Carla Maria Alves Martins

Licenciada em Ciências de Engenharia do Ambiente

Toxicological effects of a pharmaceutical, simvastatin, in *Gammarus locusta*: histopathological evaluation

Dissertação para obtenção do Grau de Mestre em Engenharia do Ambiente

Orientador: Prof. Doutora Maria Helena Ferrão Ribeiro da Costa, Professora Associada com Agregação, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa

Co–orientador: Doutor Pedro Manuel Broa Costa, Investigador Sénior do IMAR – Instituto do Mar

Faculdade de Ciências e Tecnologia

Universidade Nova de Lisboa

Outubro 2013

Toxicological effects of a pharmaceutical, simvastatin, in *Gammarus locusta*: histopathological evaluation.

Carla Maria Alves Martins

Agradecimentos

À Professora Maria Helena Costa pelo apoio e orientação nestes últimos anos da minha vida académica, e por todo o conhecimento que me transmitiu.

Ao Pedro Costa pelo apoio incondicional no laboratório e em toda a elaboração da tese, pelos conselhos que em muito contribuíram para aumentar o meu conhecimento e a minha determinação de continuar na área de investigação.

À Doutora Teresa Neuparth por me ter transmitido a sua experiência e conhecimento, e pela total disponibilidade em ajudar ao longo do decorrer do meu trabalho.

A todos os meus colegas de laboratório pelo apoio tanto a nível de trabalho como pessoal, pela amizade e pelos momentos de descontração, de onde resultaram ideias e teorias mirabolantes, mas sem dúvida interessantes.

Às meninas, Sofia, Neuza e Maria, que ao longo destes belos anos me acompanharam em viagens, aventuras, e muitos outros momentos loucos e divertidos. Obrigada por estarem sempre presentes quando precisei de apoio.

Aos meus amigos e colegas de faculdade por todos os bons momentos passados na vossa companhia, à Mariana que me aturou durante todo o último semestre nas nossas aulas quase exclusivas, e em especial ao Fábio pelo apoio, amizade, paciência, e bons cafezinhos nas pausas da tese.

Aos meus pais um especial obrigado por todo o apoio e dedicação e principalmente por me ensinarem que na vida nunca se desiste dos nossos sonhos, há que arriscar e lutar até ao fim, e que mesmo no pior dos momentos haverá sempre uma saída.

Ao meu irmão Marco por toda a paciência que teve nos dias mais complicados em que me fez rir e esquecer os problemas, e pela sua companhia imprescindível.

Abstract

Pharmaceuticals and their derivatives are considered emerging organic contaminants of aquatic ecosystems, mostly by inputs from domestic and hospital effluents. The toxicological risk associated to these substances results essentially from their ability to act on a specific metabolic process. These substances have been revealed toxic to aquatic organisms as well as to the human populations that depend on these ecosystems. However, the toxicity of pharmaceuticals in the environment is not yet well known, such as for simvastatin, which is a human drug used for the treatment of high levels of cholesterol. The amphipod *Gammarus locusta* is a species with high ecological relevance and high importance in toxicological studies, both acute and chronic. However, histopathological appraisals on these organisms are almost absent. *Gammarus locusta* (newborn) were exposed to a chronic assay with a broad range of sublethal concentrations of simvastatin. At the end of their life cycle, males and females were subjected to a histopathological evaluation using histochemical techniques for light bright-field and fluorescence microscopy, considering the hepatopancreas as the main target-organ. The comparison between the control treatment and the organisms exposed to several concentrations of the pharmaceutical permitted the selection and evaluation of qualitative and quantitative histopathological biomarkers. The results show non-monotonic dose-response curves that support the physiological alterations on the hepatopancreas epithelium reflecting alterations in the metabolism of carbohydrates, enzymatic activity and lipid storage. However, no evidence of severe lesions in this and other organs could be related to exposure. Females presented a higher ability to react to the pharmaceutical than males, when exposed to the lower concentrations of simvastatin. This can be connected with the higher energy reserves of females, associated in part to their reproductive functions. In conclusion, simvastatin affects amphipod metabolism that is responsible by alterations on cellular structures of the hepatopancreas, revealing thus more important physiological alterations than direct damage induced by the pharmaceutical.

Keyword: Amphipod, Pharmaceuticals, Histology, Histopathology, Toxicity Bioassays, Hepatopancreas

Resumo

Os fármacos e seus derivados são considerados contaminantes orgânicos emergentes que poluem os ecossistemas aquáticos principalmente através de efluentes domésticos e hospitalares. O risco toxicológico associado a estas substâncias resulta principalmente da sua actuação sobre um processo metabólico específico. Estas substâncias têm-se revelado tóxicas para os organismos aquáticos e para as populações humanas que dependem destes ecossistemas. A toxicidade associada aos fármacos, no entanto, não é ainda bem conhecida, tal como para a sinvastatina, um fármaco humano utilizado no tratamento de altos níveis de colesterol. O anfípode *Gammarus locusta* é uma espécie com elevada relevância ecológica e muito importante em estudos toxicológicos agudos e crónicos. No entanto, análises histopatológicas nestes organismos estão praticamente ausentes em estudos toxicológicos. G*ammarus locusta* (recém nascidos) foram expostos, num ensaio crónico, a uma gama alargada de concentrações sub-letais de sinvastatina. No final do seu ciclo de vida, machos e fêmeas, foram sujeitos a avaliação histopatológica recorrendo a técnicas histoquímicas de microscopia de campo claro e de fluorescência, tendo como principal órgão-alvo o hepatopâncreas. A comparação entre o tratamento-controlo e os organismos expostos às várias concentrações do fármaco permitiu a selecção e avaliação de biomarcadores histopatológicos qualitativos e quantitativos. Os resultados apresentam uma curva dose-resposta não-linear e evidenciam alterações fisiológicas no epitélio do hepatopâncreas que reflectem alterações no metabolismo de hidratos de carbono, actividade enzimática e armazenamento de lípidos. No entanto, não puderam ser associadas, em vários órgãos, lesões histopatológicas graves à exposição ao fármaco. As fêmeas apresentam uma maior capacidade de reacção ao fármaco do que os machos, quando expostas a baixas concentrações de sinvastatina. Tal pode estar ligado às altas reservas energéticas das fêmeas associadas, em parte, às suas funções reprodutivas. Em conclusão, a sinvastatina afecta o metabolismo do anfípode provocando alterações nas estruturas celulares do hepatopâncreas, o que evidencia um maior impacto fisiológico, em detrimento de lesões directamente causadas pela substância.

Palavras-chave: Anfípode, Fármacos, Histologia, Histopatologia, Bioensaios de toxicidade, Hepatopâncreas

Index

Figure Index

Table Index

Abbreviation List

ACAT – Acyl coenzyme A acyltransferase

AO – Acridine Orange stain

ATSM – American Society for Testing and Materials

B-ratio – B (Blister-like)-cells ratio

CYP – Cytochrome P450

EEA – European Environmental Agency

EOC – Emerging Organic Contaminant

F-ratio – F (Fibrillar)-cells ratio

HMG-CoA – Enzyme 3-hydroxy-3-methylglutaryl-coenzyme A

MET – Mean Epithelium Thickness

MSFD – Marine Strategy Framework Directive

MVN – Mean Non-digestive Vacuole Number

MVD – Mean Non-digestive Vacuole Diameter

PCA – Principal Component Analysis

PFC – Perfluorinated Compounds

R-ratio – R (Resorptive)-cells ratio

rRNA – Ribosomal Ribonucleic Acid

TC – Tetrachrome stain

WFD – Water Framework Directive

1. Introduction

Aquatic pollution is a serious threat with numerous consequences for communities in the aquatic environment and consequently for other animals and plants in land, as well as for humans exposed to environmental toxicants via water, air and food. The water masses are subjected to many toxicological pressures from human activities, such as industry, urbanism and agriculture; and also those deriving from the increasing production, use and release of the veterinary and human pharmaceuticals (EEA, 2010). The European Union responded to the increasing threat of water pollution by publishing, in 2000, the Water Framework Directive (WFD, Directive 2000/60/EC), which was latter complemented with the Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC). Altogether, these two directives established guidelines and standards to monitor and minimize adverse anthropogenic impacts to aquatic ecosystems, including those related to the inputs of hazardous substances. The water quality monitoring is thus an important component addressed by both directives, in order to safeguard environmental quality. However, environmental monitoring of aquatic environments is challenging, in large part due to the complexity of these ecosystems. Nonetheless, there are, nowadays, several analytical tools that may be employed to the purpose, from passive biomonitoring (i.e. in situ sampling), biomarkers to determine the effects and responses of organisms to bioassays to test substances and determine their thresholds of toxicity, as well as their effects to organisms (see Chapman, 2007).

In the past few years, a new set of aquatic pollutants has been emerging as an environmental threat, being termed emerging organic contaminants (EOCs), which are synthetic or semi-synthetic substances that present a potential risk for environmental ecosystems and human health (Farré et al., 2008). The EOC class of toxicants includes pharmaceuticals, day-care products, endocrine disruptors (including hormone-mimicking substances), surfactants, perfluorinated compounds (PFCs), flame retardants, industrial additives and agents, gasoline additives and others, including their metabolites (Farré et al., 2008). Among EOCs, pharmaceuticals pose a particular threat for affecting specific biological pathways albeit often reaching the aquatic media as biotransformed metabolites, for which toxicological effects are difficult to predict (Farré et al., 2008).

Pharmaceutical production and prescription use has been growing over the last decades, whereas urban wastewater treatment facilities are ineffective in the elimination of these compounds and their metabolites (EEA, 2010). For instance, Verlicchi et al. (2012) detected, in wastewater effluents, a wide range of pharmaceuticals, such as antibiotics, anti-inflammatory drugs and lipid regulators, however in low concentrations, presumable signifying a moderate risk for aquatic communities. However, these “low” concentrations, in the long term represented a sizable risk for aquatic organisms, which presented chronic effects derived from increase of the contaminant

individual concentration or from the pollutants mixture presence, in the aquatic system (Verlicchi et al., 2012). To the Water Framework Directive is appended the Priority Substances List, providing guidelines to monitor hazardous chemicals in water masses. Still, pharmaceuticals, as simvastatin, are not yet included in the list, probably due to the general lack of knowledge about these substances. Nonetheless, pharmaceuticals are expected to integrate the Priority Substances List in the next few years, as a result of the increasing concern on EOCs.

Simvastatin ($C_{25}H_{38}O_5$) is a commercial drug included in the statins family, prescribed to humans for the treatment of blood hyperlipidemia (Mauro, 1993). The substance is a semi-synthetic drug analogue to lovastatin, which is a fermentation product of the fungus *Aspergillus terreus* in presence of a carbon-rich medium (Mauro, 1993; Casas López et al., 2004) but has up to five times greater potency than lovastatin, as demonstrated in vitro (Desager and Horsmans, 1996). The main mode of action of simvastatin is the inhibition of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which contributes to lower squalene (a precursor of cholesterol) synthesis, as well as the synthesis of other important sub-products of mevalonate pathway as ubiquinone (coenzyme Q), dolichol (involved in protein glycosylation), and compounds that interfered in protein synthesis and tRNA modification (Gerson et al., 1989; Desager and Horsmans, 1996; Bentinger et al., 2010). In water, the drug is insoluble but it is soluble in common organic solvents such as chloroform, alcohol and methanol (Mauro, 1993). In mammals, simvastatin bioaccumulates mostly in the liver, where it is metabolized by the action of cytochome P450 (CYP) monooxygenases resulting in several metabolites among which are included simvastatin acid, which is the principal active form associated to HMG-CoA reductase inhibition (Mauro, 1993; Desager and Horsmans, 1996; Patil et al., 2007; Key et al., 2008). Desager and Horsmans (1996) also refer that simvastatin decreases acyl coenzyme A acyltransferase (ACAT) activity, an enzyme that is responsible for cholesterol absorption directly from the diet. Thus, lower simvastatin doses decrease cholesterol synthesis and absorption; however also reducing ubiquinone production, which acts as antioxidant, and interferes with the process of energy production (Walton and Pennock, 1972; Velho et al., 2006; Bentinger et al., 2010). At high dosages, simvastatin is toxic for several animals, being able to cause hepatic necrosis and multiple other lesions in various tissues. Gerson et al. (1989) studied the simvastatin effects in several mammals with ingestion of the simvastatin doses, up of the doses prescribed for human treatment (5 at 40 $mg.day^{-1}$), and conclude that higher doses are responsible for liver lesions and necrosis in renal tubule in rabbits, while the dogs may develop cataracts. Also, Horsmans et al. (1990) found a high level of hepatotoxic effect in liver of the guinea-pigs subjected to simvastatin doses of 125 $mg.kg^{-1}.day^{-1}$. In invertebrates, higher doses of simvastatin affect essentially the enzymatic system and consequently the total metabolism and energy production, as observed by Dahl et al. (2006), eliciting also a decrease of RNA content and elevated mortality in copepods. High doses of simvastatin and/or in

combination with other drugs, may even increase the risk of myopathy (lesions in muscular fibers), including rhabdomyolysis (necrosis of muscle cells with release of catalytic products to the blood stream, increasing the risk of renal insufficiency) (see Omar et al., 2001). In crustacean, simvastatin can affected the production of hormones that regulate molting, reproduction and growth (Chang, 1995; Li et al., 2003). The inhibition of HMG-CoA reductase, acts in reduction of the methyl farnesoate, a compound related to insect juvenile hormone, and consequently the reduction of vitellogenesis (Li et al., 2003). In fact, Zapata et al. (2002) observed in female insects a reduction of the reproduction; and for higher concentrations of stains the embryogenesis is blocked and females present 100% of sterility.

The amphipod *Gammarus locusta* is a marine and epibenthic crustacean that inhabits medium to low intertidal zones, having a large biogeographic distribution in European costal systems, including Portuguese costal ecosystems, where the species is abundant. The species feeds mainly on microalgae, *Ulva spp*. and *Enteromorpha spp*., however it has been verified in laboratory studies that *Gammarus locusta* can be omnivorous, herbivore, detritivore, and even prone for cannibalism (see Costa and Costa, 2000). According with the study of Neuparth et al. (2002) the ideal temperature and salinity for the rearing of the species are variable but usually within 15^0C to 20^0C with a salinity of 33 and around 15 ºC between 20 and 33 (for acute and chronic assays). *Gammarus locusta* is a species with high ecological relevance and high sensitivity to contaminants in aquatic environment, which, together with short life cycle and ease to breed in the laboratory, permits evaluating the effects of contaminants along their life cycle, following chronic exposure to toxicants (e.g. Costa and Costa, 2000; Costa et al., 2005; Neuparth et al., 2005). Nevertheless, this species, as for other amphipods, is more commonly utilized to determinate survival, growth and reproductive rates following acute exposure to toxicants in water and sediments. Several fully standardized protocols have been developed for acute and chronic tests with amphipods, such as the protocol developed for five amphipod species by American Society for Testing and Materials (ASTM, 1992). In Europe, standard protocols have been developed and published for marine and estuarine whole-sediment tests with the species *Corophium volutator* (Bat and Raffaelli, 1998) and *Gammarus locusta* (Costa et al., 1998). Numerous studies on acute and chronic exposure have been developed with amphipods; however, histopathological evaluations are almost nonexistent. Still, Correia et al. (2002) presents a study of histological description of the hepatopancreas but the work did not involve toxicity assays, being an exception amidst the otherwise lack of knowledge on amphipod histology.

Biomarkers may indicate the biological response to an environmental chemical, which are observed and measured inside of an organism indicating a deviation from the normal status that cannot be detected in the intact organism (van der Oost et al., 2003). The classic biomarkers are divided in

three types: biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility (NRC, 1987). Histopathological biomarkers are not explicitly inserted in one of these types because have characteristics similar to all. Histopathological biomarkers are based on alterations of micromorphology and cell physiology of the organisms when exposed to contaminant that are potentially indicative of exposure to toxicants. The histopathological evaluation permits indentifying the organ or tissue primarily affected by contaminants and allows the demonstration of structurals alterations that may result from contaminant interference in metabolic processes, such as energy reserve and metabolism, cell cycle, etc. (van der Oost et al., 2003). Additionally, histopathology provides tools for aquatic systems monitoring to the level of the organism health status that are easy to understand by the general public and legislators. However, the use of histopathology in monitoring programs is frequently reduced in comparison to other biomarker responses (e.g. biochemical) mostly because there is a lack of a clear knowledge on microanatomy and micropathology of organisms, especially invertebrates (Au, 2004). Histopathological biomarkers can be purely qualitative, i.e. limited to a description of lesions and alterations; semi-quantitative, permitting a more objective evaluation through the attribution of some sort of empirical scores to classify the levels of change in the tissue; and quantitative, an assessment that allows objective quantification of alterations by direct measurements of histological and cytological structures. Semi-quantitative evaluations can reveal lack of objectivity and consensus in the identification of the histopathological lesions and attribution of scores, which may impair the final assessment of the real effects or responses to contaminant (Costa et al., 2011). However quantitative evaluations circumvent most of these limitations, since measurements are made directly from histological screening. Nonetheless, all these three types of histopathological biomarkers complement the total chronic effects evaluation in an organism exposed to sublethal concentrations of the contaminant.

2. Objectives

The main objective of this thesis is to evaluate the histopathological alterations in whole mid-body sections the amphipod, *Gammarus locusta*, exposed to several concentrations (between 0 to 8 $\mu g.L^{-1}$) of an emerging pollutant, the pharmaceutical, simvastatin. The hepatopancreas, in crustaceans, is the main organ with xenobiotic metabolization functions, similar to the mammalian liver and, for this reason, it was the main organ chosen to quantitatively evaluate histopathological alterations eventually dose-dependent to pharmaceutical exposure, which implicates the identification appropriate biomarkers and correlate the results with simvastatin concentrations. As a secondary objective, sections were screened for qualitative alterations, such as hemocyte infiltration, as a sign of inflammation. Additionally, the work intend to assess differences between males and females, i.e., to assess sexual dimorphism regarding the effects and responses of amphipods exposed to simvastatin. Finally, it is intended, through microscopy observations, to contribute to the knowledge of amphipod microanatomy.

In brief, the main points to approach in this thesis are:

- To identify the histopathological alterations in hepatopancreas of amphipod *Gammarus locusta* exposed to an emerging pollutant, the pharmaceutical simvastatin;

- To identify qualitative and fully quantitative biomarkers, the latter in the hepatopancreas, for being the main xenobiotic transformation and storage organ;

- To look for potential dose-effect relationships between concentration of exposure and the histopathological measurements considered as candidate biomarkers;

- To survey potential differences, with respect to the mode of reaction to this toxicant, between males and females;

- To contribute to a histological description of the amphipod *Gammarus locusta*.

3. Materials and Methods

3.1. Experimental procedure

Amphipods, *Gammarus locusta*, were exposed to simvastatin during all life cycle (from newborn to maturation), which extended for 72 or 47 days at 18 or 20 ºC, respectively. The amphipods, fifty neonates (< 7 days-old) per replicate, were exposed in 5 L glass aquaria, salinity between 33-35 and photoperiod set at 16:8 h light:dark. A 1cm deep layer of natural sediment was placed in each aquarium the day before the start of the assay. Aeration was provided with plastic tips placed at least 1 cm above the sediment surface. Pebbles were added to provide shelter. The animals were divided by six treatment groups with four replicates. The individuals were randomly selected and placed in each aquarium, expectedly males and females in identical proportions. The treatment groups represent simvastatin nominal concentrations of 0.064, 0.32, 1.6 and 8 $\mu g.L^{-1}$ (dissolved in acetone), plus control (acetone only) and blank groups. The animals were fed daily with *Ulva lactuca*. Water changes (100%) were performed twice a week (followed by recontamination) to ensure constancy of water quality. At the end of the exposure period, at least six males and six females per treatment were sampled for histological analyses.

3.*2. Histological procedures*

Animals (females and males) were sectioned dorsoventrally through the mid-body plan and fixed in Bouin-Hollande's solution (10% v/v formaldehyde and 7% v/v acetic acid to which picric acid was added till saturation) or in Carnoy's fixative (absolute ethanol, chloroform, glacial acetic acid 6:3:1) for 24h at 4 ºC. After fixation, the Bouin-fixed samples were washed in distilled water, dehydrated in a progressive series of ethanol (70, 96 and 100%), infiltrated in xylenes and embedded in paraffin. Carnoy-fixed samples were washed in absolute ethanol and processed to paraffin as previous. Details on the procedures are described by Martoja and Martoja (1967) and Puchtler (1968).

Paraffin-embedded samples were sectioned (5 μm thickness) with a Jung RM2035 model rotary microtome (Leica Microsystems). At least two slides per individual were obtained, each containing a minimum of eight serial sections. The samples were deparaffinated in xylene, rehydrated in a regressive series of ethanol (100, 96 and 70%), washed in distilled water and stained by a tetrachrome stain (TC) (for bright-field microscopy) based on Alcian Blue, Periodic Acid-Schiff's, Weigert's Hematoxylin and Picric Acid (Costa and Costa, 2012, with few modifications) or with the Acridine Orange stain (AO) for nucleic acids (fluorescence microscopy) in histological samples (Costa and Costa, 2008).

The tetrachrome stain allows the structural visualization and detection of basic (bright pink) and acid (blue) polysaccharides with application of Periodic Acid-Schiff's and Alcian Blue histochemical dyes, respectively (Costa et al., 2012). Weigert's Hematoxylin stains the nuclei and other basophilic structures with brown/black, and the muscle fibers are stained yellow by Picric Acid. Briefly: after deparaffination and rehydration, sections were stained with Alcian Blue (30 min), followed by treatment with Periodic acid (5 min) and staining with Schiff's reagent (15 min). Samples were counterstained with Weigert's Hematoxylin (5 min) and heated (60 ºC) Picric acid (5 min). All other steps were performed at room temperature and in the dark. Intermediate washing steps were done with Milli-Q grade ultrapure water (> 16.2 MΩ cm). The slides were afterwards dehydrated in a progressive series of ethanol (70, 95 and 100%), cleared with xylene, allowed to dry completely and mounted with DPX resin.

The Acridine Orange stain was employed to enhance the contrast of cellular structures such as nuclei and endoplasmatic reticuli (see Costa and Costa 2008). Acridine Orange is metachromatic (yellowish) for double strand nucleic acids (as nuclear DNA) and ortochromatic (reddish) for single strand nucleic acids (such as ribosomal RNA), while background remains greenish. Slides were stained in Acridine Orange (0.01 % m/v AO base in 0.1 % v/v acetic acid) for 45 minutes at room temperature, in the dark, after which the slides were briefly rinsed in AO washing solution (0.5% acetic acid in ethanol) to remove the excessive dye. The slides were then dehydrated with 100% ethanol, cleared and mounted as previously described.

3.3. Histopathlogical observations

The analyses were carried out using a DMLB model microscope adapted for epifluorescence with an EL2000 UV source and an I3 filter, coupled to a DFC480 model digital camera (all from Leica Microsystems, Germany).

A preliminary qualitative histopathological screening was performed on the internal structure of the animals in order to look for alterations such as morphological changes, infiltrating hemocytes and necrotic foci. The quantitative histopathological approach was performed on the epithelia of digestive gland tubules (hepatopancreas), for this being the organ potentially involved in toxicant storage and detoxification, similarly to the vertebrate liver (Saravana Bhavan and Geraldine, 2000; Wu et al., 2008; Walker et al., 2010). Accordingly, measurements were performed on: thickness of tubular epithelia; number of vacuoles (non-digestive), non-digestive vacuole diameter and cell type ratios. Cell types were classified into F (Fibrillar)-cells with one fibrillar appearance, digestive enzymes synthesis and a developed rough endoplasmic reticulum (Al-Mohanna et al., 1985); R

(Resorptive)-cells are resorptive or absorptive cells with abundant vacuoles containing lipids and glycogen (Al-Mohanna and Nott, 1987); and B (Blister-like)-cells with a large digestive vacuole responsible for intra-cellular digestion and material elimination (Al-Mohanna and Nott, 1986). All measurements, counts and ratios were obtained from random intact sections and averaged per individual.

Measurements were made using the Image J software (National Institutes for Health, USA). The thickness of tubular epithelium was calculated by measuring the thickness of at least eight intact tubule sections (minimum of twenty measurements per sample). Vacuolar diameter was calculated by measuring the vacuole diameters of twenty-five epithelial cells per sample (minimum of twenty-five measurements per sample). The mean number of non-digestive vacuoles per cell was estimated from counting on at least twenty-five epithelial cells.

3.4. Statistical analyses

Quantitative variables were compared between treatments through the nonparametric Mann-Whitney *U* test and the Spearman rank-order correlation *R* statistic were employed at all the treatments, with differentiation between males and females. Non-parametric statistics were employed after invalidation of at least one of the assumption of parametric tests, namely homogeneity of variances (homocedasticity) and normality (determined through Levene's and Kolmogoroff-Smirnoff's, respectively. Principal component analysis (correlation-based) was employed to determine links between variables and the nominal concentrations of simvastatin. A significance level α was set at 0.05 for all analyses. All statistics were performed with Statistica (Statsoft, USA).

4. Results

4.1. Microanatomy of Gammarus locusta

Gammarus locusta presented a simple internal structure (Figure 1 A), with a mildly PAS-positive (light pink) exoskeleton, indicating chitin. Internally, the main organs are located within the organ cavity (hemal space). The hepatopancreas, intestine and gonads are the three principal organs that were observed in the histological screens through the mid-body plan. The gonads are localized on the dorsal zone, but in case of the gonads of females at more advanced maturation stages, these were observed to occupy the entire space around the intestine and hepatopancreas. Typically, females were found to be in a more advanced stage of maturation than males. The principal target organ of the present study, the hepatopancreas, is responsible for the digestive, absorption and excretory functions. The hepatopancreas was observed to be composed of four tubules (diverticula), located around and along the intestine, with a similar structure as that described by Correia et al. (2002). The tubules consisted of circular structures constituted by a single-cell layer epithelium surrounding the lumen (Figure 1 B). Both the lumen of tubules and the lumen of the gut contained AB-positive (blue) substances, indicating the presence of acidic polysaccharides, likely from the ingestion of algal material.

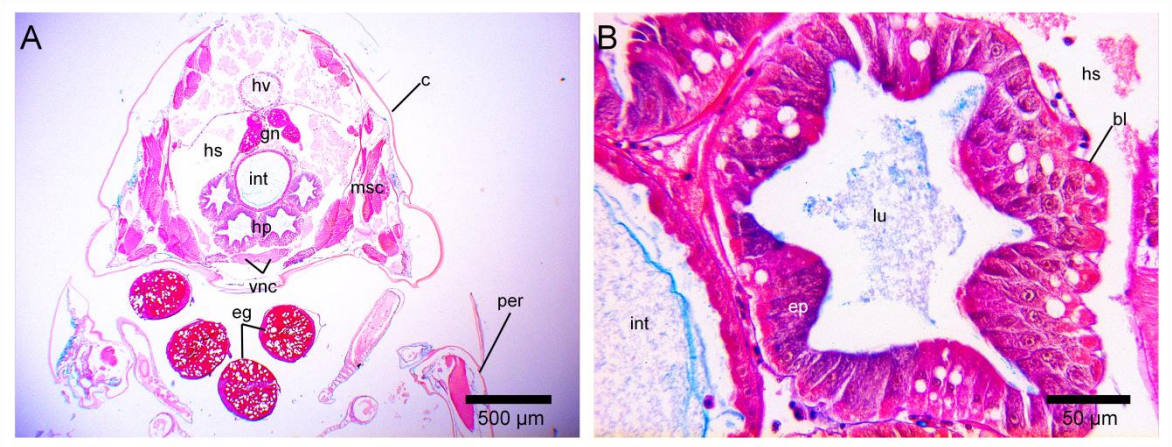

Figure 1 – *Gammarus locusta*: internal description of a blank animal, transversal mid-body section fixed on Carnoy and stained for TC stain. **A:** Description general of mature female, with eggs (eg) on marsupial sac between pereopods (per); was visible the external chitin exoskeleton (c), the muscle (msc), ventral nerve cords (vnc); on mid-body plan was visible the intestine (int) surrounded by hepatopancreas tubules (hp) and gonads (gn) and hemolymphatic vessel (hv) on dorsal zone. **B:** Hepatopancreas tubule, with a single-cell layer epithelium (ep) surrounding the lumen (lu) and lined by the basal lamina (bl). The main organs were located in the hemal space (hs)

The comparison between AO- and TC-stained samples permitted a good differentiation between the three epithelial cell types. F-cells had a fibrillar appearance, resulting from a well-developed rough endoplasmic reticulum, indicating active enzyme synthesis, since ribosomal RNA confers to the cells a reddish coloration when stained with Acridine Orange (AO) (Figure 2 A) and brownish from Weigert's Hematoxylin used in the TC stain (Figure 2 B). The F-cells normally did not have vacuoles, but sometimes one or two vacuoles could be observed, possibly associated to the differentiation into B-cells. B-cells (Figure 2 C, D), on their turn, were characterized by a single large vacuole occupying the cytoplasm almost entirely. These vacuoles contained AB-positive material (Figure 2 D), thus likely associated to digestion. R-cells had numerous vacuoles which contributed to the focal enlargement of cells and subsequently increased epithelium thickness. The cells presented a bright pink staining from the PAS-Schiff's reaction (TC stain), typically associated to glycogen storage (Figure 2 B), and were mainly AO metachromatic (yellowish) (Figure 2 A), indicating metabolically highly active cytoplasms containing densely packed materials and organelles.

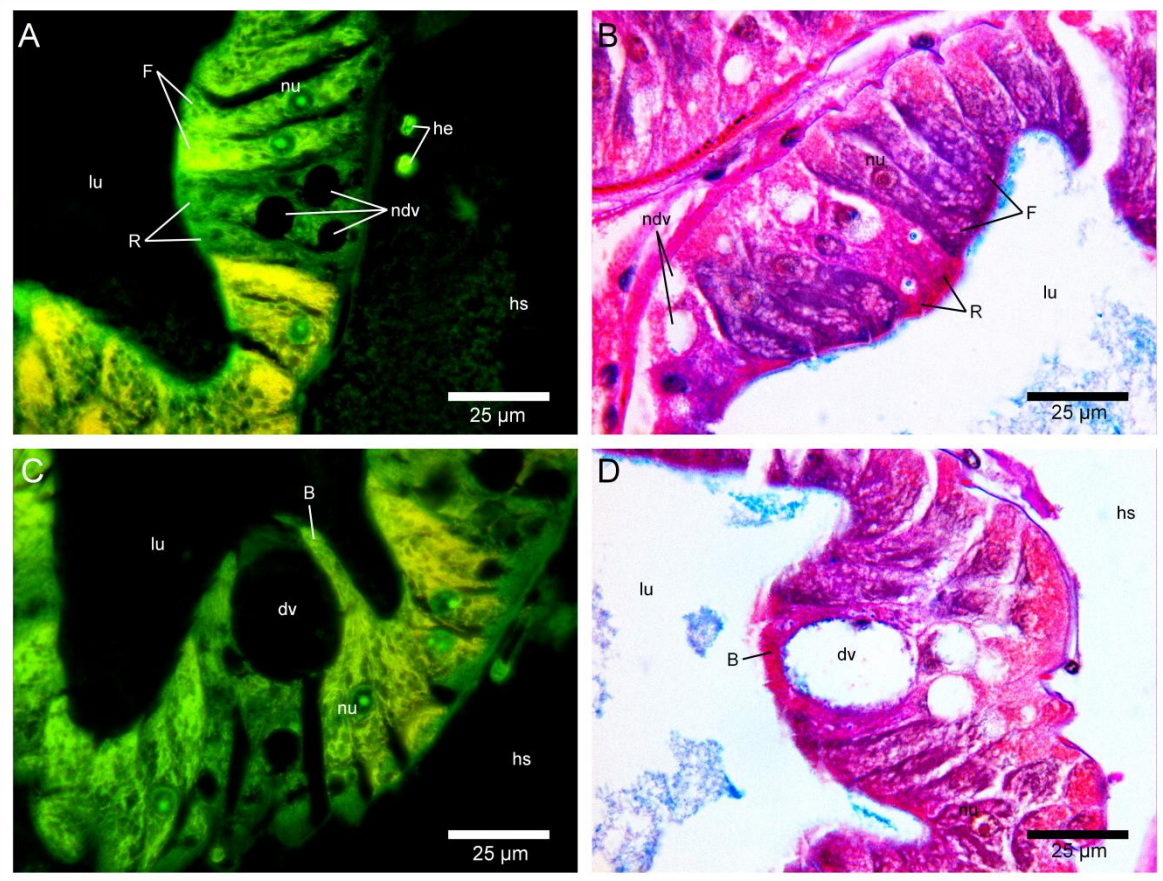

Figure 2 – *Gammarus locusta*: description of hepatopancreas epithelial cells of blank animals. **A:** Epithelial cells of an animal fixed in Bouin-Hollande's solution and stained for AO stain. The R-cells (R), with numerous non-digestive vacuoles (ndv) presenting AO metachromasia, indicative of nuclear DNA. The F-cells (F) presented AO ortochomasia indicative of nuclear RNA and a developed endoplasmic reticulum. ▶

◄Some hemocytes (he) are visible, yielding high positive AO metachomasia **B:** Epithelial cells (TC stain) of an animal fixed in Carnoy's. R-cells (R) presented a bright pink staining from the PAS-Schiff's reaction, indicative of glycogen storage, and F-cells (F) presented a brownish staining from Weigert's Hematoxylin, indicative of a well-developed endoplasmic reticulum occupying most of the cytoplasm. **C:** Epithelial cells in an animal fixed in Bouin-Hollande's solution and stained with AO. The B-cells (B) hold a large digestive vacuole (dv) responsible by intracellular digestion. **D:** Epithelial cells of an animal fixed in Carnoy's and stained with TC. The digestive vacuole (dv) in B-cells (B) revealed AB-positive undifferentiated materials. The cells' nuclei (nu) was stained brownish from Weigert's Hematoxylin reaction in panels B and D; and AO metachromatic (yellowish) in panels A and C. Hemal space (hs); lumen (lu).

4.2. Histopathological alterations

No gross histopathological lesions were observed in any of the organs analyzed (hepatopancreas, gonads and muscle) for both males and females, including blanks (B) and controls (C). Also, the qualitative histopathological evaluation showed only few cases of infiltrating hemocytes (Figure 3B, C), in animals subjected to all treatments, also including blanks (B) and controls (C), without a clear trend concerning exposure concentration. The individuals did not present necrotic foci in any of the organs subjected to histological analyses.

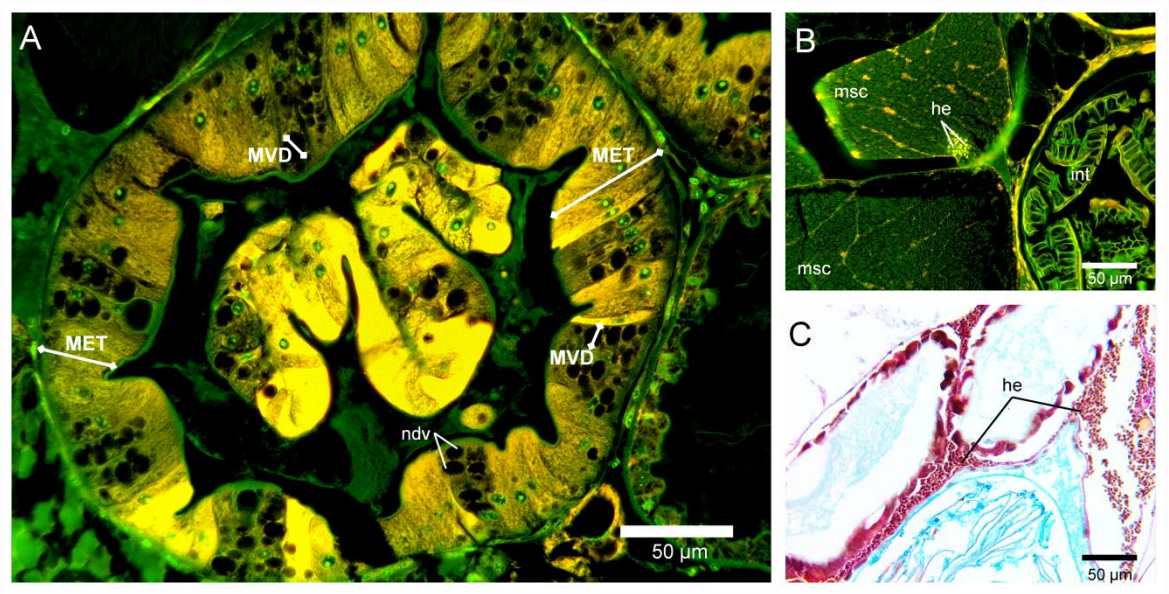

Figure 3 – Histopathological alterations on *Gammarus locusta* exposed to simvastatin. **A:** Indication of quantitative biomarkers in hepatopancreas tubule of animal exposed to 0.064 µg.L^{-1} simvastatin concentration; fixed on Bouin-Hollande's solution and stained with AO: mean non-digestive vacuole diameter (MVD) and mean epithelium thickness (MET). The mean number non-digestive vacuoles was determined by counting non-digestive vacuoles (ndv). **B:** Hemocytes (he) infiltrating in muscle tissue (msc) of an animal exposed to 0.32 µg. L^{-1} simvastatin (Bouin-Hollande's, AO stain). **C:** Hemocytes (he) aggregating in the hemal space of blank animal (Bouin-Hollande's, TC stain).

The quantitative histopathological evaluation (Figure 3 A) revealed alterations in the thickness of tubular epithelia (measured in areas where visible accumulation of vacuoles did not occur to avoid biasing from vacuolation), non-digestive vacuole diameter, number of vacuoles (non-digestive) and cell type ratio, associated to simvastatin concentrations. The mean epithelial thickness of the hepatopancreas (for both males and females) varied with the concentration of exposure, however, this variation was not monotonic, increasing up to exposure concentrations of 0.064 and 0.32 $\mu g.L^{-1}$, decreasing at higher concentrations of exposure (Figure 4 A). However, this variation was more pronounced in females (Figure 4 A). Statistical analyses revealed significant differences in the thickness of tubular epithelia between the group exposed to 0.064 $\mu g.L^{-1}$, with a mean thickness of 21.64 ± 6.14 μm and the group exposed to 8 $\mu g.L^{-1}$, with a mean thickness of 14.79 ± 1.99 μm (males and females combined) (Mann-Whitney U, $p < 0.05$).

Non-digestive vacuole diameter (Figure 4 B) decreased with increasing simvastatin concentrations. The mean vacuole diameter presented a significant difference between the control group (C) with a mean vacuole diameter of 7.29 ± 2.27 μm and the group exposed to concentration of 8 $\mu g.L^{-1}$ with a mean vacuole diameter of 4.90 ± 0.95 μm, combining females and males. Females had a more regular decrease in mean vacuole diameter than males, from controls to higher simvastatin concentration (8 $\mu g.L^{-1}$). There was a significant difference between females and males to concentration of 0.064 $\mu g.L^{-1}$, with the vacuole diameter being higher in females (Mann-Whitney U, $p < 0.05$).

On the other hand, the mean number of non-digestive vacuoles (Figure 4 C) per cell did not present significant differences between exposure treatments and controls, combining males and females. However, a few females had R-cells with more numerous vacuoles, usually between four or six per cell. Nonetheless, only controls yielded significant differences between females and males (Mann-Whitney U, $p < 0.05$). The group exposed to the simvastatin concentration of 1.6 $\mu g.L^{-1}$, and only for females, presented a significant decrease in the average number of vacuoles, compared to all other treatments.

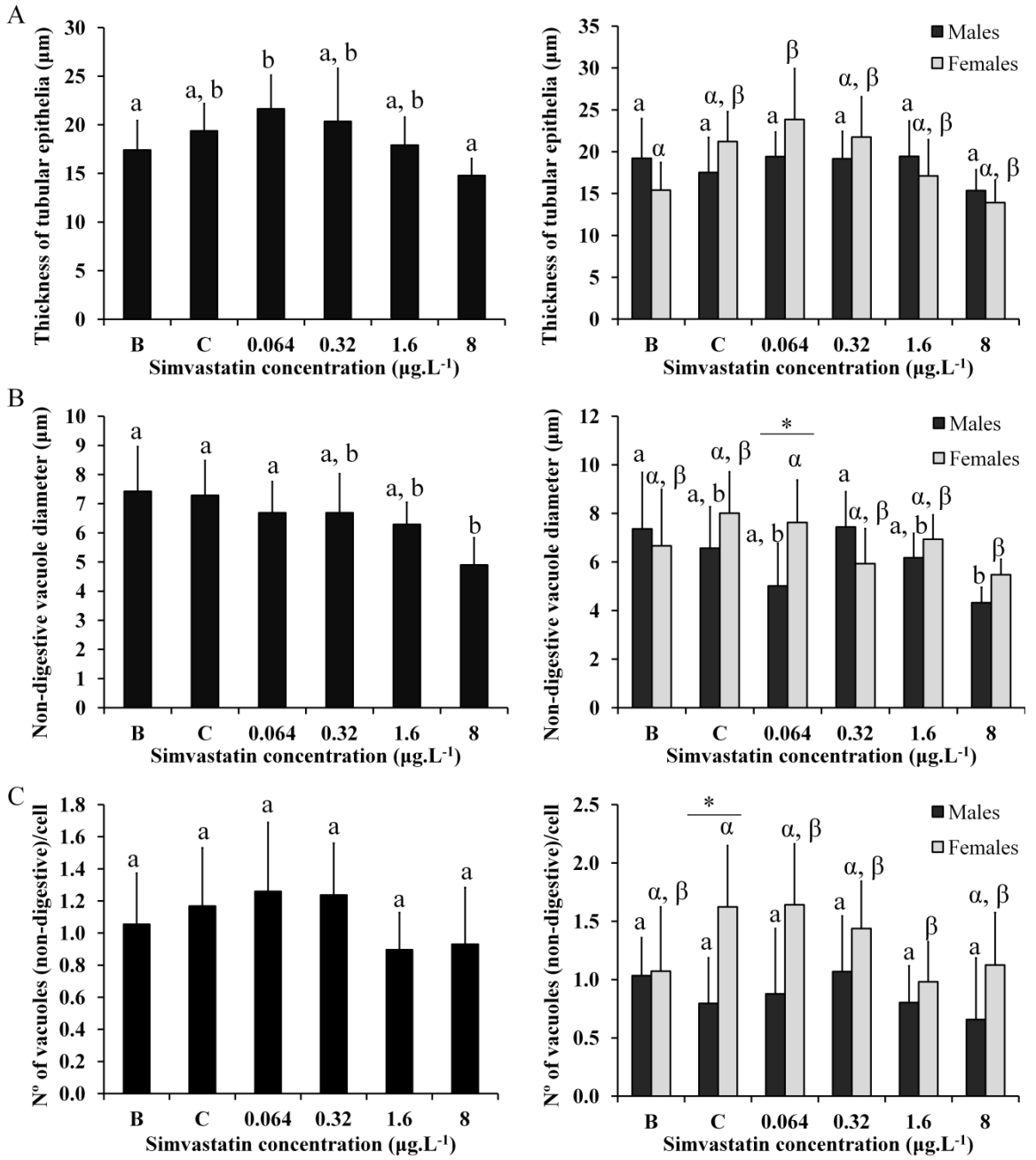

Figure 4 – Mean results per simvastatin nominal concentration, males and females combined (left) and separated (right) .**A:** Thickness of tubular epithelia. **B:** Non-digestive vacuole diameter **C:** Number of non-digestive vacuoles per epithelial cell. Different letters mean significant differences (Mann–Whitney U test, $p < 0.05$); latin letters for males results and greek letters for females results. (*) significant difference between males and females. B – blank; C – control (solvent only).

F-cells were more abundant in tubular epithelia that R-cells, and B-cells were the least numerous (Figure 5 A). Apparently, the F-cells ratio (Figure 5 D) presented few variations between treatments and control group, for either males or females. R-cells ratio (Figure 5 C) increased with simvastatin concentrations in males but not in females. The B-cells ratio (Figure 5 B) was higher in animals exposed to the concentration of 0.064 $\mu g.L^{-1}$ and decreased with the following simvastatin

concentrations, for both sexes. However, cell ratios did not reveal significant differences between treatments and control group except the F-ratio for the concentration of 0.32 $\mu g.L^{-1}$, for females (Figure 5 D). There was a significant difference between females and males in control group regarding the R-ratio, being higher in females (Mann-Whitney U, $p < 0.05$).

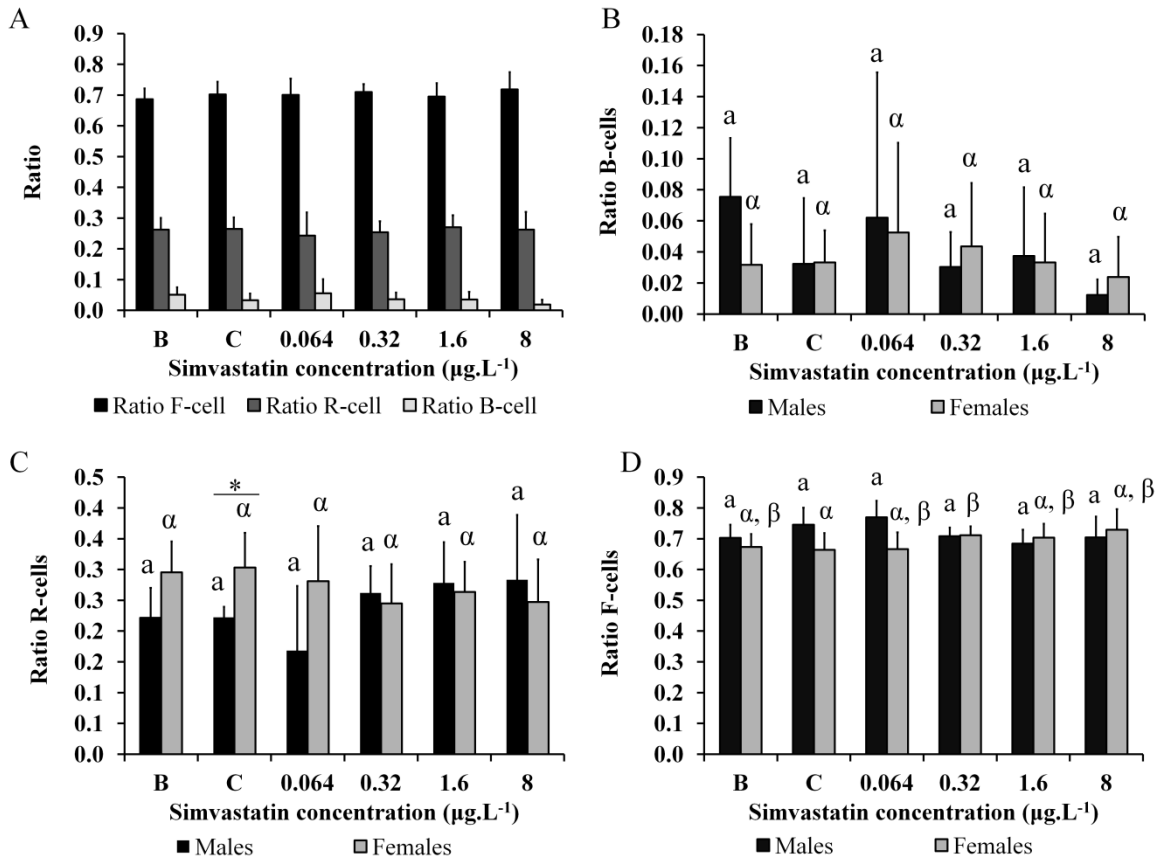

Figure 5 – Mean of cell ratios per simvastatin concentration. **A:** Cell ratios per cell type, for males and females combined. **B:** B-cell ratio. **C:** R-cell ratios. D: F-cell ratios. Different letters mean significant differences (Mann–Whitney U test, $p < 0.05$); latin letters for males ratios and greek letters for females ratios. (*) significant difference between males and females. B – blank; C – control (solvent only).

4.3. Multivariate analyses

Correlations between all variables, independently of sex and simvastatin concentrations, are presented in Table 1. The F- and R-ratios yielded the most significant (negative) correlation (Spearman‘s rank-order correlation R= -0.8527, $p < 0.05$), as expected. The mean epithelium thickness (MET) presented a significant positive correlation with mean non-digestive vacuole diameter (MVD) and number (MVN) ($R > 0.4$ and $p < 0.05$).

Table 1 - Spearman's rank-order correlation R statistics (all biological variables). Highlighted statistics are significant at $p < 0.05$.

	MET	MVD	MVN	R-ratio	B-ratio	F-ratio
MET	1.0000					
MVD	0.4121	1.0000				
MVN	0.4348	0.3438	1.0000			
R-ratio	0.2126	0.1843	0.3184	1.0000		
B-ratio	-0.1500	0.1702	0.0358	-0.2869	1.0000	
F-ratio	-0.1088	-0.2722	-0.3007	-0.8527	-0.1461	1.0000

MET – mean epithelium thickness; MVD – mean non-digestive vacuole diameter; MVN – mean non-digestive vacuoles number; R-ratio – R-cell ratio; B-ratio. – B-cell ratio; F-ratio – F-cell ratio; correlation > 0.4; correlation > 0.5.

The survival rate was variable, ranging between the averages of 15 and 51% for females; and between 7 and 43% for males. The correlations between survival rates and simvastatin concentrations only yielded a significant and negative correlation in males ($R= -0.4650$, $p < 0.05$). The survival rates for simvastatin treatments were similar to those of the control groups, without a clear trend, either dose-dependent. Females presented a maximum survival rate for 0.064 $\mu g.L^{-1}$ simvastatin and males presented a maximum survival rate for the concentration of 0.32 $\mu g.L^{-1}$.

Principal component analysis (PCA) for all individuals (Figure 6 A), independently of sex, retrieved two factors that, combined, explained over 60% of the total variance. The first principal component factor explained 37.26% of the total variance and the second principal component explained 24.24%. The F-ratio revealed a positive link with nominal concentration of simvastatin, unlike the R-ratio which presented a negative relationship. Nominal concentrations, epithelium thickness (MET) and vacuole number (MVN) were positively linked.

Females presented a first principal component factor that explained 45.42% of the total variance and a second that explained 18.94% (Figure 6 B). On total, the two factorial axes explained over 64% of total variance. Epithelium thickness (MET) and vacuole diameter (MVD) presented negative relations whit nominal concentrations of simvastatin. Males presented a fist principal component which explained 35.96% of the total variance and a second principal component which explained 27.58% (Figure 6 C).On total, the two factorial axes explained over 63% of total variance. The mean epithelium thickness (MET) and vacuole number (MVN) presented negative

correlations whit nominal concentrations. Unlike the females, the R-ratio and nominal concentrations yielded a positive relation in males.

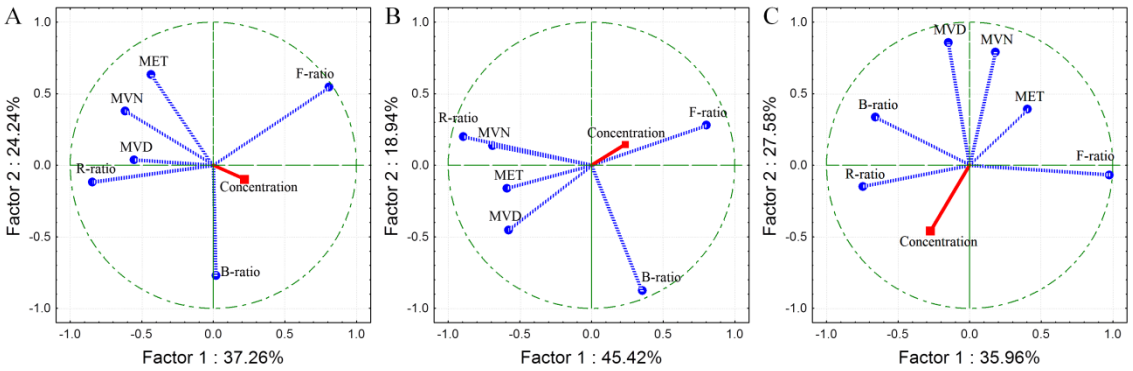

Figure 6 – Principal component analysis (PCA). **A:** PCA for both males and females together. **B:** PCA for females. **C:** PCA for males. ■ supplementary variable; ● active variable. MET – mean epithelium thickness; MVD – mean non-digestive vacuole diameter; MVN – mean non-digestive vacuoles number; R-ratio – R-cell ratio; B-ratio. – B-cell ratio; F-ratio – F-cell ratio.

5. Discussion

Simvastatin concentrations that were tested in the present study revealed a trend to induce alterations in metabolic processes, rather than severe histopathological lesions or significant morphological alterations, in the organs present in mid-body sections of the amphipods. The drug affected mainly the hepatopancreas epithelial cells, reflecting alterations that are consistent with physiological and metabolic alterations; such as the F-cell ratio that appear positive dose-dependent or epithelium thickness and non-digestive vacuole diameter that appears to be negatively dose-dependent of simvastatin concentrations (Figure 6 A). However, for some of the measured effects, the response curve is not monotonic, such as for changes in epithelial thickness which appear to increase from blanks and controls to the lower exposure concentrations, and decrease linearly to higher concentrations (Figure 4 A); and number of intracellular non-digestive vacuoles which presented the same trend of thickness. Interestingly these two biomarkers were found highly correlated (Table 1), even though the measurements were made in areas of non-vacuolated cells, in order to avoid biasing. Between males and females moderate but significant differences occurred in the surveyed histopathological biomarkers, as R-cells ratio (Figure 5 C), probably associated to sexual dimorphism in basal carbohydrate metabolism and energy reserves.

In most animals, simvastatin acts by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the enzyme that permits the conversion of Acetyl-CoA to mevalonate, a precursor of cholesterol (Mauro, 1993; Desager and Horsmans, 1996; Patil et al., 2007; Key et al., 2008). In vertebrates, best described in mammals, the organ that simvastatin affects more directly is the liver, where the drug is biotransformed to several metabolites by cytochrome P450 (CYP), such as simvastatin acid, which intervenes in cholesterol biosynthesis by HMG-CoA reductase inhibition (Mauro, 1993; Patil et al., 2007). Following the addition of squalene (a precursor of cholesterol), the mevalonate pathway results in other products: ubiquinone (coenzyme Q), an antioxidant substance that is also important in the electron transport chain of mitochondria by permitting electron transport from NADH to citochrome c; dolichol, with the function of oligosaccharide transport to proteins synthesized in endoplasmic reticulum (protein glycosylation); isopentenyl adenosine, that interferes in t-RNA modification and protein synthesis; and farnesyl diphosphate and geranylgeranyl diphosphate, important intermediate compounds for the synthesis of proteins (Gerson et al., 1989; Desager and Horsmans, 1996; Bentinger et al., 2010).

In invertebrates, namely in crustaceans, the hepatopancreas is the analogous organ to the vertebrate liver. It is responsible for xenobiotic detoxification between other functions, such as digestion, protein synthesis, secretion and enzyme synthesis, storage and carbohydrate metabolization and it is highly sensitive to polluting agents (Saravana Bhavan and Geraldine, 2000; Correia et al., 2002;

Wu et al., 2008; Walker et al., 2010). Accordingly, Walton and Pennock (1972) reported that the mevalonate pathway in invertebrates is similar to the process described for vertebrates, however, sterols, in crustaceans, are mostly acquired from diet. Furthermore, Desager and Horsmans (1996) concluded that simvastatin also decreases acyl coenzyme A acyltransferase (ACAT) activity, which is responsible for cholesterol uptake directly from the diet, so simvastatin may reduce the contents of cholesterol and similar compounds in invertebrates. Still, it must be notes that these processes are variable between taxaonomic groups. For instance, Chang (1995), in a study with the nematode *Caenorhabditis elegans* described the mevalonate pathway with production of the coenzyme Q, dolichol and precursors of proteins synthesis, but not squalene. In amphipods, the simvastatin effects are little known and the abovementioned processes are less studied, but it probably is similar to the pathways described by Walton and Pennock (1972) for crustaceans *Carcinus maenas* and *Eupagurus bernhardus*, and Chang (1995) for the nematode *C. elegans*.

There are contradictions in relation to the simvastatin effects, which probably are connected with several variables such as concentration, exposure method and assay conditions. Nevertheless, the present study showed that the drug affected cellular metabolism, with consequences at the histological level. Altogether, these results indicate that simvastatin hardly affects only the mevalonate process, but rather those directly or indirectly connected to Acetyl-CoA. As such, ATP production through the Krebs cycle, where the Acetyl-CoA is a precursor of citric acid (Junqueira and Carneiro, 2004), as well as the synthesis of triglycerides (Steinberg et al., 1961), are some of the processes that are probably altered by simvastatin exposure, which aids explaining the physiological changes endured in *G. locusta* exposed to the drug, leading to alterations in the epithelia of the hepatopancreas. The trend to increase the number of non-digestive vacuoles (which are devoid of inclusions or PAS-positive substances), in animals exposed to the lower simvastatin concentrations (0.064 and 0.32 $\mu g.L^{-1}$) suggest an increase of lipid storage (i.e. lipid that is washed-off during sample processing) in tubule epithelial cells, probably triglycerides or other hydrophobic reserve carbohydrates. This storage is probably associated to simvastatin inhibition of the HMG-CoA reductase, therefore hampering Acetyl-CoA transformation into mevalonate, then permitting an increase of Acetyl-CoA concentration in cell. Consequently, the excessive Acetyl-CoA may be used in other processes as fatty acid/triglyceride synthesis, as well was described in mammals by Desager and Horsmans (1996) where simvastatin improves the production of triglycerides and its storage in the liver (reverse cholesterol transport). In amphipods, this process occurs in hepatopancreas, which aids explaining the increased formation of lipid storage vacuoles, at least for lower concentrations of exposure. In fact, the triglycerides are the principal storage lipids in amphipods (Clarke et al., 1985).

The present results revealed a possible increase in this metabolic processes, for the two lower concentrations (0.064 and 0.32 $\mu g.L^{-1}$), which is visible through the increase in epithelium thickness (Figure 4 A) and number of non-digestive vacuoles (Figure 4 C). The increase of epithelium thickness is probably associated to endoplasmic reticulum development related to elevated enzyme production as a defense response for the metabolization and elimination of simvastatin. In relation, the trend to increase the number of non-digestive vacuoles is probably associated to up-regulated metabolic processes that permitted an increase in lipid storage, namely triglycerides. Conversely, higher concentrations of exposure (1.6 and 8 $\mu g.L^{-1}$) yielded negative responses, which may now be associated to deregulation of basal metabolic processes, especially those related to energy production and carbohydrate metabolism, leading to either decreased lipid production of increased lipid catabolism, or a combination of both. Consequently the storage of hydrocarbons in hepatopancreas (free sugars and lipids and hydrocarbons stored in R-cells) decreased such as has been observed by Dahl et al. (2006), who stated that copepods used lipid reserves to produce energy to promote survival to higher simvastatin concentrations. Also, it must be noticed that, in aerobic energy production pathways, the Krebs cycle and the electron transport chain stand out as the most critical processes, the former being able to be disrupted by the drug via changes to acetyl-CoA availability. As such, unbalancing the processes likely results in increasing intracellular levels of reactive oxygen species, leading to oxidative stress (Bentinger et al., 2010). This instability has probably been responsible, at least in part, by the reduction of the enzymatic system followed by decreasing endoplasmic reticulum and consequent decrease of epithelium thickness.

The bi-tonic dose-response curves in this study are similar to those described by Dahl et al. (2006) and Key et al. (2008). Dahl et al. (2006) described, in copepods, that RNA contents would increase for simvastatin concentrations up to 0.16 $\mu g.L^{-1}$, followed by decrease in animals exposed to higher concentrations of the drug. Interestingly, RNA contents may hold a relation with epithelium thickness, through changes in ribosome RNA (rRNA) contents, thus affecting, for instance, enzyme production and all other processes involving protein biosynthesis. Also, Key et al. (2008) disclosed acute effects of simvastatin in grass shrimp and observed that the lipid peroxidation was higher for lower concentrations of simvastatin, decreasing significantly for higher concentrations of exposure. Conversely, the diameter of non-digestive vacuoles (Figure 4 B), unlike the two biomarkers mentioned above, presents a monotonic curve, albeint presenting a trend to decrease with increasing of simvastatin concentrations, which may imply decreased storage.

The antioxidant ability in cells may be regulated by coenzyme Q, as observed for mammals by Velho et al. (2006) and Bentinger et al. (2010) and in crustaceans by Walton and Pennock (1972). In the livers of murine models exposed to lovastatin, a statin drug similar to simvastatin, Velho et

al. (2006) observed toxic effects associated to coenzyme Q inhibition that contributed to break the electron chain in mitochondrial respiration, decreasing ATP production and increasing oxidative stress. The same authors also observed a dose-dependent relationship between exposure to lovastatin and elevated intracellular Ca^{2+}, consequently increasing mitochondria membrane permeability. The increased permeability of the mitochondrial membrane increases the probability of occurring cytochrome c release, which is one of the triggers of apoptosis (Bentinger et al., 2010). In accordance, in human cells, Werner et al. (2004) found a dose-response curve between simvastatin exposure and caspase activation that induced apoptosis. However Parihar et al.(2011), on another study in rats about the effects of statins, including simvastatin, observed decreased mitochondrial oxidative stress in liver cells and decreased Ca^{2+} levels, thus preventing membrane permeability and the release of cytochrome c to the cytoplasm. The same authors observed that simvastatin contributes to lowering lipid peroxidation and decreased concentration of reactive oxygen species in the cell. In the current study apoptosis was not observed but the critical metabolic alterations in hepatopancreas may indicate that simvastatin is particularly toxic to the species by affecting multiple metabolic processes related to energy balance and oxidative stress.

The epithelium cells of hepatopancreas tubules (diverticula) have several functions, each cell type being specialized in one or multiple processes. Three main cell types were observed in this study, namely, B-, F- and R-cells; however some authors describe the existence of other epithelium cell types in the crustacean hepatopancreas, such as E- (embryonic) cells, undifferentiated cells that divide mitotically in R- and F-cells to replace dead cells (Al-Mohanna et al., 1985); and M- (midget) cells that have a similar storage function to the R-cells but are responsible for the storing of glycol-protein materials and glycogen (Al-Mohanna and Nott, 1987). Nevertheless, E- and M-cells were not observed in the present work.

The cell ratios (relatively to the total number of epithelial cells), especially those for F- and R-cells, supported the identification of simvastatin exposure effects and are most likely linked to metabolic alterations. Combining males and females (Figure 6 A), principal component analysis (PCA) revealed that the ratio of F-cells was positively correlated with simvastatin concentrations while, and at the same time, the ratio of R-cells presented a negative correlation with the concentration of the drug. Although the cell ratios, in the current results, failed to reveal much significant variations, it is important to refer that an increase of the F-cell ratio should mean an increase in the production of digestive enzymes (Al-Mohanna et al., 1985). Also, this effect may reflect changes in the digestion process since the B-cell ratio, responsible for intracellular digestion (Al-Mohanna and Nott, 1986), also presented a positive correlation with the simvastatin concentration.

The R-cells, in crustaceans, are associated to the storage of glycogen and lipids, to which are added important detoxification functions (Al-Mohanna and Nott, 1987). The presence of undifferentiated glycogen deposits in the cytoplasm (but not in vacuoles) was visible through the PAS-Schiff's reaction (in TC stained-samples). Food items are processed in the lumen of the hepatopancreas, where, through pinocytosis, B-cells assimilate food particles that will undergo intracellular digestion (Al-Mohanna and Nott, 1986), an evolutionary archaic process that persists in many invertebrate animals. After intracellular digestion, the small molecules are diffused to the haemolymph while undigested materials are exocytized to the lumen, some of this for elimination (Al-Mohanna and Nott, 1986). After, the R-cells' role is to assimilate substances and partly digested materials from both the haemolymph and the lumen of tubules, by diffusion or pinocytosis. All the material is processed in the cells' cytoplasm to originate lipids and glycogen, which are used in several metabolic processes (Al-Mohanna and Nott, 1987). The surplus lipids are stored in the non-digestive vacuoles that are numerous in R-cells (cells's main carbohydrate reserve). As mentioned above, non-digestive vacuoles were not stained by any histochemical technique, suggesting lipid contents. However, it should not be disregarded that non-digestive vacuoles can contain contaminants and/or their metabolites, as simvastatin (see Al-Mohanna and Nott, 1987). In accordance, PCA analyses revealed a negative correlation between simvastatin concentration and the R-cell ratio (Figure 6 A), which can be associated to a depletion of lipid reserves.

Moderate differences were observed between males and females suggesting some degree of sexual dimorphism in amphipods associated to higher energy reserves, namely lipids and glycogen, in females than males, which is in accordance with the findings by Sroda and Cossu-Leguille (2011) and Gismondi et al. (2012) in another amphipod, *Gammarus roeseli*. In the present study, females presented a significantly higher mean number of non-digestive vacuoles (Figure 4 C) as well as the R-cell ratio (Figure 5 C) on control group than males that suggest a higher lipid reserves in females hepatopancreas under normal circumstances. Even at the lower concentration of exposure (0.064 $\mu g.L^{-1}$), the diameter of non-digestive vacuoles (Figure 4 B) was larger in female epithelial cells. These results are in agreement with the premise that females have a higher natural capacity to store lipids, which is probably associated to the high energetic demand of reproduction (Sroda and Cossu-Leguille, 2011). In fact, females, when maturity is being reached, tend to accumulate high amounts of lipids, which are essential for egg production (Clarke et al., 1985). It should be notes that, in the present study, the females were already at a late stage of sexual maturation, with well develop gonads and, inclusively, some already held mature eggs in their marsupial sac (Figure 1 A).

The differences between males and females associated to simvastatin exposure also revealed some degree of sexual dimorphism in relation to the ability to respond of toxicological stress. In fact, the survival evaluation reveled that males are more affected by simvastatin than females, given that presents a decrease of the survival rate with increasing exposure concentrations. However, the survival rates for simvastatin did not have a clear trend (dose-dependent), which indicates that acute effects of the drug onto the species have yet to be understood. In addition, the F-cells ratio in females increased up to exposure to 0.32 $\mu g.L^{-1}$ in comparison to the control group, unlike males, who failed to exhibit significant alterations in this ratio (Figure 5 D). In fact, it has been suggested that females have a more pronounced defense ability towards contamination than males (Sroda and Cossu-Leguille, 2011; Gismondi et al., 2012). Furthermore, the increase of F-cell ratio is probably associated to elevated enzyme synthesis and secretion (see Al-Mohanna et al., 1985), some of which may participate in antioxidant defense or contaminant catabolism and elimination. In fact, amphipods females presented a large antioxidant defense that was demonstrated by Sroda and Cossu-Leguille (2011) and Gismondi et al. (2012) when found higher values of glutathione in *Gammarus roeseli* females than males. Also, in accordance, the PCA results for females (Figure 6 B) revealed a positive correlation between simvastatin concentration and the F-cell ratio, whereas was strikingly the opposite for males (Figure 6 C). The current results also showed that epithelium thickness (Figure 4 A) was unchanged in males, which indicates unaltered metabolism.

6. Conclusions

The present study revealed that simvastatin affects carbohydrate metabolism and storage in the amphipod *Gammarus locusta*. Also, it has been shown that the hepatopancreas was the main organ affected by simvastatin. The reason of this preferential localization of the alterations is probably due to the fact that hepatopancreas is a particularly active organ, having many functions such as the digestion process, protein and enzyme synthesis, storage and carbohydrate metabolization, and organism decontamination. Some physiological alterations in the hepatopancreas epithelium presented a moderate dose-dependent relation with simvastatin concentration, especially concerning epithelium thickness, probably evidencing a variation in the development of the endoplasmic reticulum and, consequently, enzyme production and activity. Also importantly, the number and diameter of non-digestive vacuoles evidenced that lipid reserves tend to deplete with the increasing of simvastatin concentration. Nevertheless, it has been shown that the dose-response curves are not-monotonic for most of the measured endpoints, rather showing that higher concentrations tend to impair metabolism, with consequences at the histopathological level.

Another important point in this study concerns the differences between males and females in relation to ability to respond to simvastatin exposure. Females appear to respond more actively than males, which is likely associated to the higher energy reserves and demand, as verified by increasing number and diameter of the non-digestive vacuoles in females.

The current findings thus suggest that, in *Gammarus locusta*, simvastatin affects primarily the cellular metabolism. As such, in the future, it would be important to develop further studies with the drug to survey other endpoints such as those related to oxidative stress, cholesterol contents, etc., and to correlate with histological findings. Yet another important approach would be the evaluation of development and growth following exposure to simvastatin, which are probably the most sensitive biological processes to changes in basal physiological functions.

7. References

Al-Mohanna, S.Y., Nott, J.A., 1986. B-cells and digestion in the hepatopancreas of *Penaeus semisulcatus* (Crustacea: Decapoda). J. Mar. Biol. Assoc. U. K. 66, 403–414.

Al-Mohanna, S.Y., Nott, J.A., 1987. R-cells and the digestive cycle in *Penaeus semisulcatus* (Crustacea: Decapoda). Mar. Biol. 95, 129–137.

Al-Mohanna, S.Y., Nott, J.A., Lane, D.J.W., 1985. Mitotic E-and secretory F-cells in the hepatopancreas of the shrimp *Penaeus semisulcatus* (Crustacea: Decapoda). J. Mar. Biol. Assoc. U.K. 65, 901–910.

ASTM, (1992). Standard guide for conducting 10-day static toxicity tests with marine and estuarine amphipods. Annual Book of ASTM Standards, Water and Environmental Technology (Vol. 11.04, E1367–90). Philadelphia: American Society for Testing and Materials.

Au, D.W.T., 2004. The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. Mar. Pollut. Bull. 48, 817–834.

Bat, L., Raffaelli, D., 1998. Sediment toxicity testing: a bioassay approach using the amphipod *Corophium volutator* and the polychaete *Arenicola marina*. J. Exp. Mar. Biol. Ecol. 226, 217–239.

Bentinger, M., Tekle, M., Dallner, G., 2010. Coenzyme Q – Biosynthesis and functions. Biochem. Biophys. Res. Commun. 396, 74–79.

Casas López, J.L., Sánchez Pérez, J.A., Fernández Sevilla, J.M., Acién Fernández, F.G., Molina Grima, E., Chisti, Y., 2004. Fermentation optimization for the production of lovastatin by *Aspergillus terreus*: use of response surface methodology. J. Chem. Technol. Biotechnol. 79, 1119–1126.

Chang, E.S., 1995. Physiological and biochemical changes during the molt cycle in decapod crustaceans: an overview. J. Exp. Mar. Biol. Ecol. 193, 1–14.

Chapman, P.M., 2007. Determining when contamination is pollution – Weight of evidence determinations for sediments and effluents. Environ. Int. 33, 492–501.

Clarke, A., Skadsheim, A., Holmes, L.J., 1985. Lipid biochemistry and reproductive biology in two species of Gammaridae (Crustacea: Amphipoda). Mar. Biol. 88, 247–263.

Correia, A.D., Pereira, A.L., Costa, M.H., Carrapiço, F., 2002. Functional anatomy of the midgut gland of *Gammarus locusta* (Crustacea: Amphipoda). J. Mar. Biol. Assoc. U. K. 82, 201–204.

Costa, F.O., Correia, A.D., Costa, M.H., 1998. Acute marine sediment toxicity: A potential new test with the amphipod *Gammarus locusta*. Ecotoxicol. Environ. Saf. 40, 81–87.

Costa, F. O., Costa, M. H., 2000. Review of the ecology of *Gammarus locusta* (L.). Polish Archives of Hydrobiology, 48, 541–559

Costa, F.O., Neuparth, T., Correia, A.D., Helena Costa, M., 2005. Multi-level assessment of chronic toxicity of estuarine sediments with the amphipod *Gammarus locusta*: II. Organism and population-level endpoints. Mar. Environ. Res. 60, 93–110.

Costa, P.M., Caeiro, S., Costa, M.H., 2012. Multi-organ histological observations on juvenile *Senegalese soles* exposed to low concentrations of waterborne cadmium. Fish Physiol. Biochem. 39, 143–158.

Costa, P.M., Caeiro, S., Lobo, J., Martins, M., Ferreira, A.M., Caetano, M., Vale, C., DelValls, T.Á., Costa, M.H., 2011. Estuarine ecological risk based on hepatic histopathological indices from laboratory and in situ tested fish. Mar. Pollut. Bull. 62, 55–65.

Costa, P.M., Costa, M.H., 2008. Biochemical and histopathological endpoints of in vivo cadmium toxicity in *Sparus aurata*. Ciencias Mar. 34, 349–361.

Costa, P.M., Costa, M.H., 2012. Development and application of a novel histological multichrome technique for clam histopathology. J. Invertebr. Pathol. 110, 411–414.

Dahl, U., Gorokhova, E., Breitholtz, M., 2006. Application of growth-related sublethal endpoints in ecotoxicological assessments using a harpacticoid copepod. Aquat. Toxicol. 77, 433–438.

Desager, D.J.P., Horsmans, Y., 1996. Clinical pharmacokinetics of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors. Clin. Pharmacokinet. 31, 348–371.

European Environment Agency (EEA), 2010. Pharmaceuticals in environment. EEA Technical report.

Farré, M. la, Pérez, S., Kantiani, L., Barceló, D., 2008. Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. TrAC Trends Anal. Chem. 27, 991–1007.

Gerson, R.J., Macdonald, J.S., Alberts, A.W., Kornbrust, D.J., Majka, J.A., Stubbs, R.J., Bokelman, D.L., 1989. Animal safety and toxicology of simvastatin and related hydroxy-methylglutaryl-coenzyme A reductase inhibitors. Am. J. Med. 87, S28–S38.

Gismondi, E., Beisel, J.-N., Cossu-Leguille, C., 2012. Influence of gender and season on reduced glutathione concentration and energy reserves of *Gammarus roeseli*. Environ. Res. 118, 47–52.

Horsmans, Y., Desager, J.P., Harvengt, C., 1990. Biochemical changes and morphological alterations of the liver in guinea-pigs after administration of simvastatin (HMG CoA reductase-inhibitor). Pharmacol. Toxicol. 67, 336–339.

Junqueira, L.C., Carneiro, J., 2004. Histologia básica, 10ª edição. ed. Guanabara Koogan S.A.

Key, P.B., Hoguet, J., Reed, L.A., Chung, K.W., Fulton, M.H., 2008. Effects of the statin antihyperlipidemic agent simvastatin on grass shrimp, *Palaemonetes pugio*. Environ. Toxicol. 23, 153–160.

Martoja, R., Martoja, M., 1967. Initiation aux techniques de l'histologie animal. Masson, Paris

Mauro, D.V.F., 1993. Clinical pharmacokinetics and practical applications of simvastatin. Clin. Pharmacokinet. 24, 195–202.

Neuparth, T., Correia, A.D., Costa, F.O., Lima, G., Costa, M.H., 2005. Multi-level assessment of chronic toxicity of estuarine sediments with the amphipod *Gammarus locusta*: I. Biochemical endpoints. Mar. Environ. Res. 60, 69–91.

Neuparth, T., Costa, F.O., Costa, M.H., 2002. Effects of temperature and salinity on life history of the marine amphipod *Gammarus locusta*. Implications for ecotoxicological testing. Ecotoxicology, 11, 61-73.

NRC: Committee on Biological Markers of the National Research Council, 1987. Biological markers in environmental health research. Environ. Health Perspect. 74, 3 - 9.

Omar, M.A., Wilson, J.P., Cox, T.S., 2001. Rhabdomyolysis and HMG-CoA reductase inhibitors. Ann. Pharmacother. 35, 1096–1107.

Parihar, A., Parihar, M., Zenebe, W., Ghafourifar, P., 2011. Statins lower calcium-induced oxidative stress in isolated mitochondria. Hum. Exp. Toxicol. 31, 355–363.

Patil, P., Patil, V., Paradkar, A., 2007. Formulation of a self-emulsifying system for oral delivery of simvastatin: In vitro and in vivo evaluation. Acta Pharm. 57.

Puchtler, H., Waldrop, F.S., Conner, H.M., Terry, M.S., 1968. Carnoy fixation: practical and theoretical considerations. Histochemie 16, 361–371.

Saravana Bhavan, P., Geraldine, P., 2000. Histopathology of the hepatopancreas and gills of the prawn *Macrobrachium malcolmsonii* exposed to endosulfan. Aquat. Toxicol. 50, 331–339.

Sroda, S., Cossu-Leguille, C., 2011. Seasonal variability of antioxidant biomarkers and energy reserves in the freshwater gammarid *Gammarus roeseli*. Chemosphere 83, 538–544.

Steinberg, D., Vaughan, M., Margolis, S., Pittman, W. the technical assistance of H.P. and R., 1961. Studies of triglyceride biosynthesis in homogenates of adipose tissue. J. Biol. Chem. 236, 1631–1637.

Van der Oost, R., Beyer, J., Vermeulen, N.P., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.

Velho, J.A., Okanobo, H., Degasperi, G.R., Matsumoto, M.Y., Alberici, L.C., Cosso, R.G., Oliveira, H.C.F., Vercesi, A.E., 2006. Statins induce calcium-dependent mitochondrial permeability transition. Toxicology 219, 124–132.

Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical compounds in urban wastewater: Removal, mass load and environmental risk after a secondary treatment – a review. Sci. Total Environ. 429, 123–155.

Walker, A.N., Golden, R., Horst, M.N., 2010. Morphologic effects of in vivo acute exposure to the pesticide methoprene on the hepatopancreas of a non-target organism, *Homarus americanus*. Ecotoxicol. Environ. Saf. 73, 1867–1874.

Walton, M.J., Pennock, J.F., 1972. Some studies on the biosynthesis of ubiquinone, isoprenoid alcohols, squalene and sterols by marine invertebrates. Biochem. J. 127, 471–479.

Werner, M., Sacher, J., Hohenegger, M., 2004. Mutual amplification of apoptosis by statin-induced mitochondrial stress and doxorubicin toxicity in human rhabdomyosarcoma cells. Br. J. Pharmacol. 143, 715–724.

Wu, J. P., Chen, H. C., Huang, D. J., 2008. Histopathological and biochemical evidence of hepatopancreatic toxicity caused by cadmium and zinc in the white shrimp, *Litopenaeus vannamei*. Chemosphere 73, 1019–1026.

Zapata, R., Piulachs, M.-D., Bellés, X., 2002. Ovarian 3-hydroxy-3-methylglutaryl-CoA reductase in *Blattella germanica* (L.): pattern of expression and critical role in embryogenesis. J. Insect Physiol. 48, 675–681.