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Importance of sterols in the mitochondrial function of *Leishmania tarentolae* promastigotes

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Wien, am 2. November 2024

Luca Erlacher

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SUMMARY

Leishmania are protozoa that cause visceral and (muco)cutaneous leishmaniasis in humans and animals. They contain a single mitochondrion, which is crucial for ATP generation. Essential constituents of cellular membranes, including outer mitochondrial membranes, are sterols. The predominant sterols in *Leishmania* are ergosterol-like triene sterols, making sterol biosynthesis pathway an ideal drug target. Therefore, the following sterol-targeting substances were investigated to determine, whether modifying sterol composition and permeability of leishmanial membranes is associated with impairment of their mitochondrial function. Amphotericin B, an antifungal and antileishmanial drug, binds to ergosterol, thereby increasing permeability of leishmanial membranes. The antifungal drug miconazole inhibits ergosterol biosynthesis by inhibiting sterol 14α-demethylase and possesses antileishmanial activity. Synthetic endoperoxides from ergosterol and dehydrocholesterol were recently shown to inhibit leishmanial viability.

The mitochondrial function of *Leishmania tarentolae* promastigotes (LtP), the flagellated form of this protozoan species, was assessed from their oxygen consumption rates after batch incubations for 0–48 hours with sterol-targeting substances. The coupling efficiency of intact LtP was studied artificially using the ATP synthase inhibitor oligomycin and the uncoupler CCCP. The physiological capacity of oxidative phosphorylation was measured in digitonin-permeabilised LtP consuming succinate as respiratory substrate and converting ADP into ATP.

Significant inhibition of LtP growth, oxygen consumption rates and respiratory control values was observed with 8 µM ergosterol endoperoxide, 20 nM amphotericin B, and 650 nM miconazole, while 2 µM dehydrocholesterol endoperoxide showed marginal effects.

In summary, targeting sterol biosynthesis pathway and/or altering sterol composition of LtP can affect their cell growth and mitochondrial bioenergetic function.

ZUSAMMENFASSUNG

Leishmanien sind Protozoen, die bei Menschen und Tieren viszerale und (muko-)kutane Leishmaniose verursachen. Sie besitzen ein einzelnes Mitochondrion, welches für die ATP-Erzeugung entscheidend ist. Zu den essenziellen Bestandteilen der Zellmembranen, einschließlich der äußeren mitochondrialen Membranen, gehören Sterole. In Leishmanien findet man überwiegend Ergosterol-ähnliche Trien-Sterole, was den Sterol-Biosyntheseweg zu einem idealen Ziel für Arzneistoffe macht. Daher wurden die folgenden auf den Sterol-Haushalt abzielenden Substanzen untersucht, um festzustellen, ob eine Modifikation der Sterol-Zusammensetzung und Permeabilität der Leishmanien-Membranen mit einer Beeinträchtigung ihrer Mitochondrien-Funktion verbunden ist. Amphotericin B, ein antimykotischer und antileishmanialer Arzneistoff, bindet an Ergosterol und erhöht dadurch die Permeabilität der Leishmanien-Membranen. Das Antimykotikum Miconazol hemmt die Ergosterol-Biosynthese durch Inhibition der Sterol-14α-Demethylase und besitzt ebenfalls antileishmaniale Aktivität. Kürzlich wurde gezeigt, dass synthetische Endoperoxide von Ergosterol und Dehydrocholesterol die leishmaniale Lebensfähigkeit hemmen.

Die Mitochondrien-Funktion von *Leishmania tarentolae*-Promastigoten (LtP), der begeißelten Form dieser Protozoen-Spezies, wurde anhand ihrer Sauerstoffverbrauchsraten nach Batch-Inkubationen für 0–48 Stunden mit auf den Sterol-Haushalt abzielenden Substanzen beurteilt. Die Kopplungseffizienz intakter LtP wurde artifiziell mithilfe des ATP-Synthase-Inhibitors Oligomycin und des Entkopplers CCCP untersucht. Die physiologische Kapazität der oxidativen Phosphorylierung wurde in Digitonin-permeabilisierten LtP gemessen, die Succinat als Atmungssubstrat verbrauchten und ADP in ATP umwandelten.

Eine signifikante Hemmung des LtP-Wachstums, der Sauerstoffverbrauchsraten und Atmungskontrollkoeffizienten wurde nach Behandlung mit 8 µM Ergosterol-Endoperoxid, 20 nM Amphotericin B und 650 nM Miconazol gefunden, während 2 µM Dehydrocholesterol-Endoperoxid nur marginale Effekte zeigte.

Zusammenfassend lässt sich sagen, dass die gezielte Beeinflussung des Sterol-Biosynthesewegs und/oder der Sterol-Zusammensetzung in LtP ihr Zellwachstum und ihre mitochondriale bioenergetische Funktion beeinträchtigen kann.

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1. INTRODUCTION

In southern regions with warm climates, such as Africa, the Eastern Mediterranean, South America, and parts of Europe, a disease called leishmaniasis poses a significant health concern for both humans and animals. Each year, an estimated 700,000 to one million people are affected (1).

This condition is caused by a protozoan parasite of the genus *Leishmania* (order: Trypanosomatida). Leishmaniasis is a vector-borne disease transmitted through the bite of the sand fly (genus *Phlebotomus*), which is native to these warm climates. Transmission occurs when a sand fly carrying the parasite bites a host, leading to the initial multiplication of the protozoa in the skin - a critical phase which determines whether the host's immune system can successfully combat the protozoa or if the disease will progress to more severe stages (2,3).

In the life cycle of *Leishmania*, two distinct morphological forms can be distinguished: the flagellated promastigote and the non-flagellated amastigote forms. Promastigotes represent the extracellular form found in sand flies, while amastigotes are the intracellular, infectious form that resides within macrophages of mammalian hosts (Fig. 1).

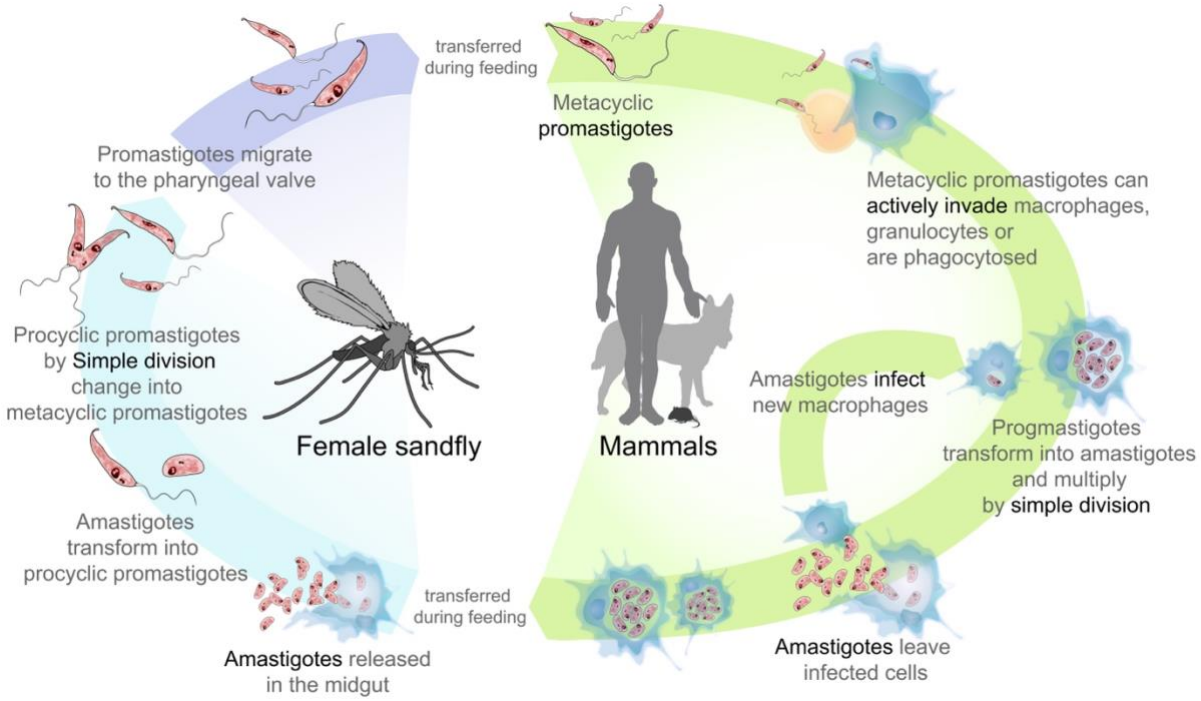

Figure 1: Life cycle of *Leishmania* (4)

The disease can be clinically categorised into three different forms: a cutaneous form, a mucocutaneous form and a visceral form. Depending on the host's immune system and the species of *Leishmania,* the different clinical forms develop (5).

Current treatments, along with substances used in the present study, such as amphotericin B and miconazole, are based on medications originally developed for other diseases that have been repurposed for leishmaniasis therapy. Also, their side effects, increasing resistance to the therapy and costs for the long-term treatment are major concomitant issues. Even though this disease affects millions of people and pets per year, a specific complication-free therapy has yet to be found (5).

As in all eukaryotic cells, the main energy-producing organelle in *Leishmania* is the mitochondrion. As shown in Fig. 2, both forms of *Leishmania* possess only one large mitochondrion (6,7). Its functionality is essential for the survival of the cell, making it an effective target for drugs aimed at combating the parasite (6).

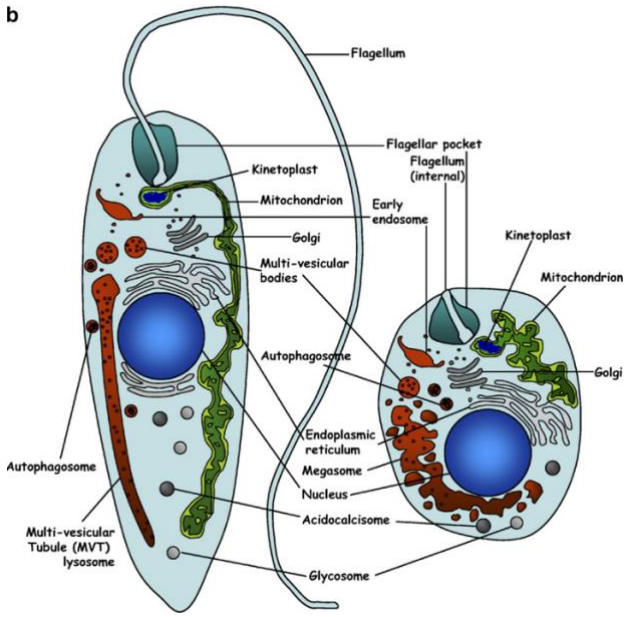

Figure 2: Schematic representation of the structure of *Leishmania* promastigotes (left) and amastigotes (right) (7)

Sterols are essential constituents of cellular membranes, including outer mitochondrial membranes, ensuring their normal structure and function.

Contrary to most eukaryotic cells, which contain cholesterol (a monoene sterol), in cell membranes of fungi and protozoa, ergosterol (a triene sterol) and ergosterol-like sterols are found. The main sterol in membranes of *Leishmania* promastigotes and amastigotes is 5-dehydroepisterol and to smaller amounts ergosterol and episterol, making sterol biosynthesis pathway an ideal drug target (8,9).

In this diploma thesis, various sterol-targeting substances were investigated to determine whether their impact on the sterol synthesis also affects the parasite's ability to utilise oxygen via mitochondrial oxidative phosphorylation or if this process is impaired by the membrane changes caused by the different substances.

The test substances used for this purpose were miconazole (MiAz), amphotericin B (AmpB), ergosterol (Ergo), ergosterol endoperoxide (ErgoEP), dehydrocholesterol (DHChol) and dehydrocholesterol endoperoxide (DHCholEP).

Miconazole is an imidazole antimycotic drug that inhibits the multi-step conversion of lanosterol to episterol, 5-dehydroepisterol and further ergosterol by binding to the haem iron in cytochrome P450. Cytochrome P450 is the coenzyme of sterol 14α-demethylase (CYP51), the enzyme responsible for the demethylation of lanosterol, and therefore, in the presence of MiAz, the parasitic sterol biosynthesis is severely affected (9–12).

Amphotericin B belongs to the group of polyene-antimycotics, a class of macrolides which also acts against *Leishmania*. Amphotericin B binds to ergosterol in the fungal membrane and creates channels, disturbing membrane permeability. As a result, intracellular particles and electrolytes leak out of the cytosol, leading to the death of the fungus. This mechanism of action is dose-dependent – at low doses, AmpB acts as a growth inhibitor, while at higher dosages, it is fungicidal (9,12–14). Interestingly, a study by Anderson et al. (2014) found that AmpB primarily acts as an extramembranous 'sterol sponge', forming aggregates that sequester ergosterol, thereby disrupting essential functions of the fungal cell membrane and compromising cell viability (15).

Ergosterol has been shown to exhibit cytotoxic effects on *Trypanosoma cruzi* by disrupting their plasma membrane permeability and, consequently, their mitochondrial membrane potential (8,16).

The mechanism of action for ergosterol peroxide has yet to be fully elucidated. However, a study by Ramos-Ligonio et al. (2012) demonstrated a strong lytic activity of ErgoEP in extracellular epimastigotes, as well as intracellular amastigotes of *Trypanosoma cruzi*, while

no cytotoxicity was observed even at 200 times higher ErgoEP concentrations in mammalian cells, such as human erythrocytes, HeLa cells and murine macrophages (17). A study by Meza-Menchaca et al. (2019) suggests that ErgoEP might exert its cytotoxic effects on *Trypanosoma* via the release of oxygen radicals after binding to CYP51 (sterol 14α-demethylase). These radicals then damage lipid membranes, proteins and nucleic acids (18). Based on the known cytotoxic effects of Ergo and ErgoEP on *Trypanosoma* and their capability of utilising cholesterol as their sterol in cases of blocked Ergo synthesis (19), it can be assumed that similar compounds, such as DHChol, a precursor of cholesterol biosynthesis, and its endoperoxide, DHCholEP, might exhibit comparable effects on *Leishmania*. Recently, it was shown that ergosterol, dehydrocholesterol and their synthetic endoperoxides (ErgoEP and DHCholEP), as well as AmpB and MiAz, inhibit the viability of *Leishmania tarentolae* promastigotes (LtP) (20).

It is hypothesised that intervening in the sterol biosynthesis pathway and/or altering the sterol content, thereby modifying membrane permeability in *Leishmania*, would impair their mitochondrial bioenergetic function. Therefore, the aim of this diploma thesis was to investigate the impact of these substances on the functionality of LtP mitochondria.

2. MATERIALS AND METHODS

2.1. Chemicals

The following chemicals were used to perform the experiments:

Name	Purity	Manufacturer
Adenosine 5'-diphosphate (ADP), dihydrate, potassium salt	–	Boehringer Mannheim
Amphotericin B	–	Cayman-Chemical Company
Bovine serum albumin, fraction V	> 96 %	Fluka
Brain Heart Infusion Broth (No.53286)	for microbiology	Sigma-Aldrich
Carbonyl cyanide m-chlorophenyl hydrazone (CCCP)	–	Sigma Chemical Co.
$CuSO_4$, pentahydrate	per analysis	Merck
Dehydrocholesterol	≥ 95 %	Sigma-Aldrich
Dehydrocholesterol endoperoxide		Provided by Prof. Lars Gille
Digitonin	–	Sigma
Dimethyl sulfoxide (DMSO)	> 99.8 %	VWR (prolabo chemicals)
Ergosterol	98 %	Thermo Scientific
Ergosterol endoperoxide		Provided by Prof. Lars Gille
Ethanol absolute	per analysis	Scharlau
Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA)	per analysis	Merck

D-(+)-Glucose, monohydrate	for biochemical purposes	Merck
Haemin (porcine)	≥ 98 %	Sigma
KCl	per analysis	Merck
KCN	≥ 98 %	Sigma
K_2HPO_4	per analysis	Merck
KOH	per analysis	Merck
D-Mannitol	≥ 98 %	Sigma-Aldrich
$MgCl_2$, hexahydrate	per analysis	Merck
Miconazole	97 %	Thermo Scientific
NaOH	per analysis	Merck
Oligomycin	–	Calbiochem
Penicillin (20,000 U/ml)/ streptomycin (20,000 µg/ml)	–	Lonza
Potassium iodide	per analysis	Merck
Potassium sodium tartrate, tetrahydrate	per analysis	Merck
Sodium dithionite	per analysis	Merck
Succinic acid	> 99.5%	Fluka
Trichloroacetic acid	per analysis	Merck
Triethanolamine hydrochloride	per analysis	Fluka

For the preparation of aqueous solutions, MQ-H_2O from a Milli-Q® Advantage A10 water purification system (Merck Millipore, Darmstadt, Germany) was used. Stock solutions of haemin (2.5 mg/ml), oligomycin (10 mM), CCCP (167 µM) and digitonin (5 mg/ml) were made in DMSO. Stock solutions of amphotericin B (10 µM), miconazole (325 µM), ergosterol (16 mM), ergosterol endoperoxide (4 mM), dehydrocholesterol (6.5 mM), dehydrocholesterol endoperoxide (1 mM) were made in ethanol (EtOH).

2.2. Cultivation of *Leishmania tarentolae* promastigotes

The entire study was conducted outside of a containment laboratory. This was possible because *Leishmania tarentolae* promastigotes (LtP) (LEXSY host strain P10, biosafety level 1, Jena Bioscience GmbH, Jena, Germany) were used, a species of *Leishmania* pathogenic only to geckos. Consequently, it posed no health risk to humans or pets, allowing for easier handling and reduced safety precautions.

The LtP culture was maintained in a growth medium consisting of 37 g/l Brain Heart Infusion Broth (BHI). To avoid contamination of the medium with bacteria, 25,000 units/l of penicillin and 25 mg/l of streptomycin were added. Additionally, 5 mg/l of haemin was included in the medium, as it is an essential iron source for *Leishmania tarentolae* (21). The LtP culture was kept at 26 ºC in an incubator (VWR INCU-Line 150R Premium, VWR International, Vienna, Austria) to maintain optimal temperature and humidity.

The culture was passaged on Mondays, Wednesdays, and Fridays to prevent nutrient depletion and overgrowth. For this purpose, sterile 50 ml polypropylene filter tubes (TubeSpin® Bioreactor 50, TPP, Trasadingen, Switzerland) were used. To prevent the cell suspension from settling, cell culture tubes were placed in a shaker (0.05 s^{-1}) inside the incubator.

2.3. Cell number determination of *Leishmania tarentolae* promastigotes

To ensure repeatable and comparable results, determining the cell number and subsequently diluting the samples to a specific cell concentration was crucial.

For this purpose, the optical density (OD) was measured with a U-1100 spectrophotometer (Hitachi, Tokyo, Japan) at a wavelength of 600 nm. The photometric measurements were conducted using 1.5 ml semi-micro-cuvettes and BHI medium or mannitol medium (see 2.7) as blank.

All samples were photometrically measured at least four times, and the resulting mean values were used to calculate the cell numbers according to the following formula based on a dissertation by Fritsche (22):

Cell number (10^6 LtP/ml) = OD_{600nm} × dilution factor × 0.969 × 124

0.969	conversion factor of g/l dry weight
124	1 g/l dry weight corresponds to 124 × 10^6 LtP/ml
dilution factor	µl LtP suspension / µl total volume

To avoid OD values being too high and, therefore, a possible deviation from Lambert Beer's law, all samples were diluted 1:8 in BHI medium and 1:21 in mannitol medium.

2.4. Incubation of *Leishmania tarentolae* promastigotes with test substances

The batch incubations with test substances (0 h, 6 h, 24 h, 48 h) and subsequent measurements were always conducted on three consecutive days, using LtP one day after cell passage.

Following the determination of cell number in untreated LtP culture, the cell suspension was diluted to achieve 4 × 10^7 LtP/ml BHI medium. The concentrations of test substances were selected on the basis of the bachelor thesis of Elisabeth Schrödl, who investigated the half-maximal inhibitory concentrations (IC_{50}) of various substances in viability assays with LtP (20). The following final concentrations were therefore used in the present experiments:

- 8 µM ergosterol endoperoxide
- 32 µM ergosterol
- 2 µM dehydrocholesterol endoperoxide
- 13 µM dehydrocholesterol
- 20 nM amphotericin B
- 650 nM miconazole

At time zero, 60 µl of the substance to be tested were added to 30 ml LtP suspension in 50 ml Falcon tubes, respectively, to obtain the desired final concentrations, as mentioned before. Two substances were tested per batch together with a control sample containing 0.2 % ethanol, the vehicle of test substances. Aliquots were taken from these samples for subsequent determination of cell numbers and oxygen consumption measurements after 0 h, 6 h, 24 h and 48 h.

2.5. Oxygen consumption measurements

In order to measure the oxygen consumption of LtP, an oxygen electrode (Hansatech Instruments Ltd, Norfolk, United Kingdom) was utilised. It consisted of three parts: the oxygen electrode disc (S1), the electrode chamber (DW1/AD) and the oxygen electrode control box (CB1D). The oxygen sensor consists of a platinum cathode and silver anode with a 50 % saturated KCl solution as an electrolyte. To protect the platinum electrode from direct contact with the liquid to be measured and to avoid a dilution of the electrolyte, a polytetrafluoroethylene membrane, which is permeable to oxygen, covered it (23).
To ensure a constant delivery of oxygen to the platinum electrode, a magnetic stirrer and a magnetic follower placed in the electrode chamber were used.
The electrode was polarised by the oxygen electrode control box at 700 mV to ensure that the desired redox reaction could take place. As soon as oxygen came in contact with the platinum electrode, the following chemical reactions occurred:

$$\mathrm{Ag} \rightarrow \mathrm{Ag^+} + \mathrm{e^-}$$
$$\mathrm{Ag^+} + \mathrm{Cl^-} \rightarrow \mathrm{AgCl}$$
$$\mathrm{O_2} + \mathrm{H_2O} + 2\mathrm{e^-} \rightarrow \mathrm{H_2O_2} + \mathrm{OH^-}$$
$$\mathrm{H_2O_2} + 2\mathrm{e^-} \rightarrow 2\mathrm{OH^-}$$

While the silver anode reacts with chloride anions in the electrolyte, the electrons released thereby are used by the platinum cathode to reduce oxygen. Since AgCl deposits on the silver electrode could deteriorate the electrode performance, frequent cleaning was necessary (23).
The voltage measured by the oxygen electrode control box was digitised using an analog-digital converter. The resulting digital signal was then recorded and displayed by the MCREC software (both devices were kindly provided by Prof. Lars Gille).

In order to keep the temperature constant during oxygen consumption measurements, a thermostat (mgw Lauda, Lauda-Königshofen, Germany) was used, keeping the electrode chamber at around 25 ºC.

2.6. Calibration of the oxygen electrode

To ensure evaluable oxygen consumption measurements, the electrode had to be calibrated beforehand. As shown in Fig. 3, 580 µl of air-saturated (at 25 °C) Milli-Q water (MQ-H_2O) were added to the electrode chamber to calibrate the electrode to 256 µM O_2. After a few minutes, a spatula tip of dithionite ($Na_2S_2O_4$) was added to chemically deplete the O_2 in solution.
This calibration was repeated twice before conducting the O_2 consumption measurements in LtP suspensions.

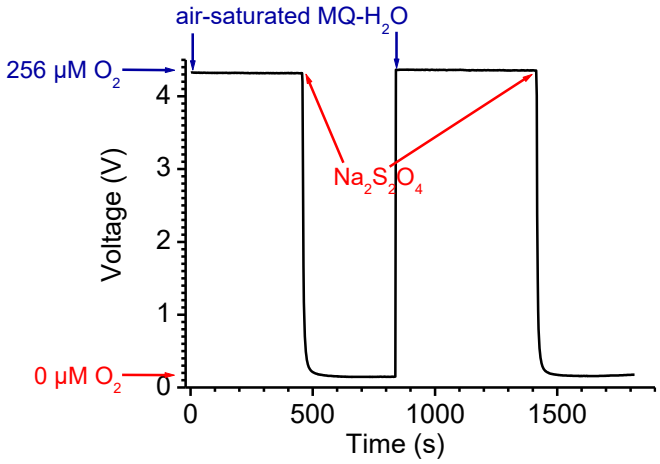

Figure 3: Representative calibration curve of the oxygen electrode

2.7. Preparation of pre-incubated *Leishmania tarentolae* promastigotes for O_2 consumption measurements

Oxygen consumption of LtP suspensions was measured in mannitol medium (0.3 M mannitol, 20 mM triethanolamine hydrochloride, 1 mM EGTA, 5 mM K_2HPO_4, pH 7.4), allowing for assessment of mitochondrial functionality while preventing further cell proliferation. In a previous study, it was shown that this medium was well-suited for studying the mitochondrial function of LtP (24).

The aliquots taken from LtP suspensions pre-incubated with test substances were washed twice in mannitol medium, i.e., LtP suspensions were centrifuged for 10 minutes at 1900 × g and 25 ºC (Sorvall LYNX 6000 centrifuge, Thermo Fisher GmbH, Vienna, Austria). After the first centrifugation, the pellets were resuspended in 5 ml of mannitol medium. After the second centrifugation, the pellets were resuspended in 1–5 ml of mannitol medium, depending on the cell number in BHI medium measured beforehand.

Following the determination of cell numbers in samples washed in mannitol medium, LtP were diluted in 1.5 ml Eppendorf tubes to 2 × 10^8 LtP/ml mannitol medium to get comparable starting conditions for oxygen consumption measurements.

2.8. Determination of protein concentration

To increase the reliability of the measurements, the protein content in each sample was also assessed. After completing the oxygen consumption measurement, 400 µl were taken from the oxygen electrode chamber and then frozen in Eppendorf tubes at -20 ºC. Once a week, the protein content of the thawed samples was determined using the biuret method.

This method uses copper ions to generate blue to violet complexes with proteins. Depending on the amount of protein in the sample, the colour varies. Therefore, photometric measurement can be used to determine its concentration.

The 400 µl of sample were replenished with 600 µl of MQ-H_2O and then precipitated with 200 µl of 3 M trichloroacetic acid. After 10 min, the solution was centrifuged at 2500 x g and 25 ºC for 10 min with a VWR-centrifuge (VWR Micro Star 17R centrifuge, VWR International, Radnor, PA, USA) to obtain the protein pellet.

After carefully decanting the supernatant, the remaining fluid in the Eppendorf tubes was removed with little paper towels. The remaining solid protein in the tubes was dissolved in one millilitre of biuret solution (12.02 mM $CuSO_4$, 31.89 mM potassium sodium tartrate, 30.12 mM potassium iodide and 0.2 M NaOH) by vortex-mixing and allowed to incubate for another 10 min to ensure complete colour reaction. The fluid was then decanted in cuvettes for the following photometric measurement at 546 nm (25).

To eliminate turbidity errors, a spatula tip of KCN was added to the biuret samples after measuring their OD in order to decolourise the copper complexes with proteins (25). Pure biuret solution, with and without the addition of KCN, was used as the blank. The protein concentration of the samples was calculated using the following formulas (25).

$$\Delta E = (E_{sample-KCN} - E_{sample+KCN}) - (E_{blank-KCN} - E_{blank+KCN})$$

$$c = \frac{\Delta E}{\varepsilon \times d} \times V_f$$

c protein concentration in the sample [mg/ml]

ε 0.20666 $mg^{-1} \times ml \times cm^{-1}$ (extinction coefficient determined from a calibration curve using bovine serum albumin as a standard)

d layer thickness of the cuvette (1 cm)

V_f dilution factor (µl total volume/µl sample volume)

2.9. Data analysis and statistics

The attained oxygen consumption results were analysed with the MCREC software. Further calculations, including statistics, as well as graphical illustration of the results, were carried out in MicroCal Origin® 6.1 (OriginLab Corp., Northampton, MA, USA).

All data represent means ± standard error of the mean (SEM) of four independent experiments. To evaluate statistically significant differences between substance-treated LtP suspensions and time-matched controls (EtOH), an unpaired two-tailed Student's t-test was done. *, ** and *** indicate significant at the level of $p < 0.05$, 0.01 and 0.001, respectively.

3. RESULTS

3.1. Influence of test substances on the cell growth of *Leishmania tarentolae* promastigotes

As an overall parameter of cytotoxicity, the number of LtP in the cell culture medium (BHI medium) was determined. Depending on the substance used and the incubation time, cell numbers of LtP deviated from the time-matched control group (EtOH) to varying degrees of significance (Fig. 4).

At 0 h incubation, no relevant deviation in cell numbers was observed with any of the test substances used, as each sample was adjusted to 4×10^7 LtP/ml BHI medium. However, after 6 h incubation, AmpB and ErgoEP caused a significant reduction in cell growth, with ErgoEP having a devastating effect on the growth of LtP. *Leishmania* treated with 8 µM ErgoEP exhibited no further cell growth up to 48 h of incubation, reducing the number of available cells for the 48 h measurement. Instead of 2×10^8 LtP/ml $1.12 \pm 0.07 \times 10^8$ LtP/ml were used. The corresponding sterol (32 µM Ergo) did not inhibit cell growth of LtP over the whole incubation period (Fig. 4A).

In contrast, while LtP incubated with AmpB for 24 h still showed a significant reduction in cell numbers compared to ethanol-treated LtP (35.7 %), cell numbers recovered after 48 h incubation, i.e., showed no significant difference to ethanol-treated LtP (Fig. 4C).

Unlike the weak effect of MiAz up to 6 h incubation, a significant inhibition of cell growth of LtP was observed after 24 h incubation (59.1%) as well as after 48 h incubation (58.9 %), indicating a delayed cytotoxic effect (Fig. 4C).

Leishmania treated with DHCholEP showed moderate reductions in cell numbers after 24 h incubation (29.0 %; Fig. 4B). However, the large scattering of the results led to high variance, and therefore, no significant difference to the ethanol-treated LtP was observed. The corresponding sterol (DHChol) did not inhibit cell growth of LtP over the whole incubation period.

As can be seen in Fig. 4, in all three control groups (EtOH), a transition from the exponential growth phase to the stationary phase takes place between 24 h and 48 h of incubation, resulting in a lower rate of cell growth.

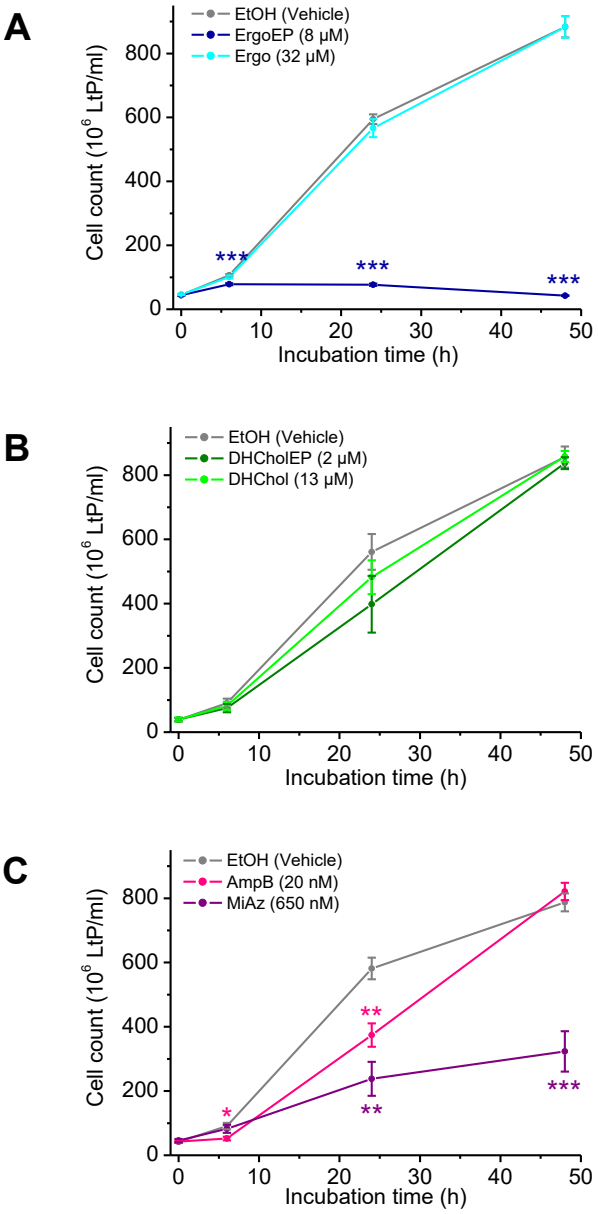

Figure 3: Cell growth of *Leishmania tarentolae* promastigotes (LtP) cultured for up to 48 h in BHI medium in the presence of **(A)** ergosterol endoperoxide (ErgoEP) or ergosterol (Ergo), **(B)** dehydrocholesterol endoperoxide (DHCholEP) or dehydrocholesterol (DHChol), **(C)** amphotericin B (AmpB) or miconazole (MiAz). Ethanol (EtOH, 0.2 % final concentration) was used as vehicle of the test compounds. Data represent means ± SEM from four independent experiments. *, ** and *** indicate significant differences to time-matched controls (EtOH) at the level of $p < 0.05$, 0.01 and 0.001, respectively.

3.2. Influence of test substances on the protein content of *Leishmania tarentolae* promastigotes

In addition to cell growth, the protein content in the samples was also examined, which should be directly proportional to the number of cells (Fig. 5). If there are any errors in OD-related cell number measurements, either due to the motility of LtP, or changes in the optical properties of treated LtP, the protein content should also decrease or increase accordingly.

Although all samples for oxygen consumption measurements, except ErgoEP samples (48 h), could be adjusted to 2×10^8 LtP/ml mannitol medium to get comparable starting conditions, a significant reduction in the protein content of DHCholEP-treated LtP in comparison to time-matched controls (EtOH) was observed after 6 h incubation (Fig. 5B). The protein content of ErgoEP-treated LtP normalised to 10^8 LtP was significantly lower in comparison to time-matched EtOH controls after 48 h incubation (Fig. 5A).

The protein content of Ergo-, DHChol-, AmpB- and MiAz-treated LtP exhibited no significant variations from time-matched controls (EtOH). However, the protein content per 10^8 LtP clearly increased during the 48 h incubation with EtOH (controls).

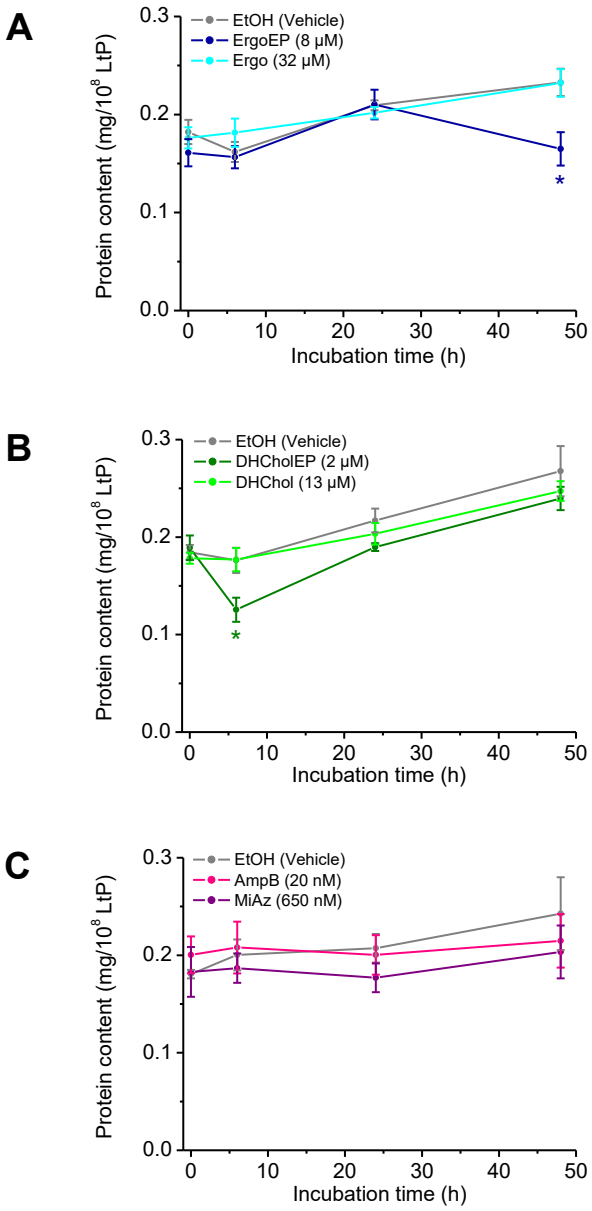

Figure 4: Protein content of *Leishmania tarentolae* promastigotes (LtP) cultured for up to 48 h in BHI medium in the presence of **(A)** ergosterol endoperoxide (ErgoEP) or ergosterol (Ergo), **(B)** dehydrocholesterol endoperoxide (DHCholEP) or dehydrocholesterol (DHChol), **(C)** amphotericin B (AmpB) or miconazole (MiAz). Ethanol (EtOH, 0.2 % final concentration) was used as vehicle of the test compounds. Protein content was determined in probes that were obtained at the end of O_2 consumption measurements in mannitol medium and normalised to 10^8 LtP. Data represent means ± SEM of four independent experiments. * indicate significant differences to time-matched controls (EtOH) at the level of $p < 0.05$.

3.3. Assessing mitochondrial function

To assess mitochondrial functionality and efficiency of oxidative phosphorylation, O_2 consumption rates of LtP were measured in an O_2 electrode chamber.

For this assessment two different approaches were employed. The first method involved permeabilising the cells to ensure that the substrates could reach the mitochondria, while the second approach utilised hydrophobic substances that targeted their sites without the need for permeabilisation.

In both experimental setups, it was necessary to close the oxygen electrode chamber to minimise the entry of room oxygen, which could alter the experimental outcomes.

For the first approach (Fig. 6), it was crucial to deliver ADP and succinate (substrate of mitochondrial complex II) to the cytoplasm and subsequently to the mitochondria. To achieve this, the detergent digitonin was used to permeabilise the plasma membrane. To guarantee a functional ATP-synthase, $MgCl_2$, a cofactor for the aforementioned enzyme, was added to the samples as a first step (26). After the addition of succinate, a major energy-delivering substrate for LtP (27), the O_2 consumption increased, reflecting the viability of its mitochondrion.

Following the addition of ADP, the O_2 consumption rose even more, reflecting the capability of LtP for oxidative phosphorylation. As a final step, KCN (an inhibitor of mitochondrial complex IV) was added to inhibit mitochondrial oxygen consumption and to determine if any other oxygen-consuming processes were occurring.

In classic bioenergetic experiments, respiratory control ratio is the most useful general measure of function in isolated mitochondria. High ratio indicates good function, and low ratio usually indicates dysfunction. Permeabilised cells can be considered as a subset of isolated mitochondria (28). Respiratory control ratios of digitonin-permeabilised LtP were calculated as the ratios of O_2 consumption rates in the presence of succinate and ADP to O_2 consumption rates in the presence of succinate.

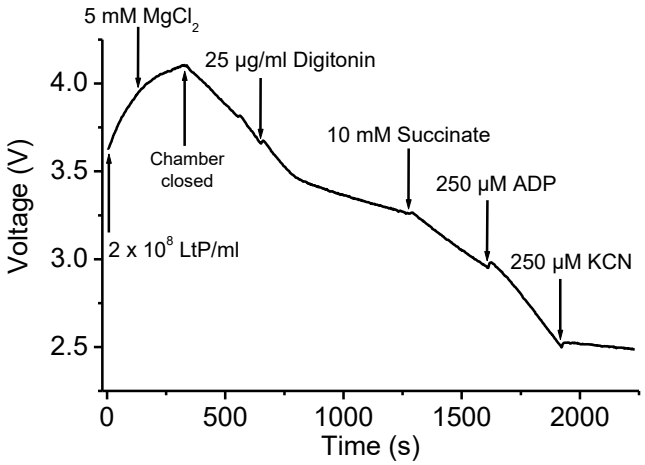

Figure 5: Example of an oxygen consumption curve of digitonin-permeabilised *Leishmania tarentolae* promastigotes (LtP) in mannitol medium. Before the measurement, LtP were incubated for 0 h with 0.2 % ethanol in BHI medium and washed twice in mannitol medium.

The second approach (Fig. 7) assessed the viability and functionality of LtP mitochondria without permeabilising the cells. Glucose was added to ensure the availability of sufficient substrates for oxygen consumption. Oligomycin was then added to inhibit the ATP synthase, resulting in a moderate decrease in oxygen consumption. The subsequent addition of the uncoupler CCCP increased backflow of protons into the mitochondrial matrix. This accelerated electron transfer through the mitochondrial respiratory chain and, consequently, the O_2 consumption. Finally, KCN was again added to confirm that the observed O_2 consumption was not of non-mitochondrial origin.

The measurement of cell respiratory control is the most useful general test of mitochondrial function in cell populations (28). Cell respiratory control ratios of intact non-permeabilised LtP were calculated as the ratios of O_2 consumption rates in the presence of oligomycin and CCCP to O_2 consumption rates in the presence of oligomycin.

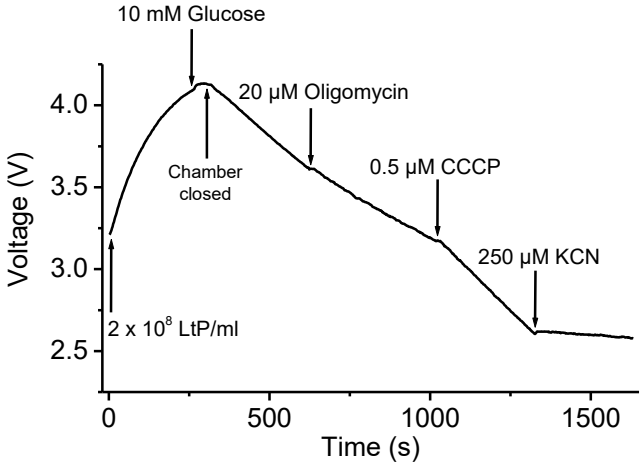

Figure 6: Example of an oxygen consumption curve of non-permeabilised *Leishmania tarentolae* promastigotes (LtP) in mannitol medium. Before the measurement, LtP were incubated for 0 h with 0.2 % ethanol in BHI medium and washed twice in mannitol medium.

3.4. Influence of ergosterol endoperoxide and ergosterol on the mitochondrial function of *Leishmania tarentolae* promastigotes

Examining the mitochondrial function in digitonin-permeabilised LtP pre-treated with either ErgoEP or Ergo reveals that significant mitochondrial dysfunctions can be demonstrated depending on the substance used (Fig. 8).

At 0 h incubation, no significant differences in bioenergetic parameters were observed. In contrast, a significant decrease in succinate-stimulated oxygen consumption rates was determined in LtP incubated with ErgoEP for 6 h and 24 h, both in the absence (Fig. 8A) and presence of ADP (Fig. 8B). Consequently, the diminished respiratory control ratios (Fig. 8C) indicated a highly significant mitochondrial dysfunction and that LtP incubated with ErgoEP can no longer utilise oxygen for ATP synthesis. In contrast, no significant differences with time-matched controls (EtOH) were observed in LtP pre-treated with Ergo.

A decrease in O_2 consumption rates of permeabilised LtP was demonstrated after 48 h for both Ergo- and EtOH-incubated LtP. Therefore, even though the ErgoEP-incubated samples had low succinate-stimulated O_2 consumption rates, starting from 6 h incubation, no significant differences to the controls (EtOH) could be shown after 48 h incubation, neither in the absence nor in the presence of ADP. Nonetheless, the respiratory control ratio decreased even further in the ErgoEP-incubated samples.

The detected oxygen consumption in permeabilised LtP pre-treated with Ergo and/or EtOH must be primarily of mitochondrial origin, as the addition of KCN, an inhibitor of mitochondrial complex IV, led to a significant inhibition of O_2 usage by the cells (84.3–95.8 %; Fig. 8D). In contrast, in permeabilised LtP pre-treated with ErgoEP, the inhibitory effect of KCN is constantly diminishing with increasing incubation time, suggesting further sources of O_2 consumption.

The mitochondrial function was also assessed artificially in non-permeabilised LtP, using the cell-permeable ATP synthase inhibitor oligomycin and CCCP, an uncoupler of oxidative phosphorylation.

Leishmania incubated for 6 h and 24 h with ErgoEP exhibited a significant decrease in O_2 consumption rates in the presence of oligomycin in comparison to time-matched ethanol controls (Fig. 9A). At all time points, significant lower CCCP-stimulated O_2 consumption rates were observed in LtP pre-treated with ErgoEP in comparison to time-matched ethanol controls (Fig. 9B), resulting in significantly lower cell respiratory control ratios (Fig. 9C), indicative for a pronounced mitochondrial dysfunction. In contrast, LtP incubated with Ergo showed no relevant differences in any of the consumption measurements.

However, after 48 h, the same descending utilisation of oxygen in LtP pre-treated with Ergo and/or EtOH was observed, as already seen in the digitonin-permeabilised cells. This effect also prevented a significant difference in O_2 consumption rates in the presence of oligomycin in LtP pre-treated with ErgoEP in comparison to controls (EtOH).

As already seen in digitonin-permeabilised LtP, the O_2 consumption of LtP pre-treated with Ergo and/or EtOH was highly sensitive to KCN (81.5–96.3 % inhibition; Fig. 9D). In contrast, in LtP pre-treated with ErgoEP, the inhibitory effect of KCN is constantly diminishing with increasing incubation time, suggesting further sources of O_2 consumption. After 48 h incubation, even negative inhibition values were determined.

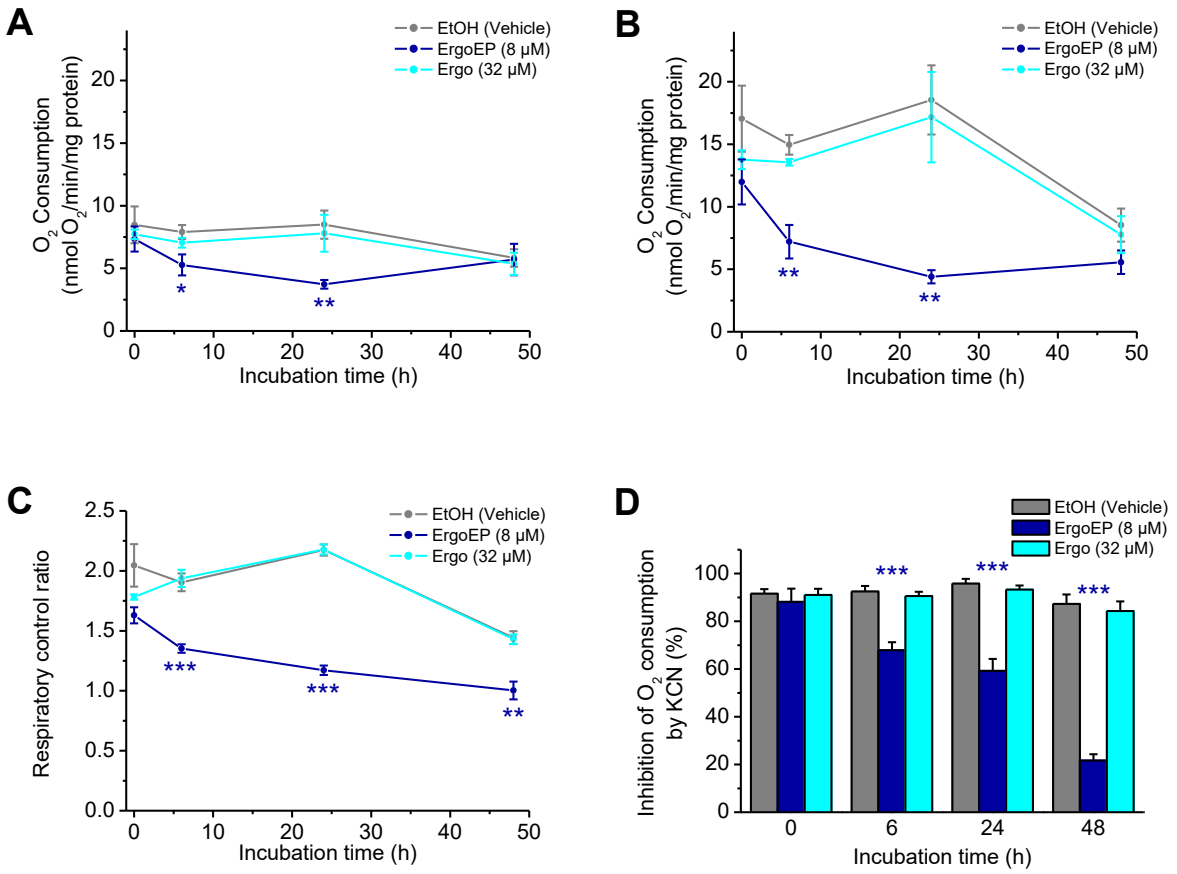

Figure 7: Mitochondrial function of *Leishmania tarentolae* promastigotes (LtP) cultured for up to 48 h in BHI medium in the presence of ergosterol endoperoxide (ErgoEP) or ergosterol (Ergo) using ethanol (EtOH, 0.2 % final concentration) as vehicle. Oxygen consumption rates of permeabilised LtP were measured in mannitol medium supplemented with 5 mM $MgCl_2$ 10 min after the addition of 25 µg/ml digitonin and consecutively 10 mM succinate **(A)** and 250 µM ADP **(B)**. Respiratory control ratios **(C)** were calculated as the ratios of O_2 consumption rates in the presence of succinate and ADP to O_2 consumption rates in the presence of succinate. The percentage of inhibition of O_2 consumption by 0.25 mM KCN **(D)** was calculated by subtracting the residual percental activity after the addition of KCN from O_2 consumption before its addition, which was set to 100 %. Data represent means ± SEM of four independent experiments. *, ** and *** indicate significant differences to time-matched controls (EtOH) at the level of $p < 0.05$, 0.01 and 0.001, respectively.

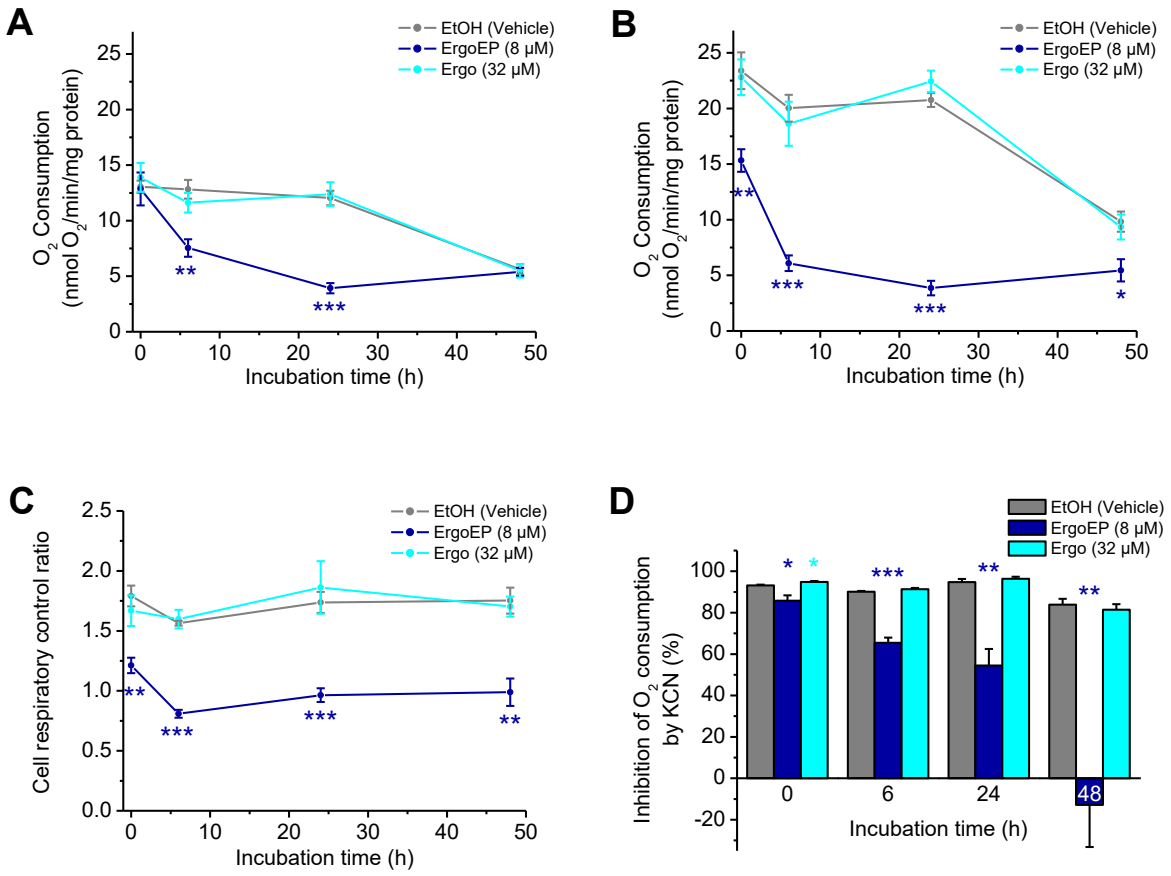

Figure 8: Mitochondrial function of *Leishmania tarentolae* promastigotes (LtP) cultured for up to 48 h in BHI medium in the presence of ergosterol endoperoxide (ErgoEP) or ergosterol (Ergo) using ethanol (EtOH, 0.2 % final concentration) as vehicle. Oxygen consumption rates of non-permeabilised LtP were measured in mannitol medium supplemented with 10 mM glucose after the addition of 20 µM oligomycin **(A)** and consecutively 0.5 µM CCCP **(B)**. Cell respiratory control ratios **(C)** were calculated as the ratios of O_2 consumption rates in the presence of oligomycin and CCCP to O_2 consumption rates in the presence of oligomycin. The percentage of inhibition of O_2 consumption by 0.25 mM KCN **(D)** was calculated by subtracting the residual percental activity after the addition of KCN from O_2 consumption before its addition, which was set to 100 %. Data represent means ± SEM of four independent experiments. *, ** and *** indicate significant differences to time-matched controls (EtOH) at the level of $p < 0.05$, 0.01 and 0.001, respectively.

3.5. Influence of dehydrocholesterol endoperoxide and dehydrocholesterol on the mitochondrial function of *Leishmania tarentolae* promastigotes

Experiments similar to those with ErgoEP and Ergo were also carried out with DHCholEP and DHChol. Over the 48 h incubation period, the digitonin-permeabilised LtP pre-treated with either DHCholEP or DHChol did not exhibit any significant differences in O_2 consumption, compared to the time-matched samples pre-treated with EtOH (Fig. 10). However, a reduction of ADP-dependent O_2 consumption rates (Fig. 10B) and in parallel respiratory control ratios (Fig. 10C) was observed after 48 h in comparison to the 0 h values in LtP pre-treated with DHChol and/or EtOH. The addition of KCN (mitochondrial complex IV inhibitor) led to an inhibition of O_2 consumption in the three experimental groups of LtP (80.9–95.7 %; Fig. 10D), indicative of a primary mitochondrial origin of O_2 consumption.

In the experimental setup with non-permeabilised LtP, significant lower CCCP-stimulated O_2 consumption rates were observed in LtP incubated for 0 h with DHCholEP (Fig. 11B), in parallel with significant lower cell respiratory control ratios (Fig. 11C). In contrast, after 6 h and 24 h incubation no significant alterations in O_2 consumption in comparison to time-matched control LtP (EtOH) were detected. In LtP pre-treated for 48 h with DHChol and/or EtOH, a noticeable decline was observed in O_2 consumption rates in the presence of oligomycin (Fig. 11A) and in CCCP-stimulated O_2 consumption rates (Fig. 11B), compared to 0 h values. At the same time, LtP pre-treated for 48 h with DHCholEP showed significantly higher CCCP-stimulated O_2 consumption rates than the time-matched control LtP (EtOH). The O_2 consumption in the three experimental groups of LtP was again highly sensitive to KCN inhibition (Fig. 11D). There were some minor, although significant, differences between the DHCholEP-treated LtP and EtOH-treated LtP after 24 h and 48 h of incubation.

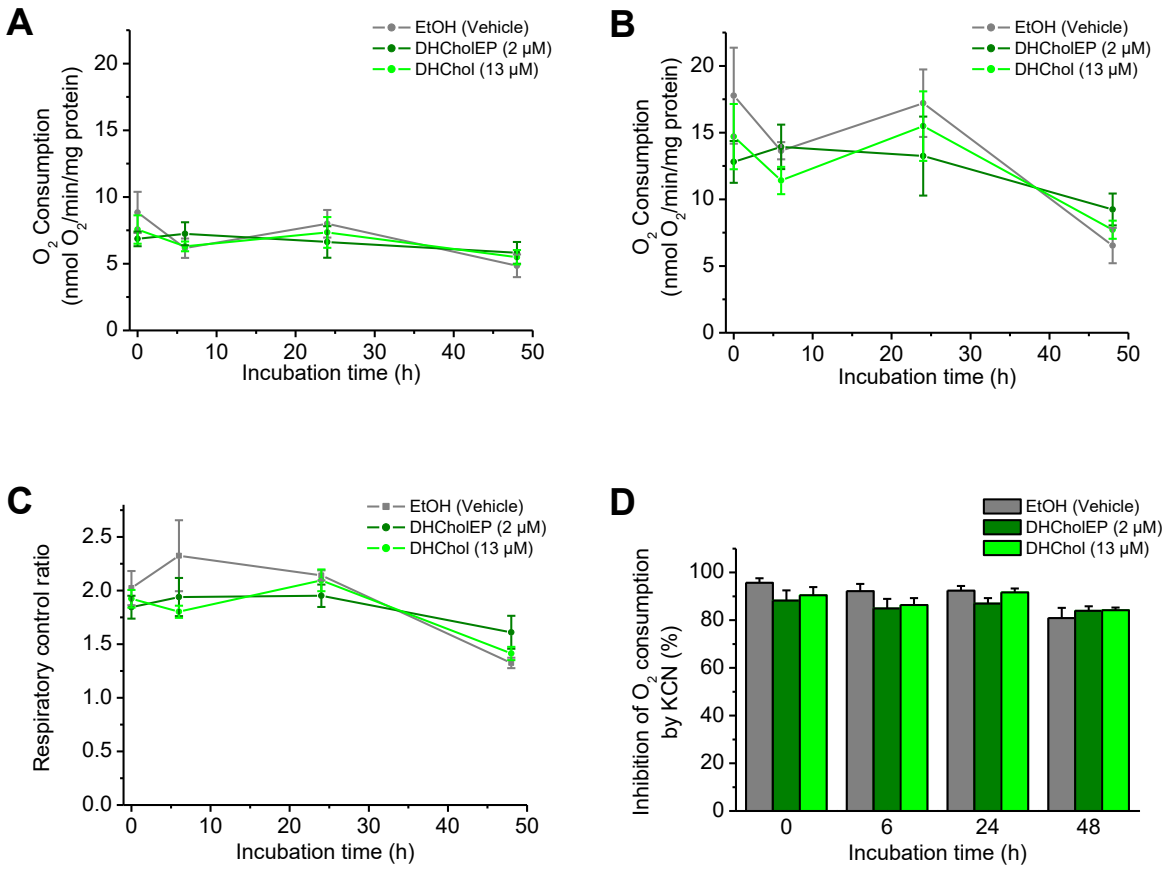

Figure 9: Mitochondrial function of *Leishmania tarentolae* promastigotes (LtP) cultured for up to 48 h in BHI medium in the presence of dehydrocholesterol endoperoxide (DHCholEP) or dehydrocholesterol (DHChol) using ethanol (EtOH, 0.2 % final concentration) as vehicle. Oxygen consumption rates of permeabilised LtP were measured in mannitol medium supplemented with 5 mM $MgCl_2$ 10 min after the addition of 25 µg/ml digitonin and consecutively 10 mM succinate **(A)** and 250 µM ADP **(B)**. Respiratory control ratios **(C)** were calculated as the ratios of O_2 consumption rates in the presence of succinate and ADP to O_2 consumption rates in the presence of succinate. The percentage of inhibition of O_2 consumption by 0.25 mM KCN **(D)** was calculated by subtracting the residual percental activity after the addition of KCN from O_2 consumption before its addition, which was set to 100 %. Data represent means ± SEM of four independent experiments.

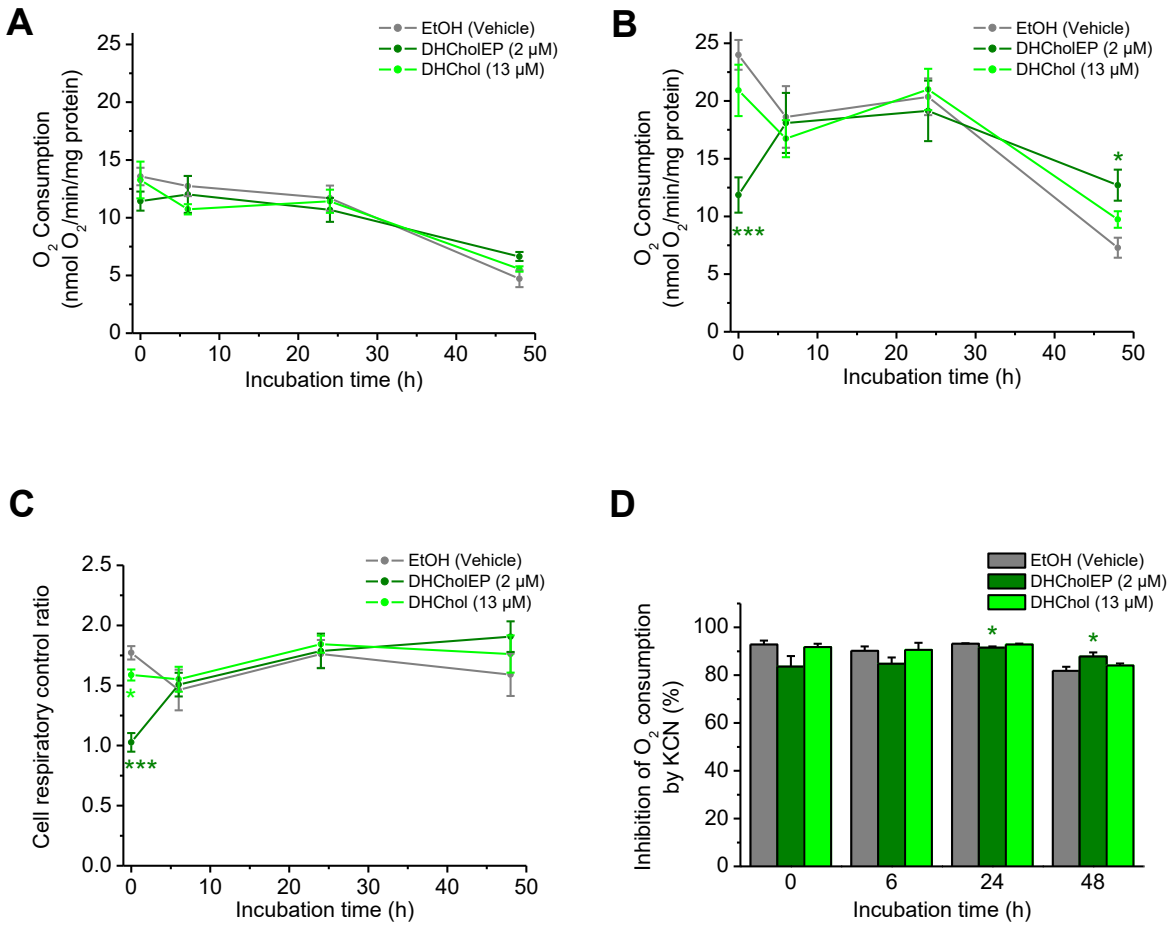

Figure 10: Mitochondrial function of *Leishmania tarentolae* promastigotes (LtP) cultured for up to 48 h in BHI medium in the presence of dehydrocholesterol endoperoxide (DHCholEP) or dehydrocholesterol (DHChol) using ethanol (EtOH, 0.2 % final concentration) as vehicle. Oxygen consumption rates of non-permeabilised LtP were measured in mannitol medium supplemented with 10 mM glucose after the addition of 20 µM oligomycin **(A)** and consecutively 0.5 µM CCCP **(B)**. Cell respiratory control ratios **(C)** were calculated as the ratios of O_2 consumption rates in the presence of oligomycin and CCCP to O_2 consumption rates in the presence of oligomycin. The percentage of inhibition of O_2 consumption by 0.25 mM KCN **(D)** was calculated by subtracting the residual percental activity after the addition of KCN from O_2 consumption before its addition, which was set to 100 %. Data represent means ± SEM of four independent experiments. * and *** indicate significant differences to time-matched controls (EtOH) at the level of $p < 0.05$ and 0.001, respectively.

3.6. Influence of miconazole and amphotericin B on the mitochondrial function of *Leishmania tarentolae* promastigotes

Examining the mitochondrial function in digitonin-permeabilised cells after incubation with AmpB and MiAz, no significant changes in succinate-stimulated O_2 consumption were observed at all time points in comparison to the time-matched ethanol controls (Fig. 12A). After 24 h, a significantly lower ADP-stimulated O_2 consumption was evident in LtP pre-treated with MiAz (Fig. 12B). The ADP-stimulated O_2 consumption rates were also lower, although not significantly ($p = 0.085$), in LtP pre-treated with AmpB in comparison to the time-matched controls (EtOH). At this time point (24 h), AmpB-treated LtP also demonstrated significantly lower respiratory control ratios (Fig. 12C). While respiratory control ratios of LtP pre-treated with MiAz were significantly lower in comparison to time-matched controls (EtOH) for up to 24 h of incubation, they were significantly higher after 48 h of incubation, due to a significant decline of respiratory control ratios in EtOH-incubated LtP. No significant alterations were observed in the inhibition of O_2 consumption by KCN (Fig. 12D).

In non-permeabilised cells, a significant decline in O_2 consumption rates in the presence of oligomycin (Fig. 13A), as well as in CCCP-stimulated O_2 consumption rates (Fig. 13B), was visible after 24 h incubation of LtP with MiAz, while no significant alterations in O_2 consumption rates were noted in LtP pre-treated with AmpB in comparison to time-matched controls (EtOH). Again, a steep decline in O_2 consumption rates over the 48 h incubation was observed. Examining the cell respiratory control ratio, no significant changes in comparison to time-matched controls (EtOH) were measured in LtP pre-treated with MiAz, while LtP pre-treated with AmpB exhibited even higher ratios after 6 h incubation. The latter was due to a decline in cell respiratory control ratio of EtOH controls at this time point (Fig. 13C).

The O_2 consumption was strongly inhibited in the presence of KCN by 82.8–93.7 % (Fig. 13D) with significantly lower values in comparison to EtOH controls in LtP incubated for 6 h and 24 h with MiAz, as well as significantly higher values in LtP, incubated for 6 h with AmpB.

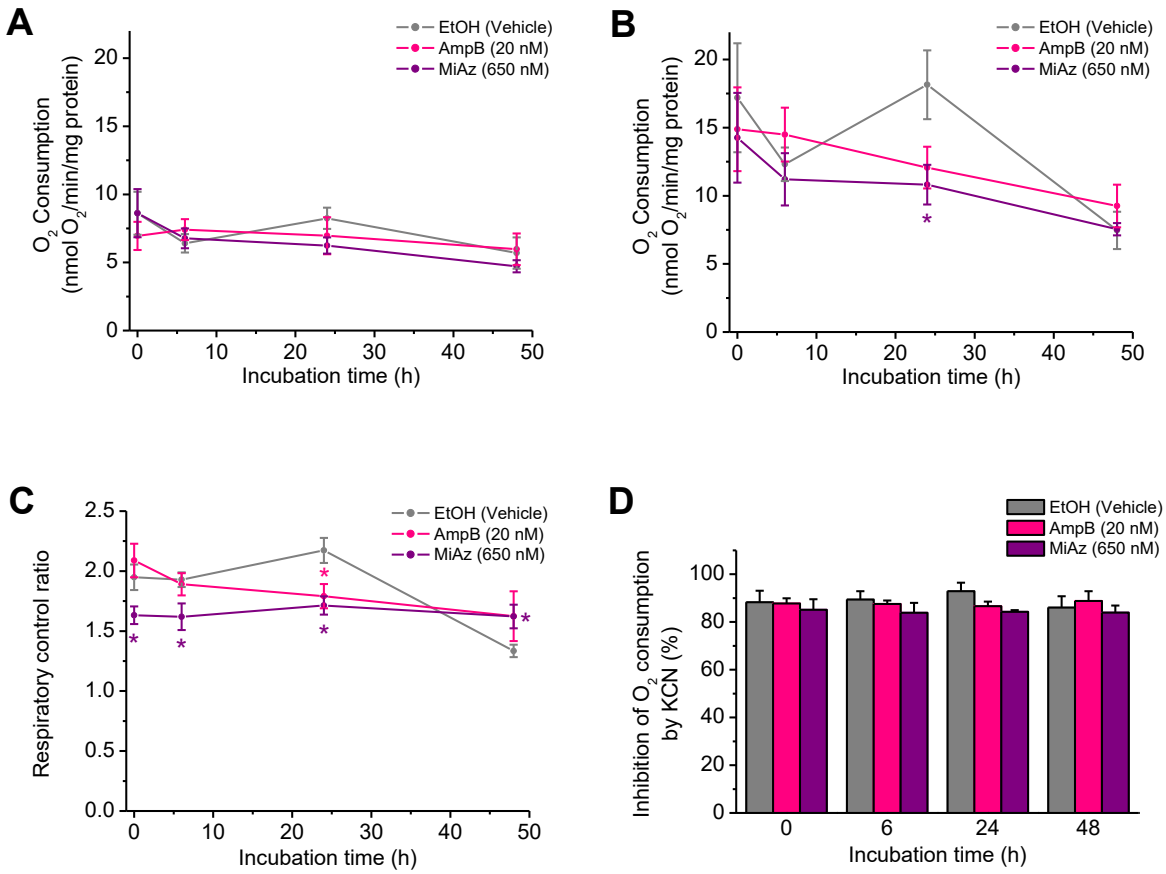

Figure 11: Mitochondrial function of *Leishmania tarentolae* promastigotes (LtP) cultured for up to 48 h in BHI medium in the presence of amphotericin B (AmpB) or miconazole (MiAz) using ethanol (EtOH, 0.2 % final concentration) as vehicle. Oxygen consumption rates of permeabilised LtP were measured in mannitol medium supplemented with 5 mM $MgCl_2$ 10 min after the addition of 25 µg/ml digitonin and consecutively 10 mM succinate **(A)** and 250 µM ADP **(B)**. Respiratory control ratios **(C)** were calculated as the ratios of O_2 consumption rates in the presence of succinate and ADP to O_2 consumption rates in the presence of succinate. The percentage of inhibition of O_2 consumption by 0.25 mM KCN **(D)** was calculated by subtracting the residual percental activity after the addition of KCN from O_2 consumption before its addition, which was set to 100 %. Data represent means ± SEM of four independent experiments. * indicate significant differences to time-matched controls (EtOH) at the level of $p < 0.05$.

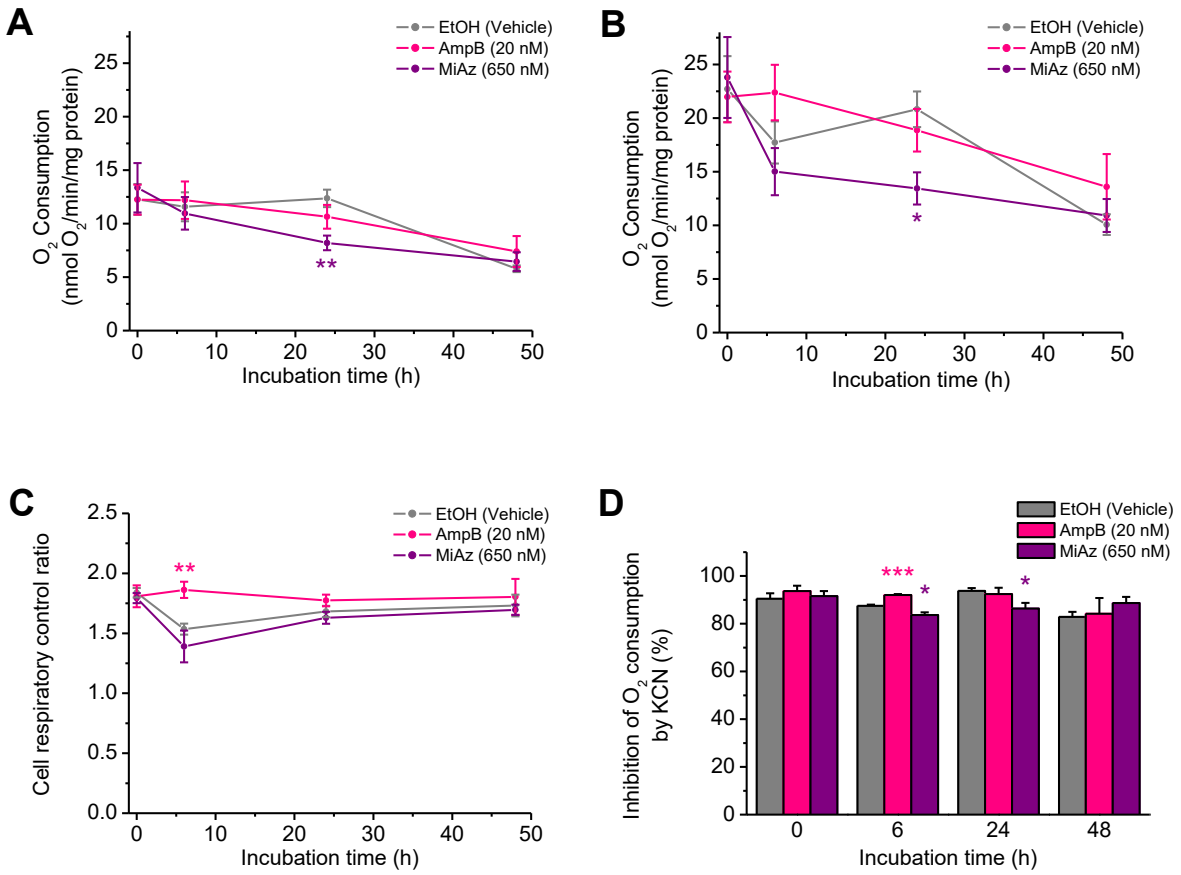

Figure 12*:* Mitochondrial function of *Leishmania tarentolae* promastigotes (LtP) cultured for up to 48 h in BHI medium in the presence of amphotericin B (AmpB) or miconazole (MiAz) using ethanol (EtOH, 0.2 % final concentration) as vehicle. Oxygen consumption rates of non-permeabilised LtP were measured in mannitol medium supplemented with 10 mM glucose after the addition of 20 µM oligomycin **(A)** and consecutively 0.5 µM CCCP **(B)**. Cell respiratory control ratios **(C)** were calculated as the ratios of O_2 consumption rates in the presence of oligomycin and CCCP to O_2 consumption rates in the presence of oligomycin. The percentage of inhibition of O_2 consumption by 0.25 mM KCN **(D)** was calculated by subtracting the residual percental activity after the addition of KCN from O_2 consumption before its addition, which was set to 100 %. Data represent means ± SEM of four independent experiments. *, ** and *** indicate significant differences to time-matched controls (EtOH) at the level of $p < 0.05$, 0.01 and 0.001, respectively.

4. DISCUSSION

Leishmaniasis remains a significant health concern in warm southern regions, affecting both humans and animals, highlighting the urgent need for effective treatment options (1). Current therapies rely heavily on the off-label use of antifungal drugs (9), which are associated with prolonged and costly treatments, adverse side effects, and the development of resistance (5). Most of these drugs target the ergosterol synthesis pathway, as ergosterol-like sterols are the main sterols in *Leishmania*, playing a crucial role in maintaining membrane fluidity (8,9). Inhibiting its production compromises the integrity of the parasite's cell membrane. The hypothesis was that targeting sterol synthesis and/or changing sterol content would also affect the mitochondrial bioenergetic parameters of the parasite due to alterations in mitochondrial membrane integrity and fluidity.

The data obtained in this study show that a consistent and significant inhibition of cell growth and mitochondrial bioenergetic function occurs with ErgoEP, AmpB, and MiAz at the concentrations used. However, impairment was observed after different periods of exposure to the test substances. This was particularly evident through changes in the respiratory control ratio and the cell respiratory control ratio. Additionally, a progressive decrease in the inhibition of oxygen consumption by KCN, an inhibitor of the mitochondrial complex IV, was observed with ErgoEP in digitonin-permeabilised as well as in intact non-permeabilised cells. This decrease in KCN inhibition with ErgoEP over time could indicate a non-mitochondrial oxygen consumption. Interestingly, in *Trypanosoma cruzi* treated with 40 µM ErgoEP, significant concentrations of carbonylated proteins were detected, which can be generated by free radicals, produced via homolytic cleavage of the peroxide bond and may be enhanced by transition metals (18). Thus, the production of highly reactive oxygen species, followed by a disruption of lipid membranes and proteins and the release of alkoxy radicals, could be an explanation for the non-mitochondrial oxygen consumption of ErgoEP-treated LtP observed in the present study. Ramos-Ligonio et al., who evaluated the trypanocidal activity of ErgoEP on *Trypanosoma cruzi*, reported an IC_{50} of 6.74 µg/ml (15.72 µM) for a 24-hour exposure (17). Although the IC_{50} value differs from that determined in the present study (8 µM), membrane disruption in these parasites was demonstrated. According to the authors, ErgoEP up to 30 µg/ml did not damage mammalian cells, such as HeLa cells and J774A.1 murine macrophages (17). In contrast, Chen et al. reported a reduced cell viability in Hep3B human hepatoma cells in the presence of ErgoEP (IC_{50} of 19.4 µg/ml) (29). Another study that determined IC_{50} values

for macrophages and LtP was the bachelor thesis of Azra Aleta, which reported IC_{50} values of 22 µM for ErgoEP in viability assays with J774A.1 murine macrophages and 4.5 µM in LtP (30). The observed differences in IC_{50} values across the various studies highlight the need for further research to assess the efficacy and safety of ErgoEP as a potential antiparasitic drug.

The differences in cell growth and mitochondrial inhibition observed in the present study could be related to the concentrations used, which were based on the IC_{50} values reported in Elisabeth Schrödl's bachelor thesis (20). The dose-response curves from that study, obtained from viability assays with LtP incubated with ErgoEP and DHCholEP, exhibited a steep decline and a large standard deviation. This variability posed challenges in accurately evaluating the IC_{50} values, as small changes in concentration could lead to significant fluctuations in outcomes, potentially resulting in an overly potent effect of ErgoEP and a weaker effect of DHCholEP. These findings suggest, that the concentration used for batch incubations of LtP with ErgoEP in the present study might have been higher than optimal, while the concentration for DHCholEP might have been lower.

Another important point to consider is that the viability assays in Schrödl's work were conducted with a starting cell density of 2×10^6 LtP/ml yeast extract medium mixed 1:1 with phosphate-buffered saline in 96-well plates without shaking the cells (20). In contrast, the present experiment was started with 4×10^7 LtP/ml BHI medium and included continuous shaking to prevent the cells from settling and becoming hypoxic in the Falcon tube. The 20-fold higher cell density resulted in a lower ratio of the amount of the lipophilic substances to the lipid/protein content of the LtP. Based on this, one would expect that the lipophilic test substances would have shown a weaker effect. However, in the present study, ErgoEP exhibited strong cytotoxic effects on LtP, whereas DHCholEP showed no significant effects. Additionally, the inability to reach the target cell number in the ErgoEP sample could potentially have been avoided by utilising a higher initial volume of LtP suspension.

At the tested concentrations, neither Ergo nor DHChol, the parent sterols of ErgoEP and DHCholEP, exhibited any significant effects on cell growth or mitochondrial bioenergetic function of LtP compared to the time-matched ethanol control. In her bachelor thesis, Azra Aleta observed additional yet unidentified products in aged stock solutions of Ergo and DHChol over time, which may be responsible for higher toxic effects on LtP in comparison to the parent substances (30). To alleviate this issue, new stocks of Ergo and DHChol were prepared every 3–4 weeks. This could explain why minimal impact on the cells of this present study was observed when Ergo and DHChol were tested at IC_{50} values determined in the bachelor thesis of Elisabeth Schrödl (20).

Given that AmpB is a well-known and frequently used drug for the treatment of leishmaniasis, a stronger inhibitory effect might have been anticipated. While cell growth decreased during the first 6 hours of incubation, LtP partially regained their growth capability after 24 hours. This progression could be attributed to the low IC_{50} values (20 nM AmpB) and, consequently, the low starting concentrations in LtP suspensions. This is further supported by two studies that evaluated the antileishmanial efficacy of AmpB: one on *Leishmania martiniquensis* and another on *Leishmania donovani* and *Leishmania major*, both of which reported 7–50 higher IC_{50} values for inhibition (31,32). However, it is important to note that these studies involved different *Leishmania* species compared to the *Leishmania tarentolae* used in the present study. Additionally, Phumee et al. utilised *Leishmania martiniquensis* isolated from patients, meaning that parasitic resistance cannot be excluded as a factor increasing the IC_{50} value (33). Another noteworthy observation with AmpB-treated LtP is the nearly linear cell growth curve, reaching cell numbers comparable to the time-matched control group (EtOH) after 48 h incubation.

It can be proposed that, in EtOH-treated control samples, but also in Ergo- and DHChol-treated LtP, a shift from exponential to stationary phase occurs between 24 and 48 hours of incubation, resulting in a lower rate of cell growth. This may be attributable to the LtP cultures reaching a critical cell density, which inhibits further growth, or to nutrient depletion (22). In contrast, in LtP incubated with AmpB, MiAz and ErgoEP, further growth is attenuated so that the stationary growth phase is not yet reached. Simultaneously with the retardation of cell growth in EtOH-treated control samples, as well as in Ergo- and DHChol-treated LtP after 48 h incubation, a decrease in oxygen consumption rates and respiratory control values was observed.

In summary, the results demonstrated strong effects on cell growth and mitochondrial bioenergetic function in LtP incubated with ErgoEP, as well as some inhibitory effects in those treated with AmpB and MiAz, while DHCholEP showed only marginal effects. In contrast, DHChol and Ergo did not show any significant inhibitory effects.

Therefore, this diploma thesis has shown that intervention in the sterol biosynthesis pathway and/or sterol composition of *Leishmania tarentolae* promastigotes can be associated not only with a reduction of cell growth but also with an impairment of their mitochondrial bioenergetic function.

5. LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AmpB	Amphotericin B
BHI	Bovine heart infusion
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
DHChol	Dehydrocholesterol
DHCholEP	Dehydrocholesterol endoperoxide
Ergo	Ergosterol
ErgoEP	Ergosterol endoperoxide
EtOH	Ethanol
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid
LtP	*Leishmania tarentolae* promastigotes
MiAz	Miconazole
OD	Optical density
SEM	Standard error of the mean
Succ	Succinate

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