

**TEMPERATURE MODULATION OF THE EFFECTS OF REPETITIVE ANOXIA ON
POTASSIUM HOMEOSTASIS IN THE BRAIN OF *Drosophila melanogaster***

by

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Abstract

Oxygen can be limited at the environmental (*e.g.* flood-prone burrows) or cellular (*e.g.* stroke, heart attack) levels. O₂ deprivation in nervous tissue depolarizes cell membranes, incrementing extracellular potassium concentration ([K⁺]_o). Consequently, [K⁺]_o can be used to assess neural failure during anoxia. The effect of temperature on the maintenance of brain [K⁺]_o homeostasis in male and female *Drosophila melanogaster* (W1118) was assessed during repeated anoxic comas induced by N₂ gas. Brain [K⁺]_o was continuously monitored using K⁺-sensitive microelectrodes while body temperature was gradually increased/decreased using a Peltier plate. Once the desired temperature was reached (16°C/17°C, 23°C or 29°C/30°C), it was maintained for the rest of the experiment and the fly was subjected to repeated anoxic bouts. Repetitive anoxia resulted in a loss of the ability to maintain [K⁺]_o baseline at ~10 mM. In both sexes, the total [K⁺]_o baseline variation ($\Delta[K^+]_o$) was augmented at 30°C ($\Delta[K^+]_{o\text{ male}} = 119.2 \pm 21.9$ mM; $\Delta[K^+]_{o\text{ female}} = 51.2 \pm 8.1$ mM), whereas 16°C stabilized [K⁺]_o baseline for the duration of the experiment ($\Delta[K^+]_{o\text{ male}} = 17.5 \pm 4.1$ mM; $\Delta[K^+]_{o\text{ female}} = 16.9 \pm 6.8$ mM). Additionally, $\Delta[K^+]_o$ in males was significantly greater (114.3 ± 10.5 mM) than in females (36.1 ± 10.5 mM) at 23°C. Under reduced dehydration, experiments performed only in males showed the same trends although the $\Delta[K^+]_o$ values were considerably reduced at 17°C ($\Delta[K^+]_{o\text{ male}} = -1.0 \pm 1.3$ mM) and 23°C ($\Delta[K^+]_{o\text{ male}} = 17.3 \pm 1.5$ mM) and increased at 29°C ($\Delta[K^+]_{o\text{ male}} = 332.7 \pm 83.0$ mM). It was concluded that 1) N₂-delivery patterns consisting of long anoxia, short normoxia and high cycle frequency increased disruption of brain [K⁺]_o baseline maintenance, 2) males were more susceptible to repeated anoxia than females at room temperature, and 3) hypothermia had a protective effect on brain K⁺ homeostasis during repetitive anoxia. Male flies are suggested as a

useful model for examining deleterious consequences of O₂ reperfusion with extensive application on therapeutical treatment of stroke or heart attack.

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List of Abbreviations

[ATP] _i	Intracellular ATP concentration
[Ca ²⁺] _i	Intracellular calcium concentration
[K ⁺] _o	Extracellular potassium concentration
AD	Anoxic depolarization
CNS	Central nervous system
Δ[K ⁺] _o	Total [K ⁺] _o baseline variation
ΔPOR	Total percentage of recovery variation
ECS	Extracellular space
HL ₃	Hemolymph-like solution
MER	Male enlarged regions
MR	Metabolic rate
n	Sample size
PGP	P-glycoproteins
POR	Percentage of recovery
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SD	Spreading depression
SEM	Standard error of the mean
Sr	Surge
TEC	Thermoelectric cooler
TTR	Time to recovery

TTS Time to surge

WLR Water loss rate

Chapter 1

Introduction

Oxygen is an important element whose presence became significant on Earth for the first time approximately 2.5 billion years ago (Farquhar *et al.*, 2000). Consequently, such an event allowed the evolutionary development of one of the most important metabolic processes in cells: aerobic respiration. Thanks to the high efficiency of aerobic respiration, the ATP demand of the cell can be met in order to maintain other metabolically important processes like synthesis of biomolecules or transport of ions across membranes. Therefore, O₂ has become an important molecule for the proper and coordinated functioning of cells, and by extension for the survival of more complex organisms like metazoans.

Oxygen availability can be limited at the environmental or cellular levels. Environmentally, animals can experience periods of hypoxia/anoxia because of flood-prone burrows or decreased partial O₂ pressure (pO₂) with increased altitude (Hoback and Stanley, 2001). Cellularly, availability of O₂ can be reduced by heavy exercise or by disrupting physiological events like heart attack or stroke (Hochachka, 1998). Furthermore, inadequate O₂ supply to nerve tissue in an organism and subsequent oxidative stress caused by reperfusion can cause detrimental consequences manifested at a systemic level. Thus, dealing with reduced/absent O₂ supply and oxidative stress during reperfusion are factors cells and organisms must face in order to maintain adequate performance and survival.

As a result of natural selective pressures, some vertebrate (Buck and Pamenter, 2006) and invertebrate (Azad and Haddad, 2009) species have developed molecular and physiological

mechanisms to tolerate and cope with low or null O₂ levels for a prolonged amount of time (from hours to months) with no apparent harmful consequences. On the other hand, most mammals cannot tolerate hypoxia/anoxia without undergoing severe cellular damage or death (Hermes-Lima and Zenteno-Savín, 2002). However, despite the importance O₂ has for proper cellular and organismal performance, we still do not have a complete understanding of the molecular and physiological processes that take place during hypoxia/anoxia or the strategies that protect tolerant species during the absence of O₂ and subsequent reperfusion. Understanding such processes will not only improve our knowledge on the subject, but also allow development of therapeutic treatments aimed to reduce cellular damage caused by physiological disruptions that limit O₂ availability in a constant or intermittent fashion. Consequently, detrimental effects of disruptive events related to constant hypoxia (*e.g.* asthma, ischemia and traumatic brain injury) and intermittent hypoxia (*e.g.* sleep apnea, central hypoventilation syndrome and intermittent vascular occlusion in sickle cell anemia) could be treated and lessened (Azad *et al.*, 2009).

1.1 K⁺ in the Nervous System: Accumulation and Clearance Mechanisms

The nervous system of any complex organism is composed mainly of two types of cells: neurons, which are in charge of sensing, integrating and responding to internal and external stimuli, and glial cells, which provide the appropriate conditions for neurons to work properly (Freeman and Doherty, 2006). Neuronal cell bodies form the outer layer of *Drosophila's* brain (cortex) and their axons and dendrites project internally to form the neuropile. Surface, cortex and neuropile glial cells constitute the glial component in the fly's brain (Pereanu *et al.*, 2005). Surface glial cells form the blood brain barrier, which acts as a selective boundary between the

brain and the hemolymph, whereas cortex and neuropile glial cells ensheath neuronal cell bodies, axonal tracts and dendritic compartments in the brain (Stork *et al.*, 2008).

In the nervous system, two elements are mainly responsible for maintaining the dynamics of ions across the cell membrane, namely ion pumps and ion channels. The brain is an energetically expensive organ that spends about 20% of the energy produced by aerobic metabolism (Won *et al.*, 2002). Most of this energy accounts for the maintenance of ion gradients across the cell membrane, a condition that is indispensable to guarantee communication among neurons. Ion pumps are important elements of the cell membrane that use the energy of adenosine triphosphate (ATP) or the concentration gradient of an ion in order to move a specific ion against its concentration gradient. In the particular case of K^+ , the Na^+/K^+ ATPase is a fundamental contributor to maintaining differential K^+ concentrations across the membrane (Ransom and Philbin, 1992). Consequently, all the potential energy stored by the action of ion pumps is transformed into electrical impulses thanks to the flow of ions through ion channels. In vertebrates, several K^+ ion channels have been described according to their biophysical properties (Verkhratsky and Steinhäuser, 2000): voltage-activated K^+ channels (K_V), Ca^{2+} -activated K^+ channels (K_{Ca}), two-pore domain K^+ channels (K_{2P}), and inwardly rectifying K^+ channels (K_{ir}). During anoxia, each one of these channels probably contributes in different proportions to increase extracellular K^+ concentration ($[K^+]_o$). ATP depletion during total lack of O_2 produces failure of the Na^+/K^+ pump and membrane depolarization (anoxic depolarization, AD). Such depolarization, produced by the flow of Na^+ down its concentration gradient, activates K_V channels that contribute to the escape of K^+ into the extracellular space (ECS) (Ransom and Philbin, 1992). Additionally, it also augments intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), probably by reverse operation of the Na^+/Ca^{2+} exchanger (Stys *et al.*, 1991), release of glutamate in the synaptic cleft and subsequent activation of NMDA receptors (Nicholls and Atwell 1990),

activation of voltage-dependent Ca^{2+} channels (Won *et al.*, 2002), and/or activation of transient receptor potential (TRP) channels (Agam *et al.*, 2000). Consequently, K_{Ca} channels will open and more K^+ will flow out of the cell. In *Drosophila's* central nervous system (CNS), slowpoke K_{Ca} channels do not necessarily need Ca^{2+} binding to be activated, but this facilitates their response to membrane depolarization (Zeng *et al.*, 2006). In the cell membrane, $\text{K}_{2\text{P}}$ channels generate a leak K^+ current also known as background K^+ current. During normal conditions, the purpose of these K^+ conductances is to stabilize the negative resting membrane potential and counterbalance depolarization (Enyedi and Czirják, 2010). Such functions are achieved thanks to their regulatory mechanisms, which involve phosphorylation and second messenger pathways (Talley *et al.*, 2003). However, with the Na^+/K^+ ATPase neutralized by the lack of ATP, K^+ flows down its concentration gradient through these channels, increasing $[\text{K}^+]_o$. Part of the knowledge acquired about regulation and gating of $\text{K}_{2\text{P}}$ channels has been gathered thanks to studies on the *Drosophila* homologue KCNK0 (Mathie *et al.*, 2010). ATP-sensitive K^+ channels (K_{ATP} , a type of K_{ir} channel) are opened by a decrease in intracellular ATP concentration ($[\text{ATP}]_i$) (Ransom and Philbin, 1992). Although they play a protective role during ischemia and hypoxia by suppressing neuronal activity through an increased K^+ -membrane permeability (Jiang *et al.*, 1994), the total disruption of the differential transmembrane concentration gradients causes K^+ outflow through these channels. One of the components of mammalian K_{ATP} channels is the sulphonylurea receptor (SUR) subunits. In *Drosophila's* heart cells, the homologue dSUR gene encodes an ATP-binding cassette transmembrane protein (Akasaka *et al.*, 2006) functionally similar to the mammalian counterpart. Furthermore, larval corpora cardiaca cells have been found to express cognates of sulphonylurea receptors and K^+ channels, proteins that comprise K_{ATP} channels in mammalian glucose sensing cells (Kim and Rulifson, 2004).

Interestingly, glial cells are also responsible for removing the excess of K^+ in the ECS (“ K^+ siphoning”) (Orkand *et al.*, 1966), and K_{ir} channels seem to be fundamental for such a role in $[K^+]_o$ regulation. These channels are open at hyperpolarized membrane potentials and their K^+ conductance decreases with depolarization (Hibino *et al.*, 2010). Such effect is the result of Mg^{2+} ions and endogenous polyamines plugging the channels at positive membrane potentials, thereby producing a decrease in outward current (Lopatin *et al.*, 1995). In *Drosophila*, neuropile glia are analogous in function to vertebrate oligodendrocytes, which ensheath axons in CNS and provide trophic support to neurons. Additionally, cortex glia resemble vertebrate astrocytes, which provide synapse modulation and trophic support to neurons (Freeman and Doherty, 2006). Evidence of “ K^+ siphoning” was found in salamander optic nerve, where isolated Müller cells and astrocytes displayed a heterogeneous distribution of K_{ir} channels, presumably to increase the efficacy of $[K^+]_o$ buffering (Newman, 1986). Moreover, K_{ir} channels are the major component described for both culture and *in situ* preparations of vertebrate oligodendrocytes (reviewed by Verkhratsky and Steinhäuser, 2000). On the invertebrate side, three cDNA encoding K_{ir} channels ($dK_{ir,I}$, $dK_{ir,II}$ and $dK_{ir,III}$) were isolated in *Drosophila* and their protein sequences showed homology with human K_{ir} channel subunits; furthermore, $dK_{ir,II}$ transcripts were found mainly in the head of adult flies and heterologous expression of $dK_{ir,I}$ and $dK_{ir,II}$ in *Drosophila* S2 cells produced typical inwardly rectifying K^+ currents (Döring *et al.*, 2002). The presence of K_{ir} channels in the head of the fly and the functional analogy of neuropile and cortex glia with vertebrate oligodendrocytes and astrocytes, respectively, supports the K^+ -buffering role of glial cells in clearance mechanisms.

1.2 Oxidative Stress and AD Repetition

In an environment with absence or reduced supply of O₂, harmful metabolites can be produced and damage generated by anoxia/hypoxia itself can be exacerbated. On the one hand, alternate energy-producing metabolic pathways like glycolysis are activated. However, glycolysis in the absence of O₂ produces lactate, a side product that decreases the intracellular pH causing acidosis. (Ransom and Philbin, 1992). Some studies suggest that acidosis mediates neuronal apoptosis following hypoxic-ischemic insults (reviewed by Won *et al.*, 2002). Insects have a low capability for anaerobic metabolism (Le Corronc *et al.*, 1999) and, for instance, do not produce detectable amounts of lactate in their flight muscles (Wegener, 1996; Wegener *et al.*, 1996). Nonetheless, accumulation of lactate, acetate and alanine has been detected in *Drosophila* flight muscles during hypoxia (Feala *et al.*, 2007), and an increase in respiratory quotient (RQ, an indicator of incremented anaerobic metabolism) was reported under a pO₂ equal to or lower than 1.2KPa (Van Voorhies, 2009). On the other hand, production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) during hypoxia/anoxia and reperfusion can cause considerable cell damage, even worse than the one caused by the lack of O₂ itself (Idris *et al.*, 2005). ROS are O₂-containing molecules with a single unpaired electron in their outer shell, which are important to maintain the physiological condition of an organism (Won *et al.*, 2002). The superoxide ion (O₂⁻) is the primary radical of mitochondrial origin; it is produced by complexes I and III when electrons leak from the respiratory chain and reduce O₂ (Jastroch *et al.*, 2010). O₂⁻ is generally cleared to H₂O by the enzymes superoxide dismutase and glutathione peroxidase (Cao *et al.*, 1988). Nevertheless, an excess produced by disturbances like reperfusion after a hypoxic episode can damage the mitochondrial electron transport chain and amplify the generation of more ROS (Radi *et al.*, 1994). Additionally, the Krebs' cycle enzyme α -ketoglutarate dehydrogenase was found to be a significant contributor to ROS production in

normally functioning mitochondria (Starkov *et al.*, 2004), probably exacerbating even more the adverse effects of reperfusion. Eventually, the high reactivity of ROS causes oxidation of DNA, lipids and proteins in the cell (Murphy, 2009). RNS are formed by the production of nitric oxide (NO) and its subsequent reaction with O_2^- to produce peroxynitrite (ONOO⁻), a damaging molecule that seems to be involved in cell deterioration during ischemia and reperfusion (Radi *et al.*, 1994). The increased $[Ca^{2+}]_i$ generated during hypoxia/anoxia activates nitric oxide synthase (NOS), an enzyme that catalyzes the conversion of L-arginine and O_2 into NO and citrulline (Alderton *et al.*, 2001). NO has an important function in a number of neuronal responses, including learning and memory (Wang and Robinson, 1997). Nevertheless, it can be neurotoxic depending on the isoforms and localization of the activated NOS (Shim *et al.*, 2001). For instance, NO produced in the neurons can diffuse freely across the membranes and cause degeneration of surrounding neurons by activation of the poly (ADP-ribose) synthetase (PARP), which depletes reserves of nicotinamide adenine dinucleotide (NAD⁺) (Zhang *et al.*, 1994) and inhibits ATP synthesis (Brookes *et al.*, 1999). Additionally, the formation of ONOO⁻ causes subsequent production of OH⁻ and NO₂, two very reactive molecules that generate tissue damage by DNA, protein and lipid oxidation, as well as DNA and protein nitration (Beckman, 1996). Besides contributing to the production of RNS, the increase in $[Ca^{2+}]_i$ experienced by neurons during metabolic challenge can also activate prooxidant pathways, like phospholipases (Au *et al.*, 1985) or xanthine oxidases (Chambers *et al.*, 1985), and increase the production of ROS by collapsing the mitochondrial membrane potential (Zhang *et al.*, 1990).

Drosophila is an anoxia-resistant organism that can endure 4 hours of total lack of O_2 and recover without any apparent locomotory impairment (Krishnan *et al.*, 1997). Since adult flies do not encounter anoxia as a selective pressure in their natural environment, such tolerance must be an adaptive remnant from the larval stage. Larvae are found in decaying plant

matter with their spiracles at the food-air interphase; consequently, the boundary layers where O₂ is obtained may be hypoxic/anoxic given that rotting plants support metabolically active bacteria and fungi that can compete for the resource (Frazier *et al.*, 2001). Furthermore, larvae bore into the core of decaying fruit where O₂ availability may not be optimal and flash floods can happen because of rain. During hypoxia, *Drosophila* nerve cells hyperpolarize, presumably by an increment in K⁺ outward conductance, in order to decrease their excitability and save energy (Gu and Haddad, 1999). Additionally, anoxia induces a state of torpor where locomotory coordination is lost (Krishnan *et al.*, 1997). Therefore, *Drosophila* enters a hypometabolic state in which ATP demand is reduced to meet the decreased ATP supply. Such reduction in O₂ consumption has been previously observed in the fly (Krishnan *et al.*, 1997; Van Voorhies, 2009) and in anoxia-tolerant vertebrates like the turtle *Pseudemys scripta* (Simon and Robin, 1970) and the crucian carp *Carassius carassius* (Johnston, 1975). Unfortunately, the mechanisms involved in reduction of the metabolic rate are not yet fully understood (Zhou *et al.*, 2008).

Despite its resistance to O₂ deprivation, it is possible to force reperfusion in *Drosophila* (Lighton and Schilman, 2007) and use it as a model to understand mechanisms governing its anoxia tolerance and its resilience when subjected to AD iteration. In order to inflict reperfusion damage, the anoxia/normoxia pattern is important. Very short bouts of anoxia (15-20 seconds of anoxia/2 minutes of normoxia, 5-10 cycles) applied on the mutant fly strain W1118 showed no effect on the metabolic rate (MR) (Van Voorhies, 2009). On the other hand, in the common cockroach *Periplaneta americana*, reoxygenation-induced hyperpolarization (an indicative of Na⁺/K⁺ ATPase reactivation) of the “fast” coxal superior motoneuron was reduced by increased frequency of repetitive hypoxia combined with short recovery time (Le Corrionc *et al.*, 1999). Furthermore, 20 minutes of anoxia and 1 minute of normoxia during 5 cycles disrupted spiracular control and subsequently increased water loss rate (WLR) in *Drosophila* Oregon-R wild

type flies (Lighton and Schilman, 2007). Therefore, the fly appears to be affected by short bouts of normoxia during long anoxic periods

1.3 Temperature and Its Role in Neural Protection during AD repetition

Temperature is an important environmental variable that strongly affects almost every aspect of metabolic performance in ectotherms (Schilman *et al.*, 2011). Consequently, *Drosophila's* metabolic rate (MR) can be easily manipulated by changing the temperature of its surroundings. Such a feature permits assessment of MR effects on neural failure during repeated reperfusion. Provided that *Drosophila's* MR has a Q_{10} of 2.2 (Schilman *et al.*, 2011), high temperatures are expected to affect damage and recovery rates during anoxia and reperfusion. In the course of anoxia, high temperature probably increases buildup of anaerobic metabolites, like alanine, acetate and lactate (Feala *et al.*, 2007). When O_2 supply is restored, ROS/RNS damage accumulation may increase as well as the activity of recovery and repair mechanisms (*e.g.* ATP production, enzymatic repair). Taking into account that the Q_{10} value for ion channel currents is approximately equal to 2 and that the Q_{10} value for ion pump activity is much greater than 2 (Buck and Pamerter, 2006), rapid reestablishment of ionic gradients is expected to happen without being radically affected by leak ion currents. Additionally, O_2 diffusivity remains barely changed with temperature whereas metabolic demand during recovery is incremented at high temperatures (Frazier *et al.*, 2001; Lighton, 2007). Therefore, O_2 may not be efficiently delivered to meet the recovery energy demand during reperfusion.

On the other hand, low temperature presumably decreases accumulation of anaerobic metabolism end products during anoxia. Moreover, during reperfusion hypothermia could also decrease buildup of ROS/RNS and slow down recovery and repair processes. Given the disparity

in Q_{10} values between ion channel currents and ion pump activity, ion pumps may not be able to counterbalance the action of background ion currents and a certain level of depolarization would be expected. However, hypothermia possibly delays consumption of endogenous antioxidant enzymes and energy metabolites (Zhang *et al.*, 2011), enhancing performance of recovery mechanisms. This aspect and the possible reduction of ROS/RNS/anaerobic metabolite buildup probably underlie the protective character of low temperatures during metabolic challenge. Although the mechanisms of hypothermia protection are not clear, mounting evidence in mammalian models shows its promising role in the therapeutic treatment of ischemia. Low temperatures (15-20°C) during mesenteric ischemia reperfusion in rats reduced histologic injury; additionally, they prevented the activation of nuclear factor kappa-B (NF- κ B) and expression of inducible nitric oxide synthase (iNOS), elements involved in inflammatory responses (Hassoun *et al.*, 2002). Furthermore, mild hypothermia (32-33°C) enhanced generation of neuronal cells and increased expression of the apoptosis regulator Bcl-2 after global ischemia in striatum of neonatal rat brain (Zhang *et al.*, 2011). Although clinical trials have not rendered conclusive results about hypothermia protection of the human brain during some metabolically challenging situations, like brain surgery (Milani *et al.*, 2011), it has been shown that a decrease in temperature reduces ischemia and traumatic brain injury damage, glutamate release and free radical production (Busto *et al.*, 1987; Globus *et al.*, 1995; Marion *et al.*, 1997; Huh *et al.*, 2000).

1.4 Neural Responses in Anoxia-Tolerant Organisms: the Ion Channel Arrest

Hypothesis

The ion channel arrest hypothesis (Hochachka, 1986) states that anoxia-tolerant organisms reduce cell sensitivity by decreasing membrane permeability to ions during anoxia. Indirect evidence supporting this hypothesis is the maintenance of membrane potential while Na^+/K^+ pump activity decreases 75% in anoxic turtle hepatocytes (Buck and Hochachka, 1993). Additionally, direct evidence shows that there is a decrease of 62% in NMDA receptor single channel open time within 15 min of the onset of anoxia in turtle neurons (Bickler *et al.*, 2000). A possible adenosine-mediated mechanism of ion channel arrest has been reviewed by Buck and Pamerter (2006). Adenosine, through adenosine receptors A1 (A1R) and A2 (A2R), has a depressant effect on neural activity. Anoxia releases adenosine that binds to G protein-coupled A1Rs. Subsequently, G-protein increases $[\text{Ca}^{2+}]_i$ by activation of phospholipase C (PLC) and inositol triphosphate (IP_3) pathways. This stimulates the action of Ca^{2+} binding protein calmodulin, which regulates diverse protein kinases and phosphatases. Consequently, variation in the phosphorylation state of ion channels can cause permeability changes, and K_{2p} channels appear to be a possible target of this mechanism. In cultured rat cerebellar granule neurons, inhibition of acid sensitive K_{2p} channels (TASK) prevented hypoxic depolarization indicating that background K^+ channel arrest was responsible for the increased tolerance to low pO_2 . An adenosine receptor (dAdoR, Wu *et al.*, 2009) and K_{2p} channels (*e.g.* KCNK0, Mathie *et al.*, 2010) have already been found in *Drosophila*, suggesting that leak K^+ channel inactivation could be, at least in part, underlying the fly's remarkable tolerance to anoxia. Furthermore, $[\text{Ca}^{2+}]_i$ increase produced by ADs could also be enhancing the activity of the previously described mechanism. Inactivation of K^+ conductances can also take place through the adenosine monophosphate-activated protein kinase (AMPK) pathway. AMPK is a key enzyme playing an active role in energy

sensing and response during metabolic stress (Turnley *et al.*, 1999). It detects variation of the AMP/ATP ratio and initiates a series of metabolic responses that favor ATP-generating catabolic pathways and inhibit energy storing mechanisms, eventually increasing the availability of ATP for basic cellular processes (Li and McCullough, 2010). AMPK was found to inhibit mouse and rat TREK channels (a type of K_{2P} channel) during hypoxia in transfected HEK-293 cells (Kreneisz *et al.*, 2009). Nevertheless, the role of AMPK as a protective agent seems to be complex, since its inhibition with compound-C prevented ouabain-induced surges in the metathoracic ganglion of the migratory locust, *Locusta migratoria* (Rodgers-Garlick *et al.*, 2011), and it was found to mediate sarcolemmal K_{ATP} channel activation and recruitment in mouse cardiomyocytes (Sukhodub *et al.*, 2007).

On the other hand, it is important to consider that lack of O_2 can activate metabolic pathways whose action can increment membrane permeability, thereby causing increased excitability and subsequent susceptibility to anoxia. NO produced during anoxia increases the production of cyclic guanosine monophosphate (cGMP) through interaction with soluble guanylyl cyclase (sGC). Consequently, cGMP increase can accomplish physiological effects by interacting with different receptor proteins like protein kinase G (PKG). PKG starts a series of signal transduction cascades by phosphorylation of different substrates (Butt *et al.*, 1994). In *Drosophila* adults, PKG-mediated activation of protein phosphatase 2A (PP2A) generated activation of K^+ conductances (presumably K_{ATP} channels), increasing whole-animal vulnerability to anoxia (Dawson-Scully *et al.*, 2010).

1.5 $[K^+]_o$ and Its Importance as an Indicator of Neural Failure

In mammals, spreading depression (SD) is a massive redistribution of ions between intracellular and extracellular compartments, coinciding with a near complete brain cell depolarization that propagates through grey matter (Somjen, 2001). Generally a benign phenomenon, SD can be elicited by mitochondrial blockers, inhibition of Na^+/K^+ ATPase, simulated ischemia, KCl application and hyperthermia (reviewed in Rodgers *et al.*, 2010). Additionally, it has also been associated with a rise in $[K^+]_o$, which is cleared when the stressor is removed (Rodgers *et al.* 2007). However, repetitive SD in the penumbra (healthy tissue around a brain infarct) further stresses the tissue, generally producing cell swelling (Andrew and MacVicar, 1994), a stable elevated $[K^+]_o$ (Branston *et al.*, 1977), dendritic beading (Obeidat *et al.*, 2000) and necrosis. Mammalian SD shares many characteristics with SD events elicited in the metathoracic ganglion of the migratory locust (*Locusta migratoria*) (Rodgers *et al.*, 2010). Moreover, studies in the locust have used $[K^+]_o$ as a way to assess neural failure in this ganglion during SD repetition (Armstrong *et al.*, 2009; Rodgers-Garlick *et al.*, 2011). Likewise, it is possible that anoxia elicits SD-like events in *Drosophila's* brain as well (Armstrong *et al.*, 2011). Consequently, assessment of brain $[K^+]_o$ can be used to evaluate the integrity of the fly's brain physiology while reperfusion damage is inflicted by repetitive anoxia. Provided that lack of O_2 causes failure of the Na^+/K^+ ATPase (Ransom and Philbin, 1992), all the ions flow down their concentration gradients through the cell membrane and nerve cells depolarize. Therefore, every time the fly is subjected to anoxia, brain cells undergo an AD and $[K^+]_o$ rises (Armstrong *et al.*, 2011). On the other hand, once O_2 supply is restored, the Na^+/K^+ pump probably re-establishes the transmembrane ionic gradient and $[K^+]_o$ returns to baseline. Inability of *Drosophila's* brain to reach the initial $[K^+]_o$ baseline after repeated ADs is reminiscent of the disruption observed in

the mammalian penumbra, evidenced as a sustained increment in $[K^+]_o$ baseline (Armstrong *et al.*, 2011).

1.6 Importance of *Drosophila* as a Study Model

Invertebrates make very useful models given that they are easily reared in the lab and have a short life cycle. Additionally, the absence of sufficient evidence of suffering in most of them (Eisemann *et al.*, 1984; Elwood *et al.*, 2009) allows vivisection and sacrifice of large numbers without hindrance from ethical and regulatory issues. Consequently, the non-sentient nature of most invertebrates has made possible implementation of semi-intact preparations, which allow pharmacological modulation of the nervous system and assessment of the effects on other physiological processes. For instance, it is possible to apply pharmacological agents on the ventral nerve cord of the migratory locust (*Locusta migratoria*), and monitor ventilatory central pattern generator performance in the metathoracic ganglion while assessing ventilation via an abdominal muscle (Armstrong *et al.*, 2009; Rodgers-Garlick *et al.*, 2011). Furthermore, significant discoveries in neuroscience have been performed using invertebrate models: the basis of action potential generation was first described in the squid giant axon (Hodgkin *et al.*, 1949) and the foundation for long-term potentiation and memory was first discovered in the sea slug, *Aplysia* (Bailey and Kandel, 1993).

Among invertebrate models, *Drosophila melanogaster* excels because of the power of its molecular and genetic tools. Additionally, the large and continuously growing community of scientists working with this model constitutes an incomparable synergy that incessantly develops new tools, increases the genetic, biological, physiological, and ethological information available, and provides help to cope with issues. Moreover, *Drosophila* has other useful

characteristics that make it a suitable all-purpose model (Haddad and Ma, 2001): 1) small number of chromosomes, 2) generation time of 10 days at 25°C, 3) generation size of 200-300 individuals per female, 4) enormous number of mutant lines and chromosomal markers, 5) molecular tools such as libraries are available, 6) there are tools for the study of cell or organ physiology, 7) P elements can be used for cloning and mapping purposes, and 8) during evolution, genes have been mostly conserved from *Drosophila* to humans. For this particular project, the ectothermic nature of the fly also allowed modifying body temperature just by changing the temperature of the fly's surroundings (Schilman *et al.*, 2011), and the tracheal respiratory system permitted immediate change of O₂ supply (O₂ is delivered 200,000-fold more rapidly in air than in aqueous media like blood) (Lighton and Schilman, 2007). Additionally, the effects of repetitive anoxia in the fly's brain are mostly decoupled from any influence of the circulatory system (Schilman *et al.*, 2011), allowing separate analysis of the different components involved in the response. The fly shares 65-70% of disease genes present in humans (Azad *et al.*, 2009) and it has been useful in establishing the relationship of these genes to a particular disease (Fortini *et al.*, 2000) and also in elucidating how these genes can induce the disease (Fortini and Bonini, 2000). Furthermore, increasing number of studies have used *Drosophila* as a model organism of brain diseases (*e.g.* Parkinson's, Alzheimer's) and central nervous system injury (reviewed by Jeibmann and Paulus, 2009). A remarkable example of how knowledge obtained from the fly can be applied on mammals was the cloning and increased expression in *Drosophila* of a gene responsible for the synthesis of trehalose-6-phosphate synthase (*tps1*) (Chen *et al.*, 2002), and subsequent successful transfection of the gene to HEK-293 cells (Chen *et al.*, 2003). In the fly, overexpression of this gene conferred increased resistance to anoxia, a trait that was also transmitted to transfected HEK-293 cells.

1.7 Anatomical, Physiological and Morphological Dimorphisms in *Drosophila*

Physiological and behavioral studies including males and females generally pool them together for data analysis (Krishnan *et al.*, 1997; Lighton, 2007; Van Voorhies, 2009), suggesting that differences between sexes are not significant enough to treat them separately. Nevertheless, *Drosophila* displays very distinct sexually dimorphic behaviors (Stockinger *et al.*, 2005) that could be produced by anatomical and morphological brain differences. Studies in the past found few small structural brain dimorphisms (Rein *et al.*, 2002; Jefferis *et al.*, 2007), the most remarkable one being the presence of 3 olfactory glomeruli in the males, 2 of which were linked to sex-specific odor processing (Kondoh *et al.*, 2003; Stockinger *et al.*, 2005). However, remarkable contribution to this field was made by Cachero *et al.* (2010), who described considerably different volumetric regions between male and female brains. Male enlarged regions (MER) were in average 41.5% larger than the female counterparts. Contrastingly, female enlarged regions (FER) were only 17.9% bigger than similar regions in males. Among the MER, three previously unidentified neuropile regions were described: the protocerebral arch, the lateral junction and the ring. Additionally, females lacked most of the neuronal processes that were contained within the MER suggesting that the increase in volume was caused by an increment in the number of neurons or their arborizations.

A factor that could underlie physiological dimorphisms in *Drosophila* is the possibility of differences in MR. Females seem to live longer than males (Lin *et al.*, 2011), suggesting dissimilarities in nutrient demand and energy allocation. Females probably invest their resources in egg production and egg laying, having a more sedentary life style that apparently does not affect their longevity. On the other hand, males seem to assign more resources to increasing mating success, thereby adopting a more active life style and probably reducing their lifespan.

Finally, body size is one of the most conspicuously dimorphic characters between males and females. Males are usually about 77% the size of a female (Lighton, 2007). Consequently, changes in the environment are expected to affect males more easily, rendering them more sensitive to external stressors. This is an important aspect to take into account when performing physiological experiments using the fly as a model.

1.8 Anoxia and Its Influence in Brain Dehydration in *Drosophila*

Water is an extremely important component of cells that provides the appropriate medium for all molecular interactions involved in the adequate functioning of any living tissue. Thus, its preservation is vital for every organism living on dry land, and different adaptations to prevent its loss have evolved. Insects breathe by means of a complex network of tubules (tracheae) that communicate with the external environment through valves (spiracles). As a generalized pattern, each body segment bears one spiracle on each flank and muscles control its degree of aperture. Consequently, the amount of oxygen delivered to the tissues can be managed depending on its availability in the environment (Hetz and Bradley, 2005). Given that the tracheal system is saturated with water vapor, the spiracular muscles are responsible for maintaining the hydric integrity of the insect and any malfunction of CNS elements that control them can result in death by dehydration (Mellanby, 1935). In *Drosophila* there is a loss of spiracular control during long anoxic periods (> 60 min) or repetitive anoxia/normoxia periods (Lighton and Schilman, 2007). Male Oregon-R flies with a mean mass of $804 \pm 10 \mu\text{g}$ underwent an increase in WLR from $61.7 \pm 3.4 \mu\text{g} / \text{h}$ to $155.3 \pm 12.2 \mu\text{g} / \text{h}$ when subjected to 20 min of anoxia and 1 min of normoxia for 5 cycles. At the end of the second cycle the increment in WLR became evident, suggesting faulty spiracular control. Such a phenomenon is remarkably

important taking into consideration that a fly would be losing almost one fourth of its mass per hour because of dehydration during repetitive anoxia. However, during normoxia there is also a baseline WLR because *Drosophila* cannot completely close its spiracles (Williams and Bradley, 1998). Consequently, depending on the humidity of the environment water can flow into or out of the fly, even during normoxia, causing different physiological effects. In the fly's brain excessive hydration may produce two effects: cell swelling and subsequent reduction of the ECS because of the hypotonic nature of water, and dilution of ions present in the ECS. On the other hand, dehydration can reduce the amount of solvent present in the ECS.

1.9 Genetic Basis of Repetitive Anoxia Resistance in *Drosophila*

Drosophila is an anoxia-tolerant organism that can cope with total lack of O₂ during 4 hours, recovering without evident locomotory impairment (Krishnan *et al.*, 1997). In the present study, the fly displayed remarkable resilience to repetitive anoxia and the subsequent oxidative stress produced by repeated reperfusion. Genetic responses during lack or reduced supply of O₂ are complex, and depend on the duration, intensity and frequency of the anoxic stimulus (Liu *et al.*, 2006; Azad *et al.*, 2009). Adult flies exposed to severe hypoxia (0.5% O₂) for 1 hour showed augmented transcription abundance of 20 genes whereas exposure during 6 hours incremented mRNA for 79 genes; additionally, flies under mild hypoxia (5% O₂, 6 hours) displayed increased transcript levels for 47 genes, 34 of which were particularly expressed under this paradigm (Liu *et al.*, 2006). Interestingly, some of the transcripts upregulated after 1 hour of severe hypoxia were not evident after 6 hours indicating the presence of feedback and timing mechanisms modulating the transition from an acute to a long term adaptation response. Furthermore, the exclusive abundance of certain transcripts during mild hypoxia suggests activation of different

molecular pathways on a stimulus-intensity basis. Besides the expected upregulation of genes related to reducing the impact of oxidative stress (*e.g.* peroxidase activity) or increasing repair mechanisms (*e.g.* response to unfolded proteins), genes involved in response to other stressors, like heat shock proteins (*e.g.* HSPs), immune response genes (*e.g.* relish) and cold response genes (*e.g.* frost), are also expressed during constant severe hypoxia; moreover, HSPs are predominantly expressed under this anoxia paradigm (Liu *et al.*, 2006; Azad *et al.*, 2009). This portrays how intricate physiological changes are during hypoxia, to a point that multiple stress response pathways become involved. On the other hand, overexpression of multidrug resistance proteins (*i.e.* Mdr49, Mdr50) and the gene 1(2)08717 is exclusive during repetitive hypoxia; furthermore, there is no importance of HSPs for this anoxia paradigm and their overexpression is only relevant for survival during constant severe hypoxia (Azad *et al.*, 2009). However, it has been shown in *Drosophila* that targeted expression of Hsp70 in glial cells confers a protective effect, evidenced as an increase in brain $[K^+]_o$ baseline maintenance, during repetitive anoxia (Armstrong *et al.*, 2011). Methodological differences could have been responsible for the contrasting results: Azad *et al.* (2009) exposed flies to 4 minutes of anoxia and 4 minutes of normoxia during 7 cycles, and ramped the O₂ supply through the transition from normoxia to anoxia (10 minutes) and vice versa (1 minute). On the other hand, Armstrong *et al.* (2011) used a pattern consisting of 2.5 minutes of anoxia and 4 minutes of normoxia for 13 cycles, and any O₂- supply variation was instantaneous. Given the dependence of gene expression on the anoxia paradigm, it is possible that a gradual decrease/increase of O₂ and differences in the anoxia/normoxia timing and cycle frequency could have caused a different response. Additionally, *Drosophila* larvae exposed to heat shock undergo upregulation of Mdr49 (Tapadia and Lakhotia, 2005), suggesting a link between HSPs and expression of multidrug resistance genes.

The mechanisms through which 1(2)08717 and multidrug resistance genes underlie *Drosophila*'s resistance to repetitive hypoxia are not well understood (Azad *et al.*, 2009). The Mdr49 gene codes for a group of transmembrane proteins (P-glycoproteins, PGP) that pump drugs out of the cell and keep their concentrations below cytotoxic levels (Tapadia and Lakhotia, 2005). Furthermore, PGP expression in the capillary endothelial cells of the human brain indicates potential importance in keeping toxins out of the system (Arceci *et al.*, 1988). Given that *Drosophila* multidrug resistance genes (dMdr) are approximately 50% similar at the nucleotide level to their mammalian counterparts (Gerard *et al.*, 1993), it is possible that their overexpression during repetitive AD could be responsible for efflux of ROS/RNS and anaerobic metabolites in the brain, thereby increasing the fly's tolerance to reperfusion. Additionally, Mdr49 is a putative target of the ADAR gene (Xia *et al.*, 2005), which encodes a double-stranded RNA-specific adenosine deaminase that alters protein structure and functions through RNA editing (Chen *et al.*, 2009). In the brain of mammals the ADAR gene is responsible for changes in functional properties of neurotransmitter receptors, ion channels and ion transporters (reviewed by Schaub and Keller, 2002). In *Drosophila*, dADAR has a regulatory function in the expression of genes encoding ROS scavengers (Chen *et al.*, 2004). Furthermore, mutant flies lacking the dADAR gene showed increased sensitivity to hypoxia and premature neurodegeneration (Xia *et al.*, 2005). This indicates that, during AD iteration, Mdr49 is indicative of the action of dADAR, which would be responsible for the increased resilience of the fly to repeated anoxic insults. On the other hand, the role of the 1(2)08717 gene is not as clear as the role of the Mdr49 gene. 1(2)08717 has homology with the human sialin protein, which is related to lysosomal storage diseases, like Salla disease (Laridon *et al.*, 2008), and has been found to be upregulated by hypoxia in cultured cancer cells (Yin *et al.*, 2006). Moreover, the sialin protein seems to regulate lysosomal pH through anion conductances or coupled movement of protons

(Wreden *et al.*, 2005). Although it is not clear if lysosomal pH regulation underlies increased tolerance to repeated hypoxia, gene mutations associated with lysosomal trafficking pathways in *Drosophila* produce synaptic dysfunction, neuronal degeneration and decrease in life span (Dermaut *et al.*, 2005; Simonsen *et al.*, 2007). Furthermore, 1(2)08717 is also a molecular target of the genes Hairy and Clock, which are responsible for regulation of hypoxia tolerance, circadian rhythms and several metabolic pathways (McDonald and Rosbash, 2001; Zhou *et al.*, 2008). Therefore, the overexpression of 1(2)08717 suggests that the action of Hairy and Clock is underlying the fly's increased tolerance to AD iteration.

1.10 Hypotheses

Drosophila is a remarkably interesting and promising study model given its tolerance to anoxia (Krishnan *et al.*, 1997) and the power of its genetic and molecular tools (Azad and Haddad, 2009). Consequently, these features allow characterization of physiological and molecular mechanisms involved in response and tolerance to anoxia, and comparison with those present in other animals. Ultimately, common response targets can be identified and manipulated, and this could be used in the development of therapeutical treatments to relieve the side effects of disruptive events like stroke or heart attack.

Anoxia/hypoxia response in *Drosophila* is paradigm dependent (Liu *et al.*, 2006; Azad *et al.*, 2009). So far studies have mainly focused on the effects of constant hypoxia/anoxia (Krishnan *et al.*, 1997; Le Corrionc *et al.*, 1999; Liu *et al.*, 2006; Lighton, 2007; Azad *et al.*, 2009, Schilman *et al.*, 2011) and just a few have considered the fly's response under a repetitive paradigm (Lighton and Schilman, 2007; Azad *et al.*, 2009; Armstrong *et al.*, 2011). Furthermore, only Armstrong *et al.* (2011) have used brain $[K^+]_o$ as a way of assessing neural failure during repetitive anoxia.

Consequently, our knowledge of the fly's mechanisms involved in responses to anoxia iteration is at an early stage and needs to be increased by, for instance, testing the effect of more variables and/or developing new tools. Repeated anoxia disrupts brain K^+ homeostasis in *Drosophila* causing an increment in $[K^+]_o$ baseline (Armstrong *et al.*, 2011). However, it is not clear 1) what kind of anoxia/normoxia pattern is more disruptive, 2) how sex can influence alteration of brain K^+ homeostasis, and 3) how temperature can modulate brain K^+ homeostasis disruption. The present study addressed these questions by measuring brain $[K^+]_o$ in male and female *D. melanogaster* (W1118) at three different temperature levels (cold, 16°C/17°C; room 23°C; high 29°C/30°C) during AD iteration. The following hypotheses were tested:

1. A N_2 -delivery pattern with long anoxia, short normoxia and high cycle frequency will cause damage to nervous tissue. This will be evidenced as the loss of the ability to maintain the initial $[K^+]_o$ baseline.
2. Based on morphological, anatomical and physiological dimorphisms, males and females are expected to show differences in $[K^+]_o$ maintenance and/or time to surge during AD repetition.
3. During AD iteration, hypothermia will help maintain the stability of $[K^+]_o$ and reduce time to recover after an experiment. On the other hand, hyperthermia will exacerbate disruption of K^+ homeostasis and increase time to recover.

Chapter 2

Materials and Methods

2.1 Effect of Temperature on Repetitive Anoxic Depolarizations: Sex-Based

Comparison

2.1.1 Animals

Male and female adult *Drosophila melanogaster* individuals (W1118, 4-6 days old after emergence from pupal stage) were used. Flies were kept under a 12 hour light/dark photoperiod in the Biosciences Complex at Queen's University. Room temperature was $22.0 \pm 0.25^\circ\text{C}$. The animals were raised on standard medium as described in Mileva-Seitz *et al.* (2008): 0.01% molasses, 8.20% cornmeal, 3.40% killed yeast, 0.94% agar, 0.18% benzoic acid, 0.66% propionic acid and 86.61% water. They were pseudorandomly chosen before every experiment, and a total of nine to fifteen male and eleven female flies were assessed per treatment.

2.1.2 Preparation of Potassium-Sensitive Microelectrodes

Potassium-sensitive microelectrodes prepared as described in Rodgers *et al.* (2007). Unfilamented glass capillary tubes with a diameter of 1 mm (World Precision Instruments) were washed with methanol (99.9%, Sigma-Aldrich) and dried on a hot plate before being pulled to form a low-resistance tip (5-7 M Ω). Subsequently, they were made hydrophobic by application of dichlorodimethylsilane (99%, Sigma-Aldrich) on a hot plate (100°C) for one hour. The tip of each electrode was filled with potassium ionophore I-cocktail B (5% valinomycin, Sigma-Aldrich) to create an artificial K⁺-selective membrane; then the electrodes were backfilled with a 500 mM

KCl solution and suspended in distilled water until needed in an experiment. Reference electrodes with a 5-7 M Ω resistance tip were made using glass filamented capillary tubes (1 mm in diameter) and backfilled with 3 M KCl before the beginning of an experiment.

2.1.3 Preparation and Setup

Flies were held using a fine-tip aspirator and were immobilized on a wax block (2 mm x 4 mm x 4 mm) using minuten pins and without anesthesia application. With a pair of microscissors, a small window (0.06 mm x 0.02 mm) was opened behind the ocelli, and brain extracellular potassium concentration ($[K^+]_o$) was measured continuously by means of a K⁺-sensitive microelectrode. To avoid desiccation, a 0.3 μ L bolus of hemolymph-like solution (HL₃, Stewart *et al.*, 1994) was applied in the head window before every experiment. Additionally, a chlorided silver wire was inserted between the fourth and fifth abdominal terga to ground the preparation (figure 1, i). Anoxic depolarizations (ADs) were delivered by repetitive N₂-induced anoxic comas alternated with periods of normoxia. Nitrogen or compressed air (8 L/min) was applied by placing the fly between two 100 mL syringes connected to a 3-way valve, allowing for a separation between syringes of 2.5 cm. The valve permitted alternate application of the gases of interest in an uninterrupted way (figure 1, ii).

2.1.4 Extracellular Potassium Recording

Reference and K⁺-sensitive microelectrodes were connected to a DUO773 two-channel intracellular/extracellular amplifier (World Precision Instruments) and calibration was performed at room temperature (22.0 \pm 0.25°C) using 20 mM and 200 mM KCl solutions. Theoretically, a 10-fold change in K⁺ concentration should produce a voltage of \sim 58 mV, and

only pairs of electrodes whose reading was 58 ± 4 mV were selected for an experiment. Both electrodes were introduced in the brain through the window previously opened and K^+ equilibrium potential (E_p) was continuously recorded. E_p was converted to $[K^+]_o$ using the Nernst equation (Rodgers *et al.*, 2007):

$$E_p = \frac{RT}{zF} \ln \frac{[K^+]_o}{[K^+]_i} \quad \text{Equation 1}$$

E_p is K^+ equilibrium potential, R is the ideal gas constant ($8.315\text{J}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$), T is the temperature in Kelvin, z is the valence of the ion (+1), F is the Faraday's constant ($96,485\text{C}\cdot\text{mol}^{-1}$), $[K^+]_o$ is the concentration of K^+ outside the cell and $[K^+]_i$ is the K^+ concentration inside the cell.

At a temperature of 22°C , replacing all the constants and substituting ln with log base 10 (\log_{10}), the equation gets transformed in the following manner:

$$E_p = 58.6 \log_{10} \frac{[K^+]_o}{[K^+]_i} \quad \text{Equation 2}$$

Given that the electrodes were zeroed in a 20 mM KCl solution, the equation above was modified to obtain $[K^+]_o$ in terms of E_p :

$$[K^+]_o = 20 \times 10^{(E_p/58.6)} \quad \text{Equation 3}$$

Equation 3 was used to convert E_p in the recordings to $[K^+]_o$, and adjustments for the different temperatures assessed were made when necessary.

2.1.5 Preliminary Nitrogen-Delivery Pattern and Variables

ADs were elicited by repetitive N_2 -induced anoxic comas (2.5 minutes each) alternated with periods of normoxia (4 minutes each). Each anoxic bout was associated with an abrupt surge in $[K^+]_o$, which returned to normal baseline during normoxia. Thirteen anoxia/normoxia

cycles were applied unless the fly died before the end of the experiment (figure 2, i). An absence of anoxic K^+ surges and a marked increase of $[K^+]_o$ during normoxia were interpreted as the death of the specimen. On the other hand, presence of anoxic K^+ surges and the inability to return to the initial $[K^+]_o$ baseline was understood as a disruption of $[K^+]_o$ homeostasis caused by the iteration of ADs. The number and duration of the anoxia/normoxia cycles were based on a pattern used previously with satisfactory results (Armstrong *et al.*, 2011). Control experiments were performed at room temperature using male flies. They consisted of an initial 2.5 minute N_2 pulse, 79.5 minutes of compressed air, and a final 2.5 minute N_2 pulse.

The response variables measured were $[K^+]_o$ before surge and time to surge (TTS). The former was defined as the $[K^+]_o$ reading at the point in which N_2 was turned on before a surge; the latter was defined, in the unconverted K^+ -electrode equilibrium potential (E_p) trace, as the time taken by the system to show a surge after N_2 was applied (figure 2, ii). Furthermore, the beginning of a surge was considered as the point in the E_p trace where there was a sustained increase of at least 1 mV after N_2 application. With the purpose of assessing $[K^+]_o$ disruption in the different treatments, the derived variable total $[K^+]_o$ baseline variation ($\Delta[K^+]_o$) was calculated. $\Delta[K^+]_o$ was defined as the difference between $[K^+]_o$ baseline before the 13th and the 1st surges.

2.1.6 Temperature Variation and Stabilization

The wax block with the immobilized fly was positioned on a plastic disc (1 mm thick and 5 mm in diameter) located on a thermo electric cooler (TEC) (figure 1, ii). The TEC is a Peltier plate that allows increasing and decreasing surface temperature by the application of direct current. It was connected to a DC power source and the excess of heat was properly dissipated

using an aluminum heat sink. Nitrogen was applied as indicated in the previous section while temperature was monitored by a thermocouple probe inserted in the fly's thorax through the mesoscutum. Three different temperatures were tested: 16°C (cold), 23°C (room) and 30°C (high). However, application of N₂ during cold and high temperature treatments heated up and cooled down the preparations, respectively. To overcome this, all the temperature measurements were performed while the preparation was subjected to a continuous stream of gas (8 L/min, N₂ during anoxia, compressed air during normoxia).

2.1.7 Tissue/TEC Temperature Correlation

In order to establish the fly's internal temperature just by knowing the TEC temperature, female flies (5 for high and 9 for low temperatures) were immobilized and placed in the setup explained in figure 1 (ii). No electrodes were used. One thermocouple probe was inserted in the fly's thorax and a second one was attached to the TEC surface using a block of plasticine (figure 3, i). The available thermocouple probe was too big to be inserted in the head, and thorax temperature was used as an indirect measure of head temperature. Evidently, there was some inherent bias when determining the real head temperature because of the considerably different volumes of the two structures. However, the results obtained support different brain [K⁺]_o baseline maintenance trends for cold, room and high temperatures. Different increasing voltages (0.5 V/2 min) were applied to the TEC and the respective thoracic and plate temperatures were registered 2 minutes after the voltage increase. The reason why there were an unbalanced number of flies in high and low temperature calibration experiments was that at low temperatures the reading in both thermocouples was not consistent between flies, making necessary an increase in the number of replicates. Nine measurements were taken per fly and a

linear regression was performed by plotting mean thorax temperature vs. mean TEC temperature (figure 3, ii). This established the following correlations:

- 16°C (thorax) = 4°C (TEC)
- 23°C (thorax) = 27°C (TEC)
- 30°C (thorax) = 48°C (TEC)

The temperature treatments were established based on two criteria: firstly, it was necessary for the chosen temperatures to be found in the fly's natural environment and secondly, the temperatures needed to be easily and consistently reached using the TEC available. The latter criterion constrained the possible range of temperatures to be tested, since temperatures lower than 4°C (thorax = 16°C) were difficult to reach. Consequently, the treatments were established mostly based on the lowest temperature reachable with the TEC.

2.1.8 Statistical Analyses

Data were plotted and analyzed using SigmaPlot 11.0 (Systat Software Inc.) and R (www.r-project.org). The values reported correspond to the mean \pm standard error of the mean (SEM). Logarithmic transformation of the data was performed when necessary in order to meet the variance assumption of parametric tests (*i.e.* homoscedasticity). Time to surge (TTS) was analyzed using a two-way ANOVA on the log-transformed data, and multiple comparisons were performed by the Holm-Sidak method. $\Delta[K^+]_o$ was analyzed within sex using a Kruskal-Wallis one-way ANOVA on ranks and Dunn's method as a pairwise multiple comparison procedure. For this variable, comparisons between sexes were performed using a one-way ANOVA (parametric, Holm-Sidak method as *post hoc* multiple comparison test), and Mann-Whitney Rank sum tests

(non-parametric). The significance level set for all the analyses was $\alpha = 0.05$. Figures show statistical groupings using letter designations: treatments with different lettering have statistically significant differences ($p < 0.05$) as opposed to treatments with the same lettering ($p > 0.05$).

2.2 Nitrogen-Delivery Pattern

Animals used and preparation of K^+ -sensitive microelectrodes as described in sections 2.1.1 and 2.1.2, respectively. Only male flies (one to four per pattern) were used for this set of experiments.

2.2.1 Preparation and Setup

Dissection and anoxic coma delivery were performed as described in section 2.1.3. The flies were held using a fine-tip aspirator and were immobilized in a wax block (2 mm x 4 mm x 4 mm) located on a plastic disc (diameter = 5 mm). Animals were held down using minuten pins and were never anesthetized during the procedure. The plastic disc was glued in the middle of a square of mesh placed on top of a 30 mL N_2 -delivery chamber. The chamber consisted of the bottom of a plastic beaker covered by a piece of canvas, which was held in place by a rubber band. To prevent any leaks, the piece of canvas was sealed to the rim of the beaker bottom using hot glue (figure 4). The rationale for using a N_2 -delivery system different from the one used in temperature experiments was based on two goals: development of a more standard setup for projects not involving the use of a TEC, and minimization of dehydration in the preparation. The first goal aimed for future lab projects using the GAL4/UAS system (Duffy, 2002) where temperature was not planned to be a response variable of interest. Additionally,

the second goal intended to improve experimental conditions to avoid bias in the results because of dehydration of the fly. Having the N₂ blown from below was expected to decrease hydric stress by avoiding direct contact with the window opened in the head, and using a small chamber (30 mL) minimized the N₂-flow rate necessary to elicit a surge (from 8 L/min to 4 L/min). Moreover, other measures were taken in order to prevent dehydration: the hole in the head was sealed with mineral oil after electrode placement and a humidifier was included in the setup (figure 4). Consequently, for this series of experiments humidified N₂ was used during anoxia, and the fly was allowed to remain at regular room-air conditions during normoxia (no humidified air was applied). This was true for all the experiments except for one control.

2.2.2 Extracellular Potassium Recording

[K⁺]_o was recorded as described in section 2.1.4 except for the calibration solutions used, which consisted of a 15 mM KCl+135 mM NaCl solution and a 150 mM KCl solution. The electrodes used in section 2.1 were calibrated using 20 mM and 200 mM KCl because these concentrations covered the range of expected amplitudes. However, experimental experience gained in such section suggested that surge amplitude was not reliable for comparison between flies because it had considerable variation, possibly caused by the relative position of the electrodes and the depth at which they were inserted. Furthermore, 15 mM and 150 mM KCl calibration solutions have been used with satisfactory results (Rodgers *et al.*, 2009; Armstrong *et al.*, 2009; Rodgers-Garlick *et al.*, 2011). Therefore the concentration of the calibration solutions was changed for these experiments. This methodological change does not affect comparability with experiments achieved using higher calibration solution concentrations as long as there is a

10-fold relationship between the solutions (see equation 2), and the concentration of the solution for zeroing the electrodes is included in equation 3.

$$[K^+]_o = 15 \times 10^{(E_p/58.6)} \quad \text{Equation 4}$$

Consequently, equation 4 was used to convert E_p to $[K^+]_o$ in this set of experiments. Additionally, the purpose of the NaCl was to balance the ionic strength of both calibration solutions in order to avoid any inaccuracy caused by ionic interactions. Hence calculations performed according to Voipio *et al.* (1994) showed that the effect of not having used NaCl in the 20 mM KCl solution resulted in a K^+ underestimation of 3.2 mM. However, according to the experience gained through the experiments, this difference is not substantial and does not significantly bias any comparison made with experiments in which NaCl was included.

2.2.3 Nitrogen-Delivery Patterns Tested and Variables

Seventeen different N_2 -delivery patterns were tested and grouped in five categories according to the following criteria (figure 5, i-v): frequency of anoxia, duration of anoxia, duration of normoxia, combination of long anoxia with short normoxia and increased cycle frequency, and controls (air and N_2). The main objective of these exploratory experiments was to find an anoxia/normoxia pattern that could cause evident $[K^+]_o$ homeostasis disruption manifested as an increase in $[K^+]_o$ baseline. Air controls aimed to demonstrate that the preparation itself did not cause significant $[K^+]_o$ variation, and N_2 controls intended to prove that repetitive anoxia was responsible for the loss of $[K^+]_o$ homeostasis. All the experiments were performed using humidified N_2 during anoxia and regular room-air conditions during normoxia. Consequently, in order to assess if there was any bias because of dehydration, one air control

experiment was performed with a continuous humidified gas flow on the fly (N_2 for anoxia, compressed air for normoxia).

The only response variable analyzed was $[K^+]_o$ baseline before surge, and was defined as the lowest $[K^+]_o$ reading before a surge was present (figure 6, i-ii). However, depending on the pattern in question, some data points were not available for all the times sampled during an experiment (every 4 minutes). Before every experiment, a 5 minute initial $[K^+]_o$ baseline was recorded and after the end, $[K^+]_o$ was recorded for 15 more minutes.

2.2.4 Statistical Analyses

Data were plotted and analyzed using SigmaPlot 11.0 (Systat Software Inc.). Because of time constraints, this set of experiments was meant to be merely exploratory and the sample sizes collected (1-2 in almost all of the patterns and 4 in the pattern that showed highest $[K^+]_o$ disruption) were not big enough to perform reliable descriptive statistics or statistical analyses. Nevertheless a qualitative analysis using line and scatter plots proved to be useful to identify the patterns that caused higher homeostasis problems, just by choosing the traces that showed a sustained increase in $[K^+]_o$ baseline after application of the cycles.

2.3 Effect of Temperature on Repetitive Anoxic Depolarizations: a More Refined Approach

Animals used and preparation of potassium-sensitive microelectrodes as described in section 2.1.1 and 2.1.2, respectively. Three to four male flies per treatment were used in the

experiments. Temperature variation and stabilization was performed as outlined in section 2.1.6. The gas flow rate was reduced from 8 L/min to 4 L/min.

2.3.1 Preparation and Setup (Oxygen Concentration Assessment)

Animal preparation implemented as described in section 2.1.3. Sealing of the head window was performed using Halocarbon oil 700 (Halocarbon products corporation/ Sigma-Aldrich), which is comparable to 15-20 S Voltalef oil (a widely used oil for live cell imaging in *Drosophila*). Halocarbon oil is a more economical, yet equally effective, substitute to Voltalef oil. It has been used in live cell imaging without any complications reported (Wood and Jacinto, 2005; Evans *et al.*, 2010), and its use was adopted in these experiments because, besides preventing desiccation through the head window, it also allows O₂ diffusion to the tissue during normoxia.

The setup was performed as described in section 2.1.3. A humidifier, consisting of an Erlenmeyer flask containing warm water, was included to prevent dehydration of the preparation (figure 7, i). The gas line went through a rubber stopper in the mouth of the flask, which had a lateral tube that connected to two slanted 20 mL N₂-delivery chambers (figure 7, ii). They were designed with the purpose of decreasing the N₂ flow rate necessary to elicit an anoxic coma. The space between them was wider at the top (1.5 cm) than at the bottom (1.0 cm), allowing proper manipulation of the electrodes and a decrease in N₂ flow rate from 8 L/min to 4 L/min.

Modification of the N₂-delivery chambers, inclusion of a humidifier, and sealing of the head window with oil allowed control of dehydration in the preparation and guaranteed stability of [K⁺]_o baseline throughout a whole experiment (120 min).

Oxygen concentration assessment of the experimental setup during anoxia was performed using a DO 1200 dissolved oxygen sensor. This sensor measures the amount of atmospheric O₂ dissolved in a membrane present on the tip of the probe, which needs to be wet to work properly. The probe was connected to a voltmeter, and different O₂ concentrations were expressed as a voltage reading (mV). Calibration was performed by insertion of the probe into an Erlenmeyer flask containing pure N₂ (O₂ = 0%), pure O₂ (O₂ = 100%) and at room-air conditions (O₂ = 21%). The respective voltages were recorded and compared with the voltage obtained after placing the probe between the slanted chambers described in figure 7 during a N₂ pulse. The setup was anoxic.

2.3.2 Extracellular Potassium Recording

The procedure was performed as described in section 2.1.4 except for the calibration solutions, which had NaCl added to one of them and a lower KCl concentration (*i.e.* 15 mM KCl+135 mM NaCl and 150 mM KCl solutions). For a discussion on the rationale for having changed the concentration and the composition of the calibration solutions, and the consequences this may have had on the comparability of the different datasets, refer to section 2.2.2. E_p was converted to [K⁺]_o using equation 2.4.

2.3.3 Nitrogen-Delivery Patterns and Variables

The anoxia/normoxia pattern chosen consisted of 3 minutes of N₂ and 0.5 minutes of compressed air during 30 cycles (figure 8, i). All the gases applied to the fly were humidified by passing them through an Erlenmeyer flask with warm water. Before every experiment, a 5 minute baseline was recorded and after the last surge [K⁺]_o was monitored for 10 more minutes

or until the fly recovered a stable baseline. Two types of controls were performed: the first one only involved continuous air flow on the fly for the whole duration of the experiment; the second one was based on a 90 minute N₂ pulse followed by compressed air during the remaining time (figure 5, v). The former (air control) showed that the preparation itself did not cause any significant [K⁺]_o disruption, and the latter (N₂ control) demonstrated that the [K⁺]_o disruption was caused by repetitive anoxia and not by anoxia itself. These controls were performed for every temperature treatment (17°C, 23°C and 29°C). Another set of air control experiments was performed only for high temperature (29°C) without application of Halocarbon oil 700 on the fly's head. The main goal was to demonstrate the importance of sealing the head window, especially at high temperatures, which can considerably increase dehydration.

Since there were a large number of cycles applied per experiment (30), only the odd surges were used to obtain data points. The response variables analyzed included [K⁺]_o baseline before surge, surge amplitude (Amp), time to surge (TTS) and time to recovery (TTR) (figure 8, ii). [K⁺]_o baseline before surge and TTS were considered as defined in sections 2.2.3 and 2.1.5, respectively. Amplitude was defined as the [K⁺]_o difference between the point of a surge where N₂ was turned off and the lowest point of the trace before the surge. Given that the N₂-delivery pattern chosen did not allow for proper baseline recovery after a surge, TTR was only measured after the last surge (30th). It was defined as the time taken by the system to reach a stable [K⁺]_o baseline after N₂ was turned off. Additionally, in order to overcome the fact that Amp was not reliable for comparisons between flies (for a brief discussion see section 2.2.2), a normalized derived variable (percentage of recovery, POR) was calculated. POR expressed the amplitude of surges 2-29 as a percentage of surge 1, which was expected to have the highest Amp value.

Furthermore, [K⁺]_o baseline and POR were statistically analyzed by establishing two corresponding derived variables: total [K⁺]_o baseline variation ($\Delta[K^+]_o$) and total percentage of

recovery variation (Δ POR). The former was defined as the difference between $[K^+]_o$ baseline before the 29th and the 1st surges, and the latter was calculated as the subtraction between POR for the 29th and the 1st surges.

2.3.4 Tissue/TEC Temperature Correlation

Male flies (n = 10 for high temperature, n = 10 for cold temperature) were immobilized and mounted in the middle of a TEC, which was located between two slanted chambers in the setup described in figure 7 (i). No electrodes were used. One thermocouple monitored the TEC temperature, whereas a second thermocouple assessed head temperature (figure 9, i). Voltage was raised at a rate of 0.4 V/2 min and the respective plate and head temperatures were recorded at the end of the second minute. With the different values obtained for flies at high temperatures (11 measurements) and low temperatures (14 measurements) a mean head temperature vs. mean TEC temperature plot was built and a linear regression on the data was calculated ($r^2 = 0.98$) (figure 9, ii). This established the following correlations:

- 17°C (head) = -1.0°C (TEC)
- 23°C (head) = 19.6°C (TEC)
- 29°C (head) = 40.3°C (TEC)

Since the treatments were established based on the lowest temperature reachable with the TEC (see section 2.1.7), it was necessary to readjust the temperatures for the cold and high treatments. Given that the head has a smaller volume than the thorax, less cold TEC temperatures were expected to cool down the head to 16°C (the cold temperature assigned during the sex-based comparison). However, the smaller head was also more susceptible to be

warmed up by the room-temperature gas flow, and much lower TEC temperatures (-4.5°C) were needed to reach the initial tissue temperature of 16°C. Accordingly, the cold treatment temperature was readjusted to 17°C (head), and the high temperature treatment was also changed to 29°C (head) just to keep the symmetry with respect to the room temperature treatment. Such variation can affect statistical analyses contrasting the sex-based comparison data set and the present one; nevertheless the latter was meant to be confirmatory of the results obtained with the former, and no direct quantitative comparisons were intended.

2.3.5 Statistical Analyses

Data were plotted and analyzed using SigmaPlot 11.0 (Systat Software Inc.). All statistical criteria and transformations applied to the data were performed as described in section 2.1.8.

A logarithmic transformation was carried out on $\Delta[K^+]_o$ and $\ln\Delta[K^+]_o$ for the different treatments was compared using a one-way ANOVA, with Holm-Sidak method as all pairwise multiple comparison procedure. To avoid mathematical issues inherent to obtaining the logarithm of a negative number, a constant was added to all values before the transformation. Untransformed data for TTS, TTR and Δ POR were analyzed using one-way ANOVAs and *post hoc* comparisons were also performed using the Holm-Sidak method.

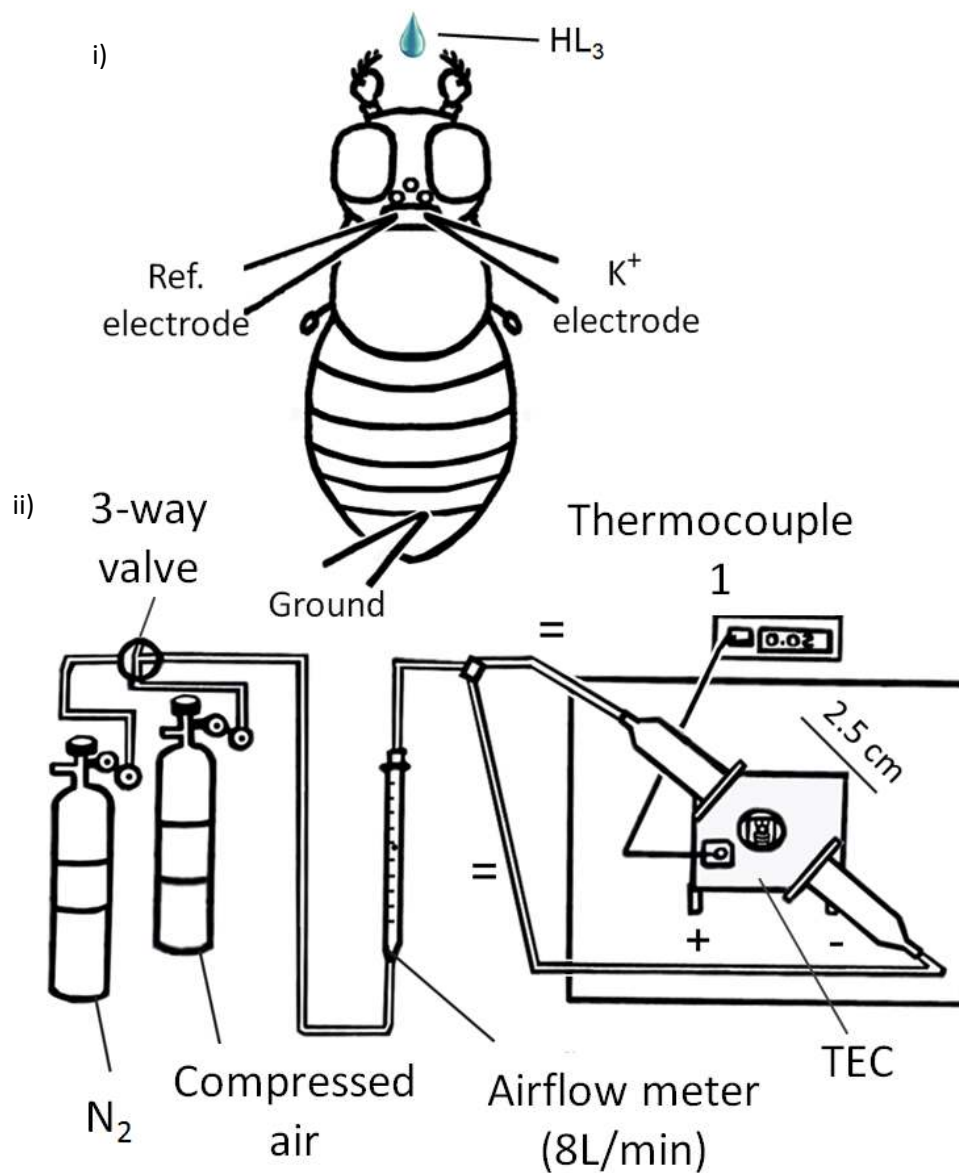


Figure 1. Sex-based comparison: animal preparation and setup. i) A window (0.06 mm x 0.02 mm) was opened at the back of the head behind the ocelli and a drop of hemolymph-like solution (HL₃, 0.3 μL) was applied to prevent dehydration. The reference and the K⁺-sensitive microelectrodes were introduced in the brain and [K⁺]_o was continuously measured. The preparation was grounded using a chlorided silver wire inserted between the fourth and the fifth abdominal terga. **ii)** The TEC was placed between two 100 mL syringes connected to compressed air and N₂ tanks. A 3-way valve allowed alternation of the gases at a rate of 8 L/min in an uninterrupted way. The TEC temperature was varied by passing direct current through its poles and was constantly monitored with a thermocouple.

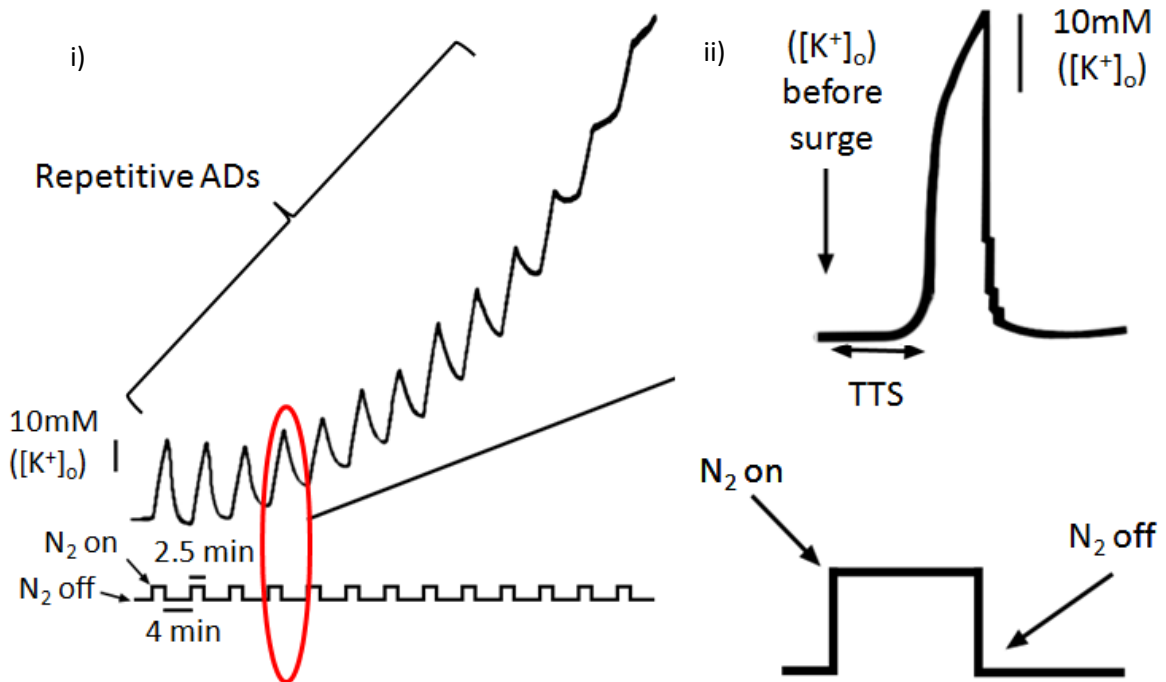


Figure 2 Sex-based comparison: preliminary N₂-delivery pattern and variables.

i) A 2.5 min/4 min anoxia/normoxia pattern was applied for 13 cycles (bottom trace) or until the fly died. Every anoxic coma was associated with a sudden [K⁺]_o surge in the brain (top trace).

ii) Enlarged version of the area enclosed by a red circle in i). Application of N₂ (bottom trace) caused a disruption in [K⁺]_o homeostasis (top trace) and return to normoxia using compressed air prompted partial recovery of [K⁺]_o baseline. Time to surge (TTS) and [K⁺]_o baseline before surge were the response variables analyzed in the experiments.

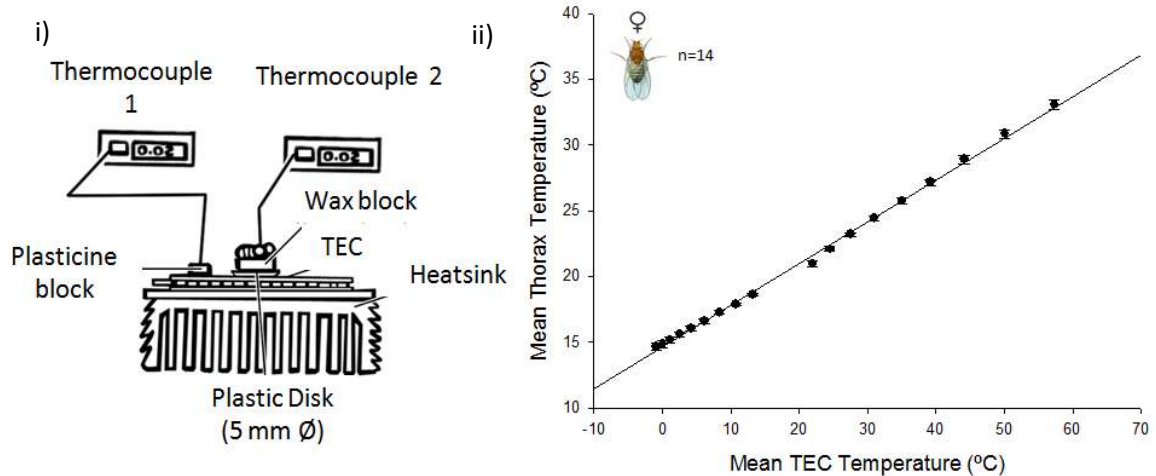


Figure 3 Sex-based comparison: tissue/TEC temperature correlation. i) Female flies were immobilized on a wax block and placed on a plastic disc (diameter = 5 mm) in the middle of a TEC. The TEC was placed between two 100 mL syringes connected to a compressed air tank (see figure 1, ii). A thermocouple (1) was attached with a plasticine block to the TEC surface and a second thermocouple (2) was used to measure thorax temperature during continuous gas flow (8 L/min). Temperature was increased (n = 5) or decreased (n = 9) at a rate of 0.5 V/2 min and 9 measurements were taken per fly. **ii)** Linear regression corresponding to mean thorax temperature vs. mean TEC temperature ($r^2 = 0.99$). The regression established the TEC temperatures necessary to reach the desired thorax temperatures (thorax_{cold} = 16°C, TEC_{cold} = 4°C; thorax_{room} = 23°C, TEC_{room} = 27°C; thorax_{high} = 30°C, TEC_{high} = 48°C). Values correspond to means \pm SEM (the error bars are hidden in the symbols).

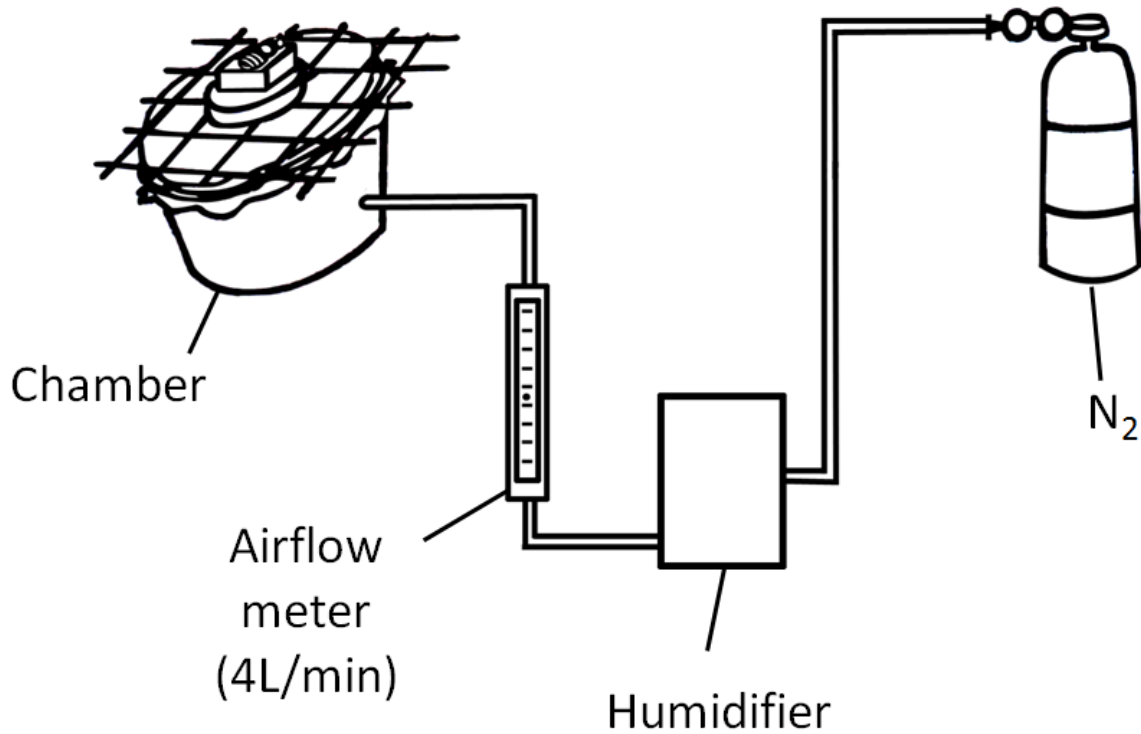
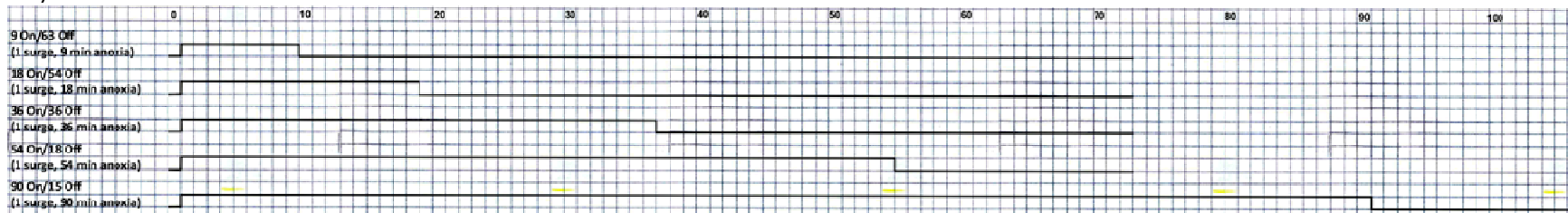


Figure 4. General setup to test for different N₂-delivery patterns. Flies were immobilized in a wax block and placed on a plastic disc (diameter = 5 mm) in the middle of a 30 mL chamber. The chamber consisted, from top to bottom, of a piece of mesh to hold the disc, a square of canvas to ensure homogeneous distribution of the N₂ surrounding the fly, and the bottom of a plastic beaker. To prevent any leaks, the square of canvas was attached to the beaker bottom using hot glue and a rubber band. Humidified N₂ was applied at a rate of 4 L/min according to the patterns illustrated in figure 5. Except for one control experiment, during normoxia no humidified air was applied and the fly was allowed to remain under room-air conditions.

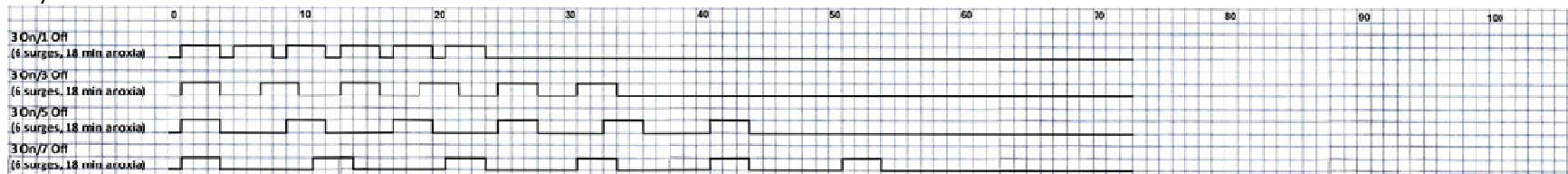
i)



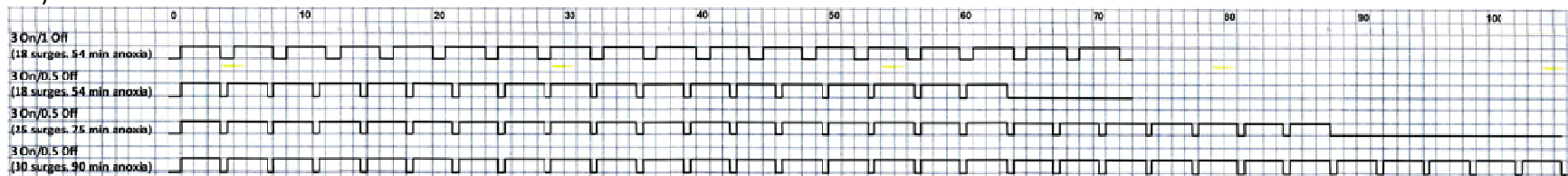
ii)



iii)



iv)



v)



Figure 5. N₂-delivery patterns tested. This set of patterns was meant to assess the effect of frequency of anoxia (i), duration of anoxia/normoxia (ii and iii, respectively), a combination of long anoxic bouts, short recovery times and increased cycle frequency (iv) and controls (air and nitrogen, v).

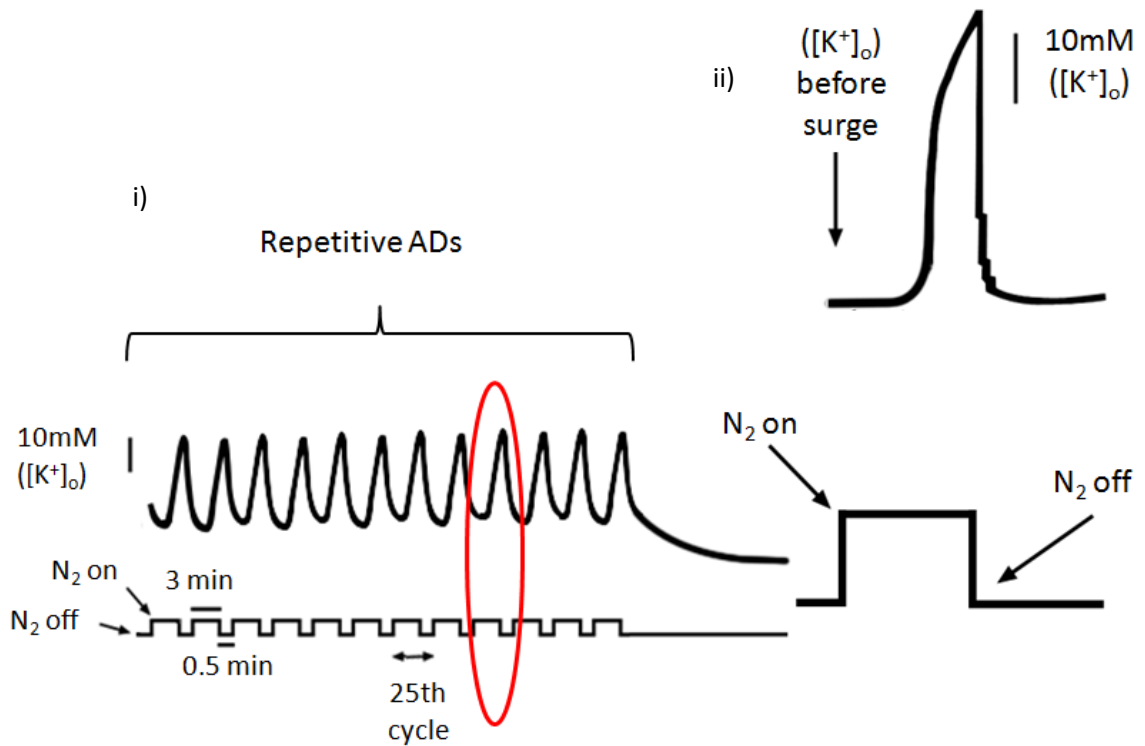


Figure 6. N₂-delivery pattern variables. i) Last 12 surges of a hypothetical $[K^+]_o$ recording during repetitive N₂-induced anoxic comas (top trace) delivered under varied anoxia/normoxia cycle patterns (bottom trace, see figure 5). Every anoxic coma was used to cause an anoxic depolarization (AD) related to a sudden $[K^+]_o$ surge in the brain. Before every experiment, a 5 minute initial $[K^+]_o$ baseline was recorded and after the last surge, $[K^+]_o$ was recorded for 15 more minutes or until the fly recovered a stable baseline. ii) Enlarged version of the area enclosed by a red circle in i. Application of humidified N₂ (bottom trace) caused a disruption in $[K^+]_o$ homeostasis (top trace) and return to normoxia prompted partial recovery of $[K^+]_o$ baseline. $[K^+]_o$ baseline before surge was the response variable analyzed in the experiments.

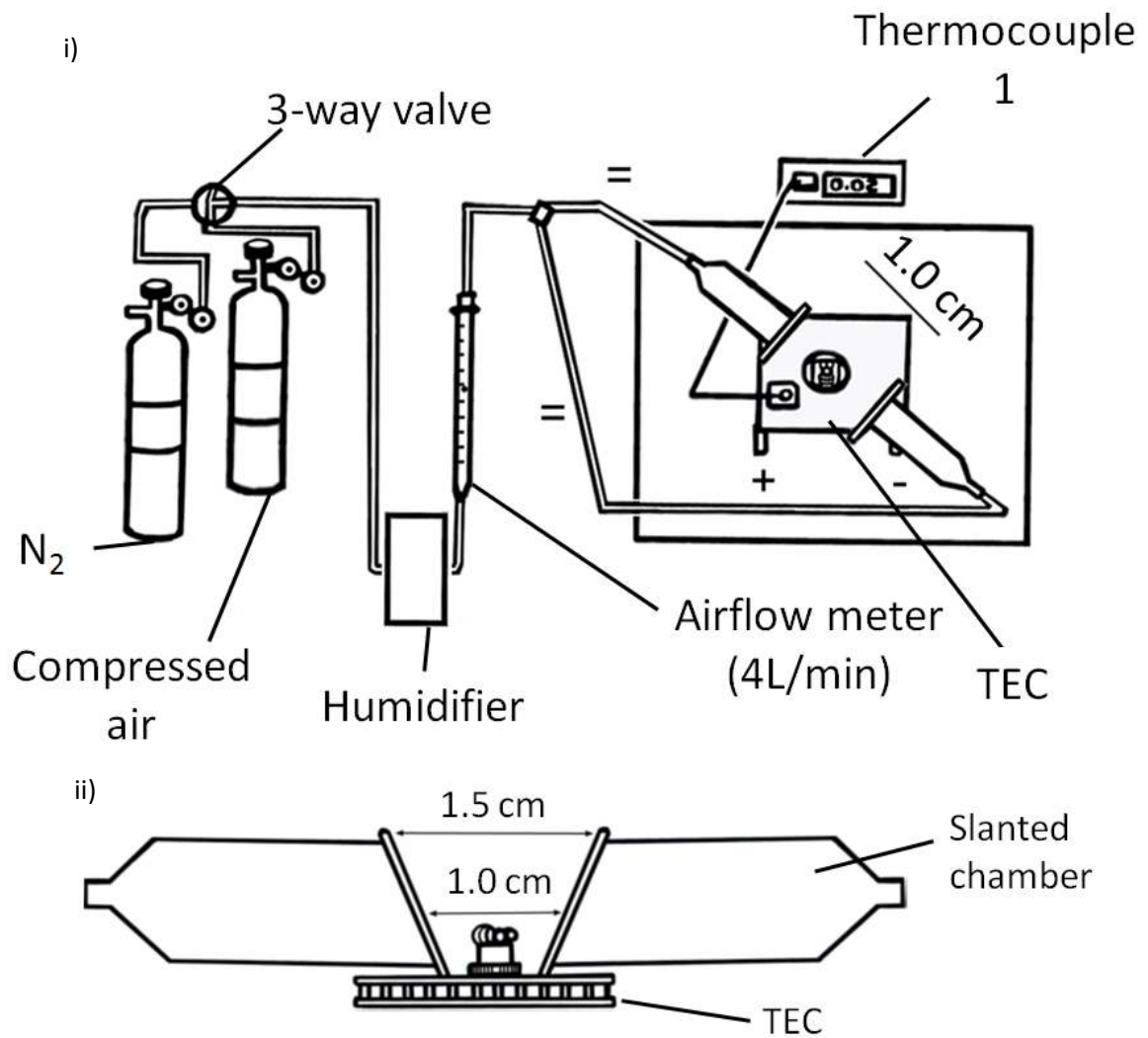


Figure 7. Refined setup to avoid dehydration of the preparation. i) A humidifier was included in the setup described on figure 1 (ii). A 3-way valve was used for alternation of the gases (N₂ and compressed air) in an uninterrupted way. The TEC temperature was varied by passing direct current through its poles and its temperature was constantly monitored with a thermocouple. ii) Magnified elevation view of the area enclosed by a red circle in i. The TEC with the fly immobilized in a wax block was placed between two 20 mL slanted chambers connected to a compressed air tank and a N₂ tank. These chambers could be arranged in such a way that, between them, there was a 1 cm gap at the bottom and a 1.5 cm gap at the top.

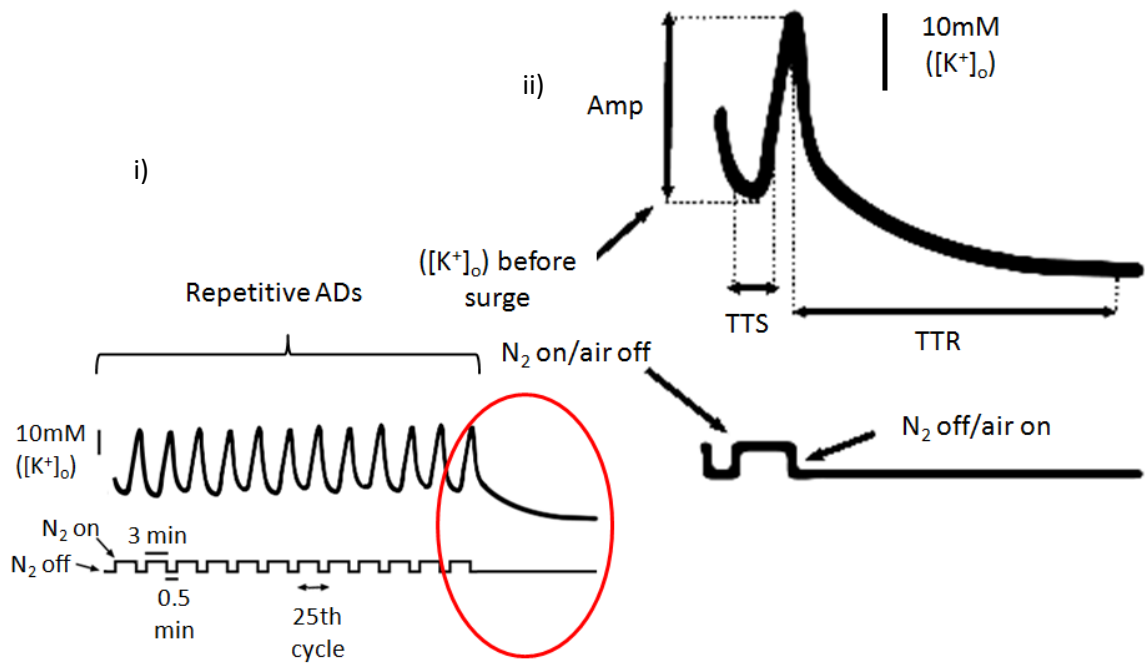


Figure 8. A more refined approach: N₂-delivery pattern and variables. i) Last 12 [K⁺]_o surges of a hypothetical cold temperature trace during repetitive anoxia. N₂-induced anoxic comas were used to cause ADs related to sudden [K⁺]_o surges in the brain (top trace). A 3 min/0.5 min anoxia/normoxia pattern was applied for 30 cycles (bottom trace). Before every experiment, a 5 min initial [K⁺]_o baseline was recorded, and after the last surge [K⁺]_o was recorded for 10 more minutes or until the fly recovered a stable baseline. **ii)** Enlarged version of the area enclosed by a red circle in i. Application of humidified N₂ (bottom trace) caused a disruption in [K⁺]_o homeostasis (top trace), and return to normoxia using humidified compressed air prompted partial recovery of [K⁺]_o baseline. Time to surge (TTS), surge amplitude (Amp), time to recovery (TTR, only after the last surge) and [K⁺]_o baseline before surge were the response variables analyzed in the experiments.

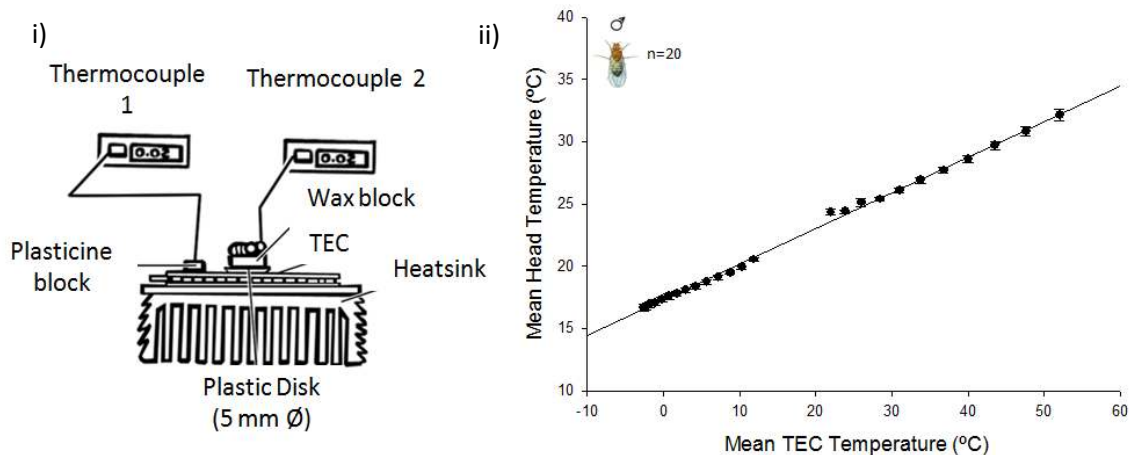


Figure 9. A more refined approach: tissue/TEC temperature correlation. i) Male flies were immobilized on a wax block and placed on a plastic disc (diameter = 5 mm) in the middle of a TEC. The TEC was placed between two 20 mL slanted chambers connected to a compressed air tank (see figure 7). A thermocouple (1) was attached with a plasticine block to the TEC surface and a second thermocouple (2) was used to measure head temperature during continuous humidified airflow (4 L/min). Temperature was increased ($n = 10$) or decreased ($n = 10$) at a rate of 0.4 V/2 min and 11 (high temperature) or 14 (cold temperature) measurements were taken per fly. **ii)** Linear regression corresponding to mean head temperature vs. mean TEC temperature ($r^2 = 0.98$). The regression established the TEC temperatures necessary to reach the desired head temperatures ($head_{cold} = 17^\circ\text{C}$, $TEC_{cold} = -1.0^\circ\text{C}$; $head_{room} = 23^\circ\text{C}$, $TEC_{room} = 19.6^\circ\text{C}$; $head_{high} = 30^\circ\text{C}$, $TEC_{high} = 40.3^\circ\text{C}$). Values correspond to means \pm SEM (the error bars are hidden in the symbols).

Chapter 3

Results

3.1 Effect of Temperature on Repetitive Anoxic Depolarizations: Sex-Based

Comparison

3.1.1 Males Are More Vulnerable to Recurrent ADs than Females at Room Temperature

Repeated N₂ pulses delivered to the flies caused anoxic comas related to a disturbance in ion homeostasis in the brain (anoxic depolarization, AD). A resulting abrupt [K⁺]_o surge occurred every time the animals went into a coma and [K⁺]_o returned to baseline when normoxia was restored. The iteration of induced ADs produced disruption of [K⁺]_o homeostasis, which eventually was manifested as the inability to maintain a stable baseline (figure 10, i-ii). At 23°C, males showed a gradual conspicuous baseline increase after the 10th surge; on the other hand, females displayed better baseline stability and [K⁺]_o increase was not pronounced (figure 10, iv). To discard the possibility that the preparation had caused any inherent [K⁺]_o homeostasis loss, control experiments lacking iteration of ADs were implemented in males (figure 10, iii). Males were chosen for these controls because they were more susceptible than females; this allowed reasonable inferences to be made about the effect of the preparation on the more resilient sex. The effect on baseline stability caused a slight and gradual increment that was smaller than the ones obtained during repetitive ADs in both sexes (figure 10, iv). Calculation of total [K⁺]_o baseline variation ($\Delta[K^+]_o$) showed a higher value for males (114.3 ± 10.5 mM) compared to females (36.1 ± 10.5 mM) and controls (10.5 ± 1.1 mM). A one-way ANOVA (Holm-Sidak method

for pairwise multiple comparisons) supported the differences established previously ($p < 0.001$ for all tests; figure 10, v). Despite the clearly different trends observed between females and controls in figure 10 (iv, v), there were no statistically significant differences ($p = 0.062$). However this value should be interpreted with care since it is too close to the significance level ($\alpha = 0.05$).

3.1.2 Hypothermia Stabilizes $[K^+]_o$ Baseline

AD iteration did not have a drastic negative effect on $[K^+]_o$ baseline maintenance at 16°C (hypothermia). This was true for both sexes, but it was more marked in males (figure 11, i) than in females (figure 11, ii). Conversely, 30°C (hyperthermia) exacerbated $[K^+]_o$ homeostasis disruption, which was evidenced as a fast and consistent baseline increase during the first 40 minutes of experiment in males (figure 11, iii) and females (figure 11, iv). As the baseline rose, the surge amplitudes diminished and became imperceptible. Such lack of response to AD repetition was interpreted as evidence of the fly's death and the experiment was stopped. This explains the lack of data points at high temperature for surges 10-13 in males and 7-13 in females, and the decreasing sample size to calculate mean $[K^+]_o$ for the last two thirds of surges at 30°C in both sexes (figure 11, v-vi). A $[K^+]_o$ baseline plot for males showed three clear trends depending on the temperature applied: at 16°C, there was an increased baseline stability; at 30°C, baseline increased fast and somewhat abruptly; and at 23°C, there was an intermediate baseline increment (figure 11, v). The same trends were evident in females, with exception of the room temperature treatment, which showed results similar to those produced by hypothermia. In spite of the results obtained at room temperature, males resisted more surges at high temperature than females (figure 11, v-vi). Comparison of $\Delta[K^+]_o$ in males showed that

values for hypothermia (17.5 ± 4.1 mM) were significantly different from room (114.3 ± 10.5 mM) and high (119.2 ± 21.9 mM) temperature treatments (Kruskall-Wallis one-way ANOVA on ranks with Dunn's method as a multiple comparison test, $p < 0.05$ for all tests; figure 11, vii). On the other hand, there were no differences between 23°C and 30°C ($p > 0.05$). A similar statistical analysis showed differences between 16°C (16.9 ± 6.8 mM) and 30°C (51.2 ± 8.1 mM) ($p < 0.05$), and grouped 16°C and 23°C (36.1 ± 10.5 mM) ($p > 0.05$), in females (figure 11, vii). Interestingly, the ANOVA on ranks also grouped 23°C and 30°C ($p > 0.05$). Between sexes, analysis of $\Delta[\text{K}^+]_o$ grouped low temperature treatments (17.5 ± 4.1 mM for males, 16.9 ± 6.8 mM for females; Mann-Whitney rank sum test, $p = 0.596$). In contrast, significant differences were found at 23°C (114.3 ± 10.5 mM for males, 36.1 ± 10.5 mM for females; t-test, $p < 0.001$) and 30°C (119.2 ± 21.9 mM for males, 51.2 ± 8.1 mM for females; Mann-Whitney rank sum test, $p = 0.013$). The previously mentioned analyses supported the trends suggested by the line/scatter plots.

3.1.3 TTS Is Inversely Related to Temperature

In males, there was no clear difference between room and high temperature treatments (figure 12, i). Additionally, the room temperature TTS trace had a slight increment during the last third of the surges. Comparatively, hypothermia increased TTS and kept TTS values constant throughout the surges, except for a slight decrease presented in the last third of the trace. This was only partially similar in females, where mean TTS displayed three trends related to the temperature treatment implemented: hypothermia increased TTS, hyperthermia decreased TTS and room temperature showed intermediate values (figure 12, ii). Furthermore, the traces were constant throughout the surges. In both sexes, the lack of data points in the final section of the high temperature experiments and the consequential decreasing sample size were caused by

premature death of the flies during the experiments (figure 12, i-ii). A bar plot of mean logTTS supported the trends suggested by the line/scatter plots, and showed that the variable was inversely proportional to temperature in both sexes (figure 12, iii). In males, TTS was significantly increased at 16°C ($19.6 \pm 2.2s$) compared to 23°C ($12.0 \pm 2.2s$) and 30°C ($8.8 \pm 1.1s$) treatments (two-way ANOVA, *post hoc* multiple comparison test performed by the Holm-Sidak method; 16°C vs. 23°C, $p = 0.009$; 16°C vs. 30°C, $p < 0.001$). On the other hand, there were no differences between 23°C and 30°C ($p = 0.111$). Within females, the same statistical analysis reported differences between 16°C ($19.4 \pm 3.0s$) and 30°C ($4.7 \pm 0.4s$), and 23°C ($13.3 \pm 1.3s$) and 30°C ($p < 0.001$ for all tests). Conversely, 16°C and 23°C were statistically grouped ($p = 0.08$). This result must be interpreted with care because the p-value was too close to the significance level ($\alpha = 0.05$) assigned. Between sexes, the only significant differences were found at 30°C ($8.8 \pm 1.1s$ for males, $4.7 \pm 0.4s$ for females, $p = 0.004$). A histogram of the untransformed response variable (mean TTS) supported all the relationships and the trends suggested by the line/scatter plot and confirmed by the statistical analyses (figure 12, iv).

3.2 Nitrogen-Delivery Pattern

3.2.1 A Combination of Long Anoxia, Short Normoxia and Increased Cycle Frequency Disrupted $[K^+]_o$ Homeostasis

In order to find an AD pattern disruptive enough to cause an $[K^+]_o$ baseline increase in the fly's brain, sets of patterns involving variation of 3 different factors were tested. Different cycle frequencies and lengths of anoxia and normoxia were evaluated in terms of $[K^+]_o$ baseline maintenance (figure 5, i-iii). Individual manipulation of each of the variables did not cause a

conspicuous baseline variation with $\Delta[K^+]_o$ ranging from 0.7-11.8 mM (figure 13). However, a combination of long anoxia, short normoxia and increased cycle frequency proved to cause $[K^+]_o$ homeostasis loss during the experiments (figure 14). With the purpose of proving that baseline disruption was actually caused by the iteration of ADs and not by the total time of anoxic exposure, control experiments consisting of a 90 minute N_2 pulse and 15 minutes of compressed air were performed. For these controls, the baseline increase was mild ($\Delta[K^+]_o$ ranged from 6.3-11.1 mM; figure 14, i, ctrl_ N_2). Additionally, an experiment lacking AD repetition was implemented to find out if the preparation itself caused any baseline disruption, resulting in a slight $\Delta[K^+]_o$ increment of 7.7 mM (figure 14, i, ctrl_ NO cont airflow). Since normoxia was delivered without application of humidified air during all the previous experiments, the role of tissue dehydration on the behavior of $[K^+]_o$ baseline had to be assessed. This goal was achieved by performing an experiment lacking AD iteration and replacing regular room air by humidified compressed air during normoxia. The outcome was a decrease in $[K^+]_o$ baseline ($\Delta[K^+]_o = -5.1$ mM; figure 14, i, ctrl_ cont airflow). In contrast, 3 minutes of N_2 application and 1 minute of normoxia during 18 cycles produced a $\Delta[K^+]_o$ ranging from 36.8-41.7 mM (figure 14, ii). Contrary to what was expected, reduction of recovery time to 0.5 minutes in the previous pattern did not cause major disruption, resulting in a $\Delta[K^+]_o$ of 6.0-6.5 mM (figure 14, iii). To surmount this unanticipated outcome, the number of cycles was increased to 25 in subsequent experiments, whereas the cycle time was maintained to 3 minutes of anoxia and 0.5 minutes of normoxia. The resulting $\Delta[K^+]_o$ ranged between 35.5-70.2 mM (figure 14, iv). Finally, the same pattern with a cycle frequency of 30 rendered similar $\Delta[K^+]_o$ results (57.4-74.8 mM; figure 14, v).

3.3 Effect of Temperature on Repetitive Anoxic Depolarizations: a More Refined

Approach

3.3.1 Protective/Damaging Effects of Temperature Are Still Evident under Reduced

Dehydration Conditions

In accordance with the results obtained in sections 3.1.1 and 3.2.1, a disruptive AD pattern consisting of 3 minutes of anoxia and 0.5 minutes of normoxia during 30 cycles was applied only on male flies. $[K^+]_o$ baseline was barely disturbed throughout every experiment at 17°C (figure 15, i). Implementation of a setup ensuring low tissue dehydration by application of humidified gas flow, sealing the fly's head with Halocarbon oil 700 and decreasing the gas flow rate, prevented permanent $[K^+]_o$ baseline disruption after AD iteration at 23°C. Consequently, all flies recovered their initial baseline values after the experiments; however, baseline disruption was evidenced during AD repetition (figure 15, ii). On the other hand, flies at 29°C never recovered after the pattern was applied (figure 15, iii). A line/scatter plot of $\ln[K^+]_o$ before surges 1-29 (only the odd surges were analyzed) showed three very clear trends during AD iteration: hypothermia stabilized $[K^+]_o$ baseline, hyperthermia caused a marked baseline increase, and room temperature caused intermediate values (figure 15, iv). Such results were consistent with the ones obtained in section 3.1.2. The logarithmic transformation of the data was necessary given the high baseline increment produced by recurrent ADs at high temperature. A one-way ANOVA carried out on the $\ln\Delta[K^+]_o$ (Holm-Sidak method as *post hoc* multiple comparison test) showed statistical differences between all treatments ($p < 0.001$ for all tests; figure 15, v), corroborating the trends suggested by the baseline plots (figure 15, iv). Considering the untransformed response variable, hypothermia caused a $\Delta[K^+]_o$ decrease (-1.0 ± 1.3 mM), while

hyperthermia and room temperature produced severe and moderate $\Delta[K^+]_o$ increments, respectively ($\Delta[K^+]_{o_high} = 332.7 \pm 83.0$ mM; $\Delta[K^+]_{o_room} = 17.3 \pm 1.5$ mM; figure 15, vi).

Assessing the influence of the preparation itself on $[K^+]_o$ baseline at every temperature was a fundamental factor to determine the real effect of repetitive ADs on $[K^+]_o$ homeostasis. To achieve this, experiments lacking AD iteration were performed and compared to their corresponding treatments at all different temperatures. Only two ADs were applied, one at the beginning and other at the end of the experiments, in order to demonstrate that the fly was responsive to anoxic comas during the whole duration of the controls. At 17°C, neither presence nor absence of AD repetition caused any obvious baseline increase (figure 16, i-ii). Plotting of baseline before surge was performed in the controls by sampling the corresponding times at which $[K^+]_o$ was sampled in the treatments. Subsequent comparison of baselines did not suggest any difference between presence and absence of AD iteration at 17°C (figure 16, iii), and a one-way ANOVA (Holm-Sidak method as pairwise multiple comparison test) performed on $\ln\Delta[K^+]_o$ supported the lack of significant dissimilarities ($p = 0.721$; figure 16, iv). A bar plot of $\Delta[K^+]_o$ revealed an actual decrease in baseline variation during cold temperature treatment (-1.0 ± 1.3 mM) and controls (-1.9 ± 1.3 mM; figure 16, v).

Concerning room temperature, an extra set of controls consisting of 90 minutes of anoxia and 15 minutes of normoxia was carried out (room_ctrl_N₂). Additionally, a regular set lacking repetitive ADs was performed as explained above (room_ctrl). The former set was intended to demonstrate that the baseline increase evidenced during AD iteration (figure 17, i) was actually produced by the repetition of ADs and not by the total anoxia time applied on the fly. Consequently, the total anoxia/normoxia times were equivalent to the times applied during recurrent ADs. Both lack of repeated ADs and a single 90 minute N₂ pulse did not cause any

apparent permanent baseline increment (figure 17, ii-iii). Line/scatter plots suggested no baseline stability disruption produced by both controls and a moderate baseline increase caused by AD iteration (figure 17, iv). Such trends were supported by statistical analyses carried out on the $\ln\Delta[K^+]_o$, grouping both controls (one-way ANOVA with pairwise multiple comparisons performed by the Holm-Sidak method, $p = 0.894$) and reporting significant differences between controls and treatment (room vs. room_ctrl, $p = 0.002$; room vs. room_ctrl_N₂, $p = 0.003$; figure 17, v). A histogram of the untransformed $\Delta[K^+]_o$ was consistent with the previous analyses and showed that absence of AD repetition caused a slight baseline decrease (-0.3 ± 0.1 mM), application of 90 minutes of anoxia produced a minor baseline increment (1.0 ± 2.6 mM) and AD iteration generated a moderate baseline increase (17.3 ± 1.5 mM) (figure 17, vi).

At high temperature, recurrent ADs produced a severe disturbance that caused a consistent and considerable baseline increment (figure 18, i), whereas controls lacking AD iteration had no apparent effect (figure 18, ii). Another set of control experiments with neither AD repetition nor oil application showed a stable baseline during approximately 80 minutes; then after a small spontaneous $[K^+]_o$ surge, baseline increased steadily and somewhat abruptly (figure 18, iii). Such set of controls was meant to assess the importance of oil-sealing the fly's head at high temperatures. Baseline plots showed three evident trends: AD repetition caused a substantial baseline increase, lack of AD iteration did not evidently affect baseline stability, and absence of recurrent ADs without oil application had an intermediate effect (figure 18, iv). Statistical analyses performed on $\ln\Delta[K^+]_o$ (one-way ANOVA with Holm-Sidak method as *post hoc* multiple comparison method) showed significant differences between all treatments ($p < 0.001$ for all tests; figure 18, v). Recurrent ADs caused a significant increase in $\Delta[K^+]_o$ (332.7 ± 83.0 mM) compared to absence of AD repetition with (2.5 ± 0.5 mM) and without (61.5 ± 16.7 mM)

oil. Additionally in the controls, lack of oil sealing proved to be considerably disrupting, producing a $\Delta[K^+]_o$ substantially higher (61.5 ± 16.7 mM) than the one obtained with oil application (2.5 ± 0.5 mM; figure 18, vi).

Line/scatter plots of high, cold and room temperature controls lacking AD iteration did not suggest a visible difference between them. Only during the last third of the surges, the traces seemed to diverge, suggesting a slightly higher baseline disturbance at 29°C, intermediate values at 23°C and a minor baseline decrease at 17°C (figure 19, i). A one-way ANOVA (pairwise multiple comparisons performed by the Holm-Sidak method) carried out on $\ln\Delta[K^+]_o$ reported no statistical differences between the different temperature controls (cold_ctrl vs. room_ctrl, $p = 0.307$; room_ctrl vs. high_ctrl, $p = 0.314$, cold_ctrl vs. high_ctrl, $p = 0.051$; figure 19, ii). However, differences between 17°C and 29°C must be interpreted with care since the p-value obtained was too close to the significance level ($\alpha = 0.05$). Despite the results obtained by the statistical analysis, a histogram of the untransformed $\Delta[K^+]_o$ suggested a trend related to temperature: hypothermia and hyperthermia caused a slight baseline decrease (-1.9 ± 1.3 mM) and increase (2.5 ± 0.5 mM), respectively, and room temperature showed intermediate values ($\Delta[K^+]_o = -0.3 \pm 0.1$ mM; figure 19, iii).

3.3.2 Hypothermia Increases TTS

Line/scatter plots of mean time to surge (TTS) showed that hypothermia decreased TTS with respect to room temperature throughout the experiments. The traces were constant and did not show marked variation. However, at 30°C the amplitude of the surges after the 13th surge became gradually and considerably smaller until the point of being almost imperceptible

(figure 15, iii). This caused a substantial increase in TTS since it took more time for every surge to cause a sustained rise of 1 mV after N₂ application (see section 2.1.5 for the definition of TTS). Thus, for the sake of comparison only the surges that had similar shape between the treatments were considered, resulting in an analysis focused on the first 9 surges (only odd surges were sampled). Plotting of the first 9 surges showed three trends: hypothermia incremented TTS, hyperthermia caused a time decrease, and room temperature caused intermediate values. Nevertheless, the traces for high and room temperature were not clearly separated (figure 20, i). A one-way ANOVA carried out on the response variable (mean TTS) reported no differences between the treatments ($p = 0.194$). Despite the lack of statistical support, a histogram of mean TTS suggested that hypothermia increased TTS ($24.0 \pm 5.4s$) compared to room temperature ($15.5 \pm 1.6s$) and hyperthermia ($13.5 \pm 0.5s$). The difference between 23°C and 29°C was not evident (figure 20, ii).

3.3.3 Temperature Is Directly Related to TTR

At 23°C and 17°C, flies recuperated their initial $[K^+]_o$ baseline after the 30th surge (figure 15, i-ii). Conversely, 30°C had a severe disruptive effect on $[K^+]_o$ homeostasis and flies never recovered (figure 15, iii). Additionally, time to recovery (TTR) during hypothermia and room temperature depended on the treatment applied since flies at 17°C experienced immediate baseline recuperation, whereas flies at 23°C recovered in two distinct phases: an initial fast stage, and a subsequent slow stage. Therefore flies at room temperature took more time to regain their initial baseline. A one-way ANOVA (pairwise multiple comparisons performed by the Holm-Sidak test) showed that TTR at 23°C ($1335.8 \pm 112.3s$) was significantly increased compared to hypothermia ($184.4 \pm 53.0s$) and controls performed at all temperatures (TTR_{cold_ctrl}

= $332.1 \pm 186.5s$, $TTR_{room_ctrl} = 134.1 \pm 43.9s$, $TTR_{room_ctrl_N2} = 451.1 \pm 79.3s$, $TTR_{high_ctrl} = 132.8 \pm 17.4s$; $p < 0.001$ for all tests; figure 20, iii). No dissimilarities were reported between controls, except for those consisting of a single 90 minute anoxic pulse ($room_ctrl_N_2$), which were different from absence of AD repetition at 23°C ($room_ctrl$, $p = 0.038$) and 30°C ($high_ctrl$, $p = 0.038$). However, the p-values that separated these groups were too close to the significance level ($\alpha = 0.05$) and should be interpreted with care. Likewise, similar conservativeness should be applied when interpreting the p-value grouping hypothermia and $room_ctrl_N_2$. ($p = 0.059$).

3.3.4 Surge Amplitude Decreases with AD Repetition

In order to make surge amplitude comparable between treatments, amplitudes for surges 2-29 (only odd surges were considered) were expressed as a percentage of the first surge (percentage of recovery, POR) and contrasted by means of a line/scatter plot. The plot suggested no differences between cold, high and room temperatures, and showed a gradual decrease in amplitude as more ADs were delivered (figure 20, iv). Calculation of ΔPOR corroborated a reduction in POR at 17°C ($55.7 \pm 2.2\%$), 23°C ($53.4 \pm 5.0\%$), and 29°C ($50.8 \pm 14.7\%$). Additionally, the lack of differences between treatments was supported by a one-way ANOVA ($p = 0.910$; figure 20, v).

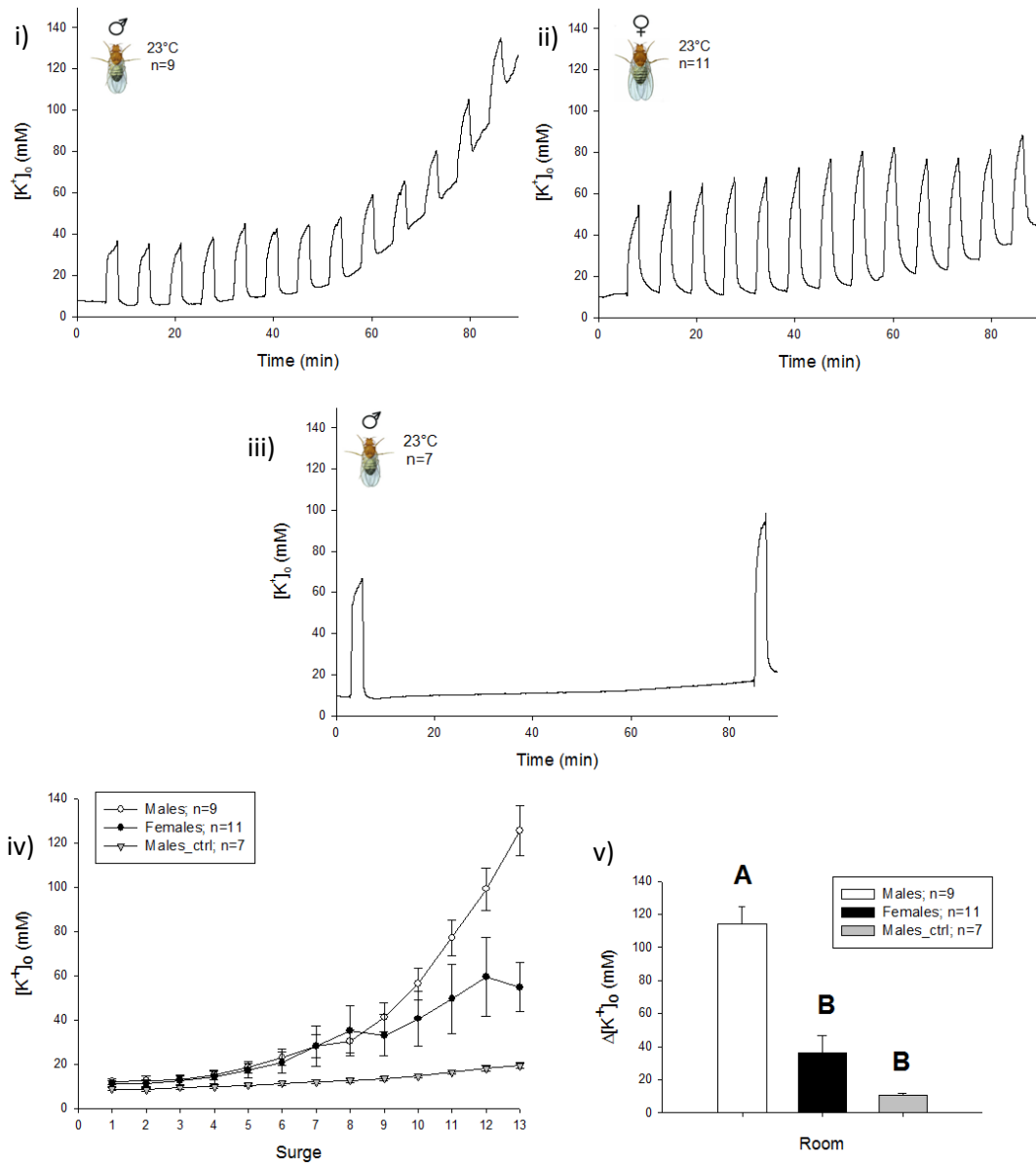


Figure 10. Susceptibility to repetitive ADs at room temperature is sex-dependent. **i)** Repetitive ADs produced sudden $[K^+]_o$ surges in the fly's brain. The iteration of surges generated a loss in $[K^+]_o$ homeostasis with an eventual baseline increase in males. **ii)** In females, the baseline increment was observed but not as marked as in males. **iii)** $[K^+]_o$ baseline in males was not conspicuously affected in the absence of repetitive ADs. **iv)** Control experiments in males (males_ctrl) showed that baseline increase is mostly caused by the ADs. Differences in baseline between males and females became more evident after the 10th surge. **v)** Compared to females and controls, there was a statistically significant increase in mean $\Delta[K^+]_o$ after repetitive ADs in males (one-way ANOVA with Holm-Sidak method as a *post hoc* multiple comparison test, $p < 0.001$). There were no differences between females and controls ($p = 0.062$). For iv and v values are means \pm SEM, $n_{\text{males}} = 9$, $n_{\text{females}} = 11$, $n_{\text{males_ctrl}} = 7$, and lettering indicates different statistical groupings.

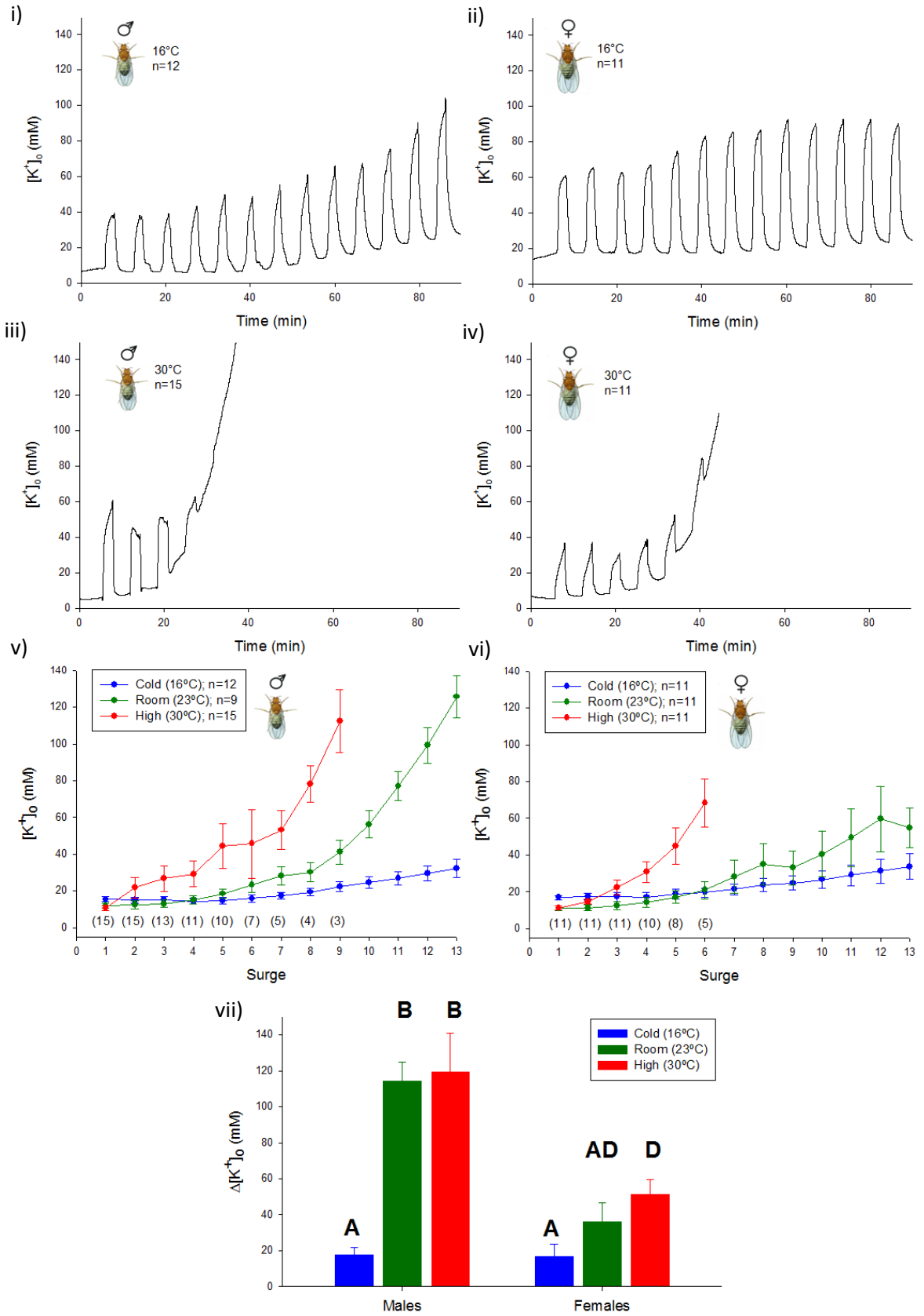


Figure 11. Protective effect of hypothermia against repetitive ADs. i) Cold temperature (16°C) reduced $[K^+]_o$ baseline disruption caused by the iteration of ADs in males. **ii)** Females also had an increase in baseline stability during hypothermia, but not as conspicuous as in males. **iii)** Hyperthermia (30°C) caused severe loss of $[K^+]_o$ homeostasis in males, producing a marked baseline increase within the first 40 minutes of the experiments. **iv)** Females showed a similar response, but the baseline increment was not as pronounced as in males. **v)** In males, there was a conspicuous effect of temperature on $[K^+]_o$ maintenance: hyperthermia exacerbated baseline loss, hypothermia preserved baseline stability, and room temperature produced intermediate values (the number of live flies available to calculate mean $[K^+]_o \pm SEM$ for every surge at high temperature is indicated in parentheses). **vi)** The same trends described in v were present in females, but differences between cold (16°C) and room (23°C) temperatures were not as marked as in males (numbers in parentheses as explained for males). **vii)** Within each sex, significant differences in mean $\Delta[K^+]_o$ were found between high and low temperature treatments (Kruskal-Wallis one way ANOVA on ranks; multiple comparisons performed by Dunn's method, $p < 0.05$). In males, there was also a significant difference between 16 and 23°C ($p < 0.05$), whereas no statistical difference was found between the same treatments in females ($p > 0.05$). Concerning the effect of high temperature, there were no significant differences when compared to room temperature within each sex ($p > 0.05$). Between sexes, differences were found at 23°C (t-test, $p < 0.001$) and 30°C (Mann-Whitney rank sum test, $p = 0.013$). Hypothermia had similar stabilizing effects on males and females (Mann-Whitney rank sum test, $p = 0.596$). For v-vii values are means $\pm SEM$, $n_{cold_males} = 12$, $n_{room_males} = 9$, $n_{high_males} = 15$, $n_{females} = 11$ (for all treatments), and lettering indicates different statistical groupings.

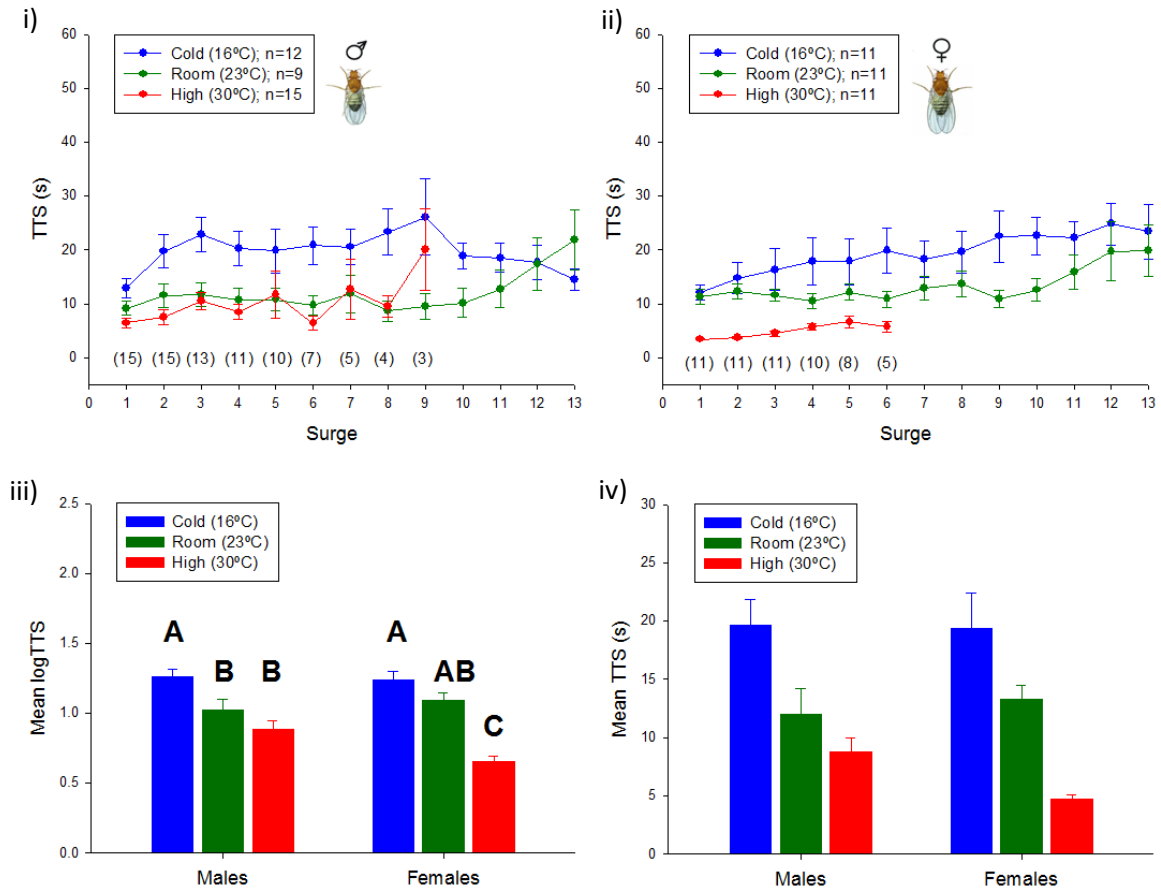


Figure 12. Temperature effects on TTS in males and females. **i)** In males, there were no apparent differences between room (23°C) and high (30°C) temperature treatments. Compared to hyperthermia and room temperature, hypothermia (16°C) caused a sustained increase in TTS. At 23°C, TTS increased continuously after the 10th surge (the number of live flies available to calculate mean TTS ± SEM for every surge at high temperature is indicated in parentheses) **ii)** Females showed three different trends through the experiments: hypothermia and hyperthermia increased and decreased TTS, respectively, whereas room temperature showed intermediate values (numbers in parentheses as explained for males). **iii)** Within sex, a two-way ANOVA (Holm-Sidak method as a multiple comparison test) on the log-transformed response variable (mean TTS per fly) showed differences between 16°C and 23°C (males, $p = 0.009$), 16°C and 30°C (males and females, $p < 0.001$), and 23°C and 30°C (females, $p < 0.001$). No statistical differences were found between 23°C and 30°C (males, $p = 0.111$), and 16°C and 23°C (females, $p = 0.08$). Between sexes, only differences at 30°C were significant ($p = 0.004$). **iv)** A histogram of the untransformed response variable shows that mean TTS is inversely proportional to temperature. For i-iv values are means ± SEM, $n_{\text{cold_males}} = 12$, $n_{\text{room_males}} = 9$, $n_{\text{high_males}} = 15$, $n_{\text{females}} = 11$ (for all treatments), and lettering indicates different statistical groupings.

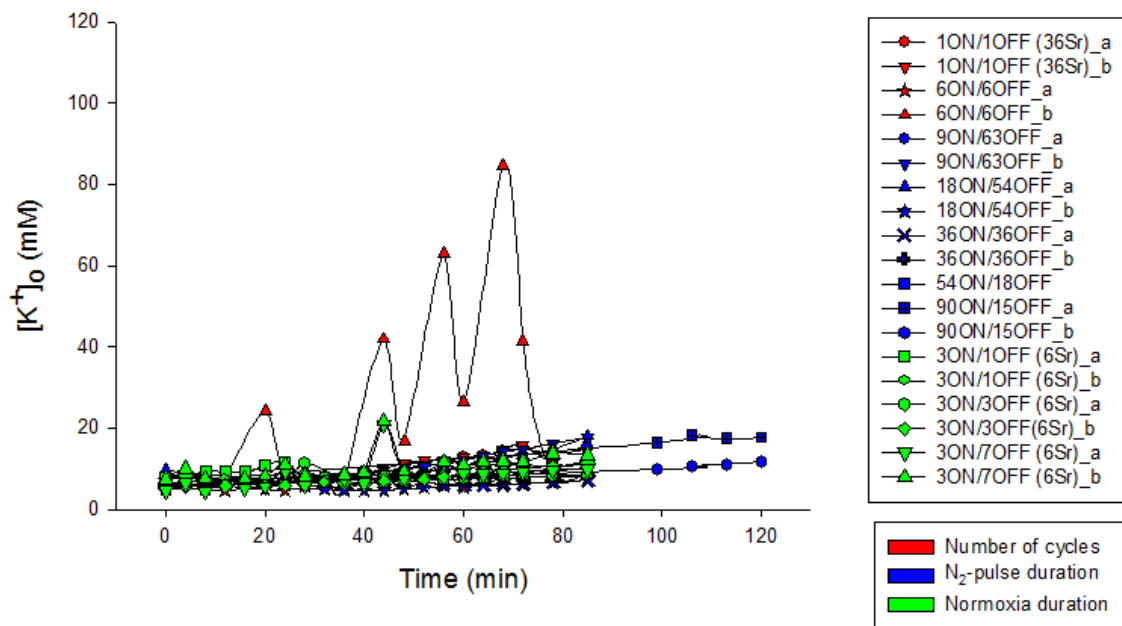


Figure 13. Effect of number of cycles, N₂-pulse duration and normoxia duration on [K⁺]_o baseline maintenance. A qualitative analysis showed that manipulation of these variables did not affect [K⁺]_o homeostasis in a conspicuous way, producing $\Delta[K^+]_o$ values that ranged between 0.7-11.8 mM. Even application of a 90 minute N₂ pulse did not cause a marked baseline increase (blue crossed squares and blue hexagons). In the figure, each trace corresponds to a single experiment. In the legend, the numbers to the left and the right of the slash symbol indicate anoxia and normoxia cycle times, respectively, and the number in parentheses designates the number of surges (Sr) elicited under the cycle time in question. Absence of a number in parentheses means that only one surge was induced. The letter at the end of a pattern indicates that the corresponding trace was a replicate experiment. For all of the patterns n = 2, except for 54ON/18OFF whose n = 1.

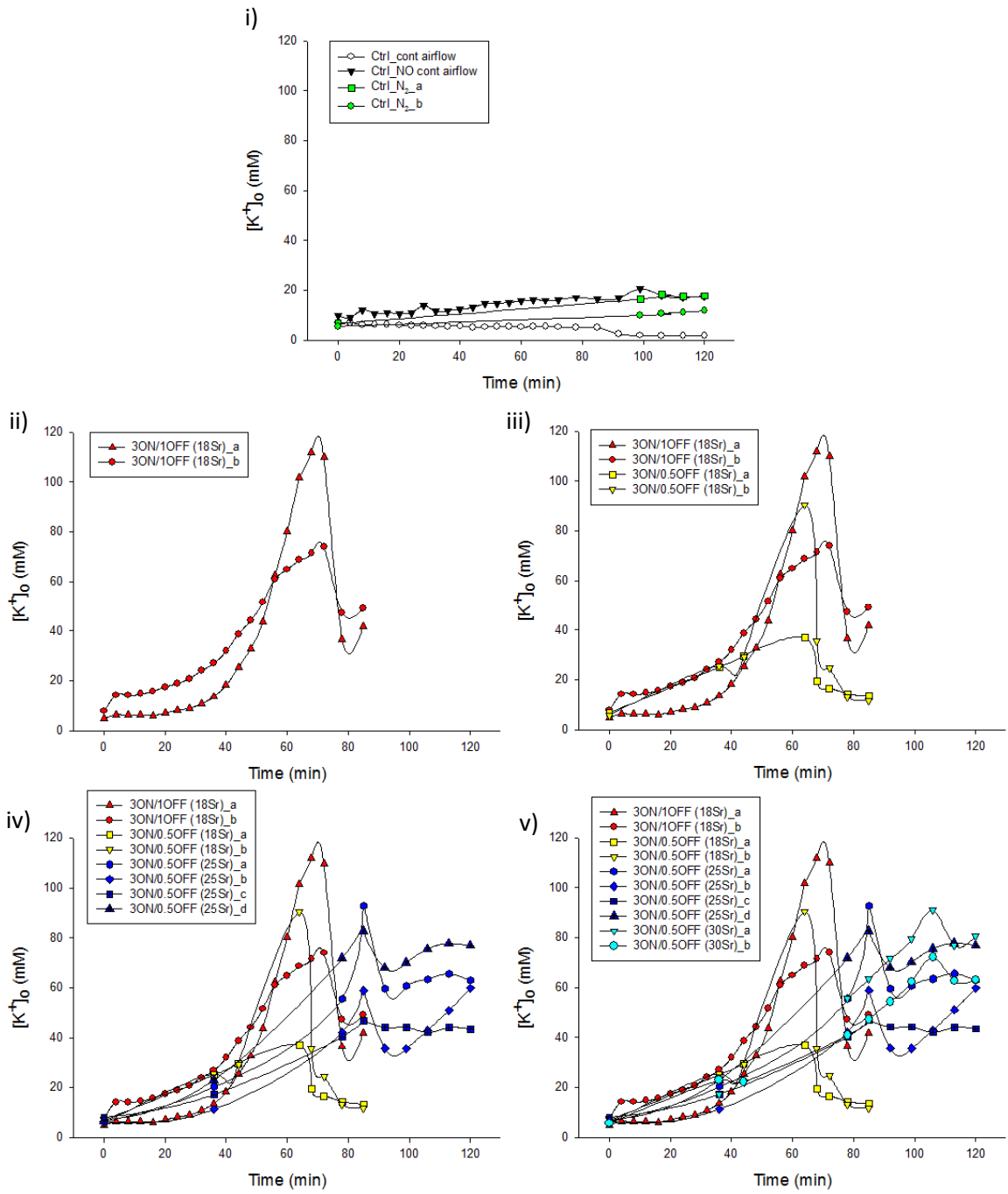


Figure 14. Effect of a combination of long anoxia/short normoxia/increased cycle frequency on $[K^+]_o$ baseline maintenance. Gradual plot of $[K^+]_o$ baseline traces obtained during different combined patterns highlights the different disruptive effects of long anoxia, short normoxia and increased cycle frequency. **i)** Two control experiments consisting of 90 minutes of anoxia and 15 minutes of normoxia (ctrl_N₂, green symbols) showed a slight $\Delta[K^+]_o$ increase (6.3 and 11.1 mM, respectively) as well as an experiment with no repetition of ADs and no humidified air applied during normoxia (ctrl_NO cont airflow, black symbols, $\Delta[K^+]_o = 7.7$ mM). Another control with a continuous humidified airflow on the preparation underwent a decrease in $\Delta[K^+]_o$ of -5.1 mM (ctrl_cont airflow, white symbols). **ii)** Experiments consisting of 3 minutes of anoxia and 1 minute of normoxia during 18 cycles produced an increase in $[K^+]_o$ baseline (red symbols, n = 2, $\Delta[K^+]_o = 36.8-41.7$ mM). **iii)** Contradictorily, reducing recovery time to 0.5 minutes did not show major effects (yellow symbols, n = 2, $\Delta[K^+]_o = 6.0-6.5$ mM). **iv)** Despite this, the same cycle pattern was conserved (3 minutes of anoxia/0.5 minutes of normoxia) and frequency was increased up to 25 cycles (dark blue symbols, n = 4) with a considerable increment in $\Delta[K^+]_o$. (35.5-70.2 mM). **v)** A pattern based on 30 cycles and the same anoxia/normoxia timing used in iv was tested with similar disruptive results (light blue symbols, n = 2, $\Delta[K^+]_o = 57.4-74.8$ mM). Figure symbols and legend description as explained in figure 13.

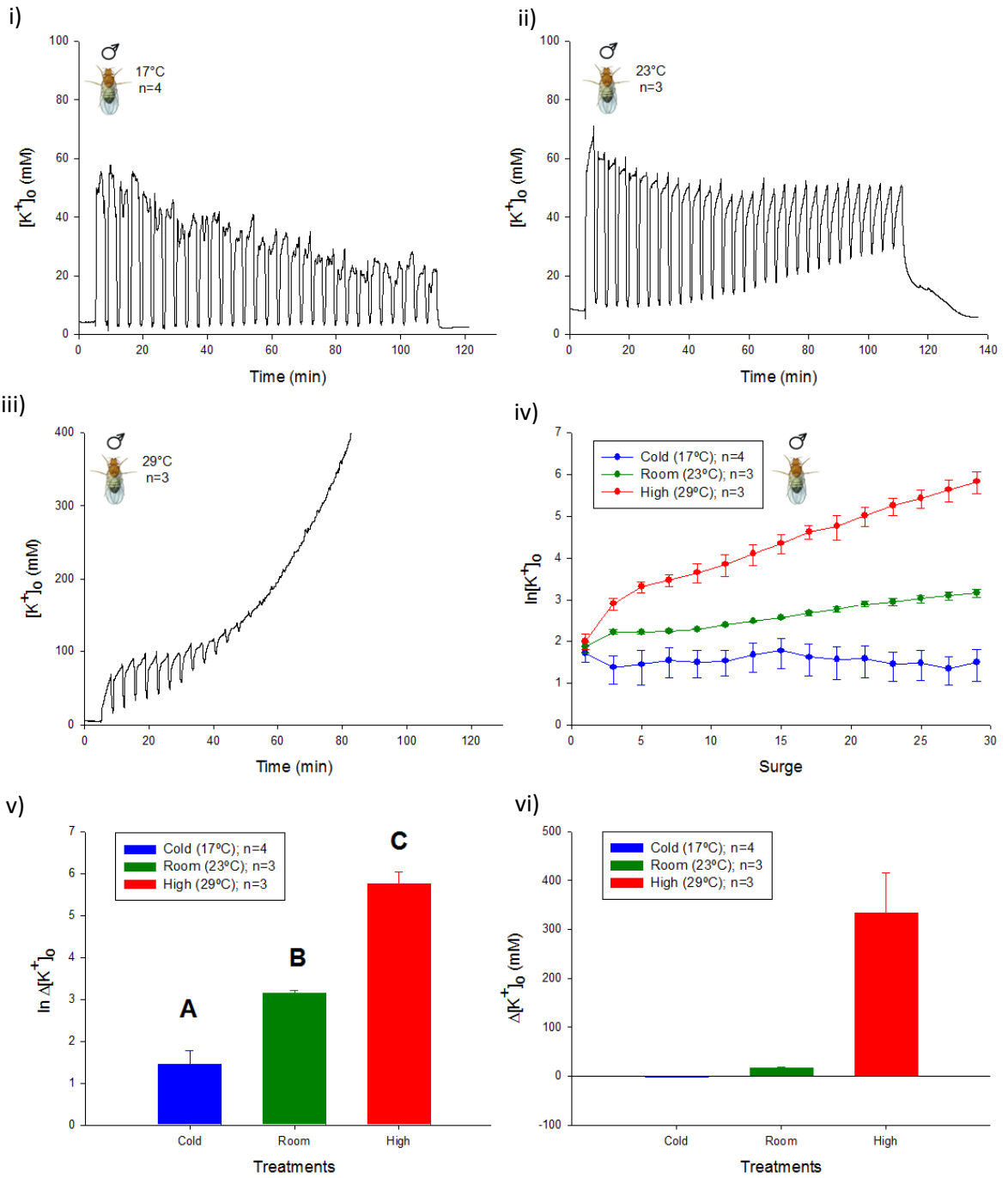


Figure 15. Effect of temperature on $[K^+]_o$ maintenance in males, during repetitive ADs and under reduced dehydration conditions. **i)** In experiments consisting of 3 minutes of anoxia and 0.5 minutes of normoxia during 30 cycles, hypothermia prevented repetitive ADs from producing major $[K^+]_o$ disruption. After the 30th surge, $[K^+]_o$ returned in about 3 minutes to values close to the initial baseline. **ii)** At 23°C, there was an increase in $[K^+]_o$ baseline caused by the iteration of ADs. When AD repetition ceased, baseline returned to values similar to the ones recorded before the 1st surge, in approximately 20 minutes. **iii)** Hyperthermia severely exacerbated the loss of $[K^+]_o$ homeostasis, and baseline reached extremely high values. Flies never recovered, and about the second half of the surges had very small amplitudes. The y-axis was reduced in order to show a range of possible physiological concentrations. Higher $[K^+]_o$ values were obtained but were considered artifactual. **iv)** Line/scatter plots of the ln-transformed response variable ($\ln[K^+]_o$) showed three distinct trends in $[K^+]_o$ maintenance: hypothermia maintained baseline stability, hyperthermia severely exacerbated loss of $[K^+]_o$ homeostasis, and room temperature produced intermediate values. The points plotted correspond to mean $\ln [K^+]_o$ before surges 1 to 29 (only odd surges were considered). **v)** Statistical analyses on the $\ln\Delta[K^+]_o$ showed significant differences among the three treatments (one-way ANOVA, pairwise multiple comparisons performed by the Holm-Sidak method, $p < 0.001$ for all tests). **vi)** A histogram of mean $\Delta[K^+]_o$ per temperature supported the trend evidenced in v. For iv-vi values are means \pm SEM, $n_{\text{cold}} = 4$, $n_{\text{room}} = 3$, $n_{\text{high}} = 3$, and lettering indicates different statistical groupings.

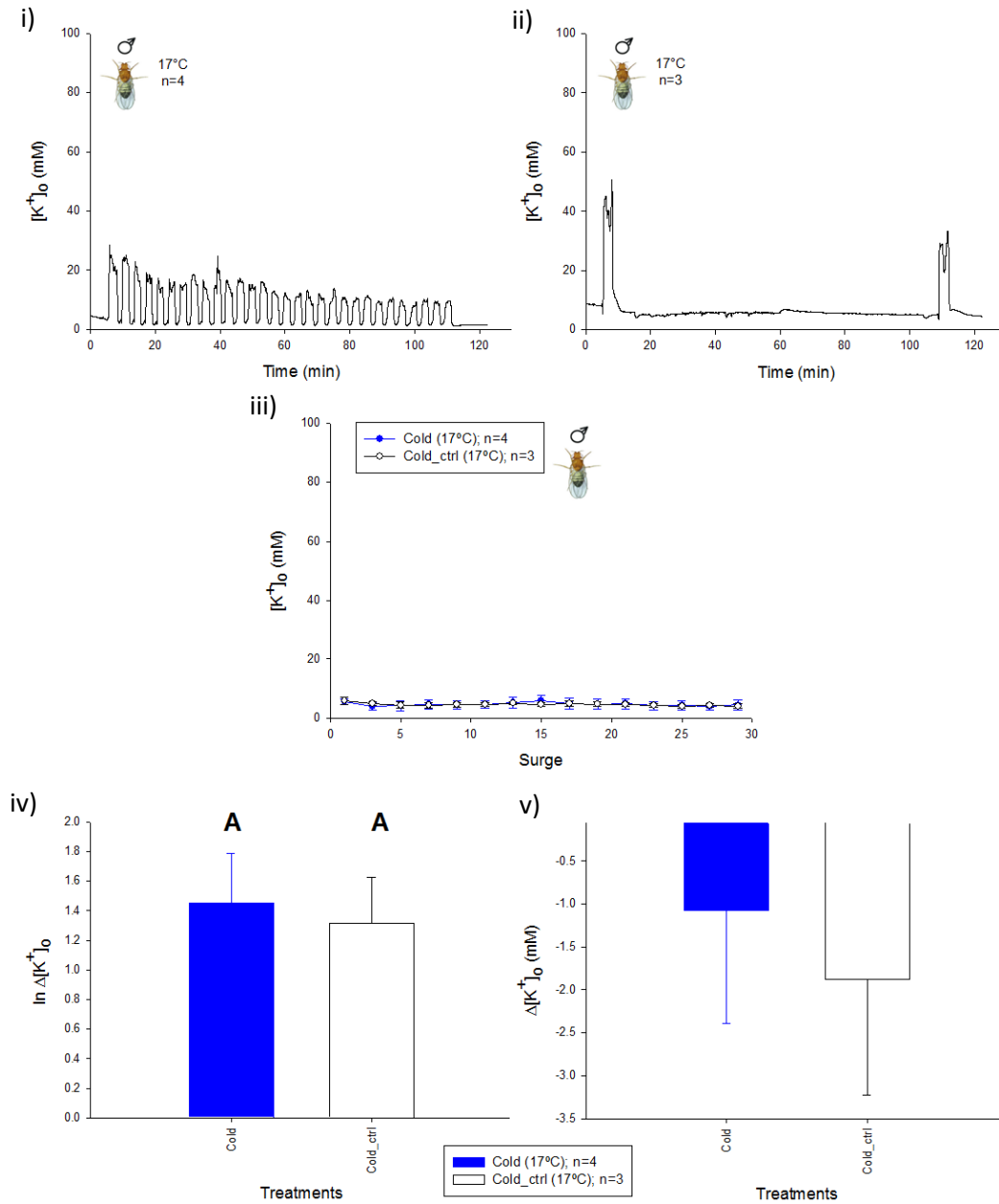


Figure 16. Comparison of cold temperature and control experiments performed in males under reduced dehydration conditions. **i)** Thirty cycles of 3 minutes of anoxia and 0.5 minutes of normoxia at 17°C did not cause a conspicuous $[K^+]_o$ baseline increment. **ii)** Control experiments with no AD iteration showed that the preparation itself did not introduce any variation in the response variable. **iii)** Comparison of mean $[K^+]_o$ baseline plots suggested no apparent difference between treatment (cold) and control (cold_ctrl). **iv)** Statistical analyses on the $\ln \Delta[K^+]_o$ confirmed that there were no significant differences between cold temperature experiments and cold controls (one-way ANOVA with Holm-Sidak method as multiple comparison test, $p = 0.721$). **v)** A histogram of the untransformed response variable showed that there is actually a slight decrease in $\Delta[K^+]_o$ at 17°C in both treatment and controls. For iii-v values are means \pm SEM, $n_{\text{cold}} = 4$, $n_{\text{cold_ctrl}} = 3$, and lettering indicates different statistical groupings.

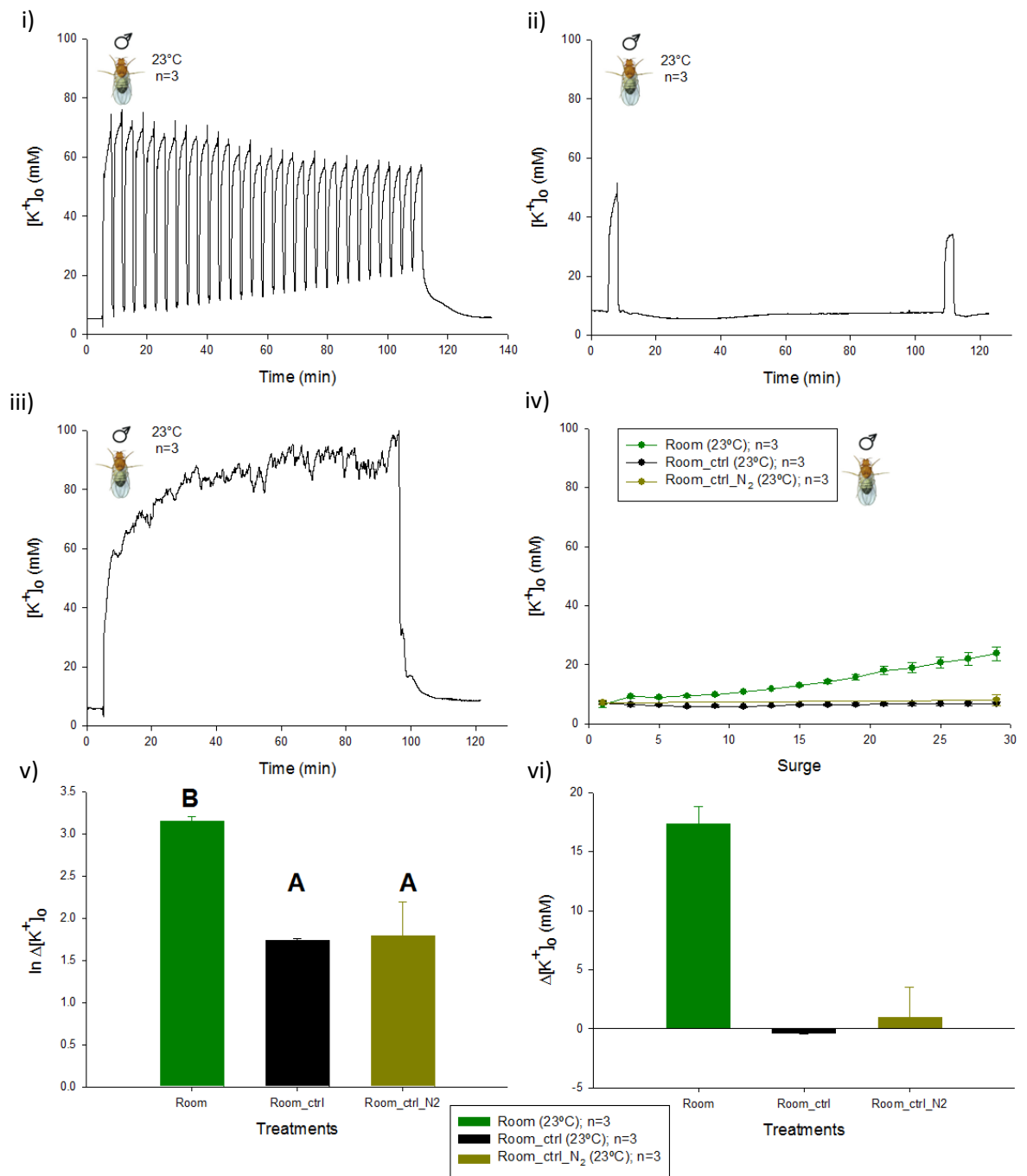


Figure 17. Comparison of room temperature and control experiments performed in males under reduced dehydration conditions. **i)** Repetitive ADs at 23°C generated a gradual increment of $[K^+]_o$ baseline. After the 30th surge, baseline was recovered in approximately 20 minutes. **ii)** Absence of AD repetition did not cause any obvious baseline change. **iii)** Control experiments consisting of 90 minutes of anoxia and 15 minutes of normoxia during 1 cycle did not cause any relevant $[K^+]_o$ increment either. **iv)** Line/scatter plots of mean $[K^+]_o$ baseline suggested that AD iteration at 23°C disrupted $[K^+]_o$ homeostasis. Conversely, lack of AD repetition (room_ctrl) or application of a long N₂ pulse (room_ctrl N₂) did not affect baseline stability. **v)** Analysis of $\ln\Delta[K^+]_o$ corroborated the difference between the room temperature treatment and the controls (one-way ANOVA with Holm-Sidak method as *post hoc* multiple comparison test; room vs. room_ctrl, $p = 0.002$; room vs. room_ctrl_N₂, $p = 0.003$). In contrast, there were no significant disparities between the two controls ($p = 0.894$). **vi)** A bar plot of the untransformed $\Delta[K^+]_o$ variable supports the statistical relationships established in v. Absence of AD iteration caused a slight baseline decrease, and a long N₂ pulse produced a mild baseline increment. For iv-vi values are means \pm SEM, $n = 3$ for the treatment and each one of the controls, and lettering indicates different statistical groupings.

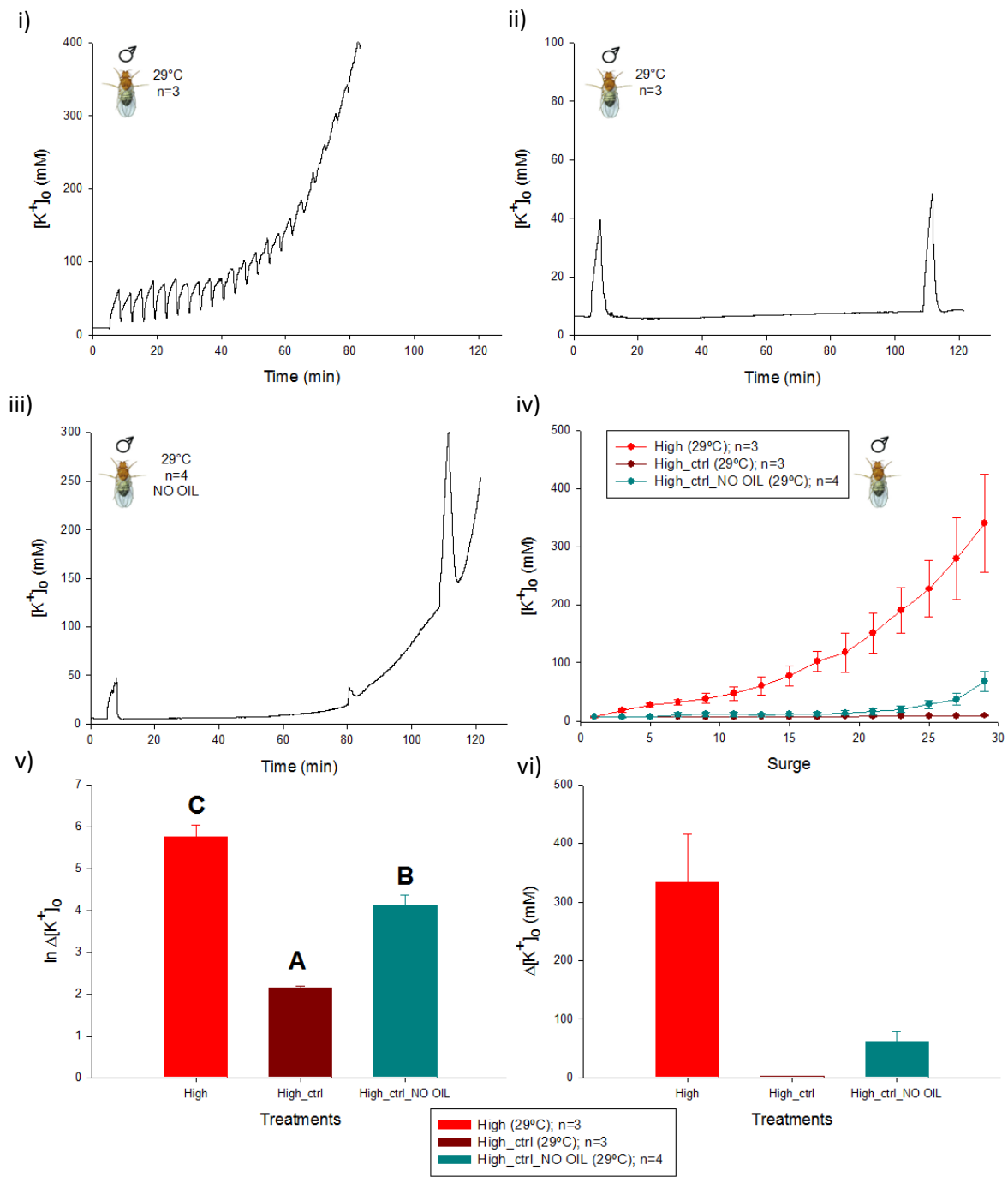


Figure 18. Comparison of high temperature and control experiments performed in males under reduced dehydration conditions. **i)** High temperature caused extreme $[K^+]_o$ baseline disruption during repetitive ADs, preventing flies from recovery after the experiments. The y-axis was reduced in order to show a range of possible physiological concentrations. Higher $[K^+]_o$ values were obtained but were considered artifactual. **ii)** Lack of AD iteration at 29°C produced very little baseline variation. **iii)** The same pattern performed in ii was implemented without sealing the fly's head with Halocarbon oil 700. All the flies had a stable baseline approximately during the first 80 minutes of experiment; then they underwent a spontaneous surge that initiated a very steady and fast increase in $[K^+]_o$ baseline. **iv)** Baseline plots of the treatment and the controls showed three trends: hyperthermia and AD iteration exacerbated loss of $[K^+]_o$ homeostasis, absence of AD repetition (high_ctrl) did not produce a conspicuous disruption in $[K^+]_o$ baseline, and lack of Halocarbon oil 700 in the head (high_ctrl_NO OIL) produced a moderate baseline increase at the end of the experiments. **v)** A one way ANOVA (Holm-Sidak method as pairwise multiple comparison test) on the $\ln\Delta[K^+]_o$ confirmed the trends evidenced in iv: there were significant differences between all treatments ($p < 0.001$ for all tests). **vi)** A histogram of mean $\Delta[K^+]_o$ supports the statistical differences found in v. For iv-vi values are means \pm SEM, $n_{high} = 3$, $n_{high_ctrl} = 3$, $n_{high_ctrl_NO\ OIL} = 4$, and lettering indicates different statistical groupings.

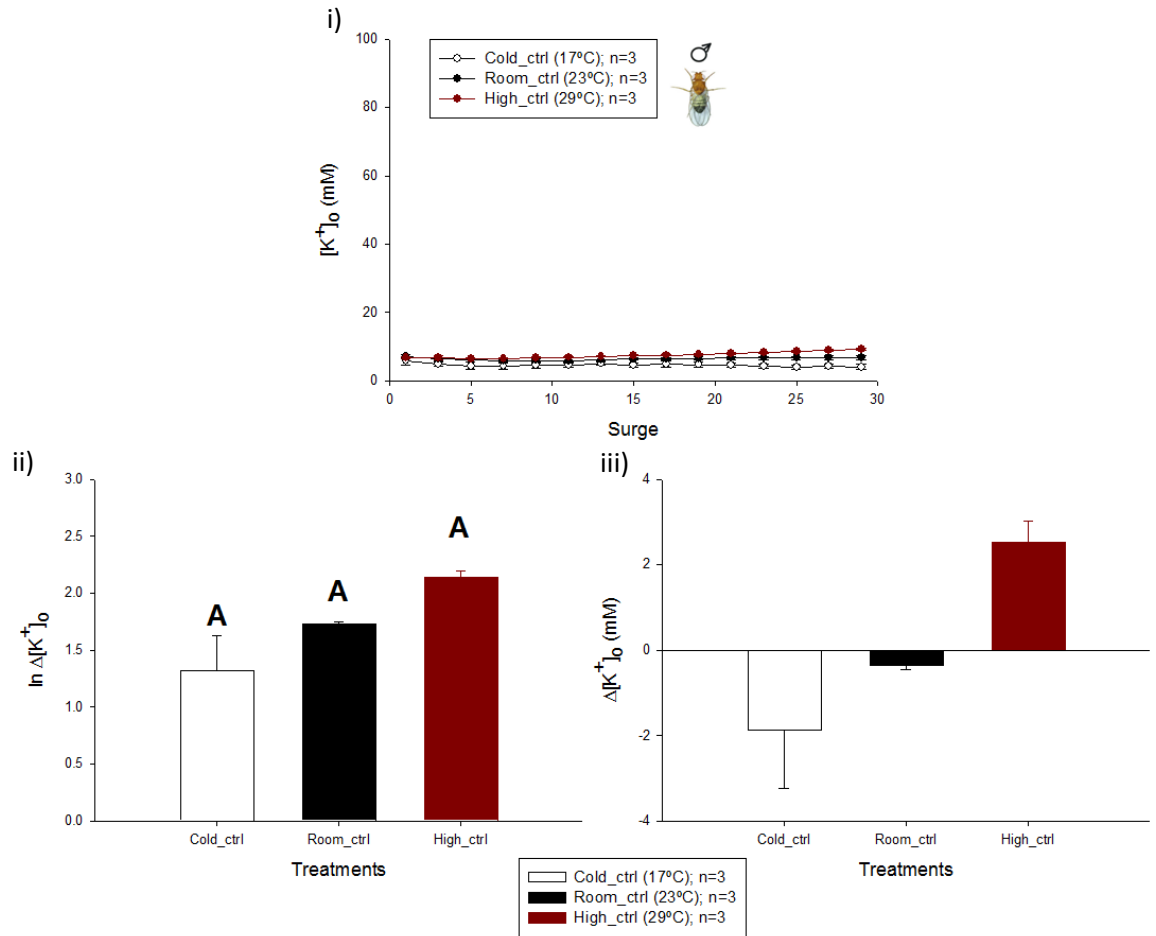


Figure 19. Comparison of control experiments performed under reduced dehydration conditions at three different temperatures. **i)** Line/scatter plots of $[K^+]_o$ baseline showed that absence of AD repetition in males at 17°C (cold_ctrl), 23°C (room_ctrl) and 29°C (high_ctrl) did not have a conspicuous disruptive effect. There was only a slight divergence after the 20th surge suggesting three trends: hyperthermia caused the highest baseline disruption, hypothermia produced a minor baseline decrease, and room temperature showed intermediate values. **ii)** Statistical analyses carried out on $\ln \Delta[K^+]_o$ corroborated the absence of significant differences among the control experiments (one-way ANOVA with pairwise multiple comparisons performed by the Holm-Sidak method; cold_ctrl vs. room_ctrl, $p = 0.307$; cold_ctrl vs. high_ctrl, $p = 0.051$; room_ctrl vs. high_ctrl, $p = 0.314$). **iii)** A bar plot of $\Delta[K^+]_o$ supported the trends suggested in i. For all plots values are means \pm SEM, $n = 3$ (for all treatments), and lettering indicates different statistical groupings.

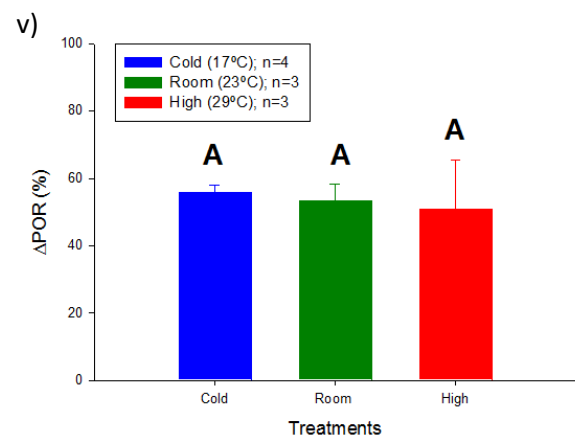
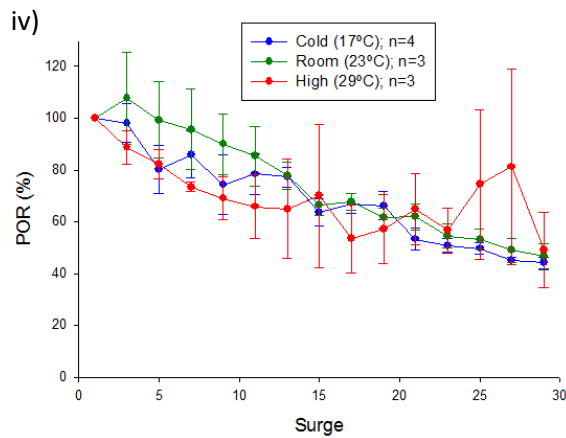
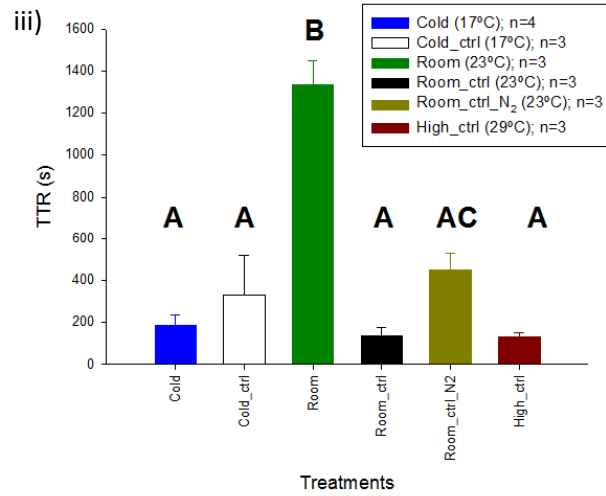
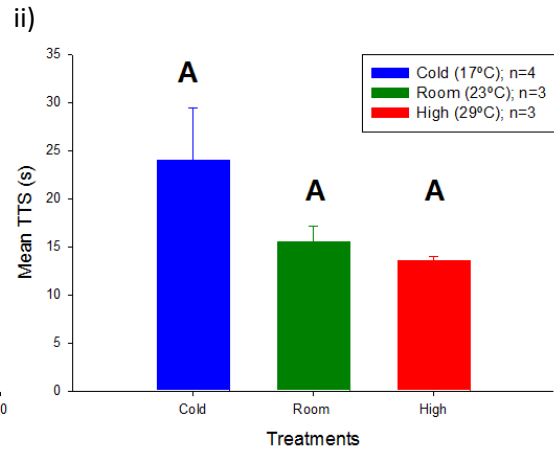
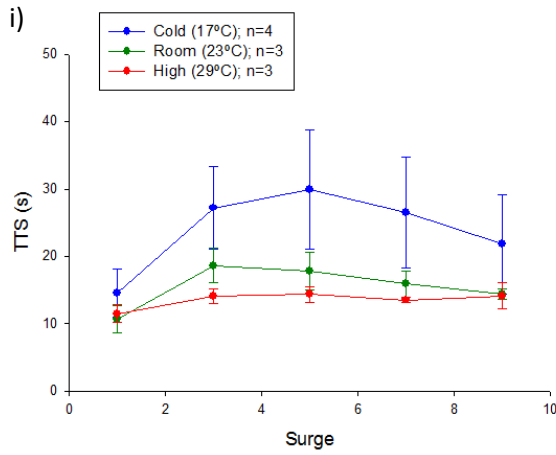


Figure 20. Influence of temperature on TTS, TTR and POR during repetitive ADs, in a setup ensuring low dehydration. **i)** Comparison of mean time to surge (TTS) for the first 9 surges (only odd surges were considered) showed three trends in males: hyperthermia (29°C) decreased TTS, hypothermia (17°C) caused an increment in TTS, and room temperature (23°C) generated intermediate values. **ii)** The corresponding mean TTS histogram suggested an inversely proportional relationship between TTS and temperature. Despite this, a one-way ANOVA reported no significant differences among the different treatments ($p = 0.194$). **iii)** Bar plot for time to recovery (TTR) after the 30th surge. At room temperature, TTR was significantly higher than during hypothermia and controls (one-way ANOVA with Holm-Sidak method as a pairwise multiple comparison test, $p < 0.001$ for all tests). There were no statistically significant differences between controls and the cold temperature treatment ($p > 0.05$). However, lack of ADs at 17°C (ctrl_cold) and a 90 minute N₂ pulse followed by 15 minutes of normoxia (room_ctrl_N₂) seemed to have caused higher TTR than AD repetition at 17°C and lack of AD iteration at 23°C (room_ctrl) and 29°C (high_ctrl). Such observation was partially supported by the statistical analysis, which separated room_ctrl_N₂ from room_ctrl ($p = 0.038$) and high_ctrl ($p = 0.038$), and grouped it with cold ($p = 0.059$), and cold_ctrl ($p = 0.402$). Despite its high value, cold_ctrl was grouped with hypothermia and all the other controls ($p > 0.1$ for all tests). **iv)** The line/scatter plot showed gradual POR reduction as recurrent ADs were applied and suggested no effect of temperature on the variable. **v)** A histogram of Δ POR and a one-way ANOVA ($p = 0.910$) reported no differences between treatments. For all plots values are means \pm SEM, $n = 3$ for all treatments (except for $n_{\text{cold}} = 4$), and lettering indicates different statistical groupings.

Chapter 4

Discussion

The present study focused on how K^+ homeostasis can be affected by repeated O_2 reperfusion in the fly's brain neuropile. Additionally, temperature and its modulating effects on possible oxidative stress were also investigated. Since only one known study has monitored brain $[K^+]_o$ in *Drosophila* during AD iteration (Armstrong *et al.*, 2011), and given that it is still not clear what mechanisms allow the fly to tolerate repeated O_2 reperfusion, it is important to broaden our knowledge on this field by testing additional variables (*e.g.* temperature). This discussion will deal initially with the repercussions dehydration may have produced in the results obtained. Subsequently, the effect of temperature on the response variables time to surge (TTS), percentage of recovery (POR) and time to recovery (TTR) will be considered. Then, general effects of AD iteration on $[K^+]_o$ baseline maintenance will be discussed. Next, comparison of $[K^+]_o$ baseline maintenance between males and females will allow analysis of dimorphic responses to temperature and repetitive anoxia. Afterwards, the effect of temperature on $[K^+]_o$ baseline maintenance will illustrate the importance of this variable in the enhancement of protective/damaging neural mechanisms. Additionally, a speculative model outlining temperature modulation of the most important molecular and physiological mechanisms taking place in the fly's brain during repetitive anoxia will be presented. Finally, future research directions and preliminary development of pharmacological techniques on the fly will be provided. This study concluded that 1) N_2 -delivery patterns consisting of long anoxia, short normoxia and increased cycle frequency augmented disruption of brain $[K^+]_o$ baseline; 2)

males are more susceptible to AD repetition than females at room temperature; and 3) hypothermia has a protective effect evidenced as a stable brain $[K^+]_o$ baseline maintenance during repeated reperfusion.

4.1 Effect of Dehydration

4.1.1 Dehydration and AD Repetition

The data sets collected in the present study had different levels of desiccation control: low in the “sex-based comparison” (no humidified gas flow; figures 10-12), partial in the “nitrogen-delivery pattern” (humidified gas flow during anoxia; figures 13-14) and complete in the “more refined approach” (humidified gas flow during both anoxia and normoxia; figures 15-20). Such refinement in preventing water loss from the preparation was the result of the evolution of the project during the year and a half that the experimental phase lasted. During AD iteration, low and partial water loss control in the preparation caused permanent slight loss of $[K^+]_o$ baseline at 16°C (both sexes) and 23°C (only females), permanent major baseline loss in males at 23°C, and permanent early disruption of $[K^+]_o$ homeostasis at 30°C (after ~40 min). On the other hand complete dehydration control produced a slight $[K^+]_o$ baseline reduction at 17°C, temporary moderate baseline loss at 23°C, and permanent delayed alteration of $[K^+]_o$ homeostasis at 29°C (after ~60-80 min). Hence, hydric stress on the tissue exacerbated the disrupting effects of AD repetition, thereby causing more rapid loss of $[K^+]_o$ homeostasis and permanent damage. Dehydration probably impaired recovery processes like removal of anaerobic end products and reactive oxygen species (ROS), and repair/restoration of protein function (Harrison and Haddad, 2011; Schilman *et al.*, 2011). Concerning time to surge (TTS),

there was no major change in values and trends between experimental conditions with low and complete control of desiccation (see section 4.2.1 for a more detailed discussion).

4.1.2 Dehydration and Controls

Regarding control experiments, two types were performed: air controls (figure 10, iii) and nitrogen controls (figure 17, iii). Since air controls did not involve AD repetition, no increased dehydration was expected (Lighton and Schilman, 2007). Nevertheless, given that *Drosophila* cannot completely close its spiracles (Williams and Bradley, 1998), some desiccation-driven $[K^+]_o$ baseline loss was evident under low ($\Delta[K^+]_o = 10.5 \pm 1.1$ mM) and partial ($\Delta[K^+]_o = 7.7$ mM) dehydration control conditions at 23°C. On the other hand, complete control of desiccation generated an insignificant baseline decrease at 17°C ($\Delta[K^+]_o = -1.9 \pm 1.3$ mM) and 23°C ($\Delta[K^+]_o = -0.3 \pm 0.1$ mM), and a slight increment at 29°C ($\Delta[K^+]_o = 2.5 \pm 0.5$ mM) (figure 19, iii). During room and cold temperature controls, the air surrounding the fly was saturated with water vapor that probably diffused to the tracheal system through the totally constricted, but not completely closed spiracles. Therefore, excessive hydration could have caused a reduction of the ECS and a subsequent baseline increase owing to the confinement of extracellular solutes in a smaller space. Additionally, excess of water in the tissues could have also produced an $[K^+]_o$ baseline decrease because of an increment in extracellular solvent. However, dilution of extracellular K^+ probably had a large effect that offset the decrease in ECS, generating a slight baseline reduction. The minor role of cell swelling in $[K^+]_o$ variation was also evident in rat optic nerve, where anoxia caused an ECS reduction of 20%, accounting for an $[K^+]_o$ increment of less than 1 mM (Ransom and Philbin, 1992). During the high temperature treatment, the water vapor surrounding the fly was dissipated more easily because of the hot TEC, possibly producing some

outwardly net flow of water vapor through the spiracles. This probably accounted for the slight baseline increase observed. Compared to the extremely high values observed during AD repetition at high temperature, this $[K^+]_o$ baseline increment because of dehydration was minimal and did not significantly bias the results obtained.

Nitrogen controls disrupted spiracular control since they involved anoxic comas lasting for 90 minutes (Lighton and Schilman, 2007). Therefore a major $[K^+]_o$ baseline increase was anticipated because of dehydration. Nonetheless, partial desiccation conditions during the nitrogen-delivery pattern caused a moderate baseline increment ($\Delta[K^+]_o = 6.3\text{-}11.1$ mM) similar to the one found in the air control ($\Delta[K^+]_o = 7.7$ mM). The application of humidified nitrogen kept the hydric integrity of the tissue during the whole anoxic coma, despite the loss of spiracular control. However, the lack of humidified air during the remaining 15 min of normoxia possibly caused the moderate baseline rise. In contrast, complete control of water loss in the preparation at 23°C preserved moisture in the tracheal system and baseline barely increased ($\Delta[K^+]_o = 1.0 \pm 2.6$ mM).

The inability of *Drosophila* to completely close its spiracles (Williams and Bradley, 1998) and the resulting loss of tracheal water vapor were not a significant cause of $[K^+]_o$ baseline increase during air controls performed under minimized dehydration conditions. However, direct desiccation of the fly's brain took place through the window opened in the head to insert the microelectrodes. Consequently, sealing the head with Halocarbon oil 700 was necessary to preserve the stability of the preparation. At 29°C, male flies with an unsealed head maintained a stable baseline approximately during the first 80 minutes of experiment. Subsequently, they underwent a spontaneous $[K^+]_o$ surge and baseline rose abruptly without any sign of recovery ($\Delta[K^+]_o = 61.5 \pm 16.7$ mM; figure 18, iii). Conversely, male flies with an oil-sealed head had an

insignificant $[K^+]_o$ baseline increment ($\Delta[K^+]_o = 2.5 \pm 0.5$ mM; figure 18, ii). Given that the gas flowing on the fly was humidified, the simplest explanation for the baseline disruption is direct desiccation of the fly's brain. Therefore, the use of oil to seal the fly's head window was crucial to conserve the integrity of the nervous tissue exposed to the environment.

4.2 Temperature Effect on TTS, POR and TTR during AD Repetition

Drosophila's brain has traditionally been divided into different neuropile regions (Cachero *et al.*, 2010). The K^+ -sensitive microelectrode was inserted behind the ocelli at an approximate angle of 70° with respect to the horizontal plane of the head and at a depth of about $1/4$ the height of the head. Taking into account the location of the microelectrode, and after comparison with the atlas of the *Drosophila* brain available at the website Flybrain of the University of Arizona (<http://flybrain.neurobio.arizona.edu>), it was possible to establish likely neuropile regions whose $[K^+]_o$ was assessed during the experiments. Such regions were, from top to bottom, the superior protocerebrum (medial and lateral), a region of unnamed neuropile, and the fan shaped body in the central body complex.

4.2.1 Time to Surge (TTS)

Lack of ATP during anoxia causes failure of the Na^+/K^+ pump and K^+ flows out of the cell (Ransom and Philbin, 1992), possibly through different types of K^+ ion channels. Given that the Q_{10} value for ion channel currents is approximately equal to 2 (Buck and Pamerter, 2006) and that *Drosophila's* MR has a Q_{10} value of 2.2 (Schilman *et al.*, 2011), temperature was expected to modulate TTS in an inversely proportional manner during AD iteration. Accordingly, low

temperature incremented the time it took for the brain to show a surge and high temperature decreased TTS. Even though there was no statistical significance between some of the treatments, the inversely proportional trend was still evident (figure 12, iii-iv; figure 20, ii). Taking into account the Q_{10} values for MR and ion channel currents, TTS values obtained for males under low and complete dehydration control conditions approximately coincided with what was expected: TTS values for hypothermia were about twice as large as the ones for hyperthermia. Additionally, the effect of dehydration seemed to lower TTS values by approximately 5s. This suggests that desiccation increased MR, and that such an increment may have accounted for augmented ROS/RNS accumulation damage, which probably caused permanent $[K^+]_o$ baseline disruption. Contrastingly, females had a 5-fold TTS increase at low temperature compared to high temperature. Prima facie, such disparity indicates that dissimilarities in MR sensitivity to temperature could have underlain the differences in $[K^+]_o$ baseline maintenance found between sexes at room temperature. However, TTS values between males and females at room temperature were similar, suggesting that MRs were not different and that the increased susceptibility of males to AD iteration was probably due to morphological and anatomical dimorphisms. Furthermore, the considerable reduction in TTS for females during hyperthermia (about 50% of TTS for males) suggests that an increment in MR may have augmented free radical accumulation damage, causing surges in females to become imperceptible after a fewer number of AD repetitions (6) compared to males (9). However, it must be taken into account that misinterpretation of the K^+ -electrode equilibrium potential (E_p) recording led to finishing the experiments in females before time (see section 4.3). These results suggest that MR between sexes is similar at low and intermediate temperatures, and that female MR has an increased sensitivity to high temperatures. Whether this observation is

correct or not, remains to be confirmed in the future since current MR studies that pooled together males and females during anoxia/hypoxia did not change temperature (Krishnan *et al.*, 1997; Van Voorhies, 2009), and those that changed temperature during anoxia only used males (Schilman *et al.*, 2011). Concerning the effect of AD iteration, females were not apparently affected and traces at different temperatures remained approximately unchanged after every reperfusion (figure 12, ii); the fly's brain maintained the same metabolic performance despite the damage caused by repeated reperfusion. Taking TTS as an indirect measure of MR, the previous result indicates that AD iteration had no effect on MR, which remained approximately constant within each treatment. Males showed similar results, but the high and room temperature traces increased at the end. This could have been the result of a decrease in K^+ membrane conductance owing to anoxia-driven inactivation of K^+ ion channels (Hochachka, 1986; Buck and Pamerter, 2006) and/or oxidative damage and subsequent loss of functionality of K^+ ion channels. These traces coincided during most of their lengths, suggesting that males had a lower MR sensitivity at high temperature. Hence, if experiments with females had not been stopped before time, and disregarding the effects of desiccation and morphological/anatomical differences, females would have been expected to show higher $[K^+]_o$ baseline loss than males at high temperature.

4.2.2 Percentage of Recovery (POR)

POR was only calculated for experiments performed under reduced dehydration conditions. In all treatments, this variable was gradually reduced by AD repetition and the rate of decrease did not depend on temperature (figure 20, iv-v). A progressive fall in amplitude with each reperfusion underlay such POR decrement. At room temperature, amplitude reduction was

the result of two aspects of the trace (figure 15, ii): a top component that caused decrement of the highest value reached by a surge, and a bottom component that produced an increment in baseline. The bottom component appears to be the result of failure of clearance mechanisms (*i.e.* Na⁺/K⁺ pump and “K⁺ siphoning”) possibly because of accumulation of ROS/RNS damage and the action of ATP-saving molecular pathways. It is important to mention that, in this case, the deterioration rate was probably slightly higher than the recovery rate. This may be the reason underlying the lack of permanent damage to the fly’s brain. Additionally, AMPK could have also decreased ATP demand by causing hypoxia-induced Na⁺/K⁺ ATPase endocytosis, a strategy already described in rat alveolar epithelial cells (Gusarova *et al.*, 2009).

On the other hand, the top component was probably underlain by progressive inactivation of accumulation mechanisms (*i.e.* K⁺ ion channels) that restricted the out flow of K⁺ and caused a gradual reduction in the maximum amplitude value of the surges. During hypoxia/anoxia, ion channel arrest could have been mediated by AMPK (Kreneisz *et al.*, 2009) and/or calmodulin (Buck and Pamerter, 2006). Although K⁺ conductance inactivation was probably the factor that regulated the behavior of the top component, it must be taken into account that other mechanisms could have increased membrane permeability, thereby causing increased excitability and subsequent susceptibility to anoxia. For instance, activation of the NO/PKG/PP2A pathway has been shown to increase whole-animal vulnerability to anoxia by activation of K⁺ conductances in the cell membrane (Dawson-Scully *et al.*, 2010). Hence, under complete control of dehydration, ion channel arrest mechanisms seemed to have been favored, possibly producing the characteristic progressive reduction of the maximum surge amplitude value after each AD.

The protective properties of hypothermia were evident during AD iteration: only the top component was conspicuous, suggesting activation of channel arrest mechanisms in order to reduce energy consumption. Furthermore, the bottom component was absent probably owing to optimal action of clearance mechanisms. The unaffected performance of clearance mechanisms suggests low ROS/RNS accumulation damage rate and decreased consumption of endogenous antioxidant enzymes (figure 15, i).

Contrastingly, the bottom component was extremely dominant during hyperthermia and overrode the protective action of the top component (figure 15, iii). Excessive accumulation of oxidative stress cell damage and ATP depletion may have been the main factors that caused failure of clearance mechanisms and the resulting dominance of the bottom component. Since cell deterioration was possibly much greater than recovery rate, $[K^+]_o$ baseline was rapidly and permanently lost.

Although POR was not calculated for experiments affected by dehydration, it is important to point out that, in these experiments, amplitudes did not behave completely as previously described. For both sexes at low temperature, the bottom component was slightly dominant, indicating that hypothermia reduced, but did not eliminate, the exacerbating effect of dehydration (figure 11, i-ii). On the other hand, the top component changed its typical trend, and showed gradual increase of the maximum surge amplitude value after each AD. This suggests that dehydration favored alternate pathways that increased K^+ membrane permeability (*e.g.* the NO/PKG/PP2A pathway) and offset the action of protective ion channel arrest mechanisms. Therefore, increased excitability and subsequent depletion of ATP reserves may explain in part why desiccation severely damaged cells and caused permanent $[K^+]_o$ baseline loss. These results were also observed in females at room temperature. At high temperature,

results obtained for males and females matched the ones observed under low dehydration conditions. The bottom component of males at room temperature was more dominant than in females because of morphological and anatomical dimorphisms (for a detailed discussion see section 4.3).

4.2.3 Time to Recovery (TTR)

TTR was only calculated on experiments performed under reduced dehydration conditions since they, with exception of high temperature experiments, were the only ones in which the initial baseline was recovered after sustained reperfusion. Baseline recovery depends on clearance mechanisms, which may be mainly affected by temperature, ROS/RNS damage accumulation and ATP depletion. At room temperature, TTR after AD repetition considerably increased compared to hypothermia and controls (air and nitrogen) probably because damage rates were slightly higher than recovery rates. Therefore, repair mechanisms required more time to reestablish the initial $[K^+]_o$ (figure 20, iii). The intermediate damage accumulated allowed identification of two stages during recovery, namely a fast phase and a slow phase (figure 15, ii). The fast phase was possibly carried out in greater part by the Na^+/K^+ pump in neurons and glial cells. The steep slope of the trace suggests intense activity of the pump to restore transmembrane ion gradients and repolarize cell membranes. Gradual hyperpolarization of cell membranes probably opened K_{ir} channels in glial cells, allowing “ K^+ siphoning” to perform most of the K^+ removal during the slow phase. Hence, there was a slow progressive transition between the two stages in which both clearance mechanisms may have worked synergistically to restore $[K^+]_o$ baseline until “ K^+ siphoning” became the main contributor. Increased contribution of “ K^+ siphoning” suggests that part of the energy used by the Na^+/K^+ pump was

probably redirected to fuel repair mechanisms. Therefore, the presence of the slow phase was possibly indicative of increased cell damage. Despite the lack of statistical difference with other controls, nitrogen controls showed slightly greater TTR values; such a result was expected considering that recovery time from anoxia depends on duration of the anoxic period (Krishnan *et al.*, 1997; Lighton and Schilman 2007). Nevertheless, the reduced increase in TTR suggested that the contribution to cell deterioration of anaerobic metabolites and free radical accumulation during 90 minutes of anoxia and subsequent reperfusion was not important. Accordingly, recovery only displayed the fast phase suggesting low accumulated cell damage.

Provided that ion pump activity has a Q_{10} much greater than 2 (Buck and Pamenter, 2006), a slight increase in TTR was observed during controls and treatments at low temperature. This indicated a mild slowdown of the Na^+/K^+ pump compared to air controls at room and high temperatures. Given that during hypothermia accumulation of cell damage owing to oxidative stress may have not played an important role, flies recovered quickly after AD repetition compared to room temperature. Additionally, only the fast phase was observed during recovery, suggesting the absence of ROS/RNS damage accumulation.

On the other hand, temperature did not appear to affect the kinetics of the Na^+/K^+ ATPase at 29°C since TTR values for air controls were similar to their counterparts at room temperature. Extreme buildup of free radical damage and ATP depletion possibly prevented flies from recovering after repeated ADs.

4.3 Sex-Based Effect

At room temperature, males had a more marked $[\text{K}^+]_o$ baseline loss than females. Comparison with the *Drosophila* brain atlas (<http://flybrain.neurobio.arizona.edu>) showed that

some of the neuropile regions described in the MER (Cachero *et al.*, 2010) partially coincided with the probable zones where $[K^+]_o$ was assessed in the present study: two regions (*i.e.* the protocerebral arch and the lateral junction) belonged to the superior lateral protocerebrum, and a third region (*i.e.* the ring) appertained in part to the region of unnamed neuropile and the superior lateral protocerebrum. The increase in volume of MER is possibly caused by an increment in the number of neurons or their arborizations (Cachero *et al.*, 2010). Consequently, taking into account that repetitive anoxia can augment ROS production up to 10-fold (Chandel and Schumaker, 2000), an increased number of neurons in the regions assessed during the present study could have augmented ROS accumulation during AD iteration. This probably caused more cell damage and the conspicuous baseline disruption observed in males. Another factor that could have underlain the sex-based differences observed in $[K^+]_o$ maintenance is a possible sexual dimorphism in MR. Differences in nutrient demand and energy allocation may affect the longevity of males, rendering them more susceptible to external stressors. However, the lack of sex-based differences in TTS at room temperature suggests that this factor was not important (see section 4.2.1). Finally, differences in size may have also influenced the increased susceptibility of males to AD repetition. Firstly, given that the tissue/TEC temperature correlation for this data set was performed using only females, and that males usually have a smaller size than females (about 77%, Lighton, 2007), it is possible that the temperature reached in the males' head was higher than in females (23°C (thorax) = 27°C (TEC)). A higher tissue temperature could have caused more accumulation of ROS/RNS, possibly accounting for the incremented $[K^+]_o$ homeostasis loss observed in males. However, the cooling effect of the gas stream, which was at room temperature ($\sim 22^\circ\text{C}$), could have actually reduced the temperature reached in the smaller male fly's head. Secondly, since these experiments were performed

under low dehydration control conditions, desiccation may have affected more males than females, causing an increased $[K^+]_o$ baseline disruption. It could be argued that since air controls performed in males showed a moderate baseline increase (10.5 ± 1.1 mM), the effect of dehydration was not remarkably significant. Nevertheless, the absence of repetitive anoxia in air controls allowed adequate maintenance of spiracular control. Hence, it was difficult to use these experiments to draw fair inferences about the hydric stability of the preparation. Consequently, dehydration may have played a main role in males by exacerbating the damage caused by AD iteration. Despite this, comparison of the effect of temperature on AD-driven $[K^+]_o$ baseline loss under low and complete desiccation control conditions showed that although the values obtained were not comparable, the trends were still preserved. According to this, it is possible to obtain under reduced dehydration conditions the same differential tendencies observed between males and females in this data set. Nevertheless, dissimilarities may not be as conspicuous because of the lack of hydric stress.

Contrastingly, males were able to resist a higher number of reperfusions than females before permanently losing their K^+ homeostasis at high temperature (figure 11, v-vi). The relationship between E_p and $[K^+]_o$ was not linear, but exponential (equation 3). This means that a small variation of high E_p values was equivalent to a large change in $[K^+]_o$, and vice versa. Consequently, when E_p reached high values at high temperature, surges that looked small and almost imperceptible could have represented moderately sized surges when converted to $[K^+]_o$. Given that the criterion to determine if a fly died before the end of an experiment was the absence of surges, experiments were stopped as soon as the surges became imperceptible. Consequently, it is possible that some surges were missed in females owing to misinterpretation of the E_p trace, and subsequent early termination of the experiments. This was also evident in

the value for $\Delta[K^+]_o$, which was significantly smaller in females at high temperature (figure 11, vii). If experiments with females had not been stopped before time, probably a greater number of surges could have been recorded, and results would have shown higher tolerance to AD iteration compared to males. In spite of this, high temperature experiments in females were still useful given that their $\Delta[K^+]_o$ values were significantly greater than the ones observed at low temperature, a result that illustrated how temperature possibly modulated ROS/RNS accumulation and subsequent cell damage during repeated ADs. Additionally, a difference in MR sensitivity to high temperatures between males and females is a factor that should not be discarded as a possible cause of the differential number of surges displayed (see section 4.2.1 for a discussion).

4.4 Temperature Effect on $[K^+]_o$ Baseline Maintenance during AD Repetition

Drosophila is an ectothermic organism, and changes to its metabolic rate can be easily performed by varying the temperature of its surroundings (Schilman *et al.*, 2011). Given that MR is affected by temperature, cellular, metabolic and physiological processes can be directly slowed down or accelerated just by controlling this variable. To overcome deterioration produced during repeated ADs, there must be a balance between damage and repair mechanisms. Any marked disruption of this equilibrium can lead to either permanent cell damage or increased insult tolerance. It has been shown previously that temperature affects deterioration and recovery rates during anoxia in *Drosophila* (Schilman *et al.*, 2011). Accordingly, in the present study this variable may have modulated accumulation of ROS/RNS and anaerobic metabolites, and repair mechanism rates during repeated reperfusion. Possible temperature-

driven unbalance between cell damage and recovery was evidenced as different levels of $[K^+]_o$ baseline loss.

During AD iteration, hypothermia preserved the integrity of K^+ homeostasis even under hydric stress (figure 11, v-vi; figure 15, iv). Low temperature affects transport mechanisms involved in ion balance (Carpenter, 1981). Furthermore, ion channel currents have a Q_{10} approximately equal to 2 whereas ion pump activity has a Q_{10} much greater than 2 (Buck and Pamerter, 2006). Such disparity can be disadvantageous at low temperatures and may cause membrane depolarization and increase in $[K^+]_o$ because of decreased activity of the Na^+/K^+ pump. However, *Drosophila* can maintain resting potentials during hypothermia better than other species, like *Apis mellifera*, and only temperatures close to chill coma ($7 \pm 0.9^\circ\text{C}$) may cause $[K^+]_o$ increment (Hosler *et al.*, 2000). Therefore, the temperatures applied during the present study (16°C and 17°C) may have slowed the Na^+/K^+ pump but did not affect the K^+ gradient maintenance. Evidence of this was the stable $[K^+]_o$ baseline recorded for 5 minutes before every experiment and during air controls, at low temperatures. During hypothermia, recovery rates were probably equal to or higher than reperfusion-driven damage rates, thereby causing increased tolerance to AD iteration. Thus, low temperature may have decreased the rate of ROS/RNS accumulation, reducing cell damage produced by DNA, lipid and protein oxidation. Furthermore, hypothermia possibly delayed the consumption of endogenous antioxidant enzymes and energy metabolites (Zhang *et al.*, 2011), enhancing the performance of recovery mechanisms. Interestingly, this effect seemed to offset the decrease in antioxidant enzymatic activity that hypothermia may have produced. Under low dehydration control conditions, low temperature significantly decreased (but did not prevent) $[K^+]_o$ homeostasis disruption and even improved the susceptibility to AD iteration shown by males. Therefore,

besides the role it could have had decreasing ROS/RNS accumulation, it also diminished the exacerbating effect of desiccation on baseline loss. Such effect was probably caused by the increased water condensation evidenced on the TEC, which may have reduced tracheal water vapor loss. Under complete desiccation control conditions, low temperature highly stabilized $[K^+]_o$ baseline and caused a slight decrease in $\Delta[K^+]_o$. The reduction was probably caused by solvent increment in the ECS produced by diffusion of water vapor from the environment to the tracheal system through the open spiracles.

Hyperthermia severely exacerbated $[K^+]_o$ baseline disruption in the fly's brain during repeated reperfusion, even under complete desiccation control conditions. Possible cell damage may have been irreparable and flies never recovered their initial $[K^+]_o$ baseline. Provided that *Drosophila's* MR has a Q_{10} of 2.2 (Schilman *et al.*, 2011), flies at 30°C had a MR at least two times greater than the MR of flies at 16°C or 17°C. Given the disparity regarding Q_{10} values for ion pump activity and ion channel currents (Buck and Pamenter, 2006), an increase in temperature could be advantageous since ion pumps can work faster to reestablish the initial $[K^+]_o$ after O_2 is restored. Accordingly, recordings showed a steady baseline before the beginning of the experiments and during air controls at high temperature. Nevertheless, increased MR possibly favored considerable ROS/RNS/anaerobic metabolite accumulation, and rapid consumption of endogenous antioxidant enzymes and energy metabolites after each reperfusion. Consequently, the damage produced by these factors may have dramatically exceeded repair mechanisms. Despite the remarkable resilience of the fly, protein, lipid and DNA oxidation probably impaired recovery of the Na^+/K^+ pump and caused cell death. Additionally, apoptotic mechanisms could have been triggered by increased $[Ca^{2+}]_i$ (reviewed by Broughton *et al.*, 2009) and accumulation of anaerobic end-products (reviewed by Won *et al.*, 2002). Furthermore, during recovery at high

temperature, ATP demand increases but O₂ diffusivity remains barely changed. Thus, O₂ cannot be efficiently delivered in order to provide the Na⁺/K⁺ pump with enough energy to clear [K⁺]_o. (Frazier *et al.*, 2001; Lighton, 2007). The combination of these factors may have been responsible for the considerable permanent baseline disruption. Moreover, the exacerbating effect of dehydration hastened the onset of baseline loss (~40 minutes) compared to reduced desiccation conditions (~60-80 minutes). Interestingly, males showed no differences between high and room temperature treatments. Such outcome was probably caused by two factors: males were more sensitive to AD iteration at room temperature, showing a high $\Delta[K^+]_o$, and high temperature experiments were stopped before time because of premature death of the fly. If experiments had continued until the end, results would have resembled more the ones obtained under reduced dehydration (figure 15, v), and a clear trend showing higher baseline loss at high temperature would have been observed.

[K⁺]_o homeostasis was moderately altered by AD iteration at 23°C. Modulation of the fly's MR by this temperature was possibly responsible for a slight increase in ROS/RNS accumulation damage compared to repair mechanisms. Such damage may have affected the performance of clearance mechanisms (*i.e.* Na⁺/K⁺ pump, K⁺ "siphoning"), producing a gradual baseline loss after each reperfusion. Under complete dehydration control conditions, flies recovered their initial baseline after sustained reperfusion (figure 17, i), suggesting that cell damage was reversible. On the other hand, increased or partial dehydration may have aggravated the deterioration caused by oxidative stress, producing permanent loss of [K⁺]_o baseline. The accumulation of free radicals may explain why patterns with long anoxia, short normoxia and increased cycle frequency affected more baseline maintenance in the fly's brain: they probably guaranteed higher ROS/RNS accumulation, reduced recovery time and amplified ROS/RNS buildup thanks to

repeated reperfusion. Although the initial pattern used (2.5 minutes of anoxia/4 minutes of normoxia, 13 cycles) did not absolutely meet these standards, it also proved to be disruptive provided that AD repetition damage was exacerbated by dehydration. Additionally, the contribution of anaerobic metabolites to K^+ homeostasis loss appeared to be not significant since a 90 minute anoxic coma only caused a baseline increase of 1.0 ± 2.6 mM in the absence of dehydration (figure 17, vi). Additionally, ROS/RNS production during anoxia and reperfusion probably had a role in the slight baseline increment as well. The effect of starvation was minimal since it has been shown that there is no mortality in W1118 flies starved for 24 hours (Van Voorhies, 2009). Nonetheless, ATP depletion may have been partially responsible for the deterioration of the Na^+/K^+ pump performance and the gradual loss of $[K^+]_o$ baseline during AD iteration.

Hypothermia is a promising therapy that will probably prove effective against the detrimental effects of ischemia in humans. Increasing evidence in mammal models and in humans supports its importance as a therapeutic agent (Hassoun *et al.*, 2002; Zhang *et al.*, 2011). Additionally, the fact that low temperature increased *Drosophila's* tolerance to reperfusion is an important finding that will allow in the future dissection of the molecular pathways that confer protection during hypothermia.

4.5 Speculative Model

Based on the literature reviewed, and taking into account the results obtained in the present study, a speculative model explaining the effects of temperature and AD iteration on molecular and physiological mechanisms involved in *Drosophila's* brain K^+ homeostasis is presented (figure 21).

In the fly's brain, the absence of ATP during anoxia (figure 21, in red) shuts down the Na^+/K^+ pump and ions flow down their concentration gradients, causing an AD. $[\text{K}^+]_o$ probably increases thanks to the contribution of several types of K^+ ion channels (*i.e.* K_{Ca} , $\text{K}_{2\text{P}}$, K_{V} , and K_{ATP}), which in the absence of a stable transmembrane differential concentration gradient act as a way for K^+ to escape from the cell. Decreased $[\text{ATP}]_i$ generated by O_2 lack may also activate K_{ATP} channels and AMPK (Li and McCullough, 2009). Additionally, harmful metabolites can be produced and damage generated by anoxia/hypoxia itself can be exacerbated. On the one hand, alternate energy-producing metabolic pathways like glycolysis may be activated by AMPK in order to fuel the Na^+/K^+ pump. However, glycolysis in the absence of O_2 produces lactate, a side product that decreases the intracellular pH causing acidosis. (Ransom and Philbin, 1992). Some studies suggest that acidosis mediates neuronal apoptosis following hypoxic-ischemic insults (reviewed by Won *et al.*, 2002). On the other hand, production of ROS and RNS during hypoxia/anoxia can cause considerable cell damage, even worse than the one generated by the lack of O_2 itself (Idris *et al.*, 2005). The superoxide ion (O_2^-) is the primary radical of mitochondrial origin. It is produced by complexes I and III when electrons leak from the respiratory chain and reduce O_2 (Jastroch *et al.*, 2010). Nevertheless, disruption of the mitochondrial proton gradient during hypoxia/anoxia increases the rate at which electrons leak, thereby causing incremented O_2^- production (Buck and Pamenter, 2006). Eventually, the high reactivity of ROS causes oxidation of DNA, lipids and proteins in the cell (Murphy, 2009). RNS are formed by the production of nitric oxide (NO) and its subsequent reaction with O_2^- to produce peroxynitrite (ONOO^-), a damaging molecule that seems to be involved in cell deterioration during ischemia (Radi *et al.*, 1994). Furthermore, ONOO^- causes subsequent production of OH^- and NO_2 , two very reactive molecules that generate tissue damage by DNA, protein and lipid oxidation, as well as

DNA and protein nitration (Beckman, 1996). Cell membrane depolarization caused by disruption of the Na^+/K^+ pump and activation of K_v channels produces an increase in $[\text{Ca}^{2+}]_i$ probably by reverse operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Stys *et al.*, 1991), release of glutamate in the synaptic cleft and subsequent activation of NMDA receptors (Nicholls and Atwell 1990), and/or activation of voltage-dependent Ca^{2+} channels (Won *et al.*, 2002). On the one hand, increased $[\text{Ca}^{2+}]_i$ augments the production of ROS by collapsing the mitochondrial membrane potential (Zhang *et al.*, 1990). Additionally, it activates nNOS, an enzyme that catalyzes the conversion of L-arginine and O_2 into NO and citrulline (Alderton *et al.*, 2001). NO increases the production of cyclic guanosine monophosphate (cGMP) through interaction with soluble guanylyl cyclase (sGC). Consequently, cGMP increment can accomplish physiological effects by interacting with different receptor proteins like protein kinase G (PKG). PKG is thought to activate K^+ ion channels through phosphorylation of PP2A, causing increased susceptibility to hypoxia/anoxia (Dawson-Scully *et al.*, 2010). On the other hand, the increment in $[\text{Ca}^{2+}]_i$ can also activate K_{Ca} and calmodulin; the latter is thought to mediate protective ion channel arrest (Buck and Pamerter, 2006). Additionally, AMPK seems to be responsible for inactivation of K_{2P} channels (Kreneisz *et al.*, 2009). Nonetheless, its role in ion channel arrest appears to be more complex given that it could also be responsible for K_{ATP} channel activation and recruitment (Sukhodub *et al.*, 2007).

Two main elements are proposed to be responsible for $[\text{K}^+]_o$ clearance in the fly's brain during reestablishment of normoxia (figure 21, in blue): the Na^+/K^+ ATPase and glial cell activity. These two mechanisms could have been responsible for the fast and slow stages of recovery at room temperature (figure 15, ii). The fast phase probably corresponded to the action of the Na^+/K^+ pump in glial cells and neurons and the slow phase may have been carried out through

“K⁺ siphoning” by glial cells (Newman *et al.*, 1984). Increased performance of the Na⁺/K⁺ ATPase may have gradually repolarized cell membranes allowing K_{ir} channels in glial cells to open. At the onset of the slow phase the pump possibly decreased its activity and ATP was likely used to repair damage caused by oxidative stress. In the meantime glial cells may have passively continued restoring the initial [K⁺]_o baseline allowing energy to be redirected to more vital processes. Consequently, this suggests that the system prioritized: 1) restoration of ionic gradients; and 2) clearance of damaging free radicals and repair of proteins. Although both processes possibly happened at the same time once O₂ levels were restored, more energy was invested in the former since depolarized membranes can trigger apoptotic mechanisms through increased [Ca²⁺]_i (reviewed by Broughton *et al.*, 2009) or alter the mitochondrial membrane potential causing amplified ROS production (Buck and Pamerter, 2006). Once the neurons reached the minimum electrical condition required to function, the final stage of clearance was likely assigned to the passive mechanism of “K⁺ siphoning” and energy was invested in enhancement of repair mechanisms. However, reaction of O₂ with free electrons in the mitochondria could have caused increased ROS/RNS accumulation, and subsequent cell damage. Repeated reperfusion may have considerably exacerbated the damage.

Temperature modulated MR, probably affecting the rates of free radical/anaerobic metabolite accumulation damage and repair mechanisms during AD iteration. Recovery rates equal to or higher than damage rates may have been responsible for the protective effect of hypothermia (figure 21, in green). Consequently, reduced accumulation of ROS/RNS/anaerobic metabolites and decreased consumption of antioxidant enzymes possibly augmented tolerance to AD repetition at low temperatures. Contrastingly, hyperthermia may have caused incremented free radical/anaerobic metabolite accumulation and consumption of antioxidant

enzymes (figure 21, in brown). Hence, cell deterioration may have been considerably greater than recovery rates, probably causing cell death and severe, permanent disruption of $[K^+]_o$ baseline.

4.6 Future Research Directions

In the present study, evidence has been provided to support a model explaining the interactions of different cellular and molecular components during repetitive anoxia in the fly's brain. Furthermore, temperature modulation of such components and their relations has also been taken into account. In order to elucidate the different roles of the model constituents, two approaches can be synergistically used: the GAL4/UAS system and pharmacology. The GAL4/UAS system allows driving overexpression or suppression of a gene of interest in different tissues of the fly (reviewed by Duffy, 2002). Targeting tissues like brain, heart, glial cells, and even different types of glial cells (Stork *et al.*, 2008; Azad *et al.*, 2009; Armstrong *et al.*, 2011) is just a small demonstration of the enormous potential of this tool. Additionally, a reporter gene (*e.g.* green fluorescent protein, GFP) included in the UAS/GAL4 construct, permits visualization of the cells where expression of the gene of interest has been driven (Stork *et al.*, 2008). Consequently, molecular dissection of the AMPK and NO/PKG/PP2A pathways can be performed by up- or downregulating expression of different pathway components, and subsequently analyzing their effect on clearance and accumulation mechanisms in the $[K^+]_o$ trace at different temperatures. Additionally, the use of different K^+ -channel mutants like shaker (sh), hyperkinetic (Hk) and ether-a-go-go (eag) (Ganetzky, 1989) will provide insight into the dynamics of accumulation mechanisms and protective ion channel arrest. Furthermore, targeted expression of RNAi allows analysis of loss-of-function phenotypes (Duffy, 2002). Consequently, such approach on neuropile

and cortex glia K^+ channels would help verify the role of “ K^+ siphoning” in the slow component of $[K^+]_o$ baseline recovery at room temperature. An alternate strategy would be to target innexins, a class of proteins that communicates cells through the formation of gap junctions (Scemes *et al.*, 2009); such connections could be responsible for moving siphoned K^+ to regions of lower concentration.

Although the molecular and genetic tools present in *Drosophila* provide irrefutable evidence of the processes being studied, it is necessary to perform numerous controls in order to properly substantiate any data set. Consequently the time required to analyze a single event within a model can be considerably increased, even more when lengthy protocols, like the ones used in the present study (~2 hours per fly), are implemented. Pharmacology provides a fast, moderately reliable exploratory approach that enables determination of the particular molecular interactions to be studied using more sophisticated methods, like the GAL4/UAS system. Although drugs can have different levels of specificity (Bain *et al.*, 2007), thereby causing potentially significant bias on the results obtained, pharmacology is still a valuable exploratory technique that can save precious time. Previous electrophysiological (Mejia *et al.*, 2010) and behavioral (Dawson-Scully *et al.*, 2010) studies have shown that whole animal pharmacology can be performed in *Drosophila* with satisfactory results. However, no former study has tried a pharmacological approach while recording brain $[K^+]_o$ during repeated anoxia. Efforts focused on developing and standardizing techniques to achieve such goal were successfully capitalized after the end of the experimental phase of the project. $[K^+]_o$ baseline and surge amplitude were modulated by injecting the fly's thorax with 50.9 nL of HL₃ mixed with different pharmacological agents (figure 22). Diffusivity of the solution was tested by injecting a different set of flies with a similar volume of food coloring and observing how fast it extended to

the brain: it took a fraction of a second for the dye to reach the head and about 5 seconds for it to uniformly spread. Application of ouabain (10 mM; figure 22, i), a Na⁺/K⁺ ATPase blocker, caused loss of [K⁺]_o baseline. Additionally, injection of tetraethylammonium (TEA, 1 mM; figure 22, ii), a general K⁺ ion channel blocker, incremented surge amplitude. Although the latter result was totally opposed to what was expected, it demonstrates conclusively that brain K⁺ homeostasis can be pharmacologically modulated. Furthermore, the N₂-delivery protocol implemented decreased the time spent in every experiment (~40 minutes per fly) and still allowed analysis of relevant response variables (*i.e.* [K⁺]_o baseline maintenance, surge amplitude, TTS, and TTR).

The synergistic combination of the powerful molecular and genetic tools available for *Drosophila* and the versatility of pharmacology will prove useful to elucidate the processes that underlie the fly's response to repeated reperfusion at different temperatures. Thus, exploratory preliminary pharmacological experiments can be performed to identify the most relevant elements of the pathways involved in the response (*e.g.*, AMPK, NO/PKG/PP2A pathways), and then reliable molecular dissections can be performed using transgenic flies. Additionally, K⁺ ion channel mutants will be useful to establish the dynamics of clearance and accumulation mechanisms. Consequently, this mixed approach will help direct efforts and save time in the laborious task of validating the model proposed in the present study.

4.7 Concluding Remarks

Based on the results obtained during the present project, the following conclusions about temperature modulation of *Drosophila*'s brain K⁺ homeostasis during repetitive ADs were drawn:

1. During AD iteration, temperature effects were mostly preserved in preparations with high hydric stress. Therefore, experiments with a low control of dehydration are still appropriate to evaluate general trends produced by the influence of temperature and ADs on the response variables.
2. To guarantee the preservation of the hydric integrity of the preparation, it is of vital importance to humidify the gases applied on the fly. Moreover, it is also recommended to seal the fly's head with Halocarbon oil 700 to avoid any direct desiccation of the brain, especially if high temperature experiments are going to be performed.
3. N₂-delivery patterns consisting of long anoxia, short normoxia and increased cycle frequency augment disruption of brain [K⁺]_o baseline. Such a loss of K⁺ homeostasis is possibly due to accumulation of oxidative stress damage and subsequent progressive impairment of clearance mechanisms (*e.g.* Na⁺/K⁺ pump, "K⁺ siphoning").
4. Males are more susceptible to AD iteration than females at room temperature, showing a greater [K⁺]_o baseline disruption. Sex-based dissimilarities may be the result of differential effects of oxidative stress and dehydration because of morphological and anatomical dimorphisms.
5. Low temperatures are protective and stabilize maintenance of brain K⁺ homeostasis during repeated reperfusion. On the other hand, high temperatures are extremely damaging and cause severe, permanent disruption of [K⁺]_o levels. This protective/detrimental effect of temperature is probably the result of modulation of MR, and subsequent decrease/increase of the rate of free radical accumulation damage and endogenous antioxidant enzyme consumption.

6. A speculative model considering the interactions and effects of anoxia, reperfusion, hypothermia and hyperthermia in the fly's brain K^+ homeostasis is presented.
7. Given that temperature had a protective effect in *Drosophila*'s brain, this novel fly model is suggested as an adequate tool to study the molecular mechanisms that influence reperfusion tolerance, with extensive application on development of therapeutical treatments in mammals.

The remarkable resilience of *Drosophila* to anoxia, and in this particular case to repeated reperfusion, is a feature that can be used to increase our understanding of animal physiology. Furthermore, the molecular pathways in the fly and in mammals are conserved (Azad *et al.*, 2009). Therefore, besides learning from the fly, we can also use its physiological and molecular strategies as a basis to develop therapies aiming to relieve the side effects of disrupting events in the nervous system (*e.g.* ischemia, reperfusion, traumatic brain injury) (see Azad and Haddad, 2009 for a review). Furthermore, the molecular basis of hypothermia protection can be elucidated, shedding light on the appropriate temperatures and delivery patterns to minimize the adverse consequences of ischemia and reperfusion.

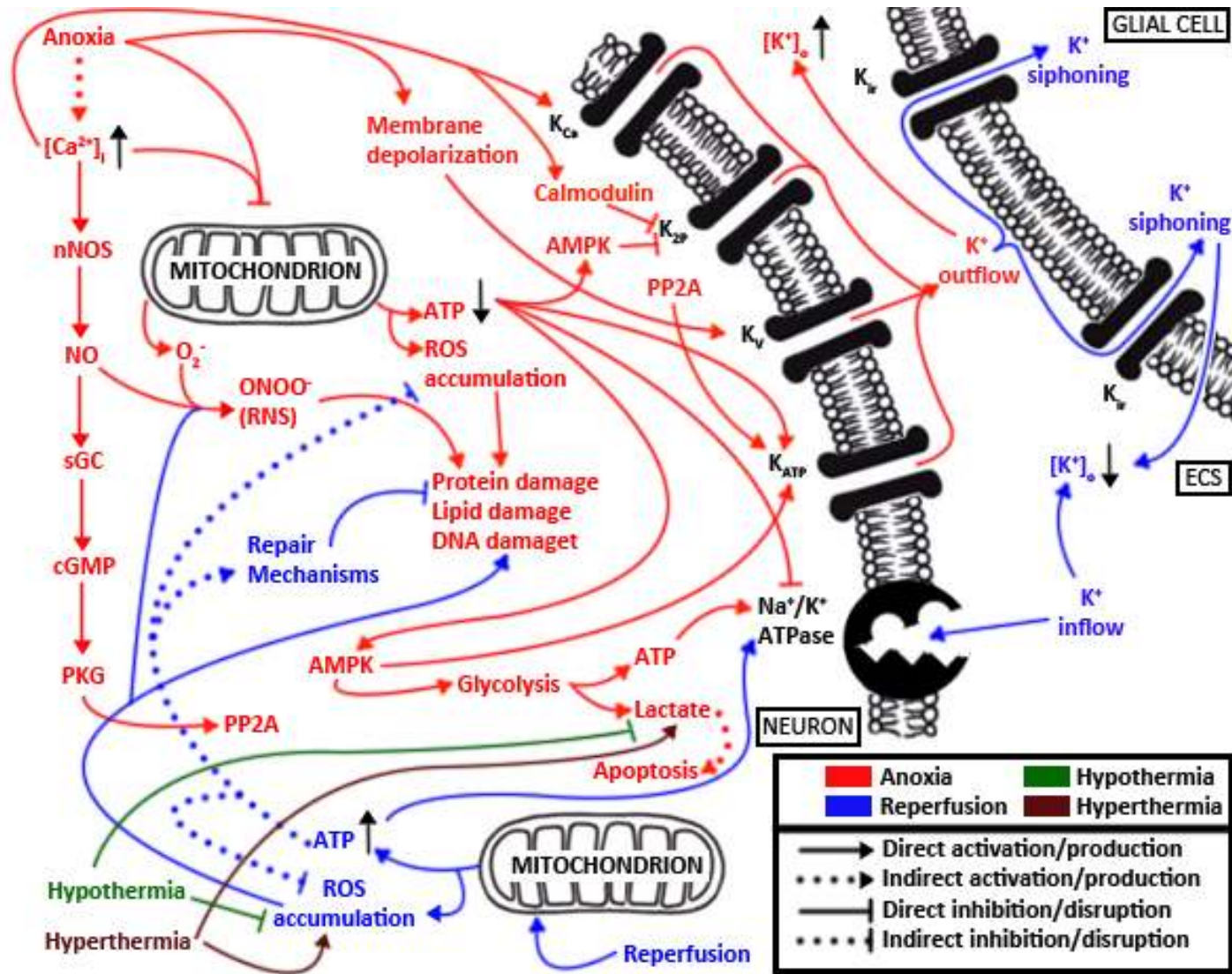


Figure 21. Temperature- and AD iteration-modulation of molecular and physiological mechanisms involved in *Drosophila's* brain K⁺ homeostasis. Oxygen lack (in red) triggers a series of excitotoxic, oxidative and metabolic processes. Under these conditions, ATP production declines causing failure of the Na⁺/K⁺ ATPase, and activation of K_{ATP} channels and AMPK. AMPK promotes glycolysis and activation/inactivation of K⁺ conductances. Anaerobic glycolysis produces ATP, which fuels the Na⁺/K⁺ pump, but also lactate, which can be indirectly involved in apoptotic mechanisms through lowering of the cell's pH (acidosis). Disrupted activity of the Na⁺/K⁺ pump depolarizes the cell membrane, subsequently activating K_v channels and augmenting [Ca²⁺]_i. On the one hand, increased [Ca²⁺]_i interrupts the electron transport chain and collapses mitochondrial membrane potential, causing ROS accumulation and subsequent oxidative stress. Additionally, it activates nNOS, which generates NO production and downstream activation of PKG. PKG is thought to activate K⁺ ion channels through phosphorylation of PP2A. Upstream, NO reacts with mitochondrial O₂⁻ to produce ONOO⁻, which, together with ROS, eventually causes tissue damage by oxidation of DNA, proteins and lipids. On the other hand, the increment in [Ca²⁺]_i can also activate K_{Ca} and calmodulin; the latter is thought to mediate protective ion channel arrest. Eventually, the whole system collapses and K⁺ flows through K⁺ ion channels, generating an [K⁺]_o surge

During reperfusion (in blue), mitochondria are reactivated and ATP levels increase. Consequently, the Na⁺/K⁺ pump commences restoration of the initial K⁺ transmembrane gradient and cell damage caused by ROS/RNS/anaerobic metabolites is repaired. Additionally "K⁺ siphoning" performed by glial cells through K_{ir} channels helps reduce [K⁺]_o. However, reaction of O₂ with free electrons in the mitochondria causes increased ROS/RNS accumulation, and subsequent cell damage. Repeated reperfusion can considerably exacerbate cell damage. Hypothermia (in green) mainly owes its protective effect to a buildup reduction of ROS/RNS/anaerobic metabolites through a decrease in metabolic rate (MR). On the other hand, hyperthermia (in brown) exacerbates such accumulation, producing irreparable damage if reperfusion is repeated.

Abbreviations (in alphabetical order): [Ca²⁺]_i (intracellular calcium concentration), [K⁺]_o (extracellular potassium concentration), AMPK (AMP-activated protein kinase), cGMP (cyclic guanosine monophosphate), K_{2P} (two-pore domain K⁺ channels), K_{ATP} (ATP-sensitive K⁺ channels), K_{Ca} (Ca²⁺-activated K⁺ channels), K_{ir} (inwardly rectifying K⁺ channels), K_v (voltage-activated K⁺ channels), nNOS (neuronal nitric oxide synthase), NO (nitric oxide), O₂⁻ (superoxide ion), ONOO⁻ (peroxynitrite), PKG (protein kinase G), PP2A (protein phosphatase 2A), RNS (reactive nitrogen species), ROS (reactive oxygen species), sGC (soluble guanylate cyclase).

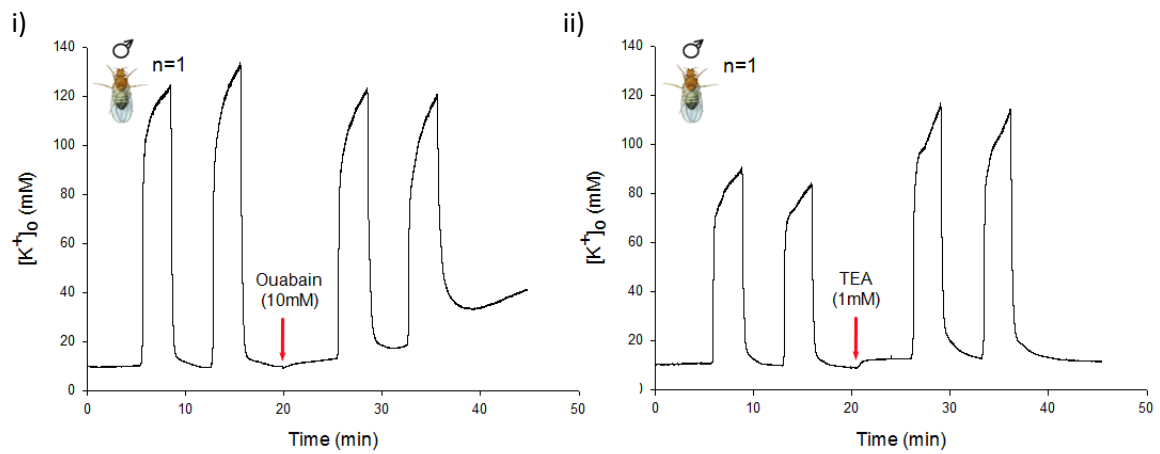


Figure 22. Pharmacological manipulation of brain $[K^+]_o$ in *Drosophila*. i) Blockage of the Na^+/K^+ pump with ouabain (10 mM, red arrow) produced a gradual, moderately steep loss of $[K^+]_o$ baseline. ii) Blockage of K^+ ion channels with tetraethylammonium (TEA, 1 mM, red arrow) caused a slight baseline increment and an increase in surge amplitude. The two first ADs were delivered before the injection and the two last ones after drug application. The pattern used was: 5 minutes of baseline, 2 pre-drug cycles (3 minutes of anoxia/4 minutes of normoxia each), 5 minutes for the drug to cause an effect, 2 post-drug cycles (3 minutes of anoxia/4 minutes of normoxia each), and 10 minutes of final baseline.

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