Bulletin of Environmental Contamination and Toxicology (2020) 104:180–184
https://doi.org/10.1007/s00128-019-02770-3

Immunohistological Biomarkers of Toxicity by a Pharmaceutical Antidepressant in the Freshwater Cichlid Fish *Cichlasoma dimerus* (Teleostei, Cichliformes)

Graciela Rey Vázquez[1,2] · Rodrigo Hernán Da Cuña[1,2] · Luciana Soledad Dorelle[1,2] · Fabiana Laura Lo Nostro[1,2]

Received: 10 August 2019 / Accepted: 13 December 2019 / Published online: 1 January 2020

Abstract

Melano-macrophage centers (MMCs) are nodular clusters of pigmented macrophages, implicated in homeostasis and destruction and recycling of endogenous and exogenous material. They can increase in size and/or frequency under environmental stress resulting in immunohistological biomarkers of water quality. Fluoxetine (FLX), a commonly prescribed antidepressant, can cause neuroendocrine, behavioral and reproductive alterations in teleost fish. In the present study, we analyzed the effects of a 2-week 50 µg/L FLX exposure on MMCs in histological sections of spleen and head-kidney (HK) of the cichlid fish *Cichlasoma dimerus*. In the spleen, FLX caused an increase in the area and a decrease in the number of MMCs. An increase in the proportion of the HK occupied by MMCs was observed in FLX-exposed fish, due to an increase in their number but not their area. The deposition rate of MMCs varies according to the hemolymphopoietic organ and would be the result of a differential response to FLX on homeostatic functions (elimination of cellular debris, iron processing and immune response).

Keywords Melano-macrophage centers · Fluoxetine · Hematopoietic tissue · Spleen · Head kidney

Melano-macrophage centers (MMCs) are nodular accumulations of pigmented macrophages ubiquitous in almost all vertebrates. In fish they are normally located in the stroma of the hematopoietic tissue of the spleen and the head kidney (HK), although they can also be found to a lesser extent in the liver, gonads, intestinal mucosa and thymus (Macchi et al. 1992; Agius and Roberts 2003). MMCs functions range from those dedicated to homeostasis, such as the destruction, detoxification and recycling of endogenous and exogenous materials, to storage of cell-derived phospholipids and iron after erythrophagocytosis, deposition of resistant pathogens, and antigen processing in immune response and the resolution of inflammation (Vigliano et al. 2006; Davies et al. 2013; Barst et al. 2015; Steinel and Bolnick 2017; Stosik et al. 2019). They can increase in size or frequency under conditions of environmental stress and have been suggested as reliable biomarkers of water quality (Wolke 1992; Sayed and Younes 2017).

Thousands of pharmaceutically active compounds are produced commercially today. Their widespread use has generated a significant discharge of these products and their metabolites in sewage systems. These substances are now considered contaminants of emerging concern (CECs), since they have been discovered in the environment and its toxicity and/or persistence can potentially alter the metabolism of organisms (Sauvé and Desrosiers 2014). Once a pharmaceutical product is consumed and excreted, it remains in wastewater as it cannot be completely removed from effluents by wastewater treatment plants (WWTP) (Blair et al. 2013). Consequently, these pollutants can reach aquatic environments, where they can affect non-target organisms, such as fish, amphibians and invertebrates (Segura et al. 2009). Fluoxetine (FLX) is a commonly prescribed antidepressant, which has been found in the final effluents of wastewater treatment plants at average concentrations of 0.012 to 1.4 g/L (Webb 2001; Kolpin et al. 2002; Christensen et al. 2009). In previous studies in teleost fish exposed to environmental concentrations of FLX, neuroendocrine and reproductive

✉ Fabiana Laura Lo Nostro
f.lonostro@gmail.com

[1] Laboratorio de Ecotoxicología Acuática, Instituto de Biodiversidad y Biología Experimental Aplicada (IBBEA), CONICET-Universidad de Buenos Aires, Buenos Aires, Argentina

[2] Facultad de Ciencias Exactas y Naturales, Departamento de Biodiversidad y Biología Experimental Aplicada, Universidad de Buenos Aires, Buenos Aires, Argentina

alterations have been demonstrated, as well as changes in behavior, food intake and body weight (Hedgespeth et al. 2014; Weinberger and Klaper 2014; Backström and Winberg 2017), including in our studied species (Dorelle et al. 2017).

The aim of this work was to evaluate the effects induced by FLX exposure in the spleen and the HK of the cichlid fish *Cichlasoma dimerus* through histological and morphometric evaluations of MMCs as immunohistological biomarkers of toxicity.

Materials and Methods

Adult specimens of *C. dimerus* were captured in Esteros del Riachuelo, a non-polluted aquatic environment in Corrientes, Argentina (27° 25′ S, 58° 15′ W), at the onset of the reproductive season. Sexually mature fish (standard length: 11 ± 2 cm and 43 ± 8 g) were kept at 26.5 ± 1°C, pH 7.3, with a 12:12 h photoperiod in well aerated 100 L aquaria, provided with external filtration and a layer of gravel at the bottom. Fish were fed once a day with pelleted commercial food (Tetra food® sticks) and were allowed to acclimate to captivity conditions for a month prior to the start of experimentation. This freshwater cichlid fish is representative of Argentinian riverine ecosystems, and is recommended by local regulations as one of the suitable fish species for use in ecotoxicological testing (IRAM 2008).

Previous to the onset of exposure, fish were placed in individual 10 L tanks under the same physical conditions and alimentary ration, except that the layer of gravel on the bottom was removed. Animals were allowed to acclimate to the new experimental conditions for a week before the experiment was started.

The test substance, FLX hydrochloride (99.9% purity), was obtained from Saporiti, Argentina. Fish (n = 10 per treatment) were exposed for 2 weeks to 0 or 50 µg/L FLX dissolved in distilled water, under a semi-static design. The concentration was chosen based on published data on the field (Mennigen et al. 2010). Water and test solution were renewed every 48 h. FLX is a fairly stable substance, declining to approximately 3% of the initial concentration over a day test period (Meijide et al. 2018). During each water renewal, small aliquots of the stock solution were added to filtered tap water in order to obtain the desired concentrations. Stock solutions were stored in the dark at 4°C.

FLX concentration in test water was measured by reverse-phase high-performance liquid chromatography (HPLC) coupled to fluorescence detection according to Meijide et al. (2018). Water samples were taken after addition of the antidepressant (time 0 h) and after 24 h of exposure. Aquarium conditions were the same used in the experiment, including the presence of fish. Samples were treated by solid phase extraction (SPE) using C18 cartridges (Thermo Scientific, USA) and eluted with 1% acidified methanol. Eluates were injected into the HPLC employing a C6-phenyl column (100 × 4.6 mm, 3 µm particle size; Phenomenex, USA) as stationary phase and the mobile phase was prepared as follows: 28% acetonitrile / 72% water with 0.4% triethylamine, pH 4. The elution flow rate was 1 mL/min. Detector was set at fixed excitation (230 nm) and emission (310 nm) wavelengths. Quantification of FLX concentration in exposure aquaria was conducted by interpolating resulting chromatographic peak area into a calibration curve (R2 = 0.99) constructed from control water with addition of known amounts of FLX. Relative standard deviation (%RSD) was 2% (inter-day variation). Instrumental detection and quantification limits were of 10 and 100 ppb, respectively.

At the end of the experiment, fish were softly narcotized with Fish Calmer® (Jungle Hypno, USA) and euthanized by decapitation. Spleen and HK were quickly removed and fixed in Bouin's fluid for 12 h at 4°C and then preserved in 70% ethanol. Samples from the middle portion of each organ were embedded in paraffin or glycol methacrylate (Leica Historesin) and sectioned at 6 µm or 3 µm, respectively. A total of 12 slides were made for each organ, 6 for each embedding medium, containing 10 sections arranged in 2 rows. The analysis was performed on 1 every 10 sections (i.e. one section per slide) to avoid considering the same MMC twice. Sections were stained with Giemsa or Masson's trichrome. Slides were examined and subsequently photographed with a Nikon Microphot FX microscope. The number and the area of the MMCs were quantified through the use of image analysis software (IMAGE-PRO® PLUS, Media Cybernetics, Silver Springs, MD, USA). Number of MMCs and total organ area occupied by MMCs were compared between control and exposed fish using a Student's t-test. Area of MMCs was analyzed using a nested ANOVA with MMCs nested to each fish. All statistical analyses were performed using Statistica 7.0 (StatSoft Inc. 2004). Data are presented as mean ± SE and the statistical significance was set at $p < 0.05$.

The experiment was conducted in accordance to international standards on the care and use of fish in research and testing according to the guide for the care and use of laboratory animals (National Research Council 2011), as well as being in compliance with the local Ethical Committee (Protocol number 018/2014, CICUAL, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires).

Results and Discussion

The fish were exposed to an actual concentration of FLX above 90% of the nominal concentration (at time 0: 46.33 ± 3.15 µg/L—92.6% -; at 24 h: 45.45 ± 2.90

µg/L—90.9%). The antidepressant was not detected in control aquaria.

The histological examination of the HK (Fig. 1d) and spleen (Fig. 1e) of individuals treated with FLX did not differ from those of control fish (Fig. 1a, b; respectively). In both organs, MMCs are seen as typically nodular structures surrounded by a thin capsule of connective tissue, which allow them to be isolated from the dense parenchyma of hematopoietic cells in which they are immersed (Fig. 1c). In control animals, the histological study of the organs indicated a qualitative difference in the number of MMCs, observing a greater number of MMCs in the spleen compared to those in the HK. The spleen also showed a higher proportion of MMCs per surface area, due to higher number of MMCs, even though they were smaller than in HK (Fig. 2).

In the spleen of FLX-exposed fish, the average area of MMCs was significantly higher than in control animals. The proportion of the organ occupied by MMCs did not change, probably due to a non significant decrease in the total number of MMCs (Fig. 2). The HK of the exposed fish showed a significant increase in the proportion of the organ occupied by MMCs due to an increase in the total number without modifying their average area (Fig. 2).

The serotoninergic system, evolutionarily conserved throughout vertebrates (Lillesaar 2011), can act as a modulating factor in the immune response through the neurotransmitter serotonin (5HT) (Jankovic 1989). A variety of stressful conditions can cause immunosuppressive effects in

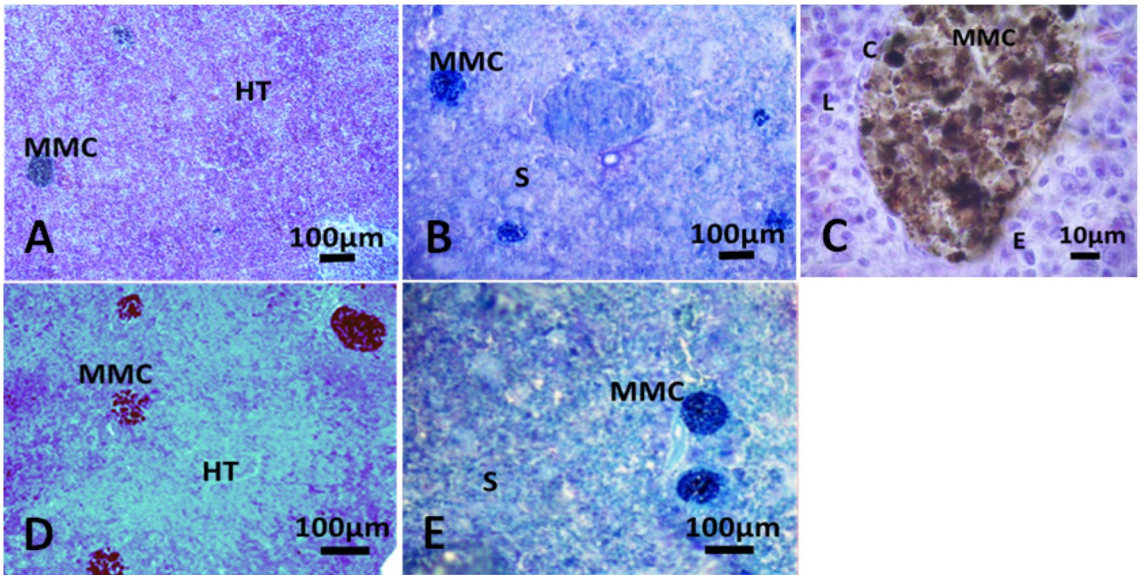

Fig. 1 MMCs in HK (**a**, **d**) and spleen (**b**, **e**) of *C. dimerus* exposed to concentrations of 0 µg/L FLX (**a**, **b**) or 50 µg/L FLX (**d**, **e**). **c** Detail of MMC in HK. Note the capsule of one layer of flat cells, well developed particularly in conspicuous MMCs: *c* capsule, *E* erythrocyte, *HT* hemopoietic tissue, *L* lymphocyte, *S* spleenic stroma. **a** and **c** Masson's trichrome stain; **b**, **d** and **e** Giemsa stain

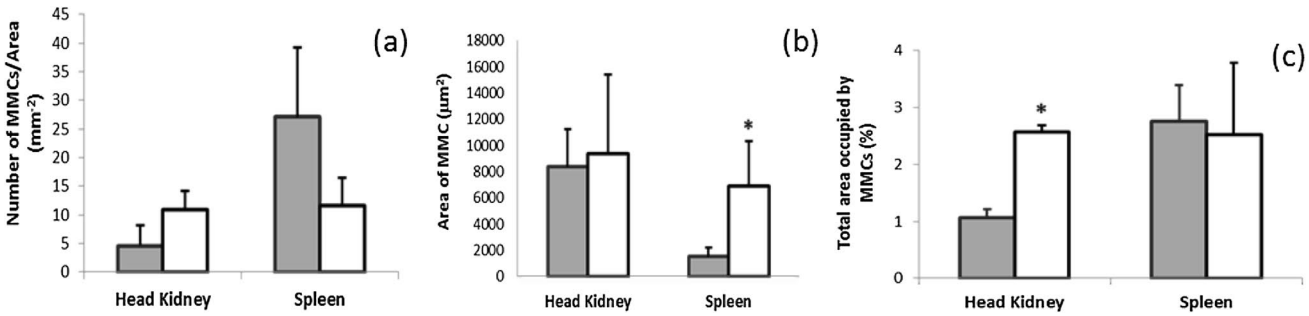

Fig. 2 Number per area (**a**), area (**b**) and total organ area occupied (**c**) by melano-macrophage centers (MMCs) in head kidney and spleen of control (grey bars) or FLX-exposed (white bars) adult *C. dimerus*. Data are expressed as mean ± SEM. Asterisks indicate significant differences between FLX and control groups ($p < 0.05$)

fish, either through glucocorticoids or through an increase in the levels of biogenic amines, particularly 5HT, which can be accompanied by suppression of the immune response (Khan and Deschaux 1997). Serotonin in fish can exert an immunomodulatory effect directly on lymphocytes by activation of specific receptors (Khan and Deschaux 1997). Given the role of 5HT and the available data, Kreke and Dietrich (2008) suggest that continuous exposure to environmental concentrations of selective serotonin reuptake inhibitors (SSRIs) may have an effect on fish immunocompetence, as exposure to SSRIs like FLX, paroxetine or sertraline caused a decrease in 5HT uptake in fish immune cells and had a negative effect on the stimulation of lymphocyte proliferation (Ferrière et al. 1999). Of these compounds, FLX (former trade name Prozac®), prescribed mainly as an antidepressant, has been widely identified as a contaminant of environmental concern (Kumar and Xagoraraki 2010).

In fish, myelopoiesis occurs in the head kidney and spleen, whereas the thymus, kidneys and spleen are the major lymphoid organs (Zapata et al. 2006). The defense system has humoral and cellular immune responses, the latter including phagocytic cells, monocites/macrophages, neutrophils, lymphocytes and natural killer cells. Macrophages, professional fish phagocytes, differentially regulate proinflammatory and homeostatic responses in vivo (Rieger et al. 2012). MMCs are thought to be functional analogues of the germinal centres present in lymph nodes of birds and mammals (Stosik et al. 2019). Macrophages within MMCs can become enlarged after active phagocytosis of heterogeneous material (cell debris, pigments, haemosiderin, etc.) (Stosik et al. 2019) resulting in larger MMCs. Number and size of macrophages in MMCs can also change in response to environmental stress and tissue degeneration (Fournie et al. 2001) leading to their use as biomarkes of exposure to pollutants. In FLX-treated fish MMCs in the spleen had a bigger size than controls, without changes in the total organ area occupied by MMCs, suggesting increased aggregation of smaller centers and leading to the non-statistical decrease in number observed. The response to waterborne FLX in HK differed, as there was an increase in the total area occupied by MMCs, likely due to an increase in number, since area of individual centers did not change. An increase in number of MMCs is likely due to an increase in phagocytic/chemotactic activities of macrophages, the result of tissue toxicity by FLX in this organ, and thus increased phagocytosis of injured cells. In addition to their role in pathogen removal and immune response, MMCs may also function neutralizing free radicals in tissues that undergo oxidative damage (Hapaaranta et al. 1996). Many studies have reported accumulation of MMCs in fish exposed to toxic chemicals (Dezfuli et al. 2006; Giari et al. 2008; Taheri et al. 2015). For instance, MMCs accumulated in higher numbers in spleen of *Oreochromis niloticus* as exposure to copper continued (Osman et al. 2009). In *Channa punctatus* exposed to fossil fuels, the number of MMCs in head kidney increased likely due to metabolic disorders (Kakkar 2011).

In the present study, the differential deposition rate of MMCs in hemolymphopoietic organs of *C. dimerus* would be a response to the stress generated by FLX in the aquatic environment. The response varies according to the organ considered and could modify homeostatic functions such as the elimination of cellular debris, iron processing resulting from erythrophagocytosis and the central role in the immune response. Analysis of MMCs can be used as a non-specific biomarker to provide information regarding the effects of toxicants on the immune response of fish.

Acknowledgments The financial support was provided by grants from Universidad de Buenos Aires (UBACyT 0672, Lo Nostro) and Agencia Nacional de Promoción Científica y Tecnologica (PICT2016-0432, Lo Nostro).

References

Agius C, Roberts RJ (2003) Melano-macrophage centres and their role in fish pathology. J Fish Dis 26:499–509

Barst BD, Bridges K, Korbas M, Roberts AP, Van Kirk K, Mcneel K, Drevnick PE (2015) The role of melano-macrophage aggregates in the storage of mercury and other metals: an example from yelloweye rockfish (*Sebastes ruberrimus*). Environ Toxicol Chem 34:8: 1918–1925

Backström T, Winberg S (2017) Serotonin coordinates responses to social stress—What we can learn from fish. Front Neurosci 11:595. https://doi.org/10.3389/fnins.2017.00595

Blair BD, Crago JP, Hedman CJ, Klaper RD (2013) Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern. Chemosphere 93:2116–2123

Christensen AM, Markussen B, Baun A, Halling-Sørensen B (2009) Probabilistic environmental risk characterization of pharmaceuticals in sewage treatmentplant discharges. Chemosphere 77:351–358

Davies LC, Jenkins SJ, Allen JE, Taylor PR (2013) Tissue-resident macrophages. Nat Immunol 14:986–995

Dezfuli BS, Simoni E, Giari L, Manera M (2006) Effects of experimental terbutylazine exposure on the cells of *Dicentrarchus labrax* (L.). Chemosphere 64:1684–1694

Dorelle LS, Da Cuña RH, Rey Vázquez G, Hocht C, Shimizu A, Genovese G, Lo Nostro FL (2017) The SSRI fluoxetine exhibits mild effects on the reproductive axis in the cichlid fish *Cichlasoma dimerus* (Teleostei, Cichliformes). Chemosphere 171:370–378

Ferrière F, Khan NA, Meyniel JP, Deschaux P (1999) Characterization of serotonin transport mechanisms in rainbow trout peripheral blood lymphocytes: role in PHA-induced lymphoproliferation. Dev Comp Immunol 23:37

Fournie JW, Summers JK, Courtney LA, Engle VD, Blazer VS (2001) Utility of splenic macrophage aggregates as an indicator of fish exposure to degraded environments. J Aquat Anim Health 13:105–116

Giari L, Simoni E, Manera M, Dezfuli BS (2008) Histocytological responses of *Dicentrarchus labrax* (L.) following mercury exposure. Ecotoxicol Environ Saf 70:400–410

Hapaaranta A, Valtonen ET, Hoffmann R, Holmes J (1996) Do macrophage centers in freshwater fishes reflect the differences in water quality. Aquat Toxicol 34:253–272

Hedgespeth ML, Nilsson PA, Berglund O (2014) Ecological implications of altered fish foraging after exposure to anantidepressant pharmaceutical. Aquat Toxicol 151:84–87

IRAM (2008) Calidad Ambiental, Calidad Del Agua. Determinación De La Toxicidad Letal Aguda De Sustancias En Peces De Agua Dulce. Método Semiestático IRAM 29112:2008

Jankovic BD (1989) Neuroimmunomodulation: facts and dilemmas. Immunol Lett 21:101

Kakkar KG, Saxena RM, Rathee NS, Joshi M (2011) Water soluble fraction of diesel fuel induced histopathological alterations in the liver of *Channa punctatus* Toxicol Int 18:14–16

Khan N, Deschaux P (1997) Role of serotonin in fish immunomodulation. J Exp Biol 200:1833–1838

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT (2002) Pharmaceuticals, hormones, and other organic waste water contaminants in streams, 1999–2000: a national reconnaissance. Environ Sci Technol 36:1202–1211

Kreke N, Dietrich DR (2008) Physiological endpoints for potential SSRI interactions in fish. Crit Rev Toxicol 37:215–24

Kumar A, Xagoraraki I (2010) Pharmaceuticals, personal care products and endocrine-disrupting chemicals in U.S. surface and finished drinking waters: a proposed ranking system. Sci Total Environ 408:5972–5989. https://doi.org/10.1016/j.scitotenv.2010.08.048

Lillesaar C (2011) The serotonergic system in fish. J Chem Neuroanat 41:294–308

Macchi GJ. Romanol A, Christiansen HE (1992) Melano-macrophage centres in white mouth croaker *Micropogonias furneri*, as biological indicators of environmental changes. J Fish Biol 40:971–973

Meijide FJ, Da Cuña RH, Prieto JP, Dorelle LS, Babay PA, Lo Nostro FL (2018) Effects of waterborne exposure to the antidepressant fluoxetine on swimming, shoaling and anxiety behaviours of the mosquitofish *Gambusia holbrooki*. Ecotoxicol Environ Saf 163:646–655

Mennigen JA, Lado WE, Zamora JM, Duarte-Guterman P, Langlois VS, Metcalfe CD, Chang JP, Moon TW, Trudeau VL (2010) Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish, *Carassius auratus*. Aquat Toxicol 100:354–364

National Research Council (US) (2011) Guide for the care and use of laboratory animals. Eighth ed. Washington, National Academies Press (US).

Osman MM, EL-Fiky SA, Soheir YM, Abeer AI (2009) Impact of water pollution on histopathological and electrophoretic characters of *Oreochromis niloticus* fish. Res J Environ Toxicol 3:9–23

Rieger AM, Konowalchuk JD, Grayfer L, Katzenback BA, Havixbeck JJ, Kiemele MD, Belosevic M, Barreda D (2012) Fish and mammalian phagocytes differentially regulate pro-inflammatory and homeostatic responses in vivo. PLoS ONE 7:e47070. https://doi.org/10.1371/journal.pone.0047070

Sauvé S, Desrosiers M (2014) A review of what is an emerging contaminant. Chem Cent J 8:15

Sayed AH, Younes HAM (2017) Melanomacrophage centers in *Clarias gariepinus* as an immunological biomarker for toxicity of silver nanoparticles. J Microsc Ultrastruct 5:97–104

Segura PA, Francois M, Gagnon C, Sauve S (2009) Review of the occurrence of anti-infectives in contaminated wastewaters and natural and drinking waters. Environ Health Perspect 117:675–684

Steinel NC, Bolnick DI (2017) Melanomacrophage centers as a histological indicator of immune function in fish and other poikilotherms. Front Immunol 8:827. https://doi.org/10.3389/fimmu.2017.00827

Stosik MP, Tokarz-Deptuła B, Deptuła W (2019) Melanomacrophages and melanomacrophage centres in Osteichthyes. Cent Eur J Immunol 44:201–205

Taheri R, Salamat N, Movahedinia A (2015) Evaluation of lymphoid tissue structure in Sole (*Euryglossa orientalis*) and Yellowfin Seabream (*Acanthopagus latus*) affected by environmental contaminants in the Persian Gulf. J Immunotoxicol 13:301–313

Webb SF (2001) A data-based perspective on the environmental risk assessment of human pharmaceuticals I-collection of available ecotoxicity data. In: Kümmerer K (ed) Pharmaceuticals in the environment—sources, fate, effects and risks. Springer, Berlin, pp 175–201

Weinberger J, Klaper R (2014) Environmental concentrations of the selective serotonin reuptakeinhibitor fluoxetine impact specific behaviors involved inreproduction, feeding and predator avoidance in the fish *Pimephales promelas* (fathead minnow). Aquat Toxicol 151:77–83

Vigliano FA, Bermúdez R, Quiroga MI, Nieto JM (2006) Evidence for melano-macrophage centres of teleost as evolutionary precursors of germinal centres of higher vertebrates: an immunohistochemical study. Fish Shellfish Immunol 21:467–471

Wolke R (1992) Piscine macrophage aggregates: a review. Annu Rev Fish Dis 2:91–108

Zapata A, Diez B, Cejalvo T, Gutierrez de Frias C, Cortes A (2006) Ontogeny of the immune system of fish. Fish Shellfish Immunol 20:126–136