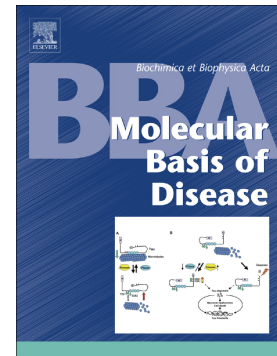


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Alternative respiratory chain enzymes: therapeutic potential and possible pitfalls

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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.

Keywords: mitochondria / mitochondrial disease / thermogenesis / AOX / reactive oxygen species

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 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^{25r}*), 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.

MATERIALS AND METHODS

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.

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 x 20 s at 4,000 rpm (PowerLyzer 24 Bench Top Bead-Based Homogenizer; MO BIO Laboratories, USA). After chloroform extraction, followed by centrifugation at 12,000 g_{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 12,000 g_{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 μ l 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 μ M 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.

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 Φ C31 recombination at a lower-expression site on chromosome 2 [38], each containing the AOX coding sequence placed under the control of the exogenously supplied GAL4 transcription factor; *tubAOX³⁵*, *tubAOX¹¹²* and *tubAOX⁷*, 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 *tubAOX¹¹²* and *tubAOX⁷* insertions, and *3xtubAOX*, homozygous additionally for *tubAOX³⁵*. Recipient line *w¹¹¹⁸* was used as a control for all transgenic lines except those created by Φ C31 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.

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 \leq 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.

Light microscopy of pupae

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

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 15,000 g_{max} at room temperature, supernatants were decanted and protein concentrations measured by NanoDrop spectrophotometry (ThermoFisher Scientific). After dilution with water and 5 x 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 x 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.

RESULTS & DISCUSSION

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 (Fig. 1, S1). Both AOX and

NDX were induced by hypoxia (Fig. 1A, S1A) or exposure to sulfide (Fig. 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 CO₂ [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 while 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 H₂S can also become pathological, with cIV as a major target. A frequent cause of disturbed H₂S 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 H₂S 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 H₂S, 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.

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 in 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 less than 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 18-29 °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.

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 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 *daGAL4* 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 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.

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 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.

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.

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 Figure 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 catalyse 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.

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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.

ACCEPTED MANUSCRIPT

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FIGURE LEGENDS**Figure 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 μ M 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 μ M sulfide or the combination of both (1.91 mg/l O₂, 100 μ M 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 \pm 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.

Figure 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 \pm SD for \geq 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 \pm SD for a total of 8-16 vials in each case, from 2-4 biological replicate experiments). For clarity, only the effects of 2 and 3 copies of the *tubAOX* transgene versus the *w¹¹¹⁸* 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 \pm SD for 8-12 vials from 2-4 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.

Figure 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 x male) as shown (means \pm SD). (D) Representative images at comparable zoom, of dying/developmentally arrested AOX-expressing pupae, cultured on low-nutrient medium.

Figure 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 \pm 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).

Figure 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 *w¹¹¹⁸*.

Figure 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 thus 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).

Figure 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.

Highlights

- AOX is induced in *Ciona* by hypoxia or exposure to sulfide
- AOX accelerates development of *Drosophila* at low temperatures
- AOX impairs metamorphosis of flies reared on low-nutrient diet
- AOX accelerates recovery of flies from cold-induced paralysis

ACCEPTED MANUSCRIPT

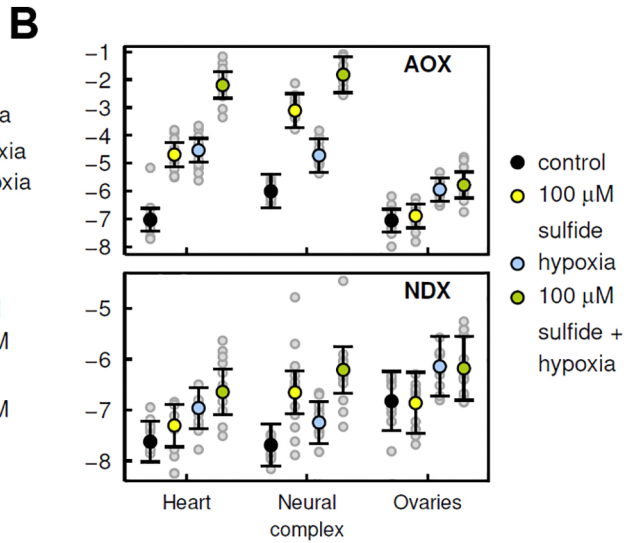
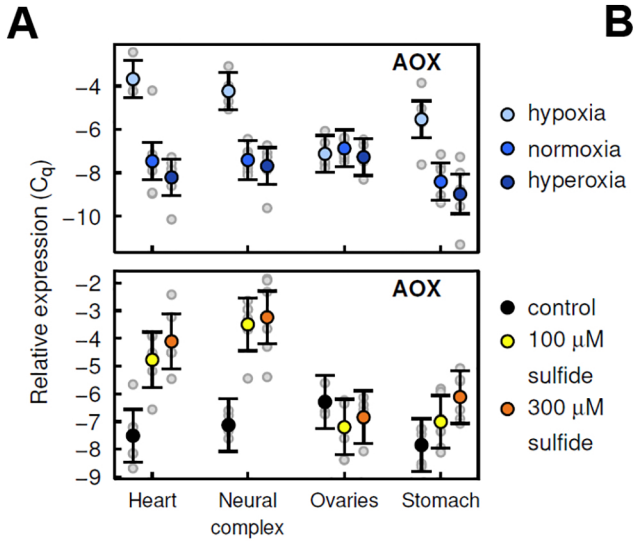


Figure 1

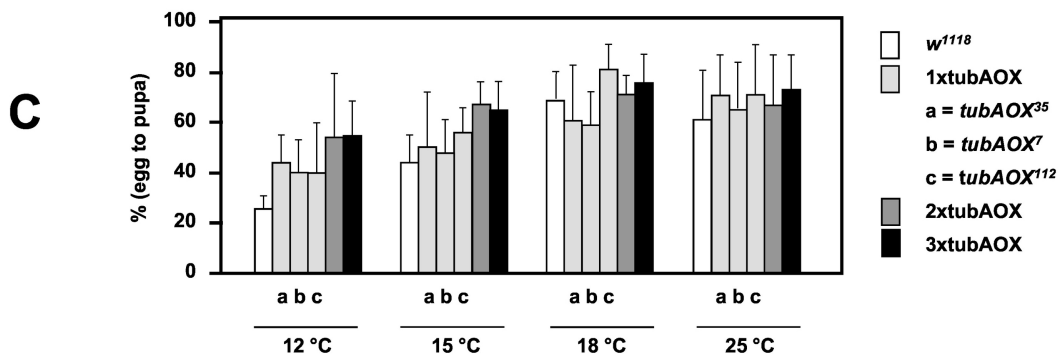
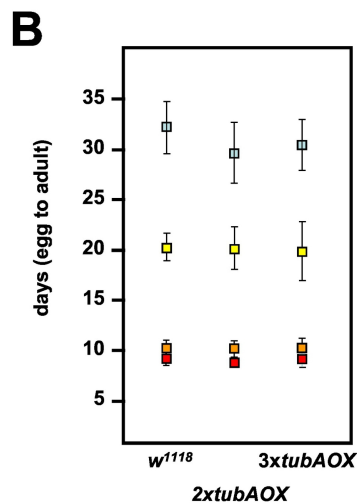
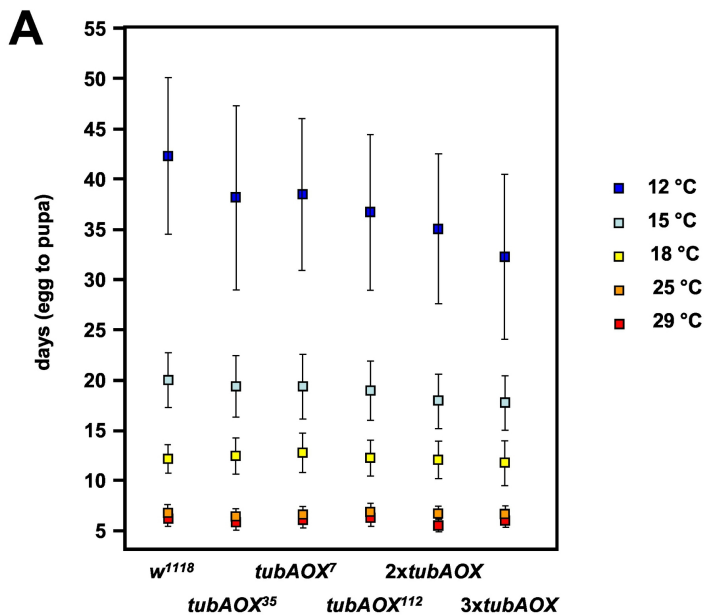
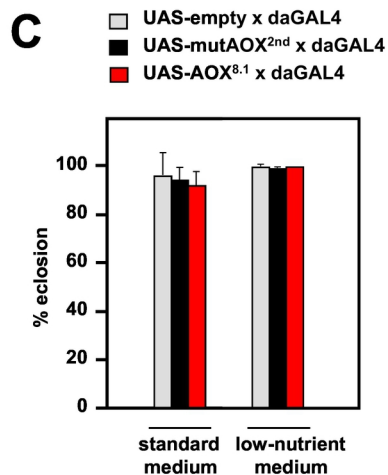
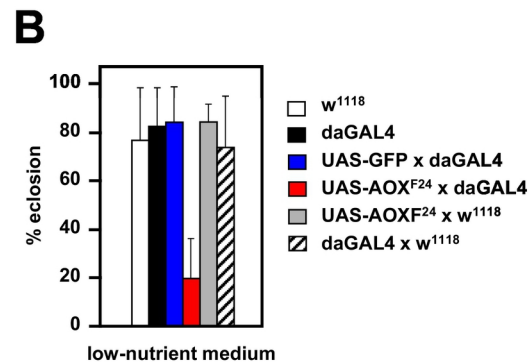
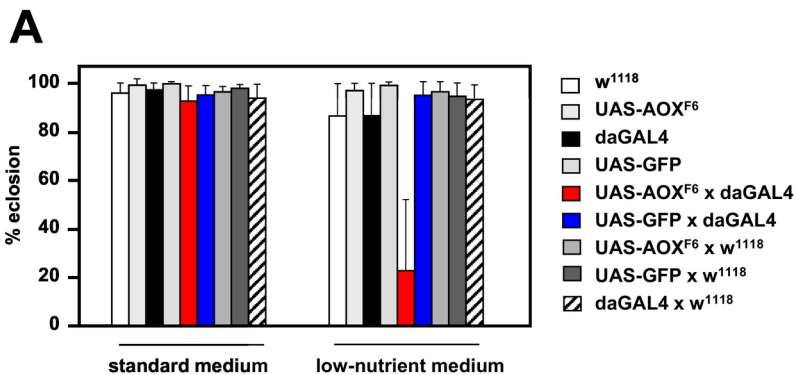


Figure 2



D

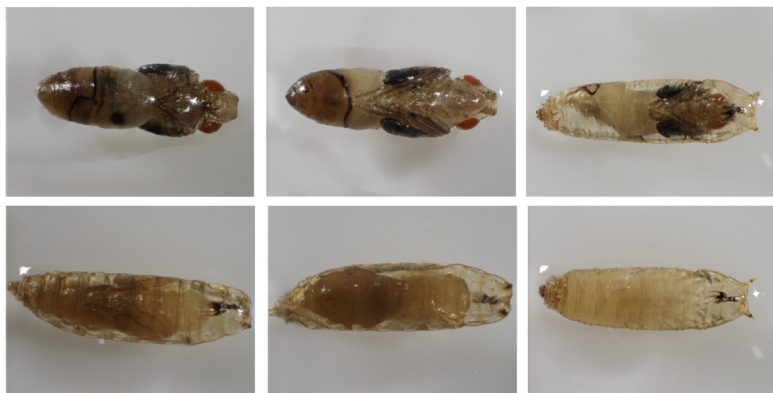
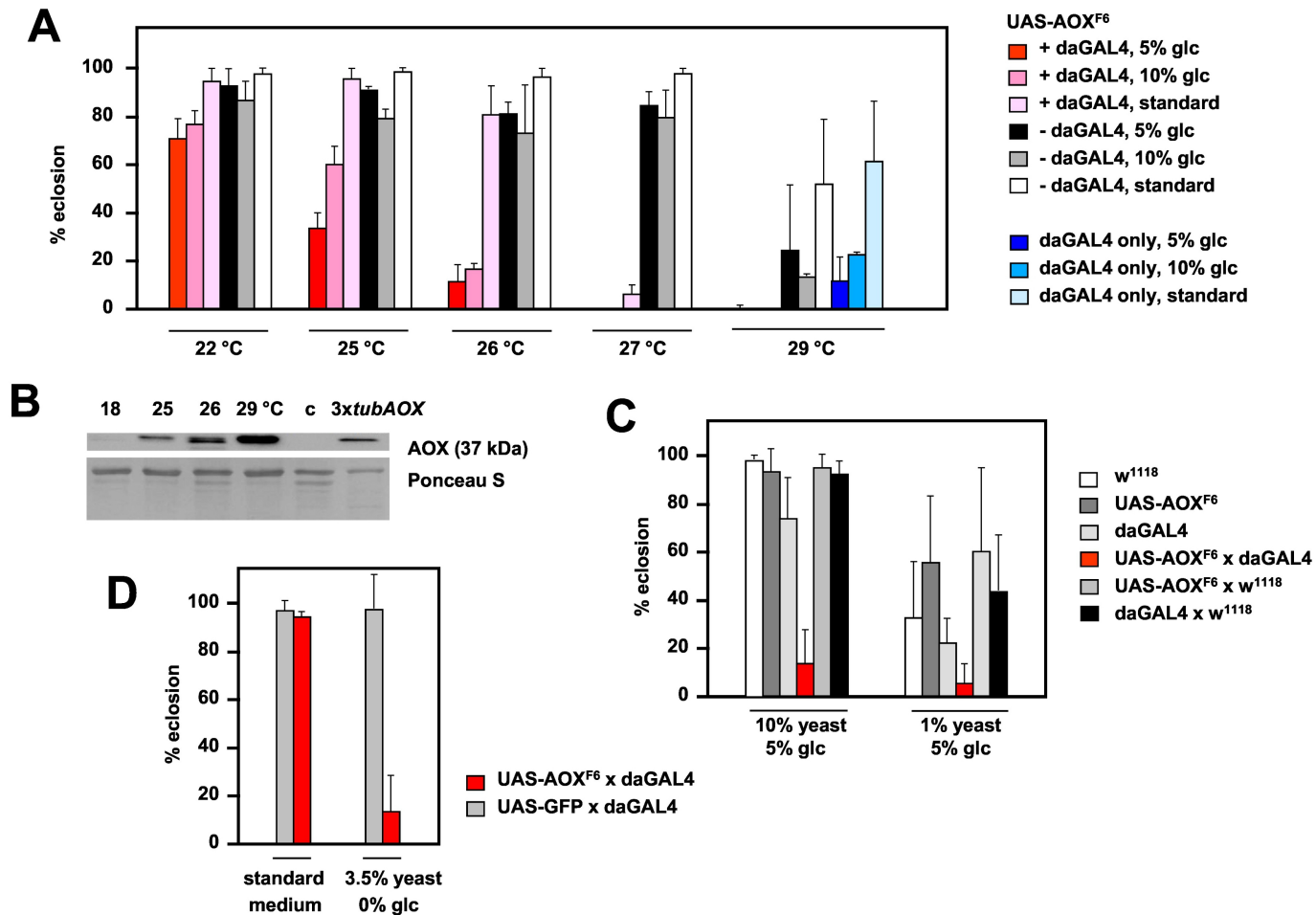


Figure 3



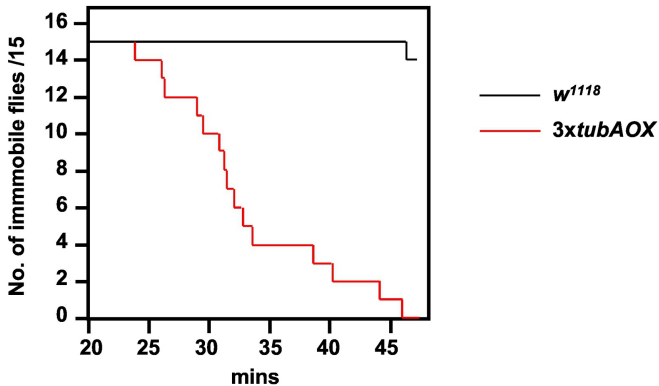
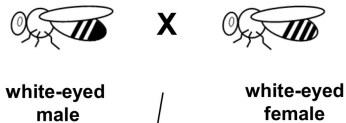


Figure 5

A first mating



white-eyed progeny

B second mating



mostly red-eyed progeny



C effective sperm competition

still mostly white-eyed progeny



D poor sperm competition

Figure 6

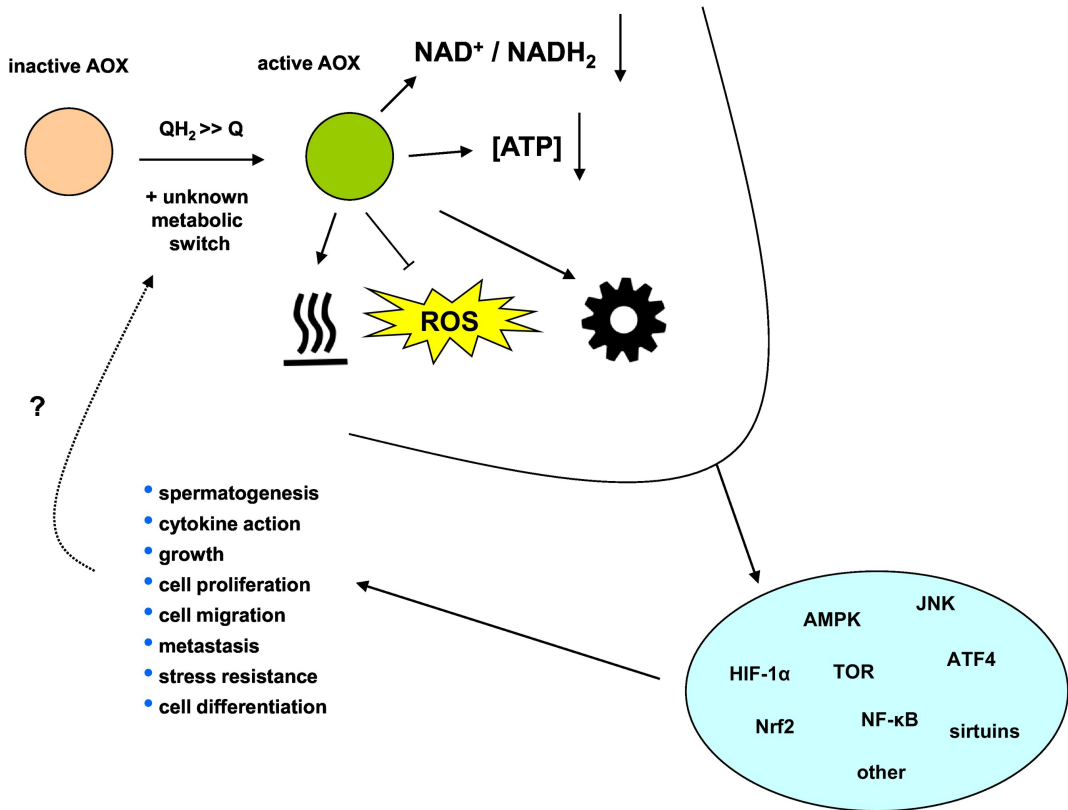


Figure 7