Environmental Science and Pollution Research (2021) 28:48823–48836
https://doi.org/10.1007/s11356-021-14136-6

RESEARCH ARTICLE

Long-term exposure of marine mussels to paracetamol: is time a healer or a killer?

Wulan Koagouw[1,2,3] · Nicolas A. Stewart[1] · Corina Ciocan[1,2]

Received: 19 January 2021 / Accepted: 22 April 2021 / Published online: 30 April 2021
© The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2021

Abstract

Pharmaceuticals pose a major threat to the marine environment, and several studies have recently described their negative effects on marine organisms. Pharmaceutical compounds are constantly being released into aquatic ecosystems, and chronic exposure, even at low concentrations, may have a major impact on marine organisms. The purpose of the present study is to evaluate the biological changes induced by one of the most widely used pharmaceuticals—paracetamol—in the blue mussel *Mytilus edulis*, after a long-term exposure at environmentally relevant concentrations. We present our data alongside and in comparison with results from a previous short-term exposure, to demonstrate the significance of exposure period on the effects of paracetamol in adult blue mussels. After 24 days of laboratory exposure, seven potential target genes were selected to examine toxicological effects in mussels' gonads and possible disruptive effects on reproductive processes. The results show the modulation of some important reproduction-related genes: *estrogen receptor-2* (*ER2*), *vitelline envelope zona pellucida domain-9* (*V9*), and *vitellogenin* (*VTG*). Variations in mRNA expression of four other genes involved in apoptosis (*HSP70*, *CASP8*, *BCL2*, and *FAS*) are also highlighted. Histopathological alterations caused by paracetamol, together with neutral red retention time response in mussels' hemocytes, are presented herein. Overall, this study highlights the exacerbated effects of low concentration of paracetamol after chronic exposure, similar to the damage induced by higher concentrations in a short exposure scenario, thus emphasizing the importance of length of exposure period when studying the effects of this substance. Additionally, this study also discusses the potential of paracetamol to inflict several major changes in the reproductive system of mussels and thus possibly affect the survival of populations.

Keywords Paracetamol · Blue mussels · Transcriptomics · Histology · Marine pollution · Pharmaceuticals · Reproduction · Bivalves

Introduction

Global consumption of pharmaceutical products, prescribed or over the counter, is projected to rise in the coming years (Fabbri and Franzellitti 2016; aus der Beek et al. 2016). Pharmaceutical consumption is estimated at more than 200,000 t per year in Russia, China, and India (Tijani et al. 2016) and is expected to be much higher for regions with high use of pharmaceuticals such as the USA and Europe. Paracetamol or acetaminophen is an active ingredient in hundreds of prescriptions and over-the-counter (OTC) medicines (Roberts et al. 2016). At present, paracetamol is used as an analgesic and antipyretic, and there is a significant prevalence for self-medication worldwide (Tariq and Din 2017), especially in the context of the COVID-19 pandemic. It is sold both as paracetamol only and, in many OTC medicines, is also combined with other active ingredients that treat cough, colds, flu,

Responsible Editor: Cinta Porte

✉ Wulan Koagouw
W.Koagouw2@brighton.ac.uk; wulan.koagouw@lipi.go.id

Nicolas A. Stewart
N.Stewart@brighton.ac.uk

Corina Ciocan
C.Ciocan@brighton.ac.uk

[1] School of Pharmacy and Biomolecular Sciences, University of Brighton, Lewes Road, Brighton, UK

[2] Centre for Aquatic Environments, University of Brighton, Lewes Road, Brighton, UK

[3] Research Center for Oceanography, Indonesian Institute of Sciences, Jakarta, Indonesia

and pain-related conditions (Wood et al. 2010). Paracetamol is recognized as the most frequently used analgesic in the UK (Bertolini et al. 2006) and is also the world's most widely marketed OTC drug (Warwick 2008), mostly because of its non-prescription availability and low cost (Jozwiak-Bebenista and Nowak 2014; Bárzaga Arencibia and Choonara 2012). Nevertheless, the consumption is set to rise significantly during the COVID-19 pandemic.

An increasing number of ecotoxicology studies show that pharmaceuticals pose a risk to aquatic organisms. This risk is evidenced, not only by the sustained persistence of pharmaceuticals in different aquatic compartments but also by their bioaccumulation in many species, as documented by Mimeault et al. (2005), Vernouillet et al. (2010), Wang and Gardinali (2013), Brodin et al. (2014), Du et al. (2015), and de Solla et al. (2016). To date, many studies focusing on the adverse outcomes of pharmaceuticals have been recorded in freshwater species (Flammarion et al. 2000; Hoeger et al. 2005; Mimeault et al. 2005), whereas only very limited data have been reported in marine organisms.

Pharmaceuticals in general can exert considerable pressure on reproductive-associated mechanisms in marine organisms, leading to knock-on ecological effects on populations and communities. Franzellitti et al. (2013) reported that fluoxetine, a common antidepressant, was associated with many detrimental effects on reproduction and other major physiological systems in Mediterranean mussels *Mytilus galloprovincialis*, even at concentrations below or approaching environmental levels. Fonseca et al. (2019) showed that tamoxifen, the oldest hormone therapy for breast cancer, can cause endocrine disruption in male *M. galloprovincialis* exposed only for 14 days. A recent study by Koagouw and Ciocan (2018) also recorded pathologies in the gonads and increased *vitellogenin* mRNA expression in blue mussels *Mytilus edulis* exposed to metformin.

Recently, several effects have been documented as a result of paracetamol exposure in bivalves. Oxidative stress has been recorded in clams *Ruditapes decussatus* (Antunes et al. 2013) and *Ruditapes philippinarum* (Antunes et al. 2013; Correia et al. 2016; Nunes et al. 2017). In the oyster *Crassostrea gigas*, Bebianno et al. (2017) reported paracetamol-induced variations in gene transcription, while Solé et al. (2010) observed changes in the feeding rate as well as oxidative stress after exposing *M. galloprovincialis* to paracetamol for 10 days. Our previous study (Koagouw and Ciocan 2019) also recorded adverse effects of paracetamol exposure in gonad tissue of *M. edulis* and the modulation of several transcripts. More recently, Piedade et al. (2020) reported that paracetamol could affect glycogen content in *Mytilus spp*. However, their results did not show any oxidative effects, and the authors pointed out that this may be a consequence of short-term exposure, recommending further exploration into the prolonged exposure effects.

As the ocean acts as the ultimate receptacle of a vast quantity of natural and anthropogenic waste that is continuously emitted from urban and industrial sources (Norse and Crowder 2005; Pereira et al. 2016), marine organisms are potentially at critical risk. A review by Ebele et al. (2017) highlights that pharmaceuticals are often persistent and frequently found in surface water at concentrations ranging from ng/L to mg/L, while low concentrations of these pollutants are detected even in drinking water (Kasprzyk-Hordern et al. 2008; Caban et al. 2015). The persistence of pharmaceuticals in the aquatic environment allows for scenarios of chronic exposure, highlighting the importance of exposure time in determining the severity of pharmaceutical impact on non-target organisms. The relative importance of contaminant concentration versus exposure duration has so far been little studied, with only a few studies focusing on the exposure length as a main parameter (Cope et al. 2008; Huang et al. 2019).

While paracetamol has been detected in various aquatic environments at concentrations ranging from 3.3 ng/L (Fairbairn et al. 2016) to 16 μg/L (Agunbiade and Moodley 2014), the levels reported in seawater vary from 3.2 ng/L (Benotti and Brownawell 2007) to more than 200 μg/L (Togola and Budzinski 2008). The continuous high consumption and production of paracetamol as well as its evident occurrence in seawater give rise to concerns regarding the impact on marine organisms, especially filter feeders. This study explores the impact of paracetamol on the gonads of marine mussels *Mytilus edulis* after a long-term exposure and discusses the potential reproductive challenges that may arise. Here, we present our data alongside and in comparison with results from a previous short-term exposure (Koagouw and Ciocan 2019), in order to demonstrate the importance of the length of exposure on the potential biological and ecological damage inflicted by paracetamol.

Mussels are excellent indicator organisms for environmental monitoring and have been intensively used worldwide to monitor marine pollution (Rittschof and McClellan-Green 2005). Representatives of Mytilidae such as *M. edulis* and *M. galloprovincialis* are also widely used as indicators in several studies on the effects of pharmaceuticals, due to their well-known physiology and their wide geographical distribution (Świacka et al. 2019). In this study, we employed neutral red retention time assay to enable observation of the effects at cellular level. Histopathological examination was performed to determine sex and any pathological conditions observed in the gonad tissue. Three genes related to reproduction—*vitellogenin* (*VTG*), *vitelline envelope zona pellucida domain-9* (*V9*), and *estrogen receptor-2* (*ER2*)—were investigated, as well as four genes involved in apoptosis: *heat shock protein-70* (*HSP70*), *caspase-8* (*CASP8*), *B-cell lymphoma-2* (*BCL2*), and *Fas cell surface death receptor* (*FAS*). This study is highly pertinent in the context of a potential increase of paracetamol in seawater following the COVID-19 pandemic.

Materials and methods

Sample collection

Blue mussels *M. edulis* were collected by hand from a single population located in Hove Beach, East Sussex, UK (50.823797, -0.173423) at low tide during April 2018. Mussels were placed on ice following collection and directly transported to the laboratory, where they were then washed and stored in an artificial seawater container (Instant Ocean® Sea Salt, USA) for acclimatization purposes. During this time, the mussels were fed each day with 500 μL of green algae *Tetraselmis* sp. culture suspension (ReefBoost, UK) per 5 L of artificial seawater. The starting water temperature (15 °C) was then slowly increased over the following 6 days to a steady experimental limit of 20 ± 2 °C.

Experimental exposure

All procedures were performed in compliance with the ARRIVE guidelines and carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63/EU on the protection of animals used for scientific purposes. The procedures have been approved by the Animal Welfare and Ethics Review Bodies (AWERB), University of Brighton. Only mussels between 30 and 50 mm in length were used in exposure experiments. Artificial seawater was prepared in compliance with the manufacturer's instructions, and tanks used for exposures contained approximately 1 L of artificial seawater per mussel. Exposures consisted of a control (artificial seawater only) and three separate treatments of paracetamol (40 ng/L, 250 ng/L, and 100 μg/L). These nominal concentrations were based on concentrations detected in the marine environment by previous studies (Togola and Budzinski 2008; Nödler et al. 2014; Bebianno et al. 2017). Twice weekly (at 72 and 96 h) artificial seawater was renewed, and the exposed groups were treated with paracetamol (BioXtra, ≥99.0%, Sigma-Aldrich). Physical characteristics of seawater (temperature, salinity, conductivity, and resistance) were monitored daily and the exposure was suspended after 24 days. All experimental tanks were set up in duplicate with 5 mussels in each tank.

A total of 10 mussels from each group were collected, measured, and dissected following the completion of the exposure. Approximately 1 cm square of each gonad was fixed in neutral buffered formaldehyde in clean tubes and preserved at 4 °C for the purposes of histological analysis. For the molecular analysis, the tissues were immediately transferred to RNA*later* (Invitrogen, UK) and kept at −80 °C.

Water analysis

The detailed protocol is outlined in Koagouw and Ciocan (2019). In brief, water samples (1000 mL) were obtained from each group 15–30 min after paracetamol was added (t_0) and immediately before artificial seawater was changed (72 and 96 h). All samples were processed through solid-phase extraction (Strata™-XL-C 100 μm polymeric strong cation 2 g/20 mL giga tube cartridge, Phenomenex, USA) after two filtrations using 1.2 μm Whatman grade GF/C microfiber glass filter paper (GE Healthcare, UK) and 0.22-μm nylon membrane filter (GE Healthcare, UK). The extract was evaporated by centrifugation under vacuum (Speedvac, Savant) and reconstituted with LC-MS grade water prior to analysis.

The concentration of paracetamol was determined by liquid chromatography-mass spectrometry (LC-MS) using a standard curve. Paracetamol separation was performed by ultra-high performance LC (Ultimate 3000, Thermo Scientific) using reversed-phase chromatography (Kinetex XB-C18, 5 μ, 100 Å, 100 × 2.1 mm, with trap column, Phenomenex, UK). Mass spectrometry (Orbitrap Q Exactive, Thermo Scientific) was conducted in positive mode using heated electrospray ionization (HESI) with a probe temperature of 200 °C. The area below the peak was defined by the reconstructed ion chromatogram of the fragment at 110.0602 *m/z* and quantitation was determined using Quan Browser data processing software (Xcalibur V:4.1.31.9, Thermo Scientific). The detailed parameters of this procedure are described in Koagouw and Ciocan (2019).

Neutral red retention time assay

The NRRT procedure was adapted from Lowe and Pipe (1994) and Lowe et al. (1995) in Mamaca et al. (2005). At the end of the exposure, hemolymph of mussels (n = 3) from each group was withdrawn using a syringe containing physiological saline solution (ratio 1:1) and then transferred to clean tubes. Thirty microliters of hemolymph-saline mixture was transferred onto the poly-L-lysine-coated microscope slide, followed by 30 μL of the neutral red working solution, and incubated in a light-proof humid chamber for 15 min at room temperature (t_0). Each slide was observed at 30-min intervals for a total of 180 min using light microscopy (Leitz Wetzlar, Germany) (40×/100×); the slide was returned to the humid chamber after each observation. The observations were terminated, and the retention time recorded when 50% of the small granular hemocytes visibly leaked their dye into the cytosol.

Tissue preparation for histological examination

Histological examination was employed for the purposes of assessing the sex of individuals and analyzing any pathological conditions developed in mussel gonads. The analysis was

performed according to Koagouw and Ciocan (2019). The 7-μm slices cut from paraffin-embedded blocks were stained with hematoxylin and eosin. Histological evaluation of tissue was conducted under light microscopy (Leitz Wetzlar, Germany) (40×/100×), and histopathological conditions were documented along with micrographs referring to each condition, using GXCam HiChrome-Lite (GT Vision, UK).

Gene expression analysis

The analyses were performed following the methodology of Koagouw and Ciocan (2019).

RNA extraction and cDNA synthesis

Total RNA from the gonads (n = 10 for each experimental group) was individually isolated using SurePrep™ TrueTotal™ RNA Purification Kits (Fisher Scientific, UK) and Monarch® Total RNA Miniprep Kit (New England Biolabs, UK) following manufacturer instructions. Qubit™ RNA HS Assay Kit (Invitrogen, UK) and Qubit® Fluorometer were used to quantify the extracted RNA concentration. The cDNA synthesis was carried out with Transcriptor High Fidelity cDNA Synthesis Kit (Roche, UK) as per manufacturer's instructions, and the complementary DNA (cDNA) concentration in each sample was measured using Qubit™ dsDNA HS Assay Kit and Qubit® Fluorometer (Invitrogen™, UK).

Quantitative real time PCR

Molecular analysis was performed to investigate potential changes in the pattern of expression of selected transcripts, as described by Koagouw and Ciocan (2019): *vitellogenin* (*VTG*), *vitelline envelope zona pellucida domain-9* (*V9*), *estrogen receptor-2* (*ER2*), *heat shock protein-70* (*HSP70*), *caspase-8* (*CASP8*), *B-cell lymphoma-2* (*BCL2*), and *Fas cell surface death receptor* (*FAS*). The same primers as in Koagouw and Ciocan (2019) were used for this study (Table S1).

Data analysis

The average cycle quantification (*Cq*) of reference genes *18S rRNA* and *EF1*, as suggested by Cubero-Leon et al. (2012), was used as normalization factor. The computation of the relative changes in the target gene expression identified by real-time qPCR applied the comparative $2^{-\Delta\Delta Ct}$ method, expressed as fold changes to the control group as defined by Livak and Schmittgen (2001).

Statistical analysis was performed using GraphPad Prism 8. One-way analyses of variance (ANOVA) followed by Tukey's post hoc multiple comparison tests were performed to identify significant differences ($p < 0.05$) between groups.

Results

Water analysis

The percentage reduction of paracetamol in treatment groups after 72 and 96 h is depicted in Fig. 1. Overall, the reduction ranged from 19 to nearly 100% after 72 h of exposure, with an average of 61%. After 96 h, the reduction was 63–83% with an overall average of 72%.

Neutral red retention time assay

The lysosome membrane integrity of hemocytes expressed as neutral red retention time is displayed in Fig. 2. All exposed groups showed a significant decrease in the ability to contain the dye within the lysosomes, relative to control; however, there was no statistically significant difference between the paracetamol treatments.

Histopathology observation

Follicle dilatation was recorded as the most widespread pathology in the exposure groups (Fig. 3). This pathological condition was observed in all exposed groups, at a prevalence of 70–80%, with the highest frequency in the 100 μg/L group. Gamete degeneration is another pathological condition that showed a high occurrence, being detected in 50–60% of individuals in all groups exposed to paracetamol. Inflammatory pathologies such as hemocytic infiltration and hemocytic aggregate were also observed in as many as 50% and 30% of individuals, respectively. Female mussels exposed to the lowest concentration of paracetamol (40 ng/L) showed a high incidence of atretic condition in their gonads, up to 40%. Parasitic infestation was observed in all treatment groups, although with low prevalence (10–20%).

The micrographs of histopathological conditions documented during observation are shown in Fig. 4.

mRNA expression analysis

The mRNA expression of each target gene is shown in Fig. 5 and is expressed here as fold changes to the control group (with control group standardized to 1); downregulation is represented by values below 1 and upregulation by values above 1. The data presented here are plotted alongside results recorded by Koagouw and Ciocan (2019), in order to ensure a direct comparison between variation in mRNA expression in short and long exposures of paracetamol.

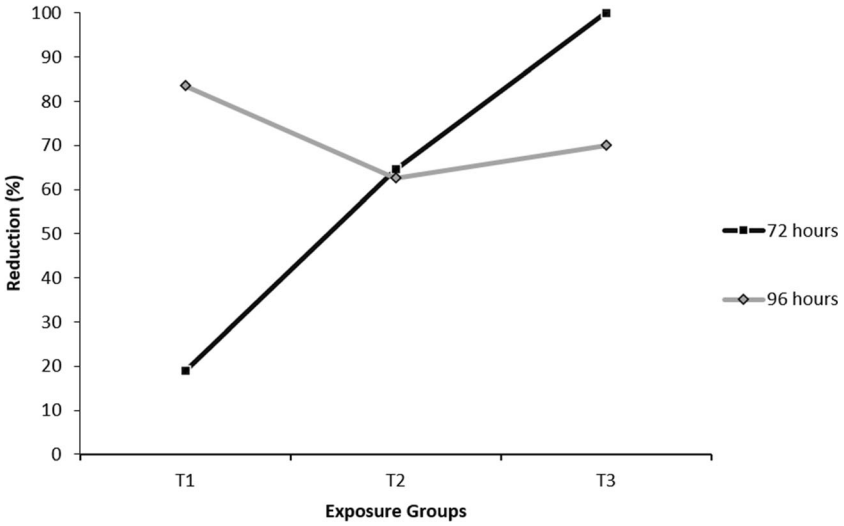


Fig. 1 Percentage decrease of paracetamol level in the water after 72 and 96 h, in three exposure tanks: T1 (40 ng/L), T2 (250 ng/L), and T3 (100 μg/L)

VTG mRNA expression was downregulated in all exposed groups by 4–11 fold changes compared to the control group, although the responses were not significantly different between the treatment groups. A similar trend was previously observed in the short exposure data (Koagouw and Ciocan 2019). A very drastic downregulation of expression was also observed in *V9* mRNA transcript following 24 days' exposure. While the response recorded for this transcript followed a dose-dependent trend in the short exposure experiment, a longer exposure to paracetamol induced a more severe gene silencing, ranging from 8 to 17 fold changes, which was similar in all exposed groups.

The mRNA expression of *ER2* showed a more severe suppression in the long exposure experiment compared to the short exposure (up to 33 fold changes compared to 4–12), while the *HSP70*, *BCL2*, and *FAS* data behaved in a similar manner: following long-term exposure, gene expression was heavily suppressed in all treatment groups. The transcription patterns were concentration dependent in short-term exposure data; however, the long-term exposure to paracetamol seems to exacerbate the effects, with *FAS* transcript downregulated by 11–100 fold changes compared to the control group. For *CASP8*, all exposed groups in the short- and long-term exposures showed a similar pattern of downregulation.

Discussion

Reduction of paracetamol in artificial seawater

A substantial depletion in paracetamol content was observed after 72 h, while after 96 h, the decrease is likely to have balanced to equilibrium, and reduction occurred at a steadier rate. The pattern of reduction here may be associated with the amount of paracetamol that could be absorbed by mussels per day. The data here, however, only show the presence and the reduction trend in the artificial seawater, and further analyses

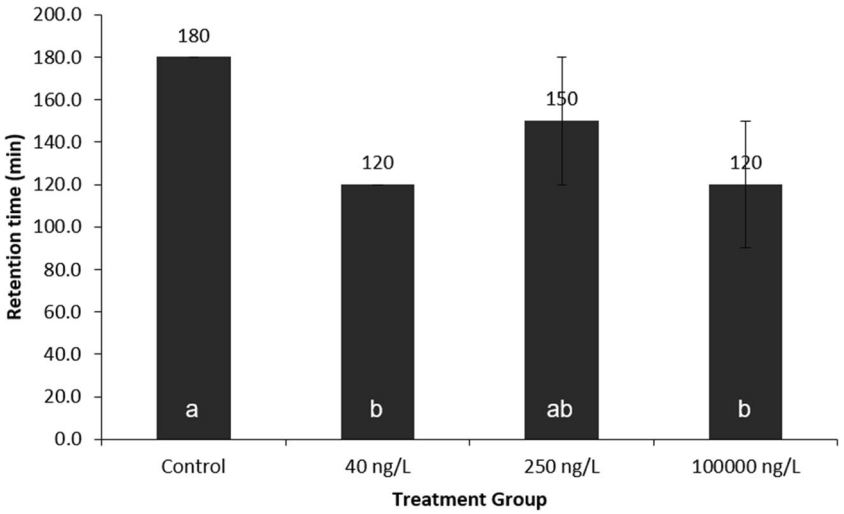
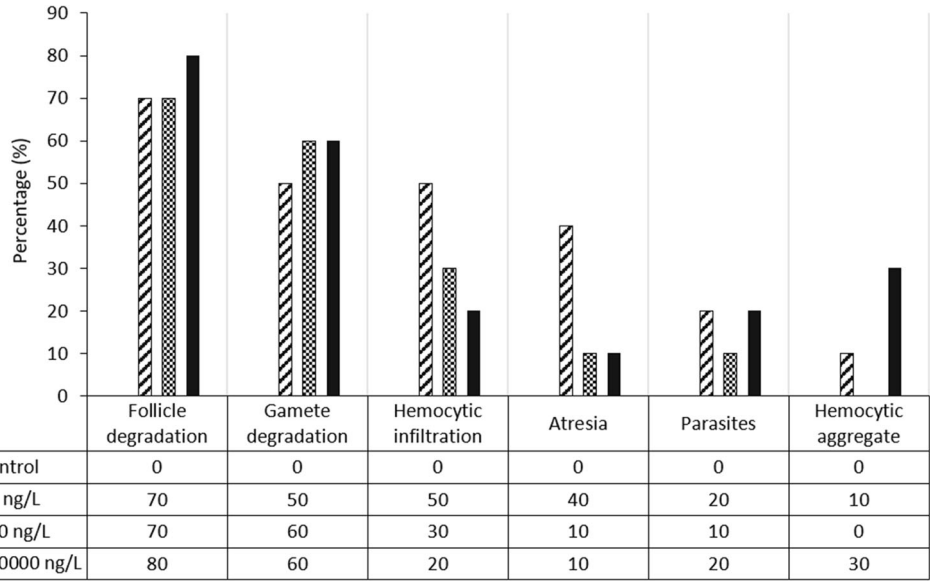


Fig. 2 Neutral red retention time of hemocytes from mussels exposed to paracetamol for 24 days ($n = 3$). Different letters represent statistically significant differences between groups. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$)



	Follicle degradation	Gamete degradation	Hemocytic infiltration	Atresia	Parasites	Hemocytic aggregate
Control	0	0	0	0	0	0
40 ng/L	70	50	50	40	20	10
250 ng/L	70	60	30	10	10	0
100000 ng/L	80	60	20	10	20	30

Fig. 3 The occurrence of histopathological conditions observed in the gonad tissue of mussels exposed to paracetamol for 24 days (n=10). The treatments were as follows: control, 40 ng/L, 250 ng/L, and 100 μg/L

to validate the quantification of the contaminant in mussels are needed, to confirm the absorption of paracetamol.

At 72 h of exposure, the reduction of paracetamol levels exhibited a monotonic response, before transitioning to a more U-shaped pattern of reduction after this point in time. U-shaped curves are very common in toxicological studies (Davis and Svendsgaard 1990; Calabrese 2008; Douron 2010) and are a very important consideration in toxicological and environmental health risk assessments, especially in case of no-observed-effect levels. The transition in the reduction pattern of paracetamol here after 72 h, therefore, may provide important information on the interpretation of ecotoxicology assessment of this pharmaceutical in mussels, when the observed effects have different patterns (monotonic vs non-monotonic) or do not display expected effects due to the different exposure time.

Neutral red retention time of mussel hemocytes

Lysosomal membrane stability is a very sensitive indicator of cellular damage, because lysosomes represent the main cellular site for sequestration and detoxification of contaminants (Dailianis et al. 2003). The principle of NRRT assay is simple: neutral red dye enters the cell and is taken up and retained by lysosomes in healthy cells. In stressed mussels, this process is measured using NRRT and depicts the integrity and capacity of the lysosomal membrane to retain the dye for a period of time. Therefore, the assay measures the ability of cytological processes to adjust to stress conditions (Lowe and Pipe 1994). In other words, mussels that have low immunity will respond by showing a lower retention time of the toxicant, in this case neutral red, as a result of the lysosomal membrane destabilization.

In this study, exposed mussels showed a shorter retention time compared to the control; however, no significant difference was recorded between different treatment groups. Parolini et al. (2009) employed neutral red retention assay in investigating the effects of three NSAIDs on the hemocytes of zebra mussel (*Dreissena polymorpha*) and demonstrated that paracetamol was the lowest in the toxicity scale compared to diclofenac and ibuprofen. Our results suggest that although paracetamol exerts an effect on mussel (*M. edulis*) hemocytes, a higher dose of this particular contaminant does not necessarily increase the biological response, probable due to its lower toxicity as suggested by Parolini et al. (2009).

Also, worth noting here is the relationship between retention time and the reduction of paracetamol in exposed groups. The percentage reduction of paracetamol in artificial seawater at 96 h was observed to be highly correlated with the retention time in each treatment group. Additionally, the lower retention time in mussels groups 40 ng/L, 250 ng/L, and 100 μg/L can be associated with the parasitic incidence in each treatment group, suggesting a strong link between the lower immune response recorded here and the susceptibility of mussels to parasitic infection.

Histological alterations in the gonads of mussels after exposure

In mussels exposed to paracetamol for 24 days, follicle dilatation was observed as the most commonly occurring pathological condition in the gonads. Such prevalence raises concerns regarding mussels' reproductive health, as suggested by Sunila (1987). It is worth mentioning that even exposures at low concentrations of paracetamol, within the range frequently reported in the marine environment, have the capacity to

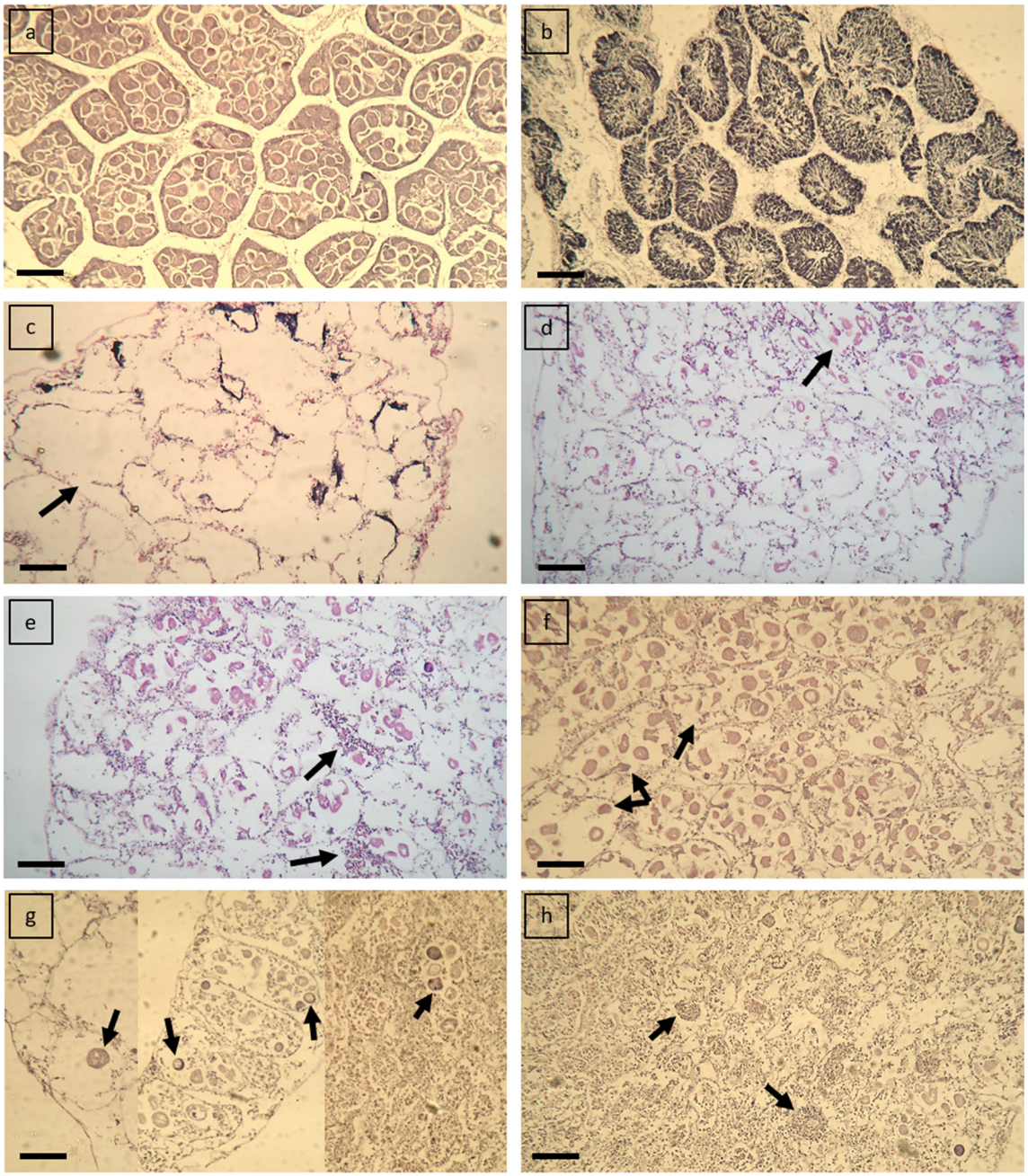


Fig. 4 Histopathological conditions in mussel gonads. Sections of 7 μm, stained with hematoxylin and eosin. **a** Normal female, **b** normal male, **c** follicle dilatation, **d** gamete degeneration, **e** hemocytic infiltration, **f** atresia, **g** parasites, **h** hemocytic aggregate. Arrows point to each pathological condition. Scale bar = 100 μm

induce widespread follicle dilatation in mussel gonads (Koagouw and Ciocan 2019).

In mammals, more than 90% of follicles undergo a degenerative process as part of their developmental cycle (Kerr et al. 2013), with some indications that this degenerative stage is induced by apoptosis of granulosa cells, which are influenced by a precarious balance of pro-survival factor withdrawal and pro-apoptotic factors (Manabe et al. 2004; Hatzirodos et al. 2014; Zhang et al. 2018). A study by García-Gasca et al. (2010) also suggested that this condition might be a useful predictor of environmental stress for coastal ecosystems, as it is directly related to the reproductive system and the success of reproduction. The high prevalence of follicle dilatation in the results presented here implies a considerable potential for paracetamol to disturb mussel reproduction and possibly to interfere with population sustainability.

Gamete degeneration was also one of the most prevalent histological conditions in mussels exposed to paracetamol. Boumela et al. (2009) suggest that the quality of gametes is important not only for gamete survival rates but also for the

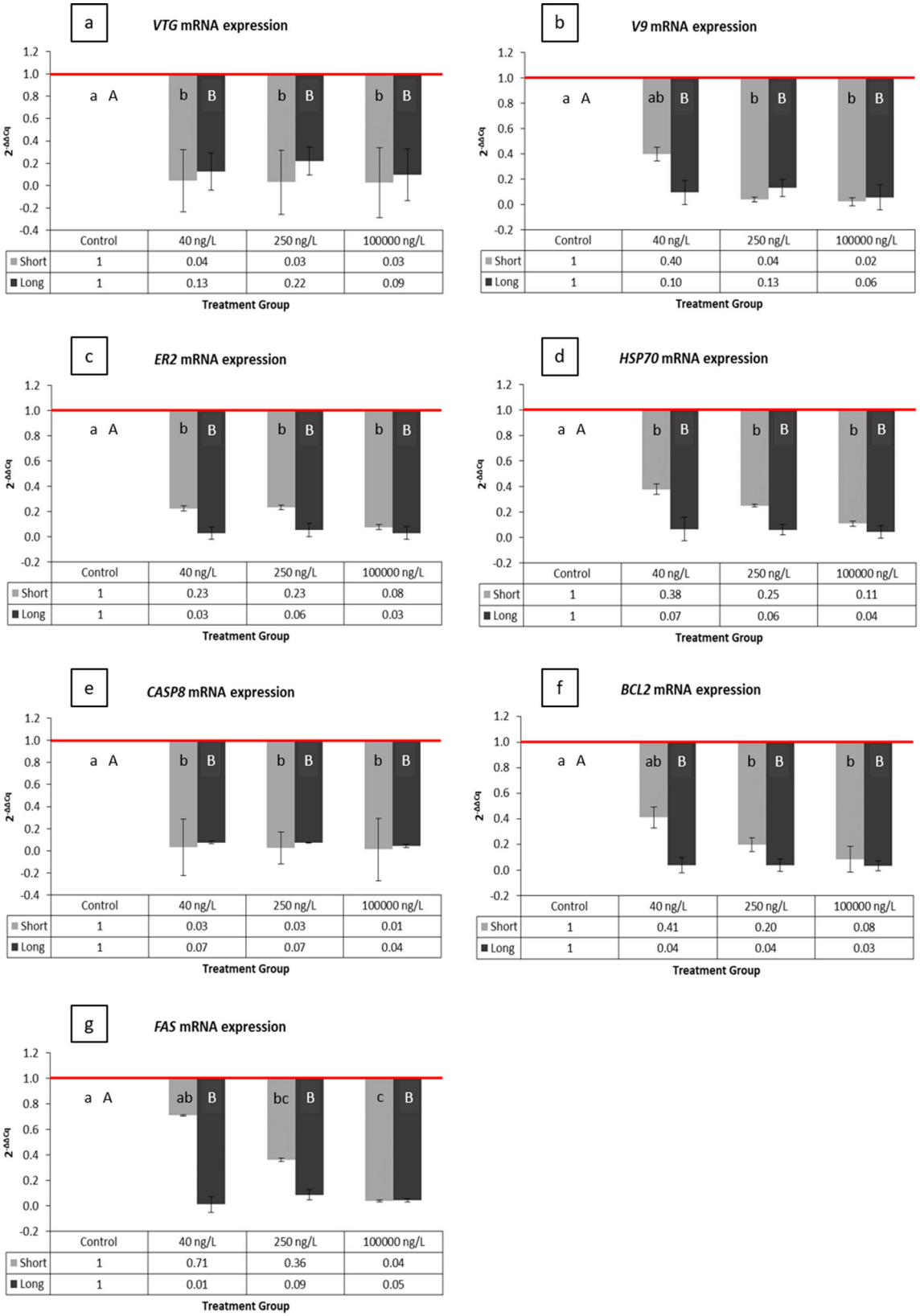


Fig. 5 Summary of mRNA expression of *VTG*, *V9*, *ER2*, *HSP70*, *CASP8*, *BCL2*, and *FAS* (**a**–**g**, respectively) in fold changes compared to control group in mussel gonads (n=10). Data plotted alongside data from short exposure experiment published by Koagouw and Ciocan (2019). Different letters (lowercase for short exposure; uppercase for long exposure) represent statistically significant differences between groups. Bars represent SD. One way ANOVA, followed by Tukey's post hoc test, $p < 0.05$

early stages of embryo development, thus representing a key to successful reproduction. Rouabhi et al. (2019) studied the reproductive cycle of Mediterranean mussels *M. galloprovincialis* from a contaminated coast in Algeria and inferred that gamete degeneration and spawning cessation due to coastal pollution and global warming could endanger recruitment of mussels and, eventually, the shellfish industry as a whole. In this study, a high incidence of gamete degeneration occurred after 24 days' exposure to paracetamol (Fig. 3). This raises concerns regarding the possibility of impaired development of mussels, and eventually the survival of populations themselves, when this pharmaceutical is present in seawater for at least that period of time.

In bivalves, hemocytic infiltration has been linked with the immune responses to stress-related events, especially infectious diseases (Allam and Raftos 2015). Several studies have shown that hemocytic infiltration is usually associated with inflammatory responses in organisms, and elicits profound detrimental effects in some species. Recent research (Gornati et al. 2016) suggested that titanium dioxide nanoparticle exposure led to hemocytic infiltration in *Mytilus galloprovincialis*. Hemocytic infiltration was also prevalent in *Mytilus galloprovincialis* used in field monitoring to assess the effects of an oil spill along the North coast of the Iberian Peninsula (Garmendia et al. 2011). These results imply that environmental contaminants are able to trigger immunological responses and can also cause immunological changes by affecting cellular energy metabolism. Hemocytic infiltration shows a very interesting pattern in our study, whereby the occurrence displays the opposite trend to the concentration (Fig. 3). A previous 7-day exposure experiment (Koagouw and Ciocan 2019) showed a dose-related trend, with a higher incidence of hemocytic infiltration in higher concentration groups, while our longer exposure data suggest a decline in the prevalence of this condition. While the adaptability of mussels to tolerate the contaminant might be a factor, this result is more likely linked to the fact that mussels exposed to the highest concentration of paracetamol are more susceptible to hemocytic aggregate condition (30%), a more severe pathology characterized by the formation of hemocyte clusters (Auffret and Oubella 1997; Garmendia et al. 2011).

An interesting result worth noting here is that all the pathological conditions were detected in almost all exposure groups, from the lowest to the highest concentration, confirming the potential damaging effect of paracetamol even at lower levels of contamination. Overall, the results here present a concerning picture, suggesting paracetamol concentrations as low as 40 ng/L can induce almost the same adverse effects caused by a concentration 2500 times higher, given a long exposure scenario.

The variation of mRNA expression of target genes in mussel gonads

After a long-term exposure to paracetamol, the mRNA expression of *VTG* was equally downregulated in all exposed groups although in a non-monotonic dose-response pattern, suggesting that the lowest concentration of paracetamol can induce a similar effect to a concentration 2500 times higher (100 μg/L). *Vitellogenin* (*VTG*) mRNA expression is a sensitive marker for early assessment of contamination by endocrine disrupting chemicals (EDCs) in vertebrates (Hutchinson et al. 2006; Barucca et al. 2006; Sugawara 2011; Kim et al. 2012). In invertebrates, although its mechanism of action, synthesis, and function are still undefined and require further study (Matozzo et al. 2008; Porte et al. 2006), several studies have identified and recorded induction of vitellogenin following exposure to EDCs (Ciocan et al. 2010; Jubeaux et al. 2012). Similar to short exposure data (Koagouw and Ciocan 2019), the long-term exposure results indicate that even the lowest concentration of paracetamol (40 ng/L) can induce extreme downregulation in *VTG* irrespective of the length of exposure to paracetamol. This sensitivity indicates the potential for this target gene to be further explored as a biomarker for ecotoxicological studies on paracetamol.

A different pattern of the *vitelline envelope zona pellucida domain-9* (*V9*) downregulation was recorded in mussels exposed to paracetamol for 24 days when compared to a shorter 7-day exposure (Koagouw and Ciocan 2019). The *V9* mRNA expression results in all 24-day treatment groups were significantly lower compared to the control ($p < 0.05$). The results presented here indicate that longer exposure to low concentration of paracetamol has the potential to inflict the same level of biological responses as brief exposures to high concentrations. Considering that pharmaceuticals are constantly released into the aquatic environment and therefore the organisms are in prolonged contact with the contaminants, our results can be considered environmentally relevant.

Vitelline envelope or zona pellucida is known to play a fundamental role in various aspects of fertilization, such as mediating the sperm binding process (Snell and White 1996) and protecting against polyspermy (Coy et al. 2008). In addition, this protein has been advanced as a potential biomarker for environmental estrogens in fish (Celius and Walther 1998; Celius et al. 1999). The paracetamol modulated *V9* expression presented herein suggests that reproductive impairment in mussels can potentially result from exposure to low concentrations similar to those detected in the natural environment.

In bivalves, since sex-related alterations may primarily be mediated by sex steroid receptors, expression variability in estrogen receptors indicates possible consequences that may occur in gametogenesis and reproductive processes (Croll and Wang 2007). In this study, *ER2* mRNA expression was downregulated in all treatment groups, in a non-monotonic dose

response pattern (Fig. 5c). The data suggest that a long exposure to paracetamol intensifies the effects by approximately 13–29 folds in all long-term exposure tanks, compared to short exposure. As estrogenic activities are facilitated through estrogen receptors by regulating the target gene expression (Gao and Dahlman-Wright 2011), the results presented here thus raise concern over the potential of paracetamol to induce disruption of estrogen-modulated transcripts. In the 7-day exposure, male mussels were highly affected and displayed a monotonic trend of response in the *ER2* gene expression (Koagouw and Ciocan 2019). The long-term exposure data suggest similar levels of downregulation regardless of the sex of mussels and paracetamol concentration and thus a more widespread effect. Several studies have documented the expression of estrogen receptors in different invertebrates (Keay and Thornton 2009; Jones et al. 2017), in addition to mussels (Puinean et al. 2006; Agnese et al. 2019; Balbi et al. 2019). However, it is also worth considering that *ER2* might display natural variation during different stages of gametogenesis (Ciocan et al. 2010); hence, further investigation is required.

The expression of several target genes involved in apoptosis was investigated after long and short exposure to paracetamol. In this study, the mRNA expression of *CASP8* was downregulated by even very low concentrations of paracetamol present in the seawater, in both short- and long-term exposures (Fig. 5e). Reduced mRNA expression of *caspase-8* (*CASP8*) may bring about changes in the deterioration process of cellular components, acting to inhibit apoptosis (Kruidering and Evan 2000; Romero et al. 2011). Ruocco et al. (2016) documented the activation of caspase-8 and changes in the expression level of *CASP8* that led to apoptosis in sea urchin embryos after exposure to oxylipins, diatom secondary metabolites. *CASP8* may also play an important role in programmed cell death, meaning its downregulation could contribute to cancer-related pathologies (Aghababazadeh et al. 2017). This suggests that paracetamol can potentially be involved in carcinogenic pathways or in any pathological condition caused by the downregulation of *CASP8* in mussels. As *CASP8* is one of the important genes involved in apoptosis, its extreme sensitivity to paracetamol poses a real threat, possibly further highlighted by apoptosis-related pathologies recorded during histological examination (Figs. 3 and 4) of the reproductive system in mussels. Moreover, the high sensitivity of *CASP8* as demonstrated here makes this transcript recommended as a potential biomarker for the monitoring of paracetamol contamination in the environment.

The expressions of *HSP70*, *BCL2*, and *FAS* in this study were downregulated in all treatment groups following non-monotonic responses after 24-day exposure, while a shorter exposure elicited a dose-dependent response (Fig. 5). Here, the different patterns of expression highlight the significance of exposure period on the modulation of these regulatory genes: they suggest that a longer period of paracetamol exposure can induce similar levels of downregulation in mussels exposed to both low and high concentrations of this substance. The dramatic changes in response of all three transcripts in the low concentration group again suggest that long-term effects are even more deleterious than short-term effects. These results should be of great concern considering the presence of paracetamol in the environment will be very likely to persist in the long term, as a result of urban waste agglomeration, and especially in the very recent special case of the COVID-19 pandemic.

As the healthy cell death balance is maintained by the modulation of apoptosis-regulatory gene transcripts through the activation or inhibition of apoptosis (Kiss 2010), alterations to this process may either lead to the progression of cell death or the survival of defective cells, which can contribute to carcinogenesis. The significant changes in expression of *HSP70*, *BCL2*, and *FAS* in this study indicate the possible threat that might be faced by mussel populations, in particular through apoptosis-related mechanisms in the reproductive organs. While the suppression of *HSP70* in paracetamol-exposed mussels as observed in this study may lead to a higher risk of apoptosis and cell death, the modulation of both *BCL2* and *FAS* presented here (Fig. 5) can induce either apoptosis or cancer-related conditions in gonad cells. This particular apoptosis-related alteration is perfectly depicted in the histopathology result, where degeneration in follicles and gametes is evident in mussels' gonads (Figs. 3 and 4c–d). The presence of the apoptosis-related pathologies, with high prevalences across all levels and durations of exposure, is consistent with the pattern of downregulation observed in target genes involved in apoptosis.

In other studies, apoptosis in molluscs (Kiss 2010) and in particular mussels (Estévez-Calvar et al. 2013; García-Gasca et al. 2010) has been proposed as a potential biomarker to monitor environmental stress in marine ecosystems. Moreover, Estévez-Calvar et al. (2013) specifically confirmed that expression of the genes involved in apoptosis can be harnessed to assess the stress-related biological responses of coastal species. Characterizing the expression of these apoptotic regulatory genes is therefore crucial as a first step towards the development of potential biomarkers.

Overall, our study demonstrates the importance of exposure duration, in that longer exposure to paracetamol could magnify its effects in adult mussels. Different responses following short- and long-term exposures to contaminants have also been recorded by several studies, in vertebrates and invertebrates. Saravanan et al. (2014) reported different responses of thyroxine in carp *Cirrhinus mrigala* after short- and long-term exposure to diclofenac and clofibric acid. The authors showed that thyroxine level decreased only in two treatment groups exposed to clofibric acid (and not in the diclofenac groups) following short-term exposure, but longer duration of exposure decreased thyroxine levels in all

concentrations of both contaminants. In invertebrates, Oliveira et al. (2017) investigated physiological and biochemical alterations in mussels *Mytilus galloprovincialis* after short- and long-term exposure to carbamazepine. The authors revealed that among all physiological parameters studied, the condition and gonadosomatic indices were mostly adversely affected by long-term exposure to carbamazepine and further concluded that this alteration could compromise the reproductive potential of organisms with implications for the survival of the population.

As the duration of exposure plays a significant role in determining the severity of effects, it is important to consider the length of contact time in assessing the effects of a given contaminant, in our case paracetamol. Our study would suggest that the assessment of a contaminant's effects should take into account not only its concentration or level in the environment but also the length of exposure encountered by organisms.

Conclusion

Paracetamol does not appear to have a consequential impact on hemocytes, especially in terms of lysosomal membrane stability. However, more dramatic results are observed in the gonad tissue, where paracetamol is seen to induce major adverse changes, such as degeneration in follicles and gametes. Such changes pose a risk to the reproductive ability of this organism and therefore a potential impact on population survival.

Paracetamol-induced damage was also recorded at the molecular level. Patterns observed in mRNA expression after long exposure to paracetamol as observed in this study demonstrate the importance of length of exposure on the biological responses elicited in blue mussels. Overall, the expression of all transcripts investigated herein suggests that longer exposure to a contaminant may result in much greater effects, potentially equivalent to those registered after exposure to much higher concentrations. These findings indicate that the presence of paracetamol in the environment even at low concentrations has the potential to cause several major changes related to the reproductive system of mussels. Further investigations exploring and confirming some related aspects, especially the mechanism of action, are encouraged.

Over recent years, there has been growing concern regarding chronic pharmaceutical pollution of the aquatic environment. As rising human populations coincide with a higher demand for pharmaceuticals, their continuous emission is very likely to persist. The situation is likely to deteriorate further in the current pandemic, as pharmaceutical companies have seen an enormous demand for pain relief medicine, particularly paracetamol. Some of these will be ingested and then excreted, but it is predicted that a large amount will be disposed of, and will most likely end up in the natural environment in vast quantities. Long exposure studies are therefore environmentally relevant, and our results demonstrate that at least for paracetamol, the effects are exacerbated in a chronic exposure scenario.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-021-14136-6.

Acknowledgements The authors would like to thank Dr. Darren McCabe, Anna Blunden, and Lisa Joliffe for their laboratory assistance and Dr. Richard Hazell for his helpful comments on the manuscript and excellent proofreading skills.

Author contribution Wulan Koagouw: conceptualization, methodology, formal analysis, investigation, resources, writing—original draft
Nicolas A. Stewart: investigation, resources, writing—original draft
Corina Ciocan: conceptualization, methodology, resources, writing—original draft, supervision
All authors read and approved the final manuscript.

Funding This work was supported by Ministry of Research, Technology, and Higher Education (MRTHE) Republic of Indonesia through Research and Innovation in Science and Technology Project (RISET-Pro). The funding body has no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate All procedures in this paper were performed in compliance with the ARRIVE guidelines and carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63/EU on the protection of animals used for scientific purposes. The procedures have been approved by the Animal Welfare and Ethics Review Bodies (AWERB), University of Brighton.

Consent for publication Not applicable

Competing interests The authors declare no competing interests.

References

Aghababazadeh M, Dorraki N, Javan FA, Fattahi AS, Gharib M, Pasdar A (2017) Downregulation of Caspase 8 in a group of Iranian breast cancer patients – a pilot study. *J Egyptian Nat Cancer Ins* 29(4): 191–195

Agnese M, Rosati L, Prisco M, Borzacchiello L, Abagnale L, Andreuccetti P (2019) "The expression of estrogen receptors during the *Mytilus galloprovincialis* ovarian cycle", *Journal of Experimental Zoology. Part A, Ecol Integrat Physiol* 331(7):367–373

Agunbiade FO, Moodley B (2014) Pharmaceuticals as emerging organic contaminants in Umgeni River water system, KwaZulu-Natal, South Africa. *Environ Monit Assess* 186(11):7273–7291

Allam B, Raftos D (2015) Immune responses to infectious diseases in bivalves. *J Invertebr Pathol* 131:121–136

Antunes SC, Freitas R, Figueira E, Gonçalves F, Nunes B (2013) Biochemical effects of acetaminophen in aquatic species: edible clams *Venerupis decussata* and *Venerupis philippinarum*. *Environ Sci Pollut Res* 20:6658–6666

Auffret M, Oubella R (1997) Hemocyte aggregation in the oyster *Crassostrea gigas*: in vitro measurement and experimental modulation by xenobiotics. *Comp Biochem Physiol A Physiol* 118(3):705–712

aus der Beek T, Weber FA, Bergmann A, Hickmann S, Ebert I, Hein A, Küster A (2016) Pharmaceuticals in the environment—global occurrences and perspectives. *Environ Toxicol Chem* 35(4):823–835

Balbi T, Ciacci C, Canesi L (2019) Estrogenic compounds as exogenous modulators of physiological functions in molluscs: signaling pathways and biological responses. *Comparative Biochem and Physiol Part C* 222:135–144

Barucca M, Canapa A, Olmo E, Regoli F (2006) Analysis of vitellogenin gene induction as a valuable biomarker of estrogenic exposure in various Mediterranean fish species. *Environ Res* 101(1):68–73

Bárzaga Arencibia Z, Choonara I (2012) Balancing the risks and benefits of the use of over-the-counter pain medications in children: an international journal of medical toxicology and drug experience an international journal of medical toxicology and drug experience. *Drug Saf* 35(12):1119–1125

Bebianno MJ, Mello ACP, Serrano MAS, Flores-Nunes F, Mattos JJ, Zacchi FL, Piazza RS, Piazza CE, Siebert MN, Gomes CHAM, Melo CMR, Bainy ACD (2017) Transcriptional and cellular effects of paracetamol in the oyster *Crassostrea gigas*. *Ecotoxicol Environ Saf* 144:258–267

Benotti MJ, Brownawell BJ (2007) Distributions of pharmaceuticals in an urban estuary during both dry- and wet-weather conditions. *Environ Sci Technol* 41(16):5795–5802

Bertolini A, Ferrari A, Ottani A, Guerzoni S, Tacchi R, Leone S (2006) Paracetamol: New vistas of an old drug. *CNS Drug Reviews* 12(3-4): 250–275

Boumela I, Guillemin Y, Guerin JF, Aouacheria A (2009) The Bcl-2 family pathway in gametes and preimplantation embryos. *Gynecologie Obstetrique & Fertilite* 37(9):720–732

Brodin T, Piovano S, Fick J, Klaminder J, Heynen M, Jonsson M (2014) Ecological effects of pharmaceuticals in aquatic systems–impacts through behavioural alterations. *Philosophical Transac Royal Soc B Biol Sci* 369(1656):20130580–20130580

Caban M, Lis E, Kumirska J, Stepnowski P (2015) Determination of pharmaceutical residues in drinking water in Poland using a new SPE-GC-MS(SIM) method based on Speedisk extraction disks and DIMETRIS derivatization. *Sci Total Environ* 538:402–411

Calabrese EJ (2008) U-shaped dose response in behavioral pharmacology: historical foundations. *Crit Rev Toxicol* 38(7):591–598

Celius T, Walther BT (1998) Differential sensitivity of zonagenesis and vitellogenesis in Atlantic salmon (*Salmo salar* L) to DDT pesticides. *J Exp Zool* 281(4):346–353

Celius T, Haugen TB, Grotmol T, Walther BT (1999) A sensitive zonagenetic assay for rapid in vitro assessment of estrogenic potency of xenobiotics and mycotoxins. *Environ Health Perspect* 107(1): 63–68

Ciocan CM, Cubero-Leon E, Puinean AM, Hill EM, Minier C, Osada M, Fenlon K, Rotchell JM (2010) Effects of estrogen exposure in mussels, *Mytilus edulis*, at different stages of gametogenesis. *Environ Pollut* 158(9):2977–2984

Cope WG, Bringolf RB, Buchwalter DB, Newton TJ, Ingersoll CG, Wang N, Augspurger T, Dwyer FJ, Barnhart MC, Neves RJ, Hammer E (2008) Differential exposure, duration, and sensitivity of unionoidean bivalve life stages to environmental contaminants. *J N Am Benthol Soc* 27(2):451–462

Correia B, Freitas R, Figueira E, Soares AMVM, Nunes B (2016) Oxidative effects of the pharmaceutical drug paracetamol on the edible clam *Ruditapes philippinarum* under different salinities. *Comp Biochem Physiol Part C: Toxicol Pharmacol* 179:116–124

Coy P, Grullon L, Canovas S, Romar R, Matas C, Aviles M (2008) Hardening of the zona pellucida of unfertilized eggs can reduce polyspermic fertilization in the pig and cow. *Reproduction* 135(1): 19–27

Croll RP, Wang C (2007) Possible roles of sex steroids in the control of reproduction in bivalve molluscs. *Aquaculture* 272(1–4):76–86

Cubero-Leon E, Ciocan CM, Minier C, Rotchell JM (2012) Reference gene selection for qPCR in mussel, Mytilus edulis, during gametogenesis and exogenous estrogen exposure. Environ Sci Pollut Res 19(7):2728–2733

Dailianis S, Domouhtsidou GP, Raftopoulou E, Kaloyianni M, Dimitriadis VK (2003) Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. *Mar Environ Res* 56(4):443–470

Davis JM, Svendsgaard DJ (1990) U-Shaped dose-response curves: their occurrence and implications for risk assessment. *J Toxicol Environ Health* 30(2):71–83

de Solla SR, Gilroy EAM, Klinck JS, King LE, McInnis R, Struger J, Backus SM, Gillis PL (2016) Bioaccumulation of pharmaceuticals and personal care products in the unionid mussel *Lasmigona costata* in a river receiving wastewater effluent. *Chemosphere* 146:486–496

Douron M (2010) "U-shaped dose-response curves: implications for risk characterization of essential elements and other chemicals", *Journal of Toxicology and Environmental Health. Part A* 73(2-3):181–186

Du B, Haddad SP, Scott WC, Chambliss CK, Brooks BW (2015) Pharmaceutical bioaccumulation by periphyton and snails in an effluent-dependent stream during an extreme drought. *Chemosphere* 119:927–934

Ebele AJ, Abou-Elwafa Abdallah M, Harrad S (2017) Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. *Emerging Contaminants* 3(1):1–16

Estévez-Calvar N, Romero A, Figueras A, Novoa B (2013) Genes of the mitochondrial apoptotic pathway in *Mytilus galloprovincialis*. *PLoS One* 8(4):e61502–e61502

Fabbri E, Franzellitti S (2016) Human pharmaceuticals in the marine environment: Focus on exposure and biological effects in animal species. *Environ Toxicol Chem* 35(4):799–812

Fairbairn DJ, Karpuzcu ME, Arnold WA, Barber BL, Kaufenberg EF, Koskinen WC, Novak PJ, Rice PJ, Swackhamer DL (2016) Sources and transport of contaminants of emerging concern: a two-year study of occurrence and spatiotemporal variation in a mixed land use watershed. *Sci Total Environ* 551-552:605–613

Flammarion P, Brion F, Babut M, Garric J, Migeon B, Noury P, Thybaud E, Palazzi X, Tyler CR (2000) Induction of fish vitellogenin and alterations in testicular structure: preliminary results of estrogenic effects in chub (*Leuciscus cephalus*). *Ecotoxicology* 9(1-2):127–135

Fonseca TG, Carriço T, Fernandes E, Abessa DMS, Tavares A, Bebianno MJ (2019) Impacts of in vivo and in vitro exposures to tamoxifen: comparative effects on human cells and marine organisms. *Environ Int* 129:256–272

Franzellitti S, Buratti S, Valbonesi P, Fabbri E (2013) The mode of action (MOA) approach reveals interactive effects of environmental pharmaceuticals on *Mytilus galloprovincialis*. *Aquat Toxicol* 140-141: 249–256

Gao H, Dahlman-Wright K (2011) The gene regulatory networks controlled by estrogens. *Mol Cell Endocrinol* 334(1):83–90

García-Gasca A, Leal-Tarin B, Ríos-Sicairos J, Hernández-Cornejo R, Aguilar-Zárate G, Betancourt-Lozano M (2010) "Follicular apoptosis in the mussel (*Mytella strigata*) as potential indicator of environmental stress in coastal ecosystems", *Journal of Environmental Science and Health. Part A* 45(1):56–61

Garmendia L, Soto M, Vicario U, Kim Y, Cajaraville MP, Marigómez I (2011) Application of a battery of biomarkers in mussel digestive gland to assess long-term effects of the Prestige oil spill in Galicia and Bay of Biscay: tissue-level biomarkers and histopathology. *J Environ Monit* 13(4):915–932

Gornati R, Longo A, Rossi F, Maisano M, Sabatino G, Mauceri A, Bernardini G, Fasulo S (2016) Effects of titanium dioxide nanoparticle exposure in *Mytilus galloprovincialis* gills and digestive gland. *Nanotoxicology* 10(6):807–817

Hatzirodos N, Irvingrodgers H, Hummitzsch K, Harland M, Morris S, Rodgers R (2014) Transcriptome profiling of granulosa cells of bovine ovarian follicles during growth from small to large antral sizes. *BMC Genomics* 15:24

Hoeger B, Köllner B, Dietrich DR, Hitzfeld B (2005) Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta f. fario*). *Aquat Toxicol* 75(1):53–64

Huang IJ, Sirotkin HI, McElroy AE (2019) Varying the exposure period and duration of neuroactive pharmaceuticals and their metabolites modulates effects on the visual motor response in zebrafish (*Danio rerio*) larvae. *Neurotoxicol Teratol* 72:39–48

Hutchinson TH, Ankley GT, Segner H, Tyler CR (2006) Screening and testing for endocrine disruption in fish-biomarkers as "signposts," not "traffic lights," in risk assessment. *Environ Health Perspec 114 Suppl* 1(Suppl 1):106–114

Jones, B. L., Walker, C., Azizi, B., Tolbert, L., Williams, L. D. and Snell, T. W. (2017) "Conservation of estrogen receptor function in invertebrate reproduction", *BMC Evolutionary Biology,* 17 (1) p. 65.

Jozwiak-Bebenista M, Nowak JZ (2014) Paracetamol: mechanism of action, applications and safety concern. *Acta Pol Pharm Drug Res* 71(1):11–23

Jubeaux G, Simon R, Salvador A, Lopes C, Lacaze E, Quéau H, Chaumot A, Geffard O (2012) Vitellogenin-like protein measurement in caged *Gammarus fossarum* males as a biomarker of endocrine disruptor exposure: Inconclusive experience. *Aquat Toxicol* 122-123:9–18

Kasprzyk-Hordern B, Dinsdale RM, Guwy AJ (2008) The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. *Water Res* 42(13): 3498–3518

Keay J, Thornton JW (2009) Hormone-activated estrogen receptors in annelid invertebrates: implications for evolution and endocrine disruption. *Endocrinology* 150(4):1731–1738

Kerr J, Myers M, Anderson R (2013) The dynamics of the primordial follicle reserve. *Reproduction* 146:R205–R215

Kim P, Park Y, Ji K, Seo J, Lee S, Choi K, Kho Y, Park J, Choi K (2012) Effect of chronic exposure to acetaminophen and lincomycin on Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*, and potential mechanisms of endocrine disruption. *Chemosphere* 89(1):10–18

Kiss T (2010) Apoptosis and its functional significance in molluscs. *Apoptosis : An Int J Programmed Cell Death* 15(3):313–321

Koagouw W, Ciocan C (2018) Impact of metformin and increased temperature on blue mussels *Mytilus edulis* - evidence for synergism. *J Shellfish Res* 37(3):467–474

Koagouw W, Ciocan C (2019) Effects of short-term exposure of paracetamol in the gonads of blue mussels *Mytilus edulis*. *Environ Sci Pollut Res Int* 27(25):30933–30944

Kruidering M, Evan GI (2000) Caspase-8 in apoptosis: the beginning of "the end"? *IUBMB Life* 50(2):85–90

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25(4):402–408

Lowe DM, Pipe RK (1994) Contaminant induced lysosomal membrane damage in marine mussel digestive cells: an in vitro study. *Aquat Toxicol* 30(4):357–365

Lowe DM, Fossato VU, Depledge MH (1995) Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: an in vitro study. *Mar Ecol Prog Ser* 129:189–196

Mamaca E, Bechmann RK, Torgrimsen S, Aas E, Bjørnstad A, Baussant T, Floch SL (2005) The neutral red lysosomal retention assay and comet assay on haemolymph cells from mussels (*Mytilus edulis*) and fish (*Symphodus melops*) exposed to styrene. *Aquat Toxicol* 75(3): 191–201

Manabe N, Goto Y, Matsudaminehata F, Inoue N, Maeda A, Sakamaki K, Miyano T (2004) Regulation mechanism of selective atresia in porcine follicles: regulation of granulosa cell apoptosis during atresia. *J Reprod Dev* 50:493–514

Matozzo V, Gagné F, Marin MG, Ricciardi F, Blaise C (2008) Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: a review. *Environ Int* 34(4):531–545

Mimeault C, Woodhouse AJ, Miao XS, Metcalfe CD, Moon TW, Trudeau VL (2005) The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, *Carassius auratus*. *Aquat Toxicol* 73(1):44–54

Nödler K, Voutsa D, Licha T (2014) Polar organic micropollutants in the coastal environment of different marine systems. *Mar Pollut Bull* 85(1):50–59

Norse EA, Crowder LB (eds) (2005) Marine conservation biology: the science of maintaining the sea's biodiversity. Island Press, Washington, pp 470

Nunes B, Nunes J, Soares AMVM, Figueira E, Freitas R (2017) Toxicological effects of paracetamol on the clam *Ruditapes philippinarum*: exposure vs recovery. *Aquat Toxicol* 192:198–206

Oliveira P, Almeida Â, Calisto V, Esteves VI, Schneider RJ, Wrona FJ, Soares A, Figueira E, Freitas R (2017) Physiological and biochemical alterations induced in the mussel Mytilus galloprovincialis after short and long-term exposure to carbamazepine. *Water Res* 117(1879-2448 (Electronic)):102–114

Parolini M, Binelli A, Cogni D, Riva C, Provini A (2009) An in vitro biomarker approach for the evaluation of the ecotoxicity of non-steroidal anti-inflammatory drugs (NSAIDs). *Toxicol in Vitro* 23(5):935–942

Pereira CDS, Maranho LA, Cortez FS, Pusceddu FH, Santos AR, Ribeiro DA, Cesar A, Guimarães LL (2016) Occurrence of pharmaceuticals and cocaine in a Brazilian coastal zone. *Sci Total Environ* 548-549: 148–154

Piedade F, Bio S, Nunes B (2020) Effects of common pharmaceutical drugs (paracetamol and acetylsalicylic acid) short term exposure on biomarkers of the mussel *Mytilus spp*. *Environ Toxicol Pharmacol* 73:103276

Porte C, Janer G, Lorusso LC, Ortiz-Zarragoitia M, Cajaraville MP, Fossi MC, Canesi L (2006) Endocrine disruptors in marine organisms: approaches and perspectives. *Comp Biochem Physiol Part C: Toxicol Pharmacol* 143(3):303–315

Puinean AM, Labadie P, Hill EM, Osada M, Kishida M, Nakao R, Novillo A, Callard IP, Rotchell JM (2006) Laboratory exposure to 17β-estradiol fails to induce vitellogenin and estrogen receptor gene expression in the marine invertebrate *Mytilus edulis*. *Aquat Toxicol* 79(4):376–383

Rittschof D, McClellan-Green P (2005) Molluscs as multidisciplinary models in environment toxicology. *Mar Pollut Bull* 50(4):369–373

Roberts E, Delgado Nunes V, Buckner S, Latchem S, Constanti M, Miller P, Doherty M, Zhang W, Birrell F, Porcheret M, Dziedzic K, Bernstein I, Wise E, Conaghan PG (2016) Paracetamol: not as safe as we thought? A systematic literature review of observational studies. *Ann Rheum Dis* 75(3):552–559

Romero A, Estévez-Calvar N, Dios S, Figueras A, Novoa B (2011) New insights into the apoptotic process in mollusks: characterization of caspase genes in *Mytilus galloprovincialis*. *PLoS One* 6(2):e17003

Rouabhi YL, Grosjean P, Boutiba Z, Rouane Hacene O, Richir J (2019) Reproductive cycle and follicle cleaning process of *Mytilus galloprovincialis* (Mollusca: Bivalvia) from a polluted coastal site in Algeria. *Invertebr Reprod Dev* 63(4):255–267

Ruocco N, Varrella S, Romano G, Ianora A, Bentley MG, Somma D, Leonardi A, Mellone S, Zuppa A, Costantini M (2016) Diatom-derived oxylipins induce cell death in sea urchin embryos activating caspase-8 and caspase 3/7. *Aquat Toxicol* 176:128–140

Saravanan M, Hur JH, Arul N, Ramesh M (2014) Toxicological effects of clofibric acid and diclofenac on plasma thyroid hormones of an Indian major carp, Cirrhinus mrigala during short and long-term exposures. *Environ Toxicol Pharmacol* 38(3):948–958

Snell WJ, White JM (1996) The molecules of mammalian fertilization. *Cell* 85(5):629–637

Solé M, Shaw JP, Frickers PE, Readman JW, Hutchinson TH (2010) Effects on feeding rate and biomarker responses of marine mussels experimentally exposed to propranolol and acetaminophen. *Anal Bioanal Chem* 396(2):649–656

Sugawara T (2011) Chapter 68 - Screening systems for endocrine disruptors. In: Gupta RC (ed) *Reproductive and Developmental Toxicology*. Academic Press, San Diego, pp 893–902

Sunila I (1987) Histopathology of mussels (*Mytilus edulis* L.) from the Tvärminne area, the Gulf of Finland (Baltic Sea). *Ann Zool Fenn* 24(1):55–69

Świacka, K., Maculewicz, J., Smolarz, K., Szaniawska, A. and Caban, M. (2019) "Mytilidae as model organisms in the marine ecotoxicology of pharmaceuticals - a review", *Environmental Pollution,* 254 (Pt B) p. 113082.

Tariq M, Din F u (2017) Poor knowledge of university students regarding paracetamol; a wakeup call for public healthcare practitioners. *Cogent Medicine* 4(1)

Tijani JO, Fatoba OO, Babajide OO, Petrik LF (2016) Pharmaceuticals, endocrine disruptors, personal care products, nanomaterials and perfluorinated pollutants: a review. *Environ Chem Lett* 14(1):27–49

Togola A, Budzinski H (2008) Multi-residue analysis of pharmaceutical compounds in aqueous samples. *J Chromatogr A* 1177(1):150–158

Vernouillet G, Eullaffroy P, Lajeunesse A, Blaise C, Gagné F, Juneau P (2010) Toxic effects and bioaccumulation of carbamazepine evaluated by biomarkers measured in organisms of different trophic levels. *Chemosphere* 80(9):1062–1068

Wang J, Gardinali PR (2013) Uptake and depuration of pharmaceuticals in reclaimed water by mosquito fish (*Gambusia holbrooki*): a worst-case, multiple-exposure scenario. *Environ Toxicol Chem* 32(8): 1752–1758

Warwick C (2008) Paracetamol and fever management. *J R Soc Promot Heal* 128(6):320–323

Wood DM, English E, Butt S, Ovaska H, Garnham F, Dargan PI (2010) Patient knowledge of the paracetamol content of over-the-counter (OTC) analgesics, cough/cold remedies and prescription medications. *Emerg Med J* 27(11):829

Zhang J, Liu Y, Yao W, Li Q, Liu H, Pan Z (2018) Initiation of follicular atresia: gene networks during early atresia in pig ovaries. *Reproduction* 156(1):23

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.