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Original Article

Rodlet cells changes in *Oreochromis niloticus* in response to organophosphate pesticide and their relevance as stress biomarker in teleost fishes

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Abstract: Rodlet cells are frequently found in teleost fishes and although their role in organisms is not completely understood. The occurrence of these cells are related to stress and may undergo changes in contaminated environments, thereby allowing their use as biomarkers. This hypothesis is tested in the present study. Thirty specimens of *Oreochromis niloticus* were divided into three groups, two groups were exposed to organophosphate pesticide methyl parathion at nominal concentrations of 4 mgl^{-1} and 8 mgl^{-1} and one group was kept as control. After ten days, the gills were removed for microscopic study and the number and area of the rodlet cells were analyzed and compared with a well-established method of assessing histological damages in fishes. No significant differences were found in the area of the cells, but there were significant differences in the number of rodlet cells among examined concentrations. The present study provides evidence for the use of this new biomarker in teleost fishes and discusses some of the potential confounding factors of this approach.

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Introduction

The Nile tilapia (*Oreochromis niloticus* Linnaeus 1758) is a cichlid fish of great economic interest in pisciculture and aquaculture (Borges et al., 2013). The species is originated from Africa and is widely distributed in water bodies of many tropical countries because of its resistance to different trophic levels (Agnèse et al., 1997; Borges et al., 2013). The Nile tilapia was reported to have a remarkable cell type called rodlet cell (RC) (Borges et al., 2013) that have also been found within the epithelia of 36 teleost fish families (Fishelson et al., 2011) from marine to freshwater habitats. The RCs are easy to distinguish when mature because of their elongated form, dense microfibrils capsule, basal nucleus and cytoplasmic corpuscles in the shape of rodlets or arrows that point to the apical area of the cell where they are discharged (Bielek, 2005; Schmachtenberg, 2007; DePasquale, 2014).

For over 120 years, scientists have attempted to discover the origin and function of the enigmatic RC. Thélohan first described these cells in 1892 and believing he had found a parasite the author named it *Rhabdospora thelohani* (Manera and Dezfuli, 2004). Plehn (1906) considered the RC to be defense cells with granules of endogenous action, thereby refuting Thélohans' hypothesis. Both assumptions have continued to coexist with different interpretations about the function and origin of these cells until the last decade (Schmachtenberg, 2007). Currently it is mainly accepted that the RC are endogenous cells involved in defense (Sfacteria et al., 2014).

The RC presence is often associated with other elements of the fish innate immune system such as mast cells, eosinophils and neutrophils (Bielek 2005). The rodlets are usually discharged at the external face of the epidermal layer and although

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their composition material is still unknown there are evidences that the antibiotic peptide piscidin and DNA reside in the rodlets (Barber and Mills Westermann, 1986; Silphaduang et al., 2006). Thereby different works have suggested that the RC comprise part of the host inflammatory defense response (Bielek, 2005; Matisz et al., 2010; DePasquale, 2014).

Regardless of its function, there are strong evidences for the potential use of the RC as biomarkers of exposure and effect as first observed by Manera and Dezfuli (2004). Biomarkers are bodily fluids, cells, tissues and physiological or behavioral changes that indicate the presence of contaminants in exposed organisms (Livingstone, 1993). Biomarkers of exposure can be used to confirm and evaluate the exposure of an individual or group to a particular substance, thereby establishing a link between external exposure and the measurement of internal exposure. Biomarkers of effect can be used to document pre-clinical changes or adverse health effects from the exposure to and absorption of chemicals (Amorim, 2003; Rüdiger, 1999; World Health Organization, 1993). Thus, the RC may be potently important for monitoring environmental quality and the health of organisms that live in polluted or stressed environments.

Although there are many studies describing the occurrence of the RC in the presence of a variety of external stressors (Dezfuli et al., 2003a; Manera and Dezfuli, 2004; Giari et al., 2008; Poltronieri et al., 2009; Matisz, 2010; Rebok et al., 2010). There is no previous work standardizing exactly how this information can be used for biomarkers proposes. Rebok et al. (2010) attempted to address this issue analyzing the liver of *Barbus peloponnesius* specimens from the River Bregalnica in the Republic of Macedonia, but it is not clear if the place they have used as reference for comparisons was indeed free of stressors. As stated by the authors “The microscopy data, however, is only partially indicative of stress effects at site A” which is the supposed polluted environment. Herein we use the pesticide methyl parathion as an environmental stressor in increasing concentrations to analyze the fish response in number and area of RC. The methyl parathion is an organophosphate pesticide that after degradation produces the compound methyl paraoxon through metabolic conversion, which is even more toxic (Araujo et al., 2006; Machado, 1999) inhibiting enzymes such as cholinesterase, carboxylases, mitochondrial oxidative phosphorylases, and acetylcholinesterase (Machado, 1999). Nevertheless, this compound is widely used in agriculture and aquaculture and also illegally as indoor pesticide (Ruckart et al., 2004). Thus to comprehend its effects in the environment is essential. Herein we evaluate the toxic effect of the methyl parathion in the gill epithelium of fishes exposed to different concentrations and compare this response to changes in size and quantity of the RC in the tissue of this organ.

Materials and Methods

Fish maintenance and experimental design: Thirty juvenile specimens of the Nile tilapia (with mean standard length of 8.79±0.82 cm, and mean weight of 17.9±4.69 g) were obtained from a local supplier. Fishes were fasted for five days prior to and during the trial to avoid interference due to the permanence of food in the gut and water (Lenon and Walker, 1964). The specimens were randomly divided into three groups housed in three 16-liter aquaria for an additional five days period of acclimation. Two groups were then exposed to nominal concentrations of 8 mgl-1 and 4 mgl-1 of methyl parathion [O,O-dimethyl-O-(-4-nitrophenyl) phosphorothioate, C8H10NO5PS] (Folisuper 600 BR ® methyl parathion, 600 g/L, Agripec) and one group was kept as control in non-contaminated water. The pesticide concentrations have being chosen based in two criteria, first in the LC_{50} dose for juveniles of *Piaractus mesopotamicus* (9.89 mgl^{-1}; Machado, 1999) and second on small-scale experimental trail (unpublished data) designed to promote toxic effects without causing death within 5 days. Throughout the periods of acclimation and exposure physiochemical parameters [nitrite, ammonia and pH (Tetratest ®)]

were monitored weekly and water temperature was maintained between 20-26(±0.5)°C using thermostats connected to heaters (40 w). Cooling was provided by air conditioning. There were neither control of photoperiod nor maintenance of the pesticide after its initial dilution. After 10 days exposure, the animals were anesthetized (immersed in ethyl benzoate at 50 mgl^{-1}) and euthanized with the disruption of the spinal cord proximal end (Silva et al., 2005). The gills then were removed for histological analyses. All procedures involving animals were in accordance with the ethical standards of the Animal Care Committee (019/09 CEP/ICS/UNIP).

Tissue processing for optical and electron microscopy: For optical microscopy, the gills were fixed in cold 2% glutaraldehyde solution buffered pH 7.2 with 0.1 M phosphate (McDowell and Trump, 1976) for 12 hrs. Two middle gill arches (one on the left and one on the right) were selected for the preparation of the histological slides. After removal of the fixation solution, the gills filaments were submitted to dehydration in increasing alcohol and Historesin (glycol methacrylate) solutions until their complete inclusion in Historesin. The material was then embedded (resin + hardener) and placed to dry at 40ºC. Histological sections 1 to 3 μm in thickness were obtained using a microtome and stained with Fuchsine and Toluidine blue. The slides were then photographed and analyzed under a light microscope (Zeiss standard 25 ICS). Samples from each group have also been processed for electron microscopy. For this analysis the tissue was fixed at 0ºC in 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.2), postfixed in 1.0% OsO_4 (Hayat, 1981) and embedded in Spurr resin (Spurr kit for electron microscopy, Sigma Chemical Co., USA). Ultra-thin sections (70 nm) were placed on copper grids and stained with 2% uranyl acetate in distilled water for 1 h, then washed in distilled water and stained with 0.5% lead citrate in distilled water. The ultrastructure was examined using a Jeol 100 CX-II electron microscope.

Gill analysis

Qualitative analysis: The method established by Poleksic and Mitrovic-Tutundzic (1994) was used to evaluate tissue damage as a control for the effects of the pesticide. This method is a rating system based on changes that are frequently encountered in injured tissue and that gives an *I* number categorized in one of four groups. An *I* value between 0 to 10 denotes a functionally normal sample; 11 to 20 denotes mild to moderate alterations; 21 to 50 denotes moderate to severe alterations, and over 100 denotes severe alterations. In this method, each alteration reported in the tissue accounts for the overall score in a different way according to its severity, thus alterations from first stage include changes such as hypertrophy and hyperplasia while the third and last stages accounts for necrosis and fibrosis of the tissue. Thus *I* values under 100 indicate that a greater number of reversible changes are present in the tissue but an *I* score higher than 100 evidences irreparable tissue damage. The observation of the sections was standardized in a way that the images could not be repeated. Twenty-five fields (1000x magnification) in alterative sections when necessary were randomly chosen for analysis. However, when histological artifacts prevented the proper interpretation of the morphological features, the field was replaced by another. Images observed in the electron microscope were used to improve the visualization of the RC and the histological alterations but not for counting them.

Quantification and measurement of rodlet cells: The same criteria for field observation used in the *I* value analyses were adopted for the RC tests in optical microscopy (25 random fields per fish - 1000x magnification). The fields were always randomly selected and the total number of RC in the secondary lamella of each selected area was manually counted. To measure the area of RC alterative sections in different concentrations were checked to select 10 cells in each tested group (including control). These cells were then photographed and analyzed using the Image J® program, which enabled the longitudinal measurement of the cell area. Only clearly characterized RC were considered, i.e., cells

showing a given trait such as peripheral nucleus, thick cell-wall and presence of the cytoplasmatic rodlets (Bielek, 2005).

Data analysis: All the statistic tests were performed in the R package software version 3.1.1 (R Development Core Team, 2008). Data were first checked for their normality with the Shapiro-Wilk normality test. When normal distribution was observed, the comparative analyses were performed with two-way ANOVA. For non-normal data the Kruskal-Wallis non-parametric test was employed with post-hoc comparison made using Mann-Whitney test with Bonferroni correction. The confidence intervals were calculated with 2000 bootstrap replicates.

The results obtained with the *I* value of Poleksic and Mitrovic-Tutundzic (1994) have being used to identify the occurrence and intensity of damage to the gill epithelium. Once the injuries were detected the RC number and area have been compared with the damage verified to evaluate their efficiency as biomarkers.

Results

Qualitative analysis: The dose-response curve of the mean *I* values obtained in all nominal concentrations follows in Figure 1. The results clearly indicate that the control fish were in healthy condition (mean *I* value: 6.8±9.6), whereas nominal concentrations of 4 and 8 mgl^{-1} caused irreparable damage to the gills (mean *I* value: 115.3±42.3 and 125.4±10.9, respectively). According to the Shapiro-Wilk the data is non-normal (*P*=0.001) with significant differences among the groups (Kruskal-Wallis *P*=0.0002).

Poleksic and Mitrovic-Tutundzic (1994) *I* value is based on the observation of a series of histologic information in the gill epithelium altered by contaminants. It indicates morphological changes such as hypertrophy and hyperplasia of the structural cells and the proliferation of mucous and chloride cells. These mechanisms may form a barrier between the irritating agent and the blood stream but will also hinder the gas exchange (Mallat, 1985; Perry, 1997).

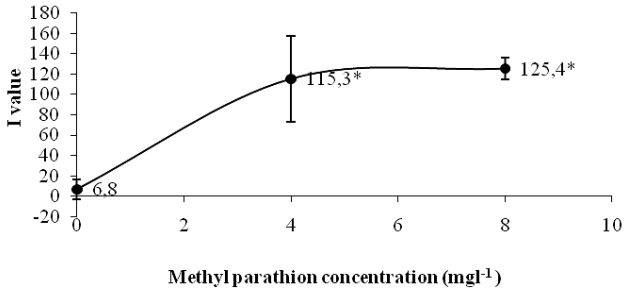

Figure 1. *I* value observed in each tested concentrations (mgl^{-1}). Numbers refer to the mean observed in control (0 mgl^{-1}), 4 mgl^{-1} and 8 mgl^{-1}. * $P<0.05$ for $\alpha = 95\%$.

Some examples of the histological alterations commonly observed in the samples exposed to the pesticide methyl parathion are displayed in Figure 2.

Quantification and measurement of rodlet cells: Figure 3 displays some of the RC observed at different concentrations. As indicated in the figure, RCs were found in all groups at different regions and stages of development. They were especially difficult to find in the gills of the control group and had been found usually close to blood vessels in fish, although in some cases, few cells were observed in the edge of the secondary lamella discharging the rodlets in the exterior (Fig. 3d). However, this behavior were more frequent in the exposed fish in which most of the RCs were found in the base of the secondary lamella.

Central tendency measures from the RC analyses are summarized in Table 1. The area of the RC follows a normal distribution but does not present any statistical significance difference among the groups, however the amount of RC shows a different pattern. The number of cells presented in the secondary lamella of each group is a non-normal dataset with a significant statistical difference (*P*=0.021) indicating that the amount of RC changes in the presence of the contaminant. Post-hoc analyses have shown that the result is due to the difference between the control and the intermediated concentration (4 mgl^{-1} *P*=0.019* and 95% confidence interval of 0.500-6.996). But no significance is found when comparing the 8 mgl^{-1} group (*P*=0.368 for control x 8 mgl^{-1}; *P*=0.697 for 4 mgl^{-1} x 8 mgl^{-1}, $\alpha = 95\%$).

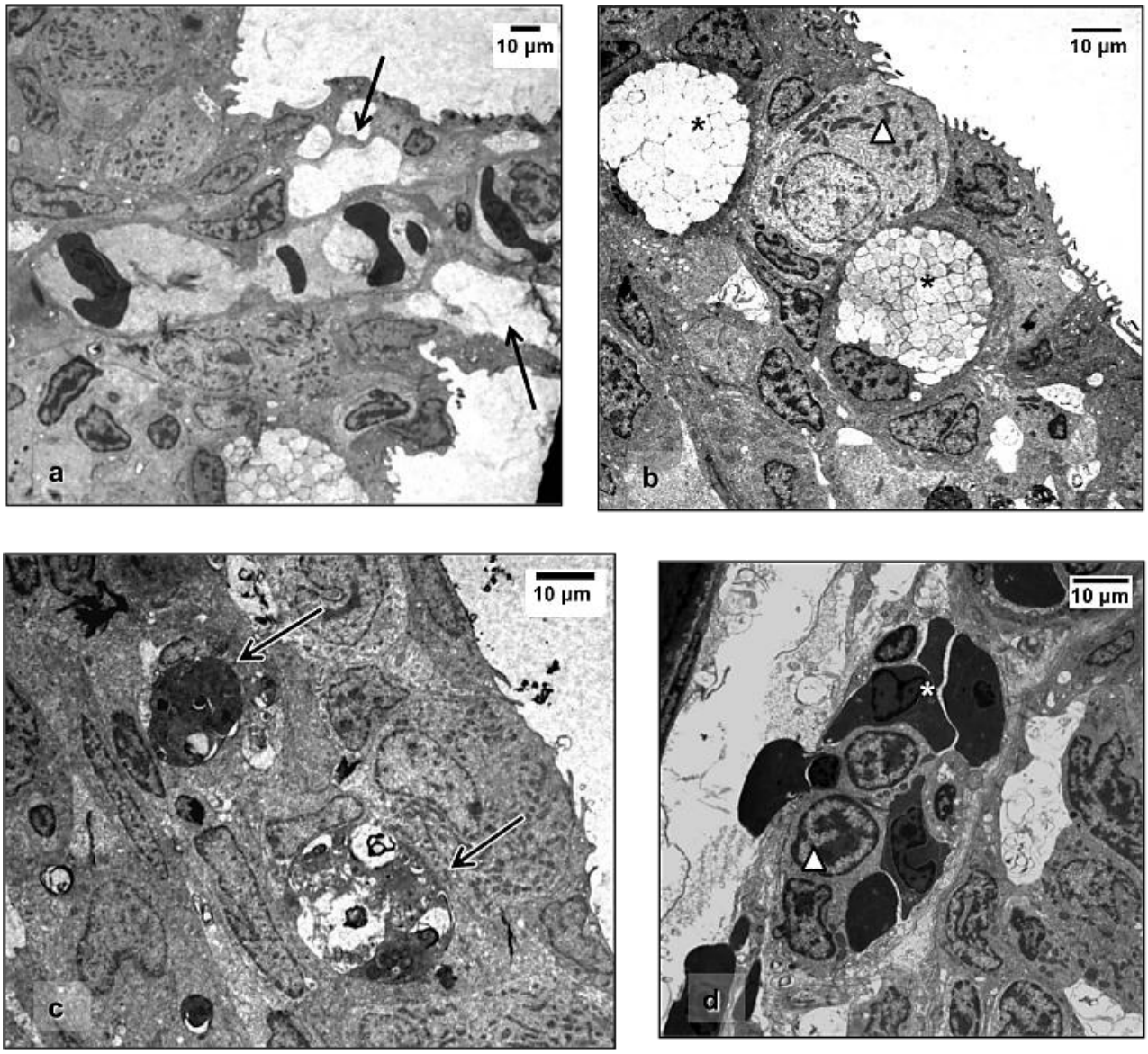

Figure 2. Histological alterations commonly observed in gills during transmission electron microcopy; (a) Necrosis in the tissue, indicated by the arrows (8 mgl^{-1}, 1000X), (b) (Δ) chloride cell (*) mucus cell (8 mgl^{-1}, 3000X), (c) Apoptosis in the cells indicated by the arrows (8 mgl^{-1}, 2500X), and (d) (Δ) monocytes and (*) erythrocytes in blood vessel (8 mgl^{-1}, 3000X).

Table 1. Central tendency measures summarizing the RC data observed in all groups. Area of the RC are in mm^2 and the significant value is featured in bold. N = 10 fishes in each studied concentration.

	RC count			RC area		
	Control	**4 mgl^{-1}**	**8 mgl^{-1}**	**Control**	**4 mgl^{-1}**	**8 mgl^{-1}**
Median	0.00	**2.00***	0.00	1 587.36	2 085.65	1 388.06
Mean	0.10 ± 0.32	**4.30*** ± 6.80	3.00 ± 7.48	1 509.28 ± 503.50	1 986.23 ± 680.20	1 535.10 ± 450.57

***** P=0.019 <0.05 for α=95%.

Discussion

In the present study, the mean *I* values found in fish exposed to both nominal concentrations of the pesticide (4 and 8 mgl^{-1}) indicate irreparable damage in the gills. The tested concentrations are close to the ones reported for regular use (Machado, 1999) but

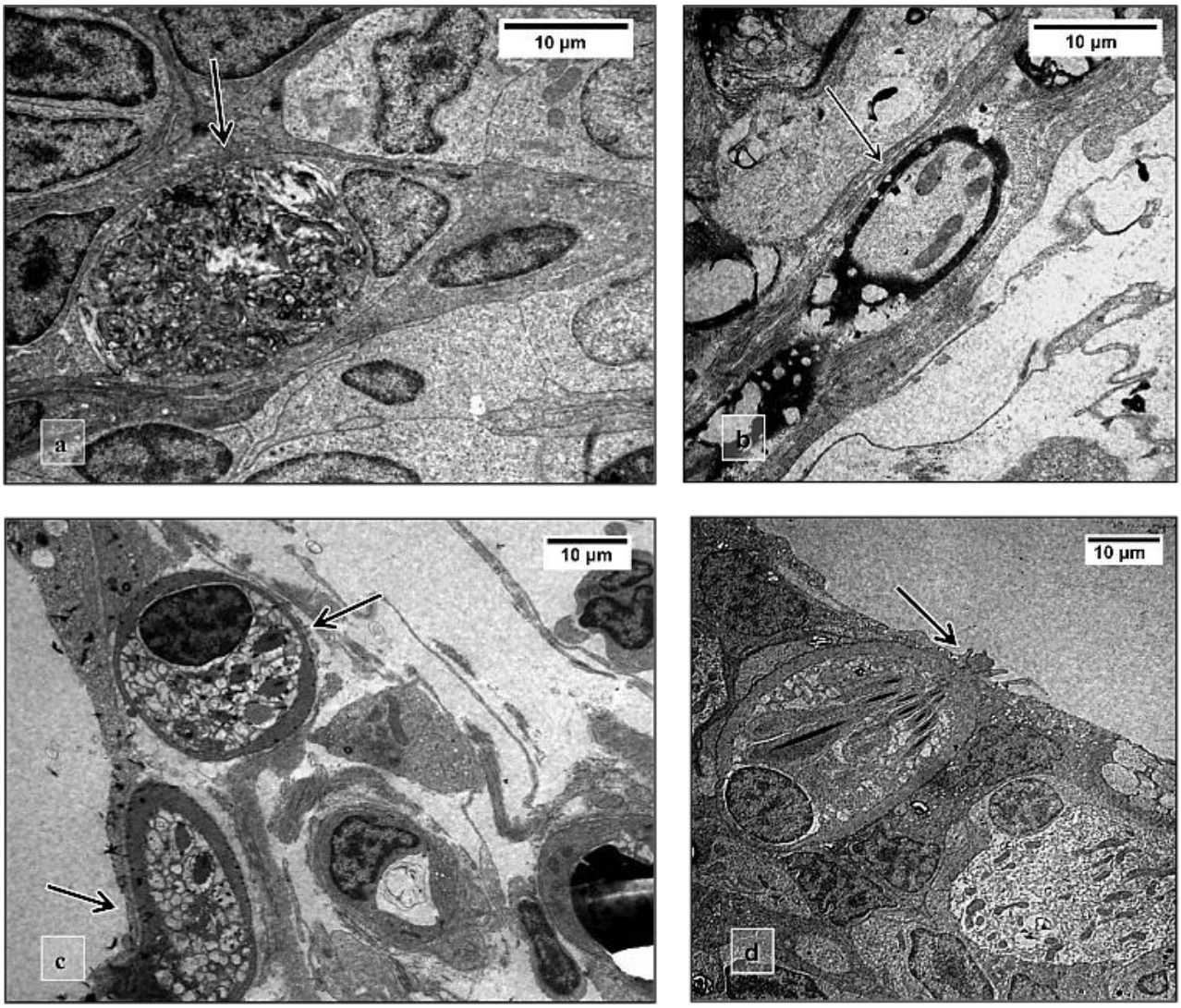

Figure 3. Rodlet cells observed in *O. niloticus* gills in transmission electron microscopy. Indicated by the arrows: (a) Immature rodlet cell (control group, 6000X), (b) Rodlet cell degenerating (8 mgl^{-1}, 7500X), (c) Mature rodlet cells (4 mgl^{-1}, 4000X), and (d) Rodlet cell degranulating rodlets in the exterior (control group, 5000X).

nevertheless the results demonstrate that even at sub-lethal doses the organophosphate can harm fishes severely compromising the pulmonary function of the epithelium.

When considering the RC data no significant difference was found among groups according to the area of the cells. It indicates that this aspect should not be used to measure the contamination of an environment by methyl parathion. However, a significant difference was found in the amount of RC. Although this was not directly correlated to the intensity of the contamination, since the highest number of RC occurred in the intermediate concentration of 4 mgl^{-1}. This result may be explained by a greater number of irreparable alterations (necrosis and fibrosis) at the higher concentration, indicating that increasing the dosage of the organophosphate agent might compromise the integrity of the RC. This hypothesis is supported by the microscopy analysis, where degenerated RC and other cell types have been observed at 8 mgl^{-1}. Previously the degradation of the RC have also been documented in a study where fishes were exposed to an herbicide (Dezfuli et al., 2003b) thus it is known that very toxic compounds may harm these cells.

In natural contaminated environments such as lakes related to urban areas, the correlation of the number of RC and the degree of contamination is more

straightforward. Borges et al. (2013) reported a correlation value of 0.98 between the number of RCs found in Nile tilapias and the trophic state index of the studied water bodies. Nevertheless in the cited work none of the *I* index found for the gills were over 100 corroborating the hypothesis that in the higher concentration of the pesticide the RCs were too compromised to be clearly identified and consequently indicate the toxic effect of the compound. That is because the capability to distinguish the RC in the tissue will vary according to the stage of development of these cells. Opposed to their characteristic oval shape and rodlets, the RCs in the very early stage of development or when degenerating are not easy to distinguish from other cell types and are probably under recorded in literature (Laurà et al., 2012).

Based on the results, it is possible to observe some of the changes in the RCs morphology due to increasing exposured concentration. In Figure 3d, the image shows the mature and easy to distinguish RC, while in Figure 3a and 3b follow the immature and degenerated RC, respectively. Because the methods used to identify the RC only accounted for mature cells and degenerated cells have been observed in the highest exposure concentration due to the pesticide; it is reasonable to assume that the number of RCs were under estimated in the higher concentration. Other variables to keep in mind when using the RCs as biomarkers are the species, used tissue and temporal factor. Fishelson et al. (2011) encountered RCs that differed strongly from other fish in lizardfishes not only regarding its structure but also about their location. In turn, Matisz et al. (2010) noticed a temporal change in the occurrence of the RCs in the optic lobe of *Pimephales promelas* exposed to trematode cercariae. They have shown that the RC densities increased at 4 and 6 weeks after the infection followed by a decline in the ninth week. Nevertheless in the present study and in many other cases in literatures, there are evidences of numerical changes in the amount of RC due to the influence of different stressors. Such as parasites, herbicides, heavy metals, viral infections, tissue damage, differences in water depth and overcrowding (Bielek, 2005; Manera and Dezfuli, 2004; Dezfuli et al., 2006; Giari et al., 2008; Mazon et al., 2007; Poltronieri et al., 2009). According to Schultz et al. (2014), even unknown disturbing agent(s) in groundwater causes an increase in the number of RC in Australian Murrays. Thus, even considering all the potentially confounding factors discussed before, the evidences indicating the use of RCs as biomarkers for most of the teleost fishes are overwhelming.

Therefore, the use of the RCs in the tissue as a biomarker is possible but when using this approach it is important to keep in mind the confounders, such as: tissue/ species used in the analyses (are RCs described in this tissue/ species); degree of contamination (is this environment/ compound too toxic for the RC) and time (is the temporal lap since contamination enough for the appearance and maturation of the RC). When these variables are considered during experimental design and interpretation of the results the amount of RCs in the tissue may be a very simple and fast way of detecting contamination.

Furthermore, although the exact function of the RCs has been debated, the current view is that they are involved in host non-specific immune response as discussed in Sfacteria et al. (2014). This idea are supported by many studies connecting the RCs appearance to a number of stressors Laurà et al. (2012) and in association to other immune system cells (Kramer et al., 2005; Reite and Evensen, 2006). Laurà et al. (2012) have registered in ultrastructural microscopy, the development of the RC from immature to complete mature stage in the intestinal epithelium of the sea bass (*Dicentrarchus labrax*) and concluded that “the RCs may be considered to be a normal component of teleost tissues, probably with a secretory function” and also that it has a probable defensive role associated with secretory activity.

Thus, despite there are some important open questions about the RC such as: What is exactly the composition of the rodlets? What is the evolutionary origin of these cells? What are their exactly importance for the host response in teleost fishes?

This is the first time that their use as biomarkers has been tested in a standardized way in order to discuss the variables and confounding factors of this approach. The results described here allied to previous workers allow the conclusion that the number of RCs in tissues exposed to stressors (such as the gills for polluted water) is indeed a good biomarker of effect. But in order to avoid misinterpretation of the results, it is recommended to use species and tissues where the occurrence of the RC were well-documented and only when the tissue is not necrotic. We also indicate the use of the RC number altogether with other histological metrics if a more detailed analysis of the environment is needed, especially in highly contaminated habitats.

Conclusions

We conclude that sublethal doses of the organophosphate methyl parathion can be harmful to the maintenance of animal life and that the number of RC is an efficient method to identify the stressor presence. This is the first time that these cells are systematically tested as biomarker and the evidences found in this work aligned to previous results indicate the usefulness of the RC as a biomarker of effect. However taking into account the higher amount of RC in the intermediate concentration and their temporal pattern of appearance (Matisz et al., 2010), this technique should be used carefully and the possible confounding factors must be taken into consideration during experimental design. The count of RCs in the exposed tissues is a simple and fast way to identify contamination but in order to establish a more precise panorama regarding the intensity of the environmental contamination this parameter must be analyzed with other aspects of tissue alteration, such as those described by Poleksic and Mitrovic-Tutundzic (1994).

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Declaration of Interest

The first author has received a fellowship from (CNPq/PIBIC process 116069/2008-2 and 117961/2009-4). The second author is employed at the Universidade Paulista (UNIP). The authors declare that they have no conflict of interest.

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چکیده فارسی

تغییرات سلول‌های rodlet در ماهی *Oreochromis niloticus* در پاسخ به سم ماهی ارگانوفسفات و رابطه آن به عنوان شاخص‌زیستی در ماهیان استخوانی

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چکیده:

سلول‌های rodlet به‌طور معمول در ماهیان استخوانی یافت می‌شوند. اگر چه نقش آنها در موجودات هنوز به‌طور کامل مشخص نمی‌باشد، بروز این سلول‌ها به‌استرس مربوط می‌باشد و ممکن است در محیط‌های آلوده تغییر کنند، از این‌رو می‌توانند به‌عنوان شاخص زیستی استفاده شوند. این فرضیه در مطالعه حاضر مورد آزمایش قرار گرفت. تعداد ۳۰ قطعه ماهی *Oreochromis niloticus* به سه گروه تقسیم شدند. دو گروه در معرض سم ماهی ارگانوفسفات دمتیل پاراتیون در غلظت‌های ۴ و ۸ میلی‌گرم در لیتر قرار گرفتند و یک گروه نیز به‌عنوان شاهد نگه داشته شدند. بعد از ده روز آبشش ماهیان برای مطالعه میکروسکوپی برداشته شدند و تعداد و مساحت سلول‌های rodlet آنالیز شدند و با یک روش ایجاد شده برای ارزیابی آسیب‌های بافتی در ماهیان مورد مقایسه قرار گرفتند. براساس نتایج هیچ تفاوت معنی‌داری در مساحت سلول‌ها مشاهده نشد، اما تفاوت معنی‌داری در تعداد سلولهای rodlet در بین غلظت‌های مورد آزمایش یافت شد. مطالعه حاضر شواهدی را برای استفاده از این شاخص‌زیستی جدید فراهم می‌کند و برخی از پتانسیل‌های فاکتورهای این روش را بحث می‌کند.

کلمات کلیدی: ارگان فسفات، شاخص‌زیستی، تیلاپیا نیل، متیل پاراتیون.