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Caffeine: a novel endocrine disruptor in molluscs?

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Contents

Abstract

Caffeine is a potent neuroactive drug widely consumed worldwide. It is not completely eliminated in waste water treatment plants (WWTPs), so as a reason it is a pseudo-persistent and ubiquitous contaminant in aquatic environment. Little is known about the effect of relevant environmental caffeine concentrations on marine organisms. Therefore, the objective of this experiment is to assay the effect of a 5-days caffeine exposure at 500 ng/l concentration in the Mediterranean mussel (*Mytilus galloprovincialis*) at transcript level of 1) potentially gene specific biomarkers, 2) reproduction, 3) oxidative stress and 4) immunological related genes.

A total of 8 mussels of each group (control and treatment) were randomly sacrificed. Samples of gills, gonad and digestive gland tissue of each bivalve were extracted for RNA extraction, cDNA synthesis and Real-Time quantitative PCR analysis.

Our results showed 1) a significant increase in *adenosine receptor A2a* (*arA2a*), but not in *dopamine receptor D2* (drD2) expression in digestive gland tissue. We also detected 2) a significant downregulation in *cyclooxygenase2* (*cox2*) and *vitellogenin* (*vtg*) expression in gonad. No differences were observed in 3) *glutathione-s-transferase* (*gst*), *heat shock protein 70* (*hsp70*), 4) *myticin B* or *mytilin B* expression.

These results suggest that 1) *arA2a* could be a potentially gene specific biomarker of caffeine contamination; 2) caffeine could be a potentially endocrine disruptor in molluscs 3) caffeine concentration of 500 ng/l (relevant environmental concentration), is not enough to increase *gst* and *hsp70* expression and therefore, to have a negative impact on mussels health status; and 4) does not affect immunological system at *myticin B* and *mytilin B* level.

Keywords: *caffeine, endocrine disruptor, mussel, Mytilus galloprovincialis, adenosine receptor, biomarker, qPCR.*

Resumen

La cafeína es una potente droga neuroactiva ampliamente consumida en todo el mundo. No es eliminada completamente en las estaciones depuradoras de aguas residuales (EDARs), así que como consecuencia, es un contaminante pseudo-persistente y ubiquista en el medio acuático. Existe poco conocimiento acerca del efecto de concentraciones relevantes de cafeína del medio en los organismos marinos. Por tanto, el objetivo de este experimento es estudiar el efecto de una exposición de cafeína de 5 días a una concentración de 500 ng/l en la clóchina (*Mytilus galloprovincialis*) a nivel de transcritos de 1) genes biomarcadores potencialmente específicos, además de genes relacionados con 2) reproducción, 3) estrés oxidativo y 4) sistema inmunológico.

Un total de 8 mejillones de cada grupo (control y tratamiento) fueron sacrificados al azar. Se extrajeron muestras de tejido de branquias, gónada y glándula digestiva de cada bivalvo para una posterior extracción de ARN, síntesis de ADNc y análisis de PCR cuantitativa en tiempo real.

Nuestros resultados mostraron 1) un incremento significativo en la expresión del *receptor de adenosina A2a* (*arA2a*), pero no del *receptor de dopamina D2* (*drD2*) en glándula digestiva. Además, detectamos 2) una disminución significativa en la expresión de *ciclooxigenasa2* (*cox2*) y *vitelogenina* (*vtg*) en gónada. No se observaron diferencias en la expresión de 3) *glutatión-s-transferasa* (*gst*), *proteína de choque térmico 70* (*hsp70*), 4) *miticinaB* y *mitilinaB*.

Estos resultados sugieren que 1) *arA2a* podría ser un gen potencialmente específico de contaminación por cafeína; 2) la cafeína podría ser un disruptor endocrino en moluscos; 3) una concentración de cafeína de 500 ng/l (concentración relevante del medio), no es suficiente para aumentar la expresión de *gst* y *hsp70* y, por tanto, para generar un impacto negativo en el estado de salud de las clóchinas; ni 4) para afectar al sistema inmunológico a nivel de miticinaB y mitilinaB.

Palabras clave: *cafeína, disruptor endocrino, mejillón, Mytilus galloprovincialis, receptor de adenosina, biomarcador, qPCR.*

Introduction

1.1. Contaminants of emerging concerns in aquatic systems

The development caused by the industrial revolution has given rise to an undoubted improvement in the quality of life. This contribution to human comfort and welfare was accompanied by an increase in the demand for anthropogenic activities, leading to the use and consequent release of new pollutants in aquatic systems (Rocha *et al*., 2018), such as current-use pesticides (CUPs), pharmaceuticals and personal care products (PPCPs), veterinary products, stimulants, food additives, corrosion inhibitors, biocides and illicit drugs (Cizmas *et al*., 2015; Brumovski *et al*., 2017) that are collectively known as emerging contaminants (ECs) (Keerthanan *et al*., 2020). Among them, pharmaceuticals and personal care products (PPCPs) have received increasing attention because some of them are biologically active drugs, so its presence even in trace amounts can negatively affect the health status of aquatic organisms (Ginebreda *et al*., 2010). The term PPCPs encompasses different substances, such as human and veterinary medicine drugs, fragrances, sunscreen agents, and cosmetic ingredients (Richardson *et al*., 2005; Lin *et al*., 2016).

Generally, the most frequently detected PPCPs in aquatic systems are antibiotics, analgesics, anticancer drugs, lipid regulators, psychopharmacological agents, contraceptive hormones, antiepileptics, anticonvulsants and β-blockers (Kümmerer, 2001; Jones *et al*., 2001; Nikolaou *et al*., 2007; Fatta-kasinos *et al*., 2011), although one of the most commonly used substance included in the list of PCPPs is the caffeine (Keerthanan *et al*., 2020).

According with Aguirre-Martínez *et al*. (2018), during therapeutics, some active PPCPs ingredients are not completely metabolized and are excreted into wastewater treatment plants (WWTPs). In addition, conventional WWTPs were not designed to remove synthetic contaminants and, consequently, removal rates of different substances range from almost 100% to less of 10% (Liu *et al*., 2013; Brumovski *et al*.,2017; Yang *et al*., 2017). Therefore, although there is a wide range of sources of PCPPs in the environment (i.e., hospitals, pharmaceutical factories, livestock farms, households and WWTPs) (Keerthanan *et al*., 2020), in general, discharges from WWTPs represents the dominant input pathway to the environment y developed countries (Verlicchi *et al*., 2012; Venkatesan and Halden, 2014).

Commonly, concentrations of usual PCPPs in freshwater are higher than in seawater, and distribution and behaviour of many PCPPs in freshwater is well documented (for

review see: Ebele *et al*., 2017; Adeleye *et al*., 2022), while in coastal and marine waters it has been much less studied (Arpin-Pont *et al*., 2014).

In the Russian part of the Baltic Sea, Chernova *et al*. (2021) detected caffeine (81% of samples), carbamazepine (81%), ketoprofen (60%), diclofenac (23%), ciprofloxacin, trimethoprim and clarithromycin in a range from 0.1 to 4452 ng/l. In the East coast of South Africa (Indian Ocean), Ngubane *et al*. (2019) reported the presence of naproxen and ibuprofen in a concentration up to 0.16 and 0.17 µg/l respectively. In the Chinese coast of the Taiwan Strait, Chen *et al*. (2021) found the presence of carbadox, carbamazepine, thiabendazole, diphendydramine, cotinine, erythromycin, oxolinic acid, oxytetracycline, naproxen, gemfibrozil, triclocarban and cimetidine in all samples. In the Eastern Mediterranean Sea, Alygizakis *et al*. (2016) detected amoxicillin, caffeine and salicylic acid with concentrations in the range of 5-127.8, 5.2-78.2 and 0.4-53.3 ng/l respectively.

In offshore waters of the Western Mediterranean Sea, Brumovski *et al*. (2017) recorded caffeine, carbamazepine, naproxen, paracetamol and sulfamethoxazole in 100% of samples in the range of 0.03-0.111, 0.32-0.89, 0.0038-0.0133, 0.468-1.70 and 0.007-0.017 ng/l respectively. Moreover, they found ibuprofen in 50% of samples in the range of 0.063-1.08 ng/l (Figure 1).

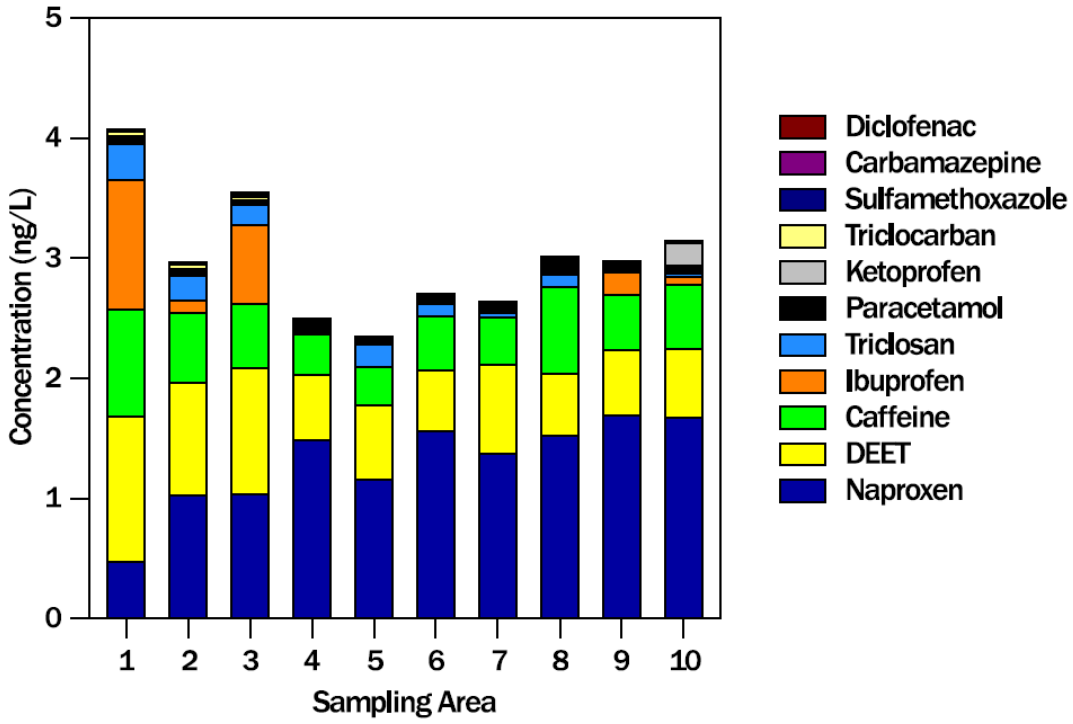

Figure 1: Detected concentrations of pharmaceuticals and personal care products found in the Western Mediterranean Sea in ng/L (November 2014) by Brumovski *et al*. (2017).

1.2. Caffeine presence in aquatic systems

Caffeine is a potent neuroactive drug that is recognized as a ubiquitous contaminant in aquatic systems (del Rey *et al*., 2011). In average, there is an estimated global consumption of 70 mg/person/day (Buerge *et al*., 2003); nevertheless 90% of adults are regular caffeine consumers with a mean daily intake of 227 mg/day (Nehlig, 2018). It is the active psychostimulant of widely consumed beverages (Acquas, 2002), so it is commonly recognized as the most popular and broadly used stimulant in humans worldwide (Lawrence *et al*., 2005; Moore *et al*., 2008; Kosma *et al*., 2014; Kleywegt *et al*., 2019).

Caffeine is an alkaloid of the xanthine group whose elemental composition is 3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione ($C_8H_{10}N_4O_2$), with a high-water solubility (13 g/l), a very low volatility and a molecular weight 194 g/mol (Silva *et al*., 2014; Edwards *et al*., 2015; Asghar *et al*., 2018). It is among the most common organic contaminants of surface waters being detected in streams, lakes, estuaries and oceans (Buerge *et al*., 2003), however, interestingly it has an estimated half-life of about 1.5 days (Lam *et al*., 2004). Therefore, it has been considered as a "pseudo-persistent" compound within surface waters (Fernández *et al*., 2010; Silva *et al*., 2014).

According with literature, after caffeine ingestion, about 1-10% is excreted in the urine after being metabolized by the body (Montagner *et al*., 2014; Beltrame *et al*., 2018). Then, the excreted caffeine enters the water cycle by reaching WWTPs. Li *et al*. (2018) reported a removal efficiency above 99.5% during the biodegradation treatment process. However, other authors reported less removal efficiency (95%) in WWTPs (Deblonde *et al*., 2011; Jacobs *et al*., 2012; Kosma *et al*., 2014).

Despite the fact that it is generally shows an excellent removal efficiency (Li *et al*., 2019), the input of caffeine into the aquatic systems is much higher than which is degraded (Zhang *et al*., 2013; Zhou *et al*., 2018). Therefore, it is constantly released to the aquatic environment via wastewater effluent and other anthropogenic activities (del Rey *et al*., 2011). As a consequence, caffeine has been considered as an indicator of anthropogenic contamination as well as a source-specific indicator for wastewater in surface waters (Buerge *et al*., 2003; Dafouz *et al*., 2018).

In fact, Weigel *et al*. (2004) detected a caffeine concentration of 293 mg/l in a WWTP influent and 0.18mg/l in its effluent; by the same way, Heberer *et al*. (2002) reported a caffeine concentration of 230 mg/l and 18 mg/l in a WWTP influent and effluent respectively. Curiously, several studies have reported higher caffeine concentrations in rivers and estuaries than in WWTPs treated effluents (Komori *et al*., 2013), suggesting

that untreated effluents are discharge reaching coastal areas (Froehner *et al*., 2011; Munro *et al*., 2019; Williams *et al*., 2019). Smith *et al*. (2015) reported a caffeine concentration of 13 mg/l in an unlicensed effluent outfall in Kuwait.

An increasing number of studies has confirmed the presence of caffeine in different aquatic environmental compartments (Brumovski *et al*., 2017; Vieira *et al*., 2022). In this context, Chernova *et al*. (2021) detected a concentration in the range of 0.8 ng/l and 181 ng/l in the Russian part of the Baltic Sea, while in the southern Baltic Sea, Szymczycha *et al*. (2020) reported concentrations ranging from below the detection limit to 1528 ng/l. In the North Sea, Weigel *et al*. (2002) reported a caffeine concentration of 16 ng/l. The highest caffeine concentration detected in sea water was 11 µg/l for the Darwin Harbour, in Northern Australia (French *et al*., 2015). Nevertheless, environmental concentrations are comprised in the range of 2-1600 ng/l, with higher values reported in WWTPs effluents, harbour seawater, estuaries and coastal waters (Figure 2) (Capulopo *et al*., 2016). Siegener and Chen (2002) reported caffeine concentrations in Boston Harbour seawater ranged from 140 ng/l to 1600 ng/.

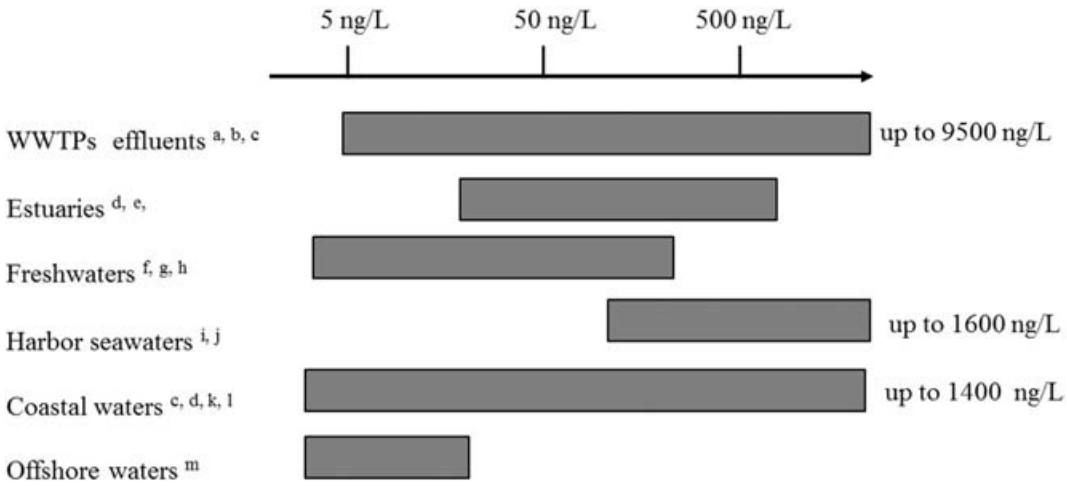

Figure 2: Levels of CF measured within surface waters and effluents from WWTPs. [a]Poiger *et al*. (2003), [b]Deblonde *et al*. (2011), [c]Comeau *et al*. (2008), [d] del Rey *et al*. (2011), [e]Fernández-Gómez *et al*. (2013), [f]Kolpin *et al*. (2002), [g]Buerge *et al*. (2003), [h]Choi *et al*. (2008), [i]Siegener and Chen (2002), [j]Weigel *et al*. (2004), [k]Peeler *et al*. (2006), [l]Singh *et al*. (2010), [m]Weigel *et al*. (2002) (cited by Capulopo *et al*., 2016).

In the Southern and Eastern Mediterranean Sea, Romagnoli *et al*. (2016) reported low caffeine concentrations ranging from 0.01 to 0.17 ng/l. Similarly, in offshore waters of the Western Mediterranean Sea, Brumovski *et al*., (2017) detected caffeine in all samples in the range of 0.03 ng/l and 0.111 ng/l.

The monitoring studies are necessary to evaluate the space-time distribution of caffeine concentration and asses the potential toxicity effect on biota (Li *et al*., 2019; Roveri *et al*., 2020).

1.3. Caffeine biomarkers in aquatic organisms: past, present and future

The increase in the prevalence of caffeine in the aquatic environment and the uncertainty of the effects on benthic invertebrates has given rise to new experiments to asses it`s repercussions on aquatic organisms (del Rey *et al*., 2011). One of the most common methods to evaluate the effect of a contaminant is based on bioindicator organisms (biomonitoring), measuring properties that can be used as an early warning system (Dailianis, 2001). In this context, bivalves such as genus *Mytilus* are a good choice as model species due to their worldwide distribution, high sensitivity to stress factors, sedentary, long-lived and filter feeding habitats (suspended matter while breathing and feeding) (del Rey *et al*., 2011; Gagné *et al*., 2011; Cappello *et al*., 2013; Boillot *et al*., 2015; Gornati *et al*., 2016). These characteristics gave rise to Mussel Watch programme in the mid-1970s, providing high-resolution temporally aligned archives of environmental variability (Schöne *et al*., 2016).

Bioindicators have been used to identify structures or processes indicating exposure or effects measured at high levels of organization (i.e., organisms, populations). In contrast, biomarkers are defined as any functional measure of exposure that is characterized at a sub-organism level (i.e., molecular, biochemical, cellular). Biomarkers provide two key attributes in environmental toxicology: 1) biomarkers characterize just de bioavailable fraction of environmental chemicals, and 2) integrate the interactive effects of complex mixtures of chemicals (Bartell, 2006).

As reported before, concentrations of caffeine typically detected in aquatic systems are in the range of low nanogram per litter. Traditional ecotoxicology endpoint studies (i.e., EC50) suggest that these levels of caffeine currently detected do not suppose a threat to aquatic organisms. However, potential sublethal levels are not detected by traditional endpoint experiments (del Rey *et al*., 2011).

Recent studies based on biomarkers and sublethal effects evidences that caffeine may enhance the production of Reactive Oxygen Species (ROS) (Li *et al*., 2019). These, give rise the activation of antioxidant enzymes mechanisms (i.e., catalase, superoxide dismutase, glutathione reductase) in an attempt to reduce the oxidative damage. Nevertheless, antioxidant enzymes are not able to eliminate the excess of ROS and, as

a consequence, there is cellular damage due to lipid peroxidation (del Rey *et al*., 2011; Aguirre-Martínez *et al*., 2015; Cruz *et al*., 2016; Capulopo *et al*., 2016; Pires *et al*., 2016a, Pires *et al*., 2016b). In this context, Aguirre-Martínez *et al*. (2013a, 2015, 2016) proposed the application of general stress biomarkers to assess the health status in animals exposed to caffeine, specifically lysosomal membrane stability of haemocytes, antioxidant enzymes activity and lipid peroxidation.

Nevertheless, the increase in ROS, lipid peroxidation or antioxidant enzymes activity is not specific of caffeine contamination. Stoliar *et al*. (2022) reported an increase in antioxidant activity in the bivalve *Unio tumidus* after exposure to ibuprofen. Besides, in *Daphnia magna*, Gómez-Oliván *et al*. (2014) observed a significant increase in lipid peroxidation and antioxidant activities after diclofenac, naproxen and ibuprofen exposure.

According with literature, ROS presence leads to genotoxicity. Aguirre-Martínez *et al*. (2015) reported DNA damage in bivalve *Corbicula fluminea* after exposed to 50 µg/l of caffeine, but not when 15 µg/l, which suggest that there is no DNA damage at relevant environmental concentrations of caffeine. Moreover, since the cause of DNA damage are ROS, this is not an exclusive biomarker of this pollutant. In fact, Aguirre-Martínez *et al*. (2013b) observed DNA damage in the crab *Carcinus maenas* exposed to a concentration of 50 µg/l of novobiocin. Although, interestingly in amphipod *Ampelisca brevicornis*, Maranho *et al*. (2014) detected DNA damage decreased after caffeine, carbamazepine, propranolol, 17α-ethynylestradiol and ibuprofen exposure.

A reduction in energy reserves has also been in relation with the presence of contaminants such as caffeine in marine organisms. The reason is an increase in the metabolic activity to fight against oxidative stress (ROS) with the induction of antioxidant enzymes (Vieira *et al*., 2022). In fact, a decrease in glycogen content was observed after exposure to relevant environmental caffeine concentrations in the bivalve *Ruditapes phillippinarum* (Cruz *et al*., 2016; Marchi *et al*., 2022), *Mytilus galloprovincialis* (Marchi *et al*., 2022), and the polychaetes *Diopatra neopalitana* (Pires *et al*., 2016a) and *Hediste diversicolor* (Pires *et al*., 2016b).

Caffeine is a potent neuroactive drug that might interact with adenosine and dopamine receptors in invertebrates (Chen, 1995; Mustard, 2013; Kucharski and Maleszka, 2005; Aguirre-Martínez *et al*., 2018), affecting the nervous systems of animals (Stiles, 1992 cited by Vieira *et al*., 2022). Moreover, acetylcholinesterase (AChE) is an enzyme involved in the proper coordination of neuromuscular transmission and in the normal functioning of the sensory neuromuscular system (Van der Oost *et al*., 2003). In this

regard, the activity of AChE has been used to assess potential neurotoxic effects of various contaminants (Maranho *et al*., 2014). Aguirre-Martínez *et al*. (2018) reported a significant decrease in AChE activity in *C. fluminea* exposed to caffeine concentration of 5 µg/l. However, they also observed an increment when exposed to 50 µg/l. Furthermore, in a previous study published by Aguirre-Martínez *et al*. (2016) with *R. phillippinarum*, the activity of AChE decreased in a dose dependent manner with caffeine concentration (range from 5 µg/l to 50 µg/l).

The usefulness of traditional biomarkers such as antioxidant enzyme activities, lipid peroxidation or lysosomal membrane stability has been well demonstrated (Aguirre-Martínez *et al.*, 2018; Godoi *et al*., 2020), however, they have limits and appear to vary in how sensitive and robust are in certain cases (Lacroix *et al*., 2014). The use of transcripts biomarkers has emerged as a result of recent progress in bivalve genomics, providing an increasing amount of available sequences in public data bases (Zhang *et al*., 2012). In that regard, the use of easy and low-cost efficient techniques to evaluate mRNA levels, such as Real-Time quantitative polymerase chain reaction (RT-qPCR) provide a great opportunity to measure changes in gene expression in response to stress and pollutants (Bourlat *et al*., 2013).

The majority of experiments focus on studying the effect of caffeine contamination on health status of organisms, however, there is a lack of specific biomarkers to detect caffeine pollution in marine environment. Moreover, most ecotoxicological studies include the use of biomarkers of early defence, related to detoxification, paying less attention in the use of biomarkers related to the neuroendocrine system, in relation with organism`s survival, growth and reproduction (Aguirre-Martínez *et al*., 2018). Therefore, this survey pretends to evaluate the effect of caffeine in traditional biomarkers related to detoxification, biomarkers related to neuroendocrine and immune system, and potentially novel specific biomarkers of this widespread contamination from a transcriptomic point of view.

2. Objectives

2.1. General objective

The principal aim of this survey is to evaluate the effect of a short-term (five days) caffeine exposure at a relevant environmental concentration in the expression of a variety of genes in *Mytilus galloprovincialis* by Real-Time qPCR analysis.

2.2. Specific objectives

- To assess the effect of caffeine exposure on potentially specific gene expression biomarkers (*adenosine receptor A2a* and *dopamine receptor D2*) in lamellae gills and digestive gland tissues.
- To assess the effect of caffeine exposure on reproduction related genes (*cyclooxygenase* and *vitellogenin*) in gonad tissues.
- To assess the effect of caffeine exposure on oxidate stress related genes (*heat shock protein 70* and *glutathione-s-transferase*) in lamellae gills and digestive gland tissues.
- To assess the effect of caffeine exposure on immunological system (*myticin B* and *mytilin B*) in lamellae gills and digestive gland tissues.

3. Material and methods

3.1. Study organisms, acclimation conditions and bioassay

A total of 40 adult Mediterranean mussels (*Mytilus galloprovincialis*) were collected in the port of Valencia, Spain (N 39º26´16´´ W 0º19´02´´) in April 2022 (Figure 3). Mussels (5.66 ± 0.85 cm) (mean ± SD) were picked from the same area on a single day and transported immediately to Universidad Católica de Valencia (UCV) in seawater tanks.

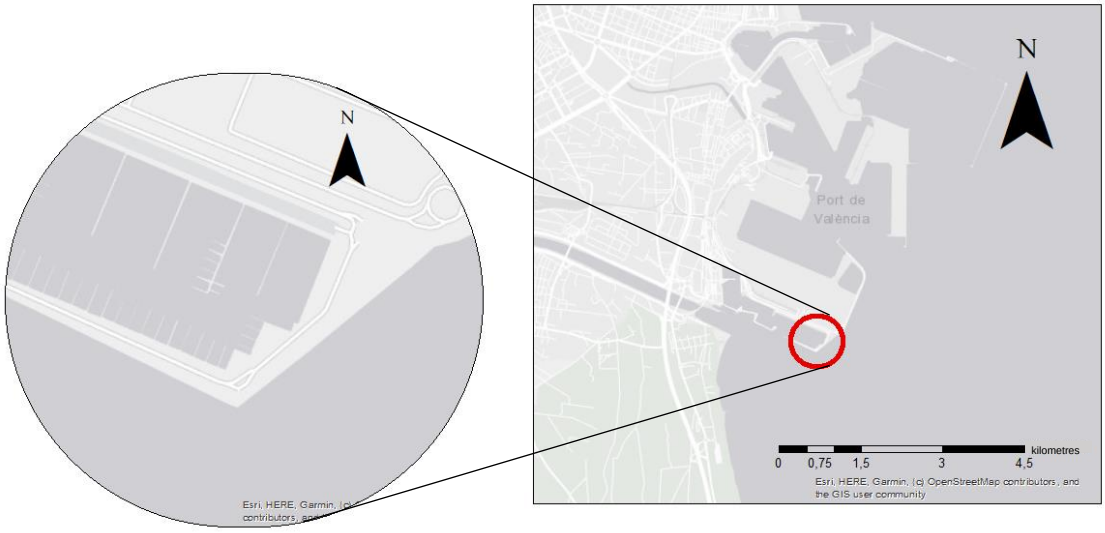

Figure 3: Location of the *M. galloprovincialis* collecting site in the port of Valencia, Spain (N 39º26´16´´ W 0º19´02´´).

In the laboratory, bivalves were randomly subdivided into 2 groups: a control group (5.73 ± 0.92 cm; 25.41 ± 12.79 g) and a treatment group (5.56 ± 0.71 cm; 23.95 ± 9.39 g) of 20 individuals each. During the 7-day acclimatation period, mussels were kept for 3 days in seawater and 4 days in Artificial Sea Water (ASW) adjusted at a salinity of 36 ppt with seawater in a 1:1 ratio. Mussels were kept at the UCV laboratory facilities in 2 different tanks with constant temperature of 18°C, continuous aeration and an internal water filtering system. Mussels were fed 3 days a week after 100% water change, with 3ml of phytoplankton gel (EasyRoti300, Easy Reefs®, Spain), 250 ml of *Isochrysis galbana* (2.27×10^7 cells/ml) and 75ml of *Tetraselmis suecica* (4.9×10^6 cells/ml) that were gently provided by Instituto de Investigación en Ciencia Marina y Medio ambiente (IMEDMAR-UCV, Spain).

After acclimatation period, mussels were transferred to plastic vessels containing 20 litres of ASW. Treatment group was exposed for 5 days to a caffeine concentration of

500 ng/l, which is the range of concentrations reported from coastal marine systems (Siegner and Chen, 2002; Comeau *et al*., 2008; Szymczycha *et al*., 2020).

Caffeine stock solution at 20 mg/l was prepared fresh using commercial caffeine (XTRAZE®, Germany) dissolved in the same ASW. Stock solution was dispensed at the beginning of the experiment and after the water change while feeding.

3.2. Sample preparation and RNA extraction

After 5 days of treatment, 8 mussels from each group (control and treatment) were randomly sacrificed and dissected in a UV light sterilized laminar flow cabinet to avoid RNases. Samples of gill lamellae, gonad and digestive gland tissue of each bivalve were extracted with forceps and scalpel (Eggermont *et al*., 2020). Subsequently, samples were collected in pre-autoclaved eppendorf tubes with 0.5 ml of RNA*later*™ solution (10427114, Invitrogen™, Spain) and immediately stored at -80°C. Due to low gonad development, no mussel gender data were taken during sampling (Figure 4).

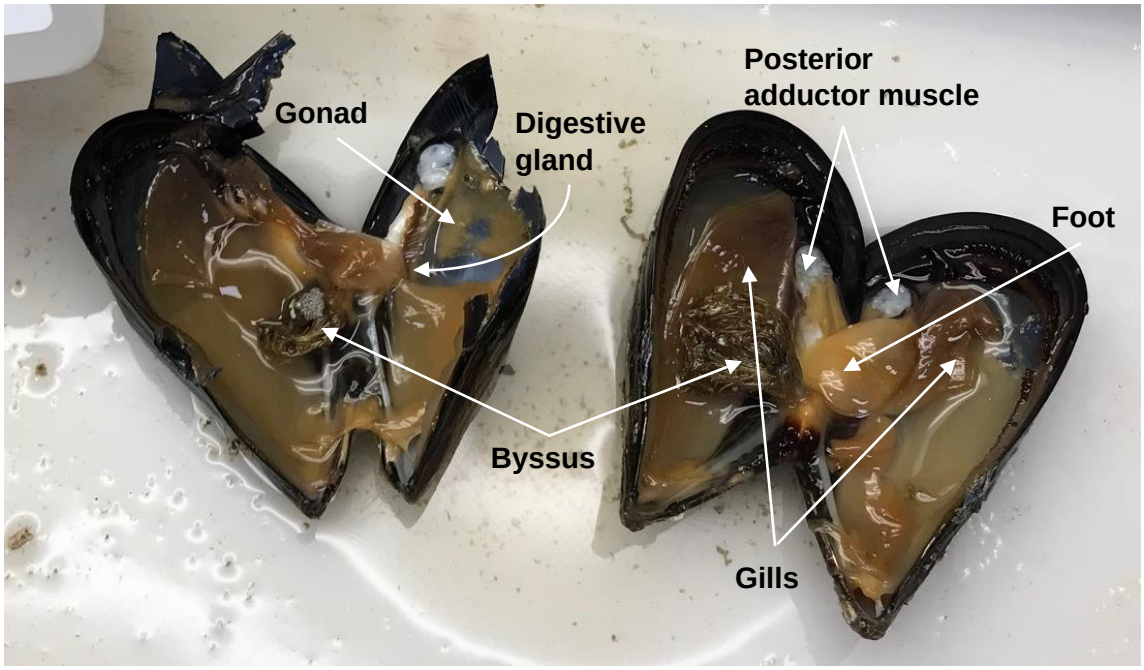

Figure 4: General anatomy of the Mediterranean mussel (*M. galloprovincialis*). Ventral view after forcing the valves to open with an awl.

The subsequent methodology described for RNA extraction and cDNA synthesis was carried-out in a UV light sterilized Class II Biological Safety Cabinet to protect RNA from RNases and obtains its maximum integrity.

Total RNA was isolated with Trizol™ solution (12044977, Invitrogen™, USA) according to the following protocol. Approximately 70 mg of tissue was ground in a 2 ml eppendorf tube with 700 µl of Trizol™ solution. After 5 minutes at room temperature and 3 minutes on ice it was centrifuged at 12.000 x g for 10 minutes at 4°C. Later, the clear supernatant was transferred to a new eppendorf tube and incubated at room temperature for 5 minutes. To separate the phases, 200 µl of chloroform were added, mixed by triple inversion for 15 seconds, and incubated at room temperature for 3 minutes. Then, it was centrifuged at 12.000 x g for 15 minutes at 4°C. At this moment, RNA was isolated by pipetting the colourless upper aqueous phase into a new eppendorf tube. Isolated RNA was precipitated by adding 500 µl of isopropanol, and mixing by triple inversion for 15 seconds. After centrifugation at 12.000 x g for 10 minutes at 4°C, the supernatant was discarded (with a micropipette) and the pellet (precipitated RNA) resuspend by vortex in 1 ml of 75% ethanol to wash the RNA. Finally, the eppendorf tube was centrifuged at 7.500 x g for 5 minutes at 4°C and the supernatant was discarded. Prior to store RNA at -80°C, it was dried at room temperature for 15 minutes (eppendorf tube was dried with a sterile swab too) and resuspend in 25 µl of RNase-free water (4387937, Invitrogen™, USA) previously heated at 55°C. RNA was not treated with DNase I.

RNA concentration and purity were assessed by spectrophotometer methods, measuring the absorbance ratio at 260/280 nm and 260/230 nm (NanoDrop 2000 spectrophotometer, Thermo Fisher Scientific, USA) (Desjardins and Conklin, 2010; Mullegama *et al*., 2018). Each RNA sample was diluted with RNase-free water to obtain an approximate concentration of 1 µg/µl.

Moreover, to asses RNA integrity and genomic DNA contamination, a standard non-denaturing agar gel electrophoresis with bleach was performed as described by Aranda *et al*. (2012) (as a substitute of a denaturing gel electrophoresis) to avoid RNases while electrophoresis running. Briefly, 0.5% v/v (500 µl of household bleach per 100ml gel), and 5µl of Real Safe nucleic acid staining (Durviz S.L., Spain) were added to 1% agarose gel electrophoresis with 1X TAE. Finally, 6X loading buffer were pipetted to a total of 12 RNA samples (2 per each group and tissue) and gel was run for 1 hour with constant voltage (100V) prior to imaging under UV transillumination.

3.3. cDNA synthesis

The first-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (4368814, Applied biosystems™, USA) according with manufacturer´s instructions. Briefly, 2X RT master mix were pipetted with 1 µl of RNA and 9 µl of RNase-

free water on PCR tubes to a final volume of 20 µl. Each master mix reaction was composed by 10X RT buffer (2 µl), 25X dNTPs (0.8 µl), 10X RT Random Primers (2 µl), MultiScribe™ Reverse Transcriptase (1 µl) and RNase-free water (3.2 µl).

Reverse transcription (RT-PCR) was conducted in a 96-well T100 PCR thermal cycler (Biorad, USA) under the following conditions: 10 min at 25°C for primers annealing, 120 min at 37°C for cDNA polymerization and 5 minutes at 85°C for enzyme deactivation.

3.4. Primer design

The selected genes for this gene expression analysis experiment in *M. galloprovincialis* were *vitellogenin* (*vtg*), *heat shock protein 70* (*hsp70*), *glutathione-s-transferase* (*gst*), *myticin B*, *mytilin B*, *adenosine receptor A2a* (*arA2a*), *dopamine receptor D2* (*drD2*) and *cyclooxygenase2* (*cox2*), also called *prostaglandin-endoperoxidase synthase2* (*ptgs2*). Gene *18s rRNA* was selected as reference gene for relative gene expression quantification (Table 1) (Pfaffl, 2001).

Table 1: Primers used in this survey.

Gene	Primer sequence	Reference
vtg	F 5` GATGAAGTCGAAGCAGAGG 3` R 5`GCATACTCCTCCCAAACAG 3`	Fernández-González *et al*., 2021
18s rRNA	F 5`CCTGCTTACCTTCCTCCAT 3` R 5`CCTGCGTGTTATGCTTTGT 3`	Rossi *et al*., 2016
hsp70	F 5`ATAACTACTGAGATATGGCAGGAA 3` R 5` TGGTCGTTGGCTATGATGT 3`	Rossi *et al*., 2016
gst	F 5` AGTTAGAGGCCGAGCTGAAG 3` R 5`TGGAAACCGTCATCATCTGTG 3`	Piscopo *et al*., 2016
myticin B	F 5´ AATGTCTTCGTTGTTCCAG 3` R 5` AATGCCAGTTTCACCTTG 3`	Balseiro *et al*., 2011
mytilin B	F 5` TGAAGGCAGGAGTTATTCTGGC 3` R 5` ACAACGAAGACATTTGCAGTAGC 3`	Balseiro *et al.*, 2011
cox2	F 5` GTGCAAACTGTAAATGGTGAAG 3` R 5´ATAGTCCAGGGAGGTTTCC 3`	Figure 6
drD2	F 5` CGTCCTTGTCGTTATGAGTG 3` R 5` CGACCATTATGTCCGCTAC 3`	Figure 7
arA2a	F 5` GTGTTTGCCCTCTGTTGG 3` R 5` GCAGAGTTGATATGGCTGAG 3`	Figure 8

Primers for *vtg*,*18s rRNA*, *hsp70*, *gst*, *myticin B* and *mytilin B* were taken from literature (Table 1). For the rest of genes, primer sequences were designed following the indications of Thornton and Basu (2015). Briefly, a real-time quantitative PCR (RT-qPCR) primer should have an amplicon length between 75bp and 150bp, a GC content between 50% and 60%, a melting temperature (Tm) between 50°C and 60°C, a size between 17 and 24 nucleotides and a 3` GC clamp. Moreover, the selected primers should avoid the formation of secondary structures (i.e., self-dimers, hairpin, hetero-dimer…) and unspecific annealing with cDNA during amplification.

The *M. galloprovincialis* genome was available in the GenBank repository (GenBank Accession No. UYJE01006747.1), however, none of the genes selected for primer design was annotated. Therefore, the strategy followed was the same for *cox2*, *drD2* and *arA2a* genes. On one hand, protein or hypothetical predicted protein sequence of each gene was aligned with other species known proteins (vertebrates and invertebrates) using ClustalW programme (Sievers *et al*., 2014) to infer the most conserved regions. On the other hand, we ran a tblasn programme with *M. galloprovincialis* genome to know the exactly nucleotides of the aminoacidic conserved regions. Then, primers were designed with PrimerQuest® tool taken the conserved regions selected.

3.4.1. Cyclooxigenase primer design

The running of tblastn programme with *M. galloprovincialis* genome revealed that *cox2* gene is composed by 7 exons and 6 introns. This gene structure allowed to design a forward exon-exon junction primer (between the third and fourth exons) (Figure 5) that ensure the absence of gDNA amplification in case of its presence.

M. galloprovincialis prostaglandin-endoperoxidase synthase 2 (VDI75842) was aligned with *Homo sapiens* COX2 (AAA58433), *Cyprinus carpio* COX2 (ASP27472), *Trachysurus vachellii* COX2 (ADG45818), *Crassostrea gigas* COX (NP_001292218.1), *Mytilus edulis* COX2 (CAG2191003.1) and *Mytilus coruscus* COX2 (CAC5375255.1) proteins. As a result, 5 protein regions were considered as “conserved” (Figure 6).

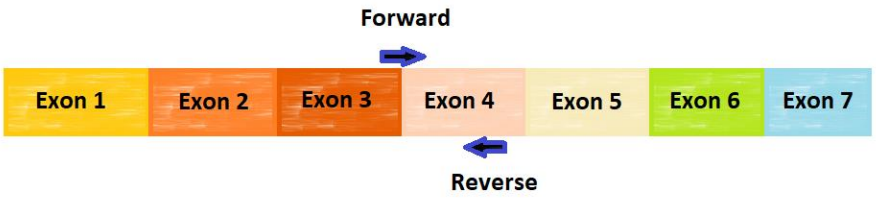

Figure 5: schematic structure of *M. galloprovincialis cox2* mRNA after splicing. Forward primer has an exon-exon junction between the third and fourth exons.

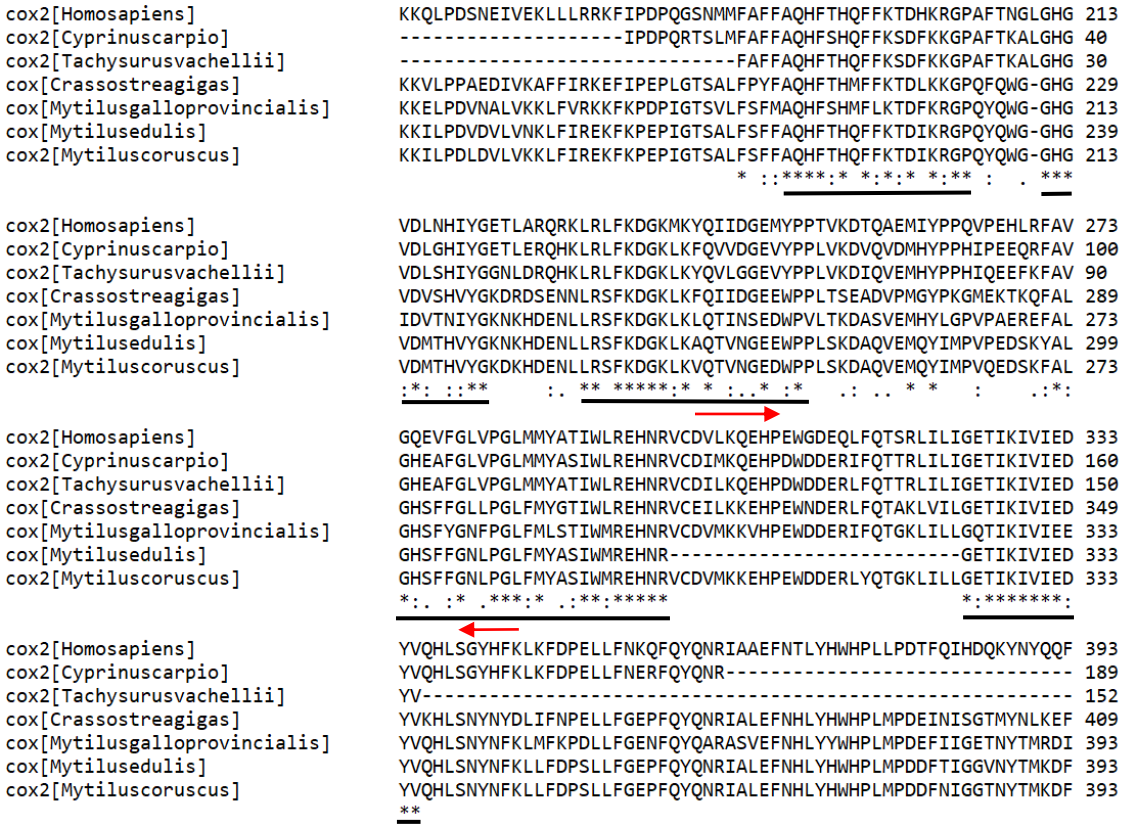

Figure 6: Alignment between COXs proteins belonging to *M. galloprovincialis* (VDI75842) *Homo sapiens* (AAA58433), *Cyprinus carpio* (ASP27472), *Trachysurus vachellii* (ADG45818), *Crassostrea gigas* (NP_001292218.1), *Mytilus edulis* (CAG2191003.1) and *Mytilus coruscus* (CAC5375255.1) with ClustalW. Considered conserved regions are underlined in black. Red arrows indicate amino acids that correspond to the annealing sequences of the designed primers (→ , Forward primer; ← , Reverse primer).

3.4.2. Dopamine receptor D2 primer design

M. galloprovincialis dopamine D2-like receptor protein (VDI63946.1) was aligned with *H. sapiens* drD2 (AAC78779), *Oreochromis niloticus* drD2 (NP_001266711), *Mus musculus* drD2 (NP_034207) and *M. edulis* drD2 (CAG2199322). As a result, 5 protein regions were considered as “conserved” (Figure 7).

Motif analysis for 580 amino acid sequence (drD2) (VDI63946.1) and 374 amino acid sequence (arA2a) (VDI19076.1) using DeepTMHMM server predicted 7 transmembrane domains (TM) in both proteins. Interestingly, we found that TM were highly conserved (Figure 7 and 8).

Furthermore, the running of tblastn programme with *M. galloprovincialis* genome revealed that neither drD2 nor arA2a have introns (Figure 7 and 8).

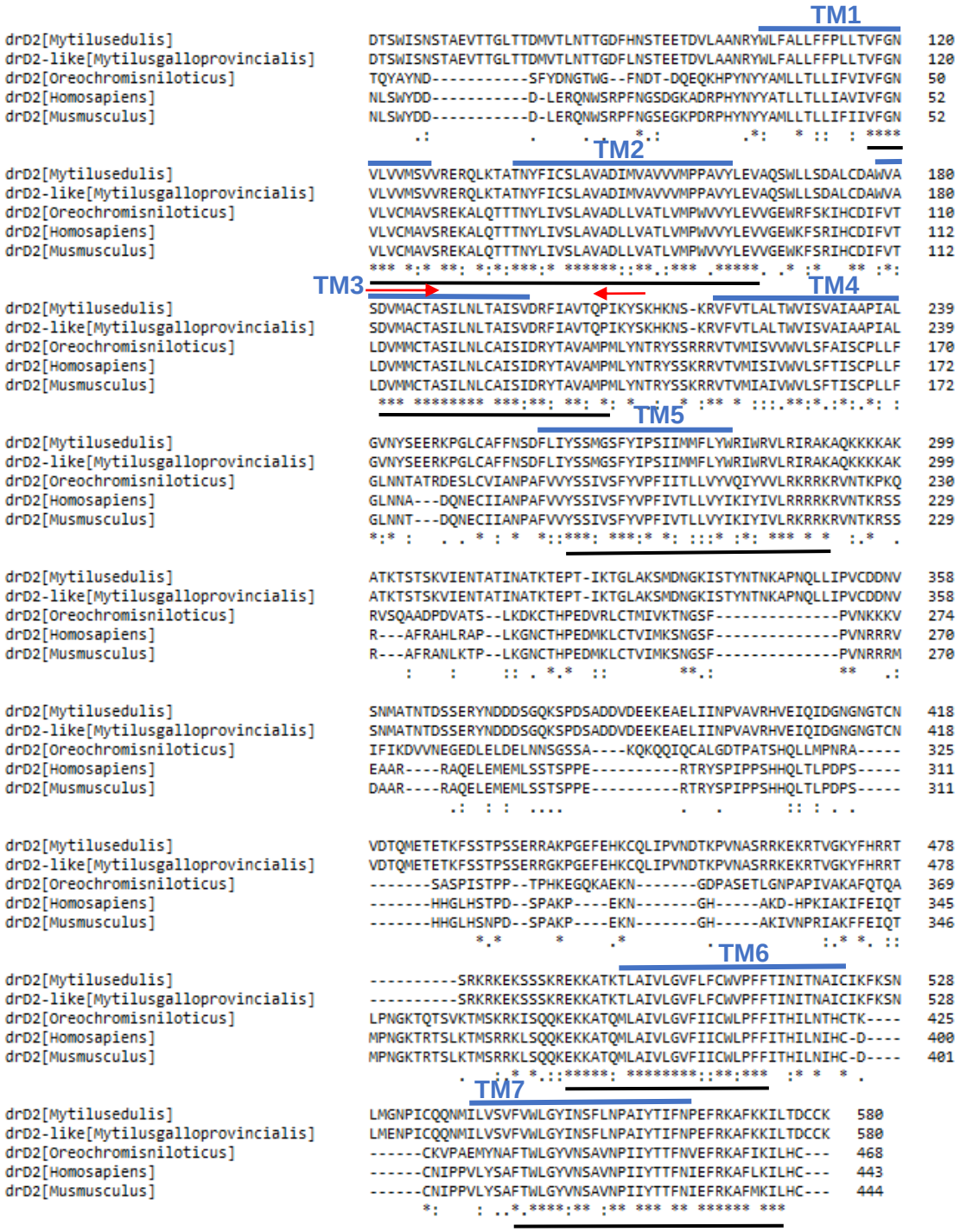

Figure 7: Alignment between drD2-like belonging to *M. galloprovincialis* (VDI63946.1) *H. sapiens* (AAC78779), *Oreochromis niloticus* (NP_001266711), *Mus musculus* (NP_034207) and *M. edulis* (CAG2199322) with ClustalW. Considered conserved regions are underlined in black. Red arrows indicate amino acids that correspond to the annealing sequences of the designed primers (→ , Forward primer; ← ,Reverse primer). Prediction of transmembrane domains was conducted using DeepTMHMM server with a very high probability.

3.4.3. Adenosine receptor A2a primer design

M. galloprovincialis arA2a (VDI19076.1) was aligned with *H. sapiens* arA2a (NP_001265429.1); *Danio rerio* arA2a (NP_001034904.1), *Gigantopelta aegis* arA2b-like (XP_041351131.1), *C. gigas* arA2b (XP_011431118.2), *Mizuhopecten yessoensis* arA3-like (XP_021353687.1) and *Pecten maximus* arA1-like (XP_033734018.1). As a result, 6 protein regions were considered as "conserved" (Figure 8).

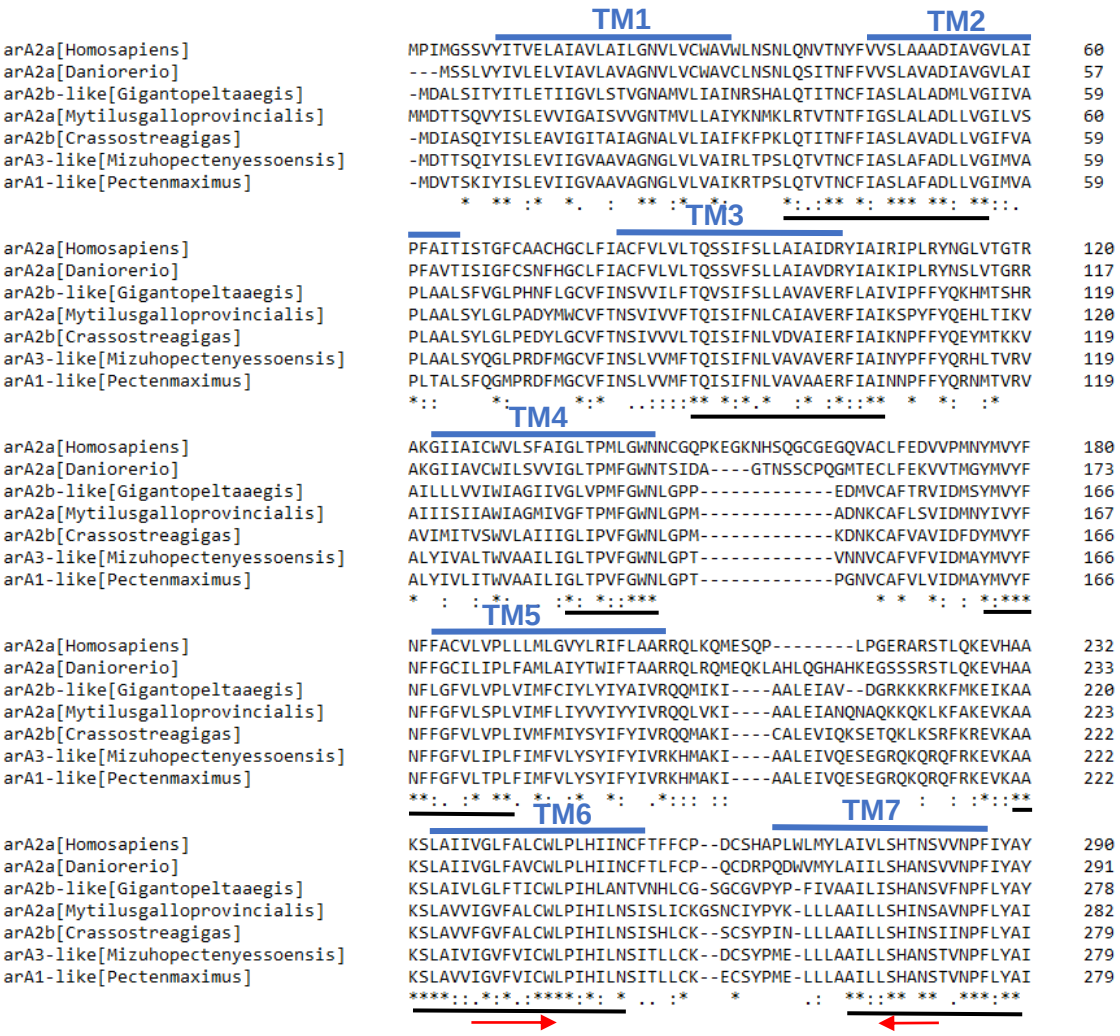

Figure 8: Alignment between arA2a belonging to *M. galloprovincialis* (VDI19076. 1) *H. sapiens* arA2a (NP_001265429.1), *Danio rerio* arA2a (NP_001034904.1), *Gigantopelta aegis* arA2b-like (XP_041351131.1), *C. gigas* arA2b (XP_011431118.2), *Mizuhopecten yessoensis* arA3-like (XP_021353687.1) and *Pecten maximus* arA1-like (XP_033734018.1) with ClustalW. Considered conserved regions are underlined in black. Red arrows indicate amino acids that correspond to the annealing sequences of the designed primers (→ , Forward primer; ← Reverse primer). Prediction of transmembrane domains was conducted using DeepTMHMM server with a very high probability.

3.5. Quantitative real-time PCR

Quantitative Real-Time polymerase chain reaction (RT-qPCR) was performed in 384 well plates in triplicate reactions using a Quant Studio™ 5 Real-Time PCR System (A34322, Thermo Fisher Scientific, USA) under conditions describe by Rossi *et al*. (2016): 5 min at 95°C for initial denaturation followed by 40 cycles of 15 s at 95°C for denaturation and 30s at 60°C for annealing/elongation. Each sample well contained a total of 10 µl, including 5 µl of GoTaq® qPCR Master Mix (A6001, Promega Corporation, USA), 0.5 µl of each primer (Forward and Reverse), 0.1 µl of CXR Reference Dye (A6001, Promega Corporation, USA), 0.5 µl of cDNA and 3.4 µl Nuclease-Free Water (A6001, Promega Corporation, USA). Previously, lyophilized primers were resuspended in Nuclease Free Water to a concentration of 100 µM.

Each primers-pair efficiency (*E*) was calculated based on the slopes of the standard curves from four serial ten-fold dilution of cDNA template to make valid comparison between different samples in a relative quantification analysis (Pfaffl, 2001; Bustin *et al*., 2009; Fraga *et al*., 2014). The slope and the correlation coefficient (R^2) were calculated plotting the cycle threshold (Ct) values (y-axis) with respect to the log of the starting cDNA target (i.e., 1, 10, 100 and 1000) (Taylor *et al*., 2010). Primers efficiency was calculated following the equation described by Kubista *et al*. (2006):

$$\text{Efficiency } (E) = 10^{(-1/\text{slope})} - 1$$

After each Real-Time qPCR analysis, we performed a melting curve analysis as a quality-control step to verify that the PCR product was specific. Melting curve analysis ($-\Delta F/\Delta T$) was plotted as the negative first derivate of the normalised fluorescence (Derivate reporter) (y-axis) with respect to temperature (x-axis).

3.6. Data analysis

For RT-qPCR data analysis, we made a relative quantification method to analyse relative changes in mRNA expression in genes of interest (Fold change; $F.C.$) in our treatment group compared to the control group. Therefore, the results were normalised with 18s rRNA gene (reference gene), which has been previously used as internal control in *Mytilus* species (Cubero-Leon *et al*., 2012; Lacroix *et al*., 2014). The method to calculate the relative change in gene expression was the original mathematical model described by Pfaffl (2001):

$$F.C. = \frac{E_{\text{Target}}^{-\Delta CT_{\text{Target}}^{(\text{Co.}-\text{Treat.})}}}{E_{\text{Ref.}}^{-\Delta CT_{\text{Ref.}}^{(\text{Co.}-\text{Treat.})}}}$$

3.7. Statistical analysis

Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. We adopted a Student *t* test in order to compare the relative gene expression levels (*F.C.*) between control and treatment group. Differences were considered statistically significant at $p < 0.05$.

In cases where data was not normally distributed or have not equal variances ($p < 0.05$), a Mann-Whitney Rank Sum Test was performed. Specifically, data analysed with this test were those corresponding to *vtg* and *cox2* in gonad tissue, *gst*, *hsp70* and *myticinB* in lamellae gills tissue, and *hsp70* and *arA2a* expression in digestive gland tissue.

Analyses were performed using SigmaPlot version 12.0 (Systat Software Inc., Richmond, CA, USA).

4. Results and discussion

4.1. RNA quality and integrity

Average total RNA concentration after RNase-free water dilution obtained were 1802 ± 679 (ng/µl) (mean ± SD). Values of purity extracted RNA assessed at A260/280 and A260/230 ratios were on 2.03 ± 0.1 and 1.62 ± 0.52 respectively.

According with Desjardins and Conklin (2010) and Mullegama *et al*. (2018), pure RNA typically yields a A260/280 ratio of 2 units. This ratio is used as an indicator to determine the level of protein contamination in the sample (Sabat *et al*., 2021).Therefore, our total RNA samples showed a very good quality in terms of protein contamination or other molecules that absorb strongly at 280 nm.

Otherwise, the values of pure RNA at A260/230 ratio should be in the range of 1.8-2.2 units, and significant lower values than expected might indicate that isolation technique may require further optimization (Desjardins and Conklin, 2010; Mullegama *et al*., 2018). In fact, according with literature, it has been observed that RNA extracted using phenol-based Trizol reagents is often contaminated with residual organic materials that can be detected by low A260/230 ratios (Sabat *et al*., 2021), such as solvents (i.e., phenol/chloroform) or chaotropic salts (guanidine isothiocyanate) (Cirera, 2013). Consequently, our RNA samples may had contaminants that absorbed at 230nm. However, there is no significant correlation between A260/230 ratio and the level of Real-Time quantitative PCR (RT-qPCR) inhibitors in RNA samples (Cicinnati *et al*., 2008), which means that our A260/230 values do not necessarily compromise the reliability of RT-qPCR reaction (Fernández-González *et al*., 2021).

Total RNA integrity assessed by 1% non-denaturing agarose gel electrophoresis with bleach showed no gDNA contamination, and no RNA degradation due to RNases activity. Furthermore, in most cases it showed 2 RNA bands, a large band and an upper faint band (Figure 9), wich are considered to be good RNA integrities in molluscs and other protostomes (Gayral *et al*., 2011; Fernández-González *et al*., 2021; Tan and Conaco, 2021).

The accuracy of gene expression analysis in RT-qPCR is strongly influenced by the quantity and quality of starting RNA, in fact, the variability of RT-qPCR results is decreased with increasing RNA quality. Moreover, the amplification of long product (over 400 bp) is also highly dependent of a good RNA quality (Fleige and Pfaffl, 2006). Total RNA integrity assessment can be measure by various methods: agarose and acrylamide

gel electrophoresis or lab-on-chip technologies like Bioanalyzer 2100 (Agilent technologies, USA) or Experion (Bio-Rad laboratories, USA). On one hand, gel electrophoresis is relatively low cost, requires a significant amount of RNA and allows to visualize gDNA contamination and RNA integrity comparing the staining intensity of 18s and 28s rRNA bands. On the other hand, lab-on-chip technologies measure the RNA Integrity Number (RIN) using an algorithm that takes into account the entire electrophoretic trace of RNA (Schroeder *et al*., 2006; Fleige and Pfaffl, 2006; Wieczorek *et al*., 2022)

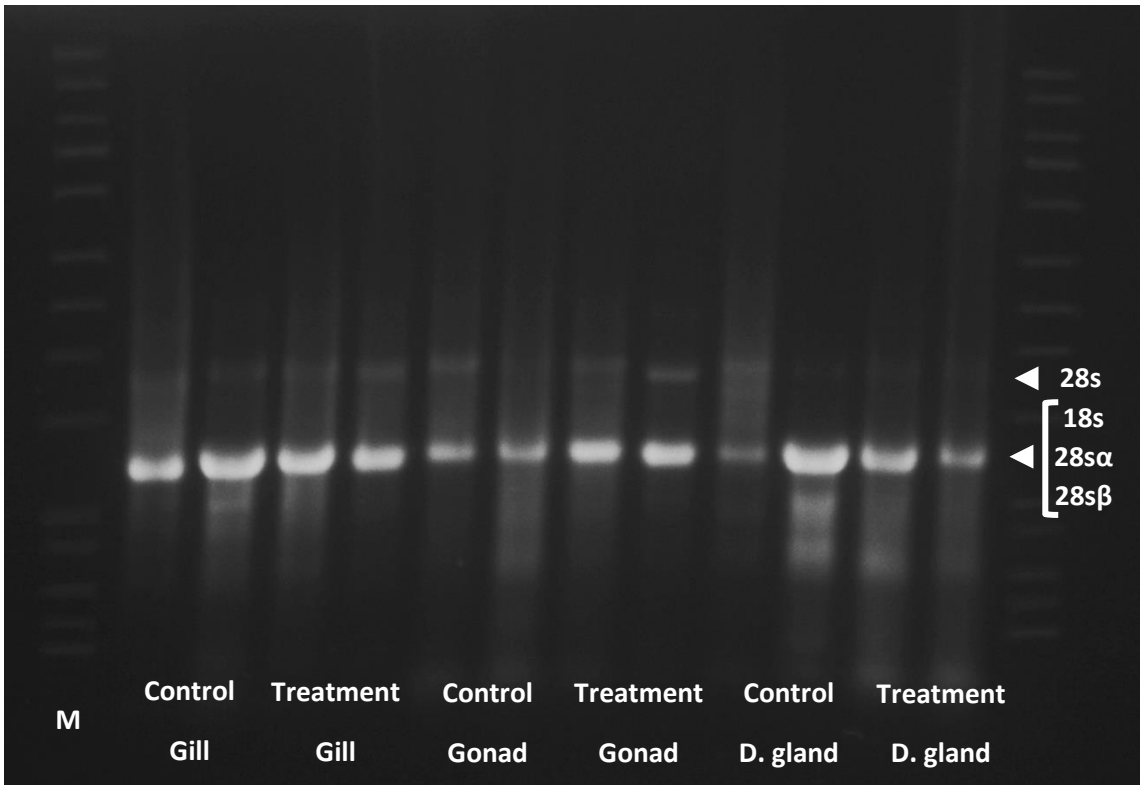

Figure 9: Mediterranean mussel (*M. galloprovincialis*) ribosomal RNA (rRNA) profile in 1% non-denaturing agar gel electrophoresis with bleach. Ribosomal RNA profile is characterized by a distinct 18s rRNA band and a faint 28s rRNA band. A total of 12 samples were characterized, 2 samples belonging to each group and tissue.

Although it is well known that in mammals a good RNA integrity is characterized by two prominent bands corresponding to 28s rRNA (upper band) and 18sRNA (lower band) when the ratio of 28s/18s is about 2 and higher (in a gel electrophoresis) (Schroeder *et al*., 2006; Fraga *et al*., 2014), this electrophoretic behaviour does not occur in molluscs and other protostomes (Tan and Conaco, 2021). This RNA behaviour, called “hidden break”, was first described by Applebaum and colleagues in 1966 when the 28s rRNA from insect tissue (*Hyalophora cecropia*) was converted to a 18s rRNA component after

brief heat-treatment. In organism with a “hidden break”, the 28s rRNA molecule is composed of 2 RNA fragments (the 5`28sα and the 3`28sβ) linked by inter-molecular hydrogen bonding. Therefore, experimental conditions during RNA extraction (denaturation or heat) commonly disrupt such weak forces, breaking the 28s rRNA molecule into 2 subunit fragments of approximately 2000 bp that comigrate with the similar-sized 18s rRNA in gel electrophoresis, thereby resulting in a single distinct band (McCarthy *et al*., 2015; Natsidis *et al*., 2019; Tan and Conaco, 2021; Adema, 2021).

Our RNA samples were extracted with chaotropic agents (guanidinium salt) which creates a perturbation in the hydrogen bond network between water molecules (Salvi *et al*., 2005). Furthermore, RNA samples were subjected to short heating process at 60°C to dissolve the RNA pellet after isopropanol precipitation. Any of this process may disrupt some inter-molecular hydrogen bonds of 28s rRNA subunit. This can explain the unusual electrophoretic profile observed in our RNA samples (Figure 9). Therefore, as described before by Barcia *et al*. (1997), *M. galloprovincialis* has a “hidden break” in its 28s rRNA subunit.

4.2. Amplification efficiency and specificity

Primers-pairs efficiency (*E*) showed a high variability (Table 2). On one hand, *hsp70*, *gst* and *mytilinB* genes taken from literature and *drD2* gene primer design had a good efficiency, all of them above recommended threshold value for reliable RT-qPCR analysis (90-100%) (Taylor *et al*., 2010). On the other hand, *vtg*, *18s rRNA* and *myticinB* genes taken from literature, and *arA2a* and *cox2* gene primers design showed bad efficiencies, specially *arA2a* (266%).

Table 2: Correlation coefficient (R^2) and efficiency of each primer-pair (expressed in percentage) following the equation described by Kubista *et al*. (2006).

Primer	**Efficiency (%)**	**R^2**
vtg	118	0,984
18s rRNA	134	0,998
hsp70	100	0,990
gst	101	0,986
myticinB	139	0,973
mytilinB	108	0,972
arA2a	266	0,934
drD2	98	0,994
cox2	120	0,980

According with Taylor *et al*. (2010), the efficiency of a PCR is a measure of the rate at wich the polymerase converts the reagents (dNTPs, oligonucleotides and cDNA) to amplicon. Therefore, theoretically the maximum efficiency is a 100%, when the number of molecules of cDNA has an increase of 2-fold per cycle (Fraga *et al.,* 2014). This efficiency corresponds to a slope in the standard curve of -3.32 as determined by Kubista *et al.* (2006). A good reaction should have an efficiency between 90% and 110%; and different PCR efficiencies is an indicative of problems with the qPCR that can cause artefactual results. Specifically, poor reaction efficiency below 90% may be caused by contaminating Taq inhibitors, high or suboptimal annealing temperatures, old or inactive Taq, poorly designed primers, or amplicons with secondary structures. In contrast, an efficiency above 110% is in relation with inhibition due to poor DNA or RNA quality, high template concentration, presence of chaotropic salts… or non-specific amplicons/primers dimers (Taylor *et al*., 2010).

Unfortunately, as indicated by Ruijter *et al.* (2021), PCR efficiency values are never published. However, Fernández-González *et al*. (2021) published *vtg* primer efficiency allowing as to compare. Under optimal conditions, this primer should have an efficiency of 102% (Fernández-González *et al*., 2021). In our samples, it had an efficiency of 118%, which means that there was an inhibition in the amplification reaction (Taylor *et al*., 2010; McCord *et al*., 2015). As described before, our RNA samples showed a good RNA integrity and quality at A260/280 ratio, although a low A260/230 ratio. Low A260/230 ratios use to be in relation with the presence of contaminants such as guanidine isothiocyanate (a chaotropic salt present in Trizol solutions) (Cirera, 2013; Sabat *et al*., 2021) which have an inhibition effect on PCR reactions (McCord *et al*., 2015).

Cicinnati *et al*. (2008) reported no significant relation between low A260/230 ratio and PCR inhibition. However, contaminants responsible for the low A260/230 ratio were not specified.

Relative quantification measures changes in gene´s expression in response to different treatments. It requires an internal reference gene to normalize for experimental error (Fraga *et al*., 2014). The ΔΔCt method (Fold change = $2^{-\Delta\Delta Ct}$) developed by Pfaffl (2001) is one of the most popular means of determining gene expression differences between samples (Bustin *et al*., 2009), however it assumes that the gene of interest and the reference gene have an optimal (100%) and identical efficiency (Pfaffl, 2001). The widespread use of the simplified equation developed by Pfaffl (2001) with low quality efficient primers could lead to inaccurate relative cDNA concentrations, yielding misleading results (Ruijter *et al*., 2021).

Our high variability amplification efficiencies (Table 2) did not allow to compare data using the ΔΔCt method. Therefore, instead we use the original equation developed by Pfaffl (2001) as described before, which allow to compare data with different efficiencies.

$$F.C. = \frac{E_{\text{Target}}^{-\Delta CT_{\text{Target}}^{(\text{Co.} - \text{Treat.})}}}{E_{\text{Ref.}}^{-\Delta cT_{\text{Ref.}}^{(\text{Co.} - \text{Treat.})}}} \quad \xrightarrow{\text{If } E = 100\%} \quad F.C. = 2^{-\Delta\Delta c_T^{(\Delta CT_{\text{Treat.}} - \Delta CT_{\text{Co.}})}}$$

(Pfaffl, 2001)

Melting curve analysis performed as a quality control revealed no unspecific PCR products, showing a single peak in all evaluated primers (Figure 10 and 11).

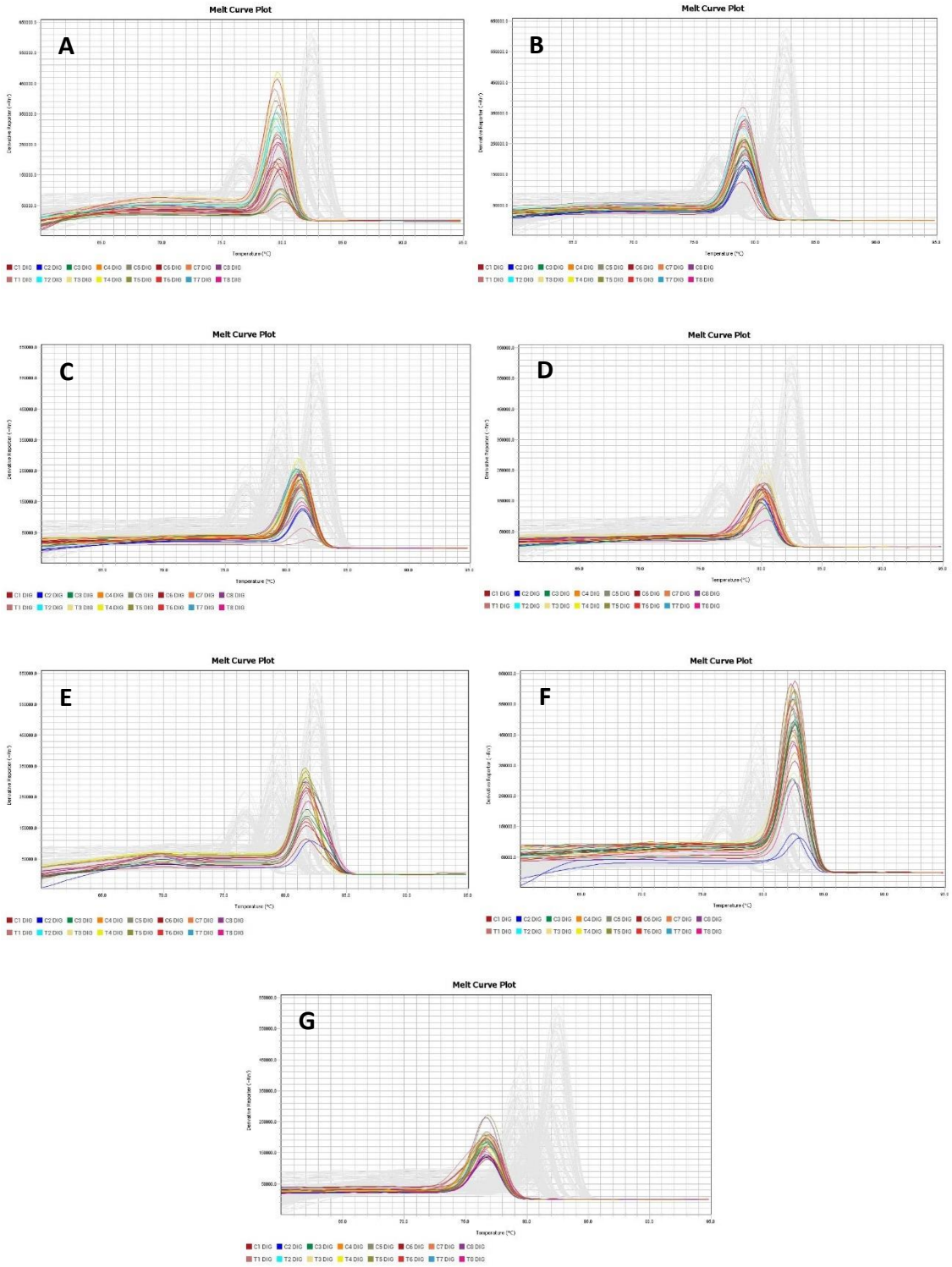

Figure 10: melting curves in *M. galloprovincialis* digestive gland tissue of A) arA2a, B) drD2, C) GST, D) HSP70, E) MyticinB, F) MytilinB and G) 18s rRNA primers.

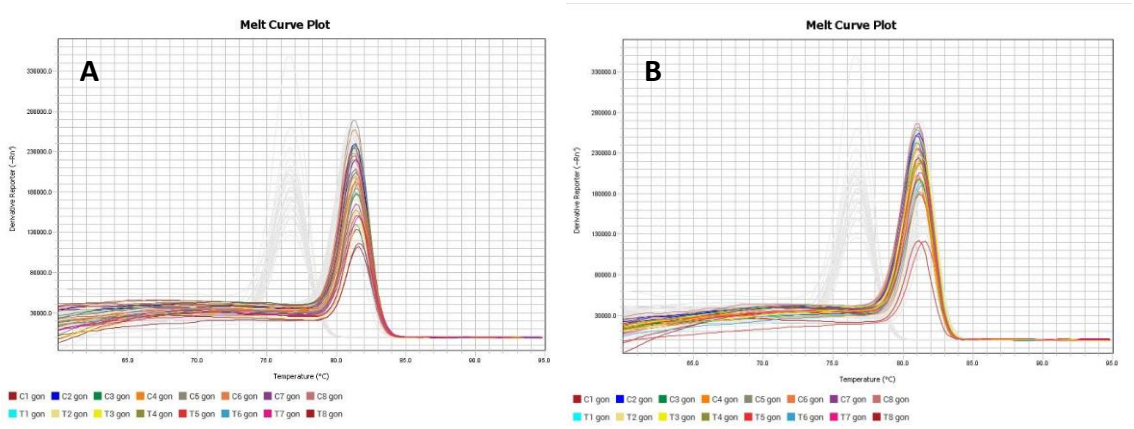

Figure 11: melting curves in *M. galloprovincialis* gonad tissue of A) Vtg, and B) COX2 primers.

Melting curve analysis is based on the fact that different amplicons have different dissociation temperatures (plotted as peaks). Fortunately, PCR artifacts such as primers dimers have shorter amplicons (less nucleotides), so typically have lower melting temperatures too. Fluorescent measurements are made while increasing the temperature of the reaction product. As a consequence, double-stranded DNA begins to denature and intercalating dyes (i.e., SYBR Green, BRYT Green…) decrease fluorescence emission (Intercalating dies generate fluorescence only when bound to double-stranded DNA) (Fraga *et al*., 2014; Ruijter *et al*., 2019).

4.3. Gene expression analysis

4.3.1. Potential gene specific biomarkers

After 5 days of exposure, *adenosine receptor A2a* (*arA2A*) and *dopamine receptor D2* (*drD2*) showed a completely distinct pattern of relative expression in digestive gland tissue among groups. Firstly, *arA2a* exhibited a strong significant increase in mRNA expression (p = 0.005). In contrast, *drD2* gene did not show differences (Figure 12). Relative expression pattern in gills did not show any differences in *arA2a* and *drD2* genes.

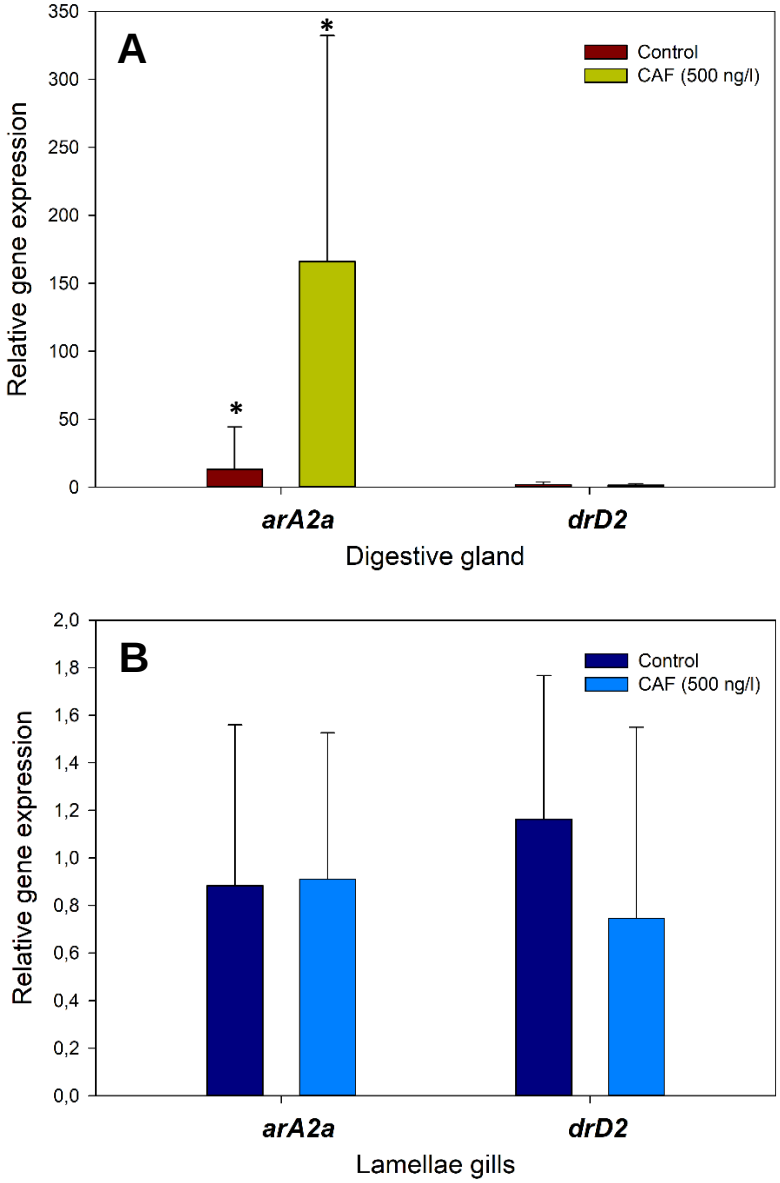

Figure 12: Comparison of relative gene expression of *arA2a* (*adenosine receptor A2a*) and *drD2* (*dopamine receptor D2*) in A) digestive gland and B) gills tissue of *M. galloprovincialis* between control group and treatment group exposed to a caffeine concentration of 500 ng/l for 5 days. Data are the mean ± SD; "*" denote significant differences among groups (p < 0.05).

To our knowledge, this is the first report about transcripts levels of any of these genes after caffeine exposure in molluscs.

According with Manalo and Medina (2018), caffeine exposure induces changes in dopaminergic systems in humans and animals. In mammals, it has been shown to interact via 3 mechanisms: by direct interaction with dopamine and adenosine receptors, by leading to dopamine release, and by convergence of dopamine and adenosine signalling pathways on common second messengers (Cauli and Morelli, 2005). In fact, caffeine is a non-selective competitive antagonist of adenosine receptors (del Rey *et al*., 2011; Mustard, 2013; Manalo and Medina, 2018), blocking them at much lower concentrations than its other targets (Mustard, 2013), especially ArA2a (rodents and mammals) (Capiotti *et al*., 2011).

Furthermore, several studies reported gene expression changes in adenosine receptors after caffeine exposure. Svenningsson *et al*. (1999) described a downregulation of *arA2a* and an increase in the expression of *arA1* in brain rats after received caffeine in their drinking water (0.3 mg/l). Capiotti *et al*. (2011) reported an increase in *ar1* expression in zebrafish (*Danio rerio*) embryos at 72 hours post-fertilization. In contrast, *arA2a* and *ar2B* did not show any significant change. In invertebrates, pharmacological studies suggests that caffeine also interact with adenosine receptors (Chen, 1995; Mustard, 2013). Although little is known about how caffeine interacts with dopaminergic system in invertebrates, it is believed that interacts via one or more of the mechanisms describe for mammals (Mustard, 2013).

Therefore, our results suggests that caffeine may interacted with ArA2a leading to an increase in its expression in digestive gland tissue. In the present study we proposed ArA2a as a potential gene specific biomarker of caffeine contamination in digestive gland. Nevertheless, more studies with different contaminants are necessary to check that none stimulates changes in its gene expression.

Assuming that the mechanism of action of caffeine on adenosine receptors is similar between vertebrates and molluscs, exposure to caffeine could stimulate de release of catecholamines such as dopamine in mussels (del Rey *et al*., 2011). This was verified by Aguirre-Martínez *et al*. (2018), who demonstrated that caffeine exposure increased dopamine levels in the bivalve *Corbicula fluminea*. It was previously demonstrated in rats, where relevant doses of caffeine increase extracellular levels of dopamine (Acquas,

2002; Solinas *et al*., 2002). In adittion, Ferré (2016) proposes that ArA2a blocking by caffeine results in a maximal DrD2 signalling. Besides, in comparison with our results, Kucharski and Maleszka (2005) reported a significant increase in DrD2 mRNA in the honeybee brain, suggesting an interaction between caffeine and dopamine receptors.

Therefore, according with literature, caffeine interacts with invertebrate´s dopaminergic system by at least the next ways: 1) direct interaction with adenosine receptors, 2) leading to dopamine release, and 3) direct or indirect (endogenous dopamine released) interaction with dopamine receptors. The actions of caffeine/dopamine are mediated via dopamine receptors, which are G-protein coupled receptors (GPCRs). In vertebrates there have been classified in 2 groups: D1-like, which activate adenylyl cyclase increasing intracellular cAMP levels; and D2-like, which inhibits adenylyl cyclase and regulates calcium and potassium channels (Mustard *et al*, 2005). Wu *et al*., (2009) reported an increase in cAMP levels through the brain in flies (*Drosophila melanogaster*) with the unique known receptor mutant.

Hence, this increase of endogenous dopamine levels and interaction (direct or indirect) with dopamine receptors due to a caffeine exposure on molluscs could lead to different consequences:

- At larval development level, Liu *et al*. (2018; 2020) reported that dopamine and serotonin (5-HT) together with its receptors might be involved in shell formation during larval development from trochophore to D-shape larvae in the oyster *Crassostrea gigas*. Specifically, dopamine induces biomineralization of calcium carbonate implying the modulation of dopaminergic system.
- At reproductive level, it is known that dopamine and serotonin are important mediators of gamete maturation and spawning in mussels (Gagné *et al*., 2011). Additionally, it has been stated that dopamine is an inhibitor of serotonin activity on gamete release (Fong *et al*., 1993; Gagné and Blaise, 2003).

4.3.2. Reproduction related genes

Our results showed a significant decrease in *vitellogenin* (*vtg*) and *cyclooxygenase2* (*cox2*) gene expression in gonad tissue mussels after five days of exposure to 500 ng/l of caffeine ($p < 0.05$) (Figure 13).

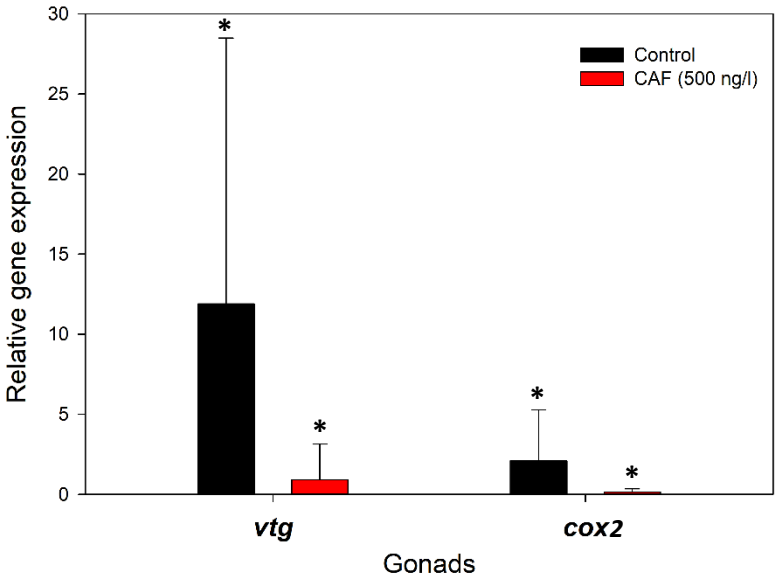

Figure 13: Comparison of relative gene expression of *vtg* (*vitellogenin*) and *cox2* (*cyclooxigenase2*) in gonad tissue of *M. galloprovincialis* between control group and treatment group exposed to a caffeine concentration of 500 ng/l for 5 days. Data are the mean ± SD; "*" denote significant differences among groups ($p < 0.05$).

Previously, Cubero-Leon *et al*. (2012), studied *cox* gene expression in the blue mussel (*Mytilus edulis*) exposed to 17β-estradiol (E2). Their findings showed a significant decrease in *cox* expression. Moreover, it is also generally known that other common contaminants such as Non-steroidal anti-inflamatory drugs (NSAIDs) (Ibuprofen, Naproxen, Diclofenac…) have the ability to promote the non-selective inhibition of COXs activity (Fent *et al*., 2006; Rao and Knaus, 2008; Gagné *et al*., 2011) including in *M. galloprovincialis* mussels (Courant *et al*., 2017). Mehinto *et al*. (2010) observed a significant decrease in *cox* expression in rainbow trout (*Oncorhynchus mykiss*) after diclofenac exposure at 1 µg/l.

Aguirre-Martínez *et al.* (2018) studied the effect of different human drugs such as caffeine on COXs activity and Vtg-like proteins in the bivalve *Corbicula fluminea*. In contrast with our results, they found a significant increase in COXs activity after caffeine exposure in all treatments (from 0,1 µg/l to 50 µg/l). *C. fluminea* were subjected to a 21

days exposure, therefore, in an initial contamination of caffeine maybe there is a first downregulation of *cox* gene due to the novel contaminant, and later there is an overexpression as a response to maintain the homeostasis.

Cyclooxygenases (COXs), also known as prostaglandin H synthases or prostaglandin endoperoxide synthases (Chandrasekharan and Simmons, 2004). COXs catalyses the first step in the synthesis of prostanoids, a large family of arachidonic acid metabolites comprising prostaglandins (Minghetti, 2004). In fact, COXs are the rate-limiting enzyme for the production of prostaglandins (Flippin *et al*., 2007). It is well known that in mammals, prostaglandins may play a pivotal role in female reproduction, particularly ovulation, implantation and menstruation (Niringiyumukiza *et al*., 2018). In molluscs, prostaglandins effect on reproduction are not clear, but results of previous studies suggest that may play a role in spawning (Deridovich and Reunova, 1993). Specifically, Martínez *et al*. (2000) reported an increase of prostaglandins during spawning in the bivalve *Argopecten purpuratus*, and a decrease when the spawning was completed. The first study to prove it, published by Morse *et al*. (1977) showed that a COXs inhibitor (aspirin) blocked the spawning in the gastropod *Haliotis rufescens*.

For these reasons, our results suggests that caffeine contamination could affect spawning in molluscs due to a decrease in *cox* gene expression.

Vitellogenin is the major precursor of the egg-yolk proteins, which provide energy to support embryonic development in oviparous organisms. It has been extensively used as a biomarker of estrogenic compounds contamination (i.e., ethinylestradiol; EE2) (Matozzo *et al*., 2008; André and Gagné, 2020), however, little is known about how other contaminants can affect vitellogenin production. In a recent survey, caffeine-ingested *Caenorhabditis elegans* showed a reduction in vitellogenin production at the adult stage; moreover, they observed a significant decline in the expression of *unc-62*, which is a transcriptional regulator (Min *et al*., 2020; Min *et al*., 2021). Similarly, Godoy *et al*. (2020) observed a reduction of E2 concentration in fish males (*Astyanax altiparanae*) exposed to 9,59 mg/l of caffeine, which is the main regulator of Vtg synthesis in the liver (Miura *et al*., 2007).

As remarked before, it is known that caffeine increases dopamine synthesis (Acquas, 2002; Solinas *et al*., 2002; del Rey *et al*., 2011; Aguirre-Martínez *et al*., 2018). Moreover,

dopamine is a negative regulator of gonadotropins (FSH and LH) secretion in fish (Carrillo *et al*., 2012), which controls the brain-pituitary-gonad (BPG) axis and regulating E2 and Vtg synthesis. E2 is a negative regulator of dopamine too (Burrow *et al*., 2019). Therefore, it is probably that caffeine reduces Vtg synthesis in fish through dopamine, gonadotropin and E2 regulation.

In molluscs, little is known about neuroendocrine regulation of reproduction. However, several studies have identified neuroendocrine and nervous system functions in molluscs that are analogous to the hypothalamic-pituitary system of vertebrates (del Rey *et al*., 2011). In fact, as in fish, studies indicate that E2 may control Vtg synthesis in the ovary (Osada *et al*., 2003; Osada *et al*., 2004; Liu *et al.*, 2014; Tran *et al*., 2016; Zhang *et al*., 2020), dopamine has a strong correlation with vitellogenesis and maybe feminization (Aguirre-Martínez *et al*., 2018), and E2 is a potential negative regulator of dopamine (Osada and Nomura, 1989; Gagné and Blaise, 2003). So according with our results, it is probably that caffeine may reduce Vtg synthesis through dopamine regulation as it is proposed in fish.

Despite the similarities, there must be consider that significant differences do not allow data to be extra poled from fish to molluscs, such as the absence of hypothalamus, pituitary or genes that encodes for gonadotropins (Sakai *et al*., 2020).

Interestingly, in relation with our results, Aguirre-Martínez *et al*. (2018) reported a slight decrease although not significant in Vtg-like proteins, and a significant increase in dopamine levels in the bivalve *C. fluminea* after 21 days of caffeine treatment. It is important to consider that our relative *vtg* expression data includes males and females due to the low gonad development during sampling (Figure 4). However, when studying sex related genes, it is desirable to separate data and analyse them separately.

“An endocrine disrupt compound/chemical (EDC) is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or its (sub)populations” (WHO/IPC, 2002 cited by Beausoleil *et al*., 2018). As described before, caffeine in an exogenous substance that blocks adenosine receptors, might stimulate dopamine release and interact direct or indirectly with dopamine receptors. This release of extracellular dopamine could decrease serotonin levels leading in a reduction of spawning success. In addition, our results suggest that caffeine may downregulates *cox* and *vtg*, which could cause a reduction in spawning success and less vitellogenin content in eggs to provide

energy to support embryonic development, which would result in lower embryo survival rates. For all this reasons, we suggest that caffeine could be a potential EDC in molluscs.

Nevertheless, further research is necessary to attempt the effect of relevant environmental concentration of caffeine on long-term exposure in reproductive physiology of marine organisms.

4.3.3. Oxidative stress related genes

The genes *glutathione-s-transferase* (*gst*) and *heat sock protein 70* (*hsp70*) that codes for enzymes with antioxidant enzymes did not show significant differences among groups (Figure 14).

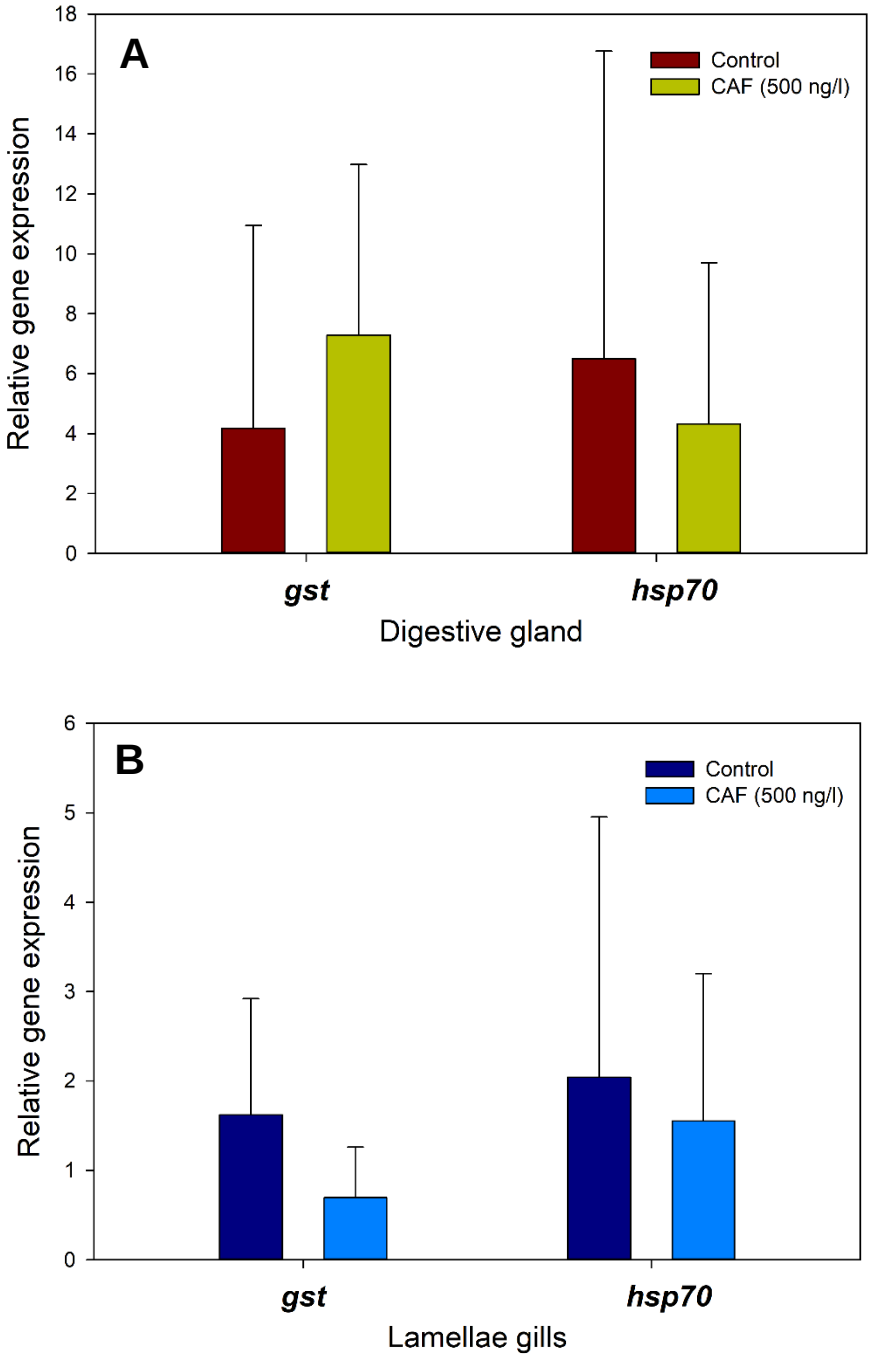

Figure 14: Comparison of relative gene expression of *gst* (*glutathione-s-transferase*) and *hsp70* (*heat shock protein 70*) in A) digestive gland and B) gills tissue of *M. galloprovincialis* between control group and treatment group exposed to a caffeine concentration of 500 ng/l for 5 days. Data are the mean ± SD.

Aguirre-Martínez *et al*. (2013a, 2015, 2016) proposed the use of general stress biomarkers to evaluate the health status in animals exposed to caffeine pollution in environment. In fact, several studies have reported the increase in ROS, and as a consequence, the increase in antioxidant enzymes activity, lipid peroxidation or decrease in lysosomal membrane stability in polychaetes (Pires *et al*., 2016a; Pires *et al*., 2016b), crabs (Aguirre-Martínez *et al*., 2013c), molluscs (Aguirre-Martínez *et al*., 2015; Aguirre-Martínez *et al*., 2016; Cruz *et al*., 2016)… after caffeine exposure.

The enzyme GST plays an important role in defence against xenobiotic-induced oxidative stress (Park *et al*., 2020). In this context, Capulopo *et al*. (2016) reported an upregulation in GST activity in digestive gland, although not in gills of *M. galloprovincialis* after 7 days of caffeine exposure at 5 and 500 ng/l. Moreover, Cruz *et al*. (2016) and Piscopo *et al*. (2021) did not observe any significant increase in GST activity in *R. phillippinarum* after caffeine exposure at 500 ng/l. Similarly, Maranho *et al*. (2014) did not detect an increase in GST activity with a caffeine concentration of 150 or 1500 ng/l in the amphipod *Ampelisca pilicornis*. Furthermore, in *Arenicola marina* and *Diopatra neopalitana* there were no differences in 0,5, 3 and 18 µg/l (Pires *et al*., 2016a). In the same manner, Aguirre-Martínez *et al*. (2013c) did not report differences in GST activity after 0.1, 5 or 15 µg/l of caffeine exposure in the crab *Carcinus maenas*.

The heat shock proteins (HSPs) have been suggested as sensitive biomarkers of sub-lethal toxicity, being synthetised in response to cellular stress (del Rey *et al*., 2011). In light of this, del Rey *et al*. (2011) did not observe any significant difference in HSP70 content in *Mytilus californianus* in mantle tissue after caffeine exposure in the range from 5 to 500 ng/l (in 10, 20 and 30 days). In gills tissue, he neither detect differences at 500 ng/l. Nevertheless, at 50 and 200 ng/l they reported an initial downregulation of HSP70 expression (10-20 days) followed by an upregulation (30 days). In another study with *M. galloprovincialis*, there was no increase in HSP70 content in gills and mantle tissue at 10, 20 and 30 days after exposure with 500 ng/l of caffeine. Although there was upregulation in gills with 50 ng/l for 20 days (Munari *et al*., 2020).

In relation with our results, according with most literature there were no differences in *gst* and *hsp70* expression at a caffeine concentration of 500 ng/l, suggesting that higher caffeine concentrations are needed to increase *gst* and *hsp70* enzymes activity and expression, and therefore, to have a negative impact in health status of *M. galloprovincialis*. Significant increases in GST activity have been reported in *Hediste diversicolor* (Pires *et al*., 2016b), *R. phillipinarum* (Cruz *et al*., 2016; Aguirre-Martínez *et*

al., 2016) and *Carcinus maenas* (Aguirre-Martínez *et al*., 2013c) but when exposed up to 18, 3, 5 and 50 µg/l respectively, which not represent the usual caffeine concentrations found in marine environment.

4.3.4. Immune system related genes

Immune system related genes did not report significant differences among groups. Interestingly, in digestive gland tissue, *myticin B* gene showed a slight increase in mRNA content after caffeine exposure, although not significant (p=0.051) due to high variability in amplification data (Figure 15).

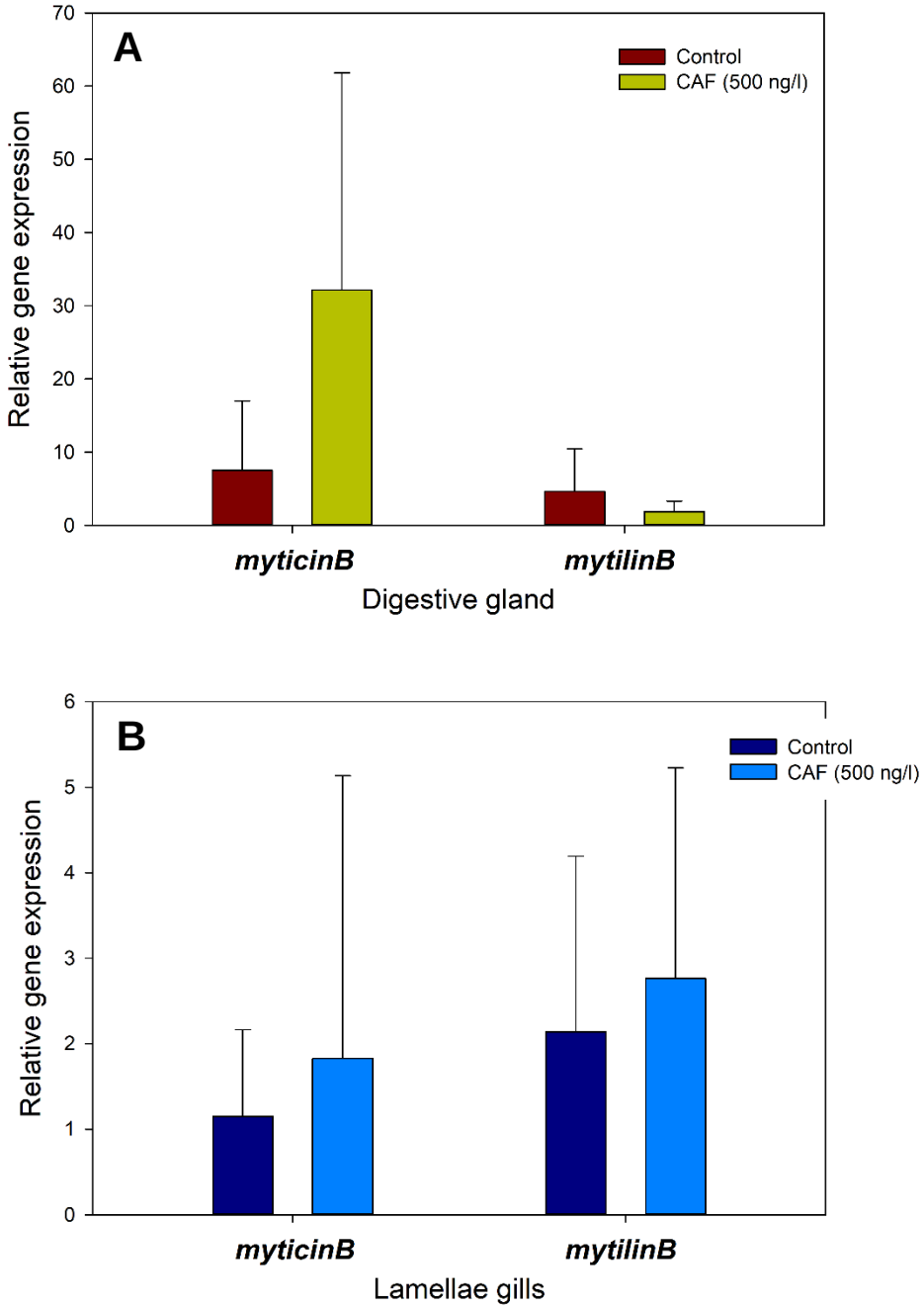

Figure 15: Comparison of relative gene expression of *myticinB* and *mytilinB* in A) digestive gland and B) gills tissue of *M. galloprovincialis* between control group and treatment group exposed to a caffeine concentration of 500 ng/l for 5 days. Data are the mean ± SD.

Immune defence systems differ between vertebrates and invertebrates. Invertebrates possess only innate immunity, while vertebrates possess innate and adaptative immunity. A ubiquitous class of molecules involved in innate immunity via direct interaction with pathogens are the antimicrobial peptides (AMPs) (Greco *et al*., 2020). Molluscs AMPs can be broadly divided in five groups: defensins, big defensins, mytilins, myticins, mytimacins and mytimycins (Watson *et al*., 2022). The AMPs mytilins, myticins, mytimacins and mytimycins have been identified and extensively studied in genus *Mytilus* (Zannella *et al*., 2017).

The antimicrobial peptide mytilin B possess a potent defensive role against Gram-positive and Gram-negative bacteria, antifungal activity (Min Jeong *et al.,* 2018) and probably an unknown antiviral role. Besides, myticins possess antibacterial and antifungal roles (Watson *et al*., 2022).

External stimuli could modulate AMPs gene expression leading to an increase or decrease in the immune system defence. In fact, Détrée and Gallardo-Escárate (2018) detected changes in gene expression of a various of genes related to immune system, such as a downregulation of *mytilin B* after 18 days exposure to microplastics. In addition, Castillo *et al*. (2017) reported an upregulation of *mytilin B* and a downregulation of *myticin A* when *Mytilus chilensis* were exposed to a higher CO_2 conditions. In this study, caffeine exposure for 5 days did not modulate mytilin B or myticin B gene expression, maybe due to low exposure time. However, despite the high interindividual variability, our results suggests that probably myticin B might be upregulated in digestive gland tissue after caffeine exposure (Figure 15A). For this reason, *myticin B* could be a potential candidate gene for future studies about gene expression analysis after caffeine exposure.

In accordance with our results, Rey-Campos *et al*. (2019) reported a high interindividual variability in the expression of AMPs in *Mytilus galloprovincialis*, demonstrating that every single individual expression profile can be very different in terms of the number of genes and their magnitude of expression (Figures 13, 14 and 15). Therefore, it would be interesting to analyse samples as a biological pool instead of individual mussels to eliminate individual differences and observe a clear pattern in a population.

5. Conclusion

1. Caffeine exposure for 5 days at a concentration of 500 ng/l in *Mytilus galloprovincialis* upregulates significantly gene expression of *adenosine receptor A2a* (*arA2a*) in digestive gland tissue, suggesting that caffeine blocks arA2a as in mammals. In contrast, there is no change in *dopamine receptor D2* (*drD2*) expression. Therefore, we proposed *arA2a* as a potentially gene specific biomarker of caffeine contamination in molluscs, although further studies with other contaminants are necessary.

2. Our results suggest that caffeine might downregulates *cyclooxygenase2* (*cox2*) and *vitellogenin* (*vtg*), leading to a reduction in spawning success (COXs) and less vitellogenin content in eggs to provide energy to support embryonic development, which would result in lower embryo survival rates. Therefore, it can be suggested that caffeine could be a potential endocrine disruptor in molluscs.

3. After 500 ng/l of caffeine exposure there is no upregulation of *glutathione-s-transferase* (*gst*) or *heat shock protein 70* (*hsp70*) suggesting that higher caffeine concentrations are necessary to lead a negative impact on mussels health status.

4. Caffeine exposure at a relevant environmental concentration of 500 ng/l do not significantly alter the immune related genes *myticin B* or *mytilin B* expression after 5 days of exposure.

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