ADAPTATION AND REGULATION OF FATTY ACID COMPOSITION IN *CANDIDA ALBICANS* IN RESPONSE TO ENVIRONMENTAL CONDITIONS

by

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ABSTRACT OF THE THESIS

ADAPTATION AND REGULATION OF FATTY ACID COMPOSITION IN *CANDIDA ALBICANS* IN RESPONSE TO ENVIRONMENTAL CONDITIONS

By PETER STIVERS

Thesis Director:
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Candida albicans is a commensal fungus that grows in the mammalian gut at a constant temperature of 37°C. Like most fungi, *Candida albicans* membranes contain mono- and polyunsaturated fatty acids that are formed by Δ9, Δ12, and Δ15 fatty acid desaturase enzymes. Studies of other ectothermic fungi have shown that desaturases are highly regulated with respect to growth temperatures and the availability of exogenous nutrient fatty acids. Experiments were performed to assess whether the lipid composition and fatty acid desaturases of *Candida* are regulated under similar conditions.

Candida was found to adapt to temperatures ranging from 15°C to 37°C, however, at 15°C an unusual 12-hour biphasic lag phase was observed before cells entered logarithmic growth. These experiments showed that cellular fatty acyl composition was dynamic and varied with both the growth phase and the growth temperature. Growth at lower temperatures in the 37°C - 20°C range resulted in higher levels of double bonds in membrane lipids, which is consistent with induced levels of desaturase activities seen in ectothermic fungi as they adapt to low temperatures. Unexpectedly, the fatty acyl double bond content in 20°C cells was

found to be higher than that at 15°C. Analysis of the relative mRNA levels for Δ9, Δ12, and Δ15 desaturases showed a correlation with the respective fatty acyl compositions found at each temperature.

Candida was found to incorporate exogenous polyunsaturated fatty acids from the culture medium at 30°C and α-linolenic acid and γ-linolenic acid accumulated to high levels in cellular lipids, resulting in repressed levels of the endogenous unsaturated species. Arachidonic acid accumulated in cellular lipids at lower levels under the same conditions, but also produced marked reductions in endogenous 18:1. Analysis of mRNAs from the 30°C supplemented cells showed that all three fatty acids repressed the Δ9 and Δ15 desaturase mRNA levels while inducing Δ12 desaturase gene expression.

In experiments to test the effects of unsaturated fatty acids on the ability of *Candida* to adapt to low temperatures all three polyunsaturated acids were found to incorporate into lipids at high levels in cells grown at 15°C. This supplementation, however, produced no change in the extent of the lag phase and growth rate of *Candida* at 15°C. Under these conditions, all three exogenous fatty acids were found to repress both Δ9 and Δ12 desaturase mRNA levels.

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List of Abbreviations

α	alpha
BCP	bromochloropropane
Δ	delta
CAI-4	strain of *Candida albicans* used in this study
CoA	Coenzyme A
DEPC	diethylpyrocarbonate
ER	endoplasmic reticulum
FAD2	gene encoding the Δ12 fatty acid desaturase
FAD3	gene encoding the Δ15 fatty acid desaturase
FADH	flavin adenine dinucleotide
Fe^{IV}	iron(IV)
γ	gamma
kD	kilo Dalton
MGA2	gene encoding the Mga2p transcription factor
Mga2p	ER-localized transcription factor that regulates *OLE1*
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine nucleotide
NFA	no fatty acids
O/N	overnight culture
OLE1	gene encoding the Δ9 fatty acid desaturase

PUFA	polyunsaturated fatty acids
RNA	ribonucleic acid
RQ	relative quantity
Rsp5p	E3 ubiquitin ligase
RT-qPCR	real-time quantitative polymerase chain reaction
SPT23	gene encoding the Spt23p transcription factor
Spt23p	ER-localized transcription factor that regulates *OLE1*
UFA	unsaturated fatty acids
URA	uracil
URI	uridine
wt%	percent by weight
YPD	yeast extract, peptone, dextrose
16:0	Palmitic acid
16:1	Palmitoleic acid
18:0	Stearic acid
18:1	Oleic acid
18:2	Linoleic acid
18:3	Linolenic acid
20:4	Arachidonic acid

INTRODUCTION

Fatty acids are lipids that play an important role in numerous cellular activities. In cells, fatty acids may be involved in energy storage, membrane formation, and cell signaling. The molecular structure of a fatty acid consists of a carboxylic acid linked to an aliphatic tail that contains 14 to 26 carbon atoms (Figure 1). Saturated fatty acids contain no double bonds in their hydrocarbon chains, whereas unsaturated species in eukaryotic cells may contain from 1 to six 6 *cis* (Z) double bonds [1].

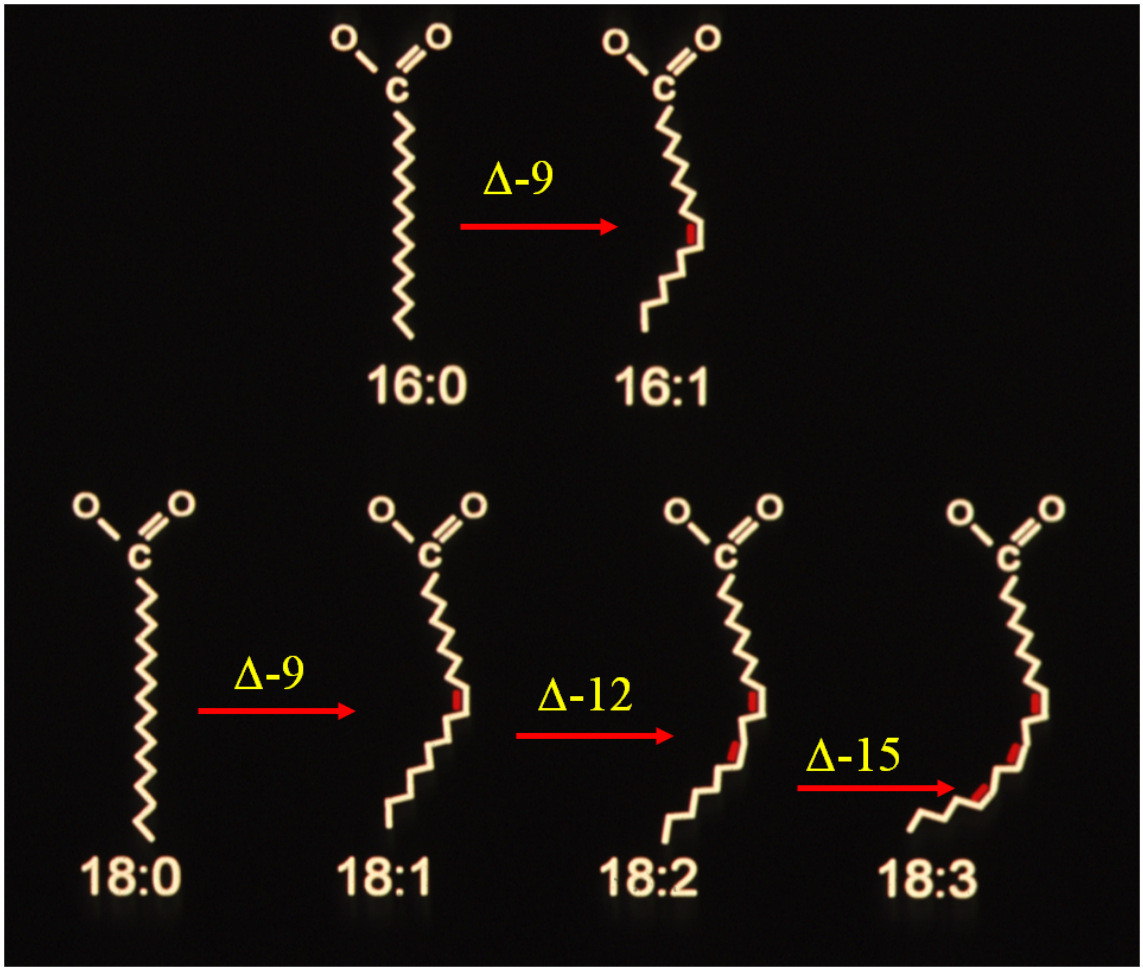

Figure 1. Major fatty acid species produced by *Candida albicans* and the fatty acyl desaturase activities involved in their synthesis.

Biological Roles of Fatty Acids

Fatty acids that are acylated to glycerophospholipids and glycerolipids form the essential hydrophobic core of cell membranes. In eukaryotic animal and fungal cells, the lipid bilayer of cell membranes is mostly composed of glycerophospholipids, in which the fatty acids are acylated to a glycerol moiety on carbons 1 and 2 and a polar headgroup is linked to carbon 3 through a negatively charged phosphate group. Due to the hydrophilic nature of the phosphate and head group, the fatty acid tails are protected from the surrounding aqueous environment, resulting in a hydrophobic core within the bilayer that allows selective permeability across the membrane.

Fatty acids are also found as energy storage molecules in lipid droplets in eukaryotic cells. In these structures, they are found as components of triacylglycerols and sterol esters. In fungal cells, when fats are needed for energy production these molecules are hydrolyzed to release the fatty acids into the cytoplasm where they can be moved to peroxosomes or mitochondria for β-oxidation. In this process two carbons are removed at a time from the hydrocarbon chain to yield acetyl CoA, NADH and $FADH_2$.

Fatty acids also play a role as signaling molecules. In mammalian cells polyunsaturated fatty acids such as arachidonic acid (20:4)[1] are released from phospholipids by phospholipase A_2 and converted by cyclooxygenases to form inflammatory signaling molecules [2]. In the fungus *Aspergillus nidulans*, unsaturated

[1] Fatty acids are designated by the numeric system XX:Y where x represents the hydrocarbon chain length and Y represents the number of double bonds in the hydrocarbon chain.

fatty acids act as precursors of signaling molecules that serve as inducers of sexual differentiation [3-6]. Polyunsaturated fatty acids such as linoleic acid (18:2) have also been shown to act as signaling molecules to induce asexual sporulation in filamentous fungi. Null mutations in the Δ12 fatty acid desaturase (FAD) that is responsible for producing 18:2 in *Aspergillus parasiticus* results in delayed spore germination and reduced conidia production [7].

Fatty Acid Biosynthesis and Desaturation

Fatty acids are initially formed as saturated species by fatty acid synthase, which is a large cytoplasmic multifunctional enzyme complex [8]. Saturated fatty acids are initially formed from the 3-carbon malonyl-CoA that is produced by acetyl CoA carboxylase. During each elongation cycle two carbons derived from the malonyl CoA molecule are added to the hydrocarbon chain with the release of CO_2. The most abundant end product of the elongation cycle is palmitate (16:0), although fatty acid species ranging from C_{12}-C_{18} fatty acids can be released from the enzyme complex.

Unsaturated fatty acids are the most abundant species in eukaryotic cells. The formation of their double bonds between carbon atoms involves the removal of a pair of protons and electrons from adjacent methylene groups by fatty acid desaturase enzymes. Animal and fungal fatty acid desaturases are intrinsic membrane proteins localized to the endoplasmic reticulum (ER). By contrast, plant desaturases that form the first double bonds in fatty acyl chains are soluble proteins located in the chloroplast.

The yeast, *Saccharomyces cerevisiae*, has only one fatty acid desaturase enzyme and can only form monounsaturated fatty acids. The *OLE1* gene encodes a Δ9 fatty acid desaturase which is a 53 kDa, 510-residue protein that forms a double bond between

carbons 9 and 10 of the hydrocarbon chain of a saturated fatty acyl CoA species (Figure 2). This enzyme consists of an N-terminal 379-residue desaturase domain that loops through the ER membrane 2 times and is linked to a 121-residue cytochrome b_5 domain. The lengths of the hydrophobic transmembrane loops of the desaturase are no more than 50 residues, which is sufficient to span the membrane twice, leaving very few amino acids exposed in the ER lumen. Therefore most of the desaturase protein, including the cytochrome b_5 domain, resides on the cytosolic surface of the ER membrane. The desaturase domain contains three clusters of histidine residues; these coordinate two iron atoms that form a diiron-oxo moiety in the reaction center of the enzyme. These histidine motifs have been found in all fatty acid desaturases [1].

Figure 2. Sequence alignments of *Saccharomyces* Ole1p (scOLE1) gene with *Candida albicans* Δ9 (caOLE1), Δ12 (caFAD12), and Δ15(caFAD15) fatty acyl desaturases. The cytochrome b_5 motifs in the carboxyl terminal region of scOLE1 and caOLE1 are delimited by the box shaded in light red.

Unsaturated fatty acids are formed in eukaryotic cells by an oxygen-dependent mechanism. In this process, electrons from reduced NADH are transferred via cytochrome b_5 reductase to cytochrome b_5. These intermediates are then transferred to the diiron-oxo reaction center in the desaturase, resulting in the formation of a highly reactive $2Fe^{IV}$ peroxo intermediate with sufficient energy to remove two electrons and two protons from adjacent carbons in the fatty acid hydrocarbon chain.

Although *Saccharomyces cerevisiae* contains only the Δ9 fatty acid desaturase and only forms monounsaturated fatty acids, most other fungi can synthesize polyunsaturated species. These are formed by other ER desaturases that sequentially insert additional double bonds in the hydrocarbon chain by a similar diiron-oxo mediated mechanism. The second and third double bonds are added via the activities of the ER Δ12 and Δ15 enzymes to produce linoleic (cis,cis, 9,12-18:2) and α-linolenic acid (cis,cis,cis 9,12,15-18:3).

Some fungi, such as *Mortierella alpina* 1S-4 [9], also express Δ5 and Δ6 desaturases that introduce double bonds between the Δ9 bond and the carboxyl group of very long chain (C_{20}-C_{26}) unsaturated fatty acids that have been lengthened through an ER membrane bound enzyme system. Both enzymes, like the Δ9 desaturase, contain cytochrome b_5 domains, but these are fused to the N-terminal end of the desaturase domain. Δ5 functionality is involved in conversion of di-homo-γ-linolenic acid (20:3 $\Delta^{8,11,14}$) to arachidonic acid (20:4 $\Delta^{5,8,11,14}$). It is also involved with the production of eicosapentaenoic acid (20:5 $\Delta^{5,8,11,14,17}$), originating from α-linolenic acid (18:3 $\Delta^{9,12,15}$)

[10]. The Δ6 desaturase enzyme forms a double bond between the sixth and seventh carbon in fatty acid chains, and is involved in the conversion of linoleic acid (C18:2 $\Delta^{9,12}$) to γ-linolenic acid (C18:3 $\Delta^{6,9,12}$).

Regulation of *Saccharomyces* Fatty Acid Desaturase Activity

The most intensively studied regulation of unsaturated fatty acid biosynthetic enzymes involves studies of the *Saccharomyces* Δ9 desaturase. These findings suggest that desaturases may be regulated by multiple biochemical factors, including the availability of nutrient unsaturated fatty acids, oxygen levels, and the presence of divalent metal ions.

Nutrient Regulation of Desaturase Activity

Bossie first demonstrated that supplementation of yeast cultures with either 16:1, 18:1 or 18:2 resulted in a dramatic repression of Ole1p enzymatic activity [11]. Each of these nutrient unsaturated fatty acids is rapidly integrated into cellular membranes, and after multiple cycles of growth can replace the endogenous 16:1 and 18:1 previously formed by the desaturase enzyme. When cells were exposed to the monounsaturated fatty acids or 18:2, *OLE1* mRNA levels rapidly dropped as much as 10-fold, followed by a loss of desaturase enzyme activity, apparently resulting from the reduced levels of Ole1p biosynthesis and rapid turnover of the existing enzyme. Northern blot analysis showed a 40% decrease in *OLE1* mRNA levels after 15 minutes of exposure to an equimolar mixture of 16:1 and 18:1 fatty acids. Steady state levels of mRNA were reached within thirty minutes following the addition of the monounsaturated fatty acids [1].

An analysis of the *OLE1* promoter region showed that there was an unsaturated fatty acid responsive element located approximately 510 base pairs upstream of the *OLE1* start codon that was essential for the activation and fatty acid mediated regulation of *OLE1* transcription [12]. A DNA fragment containing this element was found to activate transcription and confer fatty unsaturated acid mediated repression when it was placed into the inactive promoter region of a modified cytochrome c gene. Further studies by Gonzalez [13] and Vemula [14] showed that *OLE1* expression was also independently regulated by unsaturated fatty acids at the level of mRNA stability. Under stringently controlled conditions, the half-life of the *OLE1* transcript under the control of either the *GAL1* promoter or the native *OLE1* promoter was reduced from 12 minutes to less than 2 minutes when unsaturated fatty acids were added to the culture medium.

Oxygen-Mediated Regulation of Desaturase Activity

Saccharomyces cerevisiae is a facultative anaerobe and can grow in the complete absence of oxygen if unsaturated acids and sterols are provided in the growth medium. These compounds are required for anaerobic growth since they are formed by diiron-oxo desaturases, the only essential biosynthetic enzymes that require molecular oxygen. In cells growing under low oxygen levels (hypoxic conditions), *OLE1* expression is strongly increased to compensate for its reduced enzymatic activity, thereby enabling the cells to synthesize appropriate levels of unsaturated fatty acids [1, 15, 16]

Metal-Dependent Regulation of *OLE1*

Studies have also shown that *OLE1* expression responds to exposure to divalent transition metals, such as cobalt and nickel. Kwast *et al*. [17] showed that *OLE1* mRNA levels increased approximately 12-fold when cells were exposed to either 3mM cobalt or 3mM nickel chloride. The *OLE1* transcript levels were attenuated when excess iron was added to the medium, suggesting that the transition metals competed with iron in a hypothetical "oxygen sensor," which was presumed to be a heme protein associated with the mitochondrial respiratory chain. Studies of mutants defective in cytochrome oxidase and of mitochondria-deficient ρ^0 petite mutants indicated that defects in the respiratory chain attenuated, but did not completely block, the cobalt-induced response.

Molecular Regulation of *Saccharomyces OLE1* Expression

OLE1 expression in *Saccharomyces cerevisiae* is controlled by two proteins located in the endoplasmic reticulum, Spt23p and Mga2p, and by an E3 ubiquitin ligase, Rsp5p. Studies show that Spt23p and Mga2p exist in two forms, an ER membrane-bound form that is 120 kDa long, and a soluble 90 kDa form located in the nucleus and cytoplasm [18]. The inactive 120 kDa forms of Spt23p and Mga2p exist as homodimers anchored in the ER by a single membrane-spanning region near the C-terminus of each protein. Activation of either protein involves the proteolytic release of the N-terminal domain from the ER through ubiquination catalyzed by the Rsp5p enzyme, followed by cleavage near its C-terminal region by the 26S proteasome. The resulting 90 kDa soluble form of the protein is translocated to the nucleus, where its essential function is to activate transcription of the *OLE1* gene [18, 19].

Processing of the ER-bound Spt23p is regulated by the addition of unsaturated fatty acids to the medium. Experiments performed on Spt23p, under the control of the *GAL1*

promoter, showed a decrease in the processing of the membrane-bound 120 kDa form to the soluble 90 kDa form when cells were exposed to medium containing either 16:1, 18:2 or 18:3. Spt23 was effectively processed when the cells were exposed to medium containing 18:1. The fact that 18:1-containing phospholipids have higher phase transition temperatures than phospholipids containing the other fatty acyl supplements suggested that the processing of Spt23p may be sensitive to membrane fluidity [18, 19]. Activation of Mga2p, however, does not show a similar sensitivity to unsaturated fatty acids and Chellapa et al. [20], have shown that *OLE1* transcription is highly repressed in *spt23Δ;mga2Δ* cells that express a soluble 90 kD form of Mga2p. Those findings indicate that *OLE1* transcription is regulated in response to fatty acids by other mechanisms that act after the release of the protein from the ER membrane.

Fatty acid regulation in response to growth temperature.

The fatty acyl composition of phospholipid bilayers is a major determinant of the fluid properties of cell membranes. Consequently, ectothermic organisms that grow over a wide range of temperatures adapt their membrane fatty acyl content to optimize membrane-associated functions for different environmental conditions [21-23].

The introduction of a *cis* double bond into a fatty acid moiety produces a rigid kink in the hydrocarbon chain that prevents the formation of van der Waals interactions between the methylene groups of neighboring fatty acids. Thus a phospholipid bilayer with a population of highly unsaturated fatty acids (high double bond content) will exhibit a higher level of lipid mobility, or fluidity, at a given temperature than one with a similar headgroup composition and fatty acyl chain lengths, but fewer double bonds. Many ectothermic organisms alter the unsaturated fatty acid content of their membrane

lipids in response to changes in growth temperature. This is typically done by increasing the number of double bonds in fatty acyl chains of membrane lipids in cells grown at low temperatures.

Early experiments using the protozoan *Tetrahymena pyriformis* ([22, 24]), as well as the artic sculpin and goldfish [21, 25] showed decreased levels of saturated fatty acids and decreased levels of unsaturated fatty acids in membrane lipids when the organisms were adapted to lower temperatures.

Studies of the ascomycete fungus *Neurospora crassa* also showed that membrane lipids contain reduced levels of saturated fatty acids and increased levels of double bonds in unsaturated fatty acids as growth temperatures are lowered [1, 23]. The studies on *Neurospora* revealed that a shift in growth temperature from 37^{o} C to 15^{o} C resulted in a small increase in the level of unsaturated fatty acids from 82% to 87%; however, the number of double bonds/100 acyl chains increased significantly from 142 to 200. This is a consequence of the activation of the Δ12 and Δ15 desaturase activities, which resulted in the increase in 18:3 levels from 4% to 40% and an increase in the total polyunsaturated fatty acid species from 58% to 79% [23].

Although *Saccharomyces cerevisiae* does not form polyunsaturated fatty acids, Nakagawa [26] also showed that there is a small increase in total unsaturated fatty acids (from 75 % to 85%) and a corresponding decrease in saturated fatty acids in cellular lipids following adaptation from 30^{o}C to 10^{o}C. The small positive change was accompanied by an increase in 16:1 from 45% to about 56%, and a decrease in 18:1 from 28% to about 25%, suggesting that the increased levels of the shorter chain species also contributed to the maintenance of optimal membrane fluidity.

Candida Biology and Lipid Metabolism.

Candida albicans is a polymorphic fungus that is usually found in the gastrointestinal tract of humans and other warm-blooded animals. While *Candida* grows as a commensal organism in the intestine, it can also produce opportunistic infections in other parts of the body, including vaginal tissue, as well as infections in the mouth and esophagus [27-29]. These local infections are typically induced by treatments or conditions that disrupt the normal microbial flora, creating a niche for the proliferation of the *Candida* cells. In immune-compromised individuals or those with late stage HIV [30-32] *Candida* can grow as a systemic, hematogenous pathogen creating serious, life-threatening infections [27].

Candida albicans has a unique ability to exist in different morphological forms depending on its growth environment. There are three distinct forms: unicellular yeast (or blastoconidia), pseudohyphae and hyphae [28, 29, 33].

In its yeast form, *Candida* proliferates by budding to form daughter cells in a manner similar to that seen with *Saccharomyces cerevisiae*. The hyphal form grows as long cylindrical protrusions from the mother cell with septa that form compartments within the filaments [28, 29]. In the pseudohyphal form the daughter buds form elongated chains of cells that remain attached to the mother cell [34]. Unlike the hyphal forms, whose walls are linear, with no constrictions, pseudohyphal walls are constricted at the sites of the septa.

Growth of the different morphological forms of *Candida* is controlled by the environment. *Candida* grows exclusively as a unicellular form at temperatures below 30^{o}

C or at a pH of 4.0. Growth of pseudohyphae occurs at a pH of 6, or around 35^{o} C and *Candida* hyphae grow best at 37^{o} C and at a pH of 7 [34]. It has been proposed that the transition of *Candida* from the yeast to the hyphal form is a virulence factor since the cylindrical growth may aid the penetration of epithelia, thereby allowing the fungus to enter the bloodstream as well as promoting its release from engulfing macrophages [28].

Unlike *Saccharomyces cerevisiae*, *Candida albicans* and most other fungi express multiple endoplasmic reticulum desaturases in addition to the Δ9 enzyme. These produce mono- and polyunsaturated fatty acids [35]. *Candida* not only has a Δ9 desaturase, but it also has Δ12 and Δ15 enzymes, enabling the organism to convert oleic acid sequentially into linoleic (C18:2) and α-linolenic (C18:3) acids (Figure 1).

Molecular Regulation of *Candida OLE1* Expression

An examination of the existing gene data bases indicates that most fungi, including *Candida albicans*, express only one homologue of the *Saccharomyces SPT23* and *MGA2* genes. These proteins also appear to be unique to fungi and are not found in other organisms. One question that emerges is whether these *SPT23/MGA2* homologues are exclusively involved in the regulation of the multiple fatty acyl desaturases found of other fungi. Studies conducted by the Martin laboratory show that the *Candida SPT23* gene is able to repair unsaturated fatty acid auxotrophy in a *Saccharomyces spt*23Δ*; mga*2Δ strain [36]. They also showed through glucose repression studies of caSPT23 under the control of the *MAL1* promoter and by subsequent metabolic labeling studies that, when *caSpt23* expression is blocked, synthesis of mono-unsaturated and polyunsaturated acids is repressed, resulting in growth arrest and rapid cell death. These

effects can be repaired by adding unsaturated fatty acids to the growth medium, suggesting that this gene also plays an important role in *Candida* regulation of unsaturated fatty acids [36].

MATERIALS AND METHODS

Strains and Growth Media

C. albicans strain CAI-4 was exclusively used in this study. This strain is derived from a clinical isolate and contains mutations in both alleles of the *URA3* locus. *Candida* strains were streaked on a weekly basis from -80^{o}C frozen stocks onto YPD yeast medium containing 80 μM uridine (YPD Uri) to provide single colony inocula for overnight (O/N) cultures. Overnight strains were grown at 30 °C on synthetic dextrose yeast medium containing 80 μM uridine and 1 mM uracil (SD Ura+ Uri). In experiments employing fatty acid supplements, the growth medium used in liquid cultures contained 0.1% Tergitol/NP-40 (Sigma) to disperse the fatty acids. Tergitol/NP-40 is not derived from fatty acids or fatty alcohols, and is apparently not metabolized by *Candida*. Fatty acids were obtained from NuChek Prep (Elysian, MN). Growth tests were performed by monitoring *A*600 of the cultures or by hemocytometer counting.

Growth of CAI-4 Cells at 15^{o}C, 20^{o}C, 30^{o}C, 37^{o}C

CAI-4 cells were grown in an O/N culture in a 30^{o}C water bath in test tubes containing 3.0 mL Ura+ Uri medium. For experiments, cells from the O/N culture were inoculated at a concentration of 1 x 10^{7} cells/mL into 50mL medium Ura + Uri medium in a 250mL flask. Experimental cultures were grown consistently in a water

shaker bath at one of four of the following temperatures: 15°C ± .5°C, 20°C ± .5°C, 30°C ± .5°C, 37°C ± .5°C. Samples were taken for growth analysis, lipid analysis (using a gas chromatograph) and mRNA analysis at intervals for up to 50 hours. Cell samples used for growth rate determinations were used immediately after being placed on ice. Cell samples used for lipid analysis were spun down into a pellet, and washed with cold diethlypyrocarbonate (DEPC) -treated water. The washed cells were repelleted, and after the removal of 95% of the supernatant, were then stored at -80°C until further use. Cells used for mRNA analysis were pelleted and, following removal of supernatant, immediately resuspended with 1mL of RNALater. These suspensions were stored at 4°C until use for RNA extractions.

Growth curve determinations of CAI-4 cells.

For cells in cultures grown at 15°C – 30°C, cell concentrations were determined by counting 10 μL aliquots in a hemocytometer.

Because cells grown at 37°C form extensive hyphae, growth rates of these cultures were determined by wet weight analysis of hyphal pellets produced by microcentrifugation . Cells from aliquots with large volumes were initially harvested by preparative centrifugation. The resulting pellets were then resuspended in a small volume, packed by microcentrifugation and then resuspended in 1.0 mL distilled water. The total weight of the cell pellet was determined by subtracting the weight of resuspended pellet in the microfuge tube from the weight of a tared 1.5mL microfuge tube containing 1mL of cold distilled water. The density of the cell culture / mL at that time was then determined by

dividing the resulting weight by the original harvesting volume for that time point. Results are reported in milligrams per mL.

Fatty Acid Supplementation Experiments.

Cells from overnight cultures grown in SD Ura+ Uri medium + 1 % Tergitol were inoculated into 90 mL of the same medium, followed by the addition of 1 mM fatty acid supplements in no more than 250 µL ethanol. Cells were harvested at intervals by centrifugation and washed at room temperature 1x with 1% Tergitol in distilled water, followed by a 1X wash with distilled water prior to HCl methanolysis for gas chromatography.

Fatty Acid Analysis of Total Cellular Lipids

Fatty acid composition of CAI-4 cells was determined by gas chromatography analysis of fatty acid methyl esters. Harvested cells were washed with 1mL cold distilled water. These cells were pelleted and 90% of the supernatant was removed. One hundred µL of the cell suspension was then transferred to glass tubes that were rinsed with methanol:chloroform (2:1). One mL of the SAP-Meth cocktail was added to the tube, which was then sealed by a cap with a Teflon cap liner. The sealed tubes were then boiled for 20 minutes in a boiling water bath, cooled on ice to room temperature, and 1.0 mL 0.9% sodium chloride and 1.0 mL hexane ether (1:1) were added to the mixture. The suspensions were mixed by vortex and then centrifuged at max speed in a clinical centrifuge to create a hexane:ether / water phase

separation. Ninety percent of the hexane layer was then transferred to a fresh glass tube that was also rinsed with methanol:chloroform (2:1). Another 1mL hexane was added to the tube with the original mixture, and the hexane collection was performed once more. The hexane-ether solvent was then evaporated under a nitrogen steam and the resulting methyl esters were resuspended in approximately 100 µL hexane. Fatty acid methyl esters were then separated and quantified by flame detection in a Varian 3400CX gas chromatograph (GC) on a 30 m x 0.032 mm i.d. Supelcowax -10 capillary column using helium as a carrier gas.

SAP-Meth Cocktail

30.8 mL Methanol

2.5mL Dimethoxypropane

16.7 mL 3N – Methanolic HCL

RNA Extraction Methods

For the following methods all solutions were prepared using DEPC treated distilled water and were sterilized by autoclave. All plasticware was rendered RNAase free as obtained by the manufacturer or by prolonged sterilization. Cell for mRNA analysis were harvested and concentrated by microcentrifugation and immediately suspended and stored in 1.0 mL RNALater. Total RNA was extracted using either the hot phenol method or the TriZol mRNA isolation method.

TriZol mRNA isolation method:

For this method cells suspended in RNALater were pelleted by microcentrifugation and resuspended in 600µL TriZol reagent with 100 µL of glass

beads. The cells were then disrupted by 3 cycles of bead beating for one minute at 4°C, followed by cooling on ice for one minute. The Trizol volume was then brought to 1mL and the mixture was incubated at room temperature for 5 minutes followed by the addition of 100μL of bromochloropropane (BCP). The tubes were then shaken vigorously for 15 seconds and incubated at room temperature for 5 minutes. The suspensions were cleared of cellular debris by microcentrifugation at 4°C for 10 minutes. Ninety percent of the upper aqueous phase was then transferred to a fresh 1.5mL microfuge tube and 500 µL isopropanol was added to the solution (1:1). The solution was mixed by vortex for 30 seconds, incubated at room temperature for 5 minutes, and then microcentrifuged at maximum speed for 8 minutes at room temperature. The resulting RNA precipitate consisted of a white or gel-like pellet at the bottom of the tube. The supernatant was then removed and the pellet was washed 2X with 1 mL 70% ethanol, followed by centrifugation for 5 minutes to remove the ethanol RNA pellets. The pellets were then allowed to air-dry for 3 minutes, resuspended in either TE buffer or DEPC treated water, and stored at 4°C until further use.

Hot phenol method:

For this method cells stored in RNALater were pelleted by microcentrifugation for 5 minutes at maximum speed. The supernatant was discarded and the pellet was resuspended in 400 µL AE buffer, 50 µL 10% sodium dodecyl sulfate, and extracted with 500 µL AE buffer-saturated phenol. The suspensions were pelleted after incubation at 65 °C for four minutes and subjected

to 2 cycles of rapid chilling in a dry ice/ethanol bath for five minutes and re-incubation at 65 ^{o}C. After the second freeze cycle, the suspensions were thawed until just melting and microcentrifuged 5 minutes. The upper aqueous phase was then transferred into a 1.5 mL microcentrifuge tube, followed by the addition of 500 µL AE buffer saturated phenol/chloroform (1:1). This suspension was mixed by vortex and incubated for 5 minutes at room temperature, followed by centrifugation for one minute. The aqueous phase was then transferred to a new 1.5 mL microcentrifuge tube and extracted with phenol/chloroform (1:1), and again with chloroform / isoamyl alcohol (1:1); each time the upper aqueous phase was deposited into a new 1.5 mL microcentrifuge tube. A 0.1 volume of 3M NaOAC, pH5.2 and 2-3 volumes of RNase-free 100% ethanol were then added to the sample, followed by incubation at -20 ^{o}C for one hour. The resulting RNA was then pelleted by centrifugation for 5 minutes. The pellet was then washed with 500 µL RNase-free 70% ethanol by invert mixing, followed by centrifugation for 2 minutes. The pellet was then dried using a speed-vacuum for ten minutes and resuspended in 50 µL DEPC-treated TE buffer. RNA concentrations in the sample were determined by $OD_{260/280}$ readings in a spectrophotometer.

Preparation and Processing of RNA Samples for QPCR Analysis

RNAase free DNAase treatment:

In a 1.5 mL microcentrifuge tube, 50 µL resuspended pellet containing ~10 µg nucleic acid were mixed with 5µL 10X DNAase buffer (0.1 volumes) and 4 µL Turbo DNAase I (2U/ul). The sample was incubated at 37^{o} C for 30 minutes,

followed by the addition of 5.9ul DNAase inactivator (0.1 total volume). The mixture was allowed to stand for 5 minutes at room temperature, followed by centrifugation for 5 minutes at maximum speed. The supernatant was extracted into a new 1.5 mL centrifuge tube and stored as necessary. For long term storage, samples were stored at -20 ^{o}C.

Reverse Transcription (RT) reaction:

RT amplifications of DNA-free RNA samples were performed using reagents in the Applied Biosystems High Capacity cDNA Reverse Transcription Kit. The following components were combined in a 1.5 mL microfuge tube for a single RT reaction: 10 µL DNAase treated RNA sample, 2.0 µL 10X RT Buffer, 0.8 µL 25X dNTP Mix (100mM), 2.0 µL 10X RT Random Primers, 1.0 µL Multiscribe Reverse Transcriptase, and 4.2 µL Nuclease-free water. The reactions were performed with the following thermal cycling conditions: 25^{o}C for 10 minutes, 37^{o}C for 120 minutes, 85^{o}C for 5 minutes, then hold as necessary at 4^{o}C.

Preparation of Quantitative Polymerase Chain Reaction (qPCR) plates:

The following components were combined in a single well of a 46 well qPCR plate to perform single 20 µL reaction: 10 µL FAST Sybr Green, 0.5 µL forward primer, 0.5 µL reverse primer, 1 µL RNA sample (~50µg cDNA), 4 µL nuclease-free water. Oligonucleotide primers for the RT amplified transcripts were designed

using Primer Express from ABI and supplied by IDT. The primers used in this study are listed in Table 1.

Gene	5’ Primer	3’ Primer
OLE1 (Δ9 desaturase)	**TGTTGACTAAAGCCAACCCAA**	**CAGTCGGCAACAAGATCAGAAA**
FAD2 (Δ12 desaturase)	**TGATGAAAATATGTGGGTCTCGTT**	**ACCCCATTATTACCATCGACAAA**
FAD3 (Δ15 desaturase)	**CCCATGGCTTTGGGTCAA**	**GGTTGGGTCGGTGTGTTGTAA**
18S rRNA	**CGTCCCTGCCCTTTGTACAC**	**GCCTCACTAAGCCATTCAATCG**

Table 1. Primers used to generate RT-qPCR probe fragments

EXPERIMENTAL RESULTS

Previous studies of rapid cooling of ectothermic organisms typically show that membrane lipids undergo dramatic changes in their fatty acid profiles that are required to maintain an appropriate level of membrane fluidity needed for optimal growth at the new temperature. Because the native habitat for *Candida albicans* is in warm blooded animals that maintain a constant body temperature, the question arose as to whether *Candida* retained this adaptive response as it diverged from other fungi that experience a wide range of temperatures in their habitat.

Effects of Temperature Acclimation on Growth

The initial sets of experiments in this study were designed to evaluate the effects of changing temperature on *Candida* growth rates. Cells from the overnight cultures grown to stationary phase (>10^8 cells/mL) were transferred to fresh medium at varying temperatures and cell densities and were monitored over time until the cultures reached stationary phase. Figure 3 shows the effects of the different temperatures on cell growth conducted over a range from 37^{o}C to 15^{o}C. Table 2 presents the growth constants and the minimum doubling times observed for each of these conditions.

Cells that were shifted to 37^{o}C did not exhibit a detectable lag phase before assuming a doubling time of 1.37 hours. In those cultures hyphal growth from the blastocondia was evident by about 1.5 hours after inoculation. Cells that were shifted to 30^{o}C appear to exhibit a short lag phase of < 1 hour prior to assuming a doubling time of 1.22 hours. There was a lag phase of 2 hours in the 20^{o}C cultures, followed by an average

logarithmic phase doubling time of 4.74 hours. We observed that the 15^{o}C cultures exhibited a biphasic lag phase prior to the entry into logarithmic phase growth. Repeated experiments (n = 6) showed that at 15^{o}C there was an initial lag phase of about 4 hours, followed by approximately 4 hours of logarithmic growth (doubling time = 7.8 hours) followed by a second lag period of 4 hours before sustained logarithmic growth was initiated with a doubling time of 7.08 hours.

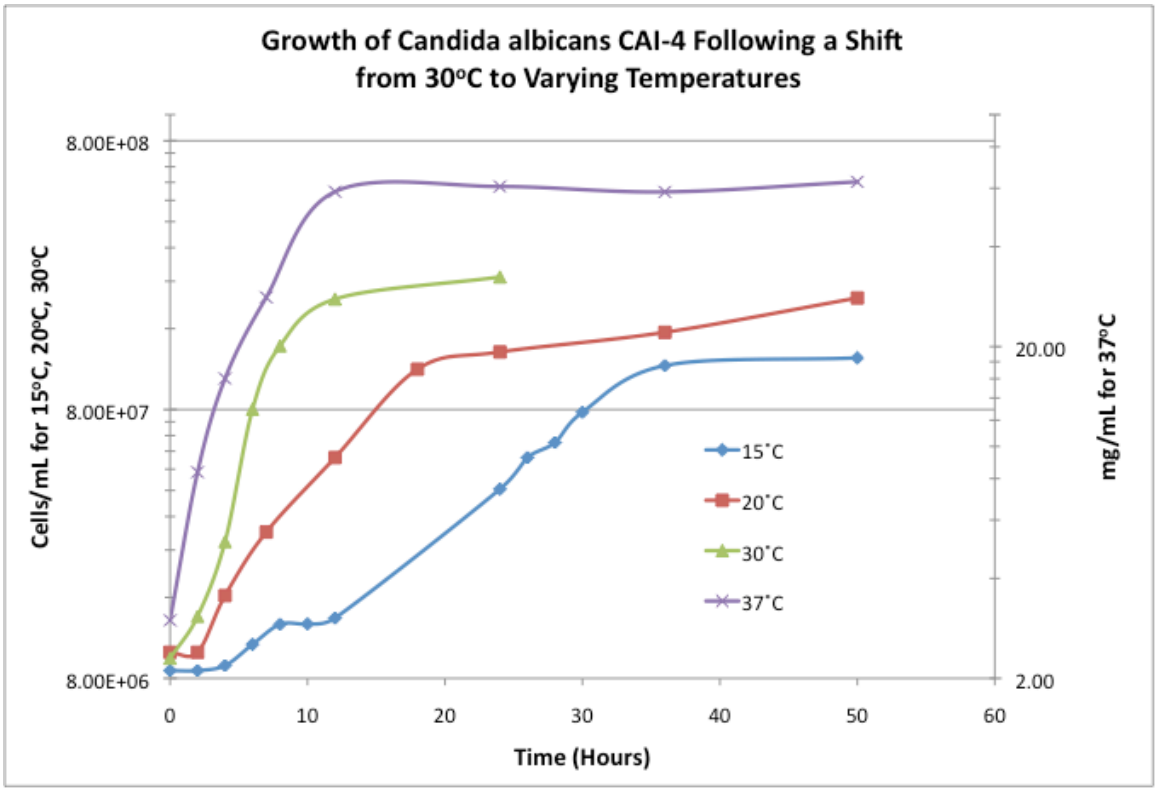

Figure 3. Growth of *Candida albicans* following a shift from 30°C to varying temperatures. Cells were shifted from stationary phase in 30°C to flasks containing fresh room temperature medium. Flasks were then placed in reciprocating water baths maintained at either 15°C, 20°C, 30°C or 37°C. Cell counts were obtained at the indicated times for 15°C, 20°C, and 30°C through stationary phase. For cells growing at 37°C, growth is plotted as cell density in mg/mL due to the formation of hyphae at that temperature. Plots show the averages of 3 independent trials for each growth

Growth Temperature (°C)	Growth Constant μ	Doubling Time (hours)
15	0.0979 ± 0.0032	7.09 ± 0.24
20	0.146 ± 0.046	4.74 ± 1.23
25	0.194 ± 0.014	3.6 ± 0.270
30	0.572 ± 0.055	1.22 ± 0.11
37	0.507 ± 0.038	1.37 ± 0.09

Table 2. Maximal logarithmic phase growth rate constants and calculated doubling times for *Candida* cells growing at different temperatures in SD Ura+ Uri medium.

Effects of Growth Temperature and Growth Phase on Cellular Fatty Acid Composition

A series of experiments was performed to observe changes in the membrane fatty acid composition that correspond to changes in growth temperature. CAI-4 cells were grown overnight to stationary phase in a 30^{o}C water bath. Cells from the overnight culture were transferred to fresh medium and were then shifted to varying temperatures. Aliquots were harvested that corresponded to a density of $2x10^{8}$ cells / mL for each time point, and fatty acid methyl esters derived from HCl methanolysis of the washed cell pellets were analyzed using gas chromatography. Figures 4-7 show the effects of the temperature shifts on the membrane fatty acid composition relative to the respective growth curves during lag, log and stationary phases of cellular growth. In these experiments we observed that the cells showed significant changes in fatty acyl content as cells progressed through the growth phases, in addition to overall differences associated with culture temperatures at each phase of growth.

Changes in Fatty Acyl Content Associated with Growth Phase at 30 ^{o}C.

Figure 4 shows the effects of the growth phase on the fatty acid composition of cells maintained at a constant temperature of 30^{o}C. The predominant fatty acid species are 18:1 and 18:2, which together comprise about 60 wt% of the total cellular fatty acids throughout the growth phases. At 0 time, 18:1 is the most abundant species at (approximately 35 wt %). As the cells enter and pass through logarithmic phase growth 18:1 levels decline and 18:2 levels increase from 27 wt% to 33 wt%. Therefore 18:1 and 18:2 are maintained at approximately equimolar levels of about 30% during the experimental period. As cells enter stationary phase, 18:1 levels increase and 18:2 levels

decrease, thus returning to the pattern observed at 0 time. By contrast, the third most abundant species, 16:0, remained at a constant level of about 12% throughout the initial and logarithmic growth phase of the experiment, and then increased its levels to about 19 wt% as the cells entered stationary phase. Levels of 18:3, the most highly unsaturated acid species, were found to increase from about 4 wt% at 0 time to about 13 wt% in logarithmic phase. 18:3 then decreased to its original level of 5 wt % as the cells entered stationary phase.

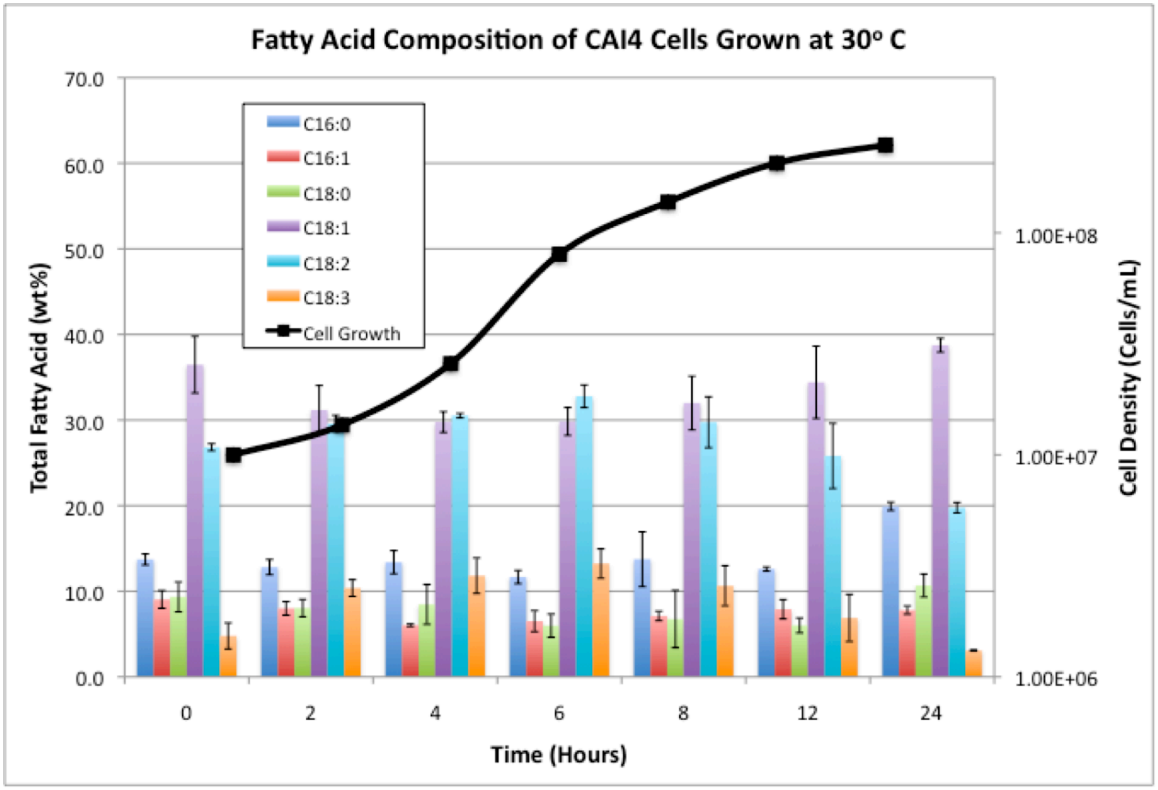

Figure 4. Fatty acid composition of CAI-4 cells grown at 30°C.
Changes in total fatty acid levels of cells grown at 30°C. Values are plotted against a corresponding growth curve at the indicated temperature. Plots show the averages of 3 independent trials.

Changes in Fatty Acid Levels Associated with Shifts in Growth Temperature.

In cells subjected to rapid cooling to either 20°C or 15°C, 18:1 levels exhibited a similar pattern of decline from through the lag and logarithmic growth phases, followed by a rebound as the cells entered stationary phase. In the cells shifted to 20°C 18:1 levels decreased from 37 wt% to 26 wt%; 18:2 levels increased from 28 wt% to 35 wt% during mid logarithmic phase, but declined to lower levels as cells returned to stationary phase. In the 15°C cultures, 18:1 decreased from 34 wt% to 26 wt%, and 18:2 increased from 28 wt% to 35 wt%, as cells entered logarithmic growth and moved into the stationary phase. Increases in 18:2 and 18:3 levels at both temperatures did not occur until the growth phase had exited the respective lag period and the cells had begun logarithmic growth (Figures 5-6).

The most dramatic changes in cellular fatty acid content occurred in cells that were shifted to 37° C. The most pronounced changes occurred at approximately 4 hours, which correlated with the induction of hyphal growth. At that point there was a pronounced increase in 18:1 levels from about 35 wt % to 47 wt %, and a concomitant decrease in 18:2 levels from about 27 wt % to 20% total fatty acids. This was accompanied by a sharp reduction in 18:3 levels, which changed from about 5 wt% total fatty acids to 1.5 wt% of the total species by late stationary phase (Figure 7).

Figure 8 shows the levels of fatty acids at comparable points in the mid-logarithmic growth phases across all temperatures. The most dramatic differences are seen at the highest temperature, 37°C, which is associated with the transition from budding to hyphal forms of the organism. In these cells there are elevated levels of 16:1

and 18:1 and reduced levels of 18:2, 18:3 and 18:0. Surprisingly, although 18:0 is a minor fatty acid species, its levels showed the closest correlation with changing temperature, exhibiting its highest levels at 15^{o}C and a progressive decline as the temperature increased to 37^{o}C.

Fatty acid levels were also compared across all temperatures at the stationary growth stages (Figure 9). These were essentially comparable at 15^{o} C and 20^{o} C, except for higher levels in 18:3 at 20^{o} C than at 15^{o}C.

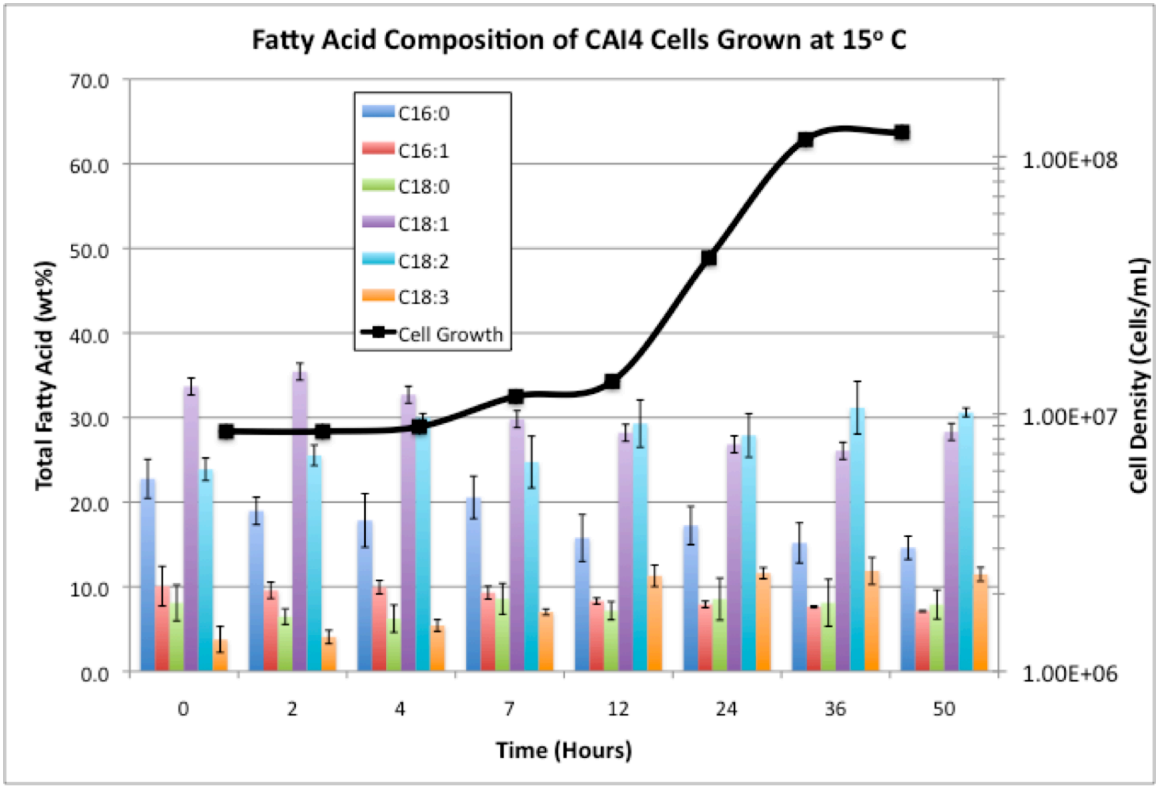

Figure 5. Fatty acid composition of CAI-4 cells grown at 15°C.
Changes in total fatty acid levels of cells grown at 15°C. Values are plotted against a corresponding growth curve at the indicated temperature. Plots show the averages of 3 independent trials.

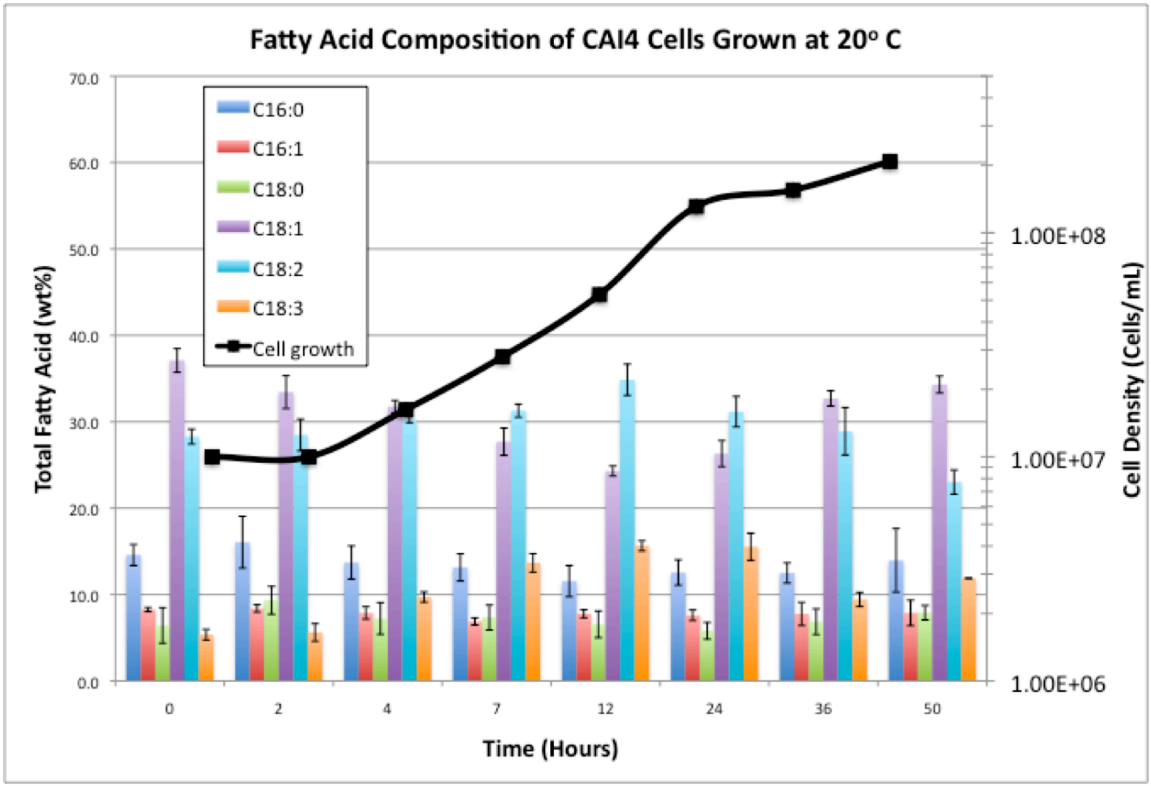

Figure 6. Fatty acid composition of CAI-4 cells grown at 20°C.
Changes in total fatty acid levels of cells grown at 20°C. Values are plotted against a corresponding growth curve at the indicated temperature. Plots show the averages of 3 independent trials.

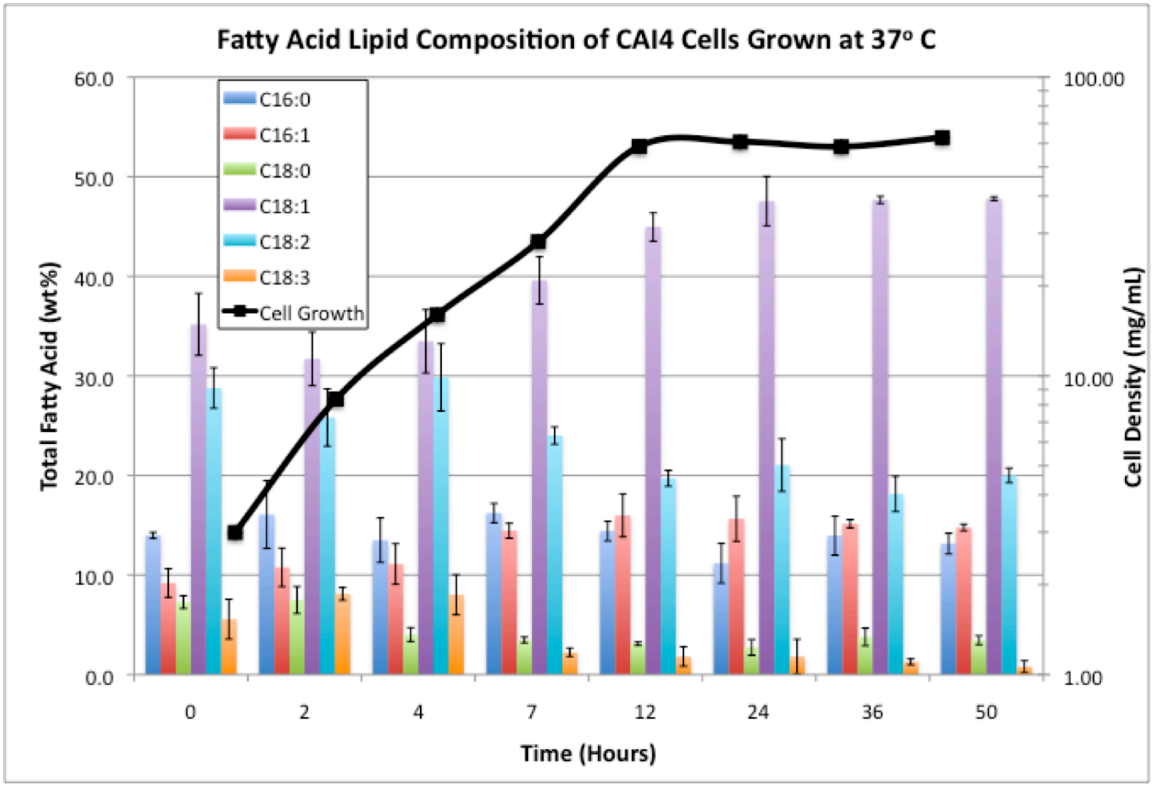

Figure 7. Fatty acid composition of CAI-4 cells grown at 37°C.
Changes in total fatty acid levels of cells grown at 15°C. Values are plotted against a corresponding growth curve at the indicated temperature. Plots show the averages of 3 independent trials.

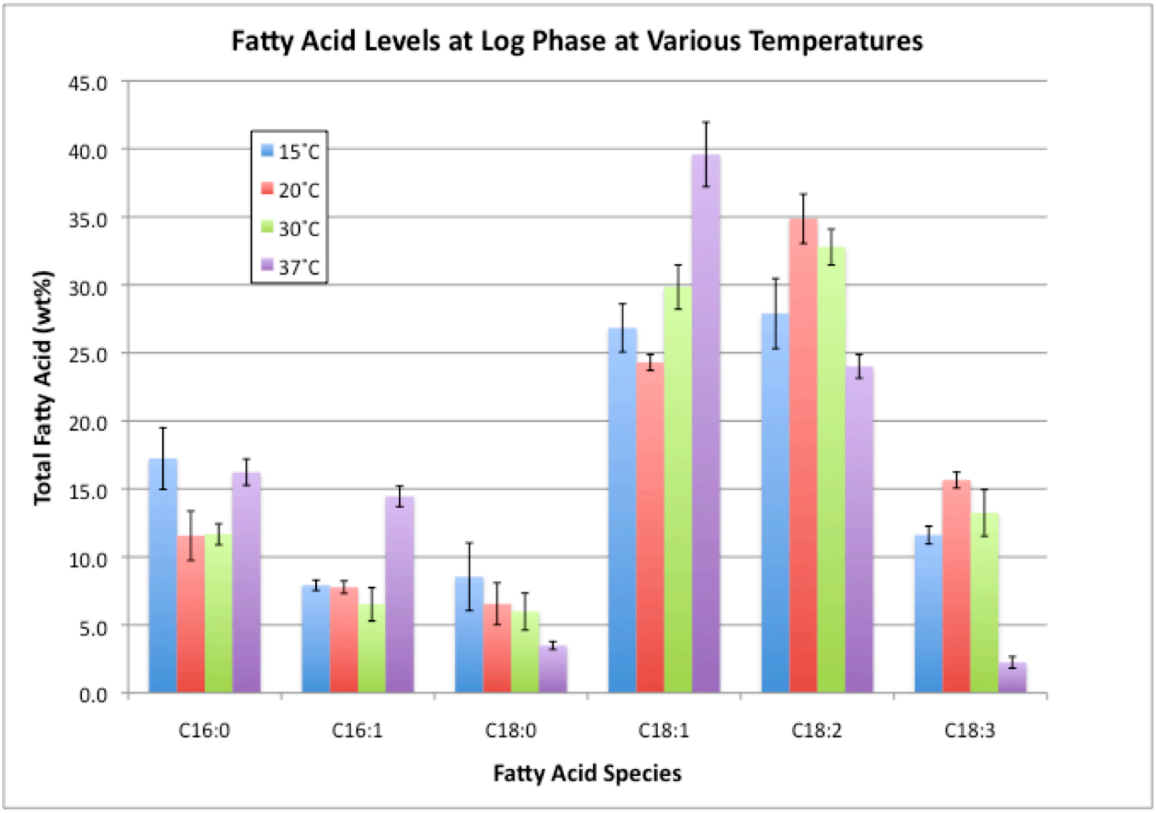

Figure 8. Fatty acid levels at log phase at various temperatures.
Comparison of different fatty acid species levels across different temperatures at mid-logarithmic phase. Plots show the averages of 3 independent trials.

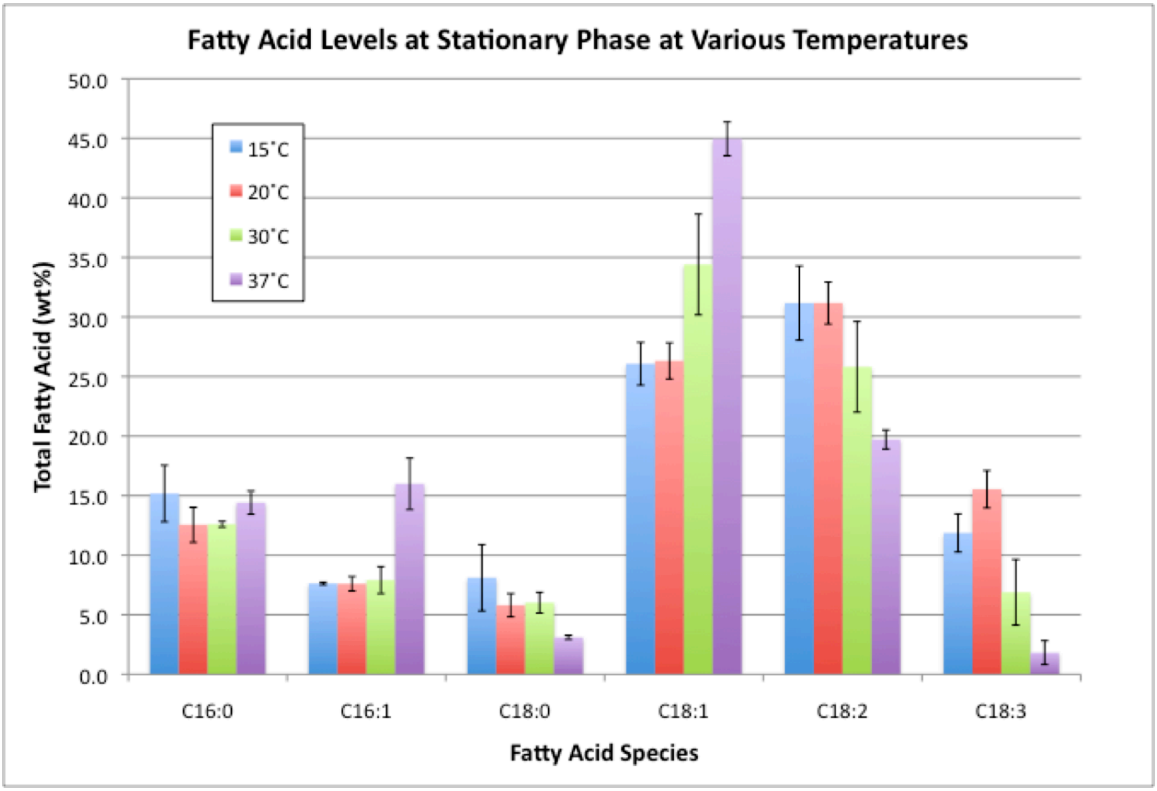

Figure 9. Fatty acid levels at stationary phase at various temperatures. Comparison of different fatty acid species levels across different temperatures at stationary phase. Plots show the averages of 3 independent trials.

Changes in Double Bond Content Associated with Shifts in Growth Temperature

For most ectothermic organisms, the adaptation to low temperature correlates strongly with proportional increases in the number of double bonds in the fatty acyl population produced by increased desaturase activities [22, 23]. Figure 10 shows the effect of growth temperature on the Double Bond Index (the number of double bonds / 100 molecules) of *Candida* cellular fatty acids at points in the growth curves corresponding to the lag phase, mid logarithmic phase and stationary phase. This number was calculated as the sum of the weight % of each fatty acid species times the number of double bonds in that species. Since all cultures were inoculated from overnight cultures adapted to 30^{o}C, all of the initial values at the different growth temperatures are similar, ranging from 112 – 120 due to the absence of significant growth during the lag period. Furthermore, cultures at all temperatures showed an increased level of double bond content at mid-logarithmic growth. The largest changes at that point were seen in the 20^{o}C and 30^{o}C cultures, which showed a 30% and 26% increase in double bonds from the initial values.

We expected that the 37^{o}C cultures would exhibit much smaller increases in the DBI during the logarithmic growth phase and in fact this corresponded to no more than a 12% increase over the initial values. We were surprised to find, however, that the DBI in the log phase 15^{o}C cultures was almost identical to that of 37^{o}C cells.

Other striking differences were observed in the double bond content of cells sampled in the stationary phase. Here the 30^{o}C and 37^{o}C adapted cells showed large

drops in their DBI from the logarithmic phase values. In the case of the 30°C culture, the values returned to those found in the initial lag phase whereas the DBI in the 37°C culture was lowered to 90% of the levels found in the initial phase. By contrast, the double bond content of the 15 °C grown cells continued to increase in the stationary phase to a value of about 132 while the 20 °C cells showed a decrease to a value of about 142 bonds/100 molecules. Given that we expected to see progressively increasing DBIs as growth temperatures were lowered, we were again surprised to see that the 20°C cultures displayed significantly higher levels of double bonds than the 15°C cells during all phases of growth.

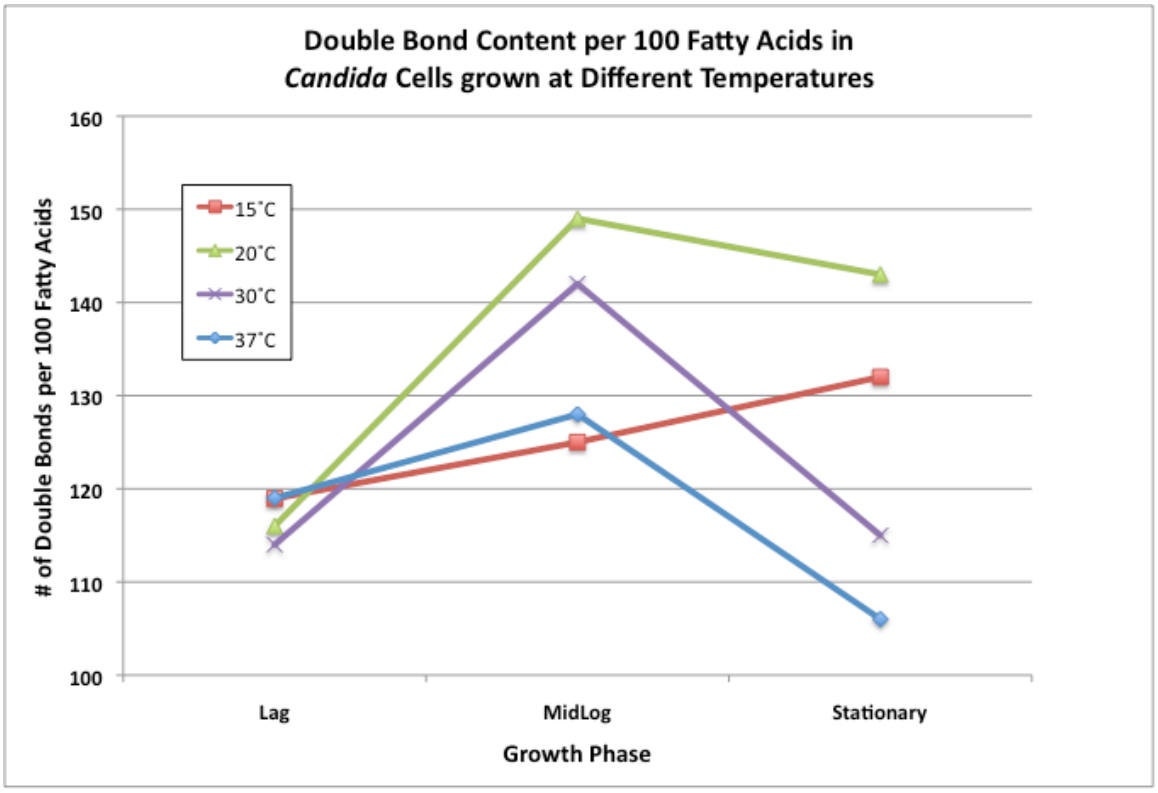

Figure 10. Double bond content of fatty acids in CAI-4 cells at varying temperatures over time. These plots show the averages of 3 independent trials for each growth temperature.

Effects of Growth Temperature on Fatty Acid Desaturase mRNA levels

To determine the effects of growth phase and temperature on gene expression, levels of the Δ9, Δ12 and Δ15 desaturase mRNAs were determined using Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-QPCR) methods. In these experiments, mRNA levels were determined relative to the 18S ribosomal RNA, which was used as the quantitative standard. These data are shown in Figures 11– 14 and are expressed as the percent of each species relative to the Δ9 transcript levels at time 0.

At all temperatures and growth conditions the Δ9 desaturase mRNA levels were found to be significantly higher than the levels of mRNAs encoding the other Δ12 and Δ15 enzymes. Typically the overnight 30^{o}C grown cells exhibited at least 5 times as many Δ9 mRNA molecules as either the Δ12 or Δ15 species. The levels of all transcripts showed significant variations with respect to the growth phases at all temperatures, however, and depending on the growth conditions, the Δ9 transcript ranged from 2 to 10-fold higher than the other species.

Figure 11 shows the effects of growth phase on mRNA levels in cells grown at 30^{o}C. All of three mRNA species tended to decrease as the cells progressed through the growth phases. The Δ9 mRNA levels remained at the initial level through 2 hours, followed by a progressive decline to 25% of starting value at late logarithmic phase. This was followed by an approximate 2.5 -fold induction of the transcript as the cells entered into stationary phase at 12 hours after inoculation. These levels then declined again, to less than 20% of the initial value in late stationary phase. The Δ12 and Δ15 transcripts, by contrast, show a progressive decline throughout the growth phases, reaching their lowest

levels at late stationary phase.

Figure 12 shows the levels of transcripts in cells grown at 20^{o}C. In these cultures the Δ9 and Δ12 transcripts show a progressive decline throughout the growth experiment such that in late stationary phase cells the Δ9 mRNA levels are reduced about 5-fold and the Δ12 transcripts are reduced about 8-fold. By contrast, the Δ15 mRNA levels exhibit a slight increase over the first four hours of the experiment, followed by a similar gradual decline in levels, with a minimum at late stationary phase.

Figure 13 shows the effects of 15^{o}C growth on desaturase mRNA levels. Here the Δ9 desaturase mRNA levels remained near the initial value during the first part of the biphasic lag phase, followed by a decline to 40% in the second phase of the lag period. These levels then became elevated to 60% of the initial value and remained at those levels throughout the remainder of the experiment. We were surprised to see that the Δ12 and Δ15 desaturase levels remained at relative levels that are lower than those observed at 20^{o}C throughout the experiment, although this appears to correlate with the higher levels of 18:2 and 18:3 that were also observed in the 20^{o}C cultures.

Figure 14 shows the relative mRNA levels in the 37^{o}C grown cultures. In these cultures, there is a 2.5 fold induction of the Δ9 mRNA at 2 hours following inoculation at the initial phase of hyphal formation. This is followed by a progressive decline in the transcripts for all three desaturases, so that in late stationary phase the Δ9 mRNA levels are 10% of the initial value, and the Δ12 and Δ15 mRNAs are not detectable.

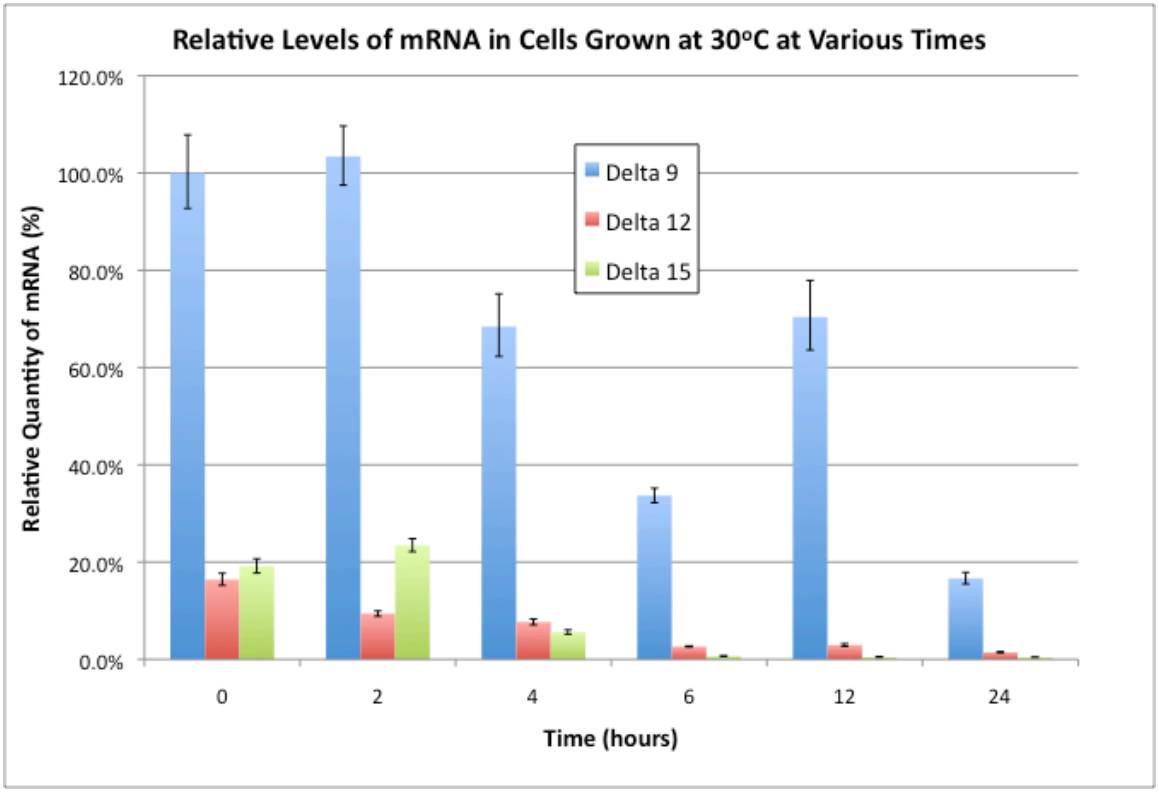

Figure 11. Comparison of mRNA levels in cells grown at 30°C over time. Relative mRNA levels are compared to the 0 time Δ9 transcript. Relative quantities are averages of 2 trials.

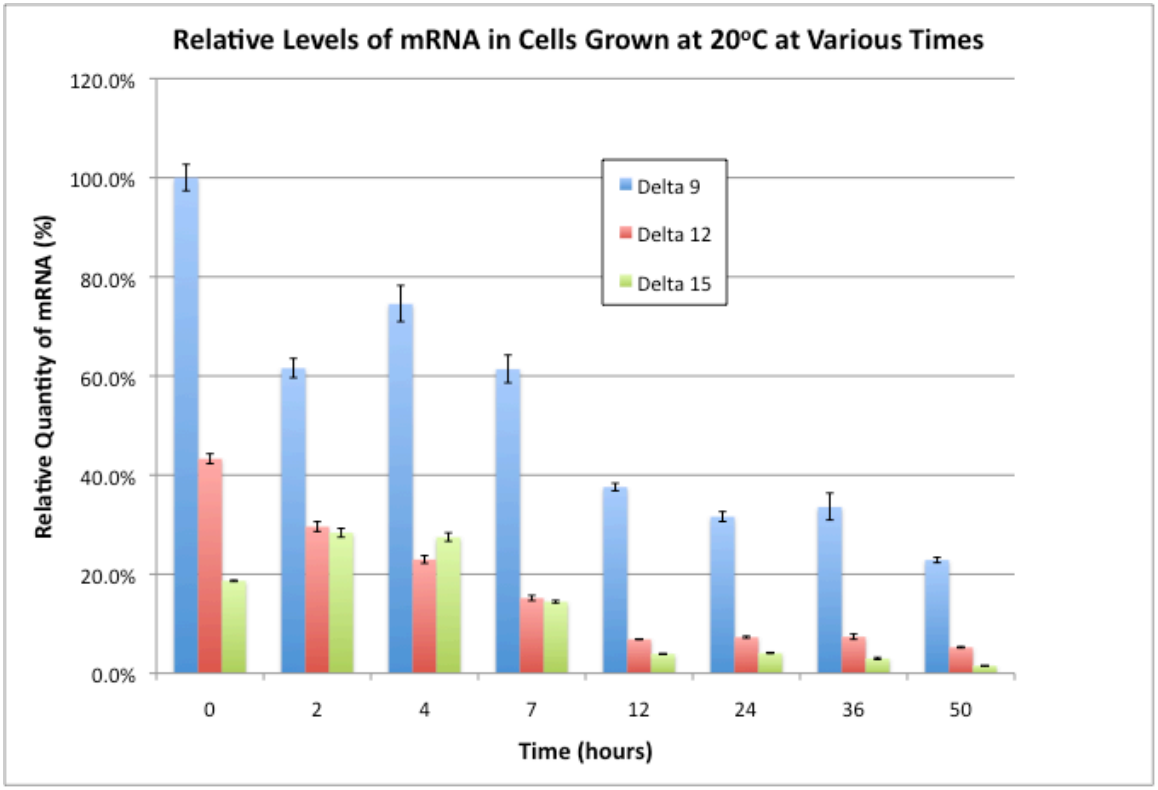

Figure 12. Comparison of mRNA levels in cells grown at 20°C over time. Relative mRNA levels are compared to the 0 time Δ9 transcript. Relative quantities are averages of 2 trials.

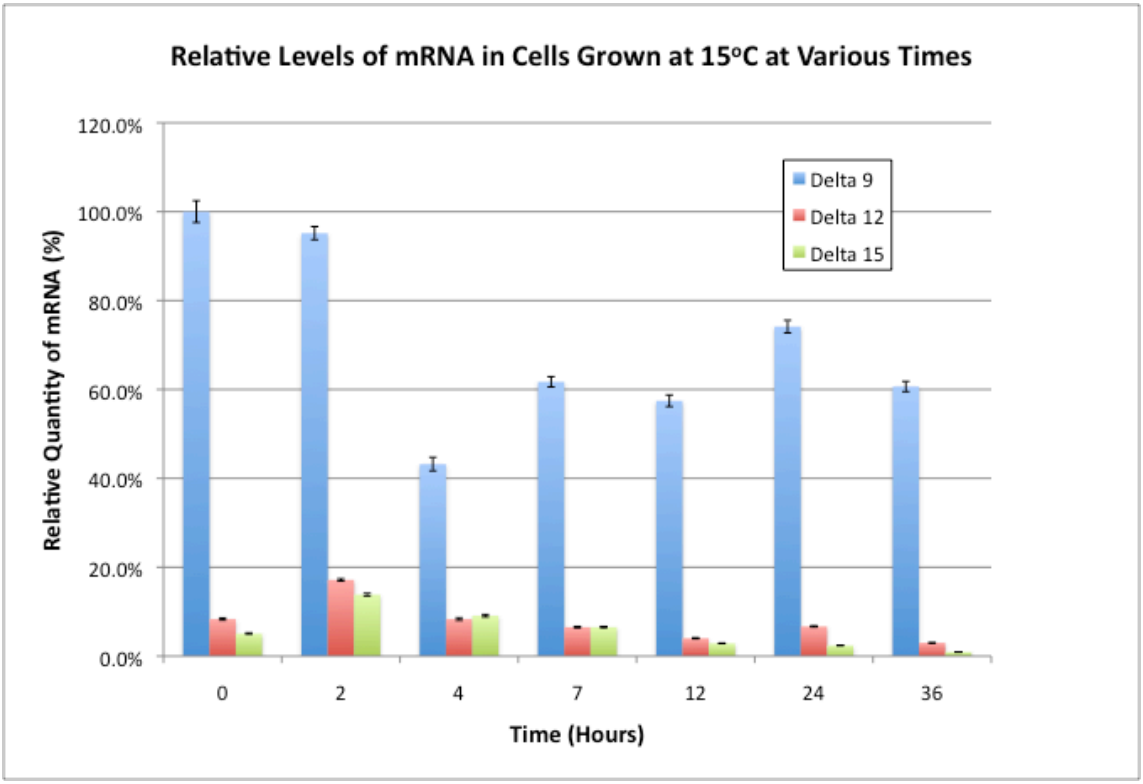

Figure 13. Comparison of mRNA levels in cells grown at 15°C over time. Relative mRNA levels are compared to the 0 time Δ9 transcript. Relative quantities are averages of 3 trials.

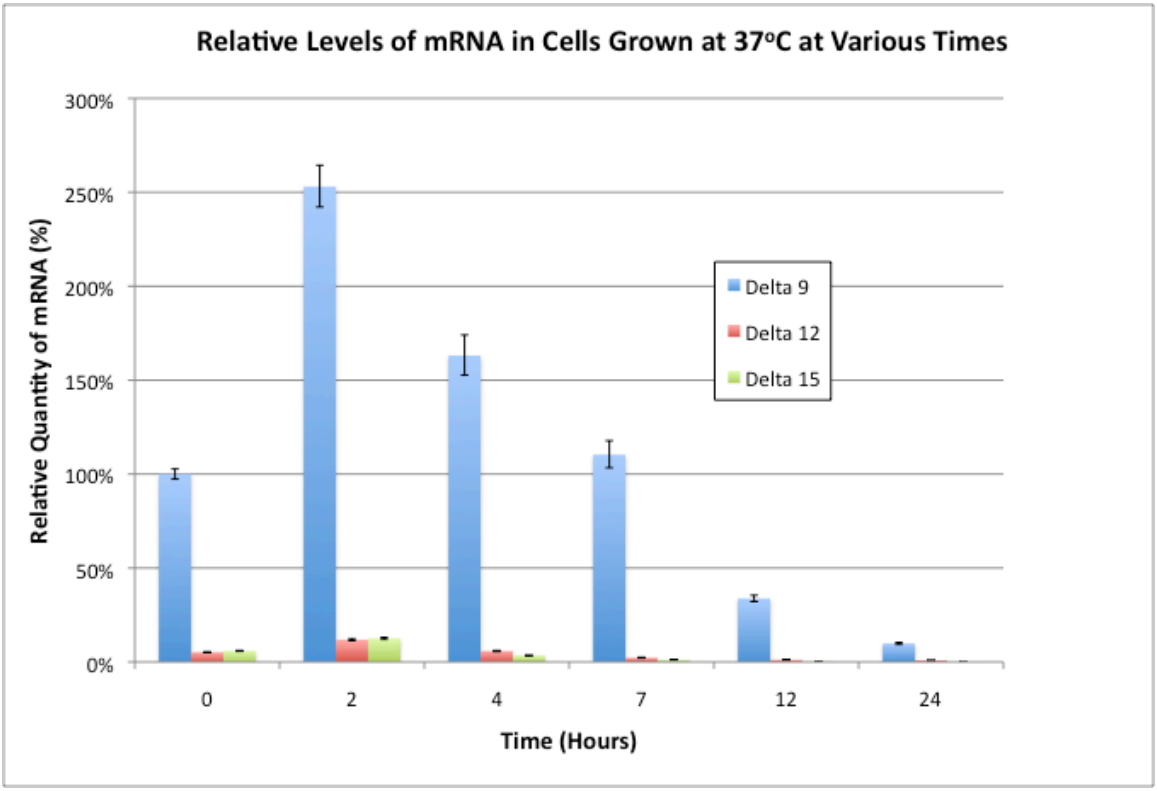

Figure 14. Comparison of mRNA levels in cells grown at 37°C over time. Relative mRNA levels are compared to the 0 time Δ9 transcript. Relative quantities are averages of 3 trials.

Effect of Nutrient Fatty Acids on Acyl Content and Fatty Acid Desaturase Activity in Cells Adapted to 30°C

In *Saccharomyces*, a wide range of exogenous unsaturated fatty acid species are rapidly internalized and incorporated into membrane lipids. These fatty acids strongly repress the Δ9 fatty acid desaturase and over the course of several generations will replace the native 16:1 and 18:1 species in the cellular membranes[1]. To test the effects of nutrient fatty acids on *Candida*, cells were grown overnight at 30°C to stationary phase in medium containing 1% Tergitol. Aliquots were then inoculated into fresh Tergitol medium followed by the addition of 1mM concentrations of either α-linolenic acid (the endogenous Δ9,12,15 18:3 species), γ-linolenic acid (Δ6,9,12 18:3 species) or arachidonic acid (Δ5,8,11,14 20:4). The effects of these nutrient fatty acids on the membrane lipid composition at times equivalent to 4 generations of growth are presented in Figures 15-17. These data are compared to data from cells transferred to Tergitol-containing medium that contained no fatty acid nutrient.

Figure 15 shows the fatty acyl distributions in cultures that were supplemented with 1mM of native α-18:3 species. There was rapid and significant incorporation of the fatty acid into the cells that was apparent during the 0 time harvesting procedure. During that time (approximately 15 minutes) the total wt% of 18:3 increased from 5 wt% to 11 wt% compared to the culture with no fatty acid. By six hours (4 generations), there were striking differences in the fatty acid composition, with the α-18:3 species now comprising 62 wt% of the total fatty acids. Those levels were about 6-fold higher than those observed in the unsupplemented control cells. This was accompanied by large reductions in the levels of 16:1, 18:1 and 18:2. Compared to the 6-hour, no fatty acid, control values, 18:2

levels were reduced approximately 6-fold, 18:1 levels were reduced 3-fold, and 16:1 levels were reduced about 6-fold. These data are consistent with the expectations that both the Δ9 and Δ12 desaturase activities were strongly repressed, resulting in the subsequent dilution of the 16:1, 18;1 and 18:2 species over the course of 4 cell divisions. Since there is no difference between the exogenous 18:3 and the endogenous species produced by the Δ15 desaturase in this experiment, the effects of the supplemented fatty acid on the Δ15 enzyme activity are not informative.

Figure 16 shows the effects of the addition of 1mM of γ-18:3 to a culture of *Candida* grown under the same conditions. These data show similar effects to those observed with α-18:3. Over a span of 6 hours, the total wt% of 18:1 and 18:2 was decreased to ~10 wt% from 35 wt%, whereas the exogenous γ-18:3 ultimately comprised about 70 wt% of the total fatty acyl species. These data also show that the endogenous 18:3 decreased from 12 wt% to being almost entirely removed from the membrane (2 wt%). This data suggest γ-18:3 exerts a strong repressive effect on all three desaturases under the above growth conditions.

Cells exposed to arachidonic acid for 6 hours showed a different effect to those with the 18:3 species (Figure 17). There was an approximate 30% decrease in 16:1 and 18:1 levels, which was not as significant as the decreases observed in cells supplemented with either 18:3 species. Levels of 18:2, however, were elevated (from 27 to 32 wt%) and levels of 18:3 were increased about 2-fold similar to the increases found in controls with no fatty acid supplementation. The level of uptake of arachidonic acid was unexpectedly low, representing only 6% of the total wt % fatty acids after 6 hours, which was about 1/10 the levels seen with the 18:3 species.

Figure 18 summarizes the effects of the exogenous fatty acid supplements on the levels of the endogenous species produced by the three desaturases in these experiments, by comparing the ratios of the native unsaturated species to the saturated species after 6 hours of exposure. (In the case of the cells exposed to α-linolenic acid only 16:1, 18:1 and 18:2 are used in the calculation because the majority of the 18:3 levels were derived from the exogenous acid). These data show dramatic reductions in the levels of internally synthesized fatty acids, which would be commensurate with strong repression of all three desaturases by the 18:3 species. By contrast, arachidonic acid appears to have little effect on the production of mono- or polyunsaturated fatty acids under these conditions.

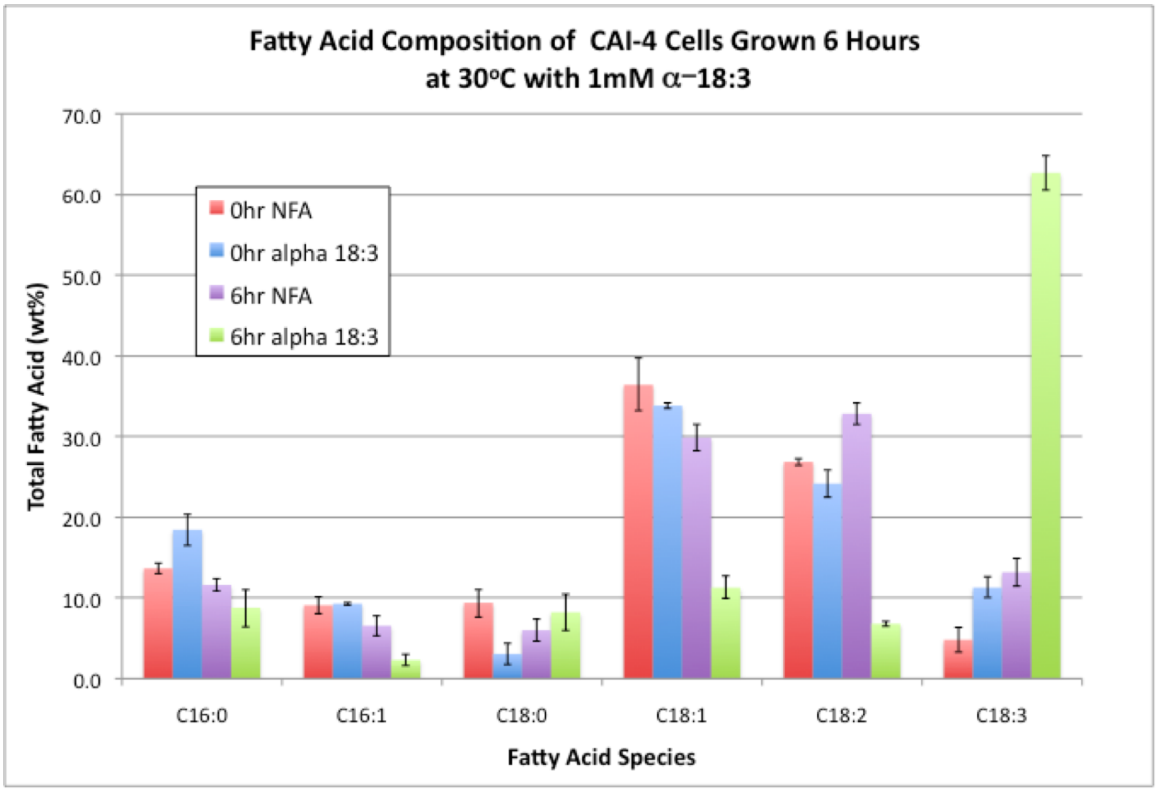

Figure 15. Comparison of fatty acid species supplemented with 1mM α-18:3 at 30°C. Comparison of different fatty acid species in cells grown with 1 mM α-18:3 (alpha 18:3) and without nutrient fatty acids (NFA) at 0 and 4 generations (6 hours) after exposure to the fatty acids. Values are an average of 3 independent cultures.

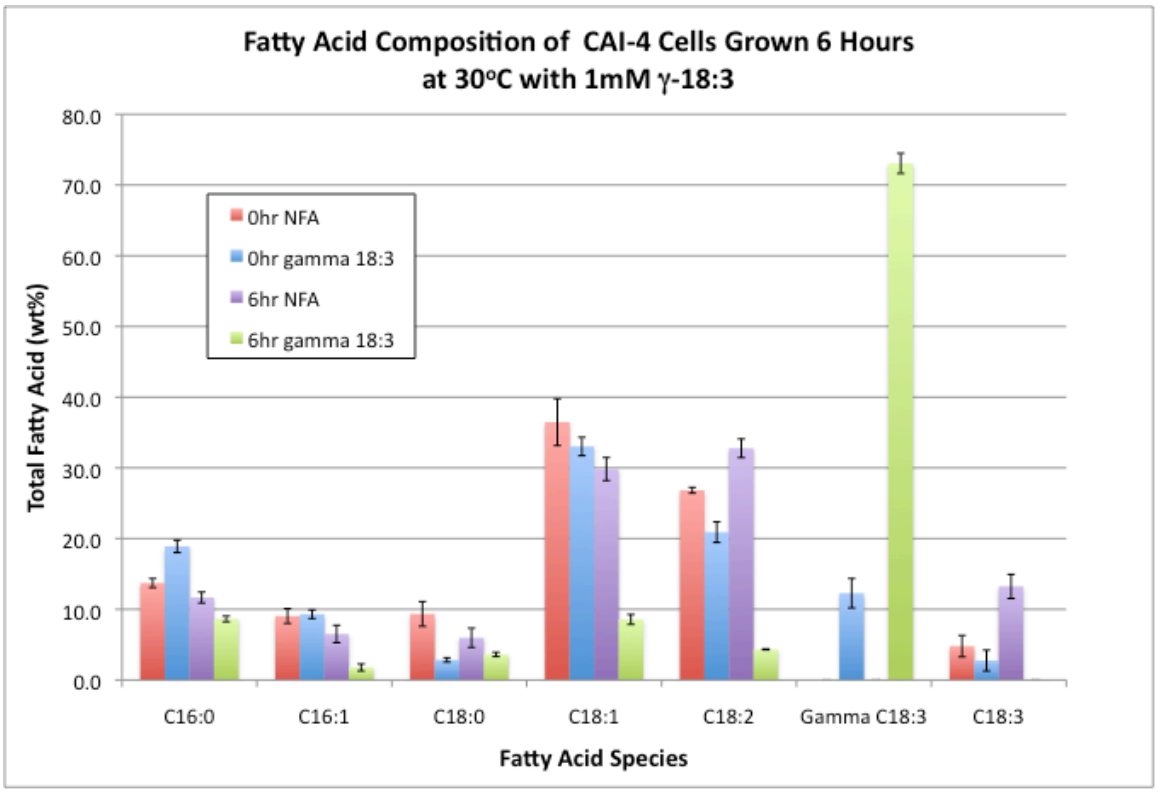

Figure 16. Comparison of fatty acid species supplemented with 1mM γ-18:3 at 30°C. Comparison of different fatty acid species in cells grown with γ-18:3 (gamma 18:3) and without nutrient fatty acids (NFA). Graph displays data at a zero hour and four generations for each condition (for 30°C four generations occurred at 6 hours in). Values are an average of 3 trials.

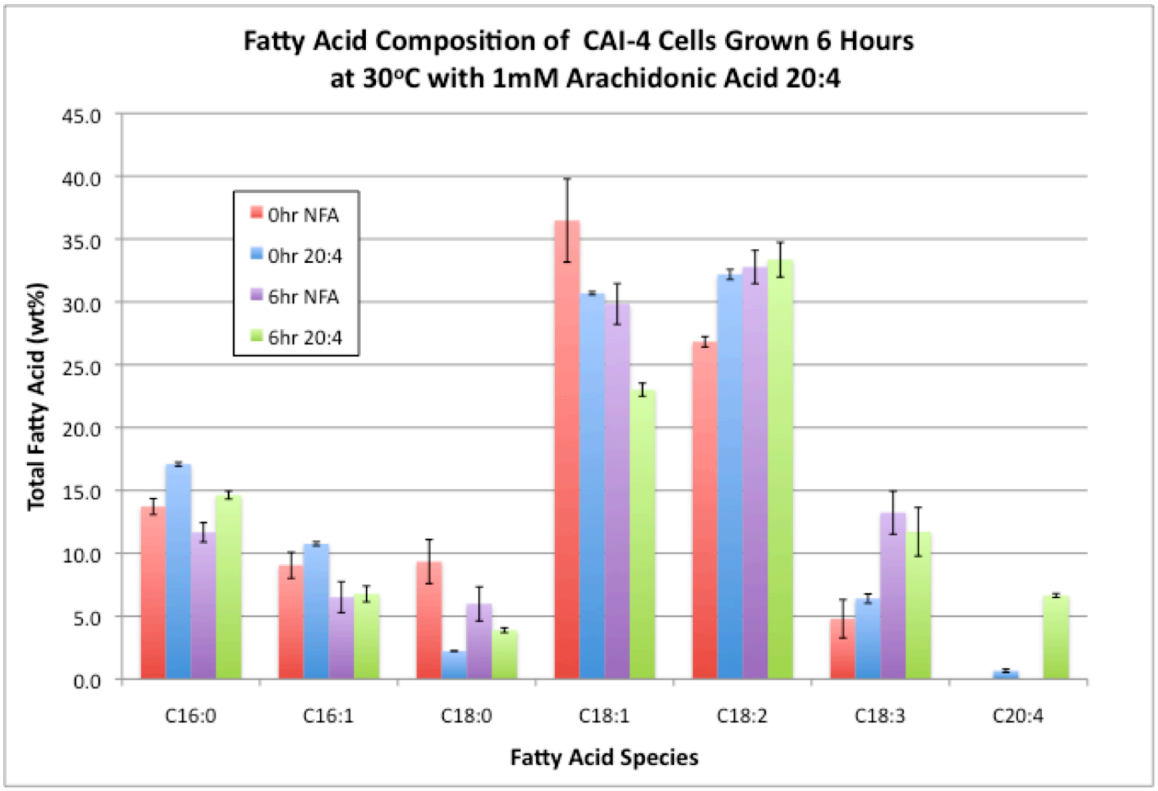

Figure 17. Comparison of fatty acid species supplemented with 1mM arachidonic acid at 30°C. Comparison of different fatty acid species in cells grown with arachidonic acid (20:4) and without nutrient fatty acids (NFA). Graph displays data at a zero hour and four generations for each condition (for 30°C four generations occurred at 6 hours in). Values are an average of 3 trials.

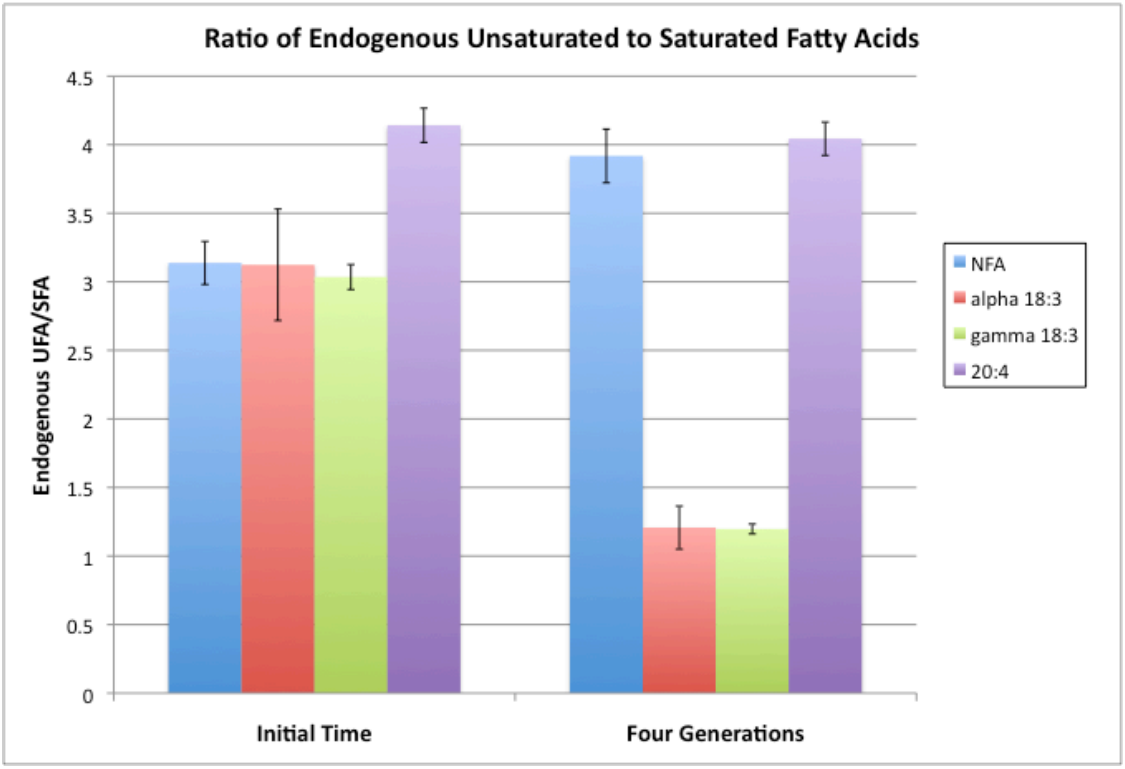

Figure 18. Ratios of endogenous unsaturated / saturated fatty acids in cells exposed to 1 mM supplements of α-18:3, γ-18:3, and arachidonic acid at 30°C. The plot shows the initial values after the addition of the fatty acids and the values after 4 generations of growth in supplemented medium. These data ± S.D. were derived from 3 different experimental cultures for each supplemented fatty acid.

Effects of Nutrient Fatty Acids on Desaturase mRNA levels at 30°C.

The effects of the nutrient fatty acids on the relative quantities (RQ) of mRNA levels at times equivalent to 4 generations of growth are presented in Figure 19. These data are compared to cells transferred to Tergitol-containing medium that contained no fatty acid supplement.

Figure 19 presents the RQ of desaturase mRNA levels in cells supplemented with either 1mM α-linolenic acid (the endogenous Δ9,12,15 18:3 species), γ-linolenic acid (Δ6,9,12 18:3) or arachidonic acid (Δ5,8,11,14 20:4). The relative quantities are determined in comparison to 100 molecules of Δ9 mRNA (100%) at time 0. In cells that were supplemented with 1mM α-linolenic acid, there was a rapid drop in the Δ9 desaturase mRNA levels to about 1% of the initial value within fifteen minutes. This was accompanied by a slight increase in the Δ12 enzyme mRNA and a reduction in the Δ15 enzyme mRNA (from 5% to 1%). After 6 hours exposure to α-18:3, the Δ9 mRNA levels are still repressed by 6-fold relative to the unsupplemented control cells. These data indicate that α-18:3 rapidly acts to repress expression of the Δ9 and Δ15 desaturase genes, while acting as an inducer of the Δ12 gene.

Cells exposed to exogenous arachidonic acid also exhibited a strong reduction in Δ9 and Δ15 desaturase mRNA levels fifteen minutes after exposure (Figure 19), although the effect on the Δ9 transcript was not as dramatic as that observed with the α-18:3 species. These levels were reduced about 5-fold (~25%) and were accompanied by a large increase in Δ12 mRNA levels (from 35% to 90%). After 6 hours of exposure, Δ9 mRNA levels were repressed 2-fold compared to those of unsupplemented cells, while

levels of the Δ12 transcript were 4-fold higher than in their NFA counterparts (from 10% to 40%). These data suggest arachidonic acid also rapidly acts to repress Δ9 and Δ15 desaturase gene expression, while acting as a strong inducer of Δ12 gene activity.

Figure 19 also depicts the effects of 1mM γ-linolenic acid on relative mRNA levels in cells grown at 30^{o}C. This exogenous fatty acid species also produces a 5-fold decrease of the Δ9 desaturase mRNA levels while producing a 2-fold increase in the levels of Δ12 mRNA. After 6 hours exposure, Δ9 mRNA are reduced about 3-fold (60% to 20%) and Δ12 mRNA levels are elevated 2-fold, compared to unsupplemented cells. Again, Δ15 desaturase mRNA levels are strongly repressed by this polyunsaturated fatty acid.

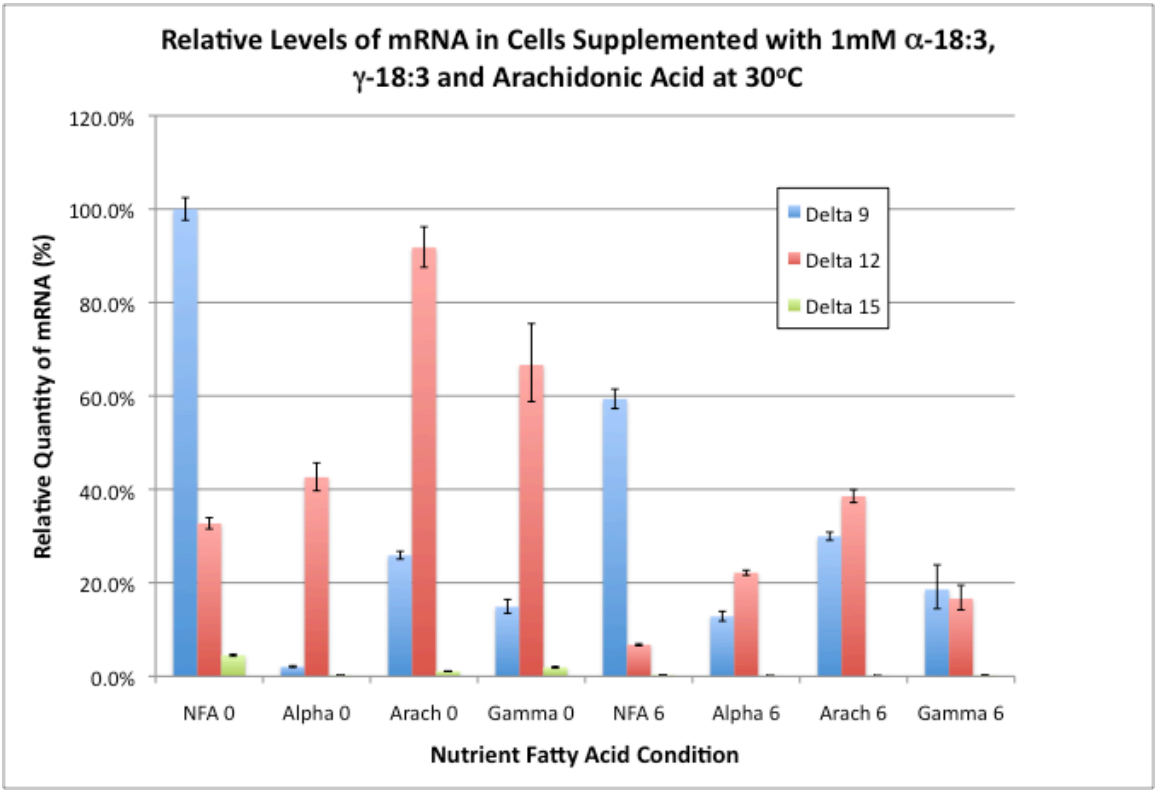

Figure 19. Comparison of mRNA levels in cells under different nutrient fatty acid conditions at 30°C. Cells were grown overnight at 30°C to stationary phase and transferred to fresh URA URI medium with either no fatty acids (NFA), 1mM α–18:3 (Alpha), 1mM γ–18:3 (Gamma) or 1mM arachidonic acid (Arach). Relative mRNA levels were observed 15 minutes in (0 hour) and 4 generations after (6 hours). All levels are compared to 0 hour Δ9 transcript. Relative quantities are averages of 2 trials.

Effects of Polyunsaturated Fatty Acid Supplementation on the Adaptation to Growth at 15°C.

Given the large adaptive changes in unsaturated fatty acid levels that take place in ectothermic fungi exposed to low temperatures, experiments were performed to test whether supplemention with highly unsaturated fatty acids would allow *Candida* to adapt more rapidly to growth at 15°C. Overnight cultures grown at 30°C were inoculated into 15°C URA+ URI + 1% Tergitol growth medium supplemented with no fatty acids, 1 mM α-18:3, 1 mM γ-18:3 or 1 mM arachidonic acid. Growth rates were determined by hemocytometer counting of replicate cultures and samples were taken for fatty acid and mRNA analysis.

Figure 20 shows a set of representative growth curves for each of the supplemented (or non-supplemented) cultures at 15°C, and Table 3 shows the calculated growth rate constants and doubling times for 3 replicate experiments for each condition. All of the cultures showed the distinctive biphasic 12-hour lag period seen with the previous experiments. We observed that the incorporation of Tergitol into the growth medium appeared to have an effect on the growth of *Candida* such that the doubling time in the unsupplemented culture increased from 7 hours to approximately 13 hours (decreasing the growth constant from .09 to .05). We were surprised to find that the nutrient fatty acids did not appear to have as much of an effect on this new doubling time, with Table 3 showing that the growth constants and doubling times remained relatively close to that of the culture grown without any exogenous UFAs. These data suggest supplementation of *Candida* with

different exogenous polyunsaturated fatty acid species does not alter the length of the lag period or increase the growth rates at the colder temperature.

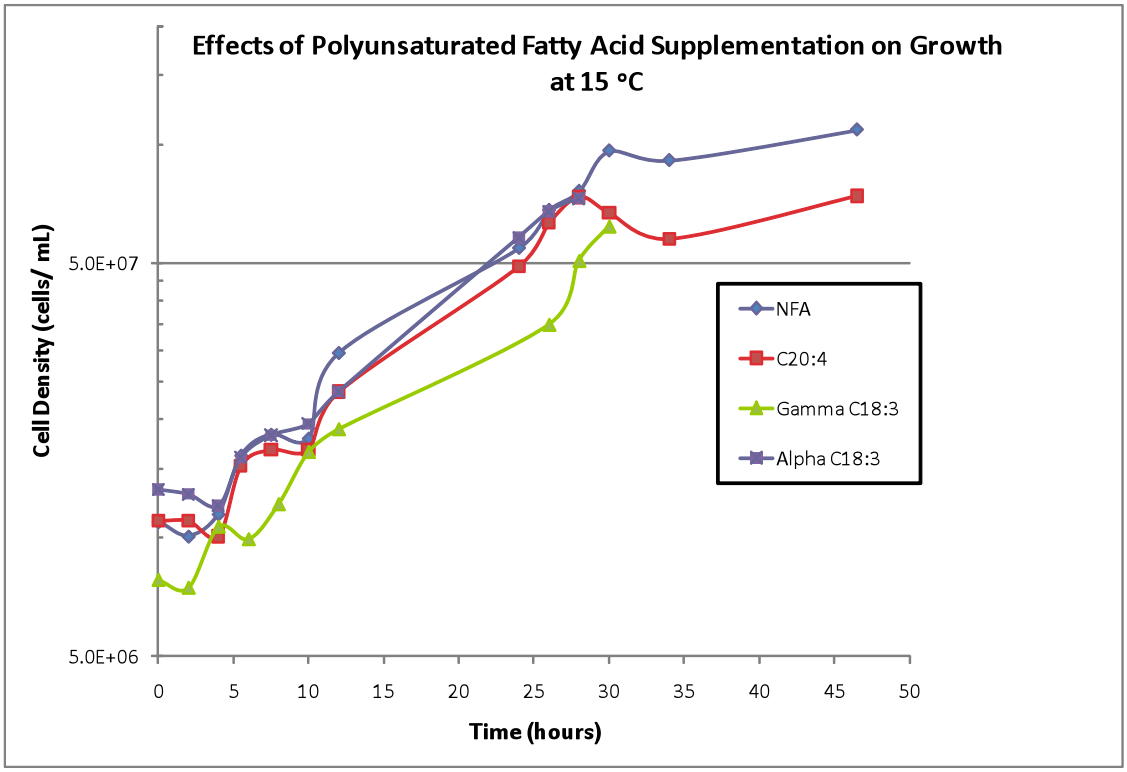

Figure 20. Effects of exogenous fatty acids on growth at 15°C. Cells were supplemented with either No Fatty Acids (NFA), 1mM α–18:3, 1mM γ–18:3 or 1mM arachidonic acid. The plots show representative growth curves for each experimental species.

Effects of Polyunsaturated Fatty Acids on Adaptation to Growth at 15 ^{o}C		
Supplement	**Growth Constant μ**	**Doubling Time (Hours)**
No Fatty Acid	**0.0979 ± 0.0032**	**7.09 ± 0.24**
No Fatty Acid + Tergitol	**0.0512**	**13.5**
α-18:3	**0.0645 ± 0.011**	**11.0 ± 1.9**
γ-18:3	**0.0467 ± 0.004**	**14.9 ± 1.2**
20:4	**0.0500 ± 0.011**	**14.3 ± 3.2**

Table 3. Calculated growth constants and doubling times for cells adapting to 15 ^{o}C under supplemented and unsupplemented conditions

Effects of Nutrient Fatty Acids on Fatty Acyl Content and Desaturase Activity at 15°C

To assess the effects of the fatty acid supplements on the lipid composition of the cells adapting to 15°C, we compared the fatty acyl content of the supplemented cells to the non-supplemented cells at different times during the growth phase. The results of those analyses are presented in Figures 21-23.

Figure 21 presents the effects of α-linolenic acid on the total fatty acid content. In the supplemented cells there is a 2-fold increase in 18:3 levels (10% total fatty acids) after fifteen minutes compared to the control cultures. After thirty hours of incubation (the equivalent of 4 generations of growth) - the total wt% of 18:3 rose to 80% of the total unsaturated fatty acids representing a 8-fold increase over the unsupplemented cells. In this case it is not clear what fraction of the observed α-18:3 is derived from the endogenous α-18:3, as opposed to the exogenous acid, because they have the same structure. At that point we observed large decreases in the levels of all of the other endogenously produced fatty acids, including an 8-fold decrease in 18:1 and a 5-fold decrease in 18:2. We were also surprised to see that there were significant decreases in the saturated 16:0 and 18:0 species as well as the unsaturated acids.

Figure 22 shows that γ-linolenic acid produces a similar effect to that seen with α-linolenic acid. After thirty hours, γ-linolenic acid comprises 70% of total fatty acids with corresponding decreases in monoenoic and dienoic fatty acids. In this case the levels of endogenous α-18:3 are very low after 30 hours of exposure, dropping from 10% in the control culture to 2% in the supplemented cells. These data indicate that both α-linolenic

and γ-linolenic acids elicit a strong repression of all three desaturase activities when cells are exposed to these fatty acids at 15^{o}C.

Arachidonic acid produced a different effect compared to either exogenous trienoic fatty acid, as shown in Figure 23. Arachidonate seems to internalize at a slower rate into the cells than either α-18:3 and γ-18:3. After thirty hours, 20:4 comprised only 15% of total fatty acids by weight. Surprisingly, 20:4 stimulated a 1.5 fold increase in α-18:3 levels and an approximate 2-fold decrease in 18:1 and 16:1, suggesting that it may act to partially repress the Δ9 desaturase enzyme activity while inducing Δ15 desaturase activity.

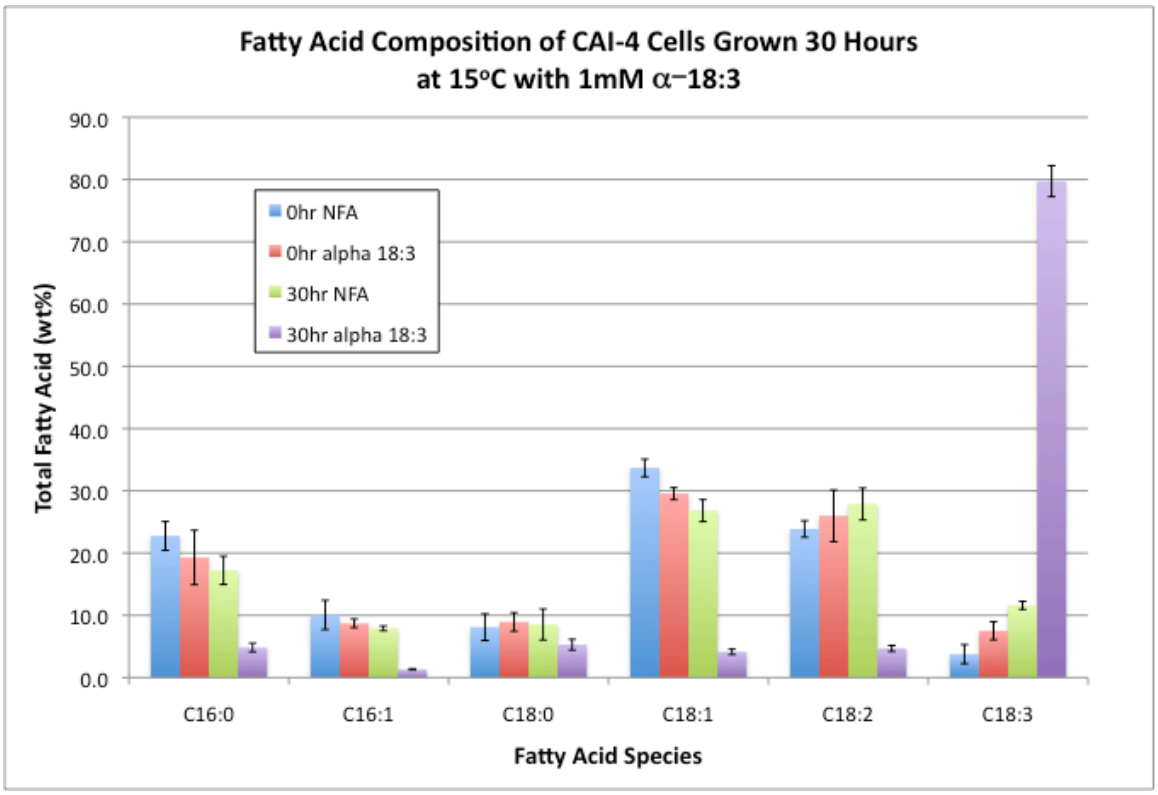

Figure 21. Comparison of total fatty acids in cells grown with 1mM α−18:3 at 15°C. Cells were then supplemented with 1mM α−18:3 (alpha 18:3). Total fatty acid levels were then observed at 15 minutes in (0hr) and at four generations (30hr). Total fatty acid values are an average of 2 trials.

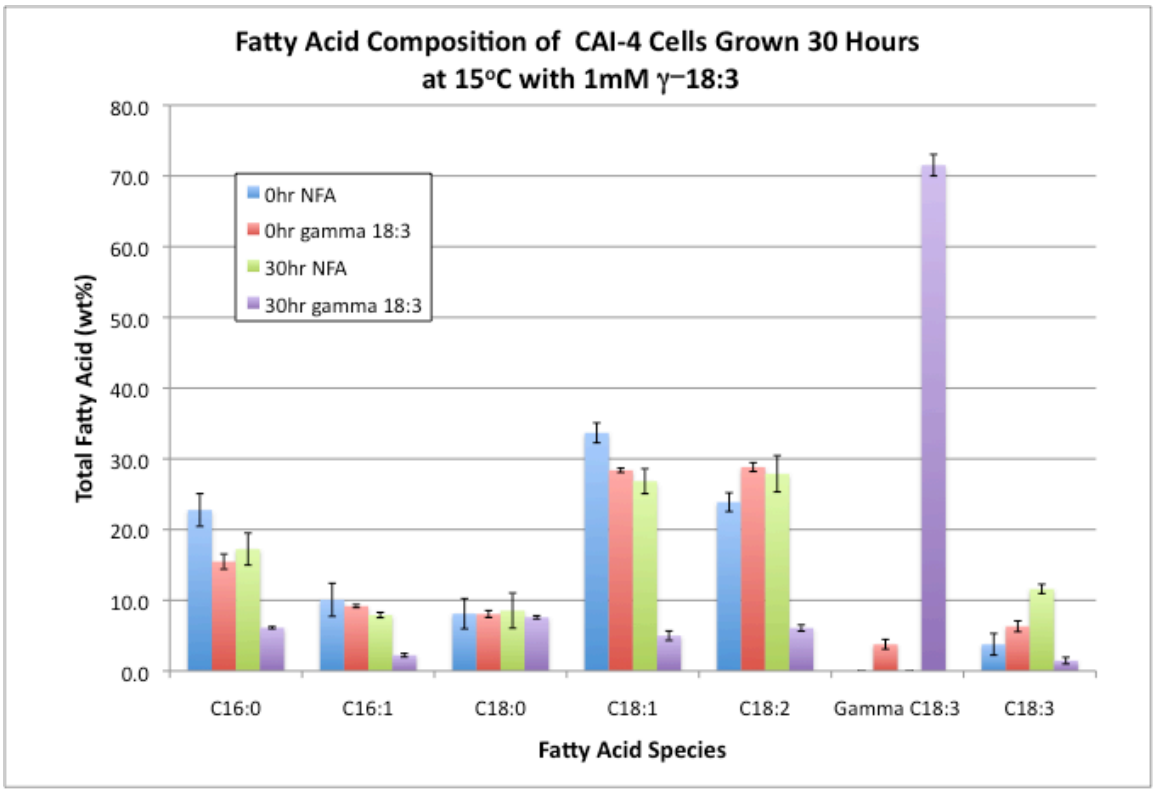

Figure 22. Comparison of total fatty acids in cells grown with 1mM γ−18:3 at 15°C. Cells were supplemented with 1mM γ−18:3 (gamma 18:3). Total fatty acid levels were then observed at 15 minutes in (0hr) and at four generations (30hr). Total fatty acid values are an average of 2 trials.

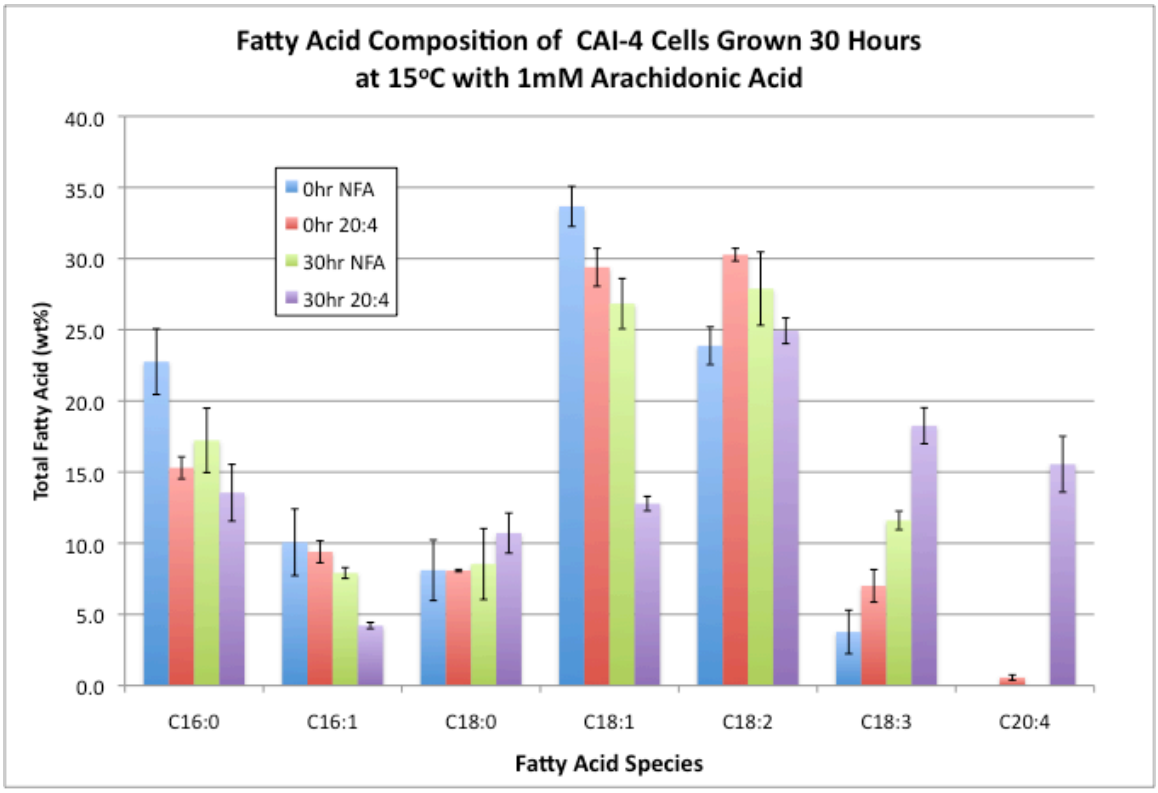

Figure 23. Comparison of total fatty acids in cells grown with 1mM arachidonic cid at 15°C. Cells were then supplemented with 1mM arachidonic acid (20:4). Total fatty acid levels were then observed at 15 minutes in (0hr) and at four generations (30hr). Total fatty acid values are an average of 2 trials.

Effects of Nutrient Fatty Acids on Fatty Acid Desaturase mRNA levels at 15°C

To determine the effects of nutrient fatty acids on the Δ9,12 and 15 desaturase mRNA levels at the low temperature, RNA was isolated from cells exposed to the unsaturated fatty acids 15 minutes (designated as 0 hours) and 30 hours after transfer to 15°C. The mRNA relative quantity (RQ) levels in the supplemented cultures are compared to those from the cultures that did not receive any nutrient fatty acids. These data are presented in Figure 24.

In cells exposed to 15°C for 15 minutes the only significant changes in mRNA levels appear to be produced by α-18:3, which represses the Δ9 desaturase mRNA levels by 50%. In cells exposed for 30 hours, however, the Δ9 and Δ12 desaturase mRNA levels are strongly repressed by all three polyunsaturated species. The strongest effect is produced by α-18:3, which caused a 3-fold reduction in the Δ9 transcripts, whereas γ-18:3 and arachidonic acid treated cells show a 2-fold reduction in that mRNA species. Under those conditions, all three polyunsaturated species appear to have repressed the Δ12 desaturase mRNA levels approximately 2-fold.

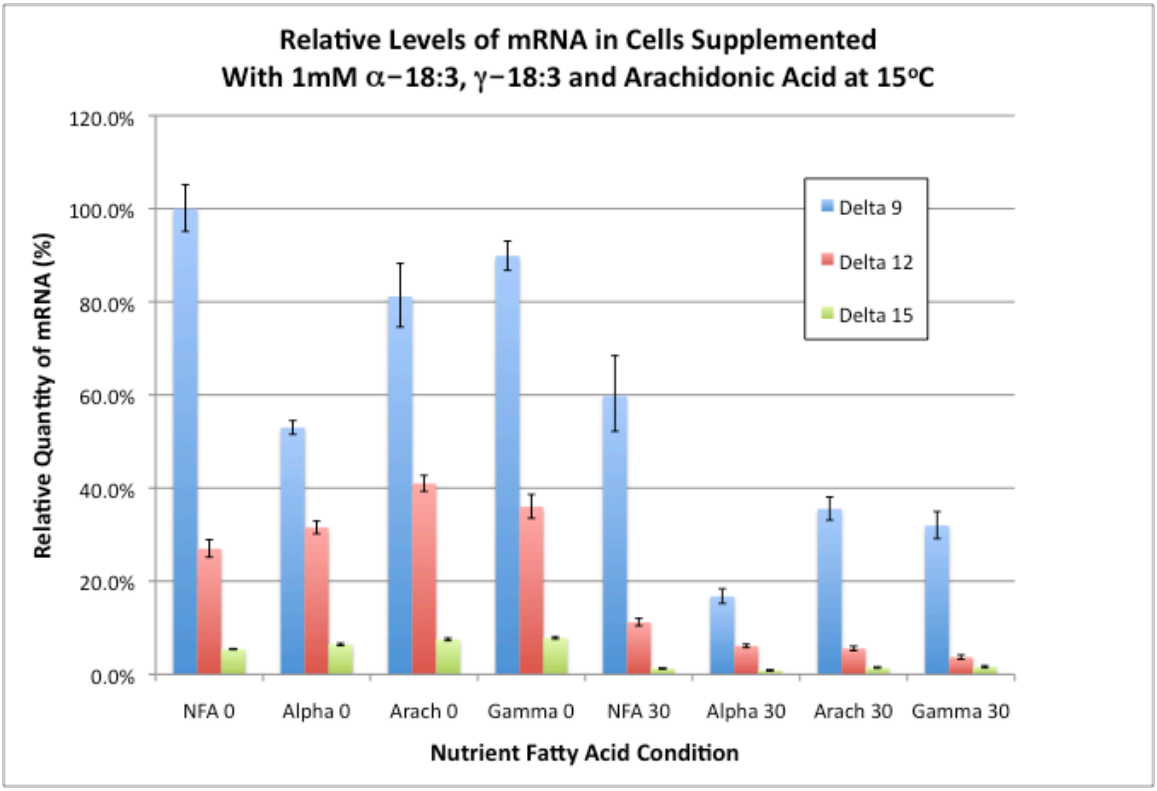

Figure 24 Comparison of mRNA levels in cells under different nutrient fatty acid conditions at 15°C. Cultures were supplemented with either no fatty acids (NFA), 1mM α−18:3 (alpha), 1mM γ−18:3 (Gamma) or 1mM arachidonic acid (Arach) and grown for 15 minutes (0 hour) or 30 hours (4 generations) before isolation of total cellular RNA. All levels are compared to 0 hour Δ9 transcript. Relative quantities are based on the averages of 2 trials.

DISCUSSION

Regulation of cellular and membrane fatty acid composition is an important component in the lipid metabolism of organisms. Almost all eukaryotic cells regulate their ability to synthesize saturated and unsaturated fatty acids, based on the availability of nutrient fatty acids that can be incorporated into membrane and storage lipids. In addition, many ectothermic organisms, such as fungi, highly regulate the saturated and unsaturated acyl content of their membrane lipids to optimize membrane fluidity in response to changing temperatures by controlling fatty acid desaturase activities.

Candida albicans is a mammalian commensal organism that derives nutrients from the contents of its host's gut as well as from surrounding tissues when it colonizes other sites in the body. Given that *Candida* is only isolated from hosts that maintain a constant environmental temperature of around 37^{o}C, it unclear whether the organism can adapt to other growth temperatures. It is also medically relevant to assess the regulatory effects that exogenous nutrients derived from the host might have on its lipid metabolism.

We found that *Candida* was capable of growing over a wide temperature range and, as expected, when the organism was exposed to lower growth temperatures the logarithmic phase growth rates slowed and it required a longer time to reach stationary phase. In the conditions used in this study, 30 ^{o}C appeared to be the optimal growth temperature for the budding form of the fungus, while 37^{o}C was optimal for hyphal growth. In these studies we observed that the highest growth

rate was at 30°C. We were surprised to observe that *Candida* actually grows at a faster rate at both temperature extremes than the ectothermic fungus *Neurospora crassa*. *Candida* has a doubling time of 1.4 hours at 37°C and 7 hours at 15°C (Table 2), while *Neurospora* was shown in previous studies by this laboratory to have a doubling time of 4 hours at 37°C and only 20 hours at 15°C [23].

We were also surprised to see that *Candida* exhibits an unusual biphasic lag phase behavior that lasts approximately 10 hours when it is grown at 15°C. It is unclear why there is this pattern of growth, but it seems to indicate that more than one essential process is involved in adapting to the lower temperature.

Growth temperature also seems to determine the cell density at which cultures will enter stationary phase. Cells grown at 20°C seem to produce about twice as many cells at the end of the experiment relative to those grown at 15°C, and an even higher cell density is seen at stationary phase for cells grown at 30°C (Figure 3).

As with most fungi, *Candida* expresses three desaturase genes that produce Δ9, Δ12 and Δ15 enzymes that form mono and polyunsaturated fatty acids. Most cellular fatty acids in *Candida* are unsaturated, comprising around 80% of the total species in cells growing throughout the range of temperatures and growth phases. The most abundant unsaturated species were found to be 18:1 and 18:2 under all conditions used in this study. These fatty acids are found in approximately equimolar quantities in logarithmic phase cells growing at 15 – 30°C, comprising 55- 60% of the total species. By contrast, linolenic acid (18:3) levels were found to be low at all of the growth temperatures, comprising less than 15% of the total species in cells grown at 15°C to 30°C, and less than 10% in cells grown at 37°C. Given that 18:1 is

the precursor for 18:2 and 18:3, the compositional data in this temperature range suggests that the activity of the Δ9 desaturase would be as much as 2-fold higher than the Δ12 enzyme, and at least 8-fold higher than the Δ15 enzyme.

Previous studies with *Neurospora crassa* showed that a major adaptation to low growth temperatures involves large increases in the levels of α-18:3 as a consequence of the activation of the Δ15 desaturase. Shifting *Neurospora* from 37°C to 15°C results in an increased level of 18:3 from 5% to over 40% of the total fatty acids, resulting in a 33% increase in the double bond index [23]. We were surprised to find that while *Candida* also expresses a Δ15 desaturase, its α-18:3 product is significantly lower (10%) when the cells arefully adapted to 15°C and that there was only a 25% increase (from 100 to 125) in the double bond index under those conditions (Figure 25). It was also unexpected to find that *Candida* cells adapting to 20°C produce much higher levels of double bonds in the fatty acids than those cells adapting to the lower temperature (Figure 10). Given that *Candida* undergoes a lag period of 10 hours before entering logarithmic phase growth at 15°C, compared to the shorter 3.5 hour lag period seen with *Neurospora*, suggests that high levels of 18:3 might play a role in the initial adaptive response.

The most dramatic changes in fatty acyl content with respect to growth temperature were found at 37°C, under conditions in which *Candida* differentiates into the hyphal state. Under these conditions, there are large increases in 18:1 and reduced levels of 18:2, resulting in approximately 1.5 fold higher levels of 18:1 throughout the growth phases. The corresponding reduction in double bond content

under these conditions would be consistent with a compensating adaptation in membrane fluidity needed for growth at the higher temperature.

Analyses of the desaturase mRNA levels during growth at the different temperatures showed correlations with the fatty acid compositional data. We expected that the ratios of Δ9 mRNA to the Δ12 and Δ15 transcripts would be the highest at 37°C, and indeed this seems to be the case throughout the entire growth period. There is a rapid induction of the Δ9 mRNA to 2.5 fold higher levels and, while this effect declines as cells continue to grow, the Δ12 and Δ15 transcripts remain at consistently low levels throughout the experiment. As cells are cooled to 20°C, the Δ9/Δ12,15 ratios are reduced, which is consistent with the production of higher levels of polyunsaturated fatty acids under those conditions. At 15°C, however, the Δ12 and Δ15 transcripts do not exhibit the higher level of induction seen 20°C, which is consistent with the reduced levels of double bonds produced at the lower temperature.

Previous studies of *Saccharomyces* in this laboratory showed that yeast rapidly import a wide range of unsaturated fatty acids, which exert a strong regulatory effect on the expression of the Δ9 desaturase enzyme. Our analyses of cells exposed to exogenous α-18:3 and γ-18:3 at 30°C showed that *Candida* can also rapidly import those fatty acids and incorporate them into membrane lipids. These studies also suggest that they exert strong regulatory effects on the different *Candida* desaturases.

Supplementation with the α-18:3 and γ-18:3 species showed that they were rapidly internalized, resulting in increased levels of those fatty acids only 15

minutes after exposure. By 6 hours exposure, the native α-18:3 comprised 60% of the total fatty acid species, accompanied by approximately 4-fold reductions in the levels of the other endogenously produced unsaturates. A similar effect was seen for the non-native γ-18:3 species, which accounted for 70% of the total fatty acids, with corresponding reductions in the other unsaturates. These rates and levels of incorporation into cellular lipids are comparable to those observed previously with *Saccharomyces*. Furthermore, in cells exposed to α-18:3 or γ-18:3 for 4 generations, the levels of endogenous monoenoic and dienoic acids were strongly reduced and in the latter case, the α-18:3 species was undetectable. These observations provide strong evidence that endogenous desaturase activity was strongly repressed, resulting in the dilution of the preexisting membrane lipid species by the exogenous fatty acid.

In contrast, cells exposed to arachidonic acid showed different effects at 30^{o}C. This species was not rapidly incorporated into cellular lipids and appeared to require as much as a six hour exposure to significantly reduce 18:1.

Analysis of desaturase mRNA levels in 30^{o}C cells exposed to the exogenous acids provided further insights into their effect on the regulation of the desaturase activities. All three fatty acids produced a rapid and strong repression of the Δ9 desaturase mRNA levels 15 minutes after addition of the exogenous species. The strongest repression was produced by the α-18:3 species, which triggered an approximate 50 fold reduction in the Δ9 transcript. By contrast, γ-18:3 and arachidonic acid produced approximately 5-fold reductions in that species (Figure 19). These two acids also rapidly induced the Δ12 gene. In this case arachidonic

acid produced a 3-fold increase and γ-18:3 produced a 2-fold in the Δ12 mRNA levels, whereas α-18:3 produced a slight increase in the mRNA.

There was a similar pattern of regulatory effects 6 hours after cells were exposed to the fatty acids. At that point the levels of all mRNA species were lower than the 0 hour values for each condition. Again, all of the fatty acids repressed the Δ9 transcript, with α-18:3 producing the strongest effect. At the same time the Δ12 mRNA levels were induced 3-fold by arachidonic acid and 2-fold by the α-18:3 and γ-18-3 species.

Given the large body of literature demonstrating that ectotherms increase unsaturated fatty acid levels to adapt cells to low temperatures, we reasoned that if high levels of unsaturated fatty acids and/or increased double bond content were essential for low temperature growth, supplying *Candida* with nutrient polyunsaturated fatty acids might enhance the adaptive response to colder temperatures. To test this hypothesis we supplemented *Candida* cultures with 1 mM α-18:3, γ-18:3 or arachidonic acid and monitored the effects on growth, fatty acyl content and desaturase mRNA levels following a shift from 30°C to 15°C.

We found that none of the exogenous fatty acids produced a detectable effect on either the lag phase pattern of growth or the logarithmic phase growth rates at the low temperature. While we did not observe any physiological effects produced by the fatty acids, we did observe relatively low levels of incorporation of α-18:3 and γ-18:3 15 minutes after exposure, but these produced very little change in the levels of the endogenous acids. Arachidonic acid also showed little incorporation at that

time. Despite that, however, we observed some increases in α-18:3 and 18:2 levels and a significant reduction in 16:0.

An analysis of cells after 4 generations (30 hours) of exposure, however, showed dramatic changes in the levels of incorporation for all three species that paralleled those seen at 30°C. Under those conditions α-18:3 and γ-18:3 were the dominant species in cells treated with those fatty acids, comprising 80% and 70% of the total species. The concomitant large reductions in all endogenous saturated and unsaturated species indicated that the exogenous species also exerted a repressive effect on all three desaturases at the lower temperature. Arachidonic acid was actually incorporated at higher levels after 30 hours of exposure compared to the incorporation seen after 6 hours in the 30°C culture, with a stronger repression of 16:1 and 18:1 and a large induction in α-18:3 levels that was not seen at the higher temperature.

RT-qPCR analysis of the isolated 15°C RNA fractions also showed that the exogenous fatty acids repressed desaturase mRNA levels, although these effects are not as pronounced as they were at 30°C. The only significant effect observed at 15 minutes was a 50% reduction in Δ9 desaturase mRNA levels in cells exposed the α-18:3. By 30 hours, however, all of the exogenous fatty acids strongly reduced all three desaturase mRNA levels, demonstrating a fatty acid mediated level of transcriptional or mRNA stability control at the lower temperature.

In summary, these experiments show that, despite its long evolutionary history of growth at constant high temperatures, *Candida* has retained its ability to adapt and grow over a broad temperature range. Surprisingly the adaptation of

Candida to low temperatures involves more than just a simple modification of membrane fatty acyl content. In addition *Candida* is shown to be capable of rapidly internalizing and incorporating into cellular lipids exogenous fatty acids that exert nutrient control over the expression of all three desaturases.

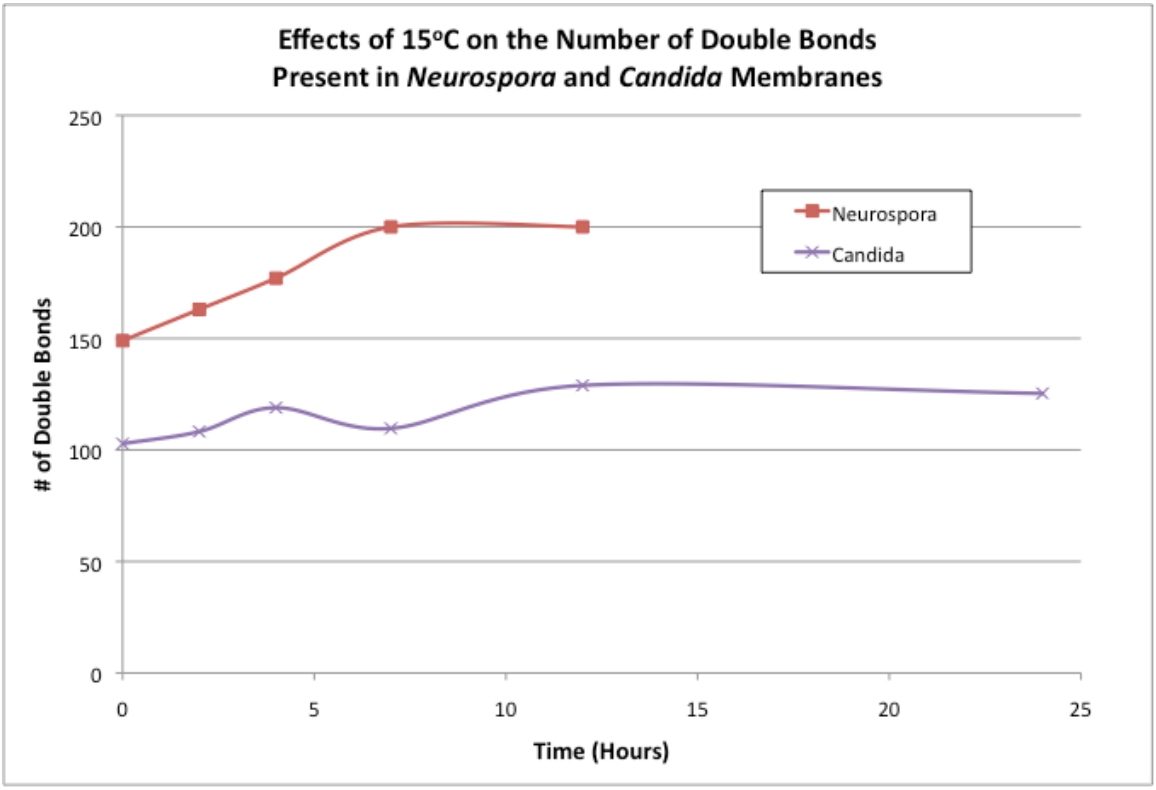

Figure 25. Number of double bonds per 100 fatty acids in *Neurospora* and *Candida* following a shift to 15°C. Previous findings from the lab for *Neurospora* are presented. *Neurospora* cells were grown at 37°C and followed with a shift to 15°C in fresh medium. Candida cells were grown at 30°C and, after transfer to fresh medium, shifted to 15°C. Plots for Candida show the averages of 3 independent trials. Plots for Neurospora show the averages of 2 independent trials.

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