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Hypocholesterolaemic pharmaceutical simvastatin disrupts reproduction and population growth of the amphipod *Gammarus locusta* at the ng/L range

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ABSTRACT

Simvastatin (SIM), a hypocholesterolaemic drug, is among the most widely used pharmaceuticals worldwide and is therefore of emerging environmental concern. Despite the ubiquitous nature of SIM in the aquatic ecosystems, significant uncertainties exist about sublethal effects of the drug in aquatic organisms. Therefore, here we aimed at investigating a multi-level biological response in the model amphipod *Gammarus locusta*, following chronic exposures to low levels of SIM (64 ng/L to 8 μg/L). The work integrated a battery of key endpoints at individual-level (survival, growth and reproduction) with histopathological biomarkers in hepatopancreas and gonads. Additionally, an individual-based population modelling was used to project the ecological costs associated with long-term exposure to SIM at the population level. SIM severely impacted growth, reproduction and gonad maturation of *G. locusta*, concomitantly to changes at the histological level. Among all analysed endpoints, reproduction was particularly sensitive to SIM with significant impact at 320 ng/L. These findings have important implications for environmental risk assessment and disclose new concerns about the effects of SIM in aquatic ecosystems.

1. Introduction

Pharmaceuticals and their metabolites, whose discharge into aquatic ecosystems has increased exponentially over the past decades, are among the emergent pollutants of concern, since they target specific pathways at rather low concentrations, and therefore behave quite differently from conventional pollutants (EEA, 2010). Despite the growing number of reports regarding acute toxicity of pharmaceuticals, these data alone are unsuitable in environmental risk assessment, as the concentrations needed to elicit acute effects are far from ecologically relevant (Fent et al., 2006; Santos et al., 2010). The current lack of knowledge holds in particular for chronic effects (Fent et al., 2006). Studies of full life-cycle effects of pharmaceuticals are lacking for most therapeutic classes, although aquatic organisms are exposed for their entire life to chronic low levels.

Simvastatin (SIM) – a hypocholesterolaemic drug – is among the most prescribed human pharmaceuticals in western European countries known to reach the aquatic environment in increasing concentrations (Ellesat et al., 2010; Grung et al., 2007; Miao and Metcalfe, 2003; Walley et al., 2005;). In vertebrates, SIM disrupts the cholesterol synthesis by inhibiting the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), responsible for the limiting step in the mevalonate pathway (Fent et al., 2006), which is in fact the primary target of hypocholesterolaemic pharmaceuticals, such as SIM (Burg et al., 2011). Hypocholesterolaemic pharmaceuticals are competitive inhibitors of HMGR, not only in vertebrates but also in arthropods (Li et al., 2003; Zapata et al., 2002a,b). However, whereas vertebrates obtain cholesterol from the mevalonate pathway, cholesterol is not synthesised *de novo* in arthropods, particularly in crustaceans (Li et al., 2003). Nevertheless, crustaceans

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synthesise the isoprenoid methyl farnesoate (MF) in the biosynthetic mevalonate pathway, which shares most of the enzymatic steps with its vertebrate counterpart (Bellés et al., 2005). Therefore, given these peculiarities, crustaceans might also be a target of this drug.

Estuaries are the first recipients of many environmental pollutants, either through diffuse or point sources (Barbier et al., 2011; Sumpter, 2005). Hence, one of the key challenges for environmental risk assessment of estuarine ecosystems is to find appropriate model organisms to efficiently evaluate the complexity of potential environmental effects of pollutants using cost-effective approaches (Verslycke et al., 2004). Among such organisms, amphipods are good candidate sentinels. These animals hold advantages, comparative to fish and mollusks, like their shorter life-cycle, logistical facilitation for brooding and testing, and ethical issues related with animal testing. As such, amphipods have been successfully used in ecotoxicology for decades (Ré et al., 2009). Among the amphipods that occur in Europe, *Gammarus locusta* has proved to be an interesting organism for ecotoxicity testing. This epibenthic species has a wide geographical distribution along the North-East Atlantic (Costa and Costa, 2000), and it is among the main prey of many fish, birds and invertebrate species, which greatly contributes to its high ecological value in coastal and transitional ecosystems such as estuaries (Costa and Costa, 1999). *G. locusta* is recognised as relevant test species due to its sensitivity to a wide variety of contaminants, ecological importance, easy culturing, but also because of its short generation time (Neuparth et al., 2002). A structural design of an approach using *G. locusta* as model organism started to be shaped in the 1990s (Correia et al., 2002a; Costa et al., 1998, 2002, 2005; Costa and Costa, 1999; Neuparth et al., 2002, 2005). These studies produced essential baseline data, launching the foundations for applying *G. locusta* for toxicity testing of emerging pollutants.

Few studies have investigated the effect of SIM in non-target aquatic organisms. In fact, to the best of our knowledge, no chronic toxicity data are available for aquatic organisms. Therefore, there is a need to focus on long-term, low-level exposure assessment of SIM to better judge its implications in aquatic transitional ecosystems, with implications for risk assessment and environmental management. The present work aims at investigating multi-level biological response in *G. locusta*, following chronic exposures to low levels of SIM. Here we integrate multiple key endpoints at individual level (survival, sex ratio, growth rate, embryo development and reproduction), with histological biomarkers in hepatopancreas and gonads. The introduction of histopathological studies, scarce for crustaceans and virtually absent from amphipod studies, was designed to assist in the interpretation of the responses observed at individual level. Finally, an individual-based population model was enforced to the toxicological data produced in the chronic bioassays, to project the ecological costs associated with long-term exposure to SIM at the population level.

2. Material and methods

2.1. Amphipod culture

The amphipods were obtained from a permanent laboratory culturing system (Neuparth et al., 2002). The culture is partially renewed once a year by introducing specimens collected from a clean site in Sado estuary, Portugal (Neuparth et al., 2002). The animals were fed with *Ulva sp.* that, together with sediments and small stones were collected from coastal areas near Porto, Portugal, in a site devoid of direct contamination sources.

2.2. Chronic toxicity bioassays

As no *G. locusta* toxicity data were available in literature for SIM, initially, a range-finding test was performed to select a SIM concentration range to be used in chronic bioassays. SIM (CAS no. 79902-63-9) was obtained from Sigma-Aldrich®. All bioassays were performed with pre-filtered seawater where no traces of SIM were found (see section 3.1). One-week old newborns were used for starting the range-finding test. Briefly, the assay was conducted in 5-L glass aquaria housing 50 newborn amphipods in a semi-static system. The density of animals per aquaria was selected based on the density of the species in the field and on our breeding stock that we keep for several years in the laboratory (Costa and Costa, 2012; Neuparth et al., 2002). The test duration was 45 days. Throughout the experiment, the photoperiod was set at 16:8 h (light:dark); 18–20 °C temperature, 33–35‰ salinity and amphipods were fed *ad libitum* with *Ulva sp* collected in the above-mentioned location (section 2.1). Since *G. locusta* is an epibenthic amphipod, a 1 cm-deep layer of natural clean sediment was placed in aquaria as small stones were used to provide shelter. Aeration was provided with plastic tips placed at least 1 cm above the sediment surface.

The experiment consisted of six treatments (four replicates each); one control (natural seawater), a solvent control (0.001% acetone), and four constant exposures to SIM final concentrations of 1.6, 8, 40 and 200 μg/L. A SIM stock solution was prepared in acetone at a 20 mg/mL concentration, aliquoted and stored at −20 °C. At each water renewal, that occurred every three days, the final SIM concentrations were reestablished. One aliquot of the stock solution was employed and serially diluted (in acetone) to achieve the SIM final concentrations in each aquaria (0.001% acetone).

Aquaria were inspected twice daily for feeding requirements, assuring that food was never in shortage, and to remove dead animals. The pH, conductivity, dissolved oxygen and ammonia concentration were monitored at each water change. Amphipods from the highest SIM concentration, 200 μg/L, did not feed during exposure and died after 9 days. From day 9 onwards, animals from 40 μg/L showed decreased food uptake (40 μg/L aquaria had systematically more *ulva* sp. available than the others), and died around day 20. Based on these results, we selected the concentrations to be used in the definitive experiments (bioassay 1 and 2).

The experimental set-up of bioassays 1 and 2 was similar to that of the range-finding test. Since we aimed at investigating the long-term chronic exposure effects of SIM, bioassay 1 started with newborns, 7–8 days-old (50 per replicate tank), and was extended for 69 days (the life-cycle of *G. locusta* is typically 35 days at 20 °C). This bioassay 1 was designed to investigate the effects of SIM in a range of key biological endpoints (survival, sex-ratio, growth, and hepatopancreas histopathology). *G. locusta* were exposed to SIM concentrations of 64 ng/L, 320 ng/L, and 1.6 μg/L plus controls (seawater only and solvent controls), four replicates per treatment. Given the results of bioassay 1, bioassay 2 was carried out to corroborate early findings and particularly to address the effects of chronic exposure in reproduction. Therefore the exposure started with 18 sub-adults per replicate tank (25–30 days-old), one week before sexual maturation, and lasted for 36 days. The reproductive traits, newborns production, fecundity, percentage of ovigerous females, and gonads histopathology, were determined for SIM concentrations of 320 ng/L, 1.6 μg/L and 8 μg/L plus seawater and solvent controls (four replicates per treatment).

At the end of the bioassays, the overlying water of each replicate was sieved through 1000 and 250 μm screens, to collect surviving adults and their newborns (the latter only in bioassay 2), respectively. Sediments were washed at least five times to assure that all organisms were collected. Body length of males and females was measured before further processing. Six males and six females per treatment were immediately processed for histopathological examination. In the bioassay 2, the reproductive traits: fecundity (number of stadium I–II embryos per female), % of ovigerous females and newborns production (number of newborns produced