DEPARTAMENTO DE CIÊNCIAS DA VIDA

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The effects of azoxystrobin and temperature on the mitochondrial activity of the green crab *Carcinus maenas*

Tito Rafael Ferreira Mendes

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica do Professor Doutor Miguel Pardal (Universidade de Coimbra) e do Professor Doutor Carlos Palmeira (Universidade de Coimbra)

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Resumo

O caranguejo verde *Carcinus maenas* é uma espécie-chave nos sistemas estuarinos, importante na manutenção da estrutura e função das suas comunidades biológicas. Este caranguejo portunídio é também uma espécie invasora com populações espalhadas pelas costas dos cinco continentes, provocando grandes impactos tanto a nível económico como a nível ecológico. Sendo um organismo euritérmico encontrado a diferentes latitudes, *C. maenas* possui tolerância para uma grande gama de temperaturas. Deste modo, o estudo dos efeitos da temperatura na fisiologia do *C. maenas* é de extremamente importante para ser possível perceber a sua tolerância térmica. Sabe-se também que a aclimatação térmica afeta profundamente a respiração mitocondrial dos ectotérmicos, uma vez que estes não conseguem regular a sua temperatura interna. Os primeiros protocolos relativos a isolamentos mitocondriais em *C. maenas*, foram descritos por Munday e Thompson em 1962. Desde essa data, não houve melhorias significativas no que toca aos protocolos, havendo uma lacuna no conhecimento, relativo a este assunto. Há também outros fatores que influenciam a atividade mitocondrial como, por exemplo, alguns pesticidas. O fungicida azoxistrobina afeta a atividade mitocondrial através da inibição da transferência de eletrões entre os citocromos b e c1, interrompendo a produção de ATP. Este modo de ação demonstra ser muito eficiente no controlo de agentes patogénicos de plantas, pertencentes às classes Ascomicetes, Basidiomicetes, Deuteromicetes e Oomicetes, sendo um dos fungicidas mais vendidos em todo o mundo. O uso de azoxistrobina acarreta problemas ecológicos às espécies aquáticas, devido à facilidade em entrar nos sistemas hídricos através de lixiviação e escoamento superficial. De acordo com os resultados, aclimatação a 5ºC causou um decréscimo no consumo de oxigénio nas mitocôndrias isoladas, comparativamente à temperatura padrão (22º). Por outro lado, aclimatação a 27ºC

provocou o desacoplamento das mitocôndrias. A alimentação foi também registada, sendo que a 5ºC a maioria dos indivíduos não se alimentou. Embora as extremas temperaturas afetem profundamente a respiração mitocondrial e o processo de alimentação, foi registada baixa mortalidade tento a 5ºC como a 27ºC, sugerindo que o *C. maenas* possui mecanismos fisiológicos para compensar as variações térmicas. No que diz respeito à azoxistrobina, não foram registados efeitos na respiração mitocondrial, devido possivelmente à baixa concentração usada (25 µg L^{-1}). A concentração usada foi semelhante às concentrações mais elevadas encontradas no ambiente que foram reportadas, mas, ainda assim, mais baixas que as utilizadas nos testes ecotoxicológicos publicados. Não foram observadas interações entre a temperatura e a concentração de azoxistrobina usada. Esta experiência ajuda a explicar as respostas mitocondriais, a alimentação e a mortalidade observadas às diferentes temperaturas e a relacioná-las ao potencial invasor do *C. maenas*. Por outro lado, a concentração de azoxistrobina usada parece não influenciar a atividade mitocondrial do *C. maenas*, no entanto, outros processos fisiológicos podem ter ficado comprometidos.

Palavras-chave: *Carcinus maenas*; Temperatura; Azoxistrobina; Ecotoxicologia; Mitocôndria.

Abstract

The green shore crab *Carcinus maenas* is a key species in estuarine systems, important in the maintenance of the structure and function of their biological communities. This European portunid crab is also an invasive species with settled populations spread on the coasts of all five continents, having great economical and ecological impacts. As an eurythermal organism that is found at different latitudes, *C. maenas* is tolerant to a wide range of temperatures. Therefore, assessing the effects of temperature on the physiology of *C. maenas* is extremely important to understand its thermal tolerance. It is also known that thermal acclimation deeply affects the mitochondrial respiration rates of ectotherms, since they cannot regulate their internal temperature. The first mitochondrial isolation protocols, regarding *C. maenas*, were described by Munday and Thompson in 1962, and since then no major improvements were made, remaining a knowledge gap regarding this topic. There are also other factors that influence the mitochondrial activity as, for instance, some pesticides. The fungicide azoxystrobin affects the mitochondrial activity by inhibiting the electron transference between the cytochrome b and the cytochrome c1, thus halting the production of ATP. This mode of action proved to be very efficient in the control of plant pathogens belonging to the Ascomycetes, Basidiomycetes, Deuteromycetes and Oomycetes classes, being one of the most sold fungicides worldwide. The use of azoxystrobin brings ecological problems to aquatic species due to entering easily in the aquatic systems through runoff and leaching processes. According to the obtained results, cold acclimation at 5ºC caused a decrease in the oxygen consumption in isolated mitochondria, comparing to a standard temperature (22ºC). On the other hand, warm acclimation at 27ºC seemed to decouple the isolated mitochondria. Feeding behavior was also registered and it was observed that at 5ºC the majority of the tested organisms

did not feed. Although extreme temperatures have deeply affected the mitochondrial respiration and the feeding behavior, low mortality was registered at both 5ºC and 27ºC, suggesting that *C. maenas* possesses physiological mechanisms to counteract thermal variations. Concerning azoxystrobin, no effects were registered on the mitochondrial respiration, possibly due to the low concentration used (25 $\mu g\ L^{-1}$). This concentration was similar to the highest reported concentrations found in the environment, but was lower than the published concentrations used in ecotoxicological tests. No significant interactions between temperature and azoxystrobin concentrations were observed. This experiment contributed to explaining the observed mitochondrial responses, the feeding behavior and the mortality at different temperature regimes and correlating them to the invasive potential of *C. maenas*. On the other hand, the assessed azoxystrobin concentration seemed to pose no threat to the mitochondrial activity of *C. maenas*, nevertheless other physiological processes may have been compromised.

Keywords: *Carcinus maenas*; Temperature; Azoxystrobin; Ecotoxicology; Mitochondria.

Chapter 1. Introduction

1.1. Study species

The shore crab *Carcinus maenas* (L.) (Decapoda, Portunidae) is an important key species for the maintenance of the structure and function of the biological communities within estuaries, mainly due to its voracious predatory behaviour (Baeta *et al*., 2005; Elumalai *et al*., 2007). This crab is an opportunistic feeder and preys on a variety of organisms, including gastropods, bivalves, polychaetes, amphipods, decapods and algae (Baeta *et al*., 2006).

Carcinus maenas has two different morphotypes. The green morphotype is the most common one corresponding to recently-moulted individuals, generally younger ones, moulting with higher frequency. The red morphotype corresponds to individuals that maintain themselves in a prolonged intermoult state, allocating energy into reproduction (Dam *et al*., 2006). Without the exoskeleton renewal, the integument pigment, Astaxanthin, begins to denature and assumes a reddish coloration (Reid *et al*. 1997). According to Dam *et al*. (2006), crabs in the red morphotype are more susceptible to anthropogenic contamination than the ones in the green morphotype.

Carcinus maenas inhabits mainly the intertidal habitats and has a wide distribution within the Atlantic coast of Europe, being found in latitudes ranging from Norway, British Isles and south to Mauritania, (Grosholz and Ruiz, 1995; Baeta *et* al., 2005). *C. maenas* is also an invasive species with populations settled in other continents. This portunid crab is considered one of the "100 world's worst invasive alien species" (Nieto *et al*., 2010). It is a highly successful global invader that has established populations in western and eastern North America, South America, South Africa, Japan and Australia (Haarr and Rochette, 2012).

1.2. Isolation of mitochondria from *Carcinus maenas*

The first mitochondrial isolation from *C. maenas* was performed by Munday and Thompson (1962a). The "sub-cellular respiring particles" were extracted from the hepatopancreas through differential centrifugation. It was established the optimum conditions for the oxidation of the succinic acid and the results were compared with the ones of other animals. At that year, the same authors published a work (Munday and Thompson, 1962b), in which the effects of the osmotic pressure on the mitochondrial activity were assessed. The activity of the succinic oxidase was higher in preparations at 0.25 M sucrose than in preparations at 0.8 M sucrose, being the last one similar to the concentration present in the intracellular fluids of *C. maenas*. Additionally, Poat and Munday (1971) determined the respiratory control of mitochondria isolated from hepatopancreas of *C. maenas* and the inhibitory effects of rotenone and oligomycin were also assessed. It was demonstrated that these drugs caused on *C. maenas* similar effects to the ones observed in mammalian organisms. Since then, no other works were developed on this species, concerning the mitochondrial activity. The existent protocols needed to be optimized and the present study aims to fulfil the gap of knowledge on this topic.

1.3. Azoxystrobin - The chemical factor

Among all pesticides, fungicides are the second most important ones, being surpassed only by herbicides (Olsvik *et al.*, 2010). Nowadays, azoxystrobin is one of the most significant agricultural fungicides due to its revolutionary mode of action. It belongs to the strobilurins group, a recently discovered class of fungicides based on natural fungicidal derivatives of β-methoxyacrylic acid, such as Strobilurin A, Oudemansin A and Myxothiazol A (Bartlett *et al.*, 2002; Boudina *et al.*, 2007; Singh *et*

al., 2010). The natural compounds Strobilurin A and Oudemansin A were firstly discovered in the Basidiomycete wood-rotting fungi *Strobilurus tenacellus* and *Oudemansiella mudida* (Sauter *et al.*, 1999; Bartlett *et al.*, 2002; Rodrigues *et al.*, 2013), leading afterwards to the development of Azoxystrobin, the first synthetic strobilurin fungicide to be produced (Singh *et al.*, 2010). In 1992, Syngenta announced this new fungicide and launched it into the market in 1996 (Bartlett *et al.*, 2002; Warming *et al.*, 2009). With sales of approximately USD $415 million in 1999 (Bartlett *et al.*, 2001; Bartlett *et al.*, 2002; Singh *et al.*, 2010), usually under the trade names of Amistar®, Bankit® and Heritage® (Sauter *et al.*, 1999), azoxystrobin was considered the world's biggest selling fungicide. Only in USA, its consumption exceeded 230 tons in 2006 (Jørgensen *et al.*, 2012).

Azoxystrobin has a broad spectrum of antifungal activity, controlling plant pathogens belonging to all four Ascomycetes, Basidiomycetes, Deuteromycetes and Oomycetes classes (Sauter *et al.*, 1999; Bartlett *et al.*, 2001; Hnátová *et al.*, 2003; Rodrigues *et al.*, 2013), only possible before through the mixture of different fungicides. Initially, it was designed to be applied on temperate cereal crops, but today azoxystrobin is also used to control diseases in more than 80 different crops, including vegetable and fruit crops (Bartlett *et al.*, 2001; Boudina *et al.*, 2007; Jørgensen *et al.*, 2012).

Strobilurins present an innovative mode of action. These fungicides affect the mitochondrial respiratory complex III (Rodrigues *et al.*, 2013) by binding specifically and reversibly to the Qo-center (ubiquinol-oxidative center) on cytochrome b (Sauter *et* al., 1999; Hnátová *et al*., 2003; Boudina *et al.*, 2007; Deb *et al.*, 2010). This results in a blockage of the electron transference between cytochrome b and cytochrome c1, both belonging to the bc1 complex present in all eukaryotes, therefore stopping the

production of ATP (Sauter *et al.,* 1999; Bartlett *et al*., 2001; Bartlett *et al*., 2002; Cedergreen *et al.*, 2006). Hence, energy-dependent processes like mycelial growth and germination of fungal spores are halted (Sauter *et al.*, 1999; Olsvik *et al*., 2010), providing to these fungicides high levels of preventative activity.

Although innovative and efficient, strobilurins' mode of action is not specific to a restrict group of organisms and, therefore, are potentially toxic not only to fungi, but also to a wide variety of non-target species. This situation is of great concern since it may endanger the structure and function of biological communities (Zafar *et al.*, 2012). Strobilurins present low toxicity in mammals, birds and terrestrial invertebrates but are very toxic to fish and aquatic invertebrates (Bartlett *et* al., 2002; Olsvik *et al*., 2010; Rasmussen *et al.*, 2012; Rodrigues *et al*., 2013). Due to runoff and leaching processes from treated fields, azoxystrobin enters easily on aquatic systems (Warming *et al.*, 2009; Deb *et al.,* 2010; Rodrigues *et al*., 2013), being the most common fungicide in stream water, according to a recent US study (Jørgensen *et al.*, 2012). In accordance to the results of EC_{50} tests, 1 mg L^{-1} of azoxystrobin is more than enough to kill 50% of a fish population or affect 50% of invertebrates and algae (EC_{50} determined by 96h flow-through exposure, 48-h stationary exposure and 72-h stationary exposure, respectively) (Jørgensen *et al.*, 2012). Concerning zooplankton, azoxystrobin causes some effects even at lower concentrations. According to Warming *et al.* (2009), daphnids changed their reproductive behaviour when exposed to azoxystrobin. For example, in Danish lakes, where the environmental concentration found was 0.026 µg L^{-1}, these organisms reproduce significantly earlier, comparing to the controls. They also stated that the daphnids increased their respiration rates by 38-43%, which is ecological relevant once it induces stress and increases the metabolic cost, leading to a reduction of fitness. Friberg-Jensen *et al.* (2010) also observed negative effects in daphnids exposed to

azoxystrobin. They stated a decrease of the activity of the filtering limbs, mandibles, heart and focal claw in clones of *Daphnia magna*. It is important to refer that the EU limit value for fungicides is 0.1 µg L^{-1} for both drinking water and groundwater (Jørgensen *et al.*, 2012). R234886 or azoxystrobin acid, the main metabolite of azoxystrobin, is also considered to be very harmful to aquatic organisms (Ghosh and Singh, 2009; Jørgensen *et al.*, 2012; Rodrigues *et al.*, 2013). Consequently, this represents a major environmental problem, being necessary additional ecotoxicological studies aiming to assess the effects of azoxystrobin and its metabolites on aquatic systems. *C. maenas* is used widely as a test organism in ecotoxicology to assess the effects of metals and organic contaminants on its physiological processes (Bamber and Depledge, 1997; Krång and Ekerholm, 2006; Elumalai *et al.*, 2007; Hagger *et al.*, 2009; Maria *et al.*, 2009; Martín-Díaz *et al.*, 2009; Costa *et al.*, 2011; Dissanayake *et al.*, 2011). Concerning pesticides, there are only few studies involving *C. maenas* (Lundebye *et al.*, 1997; Nieto *et al.*, 2010; Ael *et al.*, 2012) and none regarding azoxystrobin.

1.4. Temperature - The physical factor

Temperature is one of the most important environmental factors for aquatic ecosystems, mainly due to its impact on metabolic processes of ectotherms (Sokolova and Lannig, 2008; Iftikar *et al.*, 2010). Contrarily to endotherms that have the capacity to conserve the physiological-produced heat within their bodies, maintaining the internal temperature nearly constant, the internal temperature of ectotherms depends on external heat sources (Guderley and St-Pierre, 2002). Consequently, thermal variations lead to mitochondrial specific responses on ectotherms. For example on the short-

horned sculpin, *Myoxocephalus scorpius*, cold acclimation caused a mitochondrial density increase and a mitochondrial enzyme activity enhancement (Guderley, 1996).

On the other hand, temperature might be of great importance limiting the spatial distribution of eurythermal invasive species (Iftikar *et al*., 2010), as in the case of *C. maenas*. The assessment of the effects of thermal acclimation on isolated mitochondria from *C. maenas* could explain how this organism is able to establish itself at different latitudes.

1.5. Objectives

The main objectives of this work are: 1) to optimize the process of mitochondrial isolation from the hepatopancreas of *C. maenas*; 2) to assess the occurrence of total or partial inhibition of mitochondrial activity of *C. maenas* exposed to a sublethal concentration of azoxystrobin; 3) to evaluate the effects of thermal acclimation on the respiratory rates of isolated mitochondria; 4) to verify if the effects of azoxystrobin on isolated mitochondria are temperature-dependent.

Chapter 2. Materials and Methods

2.1. Specimen collection and maintenance

C. maenas were collected from the estuary of Guadiana (37º 13.099' N, 7º 25.968' W), Portugal, through the use of fish-baited underwater traps. According to Vasconcelos *et al*. (2007), this is the Portuguese estuary with the lowest agricultural pressure. Therefore is reasonable to assume that the crabs did not have direct contact with azoxystrobin until their capture. The organisms were collected in January, February and April 2013. Water temperatures at each time point were 13.0ºC, 13.8ºC and 21.1ºC, respectively. Only intermoult staged green adult males, with no visible traces of diseases or external parasites and ranging from 36.0 to 50.2 mm ($\bar{x}$ = 43.8 ± 0.21 mm) were collected. They were afterwards acclimated in the laboratory at 22ºC in 30 L aquaria with 34 salinity aerated seawater for, at least, 7 days before experimental use. During acclimation, the animals were fed every two days with commercial frozen clam meat (*Paphia undulata*) destined to human consumption. During acclimation partial water changes were performed every day.

2.2. Experimental design

During the experiment, the crabs were randomly selected from the stock aquaria, weighed and the carapace width was measured. Afterwards, they were transferred to individual glass flasks (9 cm ø) filled with 500 mL of artificial marine water at 34 salinity. This artificial marine water was made with artificial salt, Tropic Marin Salt, Tropical Marine Centre, and distilled water. The flasks were aerated with silicon tubes and the air was filtered with 0.22 µm Minisart® syringe filters (Sartorius Stedim, Germany). Water loss due to evaporation was replenished with ultra pure water every day.

Three treatments were performed, including control (C), solvent control (A) and contamination (Azx) treatments (two replicates for each treatment; N=12 for each replicate). The treatments lasted 10 days. The organisms were fed twice during the treatments (third and sixth days) and total medium renewal was always performed the day after feeding. The oxygen, salinity and pH values were measured right before and after medium renewals, as well as at the beginning and end of the treatments. For the Azx treatment, azoxystrobin (methyl[E]-2-[(6-[2-cyanophenoxy]-4-pyrimidinyl)oxy]-a-[methoxymethylene]benzeneacetate, PESTANAL® analytical standard solution (Sigma-Aldrich, Germany)) was added at the time of the second medium renewal in order to expose the crabs to a nominal, sublethal concentration of 25 μg L^{-1} for 3 days. The added azoxystrobin was previously diluted with acetone. In the A treatment, all flasks received approximately 34 μL of acetone during the second medium renewal, the same volume used in the contaminant treatment, in order to assess the possible effects of this solvent in the tested organisms. This procedure was repeated for three different temperatures: one standard temperature of 22ºC (American Society for Testing and Materials, 1997) and two extreme temperatures, 5ºC and 27ºC, representing the physiological extreme temperatures for *C. maenas* (Grosholz and Ruiz, 2002).

The mortality, feeding behavior and molting individuals were also registered during the experiment for all three treatments and temperatures.

2.3. Hepatopancreas extraction and homogenization

For each replicate (N=12) mitochondrial isolation was performed. The hepatopancreas of freshly sacrificed crabs were removed, washed in homogenization medium (300 mM Mannitol, 83 mM Sucrose, 5 mM Tris-Chloride, 5 mM EDTA, BSA 1%, pH 7.4) added right before the beginning of the mitochondrial isolation, and

weighed. The tissues were homogenized (4-5 pulses at 500 rpm) within a Potter-Elvehjem homogenizer in homogenization medium. All procedures described above were performed with ice-cooled materials and mediums.

2.4. Differential centrifugations and protein quantification

Homogenization of the tissues was followed by centrifugation at 2500 rpm (670*g*) for 12 min in homogenization medium, aiming to discard the cell debris and nuclei. The resulting supernatant was centrifuged at 10000 rpm (10732*g*) for 12 min, in homogenization medium and the fresh supernatant was then discard. Resuspension of the pellet was performed in washing medium (300 mM Mannitol, 83 mM Sucrose, 5 mM Tris-Chloride, BSA 0.2%, pH 7.4) added right before the beginning of the mitochondrial isolation, using a paint brush. The resuspended pellet was repelleted twice at 10000 rpm for 12 min, in washing medium, in order to purify it and to decrease the BSA concentration. All the above centrifugations were performed with a 4ºC-refrigerated centrifuge (3-16PK, 12156-H rotor, Sigma, Germany). A final resuspension of the pellet was performed in a small amount of washing medium. Mitochondrial protein content was determined by the biuret method calibrated with BSA, according to Gornall *et al* (1949).

2.5. Mitochondrial oxygen consumption

Oxygen consumption of isolated mitochondria was measured with a Clark oxygen electrode connected to a recorder in a 0.85 mL water-jacketed closed chamber, at 22ºC. The electrode was always calibrated with respiratory medium (200 mM Mannitol, 83 mM Sucrose, 10 mM $MgCl_2$, 10 mM KH_2PO_4, 10 mM Tris-Chloride, pH 7.2) before the respiratory essays. Oxygen solubility in respiratory medium (1.4

salinity) at 22ºC is 270,94 µM. Mitochondria was suspended in respiratory medium at a concentration of 1 mg mL^{-1}. Substrate oxidation rates were measured in the presence of 10 µL Glutamate/Malate (1.0 M/0.5 M) after a 3 min-incubation time and afterwards were added 2 µL of ADP (50 mM) and 2 µL of FCCP (1 mM) to observe the maximum ADP-stimulated respiration and non-coupled respiration (maximum FCCP-induced respiration) rates, respectively.

2.6. Statistical analysis

Biometrics and oxygen consumption data are presented as mean ± SE. Oxygen consumption rates (nmol min^{-1} .mg $prot^{-1}$) were calculated and used to compare the different treatments. Values obtained in the C treatments were compared to the ones obtained in the A treatments, applying a one-way analysis of variances (ANOVA), using the STATISTICA™software (StatSoft Inc., 2005; version 7.0). A two-way ANOVA was applied to compare the C treatments and the Azx treatments, being the temperature and the presence/absence of azoxystrobin the designated factors. Dunnett's tests were performed afterwards for multiple comparisons. Coupling index and mitochondrial oxidative phosphorilation limitation system index were calculated from the ratio of the maximum ADP-stimulated respiration state to the Glutamate/Malate oxidation state and from the ratio of the maximum ADP-stimulated respiration state to the FCCP-induced non-coupled state, respectively. The first index indicates the degree of coupling of the assessed mitochondria and the second index represents the limitation of the phosphorylation upon the oxidation of the substrates. The level of statistical significance was set to 0.05.

Chapter 3. Results

3.1. Mortality, molting and feeding behavior

During the experiments, the mortality was higher at the extreme temperatures and mainly at 27ºC (Table 1). Concerning the percentage of molting individuals, the highest values were registered at 22ºC. At 27ºC, values were nearly one third than that at the standard temperature, and at 5ºC no molting crabs were detected. The highest values recorded for pre-molting individuals, characterized for having a new and soft carapace formed under the old one, were also registered at both 22ºC and at 27ºC. External parasites (the parasitic barnacle *Sacculina carcini*) were only observed at 22ºC in a very low percentage (2.78%).

Regarding food consumption (Table 1), all organisms at 22ºC ate the clam meat. On the highest temperature (27ºC), only during the second feeding time a small number did not eat (4.48%), and at 5ºC more than half of the individuals did not eat the respective portion of food at both feeding times.

Table 1. *Carcinus maenas* biometrics (Mean ± SE), percentages of mortality, molting, pre-molting and parasited animals, and percentage of individuals that did not eat during the experiment.

	5ºC	**22ºC**	**27ºC**
Carapace width (mm)	43.1 ± 0.4	44.1 ± 0.3	44.1 ± 0.4
Weight (g)	17.2 ± 0.5	18.4 ± 0.4	19.1 ± 0.5
Mortality (%)	2.78	1.39	5.56
Molt (%)	0.00	6.94	2.78
Pre-molt (%)	1.39	5.56	5.56
Parasited (%)	0.00	2.78	0.00
1st feeding time (%)	68.06	0.00	0.00
2nd feeding time (%)	64.79	0.00	4.48

3.2. Mitochondrial activity

Table 2 shows the oxygen consumption rates (nmoles min^{-1} .mg $prot^{-1}$) obtained from the different treatments at 5ºC, 22ºC and 27ºC. No significant differences (Table 3) were observed between the C and the A treatments, for all temperatures (Fig. 1). Therefore, it is possible to assume that acetone had no effects on the mitochondrial activity of *C. maenas*.

Table 2. Oxygen consumption rates (nmoles min^{-1} .mg $prot^{-1}$) of the Glutamate/Malate oxidation, maximum ADP-stimulated respiration and maximum FFCP-induced respiration for each treatment (C, A and Azx) at 5ºC, 22ºC and 27ºC. The values are expressed in Mean ± SE form.

	5ºC			**22ºC**			**27ºC**		
	C	A	Azx	C	A	Azx	C	A	Azx
Glutamate/Malate oxidation	5.99 ± 1.11	8.95 ± 4.00	9.71 ± 4.78	16.94 ± 0.34	18.72 ± 4.13	15.07 ± 2.02	31.44 ± 4.94	21.76 ± 7.17	23.90 ± 0.86
ADP-stimulated respiration	10.44 ± 2.04	12.77 ± 3.40	8.68 ± 2.65	36.19 ± 0.67	36.43 ± 3.51	30.91 ± 2.50	34.37 ± 12.86	35.39 ± 18.88	33.98 ± 6.53
FCCP-induced respiration	15.78 ± 2.92	17.99 ± 4.05	8.96 ± 0.25	73.92 ± 2.88	63.46 ± 7.59	50.69 ± 6.14	34.18 ± 14.01	33.81 ± 20.46	30.72 ± 7.68

Table 3. Values of the one-way ANOVA performed for the comparison between the C and A treatments for all three temperatures (5ºC, 22ºC and 27ºC).

	5ºC	**22ºC**	**27ºC**
Glutamate/Malate oxidation	$F = 0.51$; $p = 0.55$	$F = 0.18$; $p = 0.71$	$F = 1.24$; $p = 0.38$
ADP-stimulated respiration	$F = 0.35$; $p = 0.62$	$F < 0.01$; $p = 0.95$	$F < 0.01$; $p = 0.97$
FCCP-induced respiration	$F = 0.20$; $p = 0.70$	$F = 1.66$; $p = 0.33$	$F < 0.01$; $p = 0.99$

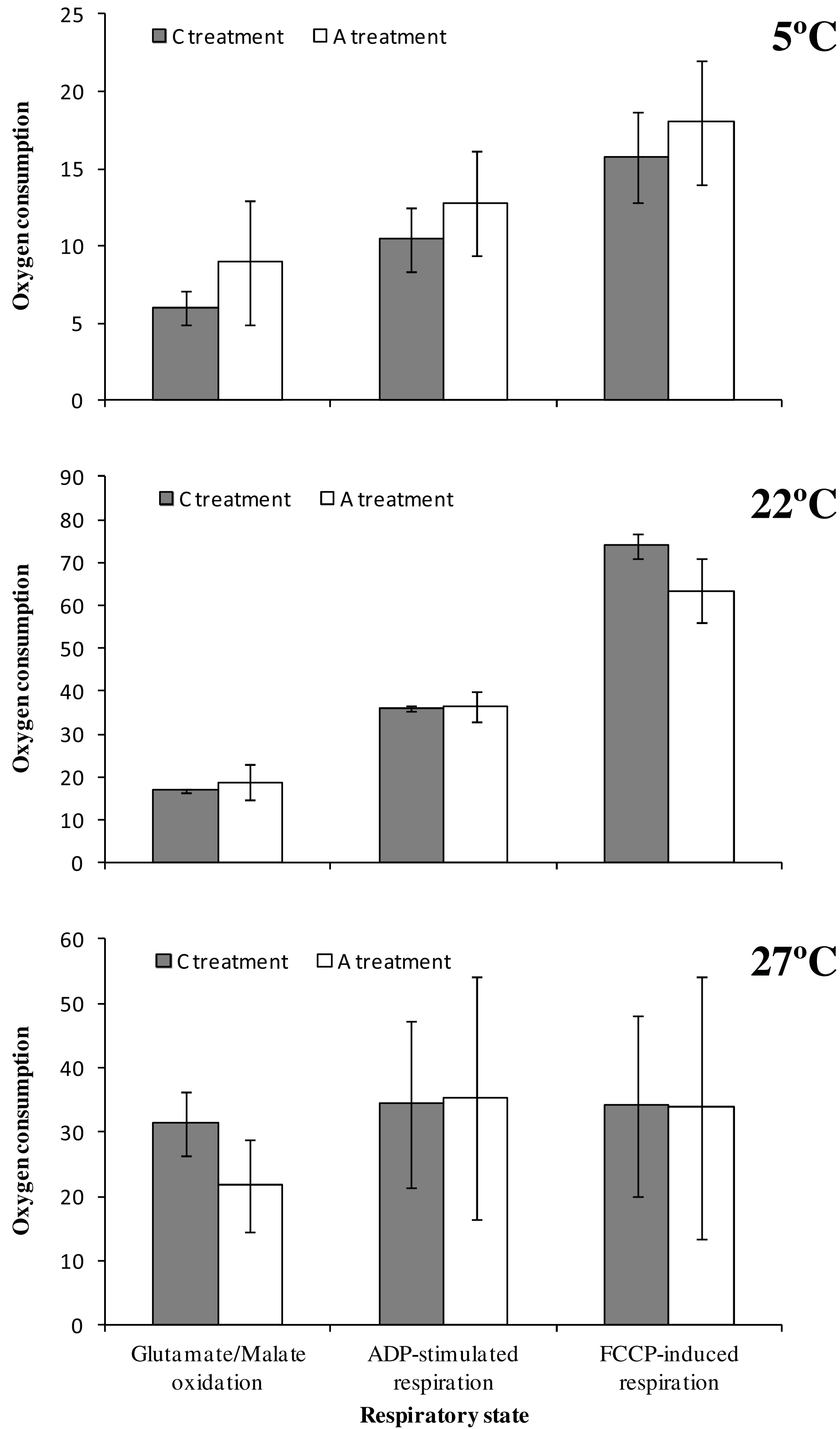

Figure 1. Mean values of oxygen consumption (nmoles min^{-1} .mg $prot^{-1}$) of Glutamate/Malate oxidation, maximum ADP-stimulated respiration and maximum FCCP-respiration at 5ºC, 22ºC and 27ºC. Gray bars represent C treatment values and white bars represent A treatment values.

Oxygen consumption rates of each respiratory state (Glutamate/Malate oxidation, maximum ADP-stimulated respiration and maximum FCCP-induced respiration) of the C and Azx treatments were compared for all temperatures (5ºC, 22ºC and 27ºC) (Fig. 2). Concerning Glutamate/Malate oxidation, significant differences (two-way ANOVA, $F=22.20$, $p=0.02$) were observed among the three temperatures (Fig. 2a). According to the post-hoc Dunnett's tests and comparing to the standard temperature (22ºC), the substrate was oxidized at significantly lower rates at 5ºC ($p=0.03$). On the other hand, the highest oxidation rates were obtained at 27ºC, being significantly different from the ones obtained at 22ºC (Dunnett's test, $p<0.01$). The oxygen consumption rates corresponding to ADP phosphorylation (maximum ADP-stimulated respiration) also significantly differed with temperature (two-way ANOVA, $F=10.46$, $p=0.01$) (Fig. 2b). At 5ºC, the respiration values were significantly lower (Dunnett's test, $p<0.01$) comparing to the respiration values of both 22ºC and 27ºC, but it was not possible to observe significant differences between the two warmest temperatures (Dunnett's test, $p=0.99$). Regarding the non-coupled rates induced by the addition of FCCP, significant differences were obtained between the extreme temperatures (5ºC and 27ºC) and the standard temperature of 22ºC (two-way ANOVA, $F=24.41$, $p<0.01$) (Fig. 2c). As expected, significantly lower values were observed at 5ºC (Dunnett's test, $p<0.01$). On the other hand and contrarily to the expected, the highest values were observed at 22ºC, being also significantly different from 27ºC (Dunnett's test, $p<0.01$).

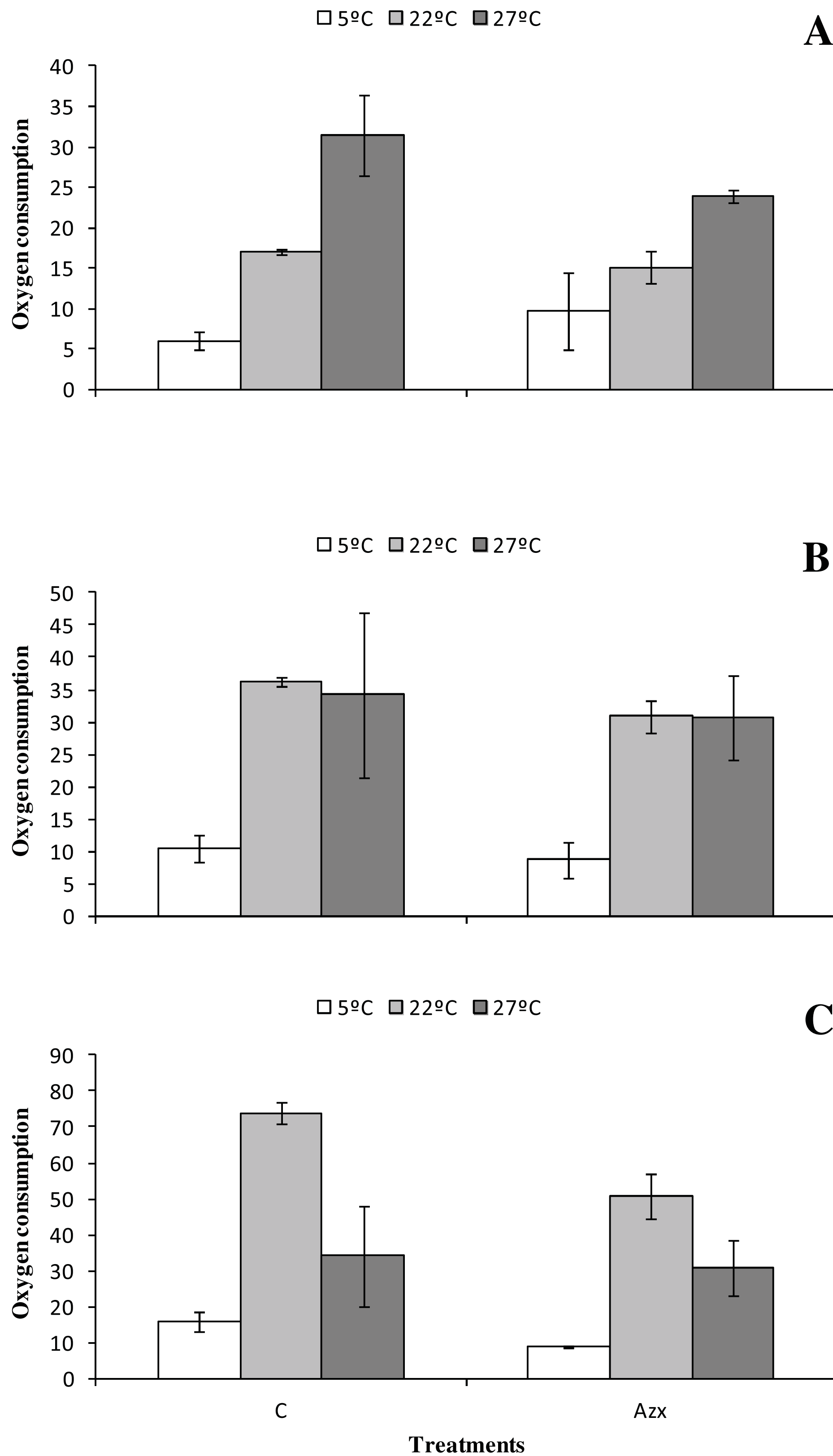

Figure 2. Mean values of oxygen consumption (nmoles min^{-1} .mg $prot^{-1}$) of C and Azx treatments at 5ºC (white bars), 22ºC (grey bars) and 27ºC (black bars). (A) Glutamate/Malate oxidation; (B) Maximum ADP-stimulated respiration; (C) Maximum FCCP-induced respiration.

For all temperatures, no significant differences were observed comparing the Glutamate/Malate oxidation rates (two-way ANOVA, F=0.58, p=0.47), the maximum ADP-stimulated respiration rates (two-way ANOVA, F=0.24, p=0.64) and the maximum FCCP-induced rates (two-way ANOVA, F=3.63, p=0.11) of the C and Azx treatments. Although no statistical differences were found, all Glutamate/Malate oxidation, ADP-induced respiration and FCCP-induced non-coupling rates were visibly lower in the Azx treatments, mainly the non-coupled state rates at 22ºC, comparing to the C treatments (Fig. 3). The exception was the Glutamate/Malate oxidation rates at 5ºC, where the Azx treatments showed higher values (Fig. 3a). It was also not possible to observe significant interactions between the temperature and the azoxystrobin factors for Glutamate/Malate oxidation (two-way ANOVA, F=1.81, p=0.24), maximum ADP-stimulated respiration (two-way ANOVA, F=0.08, p=0.92) and maximum FCCP-induced respiration (two-way ANOVA, F=1.09, p=0.40).

Coupling and mitochondrial oxidative phosphorylation limitation system indexes were calculated for each treatment of the three assessed temperatures (Fig. 4) and two-way ANOVA was performed to compare the calculated values. Concerning the coupling index and according to the obtained results, significant differences (two-way ANOVA, F=4.46, p=0.04) occurred among the three temperatures (5ºC, 22ºC and 27ºC). Mitochondria subjected to 22ºC showed significantly higher levels of coupling, comparing to 5ºC (Dunnett's test, p=0.04) and to 27ºC (Dunnett's test, p=0.02). On the other hand, no significant differences were detected regarding the applied treatments (two-way ANOVA, F=0.38, p=0.70) nor regarding the interaction between the two factors (temperature and treatment) (two-way ANOVA, F=1.07, p=0.43).

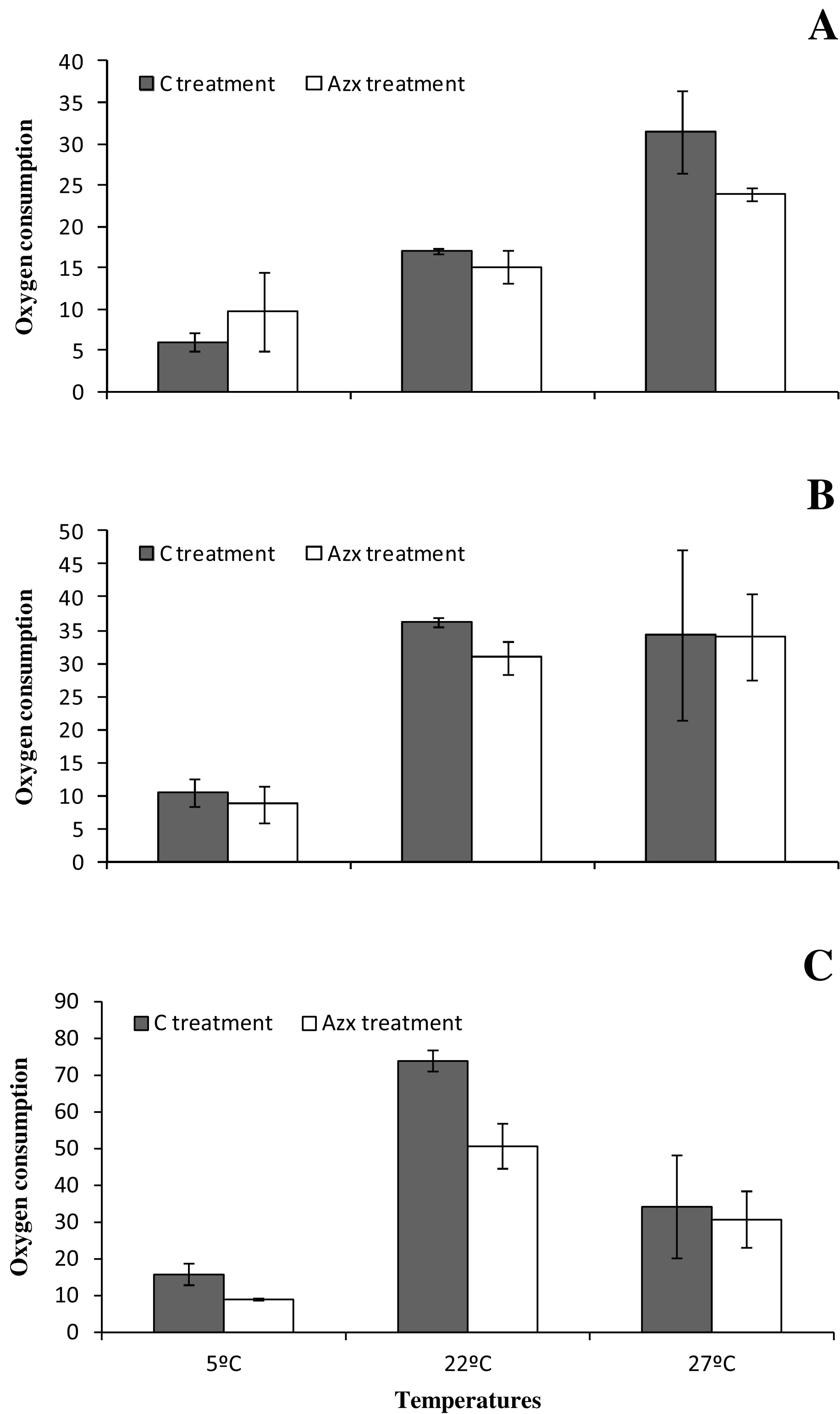

Figure 3. Mean values of oxygen consumption (nmoles min^{-1} .mg $prot^{-1}$) of C treatment (black bars) and Azx treatment (white bars) at 5ºC, 22ºC and 27ºC. (A) Glutamate/Malate oxidation; (B) maximum ADP-stimulated respiration; (C) maximum FCCP-induced respiration.

Concerning the mitochondrial oxidative phosphorylation limitation index, it was also observed significantly different values regarding the temperature (two-way ANOVA, F=15.42, p<0.01). At the standard temperature, mitochondria presented significantly lower values, comparing to both 5ºC and 27ºC (Dunnett's tests, p=0.04 and p<0.01, respectively). Once again, no significant differences were observed regarding the treatments (two-way ANOVA, F=1.59, p=0.26) or the interaction between the assessed factors (two-way ANOVA, F=0.46, p=0.76).

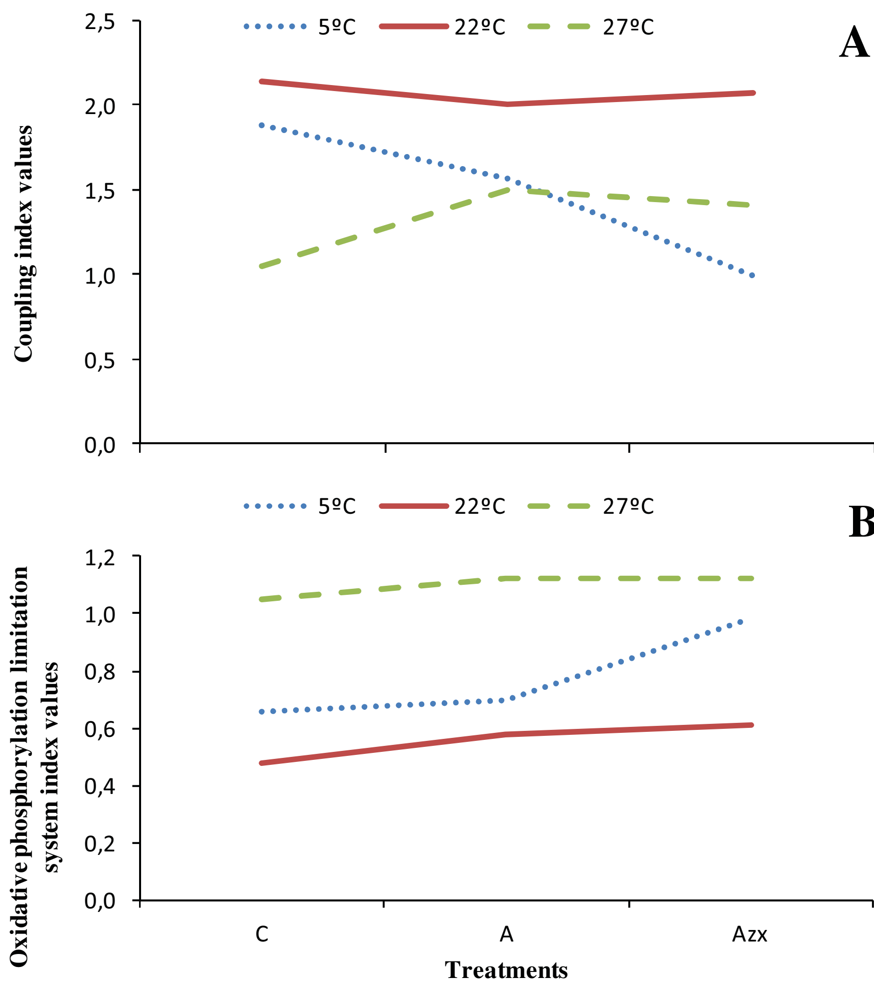

Figure 4. Mean values of the coupling index (A) and the oxidative phosphorylation limitation system index (B) for all three treatments (C, A and Azx) at 5ºC, 22ºC and 27ºC

Chapter 4. Discussion

4.1. Effects of temperature on mortality, molt, feeding behavior and mitochondrial activity of *Carcinus maenas*

Temperature is an extremely important factor in the physiology of the green shore crab *Carcinus maenas*. Since it is an ecthotermal organism, and thus being unable to regulate its internal temperature, this crab's metabolism is susceptible to fluctuations in the environmental temperature. Although it is an eurythermal species, capable of surviving within a range of temperatures between -1ºC and 31ºC (McGaw and Whiteley, 2012), the results obtained in this work seem to indicate that thermal acclimation induces changes on its mitochondrial respiration as well as on its molting and feeding behavior.

At 5ºC no molting individuals were detected. Nevertheless, the number of molts tended to increase at the two warmest experimental temperatures (22ºC and 27ºC). According to Hartnoll (2001), the molting process is influenced by several factors, being one of them the temperature. Generally, with the increase of the temperature, crustaceans tend to molt more frequently (Campos *et al.*, 2009). The process of reserves accumulation needed for molting as well as their mobilization are promoted with temperature increment, resulting in a reduction of the intermolt period duration (Hartnoll, 2001). Food also possesses an imperative role in the molting process. A nutritional deficit could lead to an increase of the duration of the intermolt period, related, once again, with lack of reserves accumulation, but also with hormonal regulation (Hartnoll, 2001; Brown *et al.*, 2003). The molt-inhibiting hormone (MIH), belonging to the crustacean hyperglycemic hormone (CHH) family is produced when the organisms are subjected to various stresses, including starvation (Hartnoll, 2001; Jeon *et al.* 2012). At 5ºC, the animals had the same amount of food than the ones subjected to 22ºC and 27ºC, but approximately 65% of the individuals did not eat during

both of the feeding times. Since they were acclimated at 22ºC before the beginning of the experience and exposed to 5ºC without a gradual acclimation, the sudden temperature change could have promoted a depression of the metabolic rates, inducing the animals to enter in a torpor (McGaw and Whiteley, 2012). Similar physiological and behavioral responses occur in fish experiencing cold-shock stress (Donaldson *et al.*, 2008), resulting, in some extreme cases, in death. Based on the described above it is possible to assume that the sum of the metabolic activity decrease and the lack of food consumption probably led to a decrease on the crab's molting process.

The mitochondrial respiration rates also changed accordingly to temperature. In the oxidative phosphorylation process, an electrochemical gradient is formed by the ejection of protons from the mitochondrial matrix to the intermembrane space, through the inner membrane. Since this mitochondrial membrane is impermeable to protons, these must pass through enzymatic complexes, namely the Complexes I, III and IV, where substrates are oxidized and electrons are transported from electron donors (the oxidized substrates) to electron receptors, being the oxygen the final electron receptor, in a series of redox reactions (Fernandéz-Vizarra *et al.*, 2009). The formed electrochemical gradient is thereby dissipated by the F_1F_0-ATP synthase and used in the production of ATP (Vayssière *et al.*, 1984; Chamberlin, 2004).

Concerning the oxidation of the Glutamate/Malate, a substrate for the Complex I, the respiration rates were lower at 5ºC and higher at 27ºC when compared to the ones of the standard temperature (22ºC). The same results were observed on the tobacco hornworm *Manduca sexta* by Chamberlin (2004), with temperature greatly affecting the substrate oxidation. In Chamberlin's work, the substrate oxidation rates were higher at 35ºC than at 25ºC and 15ºC, possibly due to an increase of the enzymatic efficiency of the electron transfer chain with the temperature.

At 5ºC, it was also observed a decrease in the maximum ADP-stimulated respiration when compared to the standard temperature, being the rates similar between 22ºC and 27ºC. Moreover, it was not possible to detect differences in the oxygen consumption rates between the substrate oxidation and the maximum ADP-stimulated respiration at 27ºC. This means that the mitochondria were uncoupled at that temperature, as indicated by the calculated coupling index and the mitochondrial oxidative phosphorylation limitation index. This decoupling is possibly due to proton leakage from the intermembrane space back to the mitochondrial matrix by a permeabilization of the inner membrane. The proton leakage leads to the electrochemical gradient dissipation, hence nullifying the F_1F_0-ATP synthase activity and halting the ATP production.

Increase of the inner membrane fluidity is one explanation for the membrane permeabilization. This effect was already detected in several organisms, like the earthworm *Lumbricus terrestris*, where membrane fluidity was changed with thermal acclimation (Crockett *et al*, 2001). According to that work, membranes from 15ºC-acclimated organisms are significantly more fluid than membranes from 5ºC-acclimated organisms, differing from the homeoviscous adaptation theory. According to this theory, organisms compensate the temperature increase with a decrease in the membrane fluidity by changing the phospholipid composition. At high temperatures, the saturated fatty acids content increases in order to maintain the membrane stability. On the other hand, at lower temperatures, organisms increase the amount of unsaturated fatty acids (Van Dooremalen *et al.*, 2011). In *C. maenas*, if the unsaturated fatty acids content increases with the temperature, in a similar process like the one of the earthworm, the membrane fluidity also increases, therefore, a reduction of the membrane stability occurs. Low membrane stability could result in loss of proton

impermeability by the mitochondrial inner membrane and dissipation of the electrochemical gradient, consequently uncoupling the mitochondria.

Other possible explanation is the expression of uncoupling proteins (UCP) and their integration in the mitochondrial inner membrane. These proteins dissipate the electrochemical gradient by promoting proton backflow (Ježek and Borecký, 1996; Porter, 2006) and are produced to prevent mitochondrial membrane damage by reactive oxygen species (ROS), such as superoxide (Dlasková *et al.*, 2010). ROS are produced in normally functioning mitochondria and result from the non-productive release of electrons from the electron transport chain, reacting with molecular oxygen (Nabben *et al*, 2008). This results in an increase of the reduced state of the electron transfer chain. In result, cells possess antioxidant mechanisms that counteract the production of ROS, like ubiquinol and vitamin E. Other mechanism is the dissipation of the electrochemical gradient by decoupling the mitochondria. Some uncoupling proteins, UCP 2 and UCP 3, had been proven to be efficient in the reduction of ROS production being the absence of UCP 1 and UCP 2 associated with ROS production increase (Dlasková *et al.*, 2010). By uncoupling the mitochondria using UCP, the organisms prevent cell damage by reducing the production and release of ROS.

The non-coupled FCCP-induced rates, representing the maximum respiration, showed differences among the three temperatures. As expected, at the lowest temperature (5ºC), the oxygen consumption rates were also the lowest, but at the highest temperature (27ºC) the oxygen consumption was also lower compared to the standard temperature (22ºC), in opposition to the expectations. A possible explanation to this mitochondrial behavior may reside in a decrease of the protein synthesis of the oxidative phosphorylation complexes, at higher temperatures (van den Bogert *et al.*, 1985). In a study performed by Murphy *et al.* (1980) using *Saccharomyces cerevisiae*

mutant strain grown at 28ºC, it was observed a significant reduction of var1 protein, a mitochondrial ribossomal protein that is part of the small subunit of mitochondrial ribossomes (Mason *et al.*, 1996). In other mutant strain grown at 36ºC, not only occurred the inhibition of the synthesis of var1 protein, but the inhibition of formation of subunit 1 of cytochrome oxidase (complex IV) as well (Murphy *et al.*, 1980). The mitochondrial ribossomes are responsible for the synthesis of some polypeptides that integrate the protein complexes involved in the oxidative phosphorylation and the synthesis inhibition of these polypeptides lead to a decrease of the oxidation of Glutamate/Malate substrate, hence decreasing the oxygen consumption by the mitochondria (van den Bogert *et al.*, 1985).

Although there were significant physiological responses by the *C. maenas* to thermal acclimation, it was registered low mortality for all temperatures. Therefore, it is possible to assume that this crab possesses mechanisms that counteract adverse thermal variations, allowing it to survive at different latitudes.

4.2. Effects of azoxystrobin on *Carcinus maenas*

Concerning the influence of the fungicide azoxystrobin on the mitochondrial respiration of *C. maenas*, no effects were observed when the organisms were exposed at 25 µg L^{-1} for three days long. This pesticide's mode of action consists in binding to cytochrome b in the mitochondrial Complex III, blocking the electron transference between the cytochromes b and c and, therefore, halting the production of ATP (Sauter *et al.,* 1999; Bartlett *et al.*, 2001; Bartlett *et al.*, 2002; Cedergreen *et al.*, 2006). Its innovative mode of action made it the world's biggest selling fungicide (Sauter *et al.*, 1999), being used worldwide. Nevertheless its great commercial success, azoxystrobin can be hazardous to non-target species. Although presenting low toxicity to mammals,

birds and terrestrial invertebrates, it was demonstrated that azoxystrobin can be very dangerous to fish and aquatic invertebrates (Bartlett *et* al., 2002; Olsvik *et al.*, 2010; Rasmussen *et al.*, 2012; Rodrigues *et al.*, 2013) and it can easily enters into aquatic systems from treated fields due to runoff and leaching processes (Warming *et al.*, 2009; Deb *et al.,* 2010). According to Jørgensen *et al.* (2012), azoxystrobin is the most common pesticide in stream waters and, even though the EU pesticide limit for both drinking water and groundwater is 0.1 µg L^{-1} (Boutron *et al.*, 2009), it has been found at 11.1 µg L^{-1} (Liess and von der Ohe, 2005) and at 29.7 µg L^{-1} (Berenzen *et al.*, 2005) in Germany. Concerning the concentration needed to kill 50% of the individuals exposed to azoxystrobin (LC_{50}), it is possible to observe different susceptibilities to this pesticide, regarding the assessed species. Fish and amphibians present LC_{50} values between 1030 and 1240 µg L^{-1} (Hooser *et al.*, 2012; Jørgensen *et al.*, 2012), while, on the other hand, aquatic invertebrates seem to be much more susceptible, with LC_{50} values between 40 and 370 µg L^{-1} (Ochoa-Acuña *et al.*, 2009; Rodrigues *et al*, 2013).

Nonetheless the concentration used in this work (25 µg L^{-1}) to assessed physiological effects on mitochondria of *C. maenas* was similar to the highest concentrations found in the environment (Berenzen *et al.*, 2005), it was also much lower than the published lethal concentrations for both aquatic vertebrates and invertebrates. This could explain the absence of azoxystrobin-induced effects on the mitochondria of the crabs exposed to this pesticide, even though the mitochondria are the physiological target of azoxystrobin. Nevertheless, even if no effects were observed in the mitochondrial respiration, other physiological processes could have been compromised by the contaminant exposure.

Chapter 5. Conclusions

5.1. Final conclusions

Although eurythermal and occupying a great range of latitudes, *C. maenas* molting, feeding behavior and mitochondrial activity are affected by thermal acclimation at extreme temperatures. Low temperatures (5ºC) seem to cause metabolic depression on this species, resulting in molting interruption, decrease in the feeding activity and significant reduction of the oxidative phosphorylation process. At higher temperatures (27ºC), the mitochondria seem to decouple and they present lower maximum respiration rates than at 22ºC.

On the other hand, at 25 $\mu g\ L^{-1}$, a sub-lethal concentration based on the highest reported concentrations found in the environment (but much lower when compared to the published lethal concentrations), azoxystrobin showed no effects on the mitochondrial activity of the green crab *C. maenas* (when exposed to this pesticide for three days long). Hereafter, a higher azoxystrobin exposure concentration is needed to observe effects of this pesticide on the mitochondrial activity of *C. maenas*.

Although this crab presented significant responses to thermal acclimation, it also revealed some physiological tolerance to both extreme thermal variations and pesticide exposure, with very low mortality rates, which could partially explain this species' capacity to settle populations at different latitudes and its great invasive potential.

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