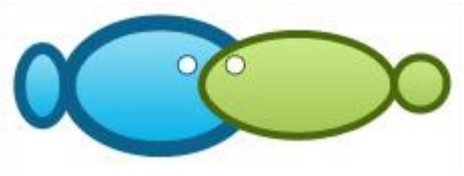

Metformin exposure induces gonadal alterations in mussels *Mytilus edulis*

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Abstract. The accumulation of pharmaceuticals in marine environments is an increasing environmental concern. Metformin, a widely used antidiabetic drug, is frequently detected in aquatic systems; however, its ecological implications are not fully elucidated. This study examines the biological effects of metformin on the blue mussel *Mytilus edulis*, employed as a bioindicator, under various concentrations that mirror both typical and elevated environmental levels. Investigations included short-term (7 days) and long-term (21 days) exposure scenarios. Results indicate that metformin markedly influences the cellular integrity and reproductive health of *M. edulis*. Notable findings include disruptions in gonadal tissue integrity and cellular response modulation, characterised by apoptosis-related and inflammatory reactions. These effects were dose-dependent and suggest metformin's potential as an emerging contaminant of concern. This research emphasises the need for comprehensive studies to fully assess the ecological consequences of metformin presence in aquatic habitats and the potential risks to marine life sustainability.

Key Words: aquatic toxicology, emerging contaminants, metformin, marine mussels, pharmaceutical pollution.

Introduction. Metformin has been widely used whether as a monotherapy or in combination with other medications in the treatment of diabetes mellitus (Ferrannini 2014). It has been on the market in Canada and Europe for 40-50 years, while in the US it was only introduced in 1995 (Samson & Garber 2015). Following its proven efficacy in improving morbidity and mortality rates in patients with type 2 diabetes, as demonstrated in the United Kingdom Prospective Diabetes Study (UKPDS), in 2009 metformin has been recommended as the first line oral treatment for type 2 diabetes by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) (Bailey 2017). Consequently, it is now the most commonly prescribed antidiabetic medication throughout the world (Samson & Garber 2015; Thomas & Gregg 2017). Jones et al (2002) reported that metformin is one of the top three prescribed compounds in the UK and that usage here was more than 100 tons per year in 2000.

Metformin is also used as treatment for polycystic ovary syndrome (PCOS) in human patients, where it works by decreasing the circulating insulin levels (Kurzthaler et al 2014). In this way, metformin attenuates insulin-induced androgenesis in the ovaries of PCOS patients. Moreover, metformin has also been successfully prescribed as a treatment for hyperinsular obesity and weight gain induced by antipsychotic therapy (Thomas & Gregg 2017). However, in a therapeutic setting, metformin toxicity has been recorded to potentiate hyperlactatemia, metabolic acidosis, and cellular hypoglycaemia (Wang & Hoyte 2019). In addition to this, despite many positive effects of metformin, including lessening hyperandrogenism and generating ovulation by acting as an insulin-sensitiser, Dupont & Scaramuzzi (2016) show evidence pointing to a direct effect of metformin on ovarian steroidogenesis.

The mechanisms underlying the effects of metformin are complex, and despite long-term clinical experience and active investigation, the exact mechanisms of action remain unclear and not fully understood. Rena et al (2017) suggest that at the molecular level, metformin may work by inhibiting mitochondrial respiration in the liver, leading to activation of AMP-activated protein kinase (AMPK) and enhancing insulin sensitivity. They also suggest AMPK-independent effects including inhibition of fructose-1,6-bisphosphatase by AMP. More recently however, studies have shown that clinically relevant concentrations of metformin act to inhibit hepatic gluconeogenesis in a substrate-selective manner, supporting a redox-dependent mechanism of action (LaMoia & Shulman 2021).

In the human body, metformin is circulated essentially unbound and is eventually eliminated unchanged (Ferrannini 2014). Graham et al (2011) reported that a notable percentage (20-30%) of a metformin dose is detected in the faeces as a consequence of the lack of absorption. This excreted proportion suggests a significant environmental release, particularly into aquatic ecosystems. These details indicate that this substance likely enters aquatic compartments in high quantities, as reported by Scheurer et al (2009), Trautwein & Kümmerer (2011), Scheurer et al (2012) and Trautwein et al (2014). Indeed, recent studies (Kot-Wasik et al 2016; de Jesus Gaffney et al 2017; Elizalde-Velázquez & Gómez-Oliván 2020; Koagouw et al 2024) showed that concentrations of metformin detected in the aquatic environment range from 0.2 ng L^{-1} to 325 µg L^{-1}, and concentrations up to 4.8 µg L^{-1} were detected in the Red Sea by Ali et al (2017).

Despite the elevated concentrations of metformin recorded in water bodies, relatively few studies have investigated the ecotoxicological effect of this pharmaceutical on marine organisms. Here we present the biological responses induced by metformin in the blue mussels *Mytilus edulis* at a range of concentrations commonly found in the aquatic environment and also, at a higher level, representing a worst-case scenario. The focus is on assessing the potential ecotoxicological impact of metformin on marine life, following short-term (7 days) and long-term (21 days) laboratory exposures. By exploring both acute and chronic exposure scenarios, this research contributes to a more comprehensive understanding of the environmental risks associated with one of the world's most widely prescribed medications.

Material and Method

Collection of mussels and acclimatisation. Blue mussels *M. edulis* were collected manually in June 2018 at low tide from Hove beach, East Sussex, UK (50°49' 25.6692" N, 0°10'24.3228" W) (Figure 1).

Figure 1. Map of the mussel collection site in Hove, UK. The exact location of the site is represented by the blue marker.

Approximately 200 mussels were taken from a single population to ensure homogeneity, kept on ice after collection, and transferred immediately to the laboratory. The collection procedure, including the number of mussels, was assessed and approved by the Animal Welfare and Ethics Review Bodies (AWERB) at the University of Brighton, to ensure no damage was caused to the population. Mussels were cleaned by removing the material attached to the shells' surfaces, and then placed in 20L containers with aerated artificial seawater for acclimatisation at 23±2°C for 6 days, to match the temperature at the collection site and the room where the exposure experiments would be conducted. Artificial seawater (Instant Ocean® Sea Salt, USA) was used for this purpose, and the solution was prepared according to the manufacturer's instructions.

Short-and long-term exposure to selected levels of metformin. All procedures were performed in compliance with the ARRIVE guidelines (Percie du Sert et al 2020) and were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. The procedures had been approved by the Animal Welfare and Ethics Review Bodies (AWERB) at the University of Brighton. Mussels selected for the exposure experiments ranged from 30 to 50 mm in size to ensure a similar developmental stage. Criteria for selection also included health and physical integrity, such as intact shells and responsive byssal threads. For all exposures, artificial seawater was prepared in 50L plastic containers, and mussels were allocated into glass tanks with a volume proportion of approximately 1 litre per mussel. The mussels were randomly allocated into four groups. Blinding methods were implemented during allocation and assessment to prevent bias. All experimental tanks were set up in duplicate with 5 mussels in each 5L tank.

The short-term exposure period was 7 days, while the long-term exposure lasted 21 days. Mussels were not fed during acclimatisation or exposure periods to avoid any influence of feed on the results of experimental metformin exposures. The metformin exposures were as follows: a control group with artificial seawater only and three exposed groups at concentrations of 100 ng L^{-1}, 80 µg L^{-1}, and 150 µg L^{-1}, which were selected based on their environmental relevance and to span a range from below to above known environmental concentrations (Elizalde-Velázquez & Gómez-Oliván 2020). The stock solution of concentration 100 mg L^{-1} was made freshly by dissolving metformin (European Pharmacopoeia Reference Standard, Sigma-Aldrich) to ultra-pure water, and only used within the period of exposure. Metformin demonstrates high solubility and stability in water across a wide range of temperatures and pH levels, thus obviating the need for frequent re-measurement of its concentration in aqueous solutions (Desai et al 2014; Yu et al 2022). The artificial seawater was changed and re-spiked with metformin every 72 to 96 hours to ensure constant metformin concentrations across exposure periods. Temperature, salinity, and conductivity of the seawater were recorded daily using calibrated digital meters (Hanna Instruments, USA).

Neutral red retention time (NRRT) assay. Due to limited capacity of laboratory operation, we were unable to conduct the NRRT assay for the mussels exposed short-term to metformin. Therefore, the assay was conducted only on hemocytes from mussels exposed long-term to study cumulative drug effects, employing the modified methods of Lowe et al (1995) as cited in Koagouw et al (2021b). The chemical solutions for the assay were prepared according to the protocol, with the neutral red dye solution freshly made by dissolving 28.8 mg of Neutral Red (Sigma-Aldrich) in 1 mL of dimethyl sulfoxide. The physiological saline solution was prepared by adding HEPES, NaCl, $MgSO_4$, KCl, and $CaCl_2$ to 1 L of reverse osmosis water, adjusting the pH to 7.3, and stirring until dissolved, as detailed in Supplementary Material S1. A working solution was made by diluting the neutral red stock in physiological saline. Haemolymph was collected with minimal stress, centrifuged, and 30 µL placed on a poly-L-lysine coated slide to attach at 20±1°C for 15 minutes in a dark, humid container. After adding 30 µL of working solution, the slide was incubated for another 15 minutes before being observed under a light microscope at 40x/100x magnification every 30 minutes for up to 180 minutes, based on established protocols by Mamaca et al (2005) and Martínez-Gómez et al (2015) for observing

significant dye uptake changes. The experiment concluded when over 50% of neutral red was observed leaking from lysosomes into the cytosol, indicating compromised lysosomal membrane integrity due to pollutant accumulation.

Histopathology. Following exposure, a total of 10 mussels from each group were euthanised according to ethical guidelines and dissected for gonad histological analysis, following procedures adapted from Koagouw et al (2022). Gonad samples (~1 cm^3) were fixed in neutral buffered formaldehyde (Sigma-Aldrich) for 24 hours, then dehydrated and cleared in an automated tissue processor (Leica Biosystems, Germany) using a 12-hour cycle of increasing alcohol concentrations and xylene, before embedding in paraffin. Sections (7 µm) were cut using a manual microtome (Thermo Scientific, UK), orientated along the anterior-posterior axis for cross-sectional analysis, and affixed to positively charged slides in a 37°C oven overnight. Paraffin was removed using dishwashing liquid (Fairy, Procter & Gamble) in hot water, a method chosen for its effectiveness and safety (Buesa & Peshkov 2009). After deparaffinization, sections were stained with haematoxylin and eosin (Sigma-Aldrich), dehydrated, and mounted in DPX (CellPath, UK) for examination under a light microscope (Leitz Wetzlar, Germany) at 40x/100x magnification. Histopathological conditions in gonad tissues were assessed by noting pathologies and counting occurrences across samples, with digital micrographs taken using a GXCam Hichrome-Lite (GT Vision, UK).

Data analysis. To determine effects of metformin concentration on lysosomal integrity of hemocytes, neutral red retention time of the three exposure groups and control group were compared using one-way ANOVA, with Tukey's post-hoc test used to determine significant differences between individual groups.

For the analysis of histopathological changes in gonad tissues, all types of observed pathologies - including follicle dilatation, gamete degeneration, hemocytic infiltration, atresia, parasites, and hemocytic aggregate - were combined into a single dataset. This pooling was intended to assess the overall impact of exposure on tissue health. A two-way ANOVA was then performed on the pooled frequencies to compare the effects across the three exposure groups and the control group. Tukey's post-hoc test was employed to identify statistically significant differences between specific groups. This analysis was carried out separately for tissues undergoing short-term (7 days) versus long-term (21 days) metformin exposure. Data were visualised using Microsoft Excel and the 'ggplot2' package in R version 4.1.2.

Results

Concentration-dependent effects of metformin on hemocyte lysosomal integrity. Metformin exposure (Figure 2) demonstrates a statistically significant concentration-dependent trend ($p < 0.05$), with higher concentrations of the contaminant drastically affecting the retention time of hemocytes. Metformin exhibits high toxicity at a concentration of 150 µg L^{-1}, as all lysosomal material burst into the cytosol in less than 30 minutes' incubation time.

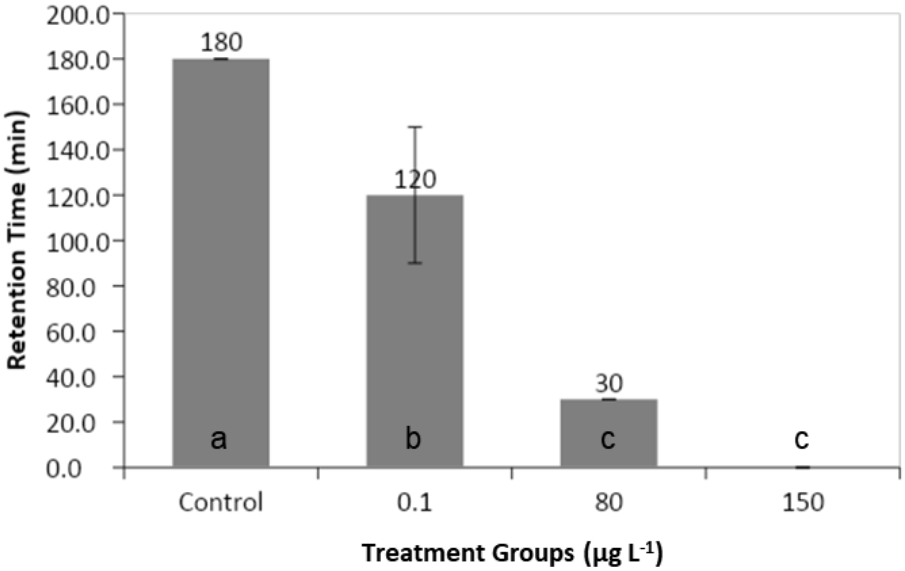

Figure 2. Neutral red retention time of hemocytes from mussels exposed to metformin for 21 days (n = 3). Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

Metformin-induced histopathological changes in mussel gonads. Short exposure to metformin induced several pathologies in mussels' gonads (Figure 3).

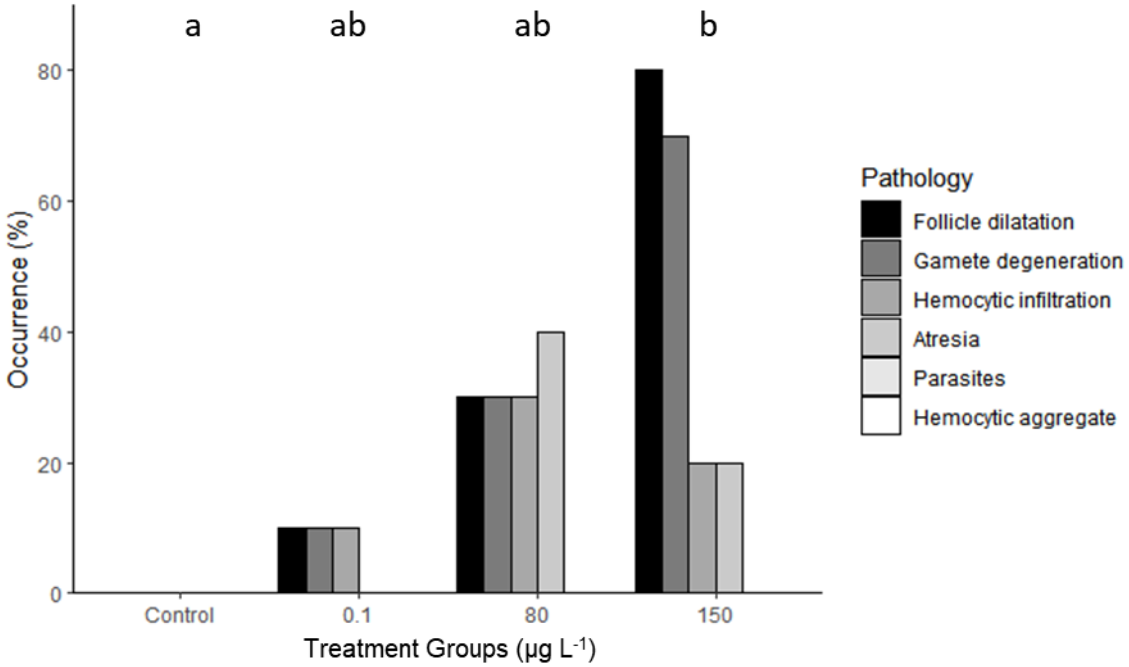

Figure 3. The occurrence of histopathological conditions observed in the gonad tissue of mussels exposed to metformin for 7 days (n = 10). The treatments were as follows: control, 100 ng L^{-1}, 80 µg L^{-1} and 150 µg L^{-1}. Different letters represent statistically significant differences between groups (two-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

Follicle dilatation was the most frequently observed pathology overall across treatment groups, followed by gamete degeneration. The occurrence of both conditions demonstrated a trend related to the concentration of metformin, where higher concentrations induced higher pathological prevalence. Follicle dilatation was also the condition with the highest incidence, up to 80% in the group exposed to metformin at 150 µg L^{-1}. Mussels in this highest concentration group displayed a very high prevalence

of pathologies in their gonads, with gamete degeneration also observed in 70% of mussels. Atresia was observed only in the two highest concentration groups, with 40% of mussels in the group exposed to 80 µg L^{-1} exhibiting this condition. Hemocytic infiltration was the only inflammatory reaction observed here, and was present in all exposed groups, including a prevalence of 30% in the group 80 µg L^{-1}.

A longer exposure to metformin evoked different prevalences of pathologies in mussels' gonads, as shown in Figure 4.

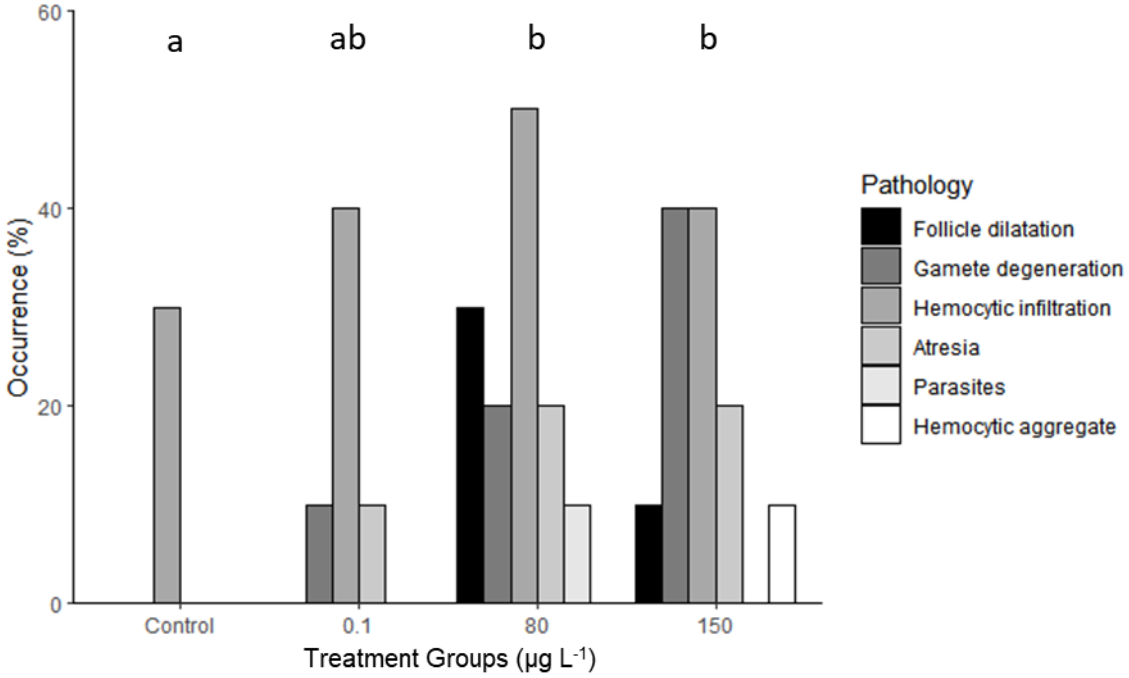

Figure 4. The occurrence of histopathological conditions observed in the gonad tissue of mussels exposed to metformin for 21 days (n = 10). The treatments were as follows: control, 100 ng L^{-1}, 80 µg L^{-1} and 150 µg L^{-1}. Different letters represent statistically significant differences between groups (two-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

In this case, hemocytic infiltration appeared in all groups and was the pathology with the highest incidence in every group. The occurrence was almost even across groups (30-50%) and the highest incidence was observed in the group 80 µg L^{-1}. Gamete degeneration and atresia were present in all exposed groups with incidences up to 40% and 20%, respectively. Parasitic infiltration and hemocytic aggregate, both of which were not present in the short exposure, were observed here after 21 days of metformin exposure, although only in 10% of the samples. The most observed pathology in the short exposure, follicle dilatation, occurred only in the two higher concentration groups here, affecting up to 30% of mussels in the group 80 µg L^{-1}. Micrographs corresponding to each histopathological condition are shown in Figure 5 and Figure 6.

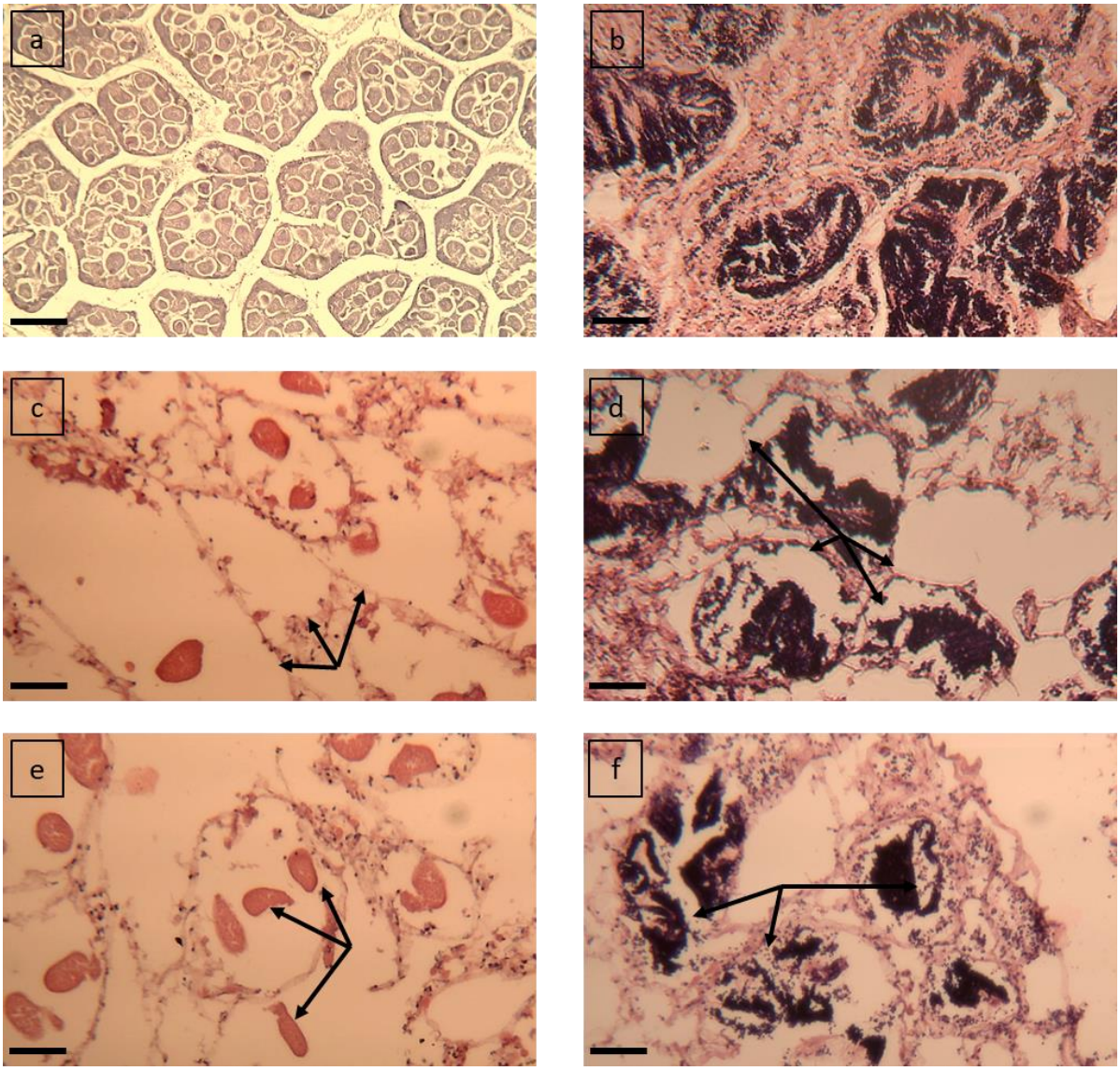

Figure 5. Histopathological conditions in mussel gonads after exposure to metformin. Sections of 7 μm, stained with haematoxylin and eosin: (a) normal histology, female; (b) normal histology, male; (c) follicle dilatation, female; (d) follicle dilatation, male; (e) gamete degeneration, female; (f) gamete degeneration, male. Arrows point to each pathological condition. Scale bar = 100 μm.

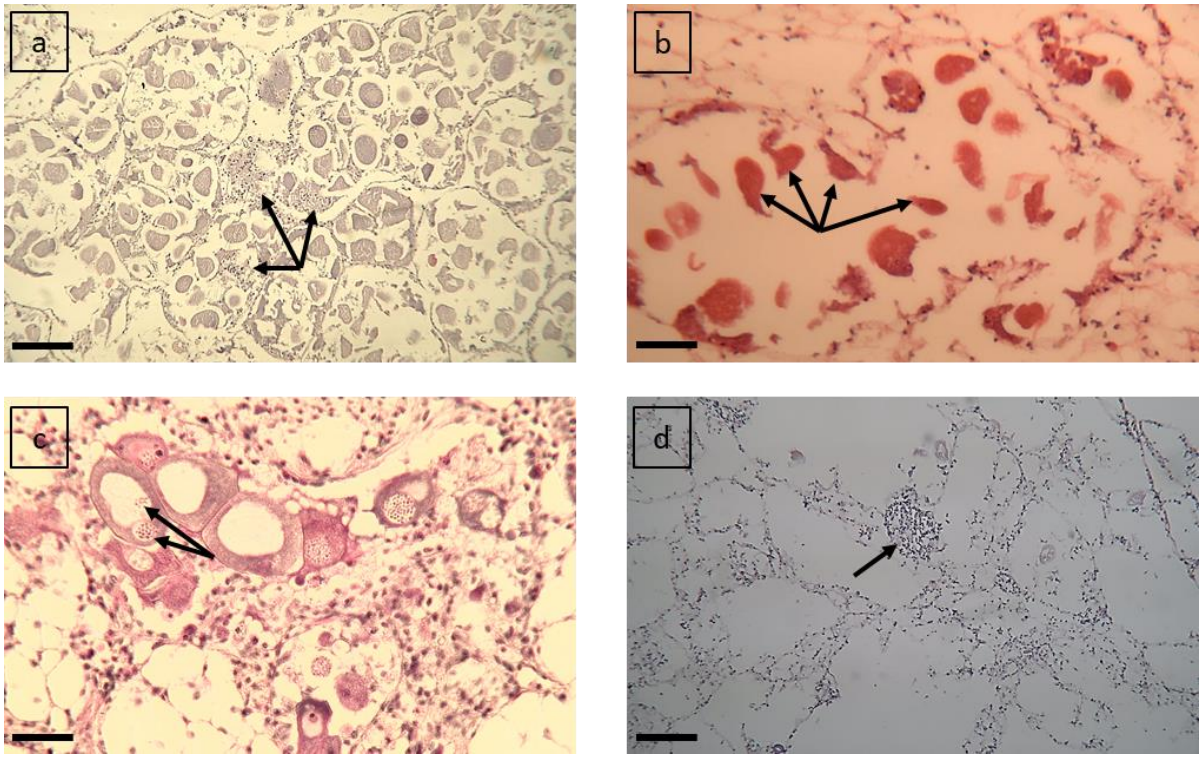

Figure 6. Histopathological conditions in mussel gonads after exposure to metformin (continued). Sections of 7 µm, stained with haematoxylin and eosin: (a) hemocytic infiltration; (b) atresia; (c) parasites; (d) hemocytic aggregate. Arrows point to each pathological condition. Scale bar = 100 µm.

Discussion

Dose-dependent toxicity of metformin on hemocyte stability. Metformin exposure showed a more evident statistically significant concentration-dependent trend, with higher concentrations of the contaminant inducing a lower retention time (Figure 2). Whilst non-exposed mussels maintained good lysosomal membrane integrity in their hemocytes, individuals exposed to 100 ng L^{-1} metformin exhibited a shorter retention time (120 minutes). Although mussels in these two groups can still be categorised as healthy according to the threshold defined by Moore et al (2006), the other two exposed groups displayed more dramatic results. Mussels exposed to 80 µg L^{-1} metformin expressed very short neutral red retention time with an average of 30 minutes and were classified as severely stressed according to the threshold. Metformin exhibited a high toxicity effect on mussels exposed at a concentration of 150 µg L^{-1}, as the disintegrated lysosomal membrane released all lysosomal content to the cytosol in less than 30 minutes' incubation time (Figure 2).

The results presented here are in agreement with the previous study by Koagouw & Ciocan (2018), which found that mussels exposed to metformin displayed destabilisation of the lysosomal membrane. The authors showed that exposure to 40 µg L^{-1} induces a lower neutral red retention time (almost 40% lower than the control), and was classified as low to mildly stressed, according to Moore et al (2006). Increasing the concentration from 40 to 80 µg L^{-1} in this study elicited a distinct stress response, with mussels being classified from moderately to severely stressed, tending towards pathology. These results indicate a concentration-dependent trend, where higher concentrations of metformin induce lower retention times and, therefore, more severe destabilisation of the lysosomal membrane.

As NRRT has been adopted as an indicator for pollution or contaminant monitoring (Hu et al 2015), the observed dose-dependent alteration caused by metformin suggests its potential impact on lysosomal membrane stability. Lysosomes play a crucial role in sequestering and processing environmental contaminants, utilizing autophagy

mechanisms to degrade extracellular and intracellular macromolecules affected by such contaminants (Lowe & Fossato 2000; Saftig & Klumperman 2009). This finding implies that metformin may compromise the integrity of cellular structures at the molecular level, which could indirectly affect the overall health and resilience of mussel populations. Furthermore, this suggests potential mechanistic actions of metformin at the cellular level that warrant further investigation, particularly into the biochemical pathways affected by the drug in non-target species such as mussels.

Impact of metformin on mussel reproductive health. The results from the histopathological analysis indicate that metformin inflicts several pathologies on the mussels' gonads after just 7 days of exposure (Figure 3). These pathological conditions, primarily related to the reproductive system, such as follicle dilatation and gamete degeneration, indicate impairment of reproductive capabilities since successful reproduction relies heavily on gamete quality. Follicle dilatation has been associated with reduced fecundity in zebrafish, *Danio rerio* (Hong et al 2018), while Williams & Bentley (2002) found that gamete health directly affects fertilization success in marine invertebrates. They contend that any changes to oocytes or sperm could diminish fertilization efficacy or cause developmental abnormalities in embryos. The pathologies observed in this study are indicative of a compromised reproductive system. Alterations during gametogenesis, particularly in the follicle stage, could disrupt gamete quality. This supports the findings of García-Gasca et al (2010), who posited that follicle dilatation could be a significant indicator of environmental stress in coastal ecosystems due to its direct connection to reproductive success.

Recent studies have linked metformin with some reproduction-related alterations in tissues (Koagouw & Ciocan 2018; Niemuth & Klaper 2015). The high prevalence of follicle dilatation and gamete degeneration in the results presented here thus imply considerable potential for metformin to cause disturbance in the reproductive systems of mussels and possibly to interfere with population sustainability. The potential long-term consequences of such reproductive impairments could lead to a decline in mussel populations, affecting both biodiversity and ecosystem functionality due to the crucial ecological roles mussels play.

It is worth noting that these two reproduction-related pathologies were observed in all exposed groups and showed a concentration-related trend (Figure 3). This indicates that the level of metformin in the environment plays a vital role in the level of toxicity of this substance to marine mussels. The very high prevalence of reproduction-related pathologies in the two groups in which metformin concentrations are in μg L^{-1} suggests a very concerning picture, should this substance reach those high levels in the environment, with follicle dilatation and gamete degeneration afflicting 80% and 70% of mussels, respectively.

There is also concern around the high percentage (20-40%) of mussels exposed to high levels of metformin showing atretic condition in their gonads (Figure 3). According to Krysko et al (2008), atresia can negatively affect fertility and may eventually lead to irreversible premature ovarian failure. Atresia was also recorded in the gonads of mussels exposed to 40 μg L^{-1} metformin in a previous study with a prevalence of 8-17% (Koagouw & Ciocan 2018). The result presented here also suggests a correlation between the concentration of metformin and the prevalence of atresia, as higher prevalences are observed in line with the increase in metformin concentration.

Another important observation is that metformin in concentrations as low as 100 ng L^{-1} could induce similar pathologies to those induced when the level is much higher (150 μg L^{-1}), albeit at lower incidence (Figure 3). This highlights the present danger faced by the marine organisms inhabiting areas where this level of metformin is actually recorded. As mentioned earlier, metformin has been quantified in the environment in a range from 0.2 ng L^{-1} to 325 μg L^{-1} (Kot-Wasik et al 2016; de Jesus Gaffney et al 2017; Elizalde-Velázquez & Gómez-Oliván 2020), and has been detected in surface water at concentrations as high as 33.6 μg L^{-1} (Elliott et al 2017), including at concentrations up to 4.8 μg L^{-1} in the marine environment (Ali et al 2017). The results here therefore

indicate a very concerning picture whereby these pathologies are actually developing in real life situations.

Whilst hemocytic infiltration was recorded in all groups in the short-term exposure, this condition was notably more prevalent after longer exposure (Figure 4). The result here indicates that longer exposure to metformin can induce inflammation as a response to stress-related events and adversely affect mussels' health. Hemocytic infiltration has been frequently recorded in animals as a result of stress-inducing experiments as documented by Arrighetti et al (2018), Velisek et al (2018) and Khan et al (2019). The results here thus call the attention to the role of exposure duration in determining the level of risk to marine organisms, as longer exposure potentially contributes to lower immunity in mussels, represented by high prevalence of hemocytic infiltration in the gonads of up to 50% of samples.

Although observed only in 10% of the samples, parasitic infiltration and hemocytic aggregate were detected here after 21 days of metformin exposure (Figure 4). Neither pathology was present in the short exposure, thus the longer exposure to metformin seems to exacerbate these events, induce the incidence of these inflammatory reactions. It is possible however that parasites may have been present in the mussels at the beginning of the experiment, and the conditions for these mussels may have deteriorated as a consequence of the contaminant effect by reducing immunity, and thus this parasitic infection became more evident. Gamete degeneration, atresia, and follicle dilatation, all pathologies directly related to reproductive mechanisms, were still present after long exposure, albeit at a moderate level of occurrence. These apoptosis-related conditions appear to be the initial or primary responses to metformin exposure, as they have a higher prevalence following short-term exposure. This is also supported by Koagouw et al (2021a) who described the modulation of certain apoptosis-related transcripts in mussels after short-term exposure to metformin. However, after longer exposure, these pathologies occurred only mildly, while inflammatory-related conditions were more dominant.

Metformin's effects via signalling cascade and ecological impacts. The observed pathological conditions in mussel gonads post-metformin exposure, particularly the transition from apoptosis-related to inflammatory reactions, highlight the complex interplay between metformin's pharmacological action and its environmental impact. This study's findings support the hypothesis that metformin induces oxidative stress, leading to apoptosis in a concentration- and duration-dependent manner. The initial apoptotic responses, characterised by follicle dilatation and gamete degeneration, are likely mediated by the disruption of mitochondrial membrane potential, a common pathway for apoptosis induction (Krysko et al 2008; Graham et al 2011). Metformin's influence on mitochondrial function could trigger the release of cytochrome c into the cytosol, activating caspases, particularly caspase-9 and caspase-3, and leading to programmed cell death (LaMoia & Shulman 2021).

Moreover, the shift towards inflammatory reactions with prolonged metformin exposure suggests an exacerbation of oxidative stress, where an imbalance between the production of reactive oxygen species (ROS) and the organism's antioxidant defences leads to cellular damage (García-Gasca et al 2010; Lee et al 2019). This oxidative stress can further activate signalling pathways involved in inflammation, contributing to the observed histopathological conditions like hemocytic infiltration and aggregation (Larguinho et al 2014; Khan et al 2019). The dose-dependent increase in oxidative stress markers, aligning with the progression of metformin concentrations, corroborates findings from other studies indicating that high concentrations of pharmaceutical contaminants can induce significant oxidative damage in aquatic organisms (Lee et al 2019; Elizalde-Velázquez et al 2021).

The apoptosis and oxidative stress induced by metformin not only provide insights into the drug's ecotoxicological effects but also highlight the potential ecological consequences. The impairment of reproductive capabilities, as evidenced by the high prevalence of follicle dilatation and gamete degeneration, can have cascading effects on mussel populations and, by extension, aquatic ecosystems (Koagouw & Ciocan 2018).

Given the role of mussels as biofilters and their contribution to nutrient cycling, these findings emphasise the urgency of developing effective strategies for managing pharmaceutical waste to mitigate the ecological risks posed by metformin and similar contaminants. This study also highlights the need for environmental policies to carefully consider the ecological risks of pharmaceutical contaminants.

However, it is important to acknowledge the limitations of this study, including the controlled laboratory conditions and the recognition that mussels in natural settings may face different exposure scenarios. Consequently, future research should aim not only to clarify the specific biochemical pathways through which metformin affects aquatic organisms, focusing on identifying potential biomarkers for early detection of oxidative stress and apoptotic responses, but also to engage in the long-term ecological monitoring of mussel populations in metformin-polluted waters. The development of more sophisticated models to predict the ecological risk of pharmaceuticals such as metformin will be imperative, alongside the investigation of potential mitigation strategies to protect aquatic life. Moreover, exploring the cumulative and synergistic effects of pharmaceutical contaminants will provide a more comprehensive understanding of their ecological impact, enabling for more robust environmental protection policies.

Conclusions. Whilst the therapeutic benefits for human health of the antidiabetic pharmaceutical metformin are well-documented, the environmental implications, particularly its impact on marine ecosystems, have remained under-researched. This study shows that metformin has the potential to alter the immune response in blue mussels by affecting the stability of the lysosomal membrane, and to initiate pathological conditions in gonads including apoptosis-related and inflammatory responses. Via these mechanisms, metformin may impact the reproductive success and population sustainability of mussels, thereby potentially disrupting the stability of aquatic food webs. Crucially, these responses are shown to be concentration- and duration-dependent, highlighting the complexity of the mechanisms through which metformin affects aquatic organisms. This study advocates for further research to clarify metformin's mechanism of action and its long-term ecological effects, facilitating the development of mitigation strategies to protect aquatic life and maintain ecosystem health. Our findings nevertheless emphasise the urgent need for management of pharmaceutical waste and the development of environmental policies that mitigate the risks posed by pharmaceutical contaminants such as metformin.

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Conflict of interest. The authors declare that there is no conflict of interest.

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