Research Article

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Dynamics of λ -cyhalothrin disappearance and expression of selected P450 genes in bees depending on the ambient temperature

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Abstract: Temperature has a significant influence on the action of pyrethroids, and their effect increases with decreasing ambient temperature. Using gas chromatography, we assessed the degradation rate of λ -cyhalothrin, active ingredients (AI) of Karate Zeon 050 CS from pyrethroid group, in bees incubated for 48 h under different temperature conditions. With RT-qPCR method, we studied expression levels of selected cytochrome P450 genes after exposure to the plant protection product (PPP). The half-life of λ -cyhalothrin decreased from 43.32 to 17.33 h in the temperature range of 21–31°C. In animals incubated at 16°C, the AI half-life was even shorter and amounted to 10.19 h. The increase in temperature increased the expression of Cyp9Q1, Cyp9Q2, and Cyp9Q3 in the group of control bees. We showed a two-fold statistically significant increase in gene expression after treatment with PPP bees. The obtained results indicate that honey bees are characterized by susceptibility to pyrethroids that vary

depending on the ambient temperature. This may be due to the different expressions of genes responsible for the detoxification of these PPPs at different temperatures.

Keywords: pyrethroid, *Apis mellifera*, expression of detoxification genes, pesticide degradation

1 Introduction

Foraging bees (*Apis mellifera*) are exposed to a variety of environmental factors, including residues from commercial plant protection products (PPPs) [1–4] and changing ambient temperatures [5]. Theoretically, gatherer bees, as heterothermic organisms, during foraging outside the hive are able to temporarily maintain the body temperature at a constant level. They spend their resting time mainly inside the hive, where, due to the presence of brood, the temperature is also constant, relatively high (33–35°C), which eliminates their exposure to low ambient temperatures. However, it often happens that on hot nights in numerous, strong families, the gatherers, being of little use in the hive, spend the night outside the nest – on the walls or hanging at the outlet. During this period, animals are exposed to unfavorable external conditions, and at the same time, they do not activate thermoregulatory mechanisms related to feeding. Similarly, bees are exposed to large fluctuations in temperature in the broodless period (from autumn to spring), which is characterized by a significantly lowered temperature in the nest. The ambient temperature and plant protection products may interact with each other [6,7], which may prove unfavorable to the functioning of a single bee. Temperature affects the stability of PPPs, their evaporation, penetration through the outer layers of the organism, and tissue penetration [8]. It also influences the physiology of the organism itself – it determines the effectiveness of the sodium/potassium pump [9], homeostasis of cell membranes [10] or the metabolic rate [11,12], which is more optimal, the more likely

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is the chance of a successful detoxification process [10]. The temperature may also determine the enzyme production responsible for detoxification and antioxidant mechanisms in bees [13], which may affect the neutralization of toxins. One of the most important groups of these compounds is the cytochrome P450 monooxygenases. They are a large and complex group of heme-thiolate proteins that are found in almost all living organisms [14]. The detoxification process mediated by cytochrome P450 enzymes is crucial in tolerating and developing pesticide resistance in many pests [15], including tolerance to pyrethroid [16], one of the most popular groups of insecticides, characterized by a complex mechanism of action [17,18]. Pyrethroids show a close dependence of their effectiveness on temperature – this dependence was called the “temperature coefficient.” Their effectiveness increases with decreasing ambient temperature [19–21]. For this reason, they are most often used in spring, during the flowering period of many nectarizing plants that feed bees.

The aim of the study was to estimate the rate of degradation of λ -cyhalothrin, active ingredient (AI) of Karate Zeon 050 CS (λ -cyhalothrin-based insecticide, CBI), in worker honeybees incubated after intoxication at 16, 21, 26, and 31°C. We also assessed whether λ -cyhalothrin is an expression inducer of cytochrome P450 isoenzymes belonging to the CYP3 family – the CYP9Q subfamily, i.e., CYP9Q1, CYP9Q2, and CYP9Q3, and whether the temperature of bees has an influence on this expression.

2 Material and methods

2.1 Insects used in the experiments

The experimental object in the study were gatherers of honey bees (*Apis mellifera carnica*) collected from the outlets of four hives located at Werynia (Poland) at the Faculty of Biotechnology of the Rzeszow University. The workers were collected from the hives between 08:00 and 09:00 a.m., 40 min against intoxication. In order to calm down, all insects were incubated at 7°C for 30 min before intoxication.

2.2 Insecticide

KarateZeon 050 CS (Syngenta Limited, Great Britain) containing λ -cyhalothrin ([α -cyano-3-phenoxybenzyl 3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethylcyclopropanecarboxylate],

type II pyrethroid insecticide) (50 g per L of the insecticide) as an AI was used in the experiments. The applied dose of the preparation was 20 $\mu\text{L L}^{-1}$.

2.3 Intoxication

Intoxication was performed using the solution obtained by dissolving 5 μL of Karate-Zeon 050 CS in 1 L of distilled water. Bees were exposed to 5 μL of distilled water (the control group) or the CBI solution, applied topically on the dorsal part of their prothorax. After intoxication, the bees in groups of 10 (always from the same hive) were placed in Petri dishes where they had unlimited access to vessels containing 50% m v^{-1} sucrose syrup. The dishes were kept in constant darkness in a laboratory incubator S 711 (Chłodnictwo-Madej, Poland) at different temperatures: 16, 21, 26, or 31°C.

After 2, 6, 12, 24, or 48 h of incubation, subsequent dishes were removed from the incubator and were frozen for further analyses. For each application temperature (16, 21, 26, and 31°C) and each termination time (2, 6, 12, 24, and 48 h), four replicates of 10 individuals were used (extraction of the residue was carried out in each case from three samples).

The insects intended for gene expression tests were treated with CBI (in the control sample – with water), in the same way as the bees used for the analysis of the λ -cyhalothrin degradation rate. For this experiment, the incubation of the insects was completed 6 h after intoxication each time.

2.4 Extraction of λ -cyhalothrin residues from bees

An analytical portion of lyophilized bees (Labconco Freezone 2.5 freeze dryer, Labconco, USA; pressure: 0.024 mbar; temperature: 50°C; time: 168 h) was shaken for 2 min with 10 mL of water and 10 mL of acetonitrile (Chempur, Poland). About 4 g of anhydrous magnesium sulfate(vi) (Chempur, Poland), 1 g of sodium chloride (Chempur, Poland), 1 g of trisodium citrate (Chempur, Poland), and 0.5 g of sesquihydrate disodium hydrogen citrate (Chempur, Poland) was added, and the contents were shaken for next 2 min and centrifuged for 5 min at 4,500 rpm. Then, 6 mL of the acetonitrile phase was transferred to a polypropylene tube containing 150 mg of the primary secondary amine (Agilent, USA) and 900 mg of anhydrous sodium sulfate(vi) (Chempur, Poland) and

vigorously shaken for 2 min and centrifuged for 5 min under the same conditions as before. Finally, 4 mL of the obtained extract was collected, transferred to a glass flask, evaporated in a helium stream of purity 5.0, and dissolved in 4 mL of petroleum ether (Chempur, Poland).

2.5 Chromatography analysis

The obtained extracts were analyzed using the Agilent 7,890 gas chromatograph (Agilent, USA), equipped with an electron capture detector (μ ECD). The chromatograph was operated via ChemStation software and equipped with an autosampler and an HP-5MS column (30 m: 0.32 mm: 0.25 mm). The following chromatographic conditions were used: μ ECD detector temperature, 290°C; injector temperature, 250°C. The oven temperature was programmed as follows: 100°C – 0 min $\rightarrow$ 10°C min⁻¹ $\rightarrow$ 180°C – 4 min $\rightarrow$ 3°C min⁻¹ $\rightarrow$ 220°C – 15 min $\rightarrow$ 10°C min⁻¹ $\rightarrow$ 260°C – 20 min. The total analysis time was 64.33 min. The injection volume was 1 μ L.

2.6 Analytical standards

A certified pesticide analytical standard was obtained from The Institute of Industrial Organic Chemistry (Poland). For linearity determinations, standard solutions in the clean matrix were prepared at the following standard concentrations: 0.01, 0.05, 0.10, 0.50, and 1.0 mg L⁻¹. Linearity was described with determination coefficients ($R^2 > 0.99$). Excellent linearity was achieved for the studied pesticide when using a matrix-matched standard. The limit of quantification values were the lowest at a spiked level (0.001 mg kg⁻¹).

2.7 Validation of the analytical method

Recoveries of two λ -cyhalothrin isomers were 72.6% (SD = 5.7%; RSD/CV = 8.87%) and 85.8% (SD = 5.7%; RSD/CV = 6.66%), which means the values obtained were within the assumed range of 70–120% [22].

2.8 RNA isolation

The total bee RNA used for the analysis was isolated from 20 adults by pretreating them with liquid nitrogen and grinding them in a mortar. The next stages of the RNA

extraction procedure were performed using the setup for RNA isolation, GenEluteMammalian Total RNA Miniprep Kit (Sigma Aldrich, USA). RNA was eluted from columns using 30 μ L of RNase-free water. RNA concentration and purity in samples were assessed using a NanoDropTM Spectrophotometer (Thermo Scientific). RNA samples with ratios OD260/280 = 1.8:2.2, OD260/230 $\geq$ 2.0 > 500 ng were used for further analysis.

2.9 cDNA synthesis

About 300 ng of RNA from each isolated sample was subjected to reverse transcription at 50°C for 1 h, using the First Strand cDNA Synthesis Kit (Roche, USA). For this purpose, we prepared a mixture consisting of 300 ng of RNA, 1 μ L of (dT)18 starter, 2 μ L of Random HEK starter, and nuclease-free water (to a final volume of 13 μ L). The mixture was incubated for 10 min at 65°C and then cooled on ice. Depending on the number of analyzed samples, we prepared a sufficient quantity of the reaction mixture, mixing its component in the right proportions. A composition for a single sample was as follows: 4 μ L of reaction buffer, 0.5 μ L of 40 U μ L⁻¹ RNase inhibitor, 2 μ L of 10 mM dNTPs, and 0.5 μ L of 20 U μ L⁻¹ reverse transcriptase. Control samples (-RT) were prepared in the same manner, with the reverse transcriptase replaced with an identical volume of nuclease-free water. The obtained cDNA was diluted 10 times with RNase-free water and stored at -20°C.

2.10 Analysis of the transcriptional activity of genes encoding cytochrome P450 isoenzymes belonging to the CYP3 family, involved in detoxification – CYP9Q1, CYP9Q2, and CYP9Q3, using the real-time qPCR technique

About 10 μ L of the reaction mixture containing 1 μ L of 10 times diluted cDNA sample, 5 μ L of LightCycler Master Mix (Roche, Switzerland), 0.25 μ L of 10 μ M solutions of both forward and reverse primers (Table 1), and 3.5 μ L of nuclease-free distilled water was prepared for the RT-qPCR analysis.

We performed a comparative qPCR analysis using Roche LightCycler® 480, according to the producer's protocol. We started with the initial sample denaturation in 40 cycles at 95°C for 10 min, with the denaturation at

Table 1: Sequences of primers of genes encoding cytochrome P450 isoenzymes belonging to the CYP3 family involved in detoxification – CYP9Q1, CYP9Q2, and CYP9Q3 [23]

Primers	Primer sequences (5'-3')
CYP9Q1	Forward: TCGAGAAGTTTTCCACCG Reverse: CTCTTCCTCCTCGATTG
CYP9Q2	Forward: GATTATCGCCTATTATTACTG Reverse: GTTCTCCTTCCCTCTGAT
CYP9Q3	Forward: GTTCCGGGAAAATGACTAC Reverse: GGTCAAATGGTGGTGAC
Reference gene eIF3-S8	Forward: GAGTGTCTGCTATGGATTGCAA Reverse: TCGCGGCTCGTGGTAAA

95°C for 10 s, primers connected at 56°C for 20 s, and strand extension at 72°C for 20 s. The reaction was stopped by cooling samples to 40°C for 10 s. To eliminate nonspecific amplification, the melting curve of the obtained products was also analyzed.

Using Roche LightCycler® 480 Software, we analyzed the reference CYP9Q transcript levels for the endogenous control eIF3-S8 (a translation initiation factor involved in cell proliferation).

2.11 Analysis of the results

The average-adjusted residue levels of the two λ -cyhalothrin isomers (summed values) were calculated and their degradation trends were expressed as exponential equation (1):

$$R = R_0 e^{-kt}, \quad (1)$$

where R_t is the concentration (residue) of any pesticide after t time (in h), R_0 is the initial concentration of the pesticide (in mg kg⁻¹), and k is the rate constant (in h⁻¹).

The half-lives were calculated using equation (2):

$$T_{1/2} = \ln(2)k^{-1}, \quad (2)$$

where k is the rate constant (in h⁻¹).

The Q_{10} coefficient for the test of degradation was calculated according to formula (3):

$$Q_{10} = (W_2/W_1)^{10/(T_2-T_1)}, \quad (3)$$

where W_2 is the degradation time obtained at a higher temperature, W_1 is the degradation time obtained at a lower temperature, T_2 is the higher temperature (°C), and T_1 is the lower temperature (°C).

The results of the gene expression analysis are presented as the arithmetic mean $\pm$ SD (standard deviation). The statistical analysis was performed using the Student's

t -test using GraphPad Prism 5. The differences with the $p < 0.05$ coefficient were considered statistically significant.

3 Results

3.1 Influence of temperature on the course of λ -cyhalothrin degradation in honey bees

Two hours after poisoning in bees kept at 31, 26, 21, and 16°C, the residues of λ -cyhalothrin were 0.049, 0.116, 0.086, and 0.132 mg kg⁻¹, respectively (Table 2, Figure 1).

The exponential equations describe very poorly ($r^2 = 0.2424$), poorly ($r^2 = 0.5187$ and 0.6117), and well ($r^2 = 0.7582$) the course of λ -cyhalothrin degradation in the bee tissues (Table 2, Figure 1). Specified degradation constants (k), respectively, 0.04 (31°C), 0.028 (26°C), 0.016 (21°C) and 0.068 (16°C) indicate the rapid detoxification of λ -cyhalothrin by bees. Based on equation (2), the AIs of PPP half-life were calculated and were 17.33, 24.76, 43.32, and 10.19 h, respectively. Based on these values, the Q_{10} coefficients were calculated as 1.42 (in the range 16–31°C), 0.40 (21–31°C), 0.49 (26–31°C), 2.43 (16–26°C), 0.33 (21–26°C), and 18.07 (16–21°C).

3.2 Transcriptional activity of genes encoding cytochrome P450 isoenzymes belonging to the CYP3 family involved in detoxification of CYP9Q1, CYP9Q2, and CYP9Q3 under various temperature conditions

In the conducted studies the expression profile of genes encoding cytochrome P450 isoenzymes belonging to the CYP3 family taking part in the first stage of detoxification of xenobiotics, i.e., CYP9Q1, CYP9Q2, and CYP9Q3 in *Apis mellifera* in response to heat stress (Figure 2) was analyzed. The results showed a statistically significant increase in the expression of CYP9Q1 and CYP9Q3 genes during the detoxification of the applied PPP at 21°C compared to the control. At a temperature of 16°C, an increase in the mRNA level of the CYP9Q1 and CYP9Q2 genes was observed, compared to the control group. Analysis of the expression of genes involved in detoxification at higher temperatures (31°C) showed an increase in mRNA of the CYP9Q3 gene.

In control bees, in most cases, there was an increase in gene expression with an increase in their incubation

Table 2: Residues (mg kg⁻¹) of λ -cyhalothrin in honey bees, 2, 6, 12, 24, and 48 h after poisoning and the parameters of their exponential degradation

Time (day)	Mean $\pm$ SD λ -cyhalothrin residue in bee samples (mg kg ⁻¹)			
	31°C	26°C	21°C	16°C
2 h	0.049 $\pm$ 0.023	0.116 $\pm$ 0.087	0.086 $\pm$ 0.037	0.132 $\pm$ 0.100
6 h	0.065 $\pm$ 0.091	0.043 $\pm$ 0.020	0.013 $\pm$ 0.005	0.055 $\pm$ 0.012
12 h	0.030 $\pm$ 0.033	0.024 $\pm$ 0.018	0.020 $\pm$ 0.004	0.004 $\pm$ 0.002
24 h	0.044 $\pm$ 0.056	0.023 $\pm$ 0.011	0.006 $\pm$ 0.002	0.010 $\pm$ 0.005
48 h	0.008 $\pm$ 0.009	0.020 $\pm$ 0.009	0.022 $\pm$ 0.002	0.003 $\pm$ 0.001
0–48 h	$R_t = 0.0666e^{-0.04t}$ $r^2 = 0.6117$	$R_t = 0.0594e^{-0.028t}$ $r^2 = 0.5187$	$R_t = 0.0266e^{-0.016t}$ $r^2 = 0.2424$	$R_t = 0.0539e^{-0.068t}$ $r^2 = 0.7582$
T _{1/2}	17.33	24.76	43.32	10.19

Figure 1: Exponential degradation trend of λ -cyhalothrin residues in bees incubated at 16°C ($n = 3$).

temperature; in the case of Cyp9Q3, in the temperature range 16–26°C, the increase was statistically significant. A similar significance was also observed for the reduction of the rate of Cyp9Q3 expression in the temperature range 26–31°C. Only in one case (16–21°C), the temperature caused a significant change in the level of gene expression (Cyp9Q3) in intoxicated bees.

4 Discussion

Pyrethroids are compounds derived from natural pyrethrins, with a nonselective, complex mechanism of action. They disturb the dynamics of sodium channels by extending their opening time [24]. They are also agonists of T-type calcium channels in the insect muscles [25], and the conduction of impulses in the muscles of bees is due to calcium ions [26]. Moreover, pyrethroids reduce the mitochondrial complex by inhibiting succinate and glutamate [27]. They also disrupt the phosphorylation of proteins [28] and modify the function of proteins, which create intracellular gap

junctions [29] and induce strong oxidative stress [30]. Despite these multiple mechanisms of action, pyrethroids are frequently used because they have low mammalian toxicity and a short half-life in their tissues. Anadón *et al.* [17] indicate that this time depends both on the form of administration (intravenous, nutritionally) and of the examined tissues. In their research, λ -cyhalothrin administered intravenously to rats disappeared in plasma after less than 8 h, and nutritionally after less than 11 h after intoxication. In the case of neuromuscular fibers, this time was extended to 26 and 34 h, respectively. In bees, this time calculated using the exponential equation was similar and ranged from 10.19 to 43.32 h (in temperature 26°C $T_{1/2} = 24.76$ h), longer than we observed in our previous studies [31], whereas in bees incubated at 26°C, the half-life was 14 h. During the research, the insects were incubated in constant darkness, the conditions that prevail at night outside the hive; for bee-gatherers, not dealing directly with work in the hive, they happen to spend resting periods. This period is characterized by a significant slowdown in the pace of metabolic changes [32]. In earlier studies, incubation has been associated with constant exposure to light and it probably contributed to the observed difference in the rate of AI decomposition.

In bees incubated in the temperature range of 31–21°C, the time of AI decomposition increased with the decrease of the ambient temperature, ranging from 17.33 to 43.32 h, and then decreased in the temperature range of 16°C $T_{1/2} = 10.19$ h (Table 2). This may be due to the heterothermal nature of bee-gatherers. Probably in the temperature range of 31–21°C, they kept their body temperature close to the ambient temperature, which resulted in nearly two times, according to the van't Hoff-Arrhenius [33] rule, the slowdown in the rate of metabolic changes estimated on the basis of changes in the decomposition rate of λ -cyhalothrin ($Q_{10} = 0.40$). At 16°C, the insects activated the mechanisms of thermogenesis, needed to maintain the temperature necessary to maintain efficiency and

Figure 2: (a) Semiquantitative evaluation of the expression of genes encoding cytochrome P450 isoenzymes belonging to the CYP3 family involved in detoxification – CYP9Q1, CYP9Q2, and CYP9Q3. Real-time PCR method. The bars show the relative expression of the gene tested in relation to the expression of the *elf3-S8* gene; the mean of three determinations with SD is marked. It differs from the control: * $p < 0.05$. (b) The heat map generated on the basis of the comparison of the transcriptional profile of genes encoding cytochrome P450 isoenzymes belonging to the CYP3 family involved in detoxification – CYP9Q1, CYP9Q2, and CYP9Q3 at various intoxication temperatures of bees (16, 21, 26, 31°C). Hierarchical clustering was performed using ClustVis, an online tool for clustering multidimensional data (BETA) (<https://biit.cs.ut.ee/clustvis/>).

readiness for feeding, which could indirectly contribute to faster AI decomposition ($Q_{10} = 2.43$ and $Q_{10} = 18.07$, respectively in the range of 16–26°C and 16–21°C). Kovac et al. [34], analyzing the temperature of individual parts of the body of bees under various thermal conditions of the environment, observed significantly greater differences at 15°C in the temperature of the body and belly of these insects than at 26 or 38°C pointing to the existence of efficient thermogenesis in worker bees at lower ambient temperatures.

Bees are organisms that can precisely regulate their body temperature, which theoretically could withstand

the temperature effect of PPPs. However, in the absence of brood (no brood tests were used in the experiment), the thermogenesis process starts only at lower temperatures, which may significantly affect the rate of detoxification of insects. Additionally, pyrethroids belong to agents where insecticidal activity increases with decreasing temperature [19,20]. It has also been shown that deltamethrin, another AI from the pyrethroids group, causes hypothermia in bees incubated at 22°C [35], which may also affect the level of thermogenesis in these animals, and thus, indirectly the efficiency of their detoxification.

Transcription of CYP9Q1, CYP9Q2, and CYP9Q3 belonging to the CYP3 group and taking part in the first stage of detoxification of xenobiotics is modulated in *A. mellifera*, incl. by tau-fluvalinate and bifenthrin (AIs from the pyrethroid group). This clan midgut P450s metabolizes pyrethroids to a form suitable for further cleavage by the carboxylesterases that also contribute to tau-fluvalinate tolerance [23]. As shown in the metabolism of cypermethrin in *Helicoverpa armigera*, carboxylesterases also metabolize hydroxylated metabolites of pyrethroids generated by P450s [36].

We also observed changes in the transcriptional activity of CYP9Q1, CYP9Q2, and CYP9Q3 in our studies. In control bees incubated at lower temperatures (16–21°C), we observed a clear, although not significantly lower than at 26 and 31°C, the transcriptional activity of genes encoding Cyp9Q1 and Cyp9Q2. In the case of Cyp9Q3, the observed increase in protein transcriptional activity along with the temperature increase from 16 to 26°C proved to be statistically significant. Li *et al.* [13] also indicated that with increasing ambient temperature in *A. cerana* and *A. mellifera*, there is an increase in oxidative stress, which induces an increase in the activity of detoxification and antioxidant enzymes. Probably, just low oxidative stress and the accompanying decreased transcriptional activities of Cyp9Q1, Cyp9Q2, and Cyp9Q3 genes in the groups incubated at lower temperatures may be responsible for the negative temperature coefficient of pyrethroids. It seems that despite the high increase in Cyp9Q1 expression at 16 and 21°C, Cyp9Q2 at 16°C and a statistically significant increase in Cyp9Q3 expression at 21°C after PPP use, the time required to activate the body's protective mechanisms is too long to prevent bees against the harmful multidirectional effects of the CBI. This means that the stress to which bees incubated at higher temperatures are exposed can practically increase their chances of survival when an additional toxic factor appears that requires the involvement of detoxification and antioxidant processes. On the other hand, the low temperature to which gatherers sleeping outside in the hive are sometimes exposed can pose a risk to them, especially in combination with hypothermic pyrethroids, which are highly effective in cooler environments.

5 Conclusion

The obtained results show that the temperature significantly influences the half-life of λ -cyhalothrin in *A. mellifera* worker-gatherers. The fastest decomposing of AI was in bees incubated at 16°C (10.19 h), and the slowest (43.32 h) – at 21°C. The fastest rate of detoxification of

bees incubated at 16°C indicates the occurrence in these insects periodically, depending on the ambient temperature, the ability to increase the rate of metabolic changes.

With increasing temperature, the expression of genes involved in pyrethroids detoxification usually increased (statistically significant for Cyp9Q3 in the range of 16–26°C) in the control bees. The treatment of bees with PPPs only in two cases (Cyp9Q1 and Cyp9Q3; 21°C) resulted in a significant increase in the expression of detoxification genes in the studied workers.

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Ethical approval: The conducted research is not related to either human or animal use.

Data availability statement: The datasets generated during the current study are available from the corresponding or the first author on request.

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