Tampere University

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Effects of Alternative Oxidase on *Drosophila* under Environmental Stress

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ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine and Health Technology
of Tampere University,
for public discussion in the auditorium F115
of the Arvo building, Arvo Ylpön katu 34, Tampere,
on 5 October 2019, at 12 o'clock.

ACADEMIC DISSERTATION
Tampere University, Faculty of Medicine and Health Technology
Finland

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The originality of this thesis has been checked using the Turnitin OriginalityCheck service.

Cover design: Roihu Inc.

ISBN 978-952-03-1240-4 (print)
ISBN 978-952-03-1241-1 (pdf)
ISSN 2489-9860 (print)
ISSN 2490-0028 (pdf)
http://urn.fi/URN:ISBN:978-952-03-1241-1

PunaMusta Oy – Yliopistopaino
Tampere 2019

To Pulla

ABSTRACT

The mitochondrion is a unique organelle with a central role in energy production and metabolic homeostasis while regulating the life and death of the entire cell. In mitochondrial dysfunction, deficiencies or abnormalities of the mitochondrial oxidative phosphorylation (OXPHOS) system impair cellular energy production. Due to its ability to bypass Complex III and IV of the OXPHOS system and divert electrons from ubiquinol to oxygen, the mitochondrial alternative oxidase (AOX) has drawn attention as a potential therapy for diseases where mitochondria are affected.

AOX provides metabolic flexibility to organisms across the eukaryote kingdoms but has been lost in vertebrates and most arthropods in the course of evolution. The AOX from the tunicate *Ciona intestinalis* has been introduced into several model organisms such as cultured human cells, the fruit fly *Drosophila melanogaster* and most recently, mice. Characterization of these transgenic model systems under standard laboratory conditions revealed no obvious detrimental effects on the survival and fitness of these organisms. To shed light on the possible reasons behind the loss of AOX in vertebrates and *Drosophila* and on the potential drawbacks of implementing the enzyme for therapeutic purposes, I exposed AOX-expressing flies to environmental stressors which the animals encounter in the wild. This included testing reproductive fitness and nutritional requirements.

The reproductive competence of AOX-expressing male flies was tested by successively mating wild-type females with AOX-expressing and wild-type males. The assay demonstrated a clear selective advantage of sperm from wild-type over AOX-expressing males, even when the AOX-expressing male was the second male to be mated, which usually competes out the sperm of the first male. Histological

examination of the testis showed a spatially deranged spermatogenesis programme in the AOX-expressing males.

The slight but significant weight loss and development delay that we regularly observe in AOX-expressing flies, led me to suspect a negative effect of the transgene on the energy metabolism of the flies. I investigated this further by rearing AOX flies on media in which one or more component of the standard laboratory diet was omitted. Diets restricted to two ingredients, dry yeast and glucose caused a decreased eclosion rate in AOX-expressing flies, ~80 % of them failing to complete metamorphosis, whereas most control flies (~90 %) developed normally. Rescue by dietary supplementation with treacle, a nutritionally complex by-product of sugar refinement, but not by yeast, sucrose or monosaccharides, pointed to an imbalance in metabolic homeostasis rather than a simple insufficiency of metabolic fuel.

Altogether these findings indicate that AOX may be activated in specific developmental contexts involving tissue reorganization and cell differentiation, such as metamorphosis and spermatogenesis, thereby potentially interfering with developmental signaling and the efficient use of nutrients. These effects need to be better understood and taken into consideration for the development of AOX as a therapeutic treatment.

Tiivistelmä

Mitokondrio on ainutlaatuinen soluelin, joka toimii keskeisenä osana solun energiantuotantoa ja aineenvaihduntaa säädellen näin koko solun elämää ja kuolemaa. Häiriöt mitokondrioiden toiminnassa, kuten oksidatiivisen fosforylaation (OXPHOS) vajaatoiminta, heikentävät solun energiantuotantoa. Mitokondrion vaihtoehtoisen oksidaasin (AOX) kyky sivuuttaa kompleksit III ja IV OXPHOS -ketjussa on tehnyt entsyymistä potentiaalisen hoitomuodon sairauksiin, joissa mitokondrioiden toimintahäiriöitä esiintyy.

AOX:n on todettu lisäävän aineenvaihdunnallista sopeutumiskykyä useissa eukaryooteissa, mutta entsyymi on kadonnut selkärankaisista ja useimmista niveljalkaisista evoluution myötä. Siitä huolimatta vaippaeläin *Ciona intestinaliksen* AOX on onnistuneesti siirretty useisiin mallieliöihin kuten viljeltyihin ihmissoluihin, banaanikärpäseen *Drosophila melanogasteriin* sekä viimeisimpänä hiireen. Normaaleissa laboratorio-olosuhteissa entsyymillä ei ole todettu olevan haitallisia vaikutuksia mallieliöiden kehitykseen tai elinkykyyn. Selvittääkseni paremmin mahdollisia syitä AOX:n katoamiselle selkärankaisista ja banaanikärpäsestä sekä sen mahdollisia rajoituksia terapeuttisena hoitona, altistin AOX-kärpäsiä haasteellisille ympäristötekijöille, joita eläin kohtaa luonnollisessa ympäristössään. Näihin lukeutui eläinten lisääntymiskyvyn testaaminen kilpailutilanteessa sekä kehittyminen ravitsemuksellisesti niukoissa olosuhteissa.

AOX-kärpäskoiraiden lisääntymiskykyä testattiin parittamalla villityypin naaraita peräkkäin sekä AOX-koiraiden että villityypin koiraiden kanssa. Koeasetelma osoitti naaraiden suosivan villityypin koiraiden siittiöitä, myös tapauksissa, joissa AOX-koiras oli jälkimmäisenä paritteleva koiras, joka yleisesti syrjäyttää ensimmäisen

koiraan siittiöt. Kivesten histologisessa tarkastelussa AOX-koiraiden spermatogeneesiprosessi osoittautui heikentyneeksi.

AOX-kärpästen painonpudotus ja hidastunut kehitys standardiolosuhteissa herätti kysymyksen siitä, vaikuttaako AOX negatiivisesti kärpäsen energia-aineenvaihduntaan. Tutkin asiaa kasvattamalla kärpäsiä niukassa ravinnossa, joka koostui ainoastaan kahdesta ainesosasta, kuivahiivasta ja glukoosista. Ravinnon ollessa rajallisempaa kuoriutuvien AOX-kärpästen osuus laski merkittävästi ja peräti ~80 % kärpäsistä kuoli muodonmuutosvaiheessa, kun kontrollikärpäsistä puolestaan ~90 % kehittyi normaalisti. Pelkän hiivan, sakkaroosin tai monosakkaridien lisääminen ravintoon ei parantanut kehitystä mutta melassin, sokerituotannossa syntyvän ravintorikkaan sivutuotteen, lisäys palautti kuoriutuvien kärpästen määrän normaaliksi, mikä viittasi aineenvaihdunnalliseen epätasapainoon yksinkertaisen energianpuutteen sijaan.

Sekä lisääntymis- että ravintokokeiden tulokset viittaavat AOX:n aktivoitumiseen tietyissä kudosten ja solujen kehitysvaiheissa kuten uudelleen organisoitumisessa ja solujen erilaistuessa muodonmuutoksessa tai spermatogeneesissä, ja mahdollisesti häiritsevän normaalia signalointia ja energia-aineenvaihduntaa. Nämä vaikutukset ja niiden parempi ymmärtäminen on syytä ottaa huomioon AOX:n mahdollisessa kehityksessä terapeuttiseksi hoitomuodoksi.

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LIST OF ORIGINAL COMMUNICATIONS

The thesis is based on the following original communications, which are referred to in the text by their roman numerals I-III:

I Saari S*, Andjelković A*, Garcia GS, Jacobs HT, Oliveira MT. Expression of *Ciona intestinalis* AOX causes male reproductive defects in *Drosophila melanogaster*. *BMC Dev Biol.* 2017. 17(1):9.

II Saari S*, Garcia GS*, Bremer K*, Chioda MM, Andjelković A, Debes PV, Nikinmaa M, Szibor M, Dufour E, Rustin P, Oliveira MT, Jacobs HT. Alternative respiratory chain enzymes: therapeutic potential and possible pitfalls. *Biochim Biophys Acta Mol Basis Dis.* 2019. 1865(4):854-866.

III Saari S, Kemppainen E, Tuomela T, Oliveira MT, Dufour E, Jacobs HT. Alternative oxidase confers nutritional limitation on *Drosophila* development. *J Exp Zool A*. 2019. 331(6):341-356.

*Equal contribution

ABBREVIATION

Aβ	amyloid-β
ACAD	acyl-CoA dehydrogenases
ACL	ATP citrate lyase
AD	Alzheimer's disease
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AOX	alternative oxidase
ATP	adenosine triphosphate
BSA	bovine serum albumin
CIC	citrate carrier
CoQ	coenzyme Q
CSF	cerebrospinal fluid
Cyt c	cytochrome c
da-GAL4	*daughterless*-GAL4
ER	endoplasmic reticulum
ETFQO	electron transfer flavoprotein-ubiquinone oxidoreductase
$FADH_2$	flavin adenine dinucleotide
GP	glycerophosphate
GS	GeneSwitch
HEK	human embryonic kidney
HIF	hypoxia-inducible factor
IC	individualization complex
MAM	mitochondria-associated ER membrane
MCU	mitochondrial calcium uniporter
MDH	malate dehydrogenase
Mfn	mitofusin
mGPD	mitochondrial glycerol-3-phosphate dehydrogenase
mROS	mitochondrial reactive oxygen species
mtDNA	mitochondrial DNA

NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NDH2	NADH dehydrogenase 2
nPG	n-propyl gallate
OPA1	optic atrophy 1
OXPHOS	oxidative phosphorylation
PBS	phosphate buffered saline
PEP	phosphoenolpyruvate
PUFA	polyunsaturated fatty acid
RC	respiratory chain
ROS	reactive oxygen species
SOD	superoxide dismutase
TCA	tricarboxylic acid
tko	*technical knockout* (*Drosophila* gene for mitoribosomal protein S12)
tRNA	transfer RNA
tub	tubulin
UAS	upstream-activating sequence
VDAC	voltage-dependent anion channel

1 INTRODUCTION

As proven by their extensive presence in the different kingdoms, alternative respiratory chain (RC) enzymes provide organisms with properties that help them to survive in less favorable conditions (McDonald & Gospodaryov, 2018). Although it has been proposed to have been crucial to the evolution of multicellular organisms, the mitochondrial respiratory chain is a complex system that is vulnerable to disruption by many kinds of stress. Alternative respiratory pathways confer metabolic flexibility that enables adaptation to challenging environments. However, these enzymes have been lost in the course of evolution from several of the most complex animal species, including insects and mammals, which suggests that the properties they provide are, in some circumstances, no longer vital or might even have become deleterious to the maintenance of metabolic homeostasis (McDonald et al., 2009). Since the alternative RC enzymes are present in mainly sessile organisms, whilst they are missing in fast-moving animals with high-energy demands, it is tempting to put forward the idea that their presence might disturb optimized energy production. However, knowing the complex role of mitochondria beyond that of being 'the powerhouse of the cell' and provider of ATP, the properties of alternative RC enzymes might impact several signaling pathways and metabolic processes regulated by the organelle.

Alternative oxidase (AOX) has been broadly studied in plants but less is known about the role and regulation of the enzyme in animals, where it was not even assumed to be present until recently. In the animal kingdom a functional AOX gene is found mainly in stationary species that are heavily susceptible to the changes in their surroundings (McDonald & Gospodaryov, 2018). However, the fact that AOX

is found in species relatively closely related to mammals raises the possibility of AOX being used as a therapeutic agent in human mitochondrial diseases.

Mitochondrial diseases are inherited metabolic disorders that still lack curative treatments, despite advances in diagnostics (Picard et al., 2016). The genetic heterogeneity of mitochondrial disorders is vast and causative mutations can arise in either nuclear or mitochondrially encoded genes (Wallace et al., 1987). Respiratory chain deficiencies are a typical result of these mutations, leading to failure in assembly of the large enzyme complexes that form the RC and provide the electrochemical potential to fuel production of adenosine triphosphate (ATP) via ATP synthase (Martinez Lyons et al., 2016). In addition to producing energy for the metabolic processes of the cell, mitochondria are central hubs for different pathways responding to environmental conditions and adjusting cellular metabolism accordingly. The responses are also modified based on tissue, cell type, developmental stage and age, which explains the extensive range of clinical phenotypes arising from mitochondrial dysfunction (Picard et al., 2016.). Our understanding of the metabolic role of mitochondria is far from complete.

Transgenic expression of AOX from the tunicate *Ciona intestinalis* has been successful in several model systems such as mammalian cells, flies and mice (Fernandez-Ayala et al., 2009; Hakkaart et al., 2006; Szibor et al., 2017). In these models it is able to alleviate phenotypes caused by mutations in respiratory chain complexes and provide resistance against RC inhibitors such as cyanide and antimycin. However, the function of *Ciona* AOX in the original host is largely unknown and compatibility of the enzyme in the metabolism of a non-native host remains hard to predict. The objectives of this thesis are to study the effects of AOX at the level of a whole organism, the fruit fly *Drosophila melanogaster*, to better understand potential detrimental effects AOX may have. I have focused my studies on its possible effects on reproductive fitness and in conditions of metabolic stress. The results will provide pivotal insight into the metabolic impact of AOX that, in the future, may constitute an impediment as well as a benefit in its clinical use.

2 REVIEW OF LITERATURE

2.1 Mitochondria

Mitochondria are organelles that function as aerobic fuel factories for the cell by providing energy in the form of ATP. They are also involved in many other tasks including cell signaling, calcium signaling and reactive oxygen species (ROS) production as well as several biosynthetic pathways such as for amino acids, fatty acids and heme. Apart from mature red blood cells, mitochondria are present in all cell types and vary greatly in number depending on the tissue. They are structured by a double-membrane consisting of an outer membrane, intermembrane space and an inner membrane that folds as wrinkled or tube-like structures called cristae inside the matrix space within (Figure 2.1). Inside the matrix they maintain their own DNA, a characteristic feature among cell organelles that traces back to the origins of mitochondria as an endosymbiont of a larger cell.

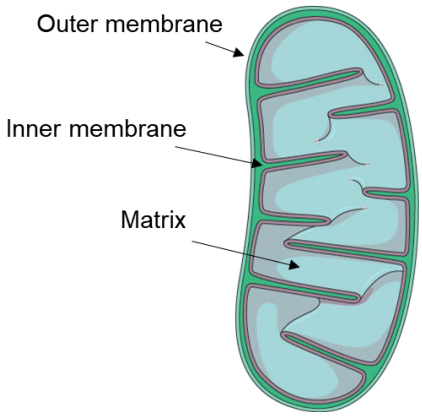

Figure 2.1. Mitochondrion.

Due to these specific characteristics, mitochondria cannot be created out of nothing but must be inherited and proliferated from existing mitochondria. Mitochondria are shaped by fusion and fission events that can result either in networks or fragmentation into of smaller compartments, depending on the tissue and metabolic state of the cell. Mitochondrial fusion has been generally detected in cells with high oxidative phosphorylation (OXPHOS) activity and is orchestrated by mitofusin proteins Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2) on the outer membrane and mitochondrial GTPase Optic Atrophy 1 (Opa1) on the inner membrane (Cipolat et al., 2004). Fission, in turn, occurs via endoplasmic reticulum (ER) contraction around mitochondria (Friedman et al., 2011).

The two mitochondrial membranes differ in their composition; the outer membrane consists of a 50:50 mixture of protein and lipids while the inner membrane is richer in proteins that include the RC complexes responsible for mitochondrial ATP production (Chrétien et al., 2018). A mitochondria-specific phospholipid known as cardiolipin is mainly localized in the inner membrane and has an important role in mitochondrial membrane dynamics, including formation of the cristae, stabilization of the RC complexes and thermogenesis (Houtkooper & Vaz, 2008; Sustarsic et al., 2018). All the above-mentioned features of mitochondria illustrate their significance in cell metabolism and the pathological heterogeneity of disorders where mitochondrial dysfunction is involved.

2.1.1 Origin & evolution

The most accepted theory about the origins of mitochondria is known as the endosymbiotic theory where mitochondria are relics of aerobic prokaryotes that were engulfed by a larger cell, presumed to be an archaeon, and were able to live inside their host as an endosymbiont (Ku et al., 2015). Endosymbiosis enabled the host cell to produce energy via respiration instead of glycolysis and fermentation leading to an increase of 5- to 10-fold in ATP production per glucose molecule (Ku et al., 2015).

Whether this symbiosis started as a parasite-host relation or as mutual syntropy that finally led to a mutual metabolism is unresolved. The process is presumed to have happened in several steps as the full integration of the mitochondrial symbiont has required creation of a protein import machinery between the two partners as well as merging of a majority of mitochondrial genes into the host genome (Roger et al., 2017).

The organization of mitochondrial DNA (mtDNA) resembles that of bacteria with its circular structure. It codes mainly protein subunits involved of the RC, which is responsible for creating the proton gradient across the inner mitochondrial membrane that drives the production of ATP. In addition, mtDNA codes for several transfer RNAs (tRNAs) and RNAs necessary for intramitochondrial protein synthesis on dedicated mitochondrial ribosomes (Martijn et al., 2018). Comparisons between alphaproteobacterial genomes and genes coding for mitochondrial proteins and RNAs show significant similarities. Until recently, mitochondria were believed to originate from ancestral lineages of Rickettsiales, pathogenic endosymbionts with several common features with mitochondria such as ubiquinol oxidase and ATP/ADP translocase. However, the exact phylogenetic position of mitochondria in the tree of alphaproteobacteria has remained under debate and in fact, recent phylogenomic analysis claim that mitochondria diverged from an even earlier proteobacterial lineage before Rickettsiales (Martijn et al., 2018).

Only a minor set of mitochondrial proteins today can be traced back to proteobacteria and genes from the mitochondrial genome have otherwise been lost or relocated into the host genome (Gray et al., 1999). There is a large variety in the size of noncoding regions of mtDNA between species but the proteins encoded have remained rather conserved. Human mtDNA contain 13 protein-encoding genes, which specify subunits of each of the major OXPHOS complexes except for Complex II (Gray et al., 1999).

2.1.2 Oxidative phosphorylation (OXPHOS)

Conducted by a series of enzyme complexes at the inner mitochondrial membrane, OXPHOS (Figure 2.2) has enabled eukaryotic cells to gain an ATP yield several-fold greater than would be provided by anaerobic glycolysis. It is highly conserved across the eukaryote kingdoms (Pierron et al., 2012). Electron transfer from nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), ultimately to molecular oxygen (O_2), pumps protons from the mitochondrial matrix into the intermembrane space, creating a membrane potential that powers ATP production by ATP synthase. Regulation of OXPHOS activity is crucial for maintaining the bioenergetic needs of the cell while preventing toxic effects, e.g. from excess production of ROS that could lead to the induction of apoptosis (Pierron et al., 2012).

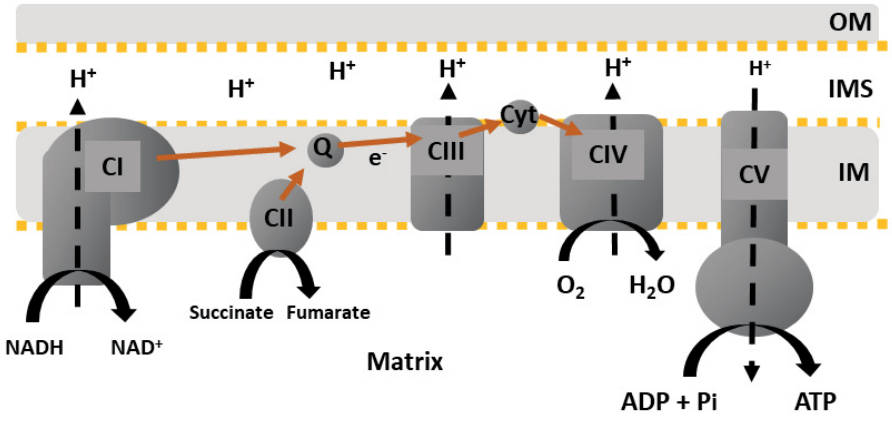

Figure 2.2. Oxidative phosphorylation. OM = outer membrane, IMS = intermembrane space, IM = inner membrane.

2.1.2.1 The core respiratory chain (RC)

At the inner mitochondrial membrane, there are four multisubunit complexes that form the RC (Figure 2.1.). Complex I (CI), Complex II (CII), Complex III (CIII) and Complex IV (CIV, also known as cytochrome c oxidase, COX) collectively provide an electrochemical potential ($\Delta\Psi$) across the inner mitochondrial membrane by pumping protons using the energy of stepwise electron transport through and between these complexes (Ghezzi & Zeviani, 2018). Two other electron shuttles participate in this process, namely ubiquinone (coenzyme Q or CoQ) and cytochrome c (cyt c). $\Delta\Psi$ created by the RC provides the proton-motive force for ATP production through ATP synthase (also known as Complex V or CV) (Ghezzi & Zeviani, 2018).

The RC complexes comprise of several protein subunits encoded by both the nuclear and mitochondrial genome and many of them require insertion of prosthetic groups to function. The process is tightly controlled and coordinated by a great number of assembly factors (Guerrero-Castillo et al., 2017). Complex I (NADH:ubiquinone oxidoreductase) is the largest of the complexes with nine prosthetic iron-sulphur (Fe-S) clusters that contribute to electron transfer (Zickermann et al., 2015). The complex oxidizes NADH to NAD^+, transfers the electrons to ubiquinone while pumping four protons through the intermembrane space (Guerrero-Castillo et al., 2017; Zickermann et al., 2015).

Complex II (succinate dehydrogenase) is the smallest of the RC complexes comprising only four subunits and is fully encoded by the nuclear genome. It functions as a part of both the RC and the tricarboxylic acid (TCA) cycle by oxidizing succinate to fumarate whilst reducing FAD to $FADH_2$. $FADH_2$ provides electrons to the ubiquinol pool via Fe-S clusters. Complex II is the only complex of the classical RC that does not pump protons across the inner mitochondrial membrane (Sun et al., 2005).

Complex III, also known as the bc1 complex or ubiquinol-cytochrome c oxidoreductase, transfers electrons from ubiquinol to cyt c coupled with proton

translocation across the membrane. The electron transfer is conducted via three subunits of the enzyme; the Rieske protein with an Fe-S cluster, cytochrome b with two hemes and cytochrome c1 with one heme (Iwata et al., 1998).

The final electron acceptor in the RC is cytochrome c oxidase (COX) or Complex IV. Using four electrons delivered sequentially by cyt c, COX reduces oxygen to water while pumping four protons from the matrix to the intermembrane space. How these two processes are coupled in the mammalian COX is still unresolved (Ishigami et al., 2017). COX activity is tightly linked to the overall activity of OXPHOS via both $\Delta\Psi$ and the ATP/ADP ratio, making it a key regulation point of the pathway (Lee et al., 2005; Pacelli et al., 2011; Villani & Attardi, 1997). The importance of COX as a regulator is highlighted by the fact that it is the only RC complex with tissue-specific isoforms with different basal activity depending on the level of aerobic energy metabolism of the tissue (Anthony et al., 1993).

In addition to electron transfer from Complex I and II, there are other electron donors outside the classical RC able to reduce ubiquinone (Figure 2.3). Mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) is the simplest of RC components and it is connected to the chain via the glycerophosphate (GP) shuttle, the main metabolic function of which is reoxidation of cytosolic NADH produced in glycolysis. The activity of mGPDH is very tissue-specific with brown adipose tissue, placenta, testes and insect flight muscles showing high activity while in e.g. mammalian muscle and liver it is almost negligible (Mrácek et al., 2009; Ohkawa et al., 1969; Sacktor & Cochran, 1958). The enzyme has been suggested to support thermogenesis in mitochondria but has also been recognized as an indirect source of ROS production regardless of its level of activity, when RC is defective (Mrácek et al., 2009; Ohkawa et al., 1969; Sacktor & Cochran, 1958). Dihydroorotate dehydrogenase (DHODH) is also localized at the intermembrane side of the inner membrane and converts dihydroororate to ororate using ubiquinone as an electron acceptor (Hines et al., 1986). On the matrix side, electron transfer flavoprotein-ubiquinone oxidoreductase (ETFQO) connects acyl-CoA dehydrogenases (ACADs)

of fatty acid β-oxidation and amino acid catabolism to the mitochondrial RC by accepting electrons from ACADs and transferring them to ubiquinone (Seifert et al., 2010).

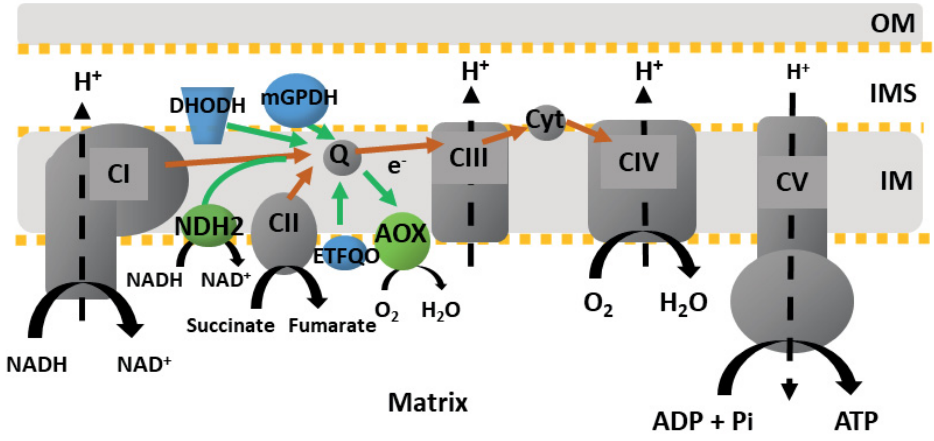

Figure 2.3. Alternative RC enzymes. Alternative RC enzymes maintain electron transfer but do not participate in proton pumping to the intermembrane space and ATP production. OM = outer membrane, IMS = intermembrane space, IM = inner membrane. Adapted from McDonald & Gospodaryov (2018), Hines et al. (1986), Seifert et al. (2010), Mrácek et al. (2009).

2.1.2.2 Alternative respiratory chain enzymes

The core RC enzymes depicted above are all present in humans but many organisms, including plants and fungi, possess additional enzymes that enable alternative pathways to the classical RC. Several theories have been proposed as to why alternative RC routes evolved. Although efficient in enabling usage of coenzymes and oxygen in ATP production, the OXPHOS system with large enzyme complexes is a rather rigid machinery when facing sudden changes in the environment. The two alternative RC enzymes that have gained most attention, alternative NADH dehydrogenase (NDH2) and AOX (Figure 2.3), are small, non-transmembrane complexes located at the inner mitochondrial membrane, providing the RC with

branching points at the sites of ubiquinone reduction and ubiquinol oxidation, respectively (McDonald & Gospodaryov, 2018). They do not contribute to pumping of protons across the membrane like most of the large core complexes but are involved in electron transfer and thereby, provide resistance to classical RC inhibitors such as rotenone, inhibitor of Complex I, and cyanide, a well-known inhibitor of Complex IV. In addition of being able to bypass functionally limited RC complexes, both AOX and NDH2 have been found to contribute to the general metabolic state of the cell and respond to changes in environment via e.g. redox homeostasis and thermogenesis (McDonald & Gospodaryov, 2018). Due to these unique features, and their absence in humans, these enzymes have become an intriguing study subject from the perspective of developing treatments for mitochondrial diseases but also to better understand the metabolic mechanisms behind cancer and aging (Kemppainen et al., 2014b; Scialò et al., 2016; Wheaton et al., 2014).

2.1.2.2.1 Alternative oxidase (AOX)

The mitochondrial AOX is localized on the matrix side of the inner mitochondrial membrane and provides a bypass-route that directly transfers electrons from ubiquinol to molecular oxygen past Complex III and Complex IV of the classical RC. AOX is a homodimer with a non-heme diiron active site and a hydrophobic region that is presumed to anchor the enzyme to the inner mitochondrial membrane. This is based on the crystal structure of AOX from the parasite *Trypanosoma brucei* (Shiba et al., 2013). Although, no crystal structures of animal AOXs have been reported, same structural elements seem to be conserved in them as well, including AOX of the tunicate *Ciona intestinalis* (Andjelkovic et al., 2015).

Several studies and transgenic models demonstrate the ability of AOX to partially maintain RC activity when either Complex III or Complex IV is inhibited by antimycin A or cyanide, respectively (Castro-Guerrero et al., 2004; Hakkaart et al.,

2006). However, kinetic models and respirometry studies indicate a lower affinity of AOX to ubiquinol compared to Complex III and the enzyme is predicted to be active only when the core RC is oversaturated or dysfunctional that would lead to accumulation of the ubiquinol pool and thus to the involvement of AOX (Castro-Guerrero et al., 2004; Hakkaart et al., 2006).

Regulation and metabolic input of AOX varies amongst organisms where it is expressed but the general significance of the enzyme seems to be providing the organism with metabolic flexibility in stress conditions caused by changes in the environment e.g light, temperature and pH (McDonald & Gospodaryov, 2018). The roles of AOX in different taxa is discussed later in more detail.

2.1.2.2.2 Alternative NADH dehydrogenase (NDH2)

Another alternative branching point in the RC is at the point of ubiquinone reduction where the alternative NDH2 is able to replace the reaction commonly catalyzed by Complex I. NDH2 provides the RC with resistance to inhibitors targeting Complex I, such as rotenone, but does not contribute to proton pumping and generation of $\Delta\Psi$. Like AOX, NDH2 is present in many different organisms but not in humans (Matus-Ortega et al., 2011; McDonald et al., 2009). Phylogenic studies show that NDH2 is present in metazoans, but with a more limited distribution than AOX, and is also expressed in archaea where AOX is absent. The understanding of the physiological significance of NDH2 is mainly based on structural analyses and very limited biochemical studies (Matus-Ortega et al., 2011). However, like AOX, NDH2 has been successfully introduced in some transgenic models where it has been harnessed to better characterize metabolic pathways involved in processes such as aging and cancer (Scialò et al., 2016).

The yeast NDH2 known as NDI1 was first introduced into Chinese hamster cells and later into human cells, both models showing resistance to rotenone as a result (Seo et al., 1998; Seo et al., 1999). In addition, expression of NDI1 has been shown

to alleviate phenotypes caused by Complex I deficiency such as neurodegeneration in a rat model of Parkinson's disease (Marella et al., 2008). It can also compensate for knockdown of Complex I subunits in a *Drosophila melanogaster* model as well as increase lifespan independent of diet (Sanz et al., 2010). NDI1 has also been implemented as a tool to study anti-cancer treatments (Wheaton et al., 2014). NDX, the NDH2 from *Ciona intestinalis,* also increased the lifespan of *Drosophila*, although the effects were weaker and the properties of NDX seemed to be more sensitive to temperature and diet (Gospodaryov et al., 2014). Whether this sensitivity is characteristic of alternative enzymes from *Ciona* and applies also to AOX is not known.

2.2 Taxonomic distribution of AOX

In the beginning, AOX was thought to be characteristic of plants, bacteria, protists and fungi but had been lost in animals during evolution. The reason behind the loss was presumed to be optimization of bioenergetic processes needed in complex, highly motile organisms. Most organisms that still possess AOX are living a rather sessile life and the enzyme is proposed to provide them metabolic flexibility and stress endurance to cope with changes in their environment. However, in recent years, extensive sequencing and *in silico* analyses have identified genes coding for AOX in genomes from various animal phyla including *Chordata* and *Arthropoda*. The functionality of most of these enzymes is yet to be confirmed but the lack of inactivating mutations in the genes implies that the expressed enzyme should be active (McDonald et al., 2009; McDonald & Gospodaryov, 2018).

2.2.1 AOX in plants

The role of AOX in metabolism is most widely studied in plants. As sessile organisms, plants are not able to change location when conditions become

unfavorable and, therefore, are subject to multiple environmental stresses such as drought and changes in temperature. AOX provides plants with metabolic flexibility that helps them to adapt to these changes. The importance of AOX in plants is emphasized by the broad expression of it throughout the kingdom and by the presence of two subfamilies of the enzyme, AOX1 and AOX2. Many plants also have multiple AOX genes in variable combinations of AOX1 and/or AOX2 subtypes. The combinations are presumed to be based on the metabolic requirements of the species of plant in question i.e. specific AOX subtypes respond and adapt the plant to specific environmental stressors (Costa et al., 2014).

Plant cells contain mitochondria and plastids that generate ROS via both respiration and photosynthesis. Oxidative stress is also brought about by environmental stresses such as drought and hypersalinity. The small size and structural simplicity of AOX protein compared to the multi-subunit complexes of the classical RC is presumed to be an advantage in enabling quick but temporary response to oxidative stress (Lushchak, 2011; Stehling & Lill, 2013; Szal et al., 2009). This may also be beneficial in hypoxic or anoxic conditions e.g. experienced by aquatic plants or during overwatering, as assembly of AOX does not require the array of cellular resources, cofactors and prosthetic groups required to build for example, Complex III (Lushchak, 2011; Skutnik & Rychter, 2009).

In plants, AOX has also been found to contribute to thermogenesis. Thermogenesis is a central process of surviving drops in temperature in the environment and is specific to the reproductive (floral) parts of plants where AOX expression has also been localized. In some species, such as skunk cabbage, *Symplocarpus renifolius,* AOX is coexpressed with plant uncoupling protein (pUCP), while in the sacred lotus *Nelumbo nucifera* no such relationship has been observed (Grant et al., 2008; Onda et al., 2008). In addition to preventing cold damage and optimizing floral development, thermogenesis is also used to attract pollinators through intensified floral scent, as well as providing a thermally attractive habitat for poikilotherms (Onda et al., 2015).

Alternative RC routes of electron transfer have also been suggested to be involved in regulation of ATP synthesis by decreasing the number of proton-pumping channels and thus favoring the direction of carbon skeletons of TCA cycle intermediates to other metabolic processes e.g. heme, fatty acid and amino acid synthesis. The activity of alternative RC enzymes is also suggested to enhance recycling of NAD^+ needed for photorespiration without any increase in oxidative stress. In plants, activity of the AOX-protein and expression of AOX-encoding genes have been reported to be induced by an increase in TCA cycle intermediates, as well as by an increase in mitochondrial ROS, suggesting that AOX has a role in enhancing TCA cycle flux while restraining phosphorylating electron transfer (Fernie et al., 2004; Gray et al., 2004). The different subtypes of AOX in plants seem to be activated by different TCA metabolites, α-ketoglutarate and oxaloacetate being the most common ones. Citrate and malate, while they increase the expression of AOX mRNA, seem to have no post-translational effects on the activity of the enzyme. These regulatory differences may explain why plants have a varying range of different AOX subtypes able to respond to different cellular stresses but unable to compensate for each other (Gray et al., 2004; Selinski et al., 2018).

2.2.2 AOX in bacteria, fungi and protists

An AOX gene has been found in several bacteria, most of which are marine organisms. The role of AOX in bacteria remains unclear but their location in an ocean environment suggests a specific function that may have significance in the marine ecosystem (McDonald & Vanlerberghe, 2005). Two AOX-expressing marine bacteria have been studied in more detail, namely *Novosphingobium aromaticivorans* and *Vibrio fischeri*. AOX mRNA expression of both bacteria was increased by specific environmental conditions; in *N. aromaticivorans* by lowered oxygen level and glucose as a carbon source; in *V. fischeri* by nitric oxide stress (Dunn et al., 2010; Stenmark & Nordlund, 2003).

Although not present in the well-studied yeasts *Saccharomyces cerevisiae* or *Saccharomyces boulardii*, AOX is expressed in several species of fungi, including other yeasts. However, in these species, including *Neurospora crassa* and *Aspergillus niger* as well as the yeast *Hansenula anomala*, AOX expression seems to be limited to metabolic states of cytochrome pathway dysfunctions (Descheneau et al., 2005; Hattori et al., 2009; Sakajo et al., 1993). In the filamentous fungus *Podospora anserina*, the disruption of the cytochrome pathway not only activates AOX but leads to a significant increase in lifespan and stabilization of mtDNA (Dufour et al., 2000) while in *Blastocladiella emersonii* AOX was recognized to be crucial for growth and sporulation (Luévano-Martínez et al., 2019). These findings support the theory that AOX activity is strongly connected to control of tissue organization and regeneration in species where it is still expressed.

Due to the central role of AOX in enhancing the survival of protists, particularly pathogenic parasites such as trypanosomes, and its absence in mammals, the enzyme has become a promising target in the development of antiparasitic drugs. Trypanosomes are transmitted by the tsetse fly and affect both cattle and humans (Fueyo González et al., 2017). *Trypanosoma brucei* causes African sleeping sickness in humans, and the trypanosome AOX (TAO) has a key role in survival of the parasite in the bloodstream (Clarkson et al., 1989). Other parasitic protist for which AOX-inhibitor development has been proposed are the intestinal parasite *Cryptosporidium parvum* (Suzuki et al., 2004) and the amphizoic scuticociliate *Philasterides dicentrarchi*, a parasite affecting farmed fish (Mallo et al., 2013).

2.2.3 AOX in animals

High-throughput sequencing data have demonstrated the presence of AOX genes in representatives of several animal phyla including *Nematoda*, *Mollusca* and *Urochordata* (Figure 2.4). The Pacific oyster *Crassostrea gigas*, from phylum *Mollusca,* was one of the first animals to be shown to express AOX (McDonald & Vanlerberghe, 2004). Based

on the latest findings, animals potentially expressing AOX now include members of arthropod subphyla such as *Hexapoda* and *Crustacea* (McDonald & Gospodaryov, 2018). Most of the data regarding the function of AOX in animals comes from *in silico* analysis of genomes and transcriptomes, but nothing is known about the post-translational regulation of AOX. Tward et al. (2019) have recently presented both AOX transcript and protein production data from the marine copepod *Tigriopus californicus* and introduced it as a potential model organism to study AOX in an animal naturally expressing the enzyme.

Due to a lack of relevant experimental data, the function and importance of AOX in the metabolism of animals remains poorly understood. However, existing data from studies of *C. gigas* and *T. californicus* suggest a role as a stress-induced enzyme also in animals. In *C. gigas*, AOX expression was found to respond to oxygen fluctuations in the surroundings (Sussarellu et al., 2013) while in *T. californicus*, AOX responded at the protein level to both extreme decrease and increase of temperature (Tward et al., 2019). Like AOX-expressing bacteria, the majority of animals expressing AOX live in an aquatic environment. The animals share several similarities in their way of life i.e. a rather sessile life that exposes the animal to environmental stresses like hypersalinity, hypoxia, changes in temperature or nutrient availability. Addition of more mobile animals such as arthropods to the list of AOX-expressing organisms has led to a reevaluation of the theory of AOX simply being an impairment for motility. Although bioenergetic efficiency may still be one of the causes, the reason behind the gene loss may also be in the requirements of metabolic adaptation and stress response mechanisms needed in the living habitat of the organism (McDonald et al., 2009; McDonald & Gospodaryov, 2018).

The presence of AOX in animals has led to the idea of applying the enzyme in human medicine, in particular in mitochondrial diseases. Animal AOXs presented a new opportunity to introduce this alternative respiratory pathway to mammalian model systems as the enzyme from an animal source is more likely to be compatible with the metabolism of mammalian mitochondria than are plant or fungal AOX

(Hakkaart et al., 2006). In addition to the medical interests, the broad expression of AOX in marine animals has increased the value of understanding AOX regulation and its impact on metabolism from the ecological perspective (Tward et al., 2019).

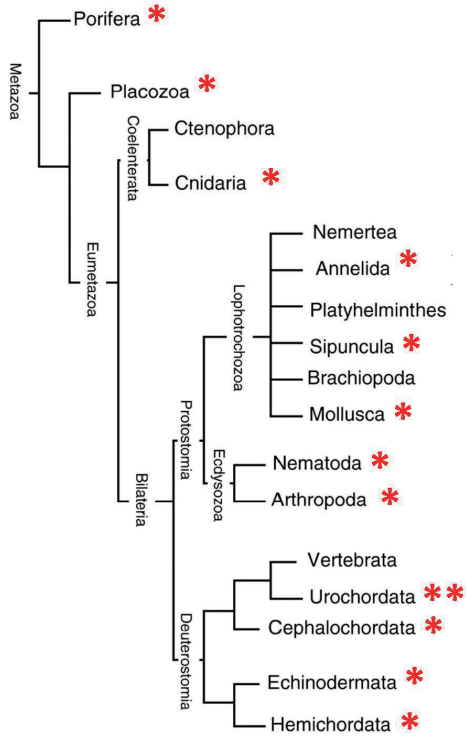

Figure 2.4. Taxonomic distribution of AOX in animals. *Transcript, protein or respiratory data demonstrating presence of AOX, **Subphylum of *Ciona intestinalis*. Adapted from McDonald et al. (2009).

2.2.3.1 Ciona AOX

The tunicate *Ciona intestinalis* belongs to the group of animals that possess both the gene for both AOX and NDH2 (known as NDX). Although *Ciona* is a well-studied model organism in the field of developmental biology, the knowledge of the function

of AOX in *C. intestinalis* is negligible and even the speciation within the genus is disputed. Nevertheless, *C. intestinalis* is phylogenetically the closest relative to humans (Figure 2.4) that still possesses the alternative RC enzymes and was therefore chosen as the best candidate gene for transgenic expression of AOX (Hakkaart et al., 2006; McDonald & Vanlerberghe, 2004; McDonald et al., 2009).

2.3 Roles of the Mitochondria

2.3.1 Metabolism: Tricarboxylic acid (TCA) cycle

The tricarboxylic acid (TCA) cycle (Figure 2.5), also known as the Krebs cycle, which operates mostly in the mitochondrial matrix, is the final metabolic hub for the breakdown of acetyl-CoA derived from carbohydrates, proteins and lipids catabolized in the cell (Akram, 2014; Owen et al., 2012). Pyruvate produced in glycolysis enters the mitochondria where it is oxidatively decarboxylated into acetyl-CoA (Figure 2.5). When entering the cycle, acetyl-CoA is condensed together with oxaloacetate to citrate by citrate synthase, after which citrate is dehydrated to an intermediate form (*cis*-aconitate) and then rehydrated to isocitrate by aconitase. Isocitrate dehydrogenase converts isocitrate to α-ketoglutarate by a decarboxylation reaction that reduces NAD^+, followed by α-ketoglutarate conversion to succinyl-CoA by α-ketoglutarate dehydrogenase. Succinyl-CoA is converted to succinate by a substrate-level phosphorylation ($GDP + P_i \rightarrow GTP$) performed by succinic thiokinase, after which succinate succinate is converted to fumaric acid by succinate dehydrogenase (succinate-coenzyme Q oxidoreductase) which is Complex II of the RC. Simultaneously, FAD is converted to $FADH_2$. RC flux is required for TCA cycle function as it maintains Complex II activity by regenerating ubiquinone and oxidizing NADH to NAD^+, which is needed in enzymatic reactions of the cycle. Fumarate is hydrated by fumarase to malate, which is oxidized in a final step to

oxaloacetate by malate dehydrogenase, with further NAD^+ reduction, bringing the cycle back to the entry point for acetyl-CoA (Akram, 2014).

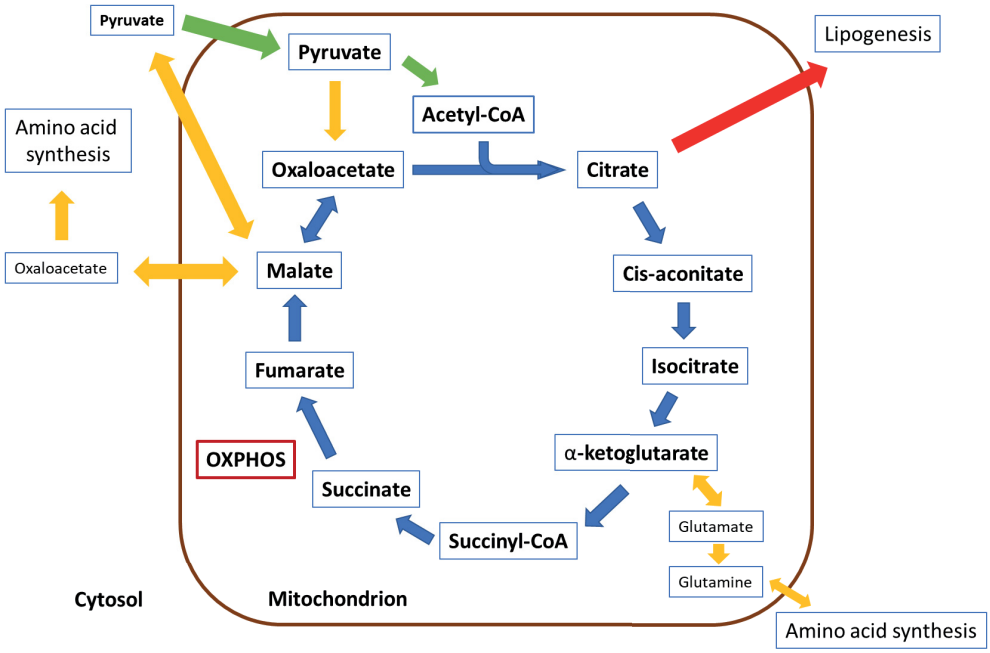

Figure 2.5. TCA cycle with anaplerotic and cataplerotic reactions. Pyruvate enters the mitochondria (green) and is decarboxylated to acetyl-CoA that is considered as the starting point of the cycle. The classical TCA cycle is presented with blue arrows. Orange arrows represent both anaplerotic and cataplerotic reactions while red arrow represents cataplerosis of citrate that is used in fatty acid synthesis. Adapted from Owen et al. (2012).

2.3.1.1 TCA cycle intermediates in metabolism

Despite the name, the TCA cycle is not a rigid circle of metabolites but a dynamic pathway with intermediate metabolites directed in and out to other metabolic processes, depending on the needs of the cell (Figure 2.5). This exchange of metabolites is divided into two different categories, namely anaplerotic and cataplerotic reactions (Owen et al., 2002). Maintenance of these reactions has gained attention, particularly in the field of cancer research, where many tumor cells, although producing energy through glycolysis, remain dependent on TCA cycle intermediate production to enable fast growth and proliferation (Martínez-Reyes et al., 2016). Anaplerotic reactions assure sufficient amounts of TCA intermediates to maintain the cycle. A classic example of this type of reaction is the carboxylation of pyruvate to oxaloacetate by pyruvate carboxylase, that occurs when malate is directed to gluconeogenesis or citrate is needed for lipogenesis (Figure 2.5). The two latter reactions are cataplerotic reactions that are needed to respond to metabolic demands of the cell but also to prevent accumulation of TCA intermediates. Another important anaplerotic pathway is conversion of glutamine via glutamate to α-ketoglutarate or vice versa, or the corresponding cataplerotic conversion of α-ketoglutarate to generate amino acids (Figure 2.5). The regulation and optimal balance between these reactions are dependent on the specific organ and tissue where they occur (Owen et al., 2002).

2.3.1.1.1 Citrate/Malate

Citrate is the first constituent of the TCA cycle formed from acetyl-CoA and oxaloacetate but it is equally required in the process of fatty acid and sterol synthesis. In these cytosolic processes, citrate is converted back to acetyl-CoA and oxaloacetate by ATP citrate lyase (ACL) and, while contributing to lipogenesis, citrate has also been found to inhibit glycolysis (Newsholme et al., 1977). Conversion of mitochondrially derived citrate to acetyl-CoA by ACL is also a central mediator of

histone acetylation in the regulation of cell growth and differentiation (Wellen et al., 2009). Citrate also functions as a chelator for divalent cations (Ca^{2+}, Zn^{2+}, Mg^{2+} etc.). Astrocytes have been found to release high concentrations of citrate into the cerebrospinal fluid (CSF) and there is evidence suggesting that citrate may modulate concentrations of Ca^{2+}, Zn^{2+} and Mg^{2+} in CSF that would in turn regulate neuronal receptors and excitation state of the neurons (Westergaard, et al., 1994; Westergaard, et al., 2017). From the mitochondrial matrix, citrate is exported to the cytosol by the mitochondrial citrate carrier (CIC) in exchange for malate or phosphoenolpyruvate (PEP). In other words, citrate efflux is dependent on the availability of these counter substrates (Owen et al., 2002; Palmieri et al., 2015).

Similar to citrate, malate plays a central role in the anaplerotic/cataplerotic reactions of TCA intermediates in both the cytosol and mitochondria. Malate can be reversibly converted to oxaloacetate by malate dehydrogenase (MDH), an enzyme that is present as a mitochondrial isoform (MDH1) and as a cytosolic isoform (MDH2). In the cytosol, malate is used for gluconeogenesis by conversion via oxaloacetate to PEP and finally to glucose. In the small intestine, glutamine is utilized in energy metabolism by entering the TCA cycle as α-ketoglutarate and exiting as malate after which, via PEP, it is converted to pyruvate by pyruvate kinase. Malate can be transported through mitochondrial membranes either in an electroneutral exchange with citrate by the CIC or using the proton motive force via the malate-aspartate shuttle (Dasika et al., 2015; Owen et al., 2002). In *Drosophila*, cytosolic malate can be converted into pyruvate by malic enzyme while generating nicotinamide adenine dinucleotide phosphate (NADPH). The activity of malic enzyme has been linked to increased lifespan, ROS tolerance and lipid metabolism (Kim et al., 2015).

2.3.2 Signaling & other roles

Mitochondria are involved in several nutrient sensing pathways by regulating the levels of metabolites that signal the metabolic state of the cell. During recent years, one of the most infamous products of mitochondrial metabolism, mitochondrial ROS (mROS), has gained attention as not only a damaging by-product, but as a central signaling molecule in regulating nutrient-sensing pathways of the cell as well as the metabolic state of mitochondria. Oxygen homeostasis of the cell is regulated by hypoxia-inducible factors (HIFs) that in turn are activated by mROS e.g. due to limited OXPHOS capacity (Brunelle et al., 2005). Metabolic reprogramming and activation of AMP-activated protein kinase (AMPK), considered a master regulator of cellular energy homeostasis, under metabolic stress conditions, has recently been shown to be dependent not only on increased adenosine monophosphate (AMP), but also in increase in mROS (Rabinovitch et al., 2017). In innate immunity, mROS has been suggested as one of the activators of inflammasomes, and immune responses are dysfunctional when ROS generation is suppressed (Zhou et al., 2011). Finally, mROS is known to regulate autophagy and apoptosis. Regulatory functions of different ROS ($O_2^{\cdot-}$, H_2O_2) are largely unknown but starvation-related autophagy is generally induced by $O_2^{\cdot-}$ formation while H_2O_2 induction is specific to deprivation of amino acids (Chen et al., 2009; Scherz-Shouval et al., 2007).

Mitochondria are also major regulators of the NAD^+/NADH ratio. NAD^+ is the product of the oxidization of NADH by Complex I in the first step of the mitochondrial RC but it is also a central cofactor in metabolic regulation of the cell. Despite the TCA cycle reducing NAD^+ back to NADH, NAD^+ levels inside mitochondria have been found to be severalfold higher compared to cytosolic levels (Cantó et al., 2015), presumably due to high requirement of NAD^+ inside mitochondria and to ensure maintenance of OXPHOS even when cytosolic NAD^+ levels are low. Evidence supporting the existence of a transport mechanisms for direct mitochondrial NAD^+ uptake has only recently been established (Davila et al.,

2018). NAD^+ levels are sensed by sirtuins that increase mitochondrial oxidative metabolism by deacetylating non-histone proteins. The NAD^+/sirtuin pathway in turn has been found to activate mitochondrial stress-signaling via the mitochondrial unfolded protein response (UPR^{mt}) and an increase in ROS defense (Mouchiroud et al., 2013). Increase in intracellular NAD^+ levels by supplementation with NAD^+ precursors has been found to enhance mitochondrial function and metabolism in mice and are currently considered as attractive treatments for human diseases (Cantó et al., 2012; Cantó et al., 2015).

Mitochondria function in close proximity with the ER at regions known as mitochondria-associated ER membranes (MAM). This interaction has many functions including lipid biosynthesis by non-vesicle lipid trafficking, bioenergetics and signaling by Ca^{2+} uptake. Lipid transport and synthesis includes phospholipid and cholesterol metabolism. By uptaking Ca^{2+} via the voltage-dependent anion channel (VDAC) and mitochondrial calcium uniporter (MCU), mitochondria function as a Ca^{2+} buffering organelle. Ca^{2+} not only activates ATP production and the TCA cycle but is also a second messenger for apoptosis which is thereby controlled by mitochondrial uptake (Szabadkai et al., 2006). There is also increasing evidence of a link between MAMs and insulin resistance, obesity and diabetes; however, the mechanisms remain unknown (Thoudam et al., 2018).

Another less studied, yet intriguing role of mitochondria, is related to the physiological properties of their lipid membranes. Cardiolipin has been found to respond to temperature which supports the recent findings suggesting mitochondria maintain a higher temperature compared to the surrounding cell. Although efficient, mitochondrial energy production machinery is not perfect and some of the energy is released as heat (Sustarsic et al., 2018). Recent studies suggest that mitochondria can heat up to 50 °C (Chrétien et al., 2018). How this would affect the properties of mitochondria and the surrounding cell remains unanswered.

2.4 Mitochondrial diseases

The precise etiology of mitochondrial dysfunction has remained puzzling in many cases, despite increasing research directed at this issue (Picard et al., 2016). Mitochondrial disorders manifest a wide range of symptoms, age of onset and severity and they are estimated to occur at a prevalence of at least one in 5000 births (Skladal et al., 2003). One reason behind the complexity is the genetic mosaicism of mitochondria, which comprise gene products encoded in both nuclear and mtDNA, with different patterns of inheritance. MtDNA is maternally inherited in thousands of copies that are prone to mutations which leads to a mixture of mtDNA genotypes known as heteroplasmy (Wallace et al., 1987). Mutations may also accumulate which usually leads to late onset of the disease. They can be somatic and tissue specific or they can occur in the germline and be inherited from the mother. The occurrence and severity of clinical symptoms caused by pathogenic mutations in mtDNA depends on the percentage of mtDNA bearing the mutation (Grady et al., 2018; Holt et al., 1988). The threshold percentage of the mutation or mutations that leads to a mitochondrial disease, varies between different tissues but has been found to be lower in tissues with high metabolic rate such as muscle and brain (Burbulla et al., 2017; Delaney et al., 2017; Holt et al., 1988). Genetics of mutations in mitochondrial genes expressed by the nuclear genome adds to the complexity as they may be recessive or dominant as well as somatic or inherited (Bourgeron et al., 1995; Thompson et al., 2016).

Maternal transmission of mitochondria plays its own role in the trajectory of mitochondrial mutations and disorders. While preserving mutations beneficial to females, this uniparental route of inheritance has led to accumulation of genetic polymorphisms deleterious to males and their fertility and reproductive fitness in particular (Innocenti et al., 2011, Perlman et al., 2015). The phenomenon is known as the "mother's curse". In humans, mitochondria-related male infertility is mainly

due to lowered activity of the RC complexes that leads to decreased motility of the spermatozoa (Ruiz-Pesini et al., 2000).

Most mitochondrial diseases involve dysfunction of the RC complexes, given the ubiquitous need for the RC, and the metabolic pathways connected to it. Dysfunction of the RC can be divided into so called isolated RC complex deficiencies, with mutations directly affecting a specific subunit or assembly factors of an enzyme complex (Hao et al., 2009; Martinez Lyons et al., 2016), or multi-enzyme RC deficiencies caused by mutations affecting the replication, transcription and translation of mtDNA (Kemp et al., 2011; Kornblum et al., 2013; Richter et al., 2018). Non-RC related mitochondrial diseases are less common but there are a few examples such as a mutation in the aconitase enzyme of the TCA cycle, that has been described to cause optic neuropathy (Metodiev et al., 2014).

2.4.1 Diagnostics and therapies

Although diagnostics for mitochondrial diseases have been improved during recent years, the incomplete understanding the molecular mechanisms behind the disorders and their heterogeneity as described above, have thus far prevented development of effective treatments and therapies.

The classical diagnostic method for mitochondrial disease is a tissue biopsy, most commonly from muscle. Defects in the RC can be detected by histochemistry by simply analyzing the muscle fiber structure. A classical method to asses RC activity is COX/SDH immunohistochemical *in situ* measurement, which measures the relative activities of partially mtDNA-encoded Complex IV and nuclear-encoded Complex II (Old & Johnson, 1989). In addition, RC function can be evaluated by measuring the activity of different RC complexes by respirometry and spectrophotometry from both cell or tissue samples (Frazier & Thorburn, 2012). The availability of next generation sequencing (NGS) has provided the possibility of whole exome sequencing that shows great promise as a diagnostic tool to recognize

mitochondrial disease without invasive biopsies, although it can only be used routinely to screen for known mutations (Lieber et al., 2013; Theunissen et al., 2018).

There is no cure for mitochondrial diseases. Nutritional supplementation such as ubiquinone/ubiquinol, is used to alleviate symptoms of some types of dysfunction but efficient treatments remain to be developed (Di Giovanni et al., 2001) and the best results in fighting mitochondrial diseases has been preventive screening by prenatal testing for known disease markers for families affected by these disorders (Nesbitt et al., 2014). In the future, the availability of extensive omics approaches presents an opportunity to build a comprehensive view of metabolic effects of mitochondrial dysfunction and enable discovery and development of both metabolic and genetic therapies for these complex disorders (Buzkova et al., 2018).

2.5 *Drosophila* as a model

Ever since the times of Thomas Hunt Morgan in the first decades of the 20th century, *Drosophila melanogaster*, commonly known as the fruit fly, has retained its importance in research, and more recently in regard to human diseases. As a model organism, the fly has several advantages including small size, large number of progeny and easy and low-cost maintenance (Yamaguchi & Yoshida, 2018). The physiology of the fly includes many features typical of a complex metazoan, such as sexual behavior, systemic responses and learning ability. The *Drosophila* genome is highly homologous with that of humans, including a major number of recognized human disease genes (Reiter et al., 2001; Yamamoto et al., 2014). However, it is low in genetic redundancy and the anatomy and tissues of the fly are well described due to a long history of basic research. This in-depth knowledge of the animal has led to the establishment of a broad range of techniques and genetic tools; balancer chromosomes unable to undergo meiotic recombination, an extensive archive of mutants, and both tissue and developmental stage-specific expression systems such as UAS-GAL4 and LexA that can be combined with several collections of RNAi fly lines. The exoskeleton of

the fly also enables easily recognizable phenotypes based on structures such as wings, eyes and bristles (Yamaguchi & Yoshida, 2018). All these are available through vast public stock centers and extensive databases as FlyBase (Thurmond et al., 2019; Yamaguchi & Yoshida, 2018).

One disadvantage of *Drosophila* is that, unlike *C. elegans* larvae or mouse germline cells, flies cannot be stored frozen but require constant maintenance as live stocks. Although many fly tissues are functionally analogous to human organs, the functions of certain tissues like the fat body, the 'liver' and adipose tissue of the fly, and the fly 'blood', known as haemolymph, are not 100 % equivalent to their mammalian counterparts and some tissues such as cartilage are completely absent (Ugur et al., 2016).

Drosophila has become a valuable tool in research on metabolic programming and reprogramming (Cox et al., 2017; Owusu-Ansah & Perrimon, 2014). The effects of nutrition on metabolism and development can be easily observed and implementing customized diets for flies is simple and cost-efficient (Birse et al., 2010; Mattila et al., 2018). The short lifecycle allows transgenerational studies, and the fly's metabolic pathways are highly conserved and therefore largely comparable to humans. Metabolism can be studied at tissue level or at the level of the whole organism (Birse et al., 2010; Valanne et al., 2019). Methods and tools for measuring common metabolites such as lipids and circulating carbohydrates are well developed, along with metabolomics protocols, making the fly an optimal model for studying 'big pictures in small scale' (Cox et al., 2017; Tennessen et al., 2014).

The picture can be further manipulated with the help of the various genetic tools listed earlier. One of the most commonly used targeted expression systems is the yeast-derived GAL4-UAS system. In one parental strain a specific promoter region drives expression of GAL4 at a specific developmental stage, in specific cells or tissues. In the other parental strain, the transgene of interest is placed downstream of the GAL4-binding upstream-activating sequence (UAS). When these two parental strains are crossed, GAL4 activates expression of the transgene in the designated

tissue of the progeny. The system may also be used to express RNAi constructs, so as to knock down genes in targeted tissues at a specific developmental time point (Brand & Perrimon, 1993; Kim et al., 2004). The temporal control and tissue specificity of transgene expression has been further fine-tuned by the introduction of an inducible GAL4 protein known as GeneSwitch, that is dependent on the presence of an activator drug, RU486, also known as mifepristone (Osterwalder et al., 2001).

2.5.1 Development

Drosophila is a holometabolous insect; in other words, during the life cycle the animal undergoes a complete metamorphosis. The life cycle of *Drosophila* consists of four phases: embryo, larva, pupa and adult (Figure 2.6). The first phase comprises 17 defined stages of embryogenesis which take place inside the eggshell (chorion), after which the 1st instar larva hatches. The second, larval phase consists of 3 instars during which nutritional resources are accumulated and stored for the pupal phase, during which there is no feeding (Yamaguchi & Yoshida, 2018). This is why the third instar larvae are of particular interest in behavioral studies on foraging and feeding (Belay et al., 2007; Dombrovski et al., 2017). The metabolic rate of *Drosophila* in metamorphosis follows a U-shaped curve, according to which energy consumption is highest in the beginning of the transformation, declines in the middle and increases again during the last phases before eclosion (Merkey et al., 2011). The energy needed for the process is stored in the form of triglycerides in the fat body. In addition to fueling the metamorphosis, the fat body, unlike most larval tissues, does not undergo remodeling but is dispersed as free-floating fat cells. These cells have been postulated to enable a stress response in the newly eclosed adult to either avoid starvation until the first feeding or protect from dehydration until full development of the cuticle (Aguila et al., 2007; Storelli et al., 2019). Once having gained its flight ability shortly after eclosion, the adult will find a mate to restart the cycle. The duration of the cycle is highly dependent on temperature; the higher the temperature, the faster the cycle.

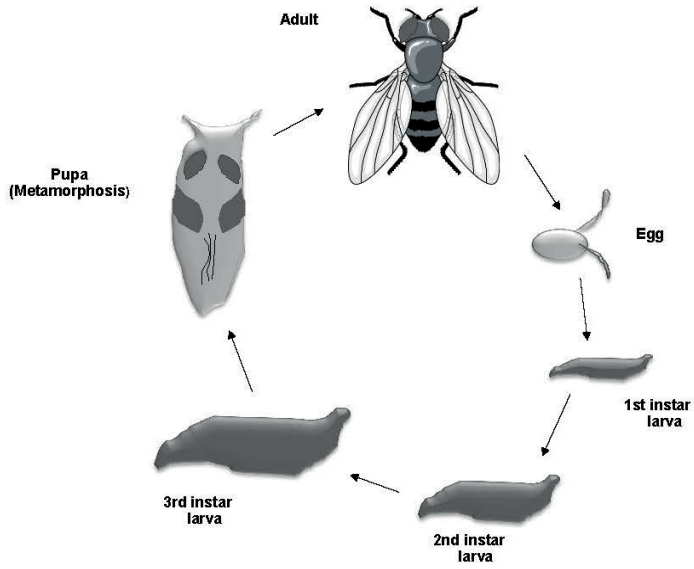

Figure 2.6. Life cycle of *Drosophila*.

2.5.1.1 Spermatogenesis

The male reproductive organs in *Drosophila* consists of two coiled, tube-shaped testes. The process of spermatogenesis starts in the germinal proliferation center at the apical tip of the testis. A stem cell undergoes mitosis producing a new stem cell and a primary spermatogonial cell. While the stem cells remain attached to the apical hub, the primary spermatogonial cell is enclosed by two somatic cyst cells and through gonial mitotic divisions produces 16 spermatocytes forming the spermatogenic unit known as the spermatogonial cyst, within which meiosis and spermatid differentiation will occur (Fuller, 1993). Spermatogenesis is regulated by non-apoptotic programmed cell death, whereby almost one third of the formed cysts are eliminated, never completing the spermatogenesis. In the remaining cysts, spermatocytes undergo meiosis and finally differentiate into 64 elongated spermatids

(Yalonetskaya et al., 2018). The spermatids then go through the invidualization process where a cystic bulge moves along the spermatids from head to tail, a process during which, in an apoptosis-like process, the spermatids lose the major part of their cytoplasm, which is collected in a 'waste bag' structure, after which the spermatids become detached from the cyst (Arama et al., 2003). Mature sperm is then released into the seminal vesicle located in the proximal end of the testis. After copulation, sperm is stored in the spermathecal and seminal receptacle of the female reproductive tract for later use (Fuller, 1993).

2.5.1.2 Nutrition

In nature, the main diet for many *Drosophila* species including *Drosophila melanogaster* is decaying fruit and vegetables. Volatiles such as ethanol attract the flies to arrive at the source on a specific time point of the decay trajectory. The flies, as both adults and larvae, take their essential nutrition from the plant and from microbes involved in the decomposition process (Markow, 2015; McKenzie & Parsons, 1972; Nunney, 1996). A recent study by Brankatschk et al. (2018) suggested that the dietary behaviour of flies is temperature dependent i.e. in a colder climate *Drosophila* prefer feeding on plants over microbes or yeast. Polyunsaturated fatty acids (PUFAs) of plant origin increase the fluidity of lipid membranes and are proposed to help acclimatize the fly's metabolism to temperature changes in the environment (Brankatschk et al., 2018). The latter study was conducted under laboratory conditions on laboratory fly strains and, therefore, comparisons with feeding behaviour in the natural habitat should be made with caution.

Under laboratory conditions, the environment is highly controlled in regard to temperature, humidity and diet. Standard laboratory diet tends to be rich in calories and treated with antimicrobial and antifungal agents, leading to a lack of diversity in microbes and micronutrients (Piper, 2017). One issue in developing an optimal *Drosophila* diet for laboratory use is the variation in nutritional needs in fly strains of

different genetic backgrounds (Aw et al., 2018; Melvin et al., 2018). For example, the optimal protein:carbohydrate ratio for development and egg laying seems to be affected by the mitochondrial haplotype of the fly strain. This, in turn, is linked to the level of mitochondrial metabolism that enables efficient intake and use of nutrients, suggesting that the genotypic background defines, at least to some extent, the metabolic response and stress caused by different macronutrients. Flies also seem to differ in their preference for different types of sugars (Aw et al., 2018). Comparison of the recently diverged *Drosophila* species, *Drosophila simulans* and *sechellia*, demonstrated significant differences in sugar tolerance with changes in genes encoding components of the mitochondrial ribosome (Melvin et al., 2018). Nutritional needs of *Drosophila*, although modest, are difficult to define but linked, at least partially, to mitochondrial metabolism.

Despite the variation in nutritional requirements between different strains, *Drosophila* presents a valuable model for studies connecting diet with phenotypic traits and clinical symptoms of diseases such as diabetes and Parkinson's disease (Bajracharya et al., 2017; Birse et al., 2010; Havula et al., 2013). In addition to conserved metabolic pathways compared to those of mammals and the availability of well-established tools for metabolic studies described above, manipulation of environmental and dietary conditions of flies in order to study genotype-phenotype relations is relatively uncomplicated.

2.6 Transgenic expression of AOX

2.6.1 AOX expression in cells

Ciona AOX was first introduced into human cells in 2006 (Hakkaart et al., 2006). Human embryonic kidney (HEK293T) cells induced to express AOX showed resistance to antimycin, an inhibitor of Complex III, and cyanide, a well-known inhibitor of Complex IV of the RC. This resistant respiration was blocked by the AOX inhibitor n-propyl gallate (nPG), implying that the resistance was AOX dependent. Addition of nPG to AOX–expressing cells in the absence of cyanide had only a mild effect on oxygen consumption, showing that in non-stressed conditions the presence of AOX was not interfering with the function of the classical OXPHOS complexes. Overall, the enzyme did not affect the proliferation of the cells under standard culture conditions, but did decrease superoxide dismutase (SOD) activity, suggesting a role as a functional antioxidant (Hakkaart et al., 2006).

In cell models with cytochrome c oxidase (COX) deficiency, resulting from RNAi knockdown of COX10 in HEK293T-derived cells, or a mutation in COX15 in patient-derived fibroblasts, AOX was able to restore respiration by maintaining electron flow and improving growth in the absence of glucose (Dassa et al., 2009). On the other hand, an increase in glucose was found to impair AOX function in this cell-line, which was deducted to be due to Complex I downregulation (Cannino et al., 2012).

2.6.2 AOX expression in *Drosophila*

AOX was first introduced into *Drosophila* as a highly expressed UAS-construct, to be expressed using the GAL4 or GeneSwitch system (Fernandez-Ayala et al., 2009). It has also been expressed constitutively under the α-tubulin promoter as *tub-AOX* (Kemppainen et al., 2014b). The enzyme, expressed by either means, caused no

detrimental phenotype in the flies under standard growth conditions. The flies developed normally and were fertile. However, when AOX expression was driven by GAL4, flies showed a slight developmental delay of 0.5 day, as well as an exaggerated posteclosion weight loss.

Transgenic AOX in *Drosophila* was inferred to be active *in vivo,* since AOX-expressing flies showed resistance to both antimycin and cyanide (Fernandez-Ayala et al., 2009). When introduced into *Drosophila* models of mitochondrial dysfunction, AOX was able to rescue COX deficiencies caused by partial knockdown of either a structural subunit of Complex IV, Cox6c *(cyclope*) or the Complex IV-assembly factor *Surf1*. AOX was also able to rescue the locomotor phenotype of a *dj-1β* hypomorph, considered as a *Drosophila* model for Parkinson's disease (Fernandez-Ayala et al., 2009). Further studies showed that even with the lower expression levels of *tub-AOX*, the enzyme was able to partially rescue deficiencies caused by knockdown of different COX subunits many of which are lethal or cause severe neurological phenotypes (Kemppainen et al., 2014b).

In *Drosophila*, downregulation of JNK signaling in the dorsal thorax causes a thoracic closure defect that was corrected by expression of AOX. However, the defect could not be overcome by AOX when it was caused by downregulating late steps of the JNK pathway, nor if downstream targets of JNK were targeted (Andjelkovic et al., 2018). AOX has also been introduced into a fly model of Alzheimer's disease (AD), that expresses human amyloid-β (Aβ) peptide that, by forming plaques, is widely considered to be the main pathological cause of AD. In the *Drosophila* AD model, AOX was able to modestly increase the shortened lifespan and blocked oxidative stress that is presumed to be involved in formation of the Aβ plaques (El-Khoury et al., 2016).

However, AOX has not been able to compensate for mitochondrial gene expression defects in *Drosophila* models. AOX expression had no effect on defects caused by mutated *POLG*, the catalytic subunit of the mitochondrial replicase pol γ, nor on defects caused by mutated mitochondrial replicative DNA helicase Twinkle.

In fact, introduction of AOX slightly worsened the larval lethality in Twinkle mutants (Rodrigues et al., 2018). AOX was also unable to rescue the phenotype of *technical knockout*^{25t} (*tko*^{25t}) mutant flies, harbouring a point mutation in the gene coding for mitoribosomal protein S12. The *tko*^{25t} phenotype consists of multiple defects, including developmental delay and sensitivity to seizures caused by mechanical stress known as bang sensitivity, neither of which AOX was able to improve (Kemppainen et al., 2014a). Although able to alleviate RC defects caused by mutations in single subunits of the enzyme complexes and decrease oxidative stress in disease models where ROS is involved, it seems that AOX cannot rescue mitochondrial dysfunction caused by mtDNA expression and translation defects that commonly lead to multiple RC deficiencies.

2.6.3 AOX expression in mice

An AOX-expressing mouse, MitAOX, was first reported in 2013 by El-Koury et al. (2013). The enzyme caused no deleterious effects on the physiology of the animals, although they were slightly lighter in weight and had mildly increased body temperature. The mice showed resistance to cytochrome chain inhibition when exposed to lethal doses of gaseous cyanide. However, the insertion of the AOX being conducted by a non-targeted lentiviral transduction resulted in insertions at multiple genomic sites and low expression levels of the enzyme. In addition, AOX was not expressed in all tissues (El-Khoury et al., 2013). Another transgenic mouse expressing AOX was later reported by Szibor et al. (2017). The gene was expressed as a single copy introduced by targeted insertion at the *Rosa26* locus and was confirmed to produce a functional, ubiquitously expressed enzyme that provided the animal with resistance to cyanide (Szibor et al., 2017).

Mutation in BCS1L, a mitochondrial inner membrane translocase required for assembly of Complex III, causes a severe pathological phenotype in humans known as GRACILE syndrome (fetal growth restriction, aminoaciduria, cholestasis, liver

iron overload, lactic acidosis, and early death during infancy). When crossed with mice with homozygous knock-in of the GRACILE mutation in BCS1L, AOX was able to alleviate multiple pathologies, e.g. increasing lifespan, preventing lethal cardiomyopathy and restoring RC function (Rajendran et al., 2019). AOX has also been shown to protect from cigarette smoke-induced tissue damage in mice (Giordano et al., 2018) and ROS-induced inflammation in a broader study on the role of Complex II in sepsis (Mills et al., 2016).

However, when AOX was introduced into COX15 knockout mice suffering from mitochondrial myopathy, instead of providing rescue, AOX expression worsened the phenotype by shortening lifespan and exacerbating the myopathy (Dogan et al., 2018). This was unexpected since AOX provided partial rescue in COX-knockdown models of *Drosophila* (Kemppainen et al., 2014b). Physiological characterization of AOX-expressing mouse model demonstrated no obvious physiological differences in body composition or cardiac performance on high-fat or ketogenic diet compared to non-transgenic mice (Dhandapani et al., 2019), while AOX-expressing mammalian cells have been found to respond to nutrient availability differently from controls (Cannino et al., 2012). On the other hand, it highlights the need for better understanding of the metabolic effects and regulation of AOX in transgenic models at both the organismal and tissue level. The therapeutic benefits of AOX seem to be limited to specific types or contexts of mitochondrial dysfunction and mapping potential limitations of the enzyme will give a better insight to the type of diseases in which it may be used in as a treatment in future.

3 AIMS OF THE STUDY

The overall aim of this study was to investigate the metabolic consequences and potential disadvantages of the presence of the alternative oxidase in higher eukaryotes/metazoans under environmental stress. The therapeutic potential of AOX demonstrated in several disease models has made it crucial to establish what effects AOX may have on the metabolic state of the organism, particularly in stress inducing conditions that are often encountered in the natural environment.

The strategy I adopted was to expose transgenic *Drosophila melanogaster* expressing AOX to environmental stress by two different approaches, namely by reproductive competition (I) and restricting the availability of nutritional resources (II and III).

1 The aim of the first part of the study was to test the potential selective disadvantage brought on by AOX expression. This was conducted by a natural selection assay, using the sperm competition paradigm and followed up by studying the physiological consequences of AOX expression on the male reproductive organs.

2 In the second part of the study, the aim was to study the consequences of AOX expression on the development of *Drosophila* reared under nutritional restriction. The objective was to identify potential detrimental effects that the enzyme might have when the organism is exposed to a nutritionally constrained environment.

3 The final aim of the study was to determine the mechanism by which AOX expression disturbs *Drosophila* development in a low-nutrient environment, with a focus on metabolic imbalances.

4 MATERIALS AND METHODS

4.1 Fly strains and maintenance

Control and recipient line w^{1118}, the transgenic, ubiquitously expressed GAL4-driven control line UAS-GFP (Stinger, insertion at chromosome 2), ubiquitously expressed GAL4-driver lines *daughterless*-GAL4 (*da*-GAL4) and *tubulin*-GeneSwitch (*tub*GS) were obtained from stock centres. GAL4-controlled transgenic lines for *C. intestinalis* AOX UAS-AOX^{F6} (chromosome 2) and UAS-AOX^{F24} (chromosome 3) were constructed and described previously (Fernandez-Ayala et al. 2009). Also, the AOX-expressing lines tub-AOX^{35}, tub-AOX^{112} and tub-AOX^{7} (chromosomes X, 2 and 3, respectively) with the ubiquitous α-tubulin promoter have been described before (Kemppainen et al., 2014b) and used to generate the lines designated 2xtub-AOX (tubAOX^{112} and tubAOX^{7} insertions) and 3xtub-AOX to which the tubAOX^{35} has been added (Kemppainen et al., 2014b). Flies were maintained and cultured on standard high-sugar diet at 18 °C (maintenance) or 25 °C (expansion/collection), with 12 h light/dark cycles, except where stated.

Table 4.1. *Drosophila* lines

Drosophila line	Tissue distribution	Chromosome(s)
w^{1118}	Genetic background	
UAS-GFP (Stinger)	Ubiquitous	2
da-GAL4	Ubiquitous (not in testis)	3
tub-GS	Ubiquitous (not in germline)	3
UAS-AOX^{F6}	Ubiquitous	2
UAS-AOX^{F24}	Ubiquitous	3
tub-AOX^{35}	Ubiquitous (not in germline)	x
tub-AOX^{112}	Ubiquitous (not in germline)	2
tub-AOX^{7}	Ubiquitous (not in germline)	3
tub-AOX^{112};*tub*-AOX^{7}	Ubiquitous (not in germline)	2, 3
tub-AOX^{35};*tub*-AOX^{112};*tub*-AOX^{7}	Ubiquitous (not in germline)	x, 2, 3

4.2 Sperm-competition assays

Sperm-competition assays were carried out by mating w^{1118} females (white eyes) first with w^{1118} males (white eyes), followed by mating with transgenic males (red eyes, *w*+) in the so-called 'offensive' approach and *vice versa* (i.e. first the transgenic male) in the so-called 'defensive' approach. The reproductive success of the male was then defined by counting the number of red- and white-eyed progeny. Approximately 50 virgin females (3-7 days old) were allowed to mate individually with 5–8 day-old w^{1118} males for 3 days. The males were discarded and the females transferred to new vials where they were allowed to mate individually with transgenic males for 3 days, after which the males were again discarded. The females were transferred to new vials, allowed to lay eggs for 3 days, and then transferred to the last set of vials for an additional 5-day egg laying. In the second approach, w^{1118} virgin females were first crossed with transgenic males and then with w^{1118} males, following the mating and egg-laying scheme of the first approach. Only vials containing progeny with both eye colors were included, to ensure dual mating of the female.

4.3 Dissection and imaging of fly testes

Drosophila testes were dissected by anesthetizing males with CO_2 prior to transferring to a dissection plate containing phosphate buffered saline (PBS). Using thin dissection forceps, the internal organs were removed from the abdomen by dislocating the external genitalia, followed by manual isolation of the testes, which were then imaged immediately using the Nikon SMZ 745 T system under white light. The images were analyzed and testis and SV thickness were measured using NIS Elements D4.20 software (Nikon Instruments Software, Netherlands). To induce AOX expression, UAS-AOX males bearing an inducible *tub*GS driver were reared in the presence of 200 μM RU486 (mifepristone, Sigma) for the number of days indicated in the figures.

4.4 Fly diet and supplementation

For low-nutrient experiments, the standard high-sugar diet was limited to two ingredients: glucose as a carbohydrate source and yeast as a source of amino acids. Specific supplements were added as detailed in Table 4.1.

Table 4.2. Composition of standard diet and restricted diet. Glucose (D(+)-Glucose anhydrous, VWR), yeast (Algist-Bruggeman, Instant SD), sucrose (D(+)-Saccharose, VWR), maize flour (Risenta), soy flour (Soyolk, Oriola Oy), treacle (Lyle's Black Treacle), wheat germ (Elovena Plus Vehnänalkio). *Glucose percentage was lowered in later diet experiments to better respond to the percentage present in the standard diet. The glucose percentage used is presented with the results from each experiment.

Ingredient	*Standard (high sugar)*	*Restricted*
Glucose %	*3*	*3 – 5**
Yeast %	*3.5*	*3.5*
Sucrose %	*1.5*	
Maize flour %	*1.5*	
Soy flour %	*1*	
Treacle %	*3*	
Wheat germ %	*1*	
Agar %	*1*	*1*

Table 4.3. Fly diet supplements.

Supplement	*Dosage*	
B-vitamins	*1 tablet / 300 ml*	*Beko Strong (Orion)*
Vitamins + minerals	*1 tablet / 300 ml*	*Multi-tabs® Family (Pfizer)*
Iron	*0.08 mg-0.8 mg/ml (Li, 2010)*	*Ammonium iron(III)citrate*
Citrate	*0.2 – 4 %*	*Trisodium citrate dihydrate*
Antibiotic	*100 µg/ml (Toivonen et al., 2001) or 15 µg/ml*	*Doxycycline*

All diets were prepared by mixing and heating the ingredients (and the supplement) and boiling for 5 min after which the mixture was allowed to cool down to 65 °C while mixing. Antimicrobials (0.1 % nipagin and 0.5 % propionic acid) were added and the mixture was divided into vials and let set for 30-45 min. Unlike other supplements, doxycycline was added on top of the dried food inside a fume hood.

4.5 Fractionation of treacle

4.5.1 Ether fractionation

To separate hydrophobic compounds from the treacle (Lyle's Black Treacle), it was diluted in water by heating and then cooled down to room temperature. To compensate for loss of material during the fractionation process, the amount of treacle (diluted to 4.5 % in water) was adjusted so as to be 1.5 times greater than in the standard diet (3 %). After cooling, the solution was poured into a separation funnel and mixed 1:1 with ether (diethyl ether, Sigma). Both fractions (ether and water) were collected and poured into their own funnels. The water-soluble fraction was washed twice 1:1 with ether and the ether-soluble fraction was washed twice with water to ensure removal of any non-soluble compounds. Both fractions were collected in beakers and left in a fume hood overnight to evaporate the ether. An equal volume of water was added to the ether-soluble fraction to prevent complete drying out of the compounds.

4.5.2 Ethanol precipitation of the water-soluble fraction of treacle

The water-soluble fraction of treacle obtained from the fractionation was divided into centrifuge tubes and mixed with ethanol at a proportion of 40 %, 60 %, 65 % or 75 %. The mixtures were allowed to precipitate at -20 °C overnight after which they were centrifuged at 14 000 g_{max} for 30 min at 4 °C. Each supernatant was collected into a beaker and the pellets were resuspended in water. The supernatant was heated but not boiled until all the ethanol had evaporated and was then replaced by water. The fractions (ether-soluble, water-soluble supernatant and pellet) were then individually combined with the ingredients of the restricted diet and prepared as fly food as described above.

4.6 Developmental assays

To test effects of the composition of the culture medium, flies of a given genotype or crosses were reared on standard high-sugar diet or on the nutritionally restricted diet with or without supplements. Crosses (10 females + 5 males) were first made on standard diet and after 24 h transferred onto the test medium for egg laying over 1-2 days. The flies were then grown at 25 °C except where stated in the Results. Eclosion rate was calculated by counting the number of eclosed adults relative to the number of pupae per vial.

4.7 Protein extraction and analysis by Western Blotting

Total protein of 10 L3 larvae or flies was extracted by crushing in lysis buffer, containing 0.3 % SDS in PBS + protease inhibitor cocktail (Roche). The samples were incubated for 15 min at room temperature and centrifuged for 10 min at 15,000 g_{max} at room temperature. The supernatants were collected and the protein concentrations were measured by NanoDrop spectrophotometry (ThermoFisher Scientific). The protein aliquots were diluted to equal concentrations (3 µg/µl) with water and 5 x SDS-PAGE loading buffer, separated on 12 % polyacrylamide gels and wet-blotted to a nitrocellulose membrane. For immunodetection, the membrane was washed in PBS-Tween (0.05 %), blocked for 3 h with 5 % nonfat milk in PBS-Tween at room temperature and then allowed to react with custom-made rabbit anti-AOX antibody (Fernandez-Ayala et al., 2009) 1:10,000 overnight at 4 °C. After 5 x 5 min washes in PBS-Tween, the membrane was incubated with secondary antibody (goat anti-rabbit, Vector Laboratories, 1:10,000) for 1 h at room temperature and washed again 5 x 5 min in PBS-Tween. Finally, the membrane was processed for imaging using Luminata™ Crescendo (Millipore) for 5 min and imaged for chemiluminescence using BioRad ChemiDoc MP. Equal loading of protein was then

confirmed by Ponceau S staining. Images were optimized for brightness and contrast and cropped, rotated and masked for clarity, but not manipulated in any other way.

4.8 Metabolite assays: Lactate/pyruvate, triglycerides

For lactate/pyruvate assays 10 L3 larvae were homogenized in 6M guanidine hydrochloride on ice and incubated at 95 °C for 5 min. The samples were then centrifuged at 4 °C at 12,000 g_{max} for 5 min and the supernatant was transferred to a fresh vial to be stored at -80 °C.

4.8.1 Lactate & Pyruvate assays

Standards and reaction master mix were prepared as instructed in the manufacturer's protocol (L-Lactate assay kit/Pyruvate assay kit, Sigma). In the assays, lactate and pyruvate are oxidized to be then recognized by a colorimetric probe. The samples were thawed and diluted 1:10 in water on ice. The reactions were performed in a black 96-well microplate-reader plate by mixing with the reagent master mix 1:5 and incubated at room temperature for 30 min. The fluorescence of the reaction was measured at Excitation/Emission 535/590 with 1 s read time (Plate Chameleon™ V, Hidex). The values were normalized to protein concentrations based on the Bradford assay (Bradford reagent, Sigma).

4.8.2 Triglyceride assay

Triglyceride assay was conducted using Triglyceride reagent (ThermoFisher Scientific). With the reagent, the triglycerides are enzymatically hydrolyzed to free fatty acids and glycerol. The glycerol is further phosphorylated to glycerol-3-phosphate which when oxidized produces hydrogen peroxide (H_2O_2). Together with 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzene sulfonate, H_2O_2 produces a

red colored dye, absorbance of which is proportional to the concentration of triglycerides present in the sample. For the assay, 10 L3 larvae were homogenized in PBS-Tween (0.05%) and incubated at 70 °C on a heat block for 5 min. After cooling, the samples were vortexed, mixed 1:20 into Triglyceride reagent in a transparent 96-well plate and incubated at 37 °C for 30 min. The absorbance was measured at 540 nm and normalized to protein concentration as above.

4.9 Respirometry using treacle fractions

The water fraction of treacle, following the ether fractionation, was dried and resuspended in water at 8 x concentration to enable a reasonable pipetting volume into the respiration chamber in the same concentration as when used as a supplement in the *Drosophila* diet.

AOX expression was induced in T-REx™-293 AOX cells (Hakkaart et al., 2006) using doxycycline (1 μg/ml) for 72 h before measurements were made. For respirometry, 5×10^6 cells were permeabilized by digitonin (80 μg/ml) in respiratory buffer A (225 mM Sucrose, 75 mM mannitol, 10 mM Tris-HCl, 1 mM EGTA, 1 mg/ml BSA (fatty-acid free, Fisher Scientific), pH 7.4) at 37 °C. Oxygen consumption was measured with a Clark-type electrode (Hansatech Oxytherm system). The substrate concentrations used were: pyruvate + malate + glutamate 20 mM each, succinate 20 mM, ADP 5 mM. To determine AOX-dependent respiratory activity antimycin A (60 ng/ml) was used to block cyt c reduction and n-propyl gallate was used at 0.2 mM for AOX inhibition to verify that the antimycin A-resistant oxygen consumption was due to AOX. All measures were corrected by subtracting the residual oxygen consumption present after full inhibition of the respiratory chain.

4.10 Mass spectrometry

To obtain a comprehensive list of the components of treacle, a set of treacle fractions was prepared as described above and analyzed by mass spectrometry. Samples were prepared by ether fractionation, after which ethanol precipitation was performed on the water fraction at 40 %, 60 % or 75 % ethanol. One ml of each fraction (3 ether fractions and pellet and supernatant from the 3 different ethanol precipitations) was aliquoted. The composition of these treacle fractions was analyzed using mass spectrometry (GC-qTOF-MS) by Dr. Esko Kemppainen from University of Turku.

4.11 Statistical analysis

For the sperm-competition assays, the statistical differences among the proportions of progeny (Clark et al. 1995) were analysed using one-way ANOVA followed by Tukey post hoc test. Similarly, statistical differences in testis thickness were analysed using one-way ANOVA followed by Newman-Keuls post hoc test, since this test is less dependent on sample normality.

For the developmental assays, pairwise comparisons were conducted with Student's T-test using Bonferroni correction when more than two groups were compared. One-way ANOVA followed by Tukey post hoc test was performed when comparing multiple levels of a single factor. The analyses were performed using GraphPad Prism Software (USA).

5 RESULTS

5.1 AOX-expression causes reproductive disadvantage to males in sperm-competition assays (I)

Postcopulatory sexual selection by *Drosophila* females occurs by a substitution mechanism where the sperm from a previous mating is commonly dislodged and replaced by the sperm from a new mating, except in cases of defective or compromised sperm (Figure 5.1). Based on this mechanism, I conducted a sperm competition assay to test the reproductive fitness of sperm produced by AOX-expressing males. Flies carrying three copies of the ubiquitously expressed α-tubulin-AOX construct (3xtub-AOX line: genotype tub-AOX^{35}/Y; tub-AOX^{112}/ tub-AOX^{112}; tub-AOX^{7}/tub-AOX^{7} (Kemppainen et al., 2014b) were compared to wild-type males of the same genetic background (*w*^{1118}). In the so-called 'defensive' approach, AOX-males (3xtub-AOX) were first allowed to mate with virgin *w*^{1118} females, which were then mated a second time with *w*^{1118} males in the absence of AOX-males. The progeny was analyzed based on the eye-color marker (red from transgenic males, white from *w*^{1118} males). The result (Figure 5.2B) was in accordance with expectations based on the previously published data where the initial progeny are of the first male but, after the second mating, were replaced by the progeny of the second male (Clark et al., 1995) i.e. the sperm from the first mating with the 3xtub-AOX male were dislodged and replaced by the sperm from the *w*^{1118} male. In contrast, when the *w*^{1118} male was presented as the first male and challenged by the sperm from the 3xtub-AOX male, the major proportion of the progeny continued to be of the *w*^{1118} male even after the second mating, suggesting a fitness-based selection against the 3xtub-AOX sperm (Figure 5.2A). The prevalence of white-eyed progeny remained even 10 days after the original mating. Control experiments

showed this to be a trait specific to AOX and not to transgenic expression in general (I/Figure S1). The fitness deficiency of sperm from tub-AOX males was dependent on the expression dose of the tub-AOX transgene, as the impairment was less drastic for males with only two copies of the tub-AOX transgene and no difference was detected between control males and tub-AOX males bearing only one copy of the tub-AOX transgene. However, expressing the GAL4-dependent UAS-AOX transgene with the ubiquitous *da*GAL4 driver caused no effect on the sperm competitiveness (I/Figure S3) suggesting that the defect is dependent on cellular expression pattern as well as the expression dose.

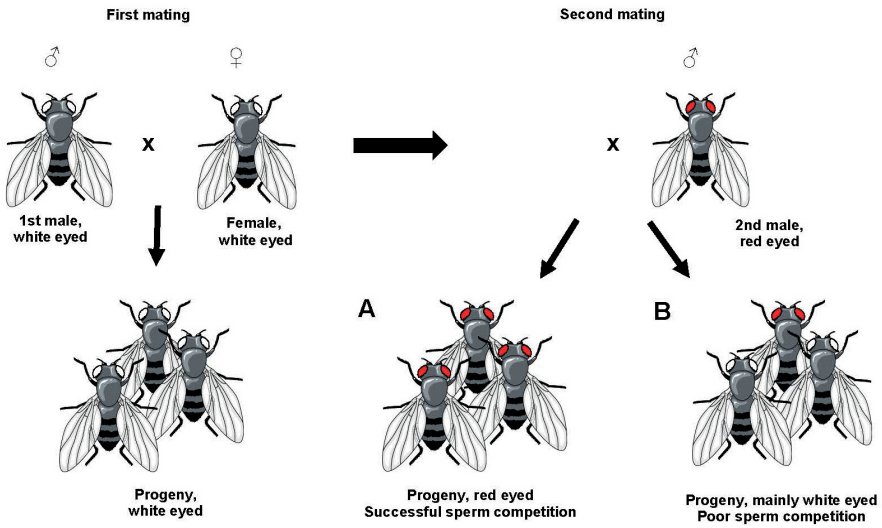

Figure 5.1. Offensive approach of sperm competition assay. In a successful case of the defensive approach of sperm competition assay (A) majority of the later progeny present the phenotype of the male from the second mating. In a case of unsuccessful sperm competition (B), majority of the progeny continues to be off the first male suggesting a counter selection against the sperm of the second male. Adapted from Clark et al. (1995) and Servier Medical Art.

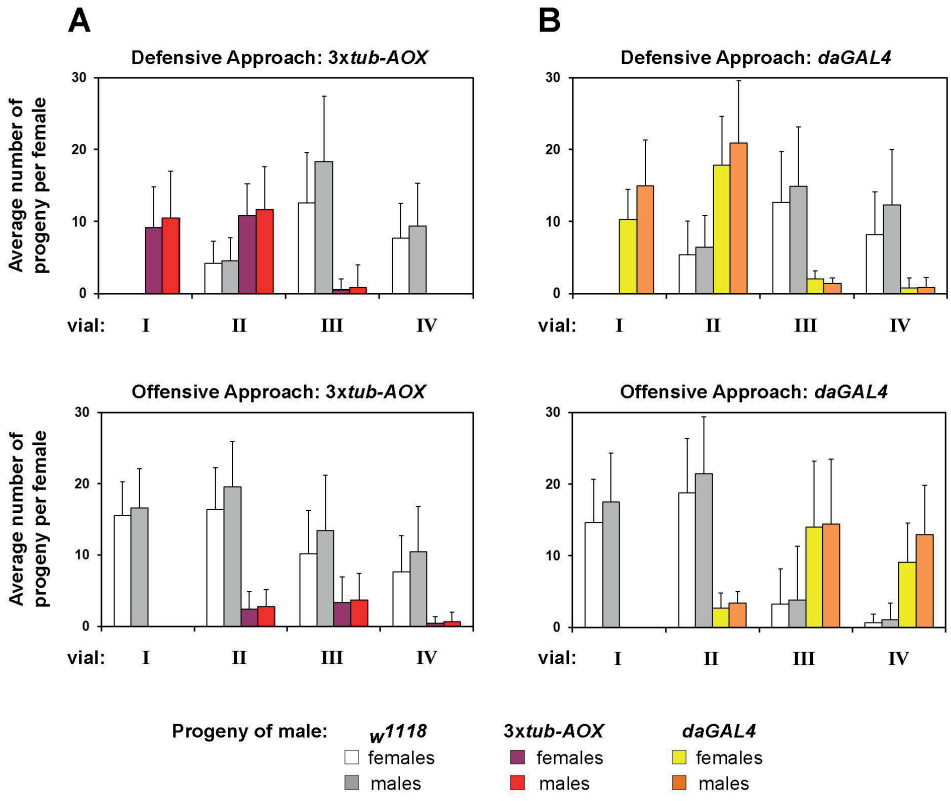

Figure 5.2. AOX-expressing males give rise to fewer progeny in the 'offensive' approach of the sperm competition assay. Females were mated with the first male in vial I, after which the male was discarded and the female allowed to lay eggs for 3 days. Mating with the second male took place in vial II. In the control experiment (B), comparing w^{1118} (otherwise wild-type) and daGAL4 males, the subsequent progeny was mostly from the second male. The opposite result was observed using AOX males, where a major proportion of the progeny in vials III and IV (A, lower panel) continued to be from the first (wild-type) male, indicating a counter-selection or decrease in the number of AOX-sperm. Numbers on the y-axis represent the mean number of red- and white-eyed offspring (error bar ±SD). Modified from Figure 1 (I).

5.2 AOX-expressing males fail to accumulate mature sperm in the seminal vesicle of the testis (I)

The viability of the male's sperm inside the *Drosophila* female reproductive tract depends on the quality and/or quantity of the spermatozoids deposited during mating. To check the functionality of spermatogenesis in the 3xtub-AOX males, the reproductive organs in adult males of different ages (days 1, 3 and 10) were dissected and quantified. In control flies, the thickness of the testes decreased more than 50 % over 10 days while the accumulating mature sperm tripled the size of the seminal vesicles (Figure 5.3C). In the case of 10-day-old 3xtub-AOX males, whitish material presumed to be mature sperm accumulated in the proximal end of the testis while the seminal vesicles, where mature sperm is normally delivered for storage prior to mating, remained almost empty (Figure 5.3A). The motility of AOX male-derived spermatozoids seemed unaffected.

The phenotype was reproduced, using UAS-AOX in combination with the tubGS driver. The driver is controlled by the same α-tubulin promoter as used in the 3xtubAOX constructs and the expression is controlled via a modified mifepristone-(RU486-) inducible GAL4 (GeneSwitch). The inducible driver enables activation of the expression of the transgene to be activated at a chosen developmental stage and the promoter expresses the gene in the same tissues as the tubAOX constructs. Indeed, the testes of tubGS-driven AOX males at 10 days of age in the presence of mifepristone remained thick, with accumulated whitish material, but with largely empty seminal vesicles at 10 days of age (Figure 5.3B). In contrast, in the absence of mifepristone the phenotype was as controls. No accumulation of whitish material at the proximal end of the testis was detected in this assay, using the control transgene GFP, catalytically inactive mutAOX or the driver line alone without a transgene (I/Figure S6 and S7). In the presence of both the drug and the driver, the seminal vesicles did show a sperm storage deficiency (I/Figure S6) but the thinning of the testis suggests no dysfunction in spermatogenesis itself. Together with the finding of no derangement of the mature sperm amount or localization in males expressing

AOX driven by daGAL4, we confirmed that the phenotype is α-tubulin promoter-specific. Note that the α-tubulin promoter is active in testis although not in the germline itself, while *daughterless* and daGAL4 are expressed in the female but not in the male germline or reproductive organs (Bo & Wensink, 1989; Cummings & Cronmiller, 1994). The observations listed above were further confirmed with immunostaining and confocal microscopy (I/Figure 3, 4 and S9, performed by Andjelkovic, A., Garcia, G.S. and Oliveira, M.T.) along with the presence of Sex Peptide, a component of seminal fluid, in the reproductive organs. This does not exclude the possibility of alterations in the composition of the seminal fluid that may play a central role in the survival or compatibility of the sperm in the female reproductive tract.

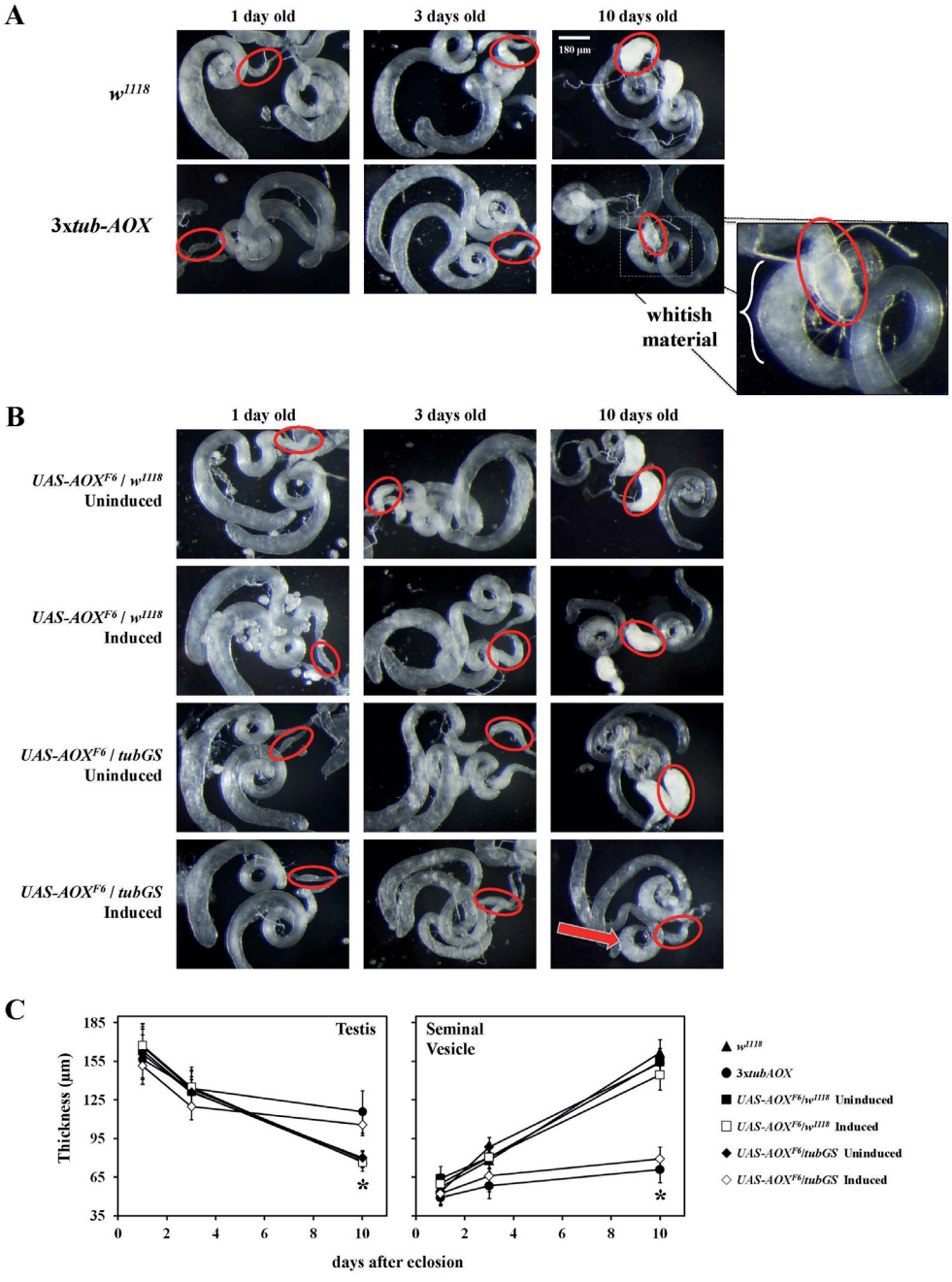
A
1 day old
3 days old
10 days old
180 µm
w1118
3xtub-AOX
whitish material
B
1 day old
3 days old
10 days old
UAS-AOXF6 / w1118
Uninduced
UAS-AOXF6 / w1118
Induced
UAS-AOXF6 / tubGS
Uninduced
UAS-AOXF6 / tubGS
Induced
C
Testis
Seminal Vesicle
Thickness (µm)
185
155
125
95
65
35
0
2
4
6
8
10
days after eclosion
w1118
3xtubAOX
UAS-AOXF6/w1118 Uninduced
UAS-AOXF6/w1118 Induced
UAS-AOXF6/tubGS Uninduced
UAS-AOXF6/tubGS Induced

Figure 5.3. Mature sperm of AOX-expressing males do not accumulate in seminal vesicles. (A) w^{1118} accumulate mature sperm in the seminal vesicles while the vesicles of 3xtubAOX males remain empty, even after 10 days. Accumulation of 'whitish material' is detected at the proximal end of the testes from 3xtubAOX –males. (B) A similar phenotype was observed when AOX was expressed using an inducible tubGS-driver, while the UAS-AOX^{F6} construct without the driver, whether with or without the inducing drug, gave the same testis morphology as wild-type males. (C) Quantification of the testis and seminal vesicle sizes confirm an increased thickness of testis but significantly shrunken seminal vesicles in the presence of AOX-expression while in the non AOX –expressing males the result is the opposite. Numbers represent the mean thickness of 10–20 dissected organs (±SD) and *One-way ANOVA ($p < 0.01$). Modified from Figure 2 (I).

5.3 AOX-expressing flies show pupal lethality on nutrient-poor diet (II)

Characterization of AOX-expressing flies in standard laboratory conditions, i.e. standard high-sugar diet at 25 °C (Fernandez-Ayala et al., 2009) previously revealed a minor developmental delay along with a slight weight loss for adult flies compared to wild-type flies. These observations led to the hypothesis that the presence of AOX may cause an impairment in the efficient use of nutrients during development of the flies. To test this proposition, a developmental assay was conducted where AOX-expressing flies (daGAL4-driven UAS-AOX) were reared on nutrient-poor or restricted diet. The composition of the restricted diet was limited to two components: yeast (3.5 %) and glucose (3-5 %) as sources for amino acids and sugar, respectively. Developmental assays showed that the egg-to-pupa survival ratio remained the same as controls in both standard and restricted diet (II/Figure 2C, III/Figure 1b). The pupa-to–adult ratio for AOX-expressing flies was also the same as controls, on standard diet (Figure 5.4A). However, this ratio was significantly different (~20 % compared to ~80-90 % for controls) on restricted diet (Figure 5.4A), suggesting that the presence of AOX causes a developmental defect at the stage of metamorphosis when nutritional resources are limited. The phenotype was observed with both UAS-AOX lines (F6 and F24) but not with flies expressing the control transgene UAS-Stinger (GFP), UAS-AOX without driver or the daGAL4 driver line on its own (Figure 5.4A,B), confirming that this is an AOX-specific effect. The phenotype was also dose-dependent as the UAS-AOX$^{8.1}$ line, with a much lower expression level of the AOX transgene did not show a decrease in eclosion rate compared to controls or flies cultured on standard diet, nor did the flies expressing the inactive mutAOX (II/Figure 3C). The pupal lethality of AOX-expressing flies on restricted diet occurred at different stages of metamorphosis instead of at a specific developmental stage (Figure 5.4C), suggesting that developmental failure was related to a general component required during or throughout pupal

development. The sex ratio of the eclosed flies remained normal suggesting that the defect affects females and males equally.

5.4 Pupal lethality of AOX-expressing flies is temperature dependent (II)

AOX is known to be involved in heat generation in organisms where the enzyme is endogenously expressed (Grant et al., 2008; Onda et al., 2008). To test whether the observed developmental phenotype was temperature sensitive, AOX-expressing flies were reared on restricted diet at different temperatures (22, 25, 26, 27 and 29 °C) (II/Figure 4A). The severity of the phenotype worsened with the increase of temperature while lowering it to 22 °C improved the eclosion rate of AOX-flies to almost normal on the nutrient-poor diet. Already an increase of 1 degree from 25 to 26 °C led to almost 100 % pupal lethality on restricted diet and at 27 °C even the flies developing on standard diet were affected i.e. barely any AOX-flies eclosed. At 29 °C even the non-AOX-expressing control flies exhibited increased pupal lethality on the restricted diet. The temperature sensitivity could be at least partially explained by the fact that GAL4, which is of yeast origin, is known to be itself temperature sensitive (Brand & Perrimon, 1993). An increased GAL4-dependent expression of AOX at the higher temperatures was confirmed by Western blotting to an AOX antibody (II/Figure 4B), in accordance with the dose-dependence of the phenotype. The phenotype appearing in control flies at high temperature does, however, suggest a thermogenic effect of the enzyme as well.

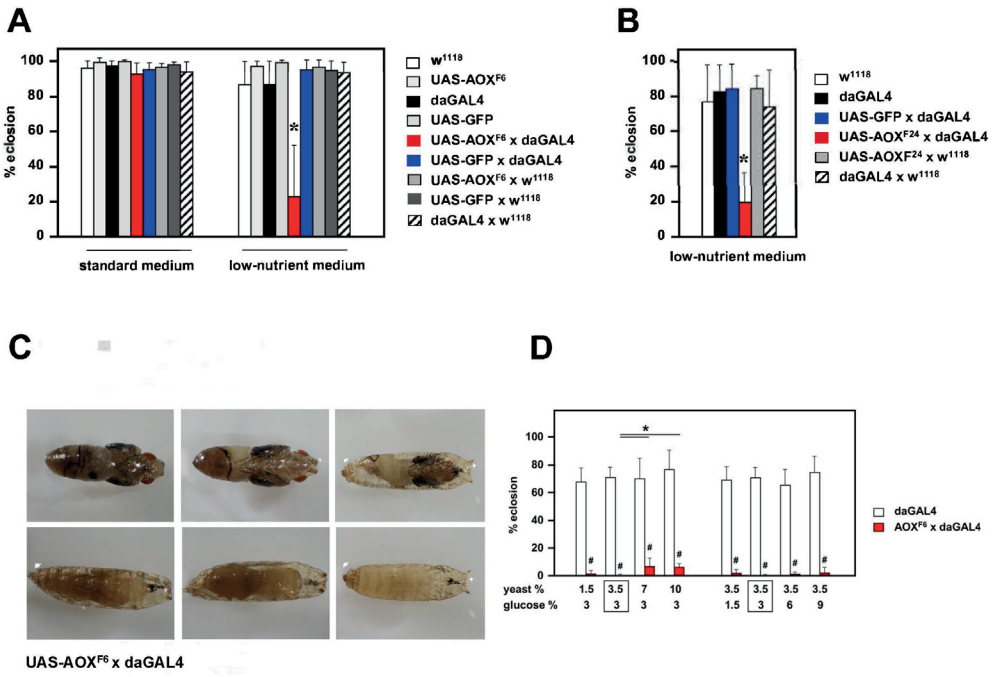

Figure 5.4. Expression of AOX leads to non-stage specific pupal lethality in nutrient-poor diet. (A) In standard medium, AOX-expressing flies (UAS-AOX^{F6} x daGAL4) eclosed in equal proportions compared to controls while in low-nutrient medium AOX-expression was associated with an increase in pupal lethality as only ~20% of the flies completed development and eclosed. (B) Another UAS-AOX line, UAS-AOX^{F24} demonstrated the same phenotype of decreased eclosion rate, verifying the phenotype to be AOX-related and not specific to the insertion site or fly line used. (C) Light microscopy images show that AOX-expressing flies died at different stages of metamorphosis in low-nutrient medium. (D) Supplementation with yeast or glucose did not rescue the pupal lethality on low-nutrient medium. The numbers (A, B, D) represent the mean percentage of pupae that eclosed per vial (±SD). *One-way ANOVA, $p<0.01$, # Student's t test, $p<0.001$. Modified from Figure 3 of (II) and Figure 2 of (III).

5.5 AOX-related pupal lethality is not rescued by increase in sugars or amino acids in the nutrient-poor diet (III)

To test whether the phenotype is simply due to an increased calorie consumption of AOX-expressing flies, the developmental assay was conducted with either increased sugar content from 5 % to 10 % or yeast content from 3.5 % to 10 %. The additional supplementation of either component did not decrease the pupal lethality (Figure 5.4D) suggesting that the developmental shortcoming is not due to simple calorie restriction or to glucose or amino acid deprivation. Removing glucose entirely worsened the phenotype while wild-type flies were able to develop normally under such conditions (unpublished data). However, retaining the glucose but decreasing yeast content to 1 % led to defects in the development of control flies as well (II/Figure 4C).

5.6 Pupal lethality is not due to decreased triglyceride storage in AOX-expressing L3 larva (III)

During the larval stage, flies accumulate biomass, so as to store sufficient fuel to survive the non-feeding phase of metamorphosis. The energy is stored as triglycerides that are broken down to provide resources for the elaborate process of tissue reorganization. To determine whether the expression of AOX results in insufficient accumulation of triglycerides in larvae, triglyceride levels were compared in AOX-expressing, wandering-stage L3 larvae reared on restricted diet and standard high sugar diet with the corresponding values from non-AOX expressing larva. No significant difference was found between the genotypes, implying that the larvae are able to accumulate triglycerides in normal amounts despite the presence of AOX (III/Figure 7A). However, regardless of the genotype, there was a significant difference in triglyceride levels when comparing larvae grown under low-nutrient conditions to those reared on nutrient-rich diet (III/Figure 7A). Although this level of storage may be enough for control flies to complete development, it is implied to

be insufficient to compensate for a possible increase in energy expenditure caused by AOX activity.

5.7 AOX-expression does not affect lactate or pyruvate levels in L3 larva on nutrient-poor diet (III)

To evaluate possible consequences of AOX-expression on the main energy metabolism pathways, such as increased depletion/accumulation of pyruvate or increase in energy production through fermentation, the levels of pyruvate (III/Figure S4A) and lactate (III/Figure 7B) were measured in L3 larvae. Once again, no difference was detected between the genotypes, indicating that AOX-expression does not affect the levels of either pyruvate or lactate. The significant difference seen between larvae fed on the two different diets (III/Figures 7B/S4) confirms a change in metabolism due to restricted availability of resources.

5.8 AOX-related pupal lethality is rescued by treacle supplementation in the nutrient-poor diet (III)

In standard high glucose diet, AOX-expressing flies survive metamorphosis at the same frequency as wild-type flies. Therefore, the complementing nutrient(s) required to overcome the pupal lethality of AOX-expressing flies on the low-nutrient diet must be present in the standard diet. In addition to yeast and glucose, the standard diet is a mixture of several complex ingredients (sucrose, maize flour, soy flour, treacle and wheat germ). To determine which ingredient(s) complement the developmental defect, each component was added to the restricted diet individually. Supplementation with sucrose did not improve the eclosion survival rate but all other components of the standard diet restored the eclosion rate to near normal (Figure 5.5A), although the effect of wheat germ was slightly weaker than that of the other

components. On the other hand, removal of individual ingredients from the standard diet did not cause increased pupal lethality (III/Figure 2A).

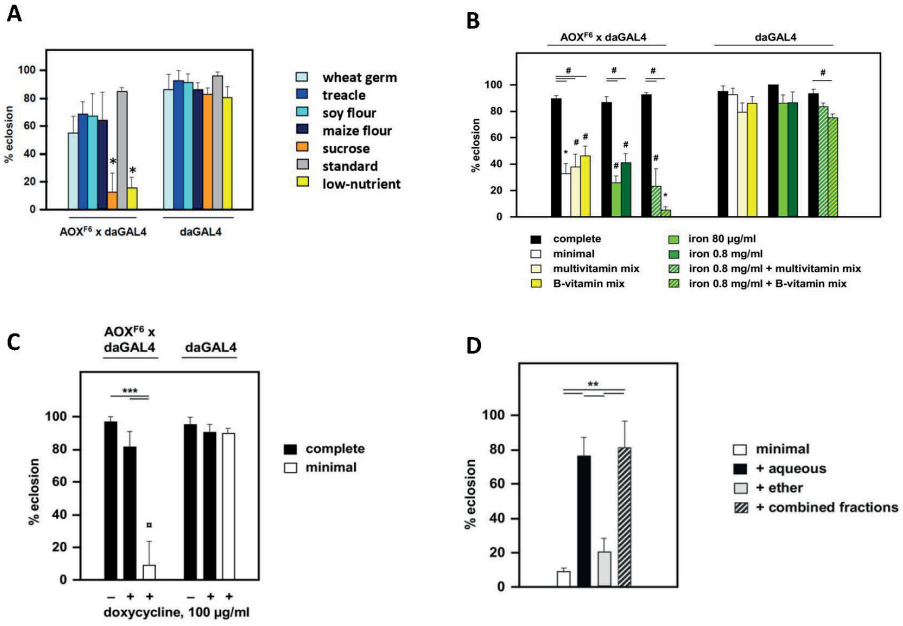

Figure 5.5. Supplementation with water-soluble fraction of treacle rescues pupal lethality of low-nutrient diet. (A) A number of complex ingredients, but not sucrose, decrease pupal lethality of AOX-flies on low-nutrient medium. that is rescued or partially rescued by several complex ingredients of standard medium. *One-way ANOVA, $p<0.01$. (B) Complementation with vitamins and/or minerals presumed to be present in treacle had no effect on the phenotype. One-way ANOVA with Tukey post hoc HSD test, $p<0.01$ (horizontal lines with #). Student's t test (individual bars with * ($p<0.001$) or # ($p<0.01$)). (C) Decrease of commensal microbiota by doxycycline did not affect eclosion rates of AOX-flies or control flies in neither complete or minimal diet. ***One-way ANOVA with Tukey post hoc HSD test, $p<0.01$. ¤Student's t test ($p<0.05$). (D) Further biochemical analysis by ether fractionation to pin down the chemical properties of the complementary compound(s) in treacle suggested the active compound(s) to be water-soluble, therefore excluding fatty acids or hydrophobic vitamins. **One-way ANOVA with Tukey post hoc HSD test, $p<0.01$. Modified from Figures 2, 3, 4 and 5 of (III).

AOX-related pupal lethality is not due to increased need for vitamins (III)

The most intriguing of the ingredients able to complement the defect was treacle. Treacle is a viscous syrup derived from sugar cane as a side product in the process of sugar refinement and is mainly composed of sugars. While almost entirely lacking in amino acids and fatty acids, treacle is known to contain several vitamins and minerals, particularly iron, which suggests that AOX flies may be depleted in one or more of these components, possibly combined with a deficiency of some specific sugars. To test whether the pupal lethality phenotype of AOX-expressing flies could be reversed by the addition of vitamins, the restricted diet was supplemented with either a mixture of vitamins and minerals or a mixture of B-vitamins. Addition of these supplements had no effect on the phenotype (Figure 5.5B) and neither did fructose (III/Figure 3B); however, it should be taken into account that the amounts used were rather arbitrary and not optimized for flies, as the supplements were obtained from prescription-free supplement tablets for human consumption.

5.9 Iron supplementation does not reverse pupal lethality of AOX-expressing flies (III)

AOX is a metalloenzyme with a non-heme diiron core at the active site. This together with the positive effects of treacle, a known source of iron, raised the question whether AOX-expressing flies might suffer from iron deficiency in the nutrient-limited environment. To test this idea, the flies were reared on restricted diet supplemented with 0.08 mg/ml or 0.8 mg/ml iron (ammonium iron(III)citrate). Iron supplementation did not have an effect on the phenotype (Figure 5.5B), whether provided alone or in combination with vitamin supplements.

5.10 Amount of commensal microbiota is not critical for development of AOX-expressing flies (III)

The role of gut microbiota in different nutritional and disease states has gained much attention in recent years and they are known to be involved in digestion and metabolism of several vitamins as well as minerals. To test whether the commensal microbiota of AOX-expressing flies was compromised, or that they were crucial to the survival of wild-type flies on low-nutrient diet, the flies were fed with 3 different diets supplemented with or without an antibiotic: standard diet, restricted diet and maize flour (1.5 %) supplemented restricted diet. Doxycycline was chosen as the antibiotic based on a previous study (Toivonen et al., 2001) where the effects of it were thoroughly tested in flies at different concentrations. Any decrease in the growth of commensal microbes produced by antibiotic treatment did not affect the development of either AOX-expressing or wild-type flies reared on standard diet or maize flour-supplemented diet (Figure 5.5C, III/Figure 4B,C). The antibiotic treatment also did not change the severity of the phenotype of AOX-flies on restricted diet (III/Figure 4B,C). The pupal lethality of AOX-expressing flies on low-nutrient diet is therefore not related to microbial growth.

5.11 Complementing compounds in treacle are water-soluble (III)

As treacle was known to restore the pupal survival rate of AOX-flies, the properties and composition of treacle were analyzed in more detail by chemical fractionation. The development assay for AOX-expressing flies was then conducted on the restricted diet supplemented with these fractions, to test which fraction(s) would potentially restore the eclosion rate to normal. The treacle was fractionated to a water-soluble fraction and a hydrophobic fraction by ether extraction. The developmental assays showed that the compound(s) of interest were retained in the water-soluble fraction of treacle (Figure 5.5D), thus excluding e.g. fatty acids, some

amino acids and fat-soluble vitamins. Treacle contains carbohydrates of different complexity and the water-soluble extract was therefore fractionated further by ethanol precipitation to precipitate less polar compounds, including higher molecular weight carbohydrates while retaining the more polar compounds in the supernatant. Interestingly, the ability of the fractions to compensate for the pupal lethality was shifted from the supernatant to the precipitate when the ethanol percentage was increased (III/Figure 5B) suggesting the importance of a compound of intermediate polarity. These fractions were sent for further analysis by mass spectrometry.

5.12 AOX activity is not affected by addition of treacle (III)

The pupal lethality of AOX flies on restricted diet is presumed to be due to activation of AOX under nutritional stress conditions. However, as the regulation of *Ciona* AOX is not understood, the possible inhibitory effects of some component of treacle on the activity of the enzyme could not be excluded. Respirometry was therefore conducted on HEK293T-REx™ cells induced to express AOX by treatment with low levels of doxycycline (Figure 5.6). The cells were permeabilized and after activation of respiration with OXPHOS substrates (pyruvate, glutamate, malate, succinate, ADP) the water-soluble fraction of treacle was added at increasing concentrations with the final concentration being equivalent to that in the standard *Drosophila* diet. Activity of AOX was then tested by antimycin-inhibition of Complex III and measuring the residual oxygen consumption. AOX-expressing cells with or without the addition of treacle showed equal antimycin-resistance (III/Figure 6B) demonstrating AOX to be fully active in the presence of treacle and excluding any inhibitory effect of the supplement. However, it should be noted that the inhibitory effects were tested on a human cell line from a single tissue, not at whole organism level, and therefore, effects of metabolization or absorption of the different components e.g. in the fly gut may affect the metabolic properties of treacle.

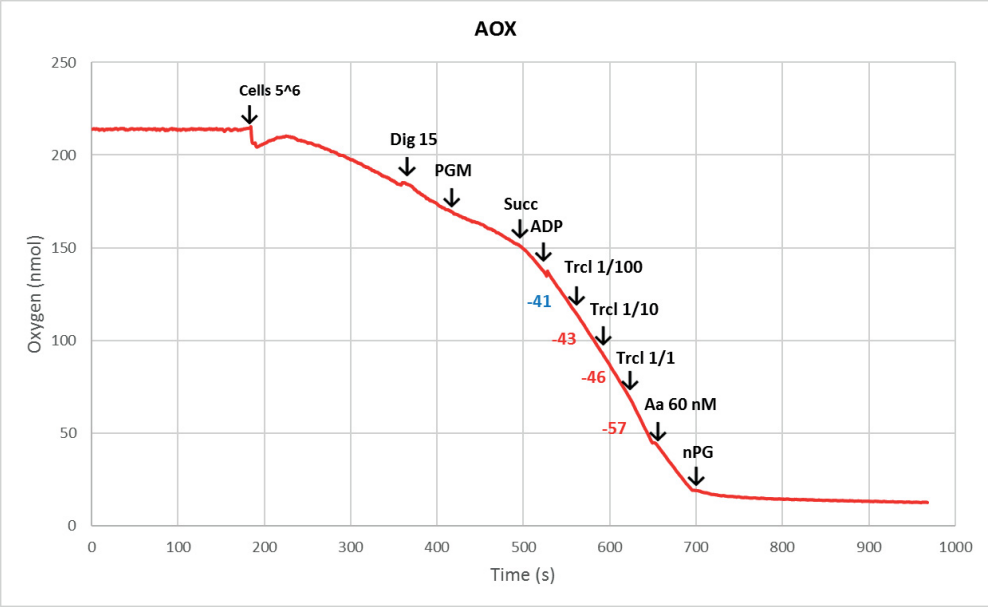

Figure 5.6. Addition of treacle does not inhibit antimycin-resistant respiration of AOX-expressing human cells. Oxygen electrode trace of doxycycline-induced HEK293T-REx™ cells shows a slight increase in oxygen consumption (decrease in oxygen) after each addition of treacle (Trcl) that is then only mildly decreased, but not abolished, by addition of antimycin. Dig = digitonin, PGM = pyruvate/glutamate/malate, Succ = succinate, Aa = antimycin, nPG = n-propyl gallate. Modified from Figure 5 of (III).

5.13 Active fractions of treacle contain several TCA cycle components (III)

The list of compounds detected in the mass spectrometry analysis of the water-soluble fractions of treacle includes several with a central role in mitochondrial metabolism (Table 5.1). Previously tested supplements such as ascorbic acid and fructose were detected, but the most interesting finding was the enrichment of TCA cycle intermediates such as citric acid, malic acid, glutamine, fumaric acid and succinic acid. In addition, there were minor amounts of fatty acids (butanoic acids, oleic acid) and amino acids, e.g. glutamine, methionine and cysteine.

Table 5.1. Quantified compounds detected in the water-soluble fraction of treacle. The compounds are listed according to their level of enrichment in the 75 % ethanol-precipitated pellet from the water fraction of treacle. This pellet fraction was demonstrated as the most active in the rescue of pupal lethality of AOX –flies on low-nutrient diet. 'Linear range' refers to whether the amount detected fell within the range of the standard used in the quantification of the listed compounds in mass spectrometry. Modified from Table 1 in (III).

Compound	*Enrichment factor in 75% EtOH (precipitate/supernatant)*	*Concentration (ng/ml) in water fractions*	*Linear range? (Y/N, [linear range])*
Citric acid	8.8	15	Y
Fructose	72	750	N [0.05-40]
3-OH-butanoic acid	>1	0.05	Y
Ascorbic acid	>1	7.5	Y
Glutamine	~1	57	N [5-40]
Malic acid	~1	16	Y
Methionine	~1	1.1	Y
Oleic acid	~1	0.5	Y
2-OH-butanoic acid	<1	0.67	N [1-40]
Arginine	<1	11	Y
Aspartic acid	<1	4.5	Y
Cysteine	<1	8.4	Y
Fumaric acid	<1	2.1	Y
Glyceraldehyde	<1	12	Y
Glyceraldehyde-3-phosphate	<1	26	Y
Lactic acid	<1	74	N [0.5-40]
Succinic acid	<1	210	N [0.5-40]
Tryptophan	<1	17	Y
Valine	n/a	0.005	N [1-40]

6 DISCUSSION

6.1 AOX expression affects sperm quality in *Drosophila*

Drosophila females have been documented to mate with multiple males both in the wild and under laboratory conditions. However, the frequency of this so-called polyandry is difficult to estimate, particularly in the wild population, as the estimates are based on molecular parentage analyses on born offspring and do not take into account unsuccessful copulation or inseminations rejected by the female reproductive tract (Giardina et al., 2017). The specific mechanism(s) of spermatid selection inside the female remain unknown but mating with multiple males has made the fly an interesting model for fertility studies, including determining the effects of transgene expression on male reproductive capacity, as successfully employed here (I).

A well-documented phenomenon when conducting sperm competition assays is the "second male advantage" where the majority of offspring come from the latest male to be mated with a given female. The exact reason for this is unknown but it is presumed that the selection is simply based on the vitality of the newly received spermatids. The composition of the ejaculate is important to the fitness and fertility of spermatids and the quality of seminal fluid has been suggested to affect the preference of some sperm over the other inside the female. Accessory gland proteins introduced into the ejaculate in the male reproductive tract are known to protect spermatids and enhance their selection (Nguyen & Moehring, 2018).

Viability of fly lines with constitutive AOX expression demonstrates that the presence of the enzyme does not lead to loss of fertility in *Drosophila* males; however, in a sperm competition setting they seem to lose the previously described "second male advantage" (Figure 5.2). The α-tubulin promoter, although supporting

expression in the testis, does not appear to direct AOX expression in the actual germ cells. The lowered fitness of ejaculate from AOX-expressing males in a competitive setting may be due to defective or incomplete seminal fluid. Seminal fluid is known to include several proteolytic enzymes and inhibitors required for proper processing of accessory gland proteins (LaFlamme et al., 2012). It is therefore plausible, that the presence of AOX in the testis tissue causes unfavorable conditions for optimal performance of these proteolytic cascades and leaves the AOX sperm more exposed to elimination. As the exact composition of wild-type *Drosophila* seminal fluid is unknown, a broader omics approach would be needed to pinpoint the potential consequences of AOX expression in testis.

6.2 AOX expression causes disorganization of the spermatogenesis machinery

Spermatogenesis in *Drosophila* is a highly coordinated process involving several biochemical pathways and intercellular signaling (Arama et al., 2003; Yalonetskaya et al., 2018). In the final process of spermatogenesis inside the testis tube, an individualization complex (IC) traverses the spermatids removing additional cytoplasm while separating them into individual spermatozoa. Dysfunction in this process lead to lowered male fertility or complete infertility (Fuller, 1993). Histochemistry of AOX-expressing testis revealed a disorganization of the spermatids, manifesting as a visible bulk of whitish material accumulating at the proximal end of the testis while the seminal vesicles remained empty of mature sperm (Figure 5.3). Since AOX does not cause male infertility, and cysts in all of the different stages of spermatid differentiation were observed in the testes of AOX-expressing males (I), it can be presumed that the differentiation of spermatids is not systematically blocked. The disruption caused by AOX is most likely targeted to the individualization process and/or the transfer of spermatids into the seminal vesicle due to e.g. insufficient peristaltic movements of the smooth muscle of the testis

walls. The disruption may be linked to the above-mentioned seminal fluid composition and deficient signaling.

One of the pathways involved in the regulation of the IC is the Lands cycle (Lands, 1958). This cycle regulates the structure of lipid membranes by catabolizing phospholipids into lysophospholipids and fatty acids that can be further metabolized into signaling molecules. Mutations in enzymes regulating the Lands cycle, although not causing loss of any actin filaments or other structures, have been found to cause disorganization of the spermatogenesis individualization machinery (Ben-David et al., 2015). Another factor affecting the function of the IC is temperature (Ben-David et al., 2015). The testis phenotype of AOX males does not cause a significant difference in the number of offspring, yet a mild decrease is already detected in vial I of the competition assay (Figure 5.2A) and due to the sensitivity of lipid membranes to temperature, it could be interesting to see how changes in temperature affect the IC of the AOX-expressing testis. The decreased number of progeny produced by AOX males may simply be due to a lower number of spermatids, as a consequence of IC dysregulation. The thermogenic effect of AOX, if activated, thus offers an intriguing plausible explanation for disorganized individualization and for decreased number of mature sperm produced.

6.3 AOX activation in conditions of limited nutrition in *Drosophila*

AOX has been characterized as a stress-response enzyme in organisms that still possess it (Grant et al., 2008; Sussarellu et al., 2013; Szal et al., 2009). In model systems, transgenic AOX expression has not caused any prominent effects in the absence of OXPHOS dysfunction under standard laboratory conditions and enzymatic studies as well as respirometry data (Dassa et al., 2009) suggest that AOX is not active under non-stressed conditions. However, in *Drosophila*, AOX expression did cause a slight delay in development and weight loss in adults over time (Fernandez-Ayala et al., 2009), suggesting impaired energy metabolism that is not

compensated by increased feeding on standard high-nutrient diet. Developmental assays, where AOX flies were reared on low-nutrient diet, showed increased pupal lethality compared to control flies (Figure 5.4A). It could be postulated that by limiting the availability of nutrients, the developmental delay observed in standard diet conditions becomes more severe, leading to premature death at the stage of metamorphosis. AOX is claimed to possess lowered affinity for ubiquinol compared to Complex III and therefore, even if active, is considered to have minimal effects on OXPHOS function and ATP production. The observation of weight loss in adults potentially contradicts this claim, as the phenotype could be caused by enhanced energy use or decreased energy production, implying interference by AOX in OXPHOS in the absence of any dysfunction or environmental stressor. One explanation to this could be that AOX may become more activated when metabolism is downregulated, such as during metamorphosis (Merkey et al., 2011). Alternatively, considering that there are effects detectable even in standard conditions, AOX may even be constitutively active at some level, but the activity has been masked by access to a nutritionally rich diet. A recent study where AOX-mice were fed with high-fat diet or ketogenic diet, suggests that the possible physiological effects of AOX are minute when the organism is exposed to a normal/high caloric diet (Dhandapani et al. 2019).

6.4 Temperature dependence of the *Drosophila* developmental defect supports proposed thermogenic properties of AOX

The developmental defect observed in AOX-expressing flies under nutrient-poor conditions was shown to be highly temperature sensitive. At a lowered temperature of 22 °C (note that the standard maintenance temperature is 25 °C), the phenotype of developmental failure was nearly abolished while an increase to 27 °C resulted in 100 % pupal lethality. AOX expression in these flies is driven by a GAL4 -driver of yeast origin that is known to be temperature sensitive (Brand & Perrimon, 1993) and

therefore, the effects on the phenotype are at least partially presumed to be due to the increased expression of AOX. Thermogenic properties of alternative enzymes have been widely studied in plants (Grant et al., 2008; Onda et al., 2015) but whether the enzyme has a similar role in *Ciona* has not yet been established. However, *Ciona* are found in marine environments of temperatures that are <20 °C where enhanced thermogenic processes may be advantageous. Although the correlation between pupal lethality and increase in temperature could be proportionate to expression level, accelerated recovery of AOX flies after cold-exposure indicates that the enzyme is active and able to boost metabolism at lower temperatures (II/Figure 5, by Andjelkovic, A.).

Recent findings suggesting that decrease in temperature switches the feeding behavior of flies from yeast to a more plant-based diet (Brankatschk et al., 2018) raises another question, namely whether the presence of AOX affect the food intake of the larva; in other words, do AOX-expressing larvae eat more (or less) to compensate for the expression of AOX? If AOX increases heat production and metabolism in larvae, it may also have an impact on nutrient sensing in a way that affects food intake. This may affect development in conditions where metabolism is normally down-regulated or modified. In turn, at higher temperatures, altered food intake or processing, in combination with an already enhanced metabolic rate, could become a problem.

6.5 AOX lowers the efficiency of nutrient utilization during metamorphosis

No universal standard diet has been developed for *Drosophila* studies and the diets used for maintenance of fly stocks in different laboratories are very variable, although most have a high sugar content combined with other ingredients from diverse sources (Piper, 2017). The standard diet described in this study is no exception. The low-nutrient diet used in my developmental assays is referred to as

'restricted' relative to the standard diet. However, as demonstrated by control fly lines used in the study, it still includes enough nutrients and calories to allow delayed but otherwise normal development of the flies. Yet, for AOX-expressing flies, this decrease in nutrients became a severe problem. One possible way to shed light on this question would be to study the food intake of both AOX-expressing larvae and adult flies to see whether the larvae require more feeding to fill their storage before pupariation and whether the adult flies require more food after eclosion.

The beginning of pupation and metamorphosis is highly regulated in *Drosophila*. Only when the larvae have stored enough nutrients to carry out metamorphosis will they stop feeding and start forming the pupa (Aguila et al., 2007). Larvae of AOX flies showed no size difference compared to controls visible by eye, and also exhibited no difference in triglyceride levels (III/Figure 7A) and no increase in larval lethality (III/Figure 1B), suggesting that they start the transformation to an adult fly with nutritional resources equal to those of wild-type counterparts reared on the same diet. Nevertheless, the majority of AOX-expressing larvae fail to complete metamorphosis. Death at different stages of metamorphosis and delayed completion of development even in cases of successful eclosion suggests that the deficiency is systemic and the flies simply run out of resources during the process. Possible reasons for this include that AOX expression results in an excessive consumption of stored nutrients during metamorphosis or starvation due to a prolongation of this non-feeding stage, caused by deranged signaling during the process. To get an extensive view on the metabolic changes in AOX-expressing larvae compared to wild-type, a metabolomics approach would be required.

6.6 Rescue of AOX-related pupal lethality on low-nutrient diet requires a combination of nutrients

Increased pupal lethality of AOX flies in low-nutrient diet was first presumed to be due to lowered energy production. When metabolism is slowed down as a result of

restricted diet, electron flow would be more easily directed towards AOX, thus bypassing proton pumps at Complex III and Complex IV, leading to lower ATP production. However, supplementing the diet with more calories, in the form of glucose or yeast, did not increase the proportion of AOX flies completing metamorphosis. Addition of dietary glucose had no effect on the phenotype and it even increased pupal lethality in control flies. Addition of other simple sugars such as sucrose, a disaccharide of glucose and fructose, or fructose alone had no effect on the eclosion rate (Figure 5.5A, III/Figure 3B). Addition of yeast which, along with microbes, is one of the main sources of amino acids and nutrients for flies in nature, unexpectedly did not restore or even improve the eclosion rate of AOX-expressing flies (Figure 5.4D). These results show that the defect is not merely a lack of calories or of specific carbon sources but a consequence of a more complex distortion in metabolism.

Pupal lethality of AOX flies was finally rescued when their diet was supplemented with complex plant-based ingredients such as treacle, wheat germ, soy or maize flour. As the addition of sugars failed to improve the eclosion rate, rescue by treacle was an interesting finding. Treacle, also known as molasses, is a by-product of sugar refinement and consists of mainly sugars and micronutrients (Valli et al., 2012). Several micronutrients are involved in mitochondrial bioenergetics including most vitamins generally known as B-vitamins, e.g. niacin, pantothenic acid and folate, as well as ascorbic acid known as vitamin C (Wesselink et al., 2018). In addition to vitamins, trace elements such as selenium and iron are essential for mitochondrial biogenesis and oxidative phosphorylation (Stehling & Lill, 2013). Iron is the cofactor of the active site of AOX. Feeding the flies with a low-nutrient diet supplemented with either a general mixture of vitamins, B-vitamins or iron did not improve the eclosion rate. Although the amounts of vitamins were approximately in the range of what has been estimated to be optimal for flies (Piper et al., 2014), it is not possible to rule out toxic effects, in particular as regards the doses and forms in which they were supplemented as or their doses, taking into account that the mixtures used were

originally manufactured for human consumption. However, doing an extensive screen of all possible vitamin and mineral supplements in different doses and forms is impractical.

In nature, flies rely heavily on microbes to provide them with essential nutrients (Wong et al., 2017) including most micronutrients discussed above, which led me to the hypothesis that AOX might disturb the composition of the microbiome. Although AOX-expressing flies raised on antibiotic-supplemented low-nutrient diet showed increased pupal lethality, the treatment did not have any effects on development of control flies (Figure 5.5C) which indicates that the AOX-related developmental defect on low-nutrient diet is not due to a scarcity of microbes on the low-nutrient diet or in the gut.

6.7 Composition of treacle suggests AOX interference in the balance of TCA intermediates

As a commercial food product, the composition of treacle is rather well-known to meet the public health standards, although not in detail at the metabolite level. Therefore, to achieve a more in-depth list of compounds or micronutrients included, samples of extracted treacle fractions were analyzed by mass spectrometry. The results were interesting as several of the compounds detected are involved in the TCA cycle and redox metabolism such as citrate and malate as well as ascorbic acid. This may support the presumption that AOX enhances TCA cycle activity and dietary supplementation of these compounds compensates for their depletion by AOX activation. Citrate and malate take part in both mitochondrial and cytosolic biosynthetic pathways (Owen et al., 2002) and depletion of either of these metabolites should have systemic effects.

Metabolite assays showed no significant difference between AOX and control larvae in their ability to store triglycerides or in lactate/pyruvate levels. However, larvae of both genotypes showed dietary differences in these metabolites i.e. they

were systematically decreased when nutrition was limited. It is plausible that in standard diet, metabolism is able to compensate for the additional stress caused by AOX but in more limited conditions there is no metabolic 'surplus' to cover this extra cost leading to lethality at pupal stage.

Minerals such as iron or calcium are not detected by the mass spectrometry and therefore, their role cannot be entirely excluded. As mentioned earlier, AOX contains a diiron core which may cause an additional burden when nutritional iron is limited while calcium plays a key role in mitochondrial signaling. A possible inhibitory effect of treacle on AOX activity was excluded, based on respirometry measurements of human cells supplemented with treacle. The results of these experiments (Figure 5.6) showed that treacle enhances oxygen consumption and hence metabolism, and does not abolish the effects of AOX through inhibition.

6.8 Hypothesis: AOX and signaling

The developmental defect caused by AOX on low-nutrient diet appears to be systemic and affects the entire process of metamorphosis. Although pupal lethality is high for these flies, a minority of them does complete the transformation to the adult stage. This points towards a threshold effect, rather than an all-or-none phenomenon related to the absence of a specific nutrient from the diet. Instead, I suggest that pupal lethality is a result of a slight metabolic imbalance that causes wasteful use of nutrient reserves. Spermatogenesis and metamorphosis are both highly coordinated processes of cell differentiation and tissue reorganization that may easily be disrupted, for example, by an insufficient signaling cascade or imbalance in redox or pH homeostasis (Arama et al., 2003).

One of the key features of AOX in alleviating phenotypes caused by respiratory chain dysfunction is the ability to decrease mitochondrial ROS (Fernandez-Ayala et al., 2009; Mills et al., 2016). However, under normal physiological conditions ROS is an important signaling molecule influencing cell proliferation, growth and death

(Brunelle et al., 2005; Chen et al., 2009). In disease states, AOX may provide protection from damage caused by excessive ROS, but under normal physiological conditions AOX may dilute a signal relayed by ROS that would help the cell to metabolically adapt to environmental changes and thus transform a previously minor stress to a lethal one. This type of possible abrogation of hormesis by AOX was also observed in COX15 knockdown mice (Dogan et al., 2018).

Imbalance of the TCA cycle and accumulation of TCA intermediates has been found to be associated with systemic acidification in *Drosophila* larvae that led to increased sensitivity to a diet with low-pH (Ghosh & O'Connor, 2014). It is plausible that AOX causes a pH imbalance either by interfering with the redox homeostasis or by enhancing turnover in the TCA cycle leading to lowered ability to buffer larval metabolism in low-pH food. In this scenario the addition of treacle may simply have increased the pH of the low-nutrient food sufficiently for AOX larvae to thrive. Furthermore, a high-protein diet is known to worsen metabolic acidosis both in humans and insects while ingredients of plant-origin improve the acid-base balance, presumably due to organic anions such as citrate and malate (Harrison, 2001; Liu et al., 2018). This may explain why treacle and different flours, but not yeast nor simple sugars, rescued the pupal lethality. It will therefore be useful to study the effects of all these additives on the pH of the food and of the larvae themselves. Effects of AOX on metabolic pH of the larvae could also be measured in the hemolymph by simple colorimetric assays. However, the fact that citrate addition alone did not rescue (III/Figure 8) and lactate levels were unaffected by AOX (III/Figure 7B), suggests that pH alterations alone may be insufficient to explain the phenotype. To obtain a truly comprehensive view on the metabolic effects of AOX, an approach using metabolomics and transcriptomics is required.

6.9 Future perspectives of AOX

AOX has alleviated the phenotypes of several disease models, both in *Drosophila* and the mouse (Fernandez-Ayala et al., 2009; Kemppainen et al., 2014b; Rajendran et al., 2019). The novelty of AOX and the myriad of unanswered questions regarding the regulation of the enzyme means that therapeutic use of AOX in treating mitochondrial diseases remains in the distant future. On the other hand, a better understanding of its effects on metabolism may broaden the scope of therapeutic uses for AOX. Already, together with NDH2, these alternative respiratory chain enzymes have proven to be unique tools to study and manipulate metabolic aberrations in detrimental conditions such as cancer (Martínez-Reyes et al., 2016) where the pathological significance of mitochondria has remained unresolved despite decades of research (Warburg, 1956).

As a candidate for therapeutic treatments, the benefits of AOX have been limited to dysfunction of specific RC complexes. Interestingly, AOX has failed to alleviate broad mitochondrial defects in protein synthesis caused by a point mutation in a mitoribosomal protein gene (Kemppainen et al., 2014a) or in mtDNA replication caused by manipulation of the mtDNA helicase Twinkle (Rodrigues et al., 2018). In contrast, it has shown great promise in alleviating inflammation and Complex III-deficiencies (Mills et al., 2016; Rajendran et al., 2019). Based on these models and the metabolic effects of AOX presented above, it could be presumed that AOX may not be optimal for treating general metabolic disorders caused by mitochondrial dysfunction. In addition, considering the tendency of male-biased accumulation of mitochondrial mutations due to the maternal inheritance of mitochondria (Innocenti et al. 2011), any detrimental effects of AOX on male fertility should be studied carefully as it may worsen already disadvantageous traits. With the potential impact on cell differentiation and tissue reorganization, AOX may also present a risk as a tumor promoting agent and any effects on nutrient sensing or redox homeostasis may even exacerbate metabolic disorders such as diabetes. Nevertheless, considering

the current lack of effective treatments for disorders with high socio-economic impact, such as Alzheimer's and Parkinson's Disease or immunological diseases, not to exclude the metabolic significance of mitochondria in global diseases such as obesity and cancer, further studies in understanding AOX, both as a tool and as a treatment, are likely to prove fruitful.

7 CONCLUSIONS

In all model systems used thus far – cultured mammalian cells, *Drosophila* and mice, transgenic AOX expression has not led to any detrimental effects under non-stressed conditions. Very little is known about the regulation of AOX in the original host, *Ciona*, let alone how the enzyme fits into and functions in a metabolic network of mammalian or *Drosophila* models where it does not naturally occur. The *Drosophila* studies presented in this thesis demonstrate that, under specific conditions, AOX also presents some deleterious effects and may introduce limitations on reproduction as well as metabolic flexibility when the organism is exposed to a stressful environment.

In the *Drosophila* testis, AOX causes derangement of the spermatogenesis machinery that seems to affect the quantity of spermatids produced but not their functionality. Spermatogenesis is a highly specific and coordinated process of cell differentiation that may lead to the AOX enzyme becoming active in the absence of OXPHOS dysfunction. It is also plausible that the metabolic state of the testis wall cells promotes activation of AOX that in turn may cause disturbances in signaling pathways controlling spermatogenesis. Whether the disorganization of spermatogenesis causes the decrease in spermatid production and release, and whether it secondarily disturbs the composition of seminal fluid that disadvantages the sperm from AOX-expressing males inside the female reproductive tract remains elusive.

In dietary studies, AOX created a disadvantage for the flies in a developmental assay where the nutrient composition of the diet was restricted to a near-minimal level. Dietary stress led to a decrease in the eclosion rate of AOX-expressing flies, most of which failed to complete metamorphosis, a stage of increased cell

differentiation and tissue reorganization. The increase in pupal lethality was not due to lower triglyceride storage or increased demand for amino acids or monosaccharides; nor was the phenotype a consequence of an altered number of commensal bacteria. The eclosion rate was restored by dietary supplementation with complex plant-based ingredients such as treacle and flours. The mass spectrometry analysis of treacle showed a composition of sugars, TCA cycle intermediates and vitamins suggesting that AOX larvae require a more complex combination of nutrients compared to controls to maintain metabolic homeostasis and enable the completion of metamorphosis.

Both the reproductive and dietary assays suggest that AOX has a greater impact on metabolism than previously expected, based on the original characterization studies in standard conditions. With a better understanding of the metabolic effects of transgenic AOX expression, the enzyme can be developed further as a potential therapy. It will also facilitate the use of AOX as a tool to manipulate cell metabolism in and via mitochondria and provide more insight into the role of the organelle in multiple diseases where its involvement is yet to be defined.

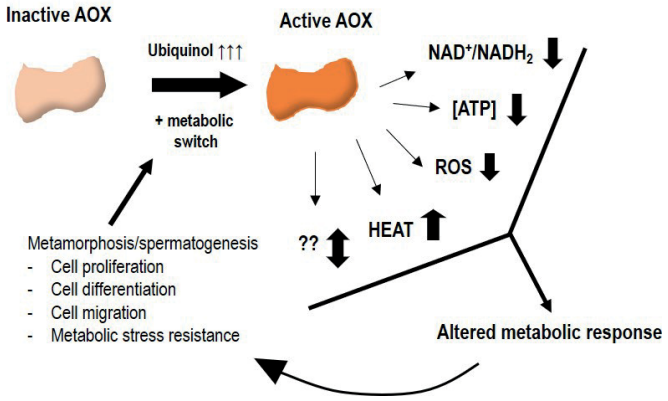

Figure 7.1. Potential effects of AOX activity in *Drosophila* spermatogenesis and metamorphosis. The figure outlines potential metabolic states that may activate AOX and potential outcomes of the activation that may distort the metabolic response.

ACKNOWLEDGMENTS

The research in this thesis was conducted in the Mitochondrial Gene Expression and Disease group, at the Institute of Biomedical Technology (BioMediTech), University of Tampere and funded by Academy of Finland, AFM Téléthon and the EU. I also want to thank Tampere University Doctoral Programme in Medicine and Life Science for education and financial support. I am grateful to my thesis committee, Professor Kirsi Pietiläinen and Professor Aurelio Teleman, for insightful discussions and ideas and genuine support for my project. I wish to thank the external reviewers, Professor Bill Ballard and Dr. Eija Pirinen, for their in-depth examination of my thesis and constructive and valuable feedback.

I am most grateful to my supervisor, Professor Howy Jacobs, for giving me the opportunity to join his research group and for his guidance and support on this journey. I wish to thank him for sharing his extensive scientific knowledge but most of all, for his endless enthusiasm for science. Your efforts to share this enthusiasm and emphasize the value of science, not only to the science community but to the public, is more important now than ever. Thank you for your encouragements and for having faith in me.

I want to express my statistically most significant (*** by all-the-ways ANOVA) gratitude to Dr. Eric Dufour, for his scientific expertise and advice (whether I agreed or not), but even more so, for his unconditional support, optimism and patience, when nothing seemed to work, when the challenges I faced felt overwhelming and I was being at my worst. You are my light of Eärendil.

Of course, I am grateful to all my co-authors and collaborators without whom the work of this thesis could not have been done. I wish to thank Dr. Marcos Oliveira, who's fault is that I started this project in the first place, for the discussions

and inspiration he has given me through the years. I would also like to thank his students for their hard work and help in this process. I want to thank Ana and Esko, for sharing this journey with me, including even some occasional laughs, Katharina and Paul, for their scientific contribution and friendship, and Marten, for sharing his scientific knowledge. I want to say a special thanks and give the biggest hug to Tea, the best laboratory manager in the world, for sharing her vast skill set and knowledge on fantastic flies and laboratory equipment and where to find them. Most of all, I want to thank you for your never-ending positivity and support as a friend.

I wish to thank sunshine in a shape of a human, Eveliina, who is not only a skilled technician and fellow sport enthusiast but exclusively approved maître d' by my cat. Thank you for being there for me. I want to thank and give an uncomfortably long hug to Jose, who, despite burning my salmon, is a good guy, most considerate friend and a pretty good scientist. I am also grateful for having worked with the most motivated student, Grazia, who resiliently conducted my cell culture experiments against all odds. Thank you for still smiling and keeping me as your friend (and sending me panettone). I wish to thank Anna and Kaisa, for going to great lengths to help me survive my administrative duties and sometimes life in general. A big thanks also to Yuliya, who bravely took over some of the mess I have produced through the years. I am grateful to Giuseppe, Çağri, Kia, Mike, Jack, Praveen, Suvi, Priit, Troy, Outi and Merja and everyone passing through and around Howylab during these years for their help and support and making the lab anything but boring. A special thanks to Birgitta and Frank, for their last-minute help in finalizing this thesis for printing.

I would not have survived this far without the support of my out-of-the-lab "posse", Alexandra, Marja, Minna, Noora M and Noora N, with whom I started my scientific journey 13 years ago and continue to share the twists and turns (and food) of life with. Thank you for putting up with me this long. I have been lucky enough to get to know the two most exceptional women, Maria and Mugen, who are always there for me to make me smile and continue to surprise me with their creativity and

good kind of quirky. Thank you for being you. I'm forever grateful for having my fellow pirate, Annika, in my life. Our adventures have gotten me through some rough seas even more than you know. You're a true treasure.

Finally, I am grateful for my wonderful and supporting family. Micke, Aino and Fanny, thank you for growing up from intolerable toddlers to interesting and most kind human beings. Thank you, Minna, for ensuring that I have at least decent skills to take care of myself in daily life. Kiitos äidilleni Sirpalle tuesta ja rakkaudesta, varsinkin silloin, kun elämä ei ole mennyt niin kuin on suunnitellut. And last, thank you to my dad Jussi, for being my rock and supporting me in whatever I wanted to do in life. I love you all.

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PUBLICATIONS

PUBLICATION I

Expression of *Ciona intestinalis* AOX causes male reproductive defects in *Drosophila melanogaster*

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BMC Developmental Biology (2017), 17(1):9
doi: 10.1186/s12861-017-0151-3

Saari et al. BMC Developmental Biology (2017) 17:9
DOI 10.1186/s12861-017-0151-3

BMC Developmental Biology

RESEARCH ARTICLE Open Access

Expression of *Ciona intestinalis* AOX causes male reproductive defects in *Drosophila melanogaster*

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Abstract

Background: Mitochondrial alternative respiratory-chain enzymes are phylogenetically widespread, and buffer stresses affecting oxidative phosphorylation in species that possess them. However, they have been lost in the evolutionary lineages leading to vertebrates and arthropods, raising the question as to what survival or reproductive disadvantages they confer. Recent interest in using them in therapy lends a biomedical dimension to this question.

Methods: Here, we examined the impact of the expression of *Ciona intestinalis* alternative oxidase, AOX, on the reproductive success of *Drosophila melanogaster* males. Sperm-competition assays were performed between flies carrying three copies of a ubiquitously expressed AOX construct, driven by the α-tubulin promoter, and wild-type males of the same genetic background.

Results: In sperm-competition assays, AOX conferred a substantial disadvantage, associated with decreased production of mature sperm. Sperm differentiation appeared to proceed until the last stages, but was spatially deranged, with spermatozoids retained in the testis instead of being released to the seminal vesicle. High AOX expression was detected in the outermost cell-layer of the testis sheath, which we hypothesize may disrupt a signal required for sperm maturation.

Conclusions: AOX expression in *Drosophila* thus has effects that are deleterious to male reproductive function. Our results imply that AOX therapy must be developed with caution.

Keywords: Mitochondria, Respiratory chain, Spermatogenesis, Sperm competition

Background

In the animal kingdom, the alternative mitochondrial respiratory chain is widely represented, as illustrated by the broad phylogenetic distribution of the gene for the alternative oxidase (AOX) [1, 2]. AOX is a single-subunit enzyme able to bypass respiratory complexes III and IV, transferring electrons from ubiquinone to oxygen without proton pumping and ATP production. It is believed to buffer stresses affecting oxidative phosphorylation, whether due to overload, environmental insults or genetic damage. However, AOX, as well as other alternative respiratory enzymes, was lost independently during the early evolution of both vertebrates and arthropods. This raises the question of what disadvantages AOX might have conferred on the survival and/or reproductive fitness of the ancestors of these taxa.

In fact, only marine invertebrates appear to still possess genes for alternative enzymes [1–3]. Nonetheless, when the AOX gene from the tunicate *Ciona intestinalis* (Ascidiacea) was expressed in cultured human cells, it promoted resistance to inhibitors of complexes III and IV [4] and compensated for the growth defects and oxidant-sensitivity of complex IV-deficient cell-lines [5]. In the fruitfly *Drosophila melanogaster*, transgenic expression of *C. intestinalis* AOX had no apparent effect on viability, reproduction and health of the organism [6]. It conferred resistance to otherwise lethal levels of complexes III and IV inhibitors [6], mitigated locomotor defects in the *dj-1β* Parkinson's disease model [6] and

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ameliorated diverse phenotypes associated with complex IV deficiency [7], defective mtDNA replication [8], or expression of human β-amyloid [9]. *C. intestinalis* AOX has also been successfully expressed in the mouse without any significant effect on major physiological parameters [10, 11]. It led to decreased production of reactive oxygen species (ROS) when the respiratory chain was blocked, was able to support cyanide-resistant respiration by intact organs, and conferred prolonged protection against lethal concentrations of hydrogen cyanide in whole animals [10, 11].

These benefits of AOX expression raise the possibility of its eventual use for treatment of human patients with complexes III and/or IV deficiencies, as a by-pass therapy [12]. However, the advantages of AOX expression in 'higher' animal systems under pathological conditions contrasts with its evolutionary loss from the genome of vertebrates and arthropods. A detailed study of the biology of the alternative pathways is therefore required before any use of this enzyme in gene therapy for mitochondrial or neurodegenerative disorders can be contemplated [13].

We set out to investigate the paradox between the beneficial by-pass and the possible maladaptive consequences of AOX expression in higher metazoans, by directly testing its effects on male reproductive function. Sperm are highly dependent on biological energy. Therefore, we reasoned that it may be a relevant target in which to test for subtle but functionally meaningful detriments of AOX expression. We challenged *D. melanogaster* males expressing AOX in competition assays with AOX-nonexpressors. AOX produced a clear impairment to reproductive function that was associated with decreased production of mature sperm cells. This suggests that possible gene therapy applications of AOX must be developed with caution.

Results

Males expressing AOX are defective in sperm-competition assays

In *Drosophila*, as in many species, females typically mate with a succession of males, storing sperm inside the female body to fertilize oocytes as they mature [14]. Sperm competition occurs by means of a substitution mechanism, in which a second male dislodges the sperm of the first male from the female's reproductive tract and replaces it with its own gametes [14, 15]; this is apparently the motive force for the correlative evolution between the male's giant sperm cells and the female's spacious sperm-storage organs [16]. Newly acquired sperm competes with and usually displaces that from previous males, unless it is functionally compromised. These mechanisms can drive evolution by maximizing male reproductive success, such as by the development of larger testes, production of more abundant, more viable or longer-lasting gametes, or by modifying the response to elevated competition risk, among other characteristics [17].

To test the reproductive success of *D. melanogaster* males expressing AOX, we performed sperm-competition assays between flies carrying three copies of the *α-tubulin-AOX* construct, as described previously (3X*tub-AOX* line: genotype *tub-AOX*^{35}/Y; *tub-AOX*^{112}/*tub-AOX*^{112}; *tub-AOX*^{7}/*tub-AOX*^{7} [7]) and wild-type males of the same genetic background (w^{1118}). In the 'defensive' approach, 3X*tub-AOX* males were first allowed to mate with virgin w^{1118} females, which were then mated with w^{1118} males in the absence of 3X*tub-AOX* males. Progeny from the competing males can be distinguished by eye-colour: those from transgenic males have red eyes, whilst those from w^{1118} males have white eyes. Controls consistently show that the eye-colour marker, as such, has no influence over the outcome (Additional file 1: Fig. S1).

The number of progeny originating from the sperm of 3X*tub-AOX* males was decreased after the females were mated subsequently with w^{1118} males (Fig. 1a, *upper panel*). This result is consistent with previously published data using diverse genetic backgrounds [14, 18–21] and with our own data using several other transgenes (Fig. 1b, Additional file 1: Fig. S1–S3): sperm of the first male is replaced (partially or completely) by the sperm of the second male. To implement a rigorous statistical analysis of the findings, we derived the parameter *P1'* which measures the proportion of progeny sired by the first male to mate in sperm-competition assays [22]. This was similar for all lines analyzed here, although 3X*tub-AOX* males sired statistically fewer offspring than some of the AOX-nonexpressor controls (Additional file 1: Table S1).

In contrast, when 3X*tub-AOX* males were challenged in an 'offensive' approach, i.e., when the virgin w^{1118} females were first crossed with w^{1118} males, then with AOX-expressing males, the number of 3X*tub-AOX* progeny never overcame the number of w^{1118} progeny (Fig. 1a, *lower panel*). In fact, even after 10 days since w^{1118} males were removed from the mating vials, eggs fertilized with the original male sperm were still preferentially laid over the ones fertilized with 3X*tub-AOX* sperm (Fig. 1a, *lower panel*, vial IV). Control experiments performed with red-eyed *daughterless-GAL4* (*daGAL4*) (Fig. 1b, *lower panel*) and *tubulin-GeneSwitch* (*tubGS*) and also *UAS-empty*^{2nd} and *UAS-empty*^{3rd} (Additional file 1: Fig. S1) males imply that the failure of AOX-expressing males to compete successfully in the offensive paradigm is specific to AOX, and is not a property of transgenic lines in general. The defect appeared to be AOX transgene-dose dependent. Males carrying only two copies of the *tub-AOX* transgene (Additional file 1: Fig. S2)

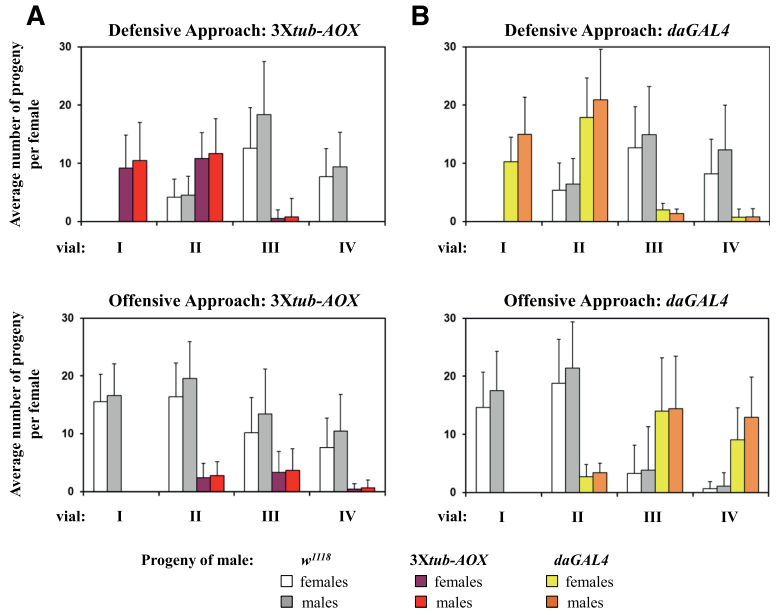

Fig. 1 AOX-expressing males are defective in sperm-competition assays. In the defensive paradigm (*upper panels*), virgin w^{1118} females (white eyes) were initially crossed individually with transgenic males (red eyes), and then with w^{1118} males (white eyes). In the offensive paradigm (*lower panels*), the virgin w^{1118} females were first crossed with w^{1118} males, and then with transgenic males (see Methods for details). The transgenic males used were 3X*tub-AOX* ($tubAOX^{35}$/Y; $tubAOX^{112}$/$tubAOX^{112}$; $tubAOX^{7}$/$tubAOX^{7}$) in **a**, and *daGAL4* (X/Y; 2/2; *daGAL4/daGAL4*) in **b**. Vials I-IV represent the mean number of white- or red-eyed progeny ± SD (error bars), eclosed after 3 days from the initial crosses (vial I), 3 days of egg-laying during second crosses (vial II), 3 days of oviposition only, subsequent to second crosses (vial III, males discarded), followed by 5 days of further oviposition (vial IV)

appeared less impaired in the 'offensive' paradigm than those carrying three (Fig. 1a), whilst those with just a single copy of *tub-AOX* were not significantly different from control males (Additional file 1: Fig. S2). These conclusions reflect the statistical analysis of the proportion of progeny sired by the second male (*P2,'* [22]), wherein 3X and 2X*tub-AOX* males were significantly impaired in the offensive paradigm compared to all control classes (Additional file 1: Table S2), although the differences between 3X and 2X*tub-AOX* (Additional file 1: Table S2) themselves were not significant. The observed phenotypes also correlate approximately with the amount of expression of the *tub-AOX* transgene in the male reproductive system, observed by immunoblotting (Additional file 1: Fig. S4).

However, expressing GAL4-dependent *UAS-AOX* using the *daGAL4* driver [6], produced no loss of sperm-competitiveness (Additional file 1: Fig. S3A, and Table S2), despite the fact that the level of AOX protein in the male reproductive system was comparable with that expressed from multiple copies of *tubAOX* (Additional file 1: Fig. S4). These data suggest that the effect of AOX on sperm competitiveness depends on the precise cellular context of its expression rather than simply its overall amount.

AOX-expressing males accumulate a decreased amount of mature sperm

Successful sperm competition in *Drosophila* depends both on the number and quality of spermatozoids and on the compatibility between the male's seminal fluid proteins and their receptors in the female reproductive tract [17, 23–25]. We checked how the production of mature spermatozoids in the 3X*tub-AOX* males is affected by dissecting the reproductive organs in adult males of increasing age. Spermatogenesis in *D. melanogaster* starts at the distal tip of the testes, taking place inside cysts. As cell differentiation proceeds, the cysts move towards the proximal end of the testes, where these organs connect to the SVs. The mature spermatozoids are then deposited in the SVs, where they are stored until mating [26]. Therefore, as adult males age, the testes tend to get thinner, because differentiation in the cysts proceeds and the fully formed spermatozoids move to the SVs, which in turn increase in size. As shown in Fig. 2 (panels a and c), the thickness of the

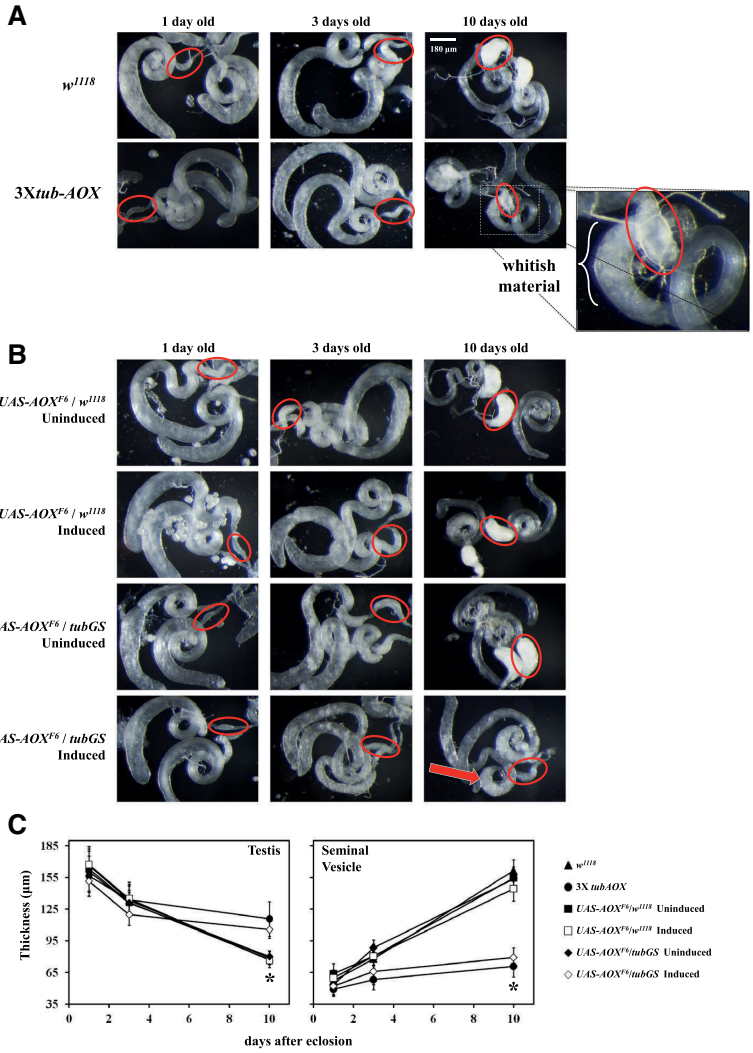

Fig. 2 Mature sperm cells do not accumulate in the seminal vesicles (SVs) of AOX-expressing males. Representative samples of dissected testes and SVs from adult males of the indicated age are shown for control and AOX-expressing lines: **a** w^{1118} (X/Y; 2/2; 3/3) and 3X*tub-AOX* ($tubAOX^{35}$/Y; $tubAOX^{112}$/$tubAOX^{112}$; $tubAOX^{7}$/$tubAOX^{7}$); **b** *UAS-AOX*^{F6}/w^{1118} (X/Y; *UAS-AOX*^{F6}/2; 3/3) and *UAS-AOX*^{F6}/*tubGS* (X/Y; *UAS-AOX*^{F6}/2; *tubGS*/3) in the absence (uninduced) or presence (induced) of 200 μM mifepristone. AOX protein induction by mifepristone was visualized by immunoblotting (Additional file 1: Fig. S5). Red circles indicate one of the dissected SVs of the representative samples of the indicated genotype. Note the underdeveloped state of the SVs in 10 day-old flies from the lines expressing AOX either constitutively (3X*tub-AOX*) or inducibly (*UAS-AOX*^{F6}/*tubGS* plus mifepristone). For an explanatory visualization of *D. melanogaster* reproductive organs, see Additional file 1: Fig. S6A. In **a**, the *inset* highlights the whitish material that accumulates in the proximal end of the testis in 3X*tub-AOX* males; in **b**, the *red arrow* points to the same material for *UAS-AOX*^{F6}/*tubGS* males treated with mifepristone. **c** Quantification of the data shown in **a** and **b**, in which the data points represent the mean thickness of 10–20 dissected organs ± SD (error bars). * indicates statistically significant difference between organs of AOX-expressing and nonexpressing males ($p \leq 0.01$). Additional file 1: Fig. S8B illustrates schematically the thickness measurement protocol used to estimate the amount of immature (in the testes) and mature (in the SVs) sperm cells per fly

testes of control flies decreases >50% in the first 10 days of adult life, whereas the SVs triple in size. In contrast, the testes of 3X*tub-AOX* males exhibit a much less pronounced decrease in thickness (~25%), whilst at the same time their SVs remain immature. In addition, we observed an accumulation of whitish material at the

proximal end of the testes of 10-day-old 3X*tub-AOX* males (Fig. 2a, *inset*), which we investigated further (see below). The mature spermatozoids produced by these males, although few in number, did not appear to have any motility defects, as judged by visual inspection.

We were able to produce a similar phenotype using the inducible GeneSwitch (*tubGS*) system, in which the same *α-tubulin* promoter as used in the *tub-AOX* constructs controls the expression of a modified GAL4 that is inducible by mifepristone (RU486). This allows time-regulated expression of a GAL4-dependent AOX transgene in exactly the same tissues as in the *tubAOX* lines. By transferring males to food-vials containing the inducing drug on the day of eclosion, any possible developmental disturbance is avoided, whilst accurate controls can be implemented, notably flies with transgene, but lacking driver and/or drug (see Additional file 1: Fig. S5 for immunoblots indicating tight regulation of AOX protein levels using this system). Similarly as for 3X*tub-AOX* males, the testes of *UAS-AOX* transgenic males driven by *tubGS* in the presence of mifepristone remained thick during the first 10 days of adult life, whilst their SVs remained small (Fig. 2b and c). These organs were as wild-type in the absence of the driver or inducing drug (Fig. 2b and c). The normal thinning of the testis over the first 10 days of adult life was also seen when both the driver and inducing drug were present, whether driving a control transgene, GFP, a catalytically inactive form of AOX, mutAOX [27] or even with no transgene at all (Additional file 1: Fig. S6A, S6C). In these cases, however, the SV remained undeveloped, but only when driver and drug were both present (Additional file 1: Fig. S6B, S6D). The whitish material near the proximal end of the testis also accumulated prominently when *tubGS* was used to express AOX (but not GFP or mutAOX), and again only under inducing conditions (Fig. 2b, *red arrow*). To confirm that this is an *α-tubulin* promoter-specific phenotype, we dissected the reproductive organs of 10-day-old males expressing *UAS-AOX* driven by *daGAL4*. The SVs had a normal morphology (Additional file 1: Fig. S7), and appeared to have accumulated a similar amount of mature sperm cells as controls, in agreement with the results of the sperm-competition assays (Additional file 1: Fig. S3).

Other reproductive organs were also evaluated morphologically (see Additional file 1: Fig. S8A for schematics of the reproductive system of *D. melanogaster* males), but no obvious alterations were observed between AOX-expressing and control males, based on visual inspection of >70 dissected flies of each genotype. Although AOX was not expressed in the accessory glands (Additional file 1: Fig. S9A), we also checked the presence of Sex Peptide in these organs by immunostaining and confocal microscopy, and again observed no difference (Additional file 1: Fig. S9B). Sex Peptide is one of the most important components of the male's seminal fluid; it has been implicated in reproductive success in sperm-competition assays [24, 28] and its action is dependent on the compatibility with receptors in the female reproductive tract [23]. Altogether, our data indicate that AOX-expressing males are defective in sperm-competition assays due to a decreased production of mature spermatozoids.

AOX is expressed in cells of the testis and seminal vesicle sheaths

Although the SVs of AOX-expressing males present perhaps the most pronounced phenotype in our study, these are purely storage organs for mature sperm cells [26], and their morphology was not diagnostic for AOX expression (Additional file 1: Fig. S6B).

Therefore, we hypothesized that the defect in sperm production caused by AOX expression most likely affects the testes, where spermatogenesis takes place. The *α-tubulin* promoter has been used previously to drive transgene expression in the fly testis in both somatic cells and the germline (from the stem-cell stage to late spermatocytes, reviewed in [29]). However, using immunofluorescent confocal microscopy (Fig. 3 and Additional file 1: Fig. S10), AOX expression from the *α-tubulin* promoter, whether directly (Additional file 1: Fig. S10) or driven by *tubGS* plus mifepristone (Fig. 3), was below the level of detection in germline cells, and seen only at very low levels in some somatic cyst cells. Nevertheless, it was abundantly expressed in the somatic cells of the testis and SV sheaths. In addition, all of the immature stages of cell differentiation appeared to be present (Additional file 1: Fig. S11), judging by the characteristic changes in nuclear morphology, mitochondrial network arrangement and cellular elongation that the germ line cells go through towards the final steps in spermatogenesis [26].

At higher resolution, it was clear that not all cells in the ensheathing tissues of the testes and SVs express AOX at a high level when driven by the *α-tubulin* promoter (either directly, as 3X*tub-AOX* or indirectly, through *tubGS*). Some cells highly expressing ATP5A, a complex V subunit traditionally used as a mitochondrial marker, appeared negative for AOX (Additional file 1: Fig. S12A). Using *UAS-StingerGFP* in combination with the *tubGS* driver, in order to mark the positive cells with GFP in the nucleus, we observed high expression in specific cells in the outermost sheath-cell layer (Additional file 1: Fig. S12B). The inner sheath-cell layer, formed of smooth-muscle cells with multiple small nuclei, appeared negative for AOX or GFP, in agreement with co-staining for AOX and actin (Additional file 1: Fig. S13A) in the testes of 3X*tub-AOX* males. Positive cells most

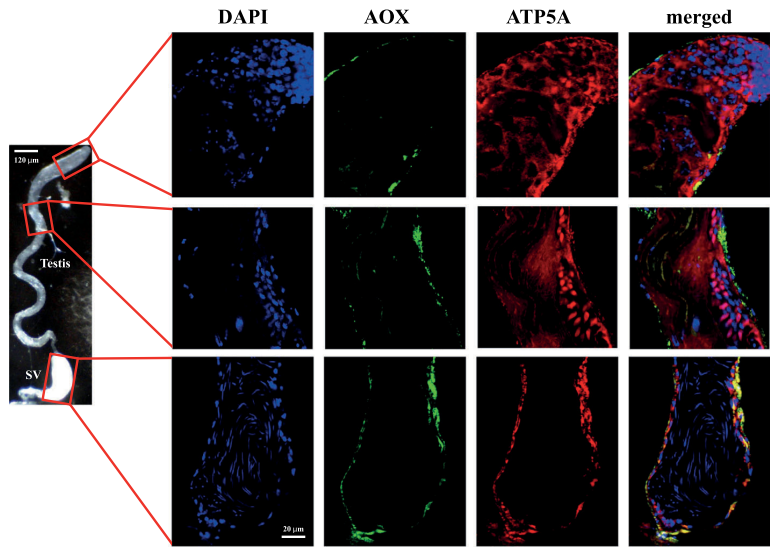

Fig. 3 AOX protein is localized in mitochondria of cells from the testis and seminal vesicle (SV) sheaths. Immunoflorescence staining and confocal microscopy for the indicated proteins and DAPI, from a 10-day old male of genotype X/Y; *UAS-AOX*^{F6}/2; *tubGS*/3, in the presence of mifepristone. AOX protein is localized to the outermost cell layer of these organs, where it overlaps that for the mitochondrial marker ATP5A, but not to germline cells at any stage of differentiation. The light microscopy image (left) is from a control male (w^{1118}), used for illustration purpose only. Spermatogenesis takes place inside cysts, starting at the tip of the testes (*upper panels* - note the concentration of nuclei that represent the first stages of this process) and moving towards the proximal end of the organ as cell differentiation proceeds (*middle panels* - note the presence of a cyst containing onion-stage spermatids with their single mitochondrial mass, called the Nebenkern). At the proximal end of the testis, mature spermatozoid cells are released from the cyst and stored in the SV (*lower panels* - note the needle-shaped nuclei that is typical of these cells). Note also the low-level AOX expression in the somatic cells of the cysts, but not in the germline. See Additional file 1: Fig. S10 for comparable images from *3Xtub-AOX* males

likely correspond with the pigment cells, which carry a single large nucleus, and are abundant around the SVs and the proximal end of the testis, where these two organs connect [30]. This is supported (Additional file 1: Fig. S13B–D) by co-staining for the *empty spiracles* gene product (ems), which is a marker for the pigment cells [31, 32]. Partial three-dimensional reconstruction of the microscopy images shows that the AOX and ems signals are located in the outermost cell layer of the sheath of the SV (Additional file 1: Fig. S13C) and testis (Additional file 1: Fig. S13D), whilst the underlying (smooth muscle) cells stain highly for ATP5A and actin. Note that the pigment cells are abundant in the anatomical region where the most pronounced morphological alterations were found in AOX-expressing males, marked by the accumulation of whitish material at the proximal end of the testis combined with a decreased amount of mature sperm cells in the SVs (Fig. 2a).

Mature-looking spermatozoids are lodged in the proximal end of the testis in AOX-expressing males

The proximal end of the *D. melanogaster* testis is also the region where the individualization process starts. This process is essential for the final stage of elongated spermatid differentiation [26], and is accomplished by an individualization complex (IC) that is assembled in the cyst region containing the spermatid nuclei and which then moves towards the tip of the tails, collecting syncitial cytoplasm and creating cystic bulges and waste bags (WBs) along the way [33, 34]. By staining the testis samples for actin and activated caspase-3, two components of the ICs, we observed no significant differences in the number of starting and established ICs in males expressing AOX driven by *tubGS*, but did observe an elevated number of WBs (Additional file 1: Fig. S14). Most noticeably, we observed in these males a dramatic change in the distribution of ICs throughout the organ. Starting ICs are usually present in the proximal region of the testis, but in 10 day-old males expressing AOX driven by *tubGS*, a significant number of them were found towards the middle region of the organ. Established ICs, which are usually found all along the testes, were concentrated at the middle and proximal end of the AOX-expressing testes. Finally, WBs appeared to be distributed in all testis regions in the AOX-expressing males, whereas in control testes they accumulated at the distal end (Additional file 1: Fig. S14C). Our observations imply that AOX expression driven by *tubGS* in the

somatic cells of the cysts, and/or in the pigment cells of the testis sheath causes internal rearrangements in this organ, disrupting the production and delivery of mature sperm.

To investigate the nature of the whitish material found at the proximal end of the testis of AOX-expressing males, we used a transgenic construct that expresses GFP-tagged Don Juan protein (DJ-GFP) in the tail of elongated spermatids and mature spermatozoids [35, 36], enabling us to evaluate the transition between these two stages. The expression of DJ-GFP revealed a high concentration of what appeared to be individualized mature spermatozoids in the proximal end of the testis of AOX-expressing males (Fig. 4, *lower panels*). In control males, few of these individualized GFP-positive cells were found in this region of the testes (Fig. 4, *upper panels*), as expected, given that they should move to the SVs after individualization. The failure of the individualized, elongated spermatids to move into the SV and the associated disturbance in the spatial distribution of ICs could account for the accumulation of the whitish material seen by light microscopy, and is the simplest explanation for the defect seen in sperm-competition assays, although the reason why they are retained in the testis is not obvious morphologically.

Discussion

In previous studies [6, 7] we reported that constitutive expression of *C. intestinalis* AOX in *Drosophila* did not affect viability or fertility under non-competitive lab conditions. Here we demonstrate that transgenic AOX expression in *Drosophila* under the *α-tubulin* promoter does cause a clear detriment to male reproductive success, using a stringent sperm-competition assay. This was accompanied by a decreased and disorganized production of mature spermatozoids. Although some sperm cells appear to complete the process of morphological differentiation, following individualization they appear to remain lodged in the proximal region of the testis, in the outer sheath of which AOX is highly expressed.

AOX, when expressed directly under the *α-tubulin* promoter or via the *tubGS* driver, is present at high abundance in the pigment cells of the testis sheath and SV, and is also found at very low levels in some somatic cyst cells. However, it is below the level of detection in the germline. The *α-tubulin* promoter has been reported to drive germline expression of transgenes up to the late spermatocyte phase [29]. Clearly, this does not apply to the construct we used [7]. Surprisingly we also observed no abnormalities during spermatogenesis in the mitochondrial network (Additional file 1: Fig. S11), the reorganization of which provides the structural platform for sperm axoneme formation [37]. On the other hand, the elevated number and altered distribution of WBs in the testes of AOX-expressing males may reflect increased recycling of dysfunctional germ cells, where differentiation has failed. We posit that the specific disturbance in spermatogenesis produced by AOX expression is most likely due to deranged signaling from somatic cells, rather than low-level AOX expression in the germline itself.

The precursor cells of the testis and SV sheaths have two distinct developmental origins: the pigment cells originate in the gonad imaginal disc, along with the testes, whereas the muscle cells and the SVs originate in the genital disc [38, 39]. When the two organs start fusing, migration of pigment and muscle cells proceeds in opposite directions with the gonad and the SVs each acquiring an outer layer of pigment cells and an inner

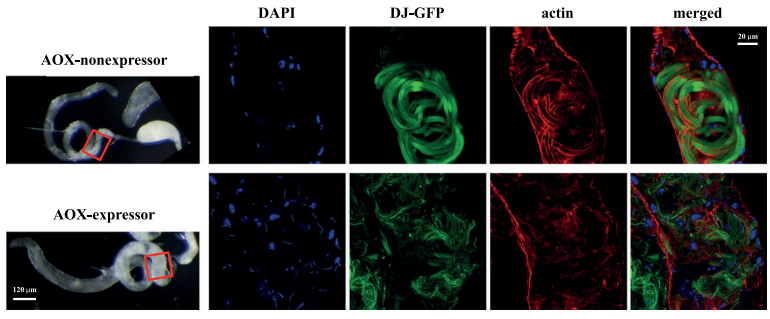

Fig. 4 Individualized sperm cells remain lodged in the testes of AOX-expressing males. Fluorescence imaging of testes of 10-day old males of genotype X/Y; *UAS-AOX*^{F6}/2; *tubGS*/DJ-GFP, under uninduced (*upper panels*) and mifepristone-induced (*lower panels*) conditions, was performed using phalloidin-TRITC to detect actin and the DJ-GFP fluorescence to detect the tails of elongated spermatids and mature spermatozoids. Note that in the proximal region of the testis (fluorescent confocal images, right, and *red boxes* on the white-light microscopy images, left) of the uninduced control sample (AOX-nonexpressor) only the bundles of not-yet-individualized elongated spermatids are observed, whereas in the same region in AOX-expressing males these cells appear individualized, tangled and/or disorganized, accounting for the accumulated whitish material shown in Fig. 2

layer of muscle [30]. The failure to produce pigment cells during development is known to lead to aberrant muscle-cell migration, causing abnormal testis morphology and male sterility, without affecting the germline or somatic cell-types that support spermatogenesis [30]. This interdependent migration implies the existence of a signaling network between the pigment and the muscle cells. Our findings suggest that communication between these cell-types might continue after testis development is complete. We suggest that AOX expression in the pigment cells might in some way disrupt their ability to deliver signals required for the completion of spermatogenesis, and/or to signal muscle contraction needed to guide individualized mature spermatozoids from the proximal end of the testis into the SV, leading to the accumulation of cysts in late stages of maturation throughout the testis. This is supported by the fact that a low percentage of testes of 3X*tub-AOX* males (~5% of total testes analyzed, Additional file 1: Fig. S15) presented malformations very similar to those reported for males unable to develop pigment cells [30]. Although the majority of testes are morphologically normal in most 3X*tub-AOX* males at eclosion, they remain thick over the first 10 days of adult life (Fig. 2B), indicating a functional defect in sperm production and delivery that would be sufficient to account for the decreased reproductive success under competitive conditions (Fig. 1). To prove that AOX expression in the sheath pigment cells was the cause of the observed sperm and reproductive defects would require a driver that enables its expression specifically in these cells. Since no such driver is currently available, we must rely instead on the strongly suggestive correlations we have documented here.

The effect of AOX on cell-cell communication could result from one of several metabolic disturbances conferred by the enzyme. If constitutively activated by local metabolic conditions in specific cells, AOX could participate significantly in respiratory electron flow, impairing ATP production or other downstream processes dependent on respiratory energy, such as the buffering of calcium (reviewed in [40]). A calcium signaling pathway has been implicated in the upregulation of Wnt-2 [41], which regulates differentiation in many cellular contexts, including the testis pigment cells during *Drosophila* development, promoting muscle cell migration and attachment [30]. *Drosophila* males mutant for Wnt-2 are sterile due to severe testis malformations [30], whereas males expressing AOX are fertile and appear morphologically normal at eclosion, with a functional defect only revealed by a stringent competition assay and by the apparent retention of sperm within the testis during the first 10 days of adult life.

An interference with cell signaling may also involve ROS. AOX has been reported to decrease mitochondrial ROS production even under conditions of normal oxidative phosphorylation activity [6, 42]. In either case, the very subtle phenotype, with all other physiological and developmental functions apparently unaffected, presents a challenge for understanding the mechanism.

Clearly, much remains to be learned about the physiological effects of AOX expression. Our findings indicate the importance of studying these effects and their mechanisms in detail, before implementing AOX in gene therapy. Some of its properties may need to be modified, before the enzyme can be safely deployed.

Conclusions

AOX offers a potential therapeutic strategy for buffering pathological stresses in mitochondria. Whilst it is, indeed, able to compensate for a number of pathological insults, it has remarkably little, if any effect on normal physiology of model organisms, including *Drosophila*. Transgenic flies ubiquitously expressing AOX were earlier found to exhibit normal development, reproduction and lifespan.

In the present study we took this analysis to a deeper level, by applying a more stringent test of reproductive capacity, the well-established sperm-competition assay. Despite being fertile, AOX-expressing males showed a specific detriment in this test, which correlates with total transgene expression dose. Morphologically, we found this to be correlated with spatially deranged spermatogenesis and failure to release mature sperm into the storage organ (seminal vesicle) in normal quantities, although the defect is inferred to arise in the somatic pigment cells of the testis, not the germline. This is the first demonstration of a developmental defect caused by AOX expression in a metazoan, and needs also to be taken into account when considering therapeutic uses of AOX in humans.

Methods

Fly stocks and maintenance

Standard lines w^{1118}, CyO and TM3,Sb balancers, *UAS-StingerGFP*, *daGAL4*, *tubGS*, and DJ-GFP were obtained from stock centres. The lines *UAS-AOX*^{F6} [6], *tub-AOX*^{35}, *tub-AOX*^{112}, *tub-AOX*^{7}, 2X*tub-AOX*, 3X*tub-AOX* [7], *UAS-AOXwt*$^{8.1}$, *UAS-AOXmut*, *UAS-empty*^{2nd}, and *UAS-empty*^{3rd} [27] were described previously. All fly lines were backcrossed into the w^{1118} background for six-to-ten generations, and were maintained in standard diet at 25 °C [6]. RU486 (mifepristone, Sigma, USA) was added to the diet at the indicated concentrations for induction of transgene expression using the *tubGS* line.

Sperm-competition assays

Sperm-competition assays were conducted using two approaches. In the defensive paradigm, approximately 50 virgin w^{1118} females (white eyes), aged between 3 and

7 days, were initially allowed to mate individually with 5–8 day-old (virgin) transgenic males (red eyes, *w+*) for 3 days. The males were discarded and the females transferred to new vials and allowed to mate individually with 5–8 day-old w^{1118} males (white eyes), for a further 3 days, after which the second males were also discarded. The females were again transferred to new vials, allowed to lay eggs for 3 days, then transferred finally for further egg laying over 5 days. In the offensive paradigm, approximately 50 virgin w^{1118} females (white eyes) were first crossed with w^{1118} males (white eyes), and then with transgenic males (red eyes, *w+*), following the same mating and egg laying scheme of the defensive paradigm. Reproductive success was measured by counting the number of red- and white-eyed progeny, which represent, respectively, the progeny of transgenic and w^{1118} males. The small number of vials derived from any single female that contained progeny exclusively of one eye colour were considered to represent only a single mating and were excluded from the analysis. Data were plotted as mean number of progeny per female of each eye colour, in the four successive mating/egg laying vial sets, ± standard deviation.

Dissection and imaging of male reproductive organs

Reproductive organs were dissected by anesthetizing males of the indicated age and genotype with CO_2 prior to transferring to a dissection board containing phosphate-buffered saline (PBS). Using thin dissection forceps, the internal organs were removed from the abdomen by dislocating the external genitalia, followed by manual isolation of the reproductive organs, which were imaged immediately using the Nikon SMZ 745 T system under white light. The images were analyzed and testis and SV thickness was measured using NIS Elements D4.20 software (Nikon Instruments Software, Netherlands).

For immunofluorescence imaging, dissected organs were fixed in 4% paraformaldehyde for 20 min at room temperature, and then transferred to PBS prior to permeabilization. The organs were rinsed twice with PBS containing 0.1% Triton X-100 and 0.1% BSA, then permeabilized and blocked with PBS containing 1% Triton X-100 and 1% BSA for 30 min at room temperature, followed by overnight incubation at 4 °C with the primary antibody diluted in PBS containing 0.3% Triton X-100 and 0.5% BSA. The samples were then rinsed twice and washed three times (30 min each) with PBS containing 0.1% Triton X-100 and 0.1% BSA at room temperature, followed by overnight incubation at 4 °C with the secondary antibody diluted in PBS containing 0.3% Triton X-100 and 0.5% BSA. Washes were performed as described above, and samples were rinsed with PBS and then Milli-Q water, prior to transferring to optically clear 35 mm glass bottom dishes (MatTek, USA) containing ProLong Gold Antifade Mounting medium with DAPI (Molecular Probes, Life Technologies). Primary antibodies used were rabbit polyclonal anti-AOX (1:10,000, [6]), mouse monoclonal anti-ATP5A (1:1000, Abcam, UK), rabbit polyclonal anti-activated caspase-3 (1:200, Cell Signaling Technology, USA), rabbit polyclonal anti-Sex Peptide (1:300, gift from Dr. Shanjun Chen) and rabbit polyclonal anti-empty spiracles (1:400, Antibody Verify, USA). Fluorescent secondary antibodies were Alexa 488-conjugated goat anti-rabbit IgG (1:1000) and Alexa 568-conjugated gat anti-mouse IgG (1:1000) (Fisher Scientific, USA). Actin was stained using phalloidin-TRITC (1 μg/ml, Sigma, USA). Samples were imaged using an Andor Spinning Disc or Zeiss LSM 780 laser-scanning confocal microscope, respectively with Andor iQ 3.0 or ZEN 2011 SP3 (black edition) software. Images were analyzed with ImageJ software (National Institutes of Health, USA). No signals were observed when the primary antibodies were omitted.

Immunoblotting

Protein extracts from whole adult males (20–30) and from dissected testes and SVs of 50–100 males were prepared by gentle grinding of tissues in 50–200 μl PBS containing 1.5% Triton X-100 and Complete, Mini, EDTA-free Protease Inhibitor Cocktail (Roche, Switzerland). The suspension was centrifuged at 16,000 g_{max} for 10 min at 4 °C, and supernatant protein concentration was measured by the Bradford method. Crude mitochondrial preparations were obtained by homogenizing 50–100 adult males in ice-cold isolation buffer (250 mM sucrose, 5 mM Tris, 2 mM EGTA, pH 7.4) followed by centrifugation at 200 g_{max} for 3 min at 4 °C. The supernatant was transferred to a new tube and centrifuged at 9000 g_{max} for 10 min at 4 °C. The pellet was resuspended in 50 μl of isolation buffer, and protein concentrations were determined using the Bradford method.

100 μg of total protein extracts and 40 μg of mitochondrial preparations were mixed with 5× Laemmli buffer (10% SDS, 50% glycerol, 25% 2-mercaptoethanol, 0.02% bromophenol blue and 0.3125 M Tris-HCl, pH 6.8), denatured at 95 °C for 5 min and resolved by SDS-PAGE on 4–20% or Any kD Criterion Precast Gels (Bio-Rad) at 120 V for approximately 1.5 h. Proteins were transferred to nitrocellulose membranes using the iBlot Gel Transfer system (Invitrogen, USA) for 7 min at room temperature. Membranes were blocked in TBST (0.15 M NaCl, 50 mM Tris-HCl, 0.05% Tween 20, pH 7.6) containing 5% dried nonfat milk for 2 h at room temperature or overnight at 4 °C. Primary antibodies (rabbit polyclonal anti-AOX [1:10,000, [6]], mouse monoclonal anti-ATP5A [1:5000, Abcam, UK], mouse monoclonal anti-PDH E1α [1:5000, Abcam, UK], and mouse monoclonal anti-GAPDH [1:5000, Abcam, UK]) were incubated for at least 1 h at

room temperature, followed by three washes (10 min each) with TBST. Secondary antibodies (HRP-conjugated goat anti-rabbit and anti-mouse IgGs [1:10,000, Bio-Rad, USA]) were incubated for at least 2 h at room temperature, and washed as described. Membranes were incubated in Luminol substrate detection system Immun-Star HRP (Bio-Rad, USA), and chemiluminescence signals were detected on Fuji Medical X-ray films (Fujifilm, Japan). Band densitometry was performed with ImageJ software (National Institutes of Health, USA).

Statistical analyses

Measurements of organ thickness were performed as schematized in Additional file 1: Fig. S8B and tested for statistical differences among the indicated groups of fly males using one-way ANOVA with Newman-Keuls post hoc test. For analysis of sperm-competition assays, the parameters *P1'* and *P2'* [22] were tested for statistical differences among the indicated groups of fly males, using one-way ANOVA with Tukey post hoc test. The analyses were performed using Prism software, version 5 (GraphPad Software, USA).

Additional file

Additional file 1: Table S1. Proportion of progeny sired by the red-eyed male when this is the first male to mate in sperm competition assays against w^{1118} males (*P1'*). **Table S2.** Proportion of progeny sired by the red-eyed male when this is the second male to mate in sperm competition assays against w^{1118} males (*P2'*). **Figure S1.** The sperm of eye-color control and *tubGS* males shows normal competitive properties. **Figure S2.** The defect in sperm-competition assays is AOX dose-dependent. **Figure S3.** AOX expressed under the *daughterless* promoter does not decrease sperm competitiveness. **Figure S4.** AOX protein levels in testes and seminal vesicles of diverse AOX-expressing lines. **Figure S5.** Induction of AOX expression using the *UAS-AOX*^{F6}*/tubGS* system. **Figure S6.** Altered testis morphology during adulthood is diagnostic for AOX expression. **Figure S7.** Mature sperm cells accumulate normally in the seminal vesicles (SVs) of males expressing AOX under the control of the *daGAL4* driver. **Figure S8.** Schematic diagrams and light microscopy of *D. melanogaster* male reproductive organs [43]. **Figure S9.** AOX is not present in the accessory glands and does not interfere with Sex Peptide production. **Figure S10.** AOX protein is localized in mitochondria of cells from the testis and seminal vesicle (SV) sheaths in *3Xtub-AOX* males. **Figure S11.** Nuclear and mitochondrial morphologies in germline cells are not altered in *3Xtub-AOX* males. **Figure S12.** AOX is expressed in the outermost cell layer of the testis and *SV* sheaths. **Figure S13.** AOX localizes to the pigment cell layer of the testis and SV sheaths of *3Xtub-AOX* males [44]. **Figure S14**. AOX-expressing males present alterations in subtesticular structures. **Figure S15.** Testis malformation in *3Xtub-AOX* males. (PDF 3255 kb)

Abbreviations

AOX: Alternative Oxidase; ATP: Adenosine Triphosphate; GFP: Green Fluorescent Protein; IC: Individualization Complex; ROS: Reactive Oxygen Species; SV: Seminal Vesicles; UAS: Upstream Activating Sequence

Acknowledgements

The authors would like to thank Eric Dufour for useful discussions and Tea Tuomela for technical assistance.

Funding

The authors acknowledge the support of the European Union (Marie Curie International Incoming Fellowship GA328988 to M.T.O. and European Research Council Advanced Grant 232,738 to H.T.J.), the Fundação de Amparo à Pesquisa do Estado de São Paulo (grant number 2014/02253–6 to M.T.O.), Finnish Cultural Foundation (to A.A.) and Academy of Finland (decisions 256,615 and 272,376), Tampere University Medical Research Fund and Sigrid Juselius Foundation (to H.T.J.).

Availability of data and materials

All primary data of the manuscript are included in the submitted figures and tables. Any additional datasets relevant to the current study are available from the corresponding author on reasonable request.

Authors' contributions

M.T.O. and H.T.J. conceived and designed the experiments, analyzed the data, compiled the figures, and drafted the manuscript with input from other authors. M.T.O., S.S., G.S.G. and A.A. performed the experiments.

Ethics approval and consent to participate

The experiments reported did not use human subjects, materials derived from humans or experimental animals covered by ethical or animal welfare legislation. Experimental use of the fruit fly, *Drosophila*, is exempt from such legislation.

Competing interests

The authors declare that they have no competing interests

Consent for publication

Not applicable.

Publisher's Note

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Received: 6 May 2017 Accepted: 14 June 2017
Published online: 03 July 2017

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PUBLICATION II

Alternative respiratory chain enzymes: Therapeutic potential and possible pitfalls

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BBA - Molecular Basis of Disease (2019), 854–866
doi: 10.1016/j.bbadis.2018.10.012

BBA - Molecular Basis of Disease 1865 (2019) 854–866

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Alternative respiratory chain enzymes: Therapeutic potential and possible pitfalls☆

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ARTICLE INFO

Keywords:
Mitochondria
Mitochondrial disease
Thermogenesis
AOX
Reactive oxygen species

ABSTRACT

The alternative respiratory chain (aRC), comprising the alternative NADH dehydrogenases (NDX) and quinone oxidases (AOX), is found in microbes, fungi and plants, where it buffers stresses arising from restrictions on electron flow in the oxidative phosphorylation system. The aRC enzymes are also found in species belonging to most metazoan phyla, including some chordates and arthropods species, although not in vertebrates or in *Drosophila*. We postulated that the aRC enzymes might be deployed to alleviate pathological stresses arising from mitochondrial dysfunction in a wide variety of disease states. However, before such therapies can be contemplated, it is essential to understand the effects of aRC enzymes on cell metabolism and organismal physiology. Here we report and discuss new findings that shed light on the functions of the aRC enzymes in animals, and the unexpected benefits and detriments that they confer on model organisms. In *Ciona intestinalis*, the aRC is induced by hypoxia and by sulfide, but is unresponsive to other environmental stressors. When expressed in *Drosophila*, AOX results in impaired survival under restricted nutrition, in addition to the previously reported male reproductive anomalies. In contrast, it confers cold resistance to developing and adult flies, and counteracts cell signaling defects that underlie developmental dysmorphologies. The aRC enzymes may also influence lifespan and stress resistance more generally, by eliciting or interfering with hormetic mechanisms. In sum, their judicious use may lead to major benefits in medicine, but this will require a thorough characterization of their properties and physiological effects.

1. Introduction

Protists, fungi and plants possess an auxiliary respiratory system in their mitochondria, which buffers metabolic stresses that arise from limitations on electron flow in the system of oxidative phosphorylation (OXPHOS). This alternative respiratory chain (aRC) comprises representatives of just two classes of enzyme: one or more alternative NADH dehydrogenases (NDX), that transfer electrons from NADH to an intermediate electron carrier, ubiquinone [1], and alternative oxidases (AOX), that complete electron transfer from ubiquinol directly to

☆ This article is part of a Special Issue entitled: The power of metabolism: Linking energy supply and demand to contractile function edited by Torsten Doenst, Michael Schwarzer and Christine Des Rosiers.

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https://doi.org/10.1016/j.bbadis.2018.10.012
Received 20 May 2018; Received in revised form 3 October 2018; Accepted 5 October 2018
Available online 17 October 2018

oxygen [2]. In terms of their net redox chemistry, NDX and AOX can functionally replace respiratory complex I (cI, NADH:ubiquinone oxidoreductase) and complexes III (cIII, ubiquinol:cytochrome *c* oxidoreductase) plus IV (cIV, cytochrome *c* oxidase) of the OXPHOS system, respectively.

In contrast to the standard OXPHOS complexes of the mitochondrial respiratory chain (RC), the alternative enzymes have five distinct properties. First, they are each composed of a single polypeptide. Second, their reaction chemistry is non proton-motive, instead dissipating the released free energy as heat. Third, they are universally coded only by the nuclear genome. Fourth, they are refractory to the commonly used OXPHOS inhibitors. Finally, their biochemical properties limit their activity to metabolic conditions where they are functionally required, at least in the case of AOX. As a result, AOX may be considered a self-regulating enzyme that does not 'short-circuit' the OXPHOS system, except when the latter is already dysfunctional. In plants, the basis of this restriction is relatively well understood, in that the enzyme only becomes active when the substrate pool of reduced quinones accumulates to elevated levels, that would be considered abnormally high in non-photosynthetic organisms [3,4]. In effect, the enzyme displays a much higher K_m for ubiquinol than does cIII. This property has been assumed to apply to AOX from other taxa, but has not yet been formally demonstrated.

In various species, AOX has been reported to become activated by metabolites such as pyruvate [3,5–7] and other organic acids [8,9], that may accumulate under conditions of RC overload or inhibition, or in response to specific metabolic stresses. In some taxa the aRC enzymes have also been shown to be regulated by heat stress [10], calcium [10,11] and purine nucleotides [7,9,12]. The molecular basis of these phenomena, or even whether regulation is direct or indirect, is mostly not known, although activation of AOX in plants is relatively well understood. In this case, AOX is believed to exist normally as a dimer, which is activated (most likely by thioredoxin) when the inter-subunit disulfide bridge is broken. Further activation is brought about by the allosteric binding of pyruvate and/or other organic acids [13]. Plant AOX is also regulated at the level of gene expression in many contexts where its presence confers predictable resistance against various metabolic stresses, including drought, heat, cold, salt and even pathogen invasion [14,15].

Much less is known about the metabolic regulation of NDX, which is found in different sub-mitochondrial compartments, and is likely to operate subject to the availability of substrate in these locations. In the yeast *Saccharomyces cerevisiae*, which lacks cI, the NDX variants Nde1 and Ndi1, which respectively catalyze NADH oxidation at the exterior- and interior-facing sides of the inner mitochondrial membrane, may be co-regulated with the rest of the OXPHOS machinery.

In plants, the aRC enzymes are also believed to maintain mitochondrial metabolic processes and redox homeostasis during daylight, at least under stress conditions [16], when excess ATP production by photosynthesis may restrict flux through the mitochondrial RC. Two other physiological traits conferred by aRCs are thermogenesis and regulation of aging. aRC enzymes do not conserve the free energy of biological oxidations by charge separation across the inner mitochondrial membrane. Instead, they dissipate the energy as heat. But this heat can also be harnessed. The most spectacular examples of aRC-based thermogenesis are in plants, where the released heat is used to volatilize insect attractants, as in the flowers of arum lilies [17,18]. In fungi, a well documented example of a life-cycle shift from OXPHOS to alternative respiration underlies the switch from vegetative growth to long-term maintenance in *Podospora anserina*. In senescent cultures the aRC acts to replenish primary electron carriers such as NAD in their oxidized form but does not support continuous growth. However, it also generates far less reactive oxygen species (ROS) than the OXPHOS system, thus limiting oxidative damage and facilitating long-term survival [19]. Defence against excess ROS may be a common function at least of AOX, since the accumulation of reduced quinols that triggers its activation potentially favours the passage of single electrons to oxygen both at cIII and, via reverse electron flow, at cI. Conversely, since ROS has also been posited to serve a signaling function, especially in regard to reverse electron transport at cI [20], AOX activation might interfere with or modify cell signaling pathways in some contexts, with important physiological consequences.

The aRC enzymes are also found in bacteria, and in representative organisms from many metazoan phyla [21], including some arthropods and some chordates [22,23]. Intriguingly, they appear to have been lost during the course of evolution in what are commonly regarded as the crown groups of metazoan evolution: the vertebrates and the more advanced insects, such as honeybees or flies [21,22] as well as, most likely, the cephalopod molluscs. Whilst at least some physiological functions of aRC enzymes in lower eukaryotes and plants are relatively well established, the roles they play in animals are almost completely unknown. They have been proposed to facilitate adaptation to environmental stresses and transitions [22], but experimental data supporting this concept remains to be gathered. In plants, some alternative NADH dehydrogenases can use NADPH as a substrate, but this issue is one of many that have not been explored in regard to the enzyme in animals.

We reasoned that, since many of the same stresses against which AOX provides a defence in plants and lower eukaryotes also occur pathologically in humans, as a result of mitochondrial OXPHOS dysfunction, deploying aRC enzymes xenotopically might help alleviate the associated disease states [24]. To test and develop this concept, we set out to examine the properties conferred upon cells and model organisms by expression of metazoan aRC enzymes.

In pioneering experiments, Yagi and colleagues had already demonstrated that yeast Ndi1 could be expressed in human cells, and could functionally replace the redox activity of cI [25–29]. By developing this concept further, they showed its potential in pathological models of cI-related diseases in rodents [30–32], informing and inspiring our work using the metazoan enzymes. For our own experiments, we selected, as a source, the group of animals closest to humans, but which still retains genes for both AOX and for NDX; namely the tunicates, a sister-group to the vertebrates.

In initial trials we were able to express in human cells the AOX from the tunicate *Ciona intestinalis* [33]. The *Ciona* AOX protein was routed to mitochondria via its own, intrinsic N-terminal mitochondrial targeting peptide. It conferred resistance to OXPHOS toxins such as antimycin A or cyanide in isolated mitochondria or in whole cells. More remarkably, when introduced transgenically into *Drosophila* or into the mouse, AOX could be expressed ubiquitously, with almost no detectable effect on phenotype [34]. Transgenic expression in *Drosophila* of the *Ciona* NDX [35], or the single-subunit NADH dehydrogenase Ndi1 from yeast [36], was also achieved with hardly any detectable effect on normal development or physiology, at least under non-stressed conditions.

Given these findings, we and others have proceeded to test how far these transgenes provide protection against physiological stresses that arise through, or are mediated, by mitochondrial dysfunction, commencing with studies in *Drosophila*. Although AOX was unable to rescue a null mutation in a subunit of cIV [34], it was able to compensate, at least partially, for the effects of cIV knockdown directed at specific subunits and tissues [37,38], as well as for the effects of a heteroplasmic mtDNA mutation affecting cytochrome oxidase [6]. More intriguingly, AOX expression was able to attenuate the pathological phenotypes of several different neurological disease models, including two *Drosophila* models of Parkinson's disease [34,39] and one of Alzheimer's disease [40]. The exact mechanisms behind these phenotypic transformations remain to be elucidated, but alleviation of excess ROS production due to blocked electron flow has been put forward as one plausible, common explanation. Conversely, AOX was unable to improve the phenotype of a fly model of mitochondrial translational disease (tko^{25t}), which exhibits developmental delay and mechanically induced seizures, associated with a decreased activity of all four OXPHOS

complexes that are dependent on mitochondrial translation products [41]. Co-expression of Ndi1 actually worsened the phenotype, suggesting that the underlying defect is insufficient ATP production rather than disturbed redox or metabolic homeostasis. AOX was also unable to rescue lethal mutations in the mtDNA helicase (Twinkle) or DNA polymerase γ, as well as the lethality produced by global knockdown of these genes [42].

Flies expressing Ndi1 showed evidence of increased lifespan, which was originally suggested to be due to a compensation of oxidative stress [36]. However, this effect appears to result from a paradoxical hormetic process, in which the constitutive expression of Ndi1 actually increases mitochondrial ROS production due to reverse electron flow through cI, which then activates stress responses that prevent oxidative damage throughout life [43].

Whilst our findings in *Drosophila* are in some ways remarkable, implementing aRC enzymes as actual therapy remains only a distant goal. The range of medical conditions in which mitochondrial RC or OXPHOS dysfunction is a contributory or essential factor is very broad, spanning from primary mitochondriopathies caused by mutations in mtDNA or in the apparatus of mtDNA maintenance and expression [44,45], through to common disease entities where mitochondrial disruption is due to ischemia/reperfusion injury, oxidative or proteotoxic stress, toxic damage or other external causes. We need to elucidate in much greater detail which of these conditions is alleviated by the expression of aRC enzymes, using the most appropriate animal models. Thus, a major thrust of current work is to test, using mouse models now available, how far AOX (or NDX/Ndi1) can negate the pathological consequences of mitochondrial dysfunction in all of these contexts. The focus of these studies will increasingly be on cardiovascular and neurological diseases, where the importance of mitochondrial dysfunction is now widely recognized.

Despite the encouraging findings to date in animal models, the potential problems in the use of aRC enzymes in therapy need to be considered in much greater detail. We need to be sure that their acute or chronic administration does not have harmful long-term consequences; in particular when the body is stressed in ways that may or may not be related to the original or underlying condition that prompted their use. Not all types of mitochondrial dysfunction are equivalent, and not all tissues respond in the same manner to a given biochemical defect or external stress.

In order to address the many issues arising from the potential use of metazoan aRC enzymes in future therapy, it is necessary to establish in finer detail their inherent properties, in particular when and how they become enzymatically active and how they affect model organisms under stress conditions where they may become activated. The wide range of medical conditions associated with mitochondrial dysfunction, and against which aRC enzyme-based therapies might be effective, combined with the diversity and complexity of their regulation already known from the plant and microbial world, mean that this task is vast and complicated.

As a first step towards this goal, we here set out to investigate aspects of the biology of aRC enzymes in the animal source that we initially selected, *C. intestinalis*. In particular, in order to obtain clues on their probable physiological functions, we tested how their expression is modified by external conditions, focusing on stressors found in their natural environment. In a parallel set of studies, we investigated how the expression of aRC enzymes, specifically AOX, can modify the physiological responses of model organisms under stressful environmental conditions, focusing initially on temperature and nutrition, and on the already well characterized *Drosophila* models which are also much more easily manipulated than their mammalian counterparts.

2. Materials and methods

2.1. Ciona specimens, maintenance and stress treatments

Ciona adults were supplied as living specimens by the Station Biologique de Roscoff. Prior to the experiments, they were acclimated to laboratory conditions for 24–48 h without feeding, at 18 °C in glass aquaria at a density of one animal per 2.5 l artificial sea water (Reef Crystals Aquarium Systems, Sarrebourg, France). To test the effects of sulfide, sodium sulfide nonahydrate (Sigma Aldrich) was added so as to reach combined concentrations of dissolved free sulfides of 100 and 300 μM. To test the effects of altered oxygen levels, normoxia was defined as the equilibrium state with atmospheric oxygen. Hypoxic conditions were established by displacement of air by nitrogen gas, and hyperoxic conditions by oversaturation using gaseous oxygen. Oxygen levels were monitored using an optical fibre probe and associated software (OxyView - PST3-V6.02, Precision Sensing GmbH, Germany) and adjustments were made so to maintain stable conditions throughout each experiment. For details of other stresses applied, see Supplementary Data File.

2.2. RNA extraction and analysis

Total RNA was extracted from frozen dissected *Ciona* organs (ovary, heart and stomach) by homogenization in 1 ml TRI Reagent (Molecular Research Center, USA) using ceramic beads, for 3 × 20 s at 4000 rpm (PowerLyzer 24 Bench Top Bead-Based Homogenizer; MO BIO Laboratories, USA). After chloroform extraction, followed by centrifugation at 12,000g_{max} for 15 min at 4 °C, RNA was precipitated from the aqueous phase by isopropanol at −20 °C for 1 h, followed by centrifugation at 12,000g_{max} for 8 min at 4 °C. Pellets were washed in 75% ethanol, air dried, and resuspended in RNase-free water at 60 °C for 10 min. RNA was prepared from frozen neural complex samples using Single Cell RNA Purification Kit (Norgen Biotek, Canada). Samples were manually homogenized in 100 μl lysis buffer using plastic pestles, followed by incubation at 42 °C for 5 min, subsequent vortexing for 15 s, and thereafter treated according to manufacturer's instructions. RNA extracts were quantified spectrophotometrically, pre-treated with RNase-free DNase I (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to manufacturer's instructions, to remove residual genomic DNA, then reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), also according to manufacturer's instructions. Quantitative PCR of cDNA used StepOne Plus PCR instrument (Applied Biosystems), with initial denaturation for 20 s at 95 °C, 40 cycles of 3 s at 95 °C, 30 s at 60 °C, and a final melt-curve step of 15 s at 95 °C, 60 s at 62 °C, and 15 s at 95 °C. Reactions using customized gene-specific primers (Supplementary Table S1) were run in duplicate, with 3.125–50 ng cDNA (depending on the target gene), 0.5 μM of each primer, and Fast SYBR Green Master Mix (Applied Biosystems). To minimize technical bias, samples from all treatments for a given gene and tissue were analyzed on the same plate, where possible. Measurements that exhibited divergent melting temperatures or amplification curves were excluded and analyses repeated. Quantification cycles (C_q) and amplification efficiencies (E) were calculated using the online tool Real-time PCR Miner and C_q values were corrected for among-plate bias and standardized against the mean of two reference genes, β-actin and RPL5.

2.3. Drosophila strains and culture

Drosophila strains used in the study were 7 transgenic lines for *C. intestinalis* AOX constructed in-house and described previously: *UAS-AOX^{F6}*, inserted on chromosome 2 [34], *UAS-AOX^{F24}*, inserted on chromosome 3 [34] and *UAS-AOX$^{8.1}$*, inserted by ΦC31 recombination at a lower-expression site on chromosome 2 [38], each containing the AOX coding sequence placed under the control of the exogenously

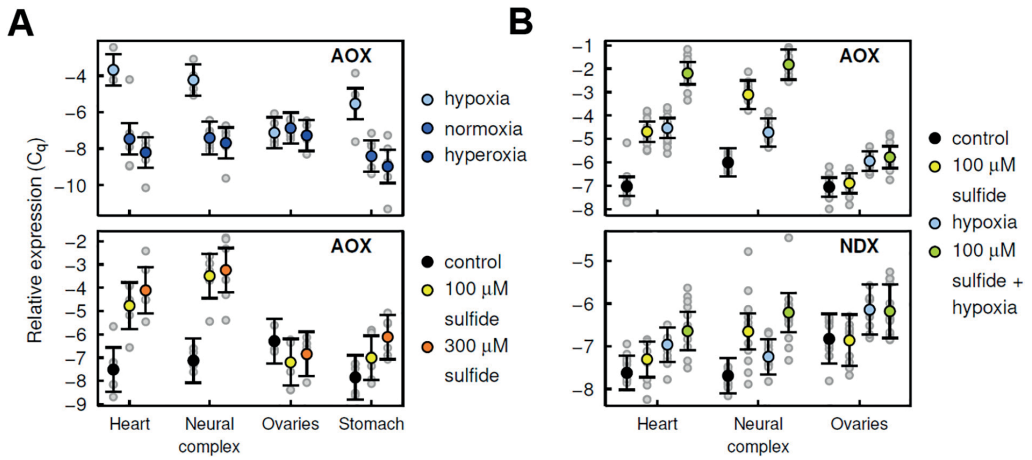

Fig. 1. *AOX* and *NDX* induction by environmental stressors in *Ciona intestinalis*.
Reverse transcription quantitative real-time PCR (RT-qPCR) analysis of the levels of *AOX* and *NDX* transcripts in RNA extracted from the indicated tissues of *C. intestinalis* adults, treated as indicated (see Materials and Methods): (A) for 6 h at oxygen levels of 1.62 (hypoxia), 7.92 (normoxia) or 15.94 (hyperoxia) mg/l or for 8 h in 0 (control), 100 or 300 µM sulfide, n = 6 for each group, in 2 aquaria; (B) for 6 h with an oxygen level of 1.96 mg/l (hypoxia), or 100 µM sulfide or the combination of both (1.91 mg/l O_2, 100 µM sulfide), n = 12 for each group, in 4 aquaria. The corrected C_q statistic, representing relative transcript levels normalized against two reference genes, was calculated as described in Materials and Methods, with data plotted as marginal model means ± 95% CI, showing data points for individual animals in grey. Panel (A) is a subset of the data shown in Fig. S1A and S1B, omitting *NDX* and indicator genes. For statistical analyses, see Supplementary Tables S2 and S3, relating to the data shown in panels (A) and (B), respectively. Note that the animals used in the experiment of panel (B) were a mixture of *C. intestinalis* types A and B, currently proposed as distinct species [77]. The primers used recognize RNA from both *C. intestinalis* types, and the data shown in the figure are normalized to type B (see Supplementary Table S3) for comparability with other experiments, where only type B was used.

supplied GAL4 transcription factor; *tubAOX*^{35}, *tubAOX*^{112} and *tubAOX*^{7}, containing insertions on chromosomes X, 2 and 3, respectively, of the AOX coding sequence under the control of the α-tubulin promoter; and *UAS-mutAOX*^{2nd}, bearing a mutated, catalytically inactive AOX variant also under GAL4 control, on chromosome 2. We also generated lines *2xtubAOX*, homozygous for both the *tubAOX*^{112} and *tubAOX*^{7} insertions, and *3xtubAOX*, homozygous additionally for *tubAOX*^{35}. Recipient line *w*^{1118} was used as a control for all transgenic lines except those created by ΦC31 recombination, for which a line with the UAS-containing vector inserted at the same site as *UAS-AOX*$^{8.1}$ and *UAS-mutAOX*^{2nd}, *UAS-empty*, was used. An additional control, *UAS-GFP* (Stinger), expressing nuclear-localized GFP under GAL4 control, was used in some experiments. Flies were maintained and cultured on standard high-sugar medium [34] at 18 or 25 °C, with 12 h cycles of light and darkness, except where indicated in specific experiments.

2.4. Developmental assays

To test effects of temperature, developmental assays were conducted at 12, 15, 18, 25, and 29 °C, as follows. 20 pre-mated females, in the presence of 10 males of the same line, were allowed to lay eggs for ≤24 h at 25 °C in a total of four vials per experiment. The eggs were counted, transferred to the indicated temperatures, and the vials monitored daily for the appearance of pupae and adults. Egg-to-pupa viability was calculated for each vial as the ratio between the total number of pupae and the total number of eggs laid. Egg-to-pupa and egg-to-adult developmental time was determined by recording the number of pupae or adults per vial on successive days relative to the day of egg laying. Experiments were performed in duplicate at 12 and 15 °C, and in triplicate at other temperatures. To test effects of the composition of the culture medium, flies of a given genotype or crosses as indicated in figure legends were grown on standard high-sugar medium or on a low-nutrient medium comprising, except where stated, 3.5% yeast and 5% glucose in standard agar with antimicrobials (niapigin and propionic acid). Pupae per vial and the number of eclosed adults were recorded.

2.5. Light microscopy of pupae

Uneclosed pupae, dissected from the pupal case if sufficiently advanced developmentally, were visualized using a Nikon SMZ 745T zoom stereomicroscope.

2.6. Protein extraction and analysis

Batches of 10 larvae or flies were snap frozen at −80 °C and crushed in an Eppendorf tube in 100 µl of lysis buffer, comprising 0.3% SDS in PBS plus protease inhibitor cocktail (Roche). Following incubation for 15 min at room temperature and centrifugation for 10 min at 15,000g_{max} at room temperature, supernatants were decanted and protein concentrations measured by NanoDrop spectrophotometry (ThermoFisher Scientific). After dilution with water and 5 × SDS-PAGE sample buffer, 63 µg protein aliquots in 20 µl were resolved on 12% polyacrylamide gels, wet-blotted to nitrocellulose membrane, washed in PBS-Tween, blocked for 3 h with 5% nonfat milk in PBS-Tween at room temperature, and reacted with custom-made rabbit anti-AOX antibody [34], 1:10,000 overnight at 4 °C. After 5 × 5 min washes in PBS-Tween, the membrane was reacted with secondary antibody (goat anti-rabbit, Vector Laboratories, 1:10,000) for 1 h at room temperature, re-washed, processed for imaging using Luminata™ Crescendo (Millipore) for 5 min, then imaged for chemiluminescence using BioRad ChemiDoc MP. Equal loading was confirmed by staining the membrane with Ponceau S for 5 min, washing with water and plain imaging. Images were optimized for brightness and contrast and cropped, rotated and masked for clarity, but not manipulated in any other way.

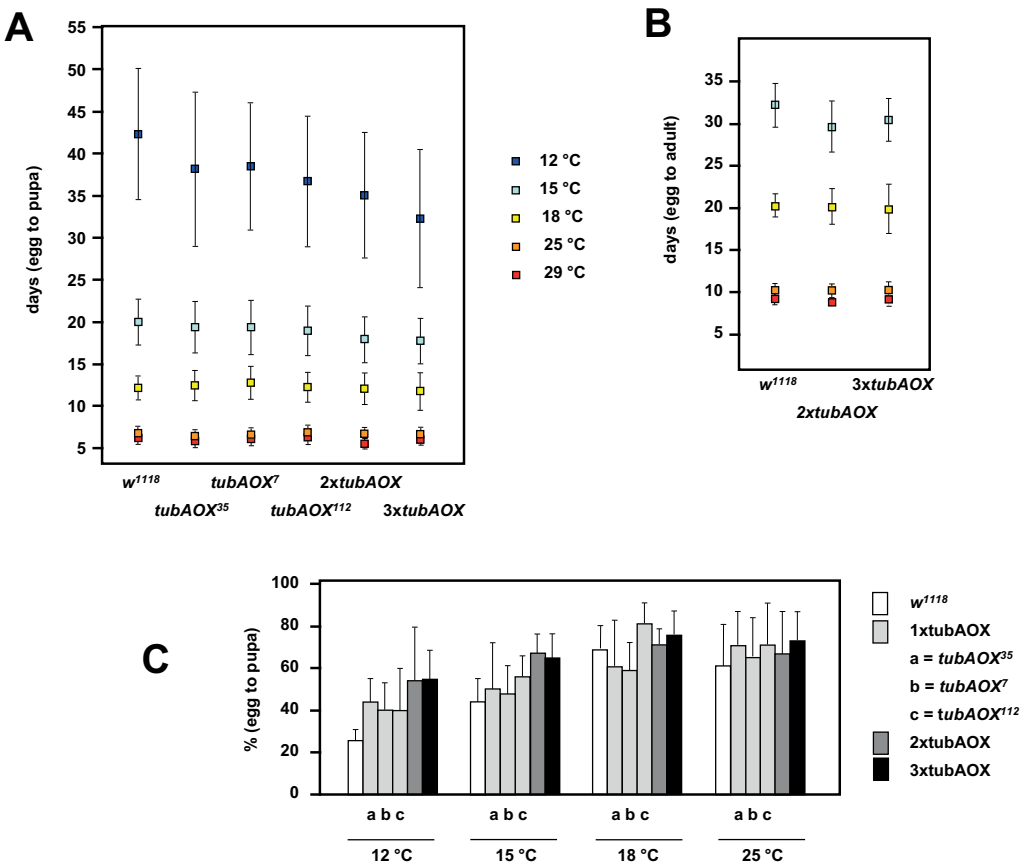

Fig. 2. AOX expression in *Drosophila* accelerates development at low temperatures.
(A) Egg-to-pupa development time of flies of the indicated genotypes and culture temperatures (means ± SD for ≥100 flies in each case, cultured in 8–12 vials in 2–4 biological replicates). For tabulated data and statistical analysis see Supplementary Table S4. (B) Egg-to-adult development time of flies of the indicated genotypes and culture temperatures (means ± SD for a total of 8–16 vials in each case, from 2 to 4 biological replicate experiments). For clarity, only the effects of 2 and 3 copies of the *tubAOX* transgene versus the w^{1118} background strain are shown in the figure. Complete tabulated data and statistical analysis are presented in Supplementary Table S5. (C) Egg-to-pupa viability of flies of the indicated genotypes, cultured at different temperatures, as shown: means ± SD for 8–12 vials from 2 to 4 biological replicates in each case. For complete tabulated data and statistical analysis see Supplementary Table S6. Note that, for clarity, data for the 29 °C stress condition is omitted from the figure. Although AOX was also beneficial at this temperature, the relationship with gene dosage was less clear than at low temperatures, compared with standard growth conditions of 18–25 °C where AOX was neutral.

3. Results & discussion

3.1. AOX and NDX are induced in Ciona by hypoxia and sulfide

To address the question of the functional roles of NDX and AOX in animals, we focused on *C. intestinalis*, the organism that has been used as the source for the aRC transgenes that we have expressed in flies, mice and human cells. We subjected *Ciona* adults to a set of stressors experienced in the natural environment, then analyzed NDX and AOX induction at the RNA level in different tissues (Figs. 1, S1). Both AOX and NDX were induced by hypoxia (Figs. 1A, S1A) or exposure to sulfide (Figs. 1A, S1B) in heart, neural complex and, to a lesser extent, in stomach. Physiologically stressful temperature or heavy-metal exposure did not induce the expression of aRC mRNAs (Fig. S1C, S1D), whilst the effects of hypoxia and sulfide were additive, at least for AOX in the tissues showing the greatest induction (Fig. 1B). These responses therefore result from independent sensing and signal transduction processes. Sulfide is a naturally occurring inhibitor of cytochrome oxidase, against which *Ciona* AOX is able to protect mammalian cells (Fig. S1E), providing a rationale for its induction in sulfide-containing seawater. AOX induction by hypoxia is more unexpected, since the enzyme requires oxygen as a substrate just as does cytochrome oxidase. However, AOX maintenance does not require the elaborate biogenetic program of the cytochrome-containing complexes cIII and cIV. In particular, the pathway for haem biosynthesis in all higher eukaryotes involves two oxygen-dependent steps: coproporphyrinogen oxidase consumes two molecules of oxygen, releasing two each of water and CO_2 [46], whilst oxygen is used as the terminal electron acceptor for the next step in the pathway, catalyzed by protoporphyrinogen oxidase [47]. AOX catalysis depends on a diiron centre rather than a haem cofactor. Therefore, AOX biosynthesis should not be affected by disturbed haem biosynthesis during prolonged hypoxia. Another possible rationale for AOX expression being responsive to hypoxia is to minimize ischemia/reperfusion injury by ROS, when the system becomes reoxygenated, but whilst the RC carriers are still in the reduced state. In a parallel study in molluscs, AOX was recently shown to be induced under

hypoxia in a freshwater bivalve [48].

Possible medical implications: What are the potential implications for this finding for the use of AOX in therapy? Toxic levels of sulfide as well as oxygen-deprivation are conditions experienced by animals in the natural environment but are also found in humans in cases of disease. Although low levels of hydrogen sulfide act in endocrine signaling and vasorelaxation [49–52], natural over-production or lack of detoxification of H_2S can also become pathological, with cIV as a major target. A frequent cause of disturbed H_2S metabolism is the alteration of the gut microbiota by antibiotic use, which has been implicated in ulcerative colitis and other intestinal diseases [51]. Sulfide can also accumulate in the body as a secondary effect of inherited disorders, notably ethylmalonyl encephalopathy, now recognized as a mitochondrial disease [53]. The ability of AOX to protect against sulfide toxicity thus has the same double-edged aspect as H_2S itself. Where sulfide is present at pathological levels, causing impaired mitochondrial respiration, AOX can potentially overcome the problem, if applied therapeutically. Conversely, if cytochrome oxidase inhibition is directly operating as a physiological sensor for H_2S, for example in vasorelaxation [52], AOX could disturb homeostatic responses. In regard to hypoxia, previous data showed comparable effects of low oxygen on the activities of AOX and cytochrome oxidase [54]. However, because the subunit-isoform composition of cIV varies between tissues [55], with some combinations exhibiting altered kinetic properties in regard to oxygen, it cannot be concluded that AOX will perform comparably to cIV in all tissues and conditions. Since solid tumours represent a pathologically important low-oxygen environment, the expression of AOX to manage other diseases may favour (or disfavour) the growth of specific tumour types, leading to unintended consequences.

3.2. AOX expression promotes temperature-dependent growth acceleration

Previous studies of *Drosophila* transgenic for *Ciona* AOX showed that ubiquitous AOX expression resulted in a slight but statistically significant developmental delay (egg-to-adult timing), and exaggerated weight loss as young adults [34]. Both of these observations are consistent with the idea that AOX might become at least partially active during development, resulting is less efficient use of stored nutritional resources. In such a case, by catalyzing the same redox chemistry as the mitochondrial cytochrome chain, but without energy conservation via proton-pumping, AOX should simply convert a greater proportion of released free energy to heat, as already demonstrated in vitro [56]. One corollary of this is that activation of AOX could potentially be beneficial at low temperatures that are otherwise sub-optimal for the completion of development, via a thermogenic effect.

To test this we made use of a set of transgenic fly lines expressing AOX under the control of the α-tubulin promoter in one, two or three diploid copies. These were cultured under different temperatures. The period of larval development, characterized by biomass accumulation, was essentially unaffected by AOX expression at the standard growth temperature of 25 °C, or at the elevated temperature of 29 °C (Fig. 2A). However, at low temperature, when the growth period was greatly extended, AOX expression resulted in a markedly increased rate of development, which was most pronounced in flies bearing three copies of the transgene, and at the lowest temperature tested, 12 °C (Fig. 2A). Pupal development was less affected by AOX (Fig. 2B): instead, the 2–3 d acceleration produced during larval development was simply maintained during metamorphosis (Fig. 2B). At low temperature we also observed a significantly higher proportion of eggs able to reach the pupal stage, also correlating with increased AOX gene dosage (Fig. 2C).

As in other contexts where rapid growth prevails (cancer cells, yeast in exponential growth phase in glucose-rich medium) ATP production in *Drosophila* larvae depends largely on glycolysis, considered as the main high-capacity pathway for generating both energy and organic intermediates for biosynthesis. Under such conditions, mitochondrial respiration nevertheless remains indispensable, since the processing of carbon skeletons for biosynthesis depends on the TCA cycle which, in turn, requires the efficient reoxidation of primary electron carriers via the respiratory chain, a condition recognized by Warburg as 'aerobic glycolysis' [57]. The capacity of the system to fulfil this role does not appear to be limiting in larvae grown under standard conditions, since AOX expression has no effect on larval growth rate at 25 °C (Fig. 2A). If the TCA cycle were being restrained by the coupling of the RC to ATP production, enzymatically active AOX should accelerate the cycle, since it produces less ATP per molecule of ubiquinol oxidized. However, at low temperature, where all chemical reactions are slowed, growth proceeds much more slowly, e.g. larvae cultured at 12 °C grow at < 20% of the rate exhibited by those grown at 25 or 29 °C (Fig. 2A). The clear growth acceleration provided by AOX expression under these conditions can thus be attributed either to its ability to alleviate limitations on electron flow, which might constrain the TCA cycle at low temperature, or else to a direct thermogenic effect raising the temperature of the mitochondria [58] and of the whole organism. In whole or permeabilized mammalian cells grown at 37 °C [78], or in mitochondrial homogenates from flies grown and assayed at different temperatures between 18 and 29 °C [34], AOX expression did not support 100% of the respiratory capacity of cIII under uninhibited conditions. Thus, we strongly favour the second mechanistic hypothesis, whereby a direct thermogenic effect actually warms larvae sufficiently to accelerate developmental processes which are sub-optimal at low temperature. Testing this will not be straightforward, however, since *Drosophila* larvae are opaque to the dyes and reporters thus far developed as intracellular temperature reporters.

Possible medical implications: based on these findings, the use of AOX to compensate RC defects potentially carries the unexpected risk that it could create metabolic conditions that promote the growth of some tumours. The role of metabolism in cancer has recently attracted a lot of interest, although it is misleading to assert that this role is always in the same direction. Rather, the relative dependence on glycolysis versus OXPHOS varies greatly between tumour types, or even between a single cancer at different stages of the disease [59]. However, rapid growth is generally associated with the high-throughput glycolytic pathway as the major source of ATP, but accompanied by a repurposing of the TCA cycle (and OXPHOS) for anabolism [59], which applies both to developing *Drosophila* larvae and to most tumour cells. Although the growth acceleration conferred upon fly larvae by AOX was seen only at low temperature, the mechanism could potentially operate under other stress conditions in cancer cells, where the processing of carbon skeletons for biosynthesis could potentially be limited by insufficiency of the RC. An obvious cause of such insufficiency would be the clonal amplification of mtDNA mutations during the establishment of the tumour, which could then limit its growth in later stages of the disease. Many tumours are indeed found to harbour deleterious mtDNA mutations. In such cases, AOX could alleviate growth constraints arising from mutations in genes for subunits of cIII or cIV or from mutations in the mitochondrial protein synthetic apparatus where cIII or cIV were the most affected products, such as the A8344 MERRF mutation [60]. Note, however, that it is also possible to construe an opposite argument, in cases where a cIII defect promotes tumour growth or metastasis through increased ROS production [61]. In such cases, AOX may relieve this effect and restrict cancer progression. The inferred thermogenic effect of AOX raises additional potential issues, as discussed further in the following section.

3.3. AOX-expressing flies show temperature dependent lethality on nutrient-poor media

The earlier observations of a slight developmental delay and mild weight loss in adult flies expressing AOX suggest that AOX-expressing flies use nutritional resources less efficiently than their wild-type counterparts. To investigate this issue further, we cultured flies ubiquitously expressing AOX, as well as a wide panel of controls, on

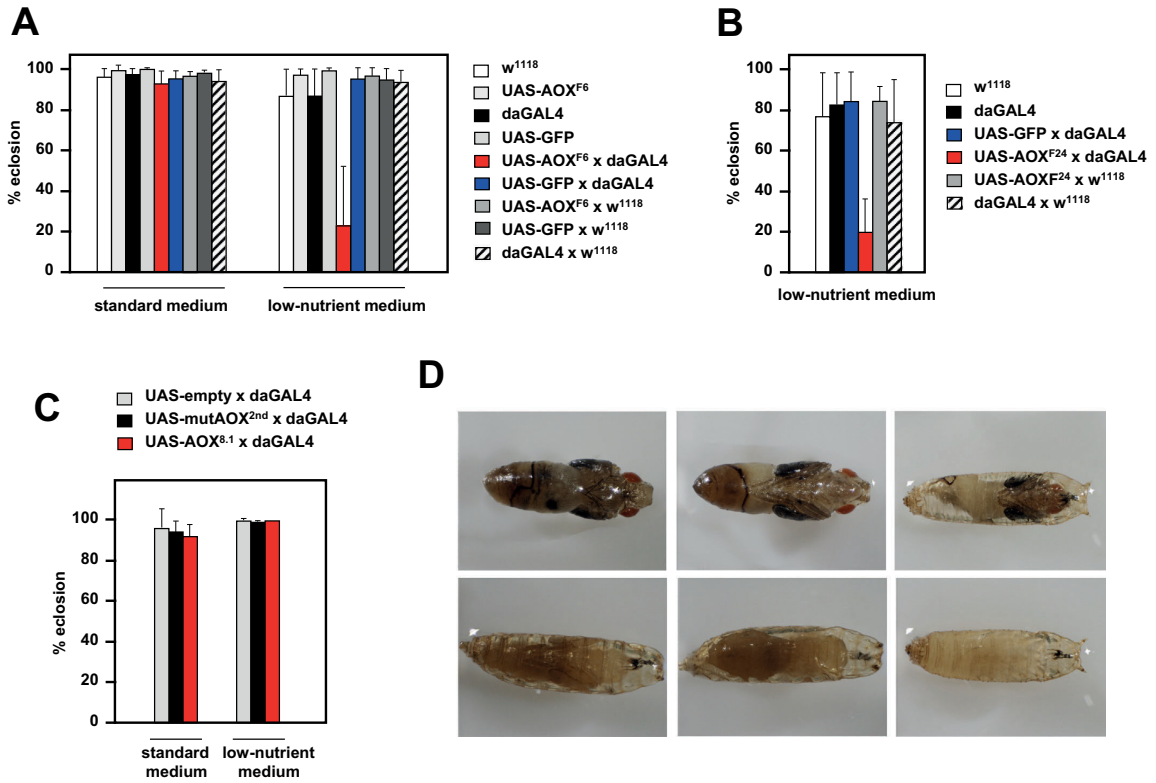

Fig. 3. AOX-expressing flies show developmental arrest on low-nutrient medium.
(A–C) Proportion of pupae eclosing on the indicated media, of the genotypes or crosses (female × male) as shown (means ± SD). (D) Representative images at comparable zoom, of dying/developmentally arrested AOX-expressing pupae, cultured on low-nutrient medium.

nutrient-poor media. On standard high-sugar medium [62], AOX-expressing flies eclosed at the same frequency as controls (Fig. 3A), whereas on low-nutrient medium, containing only 3.5% yeast and 5% glucose plus agar and antimicrobials, most (~80%) AOX-expressing flies died as pupae (Fig. 3A). Controls, including AOX transgenic but non-expressing flies, as well as flies expressing GFP, eclosed almost normally on this medium (Fig. 3A). A second AOX transgenic line behaved similarly (Fig. 3B), whereas a third, in which AOX expression is much lower [38], as well as a transgenic line expressing a catalytically inactive variant of AOX [38] did not (Fig. 3C). AOX-expressing pupae cultured on low-nutrient medium died at various different stages of metamorphosis (Fig. 3D), suggesting that they had exhausted a general component required for the completion of development, rather than becoming blocked in a stage-specific process. When the flies were cultured in tightly temperature-controlled incubators, the phenotype was found to be extremely temperature sensitive (Fig. 4A). Specifically, at 22 °C AOX-expressing flies eclosed at a near-normal frequency on low-nutrient medium, whereas at 25 °C, and more acutely at 26 °C, they mostly died as pupae, whilst at 27 °C, hardly any AOX-expressing flies eclosed even when cultured on standard medium. At 29 °C control flies also showed a decreased eclosion frequency, especially on the low-nutrient medium. The phenotype, including its modulation by temperature, was maintained even when the glucose level was raised to 10% (Fig. 4A). Although this temperature-dependence may partly be explained by the increased expression of the transgene at high temperature, under the influence of the *da*GAL4 driver (Fig. 4B), the similarities with the behaviour of control flies at high temperature suggests a contribution also from the thermogenic effect of the enzyme. Increasing the yeast concentration in the low-nutrient medium to 10% also failed to reverse the developmental phenotype, whilst decreasing it to 1% led to developmental arrest for a majority of wild-type pupae as well (Fig. 4C). Even when cultured on medium containing only yeast and agar, control flies eclosed normally, whilst > 90% of AOX-expressing flies died as pupae.

These findings imply that components of our standard high-sugar medium must be crucial in enabling AOX-expressing flies to complete development. The standard medium is a complex mixture containing several highly heterogeneous components (including yeast, as well as treacle, soya flour and maize flour). Further analysis will be needed to establish which specific nutrient(s) are essential for AOX flies.

As already indicated, *Drosophila* development involves distinct phases. During metamorphosis, the pupa does not feed, instead relying upon biomass accumulated during larval development. Metamorphosis involves drastic tissue reorganization, fuelled largely by stored triglycerides [63]. If AOX were to become activated under such conditions, ATP generation could be severely curtailed, since the pathways of triglyceride breakdown (via glycerol-3-phosphate dehydrogenase and fatty-acid β-oxidation) give rise to reducing equivalents that mostly enter the respiratory chain via ubiquinol at cIII. This may account for developmental failure, if insufficient such resources had been accumulated prior to metamorphosis. Alternatively, if AOX were activated in larvae under low nutrient conditions, impairing the efficiency of biosynthesis, this may account for insufficient amounts of stored triglycerides being laid down, and leading to developmental failure at a

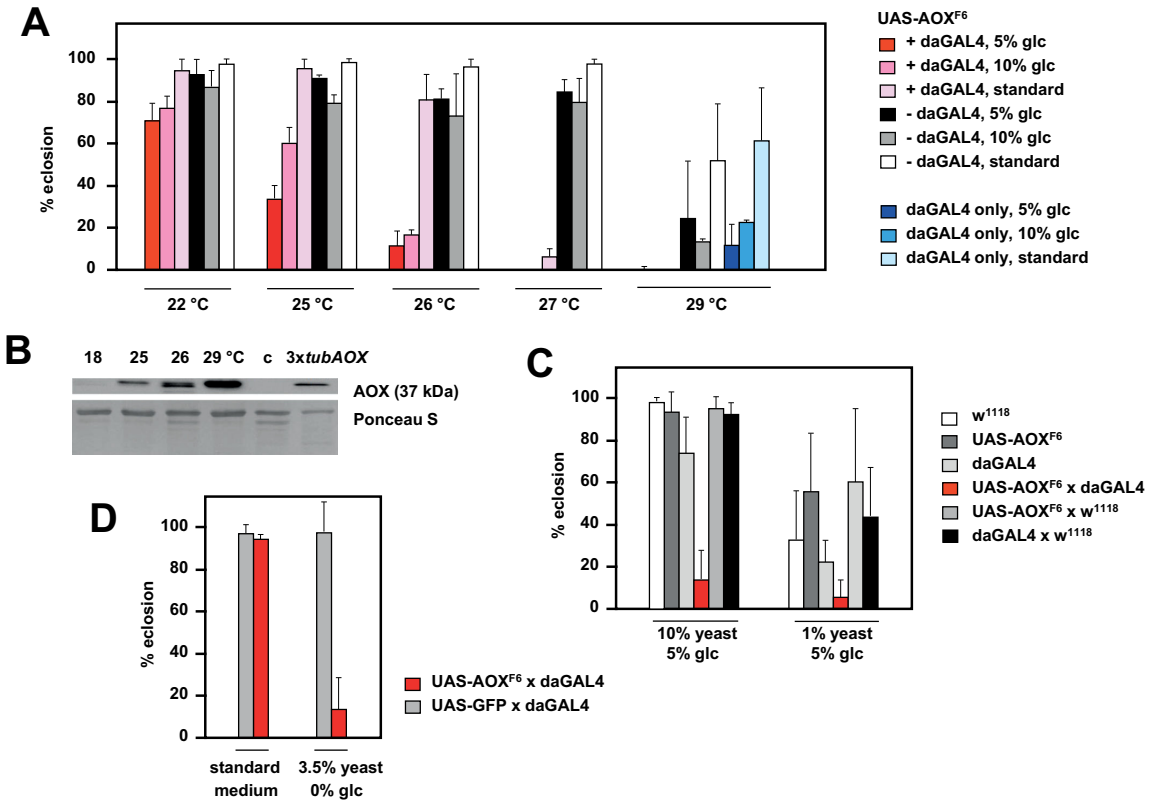

Fig. 4. Developmental arrest of AOX-expressing flies on low-nutrient medium is temperature-dependent.
(A, C, D) Proportion of pupae eclosing on different media and at indicated culture temperatures, of the genotypes or crosses (female x male) as shown (means ± SD). Where not shown, experiments were conducted at 25 °C and on media containing 3.5% yeast as well as the indicated amounts of glucose (glc). (B) Western blots of protein extracts from AOX-expressing L3 larvae (UAS-AOX^{F6} x daGAL4 cross), cultured at the indicated temperatures, negative control (daGAL4) larvae grown at 25 °C (c) and positive control 3xtubAOX adults, probed for AOX, alongside Ponceau S-stained membrane to indicate relative loading. Molecular weight of AOX, 37 kDa, extrapolated from PageRuler™ Plus Prestained Protein Ladder (ThermoFisher Scientific).

later stage.

Possible medical implications: our findings can be considered as a further example of the increasingly documented link between nutrition, mitochondrial function, and the accumulation and use of fat reserves. Dysregulation of the relevant processes is increasingly considered to underlie obesity and other metabolic disorders [56,64,65]. Here we have shown that altering the balance between mitochondrial substrate utilization and energy production can have profound implications for biological processes, leading to developmental arrest. Trying to draw exact parallels between insect and mammalian physiology may be misleading, especially given that mammals show precise thermoregulation and possess a tissue dedicated to this role, brown fat, although its importance declines with age [66]. If AOX expression leads more globally to increased fat burning, or decreased fat accumulation, its use in pathologies with a metabolic dimension may have important, and in some cases undesired consequences. An analogy may be drawn with human *CPT2* deficiency, where the inability to use long-chain fatty acids as a metabolic fuel leads either to early lethality or to sensitivity to starvation, depending on the severity of the mutation [67–69]. A similar phenotype is produced in flies homozygous for a null mutation in *Drosophila CPT2*. Once more, there is also a potential relevance in cancer, where metabolic inflexibility, i.e. loss of the ability to survive on different substrates, is a common observation [70]. Conversely, AOX may be of potential use to correct metabolic imbalances, or at least to investigate the role of mitochondrial energetics in pathology. Similar ideas have been proposed elsewhere for treatments that induce thermogenic or energetically 'futile' pathways [71]. Indeed, thermogenesis is clearly a feature of the immune response, and AOX may offer one route to intensify it and target it more effectively.

3.4. AOX accelerates recovery from cold-induced paralysis in Drosophila adults

The above findings suggest that AOX may be functionally thermogenic under specific physiological conditions. Even though the transcription of the gene was not induced in *Ciona* by cold temperature (Fig. S1), activation in the cold may be an inherent property of the enzyme. We therefore tested its effects on cold-exposed flies. In trials, 12 days at 4 °C was sufficient to kill 80–90% of control flies, whereas flies homozygous for 3 copies of the *tubAOX* transgene in the same genetic background all survived this treatment. A shorter period of cold exposure (15 h at 4 °C in food-containing vials) was non-lethal to all flies, but induced paralysis. When vials were transferred to 24.5 °C, the AOX-expressing flies started to become mobile after 25 min, whereas control flies only began to emerge from paralysis after 45 min (Fig. 5).

Possible medical implications: the simplest interpretation of these results is that AOX becomes at least partially active at low temperature, physically warming the organism to mitigate lethality at low

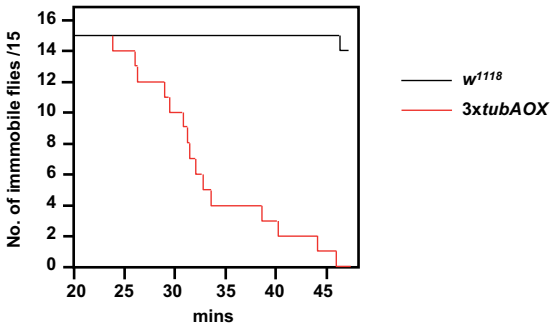

Fig. 5. AOX expression accelerates recovery from cold-induced paralysis. Number of flies remaining immobile (from batches of 15 virgin females) at the indicated times after shifting from 4 °C to 24.5 °C: flies expressing AOX (*3xtubAOX*) versus background control line w^{1118}.

temperature and accelerating recovery from cold-induced paralysis. Since a thermogenic effect of AOX in plants is well documented [17,18], our findings that it may act similarly in some animals is not implausible. However, if AOX were deployed therapeutically in homeotherms such as humans, excess heat production should activate thermoregulatory mechanisms and, in an extreme situation, might overwhelm them. Moreover, if the excess heat is not efficiently conducted away from the mitochondria, as suggested by recent studies [58], it may lead to unpredictable changes in enzyme activities and disturb, rather than restore, metabolic homeostasis. On the other hand, if properly regulated, AOX may provide a possible treatment for some forms of obesity, by increasing the amount of fat or carbohydrate that must be burned to generate a given amount of ATP, but without the potentially harmful effects arising from loss of mitochondrial membrane potential produced by chemical uncouplers or uncoupling proteins such as UCP1.

3.5. AOX has other unexpected effects on development and physiology

When undertaking the expression of *Ciona* AOX in metazoan models such as *Drosophila* or the mouse, we initially considered the possibility that widespread expression of the enzyme would be lethal, due to its ability to short-circuit mitochondrial ATP production when functionally active. We initially made the surprising observation that ubiquitous AOX expression was fully compatible with development and an ostensibly normal physiology in both flies and mice [34,72]. This, plus the known properties of the plant and algal enzyme, which is activated only under conditions of over-reduction of the quinone pool [3,4], then led us in the opposite direction. If AOX is only active under conditions of metabolic imbalance and oxidative stress, it can be proposed as a potential wide-spectrum therapeutic tool that is benign in the unstressed organism. The findings presented above now argue for an intermediate conclusion, namely that AOX, whilst displaying clear beneficial effects, does impact physiological processes in ways that were not expected, even if they can be rationalized from the known properties of mitochondria and cells. Activation of AOX by specific metabolites or energetic states can potentially interfere with specific developmental processes. The causes and consequences are not easy to predict mechanistically, since we do not have a full description of the metabolic changes that occur in animal development, nor do we yet have a clear idea of how the activity of AOX from *Ciona* is regulated. Some other examples from our recent work illustrate these points.

AOX-expressing flies of both sexes are fertile and produce normal numbers of offspring [34]. However, when tested in a direct competition assay, as illustrated in Fig. 6, AOX-expressing males were systematically out-competed by control males [73]. We traced the reason for this abnormality to a relative lack of sperm production by AOX-expressing males [73]. Surprisingly, this did not appear to be due to AOX expression in the germline, which was seen only at low levels comparable with the detection limit, but to its expression in the pigment cells of the testis sheath. Moreover, it was associated not with a block on differentiation as such, but with its spatial disorganization and consequent failure to accumulate mature sperm into the seminal vesicle. The precise mechanistic basis of the phenotype remains to be elucidated, although it logically involves deranged signaling from the mitochondria-rich pigment cells to the underlying smooth muscle, impeding the peristaltic movement of maturing sperm cysts. Why AOX would be activated in those cells, and whether the effect is attributable to decreased ATP production, impaired ROS signaling, increased temperature or some other process, remain open questions.

It is to be expected that metabolic stress can also lead to transient activation of AOX, blunting the signals that enable organisms to respond to and negate such stresses. Thus, there may be instances where AOX impairs regenerative responses and exacerbates stress-induced damage. Conversely, we have identified several contexts where AOX expression appears able to potentiate normal signaling or to quench abnormal signaling, in either case preventing a pathological outcome. Perhaps the most remarkable example is the ability of AOX to prevent a range of developmental dysmorphologies induced by abnormal nuclear receptor activity. These are provoked by the combined action of a modified GAL4 driver ('GeneSwitch'), which includes the ligand-binding domain of the progesterone receptor, plus an excess of the artificial steroid that induces it, but is brought about in the absence of any transgene. Expression of AOX, even from a GAL4-independent promoter, was able to largely prevent the appearance of these dysmorphological phenotypes, including cleft thorax or abdomen, apoptotic wing segments, malformed legs or sensory bristles [74]. AOX expression was also able to correct the most frequently observed of these abnormalities, cleft thorax, when induced by a completely separate manipulation, through the impairment of Jun N-terminal kinase (JNK) signaling in the dorsal thoracic midline during metamorphosis [75]. AOX was also found to promote cell migration in immortalized mouse embryonic fibroblasts, and to counteract the negative effects on this process of at least one protein kinase inhibitor [75].

Our studies of the properties of mouse models is much less advanced at this point, but one intriguing observation is the fact that AOX is able to block the lethal effects of lipopolysaccharide (LPS), in a mouse model of sepsis [76]. This finding was part of a wider study to understand the role of mitochondria in cytokine release by activated macrophages. If not properly controlled, this is believed to lead to septic shock and organ failure. AOX was inferred to provide a shunt for blocked electrons, normalizing mitochondrial metabolism in macrophages and preventing excess ROS production arising from reverse electron flow through cI. This study provides an important benchmark for how AOX can be used to probe the mitochondrial role in diverse pathologies, physiological and developmental processes. The lesson from studies in *Drosophila* is that a mitochondrial role should always be considered in any complex or unexplained pathological phenotype, and the availability of transgenic lines for AOX and NDX provides a way of testing this whenever a credible animal or cell-culture model exists.

Possible medical implications: it is arguably fanciful to translate knowledge directly from *Drosophila* or even the mouse to human disease, without exhaustive validation studies. Therefore, the fact that AOX impairs male reproductive competitiveness or corrects cleft thorax in flies does not mean that male infertility must automatically be considered a mitochondrial disease or that AOX could somehow be deployed to treat midline closure defects in human development, such as *spina bifida* or cleft palate. Nevertheless, the use of AOX and NDX to probe mitochondrial involvement in disease-related processes should provide powerful clues that could have unforeseen applications in medicine.

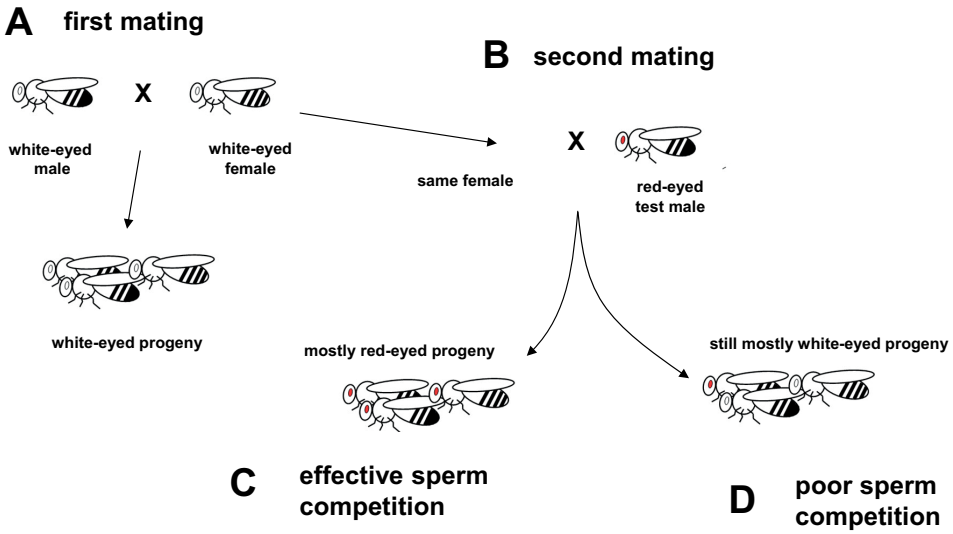

Fig. 6. Mating scheme for sperm competition assay.
The principle of the sperm competition assay is that *Drosophila* females, when mated, naturally store the sperm of the male partner, which is used to fertilize oocytes over several days, as they mature. However, females are still receptive to mating with a second male, whose sperm can then compete with that from the original male. In the scheme shown, (A) a white-eyed female is first mated to a white-eyed male, producing white-eyed progeny. In a second mating (B), the same female is re-mated with a red-eyed male. The standard outcome of such an experiment, illustrated in (C), is that the sperm from the second male displaces or out-competes that from the first. Since the red-eye allele is dominant, most of the progeny after the second mating will have red eyes. If, however, the sperm from the second male competes poorly, as illustrated in (D), most of the progeny will arise from oocytes fertilized by sperm from the original male, and will this have white eyes. As an internal control, the assay is usually done both ways around, i.e. with a second full experiment in which the first mating is performed with red-eyed males. These reciprocal setups are often described as the 'offensive' and 'defensive' paradigms. In the experiments that were conducted, the sperm of AOX-expressing males was found to compete poorly compared with controls (see [73] for details).

4. Conclusions and perspectives

Our global understanding of metabolism and how it impacts cell signaling remains limited. Thus, to predict the effects of introducing a major metabolic modification, such as the introduction of AOX (or NDX) into organisms that lack the aRC, is fraught with difficulties, and we should expect surprises. As summarized in Fig. 7, metazoan AOX, when activated by the accumulation of reduced quinol, plus other, as yet unidentified metabolic trigger(s), accelerates mitochondrial metabolism compared with an inhibited condition that it alleviates. However, it generally decelerates metabolism compared with the fully uninhibited condition, when electrons are able to pass freely to complex III, as inferred from the measured oxygen consumption of cultured cells [78]. Mitochondrial NADH oxidation and ATP production should follow similar trends, although neither has yet been specifically measured. ATP production should be the more severely affected, given that a much greater proportion of the energy released by AOX-supported respiration is converted to heat rather than being used for ATP synthesis. However, because the net effects on metabolism may be diverse and complex, total cell NAD^+ and ATP levels might remain stable or even rise. Similar but opposite considerations apply to ROS. Mitochondrial superoxide production is decreased by AOX in cells where OXPHOS is inhibited, but increased compared with fully uninhibited conditions [78]. How this affects ROS in the rest of the cell is again not clear. Much remains to be documented and tested. Because the metabolic effects of AOX expression are not fully predictable, the many cellular regulatory pathways that respond to ROS, ATP, NAD, TCA cycle intermediates and other metabolites, as well as mitochondrial heat production, are likely to be affected in complex ways, leading to the readouts observed in our studies and elsewhere. These, in turn, are very likely to affect the metabolic triggers that govern AOX activation.

About NDX we know even less at this time. For example, when expressed in mammalian cells, AOX does not interact with any of the mitochondrial OXPHOS complexes [54,72], but this has not yet been tested for NDX. A further intriguing question arises as to the functional interactions of AOX and NDX. In principle, if simultaneously active, they would catalyze a completely non proton-motive respiratory chain, although it is not known if this ever happens in a physiological situation, or whether the two enzymes are able to interact physically. An important question is how far they influence each other's activity, and which of them represents the effective control point for the aRC. In *Drosophila*, they can at least synergize functionally, e.g. in the tko^{25t} mutant [41], as already mentioned. Thus, their combined effects on metabolism may differ from that of either alone. If the aRC enzymes are to be used in clinical applications, we will need a much better understanding of all these effects, including how both enzymes are naturally regulated, how they interact, how they access and modify the quinone pool(s), what broader impact they have on metabolism, and the precise mechanisms by which they influence cell signaling.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

We thank Tea Tuomela, Eveliina Teeri, Miriam Götting, Katja Anttila and Nina Vuori for technical assistance, and Troy Faithfull for help with the manuscript.

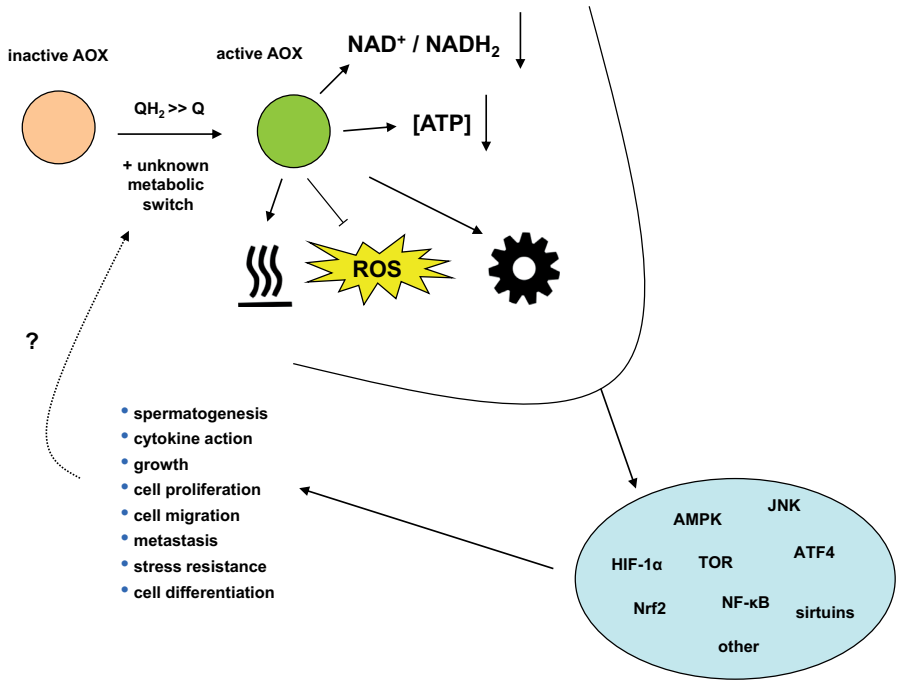

Fig. 7. Summary of known and hypothetical effects of AOX expression on cell metabolism and signaling.
Activation of metazoan AOX (orange to green circle) requires a high degree of reduction of the quinone pool, as well as other, as yet unidentified, metabolic triggers. When activated, it should result in decreased mitochondrial ATP production and NADH oxidation, compared with the fully uninhibited state, although effects in the cell as a whole, or in different physiological states, are not predictable. Heat production should increase, whilst ROS production, at least at cIII, should decrease. These and other metabolic changes (shown as the cogwheel), affecting e.g. the TCA cycle, will impact many signaling pathways (blue ellipse), some of which may act independently, others contingently, to produce the complex and sometimes unexpected readouts found in this study and elsewhere. In turn, the altered metabolic state will influence the degree of activation of AOX, in as yet unknown ways.

Funding

This work was supported by the European Union [grant numbers GA328988 and 232738]; the Academy of Finland [grant numbers 283157 and 272376]; the Fundação de Amparo à Pesquisa do Estado de São Paulo [grant numbers 2014/02253-6, 2015/02174-1 and 2016/00013-3]; the Finnish Cultural Foundation; Tampere University Hospital Medical Research Fund and the Sigrid Juselius Foundation.

Conflict of interest

MSz is a shareholder in a company set up to develop AOX-based therapies for mitochondrial diseases. The other authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2018.10.012.

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PUBLICATION III

Alternative oxidase confers nutritional limitation on *Drosophila* development

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Journal of Experimental Zoology A. (2019) 331(6):341-356.
doi: 10.012 10.1002/jez.2274

Received: 23 December 2018 | Revised: 12 April 2019 | Accepted: 17 May 2019

DOI: 10.1002/jez.2274

JEZ-A ECOLOGICAL AND INTEGRATIVE PHYSIOLOGY WILEY

RESEARCH PAPER

Alternative oxidase confers nutritional limitation on *Drosophila* development

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Funding information
FP7 Ideas: European Research Council, Grant/Award Number: 232738; Tampere University Hospital Medical Research Fund; Suomen Akatemia, Grant/Award Numbers: 272376, 283157; Sigrid Juselius Foundation; Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant/Award Number: 2014/02253-6; Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Number: 424562/2018-9

Abstract

The mitochondrial alternative oxidase, AOX, present in most eukaryotes apart from vertebrates and insects, catalyzes the direct oxidation of ubiquinol by oxygen, bypassing the terminal proton-motive steps of the respiratory chain. Its physiological role is not fully understood, but it is proposed to buffer stresses in the respiratory chain similar to those encountered in mitochondrial diseases in humans. Previously, we found that the ubiquitous expression of AOX from *Ciona intestinalis* in *Drosophila* perturbs the development of flies cultured under low-nutrient conditions (media containing only glucose and yeast). Here we tested the effects of a wide range of nutritional supplements on *Drosophila* development, to gain insight into the physiological mechanism underlying this developmental failure. On low-nutrient medium, larvae contained decreased amounts of triglycerides, lactate, and pyruvate, irrespective of AOX expression. Complex food supplements, including treacle (molasses), restored normal development to AOX-expressing flies, but many individual additives did not. Inhibition of AOX by treacle extract was excluded as a mechanism, since the supplement did not alter the enzymatic activity of AOX in vitro. Furthermore, antibiotics did not influence the organismal phenotype, indicating that commensal microbes were not involved. Fractionation of treacle identified a water-soluble fraction with low solubility in ethanol, rich in lactate and tricarboxylic acid cycle intermediates, which contained the critical activity. We propose that the partial activation of AOX during metamorphosis impairs the efficient use of stored metabolites, resulting in developmental failure.

KEYWORDS
AOX, cataplerosis, mitochondria, nutrition, TCA cycle

1 | INTRODUCTION

Animals have evolved a wide variety of physiological mechanisms to tailor their development and feeding behavior to the nature and availability of food resources. Most holometabolous insects, for

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example, lay their eggs on rich nutrient sources, which sustain rapid growth during larval development. Metabolites that are synthesized and stored during the larval stages are then used to drive the subsequent processes of cellular proliferation, migration, and differentiation. The program of metamorphosis during pupal stage is then executed in an environment where further feeding is not possible.

The metabolic processes that characterize the larval and pupal stages may be crudely described as anabolic and catabolic, respectively. Nevertheless, the biosynthesis that occurs during larval development depends on a supply of biological energy, while pupae rely on converting some of their stored resources into new biomolecules, as well as fueling and regulating the morphogenetic program. Importantly, most of the energy generation, as well as the production of relevant metabolites for biosynthesis and signaling, originate within the mitochondria. The tricarboxylic acid (TCA) cycle, in particular, is central to catabolism, anabolism, and signaling, and is in turn reliant on the mitochondrial respiratory chain.

The model organism *Drosophila melanogaster* has been extensively studied with regard to the molecular and cellular processes that underpin this developmental program. However, the metabolic events which accompany development have received less attention. *D. melanogaster* is considered a cosmopolitan species (Markow, Nazario-Yepiz, & Ramirez Loustalot-Laclette, 2017; Matzkin, Johnson, Paight, Bozinovic, & Markow, 2011), able to grow on a wide variety of food sources. These commonly include glucose and other sugars from decaying fruit, as well as yeast, which represents a rich source of amino acids and other nutrients. The major metabolic fuel for the pupal stage, accumulated during larval development, is triglycerides (Church & Robertson, 1966; Kühnlein, 2012; Merkey, Wong, Hoshizaki, & Gibbs, 2011). Lactate, the major glycolytic end-product, may also be important as a fuel, at least during the onset of metamorphosis. Lactate dehydrogenase (LDH) is required for both the synthesis and remobilization of lactate. It is highly expressed in larvae and is also induced by steroid signaling (Abu-Shumays & Fristrom, 1997). LDH activity declines during metamorphosis (Rechsteiner, 1970), reflecting the drop in its messenger RNA late in larval development (Graveley et al., 2011), with the accumulated lactate being mostly used up during the prepupal stage (Li et al., 2017). In larvae, glycolysis serves the needs of adenosine triphosphate (ATP) production and supplies carbon skeletons for biosynthesis via the TCA cycle (Tennessen, Baker, Lam, Evans, & Thummel, 2011), while in the pupa, triglycerides are catabolized mainly in mitochondria. Efficient mitochondrial respiration is therefore crucial at both stages.

In an earlier series of experiments, we found that flies expressing the alternative oxidase AOX from the tunicate *Ciona intestinalis* failed to complete development when reared on a low-nutrient agar medium containing only yeast and glucose (Saari et al., 2018). AOX branches the mitochondrial respiratory chain, bypassing complexes III and IV in a non-proton-motive reaction that oxidizes ubiquinol directly by molecular oxygen (Rogov, Sukhanova, Uralskaya, Aliverdieva, & Zvyagilskaya, 2014). The gene for AOX is present in most groups of eukaryotes, including animals (McDonald & Gospodaryov, 2018), but has been lost from specific lineages during the course of evolution, notably from vertebrates and advanced insects. The reasons for its evolutionary loss or retention are unclear. In lower eukaryotes and plants it confers resistance against stresses or metabolic disruption resulting from overload, inhibition, or damage to the standard mitochondrial respiratory chain (Dahal, Martyn, Alber, & Vanlerberghe, 2017; Dufour, Boulay, Rincheval, & Sainsard-Chanet, 2000). Such stresses include the excess production of reactive oxygen species (ROS), limitations on ATP synthesis, restraints on metabolic flux, and disturbances to cellular redox and ionic homeostasis. AOX is believed to play a similar protective role in animals (McDonald & Gospodaryov, 2018; Saari et al., 2018).

Since similar metabolic stresses arise in humans experiencing pathological dysfunction of mitochondria, we reasoned that AOX could be developed as a potential wide-spectrum therapeutic (El-Khoury et al., 2014).

As a first step, we have established the transgenic expression of *Ciona* AOX in model organisms, including both *Drosophila* (Fernandez-Ayala et al., 2009) and the mouse (El-Khoury et al., 2013; Szibor et al., 2017), to evaluate its effects on development, physiology, and pathology. In plants, AOX is enzymatically active only under conditions where the quinone pool becomes highly reduced (Castro-Guerrero, Krab, & Moreno-Sanchez, 2004; Hoefnagel & Wiskich, 1998). Thus, it contributes negligibly to electron flow under standard physiological conditions, while being available as a stress buffer whenever required. The same appears to be so for *Ciona* AOX expressed in the mouse (Dogan et al., 2018). In accordance with this, the ubiquitous expression of *Ciona* AOX in both flies (Fernandez-Ayala et al., 2009) and mammals (Szibor et al., 2017) has almost no detectable physiological effect under nonstressed conditions. However, if the standard respiratory chain is dysfunctional, for example, due to toxic inhibition (El-Khoury et al., 2013; Fernandez-Ayala et al., 2009; Szibor et al., 2017), overload (Mills et al., 2016), or genetic damage (Kemppainen et al., 2014; Rajendran et al., 2018), AOX is able to compensate the resulting phenotypes to a significant degree. However, since AOX bypasses two of the proton-pumping steps of the standard respiratory chain, it is not able to fully restore ATP production. Thus, it cannot compensate null mutations or even profound knockdown of core subunits of cytochrome *c* oxidase (complex IV), nor mutations that abolish the synthesis of a vital prosthetic group of the enzyme (Dogan et al., 2018; Fernandez-Ayala et al., 2009; Kemppainen et al., 2014).

The developmental failure of AOX-expressing flies under nutritional stress (Saari et al., 2018) implies that transgenic AOX becomes enzymatically activated during at least part of the life-cycle and/or in some crucial tissue(s), under low-nutrient conditions. In addition, since activated AOX is known to facilitate TCA cycle reactions and decrease ROS production, but also to result in less ATP synthesis, developmental failure could result from multiple, non-exclusive causes. This might explain why the addition of either glucose or yeast to the low-nutrient medium failed to improve the developmental outcome for AOX-expressing flies.

To investigate the phenomenon further, we embarked on a study to identify the missing dietary components that would enable AOX-expressing flies to complete development, as well as to pin down more accurately the timing of developmental failure and its correlates in terms of stored nutrient accumulation.

Our findings unambiguously ascribe AOX-dependent developmental failure to the pupal stage. We also found that L3 (wandering-stage) larvae reared on the low-nutrient medium are deficient in specific stored nutrients, regardless of genotype. Together, these results imply that AOX-expressing flies are unable to make proper use of this depleted metabolic store, so as to complete development. Finally, we identified a specific set of metabolites as the most likely candidates to rectify this deficiency, thus enabling AOX-expressing flies to fully undergo metamorphosis.

2 | MATERIALS AND METHODS

2.1 | *Drosophila* strains and culture

Drosophila strains used in the study were the transgenic line *UAS-AOX*^{F6} for *C. intestinalis* AOX (on chromosome 2), constructed in-house and described previously (Fernandez-Ayala et al., 2009), and the standard ubiquitous driver line *da*GAL4 (insertion on chromosome 3). Both lines were maintained as homozygotes and crossed together as indicated in figures. In addition, RNAi lines 30282 (Vienna Drosophila Resource Centre, GD library, chromosome 3, maintained over the TM3Sb balancer) and 65175 (Bloomington, TRiP line, chromosome 2, maintained over the CyO balancer), both for *ATPCL*, were crossed with *da*GAL4 to test the effects of *ATPCL* knockdown, using an internal control. Flies were maintained and cultured on standard high-sugar medium (Fernandez-Ayala et al., 2009), described here as a complete medium, with 12 hr cycles of light and darkness at 25°C, except where indicated in specific experiments. Low-nutrient medium consisted of (w/v) 1% agar, 3% glucose, and 3.5% yeast (Instant SD; Algist-Bruggerman NV, Gent, Belgium), which were boiled together then cooled to 65°C before addition of standard antibiotics nipagin (to 0.1%) and propionic acid (to 0.5%), before dispensing into food vials. All other food supplements were added to the mixture during boiling, except for doxycycline which was dispensed as a stock solution onto food plugs into which it was allowed to penetrate and dry in a fume hood (final concentrations as shown in figure legends). Food supplements that were added to the low-nutrient medium in specific experiments, were as follows: sucrose (VWR, 1.5% w/v), with or without fructose (Sigma, 3% w/v), soya flour (Soyolk; Oriola Oyj, Espoo, Finland; 1% w/v), maize flour (Risenta; Paulig Group, Helsinki, Finland; 1.5% w/v except where stated), wheat germ (Elovena Plus Vehnänalkio; Raisio plc, Raisio, Finland; 1% w/v), treacle (Lyle's Black Treacle; Tate and Lyle Sugars, London, UK; 3% w/v except where stated), multivitamin tablets (Multitabs Family; Pfizer Consumer Healthcare, Helsinki, Finland; one tablet per 300 ml of fly food or diluted as indicated in figures), B-vitamin supplement (Beko Strong; Orion Oyj, one tablet per 300 ml of fly food), Dulbecco's modified Eagle medium (DMEM) powder, containing high-glucose and glutamine but no pyruvate (Gibco; catalog #52100039), 1.24 g per 120 ml fly food, trisodium citrate dihydrate (Sigma), iron (ammonium iron(III)citrate; Sigma) or $CuSO_4$ (Sigma), used at concentrations indicated in the figures. The effects of different media on eclosion were tested by mating batches of 10 virgin females and five males overnight in food vials containing complete medium, then transferring them daily to fresh food vials containing the medium under test, for egg laying. Pupae per vial and the number of eclosed adults were recorded.

2.2 | Mammalian cells and culture

Flp-In™ T-REx™ 293 cells transformed with *C. intestinalis* AOX and their parental cell-line were cultured as previously (Hakkaart, Dassa, Jacobs, & Rustin, 2006). To induce transgene expression 1.5×10^6 cells were seeded in 10 cm^2 plates and cultured for 72 hr in medium containing 1 μg/ml doxycycline (Sigma). The medium containing doxycycline was replaced after 48 hr. AOX transgene expression was verified by western blot analysis using a customized antibody, as previously (Dassa et al., 2009).

2.3 | Respirometry

Respirometry on permeabilized cells was conducted essentially as described previously (Cannino et al., 2012), with the sequential addition of substrates and inhibitors as indicated in figure legends.

2.4 | Fractionation of treacle

Treacle was fractionated into aqueous and nonaqueous components as follows. In a fume hood, 1.8 g treacle was diluted in water to 40 ml by gentle heating. After cooling, the solution was poured into a separation funnel, mixed with 40 ml of diethyl ether (Sigma), and extracted by gentle inversion approximately 10 times. After the phases had fully separated they were isolated and each extracted twice more with the opposite solvent. A further 40 ml of water was added to the ether fraction and the two fractions were then left overnight in open beakers for all ether to evaporate. Each fraction was then made up into 40 ml fly food by adding the low-nutrient medium ingredients, for testing in the eclosion assay, or else analyzed further by mass spectrometry, as described below, or used in respirometry. For further fractionation by ethanol precipitation, 10 ml of the aqueous fraction were decanted into 50 ml centrifuge tubes, and 99% ethanol added to bring the final ethanol concentration to the desired level (between 40% and 75%). After overnight precipitation at −20°C and centrifugation at 14,000g_{max} for 30 min at 4°C, supernatants were decanted, and the ethanol evaporated, while pellets were air-dried and resuspended in 10 ml of water. Individual fractions were then made up into fly food for testing or analyzed by mass spectrometry, as described below.

2.5 | Metabolite assays

To assay triglycerides, batches of 10 L3 (wandering-stage) larvae were homogenized in 100 μl phosphate buffered saline (PBS)-0.05% Tween (Medicago, Uppsala, Sweden), using a disposable plastic pestle. Samples were heated at 70°C for 5 min, cooled to room temperature, and vortexed. Aliquots of the homogenate (5 μl) were added to 100 μl of Triglyceride Reagent (Thermo Fisher Scientific) in transparent 96-well plates. After incubation at 37°C for 30 min, absorbance at 540 nm was measured using a plate reader (Plate Chameleon™ V; Hidex) and normalized for protein content based on the Bradford assay (Bradford Reagent; Sigma). For lactate and pyruvate assays, batches of 10 L3 larvae were homogenized similarly, in 6M guanidine hydrochloride on ice, then incubated at 95°C for 5 min. Supernatants were transferred to fresh vials and stored at −80°C, then thawed on ice and diluted 1:10 with water. Standards and reaction master mix were prepared according to the manufacturer's protocol (L-Lactate assay kit/Pyruvate assay kit; Sigma). The reactions were performed in a black 96-well microplate by mixing samples 1:5 with the reagent master mix and incubation at room temperature for 30 min. Fluorescence (excitation 535 nm and emission 590 nm) was measured at 1 s intervals using the same plate reader and normalized for protein content.

2.6 | Mass spectrometry

Ten microliter aliquots of treacle fractions were mixed vigorously with 300 μl of methanol containing internal standards (0.5 ppm d8-valine, 0.5 ppm d4-succinic acid, 0.5 ppm d5-glutamic acid, 2.4 ppm heptadecanoic acid) and dried under constant nitrogen flow at room temperature. The resulting residual metabolites were converted into methoxime and trimethylsilyl derivatives by a two-step procedure. First, dried samples were dissolved in 25 μl of methoxyamine hydrochloride solution (20 mg/ml in pyridine; Sigma-Aldrich), and incubated for 1 hr at 45°C. After the addition of 25 μl of N-methyl-N-(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich) and a further 1 hr incubation at 45°C, samples were spiked with 25 μl of an alkane-standard mixture (C10–C30, 10 mg/L; Sigma-Aldrich). The analysis was performed using a gas chromatograph (Agilent 7890; Agilent Technologies, Santa Clara, CA) combined with a time-of-flight mass spectrometer (Pegasus BT; Leco Corp., St. Joseph, MI). Peak identification and data analysis used ChromaTOF software (Leco Corp.), NIST 2014 Mass Spectral Library and open-source software Guineu v2. See Supporting Information for further details.

2.7 | Statistical analysis

Student's *t* test (Microsoft Excel) was used to assess significance when performing pairwise comparisons, implementing the Bonferroni correction where more than two groups were compared. When comparing multiple levels of a single factor to each other, one-way analysis of variance (ANOVA) followed *post hoc* by the Tukey honestly significant difference (HSD) test (http://astatsa.com/) was applied. To test which of two factors was a significant determinant of a numerical outcome, two-way ANOVA (GraphPad Prism) was used. Note that *post hoc* analysis (e.g., via the Tukey HSD test) is only appropriate in cases where a significant interaction between the factors is detected by ANOVA, or where more than two levels of a given significant factor are compared. χ^2 tests to compare observed and expected outcomes were performed online (https://www.graphpad.com/). Details are given in figure legends, as appropriate.

3 | RESULTS

3.1 | Developmental failure of AOX-expressing flies occurs during the pupal stage

We first established that the developmental failure of AOX-expressing flies grown on low-nutrient medium occurs during metamorphosis, rather than during larval development or formation of the pupa (Figure 1). Matings that combined the AOX transgene with the ubiquitous *da*GAL4 driver produced a similar number of eggs as control strain matings (Figure 1a). Altering the glucose content of the medium had no significant effect on the number of eggs laid, regardless of genotype (Figure S1A). AOX expression also had no effect on the proportion of eggs that developed on low-nutrient medium as far as the pupal stage (Figure 1b and S1B). In contrast, the proportion of pupae that finally eclosed was dramatically decreased in AOX-expressing flies when cultured on the low-nutrient medium (Figure 1c).

3.2 | Complex food supplements compensate for the developmental defect of AOX-expressing flies

To identify the dietary component(s) which enables AOX-expressing flies to complete development on an otherwise low-nutrient medium, we undertook a series of experiments in which we removed individual components from the standard medium, or added individual components to the low-nutrient medium. Noting the exquisite temperature-sensitivity of the phenotype (see Saari et al., 2018), we performed most assays at 25°C, a temperature at which approximately 10–20% of AOX-expressing flies complete development on low-nutrient medium. However, as indicated in figure legends, a limited number of assays were performed at 26°C for technical reasons. The omission of any one of the complex food additives, that is, maize flour, soya flour, wheat-germ or treacle, or of sucrose, from the standard medium, had no effect on the eclosion frequency of AOX-expressing (or control) flies (Figure 2a). Conversely, the addition to the low-nutrient medium of any of the complex food additives but not of sucrose was sufficient to restore eclosion almost to control levels (Figure 2b), although wheat-germ was consistently less effective than other additives. Supplementation with glucose or yeast to varying amounts produced no rescue (Figure 2c), while the effects of both treacle and maize flour were clearly dose-dependent (Figure 2d). The published nutritional composition of

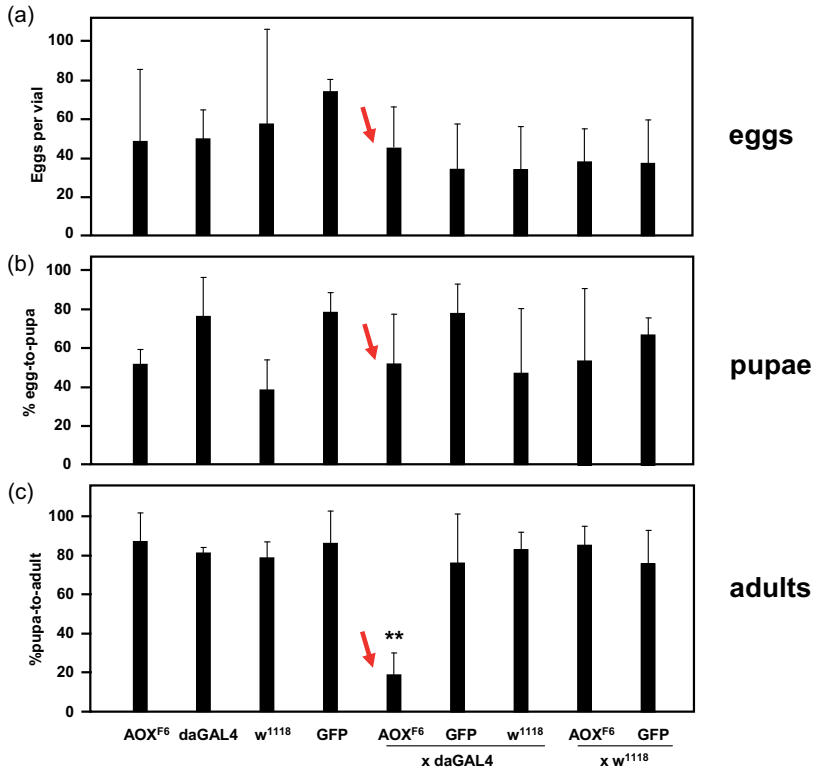

FIGURE 1 Developmental failure in AOX-expressing flies occurs during metamorphosis. Strains are denoted as w^{1118} (transgenic recipient strain), AOX^{F6} (*UAS-AOX*^{F6}, AOX transgenic strain dependent on GAL4 for expression), GFP (*UAS-GFP*^{Stinger}, control strain transgenic for nuclear-localized GFP, also GAL4-dependent), *da*GAL4 (ubiquitously expressing GAL4 driver). (a) A number of eggs laid in the indicated crosses, on low-nutrient medium containing 3.5% yeast and 5% glucose (means + *SD* of 3–6 individual vials in each case). There were no significant differences between strains or crosses (one-way ANOVA). (b) Percentage of eggs laid from the different crosses on low-nutrient medium (3.5% yeast and 5% glucose), reaching the pupal stage (means + *SD* of 3–6 individual vials in each case). There were no significant differences between strains or crosses (one-way ANOVA). (c) Proportion (%) of eclosing adult flies in three replicate crosses (3–6 vials per strain in each cross) on low-nutrient medium (3.5% yeast and 5% glucose). Means + *SD*; **Significant difference ($p < .01$) from all other classes (one-way ANOVA followed by Tukey's *post hoc* HSD test). See also Figure S1. ANOVA, analysis of variance; AOX, alternative oxidase; GFP, green fluorescent protein; HSD, honestly significant difference; *SD*, standard deviation

the complex food additives shows only modest overlap (Tables S1–S4).

3.3 | Specific food additives do not compensate for the developmental defect conferred by AOX

Next, we tested whether the addition of specific vitamins, minerals or sugars was able to restore eclosion competence to AOX-expressing flies. We selected concentrations of the various additives that have either been revealed previously to impact the phenotype of flies with relevant auxotrophies, or that are deemed effective in delivery to humans suffering an equivalent deficiency. In several cases, we tested a range of potentially effective concentrations, as well as combinations of additives. All additives and combinations of additives were found to be ineffective, including iron supplementation using two concentrations of ammonium iron(III)citrate (Figure 3a), multivitamin and B-vitamin mixes either alone (Figure 3a) or in combination with iron supplements (Figure 3a) or sugars (fructose and sucrose, Figure 3b), various amounts of copper, supplied as $CuSO_4$ (Figure 3c) and even a complex mix of metabolites used as a medium in mammalian cell culture (DMEM; Figure 3d).

3.4 | Antibiotic treatment does not modify the developmental phenotype of AOX-expressing flies

Next, we tested whether the developmental failure of AOX-expressing flies on low-nutrient medium could be related to the growth of commensal bacteria. To address this issue, we tested AOX-expressing and control flies on complete and low-nutrient medium supplemented with a wide-spectrum antibiotic, doxycycline. We used two concentrations of the drug, a stringent dose (100 μg/ml) that had a significant,

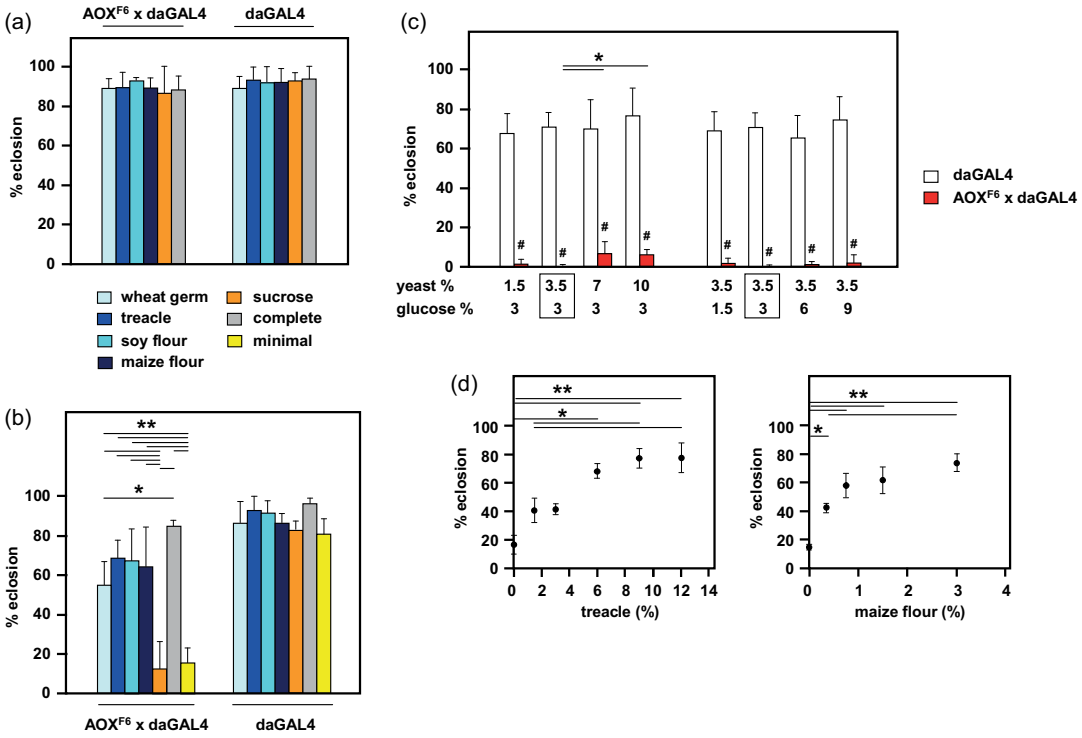

FIGURE 2 Developmental failure of AOX-expressing flies is corrected by complex dietary supplements. The proportion of pupae eclosing on the indicated media, of the genotypes or crosses (female × male) as shown (means + *SD*, $n \geq 4$). (a) Standard high-sugar medium lacking each of the indicated ingredients. Statistical analysis comparing eclosion frequency of flies of each given genotype on different media (by one-way ANOVA) and also comparing flies of the two different genotypes on each medium with each other (using Student's *t* test), revealed no significant differences. (b) Low-nutrient (3.5% yeast and 3% glucose) medium to which the indicated component from the standard high-sugar medium was added, at the concentrations indicated in Section 2. * and **Significant differences ($p < .05$ and .01, respectively) in eclosion frequencies of flies of each given genotype on different media (by one-way ANOVA followed by Tukey's *post hoc* HSD test). Pairwise comparisons of eclosion frequencies of flies of the two different genotypes on each given medium (using Bonferroni-corrected Student's *t* test), also revealed a significantly decreased eclosion rate in AOX-expressing flies for most individual added ingredients (for clarity, not shown on the figure): $p < .001$ for sucrose (or for minimal medium with no additions), $p < .05$ for wheat-germ, treacle, and soy flour. (c) Dose-response to different amounts of yeast or glucose added to, or subtracted from, the low-nutrient medium. Note that a single data set was used for the "3% glucose" and "3.5% yeast" conditions, as denoted by the boxes, this also being the standard composition of the low-nutrient medium used elsewhere in the study. *Statistically significant differences between different nutrient conditions within a genotype (one-way ANOVA followed *post hoc* by Tukey's HSD test, $p < .05$); #Statistically significant differences in a pairwise comparison of genotypes at each given nutrient condition (Student's *t* test, $p < .001$). (d) Dose-response to different amounts of two active ingredients from the standard medium, treacle, and maize flour (% w/v as shown), for the *UAS-AOX*^{F6} × *da*GAL4 cross only. * and **Significant differences between concentrations of a given nutrient (one-way ANOVA followed *post hoc* by Tukey's HSD test, $p < .05$ and .01, respectively). All flies were cultured at 25°C, except for the experiment of the panel (c), where 26°C was used, for technical reasons. ANOVA, analysis of variance; AOX, alternative oxidase; HSD, honestly significant difference; *SD*, standard deviation

detrimental effect on larval development, but nevertheless allows us to measure relative effects on the completion of metamorphosis, and a much lower dose (15 μg/ml), typically used to eliminate intracellular bacteria such as *Wolbachia* (Koukou et al., 2006). Neither of these doses of doxycycline compromised eclosion on the complete medium (Figure 4a–c). On the low-nutrient medium doxycycline also did not improve the eclosion frequency of AOX-expressing flies, while having no impact on control flies. Doxycycline also did not block the rescue brought about by supplementation with treacle (Figure 4b) or maize flour (Figure 4c).

3.5 | Specific treacle fractions contain the active components rescuing developmental failure

Since the addition of specific dietary supplements to low-nutrient medium failed to rescue the AOX-associated developmental failure, we attempted to isolate, or at least enrich for, the active material present in the complex food additives. This approach was simplest for treacle, because it contained only very low levels of particulates. The published composition of treacle (Table S1) gives only an approximate description of its biologically active ingredients, insufficient for

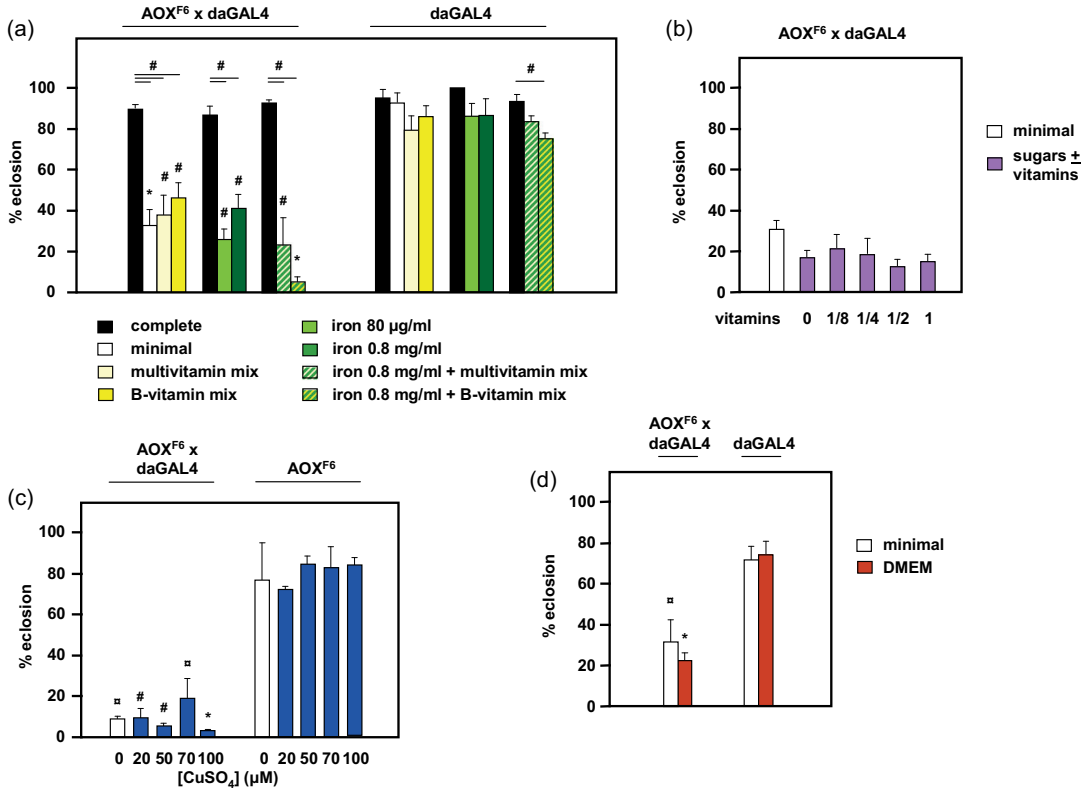

FIGURE 3 Developmental failure of AOX-expressing flies is not corrected by many specific dietary supplements. Proportion of pupae eclosing on the indicated media, of the genotypes or crosses (female × male) as shown (means + *SD*, $n \geq 4$). (a) Complete (high-sugar) medium or low-nutrient (3.5% yeast and 3% glucose) medium to which the indicated component was added at the concentrations shown, or as given in Section 2. Separated groups of bars represent trials conducted in separate series of experiments (*UAS-AOX*^{F6} × *da*GAL4 cross and *da*GAL4 controls always studied in parallel in each case). Horizontal lines annotated with symbols denote significant differences between groups within a genotype and a given experiment (one-way ANOVA with Tukey *post hoc* HSD test). Symbols above individual bars represent significant differences in pairwise comparisons between genotypes, for a given additive in a given experiment (Student's *t* test). (b) Low-nutrient (3.5% yeast and 3% glucose) medium to which multivitamins were added at the indicated dilutions from the standard amount of one tablet per 300 ml of fly food, in combination with 3% fructose and 1.5% sucrose, for the *UAS-AOX*^{F6} × *da*GAL4 cross only. There were no significant differences between the groups (one-way ANOVA). (c) Low-nutrient medium plus $CuSO_4$ at the indicated concentrations. Within each genotype, there were no significant differences between the groups (one-way ANOVA). Symbols denote significant differences between the genotypes in pairwise comparisons, at each $CuSO_4$ concentration tested (Student's *t* test). (d) Low-nutrient medium plus DMEM. Note that preliminary trials were conducted to determine a concentration of DMEM that was completely nontoxic to control flies. As for other mixed additives that had no measurable effect, it cannot be excluded that DMEM contains both positively and negatively acting components that cancel each other out. Within each genotype, there were no significant differences between the groups (Student's *t* test). Symbols denote significant differences between the genotypes in pairwise comparisons, with and without DMEM (Student's *t* test). Statistical significance in all panels is denoted by ¤, #, and *: $p < .05$, .01, and .001, respectively. ANOVA, analysis of variance; AOX, alternative oxidase; DMEM, Dulbecco's modified Eagle medium; HSD, honestly significant difference; *SD*, standard deviation

the present study. Therefore we used simple chemical fractionation to focus attention on the particular, active components. Ether extraction was used to separate treacle into aqueous and nonaqueous fractions which were then tested for their ability to support the development of AOX-expressing flies. We reproducibly found the complementing activity only in the aqueous fraction (Figure 5a). It was still active after being combined with the nonaqueous fraction, showing that the latter did not contain an inhibitor of the former. Similar fractionation of wheat-germ or maize flour was not considered feasible, due to the high level of particulates, but water/ether extraction of soya flour did produce a similar outcome, with the activity mainly in the aqueous fraction (Figure S2). To narrow down further the nature of the active component(s), the aqueous fraction of treacle, following ether extraction, was subjected to precipitation with varying concentrations of ethanol, generating supernatant and pellet fractions which were tested for effects on fly

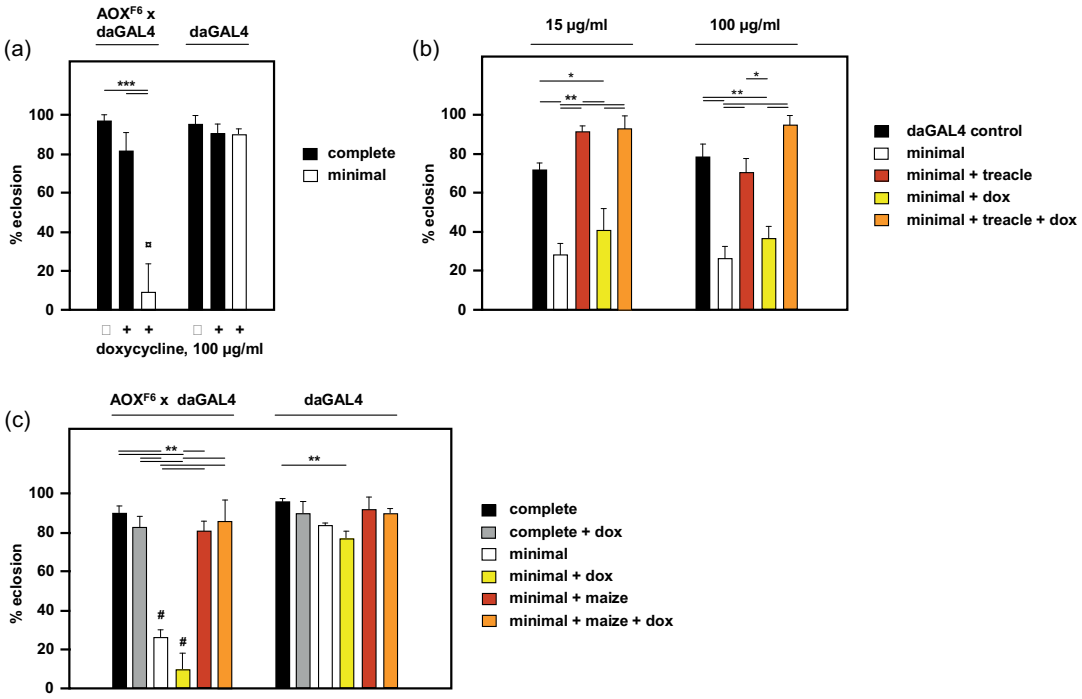

FIGURE 4 Doxycycline does not influence the development of AOX-expressing flies. Proportion of pupae eclosing on the indicated media, of the genotypes or crosses (female × male) shown (means + *SD*, $n \geq 4$). (a) Complete medium or low-nutrient medium with and without 100 µg/ml doxycycline. (b) Low-nutrient medium with and without supplementation by treacle and/or doxycycline at the indicated doses, alongside *da*GAL4 control on low-nutrient medium. (c) Low-nutrient medium with and without supplementation by maize flour and/or 100 µg/ml doxycycline. In each panel, horizontal lines annotated with asterisks (*, **, ***) denote significant differences between groups within a genotype or doxycycline concentration (one-way ANOVA with Tukey *post hoc* HSD test, $p < .05$, .01, and .001, respectively). Symbols above individual bars represent significant differences in pairwise comparisons between (a, c) genotypes or (b) doxycycline concentrations (Student's *t* test, ¤ and #: $p < .05$ and .001, respectively). ANOVA, analysis of variance; AOX, alternative oxidase; HSD, honestly significant difference; SD, standard deviation

development (Figure 5b). When precipitated with 75% ethanol, the active component(s) were recovered in the pellet fraction, whereas at 40% or 60% ethanol both the pellet and supernatant fractions showed a partial activity, albeit with high variance (Figure 5b). A 65% ethanol precipitation gave an intermediate result, with more activity in the pellet than in the supernatant (Figure 5b).

3.6 | Treacle does not contain an inhibitor of AOX

To explain the effect of treacle and other complex supplements, we considered the possibility that it contained an inhibitor of AOX, thus negating the effects of AOX expression on development. To test this possibility, we conducted respirometry on permeabilized HEK293-derived cells expressing *Ciona* AOX under the control of a doxycycline-inducible promoter (Hakkaart et al., 2006). After confirming AOX expression by western blot analysis (Figure S3A), we conducted respirometry according to a standard protocol (see Figure 6a), in which we compared oxygen consumption before and after the inhibition of complex III with antimycin. The role of AOX in antimycin-resistant respiration was confirmed by subsequent treatment with n-propyl gallate, a specific inhibitor of alternative oxidases. The addition of the water-soluble fraction of treacle to the permeabilized cells at a concentration equivalent to that in fly food, as well as 10% and 1% of this amount, had no effect on the AOX-driven respiration (Figure 6a,b). Instead, treacle addition modestly stimulated respiration in both AOX-expressing and control cells (Figure S3B).

3.7 | Flies reared on low-nutrient medium show decreased levels of triglycerides and lactate

The above findings strongly suggest one (or both) of two scenarios, relating nutrient storage and AOX. Either (a) accumulated nutritional reserves are lower in AOX-expressing larvae cultured on low-nutrient medium, compared with control larvae or with AOX larvae grown on complete medium; or (b) nutritional reserves accumulated on the low-nutrient medium are similar, regardless of

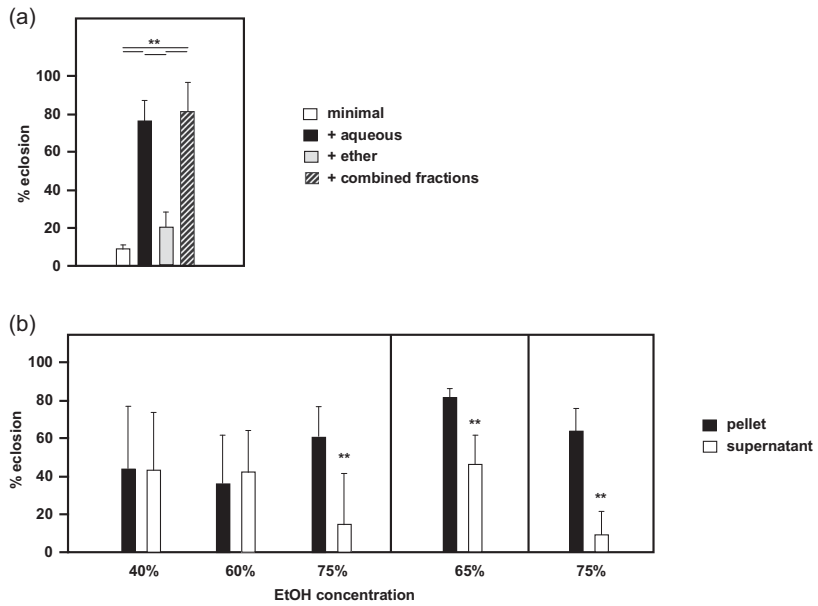

FIGURE 5 Specific treacle fractions support development of AOX-expressing flies. Proportion of pupae from *UAS-AOX*^{F6} × *da*GAL4 cross (female × male, means + *SD*, $n \geq 4$ except where indicated), eclosing on low-nutrient medium supplemented with the indicated treacle fractions. (a) Aqueous and ether fractions following ether extraction, and a 50/50 mixture of the two fractions. Horizontal lines annotated with asterisks (**) denote significant differences between groups (one-way ANOVA with Tukey *post hoc* HSD test, $p < .01$). (b) Pellet and supernatant fractions following precipitation of the ether-extracted aqueous fraction, using the indicated ethanol concentrations. Vertical bars denote separately executed series of experiments. The set of precipitations at 40%, 60%, and 75% ethanol are the combined data from all replicate vials in two separate experiments using the same extracts, $n > 5$. The symbol "**" above individual bars represent significant differences in pairwise comparisons between a given pair of supernatant and pellet fractions (Student's *t* test, $p < .01$). ANOVA, analysis of variance; AOX, alternative oxidase; HSD, honestly significant difference; SD, standard deviation

genotype, but those resources are insufficient to enable AOX pupae to complete development, due to AOX activation during metamorphosis. To test these possibilities, we assayed the relative levels of triglycerides (Figure 7a) and lactate (Figure 7b) in wandering-stage AOX-expressing L3 larvae and controls grown on complete versus low-nutrient medium. Growth on the low-nutrient medium significantly decreased the relative concentration of triglycerides (two-way ANOVA, $p < .01$) and lactate (two-way ANOVA; $p < .001$), while AOX expression had no significant effect on these metabolites. Pyruvate, being interconvertible with lactate via LDH was also decreased in larvae grown on low-nutrient medium (Figure S4, $p < .01$), but not by AOX expression. These findings support the hypothesis that the low-nutrient diet restricts the accumulation of stored nutritional resources and that it is the inability to mobilize these diminished resources that causes developmental failure in AOX-expressing pupae.

3.8 | Treacle fractionation reveals a list of candidate nutrients for compensating AOX

Treacle and its various fractions that were tested earlier in the developmental assay were analyzed further by mass spectrometry, alongside standards for 48 common metabolites. Of these, the majority was detectable in treacle, and most of these were present in the aqueous fraction thereof (Table 1). However, only four were clearly enriched in the pellet after precipitation in 75% ethanol. Since fructose, by far the most abundant of them, had already been tested in the developmental assay (Figure 3b), we proceeded to test the next most enriched, citrate, because of its metabolic role in the generation of cytosolic acetyl-CoA, the main precursor for fatty acid synthesis. However, citrate was unable to alleviate the developmental failure of AOX-expressing flies on low-nutrient medium (Figure 8), even though its conversion to acetyl-CoA by ATP citrate lyase appears to be essential for *Drosophila* development (Figure S5).

4 | DISCUSSION

In this study, we established that the developmental failure of AOX-expressing flies on low-nutrient medium occurs specifically during the pupal stage (Figure 1). Several key metabolites regarded as nutrient stores for metamorphosis were at diminished levels in flies cultured on low-nutrient medium, regardless

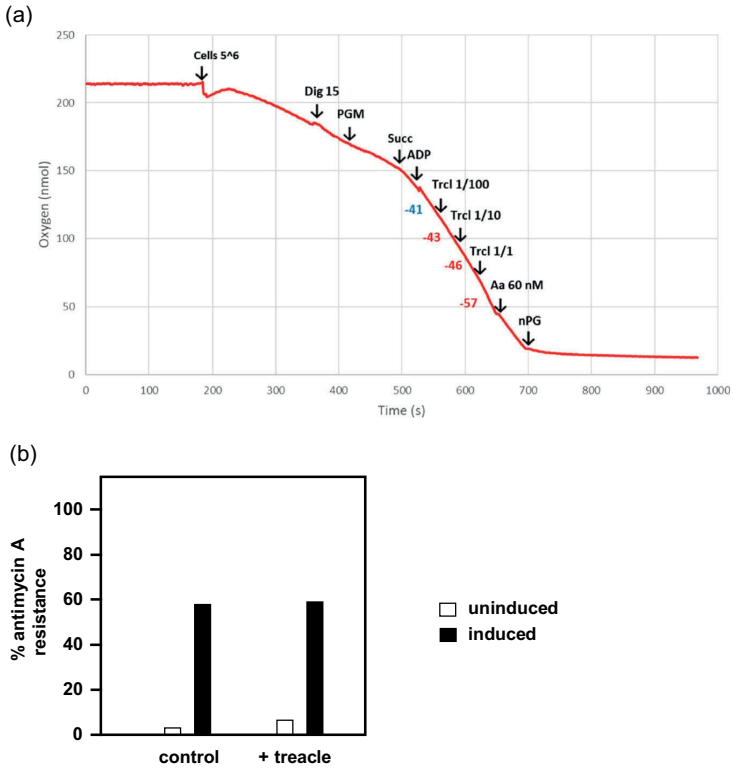

FIGURE 6 Treacle aqueous fraction does not contain an AOX inhibitor. (a) Respirometry trace for 5×10^6 AOX-expressing (doxycycline-induced) cells treated with substrates and inhibitors as indicated: Dig 15 (15 ng/ml), PGM (20 mM each), Succ (20 mM), ADP (5 mM), Trcl 1/100, 1/10, and 1/1 (treacle aqueous fraction equivalent to 1%, 10%, and 100% of the standard concentration in fly food), Aa (60 ng/ml), and nPG (0.2 mM). Numbers opposite the trace represent oxygen consumption rate before and after treacle addition (nmol/min per 5×10^6 cells), extrapolated from slope. (b) Respirometry data on permeabilized cells (averaged data from two experiments), with and without induction by doxycycline as shown. Extent of antimycin resistance with and without the addition of treacle (aqueous fraction, added to equivalent level as in fly food). See also Figure S3. Aa, antimycin A; ADP, adenosine diphosphate; AOX, alternative oxidase; Dig, digitonin; nPG, n-propyl gallate; PGM, sodium pyruvate, glutamate, and malate; Succ, sodium succinate

of genotype (Figure 7 and S4). However, the addition of sugars (glucose, sucrose, or fructose), or of food components rich in amino acids (yeast or DMEM) did not alleviate developmental failure. The same applied to specific additives, including vitamins, iron, and copper (Figure 3), and to the elimination of commensal bacteria using doxycycline (Figure 4). In contrast, various complex food additives, including wheat-germ, soya flour, maize flour, and treacle, could do so (Figure 2). Since respirometry analyses excluded the presence of any AOX inhibitor in treacle (Figure 6), it must instead contain one or more metabolites essential for completing development, whether used directly for biosynthesis or in signaling. In treacle, the complementing activity was in the aqueous fraction after ether extraction, but in the pellet after 75% ethanol precipitation (Figure 5). Mass spectrometry identified several promising candidate compounds, but the most promising, citrate, was ineffective in the developmental assay (Figure 8).

4.1 | Mechanism of developmental failure of AOX-expressing flies on low-nutrient medium

Mitochondrially determined fitness variation on different diets has been documented in *Drosophila* (Aw et al., 2018; Ballard, Melvin, Katewa, & Maas, 2007; Kemppainen et al., 2016; Melvin et al., 2018), although these tests have generally been based on varying the carbohydrate to protein ratio in the diet, or the response to overt starvation or sugar overload. The present study addresses a separate issue, since the success of AOX-expressing flies, was independent of the carbohydrate-protein ratio or the total calorific content of the food (Figure 1b), but instead affected by additives to the minimal (low-nutrient) diet.

Since AOX-determined developmental failure is specific to the pupal stage, and low-nutrient diet results in lower amounts of accumulated triglyceride, lactate and pyruvate in L3 larvae irrespective of genotype, it follows that AOX expression must compromise

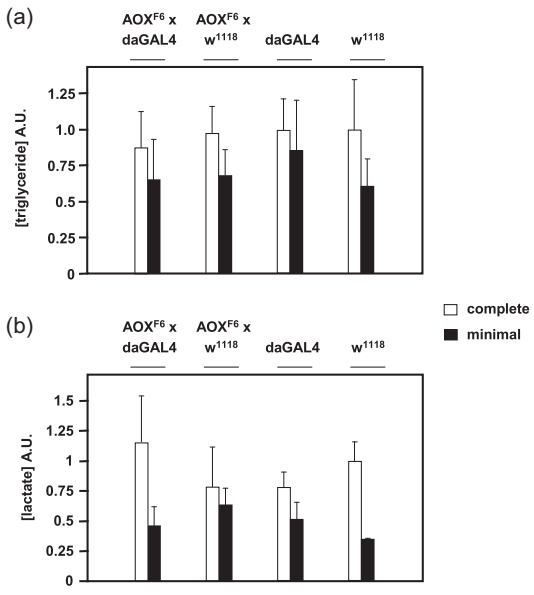

FIGURE 7 Triglyceride and lactate content of L3 larvae grown on different media. Relative amounts of (a) triglycerides and (b) lactate in L3 wandering-stage larvae from the indicated crosses, after normalization to values from control larvae (transgenic recipient strain w^{1118}, cultured on complete medium); means + *SD* of ≥ 6 batches of 10 larvae of each genotype and culture medium. Two-way ANOVA (Figure S4B, all controls vs. AOX-expressing larvae, i.e., *UAS-AOX*^{F6} × *da*GAL4 cross) found no significant differences based on genotype, but significance for diet as determinant of both lactate ($p < .001$) and triglyceride ($p < .01$) levels, as well as for pyruvate (see Figure S4A). ANOVA, analysis of variance; AOX, alternative oxidase; AU, arbitrary units; *SD*, standard deviation

the use of stored resources during metamorphosis. This, in turn, strongly suggests the enzymatic activation of AOX during metamorphosis, at least in some tissues. This could be verified by using an enzymatically incapacitated version of the enzyme expressed at the same high level as *da*GAL4-driven UAS-AOX. A previously generated control line transgenic for a mutated (inactive) AOX variant would not suffice, since the transgene was expressed at a much lower level (Andjelković et al., 2015). However, the expression of neither cytosolically expressed GFP (Saari et al., 2018) nor the GAL4 transcription factor caused developmental failure in low-nutrient medium, making it unlikely that a general proteotoxic effect is responsible.

The metabolic event that putatively switches on AOX during metamorphosis remains unknown. As indicated above, the enzyme should be catalytically inert except when the quinone pool becomes highly reduced (Dogan et al., 2018). The depletion of respiratory substrates, as observed in larvae cultured on low-nutrient medium, should logically lead to the opposite outcome, that is, to the respiratory-chain electron carriers becoming completely oxidized. However, respiratory chain shutdown might occur under such circumstances via the complex V inhibitor IF1 (Esparza-Moltó, Nuevo-Tapioles, & Cuezva, 2017), creating the conditions for AOX activation. AOX activation could, in principle, result from a number of metabolic consequences of low-nutrient diet that are hard to predict, given that rather little is known about the nutrients (other than triglycerides) that are stored in the pupa. A shortage of vitamins (e.g., folic acid, tocopherol) or mineral components of prosthetic groups such as heme is unlikely, given that these additives did not rescue the phenotype (Figure 3). Although pupal development is considered to be primarily catabolic, the precise extent and stage-specificity of substrate utilization and the involvement of different catabolic pathways (glycolysis, pentose phosphate shunt, OXPHOS) are not known in full detail. The capacity of the respiratory chain appears to be crucial both at the start of pupation and in the period immediately before eclosion (Fourche, 1967). Therefore, respiratory overload could be a factor in the activation of AOX, for example, if the CoQ pool becomes over-reduced. This may combine with a low level of stored nutrients leading eventually to ATP depletion. AOX activation could also, for example, involve partial inhibition of complex IV (Srinivasan & Avadhani, 2012) or damage to complexes I, II, and/or III (Mena, Urrutia, Lourido, Carrasco, & Núñez, 2015), resulting from oxidative or nitrosative stress, or to a relaxation of constraints on complex I that are seen in high-glucose conditions (Cannino et al., 2012).

Since the decreased larval accumulation of triglycerides and lactate/pyruvate on low-nutrient medium does not appear to pose any problem for wild-type flies and is not affected by AOX expression, it is reasonable to assume that it does not trigger a starvation response impacting ecdysteroid and insulin-like growth factor signaling. In late larval development, the fly passes through a series of checkpoints (see Tracy & Baehrecke, 2013), the most important of which is the critical weight checkpoint (Nijhout, 2003). Once passed, further growth is curtailed except under high-nutrient conditions (Tennessen & Thummel, 2011), under the control of the insulin receptor (InR) responding to the level of a crucial insulin-like peptide, dilp8 (Garelli, Gontijo, Miguela, Caparros, & Dominguez, 2012). Ecdysteroid counteracts this signal by inducing, instead, the autophagic events that occur at the onset of pupariation (see Tracy & Baehrecke, 2013). Assuming this system works normally in larvae cultured under low-nutrient conditions, the hormonal signals that initiate pupariation should not be altered due to AOX expression, which had no significant effect on nutrient accumulation. Note also that because AOX-expressing pupae die at different times during pupal stage (Saari et al., 2018) an interference with hormonal signaling is unlikely to be an explanation for the developmental failure.

In addition to its hormonal induction at the onset of metamorphosis, autophagy occurs as a response to starvation. In larvae, this is triggered via the target of rapamycin (TOR)

TABLE 1 Quantifiable compounds enriched in treacle fractions[a]

Compound	Detected[b] (Y/N)	Aqueous enriched (Y/N)	Enrichment factor[c] 75% pellet/sup	Concentration (ng/ml) in aqueous fractions[d]	Linear range[e]? (Y/N, [linear range])
Fructose	Y	Y	72	750	N [0.05–40]
Citric acid	Y	Y	8.8	15	Y
Ascorbic acid	Y	Y	>1	7.5	Y
3-OH-butanoic acid	Y	Y	>1	0.05	Y
Glutamine	Y	Y	~1	57	N [5–40]
Malic acid	Y	Y	~1	16	Y
Methionine	Y	Y	~1	1.1	Y
Oleic acid	Y	Y	~1	0.5	Y
Succinic acid	Y	Y	<1	210	N [0.5–40]
Lactic acid	Y	Y	<1	74	N [0.5–40]
Glyceraldehyde-3-phosphate	Y	Y	<1	26	Y
Tryptophan	Y	Y	<1	17	Y
Glyceraldehyde	Y	Y	<1	12	Y
Arginine	Y	Y	<1	11	Y
Cysteine	Y	Y	<1	8.4	Y
Fumaric acid	Y	Y	<1	2.1	Y
Aspartic acid	Y	Y	<1	4.5	Y
2-OH-butanoic acid	Y	Y	<1	0.67	N [1–40]
Valine	Y	Y	n/a[f]	0.005	N [1–40]
1H-indole-3-acetic acid	Y	N			
5-OH-1H-indole-3-acetic acid	Y	N			
Indole-3-lactic acid	Y	N			
Indole-3-propionic acid	Y	N			
Palmitic acid	Y	N			
Stearic acid	Y	N			
3-OH-benzoic acid	N				
Alanine	N				
Arachidonic acid	N				
Asparagine	N				
Cholesterol	N				
Decanoic acid	N				
Fructose-6-phosphate	N				
Glucose-6-phosphate	N				
Glutamic acid	N				
Glycerol-3-phosphate	N				
Glycine	N				
Homocysteine	N				
Leucine	N				
Linoleic acid	N				
Lysine	N				
Octanoic acid	N				
Ornithine	N				
Phenylalanine	N				
Phosphoenolpyruvate	N				

(Continues)

TABLE 1 (Continued)

Compound	Detected[b] (Y/N)	Aqueous enriched (Y/N)	Enrichment factor[c] 75% pellet/sup	Concentration (ng/ml) in aqueous fractions[d]	Linear range[e]? (Y/N, [linear range])
Proline	N				
Ribose-5-phosphate	N				
Serine	N				
Threonine	N				
Tyrosine	N				

Note: The raw data from this analysis have been deposited in the MetaboLights database (RRID: SCR_014663) under access code MTBLS921.

[a]Compounds listed in the following order: first those compounds enriched in the 75% EtOH fraction in order of their enrichment factor (in cases where the enrichment factor is similar, listing is in order of their abundance in unfractionated treacle); next (in alphabetical order) those compounds detected in unfractionated treacle, but which were not detected in the aqueous fractions, and finally (again in alphabetical order) those compounds which were not reproducibly detected in unfractionated treacle, but which were found in one or more specific fractions.

[b]Reproducibly detected in unfractionated treacle.

[c]The ratio of concentrations in the pellet versus the supernatant of the 75% ethanol fraction. Where one value was very low or below the detection limit, only >1 or <1 are shown, otherwise actual values to 2 sig. fig. or ~1 if the values were within a factor of 2 of each other.

[d]Based on average across all aqueous fractions (pellet plus supernatant from 40%, 60%, and 75% ethanol precipitations, two full repeats) but excluding fractions where the compound was below the detection limit. Values are quoted to 2 sig. fig and would represent concentrations in ~3% treacle, assuming losses of up to one-third during extraction.

[e]Linear range based upon concentration standards analysed in parallel. Shown only where the extrapolated concentration was outside of the linear range.

[f]Valine was at such a low level that it was reliably detected in only one aqueous fraction.

pathway, driven by an insufficient supply of amino acids (Scott, Schuldiner, & Neufeld, 2004). Conceivably, this may accelerate developmental progression, exacerbating the vulnerability of AOX-expressing pupae to subsequent developmental failure. However, such a scenario appears unlikely, since dietary supplements rich in amino acids did not alleviate the phenotype. Finally, a more prosaic explanation of AOX activation in pupae would invoke stage-specific activity of the *da*GAL4 driver, although this has not been explored experimentally.

AOX expression may also affect ROS signaling, although any such effect would only be meaningful in combination with specific stresses arising from the low-nutrient diet. ROS is recognized as a regulator of stemness (Perales-Clemente, Folmes, & Terzic, 2014) and, in *Drosophila*, influences embryogenesis (Xie et al., 2010), imaginal disc regeneration (Khan, Abidi, Skinner, Tian, & Smith-Bolton, 2017), and testis differentiation (Tan, Lee, Wong, Cai, & Baeg, 2017).

AOX limits excess ROS production (Cannino et al., 2012; Sanz, Fernández-Ayala, Stefanatos, & Jacobs, 2010) and attenuates responses to ROS (Dogan et al., 2018; El-Khoury et al., 2016; Hakkaart et al., 2006). We are not aware of any studies indicating disruption of pupal development by global treatment with

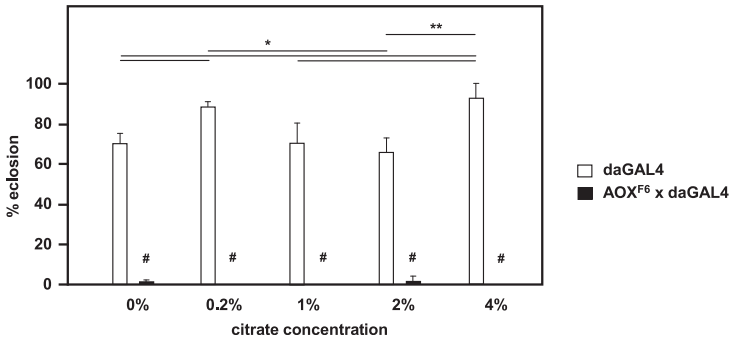

FIGURE 8 Citrate addition does not rescue the developmental failure of AOX-expressing flies. Proportion of pupae of the genotype or cross (female × male) shown, eclosing on low-nutrient medium supplemented with the indicated levels of citrate (means + *SD*, $n \geq 4$). Horizontal lines annotated with asterisks (* and **) denote significant differences between groups within a genotype (one-way ANOVA with Tukey *post hoc* HSD test, $p < .05$ and .01, respectively). Symbols "#" above individual bars represent significant differences in pairwise comparisons between genotypes for a given citrate concentration (Student's *t* test, $p < .001$). Note that this experiment was conducted at 26°C for technical reasons, also giving a more robust phenotype (Saari et al., 2018). ANOVA, analysis of variance; AOX, alternative oxidase; HSD, honestly significant difference; *SD*, standard deviation

antioxidants. However, before this hypothesis can be discarded, the metabolic changes produced by low nutrient diet will need to be profiled in detail, including those impacting ROS.

4.2 | Identity of the crucial nutrients required for the completion of development by AOX-expressing flies

Although we were able to rule out from consideration a large number of nutrients acting individually, as well as several when added together, it remains plausible that some of those already tested may act in combination to restore full developmental potential to AOX-expressing flies. A role for a combination of nutrients is also suggested by the observation that, at low ethanol percentages (40% or 60%), full activity was not recovered in either the pellet or supernatant fractions (Figure 5b). Based on the mass spectrometry findings, the active fraction of treacle is enriched in fructose, citrate, ascorbate, and the ketone body 3-OH-butanoic acid. However, this "short-list" does not include metabolites for which standards were not included, nor those that were not identifiable from the mass spectrogram. Furthermore, although these compounds were the ones that were enriched in the 75% ethanol pellet, it is entirely possible that whichever of them is the crucial one(s) is only active in combination with some other compound also present in the 75% ethanol pellet, if not actually enriched there. The candidate metabolites listed in Table 1 include other TCA cycle intermediates, among them succinate, which AOX may deplete (Mills et al., 2016), several amino acids and some glycolytic intermediates, notably lactate, which we found to be systematically decreased in L3 larvae cultured on the low-nutrient medium. Almost all of these are strong candidates to supplement those metabolites that would normally be supplied cataplerotically, thereby ensuring that a sufficient level of stored nutrients is available throughout metamorphosis, enabling AOX-expressing pupae to complete development. The importance of cataplerotic pathways is illustrated by the developmental requirement for ATPCL (Figure S5), which applies even in wild-type flies grown on standard high-sugar medium. ATPCL uses citrate, shuttled into the cytosol, to synthesize acetyl-CoA, the raw material for fatty acid, and hence triglyceride production. Given the many possible permutations of compounds and their concentrations that might be crucial in replacing such cataplerotic pathways, the development of a high-throughput approach is clearly required, to narrow down further the identity of the active substance(s).

Importantly, the effect of the missing nutrients need not be solely on the actual synthesis of triglycerides and other stored metabolites for subsequent catabolism. Some of them are known to be involved in the generation of epigenetic signals, which may program metabolism during metamorphosis according to the prevailing conditions. For example, specific catabolic enzymes may be required in higher amounts under conditions of dietary stress, enabling the pupa to survive on a lower repository of stored triglycerides. In combination with partially activated AOX, this could lead to a catastrophic depletion of resources. For example, the list of detected compounds in the active treacle fraction included succinate and several other TCA cycle intermediates that could modulate the activity of alpha-ketoglutarate-dependent histone demethylases (Berry & Janknecht, 2013), or promote histone methylation via fumarate and ROS signaling (Sullivan et al., 2013).

4.3 | Implications for use of AOX in therapeutic and biotech applications

Mice ubiquitously expressing *C. intestinalis* AOX showed no deleterious phenotypes, even under the moderate stress of treadmill exercise (Szibor et al., 2017). Metabolic profiles, at least in the heart, were not distinguishable from wild-type littermates, and there was no evidence pointing to enzymatic activation of the enzyme in vivo. AOX-expressing flies also move and behave normally (Andjelković et al., 2015; Fernandez-Ayala et al., 2009). However, possible activation of the enzyme at specific developmental stages or under nutritional stress has not been studied in the mammalian model. The mice were always fed *ad libitum* on standard chow diet, which may be considered equivalent to the rearing of flies on standard high-sugar medium, where AOX expression also had no impact on development. To conduct a parallel study on the mammalian model would require the mice to be fed a specific diet with restricted calorie intake and/or nutritional composition. In at least one case where we attempted to rescue the phenotypic consequences of respiratory chain deficiency in the mouse (*Cox15* knockout in skeletal muscle), AOX expression exacerbated rather than alleviated the phenotype (Dogan et al., 2018). In this case, interference with ROS-based stress signaling was inferred, which may apply also to the present case of nutritional stress in *Drosophila*. Clearly, before AOX is to be developed as a therapeutic, the conditions for, and consequences of, its activation need to be evaluated in detail. However, if judiciously exploited, such properties could also be beneficial, for example in allowing better regulation of the enzyme in a therapeutic setting, or even using AOX to improve adaptation to nutritional stresses.

4.4 | Implications for evolution and animal development

As discussed in Section 1, the life cycle of holometabolous insects provides a "best of all worlds" scenario, in which maximum advantage can be taken of food resources for growth and dispersal to maintain genetic fitness and adaptability, while minimizing vulnerability to predation.

In an insect species that would be naturally endowed with AOX, there would be strong selective pressure to evolve adjustments to the ways in which the onset of metamorphosis is regulated, so as to take account of potentially decreased bioenergetic efficiency during the pupal stage. This pressure might also favor the evolutionary loss of AOX itself if this inherent vulnerability to starvation outweighs the stress-resistance AOX may confer. In practice, the natural environment is likely to be much more similar to what we here describe as a low-nutrient medium than the complete medium on which we and almost all other *Drosophila* researchers culture flies in the laboratory.

Since AOX has indeed been lost during insect evolution, our findings could offer one possible explanation.

ACKNOWLEDGMENTS

We thank Eveliina Teeri for technical assistance and Andreas Bergmann and Alex Dickens from the Turku Metabolomics Centre for analytical services. This study was supported by the European Research Council (Grant No. 232738); the Academy of Finland (Grant Nos. 283157 and 272376), Tampere University Hospital Medical Research Fund, Fundação de Amparo à Pesquisa do Estado de São Paulo, grant 2014/02253-6, Conselho Nacional de Desenvolvimento Científico e Tecnológico, grant 424562/2018-9 and the Sigrid Juselius Foundation.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

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How to cite this article: Saari S, Kemppainen E, Tuomela T, Oliveira MT, Dufour E Jacobs HT. Alternative oxidase confers nutritional limitation on *Drosophila* development. *J. Exp. Zool.* 2019;331:341–356. https://doi.org/10.1002/jez.2274