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What physiological role(s) does the alternative oxidase perform in animals?

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ABSTRACT

Although the alternative oxidase, AOX, was known to be widespread in the animal kingdom by 2004, its exact physiological role in animals remains poorly understood. Here we present what evidence has accumulated thus far, indicating that it may play a role in enabling animals to resist various kinds of stress, including toxins, abnormal oxygen or nutrient levels, protein unfolding, dessication and pathogen attack. Much of our knowledge comes from studies in model organisms, where any benefits from exogenously expressed AOX may be masked by its unregulated expression, which may itself be stressful. The further question arises as to why AOX has been lost from some major crown groups, namely vertebrates, insects and cephalopods, if it plays important roles favouring the survival of other animals. We conclude by presenting some speculative ideas addressing this question, and an outline of how it might be approached experimentally.

1. Introduction

The alternative oxidase, AOX, was first identified in metazoans almost two decades ago [1]. Since that time, we have learned quite a lot about the properties that it confers on animals that naturally lack it, principally two model organisms, *Drosophila* and the mouse. Although many findings remain puzzling, the general conclusion reached is that AOX expression under standard laboratory conditions is largely benign and can even be beneficial under specific pathological or stress conditions. Such observations have even strengthened the idea that AOX might one day even be applied in humans as a therapeutic, for conditions that involve mitochondrial dysfunction [2,3].

However, far less is known of what functions AOX serves in those metazoans that have retained it over the course of evolution. AOX has been detected in most phyla of the animal kingdom, although it has been lost on multiple occasions in specific lineages, in particular from three highly successful crown groups: vertebrates, insects and cephalopod molluscs (see Fig. 1). Yet it has been retained in some of their sister groups, such as tunicates [4], copepods [5] and both gastropod and bivalve molluscs [4]. These observations raise two fundamental questions relating to AOX in animals, namely, why has it been retained so extensively, yet why has it been lost on multiple occasions.

In this short review we attempt to piece together a tentative answer to these questions. 'Short' because remarkably little is yet known, and many ideas that have been put forward are based largely on the plant and fungal literature and remain highly speculative.

Thus, we first consider the general hypothesis, supported by data, that AOX confers stress resistance on animals as it does on plants. We then discuss the relevant experimental evidence in the case of different types of stress, starting from those where most data is available or experimental approaches are feasible. We then proceed to outline possible reasons for the evolutionary loss of AOX in specific taxa, and how these ideas might be tested experimentally. Before embarking on this journey, it is appropriate to recall the basic biochemical properties of AOX as illustrated in Fig. 2. AOX catalyses the single-step reoxidation by molecular oxygen of ubiquinol, the reduced electron carrier upon which almost all mitochondrial substrate oxidation pathways converge. Unlike the complex machinery of OXPHOS complexes III and IV, to which AOX provides a by-pass, AOX is composed of a single, nuclear-coded polypeptide, which converts all of the energy released by ubiquinol oxidation into heat instead of ATP.

Note that, at this time, even less is known or understood about the physiological function(s) of the alternative NADH dehydrogenases (NDH2 or NDX) in animals, which must therefore be left to a future edition of this collection.

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2. Alleviating stress

In biology, stress may be defined as any condition that pushes cells or organisms beyond their normal operating limits. All organisms experience stress, whether imposed by environmental conditions or endogenous processes, but possess homeostatic mechanisms for accommodating or alleviating it and its effects. The most widely held belief about AOX in animals, supported from the scant literature that we have, is that it protects individuals of those species endowed with it against the most egregious consequences of various kinds of stress (Fig. 3). These have been shown or suggested to include ingestion of, or exposure to, respiratory poisons, high or low temperatures, conditions that promote the excess production of free radicals – notably reactive oxygen species (ROS) and, perhaps, other physiological stresses that have been studied more appropriately in plants and microbes (salinity, dessication, extremes of pH). Resistance against pathogens is another possible protective effect of AOX, but it has not been studied in animals, and any relevant molecular mechanism is in the realms of pure speculation.

Before discussing what has actually been reported, it is perhaps useful to rehearse what criteria might be considered experimental evidence of a role of AOX (or anything) in stress mitigation. These may be enumerated as: (1) AOX is induced by the stress, whether in the amount of the protein (or mRNA), its intracellular location in mitochondria and/or its enzymatic activity; (2) the inactivation or genetic downregulation of AOX should sensitize the organism to stress-related damage; (3) the enhancement of AOX expression or activity should increase resistance to stress. Although these are relatively simple to test, none of them is absolute. For example, AOX might be constitutively expressed or active, especially if a stress is frequently encountered either in nature or in the laboratory, or has been previously experienced, elevating longterm resistance against its recurrence by a hormetic process. Furthermore, since AOX is naturally regulated by the availability of its substrates (reduced quinones and oxygen), stressors that modulate their availability influence AOX by a common mechanism, such that the effect of any particular stress exposure *in vivo* might be masked by another. Conversely, in order to detect AOX activation using in vitro methods, activating conditions need to be applied, or else the induction of the enzyme *in vivo*, such as by a post-translational modification, might go undetected.

As regards the second criterion, we are faced again with the issue of possible hormesis. A wild organism transferred to the laboratory may experience stresses that are not obvious to the experimenter, whilst a model organism cultured longterm in the lab setting may have been selected for robust stress resistance by other means to which AOX may be redundant. Redundancy is a persistent theme in biology, and is characteristic of processes that are too important for survival to be left to a single mechanism. Thus, inactivating or downregulating AOX may not affect resistance to any (or all) given stress(es) except where one or more other processes are also inactivated. Similar arguments apply to the third criterion, for which constitutive or hormetically upregulated expression may already be maximal in terms of measurable stress resistance.

Thus, on balance, even though we start from little hard data, the ways in which this question might be addressed in future require careful analysis and judgement. Whilst 'false positives' may also occur, false negatives, where evidence for a protective effect by AOX is weak or non-existent, should never be considered as definitive.

Finally, we should bear in mind that studies of AOX transferred to animal models from plants, fungi or even other animals such as the tunicate (sister group to the vertebrates) *Ciona intestinalis*, may not function in a physiological manner in the new context. Findings may reflect the biology of the recipient organism more than that of AOX itself, whilst AOX may also interact with other proteins in the recipient in ways that are non-physiological in either species.

Whilst such *caveats* are universal in biology, they need to be uppermost when considering resistance to potentially life-threatening stresses and the properties of an enzyme about which so little is yet known.

3. Toxic stress

In considering protection against respiratory poisons, additional criteria come into view. AOX clearly protects model organisms in the laboratory setting against poisons of OXPHOS complex III or IV (cIII, cIV) such as antimycin or cyanide, respectively [6–8]. However, whether this is relevant to the biology of AOX-endowed animals in nature depends on how often animals actually encounter such poisons. Antimycins are a group of antibiotics secreted by *Streptomycetes*, but have garnered relatively little attention, since they are of no clinical use, unlike antibiotics targeted specifically on bacterial ribosomes. However, they may be widespread, and therefore of ecological relevance. Some appear to be highly effective on fungal targets [9] and have even been adopted as fungicides [10]. At least one example has been reported of antimycin-contamination of cereal grains intended for human consumption [11], implying a potential for AOX in animals to mitigate harmful effects of such toxins.

Well-studied poisons of cIV are simple chemical species such as cyanide, sulphide, carbon monoxide and azide. All of these are

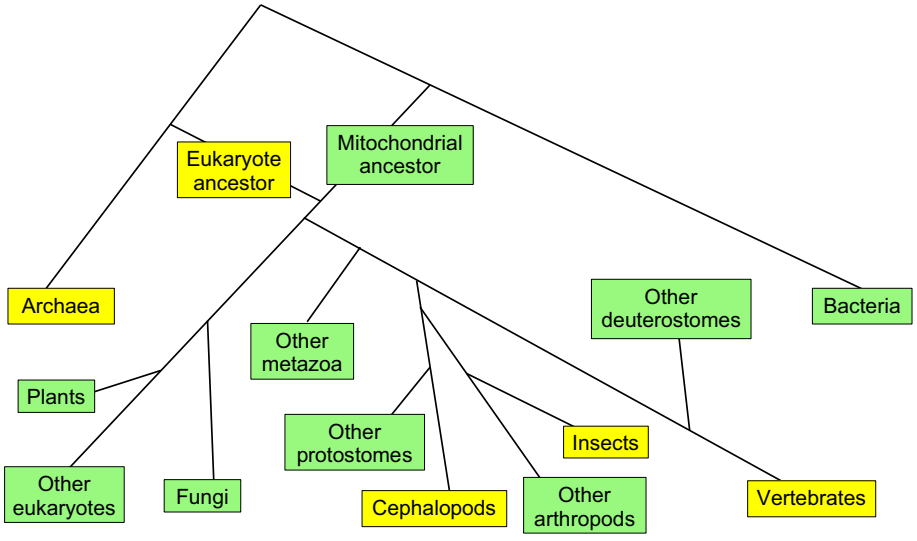

Fig. 1. Phylogenetic distribution of AOX.
Schematic diagram showing phylogenetic relationships between taxa that have (green) and have not (yellow) retained AOX. Branch lengths are purely topological and are not implied to indicate genetic distance or evolutionary time. Details, exceptions and possible cases of horizontal transfer are omitted and are dealt with in other sources. *Bona fide* AOX pseudogenes have not been reported in any of the non-AOX groups indicated.

encountered extensively in the natural environment, making AOX-based resistance highly advantageous, especially for animals that live in conditions where these chemical species may accumulate periodically. Sulphide has received particular attention, since it can accumulate to high levels in specific environments, e.g. as a result of microbial activity in anoxic waters [12], or geothermal processes [13].

In this case, AOX not only provides a by-pass of the sulphide-sensitive cIV, but can directly use sulphide as an electron donor, via sulphide-quinone oxidoreductase, SQR [14,15]. Studies in the lugworm *Arenicola marina* indicate that SQR can co-operate either with cIII/cIV under low sulphide concentrations, in effect using sulphide to drive ATP synthesis, or with AOX at higher (cIV-inhibitory) sulphide concentrations to detoxify sulphide and maintain mitochondrial redox homeostasis [16]. In the echiuran worm *Urechis unicinctus* AOX mRNA was induced in response to 50–150 μM sulphide in a time and concentration-dependent manner [17], suggesting a role in detoxification.

In *Ciona intestinalis*, the mRNAs for both AOX and for the alternative NADH dehydrogenase NDX were elevated in response to 100 or 300 μM sulphide in both heart and neural complex [18], although not in other tissues tested. AOX mRNA was induced under these conditions by 10–20 fold, as well as ~2-fold by exposure to the much lower sulphide level of 15 μM (50% of the lethal dose) in late (tailbud-stage) embryos [19]. Sulphide exposure also induced AOX at the transcript level in the marine bivalve *Anadara broughtonii* [20].

Morpholino-mediated knockdown of AOX in *Ciona* embryos significantly impaired the ability to complete development as far as the tailbud stage in sea water containing 15 μM sulphide, with only one-third as many embryos surviving as controls, whilst having no effect on development in regular sea water. Varying sulphide levels across the above range are encountered in nature, notably in ocean vents, volcanic hot springs and oxygen-deprived environments, as discussed in [18].

Cyanide is encountered less frequently in the natural environment, but is actively produced by some organisms, especially plants, as well as by some arthropods, in order to ward off or, if necessary, kill predators [21–23]. The ability to synthesize cyanogenic glucosides appears to have been acquired independently, multiple times. Since AOX itself is absent from insects and vertebrates, two major classes of herbivores, cyanogenesis might be an effective defence strategy, given that plants themselves have generally retained AOX. However, other detoxification systems for cyanide exist in nature, such as the enzyme rhodanese (thiosulphate:cyanide sulphurtransferase), which may enable insects not only to ingest cyanogenic plants but also themselves release cyanide to defend against insectivorous vertebrates [23].

4. ATP insufficiency

A common effect of many stresses experienced in nature should be a deficiency of ATP production, for which AOX, a non-protonmotive enzyme, would seem superficially to offer no benefit. However, it is important to note that, in response to ATP deficiency, AOX may sometimes be beneficial rather than harmful or neutral, depending on the precise biochemical circumstances. Where ATP yield is decreased by a limitation on electron flow through complex I, AOX, if it were able to contribute to electron flow, would exacerbate rather than alleviate the defect, by decreasing proton pumping at cIII and cIV in addition. However, AOX should not become engaged under these conditions, since the quinone pool would be under- not over-reduced. AOX could, however, worsen ATP deficiency if, in such a case of cI inhibition, there was a large switch towards FAD-linked substrate oxidation.

In the case of an ATP synthase (complex V, cV) defect, AOX would restore electron flow and redox homeostasis but not ATP production. In

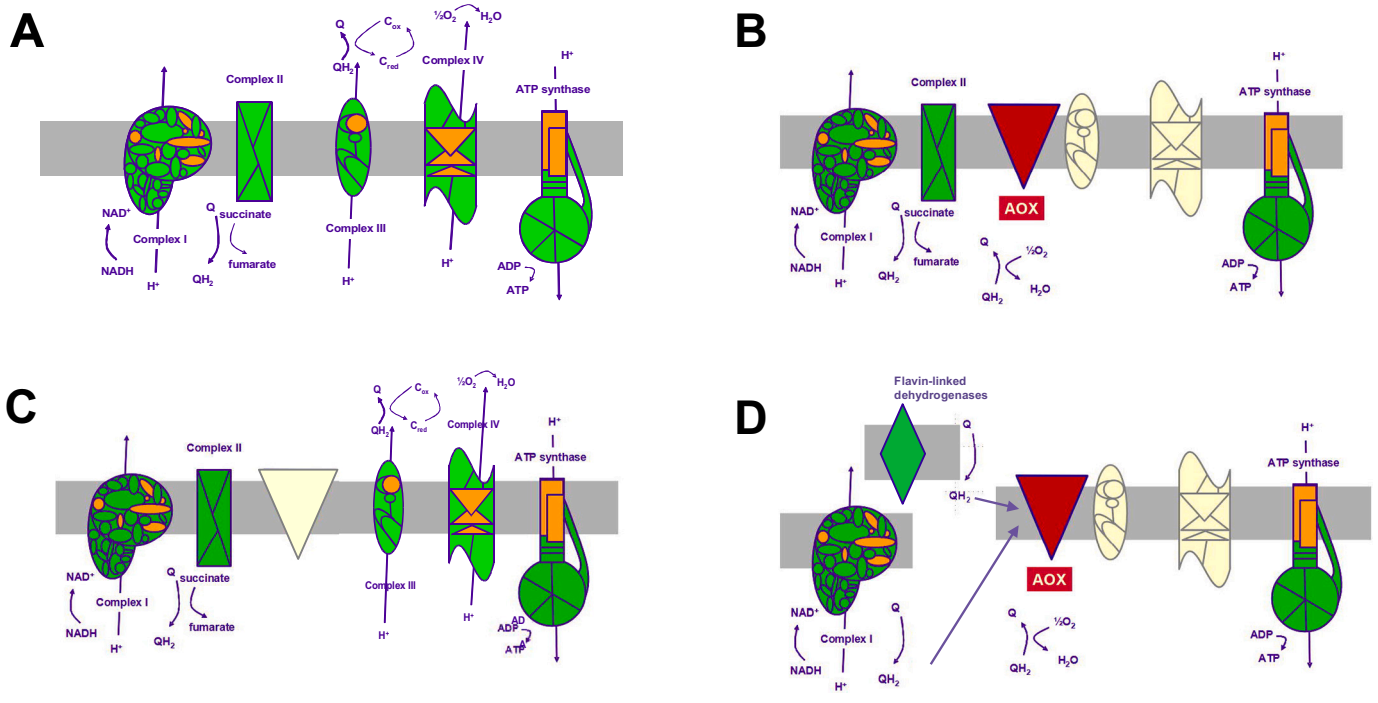

Fig. 2. Role of AOX in mitochondrial respiration.
(A) Schematic diagram of the standard OXPHOS system, with mtDNA-encoded subunits shown in orange and nuclear-coded subunits in green, indicating the proton-motive complexes I, III and IV. (B) AOX (dark red), if present, can replace complexes III and IV if the latter are inoperative (shown here in cream). AOX is non proton-motive but performs the same redox chemistry as complexes III and IV combined, but thermogenically. (C) AOX has a much higher Km for ubiquinol (QH_2) than does complex III, so under standard physiological conditions is itself essentially inoperative (shown in cream) and contributes only minimally to electron flow. (D) Note that, in the standard OXPHOS system complex II is, in reality, just one of several flavin-linked dehydrogenases supplying electrons to complex III in the form of QH_2, and in combination with AOX these constitute a completely non-proton-motive electron transport system.

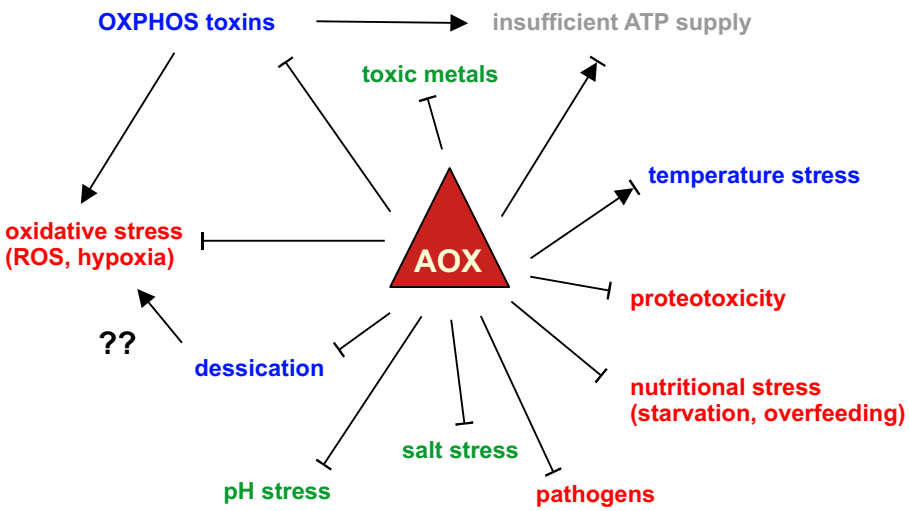

Fig. 3. Stresses to which AOX may confer resistance. Stresses shown in blue are supported by data on AOX-endowed invertebrates, those shown in red only by data on AOX-expressing model organisms, and those shown in green are based purely on analogy with plants and/or fungi. Those based on hypothetical criteria alone are shown in grey. Barred arrows indicate that AOX may also sensitize as well as alleviate the given type of stress, as discussed in the text. Note that almost all implied effects of AOX in animals remain conjectural.

line with such predictions. AOX expression was unable to rescue the phenotype of the *Drosophila tko*^{25t} mutant [24], affecting the capacity of mitochondrial protein synthesis and leading to decreased activity of all of the OXPHOS complexes containing mitochondrially synthesized polypeptides. However, in a case where ATP supply was insufficient due specifically to a limitation on electron flow at cIII and/or cIV, AOX should be able partially to alleviate the effects, by facilitating additional electron flow through cI.

In addition to cases of respiratory poisons already considered, some other specific conditions may lead to ATP deficiency, in which AOX could act as a modifier. These would include any stress leading to an excess expenditure of ATP, such as a need for clearance of toxins that do not specifically impair OXPHOS, resource depletion or predator stress that dictate excessive foraging or escape behaviour, injury, and disease- or diet-related tissue damage necessitating rapid or prolonged regeneration. Diverse mineral or vitamin deficiencies that impinge on catabolic pathways, including a deficiency of ubiquinone (coenzyme Q), would impair ATP supply, but may also be alleviated (or exacerbated) by AOX, depending on the precise context. AOX may also, in some cases, have two opposing effects, for example, decreasing oxidative stress (as considered in the next section) but also compromising ATP supply, with different and complex consequences for short- and long-term survival, different tissues or developmental stages.

The influence of AOX on ATP deficiency, whether due to toxins or other causes, is predictable, although precise mechanisms remain to be fully explored. This presents as an enticing opportunity to gain basic information on a remarkable genetic system. In the next section, we consider oxygen stress. Mitochondria, the cell's major consumers of oxygen, play a major role in sensing changes in oxygen levels and adapting cellular metabolism accordingly.

5. Oxygen stress

The term ‘oxygen stress’ here covers two related but distinct phenomena. First is the metabolic stress imposed on aerobic organisms by an insufficient level of oxygen (hypoxia) or by its complete absence (anoxia). The second phenomenon is the production of highly reactive free radicals usually associated with the initial transfer of unpaired electrons to molecular oxygen to generate the superoxide anion $O2^{-\bullet}$, from which other reactive oxygen species (ROS) are derived. ROS production in mitochondria may also be exacerbated by conditions of hyperoxia or by the presence of metal cofactors, notably Fe^{2+}. ROS in mitochondria are generally produced via side-reactions of the respiratory chain complexes I, II and III, when normal electron flow is interrupted and usually short-lived intermediates with unpaired electrons start to accumulate. This may occur as a by-product of toxic inhibition of any of the respiratory chain complexes, including cV, by system overload, i.e. too high a concentration of NADH or other substrates for dehydrogenases feeding electrons to cIII, or by too low a rate of utilization of ATP, restricting electron flow. The generally accepted view is that low levels of ROS act as signalling molecules, leading to a corrective response, whilst excessive ROS production can damage proteins, nucleic acids and lipids which may overwhelm the repair capacity of the cell and is postulated as a major cause of organismal aging [25].

In the alpha-proteobacteria, the ancestral lineage of mitochondria, AOXs have been claimed to have a higher Km (lower affinity) for oxygen than do the heme-linked oxidases [26], to which mitochondrial cIV is related. However, there is no reliable literature supporting this assertion as regards AOX in eukaryotes. The *Ciona* enzyme, when expressed in the mouse, carried a similar proportion of electron flow when exposed to widely differing oxygen levels [7]. However, in *Ciona* adults, AOX mRNA was induced by hypoxia in the same tissues as by sulphide [19], and the two effects were additive, and thus likely to be mechanistically separate. Hypoxia also induced AOX in several bivalve species [20,27,28], including the Pacific oyster *Crassostrea gigas* [28], where it was again tissue specific, being greatest in gills and digestive gland.

Since AOX is an oxygen-dependent enzyme, these findings seem paradoxical. However, they can be reconciled if AOX is part of a wider metabolic programme under hypoxic conditions, in which ATP is principally supplied by glycolysis, whilst the alternative respiratory system enables the TCA cycle and the rest of intermediary metabolism to remain engaged. Another possible explanation is suggested by the relative simplicity of AOX and its biosynthesis, compared with cIII and cIV. The latter requires a multi-step process dependent on two genomes in separate compartments and an intricate post-translational assembly programme involving dozens of polypeptides. Increased synthesis of a single polypeptide would seem a more appropriate and rapid response to a sudden or transient drop in oxygen levels, so as to maintain redox homeostasis and metabolism. This is reflected in the concomitant decrease in transcript levels of OXPHOS system genes during hypoxia in the freshwater bivalve *Diplodon chilensis* [27].

In model organisms subjected to respiratory chain inhibition, or in cells with pathological mutations affecting OXPHOS [29], the expression of AOX dampens ROS production, which may theoretically take place at cI, II or III, depending on the precise site and nature of OXPHOS inhibition or limitation. Whilst it cannot be excluded that AOX might have an accessory role in ROS metabolism, the *Ciona* enzyme at least appears not to use peroxide as a substrate, and the consensus is that it acts by alleviating the accumulation of reduced electron carriers upstream of the blocked site, in particular preventing reverse electron

transport (RET) through cI, which is potentially a major source of ROS [30,31]. Since these processes are covered extensively in other reviews in this collection, we confine our remarks here to concrete examples of a role of AOX in ROS mitigation in animals naturally endowed with the enzyme. Unfortunately, there are very few such examples.

In the oyster *Crassostrea virginica*, one of two splice-isoforms of AOX, each encoding variant polypeptides, was specifically induced under the stress of prolonged air exposure [32], although the exact nature of the inducing stimulus is unclear. In another oyster species, *C. gigas*, respirometry using inhibitors showed that AOX took a greatly increased proportion of the electron flow to oxygen (~40% instead of ~10%) 1 h after re-oxygenation, following a 12 h period of hypoxia [28], during which this parameter did not change. This may reflect a mechanism to minimize the burst of ROS production that is associated with re-oxygenation [33], akin to protective mechanisms in the mammalian heart during ischemia-reperfusion. AOX has thus been proposed to play a major role in resistance to oxidative stress in marine invertebrates [34].

These 'tip of the AOX iceberg' studies illustrate the complex mechanism by which AOX responds to oxygen stress. Temperature stress often works in parallel to oxygen stress and we consider the influence of AOX in responding to or mitigating it, in the following section. Arguably, temperature stress is one of the greater organismal stressors that impacts the ability to maintain normal function in today's ecosystems and those of the near future. This is particularly true of organisms with limited vagility, but also affects the distribution of highly mobile species and their resilience to environmental fluctuations.

6. Temperature stress

The involvement of AOX in enabling organisms to respond to temperature stress is strongly suggested by the fact that the enzyme is by its nature thermogenic, as seen vividly in plants [35], and by the recognition that mitochondria, at least in mammals, are already operating at a temperature well above that of the cellular environment [36]. Although the 10–12 °C temperature excess of mitochondria reported in mammalian cells has not been explored in AOX-bearing invertebrates, the fact that the structure, composition and biochemistry of mitochondria are so highly conserved across the animal kingdom and beyond implies that mitochondria in so-called ectothermic organisms are also likely to operate at elevated temperatures. Moreover, since AOX constitutes a simple switching mechanism to alter the balance of heat and ATP production, it is likely that it acts to preserve both, by mitigating changes in mitochondrial temperature and possibly external temperature as well, under varying environmental conditions. However, concrete evidence that it does so is still lacking.

There is an extensive literature on invertebrate strategies for handling extreme cold, e.g., see [37], but little attention has been paid to possible thermogenic mechanisms, perhaps driven by the description of such animals as 'ectotherms', ignoring the fact that biological reactions generate heat. However, estimates of the thermodynamic efficiency of 'fully coupled' mitochondria indicate that up to half of all the input energy is released as heat, rather than being converted to ATP [38,39].

Several examples present tentative evidence indicating possible regulation of intracellular heat production in AOX-bearing animals. One striking observation is the reported rate of protein synthesis after fertilization in the Antarctic sea urchin *Sterechinus neumayerii* [40], which lives in a fairly constant temperature environment of around −1 °C. As is almost universal in animal embryos, fertilization is followed by the rapid activation of protein synthesis using stored maternal mRNAs, to serve the needs of embryogenesis and convert the relatively defenceless sessile egg into a swimming blastula able to escape predation and locate food sources. The protein synthesis rate in *S. neumayerii* after fertilization is comparable with that seen in temperate species [40], raising the interesting possibility that thermogenesis is involved, though not necessarily via AOX.

Other mechanisms of thermogenesis have been documented in invertebrates, such as via ATP hydrolysis in smooth muscles of the sea cucumber *Ludwigothurea grisea* [41]. Many studies have reported that diverse invertebrates and fish species living in polar waters exhibit increased mitochondrial density [42], which has generally been interpreted as an adaptation to maintain activity by providing additional ATP to compensate for low temperature. However, the possibility that this also serves to raise intracellular temperature has not been widely considered nor has this been measured.

Temperature stress, hypoxia and ROS production are intimately linked in the marine environment, reflecting the fact that the oxygen concentration in sea water falls markedly as temperature rises [34]. Thermal stress also leads to a large increase in mitochondrial ROS production [43]. It has been suggested that the activation of the AOX pathway at high oxygen levels (e.g. in cold waters) represents a mechanism to remove oxygen, thus exerting an additional brake on ROS production [34]. However, this rests on the unsubstantiated idea that metazoan AOX has a low oxygen affinity. Nevertheless, if AOX is stimulated directly by low temperature, it should have such an effect in cold water that was overly replete with oxygen. Moreover, since AOX is by its nature thermogenic, with the energy that would otherwise have been conserved by proton-pumping at cIII and cIV instead released as heat, any resulting local warming would decrease the concentration of dissolved oxygen and mitigate ROS production. Thus, a key question to ask is whether AOX activity (or its expression) in metazoans shows temperature dependence. Once again, the literature is scant.

A study conducted in the copepod *Tigriopus californicus* found that the protein(s) detected by Western blotting against an antibody to a plant AOX was elevated in animals exposed for 24 h either to low (6 °C) or high temperature (28 °C), compared to the control condition of 15 °C [5]. However, since the full-length AOX polypeptide from this species has not been characterized, the result should be interpreted cautiously. In contrast, *Ciona* adults subjected to a temperature up- or down-shift to 8 or 25 °C from the control condition of 18 °C showed only minimal and non-significant differences in AOX expression at the RNA level. However, the temperature profile of activity of the enzyme is unknown, so any thermoregulation thereof remains to be investigated. When expressed in *Drosophila*, *Ciona* AOX accelerated recovery from cold-induced coma and increased the rate of larval development in the cold [18]. Next, we consider starvation and metabolic overload. Electrons can enter the mitochondrial electron transport system at multiple points along the chain, upon which AOX should have distinct effects.

7. Starvation and metabolic overload

Because AOX tilts the balance between mitochondrial heat and ATP production, and does so in a way that depends on the exact metabolic fuels being used as substrate, it should have an important role in how animals respond to nutritional stresses. Different responses are appropriate if nutrition is insufficient, is over-abundant or over-consumed, or if large fluctuations occur in the availability or type of nutritional resources available. Few organisms are limited to a single dietary source, and even obligate carnivores or herbivores show a degree of metabolic flexibility that allows the exact balance of different fuels – carbohydrates, fats and sometimes other biomolecules – to vary without significant harm or consequences. As with the stresses already considered, there is almost no relevant scientific data regarding AOX to build upon, even from model organisms reared in artificial laboratory environments. Thus, virtually all considerations are, for now, purely hypothetical.

For rapid growth, e.g. in yeast, cancer cells or the *Drosophila* larva, glycolysis is the predominant pathway used for ATP production. This is traditionally explained on the basis of its higher kinetic capacity and because it spares carbon skeletons for use in biosynthesis. However, the respiratory chain remains essential to enable the TCA cycle, which supplies or processes many of those carbon skeletons. In contrast, only low level biosynthesis is needed to sustain a fully differentiated tissue, whilst a plentiful energy supply is needed for physiological function. In

such tissues, for example, the nervous system or muscle, OXPHOS is the major source of energy, and most carbon skeletons are combusted completely.

If AOX is available it should facilitate the burning of excess substrates, particularly those metabolized via flavin-linked dehydrogenases that directly reduce ubiquinone. This may apply particularly to lipids, whether consumed in the diet or arising *de novo* from excess consumption of carbohydrates. Thus, AOX might be predicted to alleviate some of the effects of fat-rich diets or ad libitum feeding in general. Importantly, in mouse cells, *Ciona* AOX only becomes engaged on cI-linked substrates in the presence of inhibitors of cIII or cIV [44], whereas succinate-driven, non-phosphorylating respiration is supported by AOX even if there is no inhibition of cI [44]. This indicates that the effect of AOX is, indeed, highly dependent on the available substrates, and implies that it may enable metabolic adaptation in order to maintain redox homeostasis and normal growth under conditions of intermittent or excess feeding or fluctuations in the balance of available nutrients.

Despite these expectations, our own studies with AOX-expressing mice reared on fat-rich diets were inconclusive: effects were quantitatively minor and potentially compromised by behavioural issues [45]. In *Drosophila*, we observed that AOX-expressing males reared on standard diet showed a more pronounced weight loss as young adults than did wild-type flies [6], consistent with the idea that they were burning up more fuel to produce the same amount of ATP, and leaving fewer carbon skeletons remaining for tissue maintenance. However, the amount of food consumed was not restricted or measured in these experiments and metabolite levels were not determined, so it is not possible to distinguish whether AOX flies were starving or were actually healthier than wild-type. AOX expression did impair development in nutritionally limited flies [18]. Clearly a more wide-ranging and rigorously controlled study is needed to understand properly whether and how AOX-endowed animals are able to withstand nutritional stresses.

Next, we consider proteotoxic stress. Because of its thermogenic properties, AOX may influence both the formation and clearance of different types of protein aggregates.

8. Proteotoxic stress

The recognition that protein aggregates and the failure to clear and degrade them are frequently seen in cases of neurodegenerative disease has led to a great deal of attention being devoted to understanding how they arise, how they are removed, and how these process might be slowed or reversed by different kind of intervention. Many different kinds of protein aggregate have been studied in this regard, notably those that tend to form amyloid fibrils, as well as expanded polyglutamine repeats, and proteins that are able to self-catalyse their conversion and polymerization into a toxic, misfolded state (prions). Protein chaperones play a major role in preventing amyloid and aggregate formation or resolving such products once formed, placing a large additional burden on cellular protein synthesis, which already consumes the greater part of the cell's ATP supply [46]. To bring about disaggregation of target proteins, many chaperones require additional ATP, whilst resolving protein aggregates in the endoplasmic reticulum also consumes NADPH, potentially disturbing cellular redox homeostasis. A potential role for AOX in these processes is suggested by the observation that its expression in human cells limits the accumulation of the Alzheimer's disease-linked beta-amyloid (Aβ) peptide when respiration is inhibited [47] and also alleviates the severe phenotypic consequences of expression of the most pathogenic human Aβ variant when expressed in neurons in *Drosophila* [47].

The underlying mechanisms remain unknown. One possibility is that amyloid formation is counteracted by the additional heat generated when AOX is activated. This might be especially important in cases where the first steps in misfolding or amyloid formation occur inside mitochondria, as has been proposed elsewhere for the Aβ peptide [48]. Another potential mechanism by which AOX might limit proteotoxicity is by preventing excess ROS production, which has been postulated as a key contributor to deranged protein folding [49]. Prions, amyloids and protein aggregates are not simply a feature of the mammalian nervous system, but are widespread in biology [50], raising the intriguing possibility that mitochondrial thermoregulation and/or ROS attentuation involving AOX might contribute to fitness in many contexts where proteotoxicity is a threat.

Hard data on the role of AOX in other biological processes in animals remains very limited. Nevertheless, they provide a tantalising window into the wider physiological roles of mitochondria, some of which may be lineage- or tissue-specific. One such area concerns the role of mitochondria in immunity, to which we now turn. In an era plagued by emerging infectious diseases understanding such processes assumes great importance.

9. Pathogen resistance

The ability of AOX to attenuate inflammatory processes when expressed in the mouse [51] and its known role in promoting antiviral immunity in plants [52] raises the question of whether it could play roles in pathogen resistance and immune modulation in those animals where it is naturally present. The precise mechanisms by which AOX impinges on immune processes in the two cases cited remain only partly understood, and the underlying evolutionary rationale is far from obvious. One hypothesis is that the inflammatory response involves the inhibition of OXPHOS [53], depriving pathogens of host-derived ATP. This recalls the response of plants to diverse pathogens (and herbivores), which elicit a response that involves the release of the stress hormone salicylic acid. This agent has also been reported to act as an inhibitor of OXPHOS [54] and to promote the concomitant induction of AOX, which is immune to the inhibitor. Whilst salicylic acid is not produced by animals, and AOX is absent in mammals, the pathways of innate immunity are common to all metazoans. So the possibility that AOX provides a way for animals that possess it to maintain mitochondrial redox homeostasis whilst depriving pathogens of energy and metabolites clearly deserves to be explored experimentally.

In mammals, mitochondrial uncoupling by members of the mitochondrial uncoupler protein family might perform an analogous function, manifesting as the near universal phenomenon of fever. Traditionally fever has been seen as a mechanism to raise body temperature so as to limit pathogen growth, but this flies in the face of overwhelming evidence that bacteria are far less susceptible to the effects of minor changes of temperature than are mammals. On the other hand, fever resulting from greatly increased mitochondrial heat production limited to the site of an infection makes much more sense. If this is so, one prediction would be that AOX-endowed animals should also experience fever, as well as mammals and insects [55]. Almost all such studies on invertebrates thus far have been conducted on specific arthropods [56], where the presence or absence of AOX in the genome is yet unknown.

The major antiviral pathways in mammals are the intracellular activation of the protein kinase EIF2AK2 (PKR) by dsRNA, which arrests cytosolic translation, lowers the threshold for apoptosis, and promotes the secretion of interferons, hormones that pre-activate EIF2AK2 and other antiviral defences in adjacent or distant cells. Although any relevance to mitochondria is uncertain, a prolonged interruption of protein synthesis will impair the assembly of new OXPHOS complexes and likely lead to proteotoxic stress within the organelle itself. Activation of AOX, and its metabolic co-operation with NDX and with flavin-linked dehydrogenases that feed electrons directly to ubiquinone, should assist such cells to maintain redox homeostasis under such circumstances.

The effects of PKR inside mitochondria remains to be elucidated in detail, but mitochondrially derived dsRNAs are a major class of effectors that interact with PKR, and a substantial fraction of PKR is found within mitochondria, where it is responsive to changes in mitochondrial transcription [57] even in uninfected cells. Although these findings do not

implicate AOX, they do indicate a link between an antiviral immunity and mitochondrial metabolism.

Another potential link between mitochondria and immunity is the pathogenicity of some intracellular bacteria. The immune system has to distinguish such intracellular parasites from the cell's own mitochondria. In theory, a backup system to preserve mitochondrial metabolism, redox homeostasis and heat output in the presence of an OXPHOS inhibitor would seem an attractive mechanism, but there is no data supporting this.

Part of the problem in elucidating the role of AOX in any of these processes is the fact that the immune systems of AOX-endowed invertebrates has not been fully characterized and may exhibit unique, taxon-specific features e.g. see [58].

10. Other stresses

Drought resistance is another property conferred by AOX in plants, although its molecular basis is not fully understood [59]. Surprisingly, given their very different physiology and mode of development, animals may also be protected from drought and desiccation by AOX, based on a recent study in a model tardigrade, using the AOX inhibitor BHAM [60]. Specifically, BHAM was shown to inhibit survival during dehydration and/or the desiccated tun stage but not during rehydration. The hypothesis that the protective effect of AOX is due to its antioxidant-like properties, via maintenance of electron flow from ubiquinol to oxygen, was not fully supported by parallel studies using the mitochondrial ROS scavenger mito-TEMPO, suggesting that AOX protects against desiccation by a different or more complex mechanism [60]. AOX also promotes resistance to salt stress in plants by a ROS-linked mechanism [61]. A possible role of AOX in animal survival in hypersaline environments has not been tested. Since hypersaline marine environments may also manifest temperature and oxygen anomalies, any specific role of AOX under salt stress will need to be investigated carefully under laboratory conditions. The most studied model invertebrate with regard to salt resistance is the brine shrimp *Artemia salina*, the genome of which does not have an identified homologue of AOX. At least this indicates that AOX is not *required* for the ability of animals to withstand a hypersaline environment, but does not exclude it from having such a role.

AOX mitigates several other types of stress experienced by plants [62], with attenuation of ROS production as a common mechanism. Some, such as high light exposure [63] have no obvious counterpart in animals, whereas others such as phosphorus deficiency, pH stress or exposure to toxic metals such as aluminium or cadmium, might conceivably do so. Studies on *Ciona*, however, did not reveal AOX induction in response to heavy metals or mild acidification [18]. Polar echinoids, which should be amongst the most vulnerable animals to ocean acidification consequent upon changing atmospheric CO2 levels, appear to be largely resistant to this stress [64], although it is not known if AOX plays any role in this.

Integrating research to distil underlying biochemical and cellular principles by which AOX influences responses to environmental stress remains a formidable challenge worthy of focused attention. Such studies must aim also to spotlight why AOX appears to have been independently lost in three crown groups. Occam's razor posits that a single explanation should be considered the most likely, but we should not discount the possibility of two or three independent mechanisms underpinning AOX loss. Accordingly, we now consider the spectrum of available evidence indicating the potential evolutionary pressures for AOX loss.

11. Evolutionary pressure for AOX loss

It is by definition impossible to verify a rationale for rare events in evolution, especially those occurring in ancient environments of which we know little. However, in the case of AOX we can at least infer possible disadvantages of its retention, based on studies in model organisms. Such disadvantages might have played a part in the evolutionary loss of AOX in specific crown groups, although it is important to note that laboratory studies are mostly conducted under conditions that may be very far from those ever experienced in nature. However, before evaluating these studies, it is worth considering two relevant scenarios.

The most obvious character that distinguishes vertebrates, cephalopods and insects from other animals is that they each use muscle power, albeit in slightly different ways, to enable them to move fast. This has two relevant consequences. The first is that, unlike all the other animals that have retained AOX, and which are sessile (e.g. adult tunicates), pelagic (e.g. krill) or slow-moving (e.g. adult echinoderms), they are able to deal with a stressful environment simply by switching rapidly to a different one. Thus, *Ciona*, has to detoxify a harmful accumulation of sulphide to survive, but fish can just swim away to healthier waters. Although this doesn't provide a pressure to lose AOX, it removes or minimizes any pressure to retain it, and its genetic decay by the accumulation of inactivating mutations would be expected over evolutionary time. Although, for reasons stated, this argument is hard to prove (or disprove), there are some possible counter-examples, such as cephalochordates like the sandworm *Branchiostoma*, that do possess AOX (NCBI protein database accessions XP_035700253 and XP_019614441). These primitive chordates are benthic, sand-dwelling filter feeders like *Ciona*, but are able to move to new locations if disturbed. Apart from this case, all of the animals known to have retained AOX live in the marine environment, although so do cephalopods and even some vertebrates, so this alone seems insufficient as an explanation for the selective retention or loss of AOX.

A similar, though even stronger argument, comes from the observation that almost all of the fast moving animals that have lost AOX use movement not merely to avoid unhealthful environments, but to access sources of food or escape from being a source of food themselves. This raises the crucial question of whether AOX impairs the speed or agility with which animals can catch prey or avoid being caught. It appears to be a fundamental property of AOX that it only contributes substantially to electron flow when the relative reduction state of the quinine pool reaches a sufficient threshold, due to its much higher Km for ubiquinol than that of cIII. However, the contribution of AOX to electron flow at lower concentrations of the reduced carrier, e. g., in the absence of any inhibitor, though minor, is not zero [44]. Thus, it could conceivably impact the net output of ATP, affecting survival fitness in critical predator-prey situations where ATP output needs to be maximized. More importantly, this process requires full mobilization of catabolic pathways that should alone lead to a relative accumulation of primary and secondary electron carriers in the reduced form. This means that an animal endowed with AOX may reach a critical threshold where increased primary catabolism of glucose and other substrates leads to the partial engagement of AOX and thus to a drop in ATP production under conditions where it needs to be at its highest to ensure survival. However, appearing to contradict this assertion, AOX-expressing mice performed as well on standard treadmill and grip-strength assays as controls [8] Perhaps a more rigorous endurance or performance assay might yet reveal a difference indicating a survival disadvantage of AOX under 'fight-or-flight' conditions. For now, the idea remains speculative.

A further possibility is that AOX is partially redundant, both for the relief of metabolic blockade and for themogenesis, with the uncoupler protein (UCP) family. UCP is part of the wider mitochondrial carrier superfamily, whose dozens of members are involved in the transport of ions, substrates, nucleotides and other moieties into and out of mitochondria. UCP1 itself, the eponymous uncoupler protein with an experimentally verified role in thermogenesis, is found only in vertebrates [65], and the physiological role(s) of other members of the UCP family is not clear. Although potentially thermogenic, they are believed to play other physiological functions including the relief of abnormally high mitochondrial membrane potential, the facilitation of calcium transport, protection against ROS and adenine nucleotide homeostasis [65].

The expansion and diversification of the UCP gene family amongst metazoans [66] may have provided an alternative 'relief valve' for handling elevated membrane potential arising from respiratory dysfunction. On the other hand, an opposite argument could also be made, that AOX and NDX provide greater flexibility in responding to metabolic overload or inhibition by virtue of being a two-component system, correcting defects limited to cI (by NDX) or cIII/IV (by AOX). Importantly, the conditions under which UCPs and AOX provide protection are not identical. UCP action can adjust membrane potential anomalies that are not caused by respiratory impairment, even in cells that are not respiring. Conversely, UCP permits electron flow that would otherwise be restrained by high membrane potential, but cannot restore such flow if it is impaired by physicochemical or mutational damage to the respiratory enzyme complexes. Thus, AOX retention by species that also possess UCPs, such as echinoderms [67], is not unexpected, and the replacement of AOX by UCP family members for thermoregulation is not strongly supported by existing data.

One may also ask whether those species that have retained AOX have a common dietary source (e.g. toxic micro-organisms) that might explain a continued need for the enzyme. However, considering both echinoderms that have retained AOX and vertebrates that haven't, each group includes filter-feeders subsisting on micro-algae, vegetarians that eat kelp, and carnivores that even consume other echinoderms or vertebrates. A dietary explanation for AOX retention or loss thus seems very unlikely.

Model organism studies do, however, reveal some disadvantages of AOX expression that could lead to explanations of its loss over evolutionary time. However, it should be born in mind that these studies have been conducted in the highly artificial context of driving the expression of a foreign transgene to high levels in taxa that have not experienced an endogenous copy for up to a billion years. Adaptation to such expression would be expected to mitigate harmful effects during the course of subsequent evolution, so conclusions regarding the fitness costs of AOX may be exaggerated or invalid. Two clear examples are probably the negative effects of AOX expression in *Drosophila* on spermatogenesis, reflected in male reproductive success [68] and on the completion of development under conditions of nutrient limitation [18,69,70].

AOX-expressing *Drosophila* males produce much less mature sperm than controls, and spermatogenesis is spatially disorganized [68]. The expression system that was used in the study drives high-level transgene expression in the pigmented cell layer of the sperm sheath, rather than in the smooth muscle of the testis or in the germline itself. Sperm production in mammals and birds has long been noted to occur in body compartments maintained at lower temperature than the rest of the body, leading to the suggestion that AOX expression in the fly testis might disturb sperm production thermogenically. This would imply that even in a poikilotherm such as *Drosophila*, excessive mitochondrial heat production is somehow damaging to spermatogenesis, and is limited in wild-type flies by inhibition of electron flow through cIII and or cIV, which AOX expression restores. However, one can imagine diverse ways in which this issue could be adaptively overcome without the need to delete AOX altogether. Importantly, in another study using flies expressing AOX under the control of the *da*GAL4 driver, which does not derange sperm production or competitiveness [68], females mated to AOX males produced more offspring than with control males, and AOX females further enhanced the effect [71], which has been suggested to be due to the decreased ROS production in AOX-expressing flies. This expression system also had no impact on sperm competition.

The nutritional limitation on development is highly temperature sensitive [18], suggesting a possible role there also for thermogenesis. Under any condition in which AOX is enzymatically activated, even partially, the balance between heat and ATP production is disturbed. Moreover, to produce the same amount of ATP more nutrients must be fully consumed, producing more heat but also restricting the availability of carbon skeletons for biosynthesis, unless more nutrients can be mobilized. Recent findings [70] indicate, in agreement with these assumptions, that AOX-expressing larvae are effectively running low on exogenous nutrients, notably specific amino acids, and mount a response that is indicative of a starvation condition. This might mean that AOX is detrimental under some specific environmental conditions, especially if nutrient limitation is experienced, even periodically or only during specific developmental stages.

Studies with AOX-expressing mice have also yielded some unexpected findings, suggesting that AOX expression abrogates some important signalling processes of physiological importance. In both a surgical model of cardiac ischemia-reperfusion [72] and in a genetic model of mitochondrial skeletal myopathy [73], AOX expression disrupted intra- and/or inter-cellular signals of mitochondrial respiratory dysfunction, in both cases seemingly connected with the production of ROS. At the cellular level this resulted in a failure of repair mechanisms that ultimately led to an exacerbation of tissue damage and functional deficit. A similar mechanism may underlie the phenotypic worsening produced in a mouse model of inflammatory cardiomyopathy by cardiomyocyte-specific expression of AOX [74]. Physiological inhibition of cIV also seems to be linked to acute hypoxia signalling in the vasculature of the lung, based on its abolition by AOX [75], whilst the inflammatory crisis resulting from the presence of excessive amounts of lipopolysaccharide in the bloodstream, which mimics the events occurring in sepsis, is actually normalized by AOX expression [51]. The effect of AOX has also been attributed to the abrogation of signalling arising from respiratory chain inhibition, manifesting as increased ROS due to RET.

The derangements of signalling implied by these observations have thus far been demonstrated only in mammals. Thus, the underlying signalling processes may have evolved after the loss of AOX rather than before. The absence of AOX thus may have allowed them to arise, despite not being the initial reason for AOX gene loss. Equivalent phenomena have not been exhaustively searched for in insects or cephalopods, so it will be interesting to see if any have arisen independently or perhaps ancestrally. Certainly, the ability to use ROS or metabolite accumulation due to respiratory chain inhibition as a physiological signal would have been far more limited in a world where AOX remained universal.

12. Future perspectives

The availability of transgenic lines of *Drosophila* expressing *Ciona* AOX in widespread but distinct expression patterns offers a convenient way to test some of these predictions in a model organism. As indicated, the two main AOX-expressing lines – driven, respectively, by the α-tubulin promoter or under the control of the 'ubiquitous' *da*GAL4 driver – have opposite effects on reproductive fitness. The transgenic line that enhanced fecundity is also the one that manifested temperature-dependent developmental failure on nutrient-poor media, making it possible to evaluate how these two simple environmental factors influence the fitness effects of AOX in a laboratory setting. *Drosophila* is an ideal model organism in which to conduct such studies, e.g. see [76], because of its short generation time, ease of culture and cosmopolitan diet. These have enabled population level studies (Fig. 4) to examine the long-term effects of different diets on genotype frequencies, including not only rich versus poor diets, but those with different ratios of protein and carbohydrates, or based around natural fruits with varying content of different nutrients. Such an approach should make it possible not only to test the specific conditions under which AOX expression is harmful or beneficial, but also whether and how it contributes to metabolic flexibility and resistance to thermal stress, under conditions where the nutritional and temperature environment is varied in specific ways, in population cages.

An important additional dimension to such a study is afforded by the ease with which genetically different mtDNAs may be introgressed into any given nuclear background, exerting different effects on physiology and development, once again with distinct temperature and diet-

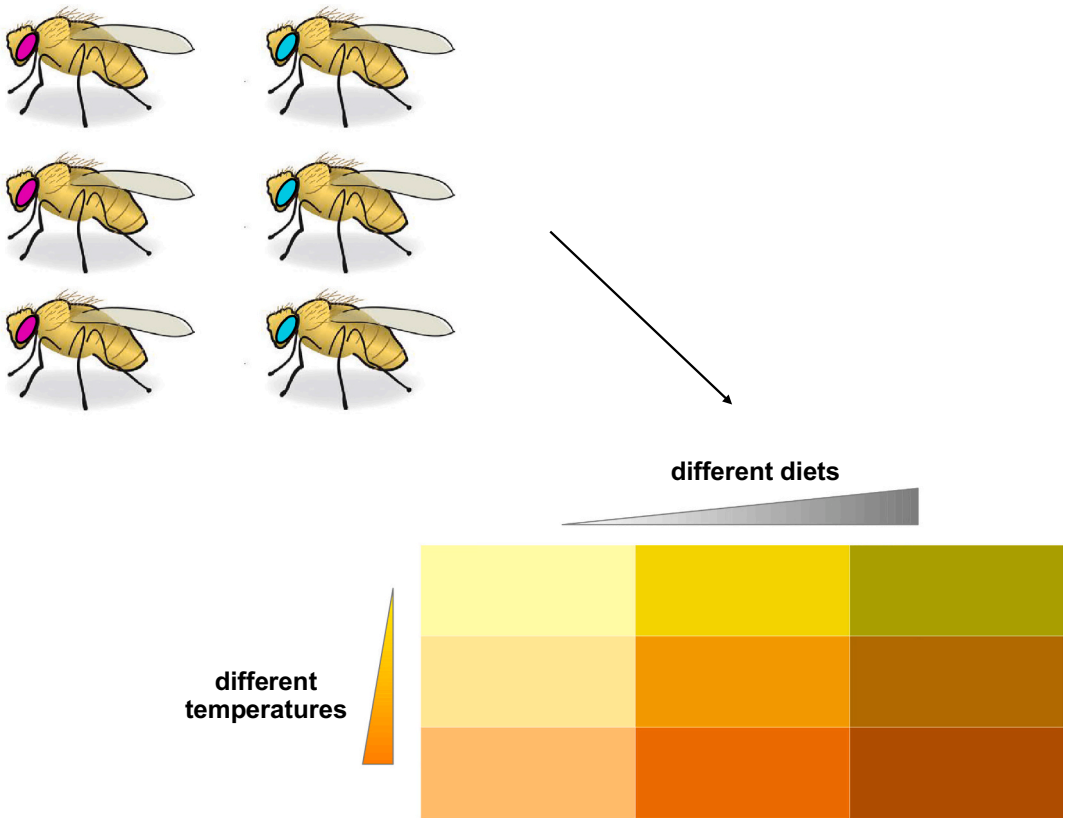

Fig. 4. Testing the effect of AOX on fitness in *Drosophila*.
In a population-cage experiment, flies of two or more genotypes, shown schematically by (non-physiological) cyan and magenta eye-colour, are co-cultured in bulk over many generations in cages containing media of different composition and at different temperatures, as indicated. Genotype frequencies are tracked by PCR at regular intervals. Note that neither eye colour nor any other external marker is used in such an experiment, and is shown here only for illustrative purposes. In such an experiment, the selective value of AOX under the different conditions tested would be revealed by the direction and rate of change of genotype (i.e. positive or negative for AOX) at the population level over successive generations.

dependence [77,78]. For example, the mtDNA of strain KSA2, collected from nature, contains a polymorphism in the cytochrome *b* gene that appears to be phenotypically silent in a wild-type nuclear background, but synthetically lethal with the tko^{25t} nuclear mutation [79]. The KSA2 mtDNA polymorphism affects cIII activity in the tko^{25t} nuclear background, where it also gives rise to an increased hemocyte count and to melanotic nodules in the larval stages [78], both considered a signature of the pre-priming of innate immunity by oxidative stress. We speculate that, by limiting electron flow through cIII, the KSA2 polymorphism may increase ROS production at cIII and/or at cI via RET, and that the resulting pre-priming of innate immunity confers a survival advantage. We would predict that constitutive AOX expression should mitigate or even nullify such effects in nature, though may still confer advantages in a controlled laboratory environment. Similarly, two other wild-type strains (Dahomey and Alstonville), differing by a polymorphism in a cI subunit, showed opposite competitive advantages on different laboratory diets or on diets including different natural fruits [80]. These outcomes were partly determined by differences in developmental time and partly by fecundity. AOX may disturb or accentuate these traits, based on its own effects on both of these parameters, as well as on sperm fitness, ROS production and metabolism. Conducting such experiments may lead to a clearer definition of the genetic and environmental conditions under which AOX expression impacts fitness.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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