EFFECT OF CADMIUM AND SEASONALITY ON CRITICAL TEMPERATURES OF AEROBIC METABOLISM IN EASTERN OYSTERS, *Crassostrea virginica* Gmelin 1791

by

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ABSTRACT

RITA BAGWE. Effect of cadmium and seasonality on critical temperatures on aerobic metabolism in eastern oysters, *Crassostrea virginica* Gmelin 1791.
(Under the direction of DR. INNA SOKOLOVA)

Cadmium (Cd) and elevated temperatures are common stressors in estuarine and coastal environments affecting intertidal mollusks such as oysters. Cd and elevated temperature can raise the metabolic demands of oysters due to the increase cost of detoxification and damage repair and induce tissue-wide hypoxemia. According to the concept of the oxygen- and capacity-limited thermal tolerance (OCLTT), this is expected to lead to the shift of the critical temperature (Tc) of aerobic metabolism (indicated by the onset of partial anaerobiosis) restricting the thermal tolerance limits of the organism. We tested this hypothesis by determining the onset of anaerobic metabolism, changes in the cellular energy budget and the extent of oxidative damage during acute temperature rise (from 20°C to 36°C) in eastern oysters *Crassostrea virginica* under control conditions and after prolonged Cd exposure (50 µg Cd l^{-1} for 30 days). In summer and winter control oysters, levels of anaerobic end products increased at 28°C and 24°C, respectively, indicating an earlier onset of tissue hypoxemia in winter-acclimated oysters. Cd exposure shifted Tc to lower levels in summer oysters (from 28 to 24°C) oysters Tc was 24°C in summer However, in winter there was no temperature-induced accumulation of anaerobic end products in Cd-exposed oysters suggesting that the effect of Cd exposure on Tc varies between the seasons and/or that the studied range of temperatures was insufficient to detect Tc in the winter Cd-exposed group. Acute warming had no negative effects on tissue and cellular energy status of oysters in summer or winter but led to an increase of oxidative damage to proteins in winter control oysters. This suggests that oysters are well

adapted to acute temperature fluctuations in the intertidal zone and maintain energy balance despite the limitations of aerobic metabolism and partial transition to anaerobiosis.

DEDICATION

I dedicate my doctoral dissertation to my

Son Sahil, for his love, patience and sacrifice,

My parents, for instilling the importance of hard work and higher education,

My sister Rekha, for her constant support and encouragement,

To my niece Rayna, for her love and warmth,

and to my best friend, philosopher and guide late Dr. Sudhakar Karmarkar.

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ABC	ATP-binding cassette
ANOVA	Analysis of Variance
ADP	Adenosine diphosphate
AEC	Adenylate energy charges
AFP	Antifreeze peptides
AFGP	Antifreeze glycopeptides
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
BMR	Basal metabolic rates
Cd	Cd
$Cd^{2+}Cl_2$	Cd chloride
Cu	Copper
DEB	Dynamic energy budget
DNA	Deoxyribose nucleic acid
DNPH	Dinitriphenylhydrazine
EDTA	Ethylenediaminetetraacetic acid
ETS	Electron transport system
FADH	Flavin adenine dinucleotide
HClO	Hypochlorous acid
HVA	Homeoviscous adaptation
HSPs	Heat shock proteins
H_2O_2	Hydrogen peroxide

GTP	Guanine triphosphate
GSH	Glutathione
GSSG	Glutathione disulphide
IARC	International agency for research on cancer
IMM	Inner mitochondrial membrane
INA	Ice nucleating agents
SMR	Standard metabolic rates
Tc	Critical temperature
MT	Metallothioneins
MXR	Multixenobiotic resistance proteins
NADH	Nictotinamide adenine dinucleotide dehyrogenase
NADP	Nictotinamide adenine dinucleotide phosphate
O_3	Ozone
OCLTT	Oxygen and capacity limited thermal tolerance
PLA	Phospho-L-arginine
R-PLA	Relative phosho-L-arginine
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
UV	Ultraviolet
Zn	Zinc

CHAPTER 1: INTRODUCTION

1.1 Physiological responses and adaptations of aquatic animals to environmental temperature:

Temperature is an important abiotic factor that affects the physical environment as well as all life processes in an organism (*5*). Temperature can directly affect the physical environment of an organism due to physico-chemical changes such as a decrease in the solubility of the gases including oxygen and increase in solubility of inorganic salts and other substances (*6*). Increasing temperature also decreases the viscosity of the fluids and reduces pH due to increasing auto-dissociation of water (*6*). Changes in temperature from 0°C to 100°C cause phase transition of the water from solid state ice through liquid phase of water and finally to gaseous state of water vapor (*6*). Many of these temperature-associated changes affecting the physical environment elicit an adaptive physiological response in aquatic organisms (*6*). Temperature can also directly affect the structure and function of macromolecular structures of the cell (such as lipids and proteins) and the rates of biochemical reactions (*6*).

Most aquatic organisms (including invertebrates and fish) are ectotherms and poikilotherms[1] and thus are unable to maintain a constant body temperature that is

[1]Ectotherms are the organisms that predominantly regulate their body temperature using external heat sources (6). Ectothermic thermoregulation is rarely efficient enough to maintain a constant body temperature; therefore, most ectotherms are also poikilotherms – i.e. organisms whose body temperature changes with the changing environmental temperature.

different from the environmental termperature (6). Therefore, changes in environmental termperature directly affect the rates of all physiological and biochemical processes in these organisms and have strong implications for their fitness and survival (*6*). Depending on the habitat, aquatic ectotherms have adapted to withstand different degrees of fluctuation in environmental temperature (*6*). While some stenotherms from thermally stable environments such as Arctic and Antarctic waters can tolerate only a few degrees change in environmental temperature, eurythermal ectotherms (such as intertidal invertebrates, freshwater fish and terrestrial vertebrates and invertebrates from temperate zones) can adjust to a wide range of temperatures spanning over 20-40°C (*6*). Regardless of the breadth of their thermal niche, all ectothermic poikilotherms must be able to adjust their cellular processes to maintain the normal physiological function, grow survive and reproduce within the typical range of the environmental temperatures (*2*). This is especially critical for the organisms with low motility that cannot escape unfavorable temperature conditions and have to rely on their physiological and biochemical tolerance mechanisms to survive the thermal change *in situ* (*6*). Due to the all-encompassing nature of the temperature effects on animal physiology, temperature adaptations and acclimatory adjustments involve changes in all key cellular structures – from membranes and organelles to individual enzymes and small molecular weight molecules that create the biochemical mechanisms involved in the temperature adaptations of ectotherms.

Adjustments of the structure and composition of cellular membrane (including the plasma membrane and the organelle membranes) play a key role in adaptation and acclimation to environmental temperature (*6*). Phospholipids form the bulk of the cell and organelle membranes, and a change in the temperature is immediately reflected in

changes in the viscosity and phase order of the membranes, which may in turn affect the enzymes, transporters and channels embedded in the membrane (*6*). Low temperatures decrease the conformational flexibility of the membranes, whereas high temperatures increase the flexibility, resulting in excessively rigid or fluid membranes and eventually membrane disruption that can lead to cell dysfunction or death (*3, 6*). In living cells however, such changes are avoided by a series of adaptive mechanisms called homeoviscous adaptations (HVA) that maintain optimal membrane viscosity and phase order (*6-7*). This is achieved by adjustment in the fatty acyl chain composition (especially the proportion of polyunsaturated to saturated fatty acid chains) and/or changes in cholesterol concentration (*3, 6-7*). Typically, cold-acclimated or –adapted animals have a higher proportion of unsaturated fatty acid chains in their membranes that helps to maintain the overall membrane fluidity at low temperatures (*6*). A group of enzymes called desaturases plays a key role in this adaptive mechanism by introducing a double bond between 9-10 carbon positions from the carboxy terminal in fatty acids like stearic or palmitic acid in response to decreasing temperature (*8*). HVA during increasing temperature results in the opposite changes in the membrane composition, i.e. an increase in the proportion of saturated fatty acid tails in the membrane phospholipids. HVA also can involve changes in the cholesterol content of the membrane; cholesterol tends to increase the membrane order and its concentration in the membrane decreases in the cold and increases in the warm-acclimated or adapted organisms (*6*).

Extremely high temperatures can also result in the denaturation of proteins in animals. In order to counteract this, animals have evolved protective proteins known as

heat shock proteins (HSPs), which are molecular chaperones in the cell (*6*, *9*). HSPs are classified based on their molecular weight and broadly divided into two categories of large (HSP60, HSP70, HSP90, HSP100) and small HSPs (HSP10, HSP27) (*3*, *6*, *8*). Their structure and function are highly conserved among eukaryotes from yeast to mammals. HSPs are either constitutive (helping in the proper protein folding during the *de no*vo protein synthesis or protein transport) or can be induced by stressors that cause denaturation of existing proteins (*9*). The primary stress-induced HSPs are HSP70 family and small HSPs (*2-3*, *6*). Generally, stressors such as elevated temperatures, UV, hypoxia, hyperoxia, pH change, exposure to heavy metals, toxins and free radical can damage the tertiary protein structure resulting in partially unfolded proteins(*6*). Such improperly folded proteins can bind to each other forming aggregates or to other cellular components impairing their function. HSPs minimize the aggregation of these unfolded proteins, facilitate repair and refolding and limit their interactions by binding to the exposed part of unfolded proteins (*6*, *9*). The proteins that are severely damaged and beyond repair are targeted by HSPs for ubiquitination and proteolytic degradation (*9*). Notably, heat shock response is energetically very expensive because HSPs synthesis as well as their chaperoning functions require ATP (*9*).

Temperature also directly affects the enzyme activity and thus, the rates of the biochemical reactions in the cell. Two commonly used indices to evaluate effect of temperature on biochemical processes are activation energy (E_a) and the Q_{10} temperature coefficient (*6*). Activation energy is the amount of energy needed to cross the energy barrier of a chemical reaction so that the reaction can proceed (*6*). An increase in temperature increases the kinetic energy of molecules and thereby increases the

proportion of the molecules in the reactant mixture which have sufficient energy to overcome the activation energy threshold resulting in increased rate of biochemical reactions (*6-7*). High activation energy of an enzyme means that its catalyzed reaction is highly temperature sensitive, whereas low activation energies are characteristic of the enzymatic reactions that experience less change in the rate with changing temperature(*6*). The Q_{10} coefficient is used to express the degree of the temperature dependence of a reaction and equals to the –fold change in the reaction rate for every 10°C rise in the temperature; thus, if Q_{10}=2, the rate of a reaction doubles for every 10°C increase in temperature, triples if Q_{10}=3 (*6*). For most biochemical and physiological reactions like enzymatic activity and rates of respiration, Q_{10} is around 2-3 within the normal physiological range of temperatures, and temperature increase accelerates almost all biochemical and physiological processes (*6*). However, beyond this normal thermal limit, Q_{10} value may fall below 1 at high temperature indicating damage to the biochemical molecules involved in the processes and potentially leading to drastic reduction or complete abolition of the chemical reactions (*6*). Due to this thermal sensitivity of enzymatic reactions, cold adaptation and acclimation in aquatic ectotherms often involve an increase in enzyme concentration and/or expression of more catalytically efficient isoforms to compensate for the reduced enzyme activity at low temperatures(*6*). While the above-described adaptations are effective in maintaining the membrane order and the proper protein function in ectotherms experiencing moderate temperature fluctuations, extremely low temperatures present an additional major challenge due to the necessity to prevent and/or tolerate freezing at sub-zero temperatures (*6*). Animals evolved to do so by exhibiting either freeze tolerance or freeze avoidance strategies (*6*). Freeze-tolerant

animals have evolved ice nucleating agents (INAs) and cryoprotectants to survive subzero temperatures (*6*). INAs allow ice crystals to be formed slowly and confine the crystals to the extracellular fluids (*6*). This is achieved by encouraging formation of ice crystals in the extracellular fluid using hydrophilic ice nucleating proteins while keeping intracellular water highly ordered due to its adherence to macromolecules (*6*). The high molecular order of intracellular water prevents ice formation. Cells of the freeze-tolerant organisms can also be protected from ice injury by colligative or non-colligative cryoprotectants(*6*). Colligative cryoprotectants like polyhydric alcohols, sugars, sorbitol and ribitol are present in high concentrations to raise osmotic concentrations of body fluids thereby limiting the formation of extra and intracellular ice (*6*). Non-colligative cryoprotectants like trehalose and proline are membrane protectants present in lower concentrations which bind in place of water and prevent long-term damage to the membrane (*6, 8*).

Freeze-intolerant animals adapted to low temperatures avoid freezing of intra- and extracellular fluids owing to a phenomenon called supercooling and by having specific antifreezes(*6*). Supercooling allows the fluids to be cooled down below the freezing point of the surrounding water without being frozen; this is achieved by high concentrations of antifreezes such as glycerol, sugars, and polyols in the blood and intracellular fluids to lower the supercooling point (*6*). Some cold-adapted freeze-intolerant organisms such as polar marine fishes also have antifreeze peptides (AFP) and antifreeze glycopeptides (AFGP) which suppress the freezing point of the body fluids below their melting point, a phenomenon called “thermal hysteresis” (*6*). These antifreeze molecules act by

preventing the new water molecules to be added between to the edge of an ice crystal and thereby inhibiting the crystal growth (*6*).

1.2 Energy homeostasis in temperature adaptation and stress tolerance:

1.2.1 An overview of ATP-generating pathways in animals:

Energy homeostasis plays a key role in tolerance and adaptation to a variety of environmental stressors including temperature (*9*). For survival of the organisms and their populations, the energy demands must be met with sufficient energy supplies to maintain cellular order and with sufficient net energy gain to invest in somatic growth and reproduction (*9*). Organisms are thermodynamically open systems relying on external energy sources to meet their energy requirements; in case of animals, that energy comes in the form of organic food compounds assimilated from external sources [9].

In animals, the energy is stored in three major forms including: (a) high energy phosphate compounds, (b) transmembrane proton gradients and (c) metabolic substrates including high energy phosphates such as phosphagens (*10-11*). All three forms are interconnected and energy is conserved in the cell in the form of ATP as these substrates are catabolized or the proton gradient is dissipated during mitochondrial oxidative phosphorylation (*11*). The chemical energy obtained from the food can be conserved in the form of ATP by aerobic or anaerobic metabolism. Excess energy gets stored in the form of lipids and carbohydrates, and these compounds also serve as buffers against the inevitable fluctuations of the food availability and intake (*9*). In most free-living eukaryotes under normal physiological conditions, oxidative phosphorylation taking place in mitochondria produces the majority of the ATP used as an energy source for ATP-demanding cellular reactions (*10-11*). In aerobic metabolism, ATP is produced via

mitochondrial oxidative phosphorylation supplied by the energy-rich substrates from glycolysis, citric acid cycle, oxidation of amino acids or β-oxidation of fatty acids(*6*). It requires a continuous supply of oxygen via ventilation and distribution of oxygen to cells, tissues and organs to drive the oxygen-dependent oxidation of the organic carbon to H_2O and CO_2 (*6, 12*). In contrast, anaerobic metabolism involves O_2-independent catabolism of substrates. The turnover of ATP varies significantly in both these processes. Anaerobic fermentation of glucose to lactate results in a net gain of 2 ATP molecules as compared to 36 ATP molecules produced per glucose molecule in aerobic oxidative phosphorylation(*8*).

The simplest and the most rapid pathway of anaerobic ATP generation are via the phosphagens (*6*). A phosphagen is a reserve energy compound found in animal tissues that contains energy in the form of a high-energy phosphate bond. During situations when rapid ATP flux is required (e.g. during exercise), the high energy phosphate group can be enzymatically transferred from phosphagens onto ADP, thereby generating ATP in a rapid one-step reaction (*6*). Phosphagens are then again replenished during recovery when excess ATP is available. Most animal phosphagens are phosphorylated derivatives of guanidinium compounds and their concentration is up to 10 times higher than that of ATP in metabolically active tissues such as brain and muscle (*6, 10*). The vertebrate phosphagen is creatine phosphate while in invertebrates a variety of phosphagens are found including creatine phosphate, arginine phosphate, lombricine phosphate and tauromycine phosphates (*6, 10, 13*). Each phosphagen type is substrate specific and has its associated kinase enzyme (e.g. creatine kinase, arginine kinase, etc.) that catalyzes the conversion of ADP to ATP (*6, 10, 13*). Phosphagen stores can be used under both aerobic

and anaerobic conditions as a buffering system for ATP (*6, 10*). In addition to immediate provision of necessary ATP for cellular functioning during rapid ATP flux, phosphagens also regulate inorganic phosphate levels, and are involved in proton buffering and in the regulation of glycogenolysis (*6, 10*).

Glycolysis is another anaerobic pathway of ATP generation that is ancient and highly conserved in all eukaryotes. Glycolysis is an enzymatically catalyzed 10-step reaction involving substrate-level phosphorylation of ADP (*6, 8*). It can be broadly divided into two stages. At the first stage involving the first five reactions, one molecule of hexose (glucose) is converted into two molecules of triose (glyceraldehydes-3-phosphate). It is the energy investment stage where glucose is phosphorylated and cleaved into trioses consuming 2 ATP (*6, 12*). In the second stage involving the last five reactions, two molecules of glyceraldehyde-3-phosphate are converted to pyruvate generating 4 ATP. Therefore, the overall net gain of ATP in glycolysis is 2 ATP per molecule of glucose (*6, 12*). Oxidation of glucose in glycolysis also yields two reduced co-enzymes, nicotine amide adenine dinucleotide (NADH) (*12*). Under aerobic conditions, NADH can be transported through the mitochondrial membrane by the a shuttle system and serve as an electron donor in the electron transport system (ETS) generating the proton gradient used for the formation of ATP from ADP (*6, 12*). Under anaerobic conditions, NAD^+ is regenerated from NADH by a terminal dehydrogenase such as lactate dehydrogenase in vertebrates (*12*).

The terminal dehydrogenase in the reaction that involves conversion of pyruvate and regeneration of NAD^+ varies across different taxonomic groups of animals. In vertebrates and some invertebrates such as arthropods or freshwater mollusks, pyruvate is

anaerobically reduced to lactate by lactate dehydrogenase.(*6*) In other organisms, pyruvate can be converted to alternative end products such as ethanol (e.g. in carp and goldfish), the imino acid derivatives called opines (including alanopine, tauropine, nopaline, strombine, lysopine and octopine in many marine mollusks) or short-chain organic acids (such as succinate, acetate or propionate in many hypoxia-tolerant invertebrates) (*6, 14*). With the exception of the opines, these alternative anaerobic pathways yield more ATP per unit glucose compared to the lactate pathway, and/or result in less toxic or more easily excretable end products reflecting an adaptation to survive prolonged hypoxia or anoxia (*6*) .

Aerobic oxidative phosphorylation is evolutionarily the newest acquisition among the energy conserving pathways. Oxidative phosphorylation occurs only in the mitochondria of eukaryotes and can be fueled by different substrates including carbohydrates, fatty acids or amino acids. Under aerobic conditions, pyruvate derived from glycolytic breakdown of carbohydrates is completely oxidized to carbon dioxide. Pyruvate is first decarboxylated and converted to acetyl-coenzyme A (acetyl-coA), which is the substrate for entry into the mitochondrial matrix-associated Krebs cycle (*6, 12*). Acetyl-coA is reduced in the Krebs cycle with the concomitant generation of guanine triphosphate (GTP), NADH and $FADH_2$ equivalents. NADH and $FADH_2$ are then reoxidized to NAD^+ and $FADH^+$, respectively, in the electron transport system (ETS) present on the inner mitochondrial membrane (IMM) (*6*). The ETS has four enzyme complexes which participate in the transfer of electrons, and in that process H^+ ions are expelled across the IMM from the matrix into the inter membrane space creating a proton gradient and electrical potential across the IMM(*6*). The free energy stored in this

electrochemical gradient (also known as the proton motive force) is used for the synthesis of ATP from ADP and Pi through proton-pumping ATP synthase or F_1F_o-ATPase (*6*). Carbohydrates and fats are the most common energy yielding substrates for oxidative phosphorylation under the normal conditions in animals. Aerobic oxidation of carbohydrates yields 36 ATPs, 6 CO_2 and 4 H_2O molecules for each glucose molecule oxidized (*12*). Fats provide the highest ATP yield of all energy substrates yielding multiple acetyl-CoA molecules in the process of β-oxidation of the fatty acid backbone, that are then fed into the Krebs cycle (*6*, *15*). Each round of β-oxidation produces one molecule each of NADH, $FADH_2$ and acetyl Co-A and reduces the length of the fatty acid chain by two carbons; the process is repeated until the fatty acid chain is completely oxidized. Acetyl Co-A is then oxidized in mitochondria via the Krebs cycle and mitochondrial ETS, while NADH and $FADH_2$ are directly fed to the ETS and fuel oxidative phosphorylation to yield ATP (*12*). Thus, oxidation of the palmitic acid (a 16 carbon fatty acid) involves 7 rounds of β-oxidation resulting in a total of 129 ATP $mole^{-1}$ compared to 36 moles ATP per mole glucose (*12*).

Proteins are rarely used for metabolic fuel and whenever they are, they are metabolized to amino acids that are ultimately fed into the Krebs cycle. Amino acids are fed into the metabolic pathways according to their number of carbon atoms (*6*). All three-carbon (C3) amino acids are utilized during gluconeogenesis and ultimately degraded to pyruvate. The C4 & C5 amino acids are fed into Krebs cycle via glutamate and α-ketoglutarate. Oxidation of proteins yields different amounts of ATP depending on the amino acids. For example, oxidation of proline, glutamate and alanine yields 30, 27 and 15 mole ATP $mole^{-1}$ respectively (*8*). While the metabolic end products of complete

oxidation of carbohydrates and fats are CO_2 and H_2O, oxidation of protein results in additional nitrogenous end products in form of urea (commonly found in fishes, amphibians and mammals), ammonia (commonly found in aquatic animals) or uric acid (reptiles, birds and insects) (*6, 8*). The nitrogenous wastes must be excreted from the organism in an energy-dependent manner, thus further reducing the net energy gain from the oxidation of amino acids.

1.2.2 Basal metabolic rate:

Basal metabolic rate (BMR) of an organism - also called a cost of survival or basal maintenance cost - is an important concept in bioenergetics. BMR is a measure of the lowest amount of energy required for sustaining metabolic processes and maintaining thermodynamic non-equilibrium state of living (*11*). BMR or SMR does not include the costs of growing, feeding, processing food, thermoregulation, or physical activity (*11*).

Basal costs of metabolism can be measured as basal or standard metabolic rates (BMR or SMR in endotherms and ectotherms, respectively) either directly as heat production or indirectly by measuring oxygen consumption by a whole organism under a set of basal or standard conditions (*8*). In endotherms, BMR is determined at a normal body temperature in the thermoneutral zone, while in ectotherms SMR is measured at a specific temperature that can vary depending on the acclimation conditions (*11*). To measure BMR, an endotherm should be in an adult stage of development, in post-absorptive stage (overnight fasting), normothermic, unstressed in any other way and at rest (in supine position but fully awake) (*8*). In ectotherms, SMR determination is less standardized and varies among species. Typically, it is determined as the oxygen consumption or heat production by a resting organism in a post-absorptive state measured

at the temperature at which it was acclimated. In some active organisms such as fish, SMR is obtained by extrapolation of the activity-dependent metabolic rates to zero activity levels (*6*).

In mammals, 70% of oxygen consumed in BMR is used for mitochondrial ATP production. Of this aerobically produced ATP, 20–25% is used for protein synthesis, 20–25% for maintenance of transmembrane Na^+ gradients, 5% for Ca^{2+} gradient, 7% for gluconeogenesis, 2.5% for ureogenesis, 5% for actinomyosin ATPase and the remainder of the 70% is used for nucleic acid synthesis and substrate cycling (*11, 16*). Of the 30% of oxygen consumption that is not used for ATP synthesis, 5-10% is used for non-mitochondrial oxygen consumption (e.g. by cytosolic oxygenases) and the remaining 20-25% - for counteracting the mitochondrial proton leak (*8, 11, 17*). In hepatocytes of ectothermic vertebrates (crocodiles) ATP turnover was responsible for 13-50% of oxygen consumption, while proton leak and non-mitochondrial respiration accounted for 10-30% and 30-65% of respiration, respectively (*18*). In the marine ectothermic oyster, *C. virginica* mitochondrial proton leak, non-mitochondrial respiration and protein synthesis represents 22-38%, 12-31% and 7-23% of the total energy cost (*19*).

In most organisms, energy allocations to support BMR (and thus survival) are prioritized over the allocations to other fitness-related aspects of the energy budget such as growth, activity or reproduction (*6*). Due to the inherently limited energy assimilation aerobic capacity of any organism, increases in BMR (e.g. due to the environmental stress) lead to trade-offs in energy allocation to growth, reproduction or activity, which in turn can negatively affect an organism's fitness. These trade-offs play an important role in

setting the physiological limits of stress tolerance (including thermal tolerance) of aquatic ectotherms and will be discussed in the next section (see 1.2.3).

1.3 The role of aerobic scope in determining the thermal tolerance limits of aquatic ectotherms:

The energy balance of an organism can be summarized by the following equations:

$$\mathbf{C = P + R + F + U,} \quad [1]$$

where $$\mathbf{C = A + F}. \quad [2]$$

In equations [1] and [2], **C** is the energy from the consumed food in the form of carbohydrates, lipids or proteins, **P** = production or the amount of energy incorporated into various tissues, reproductive products and reserve energy storages; **R** represents respiration which is the amount of metabolic heat lost; **F** is the energy eliminated from the body via feces; **U** is the energy excreted from the body as end products of nitrogen metabolism (such as urea, ammonia or uric acid) and **A** is the energy assimilated in the body (*7, 9, 20*). Balance of energy gained from the environment (**C**) against the energy lost via various metabolic processes **(R, F & U**) is important for the survival of an organism (*9*). All the terms in the equations [1] and [2] are calculated per unit time and reflect the fact that the net energy balance of an organism depends not only on the amount of food available but also on its rate of acquisition and metabolic conversions that channel the energy flux to different processes (*9*).

At the whole-organism level, the energy obtained by the uptake of food and oxygen is assimilated into the ATP pool (via the aerobic and anaerobic metabolic pathways described below) from which it is used for maintenance, growth and reproduction (*14*). Under the optimal conditions, organisms maintain their energy budget in such a way that there is an excess amount of energy and metabolic capacity left after

meeting the basal energy needs of the organism (i.e. the costs of maintenance of cellular homeostasis and basal systemic activities such as ventilation, circulation and excretion) (*9*). This excess metabolic capacity is called **aerobic scope** and is directly linked to the amount of energy that an organism can invest in fitness-related functions such as growth and reproduction, and/or store in the form of energy-rich substrates such as fats and glycogen that can serve as buffers during the periods of high energy demand (*14*).

Aerobic scope scope varies with the changing environmental temperature (*9, 21*) and determines the width of the thermal tolerance window in aquatic organisms. The relationship between the aerobic scope and thermal tolerance was summarized in a physiological model of oxygen and capacity limited thermal tolerance (OCLTT) in aquatic ectotherms proposed by Pörtner later modified by Sokolova et al. (Fig.1) (*2-3, 9*). According to this concept, environmental temperature range can be subdivided into the **optimal, pejus, pessimum** and **lethal** ranges, each associated with a distinctive change in the aerobic scope and the resulting change in physiological and ecological performance of an organism. At the optimal environmental temperatures (i.e. in the non-stressful situation) the aerobic scope is maximum and the energy balance is positive, with a considerable amount of energy available to invest into growth, reproduction and other fitness-related functions. This is the preferred body temperature (T_{pref}) by most of the organisms and very close to the optimal performances of many of the physiological processes (Fig.1) (*6*). The range of the environmental temperatures that are close to the preferred body temperatures and where the performance of an organism is 100-80% of the maximum, is known as the **optimal thermal performance window.**

As the temperature deviates from the optimum range, the aerobic scope of an organism diminishes but remains positive. This temperature range is known as the **"pejus"** range (pejus = getting worse). Transition temperatures from the optimum to the pejus range are designated Tp_1 and Tp_{11} (in the lower and higher temperature range, respectively) (Fig.1). In the pejus range, the cost of the basal maintenance is elevated (as reflected in the higher standard or basal metabolic rate, SMR or BMR) (*2-3, 7*). Organisms employ various strategies at the cellular, physiological and behavioral levels to deal with the moderate stress in the pejus range (*9*). At cellular levels DNA and protein repair machinery are switched on to control the damage set by moderate stress. Moderate stress also induces cell cycle arrest and cells switch on cell damage control mechanisms (*9*). Acceleration of ventilation to increase oxygen uptake and stimulation of feeding is seen as a compensatory mechanism to overcome stress. Some animals will try to escape or avoid stressful situations. Such adaptations to stressful situations are energetically expensive and requires diversion of part of energy to machineries involved in cellular maintenance and repairs from energy allocated to growth and reproduction (Fig.1) (*9, 21*). In this situation energy homeostasis is achieved by cutting into the energy budget allotted to other processes (growth, development, reproduction and energy reserves) (*21*). These trade-offs in energy budget ensures the survival of the organism by allocating maximum energy for maintaining BMR or SMR (*2-3, 9, 14*). As the temperature further deviates from the organismal optimum and pejus ranges, the aerobic scope decreases along with a progressive increase of the ATP demand to meet the cost of BMR.

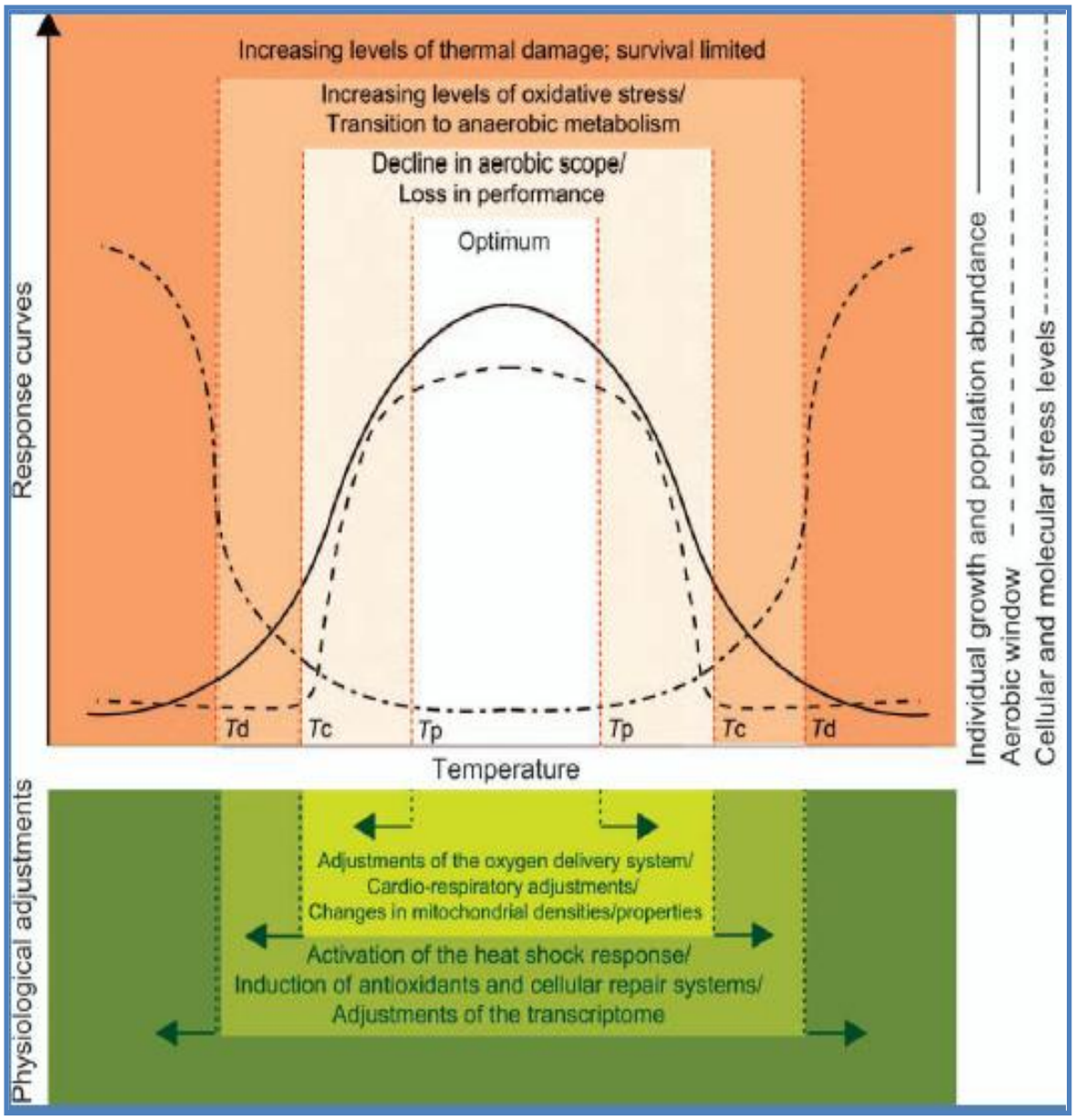

FIGURE 1: Oxygen-and capacity-limited thermal tolerance model in ectotherms. In aquatic ectotherms organismal, physiological and cellular functions are dependent on environmental temperatures. Tolerance to environmental temperature is largely dependent on organism's ability to maintain aerobic scope. At optimal temperature animals have maximum aerobic scope and energy to invest in BMR/SMR, individual growth and reproduction. Any deviation from optimal temperatures to higher or lower temperature results in upper or lower pejus temperatures Tp, where the aerobic scope decreases. At and beyond Tp there is increase in loss of performance, decline in individual growth and population abundance. Organism makes changes in oxygen delivery system and mitochondrial densities to shift the threshold of Tp. Further increase or decrease in temperature results in upper or lower critical temperatures Tc. At Tc there is severe decrease in aerobic scope and the organism switches on anaerobic metabolism in order to supplement the energy requirement to maintain BMR/SMR. Beyond Tc cellular and molecular stress follows loss of aerobic scope. It is manifested as an increase in levels of oxidative stress, activation of the heat shock response, induction of antioxidants and cellular repair machinery. Further change results is denaturation temperature Td, which causes thermal stress, denaturation of proteins and offers only time limited survival(*1-3*).

At certain threshold temperatures, known as the **critical temperatures** (**Tc**), aerobic scope of an organism disappears and all available aerobic capacity is devoted to the basal maintenance (Fig.1). Typically, the organism also engages partial anaerobiosis to compensate for the insufficient aerobic ATP production. At high Tc, the disappearance of the aerobic scope is due to the increased oxygen demand causing insufficient oxygenation of the body fluids, whereas at low Tc the effect is due to decreased anaerobic mitochondrial capacities rather than ventilation or circulation (*2, 7, 22-24*). Severely diminished aerobic scope and transition to partial anaerobiosis indicates transition into the "**pessimum**" or thermal survival range (*2-3, 9, 14*). In this range, the organism often switches from compensation to conservation mode of energy metabolism characterized by metabolic rate depression to conserve the energy and to divert it to maintain the BMR (*2, 7, 22, 24-25*). This allows animals a time limited survival ranging from few days to several months. However, to ensure normal performance and eventual recovery, the environmental temperature must return back to at least pejus or optimum range (Fig.1).

Extreme environmental temperatures beyond the pessimum range can be lethal to an organism even during short term (minutes to hours) exposures and are called the lethal range (with L_{l} and L_{ll} being the lower and higher lethal temperatures) (Fig.1). Upper lethal temperature results in denaturation of macromolecules, especially thermolabile proteins, and increases the fluidity of the cell membranes (*26*). Lower temperatures result in low rates of biochemical processes, freezing of intracellular water and rigidity of cell membranes (*3, 9, 14, 26*). In this range the energy balance is negative, and the organism's

short-term survival depends on emergency protective mechanisms (such as HSPs and antioxidants) (Fig.1).

The change of aerobic scope and adjustments of the aerobic metabolism in response to changes in environmental temperatures set the thermal tolerance limits in aquatic ectotherms such as fish, crustaceans, annelids and mollusks (*9*). In these organisms the onset of partial anaerobiosis at the pessimum range is indicated by the accumulation of the anaerobic cytosolic end products (lactate or alanine) and/or mitochondrial anaerobic end products like acetate, succinate and propionate (*9*). The transition to anaerobiosis in these organisms is not due to damage to neuronal or mitochondrial capacities but due to limitations in the capacity of oxygen uptake and transport mechanisms [9]. It is more prominent in aquatic ectotherms due to lower oxygen content and slower diffusion of oxygen in water as compared to air (*9*). Other factors involved in the temperature-induced metabolic disturbance involves temperature-induced changes in the membrane fluidity which affects the metabolic regulation, seasonal differences in mitochondrial properties and density and temperature-dependent transport capacity of respiratory pigments, which can contribute to organism's transition to partial anaerobiosis (*9*). The optimal thermal performance window, thermal tolerance range and thermal survival zone may vary between species, populations from different latitudes, different life stages and organisms of different body sizes (Fig.1) and can be shifted by the acclimation (*2, 6*). However, the degree of plasticity of the thermal tolerance window is limited, and a temperature rise (e.g. such as is predicted by the global climate change scenarios by the IPCC (Intergovernmental panel on climatic

change), 2007 can lead to the shifts in the thermal tolerance windows that push organisms to their eco-physiological limits (*24*).

It is worth noting that the thermal tolerance windows of aquatic ectotherms can be strongly affected by environmental factors that reduced the aerobic scope due to an increase of the basal maintenance costs, reduction of the aerobic metabolic capacity, or both [9]. Pollutants are among the major factors that can change metabolism and energy allocation in aquatic organisms [9]. Thus, presence of xenobiotics (chemical substances that are foreign to an organism) including toxic metals increases the energy requirements for the basal maintenance as the organism has to allocate additional energy to detoxify the pollutant and/or to repair the damage caused by it (*27*). Toxic metals such as Cd, Zn and Cu can affect cellular processes such as ion homeostasis, protein stability and mitochondrial efficiency leading to an increased amount energy which has to be diverted for maintenance, detoxification and compensation for the impaired oxygen supply (*7, 28*). Moreover, toxic metals including Cd can also directly interfere with aerobic metabolism due to their negative effects on mitochondrial function (*7*). These effects may be not be detrimental to the organism as long as the energy supply from food and oxygen supply via ventilation and circulation are sufficient, and cellular processes can provide sufficient ATP to maintain the elevated basal metabolism (*28*). However, metal-induced increase in basal maintenance costs are likely to have a negative effect on the aerobic scope of aquatic organisms (i.e. the amount of energy and aerobic capacity that can be used on fitness-related functions such as growth and reproduction) and thus can narrow the thermal tolerance window (*9*). Although the negative effects of toxic metals on thermal tolerance and specifically on the critical temperatures indicating transition into the

pessimum temperature range has been predicted by the modified OCLTT theory (*2-3, 9*), this prediction has not been experimentally tested in marine ectotherms. Therefore, a goal of this research was to determine whether exposure to environmentally relevant concentration of a common metal pollutant, Cd, affects Tc in a model marine ectotherm (the eastern oyster *C. virginica*), and whether these effects are modified by seasonal acclimatization to different thermal regimes.

1.4 Metal pollution and its effect on aquatic organisms:

1.4.1 Characteristics of metals as pollutants in aquatic environments:

Trace metals including cadmium (Cd) are among the common persistent pollutants in estuarine and coastal ecosystems, and their presence affects the biodiversity in these habitats due to the negative effects of metals on fitness and survival of the organisms (*9, 14*). Metals bioaccumulate in marine organisms including mollusks and can be transferred to the higher levels of the food chain ultimately reaching the top-level consumers (*29*). For some metals (such as mercury), biomagnification can also occur along the food chain enhancing the toxic effects. In the environment, metals are distributed based on their chemical nature, medium available for their dissolution and availability of binding sites (*30*).

Exposure of aquatic organisms to metals can occur through digestion of contaminated food or sediments, through the direct uptake of the waterborne metals, or through combination of both pathways depending upon the distribution and bioavailability of metals in different components of an ecosystem (*30*). Metal present in water can react with different organic and inorganic substances forming complexes which can be taken up by an organism and later transferred to the higher levels in the

food webs (*30*). Response of the organisms to metal pollution depends upon 1) the nature of metal (essential or non-essential), 2) the concentration, 3) the exposure time (acute, chronic), 4) physiological state of organism, and 5) possible synergistic combination with the other stressors in the environment such as temperature, dissolved organic matter, salinity and others (*14*).

Aquatic organisms are protected from the harmful effects of the environmental metals by physical barriers such as skin or cell membranes. However, metals can gain entry into the organism through permeable epithelia of the organs that specialize on the uptake of essential nutrients, minerals, ion and gas exchange such as gills and gut (*30*). In these tissues, cell membranes serve as the ultimate physical protective barrier for hydrophilic substances (such as many dissolved metals) (*30*). Excessive mucus production in gills can also limit the uptake of metals; however, it comes at a cost of the reduced efficiency of oxygen uptake (*7*). Both essential and non-essential trace metals can be taken up into the tissues, and their uptake requires transporters to ferry them across the membrane and into the intracellular compartment of the cell (*30-31*). Metals gain entry into the cell via specialized transport proteins, carriers, channels or pores that are found embedded in the gill and gut cell membrane and are specialized for uptake of specific essential metals (*30*). For example, Cd gains entry into the organism via Ca^{2+} transporters due to its resemblance of the hydrated ionic radius and charge to Ca^{2+} ions (*30*).

All metals have the potential to become toxic at high concentrations, and therefore, it is crucial for an organism to control levels of free metals in the body (*30*). Essential metals such as zinc (Zn) and copper (Cu) are required as an essential

component of many enzymes. For essential metals, low concentrations can result in deficiency or pathology, while excessive accumulation above the optimal levels results in toxicity and has to be eliminated from the body(*30*). In contrast, non-essential metals like cadmium, lead or mercury have no known biological function in animals and are toxic at very low levels (*30*). Depending upon the physiology of an organism, trace metal may be excreted or stored in the tissues. The non-excreted, accumulated metals can be divided into two groups: 1) metals that are biologically and metabolically active and can be used for essential metabolic purposes and/or in extreme cases can cause toxic effects on biomolecules; 2) metals that have been detoxified by permanent or temporary binding to a site from which escape is limited (*30-31*). Toxicity of essential and non-essential metals primarily depends upon the concentration of the biologically and metabolically active metal in the cells and tissues.

1.4.2 Environmental sources of cadmium and mechanisms of its toxicity:

Cadmium (Cd) is a non-essential, persistent, highly toxic trace metal found in estuaries and coastal areas. Since industrial development its accumulation in the environment is due to its wide use in manufacturing of paints, inks, plastic stabilizers, nickel-cadmium batteries, pigments and alloys (*27, 32-33*). Cd also accumulates in the environment due to the gradual process of soil erosion, heavy metal mining, abrasion of rocks and soils, forest fires and volcanic eruptions (*32-34*). Anthropogenic sources are contributing three to ten times more Cd in the environment compared to the natural processes such as volcanic activity or leaching from Cd-rich soils (*33*). Cd is of high environmental concern because it affects various physiological functions in aquatic organisms. Cd is predominantly present in sea water as an uncharged $CdCl_2$ complex at a

trace level but readily bioaccumulates in marine invertebrates especially in mollusks, where it may reach toxic concentrations (*35-36*). Cd can be taken up by an organism directly from water or through contaminated food (*7, 36*). The free metal ion form of Cd (Cd^{2+}) is the most bioavailable for the aquatic species. Toxicity of Cd depends upon environmental factors such as salinity, water hardness, chelating agents (EDTA) and high organic contents (*36*). Thus, Cd uptake from water is dependent on the presence of other divalent metal ions such as calcium or magnesium which compete with Cd^{2+} for the uptake sites (*36*). On the other hands, some hydrophobic complexes of Cd (e.g. with xanthates and dithiocarbamates) can act as metal carriers across biological membranes and lead to an increased uptake of Cd (*36*). Environmental temperatures also increase Cd uptake due to the elevated solubility of Cd compounds and higher concentrations of free, bioavailable Cd^{2+} ions (*7*).

In marine mollusks such as oysters, Cd is predominantly taken up directly from water and less than 1% is obtained through food (phytoplankton) (*36*). Marine organisms including mollusks typically contain higher Cd concentrations as compared to their freshwater and terrestrial counterparts (*36*). Gills are the main site for the uptake of the water-borne metals in aquatic organisms (*29*). Thus, in a freshwater bivalve, *Pyganodon grandis* gills contribute to nearly 50% of the total Cd burden in followed by the mantle, the digestive glands and the foot (*37*). Similarly, in marine bivalves *Mytilus edulis, Mercenaria mercenaria* and *C.virginica,* Cd uptake occurs mainly via gills (*29*). Cd may enter the gills by passive diffusion or by facilitated diffusion through calcium channels (*29, 36*). Further transport of Cd into blood possibly involves Na+ / Ca^{2+} exchange

mechanism, where Cd^{2+} replaces Ca^{2+} (*36*). Metal uptake can be also occur in the digestive tract by endocytosis (*29*).

Cd has no known biological function in animals and thus is toxic in trace amounts (*35*). The Agency for Toxic Substances and Diseases Registry (ATSDR) lists Cd among the top seven of the 275 most hazardous substances found in the environment (*38*). Cd has also been classified as a Category 1 carcinogen in humans by the International Agency for Research on Cancer (IARC) (*32*). Once accumulated, Cd persists for a long time due to its long biological half-life (e.g. 15-30 years in humans) (*33, 39*). In marine bivalves Cd mostly accumulates in the soft tissues (*25*).

Cd is a class II B metal that has high affinity to nitrogen and sulfur groups (*29*). It shares common toxic mechanisms and elicits the same protective response as other class B and borderline metals such as lead, mercury and copper (*32*). In the cell, Cd accumulates in the cytoplasm, lysosomes and mitochondria where it can cause injury to membranes, proteins and DNA (*35*). Cd has several mechanisms of toxicity including substitution of essential metals, interference with calcium signaling and uptake, impairment of DNA repair and induction of oxidative stress (*32*). Cd inhibits the functioning of proteins by binding to the sulphydryl (thiol) groups of amino acids like cysteine and glutathione (*32*). Cd can also exert toxicity via replacement of essential cations (Zn^{2+} and Cu^{2+}) which serve as cofactors in a number of enzymes (*32*). Zinc and Cd have the same oxidation state of +2 and when ionized are almost of the same size(*32*). Cd can replace zinc in enzymes and the proteins that contain Zn finger motifs and are implicated in the maintenance of genomic stability, DNA repairs and DNA damage signaling (*32*). Substitution of Zn for Cd in these proteins interferes with their essential

functions(*32*). Cd also interacts with other metalloproteins in the cell including those containing iron, calcium, copper and zinc, substituting the essential metals in their active centers and influencing enzymes in the metabolic pathways (*32*).

Cd impairs cellular metabolism due to the direct negative effects on mitochondrial function (*25, 37*). Mitochondria accumulate high levels of Cd and are key intracellular targets for the Cd toxicity (*25, 35*). Cd is transported into mitochondria by a Ca^{2+} uniporter mechanism (*40*). In oysters, mitochondria can accumulate up to 250-300 ng Cd mg^{-1} proteins in gills during 3 weeks of exposure to 25 µg L^{-1} (*35*). Cd has strong inhibitory effects on mitochondrial functions, aerobic metabolism, cellular energy budget and functional collapse of mitochondria leading to apoptosis (*25*). *In vivo* concentrations of Cd as low as 10^{-7} – 10^{-6} M result in uncoupling and impaired ability to produce ATP leading to disturbances in the mitochondrial bioenergetics and ultimately cell death (*35*). Cd exposure also leads to inhibition of electron transport chain and ATP synthesis in mitochondria (*41*). At extreme concentrations, Cd can leads to the collapse of mitochondrial membrane potential, mitochondrial swelling and loss of pre-accumulated calcium (*34*). Notably, intrinsic sensitivity to mitochondria to the negative effects of Cd strongly increases with increasing temperatures. Thus, the apparent inhibition constant for Cd (i.e., the concentration of Cd required to inhibit 50% of mitochondrial respiration) is reduced by two orders of magnitude when the temperature increases from 15°C to 35°C in oysters (*7*).

Cd toxicity is also associated with oxidative stress, which is a major hallmark of Cd exposure in the cell (*32-33*). Cd binds to the inner membranes of mitochondria and disturbs the integrity of mitochondrial membranes and enhances lipid peroxidation (*34*).

Cd toxicity to mitochondria results in ROS production due to the inhibition of complex II & III of the ETS (*7, 25, 34, 41*). Cd exposure results in accumulation of semi-ubiquinones at the Q_0 site of complex III. Semi-ubiquinones are responsible for transfer of one electron to molecular oxygen resulting in ROS and thus oxidative stress (*34*). Cd is also involved in the release of iron from the biological molecules which in turn enhances oxidative stress (*33, 42*). ROS affects the mitochondrial ETS, membrane potential, mutation in MtDNA, free radical attack on membrane phospholipid causing lipid peroxidation and activates events leading to apoptosis (*34*).

Cd also affects the cell by interfering with the cell cycle progression, differentiation, proliferation, DNA replication and repair system and apoptotic pathways (*32*). It is not directly genotoxic as it cannot bind to the DNA directly or stably interact with DNA (*32*). Cd affects the genome stability indirectly via production of ROS, by inhibiting the DNA repair systems, causing single stranded breaks in DNA (SSB), chromosomal abberation and depleting the activity of cellular antioxidants (*32-33, 41*).

In addition to the direct toxic effects of Cd, this metal can also have indirect negative effects on the organism's fitness due to the increased basal maintenance costs and reduced aerobic scope in Cd-exposed organisms (*19*). Increased BMR and reduced aerobic scope are caused by Cd-induced increase in the rates of proton leak that lower mitochondrial efficiency, reduced ATP yield, elevated energy costs for metal detoxification and damage repair due to upregulation of metallothioneins, HSP, metabolism, antioxidants and maintenance of ion homeostasis that divert energy from fitness-related functions such as growth and reproduction (*33, 41, 43*).

1.4.3 Detoxification and defense mechanisms against cadmium-induced injury:

Physiological and cellular defense mechanisms involved in detoxification of Cd can be divided into three groups: 1) elimination mechanisms excreting Cd from the cells; 2) binding mechanisms that remove Cd from the free ion pool thereby reducing its toxicity; 3) general stress protection mechanisms that deal with the consequences of Cd-induced cellular damage (*32-33, 44*). These cellular protection systems are very efficient in reducing metal toxicity, but their capacity is limited, and high metal accumulation can overwhelm them resulting in the destabilization of lysosomal membranes and interaction of free metal ions with proteins and DNA [36].

Elimination and binding mechanisms are the first line of defense against Cd toxicity (*45*). Multixenobiotic resistance proteins (MXR) and metallothioneins (MTs) play an important role in Cd elimination and binding respectively. MXRs belong to the superfamily of ATP-binding cassette (ABC) transporters (*45*). MXR can bind to and efflux a wide variety of structurally and functionally diverse substrates against the gradient of concentration using energy of ATP (*45-46*). Their role in the cell is to prevent the entry of xenobiotics and remove those that have entered so that the damage to cell is minimal (*45*). MXRs are pumps are mostly involved in the transport of organic pollutants but can also remove trace metals like Cd and Hg bound to organic ligands such as glutathione (*47*).

Binding to metallothioneins (MTs) is a key detoxification mechanism for Cd and other toxic metals (*7*). MTs are non-enzymatic cytosolic proteins with low molecular weight (typically 6-10 kDa) that regulate homeostasis of essential metals such as copper and zinc, and bind to toxic metals such as Cd and mercury (*33, 39, 44, 48-49*). MTs

contain multiple cysteine residues, but no aromatic amino acids (*50*). Thiol groups of cysteines on MTs serve as the binding sites for class B metals including Cd (*30, 33, 44*). Metallothioneins (MT) reduce availability of free Cd in the cytoplasm by removing it from the biologically active pool and protecting sensitive target molecules (*33, 39, 44*). MTs also serve as free radical scavengers (*33, 39, 44, 48-49*). Recent studies show that MTs may also be involved in transfer of metal ions from the cytosol to lysosomes, and from there to insoluble metal-containing granules aiding in metal elimination (*35*). Exposure of cells and tissues to Cd causes upregulation of genes involved in MT protein synthesis resulting in an adaptive response of the cell to Cd toxicity (*30, 33*).

Heat shock proteins (HSPs) are the second line of cellular defense against the toxic effects of Cd and other trace metals. HSPs can be induced by a number of factors such as heat, hypoxia, heavy metals, oxygen radicals, radiation and osmotic changes. Protein denaturation or any type of protein damage serves as trigger for induction and upregulation of HSPs (*33*). HSPs act as molecular chaperones assisting in the correct folding of misfolded proteins with exposed hydrophobic and/or promoting selective degradation of misfolded or denatured proteins (*51-52*). HSPs assist to repair and protect cellular proteins from stress induced damage and to minimize protein aggregation (*52-53*). Several members are also expressed in unstressed cells where they act as a molecular chaperone to assist in protein maturation (*53*). Induction of HSPs in response to Cd is largely due to denaturation and oxidation of proteins (*32*). HSPs are broadly divided into five families based on their molecular weights, amino acid sequence homologies and functions: HSP 100, HSP90, HSP 70, HSP 60 and the small HSP family (*51, 53*). Among these, HSP70 is the most abundant family that includes both stress

inducible and constitutively expressed proteins (*52-53*). HSP70 is most highly conserved and the first to be induced by a variety of stressors including Cd (*51*). HSP90 and HSP70 induction by Cd and other stressors have been reported in mollusks including *Mytilus edulis, Mytilus galloprovinciallis, Crassostrea gigas* and *Crassostrea angulata* (*53-54*).

Glutathione (GSH) is another ubiquitous molecule that functions as an antioxidant and is essential for detoxification of metals including Cd (*33, 55-56*). GSH is the reduced form whereas glutathione disulphide (GSSG) is the oxidized form (*32*). GSSG is formed by joining of two molecules of GSH in an oxidation reaction of –SH group of cysteine resulting in formation of disulphide bridge between cysteine residues (*4*). In marine invertebrates levels of GSH and ratio of GSH/GSSG have been used as a biomarker of heavy metal stress (*32, 55*). Reduction in the levels of GSH on exposure to Cd is due to conjugation reaction of metals to GSH that detoxifies the metals but reduces the total amount of GSH available (*55-56*). Cytoplasmic Cd can also bind to GSH forming bisglutathionato-Cd-complexes (Cd-GS2) that are removed from the cytosol to lesser sensitive compartments of the cell (*34*).

Antioxidant protection mechanisms also play a key role in preventing Cd-induced cellular injury. Because determination of the effects of Cd and temperature stress on oxidative damage is a focus of my dissertation research, below I discuss the mechanisms and markers of oxidative damage, as well as antioxidant defense mechanisms in details.

1.5 Oxidative stress and its effect on biomolecules:

1.5.1 Causes of oxidative stress:

Cd and temperature stress are known to cause oxidative damage in a variety of organisms including mollusks. Oxidative stress is an imbalance between the production

of oxidants and an organism's ability to detoxify the reactive intermediates by antioxidants (*4, 57-59*). Chemically any substance that can accept an electron or readily transfer an oxygen atom is known as an oxidizing agent or an oxidant(*4*). In contrast, any chemical that loses an electron or accepts a hydrogen atom is referred to as a reducing agent (*4*). Reactive oxygen and nitrogen species (ROS and RNS) that are generated in the process of cellular metabolism and/or in response to metal toxicity are strong oxidizing agents and are often referred to as **pro-oxidants**, while biological molecules that neutralize these pro-oxidants by reducing them are called **antioxidants** (*4*). Oxidants can Oxidants be further classified into radicals and non-radicals (*4*) (Table 1). Compounds capable of independent existence and containing at least one unpaired electron in their orbit are termed **radicals** (*4, 60*). This includes compounds such as biradical ($O_2^{\bullet\bullet}$), superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$) and nitric oxide ($NO^{\bullet}$) (*4*). Radicals are highly reactive due to their ability to accept or donate electrons to attain stable electronic configuration. **Non-radical** substances such as one form of singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), ozone (O_3), aldehydes (HCOR), and peroxynitrite (ONOOH) can also act as oxidants (*4*). Various exogenous and endogenous sources can lead to formation ROS and RNS in the cell (*4*). Exogenous sources include gamma and UV radiation, ultrasound, drugs, pollutants, xenobiotics and toxins, whereas endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes and inflammatory cell activation (*4, 60*).

Mitochondria is a major organelle responsible for ROS production (*4*). Reduction of oxygen to water to form ATP takes place via electron transport system (ETS),

increasing chances of formation of mitochondrial ROS at complexes I and III (*4*, *32-33*, *61*). Superoxide is the main radical produced by mitochondria and it is generated by the incomplete reduction of molecular oxygen in the mitochondrial ETS (*62*). The dismutation of superoxide radicals results in production of H_2O_2, which can cause damage to the cell even at a relatively low concentrations (*4*).

TABLE 1. Classification of most important oxidants in biological systems [modified after Kohen and Nyska(*4*)].

Classification of cellular oxidants	
Oxygen and nitrogen radicals	Oxygen and nitrogen non-radicals
Oxygen (bi-radical) ($O_2^{\bullet\bullet}$)	Singlet oxygen ($^1O_2$)
Superoxide anion ($O_2^{\bullet-}$)	Hydrogen peroxide (H_2O_2)
Hydroxyl ($OH^{\bullet}$),	Hypochlorous acid (HClO)
Peroxyl ($ROO^{\bullet}$)	Ozone (O_3)
Alkoxyl ($RO^{\bullet}$)	Aldehydes (HCOR)
Nitric acid ($NO^{\bullet}$)	Peroxynitrite (ONOOH)

The reactivity of the $OH^{\bullet}$ radical is the highest; it is short lived and has a strong affinity towards other molecules (*4*). Under normal physiological conditions superoxide and nitric oxide radicals react forming peroxynitrite, which can cause effects similar to $OH^{\bullet}$, oxidizing the sulfhydryl groups and causing damage to biological structures (*4*). Most ROS and RNS are short lived and they react quickly with other molecules(*4*). For example, highly reactive species like $OH^{\bullet}$ causes damage at the site where it is produced and if there are no susceptible sites in the vicinity the effects are not seen (*4*). In cases of

radicals like superoxide and non-radicals like HClO, the longer half-lives allows them time to diffuse and reach long-distance sensitive sites to cause oxidative damage (*4*). For example, superoxide radicals that are produced in mitochondrial membrane typically diffuse into the mitochondrial matrix and can react with mitochondrial proteins and lipids as well as the transition metals bound to the mitochondrial genome (*4*).

Free metals in the cytoplasm can enhance oxidative stress due to ROS or RNS. Most transition metals contain unpaired electrons and can convert a relatively stable oxidants into powerful radicals (*4*). Metals like copper and especially iron participate in Fenton reaction and metal-mediated Haber-Weiss reactions (*4*) (Figures 2 & 3). Typically, metals that are bound to the surface of macromolecules like proteins and DNA participate in these reactions whereas metals which are deep within the proteins (e.g. in a catalytic site or in cytochromes) are not available for these reactions (*4*). To participate in the Fenton reaction, iron which is present in cell in the form of ferric ions (Fe^{+3}) have to be reduced to ferrous ions (Fe^{+2}) (Figure 3) (*4*). This reduction can be achieved by superoxide radicals leading to continuous production of highly toxic hydroxyl radical via Haber–Weiss reaction (Figure 4) (*4*). It is estimated that around 1-3% of the mitochondrial oxygen consumption in mammals is converted to ROS, while in invertebrates, this percentage can reach 10-30% (*63-64*).

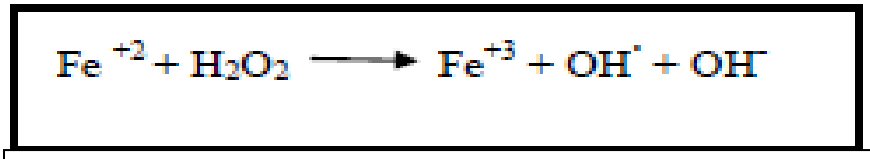

FIGURE 2: Fenton reaction. Iron participate in Fenton reaction where ferric ions (Fe+3) are reduced to ferrous ions (Fe+2) by hydrogen peroxide leading to production of highly toxic hydroxyl radicals (*4*).

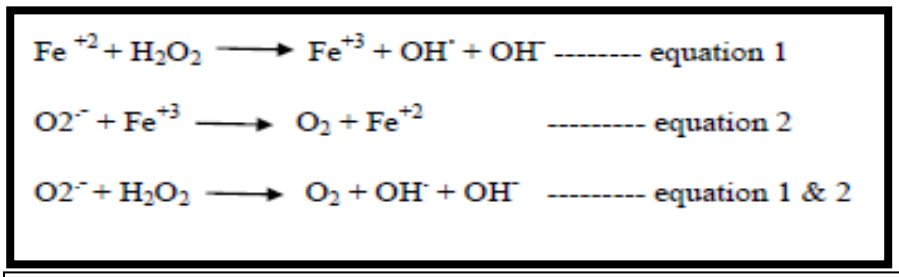

FIGURE 3: Haber-Weiss reaction. It is a metal mediated reaction in which ferric ions are reduced by superoxide radicals to ferrous ions. Ferrous ions then react with H_2O_2 formed due to enzymatic dismutation to superoxide radicals to yield $OH^{\cdot}$ via Fenton type reaction. The sum of equation 1 & 2 results in Haber-Weiss reaction. Thus the interaction of superoxide radical and H_2O_2 leads to production of hydroxyl radicals (*4*).

1.5.2 Effects of oxidative stress on biomolecules:

Biological targets of ROS/ RNS include both organic and inorganic molecules in the cell including DNA, proteins, lipids, amino acids, sugars and metals (*4*, *58-59*). Protein oxidation is a covalent modification of proteins by ROS or secondary byproducts of oxidative stress (*58*). Oxidative attack on proteins results in site-specific amino acid damage, fragmentation, denaturation and damage to their tertiary structure, loss of enzymatic activity due to oxidation of iron-sulfur centers, altered electrical charge and aggregation of cross-linked products (*58*). Oxidation of amino acids such as histidine, lysine, proline, arginine and serine results in formation of carbonyl groups (aldehydes and ketones) (*4*, *58*, *65*). Concentration of the carbonyl groups in cellular proteins serves as a general marker for the oxidative damage to proteins (*58*). Most of the assays for detection of carbonyl group use dinitriphenylhydrazine (DNPH), which leads to formation of a stable dinitrophenyl hydrazone products, which can be spectrophotometrically analyzed at 370nm (*4*, *58*). Indirect oxidative modification of amino acyl side chains of protein can also occur through its interactions with oxidatively modified lipids, amino acids, sugars and glutathione (*58*). For example, end products of lipid peroxidation such as 4-

hydroxynonenal (4-HNE) and malondialdehyde (MDA) can bind with amino acids resulting in formation of aldehyde moieties on amino acids (*58*).

Membrane lipids are also among the key targets for ROS and RNS in the cell due to the presence of high concentrations of unsaturated fatty acids (*4*). ROS and RNS can induce a chain reaction in which one molecule of free radicals can oxidize many molecules of lipids (*32, 44*). It may cause modification of lipid composition, cross linking and polymerization of membrane components (*32*). These changes can in turn cause disturbance to membrane organization, structure, integrity, permeability and functionality (*66*). Lipid peroxidation products are also carcinogenic and mutagenic. If oxidation of the membrane lipids occurs in mitochondria, it can lead to impaired enzymatic activity and ATP production (*32*).

Lipid peroxidation takes place in three steps: initiation, propagation and termination (*4*). Initiation involves an attack of ROS on double bonded methylene group of lipids extracting a hydrogen atom and weakening the bond between carbon and hydrogen (*4*). In order to stabilize, fatty acid radical rearranges its molecular structure to forms conjugated dienes (*4*). In presence of oxygen, fatty acid radical ($ROO^{\bullet}$) will form during the propagation stage (*4*). These radicals are in turn capable of extracting hydrogen atom from the neighboring fatty acid molecule leading to a chain reaction(*4*). Single initiation event can lead to peroxidation of all the unsaturated lipids in the membrane (*4*). Finally, the termination can take place when $ROO^{\bullet}$ reacts with another radical or antioxidants (*4*). The degraded products of lipid peroxidation are aldehydes such as malondialdehyde (MDA) (*4, 32, 67*). MDA is among the first lipid peroxidation products and it is widely used as an indicator of oxidative stress (*4*).

ROS/RNS can also lead to DNA damage including modification of DNA bases, DNA strand breaks, extraction of hydrogen from deoxyribose sugars, loss of purines, DNA-protein cross linkage and damage to DNA repair mechanisms (*4, 44, 65*). Of all the ROS, hydroxyl radicals cause most damage to DNA by attacking the C-8 position of guanine leading to formation of 8-hydroxydeoxyguanosine (8-OHdG) (*4*). Transition metals like iron that catalyze the production of $OH^{\bullet}$ via Haber-Weiss reaction, have high binding affinity to DNA and in the presence of ROS can cause extensive damage to DNA leading to double strand breaks (*4*).

1.5.3 Antioxidant defense system:

To prevent the interaction between the radicals and biologically important molecules, antioxidants are usually present at the sites where these radicals are produced (*4*). Cells have evolved multiple mechanisms for protection against ROS and RNS including enzymatic and non-enzymatic antioxidants (*9*).

Enzymatic antioxidants involve superoxide dismutase (SOD), catalase and glutathione peroxidases (GSH peroxidases) (Figure 4) (*59*). SOD is present in different locations in the cells as a metalloprotein bound to different metals like copper, zinc, manganese and iron. SOD enhances dismutation of superoxide radicals to hydrogen peroxide (H_2O_2), which is further removed by the activity of enzyme catalase and glutathione peroxidase (*4, 9, 65*). Catalases can effectively remove H_2O_2 present at high concentrations by converting two molecules of H_2O_2 to produce an oxygen molecule and two molecules of water (*4*). In contrast, glutathione peroxidases (GPx) have high affinity for H_2O_2 and can remove it even at low concentrations (*4*). Compared to catalases, GPx

activity is an expensive process as it consumes two molecules of glutathione (GSH) for removal of one molecule of H_2O_2 (*4*).

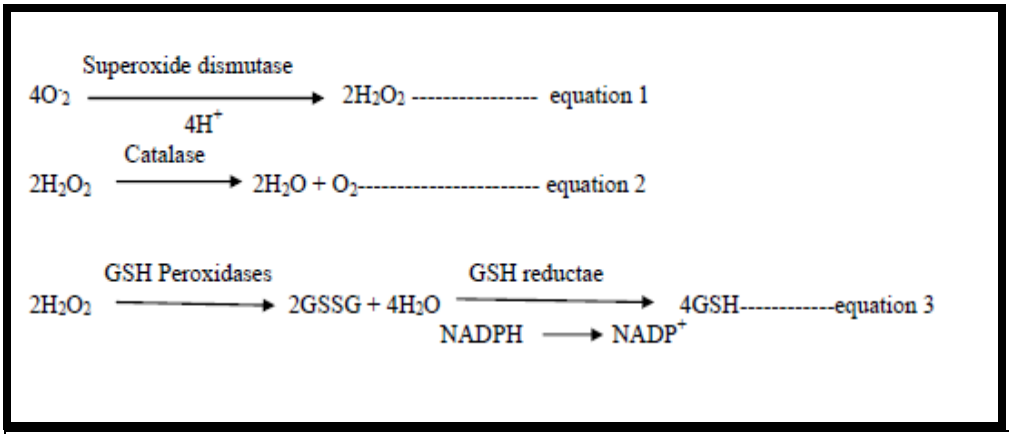

FIGURE 4: Antioxidant enzymes in mitochondria. SOD is capable of spontaneous dismutation of superoxide radicals to H_2O_2 (equation 1), which can be further be removed by catalases to produce an oxygen molecule (equation 2). In contrast to catalase, GSH peroxidases possesses high affinity to H_2O_2 and oxidize glutathione, GSSG. GSSG is further reduced to GSH reeducates by reduced NADH reductase (equation 3) (*4*).

Non-enzymatic antioxidants are classified as hydrophilic and lipophilic based on their solubility (*4*). Hydrophilic non-enzymatic antioxidants include vitamin C (ascorbic acid), glutathione, lipoic acid and uric acid, and lipophilic antioxidants include Vitamin E (tocopherol), β-carotenoids (vitamin A precursor) and ubiquinol (Co-e-Q) (*4, 44, 59*). Some small molecules such as melatonin can also act as a scavenger for a variety of pro-oxidants (*4, 9, 33, 65*). Glutathione is a low molecular weight tripeptide and an important non-enzymatic antioxidant in the cell (*4*). GSH can scavenge ROS like $OH^{\bullet}$, $ROO^{\bullet}$, $RO^{\bullet}$ and HClO and prevents copper from participating in Haber-Weiss reactions by acting as a chelating agent (*4, 32*). GSH also acts as a cofactor for enzyme GSH peroxidases and donates necessary electrons for the decomposition of H_2O_2 (*4, 32*). In the process of antioxidant action, GSH is typically oxidized to glutathione (GSSG) and has to be

regenerated by GSH reductase in a NADPH-dependent reaction (*4, 57*). Ratio of GSH/ GSSG is often used as an indicator of oxidative stress in the cell (*4, 9, 44, 65*).

1.6 Eastern oysters (*Crassostrea virginica)* as a model to study metabolic effects of Cd:

The eastern oyster, *Crassostrea virginica* is a common eurythermal bivalve inhabiting estuarine ecosystems along the east coast of North America from Gulf of St. Lawrence to the Gulf of Mexico (*68*). Oysters are both a "colonizer" and an "ecosystem engineer" species and an important indicator of estuarine ecosystem health [69]. They have high fecundity rates and can colonize new niches opened by changes in the physical environment (*69*). They then modify their physical environment to make it suitable for the long term survival. Oysters qualify as an "ecosystem engineers" because of their reef building capabilities. Oyster reefs provide shelter to more than 300 species (*69*). Their filter feeding helps in cleaning of water by filtering out the phytoplankton and suspended particles from the water, and supports higher-level consumers by converting microalgae and detritus to animal biomass [69]. Oysters are an important link in the food chain and are prey to numerous animals including birds, sea stars, whelks, crabs and humans [69]. In recent years, over-harvesting, loss of habitat, decline in the water quality, increase in anthropogenic pollution, global warming, and increased incidence of disease have led to a drastic reduction in oyster populations and the health of the estuarine ecosystems in which they reside (*70*). Approximately 91% of oyster population has declined due to massive destruction of oyster reefs by dredging, eutrophication and disease (*71*). Oyster production now predominantly depends upon aquaculture (*71*).

Oysters are facultative anaerobes and can transition between aerobic and anaerobic metabolism depending on the environmental conditions such as oxygen

availability or temperature. When oxygen supply from ventilation and circulation is sufficient to fully support the mitochondrial function, oysters predominantly depend on aerobically produced ATP (*23*). However, if oxygen supply becomes limited (due to the environmental hypoxia or tissue hypoxemia caused by a mismatch between oxygen demand and supply), oysters transition to partial or full anaerobiosis (*23*). Oysters can produce ATP via a variety of anaerobic mechanisms including glycolysis and hydrolysis of high energy phosphates such as phospho-L-arginine (PLA). PLA is used in a one-step energy conversion reaction catalyzed by arginine kinase to rapidly replenish ATP during the short-term bursts in ATP demand, such as exercise (*8*). In contrast, during the long-term hypoxia or hypoxemia, oysters depend on anaerobic catabolism of carbohydrates and amino acids such as aspartate (*72-75*). The anaerobic glycolysis of oysters yields alternative end products such as L-alanine and opines in the cytosol while succinate, acetate and propionate are produced in the mitochondria (*74, 76*). The mitochondrial pathways producing succinate, acetate and propionate yield more ATP per unit glucose metabolized and are more compatible with the long-term energy and acid-base homeostasis compared with lactate or opines (*72-75*). Thus, production of succinate yields 4.7 ATP molecules per glycosyl unit, while propionate yields 6.4 ATP per glycosyl unit compared with 3 molecules of ATP per glycosyl unit produced in the lactate pathway. However, even the most efficient anaerobic pathways yield considerably less ATP production compared to aerobic pathways (38 ATP molecules per glycosyl unit), and partial or complete anaerobiosis cannot support survival of an organism for a long period of time (*8*). Onset of partial anaerobiosis indicated by accumulation of anaerobic end products such as L-alanine, acetate, succinate or propionate is therefore an indicator

of a transition to the pessimum physiological range and time-limited survival (Figure 1) (*77*). In the case of the environmental temperature, accumulation of anaerobic end products indicates Tc and transition from the pejus to the pessimum temperature range in oysters (Figure 1).

Oysters are exposed to multiple environmental stressors in their habitats including fluctuating temperatures during diurnal and tidal cycles, seasonal temperature changes, variation in food and oxygen availability, salinity and pollutants (including Cd) (*23*). Oysters are ectothermic organisms, and their body temperature changes with the environmental temperature (*23*). Like all intertidal organisms, oysters can experience rapid changes in body temperatures as large as 10-20°C during low summer tides and even greater temperature shifts during seasonal cooling and warming (*19, 41*). Oysters also are often exposed to trace metals including Cd in polluted estuaries and have ability to strongly accumulate Cd up to 0.4-40 μg^{-1} dry weight in natural populations and up to 300-400 μg^{-1} dry weight during acute exposures to waterborne Cd (*35*). This makes eastern oysters a useful model for the study of interactive effects of Cd and temperature (*41*).

Cd strongly affects metabolic function and energy balance in *C. Virginia* [9]. Cd has a direct effect on the aerobic capacity of oysters due to its negative impacts on oyster mitochondria including decreased in phosphorylation efficiency, elevated proton leak and uncoupling and thus impaired ATP production (*43*). Cd exposure also can inhibit anaerobic metabolic pathways in oysters suggesting global impairment of energy metabolism (*14*). It is known to result in cellular energy deficiency and oxidative stress and at higher concentrations of Cd can cause collapse of mitochondrial functioning

leading to apoptosis (*25*). Cd exposure in oysters also results in the elevated standard metabolic rate (SMR) reflecting higher costs of basal maintenance due to the increased rates of proton leak, elevated costs of metal detoxification due to upregulation of metallothioneins, HSP, antioxidants, and maintenance of ion homeostasis (*43*). These data strongly suggest that exposure to Cd results in the reduced aerobic scope and therefore is likely to sensitize oysters to temperature stress by narrowing their thermal tolerance window [9]. Earlier studies support this hypothesis showing that concomitant exposure to Cd and elevated disrupts balance between energy demand and energy supply and results in physiological stress and high mortality of oysters (*63*). However, up to date there have been no studies to directly test the effects of Cd exposure on the thermal tolerance window on marine ectotherms and determine whether Cd exposure reduces thermal tolerance by shifting the threshold critical temperatures (Tc). My dissertation research aim to close this gap by determining whether exposure to environmentally relevant concentrations of Cd shifts the upper Tc of oysters to lower values thereby narrowing the thermal tolerance window of these organisms, and whether the effects of Cd on thermal tolerance are modified by seasonal acclimatization to different thermal regimes. This study will provide important insights into the physiological mechanisms of tolerance to temperature and Cd stress in marine bivalves and the potential implications of temperature stress (such as expected in the case of the global climate change and/or seasonal thermal fluctuations) for performance and survival of oyster populations in polluted estuaries.

1.7 Aims and hypotheses of the study:

The overall goal of this project was to determine the effects of Cd exposure and seasonal acclimatization on the thermal tolerance window determined by the crtitical temperatures of aerobic metabolism (Tc) in a model marine ectotherm, the eastern oyster *Crassostrea virginica*. To achieve this goal, I have tested the following hypotheses:

1) Exposure of oysters to Cd and/or cold acclimatization in winter will result in a shift of the upper critical temperatures of aerobic metabolism to lower values as determined by the earlier accumulation of anaerobic end products and/or impaired cellular energy status.

2) Transition to anaerobiosis at elevated temperature will be associated with elevated oxidative damage reflecting mitochondrial dysfunction at elevated temperatures, and this oxidative damage may be enhanced by Cd exposure.

3) Oysters collected in winter season will show higher oxidative damage in response to acute temperature stress and/or Cd exposure as compared to their warm-acclimatized summer counterparts.

These hypotheses were tested in three Research Aims: Research aim 1: To determine effects of Cd exposure and season on Tc in oysters as determined by accumulation of anaerobic end products (as an early markers of Tc).

Research aim 2: To determine the effects of seasonality and Cd exposure on the cellular energy status of oysters (as a late marker of transition into the pessimum range).

Research aim 3: To determine the effects of seasonality and Cd exposure on oxidative stress during acute temperature rise in oysters.

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental design:

Adult North Carolina oysters were purchased from J & B Aquafood (Jacksonville, NC) and shipped to UNC Charlotte within 24 hours of collection. Summer and winter oysters were collected in May and December, respectively, and the exposures were conducted separately in each season using the same experimental design. After a preliminary acclimation period of 7 days at 20°C, the oysters were randomly divided into two groups and were exposed to either clean artificial seawater (ASW; control group) or ASW with 50 µg L^{-1} of Cd (Cd-exposed group) at 20 °C for a period of 30 days. For the Cd-exposed group, ASW in tanks was supplemented with 50 µg L^{-1} Cd added as $CdCl_2$. Cd was replenished twice a week to maintain the concentration of 50 µg L^{-1}. Water was changed in the control and Cd-exposed tanks weekly. Salinity was maintained at 30$\pm$2 ppt in all exposures. Oysters were fed *ad libitum* on alternate days with DT's Live Marine Phytoplankton (Premium Reef Blend) according to the manufacturer's recommendation. After the 30-day acclimation period, oysters were transferred into two 120 L tanks each containing ASW (20°C, 30 ppt salinity) with the same concentration of Cd as during the acclimation (0 and 50 µg L^{-1} for control and Cd-exposed oysters, respectively). Temperature in the tanks was controlled by heating rods and a circulating water bath. Oysters were allowed to acclimate in the tanks for 24 h at 20°C, after which a subsample (8-16 oysters) was collected. The temperature was then increased by 4^{o}C at a rate of 1^{o}C

per hour, and once the target temperature (24^{o}C) was reached, the oysters were kept at it for 48 h. This time is sufficient to reduce the acute effects of rapid temperature change but not sufficient for full thermal acclimation (Sokolova, personal communication). After 48 h of exposure, another subsample of 8-16 oysters was collected, and the temperature was again increased by 4^{o}C as described above. As a result of this stepwise warming procedure, subsamples of oysters were collected at 20°C, 24°C, 28°C, 32°C and 36°C. All experimental oysters experienced gradual warming starting from 20°C with a 48 h of exposure after every 4°C temperature increment until their target temperature of collection was reached. Water was changed in experimental tanks every 48 h using clean or Cd-supplemented water equilibrated to the same temperature as the current exposure temperature in experimental tanks. During every water change, Cd was added to the tank with Cd-exposed oysters to avoid Cd depletion and maintain Cd concentration at 50µg L^{-1}. Throughout the 30-day acclimation and experimental exposures, the average measured Cd concentration in Cd-exposed tanks was 45.6 µg Cd L^{-1}, while Cd levels in the control tanks were below the detection limits of the method used (<0.5 µg Cd L^{-1}).

Immediately following the collection, experimental oysters were dissected and their gills, hepatopancreas, muscle and mantle tissues were shock frozen in liquid nitrogen (*19, 78*). All tissues were stored in liquid nitrogen until further analyses.

2.2 Tissue metabolite determination:

Tissue metabolite concentrations were measured in deproteinized perchloric acid (PCA) extracts of oyster tissues. For extraction, 200-300 mg of tissues was ground to a fine powder in mortar and pestle under the liquid nitrogen. Tissue powder was mixed with 1.5 ml of ice cold 0.6 M perchloric acid (PCA) containing 150 mM EDTA to

maximize ATP extraction. The extracts were sonicated for 20 s at the maximum power (10 W), and centrifuged for 2 minutes at 10,000 x g at 4ºC to remove precipitated proteins. The extract was neutralized by adjusting the pH to a range of 7-7.5 with 5 M potassium hydroxide (KOH) and 5 M hydrochloric acid (HCl). Samples were centrifuged to remove precipitated potassium perchloride at 10,000 x g for 5 minutes at 4ºC. Supernatant was collected and stored at -80ºC till further analyses (*25, 77, 79*).

Concentration of metabolites (L-alanine, acetate, succinate, adenylates, phosphagen/aphosphagen, glycogen and glucose) in oyster tissues were measured in neutralized PCA extracts spectrophotometrically using enzymatic assays described elsewhere (*77, 80-81*). Briefly, the assay conditions were as follows:

L-alanine: 80 mM Tris buffer, pH 7.6, 7 mM 2-oxoglutarate, 0.24 mM NADH and 260 U ml^{-1} of lactate dehydrogenase, 10,000 U ml^{-1} alanine amino-transferase (glutamate pyruvate transaminase); absorbance (340 nm) was measured at the start and after 30 min of the reaction (*25, 77*);

Acetate: 100 mM triethaloamine (TRA) buffer pH 7.6, 0.2 M magnesium chloride, 18 mM NADH, 91 mM ATP, 150mM PEP phosphoenolpyruvate (PEP), 5 U ml^{-1} of pyruvate kinase (PK), 5 U ml^{-1} lactate dehydrogenase (LDH) each, 50 U ml^{-1} of acetate kinase (AK); absorbance (340 nm) was measured at the start and after 40 min of the reaction (*25, 77*);

ATP: 38.5 mM triethanolamine hydrochloride (TEA) buffer pH 7.6, 0.04 mM NADP, 7 mM $MgCl_2.6H_2O$, 0.462 U ml^{-1} glucose-6-phosphate dehydrogenase, 50 mM glucose, 1.8 U ml^{-1} hexokinase; absorbance (340 nm) was measured at the start and after 30 min of the reaction (*77*);

ADP and AMP: 58 mM TEA buffer pH 7.6, 3 mM phosphoenolpyruvate (PEP), 0.09 mM NADH, 24 U ml^{-1} lactate dehydrogenase (LDH), 18 U ml^{-1} pyruvate kinase (PK), 16 U ml^{-1} myokinase (MK); absorbance was measured at 340 nm;

L-arginine: 170 mM glycilglycin pH 7.6, 13 mM $Mgcl_2.6H_2O$, 6.5 mM pyruvate, 0.26 mM ADP, 0.79 mM NADH, 1 U ml^{-1} octopine dehydrogenase; absorbance (340 nm) was measured at the start and after 30 min of the reaction (*25, 77*);

D-glucose: 38.5 mM TRA buffer, pH 7.6, 0.04 mM NADP, 7 mM $MgCl_2*6H_2O$, 0.462 U ml^{-1} glucose-6-phosphate dehydrogenase, 1.8 U ml^{-1} hexokinase (*7*).

For determination of phospho-L-arginine (PLA) levels, PLA in the sample was subjected to acid hydrolysis yielding L-arginine. L-arginine was determined as described above and PLA levels were calculated as a difference in L-arginine contents of the sample before and after the acid hydrolysis (*25, 77, 82*). Glycogen concentration was measured in PCA extracts after enzymatic hydrolysis of glycogen to D-glucose by glucoamylase ((*83*)) and determined by the difference in the D-glucose levels in the tissue extract before and after glucoamylase treatment. Succinate was measured in PCA extracts using succinic acid kit (Boehringer Mannheim, R-Biopharm kit Darmstadt, Germany) according to the manufacturer's instructions (*25, 77*). Concentrations of glycogen were expressed in mg g^{-1} wet tissue mass, and all other metabolites – as µmol g^{-1} wet tissue mass.

2.3 Lipid and protein analysis:

Tissue lipid content was measured using a standard method of chloroform extraction (*84-85*). Oyster tissues (~50-100 mg of wet mass) were homogenized in chloroform/methanol mixture (2:1 v:v) using tissue to chlorophorm/methanol ratio of

1:20 (w:v). Samples were sonicated for 1 min (output 69 W, Sonicator 3000, Misonix, Farmingdale, NY, USA), vortexed and centrifuged for 5 min at 13,000 x *g*. The supernatant was transferred into a new tube and the chloroform/methanol extraction was repeated on the tissue pellet. The supernatants of two extractions were pooled, mixed with water (25% of the total volume of supernatant), and centrifuged for 5 min at 13,000 x *g*. The lower phase (chloroform) was transferred to a pre-weighed tube and evaporated to determine the mass of the extracted lipids.

For protein determination, 100-200 mg of oyster tissues were homogenized in ice-cold homogenization buffer (100 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100) using hand-held Kontes Duall tissue grinders (Fisher Scientific, Suwanee, GA, USA). Homogenates were sonicated 3×10 s (output 69 W, Sonicator 3000, Misonix, Farmingdale, NY, USA) to ensure complete release of the proteins, with cooling on ice (1 min) between sonications. Homogenates were centrifuged for 10 min at 20,000 x *g* and 4°C, and supernatants were used for protein determination. Protein content was measured using the Biuret protein assay. Concentrations of lipids and proteins were expressed in mg g^{-1} wet tissue mass.

2.4 Oxidative stress markers:

Tissue levels of malondialdehyde (MDA) were determined by thiobarbituric acid assay (TBARS) (*86-87*). Briefly, tissues powdered under liquid nitrogen were homogenized in 4 volumes of 50 mM potassium phosphate buffer (pH 7.0 at 20°C), sonicated for 3 to 5 seconds (output 7, Sonic Dismembrator Model 100, Fisher Scientific, Suwanee, GA) and centrifuged for 5 min at 13 000 x g and 4°C. Sample supernatants as well as blanks and MDA standards of known concentrations were mixed with 0.375 %

thiobarbituric acid (TBA) and 2% butylated hydroxytoluene (BHT) in the following ratio: 1:14:0.14, heated for 15 min at 100°C and again centrifuged (13 000 g, 5 min, room temperature). The formation of a pink chromagen by reaction between MDA and TBA was measured spectrophotometrically at 532 nm. Tissue levels of MDA were expressed in μmol g^{-1} wet mass.

Protein carbonyl groups were measured spectrophotometrically as described elsewhere (*88*). Tissues were ground under liquid nitrogen and homogenized in the buffer containing 50 mM HEPES, 125 mM KCl, 1.1 EDTA and 0.6 mM $MgSO_4$ (pH 7.4) and protease inhibitors [leupeptin (0.5 μg mL^{-1}), pepstatin (0.7 μg mL^{-1}), phenylmethylsulfonyl fluoride (40 μg mL^{-1}) and aprotinin (0.5 μg mL^{-1})]. Samples were centrifuged at 100,000 × g for 15 min, supernatant was collected and incubated at room temperature with 10 mM 2,4-dinitrophenylhydrazine (DNP) in 2 M HCl. The blanks were incubated with HCl without DNP. After incubation, proteins were precipitated by adding 100% TCA and centrifuged at 11,000 × g for 10 min. The pellet was collected, washed with ethanol ethylacetate (1:1) and resuspended in 6 M guanidine hydrochloride in 20 mM in KH_2PO_4 (pH 2.5) until dissolved. The absorbance was measured at 360 nm on a spectrophotometer (Cary 50, Varian) using guanidine HCl solution as reference. The amount of carbonyls was estimated as a difference in absorbance between samples and blanks using a molar extinction coefficient of carbonyls $\varepsilon = 22000\ cm^{-1} * M^{-1}$. Amount of carbonyls was expressed per mg total protein measured in the same samples using Bradford method.

2.5 Tissue Cd concentrations:

Mantle samples (~1-2mg dry weight) were freeze dried and digested in Teflon bottles with 52.5% nitric acid (trace metal grade; Fisher scientific, Suwanee, GA,USA) using 3-5 cycles of microwave heating (20-30s) with between cycle cooling on ice for 10-12 minutes until tissues were fully digested. Cd concentrations were determined in the tissue digests with an atomic absorption spectrophotometer (Perkin-Elmer AAnalyst 800, Shelton, CT, USA) equipped with graphite furnace and Zeeman background correction. Water samples from experimental tanks were acidified with nitric acid (1% final concentration), and Cd levels measured by AAS. The detection limit of this method was 0.5-1 µg Cd L^{-1} sample or 2.5-5 x 10^{-3} µg Cd g^{-1} wet tissue weight.

2.6 Derived indices:

The energy state of organisms is assessed by the individual concentration of all three phosphoadenylate nucleotides (ATP, ADP & AMP), a total concentration of adenylates (TANP), adenylate energy charge (AEC) and the proportion of phosphagen in the total phosphagen/aphoshagen pool (RPLA) (*89*). Adenylate energy charge (AEC) was calculated using the formula:

AEC = ([ATP] + 0.5 [ADP]) / ([ATP] + [ADP] + [AMP])

Total adenylate nucleotide pool was calculated as a sum of the concentrations of the respective adenylates:

TANP = [ATP] + [ADP] + [AMP]

The relative amount of phosphagen (R_{PLA}) in the total phosphagen/aphosphagen pool was calculated as follows:

$\mathbf{R_{PLA}}$ = [PLA] / ([PLA] + [L-arginine])

Where [PLA] and [L-Arginine] are tissue concentrations of phospho-L-arginine (phosphagen) and L-arginine (aphosphagen), respectively, measured in μmol g^{-1} wet mass (*90*).

2.7 Statistical Analyses:

Statistical analysis was performed using generalized linear model (GLM) analysis of variance (ANOVA) after testing for normality of the data and homogeneity of variance and was followed by post hoc procedures (Fisher's Least Significant Difference test for unequal N). To determine the effects of temperature, Cd exposure and season on tissue metabolite concentrations, adenylate and phosphagen/aphosphagen levels as well as energy-related parameters (TANP, AEC and R_{PLA}), three-way ANOVAs were used with 'Temperature' and 'Cd exposure' as fixed factors, and "Season' as a random factor. To determine the effects of temperature, Cd exposure, season and tissue source on glycogen, glucose, lipid and protein concentrations, four-way ANOVAs were used with 'Temperature', 'Tissue' and 'Cd exposure' as fixed factors, and "Season' as a random factor. All models included the main factor effects and all factor interactions. Factor effects and differences between the means were considered significant if the probability of a Type I error was less than 0.05 ($P \leq 0.05$). Data are presented as percentages or means ± S.E.M. (Standard error of the mean) unless indicated otherwise. Statistical Analysis Software (SAS 9.2) (SAS Institute, Cary, NC, USA) was used for the statistical analyses, and graphs were constructed using Prism GraphPad software (GraphPad Software, Inc., La Jolla, CA).

CHAPTER 3: RESULTS

3.1 Cd concentrations in oyster tissues:

Cd exposure resulted in a significant accumulation of Cd in the mantle tissue of oysters (Fig. 5). Accumulated Cd burdens were higher in winter Cd-exposed oysters compared to their summer counterparts ($P<0.05$) but were not significantly affected by the acute temperature rise (Fig. 5).

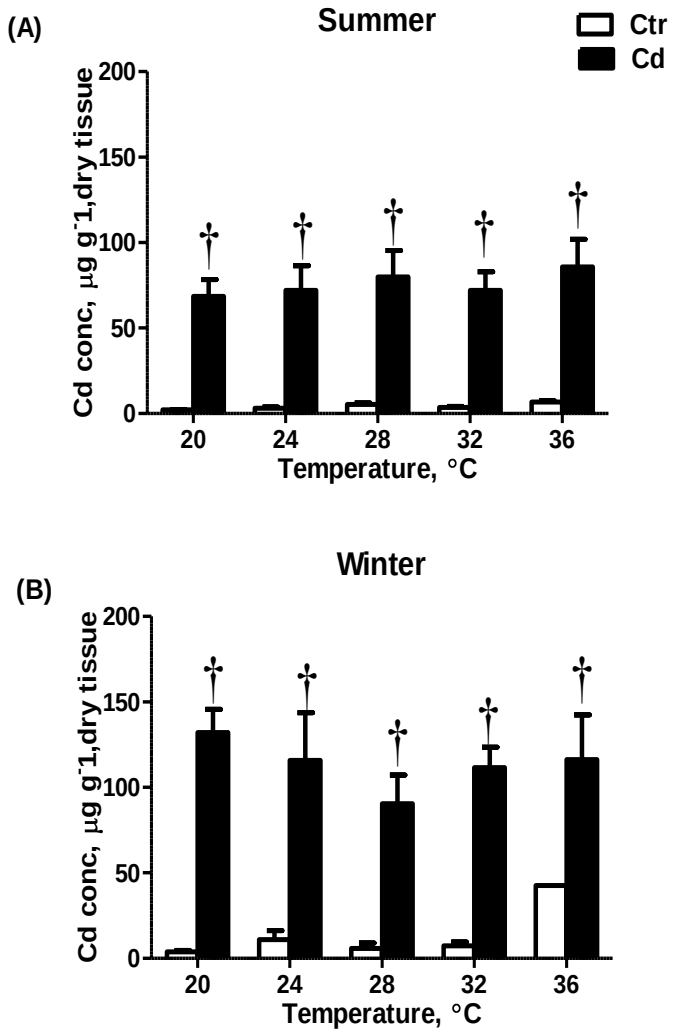

FIGURE 5: Total accumulation of Cd in mantle tissue in control (Ctr) and Cd-exposed (Cd) eastern oysters, *C.virginica* during summer (A) and winter (B) seasons. Daggers show the values that differ between control and Cd-exposed oysters ($P<0.05$). Summer: n = 8-12; winter n = 8 except the control oysters at 36°C where n = 3.

3.2 Anaerobic end product accumulation:

Concentrations of anaerobic end products (L-alanine, acetate and succinate) were differentially affected by the studied factors (exposure to Cd, acute temperature rise and seasons) (Table 1). The effects of season were significant for all studied anaerobic end porducts whereas the effects of other factors and their interactions were different for L-alanine, succinate and acetate (Table 1).

L-alanine levels in gill tissues of oysters were significantly affected by Cd exposure and season, as well as by the interactions of the exposure temperature and season and the three factor interactions between temperature, season and Cd exposure (Table 1). At the acclimation temperature (20°C), control oysters had lower L-alanine levels in winter than in summer ($P<0.05$), whereas in their Cd-exposed counterparts L-alanine levels were similarly high in summer and in winter (Fig. 6). Acute exposure to elevated temperatures resulted in a significant increase of L-alanine levels in gills of control and Cd-exposed oysters (Fig.6A). However, the pattern of the temperature response was different in oysters collected in different seasons, as well as in control and Cd-exposed oysters. In control oysters collected in summer, significant L-alanine accumulation occurred at 28°C and above, while in their Cd-exposed counterparts L-alanine levels were elevated at 24°C and above (Fig. 6A). In winter, L-alanine levels in gills of control oysters increased at 24°C and above, although this trend was statistically significant only at 24 and 32°C (Fig.6B). In Cd-exposed oysters collected in winter, L-alanine levels were elevated at the acclimation temperature (20°C) and did not change in response to the acute temperature rise except for a slight transient decrease at 28°C (Fig.6B).

Acetate levels in oyster gills were significantly affected by studied factors (Cd exposure, temperature and season) and their interactions (Table 1). At the acclimation temperature (20°C), tissue acetate levels were similar in summer and winter and in control and Cd-exposed oysters ($P<0.05$; Fig.7). Acute temperature rise induced significant acetate accumulation in control oysters in winter (at 24°C and above) but not in summer (Fig. 7A & B). In Cd-exposed oysters, elevated temperatures did not lead to acetate accumulation in winter or summer (Fig. 7A & B).

Succinate levels in oyster gills were significantly affected by the season but not by temperature or Cd exposure (Table 1). Tissue levels of succinate were higher in summer compared to winter in control and Cd-exposed oysters at the acclimation temperature (20°C). No significant succinate accumulation was found in response to elevated temperatures in control or Cd-exposed oysters (Fig. 7 C & D).

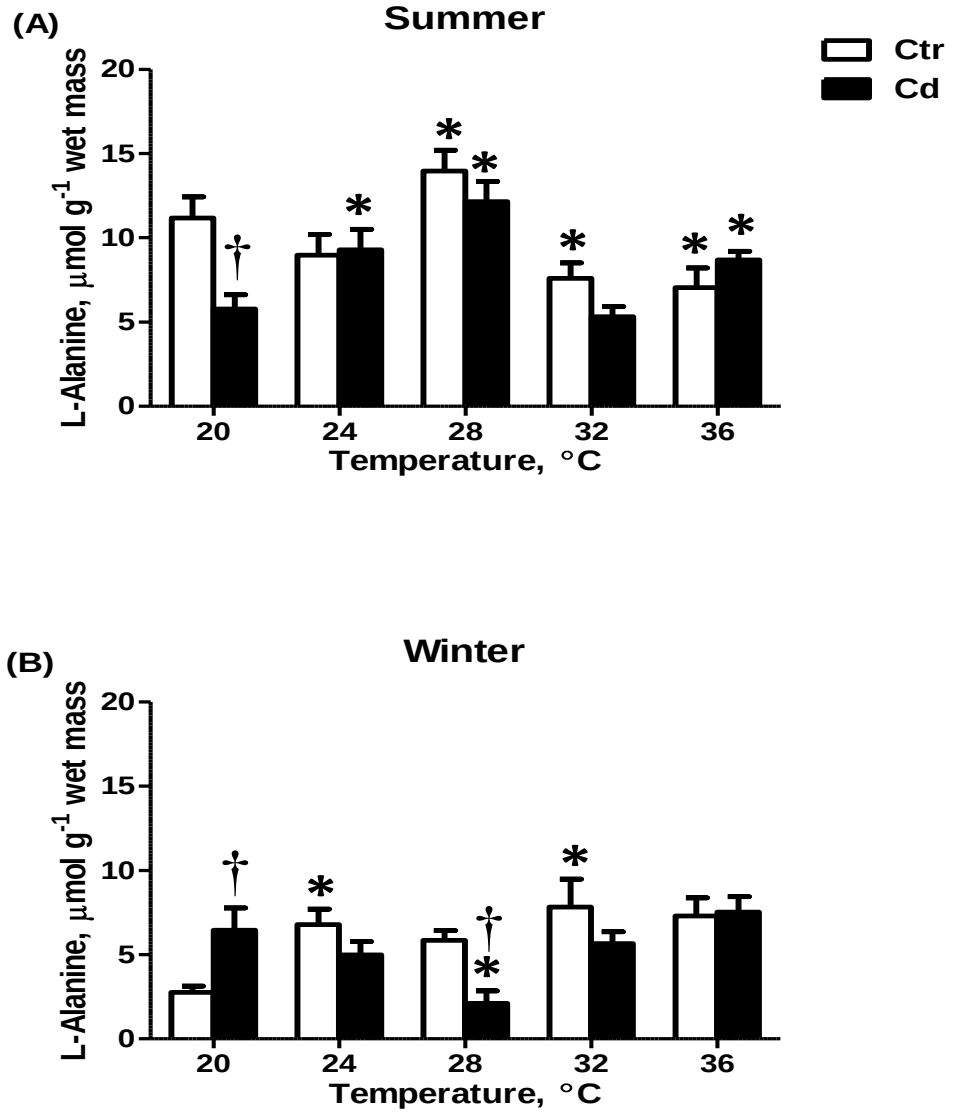

FIGURE 6: Accumulation of L-alanine (an end product of cytosolic anaerobiosis) in gills of control (Ctr) and Cd-exposed (Cd) oysters *C.virginica* during summer (A) and winter (B) seasons. Asterisks indicate values significantly different from the respective groups of control or Cd-exposed oysters at 20°C ($P<0.05$). Daggers show the values that differ between control and Cd-exposed oysters at the same temperature ($P<0.05$). Summer: n = 9-13; winter: n = 6-8 except at 36°C where n = 2.

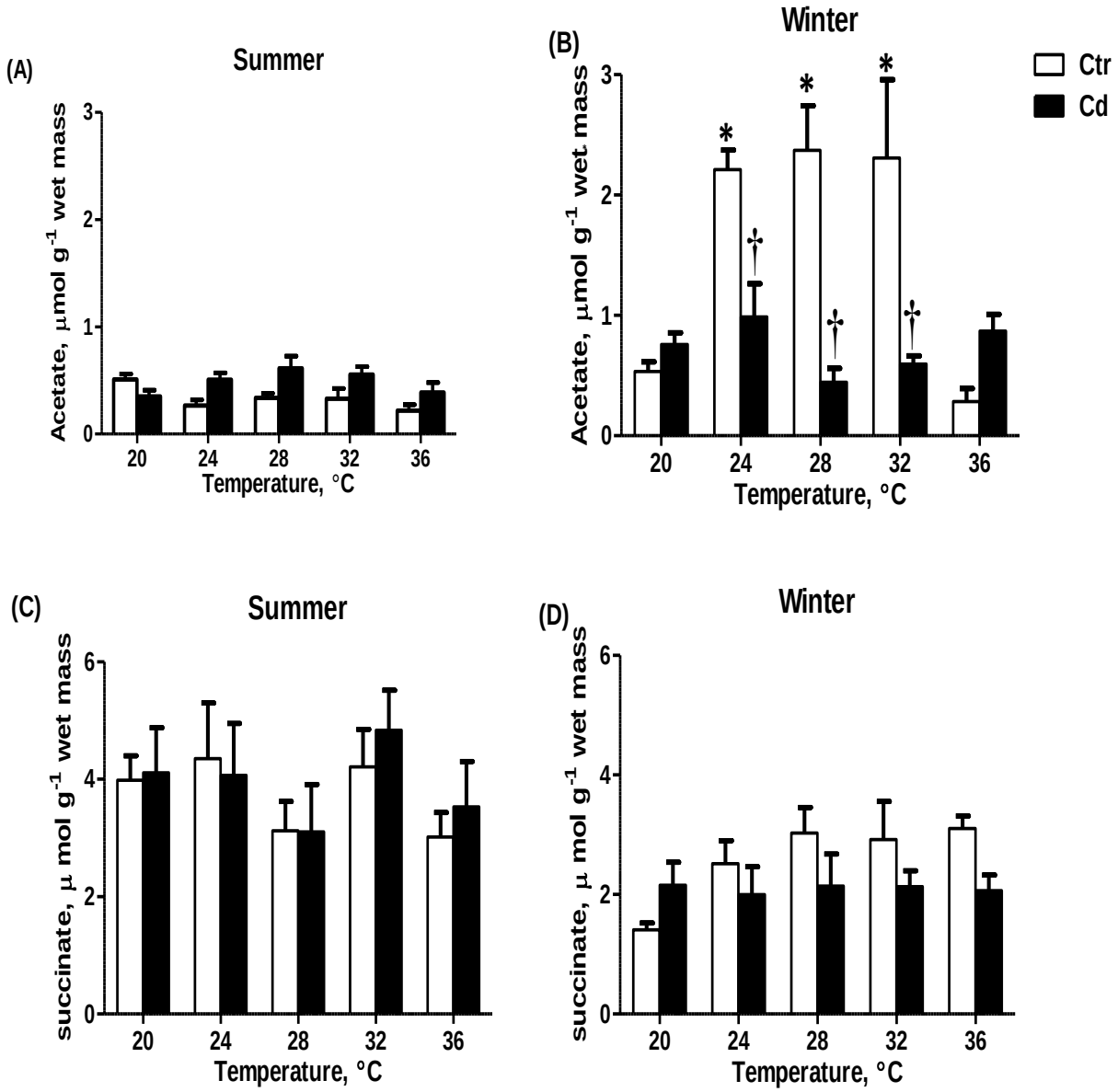

FIGURE 7: Accumulation of acetate (A & B) and succinate (C &D) (mitochondrial anaerobic end products) in gills of control (Ctr) and Cd-exposed (Cd) oyster, *C.virginica* during summer and winter seasons. Asterisks indicate values significantly different from the respective groups of control and Cd-exposed oysters at 20°C ($P<0.05$). Daggers show the values that differ between control and Cd-exposed oysters (($P<0.05$). Summer: n= 9-13; winter: n = 6-8 except at 36°C where n=2.

3.3 Cellular energy status:

3.3.1 Adenylates:

ATP concentrations in the gills of *C.virginica* were significantly affected by the season as well as interactions between Cd exposure and season (Table 2). At the acclimation temperature (20°C), the steady-state ATP levels were similar in control and Cd-exposed oysters in winter and summer ($P>0.05$). Acute temperature rise did not lead to a depletion of tissue ATP levels (Fig. 8). In fact, tissue ATP levels were slightly elevated at 28°C and 36°C in the gills of summer-acclimatized control oysters (Fig. 8A). No temperature-induced change in tissue ATP levels were found in Cd-exposed oysters in summer, or in control and Cd-exposed oysters in winter (Fig. 8).

Season and interaction of season and temperature significantly affected ADP levels in oyster tissues (Table 2). At the acclimation temperature of 20°C, tissue ADP levels were notably higher in winter than in summer oysters albeit the difference was only significant in Cd-exposed oysters ($P<0.05$). Acute temperature rise had no effect on tissue levels of ADP in oysters, except for slight but significant decline at 28°C and 36°C in summer control oysters (Fig 8C).

Tissue AMP levels of oysters were significantly affected by the factors ‘Season’ and ‘Temperature’ (Table 2). Similar to ADP, tissue AMP levels were notably higher in winter than in summer oysters at 20°C, and this difference was significant in control and Cd-exposed groups ($P<0.05$). Acute temperature rise had no effect on tissue levels of ADP in winter oysters, but induced a significant decline in AMP levels in summer in Cd-exposed oysters at 24°C and above, and in control oysters – at 36°C (Fig. 8).

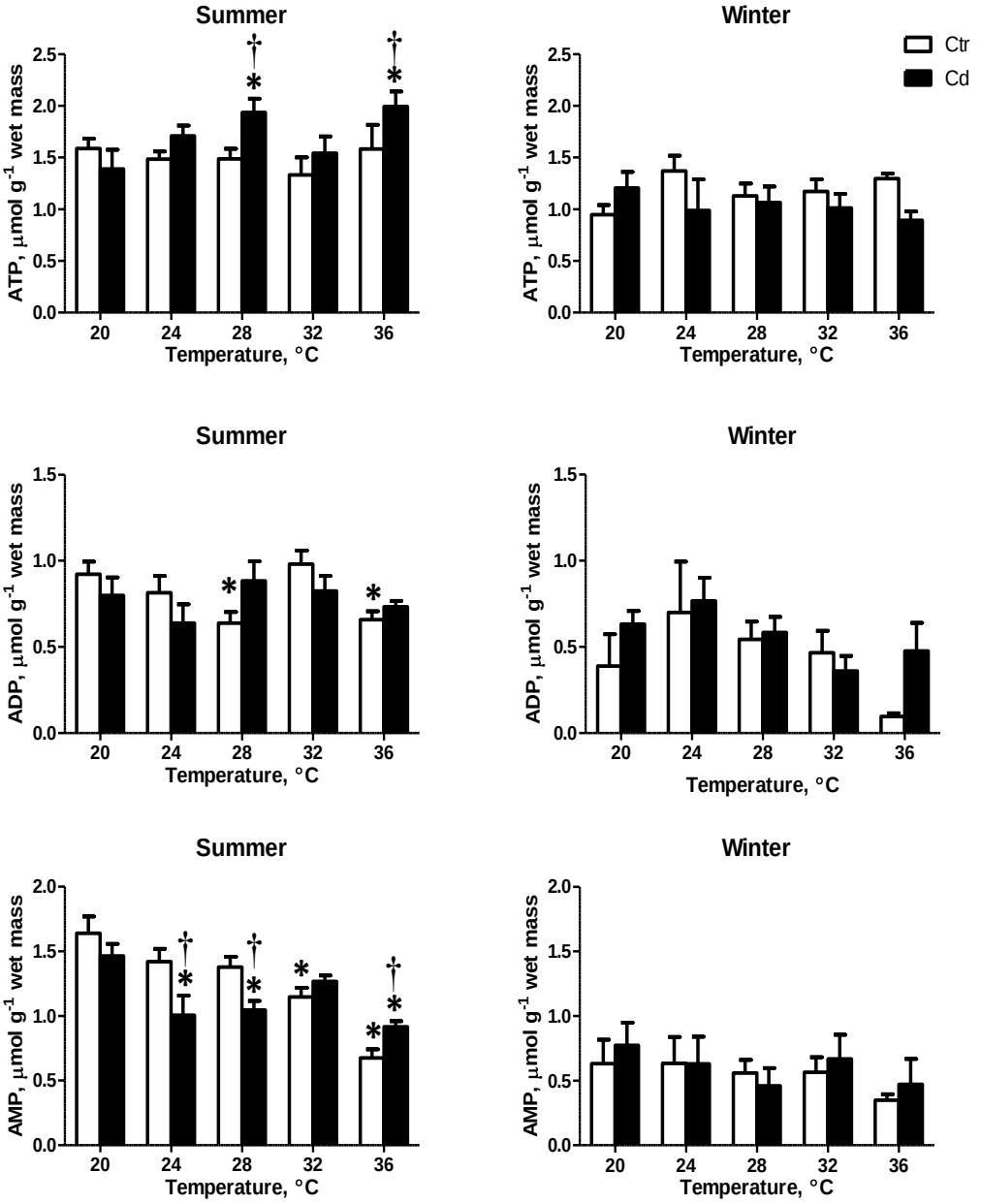

FIGURE 8: Effects of acute warming on the levels of adenylates (ATP, ADP & AMP) in the gills of eastern oysters, *C.virginica*. ATP (A & B), ADP (C & D) and AMP (E & F) in gills of control (Ctr) and Cd-exposed (Cd) during summer and winter seasons. Asterisks indicate values significantly different from the respective groups of control and Cd-exposed oysters at 20°C ($P<0.05$). Daggers show the values that differ between control and Cd-exposed oysters ($P<0.05$). Summer: n = 9-13; winter: n = 6-8 except at 36°C where n = 2.

3.3.2 Adenylate energy-related indices:

Energy-related indices (AEC, TANP & ADP/ATP ratio) in gill tissue of oysters were calculated to assess the cellular energy status of the oysters exposed to Cd and temperature stress in summer and winter seasons. Adenylate energy charge (AEC) was significantly affected by season, temperature and interaction of season and Cd exposure (Table 3). Winter control and Cd-exposed oysters had higher AEC than their summer counterparts when compared at the acclimation temperature of 20°C ($P<0.05$). AEC tended to increase slightly during the acute temperature rise, likely reflecting a decline in ADP and AMP levels, and this increase was significant in summer and winter control oysters at 36°C and in summer Cd-exposed oysters at 24°C and above (Fig. 9A & B).

The total levels of adenylates (TANP) in oyster gills were significantly affected by the season but not temperature of Cd exposure (Table 4). TANP was significantly higher in summer than in winter in control and Cd-exposed groups at the acclimation temperature of 20°C ($P<0.05$) and remained stable during the acute temperature rise except the summer control oysters where a slight but significant decline was seen at 28°C and above (Fig. 9 C & D).

ADP/ATP ratio was significantly affected by the interaction among the factors ‘Season’ x ‘Temperature’ and ‘Season’ x ‘Cd exposure’ (Table 3). Acute temperature rise had no effect on ADP/ATP ratios in any of the experimental groups (Fig. 9E & F).

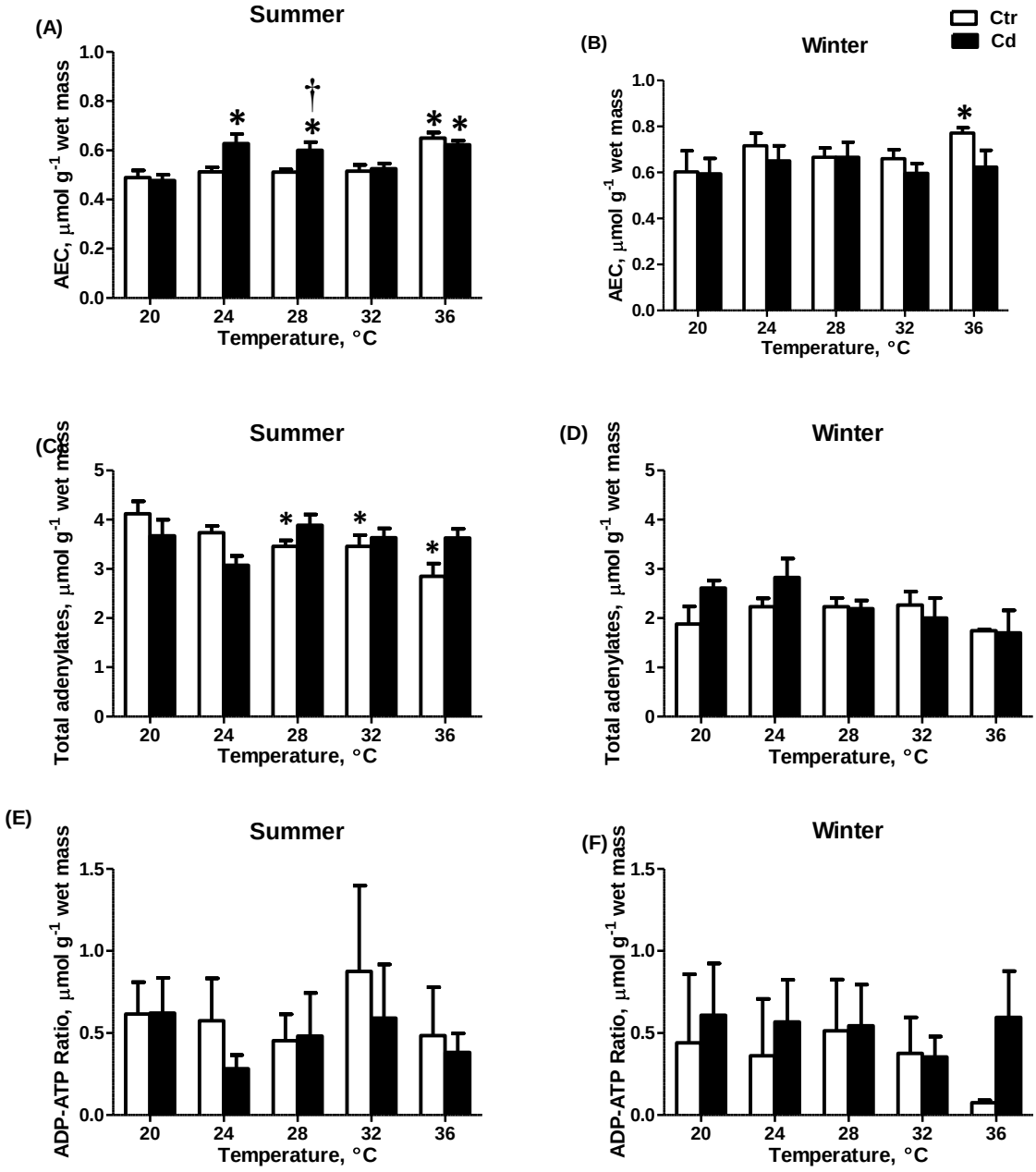

FIGURE 9: Effect of of acute warming on the energy related indices including AEC (A & B), total adenylates (C & D) and ADP/ATP ratio (E & F) in the gills of control (Ctr) and Cd-exposed (Cd) oyster, *C.virginica* during summer and winter seasons. Asterisks indicate values that are significantly different from the respective groups of control and Cd-exposed oysters at 20°C ($P<0.05$). Daggers show the values that differ between control and Cd-exposed oysters at the same temperature ($P<0.05$). Summer: n = 7-16, winter: n = 3-8 except at 36°C where n = 2.

3.3.3 Phosphagens:

PLA levels were significantly affected by the season and the interaction of season and Cd exposure (Table 4). PLA levels were considerably higher in winter oysters compared to their summer counterparts ($P<0.05$ at the acclimation temperature of 20°C). Acute temperature rise had no effect on the tissue PLA levels in any of the experimental groups (Fig. 10A & B). In contrast, L-arginine levels were significantly affected by Cd exposure, season and the interactions 'Cd exposure' x 'Season' and 'Cd exposure' x 'Temperature' (Table 4). L-arginine levels were similar in winter and summer oysters at the acclimation temperature ($P>0.05$). In summer, L-arginine tended to accumulate with increasing temperatures but this trend was only significant in control oysters at 28°C and above (Fig. 10C). No temperature-induced changes in tissue L-arginine content were seen in winter acclimatized oysters (Fig. 10D).

The total amount of phosphagen and aphosphagen ([PLA] + [L-Arginine]) was considerably higher in winter oysters compared to their summer counterparts and did not change in response to the acute temperature rise (Table 5, Fig. 11). The relative proportion of phosphagen in the total phosphagen/aphosphagen pool (R_{PLA}) was also significantly higher in the winter oysters compared to the summer-acclimatized ones ($P<0.05$). In winter, acute temperature rise had no effect on R_{PLA} (Fig. 11). In summer, acute warming led to a decrease in R_{PLA} in control oysters but not in their Cd-exposed counterparts (Fig. 11).

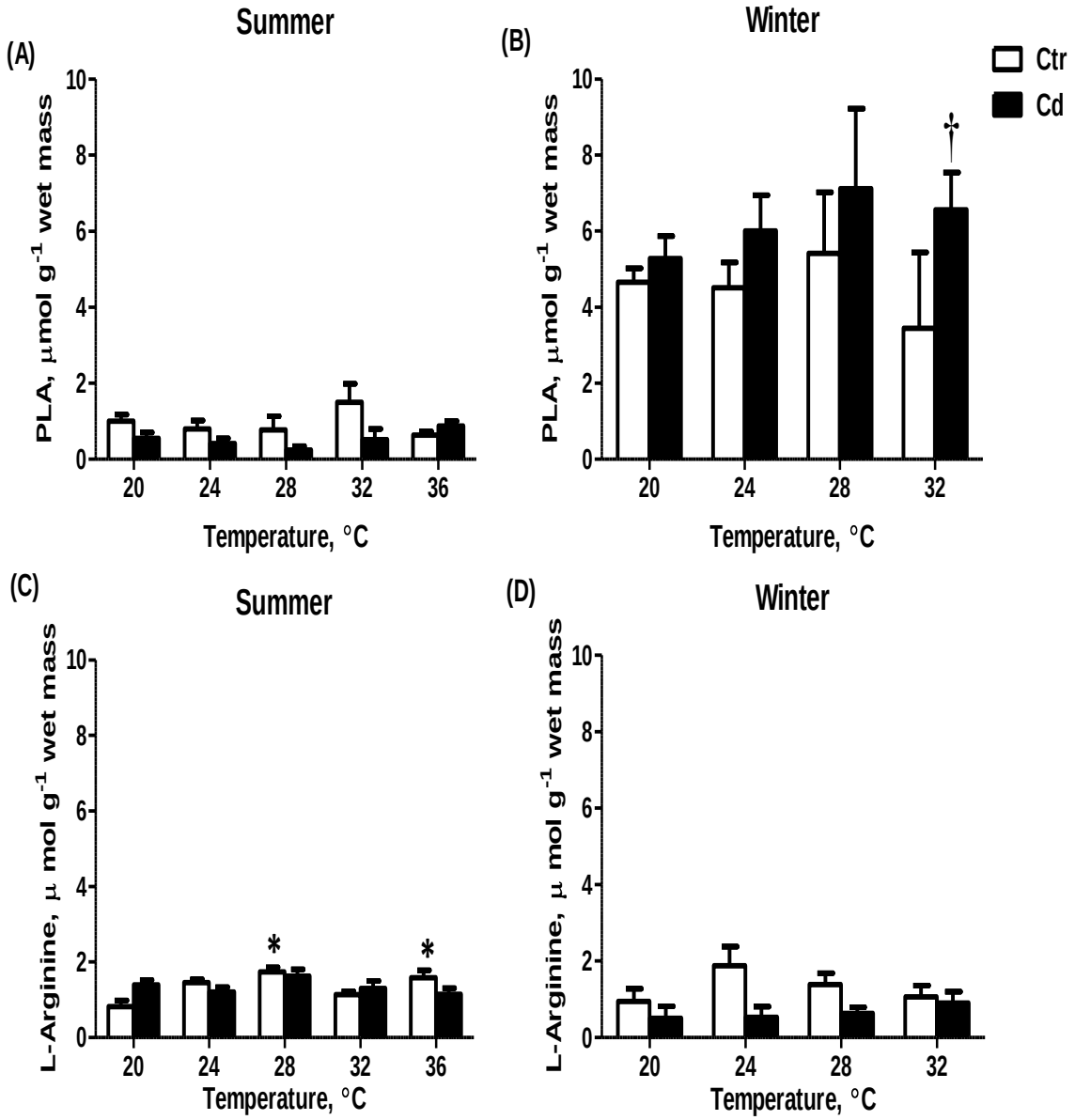

FIGURE 10: Effect of of acute warming on the levels of phospho-L-arginine (PLA) (A & B) and L-arginine (C & D) in the gills of control (Ctr) and Cd-exposed (Cd) eastern oysters, *C.virginica* during summer (A & C) and winter (B & D) seasons. Asterisks indicate values that are significantly different from the respective groups of control and Cd-exposed oysters at 20°C ($P<0.05$). Daggers show the values that differ between control and Cd-exposed oysters at the same temperatures ($P<0.05$). Summer: n = 7-8; winter: n = 4-8.

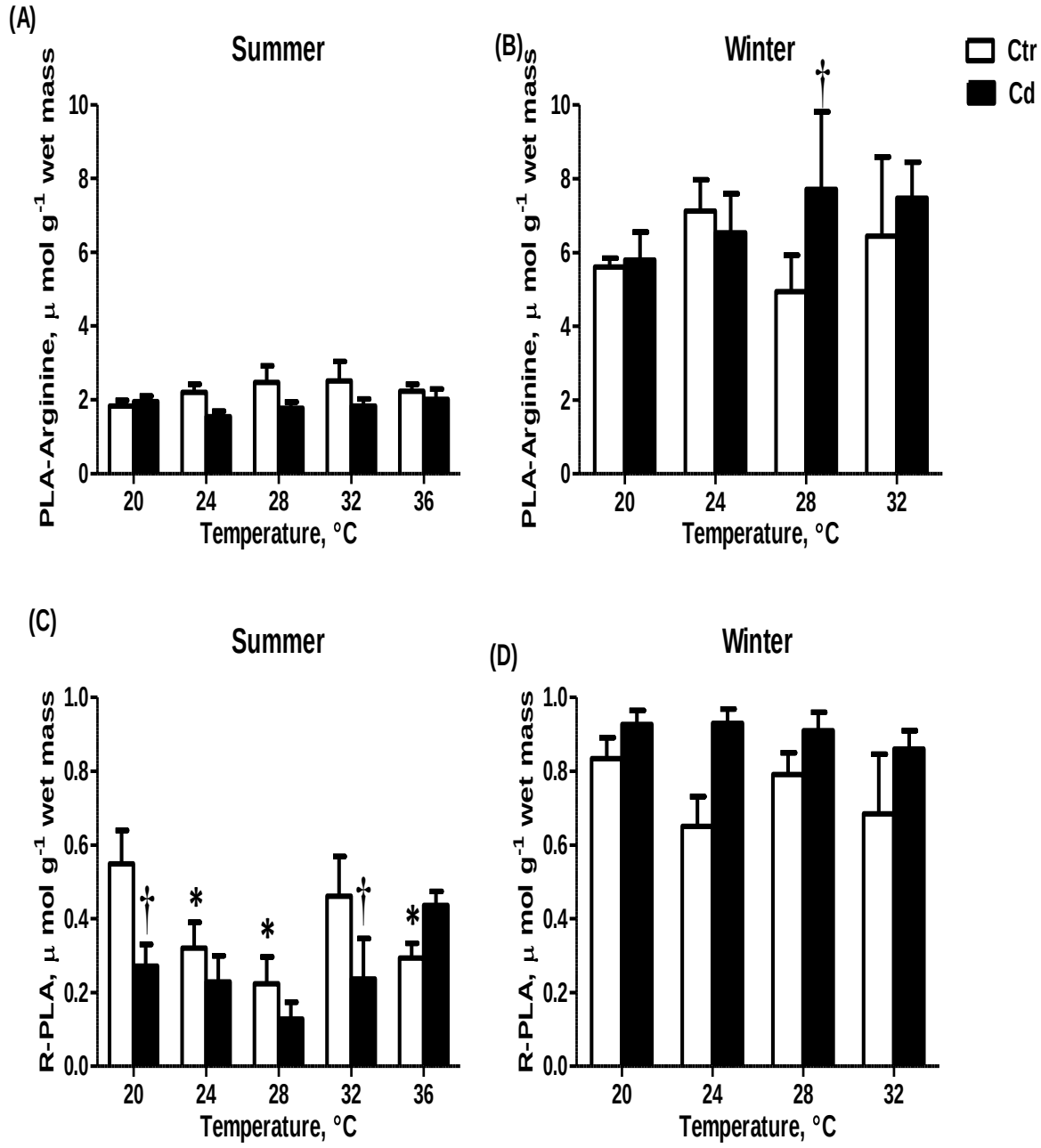

FIGURE 11: Effect of of acute warming on the total levels of phosphagen/aphosphagen (A & B) and R_{PLA} (C & D) in the gills of control (Ctr) and Cd-exposed (Cd) eastern oysters, *C.virginica* during summer (A & C) and winter (B & D) seasons. Asterisks indicate values significantly different from the respective groups of control and Cd-exposed oysters at 20°C ($P<0.05$). Daggers show the values that differ between control and Cd-exposed oysters at the same temperatures ($P<0.05$). Summer: n = 7-8; winter: n = 4-8.

3.4 Tissue energy stores:

3.4.1 Glycogen and glucose:

Glycogen and free glucose levels were measured in different oyster tissues (gills, hepatopancreas and muscle) at three selected temperature points 20, 28 & 36°C . Factors 'Season', 'Tissue' and their interaction significantly affected glycogen levels in oysters (Table 6). Overall, tissue glycogen stores were the highest in the gill compared to other tissues in summer oysters ($P<0.05$) but not in their winter counterparts. Acute temperature rise had no effect on tissue glycogen stores in gills of winter or summer oysters (Fig. 12A & B) ($P>0.05$). In hepatopancreas, there was a significant transient increase in the glycogen stores at 28°C in control and Cd-exposed oysters in summer ($P<0.05$). This increase was not seen in winter oysters where glycogen content of the hepatopancreas remained stable at all studied temperatures (Fig 12C & D). In the muscle, acute temperature rise did not affect the glycogen levels except a small but significant decrease in winter-acclimatized control oysters at 36°C ($P<0.05$) (Fig. 12E & F).

Tissue glucose levels in oysters were significantly affected by the factors 'Cd exposure', 'Tissue' and the interaction between temperature, Cd exposure and season (Table 7). Tissue glucose content was generally low (<1 μmol g^{-1} wet mass) and tended to be higher in Cd-exposed oysters compared to their control counterparts in the muscle and hepatopancreas but not in the gills. Acute temperature rise did not affect tissue levels of free glucose in any of the experimental groups (Fig. 13).

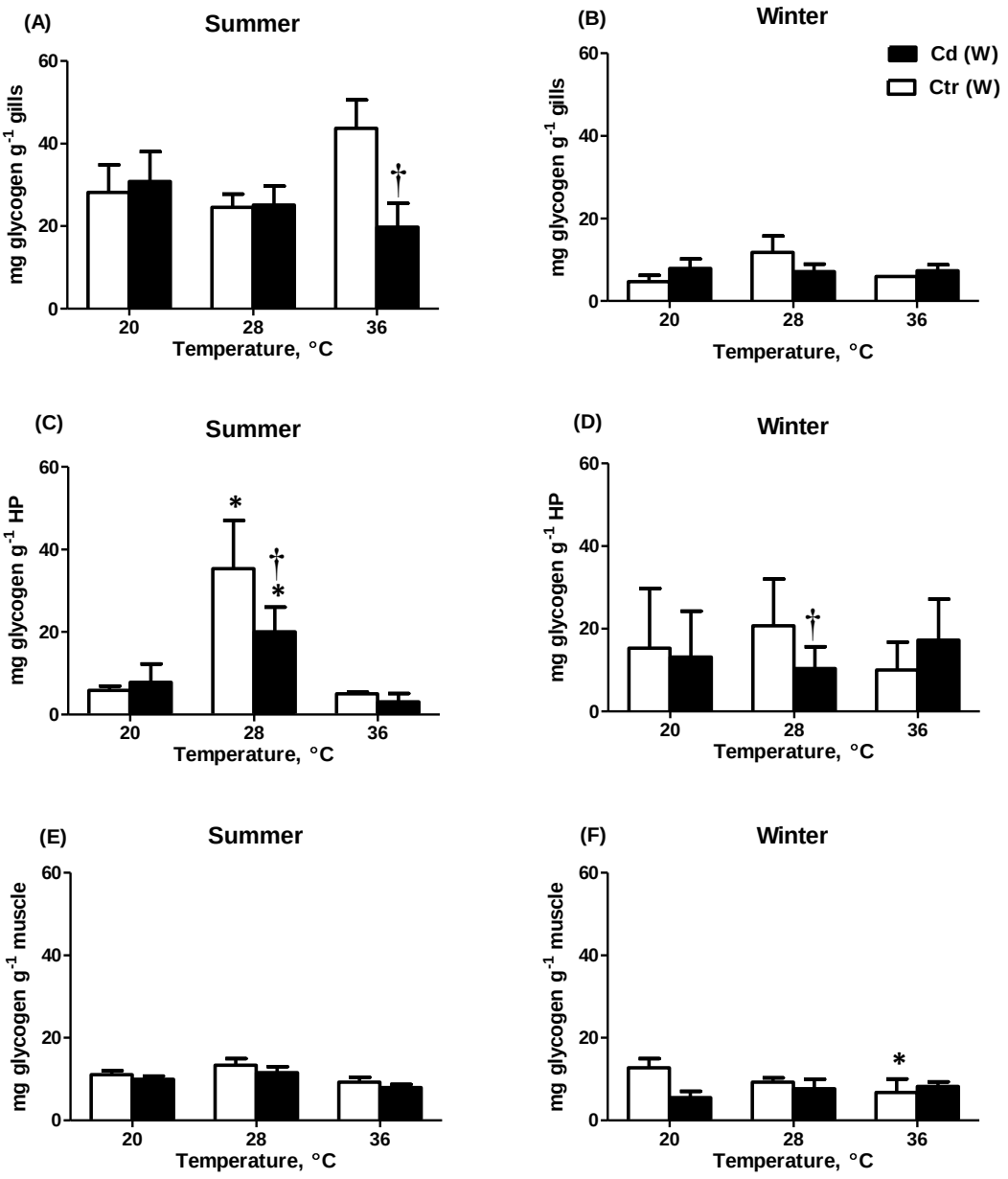

FIGURE 12: Effects of acute warming on glycogen stores in gills (A & B), hepatopancreas (C & D) and muscle (E & F) of control (Ctr) and Cd-exposed (Cd) eastern oysters, *C.virginica* during summer and winter seasons. Asterisks indicate values significantly different from the respective groups of control or Cd-exposed oysters at 20°C ($P<0.05$). Daggers show the values that differ between control and Cd-exposed oysters at the same temperatures ($P<0.05$). Summer: n = 3 -14 except hepatopancreas at 36°C where n = 2; winter n = 3-8 except gills at 36°C where n = 1.

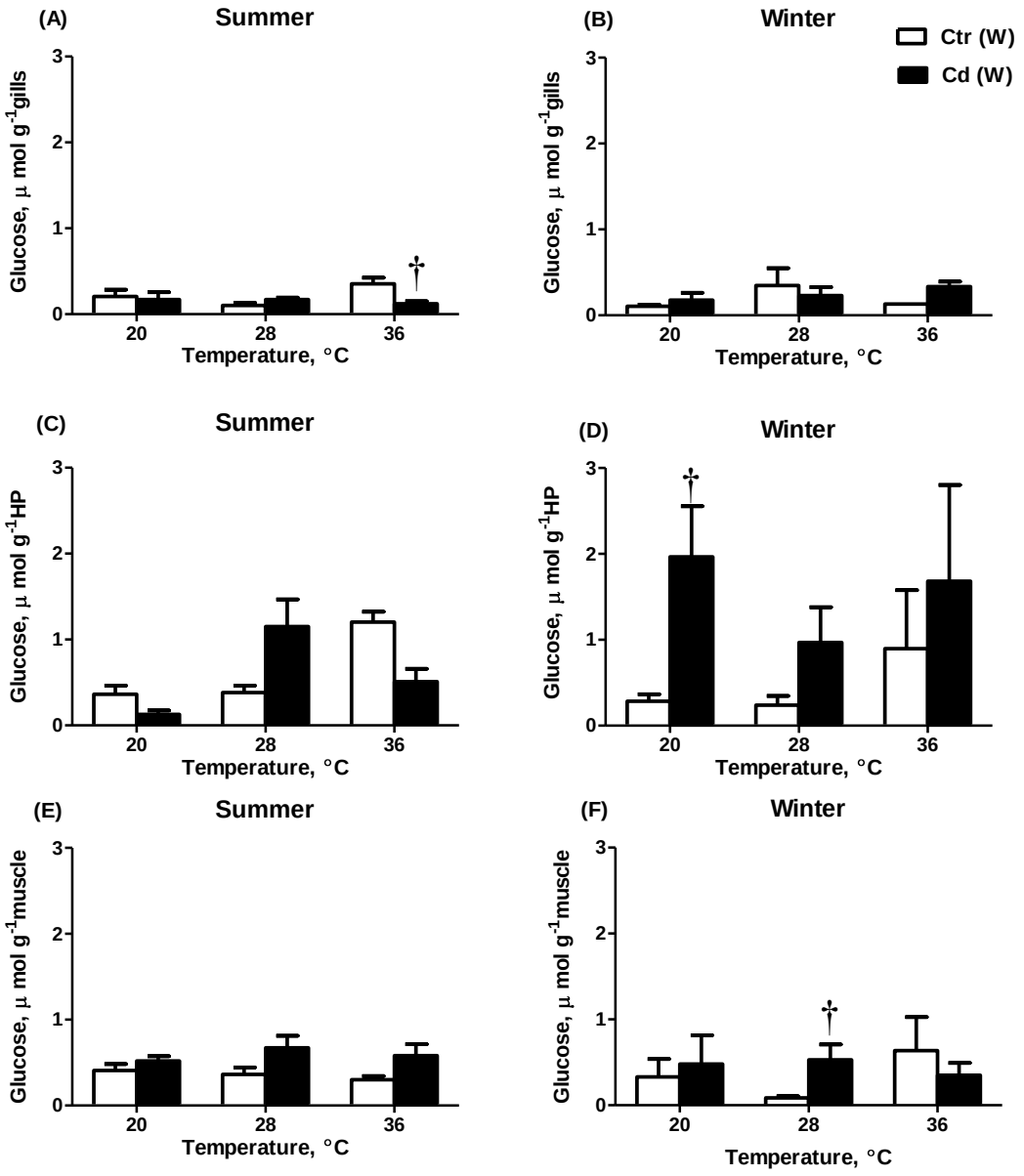

FIGURE 13: Effects of acute warming on free glucose levels in gills (A & B), hepatopancreas (C & D) and muscle (E & F) at the experimental temperatures in the gills of control (Ctr) and Cd-exposed (Cd) eastern oysters, *C.virginica* during summer and winter seasons. Asterisks indicate values significantly different from the respective groups of control and Cd-exposed oysters at 20°C ($P<0.05$). Daggers show the values that differ between control and Cd-exposed oysters ($P<0.05$). Summer: n = 3 -15; winter n = 3-8 except gills at 36°C where n = 1.

3.4.2 Lipids:

Lipid levels in oyster tissues were significantly affected by the tissue type, season and interactions between tissue and season, and tissue and experimental temperature (Table 8). At the acclimation temperature (20°C) tissue lipid levels were similar in control and Cd-exposed oysters in summer and in winter ($P>0.05$) (Fig. 14). However, the response to the acute temperature rise differed between the winter and summer oysters (Fig. 14). In summer, acute warming led to an increase in tissue lipid content of control and Cd-exposed oysters which peaked at the intermediate temperature (28°C) (Fig. 14). In winter, temperature had no effect on the tissue lipid content (Fig. 14).

3.4.3 Proteins:

Protein content of oyster tissues was significantly affected by the tissue type, experimental temperature and interactions between season and experimental temperature (Table 9). Overall, tissue protein levels were similar in control and Cd-exposed oysters in summer and in winter when determined at the acclimation temperature (20°C) ($P>0.05$) (Fig. 15). Similar to lipids, protein content in tissues of summer oysters showed a transient increase at 28°C, while in their tissue counterparts acute warming had no effect on the protein content except for an increase at 36°C found only in hepatopancreas of Cd-exposed winter oysters (Fig. 15).

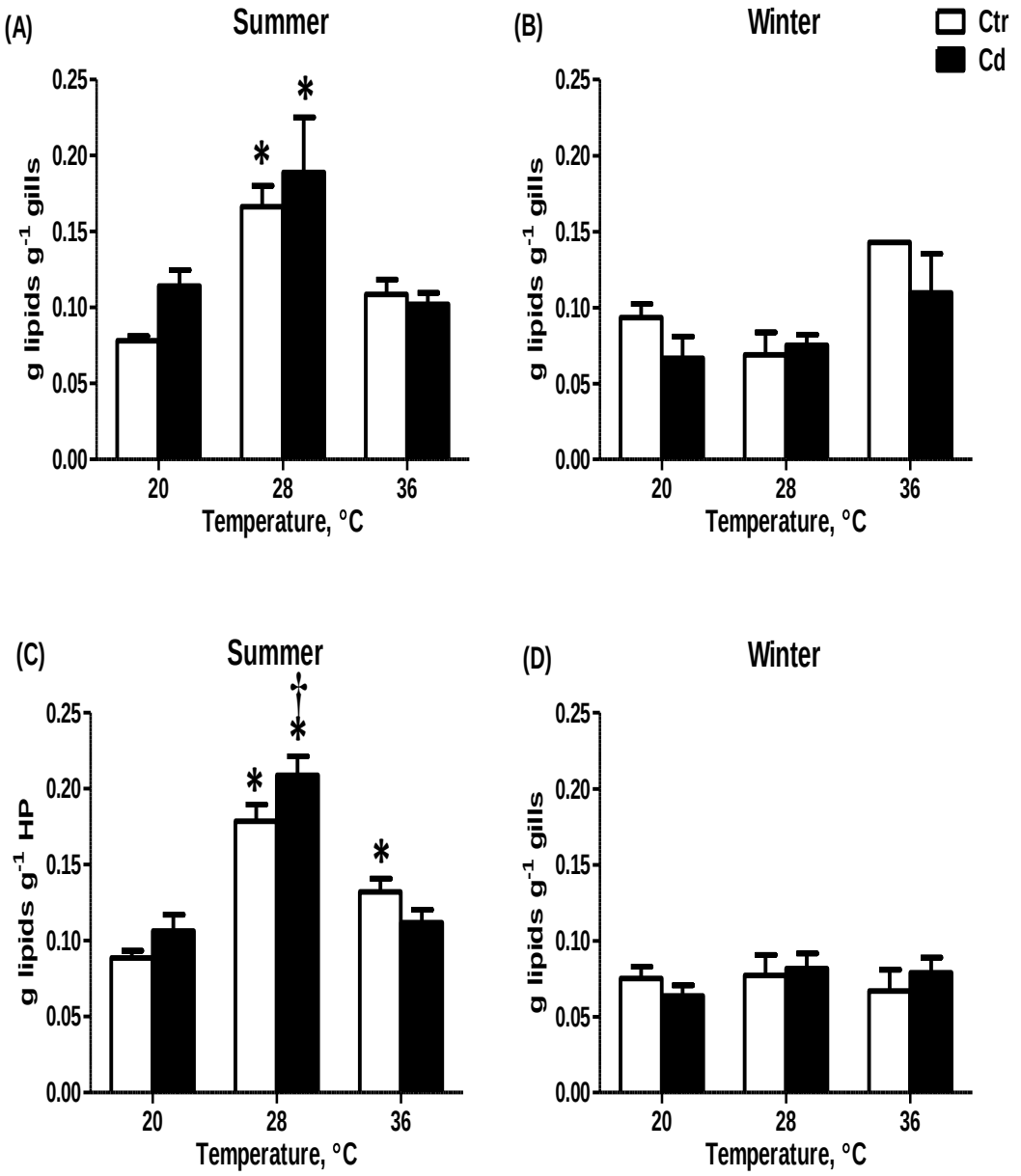

FIGURE 14: Temperature-induced changes in lipid stores in gills (A & B) and muscle (C & D) of control (Ctr) and Cd-exposed (Cd) eastern oysters, *C.virginica* in summer and winter seasons. Asterisks indicate values that are significantly different from the respective groups at 20°C ($P<0.05$). Daggers show the values that differ between control and Cd-exposed oysters at the same temperature ($P<0.05$). Summer: n = 7-8; winter n = 3-8 except at 36°C where n = 1.

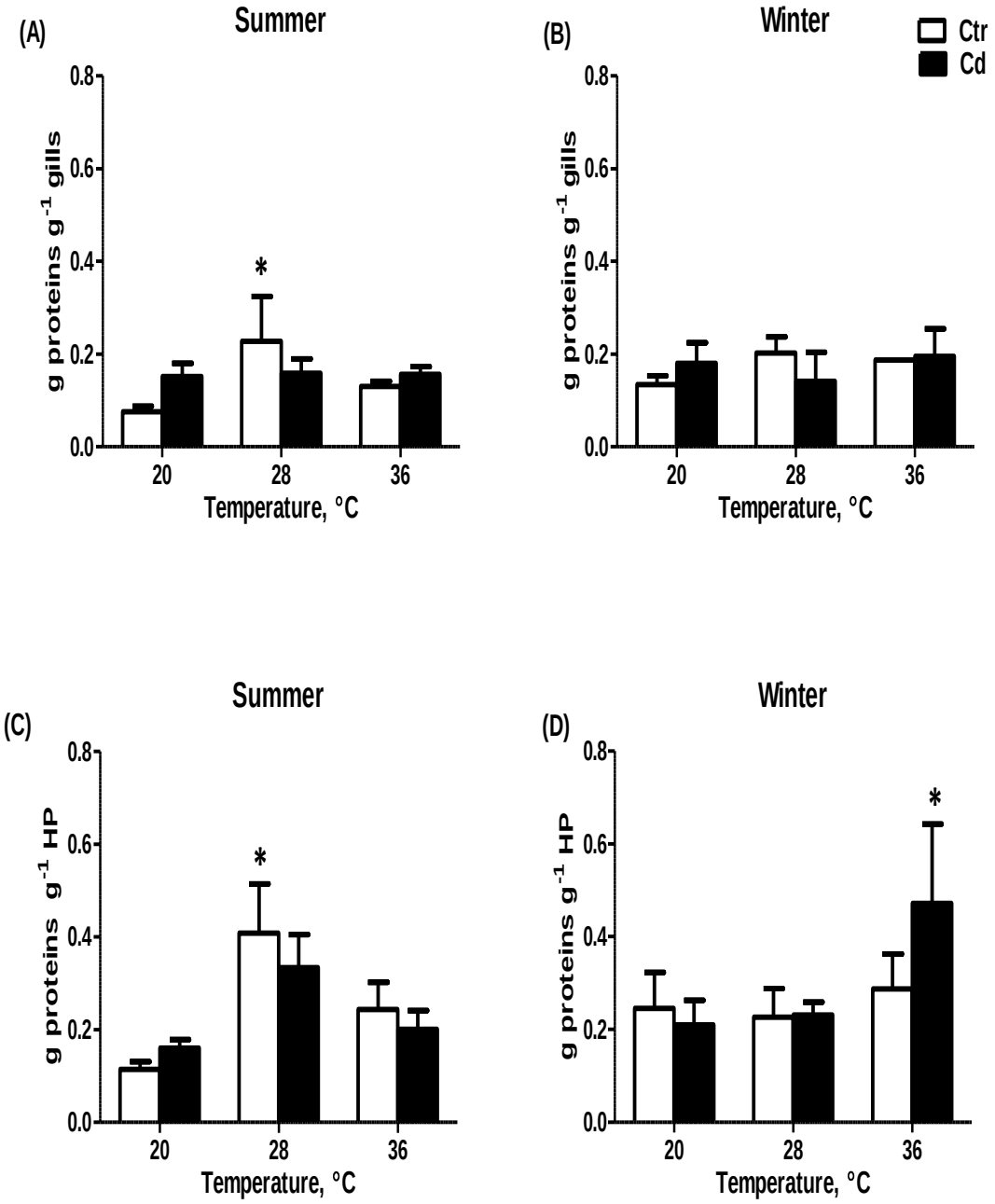

FIGURE 15: Temperature-induced changes in protein content in gills (A & B) and HP (C & D) in control (Ctr) and Cd-exposed (Cd) eastern oysters, *C.virginica* during summer and winter seasons. Asterisks indicate values that are significantly different from the respective groups at 20°C ($P<0.05$). Daggers show the values that differ between control and Cd-exposed oysters at the same temperature ($P<0.05$). Summer n = 6-9; winter n = 3-8 except at 36°C where n = 1.

3.5 Oxidative stress:

The two studied markers of oxidative damage (protein carbonyls and MDA) showed different responses to acute temperature stress and Cd exposure in oysters. MDA levels in oyster mantle were significantly affected by Cd exposure and season, and their interactions, but not the experimental temperature (Table 10). In contrast, carbonyl content of the mantle tissue was affected by season and experimental temperature and their interactions, but not by Cd exposure (Table 10). Overall, the levels of the oxidative stress biomarkers were higher in winter oysters compared to their summer counterparts (Fig. 16). Acute temperature rise did not affect tissue MDA content of oysters regardless of the season or Cd exposure (Fig. 16). In contrast, mantle carbonyl content increased with increasing temperatures in winter oysters; this increase was significant at 28°C and above in control oysters and at 36°C in the Cd-exposed group (Fig. 16). In summer, acute temperature rise did not affect the tissue carbonyl content in control or Cd-exposed oysters (Fig. 16).

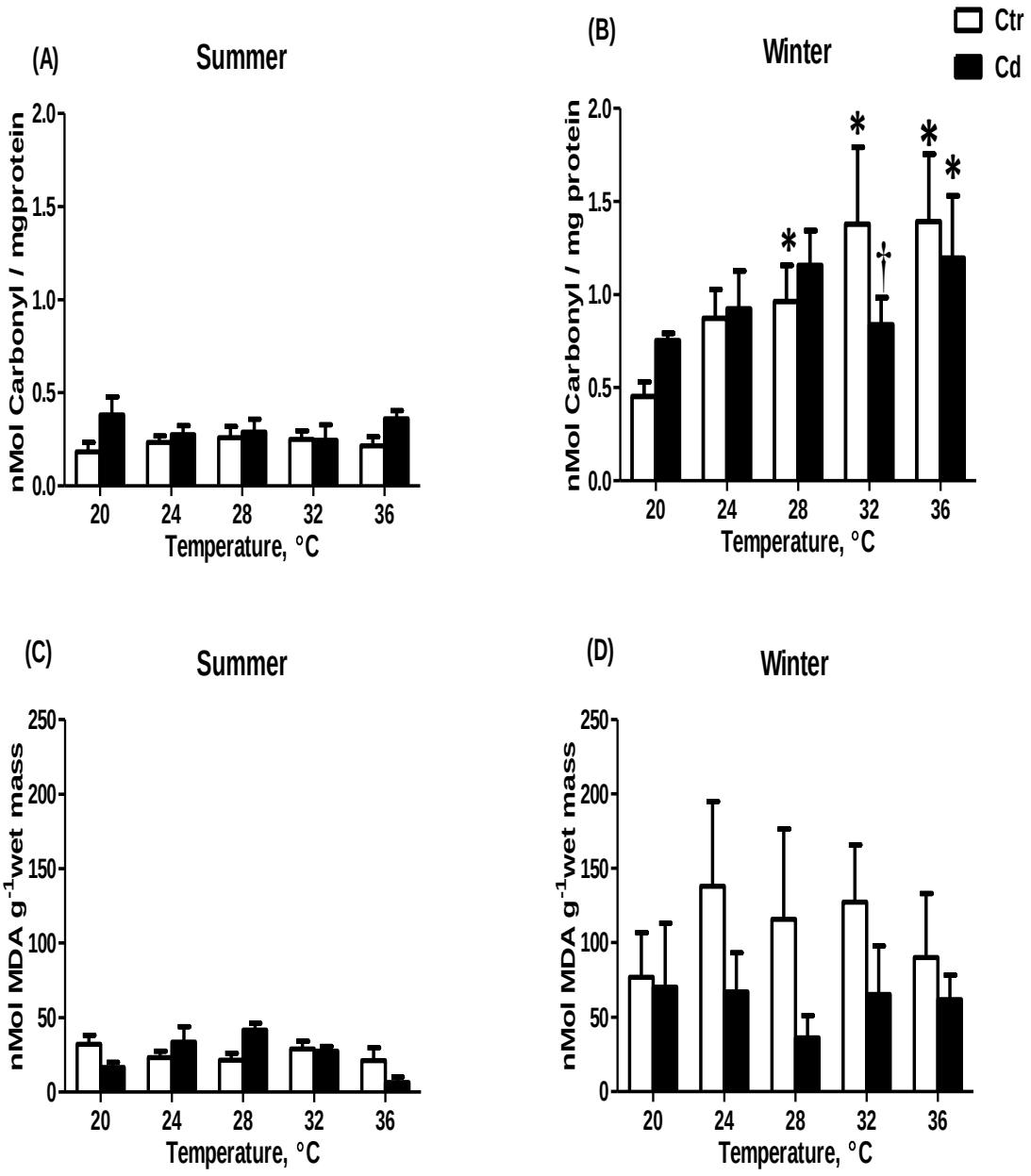

FIGURE 16: Effect of Cd exposure and acute temperature rise on the levels of protein carbonyls (A & B) and malondialdehyde (C & D) in the mantle in control (Ctr) and Cd-exposed (Cd) eastern oysters, *C.virginica* during summer and winter seasons. Asterisks indicate values that are significantly different from the respective groups at 20°C ($P<0.05$). Daggers show the values that differ between control and Cd-exposed oysters at the same temperature ($P<0.05$). Summer: n = 8-12; winter: n = 8 except the control oysters at 36°C where n = 3.

CHAPTER 4: DISCUSSION

4.1 Effects of Cd exposure and seasonality on the critical temperatures of aerobic metabolism in oysters:

According to the concept of oxygen and capacity-limited thermal tolerance, acute temperature rise leads to the reduction of the aerobic scope in aquatic ectotherms and eventually its loss beyond the lower and upper Tc (*90-94*). Critical temperatures of aerobic scope (Tc) in aquatic ectotherms including oysters can be determined by the transition to partial anaerobiosis and resultant accumulation of anaerobic end products. In invertebrates and fish, the temperature-induced anaerobiosis occurs even in fully aerated water and indicates a time limited survival at and beyond Tc (*24*). Notably, oxygen limitation sets in prior to functional failure of the mitochondria which develops beyond the Tc (*24*). Earlier studies showed that mitochondrial enzymes and isolated mitochondria of oysters and other bivalves have high thermal tolerance and can function at temperatures high temperatures well beyond Tc (*90, 95*). This indicates that mitochondrial dysfunction is not a likely explanation for the onset of anaerobiosis at Tc. In contrast, the functional limitations of the oxygen uptake and delivery systems coincide with Tc and transition to the pessiumum range of environmental temperatures. Thus, in a marine worm *Sipunculus nudus* a decrease in ventilatory performance and oxygen tension (PO_2) in coelomic fluid occurred before anaerobiosis sets in (*24*). Similarly, in the spider crab *Maja squintado* a decrease in ventilatory, circulatory and PO_2 levels preceded the

onset of anaerobiosis at Tc (*24*). Therefore, the onset of anaerobiosis at Tc is likely due to the insufficient capacity of the ventilatory and circulatory systems to deliver oxygen to the tissues.

In the present study, we found accumulation of the cytosolic anaerobic end product (L-alanine) and mitochondrial anaerobic end products (acetate and succinate) in the gills of oysters (*C.virginica)* indicative of the temperature-induced transition to anaerobiosis. In the summer control oysters, L-alanine was the first anaerobic end product to accumulate at the temperature of 28°C and beyond indicating the Tc is close to 28°C in this group. This is consistent with the earlier studies in mollusks that showed that cytosolic L-alanine is an early anaerobic end product and the onset of the mitochondrial anaerobiosis follows at the latter stages (*77, 96*). L-alanine accumulation indicates that cytosolic anaerobic pathways become engaged in ATP production at temperatures of 28°C and higher in summer control oysters. No accumulation of mitochondrial anaerobic end products (succinate and acetate) was seen at any of the experimental temperatures in control oysters in summer suggesting that the mitochondrial anaerobiosis was very low or absent. Earlier studies have shown that *C.virginica* has an optimal range of aerobic scope between 20°C and 24°C, and 28°C represents a stressful temperature that may be close to the pessimum range (*23*). At 28°C and above, oysters enter the "no scope for growth" area as they do not deposit shell material and are not capable of withstanding sublethal Cd stress (*23*). There is a concomitant decrease in mitochondrial density at 28°C suggesting a decrease in aerobic capacity of oysters (*23, 63*). These findings agree with the critical temperatures of 28°C found in the present study and indicate that *C. virginica*

may be unable to survive in the environments where summer temperatures exceed 28°C for a prolonged period of time.

Winter acclimatization resulted in a shift of Tc to the lower values as indicated by an earlier onset of anaerobiosis at 24°C in winter control oysters compared to 28°C in their summer counterparts. Notably, in winter control oysters both L-alanine and acetate accumulated at the temperatures of 24°C and above. Acetate accumulation indicates that in winter, acute temperature rise leads to an onset of mitochondrial anaerobiosis in addition to engaging of the cytosolic anaerobic pathways. Overall, our data show that aerobic metabolism of winter season acclimatized control oysters is more sensitive to warming than their summer counterparts and winter acclimatized oysters reach Tc at lower temperatures.

A downward shift of Tc in winter oysters is consistent with the previously described downward shifts in Tc in cold-acclimated or cold-adapted ectotherms. Thus, in a marine lugworm *Arenicola marina* the Tc shifts to lower temperatures and the thermal tolerance window narrows in winter compared to summer, in parallel with an increase in mitochondrial densities in winter worms (*91*). Both low and high Tc values were lower in a cold adapted, sub-Arctic population of *A. marina* from the White Sea compared to their temperate North Sea counterparts (*91*). Similarly, the upper Tc were considerably higher in the eelpout from the temperate North Sea compared to the Antarctic (23°C and 9°C, respectively) (*97*). In an intertidal gastropod *Littorina saxatilis*, Tc shifts were also induced by the acclimation temperatures; the Tc for warm acclimated (13°C) *L. saxatilis* were 28°C and 32°C for the snails from North Sea and White Sea populations, respectively, while in their cold acclimated (4°C) counterparts the Tc shifted to 18°C and

28°C, respectively (*90*). Notably, *Littorina saxatilis* from the North Sea and White Sea populations had different Tc even at the same acclimation temperature indicating long-lasting (possibly genetic) differences in metabolic physiology.

It is worth noting that in our present study both summer and winter oysters were acclimated in the laboratory at 20°C for 3-4 weeks prior to the determination of Tc which is considered sufficient for full thermal acclimation of these organisms (Sokolova, personal communication). Despite the similar acclimation temperatures, upper Tc was lower in winter oysters compared to their summer counterparts. This suggests some seasonal differences in physiology of oysters that can affect their aerobic scope (such as differences in the reproductive status or mitochondrial capacity) are not fully overcome by the prolonged thermal acclimation. Lower Tc in winter oysters may be due to the persistent differences in the mitochondrial density and capacity despite the prolonged acclimation at the same temperature. Earlier studies have shown that seasonal variation has a strong effect on the mitochondrial densities and functions in *C.virginica* (*24, 98*). In summer the oyster gills have reduced mitochondrial densities as compared to their winter counterparts (*98*). Increased mitochondrial density in winter increases the aerobic capacity and compensates for lower enzymatic rates and slower rates of oxygen diffusion in winter but it is also accompanied by an increase in the amount of energy spent to prevent proton leakage (*98*). This elevated energy demand to counteract the mitochondrial proton leak can lower the upper Tc (*24*). In contrast, in summer, a reduction in the mitochondrial density leads to reduction in baseline oxygen demand, thereby allowing the upper critical and pejus temperature to shift to higher values (*24*). Future studies are needed to determine whether these differences in the mitochondrial

density between summer and winter oysters are sustained during the long-term laboratory acclimation. However, differences in the concentrations of PLA, adenylates, glycogen and lipid content between winter and summer oysters found in this study are consistent with the hypothesis that seasonal differences in energy metabolism persist despite the similar thermal acclimation regime.

Cd exposure affected Tc in oysters although these effects were different in summer and winter. Cd exposure in summer oysters led to a downward shift of Tc to 24°C as shown by the significant accumulation of L-alanine. Notably, similar to the control summer oysters, there was no accumulation of acetate or succinate during the acute warming in Cd-exposed oysters indicating that mitochondrial anaerobic pathways are not involved at Tc in summer. The downward shift of Tc in Cd-exposed oysters in summer is consistent with an earlier onset of temperature-induced tissue hypoxemia in Cd-exposed oysters (*23*) and indicates that Cd can sensitize oysters to acute temperature rise during the summer months causing a decrease in the thermal tolerance and the aerobic scope. In winter Cd-exposed oysters Tc could not be determined based on accumulation of the anaerobic end products. The inability of winter Cd-exposed oysters to switch over to anaerobiosis may be indicative that some key anaerobic pathways are inhibited by Cd (*77*). However, this hypothesis is contradicted by our finding that in summer, Cd-exposed oysters transition to partial anaerobiosis at the temperatures of 24°C and above. Alternatively, the absence of temperature-induced accumulation of anaerobic end products in Cd-exposed oysters in winter may indicate that in the winter Cd-exposed oysters Tc may be reached earlier (at or below 20°C) than the experimental temperatures

of this study. This is consistent with the elevated levels of L-alanine and acetate in winter Cd-exposed oysters at the acclimation temperature (20°C).

A downward shift of Tc in Cd-exposed oysters found in summer indicates that Cd exposure reduces the aerobic scope of oysters. This may be due to the metal-induced increase in the basal cost of maintenance, impaired oxygen supply to the tissues, interference of Cd with the mitochondrial function or combination of these factors (*7, 28, 78, 99*). Earlier studies also showed that oysters exposed to Cd have elevated standard metabolic rate (SMR) due to increase in the cost of energy spent on the synthesis of protective proteins such as HSPs, MT and antioxidants (*7, 78, 100-101*). Cd exposure and elevated temperatures also resulted in elevated energy (oxygen) demand and increased ventilating activity in oysters (*23, 102*). Despite the high ventilation activity, increasing temperatures led to a progressive decrease in hemolymph PO_2 in Cd-exposed oyster at 24°C and 28°C compared to their control counterparts (*78*). Cd exposure did not affect heart rate in oysters in the temperature range between 20 and 28°C suggesting that oysters cannot compensate for elevated oxygen demand by upregulating the cardiac function (*78*). Notably, the cardiac hemolymph flow in the atrium of the heart was affected in control and Cd-exposed oysters at 28°C suggesting that elevated temperature result in impaired circulation (*78*).

These data indicate the impairment of ventilatory and/or circulatory system of oysters by Cd, especially at elevated temperatures, and are consistent with earlier studies in aquatic ectotherms that show high sensitivity of ventilatory and circulatory systems to Cd. Gills are particularly sensitive to waterborne Cd because of their larger surface area to facilitate gas and ion exchange and is also a major site of metal uptake in aquatic

animals (*78, 98*). Moreover, gills can be slow in producing protective proteins such as MT and thus Cd exposure can result in a greater cellular damage to the gills compared to other tissues (*78*). Thus, in crabs, *Callinectes similis* exposure to Cd resulted in decrease in the percentage of oxygen extraction and lower efficiency of O_2 uptake at the respiratory surfaces (*103*). Cd exposure also suppressed the oxygen carrying capacity of hemolymph in dogwhelks, *Nucella lapillus* (*104*).

Metal-induced onset of partial anaerobiosis has also been documented in other aquatic ectotherms. In a carp, *Cyprinus carpio* exposure to sublethal and lethal Cd led to the onset of partial anaerobiosis as indicated by the upregulation of lactate dehydrogenase and accumulation of anaerobic end products (pyruvate and lactate) (*105*). At lethal Cd levels transition to anaerobiosis was due to the gill damage whereas at sublethal Cd levels, limited aerobic capacity and elevated energy demands due to cellular detoxification and repairs contributed to the onset of anaerobiosis (*105*). In crabs *Scylla serrata,* Cd exposure resulted in onset of partial anaerobiosis indicated by lactate accumulation which was proposed to reflect the impairment of mitochondrial enzymes (*105-106*). Similar transition to partial anaerobiosis in response to metal exposure was seen in yellow perch *Perca flavescens* and tilapia *Oreochromis mossambicus* (*107-108*). Exposure to Cd or chromium (Cr) in marine prawn *Macrobrachium rosenbergii* and in stonefly nymph *Clioperla clio* reduced the CTmax (critical thermal maximum of the onset of the neural damage) and decreased O_2 concentrations indicating disruption of energy metabolism (*109-110*). Similar decrease in CT_{max} was seen in coho salmon *Oncorhynchus kisutch* and steelhead trout *Salmo gairdneri* on exposure to Ni and in muskellunge fry *Esox masquinongy* on exposure to arsenic (*7, 111*). These data are

consistent with our finding of an earlier onset of temperature-induced anaerobiosis and the narrowed window of thermal tolerance in Cd-exposed oysters found in summer.

4.2 Bioenergetics status in oysters during acute temperature rise and Cd exposure:

Tissue levels of adenylates and phosphagens, energy stores (lipids and glycogen) and energy-related indices such as AEC provide important indications of the energy levels in an organism and the potential stress-induced disturbance of energy balance. Adenylate energy charge (AEC) is a common parameter used to assess the effect of environmental perturbation on the physiological status of an organism and to measure the stress-induced energy deficiency (*89, 112-113*). It can vary from 0 to 1 and correlates with the potential energy available for the cell metabolism (*112*).

In our present study, acute temperature rise did not reduce tissue levels of high energy phosphates (ATP and PLA), adenylate energy charge (AEC) or ADP/ATP ratio in the gills of summer control oysters despite their transition to partial anaerobiosis at and above 28°C. There was decline in ADP and AMP levels at elevated temperatures (at and above 28°C and 32°C for ADP and AMP, respectively), as well as a decrease in the total adenylate concentrations suggesting that cellular energy status (including AEC and ADP/ATP ratios) was maintained by the breakdown of ADP by nucleoside-diphosphate kinases and by AMP deamination. PLA levels also remained unchanged even though there was a transient increase in the levels of free L-arginine. In winter control oysters there were no changes in tissue adenylate concentrations or energy related indices (TANP & ADP/ATP ratio), although there was increase in AEC at the extreme temperature of 36°C. Given that oysters cannot survive at this temperature for a long period of time, an increase in AEC is unlikely to indicate an improved energy status but most likely reflects

a mismatch between the rates of ATP consuming and producing reactions during extreme thermal stress. A similar mechanism likely explains an increased in AEC and ATP levels in Cd-exposed summer oysters beyond their critical temperatures (at 28°C and above). Overall, in all experimental groups of oysters the steady-state ATP levels and energy status (indicated by AEC and ATP/ADP ratios) were sustained during the acute temperature stress by aerobic metabolism and anaerobic glycolysis without a significant contribution from PLA breakdown. Notably, phosphagen reserves were considerably lower in summer Cd-exposed oysters compared to their winter counterparts suggesting that these animals potentially have a lower ability to rapidly respond to elevated energy demands, e.g. during reproduction in summer .

In aquatic organisms, AEC value is maintained during moderate environmental perturbations but can vary with the season, life stage, activity level and exposure to the stressful conditions such as metal pollution, decreased salinity, anoxia, hypoxia or starvation (*89, 112*). Seasonal variations in AEC and TANP have been reported in various invertebrate species (*113-114*). AEC is also known to vary with the reproductive status and season (*115*). However, AEC is not sensitive to low levels of stress, and some studies suggest that tissue ATP levels may be a better indicator of stress-induced shift of the cellular energy status (*113*). In some aquatic organisms, the AEC levels remain constant despite the significant changes in the levels of adenylates, phosphagens, glucose and glycogen (*114*). Thus, exposure of the blue mussels *Mytilus edulis* and the sea bass *Dicentrarchus labrax* to sublethal Cd levels did not affect AEC but led to a significant change in the levels of ATP and total adenylates (*112*). In glass shrimp, *Palaeomonetes paludosis* Cd exposure (0.4, 10 & 30µg l^{-1}) led to a significant decrease in ATP and

TANP after an initial rise in the adenylate concentrations on 32 days exposure (*113*). In contrast, in some freshwater crustaceans and mollusks (a crayfish, *Procambarus acutus acutus,* the papershell clam *Anodonta imbecillis* and the Asian clam, *Corbicula fluminea),* Cd exposures led to significant changes in tissue levels of adenylates and a decrease in AEC levels (*89*). This suggests that different organisms can use different strategies to defend cellular energy status and demonstrate different susceptibility to the stress-induced shits in adenylate concentrations. However, regardless of whether AEC or ATP concentrations are used as a marker, our data suggest that Cd exposure and acute temperature stress result in the minimal shifts of the of cellular energy status in oysters *C. virginica* under the conditions of this study.

Glycogen and lipid concentrations reflect the amount of metabolic reserves in tissues of marine ectotherms that can be diminished during stress and/or periods of elevated energy demand such as spawning and gametogenesis (*112*). Earlier studies in *C.virginica* showed that long-term combined exposure to elevated temperatures and Cd resulted in a significant depletion of tissue glycogen stores, whereas each of these stressors alone had no effect (*7*). Exposure to Cd, Cr and/or tributylin (TBT) in fishes (sea bass *Dicentrarchus labrax* and rainbow trout *Oncorhynchus mykiss*), gastropods (dogwhelk *Nucella lapillus*) and crustaceans (*Scylla serrata, Tigriopus japonicus* and *Daphnia magna*) resulted in depletion of tissue glycogen and/or lipid reserves (*106, 112, 116-117*). Mobilization of glycogen in Cd-exposed organisms likely reflects activation of glycogen phosphorylase kinase by Cd (*112*). In a marine clam, *Ruditapes decussates* Cd exposure also led to the depletion of tissue energy stores, starting with glycogen followed by lipoproteins and lastly proteins (*118*). In contrast, in our present study there was no

decrease in glycogen or lipid stores in control or Cd-exposed oysters in response to acute temperature rise; in fact in summer oysters there was a transient significant increase in glycogen and lipid levels at 28°C in hepatopancreas and/or gill tissues. The discrepancy between our findings and earlier published research may be due to the moderate Cd concentrations and short-term temperature stress used in the present work which may have been insufficient to induce strong energy deficiency in oysters.

Overall, our data suggest that ATP turnover was sustained by aerobic metabolism and partial anaerobiosis during acute temperature rise in both the control and Cd-exposed oysters. There was no decrease in the levels of ATP or PLA breakdown indicating their negligible contributions to maintain ATP turnover. Tissue energy stores were maintained or even temporarily increased indicating that metabolic adjustments were effective in protecting energy status of oysters during acute temperature stress regardless of the season or Cd exposure.

4.3 Oxidative stress in oysters as a late marker of transition to the pessimum range:

Stressors such as Cd and elevated temperature can lead to a imbalance between the ROS production and removal due to the excessive ROS generation in mitochondria, inhibition of cellular antioxidant defenses or both (*7*). This can result in oxidative damage to macromolecular structures such as nucleic acids, lipids and proteins. Effects of individual stressors including trace metals and temperature stress in causing oxidative damage have been extensively studied in aquatic ectotherms including mollusks (*14, 119-121*); however, the combined effects of these two stressors on oxidative damage in oysters is not fully understood.

Our study show that oxidative damage in response to the combined Cd stress and acute warming depends on the seasonal acclimatization in oysters. Acute temperature rise did not cause oxidative stress in summer control oysters as indicated by the stable levels of protein carbonyls and MDA in the mantle tissues. In contrast, in winter oysters acute warming led to oxidative damage to proteins reflected in the accumulation of carbonyl content of the mantle tissues with increasing temperatures. No MDA accumulation was found indicating that lipid peroxidation was not enhanced at elevated temperatures. These results agree with the previously published data that show elevated oxidative stress in heat-exposed marine invertebrates and the higher susceptibility of cold-acclimated or cold-acclimatized organisms to oxidative damage (*122*). Thus, in a lugworm *A. marina* sudden warming exacerbates the ROS formation, and the rate of ROS generation and its thermal sensitivity is influenced by seasonal temperature fluctuations (*123*). Studies in oysters also have shown that oxidative stress in mitochondrial aconitase (a Krebs cycle enzyme often used as an oxidative stress marker) was more pronounced at elevated temperature of 30°C compared to 20°C (*63*). In the blue mussels *Mytilus edulis* antioxidative enzyme activities decrease in winter making the organism more susceptible to ROS-induced damage (*124*).

Metals such as Cd are also strong inducers of oxidative stress (*125*). In marine bivalves, Cd causes oxidative cellular damage by interfering with the antioxidant defense system, inducing lipid peroxidation and ultimately leading to apoptosis (*126-127*). Notably, environmental temperature can modify the pro-oxidant effects of Cd. Thus, in oysters *C. virginica* Cd exposure of isolated mitochondria did not lead to a damage to aconitase at 20°C suggesting that mitochondrial antioxidants offer adequate protection

against Cd-induced ROS damage at this temperature (*63*). In contrast, at 30°C antioxidant defense system was incapable of coping with Cd-induced ROS and considerable damage occurred to aconitase enzyme (*63*). These findings suggest that at elevated temperatures Cd-induced ROS increases in oysters and can overwhelm the antioxidant defenses (*63*) and stress the importance of considering temperatures when assessing the metal toxicity in marine ectotherms.

Absence of the temperature-induced accumulation of carbonyls or MDA in Cd-exposed oysters found in the present study contrasts with the earlier findings in *C.virginica.* Previous studies show that Cd-exposed oysters can accumulate high levels of lipid peroxidation products when acclimated at elevated temperatures (28°C) (*23, 41*). It is worth noting that the earlier studies on the combined effects of temperature and Cd on oxidative stress in oysters used long-term exposure regimes (3-7 weeks) at elevated temperature and Cd, in contrast to the present study where oysters were exposed to acute short-term warming. Interestingly, carbonyl accumulation induced by the acute warming in the present study was lower in Cd-exposed oysters compared to their control counterparts in winter. Moderate exposures to metals (including Cd) can upregulate cellular defense mechanisms in oysters including antioxidants and heat-shock proteins (*119, 128*) possibly offering cross-protection against the elevated ROS production during the acute, short term warming in Cd-exposed oysters (*128*). Overall, our studies suggest that acute temperature rise does not elicit major oxidative stress in oysters, and Cd exposure may partially antagonize the effects of acute warming on redox balance.

4.4 Conclusions and perspectives:

To the best of our knowledge, the present study provides the first experimental evidence that exposure to environmentally realistic Cd concentrations affects the thermal tolerance window in a model marine ectotherm, the eastern oyster *C. virginica*, by inducing a downward shift of the upper Tc values (Fig. 17). This shift was clearly pronounced in summer oysters but was not detected in the winter, possibly due to the overall downward shift of Tc in winter oysters pushing the thermal tolerance envelope close to the lowest temperatures tested in this study. Cd-induced shift in Tc likely reflects the reduction of the aerobic scope of oysters due to the elevated energy demand and insufficient oxygen supply by ventilation and circulation systems. Despite the onset of partial anaerobiosis at and beyond the Tc, oysters were capable of maintaining their tissue energy stores and cellular energy balance likely reflecting the effective metabolic adaptations to acute heat stress in this intertidal species. Similarly, the temperature-induced oxidative damage was minimal and only detected in winter oysters indicating that these organisms are well protected against the temperature-induced increase in ROS generation at least during the warmer season when an acute heat stress is most likely. A decrease in thermal tolerance in Cd-exposed oysters may have implications for survival of oyster populations in polluted estuaries during the acute heat stress such as observed in the intertidal zone during the summer low tides and/or during the chronic exposure to elevated temperatures such as can be expected during the global climate change in oyster populations of the southeastern United States that live close to the geographical distribution limit of this species.

Future studies should focus on the understanding of the effects of multiple stressors including pollutants that affect energy demand, oxygen supply and/or mitochondrial capacity on the thermal tolerance windows of marine ectotherms under the environmentally realistic exposure scenarios. Such studies are important from the viewpoint of the fundamental thermal physiology in order to test the applicability of OCLTT hypothesis for predicting the stressor interactions and factors affecting the physiological limits of thermal tolerance in aquatic ectotherms (*7*). Studies focusing on the interactive effects of pollutants and long-term acclimation or adaptation to different temperature regimes and placing the thermal tolerance limits in the context of the environmentally realistic long-term exposure scenarios would be required to complement the studies of the effects of the acute temperature rise These studies can be used for developing quantitative physiological models to predict the effects of pollutant–temperature interactions in marine ectotherms and to facilitate the ecological risk assessment of ectotherm populations facing the global climate change in polluted environments (*7*).

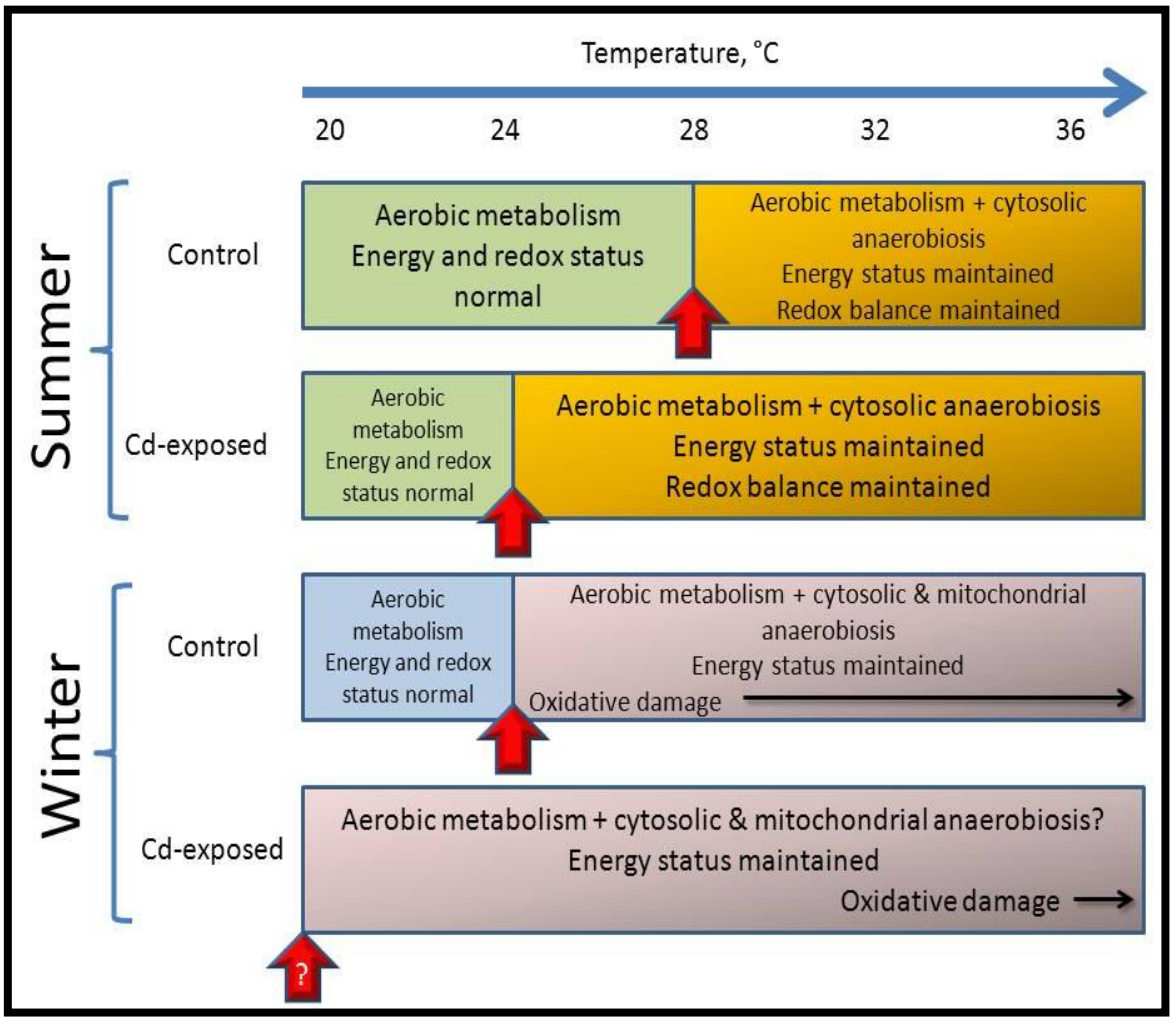

FIGURE 17: Summary of the seasonal and Cd-induced effects on the thermal tolerance window of *C. virginica*. Green and blue boxes represent the optimal and pejus ranges of the environmental temperatures, while orange and purple boxes represent the pessimum range where the time-limited survival only is possible. Red block arrows indicate the critical temperatures of aerobic metabolism (Tc). The question mark in the block arrow indicates that Tc could not be reliably determined in this study for Cd-exposed winter oysters.

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APPENDIX A: ANOVA TABLES

TABLE 1: ANOVA: Effects of Cd exposure, season and temperature exposure on concentrations of the end products of cytosolic (L-alanine) and mitochondrial (acetate and succinate) anaerobiosis in the gills of the eastern oysters *C.virginica*. Significant effects are highlighted in bold.

	L-Alanine		**Acetate**		**Succinate**	
Factors / Interactions	**F**	**P**	**F**	**P**	**F**	**P**
Cadmium concentrations	**$F_{1,158}$ = 4.5**	**0.0354**	**$F_{1,174}$ = 18.12**	**<.0001**	$F_{1,161}$ = 0.24	0.6248
Season	**$F_{1,158}$ = 37.56**	**<.0001**	**$F_{1,174}$ = 88.18**	**<.0001**	**$F_{1,161}$ = 22.96**	**<.0001**
Temperature	$F_{4,158}$ = 2.17	0.0743	**$F_{4,174}$ = 8.45**	**<.0001**	$F_{4,161}$ = 0.76	0.5511
Cadmium concentrations * Season	$F_{1,158}$ = 0.5	0.4824	**$F_{1,174}$ = 38.45**	**<.0001**	$F_{1,161}$ = 1.23	0.2682
Temperature * Cadmium concentration	$F_{4,158}$ = 1.3	0.2736	**$F_{4,174}$ = 8.08**	**<.0001**	$F_{4,161}$ = 0.31	0.8716
Temperature * Season	**$F_{4,158}$ = 10.14**	**<.0001**	**$F_{4,174}$ = 6.88**	**<.0001**	$F_{4,161}$ = 1.43	0.2252
Temperature * Cadmium concentration * Season	**$F_{4,158}$ = 4.72**	**0.0013**	**$F_{4,174}$ = 13.41**	**<.0001**	$F_{4,161}$ = 0.43	0.7892

TABLE 2: ANOVA: Effects of Cd exposure, season and temperatures and their interactions on the levels of adenylates (ATP, ADP and AMP) in the gills of the eastern oyster *C.virginica*. Significant effects are highlighted in bold.

	Adenylates					
	ATP		**ADP**		**AMP**	
Factors / Interactions	**F**	**P**	**F**	**P**	**F**	**P**
Cadmium concentrations	$F_{1,169} = 0.18$	0.6694	$F_{1,147} = 0.81$	0.3703	$F_{1,147} = 0.29$	0.5899
Season	**$F_{1,169} = 39.06$**	**<.0001**	**$F_{1,147} = 27.3$**	**<.0001**	**$F_{1,147} = 125.37$**	**<.0001**
Temperature	$F_{4,169} = 0.8$	0.5282	$F_{4,147} = 1.61$	0.1759	**$F_{4,147} = 8.13$**	**<.0001**
Cadmium concentrations * Season	**$F_{1,169} = 5.4$**	**0.0213**	$F_{1,147} = 1.89$	0.1712	$F_{1,147} = 2.22$	0.1382
Temperature * Cadmium concentration	$F_{4,169} = 0.34$	0.8501	$F_{4,147} = 1.36$	0.249	$F_{4,147} = 2.07$	0.0873
Temperature * Season	$F_{4,169} = 0.67$	0.6129	**$F_{4,147} = 2.5$**	**0.0449**	$F_{4,147} = 1.76$	0.141
Temperature * Cadmium concentration * Season	$F_{4,169} = 2.1$	0.0832	$F_{4,147} = 1.05$	0.3839	$F_{4,147} = 0.74$	0.565

TABLE 3: ANOVA: Effects of Cd exposure, season and temperatures and their interactions on the levels of adenylates energy related indices (AEC, total adenylates and ADP/ATP ratio) in the gills of the eastern oyster *C.virginica*. Significant effects are highlighted in bold.

	AEC		**Total Adenylates**		**ADP/ATP Ratio**	
Factors / Interactions	**F**	**P**	**F**	**P**	**F**	**P**
Cadmium concentrations	$F_{1,132} = 0.19$	0.6644	$F_{1,133} = 0.9$	0.3438	$F_{1,133} = 0.23$	0.6356
Season	$\mathbf{F_{1,132} = 7.91}$	**<.0001**	$\mathbf{F_{1,133} = 10.13}$	**<.0001**	$F_{1,133} = 2.92$	0.0899
Temperature	$\mathbf{F_{4,132} = 5.64}$	**0.0003**	$F_{4,133} = 1.97$	0.103	$F_{4,133} = 1.45$	0.2212
Cadmium concentrations * Season	$\mathbf{F_{1,132} = 6.89}$	**0.0097**	$F_{1,133} = 0.29$	0.5902	$\mathbf{F_{1,133} = 8.14}$	**0.005**
Temperature * Cadmium concentration	$F_{4,132} = 1.11$	0.3546	$F_{4,133} = 0.3$	0.8756	$F_{4,133} = 1.26$	0.2894
Temperature * Season	$F_{4,132} = 0.37$	0.8271	$F_{4,133} = 0.83$	0.4775	$\mathbf{F_{4,133} = 2.5}$	**0.0459**
Temperature * Cadmium concentration * Season	$F_{4,132} = 0.74$	0.5663	$\mathbf{F_{4,133} = 2.83}$	**0.0274**	$F_{4,133} = 0.99$	0.4132

TABLE 4: ANOVA: Effects of Cd exposure, season and temperatures and their interactions on the levels of phosphagen and aphosphagen in the gills of the eastern oyster *C.virginica*. Significant effects are highlighted in bold.

	PLA		L-Arginine	
Factors / Interactions	**F**	**P**	**F**	**P**
Cadmium concentrations	$F_{1.,112} = 3.44$	0.0663	$\mathbf{F_{1,116} = 10.04}$	**0.0022**
Season	$\mathbf{F_{1.,110} = 142.86}$	**<.0001**	$\mathbf{F_{1,116} = 10.35}$	**0.0037**
Temperature	$F_{4,112} = 0.29$	0.8828	$F_{4,116} = 1.93$	0.118
Cadmium concentrations * Season	$\mathbf{F_{1.,112} = 9.35}$	**0.0028**	$\mathbf{F_{1,116} = 10.62}$	**0.0016**
Temperature * Cadmium concentration	$F_{4,112} = 0.36$	0.8392	$\mathbf{F_{4,116} = 2.71}$	**0.0367**
Temperature * Season	$F_{3,112} = 1.14$	0.3378	$F_{3,116} = 0.8$	0.3791
Temperature * Cadmium concentration * Season	$F_{3,112} = 0.76$	0.5215	$F_{3,116} = 0.54$	0.6628

TABLE 5 : ANOVA: Effects of Cd exposure, season and temperatures and their interactions on the total phosphagen/aphosphagen pool and R_{PLA} in the gills of the eastern oyster *C.virginica*. Significant effects are highlighted in bold.

	[PLA] + [L-Arginine]		**R_{PLA}**	
Factors / Interactions	**F**	**P**	**F**	**P**
Cadmium concentrations	$F_{1,115} = 0.28$	0.5977	$F_{1,113} = 1.43$	0.2342
Season	**$F_{1,115} = 118.73$**	**<.0001**	**$F_{1,113} = 228.04$**	**<.0001**
Temperature	$F_{4,115} = 0.47$	0.759	$F_{4,113} = 2.62$	0.0387
Cadmium concentrations * Season	$F_{1,115} = 2.65$	0.1065	**$F_{1,113} = 24.1$**	**<.0001**
Temperature * Cadmium concentration	$F_{4,115} = 0.54$	0.7058	**$F_{4,113} = 3.13$**	**0.0174**
Temperature * Season	$F_{3,115} = 0.42$	0.7418	$F_{3,113} = 2.59$	0.056
Temperature * Cadmium concentration * Season	$F_{3,115} = 1$	0.3948	$F_{3,113} = 0.4$	0.7539

TABLE 6: ANOVA: Effects of Cd exposure, season, experimental temperatures and tissues and their interactions on the levels of glycogen stores. Significant effects are highlighted in bold.

	Glycogen	
Factors / Interactions	**F**	**P**
Cadmium concentrations	$F_{1,243} = 3.21$	0.0743
Season	$\mathbf{F_{1,243} = 20.44}$	**<.0001**
Temperature	$F_{2,243} = 2.83$	0.0607
Tissue (gills, hepatopancreas, muscle)	$\mathbf{F_{2,243} = 9.21}$	**0.0001**
Season*Cadmium concentrations	$F_{1,243} = 0.85$	0.358
Temperature*Cadmium concentrations	$F_{2,243} = 0.97$	0.3797
Tissue*Cadmium concentrations	$F_{2,243} = 0.1$	0.9074
Season*Temperature	$F_{2,243} = 1.05$	0.3509
Season*Tissue	$\mathbf{F_{2,243} = 17.07}$	**<.0001**
Tissue*Temperature	$F_{4,243} = 2.82$	0.0259
Temperature * Season*Cadmium concentrations	$F_{2,243} = 1.87$	0.1561
Tissue*Cadmium concentrations*season	$F_{2,243} = 0.48$	0.6169
Tissue*Cadmium concentrations*Temperature	$F_{4,243} = 1.99$	0.0959
Season*Tissue*Temperature	$F_{4,243} = 3.05$	0.0177
Season*Tissue*Temperature*Cadmium concentrations	$F_{4,243} = 0.54$	0.7046

TABLE 7: ANOVA: Effects of Cd exposure, season, experimental temperatures and tissues and their interactions on the levels of glucose. Significant interactions are highlighted in bold.

	Glucose	
Factors / Interactions	**F**	**P**
Cadmium concentrations	$\mathbf{F_{1,241} = 4.81}$	**0.0292**
Season	$F_{1,241} = 1.26$	0.2634
Temperature	$F_{2,241} = 0.98$	0.3756
Tissue (gills, hepatopancreas, muscle)	$\mathbf{F_{2,241} = 12.13}$	**<.0001**
Season*Cadmium concentrations	$F_{1,241} = 3.31$	0.0703
Temperature*Cadmium concentrations	$F_{2,241} = 1.01$	0.3646
Tissue*Cadmium concentrations	$F_{2,241} = 2.18$	0.1158
Season*Temperature	$F_{2,241} = 1.06$	0.3483
Season*Tissue	$F_{2,241} = 1.97$	0.1418
Tissue*Temperature	$F_{4,241} = 0.57$	0.6823
Temperature * Season*Cadmium concentrations	$F_{2,241} = 1.22$	0.2957
Tissue*Cadmium concentrations*Season	$\mathbf{F_{2,241} = 3.78}$	**0.0242**
Tissue*Cadmium concentrations*Temperature	$F_{4,241} = 0.45$	0.7734
Season*Tissue*Temperature	$F_{4,241} = 1.28$	0.2796
Season*Tissue*Temperature*Cadmium concentrations	$F_{4,241} = 1.27$	0.2833

TABLE 8: ANOVA: Effects of Cd exposure, season, experimental temperatures and tissues and their interactions on the levels of lipids. Significant effects are highlighted in bold.

	Lipids	
Factors / Interactions	**F**	**P**
Cadmium concentrations	$F_{1,134}=0.16$	0.6894
Season	$\mathbf{F_{1,134}=59.54}$	**<.0001**
Temperature	$\mathbf{F_{2,134}=20.44}$	**<.0001**
Tissue (gills, hepatopancreas)	$F_{1,134}=0.35$	0.556
Season*Cadmium	$F_{1,134}=2.90$	0.0907
Temperature*Cadmium	$F_{2,134}=1.44$	0.2413
Tissue*Cadmium	$F_{1,134}=0.21$	0.6484
Season*Temperature	$\mathbf{F_{2,134}=24.68}$	**<.0001**
Season*Tissue	$\mathbf{F_{1,134}=5.69}$	**0.0184**
Tissue*Temperature	$F_{2,134}=1.72$	0.1822
Temperature * Season*Cadmium	$F_{2,134}=1.24$	0.2937
Tissue*Cadmium	$F_{1,134}=1.21$	0.2734
Tissue*Cadmium*Temperature	$F_{2,134}=0.15$	0.8577
Season*Tissue*Temperature	$F_{2,134}=2.10$	0.1264
Season*Tissue*Temperature*Cadmium	$F_{2,134}=0.58$	0.58

TABLE 9: ANOVA: Effect of Cd exposure, season, experimental temperatures and tissues and their interactions on the levels of proteins. Significant effects are highlighted in bold.

	Proteins	
Factors / Interactions	**F**	**P**
Cadmium concentrations	$F_{1,132} = 0.08$	0.7725
Season	$F_{1,132} = 0.85$	0.3582
Tissue (gills, hepatopancreas)	$\mathbf{F_{2,132} = 9.8}$	**0.0022**
Temperature	$\mathbf{F_{1,132} = 3.25}$	**0.0419**
Season*Cadmium	$F_{1,132} = 0.23$	0.6309
Tissue*Cadmium	$F_{2,132} = 0.02$	0.8835
Temperature*Cadmium	$F_{1,132} = 0.90$	0.4078
Season*Tissue	$F_{2,132} = 0.03$	0.8553
Season*Temperature	$\mathbf{F_{1,132} = 3.21}$	**0.0437**
Tissue*Temperature	$F_{2,132} = 0.77$	0.4671
Tissue*Cadmium*Season	$Fl_{,132} = 0.49$	0.4861
Temperature * Season*Cadmium	$F_{2,132} = 0.56$	0.5716
Tissue*Temperature*Cadmium	$F_{2,132} = 0.31$	0.7352
Season*Tissue*Temperature	$F_{2,132} = 1.16$	0.317
Season*Tissue*Temperature*Cadmium	$F_{2,132} = 0.44$	0.6457

TABLE 10: ANOVA: Effects of Cd exposure, season and temperatures and their interactions on the levels of oxidative stress markers in mantle tissue. Significant effects are highlighted in bold.

	Carbonyl		**Malondialdehyde**	
Factors / Interactions	**F**	**P**	**F**	**P**
Cadmium concentrations	$F_{1,140} = 0.01$	0.7575	$\mathbf{F_{1,132} = 7.1}$	**0.0087**
Season	$\mathbf{F_{1,140} = 101.1}$	**<.0001**	$\mathbf{F_{1,132} = 40.49}$	**<.0001**
Temperature	$\mathbf{F_{4,140} = 2.63}$	**0.0372**	$F_{4,132} = 0.67$	0.6157
Cadmium concentrations * Season	$F_{1,140} = 0.69$	0.4080	$\mathbf{F_{1,132} = 6.91}$	**0.0096**
Temperature * Cadmium concentration	$F_{4,140} = 1.54$	0.2151	$F_{4,132} = 0.18$	0.947
Temperature * Season	$\mathbf{F_{4,140} = 2.66}$	**0.0354**	$F_{4,132} = 0.38$	0.8232
Temperature * Cadmium concentration * Season	$F_{4,140} = 0.90$	0.4680	$F_{4,132} = 1.23$	0.3015

TABLE 11: ANOVA: Accumulation of Cd in mantle tissue in summer and winter seasons. Significant effects are highlighted in bold.

	Cadmium concentrations	
Factors / Interactions	**F**	**P**
Cadmium concentrations	$\mathbf{F_{1,99} = 119.94}$	**<.0001**
Season	$\mathbf{F_{1,99} = 19.29}$	**0.003**
Temperature	$F_{4,99} = 10.4$	0.8077
Cadmium concentrations * Season	$F_{1,99} = 13.15$	0.079
Temperature * Cadmium concentration	$F_{4,99} = 0.3$	0.8802
Temperature * Season	$F_{4,99} = 0.44$	0.7776
Temperature * Cadmium concentration * Season	$F_{4,99} = 0.66$	0.6202