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# Mate finding in the isopod parasite *Ceratothoa oestroides* (Risso, 1816) in an aquaculture environment: A case study in the sea bass *Dicentrarchus labrax* (Linnaeus, 1758)

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ABSTRACT

The purpose of this *in situ* study was to determine the pattern of protandrous hermaphrodite *Ceratothoa oestroides* infestation in sea bass with respect to parasite sexual behaviour. Sea bass (N = 618) were divided into six net cages which presented three experimental groups. Group 1 represented infected fish, where female parasites were manually removed and male parasites were left in the buccal cavity of fish. Group 2 represented infected fish where both female and male parasites were removed, and group 3 represented uninfected fish (i.e., the control group). The duration of the experiment was 865.47 degree days (ºD), and average sea bass mortality rate was 11.97 %. No mature *C. oestroides* females were found at the end of the experiment. Fish in cages with removed female parasites (group 1) had a statistically higher number of parasites in pairs at the end of the experiment, compared to the group where all parasites - both males and females - were removed (group 2) and control group (group 3) ($P < 0.005$). Female parasites from group 1, being larger and older, had more success in mate finding. These results suggest that the female parasite uses some method of communication to attract the pulli II, i.e., the future male parasite, necessary for reproduction. Because there is a lack of parasite biology studies, especially *in situ*, our data on reproduction and mating behaviour of *C. oestroides* could help in the development of overall parasitic removal procedures and health management in aquaculture.

## 1. Introduction

Global aquaculture production for sea bass (*Dicentrarchus labrax*) has expanded from 70 694 tons in 2000 to 191 000 tons in 2016. This expansion is most dominant in the Mediterranean Sea, where production of sea bass *D. labrax* and sea bream *Sparus aurata* has increased rapidly in the last decade (Faggio et al., 2014a, 2014b; FAO, 2020). In Croatia, sea bass production has been influenced by a growing number of parasites in the farmed area (Šarušić, 1999; Čoz-Rakovec et al., 2002; Mladineo, 2006; Čolak et al., 2019).

Arthropod parasites of fish are copepoda, branchiura and isopoda; ca. 450 species of isopods are aquatic (marine and freshwater) fish parasites (Vagianou et al., 2017). The main marine parasite species of interest to the fish farmers in Greece, Turkey, Lebanon, Algeria, Tunisia, Spain, France, Montenegro and Croatia are from the family Cymothoidae (Trilles, 1969; Trilles et al., 1989; Trilles, 1994; Šarušić, 1999; Charfi-Cheikhrouha et al., 2000; Horton and Okamura, 2001; Mladineo, 2003; Bariche and Trilles, 2005;Zouhir et al., 2007; Matašin and Vučinić, 2008; Hernández-Moreno et al., 2011; Vagianou et al., 2017; Čolak et al., 2017, 2019).

A peracarid isopod, *Ceratothoa oestroides* (Risso, 1816) belongs to family Cymothoidae. Although other fish-associated isopods have separate sexes, cymothoids are protandrous hermaphrodites with multiple life stages (Brusca, 1981; Bunkley-Williams and Williams, 1998). The ectoparasite *C. oestroides* develops from eggs in brood pouches of the female, where it stays until it is developed into pulli II larvae. Afterwards, it starts to swim looking for a fish host. This parasite passes through separate developmental stages, beginning as a male when during the puberty it loses its swimming capacity. After a transitional stage, it becomes a female. Females inhibit the sexual transformation of their associated males and afterwards maintain a stable nested female-male pair in a single host (Trilles, 1969; Bunkley-Williams and Williams, 1998; Mladineo, 2003; Čolak et al., 2017). Once settled in the buccal cavity of a fish, the parasite is incapable of active migration to

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another host.

However, it is still unclear how these parasites find their mate. Mladineo (2003) reported that even when fish were exposed to super-infection by pulli II, only one pair of parasites was settled in the fish mouth. Finding parasite mates is demanding in open sea conditions, where visual contact is impossible and sound or chemical information is often weakened by distance (Thiel and Breithaupt, 2011).

Chemical signals are important for marine crustaceans, especially in parent–offspring communication, mate finding, and mate choice, which are all mediated by chemical signals. Chemicals seem to be ubiquitous messengers because they convey information such as sex, sexual receptivity, species identity, health status and molt stage (Thiel and Breithaupt, 2011; Wyatt, 2011).

In crustaceans, chemical signals can be released to the surrounding marine environment, as soluble or volatile pheromones, "distance pheromones". Urine is often, but not always, the carrier of this chemical signal, acting as a source of information on sexual maturation (Kamio et al., 2002; Thiel and Breithaupt, 2011). Females are generally the pheromone emitter and males the pheromone receiver. There is a strong pressure on the males to detect female pheromones and track down the female that usually remains stationary while signalling (Phelan, 1997).

However, knowledge about pheromones in general and its effects in marine invertebrates is very scarce. Up to date, the greatest knowledge of chemical communication comes from studies on decapod crustaceans including crabs, lobsters, and shrimps, while information on many other crustacean species remains unknown (Thiel and Breithaupt, 2011).

Since the existing data on pathogens in Mediterranean aquaculture is also sparse, including knowledge on mating behaviour of cymothoids, in this paper we have studied *C. oestroides* mating behaviour. Moreover, Mediterranean aquaculture is poorly and non-uniformly regulated, and future management measures should incorporate all available data on fish parasites that infect the whole production (Chapela-Perez and Ballesteros, 2011; Arechavala-Lopez et al., 2013).

It is suggested that *C. oestroides* finds its host by swimming, and by passive transport in marine currents, and the parasite attaches when it finds a suitable host fish (Mladineo, 2002). Therefore, the purpose of this study was to determine the pattern of *C. oestroides* infestation in sea bass to reveal if settlement of the male in the buccal cavity of fish is random. Furthermore, we attempted to determine whether female *C. oestroides* uses communication to find a mate. This study is also the first attempt, in a farming environment condition, to explain the mating behaviour of *C. oestroides,* which could help in the development of parasite traps, as well as in overall parasitic removal procedures and health management in aquaculture.

## 2. Materials and methods

This *in situ* experiment was carried in the Middle Eastern Adriatic Sea, at a farm operating since the 1980's with a long history of *C. oestroides* infestation on sea bass (Fig. 1). For the better control and prevention of *C. oestroides* infestation this farm implements the following procedures on a regular basis: separation of fish generations, exclusion of the infected individuals during vaccination procedures and use of antiparasitics. However, for this experiment only fish untreated with antiparasitics were used.

In the first phase of the experiment, all fish were introduced in one floating net cage (16 m diameter and 2000 $m^3$ volume; i.e., cage 1) in March 2018 and were monitored on a daily basis. The cage was stocked with 200 000 sea bass fingerlings of the same origin and age, with average mass 2.76 g. At the end of July 2018, all fish were removed from the cage for vaccination, and they were visually inspected. The prevalence of fish infected with *C. oestroides* was determined and infected fishes were separated from the cage. The parasite *C. oestroides* was identified according to Trilles (1972). On 100 randomly taken healthy fish samples (i.e., uninfected fish), total length and mass were measured. Afterwards total length was measured on sea bass infected by parasites (i.e., infected fish). Sex, length, maturity, and fecundity of the parasite were also recorded.

In the second phase, 410 infected and 208 uninfected sea bass were isolated. The fish (N = 618) were divided into six net cages (5 × 5 m diameter and 225 $m^3$ volume) located at the same location as the commercial floating net cages at a farm. These six net cages represented three experimental groups (each containing approximately 200 sea bass individuals) with two replicates (Table 1). Group 1 (C1 and C2) represented infected fish where female parasites were manually removed and male parasites were left in the buccal cavity of fish. Group 2 (C3 and C4) represented infected fish where both female and male parasites were removed. In order to evaluate fish predisposition for infestation with *C. oestroides*, group 3 (C5 and C6), which represented uninfected fish (i.e., control group), was added.

Mortality, sea surface temperature (SST) and oxygen were also recorded, and degree days (ºD) were calculated during the experiment. Sea bass were fed *ad libitum* with the same extruded commercial food during the experiment.

At the end of the experiment, 36 days after the 2nd phase, visual inspection of all fish was carried out. The *C. oestroides* were removed with tweezers. The total length (mm) of parasite was measured with an ichthyometer (precision 1 mm) following Čolak et al. (2017).

The sex of parasites was identified and sexual maturity and fecundity of *C. oestroides* females were determined according to the presence and number of larvae in the marsupium. Female parasites with larvae containing visible pigmented eyes were classified as sexually mature.

Prevalence of parasites in the total population of sampled fish was calculated for each cage according to Bush et al. (1997):

$$Prevalence = \frac{a}{a + b} \times 100$$

where $a$ = number of individuals in the sample with parasite; $b$ = number of individuals in the sample without parasites.

After the biometric data were collected statistical analyses were performed. Comparisons of parasite frequency on fish among cages were made using the chi square test, and p values were adjusted using the Bonferroni correction for multiple comparisons. Parametric analysis of variance on untransformed data was used to compare male and female parasite length and fish length among cages, and the Holm-Šídák test was used for post-hoc testing of pairwise differences. The criterion for statistical significance was $P < 0.05$.

## 3. Results

From March, during the first phase of experiment, when sea bass fingerlings were introduced in cage 1, to the end of July 2018, the average sea surface temperature (SST) was 17.7 °C, the duration was 2 566 °D, while oxygen level was always above 5 ppm.

In the second phase of the experiment (end of July 2018), during the vaccination procedure, average values of total length (cm) of infected and uninfected fish from cage 1, and total lengths of female and male parasite from infected fish were measured (Table 2). In total, prevalence of fish infected with *C. oestroides* was 0.51 %. The duration of the second phase of the experiment was 865.47 °D, while average SST was 23.4 °C.

Values are mean ± SD. Asterisk indicates a significant difference ($P < 0.05$) in lengths between uninfected and infected fish.

At the end of the experiment (in September) fish mortality was 10 % in C1, 17 % in C2, 11.88 % in C3, 9.17 % in C4, 10.68 % in C5 and 13.33 % in C6 (average mortality rate was 11.97 %), and there were no statistical differences in mortality between cages. There was no mature *C. oestroides* female found and therefore parasite fecundity was 0. A significant difference was found in prevalence of fish infected with the female parasite, among the experimental groups ($P < 0.05$). Fish from


**Fig. 1.** Study area.

**Table 1**
Experimental design.

| Fish (sea bass) from the same net cage (i.e., cage 1) | | | | | |
|---|---|---|---|---|---|
| Infected fish with *C. oestroides* | | | | Uninfected fish | |
| Group 1 - removed female parasites (N = 200) | | Group 2 - removed male and female parasites (N = 210) | | Group 3 – uninfected fish (N = 208) | |
| C1 | C2 | C3 | C4 | C5 | C6 |

**Table 2**
Total lengths of sea bass and *C. oestroides* at the beginning of the second phase of the experiment.

| | Length of fish (cm) | Length of female parasite (cm) | Length of male parasite (cm) |
|---|---|---|---|
| Uninfected fish | 14.40 ± 1.06* | – | – |
| Infected fish | 13.39 ± 1.06* | 1.52 ± 0.31 | 0.55 ± 0.16 |

group 1 (female parasites removed and males were left, C1 and C2) had a statistically higher number of female parasites compared to the group 2 and group 3 (cages where all parasites - both males and females - were removed and control group, C3–C6) ($P < 0.001$). No statistical difference was found in the number of fish with female parasites, between group 2 and group 3 (Fig. 2a).

A significant difference was also found in the prevalence of fish with male parasites among the experimental groups ($P < 0.001$) (Fig. 2b). At the end of the experiment, fish in cages with removed female parasites (group 1) had a statistically higher number of males compared to the cages where both male and female parasites were removed (C3 and C4; group 2) and the control group (C5 and C6; group 3).

A significant difference was also found in number of fish with parasites of both sexes (male and female, i.e., parasites in pair) compared to the fish with only one parasite sex (male or female) or fish without parasites (Fig. 3). At the end of the experiment, fish in cages with removed female parasites (C1 and C2; group 1) had a significantly higher number of parasites in pairs compared to the group where all parasites - both males and females - were removed, and the control

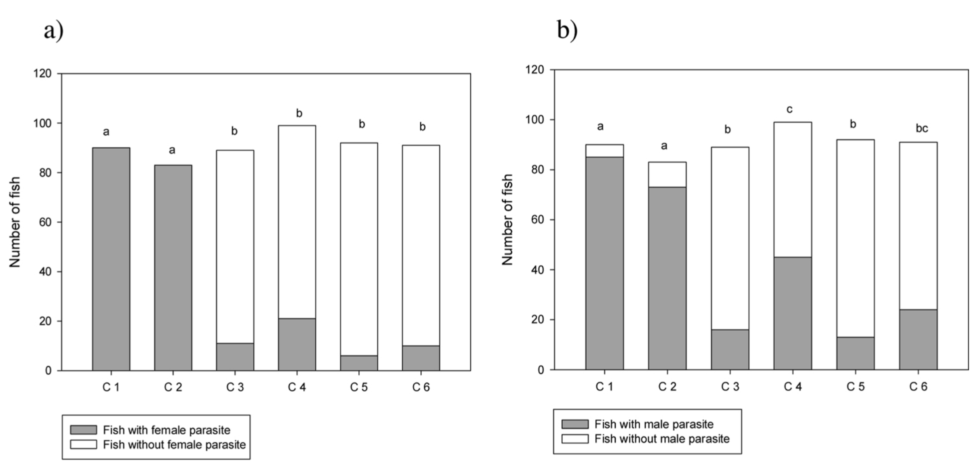


**Fig. 2.** Prevalence of female (a) and male (b) parasite *C. oestroides* in farmed sea bass after 865.47 °D (at the end of the experiment). Different letters indicate the statistically significant differences ($P < 0.001$) among cages.

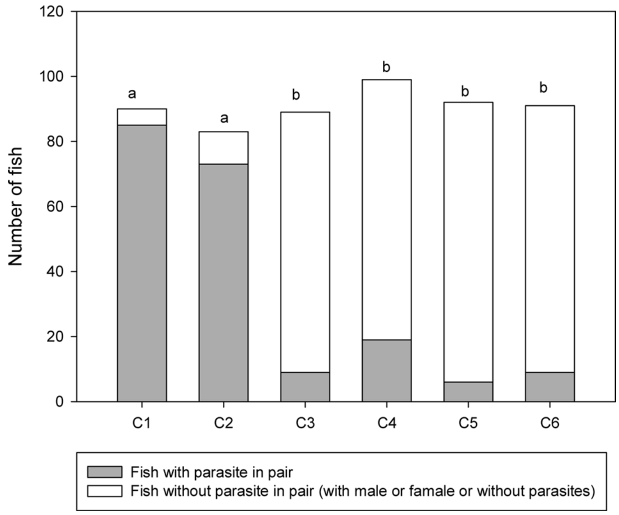


**Fig. 3.** Prevalence of *C. oestroides* pairs in farmed sea bass after 865.47 °D (at the end of the experiment). Different letters indicate the statistically significant differences (P < 0.001) among cages.

group (C3–C6; group 2 and 3) (P < 0.005). On the other side, there was no significant difference in the number of fish with only one parasite (male or female) or without parasites, between group 2 (C3 and C4) and group 3 (C5 and C6).

Length of removed female parasites at the beginning of the second phase showed no significant differences between groups 1 and 2 (C1, C2 *vs.* C3 and C4, respectively) (1.53 ± 0.29 cm – C1; 1.51 ± 0.23 cm – C2; 1.52 ± 0.31 cm – C3; 1.51 ± 0.35 cm – C4).

At the end of the experiment, total length of females from group 1 i.e., cages C1 (1.24 ± 0.2 cm) and C2 (1.27 ± 0.2 cm) were significantly higher than in the other groups (2 and 3; P < 0.05; 0.75 ± 0.36 cm – C3; 0.82 ± 0.24 cm – C4; 0.92 ± 0.29 cm – C5; 0.98 ± 0.37 cm – C6). There were no differences found in mean total length of female parasites between the cages from the same group (C1 compared to C2, and among the cages C3–C6; Fig. 4a).

No significant difference was found in the mean lengths of male parasites among groups (0.47 ± 0.15 cm – C1; 0.51 ± 0.15 cm – C2; 0.41 ± 0.14 cm – C3; 0.44 ± 0.11 cm – C4; 0.46 ± 0.18 – C5; 0.42 ± 0.19 cm – C6) (Fig. 4b).

Parasite sex ratios at the end of the experiment are shown in Table 3. Number of female parasites exceeded those of male in cages C1 and C2, while in cages C3–C6 males were dominant.

**Table 3**
Ratios of the number of sea bass with female/male *C. oestroides* after 865.47 °D.

| | C1 | C2 | C3 | C4 | C5 | C6 |
|---|---|---|---|---|---|---|
| Number of fish with female parasites | 90 | 83 | 11 | 21 | 6 | 10 |
| Number of fish with male parasites | 85 | 73 | 16 | 45 | 13 | 24 |
| Female/Male Ratio | 1.06 | 1.14 | 0.69 | 0.47 | 0.46 | 0.42 |

## 4. Discussion

In aquaculture, the control of parasites is an important goal for aquatic animal health management, due to significant economic losses. Farming technology has an influence on the composition of parasitic fauna on farms. Fish cultured in floating net cages are exposed to parasitic infections from wild and farmed populations (Fioravanti et al., 2006; Murray, 2009). *C. oestroides* is a crustacean isopod, living as a parasite in the natural marine environment and transmittable to farmed fish from wild fish (Šarušić, 1999). After its first occurrence in farmed fish, the conditions are fulfilled that also the farmed fish can be a source of infection of wild fish. These parasites live as a pair in the buccal cavity of fish. In open sea conditions, visual communication is blurred and challenging; hence not in the favour for parasite mate finding. Males must detect minor concentrations of female pheromones and localize a female, that usually remains stationary in the fish host while signalling (Phelan, 1997). Establishment of a parasite population within the fish farm area will occur if the net reproductive rate is greater than one, which depends on both the efficiency and success of their mating behaviour, and also on parasite prevalence (Peeler et al., 2007).

Biological knowledge of the host and its parasite is necessary, as well as the influence of the environment, for a better understanding of parasite sexual behaviour (Mladineo, 2003). In this research, we used fish with average length and weight of 14.4 ± 1.06 cm and 30.64 ± 6.69 g (mean ± SD), respectively. The main losses in *C. oestroides* infestation have been noticed in juvenile fish populations up to 20 g of weight (Šarušić, 1999; Horton and Okamura, 2001; Čolak et al., 2017) and at this weight we did not expect large losses due to parasitosis. However, mortality during this experiment was high (from 9.17 to 17.00 %) throughout the whole period of research, which may be due to handling of the fish during preparation of experimental groups, and during parasite removal. There was a significant difference found in mean total lengths of uninfected and infected fish, confirming the possibility of growth reduction of infected fish, even in specimens with average weight of 30 g (Šarušić, 1999; Horton and Okamura, 2001; Čolak et al., 2017).

In Mediterranean cage farming, the prevalence of fish infected with *C. oestroides* ranged from 1 to 68 % while in our study it was lower (0.5

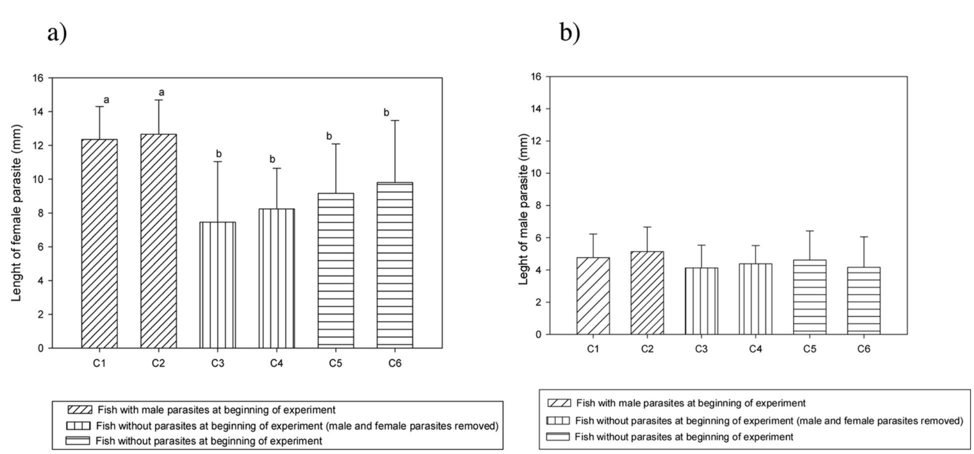


**Fig. 4.** Total length of female (a) and male (b) *C. oestroides* in farmed sea bass after 865.47 °D (at the end of the experiment). Different letters indicate the statistically significant differences (P < 0.001) among cages.

%) (Horton and Okamura, 2001; Mladineo, 2002). This difference in parasite prevalence was expected, since the farm where the experiment was done separates fish generations, excludes infected individuals during vaccination procedures, and uses antiparasitics.

Crustacean growth, including molting and reproduction, are dependent on temperature and photoperiod (Aiken, 1969). In our research at 2 566 °D, total lengths of female and male parasites were within the ranges observed in previous studies (Hadfield et al., 2016). At the end of the experiment, after 865.47 °D, no mature female parasites were present, which could imply that maturity and therefore fecundity of *C. oestroides* requires more degree days. Our observed positive correlations between parasite total length and degree days, as well as between total length and fecundity of *C. oestroides,* were previously reported (Kolega et al., 2018).

Most research on crustacean sexual behaviour has been conducted in controlled laboratory conditions (Thiel and Breithaupt, 2011). Although *C. oestroides* is a large parasitic isopod, lives in pairs, and therefore is very appropriate for mating behaviour studies, it and other cymothoides are still understudied in both the laboratory and natural environment (Hadfield et al., 2016). Hence, our study was performed *in situ* (within farming conditions) to determine whether female *C. oestroides* uses communication to find a mate. At the end of the experiment, significantly more fish had a female parasite in group 1 (group with only male parasites left in fish at the beginning of the experiment) than in groups 2 or 3 (both groups without parasites). This suggests that *C. oestroides* males (which remained in fish in group 1) develop into females in the absence of females, which was also found earlier (Raibaut and Trilles, 1993). No statistical difference was found in the number of fish with female parasites between group 2 (cages where all parasites were removed from fish at the beginning of experiment) and group 3 (uninfected fish - control group), indicating that there is no evidence of sea bass susceptibility to this parasite, which contradicts an earlier suggestion (Horton and Okamura, 2001).

At the end of the experiment, mean total lengths of female parasites from group 1 were significantly higher than in the other groups (2 and 3). This was expected since females from group 1 were the only parasite present at the beginning of the experiment, although they were males at that time. There was no difference found in mean total lengths of male parasites from all experimental groups, indicating that they settled on their host at similar times.

Female parasites from group 1, which were larger and older, had more success in mate finding. Therefore, fish from group 1 had a significantly higher number of parasite pairs compared to the groups without parasites (group 2 and 3). This result suggests that the female parasite uses some method of communication to attract the pulli II, i.e. the future male parasite. This attraction enables successful mate finding, necessary for reproduction. For future research it would be interesting to found out the minimum degree days until *C. oestroides* completes its reproductive cycle.

Marine and freshwater crustaceans communicate via chemical substances. However, besides the lack of *in situ* experiments with crustacean parasites, most studies on chemical communication come from research on decapod crustaceans, including crabs, lobsters, and shrimps (Thiel and Breithaupt, 2011; Wyatt, 2011). In addition, research on crustacean pheromone disruption in marine ecosystems is still very scarce, but is important and needed for sustainable management in aquaculture as well as for understanding ecological consequences of anthropogenic disturbances of aquatic ecosystems (Thiel and Breithaupt, 2011; Wyatt, 2011). Furthermore, Thiel and Breithaupt (2011) found that pheromones, when identified, may be artificially synthesized to be used in mass attraction of target organisms. For aquaculture health management, this would be very useful, since then certain parasites could be lured into traps (Mordue and Birkett, 2009).

## 5. Conclusion

Fish in cages with male parasites (group 1) had significantly more parasites in pairs at the end of the experiment than the group where all parasites - both males and females - were removed, and than the control group. This suggests that males from group 1 become female parasites, and being larger and older, had more success in mate finding. Results of our study indicate that the female parasite uses some method of communication to attract the pulli II, i.e. the future male parasite, which enables successful mate finding. Considering the lack of parasite biology studies *in situ*, data on reproduction and mating behaviour of *C. oestroides* could help in the development of overall parasitic removal procedures and health management in sea bass aquaculture.


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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**Slavica Čolak:** Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Danijel Mejdandžić:** Formal analysis, Investigation, Writing - original draft. **Toni Števanja:** Formal analysis, Investigation, Writing - original draft. **Matko Kolega:** Formal analysis, Investigation, Writing - original draft. **Renata Barić:** Formal analysis, Investigation, Writing - original draft. **Tomislav Šarić:** Formal analysis, Writing - original draft, Writing - review & editing. **Bruna Petani:** Writing - original draft, Writing - review & editing. **Ivan Župan:** Writing - original draft, Writing - review & editing. **Bosiljka Mustać:** Writing - original draft, Writing - review & editing.


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