data imply that each of these long-lived yeast species also exhibited extended CLS when cultured in the absence of LCA under non-CR conditions (although to a lesser extent than that under CR conditions). Thus, consistent with its sought-after effect on longevity regulation pathways, our LCA-driven multistep selection process under laboratory conditions yielded long-lived yeast species whose greatly delayed chronological aging was caused by the selection of a mutant allele or alleles that activate so-called housekeeping longevity assurance pathways. These housekeeping longevity assurance pathways modulate longevity irrespective of the number of available calories and do not overlap with the adaptable longevity pathways that are under the stringent control of calorie availability [167].

To test the validity of other aspects of our hypothesis on the ecosystemic evolution of longevity regulation mechanisms, we assessed how a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 influence a compendium of the housekeeping longevity-related processes that, as we recently revealed [167], are modulated by LCA in chronologically aging yeast. We found that in the absence of LCA this allele or alleles alter the age-dependent dynamics of mitochondrial respiration under both CR and non-CR conditions. This finding further confirms our conclusion that, consistent with its sought-after effect on longevity regulation pathways, our LCA-driven multistep selection process under laboratory conditions yielded long-lived yeast species whose greatly delayed chronological aging was caused by the selection of a mutant allele or alleles that activate so-called housekeeping longevity assurance pathways. As we previously demonstrated, these housekeeping longevity assurance pathways modulate longevity irrespective of the number of available calories and do not overlap with the adaptable longevity pathways that are under the stringent control of calorie availability [167].

We also revealed that under CR conditions in the absence of LCA a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species: 1) enhance the resistance of yeast to chronic oxidative, thermal and osmotic stresses; 2) suppress mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide; and 3) attenuate lipid-induced necrotic cell death induced by exogenously added palmitoleic acid. All these processes are known to be

governed by the housekeeping longevity assurance pathways that modulate longevity irrespective of the number of available calories [167].

We also revealed that the addition of LCA to long-lived yeast species 3, 5 and 12 cultured under CR conditions 1) further extends their CLS, although to a lesser degree than that of WT; 2) causes further changes in the age-dependent dynamics of mitochondrial respiration, although not as dramatic as those seen in WT; 3) further enhances the resistance of yeast to chronic oxidative, thermal and osmotic stresses, although to a lesser extent than those observed in WT; 4) further suppresses mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide, although to a lesser degree than those seen in WT; and 5) further attenuates lipid-induced necrotic cell death induced by exogenously added palmitoleic acid, although to a lesser extent than those observed in WT. We therefore concluded that, although a mutant allele or alleles selected during the LCA-driven multistep selection process of long-lived yeast species 3, 5 and 12 greatly impact a compendium of the LCA-sensing housekeeping longevity assurance processes, this allele or alleles do not activate such processes sufficiently enough to attain the maximal CLS achievable under life-extending CR conditions in the presence of LCA.

Altogether, the findings presented in this Master's thesis further confirm the validity of our hypothesis in which the evolution of longevity regulation mechanisms within an ecosystem can be driven by the xenohormetic, hormetic and cytostatic selective forces

created by a lasting exposure of an organism to an anti-aging natural compound released by other organisms composing this ecosystem.

Future studies may involve an empirical test of the antagonistic pleiotropy and life history optimization theories of aging [232, 233]. One may consider analyzing the trade-offs between early-life fitness and longevity by measuring the relative fitness of the selected long-lived yeast species in a direct competition assay with the parental WT strain [234]. The assay could be carried out under CR conditions, which mimic the natural stressful environment of cyclical starvation, as well as under non-CR conditions in a more favourable environment [234, 235]. It would be very interesting to see if any of the three laboratory-evolved long-lived yeast species exhibits reduced fitness in the direct competition assay with WT cells.

We expect that these experiments using the three laboratory-evolved long-lived yeast species will reveal the trade-offs between early-life fitness and longevity. The subsequent studies of the molecular and cellular mechanisms underlying the anticipated trade-offs between early-life fitness and longevity would first assess if any of the laboratory-evolved long-lived yeast species has reduced fecundity, which could be monitored as their mating and sporulation efficacies. Furthermore, one could then elucidate if the decreased relative fitness of each of these species under field-like CR conditions of cyclical starvation is due to a reduction in maximal growth rate early in life.

Finally, it would be important in the near future to test if the greatly extended longevity of the laboratory-evolved long-lived yeast species is a monogenic trait. That could provide important information complementary to the ongoing effort of sequencing the entire genomes of these yeast species in an attempt to establish, for the first time, the molecular landscape of the evolution of longevity under laboratory conditions.

This future work may result in a unified hypothesis of the natural selection forces and underlying molecular mechanisms that drive the evolution of yeast longevity and maintain a finite yeast lifespan within ecosystems.

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