

Contents lists available at ScienceDirect

Current Research in Insect Science



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

# Antioxidants, oxidative stress and reactive oxygen species in insects exposed to heat

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ARTICLE INFO

Keywords: Heat tolerance Oxidative stress Reactive oxygen species Thermal stress

# ABSTRACT

In response to high temperatures, insect metabolic rates increase, favoring the release of higher amounts of reactive oxygen species (ROS). These ROS need to be counteracted by antioxidants to avoid oxidative stress, which can lead to cell damage and death. In this manuscript, we review evidence in insects showing the effects of high temperatures on ROS production, the antioxidant defenses reported in insects in response to high and extremely high temperatures and the extent to which they contribute to preventing oxidative damage. Endogenously produced antioxidants can be enzymatic or non-enzymatic and are involved in heat responses in at least seven insect orders. Our review indicates that evidence is very limited for the effect of high temperature on ROS production, but it clearly shows that at least one antioxidant is upregulated during short-term heat exposure. However, the effects of antioxidants in effectively reducing oxidative damage in biomolecules are still poorly supported by evidence. Dietary-dependent antioxidants show strong potential for coping with heat stress, but evidence is limited, although numerous plants produce antioxidant compounds and a great number of insect species feed on plants. The role of antioxidants in heat acclimation and adaptation is promising but evidence is still very limited in insects. Antioxidants also protect from other prooxidant conditions such as pesticide exposure, nutrient stress, or new biotic interactions, which often act in combination. Potential trade-offs between antioxidant use to different functions could define insect survival and pace of life in response to multiple stressors, including high temperatures. Our literature review indicates that there is only limited evidence of the role of antioxidants in preventing oxidative damage caused by heat, opening the possibility that ROS production might be mitigated by the action of uncoupling proteins or degradation of mitochondria. Finally, we conclude by proposing promising research avenues to gain a deeper understanding of the role of ROS and antioxidants in the oxidative balance of insects exposed to mild and extreme heat.

#### 1. Introduction

Climate warming is one of the main causes of insect declines worldwide, with negative consequences for human wellbeing and ecosystem functioning (Halsch et al., 2021). The underlying negative effects of high temperatures on insects are the associated physiological challenges that impair functional homeostasis, increasing mortality and compromising reproductive performance (Hoffmann, 1984; Neven, 2000). As a first line of defense, behavioral avoidance of stressfully high environmental temperatures is observed in highly sensitive insects and prevents the need for using costly physiological responses (Bai et al., 2023). However, limited availability of cool microhabitats or refuges frequently challenges the insect physiological machinery. In ectothermic aerobic organisms, such as insects, high ambient temperatures stimulate respiration and increase metabolic rate (Chown et al., 2002), consequently increasing the cellular demand for adenosine triphosphate (ATP) in mitochondria (Ali et al., 2017; Jena et al., 2013; Sokolova, 2023). Despite respiration being an essential process, the use of molecular oxygen (O<sub>2</sub>) by aerobic organisms inexorably results in the formation of Reactive Oxygen Species (ROS). ROS normally play an important role in cell functioning and signaling, regulating fundamental processes in insects, including immune responses, symbioses, dormancy

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https://doi.org/10.1016/j.cris.2025.100114

Received 30 January 2024; Received in revised form 9 May 2025; Accepted 20 May 2025 Available online 22 May 2025

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Current Research in Insect Science 7 (2025) 100114

and arousal, by changing the activity of specific enzymes (Zug and Hammerstein, 2015; Chen et al., 2021). However, due to their chemical instability, when ROS are overproduced, they can react with biomolecules (i.e., lipids, proteins or nucleic acids), damaging them and threatening their functionality (Matsumura et al., 2017; Liang et al., 2023) (Fig. 1).

In response to the increased ROS production, insects can make use of antioxidant defenses via increased production of endogenous antioxidants or higher ingestion of dietary ones (Ahmad, 1992; Felton and Summers, 1995; Ju et al., 2014; Halliwell, 1999, 2011; Abele et al., 2002; Slocinska et al., 2016). However, antioxidants are unlikely to protect the organism over prolonged timeframes or under severe heat conditions, thus the main action of antioxidants is probably delaying the damage rather than completely preventing it (Halliwell, 1999, 2011), but our understanding of this process is hamstrung by the fact that evidence regarding the protective action of antioxidants under heat conditions has not been synthesized yet. Under environmentally adverse situations, such as heat stress, the increased production of metabolic ROS can exceed the capacity of antioxidant defenses, leading to a state of oxidative stress (an unbalance between reactive oxygen species and antioxidants, in favor of the former) and opening the requirement for a counteracting response, or otherwise increasing the risk of suffering oxidative damage to biomolecules and death (Jena et al., 2013; Tomanek 2015; Ali et al., 2017). The negative effect of heat stress on insects



**Fig. 1.** In response to high temperatures, insect metabolic rates increase, elevating the rates of reactive oxygen species (ROS) production, some of which are more damaging than others given their potential to generate oxidative stress. (a) Limiting ROS production upon heat exposure can avoid the negative effects of oxidative stress, but the proposed mechanisms are still hypothetical in insects. (b) Sources of antioxidant defenses (endogenous and exogenous). The numbers indicate the following antioxidants: 1=Aldehyde dehydrogenase (ALDH), 2=Catalase (CAT), 3=Ferritin, 4=Glutathione reductase (GR), 5=Glutathione (GSH), 6=Glutathione-S-transferase (GST), 7=Lactate dehydrogenase (LDH), 8= Superoxide dismutase (SOD), 9=Transferrin; exogenous: 10=Ascorbic acid, 11=Carotenoids, 12=Melanins, 13=Ommochromes, 14=Polyunsaturated fatty acids; others (in gray, sources that need further evidence): 15=Polyphenols, 16=Uric acid, 17=Allantoin, 18=Bilirubin, 19=Ophthalmic acid, 20=Polyamines, 21=Tocopherol, 22=Xanthurenic acid. (c) Antioxidants from different sources contribute to combat excessive ROS caused by exposure to high temperature (c1), but when ROS production exceeds the insect's antioxidant capacity (AOX) (with only two scenarios presented here, but see Fig. 2), oxidative damage can occur in lipids, proteins and nucleic acids (c2). The effect of high temperatures on oxidative balance will likely define individual survival and reproduction through the costs and benefits of modulating response mechanisms to high temperatures and other stressors, contributing with physiological machinery to acclimation and adaptation and hence insect evolution under warmer conditions (c3).

depends on the balance of ROS production, antioxidant availability and resulting oxidative damage exerted on biomolecules. However, these components of the oxidative balance and their resulting consequences are rarely evaluated together, limiting our understanding of the efficacy of antioxidant responses (Costantini and Verhulst, 2009).

In this review, we synthesize the evidence showing the effects of high temperature on ROS production in insects, the most common reported patterns of antioxidant responses in insects exposed to heat and extreme heat conditions, and the extent to which antioxidants effectively prevent oxidative damage to biomolecules in response to heat. We further discuss the potential role of other mechanisms that may influence the oxidative balance, and the resulting damage, that remain understudied but are potentially relevant in response to heat. Then, we discuss the role of oxidative balance in heat adaptation and acclimation and how antioxidants might be used or limited in current times of global change, when high temperatures interact with other stressors and where antioxidant use might be limiting due to its costly nature (Monaghan et al., 2009). Finally, we identify key aspects of the response to heat that still need to be investigated to understand the role of ROS, antioxidants and oxidative stress in insect heat tolerance.

# 2. Reactive oxygen species: from indispensable to damaging in insects exposed to high temperatures

During aerobic metabolism, the normal process of conversion of energy into ATP in mitochondria of most animal cells,  $O_2$  is required. Because  $O_2$  has two uncoupled electrons, it is reduced (in this case, frequently gains one electron at a time), leading to the formation of ROS. The precursor of most ROS, superoxide anion  $(20 e^{2-})$  cannot cross cell membranes, but rapidly reacts to produce hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) that can do it, and later on, results in the highly reactive hydroxyl radical (OH•) (Bergamini et al., 2004; Juan et al., 2021). This chemical reaction of gaining electrons from other molecules that have lost them, known as a redox reaction, can break chemical bonds, resulting in the production of free radicals, with ROS as the subgroup of those that contain oxygen (Halliwell, 2011).

At low concentrations, ROS such as superoxide anion and hydrogen peroxide are indispensable in cell signaling, acting as second messengers that spread the signal to different parts of the cell, modulating signaling proteins and transcription factors required to produce cell responses including proliferation, differentiation, adhesion, or apoptosis, with consequences on cell functioning and survival (Bergamini et al., 2004; Li et al., 2016). In insects, ROS production is hormonally regulated by metabolic neurohormones, such as the adipokinetic hormone (AKH), which also plays a significant role in the regulation of antioxidant production (see Section 3.1) and uncoupling proteins (see Section 5) (Slocinska et al., 2013; Kodrík et al., 2015). ROS also contribute to temperature sensing and to the activation of temperature defense mechanisms, such as heat shock proteins and ROS-scavenging enzymes (Suzuki and Mittler 2006). ROS regulate diapause promoting extended lifespan (Zhang et al., 2017) and mediate the enhanced expression of genes encoding the antioxidants superoxide dismutase (Cu/Zn-SOD) and catalase via redox reactions (see Section 3.1) (Matsumura et al., 2017; Matsumura et al., 2023). However, evidence of the involvement of ROS in protection against high temperatures in insects is still scarce compared to evidence in plants and mammals (Ahn and Thiele 2003; Suzuki and Mittler 2006). Instead, for being highly reactive, ROS at high concentrations have the potential to damage biomolecules such as lipids (producing toxic byproducts that induce oxidative chain reactions that alter fluidity and permeability of cell membranes), proteins (altering the structure of protein skeleton due to fragmentation, dimerization, unfolding and conformational changes that when major cannot be repaired), and nucleic acids (producing damage to bases or sugars, single and double strand breaks, and formation of abasic sites, leading to mutagenesis, carcinogenesis, cellular senescence, apoptosis, and necrosis) (Bergamini et al., 2004; Juan et al., 2021).

Intracellular ROS are mainly produced during mitochondrial respiration and their release to the cytosol is tightly controlled by different proteins (Bae, 2011). As exposure to high temperatures increases metabolic rates in insects (Gillooly et al., 2001; Irlich et al., 2009), there is also a high potential of producing ROS and oxidative damage (Fig. 1; Table 2). This is observed (at least during a phase) in 11 out of 13 cases where ROS have been measured in insects exposed to high temperatures (Tables 1 and 2), indicating that increased ROS production is a common result of exposure to high temperatures in insects. For example, In Bemisia tabaci (Hemiptera: Aleyrodidae) adults, exposure to high temperatures such as 39 or 41 °C increases the levels of ROS, when antioxidant genes (see Section 3.1) are silenced (the probe reacts with hydroxyl, peroxyl and other unspecified ROS within the cell), eventually decreasing adult survival and fecundity (Liang et al., 2022). Also, in Bombyx mori (Lepidoptera: Bombycidae), larval exposure to 30 °C increases ROS levels (unspecified ROS) and decreases survival, compared to larvae exposed to 24 °C (Li et al., 2018). In three of the available cases, ROS increase but then decline at higher temperatures. In this line, armyworms Mythimna separata (Lepidoptera: Noctuidae) exposed to 40 °C during the larval stage show higher ROS levels in plasma (used probe reacts with hydrogen peroxide, peroxyl radical, nitric oxide, and peroxynitrite anion), hemocytes and fat body than control larvae reared at 25 °C, although this pattern is not observed at other stressfully high temperatures such as 38 or 42 °C (Matsumura et al., 2017). Only in one case, Musca domestica (Diptera), ROS production is lower at the higher tested temperature (28 vs 20 °C), although a gradient of temperatures (i. e. more than a control and a single experimental temperature) is necessary to bring conclusions about general effects of heat on ROS production. Despite most available evidence showing that high temperatures lead to increased ROS levels, further studies across temperature gradients are still needed to evaluate the real threat that ROS imply in heat-exposded insects, as well as oxygen consumption, the rate of ROS release, and the resulting oxidative damage caused by ROS to different biomolecules (Table 3; Hraoui et al., 2021).

#### 3. Antioxidants used by insects in response to high temperatures

The protective role of antioxidants in insect responses to heat stress might be necessary if ROS are overproduced in heat-exposed individuals, as suggested by the (limited) available evidence. Nevertheless, antioxidants have been described to play a role in response to heat and extreme heat conditions in seven insect orders (Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Psocoptera and Thysanoptera). From the available evidence, 82 % of these studies have been conducted in species of temperate zones, with a clear underrepresentation of tropical species, and only 2 % of them have studied species with aquatic habits, despite aquatic taxa have shown higher physiological plasticity to heat conditions than terrestrials (Gunderson and Stillman, 2015) (Tables 1 and 2). Finally, only 16 % of the studies report responses for more than one developmental stage of the same species, being this information key to understand whether the participation of antioxidants in heat tolerance may be more important in one stage than others. In most studies, experimental designs consist in evaluating mainly enzymatic antioxidants (see below) (i.e., concentration and activity, gene expression - very few studies) in laboratory-reared insects exposed to different field-realistic and extreme temperatures (Table 1). However, the reviewed studies frequently use experimental settings that might leave out ecological conditions relevant for understanding insects' response to heat (i.e., type of substrate, space for allowing proper behavioral responses, group density, humidity, heat exposure timeframe), and do not test directly the role of antioxidants at preventing oxidative damage triggered by heat conditions (rather, evaluate covariation between heat and antioxidants). Interestingly, only 29 % of those studies evaluate ROS production in exposed insects to confirm the need and the benefit of modulating antioxidants in response to heat. The resulting oxidative damage to biomolecules to evaluate the efficiency of

#### Table 1

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Antioxidant responses in insects exposed to increasing temperatures. Evaluated responses also include the level of hydroperoxide  $(H_2O_2)$  as a measure of ROS production, aldehyde dehydrogenase (ALDH), besides the endogenous antioxidants superoxide dismutase (SOD), catalase (CAT), peroxidases (POD), glutathione-S-transferase (GST), total antioxidant capacity (TAC), and metal binding proteins (MBP). At high temperatures, reported mechanisms (gene expression, concentration or activity) either increase (-), decrease and then increase in a U-shape pattern (U), increase and then decrease in a threshold pattern () or remain unchanged (=) in different insect tissues.

$\begin{array}{l} \text{Temperature} \geq \\ \text{control} \end{array}$	$H_2O_2$	ALDH	SOD	CAT	POD	GST	TAC	MBP	Order	Species	Tissue	Zone	Stage	Habit	Reference
32, 35, 38			+	+	_	-	+		Coleoptera	Hippodamia variegata	Whole body	Temperate	Adult	Flyer	Yang et al. 2022
25, 40		+	+			-			Coleoptera	Monochamus alternatus	Whole body	Temperate	Larvae	Terrestrial	Li et al. 2020
32, 35, 38			+	+	-	-	-		Coleoptera	Propylaea	Whole body	Temperate	Adult	Flyer	Yang et al. 2022
										quatuordecimpunctata					
28, 40, 42, 44									Coleoptera	Ophraella communa	Whole body	Temperate	Egg, Larvae, Adult	Terrestrial, Flyer	Chen et al. 2018
25, 37	+							$+^{5}$	Coleoptera	Protaetia brevitarsis	Fat body, hemopymph	Temperate	Larvae	Terrestrial	Kim et al. 2008
27.40			+						Diptera	Bactrocera dorsalis	Whole body*	Temperate	Adult	Flver	Gao et al. 2013
20, 28	_		=	_		_1			Diptera	Musca domestica	Whole body	Temperate	Adult	Flver	Farmer and Sohal 1987
20, 25, 28			+	+	+	+			Hemiptera	Aphis pomi	Whole body	Temperate	Nymph.	Terrestrial	Durak et al. 2021
-, -, -									I I I I	I I I			Adult		
27, 34			+	-	+	+			Hemiptera	Bemisia tabaci	Whole body	Temperate	Adult	Flyer	Li et al. 2017
20, 25, 28			+	+	+	+			Hemiptera	Cinara cupressi	Whole body	Temperate	Nymph, Adult	Terrestrial	Durak et al. 2021
20, 25, 28			+	+	+	+			Hemiptera	Macrosiphum rosae	Whole body	Temperate	Nymph, Adult	Terrestrial	Durak et al. 2021
27, 40						+			Hemiptera	Diaphorina citri	Whole body	Temperate	Adult	Flver	Xiong et al. 2019
25, 30, 35			+	+	+	+			Hymenoptera	Aphidius gifuensis	Whole body	Temperate	Pupae, Adult	Flyer	Kang et al. 2017
35, 45			+	=	=	$=^{2}$			Hymenoptera	Apis cerana	Whole body	Temperate	Larvae	Terrestrial	Li et al. 2019
35, 45			+	=	_	$+^{2}$			Hymenoptera	Apis mellifera	Whole body	Temperate	Larvae	Terrestrial	Li et al. 2019
25, 32.5, 35, 37.5, 40, 41			?	?	?	?			Hymenoptera	Aphelinus asychis (males)	Whole body*	Temperate	Adult	Flyer	Liu et al. 2022
25, 32.5, 35, 37.5, 40, 41			?	+	+	?			Hymenoptera	Aphelinus asychis (males)	Whole body	Temperate	Adult	Flyer	Liu et al. 2022
25, 32.5, 35, 37.5, 40, 41			U	U	U	U			Hymenoptera	Aphelinus asychis (females)	Whole body*	Temperate	Adult	Flyer	Liu et al. 2022
25 32 5 35 37 5			U	+	+	IJ			Hymenoptera	Aphelinus asychis (females)	Whole body	Temperate	Adult	Flver	Liu et al. 2022
40, 41			U			U			inginenopteru	ripiteinius usyeinis (teinutes)	Whole body	remperate	- Iddit	11901	
25, 40		+	+		+	+			Lepidoptera	Glyphodes pyloalis	Midgut	Temperate	Larvae	Terrestrial	Liu et al. 2017
30, 35, 40, 45			+			+	+		Lepidoptera	Mythimna separata	Whole body	Temperate	Adult	Flver	Ali et al. 2016
28, 33, 36, 39				+					Lepidoptera	Chilo suppressalis	Whole body	Temperate	Larvae	Flver	Cui et al. 2011
25, 38, 40, 42, 44									Lepidoptera	Mythimna separata	Plasma, hemocytes, fat body	Temperate	Larvae	Terrestrial	Matsumura et al. 2017
28, 40							_3		Lepidoptera	Bombyx mori	Hemolymph, silk	Tropic	Larvae	Terrestrial	Aneesha and Sreeranjit Kumar 2022
25, 40						=		_4	Lepidoptera	Ostrinia furnacalis	Whole body	Temperate	Larvae	Terrestrial	Chen et al. 2019
21, 26, 33			U	=	+	+			Thysanoptera	Frankliniella occidentalis	ND	Temperate	Adult	Flyer	Li et al. 2014

Notes: <sup>1</sup> glutathione (GSH), <sup>2</sup> glutathione reductase (GR), <sup>3</sup> Ascorbic acid, <sup>4</sup> Ferritin and Transferrin, <sup>5</sup> Transferrin, \* gene expression, ? unclear pattern.

## Table 2

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Protection offered by antioxidants against oxidative damage to biomolecules in insects exposed to heat stress. With temperatures above the control temperature, the level of hydroperoxide  $(H_2O_2)$  as a measure of ROS production, malondialdehyde (MDA, a marker of oxidative damage to lipids) and antioxidants, are found to increase (+), decrease (-), decrease and then increase in a U-shape pattern (U), increase and then decrease in a threshold pattern () or remain unchanged (=). Antioxidants reported were superoxide dismutase (SOD), catalase (CAT), peroxidases (POD), glutathione-S-transferase (GST) and total antioxidant capacity (TAC). The shaded area represents cases where the antioxidant response effectively mitigates or reduces oxidative damage. Unchanged or decreased levels of oxidative damage to biomolecules, mainly MDA (but see superscripts added) are considered suggestive of antioxidant protection.

$\begin{array}{l} \text{Temperature} \geq \\ \text{control} \end{array}$	$H_2O_2$	MDA	SOD	CAT	POD	GST	POX	TAC	Protection	Order	Species	Tissue	Zone	Stage	Habit	Reference
21, 24	+	+	+	+	+	+	+		No	Coleoptera	Galerucella placida	Whole body	Tropic	Adult	Flyer	Das et al. 2018
25, 37, 45		+	+		=			+	No	Diptera	Bactrocera cucurbitae	Whole body	Temperate	Adult	Flyer	Jia et al. 2019
27, 35, 37.5, 40		+	+	+	U	+		+	No	Diptera	Bactrocera dorsalis	Whole body	Temperate	Adult	Flyer	Jia et al. 2011
26, 33, 35, 37, 39, 41, 43		+	+	+		+4			No	Hemiptera	Corythucha ciliata	Whole body	Temperate	Adult	Flyer	Ju et al. 2014
25, 27, 30, 33, 36	+		+	+	+				No	Homoptera	Myzus persicae	Whole body	Temperate	Adult	Terrestrial, Flyer	Khurshid et al. 2021
25, 27, 30, 33, 36			+	+	+				No	Homoptera	Myzus persicae	Whole body*	Temperate	Adult	Terrestrial, Flyer	Khurshid et al. 2021
25, 35, 40	$+^1$	+	+	+			+		No	Lepidoptera	Bombyx mori (SK7)	Haemolymph	Tropic	Larvae	Terrestrial	Makwana et al. 2021a
24, 34		+	+	+	+	+	+		No	Lepidoptera	Chilo suppressalis	Whole body	Temperate	Larvae	Terrestrial	Shamakhi et al. 2018
25, 37		+	+	+					No	Lepidoptera	Ectomyelois ceratoniae	Whole body	Temperate	Egg, Larvae, Pupae, Adult	Terrestrial, Flyer	Farahani et al. 2020
25, 35, 37, 39, 41, 43	+				+	+		+	No	Coleoptera	Propylaea japonica	Whole body	Temperate	Adult	Flyer	Zhang et al. 2015
25, 36, 38		+							No	Diptera	Bradysia difformis	Whole body	Temperate	Adult	Flyer	Zhu et al. 2017
25, 36, 38		+			+	+			No	Diptera	Bradysia odoriphaga	Whole body	Temperate	Adult	Flyer	Zhu et al. 2017
28, 35, 40	+	+	=	+		+			No	Lepidoptera	Antheraea mylitta	Testes	Tropic	Pupae	Terrestrial	Jena et al. 2013
25, 35, 40	+			+			+		Partial	Lepidoptera	Bombyx mori (sarupat)	Hemolymph	Tropic	Larvae	Terrestrial	Makwana et al. 2021b
27.5, 37.5, 40, 42.5, 45					-	?			Partial	Psocodea	Liposcelis bostrychophila	Plasma, hemocytes, fat body	Temperate	Adult	Terrestrial	Miao et al. 2020
25, 35, 40	+	=	+				+		Yes	Lepidoptera	Bombyx mori (SK6)	Hemolymph	Tropic	Larvae	Terrestrial	Makwana et al. 2021a
25, 35, 40		=	+	+			+		Yes	Lepidoptera	Bombyx mori (nistari)	Hemolymph	Tropic	Larvae	Terrestrial	Makwana et al. 2021b
20.2, 21.1, 29.3	=	$=^3$	+	=		$+^{5}$		=	Yes	Diptera	Tanytarsus minutipalpus	Whole body	Tropic	Larvae	Aquatic	Bianchini et al. 2017
30		$=^{2}$	=	+				=	Yes(?)	Diptera	Belgica antarctica	Whole body*	Temperate	Larvae, Adult	Terrestrial	López-Martínez

Notes: <sup>1</sup> H<sub>2</sub>O<sub>2</sub> and Nitric oxide (NO), <sup>2</sup> MDA and carbonyls, <sup>3</sup> MDA and DNA damage, <sup>4</sup> Glutathione reductase (GR) and Glutathione (GSH), <sup>5</sup> GR, Glutatione Peroxidase (GPx) and GSH, \*gene expression, ? unclear pattern.

#### Table 3

Testable hypotheses that remain to be evaluated to understand the role of ROS, antioxidants and oxidative stress in tolerance to high temperatures in insects.

Hypothesis	Experimental approach	Prediction			
High temperatures increase mitochondrial oxygen consumption, resulting in higher ROS production and oxidative stress.	Expose insects to elevated temperatures and evaluate mitochondrial oxygen consumption, ROS production and oxidative damage to lipids, proteins and nucleic acids.	Mitochondrial oxygen consumption, ROS production and oxidative damage to biomolecules increase with increasing temperatures.			
Antioxidants provide heat tolerance in insects because they avoid oxidative damage, thereby enhancing survival under thermal stress.	Expose insects to elevated temperatures to evaluate oxidative damage and heat tolerance in individuals with knockdown of genes for production of antioxidants and control individuals.	Knockdown individuals will show higher oxidative damage and lower heat tolerance than control individuals.			
Dietary antioxidants help prevent oxidative damage and provide heat tolerance in insects exposed to high temperatures.	Manipulate dietary antioxidants and compare oxidative damage to biomolecules and heat tolerance with control-fed insects.	Individuals supplemented with antioxidants will show reduced oxidative damage and increased heat tolerance compared to non-supplemented individuals when exposed to high temperatures.			
Uncoupling proteins (UCP) play a crucial role in mitigating ROS production and reducing oxidative damage induced by heat stress.	Determine ROS production and oxidative damage in individuals with knockdown of genes for UCP and control individuals exposed to high and control temperatures.	Knockdown insects will exhibit higher ROS production and oxidative damage than control insects when exposed to high temperatures.			
Oxidative damage defines the type of interaction (i.e., additive, antagonistic, or synergistic) between high temperature and other stressors, with the antioxidant defense modulating their overall impact.	Assess ROS production, antioxidant activity, oxidative damage and mortality in insects exposed to high temperatures combined with other stressors.	Synergistic lethal combinations of stressors occur due to oxidative stress, either due to high ROS production or low antioxidant levels.			
ROS production and antioxidant responses to high temperature are heritable traits.	Determine ROS production and antioxidant activity in parents and offspring exposed to different temperatures from a full- sib / half-sib experimental design	Insects with high ROS production and antioxidant activity in response to high temperature are expected to have offspring with similar responses.			
Antioxidant responses to high temperature are adaptive, enabling organisms to cope with increasing oxidative stress.	Estimate antioxidant activity, oxidative damage and heat tolerance across the phylogeny of several closely related species occurring along a latitudinal (or elevational) gradient.	Antioxidant activity counteracts oxidative stress in response to high temperatures in a latitudinal pattern, revealing local adaptation rather than phylogenetic inertia.			
Parental effects define antioxidant responses and oxidative stress in response to high temperature.	Evaluate antioxidant activity in families where the mother, the father, or both parents are exposed to different temperatures.	Mothers and fathers exposed to high temperatures produce offspring with higher antioxidant activity and lower oxidative stress in response to heat.			

the antioxidant response was quantified in 36 % of the total reviewed studies, with most of them evaluating damage to only one biomolecule and in only one biological substrate (i.e., tissue; Table 2).

According to their source, antioxidants are either endogenously produced or obtained from the diet (Fig. 1). The first line of defense, and the most studied antioxidants in insect responses to heat, are the endogenously produced enzymes superoxide dismutase (SOD), catalase (CAT), peroxidases (POD), glutathione reductase (GR), and glutathione-S-transferase (GST), that have been frequently found to increase in insects exposed to high and extremely high temperatures (Tables 1 and 2; Fig. 1). As a second line of defense, enzymatic compounds such as aldehyde dehydrogenase (ALDH) increase in response to heat. ALDH probably plays a role at scavenging aldehydes resulting from oxidative damage to lipids, as this is one of its conserved functions across species which can contribute to mitigating further oxidative chain reactions (Table 1; Fig. 1) (Singh et al., 2013). Glutathione (GSH) and metal-binding proteins such as transferrin and ferritin are endogenous, but non-enzymatic compounds with antioxidant capacity that appear to contribute to the response of insects to heat (Table 1; Fig. 1) (Kim et al., 2008; Ju et al., 2014; Chen et al., 2019). They can be recycled by the action of different mechanisms, but further research is required to understand their participation in this response. Finally, antioxidants obtained from the diet, such as ascorbic acid, may also contribute to mounting a response to heat, but their role has received little attention.

#### 3.1. Endogenous antioxidants

During the aerobic metabolism, in mitochondria, the endogenously produced enzyme superoxide dismutase (SOD) neutralizes ROS by catalyzing the reaction that converts the superoxide anion ( $20^{\circ^{2-}}$ ; a free radical) to hydrogen peroxide (H2O2), tough this reaction does not directly consume ATP, cellular processes required for synthesizing SOD do (RXN. 1). Although H<sub>2</sub>O<sub>2</sub> is not a free radical, it leads to the production of the highly reactive and toxic hydroxyl radical (OH•) through non-enzymatic reactions catalyzed by iron (Fe), that (in healthy cells) do not directly require or produce ATP, yet the uptake and storage of Fe ions do consume ATP (RXN. 2). SOD is a family of enzymes comprised by three members: SOD1 or Cu/Zn-SOD, SOD2 or Mn-SOD, and SOD3 or EC-SOD. Particularly, Cu/Zn-SOD is located at the cytoplasm, nucleus, peroxisomes, and the external mitochondrial membrane and, due to the type of transcription factors that regulate its expression, this can be induced by stimulus such as thermal shock and UV radiation (Fig. 1). Peroxidases are endogenously produced enzymes responsible for eliminating H<sub>2</sub>O<sub>2</sub>, and they are classified into two groups, catalases (CAT) and peroxidases (POD) (Fig. 1). Catalases decompose H<sub>2</sub>O<sub>2</sub> directly into O2 and water, using a molecule of H2O2 as reducing agent and another molecule of H<sub>2</sub>O<sub>2</sub> as oxidizing agent (RXN. 3). Similarly, peroxidases use one H<sub>2</sub>O<sub>2</sub> as oxidizing agent, but a different molecule as reducing agent (hydrogen sulfide in the example) (RXN. 4). Hence, transforming  $H_2O_2$ into H2O involves enzymatic reactions that require catalases or peroxidases, but do not directly involve ATP, and the energy released from them is not used to phosphorylate ATP. These enzymatic reactions prevent the domino effect caused by ROS, and then, decrease the opportunity for damage to lipid membranes to occur:

 $\begin{aligned} & 2O^{\bullet 2-} + 2H^+ \to H_2O_2 + O_2\bullet \text{ (RXN. 1)} \\ & H_2O_2 + Fe^{2+} \to Fe^{3+} + OH\bullet + OH^- \text{ (RXN. 2)} \\ & 2H_2O_2 \to 2H_2O + O_2 \text{ (RXN. 3)} \\ & SH_2 + H_2O_2 \to S + 2H_2O \text{ (RXN. 4)} \end{aligned}$ 

Also endogenously produced, the tripeptide glutathione (GSH) is the most abundant cellular antioxidant found in the cytosol of animal, plant, and microbial cells; it is synthesized exclusively in the cytosol, and then it is distributed to other cellular organelles (Fig. 1). Among other

functions, such as protein and nucleic acid synthesis, GSH is involved in the reduction of H<sub>2</sub>O<sub>2</sub> produced inside mitochondria, as this organelle lacks catalase, so GSH plays a vital protective role (Meister, 1988). In vertebrate cells, heat stress increases ROS production accompanied by GSH depletion (Mitchell et al., 1983; Russo et al., 1984). Although glutathione is not an enzyme, it requires the action of several enzymes to perform its antioxidant function. In mammals, glutathione peroxidase (GPx) plays a major role to reduce H<sub>2</sub>O<sub>2</sub> and lipid hydroperoxides, but in insects this enzyme seems to be practically absent and such fundamental antioxidant function is performed by glutathione-reductase (GR) and glutathione-S-transferase (GST), which reduce H2O2 and lipid hydroperoxides resulting from damage to lipid membranes (Ahmad, 1992; Felton and Summers, 1995; Forman et al., 2009). Endogenous antioxidants can be found in the mitochondria, cytoplasm or the extracellular space (Parker et al., 2004), with those of mitochondrial origin being the main ones (Halliwell, 1999). They are generally found at high levels in metabolically active tissues (Felton and Summers, 1995). Therefore, in insects exposed to high temperatures, the endogenous antioxidants are expected to be particularly higher in tissues such as the fat body; however, only 7 % of the studies reviewed here quantified antioxidants in this organ (Tables 1 and 2).

Aiming at synthesizing the results of the studies reviewed here, we first describe three main potential scenarios of the response of endogenous antioxidants to heat in insects (increase, decrease or remain constant; Fig. 2) exposed to permissive and realistic ranges. The first scenario corresponds to an increase in antioxidants in response to heat. This positive response of the antioxidants may protect against oxidative damage induced by heat only if those increased antioxidants are successful at neutralizing the overproduction of ROS (Fig. 2a), but if the rate of ROS production exceeds the neutralizing capacity of the antioxidants, oxidative damage can occur despite the increase in the antioxidants (Fig. 2b). The second scenario presents a decrease in antioxidants in response to heat. This negative response of the antioxidants can offer an effective protection against damage, in the case that antioxidants available are getting used up to successfully counteract the overproduction of ROS (Fig. 2c), but if the consumption of the antioxidants available is not enough to successfully neutralize the overproduction of ROS, oxidative damage will occur (Fig. 2d). Finally, the third scenario shows an unchanged level of endogenous antioxidants, that might occur when genuinely there is no response at all of the antioxidants to heat or when antioxidant production equals use, and thereby, oxidative damage will arise (Fig. 2e).

Studies reviewed here suggest that a gradual increase in the temperatures at which insects are exposed may result in a range of patterns that, besides including the three scenarios described above, may also combine then resulting in two additional patterns: u-shaped and threshold responses. Below, we present studies selected from Table 1

that illustrate the five main identified patterns of antioxidant responses of insects to heat stress. In response to increasing temperature (or increased time of exposure to a high temperature), most studied insects show increased levels in at least one antioxidant enzyme (Table 1 and 2; Fig. 2a, b). For example, in Mythimna separata larvae (Lepidoptera) at 40 °C, relative expression of mRNAs encoding CAT in the fat body start increasing after 15 min of exposure and keep increasing during the next 4-5 h; mRNAs encoding SOD reach the highest levels after 30-60 min of exposure, suggesting an upregulation in the activity of such enzymes (Matsumura et al., 2017). Similarly, in the highly invasive Corythucha ciliata (Hemiptera), which is naturally exposed to high temperatures of over 40 °C (Ju et al., 2014), when individuals are experimentally exposed to heat in both the field and the laboratory, SOD, CAT and GR activities, as well as GSH concentration, increase gradually from 26 to 43 °C during 6 h of heat exposure. However, in this last case, it cannot be distinguished if increased enzyme activity is an active response to high temperature or simply a passive effect of temperature on enzyme activity. Unfortunately, only a few studies evaluating antioxidants measure gene expression besides enzyme activity, and just a couple of them report the use of a control region (Tables 1 and 2).

In other insect species, as temperatures get warmer, the activity of antioxidant enzymes increases up to a threshold above which it starts decreasing (Table 1 and 2). This pattern of response is similar, to some extent, to a biphasic hormetic response (Rix and Cutler, 2022). Despite this pattern being less common, in Bradysia odoriphaga and B. difformis (Diptera), the activity of antioxidant enzymes CAT, POD and SOD shows an increase at 36 °C but starts decreasing at 38 °C (Zhu et al., 2017). Also, in Liposcelis bostrychophila (Psocoptera), an important pest of stored products, CAT and SOD activity tend to increase in a temperature gradient from 27.5 to 42.5 °C, but then decrease towards 45 °C (although remaining upregulated with respect to the control) (Miao et al., 2020). Considering that enzyme activity is temperature dependent, patterns described here may respond to changes in the geometry of binding substrates, cofactors that prevent the proper function of the enzyme above the optimal temperature, or denaturation of proteins that prevent enzyme activity at extremely high temperatures (Fields 2001), yet these alternatives still require empirical examination.

Negative responses of antioxidants (i.e., decrease) to increasing temperatures (Table 1 and 2; Fig. 2c and 2d) are not common but have also been reported in insects, as POD, CAT, GST, GSH, or total antioxidant capacity (TAC) sometimes decrease in heat-exposed insects (Table 1 and 2). For example, in *Propylea quatuordecimpunctata* (Coleoptera) a decrease in POD, GST and TAC is observed as temperature increases from 32 to 38 °C (Yang et al., 2022). Such a decrease in antioxidants in response to high temperatures can occur at least in two scenarios: First, other coping responses such as heat shock proteins are present. Second, extremely high ROS levels might be suppressing



**Fig. 2.** Potential scenarios for changes in ROS production and antioxidant capacity in response to warming temperatures. In response to heat, the insect metabolic rates increase together with the production of reactive oxygen species (ROS). In consequence, insects show a battery of endogenous (and likely exogenous) anti-oxidant mechanisms (AOX) that can provide a hypothetical range of protection that goes from total protection to no-protection against oxidative damage to bio-molecules. The resulting protection depends on the dynamic of the antioxidant response that can be positive (a, b), negative (c, d) or unchanged (e) along the thermal gradient, from control up to reach sublethal high temperatures. Blue areas represent the extent of predicted protection offered by the antioxidant response and pink areas represent the extent of damage expected in absence of antioxidant protection.

antioxidant activity (Matés 2000), depleting antioxidant defenses or inactivating the antioxidant enzymes through structural changes in proteins (Tabatabaie and Floyd, 1994).

U-shape antioxidant responses to warming temperatures (Table 1 and 2) could correspond to a combination of an initial negative response of antioxidants (i.e., decrease) to a moderate rise in temperature, followed by a subsequent positive response of antioxidants to increased heat. For example, in *Aphelinus asychis* (Hymenoptera) females exhibit a U-shape response in the activity of SOD, CAT, POD, and GST when exposed to temperatures ranging from 25 to 41 °C (Table 1; Liu et al., 2022).

The efficacy of the antioxidant responses described for preventing oxidative damage remains unclear due to the lack of studies evaluating oxidative damage along with antioxidants (see Section 4). An effective protection has only been described in two species, Bombyx mori (K6 and Nistari) (Lepidoptera) and Tanytarsus minutipalpus (Diptera) (Bianchini et al., 2017; Makwana et al., 2021a), out of 17 studied species of five orders (Table 2). This apparent lack of a complete protective response by antioxidants suggests that the rate of ROS production under heat conditions is higher than the production of endogenous antioxidants, inevitably resulting in increased oxidative damage to biomolecules (Table 2). For example, maintaining an effective antioxidant protection along the whole sublethal thermal range (or during prolonged time), even during extreme heat, would require the rate of antioxidant production to surpass the rate of ROS production, which seems unlikely to occur under extremely challenging conditions. Also, some ROS are potentially more damaging than others, such as the hydroxyl radical that is extremely reactive with most biological molecules and exerts a higher pressure on the antioxidant response (Bergamini et al., 2004; Barbehenn et al., 2013). The only hypothetical scenario where a complete protection by antioxidants seems likely is when heat triggers either a metabolic depression (as has been modeled for other invertebrate species, Liao et al., 2021), or an increase in mitochondrial uncoupling or degradation. This would lead to a potential decline in the rate of ROS production, as has been shown under other environmentally stressful conditions (Ali et al., 2012; Liao et al., 2021). However, heat damage (in metabolic processes or mitochondria) should be distinguished from a potentially adaptive scenario. The occurrence of a drop in metabolic rate or in the rate of ROS production (and potentially on the endogenous antioxidants) would depend on the existence of a thermal threshold that may vary by species, phenology, and ecological context (Kingsolver et al., 2011; Li et al., 2012; Zhang et al., 2015a; Zhang et al., 2015b; Dampc et al., 2020). An estimation of the oxidative damage occurred and the impact on developmental, reproductive and/or survival performance would be required to confirm the existence of such a mechanism. Further studies in genetically modified insects for antioxidant expression (i.e., knockdown genes linked to antioxidant response) should evaluate oxidative damage caused by heat to test the actual potential of specific endogenous antioxidants in preventing oxidative damage (Table 3).

#### 3.2. Non-enzymatic antioxidants

Two metal-binding proteins, transferrin and ferritin, have important antioxidant function in insects, as they prevent iron from reacting with  $H_2O_2$  and forming the highly reactive hydroxyl radical (OH•), thereby preventing noxious chain reactions that expand the scope of oxidative damage (Felton and Summers, 1995). They may be particularly important when dealing with high temperatures because iron levels are known to increase in the hemolymph in response to temperature stress (Chen et al., 2019). Transferrin and ferritin are common metal-binding proteins in insects. Insect transferrin is homologous to that of vertebrates, but in insects this role can also be played by ferritin, which is synthesized in the fat body and it functions to store and transport iron (Felton and Summers, 1995; Geiser and Winzerling, 2012). Ferritin is a large protein shell that can store thousands of iron atoms in a non-toxic way

while maintaining them available for further body functions (Geiser and Winzerling, 2012). Ferritin is known to prevent neurons to suffer from iron-mediated oxidative stress in Drosophila melanogaster (Diptera) (Mukherjee et al., 2020), among many other functions (Geiser and Winzerling, 2012). Transcriptomic analysis revealed that both ferritin and transferrin are upregulated at 40 °C compared to 25 °C in Ostrinia furnacalis (Lepidoptera), and low levels of ferritin are associated with an increase in hemolymph iron levels during heat stress (Chen et al., 2019). These results could be linked to the formation of iron sulfur clusters (Fe-S) that block ferritin synthesis, but increase ferritin secretion, allowing the excess of iron to be eliminated from the cells through sequestration by the protein (Geiser et al., 2006; Arosio et al., 2009). Moreover, the described response may have consequences on the function of some enzymatic antioxidants, such as SOD2 or MnSOD, because increased levels of iron can replace Mn in this enzyme structure, consequently inactivating it (Marelja et al., 2018), suggesting that the interaction between different antioxidant pathways should be considered when analyzing their potential to protect insects against heat. Transferrin mRNA is also upregulated in the fat body and the haemolymph of Protaetia brevitarsis (Coleoptera) after 24 h at 37 °C (vs 25 °C) (Kim et al., 2008). Despite evidence still being scarce (Table 2), the antioxidant roles of ferritin, transferrin and other metal-binding proteins to avoid oxidative stress and enhance heat tolerance are potentially widespread in insects, as similar roles have been described for metal-binding proteins in antioxidant responses to heat in other invertebrates and plants (Zang et al., 2017).

Another powerful antioxidant, uric acid, is the main end product of nitrogen metabolism in insects and is usually excreted in fecal pellets. For example, in termites Reticulermes speratus (Blattodea: Rhinotermitidae), it is the most abundant antioxidant and protects from the detrimental effects of UV radiation (Tasaki et al., 2017). In the hematophagous bug Rhodnius prolixus (Hemiptera), uric acid is the most abundant antioxidant in the hemolymph and prevents from oxidative damage caused by blood feeding (Graça-Souza et al., 2006). Uric acid could be involved in the insect response to high temperatures, as urate-null Drosophila melanogaster mutants are hypersensitive to experimentally induced oxidizing conditions (Hilliker et al., 1992). However, the direct role of uric acid in response to heat exposure remains to be studied in insects. Similarly, polyphenols such as catechins and phenolic acids from plants act as antioxidants and are involved in heat tolerance, although their role in insects is only known for Drosophila melanogaster (Huang et al., 2023). The roles of these and other antioxidants such as allantoin, xanthurenic acid, ophthalmic acid, bilirubin, polyamines and tocopherol still need to be evaluated in insect responses to heat.

# 3.3. Dietary dependent antioxidants used in response to high temperatures

Ascorbic acid (Vitamin C) is not synthesized in insect cells and needs to be obtained from the diet (Krishnan et al., 2009; Subedi et al., 2018). This compound is one of the main low molecular weight free radical scavengers that plays an important defensive role against oxidative assaults from diverse origin (Ahmad, 1992; Barbehenn, 2003). Evidence in *Bombyx mori* (Lepidoptera) shows a role for this antioxidant in the thermal response, as larvae supplemented with ascorbic acid in the diet improved general biochemical performance and were more physiologically active when exposed to extreme temperatures of 40–42 °C (Aneesha and Sreeranjit Kumar, 2022). In *Antheraea mylitta* (Lepidoptera), ascorbic acid content was found at higher concentration in testes of pupae exposed to 35 °C and 40 °C (compared to a control at 28 °C) during up to 5 days, indicating an important role in response to heat (Jena et al., 2013). However, the contribution of ascorbic acid in heat tolerance is unknown in most insect orders.

Carotenoids are lipid-soluble antioxidants, also used for pigmentation and are precursors ( $\beta$ -carotene) of vitamin A (Dhinaut et al., 2017; Feltwell and Rothschild, 1974). Carotenoids cannot be synthesized by insects and must be derived from ingested products (Klowden, 2013). These pigments scavenge free radicals produced by immune activity through electron transference, hydrogen ion removal and addition of radical species. Carotenoids are present in insect eggs, fat body, hemolymph, and tissues (Goodwin, 1984). Insects rely on carotenoids for many physiological functions, with beneficial effects on survival, growth and immunity (Babin et al., 2015). Moreover, carotenoids are involved in the expression and regulation of ferritin and SOD genes (Tan et al., 2020). However, it is not clear how dietary carotenoids contribute to insect responses to heat, leaving an open field for future research.

Another important antioxidant, melatonin, is synthesized from the essential aminoacid tryptophan and has an important antioxidant role (among many other functions). Melatonin exerts an antioxidant function by donating electrons, and so, neutralizing ROS. Moreover, the resulting metabolites (secondary and tertiary) of this redox reaction also have antioxidant functions, and can promote the activity of antioxidant enzymes (Tan et al., 2015; Purushothaman et al., 2020). When tryptophan (T5H-1) gene expression is knocked down in Apis cerana cerana (Hvmenoptera), most antioxidant genes are upregulated under adverse conditions, that include exposure to 45 °C, and increased levels of ROS and oxidative damage are found (Fan et al., 2021). Interestingly, when knocked down individuals are supplemented with melatonin and exposed to cold stress, levels of malondialdehyde and hydrogen peroxide decrease and survival increases, suggesting that melatonin also plays an important role in antioxidant protection against cold stress, and potentially against heat as well, but it still needs to be tested (Fan et al., 2021). As melatonin can be found in plants, where it is readily bioavailable (Tan et al., 2003), melatonin-rich diets might contribute to enhance survival in warm habitats, but further research is needed to understand the precise role of this antioxidant in insect heat tolerance. Similarly, polyunsaturated fatty acids are also present in insect diets and they play a fundamental role in the mitochondrial metabolism and oxidative stress in D. melanogaster (Champigny et al., 2018), being also potentially relevant in warming habitats.

Melanins and ommochromes also depend on the diet, as they are synthesized from the essential aminoacids tyrosine and tryptophan, respectively (Ushakova et al., 2019). These compounds not only function as antioxidants, but are also used as pigments, are fundamental in the insect immune system and the excretion of toxic metabolic byproducts (González-Santoyo et al., 2021; González-Santoyo and Córdoba-Aguilar, 2012). The antioxidant function of these pigments has never been considered to contribute against stressful heat, but a mechanistic link between heat tolerance and coloration could exist in insects mediated by the antioxidant activity of these common pigments. Given the multiple functions of dietary antioxidants in insects, trade-offs between allocation to heat tolerance and other life-history traits such as coloration, immunity or detoxification could be expected in insects. For example, evidence in the damselfly Coenagrion puella (Odonata) shows that the insecticide chlorpyriphos becomes lethal for individuals exposed to a heat wave (four days at 30 °C vs. 22 °C) only when starved, suggesting either an energetic trade-off, a role played by dietary antioxidants, or both (Dinh et al., 2016). Whether insects naturally exposed to conditions of high ROS production are less tolerant to heat due to greater demand for antioxidants or whether this baseline high ROS production works as a priming mechanism by upregulating antioxidant defenses are still open questions.

#### 4. Oxidative damage caused by high temperatures

Exposure to high temperatures can result in oxidative damage to different biomolecules, namely lipids, proteins or nucleic acids. The effects of high temperature on lipids are the most commonly evaluated, observed as increased levels of malondialdehyde (MDA), a product of lipid peroxidation. Despite most available evidence coming from controlled laboratory conditions, oxidative damage to lipids has been observed in insects exposed to thermal stress under natural conditions (Ju et al., 2014). When lipid peroxidation is not prevented, it triggers a

chain reaction that includes the production of highly reactive and toxic aldehydes that can be sequestered by the enzyme aldehyde dehydrogenase (ALDH), buffering further oxidative reactions (Singh et al., 2013). ALDH is found at higher levels in insects exposed to extreme heat, indicating oxidative damage to lipids. Evidence about the behavior of ALDH under heat is provided by comparative transcriptomic analysis in Monochamus alternatus (Coleoptera) and Glyphodes pyloalis (Lepidoptera), where ALDH genes are critically upregulated after 3–4 h at 40 °C (Li et al., 2020; Liu et al., 2017) (Table 2). Interestingly, in Corythucha ciliata (Hemiptera), the enzyme lactate dehydrogenase (LDH), that allows continuation of ATP production from glycolysis by recycling NAD+ under oxygen limiting conditions (Khan et al., 2020), also increases from 30-37 °C in field-exposed individuals, indicating that anaerobic respiration may act together with antioxidant mechanisms to prevent oxidative stress. However, no change is observed in this enzyme under laboratory conditions between 26 and 43 °C (Ju et al., 2014).

Despite antioxidant activity frequently increasing with temperature, at least transiently, oxidative damage to biomolecules is rarely prevented (Table 2), although we found two exceptions: Tanytarsus minutipalpus and Bombyx mori (SK6 and Nistari). In T. minutipalpus (Diptera), larvae collected in the field during the warmest hours of the day exhibited higher activity of SOD and GR, and elevated concentrations of GSH and oxidized GSH (maintaining a stable ratio) compared with larvae collected out of the heat peak. This increase in endogenous antioxidants occurred in absence of an increase in the levels of lipid peroxidation and DNA damage, suggesting an effective protection of antioxidants against oxidative damage induced by heat stress (Bianchini et al., 2017). Similarly, in Bombyx mori (SK6 and Nistari, Lepidoptera), larvae exposed to high temperatures in laboratory conditions increased H<sub>2</sub>O<sub>2</sub> production and enzymatic antioxidant activity (CAT, SOD), resulting in an effective protection against oxidative damage, as can be inferred by the absence of an increase in the levels of lipid peroxidation (Makwana et al., 2021b). More studies simultaneously quantifying ROS production, antioxidants, and oxidative damage during a heat challenge at different temporal scales are required to understand the extent to which antioxidants contribute to mitigate or prevent damage caused by heat exposure in insects.

Most studies presented in Table 2 have measured oxidative damage by quantifying lipid peroxidation, which is far from representing the overall oxidative damage suffered by cells exposed to environmental stress. Other biomolecules, such as DNA and proteins often suffer more severe and earlier damage from oxidative stress (Halliwell and Whiteman, 2004), threatening cell functioning and survival, but effects such as protein carbonylation or DNA oxidation are mostly unstudied in heat-exposed insects (but see Bianchini et al., 2017 and Makwana et al., 2021b). Aiming at improving the current understanding on the extent to which antioxidants contribute to mitigating the negative effects of heat exposure on insects, future studies need to go beyond just quantifying oxidative damage to lipids and incorporate simultaneous measures of damage to other biomolecules.

#### 5. Mitigating ROS production: an alternative to antioxidants?

Besides antioxidants being an important mechanism in response to ROS production, mitigating ROS production itself might also contribute to avoid oxidative damage and improve heat tolerance once behavioral avoidance has not been possible. Mitochondria are where most ROS are produced and changes in mitochondrial function are expected in response to high temperature, given that excessive production of ROS can increase the energetic cost of antioxidant defenses due to an increased demand for antioxidant defense actions (Sokolova, 2023). In all aerobic organisms, ROS are produced in the mitochondrial respiratory chain during the routine process of transduction of energy from nutrients to ATP usable for growth, reproduction, and self-maintenance. However, the rate of ROS production during this process is variable (Brand, 2000; Brand and Nicholls, 2011) due to the limitation of catalytic activity (i.e., the efficiency of electron transfer through the electron transport system) or the availability of NADH. If the mitochondrial respiratory chain complexes are not able to accept electrons, cellular energy demands require an increase in the production of ATP or there is an excess of NADH, so that the electrons can leak and react with oxygen (Blier et al., 2014). As an alternative to antioxidants, organisms may reduce the rate of ROS production by altering mitochondrial function through uncoupling proteins, or even degrading mitochondria in some cells during stressful periods (Alves-Bezerra et al., 2014; Jørgensen et al., 2021; Lebenzon et al., 2022; Slocinska et al., 2016, 2012). Both mechanisms and their involvement in response to high temperature are described below.

An uncoupling of the substrate oxidation from ATP synthesis can occur thanks to the action of uncoupling proteins (UCP), which are transmembrane proteins that occur in the mitochondrial membrane of eukaryote cells, leading to reduced ATP but also reduced ROS production (Slocinska et al., 2016; Mailloux and Harper 2011). This mechanism may be costly in terms of ATP production, but ROS decrease also reduces the risk of entering an oxidative unbalance and its further noxious consequences (i.e., oxidative damage) (Sokolova, 2023). Two uncoupling proteins, UCP4 and UCP5, occur in insects (Slocinska et al., 2016). The main role of UCP4 is to contribute to the reduction of mitochondria-produced ROS. Interestingly, in insects, UCP4 decreases superoxide anion levels, via uncoupling respiration from ATP synthesis, resulting in thermogenesis. For example, in Drosophila melanogaster, the action of UCP4 generates heat and maintains body temperature above ambient temperature, just as has been documented in mammals (Da-Ré et al., 2014; Slocinska et al., 2013). Moreover, UCP4 is essential for larval development at low but not at high temperatures, suggesting a role in thermal adaptation and cold tolerance in insects (Slocinska et al., 2016). Although UCP4 is expected to contribute to cold rather than heat tolerance, the role of UCP in preventing ROS production and oxidative damage caused by heat deserves further attention. One possibility, understudied up to date, is that lipid peroxidation subproducts may stimulate UCP activity (Slocinska et al., 2016). Knockdown of UCP genes could contribute to determine their role in mitigating ROS production and preventing oxidative stress at high temperatures, and could be focused on the insect tissues where these mitochondrial proteins cover particular importance, such as muscle or the fat body (Sanchez-Blanco et al., 2006) (Table 3).

Mitochondrial degradation, or mitophagy, is another alternative to reduce metabolism and potentially cope with oxidative stress. It has been described in muscle cells of diapausing chrysomelid beetles, *Leptinotarsa decemlineata* (Lebenzon et al., 2022). Despite representing an efficient mechanism to reduce metabolic rate at cold temperatures, mitophagy has not been evaluated as an alternative response to reduce ROS production or oxidative stress in insects exposed to high temperatures. Besides mitophagy, further studies should evaluate ROS production and antioxidant responses during heat coma, a reversible state of neural shutdown that protects the insect nervous system against heat damage (Rodgers et al., 2010).

#### 6. Antioxidant responses to combined stressors

In times of global change, increasing temperatures act together with a variety of other stressors with the potential to produce ROS, drive antioxidant responses and induce oxidative stress. The impact of environmental stressors might become more lethal at higher temperature, resulting in additive or synergistic interactions between them when combined (Coors and Meester, 2008; Verheyen et al., 2019). For example, in the aphid *Mysus persicae* (Homoptera), the neonicotinoid insecticide imidacloprid becomes 8-fold more lethal at 28 °C compared to 20 °C (Silva et al., 2025). This synergistic effect between high temperature and the pesticide is accompanied by a drastic increase in oxidative stress after 12 h of exposure (H<sub>2</sub>O<sub>2</sub> levels in whole body macerates), although this effect was not as evident in lipid peroxidation

(MDA levels) and did not persist after 48 h. Antioxidants and ROS were not evaluated in this study. Another stressor, water turbidity, increases oxidative stress in larvae of caddisflies *Stenopsyche marmorata* (Trichoptera) at 25 °C but not at 10 °C, indicating a potential synergistic effect of turbidity and high temperature, despite the observed increases in SOD and CAT (Suzuki et al., 2018).

In contrast, exposure to high temperatures may lead to improved performance upon exposure to other sources of stress, as cellular mechanisms used against thermal stress might share signaling pathways or protective mechanisms with responses against other stressors (Sinclair et al., 2013). For example, in damselflies Enallagma cyathigerum (Odonata), exposure to the insecticide chlorpyrifos causes oxidative damage to lipids, but this pesticide interacts antagonistically with high temperature, as the effect is lower at 24 °C than at 20 °C, apparently by means of SOD and CAT activities driving cross-tolerance to stressors (Janssens and Stoks, 2013; 2017). Also in E. cyathigerum, predation risk has the potential to increase oxidative stress, but the effect is lower at 24 °C compared to 20 °C, also associated with increased SOD and CAT activities (Janssens and Stoks, 2017). In Bemisia tabaci (Hemiptera: Alevrodidae), SOD activity increases at elevated temperature but it decreases when high temperature is combined with elevated CO<sub>2</sub> (Li et al., 2017). However, the opposite occurs with CAT, which increases in individuals exposed to both stressors, indicating that different components of the antioxidant response show different patterns when faced with multiple stressors. However, the extent to which oxidative stress is prevented was not evaluated in this system. Despite evidence indicating that oxidative stress is a potential mechanistic driver of synergistic effects between high temperature and other stressors, the complete picture still needs evaluations of ROS production, activity of different antioxidants and resulting oxidative stress and mortality (Table 3).

#### 7. Antioxidants in heat acclimation and adaptation

Heat acclimation is a phenotypic response that allows heat-exposed individuals (or their offspring) to improve heat tolerance compared to unexposed individuals (Collier et al., 2019). It differs from heat adaptation, which involves a genetic change favored by natural selection over generations. Although evidence in insects is limited, antioxidants are involved in heat acclimation in Cnaphalocrocis medinalis (Lepidoptera: Pyralidae). In this species, transcriptomic analysis revealed that five generations of exposure led to the upregulation of GST upon exposure to 41 °C in heat acclimated (at 39 °C) but not in unacclimated larvae (at 27 °C) (Quan et al., 2020). In Bemicia tabaci (Hemiptera: Aleyrodidae), parental exposure to high temperatures strongly defines the antioxidant activity of SOD, CAT, POD and GST in offspring, indicating that parental effects play a role in the production of endogenous antioxidants as mechanisms of heat acclimation, suggesting a potential transgenerational adaptive response to heat (Li et al., 2017). However, the extent to which ROS production and oxidative stress are associated with antioxidants expressed after heat acclimation is still unexplored.

Parental effects can also be driven by bacterial endosymbionts, some of which are known to provide increased heat tolerance in host insects. For example, in aphids (Hemiptera), heat tolerance depends on the tolerance of the endosymbiotic bacterium Buchnera (Zhang et al., 2019). In Aedes albopictus (Diptera: Culicidae), the obligate intracellular endosymbiont Wolbachia is transferred from the mother and provides the host increased SOD and GPx levels, although the resulting thermal tolerance has not been evaluated (Brennan et al., 2008). Considering that GPx is not produced by insects, one interesting possibility is that the obligate intracellular endosymbiont is responsible for the production of this enzymatic antioxidant. Moreover, in Diaphorina citri (Hemiptera), Wolbachia was the only symbiont to benefit from heat-shock treatment (Dossi et al., 2018) and it is a potential candidate to be studied in this context. However, whether antioxidants provided by symbionts confer increased heat tolerance remains unknown in insects, although it has been reported in other invertebrates (Krueger et al., 2015).

Local adaptation to high temperatures can be detected across environmental gradients and could result from differential antioxidant responses. For example, chrysomelid beetle species from Costa Rica show local adaptation and low phenotypic plasticity in heat tolerance across elevational gradients, with no phylogenetic signal determining species heat tolerances (García-Robledo et al., 2016). Opposite evidence in the genus Drosophila (Diptera) indicates that phylogenetic signal rather than distribution explains heat tolerance (Kellermann et al., 2012). Mitochondrial ROS production and antioxidants might play a role in the observed adaptive responses to heat, or might alternatively show phylogenetic inertia (Blier et al., 2014), but these possibilities remain to be experimentally tested in insects across natural environmental gradients (Table 3). Locally adapted populations or species would be ideal study systems. Further studies are needed to identify genetic and non-genetic effects in transgenerational responses to heat, and the role of ROS production and antioxidant defenses involved in both effects (Table 3).

## 8. Conclusions

Exposure to high temperature accelerates insect metabolism, releasing ROS with potential to cause oxidative stress and damage to biomolecules, mainly lipids, DNA and proteins, resulting in insect death. Antioxidants are potentially main mechanisms used by insects to cope with such a stress. Whereas some insect antioxidants are endogenous and increase in insect tissues in response to heat and extreme heat, others are obtained from diet, but their role in response to heat remains mostly unknown. While antioxidants might be key protective mechanisms in times of global change, where high temperatures interact with other stressors, their role in preventing oxidative damage to different biomolecules is mostly unknown and might be limited by life-history trade-offs. Nevertheless, the limited evidence does not support the idea that oxidative stress caused by high temperature can be counteracted with antioxidants. Alternative mechanisms involved in prevention of ROS production, such as mitochondrial uncoupling or degradation, must work complementarily to avoid heat-caused oxidative damage. While antioxidants are likely to be involved in heat acclimation and adaptation, evidence is limited to a few insect species and many questions remain open (Table 3).

Box 1: Glossary

# List of abbreviations

AKH Adipokinetic hormone ALDH Aldehyde dehydrogenase CAT Catalase GSH Glutathione GR Glutathione reductase GST Glutathione-S-transferase H<sub>2</sub>O<sub>2</sub> Hydrogen peroxide OH• Hydroxyl radical LDH Lactate dehydrogenase POD Peroxidases ROS Reactive Oxygen Species 2O•<sup>2–</sup> Superoxide anion SOD Superoxide dismutase TAC Total Antioxidant Capacity UCP Uncoupling proteins

# Outlook

By reviewing current evidence of antioxidants used by insects in response to high temperatures, we identified main hypotheses that remain to be evaluated to have a better comprehension of the antioxidant mechanisms used by insects to tolerate and adapt to increasing temperatures (Table 3).

## Credit author statement

Conceptualization: all authors. Writing: all authors. Writing, review and editing: all authors.

#### 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.

- Adaptation: Process by which a population or species develops higher fitness in an environment; it is the result of natural selection acting upon heritable variation over several generations.
- Aerobic metabolism: Process involves the breakdown of nutrients (such as carbohydrates, fats and proteins) to produce energy in the presence of oxygen.
- Antioxidant: Compound that retards or prevents oxidative damage to biomolecules such as lipids, proteins, carbohydrates and DNA.
- Free radical: An atom or a molecule that contains a free electron (usually represented by a dot) that renders it highly chemically reactive.
- Heat shock proteins: Group of proteins that help in the folding and repair of denatured proteins during stress.

Lipid peroxidation: Oxidative damage of lipids.

Oxidative damage: Damage to lipids, proteins and DNA caused by oxidative stress.

Total antioxidant activity/capacity (TAA/TAC): Measure of the ability of all the antioxidants to neutralize ROS.

Acclimation: A plastic phenotypic response that enables an organism to tolerate a change in a single factor (e.g., temperature) of its environment.

Heat stress: Physiological stress induced by an increase in temperature that can impair functioning and cause injury or death.

Metabolic depression: Significant slowdown in metabolic processes.

Metabolic rate: Rate at which organisms obtain, transform, and use energy in biological processes (i.e., survival, growth, and reproduction).

Oxidative stress: Unbalance between pro-oxidants and antioxidants in favor of the former, that when sustained over time may cause oxidative damage to cells or tissues. Prooxidant: Substance with potential to cause oxidative stress.

**Reactive Oxygen Species (ROS):** Oxygen containing molecules that are highly reactive with other biomolecules; some are free radicals (e. g.,  $20^{\bullet^2-}$ ) whereas others are not (H<sub>2</sub>0<sub>2</sub>). **Synergistic effects:** When two stressors have much higher effect when combined than the addition of their effects when separated.

Trade-off: When an increase in a fitness related trait has simultaneous detrimental effects in another fitness-related trait, thus preventing the organism from maximizing payoffs obtained from increasing both traits (e. g., high fecundity shortens life expectancy).

Uncoupling proteins (UCPs): Group of mitochondrial proteins that alter the rate of ATP synthesis linked to the electron transport chain. UCPs uncouple the oxidative phosphorylation process and allow the flow of protons through the mitochondrial membrane, decreasing ROS production.

#### Acknowledgements

AH was supported by a CONAHCYT-SECIHTI postdoctoral grant (CVU 597708). Dr. Brent Sinclair and two anonymous reviewers provided insightful comments that enriched this manuscript.

#### Data availability

No data was used for the research described in the article.

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