Yeast Sensors Reveal Chloroquine as Yeast Fertility Drug

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This dissertation is dedicated to my father, A.P.S. Olivier, and my mother, C.L. Olivier.

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Chapter 1

Introduction

1.1. Motivation

Preliminary results obtained by Kock and co-workers (personal communications) have shown that the antimalarial drug, chloroquine (CQ) may stimulate yeast sexual reproduction. This is supported by literature that demonstrates that CQ stimulates gametocytogenesis (formation of the sexual phase) of *Plasmodium falciparum* [1,2], as well as in humans and other mammals [3,4,5]. Considering that gametocytogenesis is a key stage in the infective life cycle of the malaria causing parasite [6], the potential medical implications of this find are profound.

Consequently, the aim of this study became to determine the influence of CQ on the sexual phase of various yeasts, thereby determining the conserved status of CQ as a fertility drug.

1.2. Yeast Sensors

The anti-mitochondrial antifungal assay (^{3}A) system was developed to screen for anti-mitochondrial drugs as well as drugs that inhibit or stimulate sexual reproduction in yeasts and possibly other fungi [7,8]. The assay is based on the fact that the sexual phases of yeasts have higher mitochondrial activity and that selective inhibition of mitochondria can be detected as selective inhibition of the sexual phase [9,10,11]. Ideally, the bio-sensor (indicator organism) used is an ascomycetous yeast with a pigmented sexual phase that makes visual detection of the inhibition of sexual reproduction possible. Two such sensors that have successfully been used are *Eremothecium ashbyi* [7], with yellow pigmented sexual structures and *Nadsonia fulvescens* [10,11] - with brown pigmented sexual structures. It should be cautioned

Figure 1.1 The anti-mitochondrial antifungal assay shows the effect of different concentrations of fluconazole (Etest® strip) on yeast growth and sexual reproduction of *Nadsonia fulvescens*. The light zone, surrounding an inhibition zone indicates selective inhibition of sexual reproduction, while the brown zone indicates that sexual reproduction has occurred. These results indicate possible anti-mitochondrial activity. B, brown zone; in, inhibition zone; L, light zone. Taken with permission from Swart *et al*. [11].

that inhibition of the sexual phase does not conclusively prove anti-mitochondrial activity, due to the fact that these drugs may act on any number of physiological processes involved in sexual reproduction (for example meiosis or ascus formation) [8]. This is therefore only a preliminary screening method to be followed up with more detailed studies.

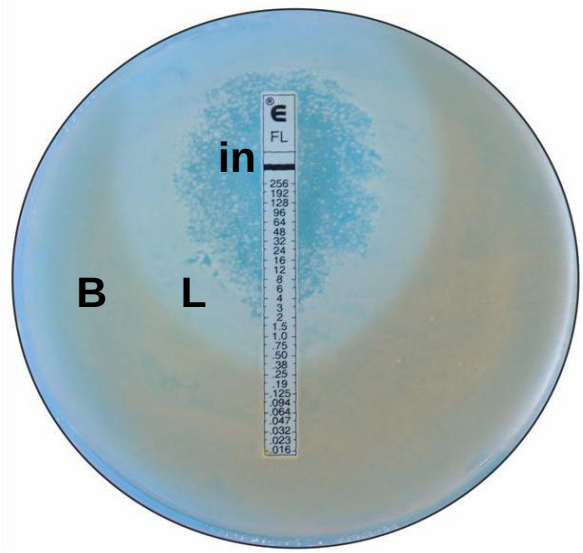

Figure 1.1 (See opposite page).

Bio-assays are constructed by streaking out the yeast as a lawn culture on an agar plate, and the compound to be tested is inoculated in the centre of the plate, or added as a concentration gradient along an Etest® strip [7,8]. During incubation the compound will form a concentration gradient from the centre to the periphery of the plate (Figure 1.1). Positive results for possible anti-mitochondrial activity are indicated by a loss of pigmentation (lighter zone) at high (sub-lethal) concentrations of the compound, with a pigmented zone at lower concentrations (i.e. periphery of the plate). Theoretically, this assay may also be used to detect yeast fertility drugs, in which case inverse results are expected (i.e. a pigmented zone near the centre of the plate and a lighter zone near the periphery).

Figure 1.2 According to the anti-mitochondrial antifungal hypothesis, the strictly respiring yeasts will be more sensitive towards anti-mitochondrial drugs than yeasts that can both respire and ferment, due to their higher dependence on energy from mitochondrial respiration. The hypothesis also states that the sexual/fruiting stages of both kinds of yeasts will be more sensitive towards anti-mitochondrial drugs than the vegetative stages due to the higher energy demand for sexual reproduction. FRUIT, fruiting/sexual phase; RESP., strictly respiring yeasts; RESP. + FERM., yeasts that can both respire and ferment; VEG, vegetative stages of yeasts. Taken with permission from Kock *et al*. [12].

The anti-mitochondrial antifungal hypothesis forms the basis for this assay (Figure 1.2). It compares the effects of anti-mitochondrial drugs on the mitochondrial activity and 3-hydroxy (3-OH) oxylipin production (via β-oxidation) in the sexual (fruiting) and vegetative stages of both strictly respiring yeasts and yeasts that can both respire and ferment [12]. According to this hypothesis, strictly respiring yeasts will be most sensitive to changes in mitochondrial activity due to the higher dependency on mitochondrial respiration. The sexual phases will also be more sensitive to changes in mitochondrial activity, due to the higher energy demand of sexual reproduction [8].

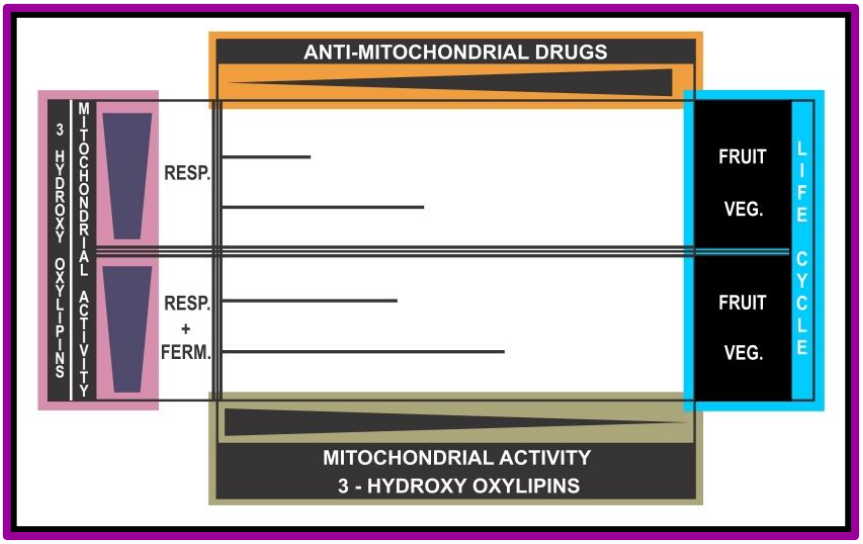

Figure 1.2 (See opposite page).

The results displayed in Figure 1.3 demonstrate how this assay enables detection of anti-mitochondrial drugs by observing changes in the pigmentation of *E. ashbyi*, *Lipomyces yamadae* and *N. fulvescens* sensors [9,10,11,13]. While normal development of sexual structures was associated with fully pigmented cultures that had not been treated with an anti-mitochondrial drug (Figure 1.3 (a,b,e,f,i,j)), the inhibition of sexual structure formation was very strongly associated with a loss of

Figure 1.3 The yeast sensors of *Eremothecium ashbyi*, *Lipomyces yamadae* and *Nadsonia fulvescens* in response to acetylsalicylic acid (ASA). (**a**) Scanning Electron Micrograph of *L. yamadae* sensors taken from the brown zone (B) in (**b**). Similar results were obtained from the brown zone (B) in (**c**). (**b**) Ethanol (EtOH) control plate showing a brown lawn (B) with no selective inhibition of the sexual phase. (**c**) The presence of a light zone (L) indicates the selective inhibition of the sensors by ASA. (**d**) Scanning Electron Micrograph of yeast cells from the light zone (L) in (**c**) indicating the inhibition of ascus formation. (**e**) Scanning Electron Micrograph of *E. ashbyi* sensors and hyphae taken from the yellow zone (Y) in (**f**). Similar results were obtained from the yellow zone (Y) in (**g**). (**f**) Ethanol control plate showing a yellow lawn (Y) surrounding an inhibition zone (in), indicating no selective inhibition of the sensors. (**g**) The presence of a light zone (L) surrounding the inhibition zone (in) indicates the selective inhibition of the sensors by ASA. (**h**) Scanning Electron Micrograph of hyphae without sensors from the light zone (L) of (**g**). (**i**) Transmission Electron Micrograph of a *N. fulvescens* sensor which contains an ascospore taken from the brown zone (B) in (**j**). Similar results were obtained from the brown zone (B) in (k). (**j**) Ethanol control plate showing a brown zone (B) surrounding and inhibition zone (in) indicating no selective inhibition of sensors. (**k**) The presence of a light zone (L) surrounding the inhibition zone (in) indicating the selective inhibition of sensors by ASA. (**l**) Transmission Electron Micrograph of a sensor with inhibited ascospore formation from the light zone (L) in (**k**). Asp, ascospore; B, brown zone; Hy, hyphae, in, inhibition zone; L, light zone; Sens, sensor (ascospore); Y, yellow zone; Yc, yeast cell. Taken with permission from Swart *et al*. [13].

sensor pigmentation at sub-lethal concentrations of the anti-mitochondrial drug (Figure 1.3 (c,d,g,h,k,l)).

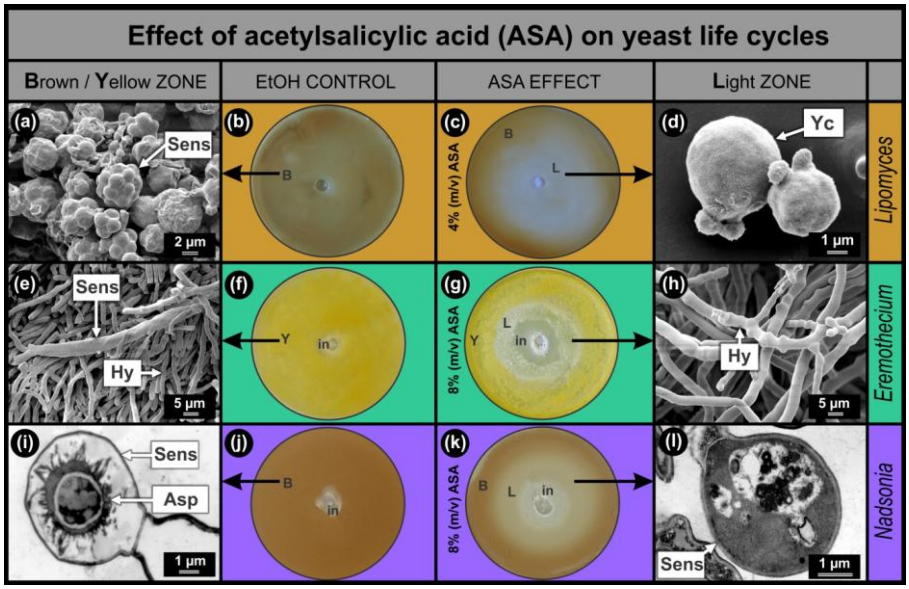

Figure 1.3 (See opposite page).

1.3. Application of Yeast Sensors

Sensors of the yeasts *E. ashbyi*, *L. yamadae* and *N. fulvescens* were used to screen for anti-mitochondrial activity of known and unknown anti-mitochondrial drugs [13]. The results are presented in Table 1.1. It was found that all the Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), antifungal - and anticancer drugs tested gave positive hits for anti-mitochondrial activity. Note that, despite having tested positive for anti-mitochondrial activity with the bio-assay, which is supported in literature [14,15], diflunisal and fenoprofen are not indicated as having any mitochondrial liability in the Food and Drug Administration (FDA) Black Box Warning. This data should prompt the FDA to re-evaluate the Black Box indications of these drugs.

Table 1.1. Compounds screened with the yeast sensors of *Lipomyces yamadae*, *Eremothecium ashbyi* and *Nadsonia fulvescens* and compared to the Food and Drug Administration (FDA) Black Box Warnings for mitochondrial liabilities of drugs in use today. Taken with permission from Swart *et al*. [13].

Compound Tested	Selective Inhibition of Sensors			Black Box
	Lipomyces	*Eremothecium*	*Nadsonia*	
NSAIDs				
Acetylsalicylic acid (ASA)	+	+	+	
Diflunisal	+	+	+	-
Diclofenac	+	+	+	+
Fenoprofen	+	+	Nt	-
Ibuprofen	+	+	+	+
Indomethacin	+	+	+	+
Naproxen	+	+	Nt	+
Peroxicam	+	+	Nt	+
Salicylic acid	+	+	+	
Sulindac	+	+	Nt	+
ANTI-MALARIAL DRUGS				
Artemisinin	Nt	+	Nt	
Chloroquine	-	-	-	
Quinine	-	-	Nt	
Thapsigargin	Nt	+	Nt	
ANTIFUNGAL DRUGS				
Caspofungin	+	+	+	
Fluconazole	+	+	+	
Itraconazole	+	+	+	
Ketoconazole	+	+	+	
Posaconazole	+	+	+	
Voriconazole	+	+	+	
ANTICANCER DRUGS				
Lonidamine	+	+	Nt	
CD 437	+	Nt	Nt	
Betulinic acid	+	Nt	Nt	
CLASSIC RESPIRATORY INHIBITORS				
Antimycin A	+	+	+	
Oxygen limitation	+	+	+	
Rotenone	+	Nt	Nt	

(+ = inhibitory effect; - = no inhibitory effect; Nt = not tested)

Figure 1.4 Comparison of the effects of the anti-mitochondrial drug acetylsalicylic acid (ASA; aspirin) and the antimalarial drug chloroquine (CQ) in an *Eremothecium ashbyi* bio-assay. (**a**) A mature bio-assay of *E. ashbyi* (after four days of incubation) treated with the anti-mitochondrial drug ASA. A clear inhibition zone is observed in the centre of the plate and a light (not pigmented) zone at sub-lethal concentrations of the drug, indicating selective inhibition of the sexual phase. At a lower concentration of the drug a yellow zone can be observed indicating that the sexual phase was not inhibited at this concentration. (**b**) An immature bio-assay of *E. ashbyi* (after one day of incubation) treated with CQ. An inhibition zone can be observed in the centre of the plate at lethal CQ concentration. At sub-lethal concentrations of CQ a yellow zone can be observed, indicating the presence of sexual structures. At a lower concentration of CQ a light (not pigmented) zone can be observed indicating the absence of sexual structures. in, inhibition zone; L, light zone; Y, yellow zone. Taken with permission from Kock and co-workers (unpublished data).

1.4. Chloroquine: A Fertility Drug

Preliminary results by Kock and co-workers indicated that CQ may have a stimulatory effect on sexual reproduction in *E. ashbyi* (Figure 1.4). This was visualized as a distinctly yellow pigmented zone on the bio-assay plate at sub-lethal concentrations of CQ (Figure 1.4 (b)). Here the bio-assay was still in development, i.e. mature yellow asci were still to be formed at low CQ concentrations.

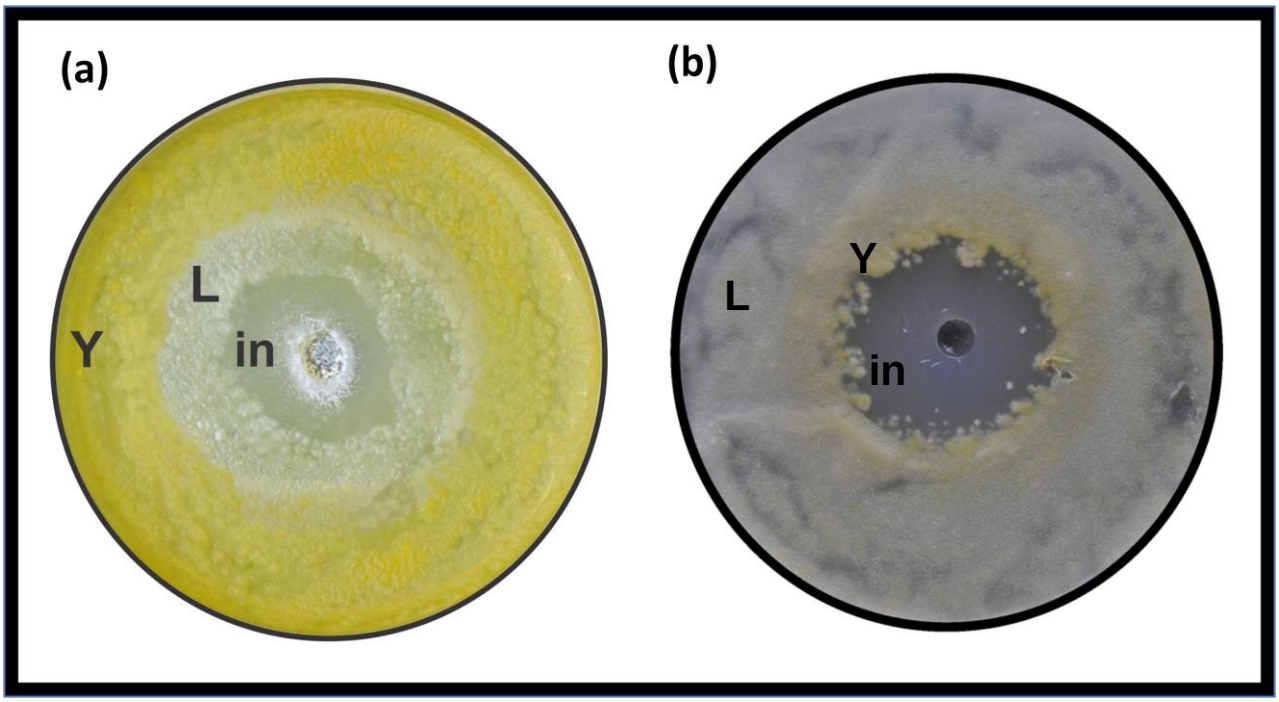

Figure 1.4 (See opposite page).

Published research indicates that CQ also increases the vitality and conception rate of bovine and porcine spermatozoa [3,4] and increases the birth weight of human infants when the mothers are treated with CQ during pregnancy [5]. The greatest medical significance of this fertility inducing action can be observed in the malaria causing parasite *P. falciparum*. Research shows that CQ treatment stimulates the formation of the gametocyte (sexual; haploid) phase of *P. falciparum* [1,2]. The parasite spreads by being transferred from the human host to the

mosquito vector in the gametocyte phase [6]. In the mosquito vector the gametocytes undergo karyogamy to form ookinetes (diploid) that later release sporozoites (haploid) which are transferred from the vector to the new host [16]. Hence, considering that the gametocyte phase is a pre-requisite for the spread of *P. falciparum*, CQ may act to increase the "gametocyte load" carried by the mosquito vectors and in so doing stimulate the spread of malaria.

There have been recent suggestions in literature that CQ may be used as an antifungal drug (17). This is ill advised, considering the long half-life of CQ in the human body (18). If a dose of CQ is large enough to kill or inhibit the majority of the fungal infection – without seriously harming the patient – CQ-metabolism within the body will eventually lower the dose to sub-lethal levels. In addition, prolonged treatment with CQ may enhance resistance among *Plasmodium* strains [19] thereby causing therapeutic doses of CQ to be sub-lethal for the parasite. If CQ is indeed a yeast fertility drug, exposure of the pathogenic fungi to sub-lethal concentrations of CQ may induce sexual sporulation thereby forming resistant spores and possibly aggravating, prolonging and propagating the infection [20]. The regimes for using CQ to combat malaria must urgently be re-examined.

1.5. Conclusions

Yeast sensors have been successfully employed to detect anti-mitochondrial drugs and have indicated that CQ may act as a yeast fertility drug. According to the Anti-mitochondrial Anti-fungal Hypothesis, inhibition of yeast sexual reproduction indicates the inhibition of mitochondrial activity, but the reverse may also be true and

stimulation of yeast sexual reproduction may indicate an increase in mitochondrial activity.

With this as background, the aim of this project was to determine if CQ is in fact a yeast fertility and/or pro-mitochondrial drug. If CQ is indeed a pro-fertility drug, the implications are profound.

1.6. Purpose of Research

Determining whether CQ is a yeast fertility and/or pro-mitochondrial drug, will involve the following:

1. Determining the effect of CQ on the sexual reproduction of yeast sensors.
2. Applying CQ in an attempt to rejuvenate the sexual phase of yeasts that have lost the ability to sporulate.
3. Compare the pro-fertility effects of CQ on yeasts to that of CQ on other eukaryotes, as described in literature, to determine the conserved status of this phenomenon.

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Chapter 2

Yeast Sensors Reveal Chloroquine as a Yeast Fertility Drug

Parts published in:

- Sensors Vol. 12(10), pp. 13058-13074, 26 September 2012, **Yeast sensors for novel drugs: Chloroquine and others revealed** (ISI-accredited scientific journal)
    - highlighted in Global Medical Discovery (http://globalmedicaldiscovery.com/key-scientific-articles/yeast-sensors-for-novel-drugs-chloroquine-and-others-revealed/)
- Canadian Journal of Microbiology Vol. 59, pp. 413-416, 22 April 2013, **The "firing cannons" of *Dipodascopsis uninucleata* var. *uninucleata*** (ISI-accredited scientific journal)

The following sections refer to work done during my B.Sc. Hons. year: 2.3.11; 2.3.12; 2.3.13; 2.3.14; 2.3.15.

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- Federation of European Microbiological Societies (FEMS) 2011
  4th Congress of European Microbiologists
  Geneva, Switzerland
  NanoSAM Investigates Smart Spore Movement
  Olivier APS, Swart HC, Coetsee E, Swart CW, Van Wyk PWJ, Schoombie SW, Pohl CH, Kock JLF

- South African Society for Microbiology (SASM) 2011
  Cape Town, South Africa
  Smart spore movement in yeast
  Olivier APS, Swart HC, Coetsee E, Swart CW, Van Wyk PWJ, Schoombie SW, Smit JB, Pohl CH, Kock JLF

- NanoAfrica 2012
  4th International Conference on Nano-science and Nanotechnology (South African Nanotechnology Initiative) 2012
  Bloemfontein, South Africa
  NanoSAM reveals mechanics of spore dispersal in yeasts
  Olivier APS, Swart HC, Coetsee E, Swart CW, Van Wyk PWJ, Schoombie SW, Pohl CH, Kock JLF

- BIT's 3rd Annual International Conference of Medichem, 2012
  Beijing, China
  A novel nano^{3}A assay to expose a new antifungal target and structural leads
  Olivier APS, Swart CW, Pohl CQ, Kock JLF

2.1. Abstract

In previous studies, the sexual structures of yeasts (which are characterized by high mitochondrial activity) were utilized as sensors to screen for novel anti-mitochondrial drugs. In the course of these studies it was found that the anti-malarial drug chloroquine (CQ) appeared to be pro-mitochondrial. In this study the pro-mitochondrial effects of CQ were further explored using *Eremothecium ashbyi*, *Dipodascopsis uninucleata*, *Lipomyces yamadae* and *Scheffersomyces stipitis* as yeast sensors. In all cases it was found that CQ stimulated the formation of sensors (sexual structures, i.e. asci with ascospores), as well as release of ascospores from mature asci, thus indicating increasing mitochondrial activity. Furthermore, CQ rejuvenated the sexual phase of a non-sporulating strain of *S. stipitis* used in this study. In addition, it was proven that sub-culturing, in contrast to freezing as a method of yeast preservation, leads to inhibition of yeast sexual structure formation, and thus inhibition of mitochondrial activity. The use of yeast sensor bio-assays may fast-track the discovery of other pro-mitochondrial compounds and act as a launch pad to study this class of drugs using *in vivo*, *in vitro*, *in silico* and “omics” methods.

2.2. Introduction

Novel targets and drugs are constantly being sought to effectively combat fungal infections and diseases such as cancer. In this study, we regard the mitochondrion as such a target. Yeast bio-assays, with the sexual structures (asci and ascospores) serving as sensors, have been developed to select new anti-mitochondrial antifungal and anticancer drugs as well as drugs that may adversely affect human mitochondria and consequently health [1-5]. These bio-assays are derived from the Anti-mitochondrial Antifungal Hypothesis. This hypothesis was formulated by Kock and co-workers in 2007 [1] and established a link between: (i) yeast sexual reproduction, (ii) the production of 3-hydroxy (3-OH) oxylipins (via mitochondrial β-oxidation), (iii) mitochondrial activity and (iv) sensitivity towards anti-mitochondrial drugs. For a synopsis of the development of this field of research, which includes yeast sensor smart mechanics linked to Auger nanotechnology, the reader is referred to the video lectures presented by Kock and co-workers in *e*-conference format [6,7].

According to the hypothesis [1], yeasts are divided into two groups: (i) strictly aerobically respiring fungi and (ii) fungi that can obtain energy through aerobic respiration as well as fermentation. As the concentration of anti-mitochondrial drugs added to the yeasts increases, a decrease in the mitochondrial activity and 3-OH oxylipin production in both groups of organisms are expected. The aerobically respiring yeasts should be more susceptible to high anti-mitochondrial drug concentrations than the group that can make use of fermentation as well. The Anti-mitochondrial Antifungal Hypothesis also indicates that the sexual stages of both groups of yeasts should be more sensitive towards anti-mitochondrial drugs than the asexual stages. Furthermore, decreased levels of 3-OH oxylipins as well as

mitochondrial activity are suggested in asexual cells when compared to sexual cells in both groups.

This hypothesis was visualized by the establishment of the Anti-mitochondrial Antifungal Assay (^{3}A) using the sensing yeasts *Eremothecium ashbyi* and *Nadsonia fulvescens*, mimicking the effects expected from the hypothesis [4]. These yeasts can both ferment and aerobically respire and should therefore be less sensitive to anti-mitochondrial drugs compared to yeasts that can only aerobically respire. Consequently, these bio-assays may not be sensitive enough to detect anti-mitochondrial compounds with low activity, especially since only relatively high drug concentrations could be detected by these bio-assays [4-6].

Previous studies aimed at finding more effective yeast sensors to screen for anti-mitochondrial drugs, focused on the non-fermenting yeast *Lipomyces yamadae* [5]. In the course of these studies three important goals were met, namely: (i) to develop a more sensitive ^{3}A system by using a freshly isolated *Lipomyces* strain that can only respire aerobically, (ii) to screen various compounds as well as oxygen limitation with this new yeast bio-assay and (iii) to compare screening results obtained from the *Lipomyces* bio-assay with that of *Eremothecium* and *Nadsonia* [8-10]. In the course of these studies, preliminary data suggested that, while not an anti-mitochondrial drug, the antimalarial drug chloroquine (CQ) may in fact stimulate yeast sexual reproduction (Kock personal communications).

With this as background, the aims of this study became: (i) to use various yeast sensors to evaluate the effect of CQ on yeast sensor development and hence mitochondrial activity and (ii) to apply CQ as a fertility drug to induce sexual reproduction in *Scheffersomyces stipitis* – a yeast strain that has lost the ability to

reproduce sexually. In addition to these primary goals it was decided to determine the effect of sub-culturing of yeasts, as opposed to freezing as a method of preservation, on the sexual phase. For each of the sensors used in this study, the sensor structure as well as the ascospore release mechanics (where applicable) are discussed.

This ^{3}A system of drug screening is a holistic approach that is advantageous compared to specialized *in vitro* screening methods since it takes the cell as a whole (from an intra-omics perspective) into consideration. Yeast mitochondria are similar to mammalian mitochondria and can therefore act as a model to predict the effect of drugs on mammalian mitochondria, especially new drugs with mitochondrial liabilities [4] and drugs with possible pro-mitochondrial effects in yeast.

2.3. Experimental Section

All experiments were performed at least in triplicate.

2.3.1. Lipomyces: Strain Used, Cultivation and Preservation

Lipomyces yamadae (UOFS Y-2824) is preserved in the UNESCO MIRCEN culture collection at the University of the Free State, South Africa. Two separate cultures of *L. yamadae* were used: (i) a culture that had been preserved at -70 °C for one year prior to the experiment and (ii) a culture that had been preserved by continuous sub-culturing (every three months at 25 °C) for one year prior to the experiment. The cultures were streaked out on Yeast Malt (YM) agar plates and incubated at 25 °C to obtain growth for bio-assay experiments.

2.3.2. Lipomyces: Bio-assay Preparation and Effect of Preservation in Combination with Chloroquine (CQ) on Sexual Phase

A *L. yamadae* culture that had been sub-cultured for one year (every three months at 25 °C), was suspended in sterile distilled water (dH_2O), where after 200 µL of the suspension was spread out on YM agar plates. Central wells were made in the plates with sterile Pasteur pipettes. To these wells 46 µL of 8% (w/v) CQ (Sigma-Aldrich, Johannesburg, South Africa) suspended in 96% ethanol (EtOH; Merck, Darmstadt, Germany) was added. An EtOH control (46 µL EtOH alone per well) treatment was included [2]. All plates were incubated at 25 °C until pigmentation was clearly visible.

After incubation, samples were drawn from two sampling zones of each plate, corresponding to high (*i.e*., sub-lethal) and lower concentrations of CQ respectively. Each sampling zone was sampled at five different points spaced equally across the specific sampling zone tested on the plates. Each experiment was performed on separate bio-assay plates in triplicate. A total of 10 visual fields (40 x magnification) were analyzed per sample point and the percentage sensors (*i.e*., asci with ascospores) relative to the total number of asexual cells plus sensors of *L. yamadae* was determined using Light Microscopy (LM; Axioplan, Zeiss, Göttingen, Germany) coupled to a Colourview Soft Digital Imaging System (Münster, Germany). Therefore, a total of 1 (sampling zone) × 5 (sample points in each sampling zone) × 10 (fields per sample point) × 3 (triplicate) = 150 fields were analyzed per sampling zone to obtain a representative relative sensor count (Figure 2.1A). This data was compared to an identical experiment performed on freshly isolated *L. yamadae* by Marleen Maartens (unpublished data) to determine the effects of storage and sub-

culturing, in combination with CQ, on sensor development.

2.3.3. Lipomyces: Confocal Laser Scanning Microscopy (CLSM)

To evaluate whether the sensors of the yeast *L. yamadae* are associated with increased mitochondrial transmembrane potential ($\Delta\Psi_m$), cells of this organism (grown for 30 days at 25 °C on EtOH control as well as CQ in EtOH bio-assay plates), were washed with Phosphate Buffered Saline (PBS; Oxoid, Hampshire, England) and treated with 31 µL of a 0.33% (v/v) in PBS solution of the fluorescent mitochondrial stain Rhodamine 123 (Rh123; Molecular Probes, Invitrogen Detection Technologies, Eugene, OR, USA). These were incubated for 1 h in the dark at 21 °C, washed with PBS and subsequently fixed in Dabco (Sigma-Aldrich, Johannesburg, South Africa) on microscope slides. The cells were then viewed with Confocal Laser Scanning Microscopy (CLSM; Nikon TE 2000, Tokyo, Japan) to visualize $\Delta\Psi_m$ [8]. Yellow-green fluorescence indicated areas of increased $\Delta\Psi_m$ and therefore elevated mitochondrial activity.

2.3.4. Lipomyces: Effect of Preservation on Sexual Phase

Cultures of *L. yamadae* preserved at -70 °C for one year as well as cultures preserved by sub-culturing (every three months at 25 °C) for the same period of time, were streaked out on YM agar plates and incubated at 25 °C until pigmentation was observed. Neither culture was treated with EtOH or CQ in EtOH. The percentage sensors formed for each culture was determined as described in Section 2.3.2, however all 150 visual fields originated from the pigmented zones of the cultures.

2.3.5. Lipomyces: Nano Scanning Auger Microscopy (NanoSAM) and Argon (Ar^+) Etching

This was performed according to the method of Swart and co-workers [9]. Cells from the dark brown zone of the *L. yamadae* CQ bio-assay were chemically fixed with 3% (v/v) glutardialdehyde (Merck, Darmstadt, Germany) in a sodium phosphate buffer (Merck, Darmstadt, Germany) for 3 h. The suspension was rinsed with sodium phosphate buffer followed by fixation with 1% (w/v) osmium tetroxide (Merck, Darmstadt, Germany), also in sodium phosphate buffer for 90 min. The suspension was again washed with buffer and dehydrated in a graded series of EtOH: 30%, 50%, 70% and 90% for 30 min each, with centrifugation between each step, and 2 × 100% EtOH for 90 min each, with centrifugation between each step. The dehydrated yeast cells were submitted to critical point drying, mounted on aluminum stubs and coated with a layer of gold - making it electron conductive. These preparations were then subjected to Nano Scanning Auger Microscopy with Argon ion etching (NanoSAM Ar^+). Here, a PHI 700 Nanoprobe (Chigasaki, Japan) with SAM and Scanning Electron Microscopy (SEM) facilities was used. The field emission electron gun employed for SEM and SAM analyses was set at 2.45 A filament current, 4 kV extractor voltage and 238.1 µA extractor current, thereby obtaining a 20 kV, 10 nA electron beam for the Auger analyses and SEM imaging. The diameter of the electron beam was 12 nm. The electron gun unit's upper pressure was 8.8E-10 Torr and that in the main chamber was 2.29E-10 Torr. All measurements were performed using aperture A. An 8 µm Field of View (FOV) was used for SEM and the number of frames used was 4. The Auger point analyses were acquired by using 10 cycles per survey, 1 eV per step and 20 ms per step. The Nanoprobe was equipped with an Ar^+ ion sputtering gun that operated at a beam voltage of 2 kV, an ion beam current

of 2 µA and a raster area of 1 × 1 mm. This gave a sputter rate of about 27 nm/min. The ion emission current operated at 15 mA. Finally, an alternating sputter mode with 1 min sputter intervals and 2 min sputter time was used with no rotation.

2.3.6. Eremothecium: Strain Used, Cultivation and Preservation

Eremothecium ashbyi (UOFS Y-630) is preserved in the UNESCO MIRCEN culture collection at the University of the Free State, South Africa. This yeast was cultivated on YM agar plates at 25 °C to obtain cells for the experiment.

2.3.7. Eremothecium: Bio-assay Preparation and Effect of Chloroquine (CQ) on Sexual Phase

The same protocol used to construct the bio-assay of *L. yamadae* (Section 2.3.2) was used. The bio-assay plates were incubated at 25 °C until pigmentation was observed. After incubation, samples were drawn from two sampling zones of each plate, corresponding to areas of high (sub-lethal) and lower concentrations of CQ respectively, as seen in Figure 2.2B. Each sampling zone was sampled at three points spaced equally across the specific sampling zone tested on the plate. Experiments were performed in triplicate on EtOH and CQ in EtOH bio-assay plates. A total of 25 fields in each sampling zone were analyzed by using a haemocytometer (40 X magnification) and LM coupled to a Colourview Soft Digital Imaging System. This was performed by counting the number of discharged sensors (ascospores released) as well as immature sensors (sensors filled with ascospores). Therefore a total of 1 (sampling zone) x 3 (sample points) x 25 (visual fields) x 3 (triplicate) = 225 fields were analyzed per sampling zone to obtain a representative relative discharged sensor count that depicts the percentage discharged sensors.

2.3.8. Eremothecium: Light Microscopy (LM)

Cells from the EtOH control as well as CQ in EtOH bio-assay plates were studied using LM coupled to a Colourview Soft Digital Imaging System.

2.3.9. Dipodascopsis: Strain Used, Cultivation and Preservation

Dipodascopsis uninucleata var. *uninucleata* (UOFS Y-0128) is preserved in the UNESCO MIRCEN culture collection at the University of the Free State, South Africa. This yeast was cultured on YM agar plates at 25 °C to obtain cells for the experiment.

2.3.10. Dipodascopsis: Bio-assay Preparation and Effect of Chloroquine (CQ) on Sexual Phase

The same protocol was used as in Section 2.3.7, however bio-assay plates were incubated for 72 h at 25 °C and three sampling zones were selected per plate (Figure 2.3A(a,b)) corresponding to high, intermediate and low CQ concentrations respectively.

2.3.11. Dipodascopsis: Lyticase Treatment

To visualize the presence of positive pressure inside sensors, the cell walls of sensors of *D. uninucleata* were partially digested with lyticase (Sigma-Aldrich, Johannesburg, South Africa). Lyticase is a β(1-3) glucanase and thus catabolises the β(1-3) glucans of the yeast cell wall [11]. Cell growth was scraped from YM agar plates that were previously incubated for 96 h, and dispersed in an enzymatic reaction mixture of 100 mg 50 T Lyticase in 2 mL potassium phosphate buffer (Merck, Darmstadt, Germany) at pH 5.8. The samples were left undisturbed at 25 ºC

for 2 h to allow optimal digestion of the sensor walls.

2.3.12. Dipodascopsis: Light Microscopy (LM)

Mature sensors of *D. uninucleata* that were treated with Lyticase, as well as untreated sensors, were stained with safranin [0.2% (w/v) in dH_2O; Merck, Johannesburg, South Africa] and viewed with LM coupled to a Colourview Soft Digital Imaging System in order to observe polar and hydrophilic components of the sensors and ascospores.

2.3.13. Dipodascopsis: Confocal Laser Scanning Microscopy (CLSM)

To evaluate whether the sensors of the yeast *D. uninucleata* are associated with increased $\Delta\Psi_m$ the same protocol was applied as in Section 2.3.3 on EtOH control cultures as well as CQ in EtOH bio-assay cultures grown for 72 h at 25 °C.

To visualize the presence of positive pressure inside sensors, both control and enzymatically treated sensors of *D. uninucleata* (Section 2.3.11) were stained with the fluorescent dye Calcofluor white (Fluka, Johannesburg, South Africa) which has a high affinity for (amongst other polymers) chitin – one of the major components of the sensor wall [12]. The stained cells were viewed with CLSM at 407 nm laser light as well as epifluorescence UV light, to visualize the structural components of the sensor wall.

2.3.14. Dipodascopsis: Effect of Water Activity (a_w) on Ascospore Release

To determine if sensor pressure and hence ascospore release in *D. uninucleata* is the result of osmotic uptake of water, 72 h old cell culture was scraped from the surface of an YM agar plate and dispersed into three separate solutions in sterile test

tubes. The control solution was 10 mL dH_2O. The hypertonic solutions were respectively 10 mL of 50% (w/v) sucrose (Merck, Johannesburg, South Africa) in dH_2O and 10 mL of 10% (w/v) sodium chloride (NaCl; SAARCHEM, Krugersdorp, South Africa) in dH_2O, with the aim of significantly reducing the water activity (a_w) of the surrounding medium. The water activity (a_w) of the sucrose and NaCl solutions were 0.927 and 0.940 respectively [13]. At time intervals of 0.5 h and 24 h a drop of each solution was placed in a haemocytometer and the total sensors and discharged sensors were counted to calculate the percentage discharged sensors per sample (n = 228). This experiment was performed in triplicate. A Student t-Test was performed to determine if there was a statistically significant difference in the percentage discharged sensors between the different treatments.

2.3.15. Dipodascopsis: Nano Scanning Auger Microscopy (NanoSAM) and Argon (Ar^+) Etching

Cells from the EtOH control plates as well as the CQ in EtOH bio-assay plates were first prepared and then subjected to NanoSAM with Ar^+ etching as described in Section 2.3.5.

2.3.16. Dipodascus: Strain Used, Cultivation and Preservation

Dipodascus geniculatus (UOFS Y-1144) is preserved in the UNESCO MIRCEN culture collection at the University of the Free State, South Africa. This yeast was cultured on YM agar plates at 25 °C for 48 h to obtain cells for the experiment.

2.3.17. Dipodascus: Light Microscopy (LM)

To visualize the polar and hydrophilic components of the sensors and ascospores, sensors of *D. geniculatus* were stained with safranin and viewed with LM coupled to a Colourview Soft Digital Imaging System as described in Section 2.3.12.

2.3.18. Scheffersomyces: Strain Used, Cultivation and Preservation

Scheffersomyces stipitis (UOFS Y-1314) is preserved at -196 °C in the UNESCO MIRCEN culture collection at the University of the Free State, South Africa. This yeast was cultured on YM agar plates at 25 °C for 48 h to obtain cells for the experiment.

2.3.19. Scheffersomyces: Bio-assay Preparation and Effect of Chloroquine (CQ) on Sexual Phase

The same protocol was applied as in section 2.3.2 to cultures that were incubated for eight weeks at 25 °C.

2.3.20. Scheffersomyces: Confocal Laser Scanning Microscopy (CLSM)

To determine whether CQ increased $\Delta\Psi_{m,}$ the same protocol was applied as in Section 2.3.3 to *S. stipitis* cultures from the EtOH control as well as the CQ in EtOH bio-assay cultured for eight weeks at 25 °C (Section 2.3.19).

2.4. Results and Discussion

2.4.1. *Lipomyces yamadae*

Sensor Bio-assay

Chloroquine effect: The anti-malarial drug CQ had a stimulatory effect on the sensor development in the *L. yamadae* bio-assay (Figure 2.1A(a,b)). With increased drug concentration (from the periphery towards the centre, *i.e.*, sub-lethal zone) a drastic increase ($p < 0.05$; Two tailed t-Test) from 44.3% ± 10.0% (Figure 2.1A(a)-2) to 69.8% ± 9.8% (Figure 2.1A(a)-1) in sensor development of freshly isolated culture was observed. This is visualised by the development of a concomitant darker brown zone closer to the well.

After one year of preservation by sub-culturing, a similar trend was experienced. Here, the percentage sensors in the darker brown zone was 25.6% ± 7.6% (Figure 2.1A(b)-1), while the percentage sensors in the lighter brown zone was 16.6% ± 9.1% (Figure 2.1A(b)-2), again indicating a significant ($p < 0.05$; Two tailed t-Test) stimulatory effect of CQ on the sensor development and expected mitochondrial activity after preservation. Strikingly, this stimulatory effect has also been reported in literature where sub-lethal doses of CQ elevate the levels of *Plasmodium* gametocytes (*i.e.*, start of sexual phase) in the host's blood by favoring asexual merozoite transition to sexual gametocytes, thereby affecting greater transmission of the parasite via the mosquito to the host. This effect was shown to be enhanced in resistant *Plasmodium* strains [14]. It is interesting to note that CQ also influences mitochondrial activity by stimulating respiration in bovine spermatozoa [15].

Figure 2.1A Colour sensors of *Lipomyces yamadae* sensing chloroquine (CQ) and the effect of preservation. (**a**) Bio-assay preparation without prior preservation of indicator yeast (*i.e*., freshly isolated *L. yamadae*). Column 1 represents the percentage sensors relative to asexual cells counted in the darker brown zone (indicated as 1). Column 2 represents the percentage sensors obtained in the lighter brown zone (indicated as 2). (**b**) Bio-assay preparation after one year of preservation by sub-culturing. Column 1 represents the percentage sensors relative to asexual cells counted in the darker brown zone (indicated as 1). Column 2 represents the percentage sensors in the lighter brown zone (indicated as 2). The bars indicate the standard deviation. (**c**) Sensor disassembly by Argon (Ar^+) etching visualized by Nano Scanning Auger Microscopy (NanoSAM). Green = gold (Au); Blue = carbon (C). (**d**) Increased fluorescence (green) surrounding ascospores inside sensors that had been treated with the mitochondrial stain Rhodamine 123 (Rh123) and observed by Confocal Laser Scanning Microscopy (CLSM). Parts of this figure (**a**) are available in a video lecture posted on the website of Translational Biomedicine and are reproduced with permission [6]. Asp, Ascospore; Sens, sensor.

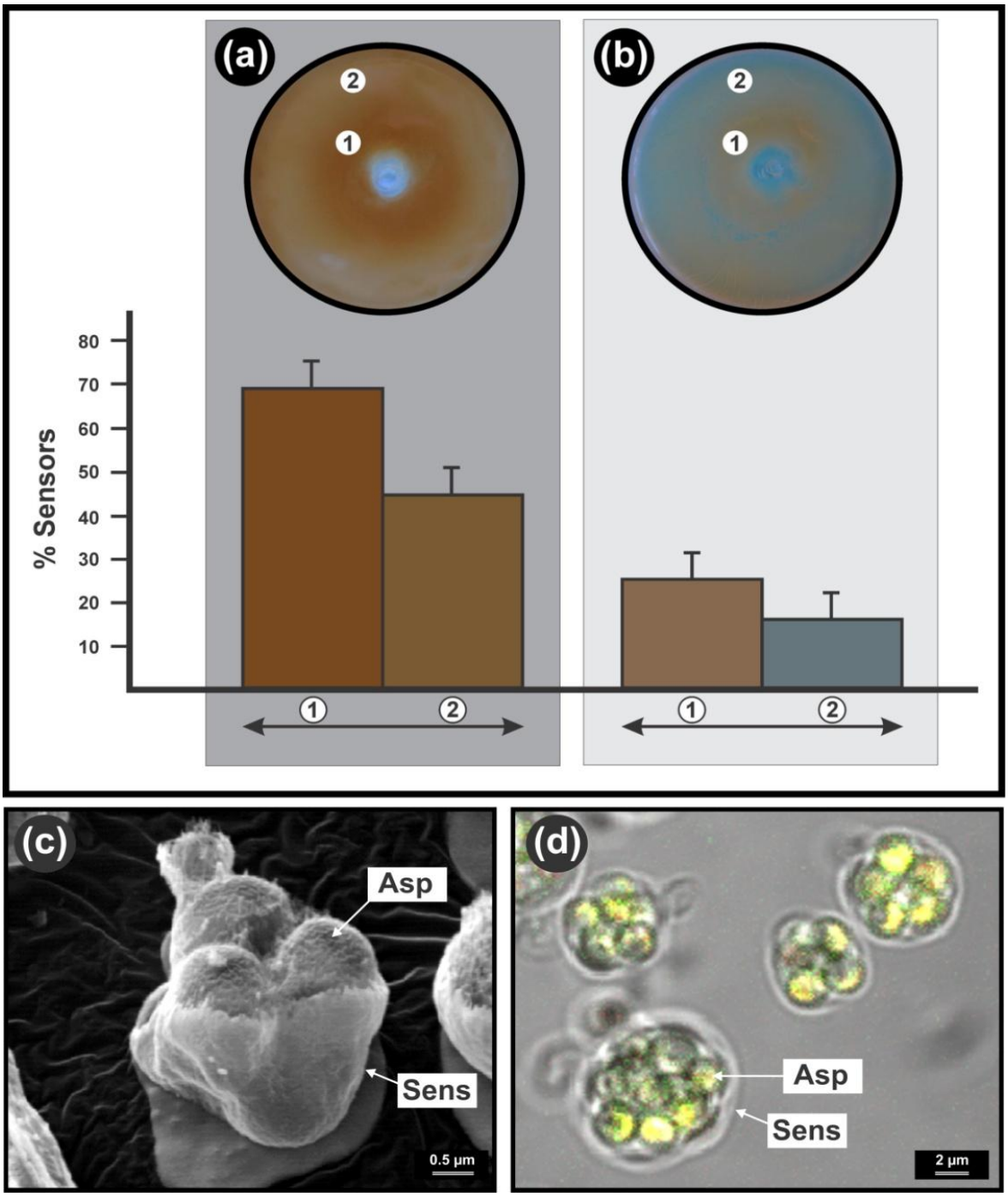

Figure 2.1A (See opposite page)

Preservation effect: When comparing bio-assays before and after one year of preservation by continuous sub-culturing, it is clear that this preservation method inhibited sensor development (Figure 2.1A(a,b)). The results indicate a 2.7 times

significant decrease (from 69.8% ± 9.8% to 25.6% ± 7.6%; $p < 0.05$; Two tailed t-Test) in the ability to produce sensors in response to CQ at sub-lethal concentrations over one year of preservation (compare Figure 2.1A(a)-1 to Figure 2.1A(b)-1). Strikingly, the same level of decrease was observed at lower concentrations of CQ (compare Figure 2.1A(a)-2 to Figure 2.1A(b)-2) again with a 2.7 times significant decrease in the ability to produce sensors (from 44.3% ± 10.0% to 16.6% ± 9.1%; $p < 0.05$; Two tailed t-Test). This is also visualised by the concomitant decrease in sensor associated pigmentation. Similar differences in sensor development were recorded when comparing sensor development at lower CQ concentrations at the periphery of respective plates (Figures 2.1A(a)-2 and 2.1A(b)-2)) with the periphery of control plates, *i.e.*, when only EtOH was added to bio-assays before and after one year of preservation (results not shown).

The inhibitory effect of continuous sub-culturing, compared to when cultures were preserved at -70 °C, on sensor formation was confirmed quantitatively. The percentage sensors formed by *L. yamadae* cultures preserved at -70 °C for one year, was compared to that of *L. yamadae* cultures preserved for the same time period by continuous sub-culturing on YM agar slants at 25 °C. The culture preserved at -70 °C (Figure 2.1B(a)-1) displayed an average percentage sensors formed of 48.5% $\pm$ 21.2% after incubation on YM agar plates (30 days at 25 °C). The sub-cultured yeast (Figure 2.1B(b)-2) showed an average percentage sensors formed of 13.8% $\pm$ 8.3% under the same cultivation conditions (30 days at 25 °C). This was visualized as a significant decrease in sensor pigmentation after one year of sub-culturing (Figure 2.1B(a,b); $p < 0.05$; Two tailed t-Test).

Figure 2.1B Colour sensors of *Lipomyces yamadae* after two methods of preservation. (**a**) Plate culture preparation after the indicator yeast had been preserved at -70 °C for one year. Column 1 represents the percentage sensors relative to asexual cells counted across the dark brown zone. (**b**) Plate culture preparation after the indicator yeast had been preserved with continuous sub-culturing at 25 °C for one year. Column 2 represents the percentage sensors relative to the asexual cells counted across the darker brown zone. The bars indicate standard deviations.

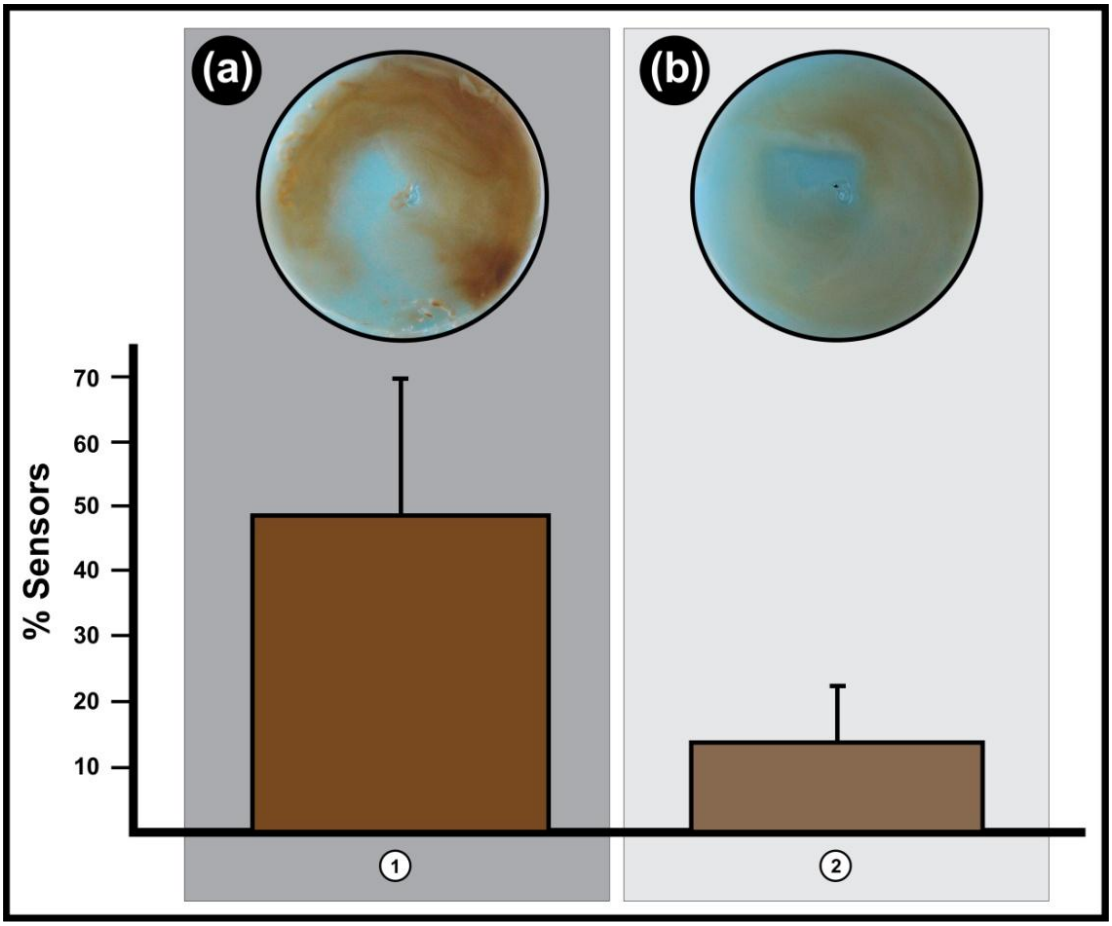

Figure 2.1B (See opposite page)

The above results suggest that preservation through sub-culturing is also anti-mitochondrial. Interestingly, according to literature [16] sub-culturing over a long period may have mutagenic effects on yeasts leading to changes in morphological characteristics and loss in the ability to sporulate.

This may explain the decrease in pigmented sensor development in the strictly respiring *L. yamadae* after one year of this type of preservation. Preservation by sub-culturing over extended periods can therefore be considered anti-mitochondrial where the sexual structures (*i.e*., sensors and ascospores) of yeasts, that can only obtain energy from aerobic respiration, should be the most sensitive.

Sensor Structure

Sensors are characterised by various smooth spherical ascospores of about 2 µm in diameter all enveloped by a sensor wall (Figure 2.1A(c,d)). Sensor structure is exposed by sensor disassembly using Ar^+ etching and NanoSAM in SEM mode (Figure 2.1A(c)). Here Auger nano-probing shows remnants of gold (in green) on the sensor wall (from gold sputtering during NanoSAM preparation) as well as elemental carbon (in blue) inside the sensor and associated with ascospores. As expected [4] these sensors have increased mitochondrial activity indicated by increased fluorescence associated with ascospores (Figure 2.1A(d)). Here no smart mechanics, as is evident in certain other sensing yeasts, were observed.

2.4.2. Eremothecium ashbyi

Sensor Bio-assay

Similarly to *L. yamadae*, CQ also stimulated pigmented sensor development in the *E. ashbyi* bio-assay (Figures 2.2A(a,b)). At sub-lethal concentrations of the drug, yellow pigmentation started to develop next to the inhibition zone (Figure 2.2A(b)). This was not visible in the EtOH control (Figure 2.2A(a)). This is indicative of increased development of sickle-shaped sensors filled with ascospores (Figure 2.2A(c)) as well as increased mitochondrial activity [2]. It is important to note that the yellow zone appeared within 24 h after which the whole plate coloured yellow. Therefore, if the plate was inspected after 24 h, no effect of CQ would have been observed as was reported [6].

Figure 2.2A Colour sensors of *Eremothecium ashbyi* sensing chloroquine. (**a**) Ethanol (EtOH) control plate showing no yellow pigmentation and thus no stimulation of sensor development. (**b**) Bio-assay plate treated with chloroquine (CQ) suspended in EtOH showing yellow pigmentation at sub-lethal concentrations that is indicative of sensor development and pro-mitochondrial activity. (**c**) Light Microscopy (LM) micrograph of sensors comprised of asci filled with sickle shaped ascospores. Asp, ascospore; Sens, sensor.

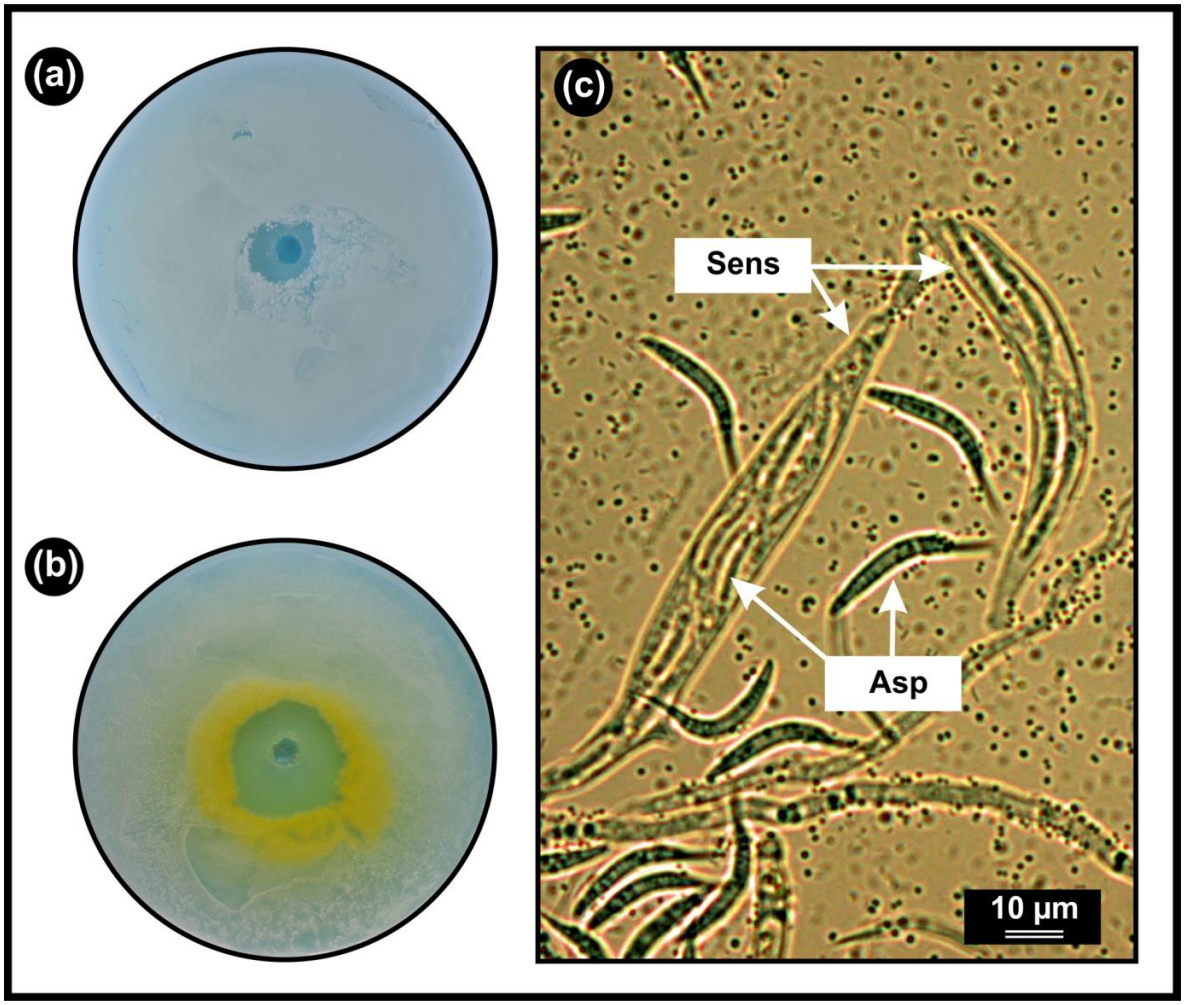

Figure 2.2A (See opposite page)

The effect of CQ was quantified in terms of percentage discharged sensors. At sub-lethal levels of CQ, as indicated by the formation of a yellow pigmented ring on the bio-assay plate (Figure 2.2B-1) the percentage discharged sensors was 66.0% ± 30.3%, while at a lower concentration of CQ (Figure 2.2B-2) it was 16.4% ± 13.1%, similar to the percentage discharged sensors on the EtOH control bio-assay plates (results not shown). This difference was statistically significant ($p < 0,05$; Two tailed t-Test).

Figure 2.2B Colour sensors of *Eremothecium ashbyi* sensing chloroquine (CQ) by ascospore discharge. Column 1 represents the percentage discharged sensors relative to immature sensors (*i.e*., sensors with ascospores) counted in the yellow zone (indicated as 1). Column 2 represents the percentage discharged sensors relative to immature sensors counted in the light zone (indicated as 2). The bars indicate standard deviation.

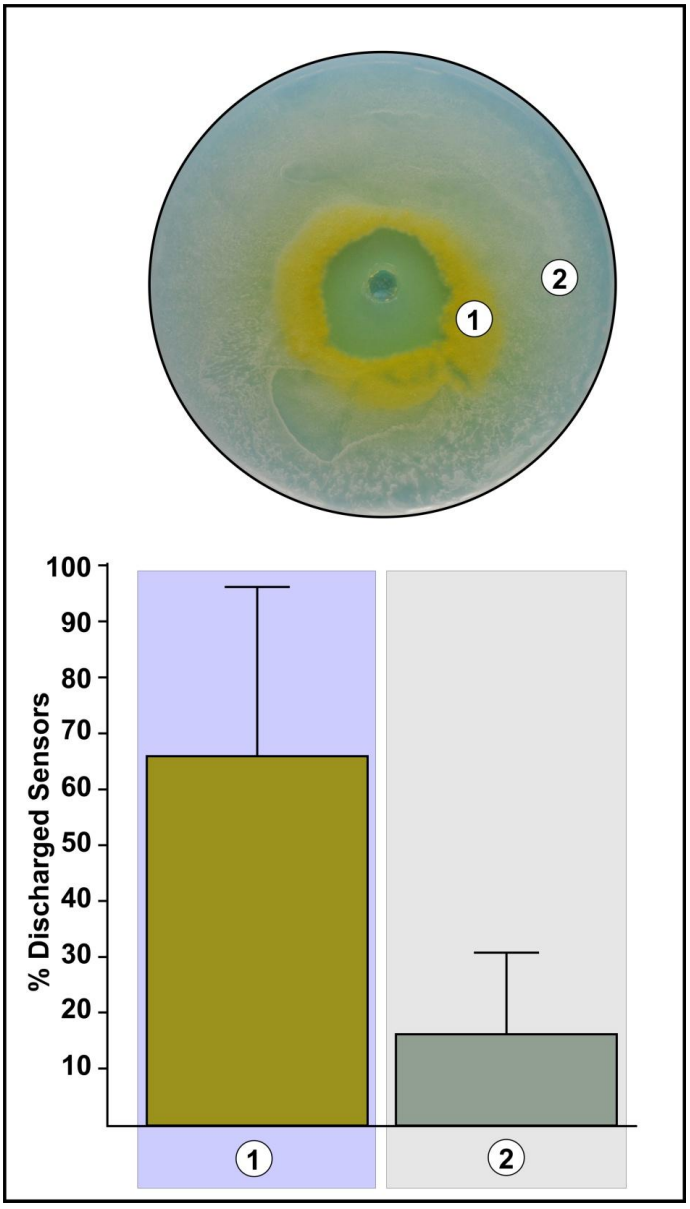

Figure 2.2B (See opposite page)

Sensor Structure and Smart Mechanics

The sensors are mostly intercalary in long chains and may be ellipsoidal or fusiform in shape (Figure 2.2A(c)). Each sensor contains between eight and 16 hyaline, narrow, sickle-shaped ascospores. These ascospores have rounded tips and basal spines. Smart mechanics inside the sensor is made possible by water movement across V-shaped fins situated on the blunt end of the ascospore [6].

These fins are covered with anti-mitochondrial drug sensitive 3-OH oxylipins, which are formed by β-oxidation. Sensor-ascospore smart mechanics can be viewed as a movie in video lecture format published by the Kock-group [6,7].

2.4.3. Dipodascopsis uninucleata

Sensor Bio-assay

When CQ was added to the bio-assay of *D. uninucleata* (Figure 2.3A), an inhibition of sensor discharge at sub-lethal concentration was found (Figure 2.3A(c)-1CQ). However this same effect was found in the EtOH control plates (Figure 2.3A(c)-1Contr.). At lower concentrations (Figure 2.3A(c)-2CQ), this drug enhanced ascospore release compared to samples from a similar position on the control plate (Figure 2.3A(c)-2Contr.) resulting in increased discharged sensors from 0.0% to 2.4% ± 3.8%. When comparing the EtOH control (Figure 2.3A(c)-3Contr.) to CQ bio-assay cultures (Figure 2.3A(c)-3CQ.), a significant increase ($p < 0.05$; Two tailed t-Test) in ascospore discharge from sensors was found to occur at the periphery of the plates *i.e.*, from 2.9% ± 5.7% to 19.6% ± 9.7%. It is interesting to note that a decrease in the concentration of EtOH in the absence of CQ (Figures 2.3A(c)-1Contr. to 2.3A(c)-3Contr.), also enhanced ascospore release but at a significantly lower level compared to when CQ was added ($P < 0.05$; Two tailed t-Test). Unfortunately these sensors are not pigmented and sensor formation had to be followed by means of time consuming microscopic investigations. Another drawback is that, due to the hyphal nature of this yeast, the percentage sensors formed relative to single vegetative cells could not be quantified as was the case with *L. yamadae*.

Figure 2.3A Sensors of *Dipodascopsis uninucleata* sensing chloroquine (CQ) by ascospore discharge. (**a**) Contr: Bio-assay plate showing three zones (1,2,3) that coincide with a decrease in concentration of ethanol (EtOH) alone. (**b**) CQ: Bio-assay plate showing three zones (1,2,3) that coincide with a concomitant decrease in the concentration of CQ in EtOH. (**c**) A graph showing changes in percentage discharged sensors over a decreasing gradient (from 1 to 3) of EtOH alone (Contr.) and EtOH in combination with chloroquine (CQ). The bars represent standard deviation. (**d**) A Confocal Laser Scanning Microscope (CLSM) micrograph of a Rhodamine 123 (Rh123) treated fluorescing sensor of *D. uninucleata.* The yellow-green fluorescence indicates elevated mitochondrial activity. (**e**) Disassembly of the sensor by Argon (Ar^+) etching and visualized with Nano Scanning Auger Microscopy (NanoSAM) in Scanning Electron Microscopy (SEM) mode showing microfibrillar composition of the sensor wall at the sensor tip. (**f**) Scanning Electron Microscopy micrograph of an ascospore showing longitudinal grooves necessary for smart sensor mechanics. 1,2,3, areas on bio-assay plates; Asp, ascospore; Contr., control; CQ, Chloroquine in EtOH; in, inhibition zone; Mf, microfibrils; Sens, sensor; T, tip of sensor. Part of this figure (**e**) is available in a video lecture posted on the website of Translational Biomedicine and are reproduced with permission [6].

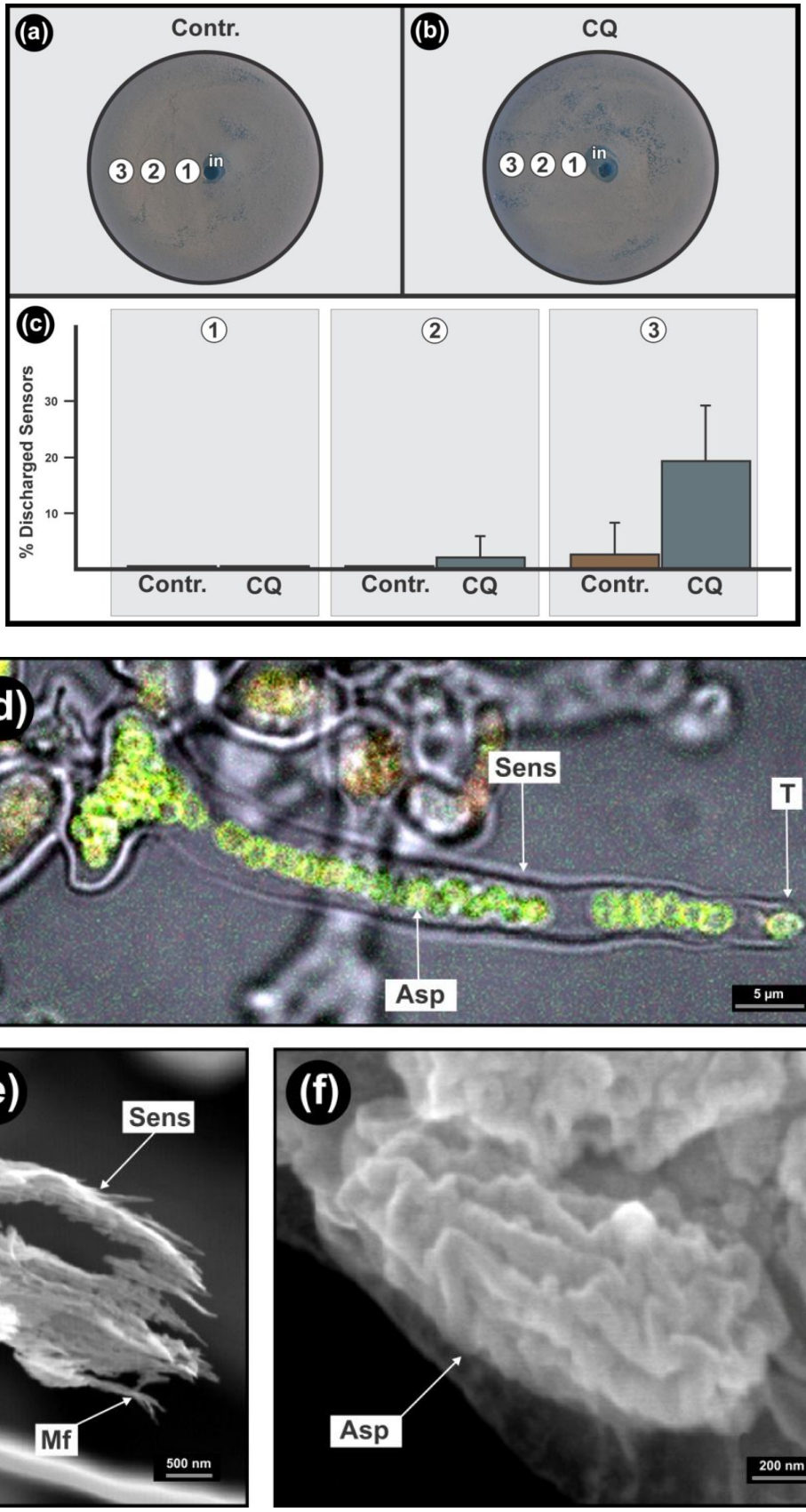

Figure 2.3A (See opposite page)

Figure 2.3B The sensor of the yeast *Dipodascopsis uninucleata* var. *uninucleata* (**a**-**d**) compared to the sensor of *Dipodascus geniculatus* (**e**). (**a**) Confocal Laser Scanning Microscopy (CLSM) micrograph of a sensor stained with Orange G, showing an ascospore column, surrounded by a transparent sheath structure [5]. (**b**) Nano Scanning Auger Microscopy - Scanning Electron Microscopy (NanoSAM-SEM) mode image of a sensor subjected to Argon (Ar^+) etching, showing ascospores inside the sensor and coiled microfibrillar structures (orientated at an angle to ascus length towards sensor tip) in the ascus wall. (**c**) Sensor stained with safranin, showing ascospores in the central channel, surrounded by a sheath-structure. (**d**) Sensor stained with safranin showing the empty central channel, surrounded by a sheath-structure, and the collapsed central channel near the base of the sensor. (**e**) Sensor of *Dipodascus geniculatus* stained with safranin showing a sensor with no central channel and the absence of an inner lined sensor sheath. A, ascus (sensor); Cc, central channel; CCc, collapsed central channel, Mf, microfibrillar structures; S, ascospore(s); Sh, sheath structure.

Selective fluorescent and non-fluorescent staining reveals that the ascospores of *D. uninucleata* are suspended in a central channel along the centre of the sensor (Figure 2.3A(d) and Figure 2.3B(a)).

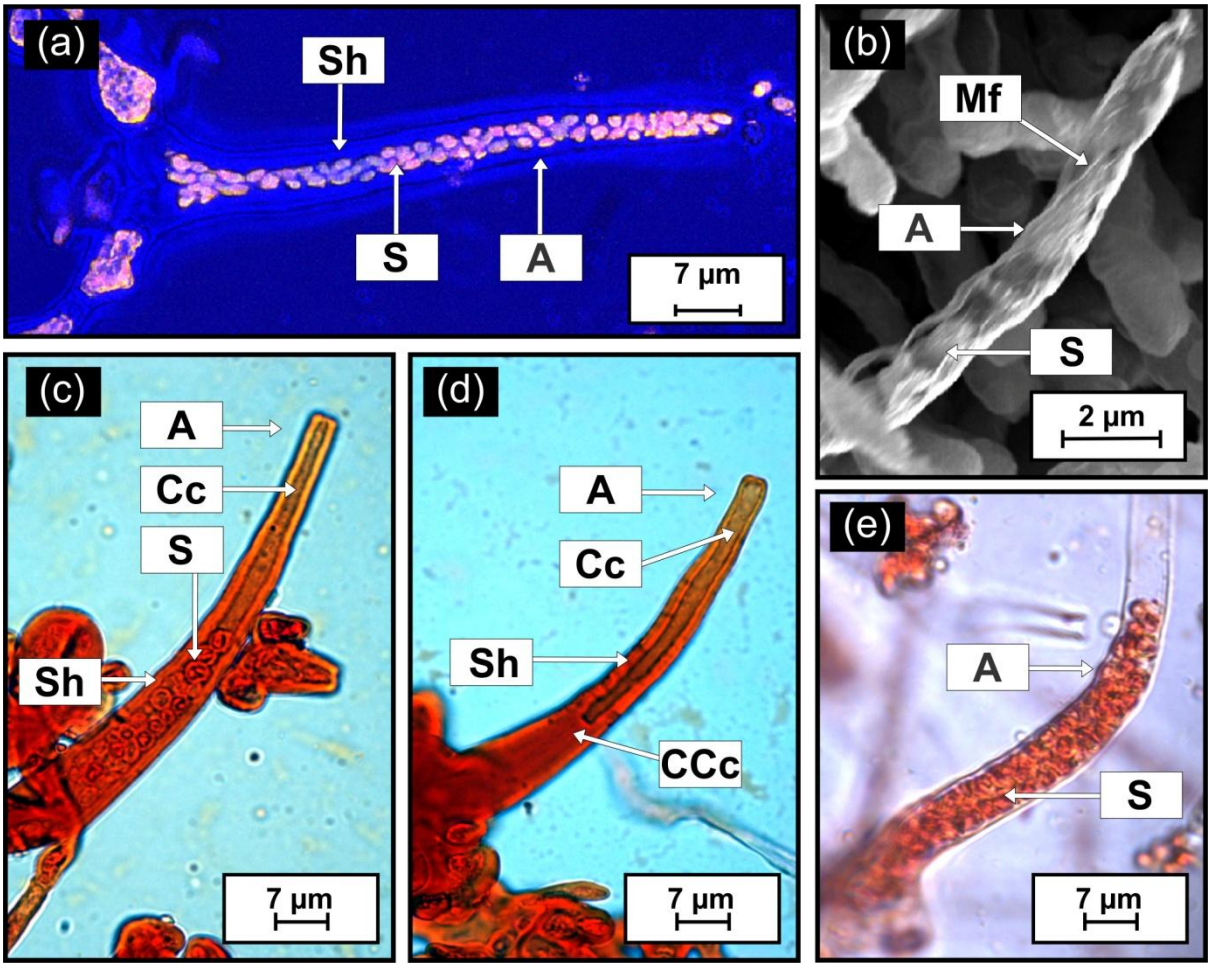

Figure 2.3B (See opposite page)

The sensors were disassembled using Ar^+ etching at a rate of 27 nm/min into the sensor [5]. Nano Scanning Auger Microscopy (NanoSAM) in SEM mode revealed microfibrillar strings coiled around the ascospores (see Figure 2.3A(e) and Figure 2.3B(b)). Exposing the sensor interior with NanoSAM revealed the elongated, grooved ascospores (Figure 2.3A(f)), which measure approximately 1 µm in diameter

and 2 µm in length. The ascospores are individually released from the sensor, presumably with the aid of lubricating β-oxidation products such as 3-OH oxylipins [1]. It is also suggested that the interaction between the surface grooves of the ascospores and the microfibrillar structures on the interior walls of the sensor cause the rotary movement observed in released ascospores. Staining with safranin in this study, clearly exposed a well demarcated sheath-like structure, as previously anticipated, surrounding the ascospore column inside the sensor (Figure 2.3B(c)). Strikingly, as the spores were released the central channel collapsed from the bottom of the sensor towards the tip as the walls of the sheath moved closer towards each other. This was affected by the sheath that expanded to fill the empty central channel (Figure 2.3B(d)). This is in sharp contrast to the sensor architecture found in *Dipodascus geniculatus.* Here no such sheath, lining the entire sensor interior could be detected (Figure 2.3B(e)). Each ascospore is enveloped by its own separate sheath [17].

Our results suggest that the outer part of the sheath of *D. uninucleata* is surrounded by a chitinous wall (Figure 2.3C(a) - insert) [12]. When the sensor wall was digested enzymatically [18] the sensor with sheath started to inflate, forming a bulging sensor at the point of cell wall digestion as observed by CLSM (Figure 2.3C(a)). This was not the case in the untreated control (Figure 2.3C(a) - insert). The bulging parts of the sensor could also be observed when asci were stained with safranin (Figure 2.3C(b)). Here no central channel was detected, probably as a result of the deformation of the sensor upon lyticase digestion and consequent disturbance of the cell wall (Figure 2.3C(b,d)). In Figure 2.3C(c) bulging and eventual rupturing of the sensor wall is visible after lyticase treatment and subsequent safranin staining. Again the central channel could not be observed.

Figure 2.3C Stained sensors of *Dipodascopsis uninucleata* var. *uninucleata* treated with a cell wall degrading enzyme. (**a**) Confocal Laser Scanning Microscopy (CLSM) image of a sensor digested with lyticase and stained with Calcofluor White (the latter suggesting also chitin in the sensor wall), fluorescing blue, and bulging due to positive pressure. Insert: Sensor without lyticase treatment showing ascospores inside and fluorescing cell wall. (**b**) Sensor digested with lyticase and stained with safranin, showing bulging, an inflated sheath-structure and a central channel that is collapsed probably due to positive pressure and enzymatic cell wall disturbance. (**c**) Sensor digested with lyticase and stained with safranin, rupturing due to positive pressure. (**d**) Sensor digested with lyticase and stained with safranin, showing an inflated sheath-structure and collapsed central channel. A, ascus (sensor); B, bulging; C, chitin; Cc, central channel; CCc, collapsed central channel; R, rupture; S, ascospore(s); Sh, sheath structure.

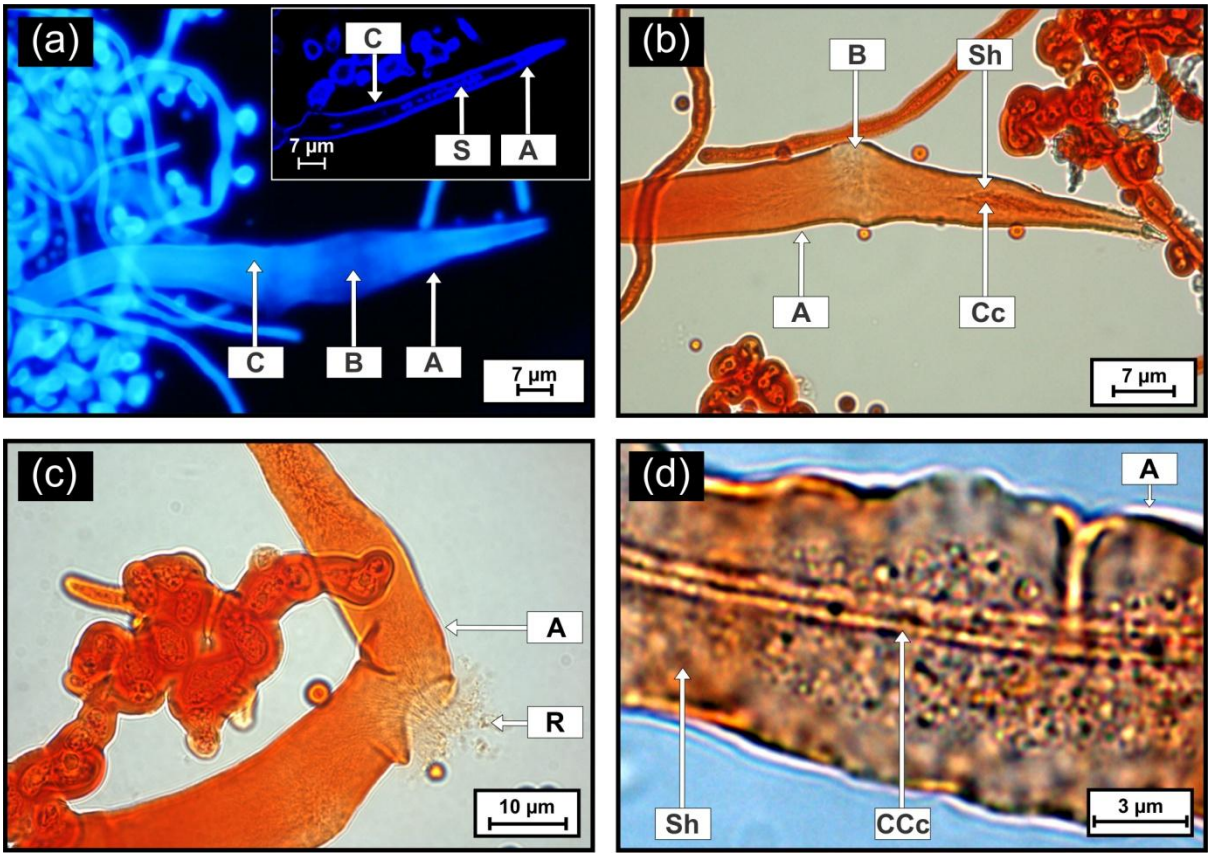

Figure 2.3C (See opposite page)

These smart sensor mechanics are discussed in detail in literature [6,7,19]. It was concluded that since CQ had an enhancing effect on ascospore discharge from sensors, it should be pro-mitochondrial, probably also enhancing β-oxidation at lower concentrations - especially since blocked sensors that were filled with well developed, unreleased ascospores, were formed at higher concentrations of this drug (results not shown). Whether CQ has a direct or indirect effect on mitochondrial activity should now be investigated.

When the sensors were suspended in solutions with different water activities (a_w) for 24 h at 25 ºC, a significant decrease ($p<0.05$; Two tailed t-Test; n=228) in ascospore release was observed from 69.6% ± 15.6% (in pure dH_2O solution) to

35.2% ± 15.7% (in 50% sucrose in dH_2O solution; a_w = 0.927) and 38.1% ± 5.4% (in 10% NaCl in dH_2O solution; a_w = 0.940). Ascospore release was quantified as 30% ± 15% at the start of incubation in all suspensions tested.

2.4.4. Scheffersomyces stipitis

Sensor Bio-assay

At sub-lethal concentrations of CQ (Figure 2.4-1), sensor development was observed with the *S. stipitis* bio-assay. The percentage sensors formed relative to the asexual cells was 0.5% ± 0.7%. This low number of sensors formed is significant when considered that no sensor formation was found at either low concentrations of CQ (Figure 2.4-2) or on the EtOH control plates (results not shown). However, whether CQ acts directly on the mitochondria or indirectly (*i.e*., through acting on the cellular machinery of sexual reproduction) is still unclear.

Sensor Structure

Confocal Laser Scanning Microscopy (CLSM) images of *S. stipitis* cultures exposed to sub-lethal concentrations of CQ, showed the presence of the distinctly hat-shaped ascospores of this yeast (Figure 2.4-1Top - insert). The sensors of *Scheffersomyces* were found to be sequestrate (*i.e*., making use of passive ascospore release).

Figure 2.4 Sensors of *Sceffersomyces stipitis* sensing chloroquine (CQ). Column 1 represents the percentage sensors relative to the asexual cells counted at sub-lethal concentrations of CQ (indicated as 1). The bars represent the standard deviation. Above column 1 the Confocal Laser Scanning Microscopy (CLSM) micrograph shows the presence of two hat-shaped ascospores of *S. stipitis* inside a sensor that had been stained with the fluorescent mitochondrial stain Rhodamine 123 (Rh123). Insert: hat-shaped ascospores of *S. stipitis* inside a sensor. The yellow-green fluorescence indicates increased mitochondrial transmembrane potential ($\Delta\Psi_m$). Column 2 represents the percentage sensors relative to the asexual cells counted at low concentrations of CQ (indicated as 2). Above the column (indicated as 2), a CLSM micrograph of cells stained with Rh123, show that no sensors with ascospores were formed.

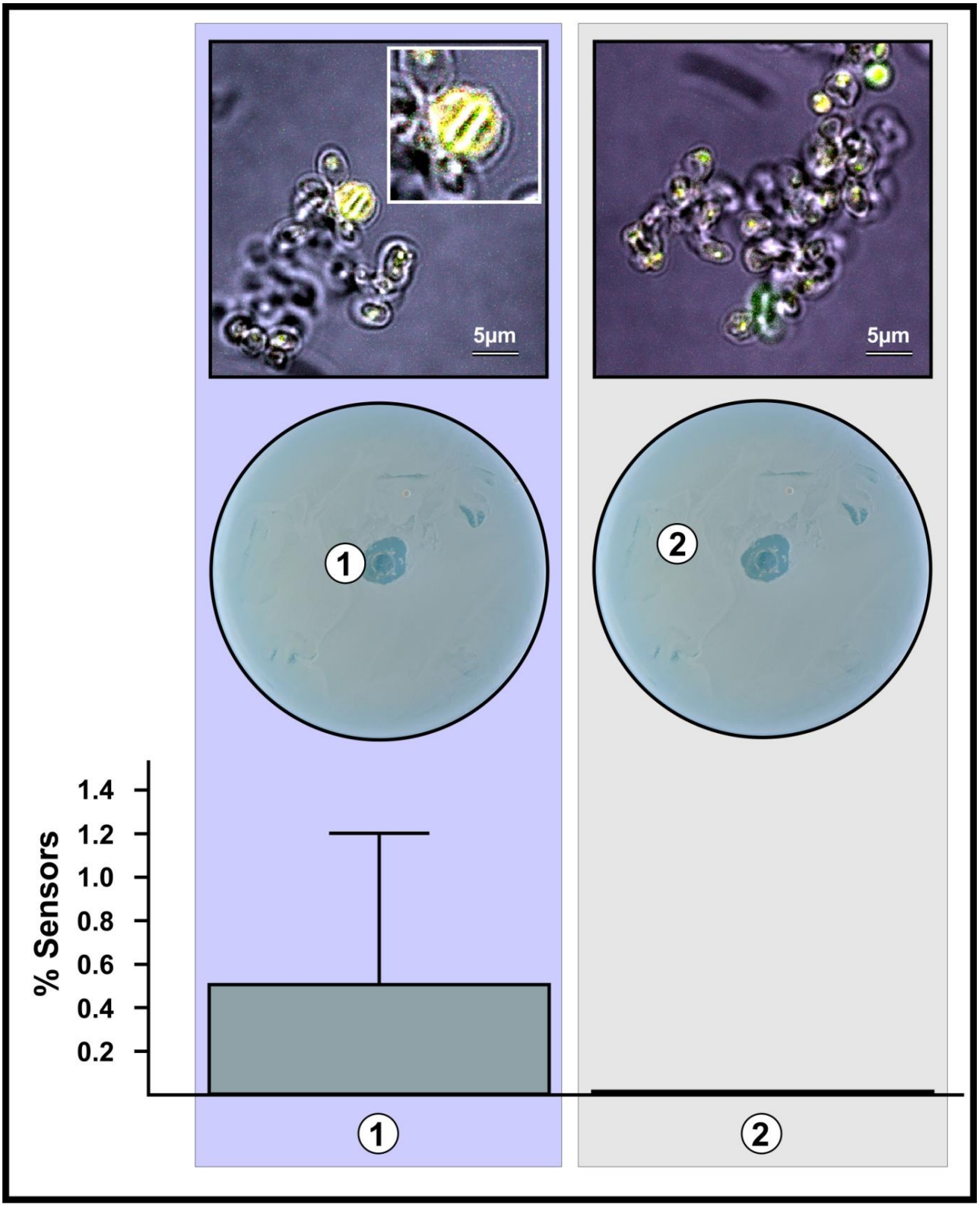

Figure 2.4 (See opposite page)

2.5. Conclusions

2.5.1. Effect of Chloroquine (CQ)

In previous studies aimed at the development of yeast sensor bio-assays for anti-mitochondrial drugs [5], it was found that CQ gave negative results for anti-mitochondrial activity in the ^{3}A system. However, upon closer inspection it was found that CQ stimulated sexual reproduction in various yeast sensors such as *Lipomyces, Dipodascopsis, Eremothecium* and *Scheffersomyces*. The potency of CQ as a yeast fertility drug was demonstrated by its ability to rejuvenate the sexual phase of a *Scheffersomyces* strain that had apparently become non-sporulating. It was also found that stimulation of yeast sexual structures coincided with increased mitochondrial activity, leading to the hypothesis that CQ stimulates mitochondrial respiration, thus driving the formation of yeast sexual structures.

Care should however be taken with interpreting these results. Drugs, and any other physiological factors, that stimulate sensor development may not necessarily influence mitochondrial activity directly. They may influence any number of the complex physiological processes involved in sensor development such as meiosis, the formation of secretory vesicles, septin, dityrosine layers, membranes and cell walls as well as pigment production in the cases of *Eremothecium* and *Lipomyces*. The up-regulation of such energy-intensive processes may indirectly increase mitochondrial respiration as a means of catering to the higher energy demand of sensor formation (*i.e.*, various ascospores are formed inside the sensors).

It is therefore important that a positive hit obtained with these yeast bio-assays be followed up by further *in vitro*, *in vivo*, *in silico* and “omics” research, to elucidate the

stimulatory action of these drugs and physical effects at molecular level [4]. For instance, when a positive hit for a pro-mitochondrial compound is encountered, more *in vitro* mechanistic studies can be applied on oxidative phosphorylation, permeability transition, $\Delta\Psi_m$ status and other mitochondrial drug targets. This may then be followed up by different tests, using, amongst others, NanoSAM imaging, enzyme-linked assays of the different respiratory complexes, different histochemical and immuno-histochemical methods as well as animal models to finally predict clinical outcomes [4]. Of particular interest will be research concerned with the application of CQ as a pro-mitochondrial/fertility drug and the use of NanoSAM linked bio-assay systems for the identification of new pro-mitochondrials. This approach may give new careers to conventional drugs used today to treat specific disorders (drug “reprofiling”) [5].

The finding that CQ acts as a yeast fertility drug is of great importance to the field of malarial research and cellular invigoration (increasing mitochondrial activity). This should be followed up urgently. For many years, CQ has been considered the gold standard for the treatment of malaria. However resistance build-up by the infective parasite hampers its application. This drug not only targets the asexual stage of the parasite, but also induces gametocytogenesis of *Plasmodium* in the host, which is a prelude to sexual reproduction occurring in the mosquito vector - a process that renders the vector infective. Chloroquine can therefore also favour malaria infection, an aspect that needs urgent attention [14]. The recent advocation in literature for the use of CQ as an antifungal drug, is a cause of concern [20]. If the dose of CQ – safely administered to the patient – were high enough to kill most of the infective fungal cells, the body will eventually metabolize the CQ to sub-lethal concentrations (21). Exposing infective fungal cells to sub-lethal doses of CQ may lead to

sporulation, or the formation of other virulence factors, which would aggravate and prolong the infection (22). Another important aspect of the pro-fertility action of CQ is its ability to induce sexual reproduction in seemingly non-sexual yeasts (*i.e.*, *Scheffersomyces stipitis* that has lost the ability to reproduce sexually). This effect has also been demonstrated in humans, where CQ has been reported to increase the birth weight of infants during the first pregnancy of mothers who received CQ as prophylactic treatment [23]. Thus it is evident that CQ acts as a fertility drug not only in yeasts but also protozoa (*i.e.*, *Plasmodium*) and in mammals (*i.e.*, humans). Further investigation of CQ's pro-fertility mechanism of action, its risk regarding widespread use as an anti-malarial, as well as possible novel medical applications should be encouraged.

2.5.2. Preservation Effect

An important spin-off of this research project is the confirmation that sub-culturing as a method of preservation, inhibits sexual reproduction in yeast cultures over time [16]. This strongly contrasts preservation by freezing at -70 °C (and presumably also at -196 °C) which has little inhibitory effects. This may be explained in terms of energy conservation. The relatively stable environments of the agar slants, used for sub-culturing, most likely negates the need for continuous genetic adaptation. This implies that sexual reproduction (as a means of improving genetic composition) becomes redundant. Due to the high energy demands of sexual reproduction (as mentioned above), sexual structure formation is probably continuously down-regulated in sub-cultured yeasts, as a means of conserving energy. This process, if allowed to continue for a sufficient period of time, may lead to a complete loss of the sexual phase in yeasts. In such cases the cellular/genetic machinery for sexual

reproduction may or may not be present in the apparent anamorphs. Yeast cultures that are stored at sub-zero temperatures are inactive and thus the loss of sexual reproduction is prevented from taking place during the storage period.

These findings have important implications for both the biotechnology industry as well as yeast culture collections, both of which routinely make use of sub-culturing as well as freezing as methods of preservation [24,25]. The loss of the sexual phase, which coincides with sub-culturing, may lead to problematic – or incorrect - characterization of yeast strains, considering that the sexual structures are commonly used in characterization [25].

2.5.3. Concluding Remarks

The importance of these findings cannot be overstated. The fertility effects of CQ on the parasite *Plasmodium* is a strong indicator that the ways in which this "gold standard" antimalarial drug is used must be re-evaluated. As far as literature shows, CQ is the only known drug to have a proven fertility effect on such a variety of organisms, making it the "founding member" of a novel class of fertility drugs. The strong indications that CQ is also pro-mitochondrial in nature opens up the potential field of pro-mitochondrial drug research. The potential medical implications range from drug reprofiling to the application of pro-mitochondrials for the treatment of important medical conditions. However, the exact nature of CQ's effect on mitochondrial activity must first be established before such avenues of research can be further explored.

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Chapter 3

Main Conclusions

3.1. Introduction

Previous studies aimed at using yeast sensors to identify anti-mitochondrial drugs [1] indicated that the antimalarial drug chloroquine (CQ) may stimulate yeast sexual reproduction (unpublished data). Consequently, various yeast sensors that are distantly related within the ascomycetous yeasts (Figure 3.1) were used to investigate this phenomenon [2]. The results were compared to the known effects of CQ on other eukaryotes to determine the conserved status of this pro-fertility phenomenon.

3.2. Conserved Status

The yeast sensors used in this study were from *Eremothecium ashbyi* (family Saccharomycetaceae), *Dipodascopsis uninucleata* var. *uninucleata* and *Lipomyces yamadae* (family Lipomycetaceae) and *Scheffersomyces stipitis* (family Debaryomycetaceae) [2]. These distantly related yeasts, which include species that actively release ascospores [3,4] as well as sequestrate species, all displayed elevated sexual reproduction after CQ treatment. Similarly, reports show that CQ stimulates gametocytogenesis (transition to the sexual phase) in the malaria causing protozoan parasite *Plasmodium* [5,6], and increases fertility in mammals, including humans [7,8,9]. Despite the fact that yeasts, *Plasmodium* and mammals are only distantly related (Figure 3.2) [10], CQ leads to enhanced fertility in all these groups, demonstrating the conserved status of the phenomenon among the eukaryotes.

Figure 3.1 Phylogenetic relationships of ascomycetous yeast genera and families based on Large Subunit (LSU) ribosomal RNA (rRNA), Small Subunit (SSU) rRNA and translation elongation factor-1α sequences. The genera of the yeast species used are depicted as **a**, **b** and **c**. (**a**) *Eremothecium* in the family Saccharomycetaceae. (**b**) *Scheffersomyces* in the family Debaryomycetaceae. (**c**) *Dipodascopsis* and *Lipomyces* in the family Lipomycetaceae [2].

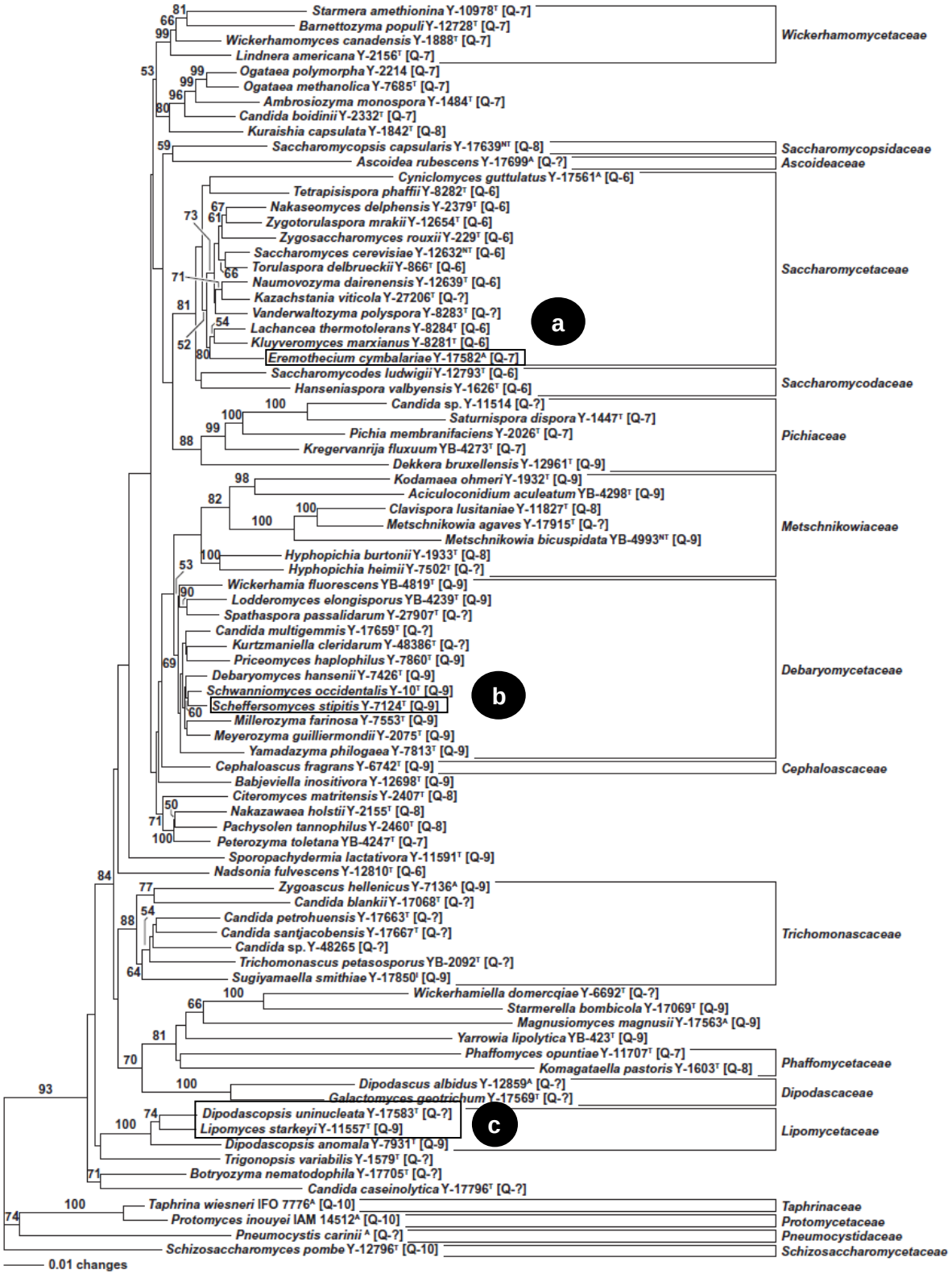

Figure 3.1 (See opposite page)

3.3. Implications of Research

Considering that *Plasmodium* gametocytes (sexual phase) are transferred from host to vector to facilitate infection [11], elevation of gametocytogenesis by CQ, which is considered the “gold standard” for malaria treatment and prevention [12], may stimulate the spread of infection by increasing the gametocyte load transferred to the vector. Additionally, elevated sexual reproduction in *Plasmodium* may lead to faster genetic adaptation, accelerating the rise of resistant parasites [13]. Fortunately, other treatment posibilities are currently being explored, such as the PfSPZ vaccine [14] which was recently found to be safe for human use. The effectiveness of this vaccine is yet to be determined. If this vaccine were to be deployed into widespread use, the pro-gametocytogenesis effects of CQ will possibly be cancelled out, due to the fact that the vaccine targets the sporozoite phase.

What is also cause for concern is the proposed use of CQ to treat fungal infections [15]. While applying high doses of CQ may eliminate a large proportion of the infective fungal cells, it is reasonable to assume that the body will metabolize the CQ to sub-lethal levels [16]. Exposure of pathogenic fungi to sub-lethal concentrations of CQ may result in the formation of spores which are resistant to treatment and may re-activate the infection [17].

Chloroquine and similar compounds represent a novel class of drugs in the sense that no other known fertility drug has, as far as the author is aware, such a highly conserved status among eukaryotes. Future research in this area requires the search for other pro-fertility drugs with the same conserved status across the Eukarya. The mechanism of action regarding fertility induction by CQ should also be determined.

Figure 3.2 Phylogenetic tree of the Eukarya based on whole ribosomal RNA (rRNA) sequences showing the relationship between the mammals (Animals), yeasts (Fungi) and *Plasmodium* (Apicomplexans). Adapted from Reece *et al.* [10].

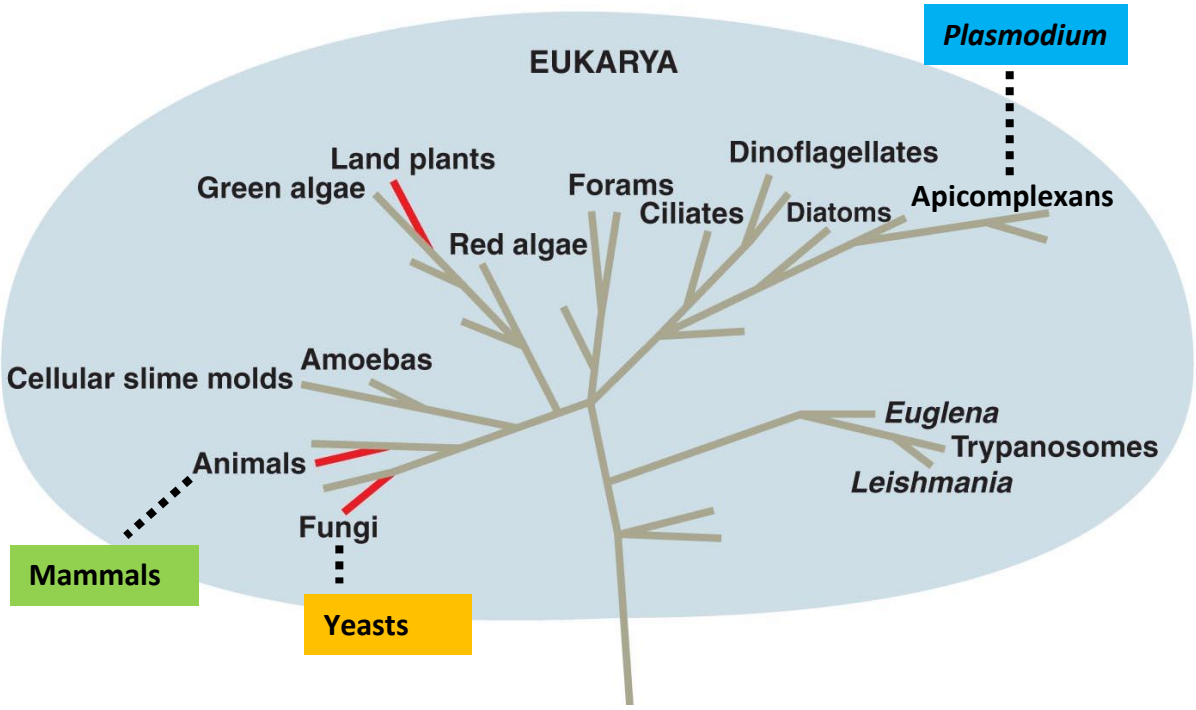

Figure 3.2 (See opposite page)

3.4. Hypothesis

The effects of CQ on cellular metabolism are varied and still largely unknown [12], however the inhibition of glutathione (GSH) by CQ is a well studied effect [18,19]. Glutathione is responsible for many housekeeping functions in living cells, the most important of which is the removal of reactive oxygen species (ROS) from the cellular environment [20]. The increase in ROS leads to oxidative stress when the ROS is not removed from the cell efficiently [21]. Considering that sexual reproduction is often a response to stress in the cellular environment [22], this increase in oxidative stress may be the trigger to CQ-induced sexual reproduction. The synthesis of GSH requires the input of ATP and GSH homeostasis (maintaining the balance between

reduced and oxidised GSH) requires the input of NADH [23]. By inhibiting GSH synthesis, CQ would indirectly make a large quantity of ATP and NADH available for other cellular functions, such as sexual reproduction, which is by nature an energy intensive process [24,25].

The proposed hypothesis states that CQ inhibits the synthesis of GSH, thereby elevating ROS levels, as well as making ATP and NADH available to the cell. The ROS causes oxidative stress that induces sexual reproduction while the available ATP and NADH increases the cell's energy supply, consequently fuelling sexual reproduction.

It is cautioned, however, that this hypothesis does not fully take into account the possible unknown effects CQ may have on other physiological aspects of sexual reproduction. This highlights the importance of further investigations into the pro-fertility effects of CQ.

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Summary / Opsomming

Summary

Previous unpublished research by Kock and co-workers indicated that the antimalarial drug chloroquine (CQ) stimulates yeast sexual stages (biosensors). Consequently several indicator yeasts (*Eremothecium ashbyi*, *Dipodascopsis uninucleata* var. *uninucelata*, *Lipomyces yamadae* and *Scheffersomyces stipitis*) were exposed to concentration gradients of CQ in the Anti-mitochondrial Antifungal Assay (^{3}A) system and their ascospore release mechanics were subjected to Auger architectomics. Auger architectomics is the study of the structure and atomic composition of cells by making use of Nano Scanning Auger Microscopy (NanoSAM) as well as other techniques (http://en.wikipedia.org/wiki/Auger_architectomics).

Investigation of the ascospore release mechanics revealed that *L. yamadae* and *S. stipitis* were sequestrate (making use of passive ascospore release) while *E. ashbyi* and *D. uninucleata* made use of active ascospore release. The sensors of *L. yamadae* have smooth, spherical ascospores that are released by destruction of the sensor wall. The spherical sensors of *S. stipitis* each contain two brimmed ("hat"-shaped) ascospores that are released when the sensor wall breaks apart. The sensors of *E. ashbyi* are mostly intercalary in long chains with up to sixteen sickle-shaped ascospores in each ellipsoidal sensor. The V-shaped fins at the base of each ascospore of *E. ashbyi* are coated with 3-hydroxy (3-OH) oxylipins, making them hydrophobic. This facilitates the movement of ascospores by water flow. The tapered tips of the ascospores pierce through the sensor wall to allow release (http://vimeo.com/61521401). In *D. Uninucleata*, the inside surface of the sensors are lined with flexible sheaths, surrounding the ascospores inside the central channel. Inflation of the sheaths due to water uptake, generates turgor pressure that

forces the ascospores out of the sensor. This is in sharp contrast to the morphologically similar yeast *Dipodascus geniculatus*, where the inflation of sheaths surrounding each individual ascospore is responsible for ascospore release. In all cases the sensors with ascospores were observed to have increased mitochondrial activity compared to surrounding cells.

It was found with the ^{3}A system that CQ is indeed a potent yeast fertility drug, having pro-fertility effects on all the yeast sensors used, including yeasts with decreased ability to sporulate due to preservation by sub-cultivation. Chloroquine caused increased formation of mature sensors of *L. yamadae* and *S. stipitis*, and increased the rate of ascospore release from the sensors of *E. ashbyi* and *D. uninucleata.* This data becomes even more compelling when considered that the *S. stipitis* strain used had lost the ability to sporulate prior to this study. An investigation of the relevant literature showed that the pro-fertility effects of CQ are highly conserved in the Eukarya, having similar effects on mammals (including humans) as well as the malaria causing parasite *Plasmodium falciparum*. This highlights the need to re-evaluate future and current CQ based treatment regimes.

Key Words: Ascospore release, auger-architectomics, chloroquine, fertility drug, pro-mitochondrial, yeast bio-assay, yeast sensors.

Opsomming

Inligting van ongepubliseerde studies deur Kock en medewerkers het aangedui dat die malaria teenmiddel chloroquine (CQ), die seksuele fases (sensors) van gis stimuleer. Gevolglik is verskeie sensorgiste (*Eremothecium ashbyi*, *Dipodascopsis uninucleata* var. *uninucleata*, *Lipomyces yamadae* en *Scheffersomyces stipitis*) blootgestel aan konsentrasie gradiente van CQ in die Anti-mitokondriale, Antifungale Toets (AAT) sisteem, wat gekoppel is aan Auger argitektomika. Auger argitektomika is die studie van ‘n sel se struktuur en atomiese samestelling deur gebruik te maak van Nano Skandering Auger Mikroskopie (NanoSAM) en ander tegnieke. (http://en.wikipedia.org/wiki/Auger_architectomics).

Ondersoek na die askospoorvrystellingsmeganika het uitgewys dat *L. yamadae* en *S. stipitis* gebruik maak van passiewe askospoorvrystelling, terwyl *E. ashbyi* en *D. uninucleata* gebruik maak van aktiewe askospoorvrystelling. Die sensors van *L. yamadae* besit gladde, sferiese askospore wat vrygestel word na afbreek van die sensorwand. Die sferiese sensors van *S. stipitis* huisves elk twee gerandte (“hoed”-vormige) askospore wat vrygestel word wanneer die sensorwand opbreek. Die sensors van *E. ashbyi* is meestal in kettings gerangskik met soveel as sestien sekelvormige askospore in elke ovaalvormige sensor. Die V-vormige vinne op die basis van elke askospoor is bedek met 3-hidroksie (3-OH) oksielipiene, wat die vinne hidrofobies maak en die beweging van die askospoor in stromende water bewerkstellig (http://vimeo.com/61521401). Die skerp punte van die askospore van *E. ashbyi* steek deur die sensorwand om die spore vry te stel. Die binneste oppervlak van die *D. uninucleata* sensorwand is uitgevoer met ‘n buigbare skede wat die askospore binne in ‘n sentrale kanaal omsluit. Die skedes swel op weens die

osmotiese opname van water, wat tot verhoogde turgordruk in die sensor lei wat askospoorvrystelling bewerkstellig. Dit is in skerp kontras met die morfologiese verwante gis *Dipodascopsis geniculatus*, waar die swelling van skedes rondom elke individuele askospoor verantwoordelik is vir askospoorvrystelling. In alle gevalle het die sensors en askospore verhoogde mitokondriale aktiwiteit getoon in vergelyking met die omliggende selle.

Dit is met die ^{3}A sisteem bevind dat CQ ‘n sterk gisfertiliteitsmiddel is, insluitend vir giste met gedempte vermoë om askospore te vorm weens die effek van subkultivering. Chloroquine het gelei tot verhoogde vorming van volwasse *L. yamadae* en *S. stipitis* sensors en het askospoorvrystelling gestimuleer in *D. uninucleata* en *E. ashbyi*. Die belang van dié inligting word meer duidelik wanneer in ag geneem word dat *S. stipitis* die vermoë verloor het om askospore te vorm voor die aanvang van die studie. Ondersoek van die relevante literatuur het bewys dat die pro-fertiliteitseffek van CQ hoogs gekonserveerd is in die Eukarya, met soortgelyke uitwerkings op soogdiere (insluitend mense) asook die malaria parasiet *Plasmodium falciparum*. Die inligting wat in die studie verkry is beklemtoon die behoefte om mediese behandeling wat op CQ gebaseer is, te herassesseer.

Sleutelwoorde: Askospoor vrystelling, Auger-argitektomika, chloroquine, fertiliteitsmiddel, gis-biologiese-toets, gissensor, pro-mitokondriaal.