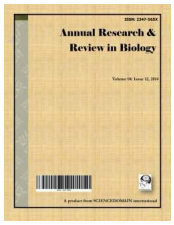

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Analysis of Tissue Alterations and Quantitative Histopathological Indices in *Rhamdia quelen* (Quoy & Gaimard, 1824) and *Metynnis maculatus* (Kner, 1858) During Treatment of Ichthyophthiriasis

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TOZ and AO designed the study, wrote the protocol and interpreted the data. Authors AH and GGCW managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The present study aimed to describe the histological alterations observed in gills of *Rhamdia quelen* and *Metynnis maculatus* during treatment of white spot disease and to compare three quantitative indices of gill alterations originally developed by other authors for this type of evaluation.
Study Design: Animals were collected and analysed on days zero, 10, 20, 30, 40 and 100 in relation to the beginning of treatment of ichthyophthiriasis.

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Place and Duration of Study: Integrated Group for Aquaculture and Environmental Studies, Department of Animal Sciences, Federal University of Paraná, between January and April 2014.
Methodology: For the analyses, animals presenting clinical signs of a ciliate *Ichthyophthirius multifiliis* infestation were maintained in a laboratory. Immediately after disease identification, the fish were medicated and monitored for the occurrence and evolution of gill histological alterations. For histopathology, 15 sick fishes (presence of white spot) of each species were collected from each tank on day zero. On days 10, 20, 30 and 40 after diagnosis, 10 individuals of each species were collected. After collection, the fish were subjected to spinal cord section and subsequent biometric analysis. Next a gill arch from the right side of each fish were collected and fixed in Davidson solution for 48 hours (ALFAC) [1]. The biological material was then subjected to a routine histotechnical procedure. Animals were observed daily for behavioural analysis, and the mortality was recorded.
Results: Twelve histological alterations were identified and described throughout the collections. However, it was not possible to establish an unequivocal causal relationship between the observed alterations and the parasitic disease. The application of the different indices suggests that the dynamics of the gill alterations occurred differently for *R. quelen* and *M. maculatus*.
Conclusion: The robustness and suitability of the indices as a tool for assessing the severity of the damage caused by the parasitic disease to gill tissue in the two species allow us to better understand of the temporal evolution of the disease are discussed and questioned.

Keywords: Parasitic disease; tissue alterations; histology; bernet index; cardoso index.

1. INTRODUCTION

Ichthyophthiriasis is a parasitic disease caused by the ciliate protozoan *Ichthyophthirius multifiliis*, commonly known as “ich”. The trophozoite stage of *I. multifiliis* is an obligate parasite that infests the gills and skin of fish [2]. This parasite is histiophagous and hematophagous and feeds on secretions; thus, it can cause significant tissue lesions [3].

Infested animals tend to rub against surfaces or even each other, leading to skin lesions and thus enhancing mucous secretion [4]. When found in excess in the gills, this type of secretion may hinder gas exchanges and even lead to host death [2]. Moreover, injuries caused by such friction may serve as the port of entry for secondary infections, further increasing the fish morbidity [3].

Histology is a tool that is widely used in investigations of fish pathologies [5-8]. The use of histopathological biomarkers allows the visualisation of the exposure effects of these animals to several agents, either biological or chemical [9]. Other advantages of histopathology are the low cost of analysis and the possibility of acquiring rapid results. However, the lack of standardised methods in result analysis and interpretation may hinder the comparison of studies performed by different authors or even lead to inaccurate conclusions and incorrect results.

This study aimed to describe tissue histopathologies in *Rhamdia quelen* (South American catfish) and *Metynnis maculatus* (spotted metynnis) during treatment of ichthyophthiriasis and to compare three indices applied in the evaluation of tissue impact in fish.

2. MATERIALS AND METHODS

Specimens of *R. quelen* and *M. maculatus* were acquired from the Supply Centre of Paraná (Central de Abastecimento do Paraná S/A - Ceasa) in Curitiba, Paraná, Brazil and were transported in plastic bags containing 1/3 water and 2/3 oxygen to the Laboratory of Aquatic Organisms Research (Laboratório de Pesquisa com Organismos Aquáticos - LAPOA) of the Integrated Group of Aquaculture and Environmental Studies (Grupo Integrado de Aquicultura e Estudos Ambientais - GIA) of the Federal University of Paraná (Universidade Federal do Paraná - UFPR).

In the laboratory, animals underwent a routine handling for temperature and pH acclimatisation before undergoing a prophylactic bath with iodised salt (NaCl) (6 g/L) for two hours. After the bath, fish were transferred to polyethylene tanks (1,000 L) with closed physical and biological filtration systems. The tanks were maintained under constant aeration and controlled temperature (23℃±2℃). Animals were fed daily *ad libitum* with pelleted commercial feed containing 35% crude protein.

After 20 days in the laboratory, the fish began to show signs of behavioural alterations characteristic of *I. multifiliis* infestation (animals were grouped next to the air source, rubbing vigorously against each other and rejecting the food provided). This tank (tank 1) was filled with 500 *R. quelen* (3.96±1.8 g (mean ± standard deviation), 6.85±0.9 cm) and 500 *M. maculatus* (7.60±3.2 g; 6.56±1.0 cm). After 10 days, *M. maculatus* stored in a second tank (tank 2) (n= 261, 16.7±5.3 g; 9.01±0.9 cm) started to show the same signs and mortality.

After the first clinical signs were recorded, the presence of disease was confirmed by mucus-containing gill and skin scrapings collected from moribund fish, which were analysed by light microscopy. Drug treatment was initiated immediately. In tank 1, the treatment consisted of 6 g/L of malachite green, 3 g/L of methylene blue, 3 g/L of magnesium sulphate, 2 g/L of potassium chloride, and 10 g/L of copper sulphate solution added in a ratio of 1 mL of solution/40 L of water every 48 h for 20 days. Before each application, a partial replacement (25%) of the tank water (200 L) was performed. In tank 2, secondary infections caused by bacteria and fungi were diagnosed. Thus, the treatment described above was complemented with oxytetracycline (in the water) at 0.91 g/kg live weight, once a day for 10 days.

During treatment, the dissolved oxygen concentration, temperature (portable digital oximeter, YSI 550A, USA) and pH (bench pH meter AZ86505, Brazil) were monitored in the water of the tanks. Analyses of nitrite and total ammonia (N-TA = NH_3+NH_4^+) were performed by spectrophotometry by the indophenol method (APHA, 2005), followed by determination by spectrophotometry (Spectronic 20 Genesys, England). Gaseous ammonia (N-NH_3) was analysed according to the method described by [10]. Animals were observed daily for behavioural analysis, and the mortality was recorded.

For histopathology, 15 sick fishes (presence of white spot) of each species were collected from each tank on day zero. On days 10, 20, 30 and 40 after diagnosis, 10 individuals of each species were collected. After collection, the fish were subjected to spinal cord section and subsequent biometric analysis (Ethics committee for animal from the biological science section of the UFPR – 23075.064636-2015-04). Next a gill arch from the right side of each fish [11,12] were collected and fixed in Davidson solution for 48 hours (ALFAC) [1]. The biological material was then subjected to a routine histotechnical procedure in a Tissue Processor (Leica TP1020) and sectioned in a Rotary Microtome (Leica RM2125RT Germany) into 5-µm-thick sections. Permanent slides were mounted and stained in an Automated Stainer (Leica XL, Germany) with Harris hematoxylin and eosin [13].

Since the breakthrough of the disease was not the result of an induced experiment, but a result of a routinely occurring situation during the fish culture and as the fish of both tanks were affected, there was no way to establish a control group to compare the evolution of the histopathological alterations throughout the treatment. To overcome this situation, the alterations identified in the fish themselves 100 days after the beginning of the treatment was established as comparison parameter.

Quantitative analysis of the tissue alterations was performed according to the methods proposed by Bernet, Schmidt [14] and Cardoso [5]. The Bernet, Schmidt [14] method is based on the reaction pattern (rp), the pathological relevance of the observed alteration (w) and the extent of the lesions (α). When lesions of a single organ are analysed, two indices may be used. The first is the organ index (I org), which represents the degree of damage in the analysed organ. The second, called the reaction index (I org rp), aims to assess the severity of the identified alterations.

$$I_{org} = \sum_{rp} \sum_{alt} (\alpha_{or\ rp\ alt} \times w_{or\ rp\ alt})$$

$$I_{org\ rp} = \sum_{alt} (\alpha_{or\ rp\ alt} \times w_{or\ rp\ alt})$$

where org=organ, alt=alteration, rp=reaction pattern, α=lesion extension, and w=pathological relevance of the alteration.

The Impact index proposed by Cardoso [5], in turn, does not consider the severity of the lesions, only their occurrence and prevalence; further, it may be applied to several tissues. For evaluation of the gills, five-filament lamellae are analysed, and any alterations observed are recorded. Thus, the GII (Gill Impact Index) may be calculated individually for each pathogenesis and may exhibit values ranging from zero (minimum value, indicating the absence of histological alterations) to 1.0 (maximum value, indicating alterations in all the analysed filaments).

$$GII = (N_1/N_2)$$

where N_1=number of altered lamellae, and N_2=number of lamellae analysed.

Then, the sum of the gill impact indices from all lamellae analysed was obtained.

$$TGII = \sum_{i=1}^{5} GII_n$$

where TGII=total gill impact index, and GII_n=gill impact index of lamella n.

2.1 Statistical Analysis

The data were subjected to the Shapiro-Wilk normality test ($p<0.05$). The data did not fit a normal distribution; thus, they were analysed using the nonparametric Mann-Whitney, Kolmogorov-Smirnov and Kruskal-Wallis tests. The data from *M. maculatus* from tanks 1 and 2 were treated as a group, as they showed no significant differences.

To enable a direct comparison among the histological indices, the index values were transformed into percentages and were subsequently analysed using the Kolmogorov-Smirnov and Kruskal-Wallis tests, where p-values ≤0.05 were considered significant. All statistical analyses were performed using Statistica Software, version 8.0®.

3. RESULTS

The temperature was maintained near 25℃, the concentrations of dissolved oxygen were approximately 5 mg/L, and the pH was approximately 7. The gaseous ammonia did not exceed 0.05 mg/L, and the nitrite was maintained below 0.3 mg/L. In general, the environmental conditions were considered appropriate for both species [15].

In both cases (tanks 1 and 2), the treatments were highly effective against ichthyophthiriasis (Table 1). In tank 1, the mortality ceased completely after 7 days, while in tank 2, the same phenomenon occurred on the sixth day. In both tanks, one day before the last recorded death, the fish were swimming and feeding normally.

The gill, on day zero, revealed the presence of *I. multifiliis* trophozoites, characterised by a horseshoe-shaped nucleus. At the time treatment was initiated in tank 2, the animals displayed severe ulcerations on the body surface that were deep enough to expose the muscle tissue. After five days of treatment, the lesions were only skin deep, and on the 10th day, it was no longer possible to detect animals with visible macroscopic lesions.

Histological analysis allowed the detection of parasites in the animals only on day zero, i.e., before treatment was initiated. No macroscopic alterations were observed in the gills of *M. maculatus* and *R. quelen*. Altogether, 12 tissue alterations and the presence of parasites were identified in the gills analysed during 100 days of observation (Table 2).

The presence of parasites in the gills was observed only in the first sample collection (day zero) in both species. The parasites were surrounded by a layer of epithelial tissue. Lamellae in regeneration, epitheliocystis and haemorrhage were observed only in *M. maculatus*. Desquamation, in turn, was detected only in *R. quelen*. There was a higher prevalence of epithelial detachment, edema and hyperplasia (Table 3).

These three alterations, however, were observed in all collections, and they did not display any pattern of prevalence distribution associated with disease evolution or with the treatment applied.

3.1 Application of Tissue Alteration Indices

To assess the evolution of tissue alterations caused by the disease, indices of histopathological alterations were compared separately for each species. Analysis of the variation curves of the indices suggests a downward trend in the rates of tissue alterations in the first 10 days of treatment. After this period, however, the indices started to vary differently from each other and between the two species. Significant differences ($p<0.05$) between the beginning (day zero) and the end of the monitoring period (100 days) were observed only when I_{org} was used for *M. maculatus*. In the remaining cases, this difference was not significant.

In the case of *R. quelen*, a high, significant correlation ($r^2>0.85$, $p<0.05$) was observed between the tissue alteration indices. As for *M. maculatus*, a high correlation was observed only between $I_{org\ rp}$ and TGII (Table 4).

After transformation into percentages, based on the relationship between the maximum and minimum values quantified for each index, the

respective transformed indices were directly compared using the Kolmogorov-Smirnov and Kruskal-Wallis tests. Out of the 12 possible combinations, significant differences among the three indices of tissue alterations were observed for nine combinations. In a few cases, depending on what statistical test was used, the results were opposite (Table 5).

Nevertheless, when the respective indices assigned to each species were compared using the Mann-Whitney test, the analysis indicated that specimens of *M. maculatus* always displayed higher indices of tissue alteration compared to those of *R. quelen* (Table 6).

4. DISCUSSION

In addition to their involvement in respiration, the gills play a role in the system responsible for maintaining the osmotic balance of fish; thus, their integrity is crucial for maintaining the vital functions of fish. Accordingly, parasitic diseases of the gills, such as that caused by *I. multifiliis*, may cause structural or functional damage in their hosts that can lead to death [16].

In the present study, no parasite encapsulation was observed. Instead of a capsule made of connective tissue, the presence of a thin layer of epithelial tissue was observed around the parasite.

The combined administration of copper sulphate, methylene blue and malachite green, the antiparasitic properties of which have been reported by several authors [17,18], was effective in the treatment of ichthyophthiriasis. According to Eiras [19], the presence of "ich" causes skin and gills lesions leading to death in a short time. The treatment used in the present study quickly eliminated the parasites present in the gills of both fish species, reducing mortality rapidly and definitively, so that between the 8th and the 100th day, no death was recorded.

However, even with the complete elimination of the parasites at the beginning of treatment and the complete control of mortality after one week, 12 gill alterations were identified throughout the six collections, raising questions about the existence of a causal relationship, direct or indirect, between these alterations and the occurrence of the parasitic disease. Furthermore, it was unclear whether the applied indices of the tissue alterations reflected the severity of the damage caused by the parasites.

Table 1. Temporal variation in the number of dead individuals and the behaviour of fish infested by *Ichthyophthirius multifiliis* during drug treatment

Day	Tank				
	1			2	
	Mortality		Behaviour	Mortality	Behaviour
	***M. maculatus* (n=500)**	***R. quelen* (n=500)**		***M. maculatus* (n=261)**	
1	3	0	Fish swimming in groups on the tank surface, near the water inlet. Decreased food intake	4	Anorexia
2	2	1		40	Anorexia. Fish on the water surface, swimming next to each other
3	8	7	Fish swimming in groups on the surface and anorexia	0	Anorexia. Fish swimming actively in the water column
4	0	0		5	Fish feeding and swimming normally
5	2	0		1	
6	0	0	Fish swimming in a less grouped manner	0	
7	1	0	Fish feeding and	0	
8	0	0	swimming normally	0	

Table 2. Alterations observed in gill tissue of *Rhamdia quelen* and *Metynnis maculatus* infested with *Ichthyophthirius multifiliis*

Tissue alteration	Description
Aneurism	Abnormal accumulation of blood within the blood vessels, due to the loss of vascular integrity.
Congestion	Increased blood volume in a particular region.
Epithelial desquamation	Destruction of the simple squamous epithelium that surrounds the gill lamellae, with cell disruption and separation likely as a result of the cell death process.
Detachment of the lamellar epithelium	Separation of the simple squamous epithelium lining the lamellae from its basement membrane, forming large areas of interstitial fluid accumulation.
Detachment of the lamellar epithelium with eosinophilic fluid accumulation (edema)	Extravasation of eosinophilic fluid into the sub-epithelial extracellular space, causing a physical separation between the gill epithelium and its basement membrane.
Epitheliocystis	It is usually a benign infection, although proliferative lesions are also reported. *Rickettsia* sp. or *Chlamydia* sp. may be the causative agents of epitheliocystis; however, the actual taxonomic status of these organisms remains unknown.
Fusion of gill lamellae	As a result of hyperplasia of the lining epithelium, there is fusion of gill lamellae, causing a reduction in the area of gas exchange.
Haemorrhage	Blood extravasation from the vascular bed.
Hyperplasia	Increase in the number of cells, with no morpho-functional alteration, where fusion of lamellae and, more rarely, of filaments may occur.
Hypertrophy	Increase in cell volume, without mitosis or meiosis.
Lamellae in regeneration	A process in which there is replacement of altered lamellae by new intact ones.
Vacuolisation	Presence of intracytoplasmic vacuoles in cells of the gill epithelium.

Table 3. Frequency (%) of specimens of *Rhamdia quelen* and *Metynnis maculatus* with gill alteration after infestation by the parasite *Ichthyophthirius multifiliis*

Alteration	Day of collection											
	R. quelen						*M. maculatus*					
	0[1]	10[2]	20[2]	30[2]	40[2]	100[2]	0[3]	10[4]	20[4]	30[4]	40[4]	100[4]
Aneurysm	20	0	0	10	0	0	23	40	25	20	5	5
Congestion	67	20	0	0	0	40	3	0	0	0	0	5
Desquamation	20	0	0	0	0	10	0	0	0	0	0	0
Detachment	100	80	90	100	100	80	70	85	100	90	85	45
Edema	93	40	80	60	90	80	70	90	95	95	85	50
Lamellae in regeneration	0	0	0	0	0	0	7	0	5	0	5	0
Epitheliocystis	0	0	0	0	0	0	7	0	0	0	0	0
Fusion	53	0	0	0	0	10	23	10	15	10	5	0
Haemorrhage	0	0	0	0	0	0	0	0	0	0	20	0
Hyperplasia	93	60	50	80	10	40	77	55	45	25	10	20
Hypertrophy	27	0	20	10	10	10	17	0	5	0	0	0
Parasite	100	0	0	0	0	0	93	0	0	0	0	0
Vacuolisation	0	20	10	0	0	0	3	0	0	0	0	0

[1] n=15; [2] n=10; [3] n=30; [4] n=20

Studies on infestation of fish with “ich” have histopathological alterations of the gills reported the occurrence of several associated with *I. multifiliis*.

Table 4. Coefficient of determination (r^2) of the correlations between the different indices of tissue impact ($I_{org\ rp}$, I_{org} and IIBRT) applied to *Rhamdia quelen* and *Metynnis maculatus*

Rhamdia quelen			*Metynnis maculatus*		
	$I_{org\ rp}$	I_{org}		$I_{org\ rp}$	I_{org}
I_{org}	0.90*		I_{org}	0.40*	
TGII	0.93*	0.85*	TGII	0.94*	0.52*

**Significant correlation (p<0.05)*

Table 5. Comparison of the results obtained by applying the transformed (in %) Bernet ($I_{org\ rp}$ and I_{org}) and Cardoso (GII) indices for *Rhamdia quelen* and *Metynnis maculatus* infested with *Ichthyophthirius multifiliis* (α =0.05)

Species	Test			
	Kolmogorov-Smirnov		Mann-Whitney	
M. maculatus	$I_{org} > I_{org\ rp}$		$I_{org} = I_{org\ rp}$	
	TGII > $I_{org\ rp}$	TGII> I_{org}	TGII > $I_{org\ rp}$	TGII > I_{org}
R. quelen	$I_{org} < I_{org\ rp}$		$I_{org} > I_{org\ rp}$	
	TGII >$I_{org\ rp}$	TGII = I_{org}	TGII >$I_{org\ rp}$	TGII = I_{org}

Table 6. Degree of lesions observed for the Bernet ($I_{org\ rp}$ and I_{org}) and Cardoso (TGII) indices (p<0.05) applied to *Rhamdia quelen* (Rq) and *Metynnis maculatus* (Mm) infested with the parasite *Ichthyophthirius multifiliis*

	Kolmogorov-Smirnov		Mann-Whitney	
	$I_{org\ rp}$	I_{org}	$I_{org\ rp}$	I_{org}
$I_{org\ rp}$		Mn>Rq		Mn>Rq
I_{org}	Mn>Rq		Mn>Rq	
TGII	Mn>Rq	Mn>Rq	Mn>Rq	Mn>Rq

Juvenile of *Ictalurus punctatus* infested with "ich" exhibited an epithelial hyperplasia, focal areas of cellular disruption and necrosis [20]. According to [21], the nature and severity of histopathological alterations observed in fish infested with *I. multifiliis* may vary greatly, as the degree of severity of the alterations is affected by factors that the host was previously exposed to, such as stress, and by the nutritional status of the host. The results obtained in the present study suggest that the severity of tissue alterations may also be species-specific. Even after subjection to similar environmental conditions considered appropriate for both species and to a proper diet, *M. maculatus* consistently exhibited higher indices of tissue alterations than those of *R. quelen*.

According to Pádua, Shikawa [22] massive infestations of the gills caused by this parasite may induce severe epithelial hyperplasia and cause hyperplasia of mucus-producing cells and fusion of secondary lamellae. In more severe situations, according to the author, areas of necrosis may be observed in the gills of infected animals. As stated by Moreira, Vargas [23], the reduction in the interlamellar spaces that occurs due to the presence of parasites significantly limits the area where gas exchange takes place. For this reason, the organism reacts by increasing the number of local epithelial cells to improve oxygen uptake. The data obtained here corroborate the report that epithelial hyperplasia may be found during parasitic infestations. However, as this alteration was observed in all sample collections, it is likely that factors other than "ich" may lead to the emergence of such alterations.

None of the studies cited here, which were based on histological analyses of the gills to evaluate the effects caused by parasitic diseases in fish, reported any information about the degree of histological alteration in the study population before the onset of the disease or after its complete control. Based on this lack of data and on the findings observed in the present study, it is not possible to confirm a causal relationship between the disease and the alterations observed by the respective authors.

Another important point is that there are differences in how the histological alterations are treated and analysed by different authors.

Several previous studies considered only the observation and description of the alterations, without applying any quantitative index to assess the possible damage caused or to describe the evolution of these alterations [16,24-26]. Other studies, such as that of Jacob, Nandini [27], use the index proposed by Bernet, Schmidt [14]; however, the authors do not mention which of the indices proposed by Bernet was applied. Finally, none of the studies cited here that used the Bernet index presented the numerical results obtained with this index or described its temporal variation.

In the present study, it was possible to observe that between days zero and 10, there was a trend toward lower indices of tissue alterations for both species. This trend was expected and was corroborated by behavioural and mortality analyses of fish from the tanks, in addition to the non-identification of parasites in the gills immediately after the beginning of treatment. Thus, if this trend was maintained and the indices were influenced exclusively by the parasitic disease, a sequential and significant decrease in tissue alteration indices over time would be expected.

However, 30 days after the beginning of treatment, although no evidence of parasite recurrence was observed, the indices - with a single exception (I_{org} calculated for *M. maculatus*) - began to increase, which leads to the assumption that the disease was the factor responsible for the appearance of the tissue alterations observed.

The hypothesis that routine situations that are considered normal for the management of captive fish may influence the appearance of gill alterations cannot be ruled out. For instance, aneurysm is cited as an alteration that may be associated with physical or chemical trauma. According to Santos [28] the alteration is usually observed after handling captive fish; however, it may also be associated with parasitic lesions, metabolic wastes or chemical contaminants present in water. Nevertheless, no biological or environmental factor was identified that could explain the decrease in the tissue alteration indices after the 10^{th} day of observation. Finally, as there were no more deaths even after increased indices of gill alterations, it is not possible to associate the indices or any tissue alteration observed with an increased risk of fish death.

5. CONCLUSION

As shown here, it is critical that quantitative indices of tissue alterations are employed in histopathological studies of parasitic diseases of fish. However, based on the results obtained in the present study, it is not possible to validate or recommend the use of any of the indices used here. Despite a few high, significant correlations, especially when comparing $I_{org\ rp}$ and TGII, most often, these indices showed conflicting results. Thus, in the present study, none of the indices were shown to be sufficiently robust and reliable as a tool to assess the degree of severity of tissue alterations caused by ichthyophthiriasis or for understanding the evolution of the effects of this disease. Accordingly, large gaps remain in the analysis and interpretation of results in histopathological studies associated with this topic.

ETHICAL APPROVAL

Ethics committee for animal from the biological science section of the UFPR – 23075.064636-2015-04.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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