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Authors: Musil, Martin, Let, Marek, Riebel, Martin, Balzani, Paride, and Kouba, Antonín

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Non-native three-spined stickleback, a small but voracious predator of invasive crayfish

Martin MUSIL¹ , Marek LET¹ , Martin RIEBEL^{1,2} , Paride BALZANI¹  and Antonín KOUBA^{1*} 

¹ University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodňany, Czech Republic; e-mail: musilm@frov.jcu.cz, mlet@frov.jcu.cz, balzani@frov.jcu.cz, akouba@frov.jcu.cz

² Palacký University Olomouc, Faculty of Science, Department of Ecology and Environmental Sciences, Olomouc, Czech Republic; e-mail: riebmar@seznam.cz

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Abstract. Numerous fish and crayfish species are invasive in freshwater ecosystems, where they interact. In this study, we performed two experiments to investigate adult three-spined stickleback *Gasterosteus aculeatus* predation on early juvenile invasive crayfish in Europe. The first experiment focused on evaluating predation upon early juveniles of three invasive species (the marbled crayfish *Procambarus virginalis*, the spiny-cheek crayfish *Faxonius limosus*, and the signal crayfish *Pacifastacus leniusculus*) with varying exposure times (one, three and six hours), revealing crayfish species-specific vulnerabilities and the role of exposure duration. Marbled and spiny-cheek crayfish juveniles were more susceptible to three-spined stickleback predation than signal crayfish. Nevertheless, larger signal crayfish suffered more damage caused by the fish predator. The second experiment assessed the role of size in predation efficiency, using three different size groups of marbled crayfish juveniles as prey of adult three-spined sticklebacks during three hours of exposure. In this second experiment, we found the predation level to be size-dependent, with the smallest group of juveniles (20–80 mg) being preyed upon the most, and the largest group (250–350 mg) the least. The efficient also predation of the three-spined stickleback on large juvenile crayfish underscores its potential ecological impacts also on native crayfish.

Key words: behavioural ecology, biological invasion, non-native crayfish, non-native fish, predation

Introduction

Numerous studies on the interactions between fish and crayfish have been conducted (Dorn & Mittelbach 1999, Nyström et al. 2006). In general, it has been reported that fish predate on different sizes of crayfish, compete for resources (e.g. food or shelter), and inhibit crayfish foraging behaviour. In addition, crayfish prey on fish eggs and juveniles and compete for resources (Reynolds 2011). Several invasive species of fish and crayfish are recognised to represent a severe threat to freshwater ecosystems

(van der Veer & Nentwig 2015, Soto et al. 2023a), potentially with socioeconomic impacts (Kouba et al. 2022, Soto et al. 2023b), and with implications for fish and crayfish interactions.

The three-spined stickleback *Gasterosteus aculeatus* Linnaeus, 1758 is a fish with a Holarctic native distribution, where it lives in coastal waters, river mouths, streams, and lakes (Hureau 1986) and is thus one of the most widespread freshwater fish species in the northern hemisphere (Fang et al. 2018). In some European countries such as the Czech

* Corresponding Author

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Republic, Switzerland and some inland areas in Italy, France, Spain, Germany, and Poland, this species is considered as invasive (Mäkinen et al. 2006, Clavero et al. 2009, Lucek 2016, Gugele et al. 2020). The three-spined stickleback is an active predator whose hunting activity spans from early morning hours throughout the day (Bretzel et al. 2021). Its exceptional predatory capabilities are further enhanced by its ability to feed in darkness, which is uncommon among freshwater fishes (Mussen & Peeke 2001). European populations commonly exceed a standard length of 50 mm (Kováč et al. 2002, Leinonen et al. 2011), and it is classified as a mesopredator. It chiefly consumes zooplankton, subsequently promoting the growth of phytoplankton or macroscopic algae and exerting top-down effects on the trophic web of lakes and lagoons (Norlin et al. 2005, Harmon et al. 2009). Furthermore, moderate eutrophication facilitates three-spined stickleback population growth due to higher food availability and reduced predation pressure (Jakobsen et al. 2004).

Despite their relatively small size, the three-spined stickleback can impact populations of larger fish species, primarily through competition for food and predation on their eggs and fry (Baer et al. 2021, Bretzel et al. 2021). An illustrative example of competition for food can be observed in Lake Constance, where the introduced three-spined stickleback competes for zooplankton, reducing the numbers of native Wartmann's whitefish *Coregonus wartmanni* (Bloch, 1784) (Rösch et al. 2018). Moreover, three-spined sticklebacks predate on juveniles of both coastal predatory fish in the Baltic Sea (Bergstrom et al. 2015) and on native predatory fish in inland waters, such as the Eurasian perch *Perca fluviatilis* Linnaeus, 1758 and northern pike *Esox lucius* Linnaeus, 1758 (Bystrom et al. 2015). The growth and survival of the juveniles of two vulnerable sturgeon species, the Persian sturgeon *Acipenser persicus* Borodin, 1897 and the Russian sturgeon *Acipenser gueldenstaedtii* von Brandt & Ratzeburg, 1833, are under pressure for growth and survival from three-spined sticklebacks in the Caspian Sea (Niksirat et al. 2010).

A recent study investigating the trophic relationships at localities where several invasive crayfish species living in syntropy with three-spined sticklebacks showed that this fish occupied a top predator position there (Veselý et al. 2021). However, there is little information available on the predation of three-spined sticklebacks on freshwater crayfish, as observed for other invasive mesopredator fish species, such as the round goby *Neogobius*

melanostomus (Pallas, 1814) (Franta et al. 2021, Roje et al. 2021). Therefore, we conducted two experiments to elucidate these trophic relationships. In the first experiment, we evaluated the predation of adult three-spined sticklebacks on early juveniles of three different crayfish species (the spiny-cheek crayfish *Faxonius limosus* (Rafinesque, 1817), the marbled crayfish *Procambarus virginalis* Lyko, 2017, and the signal crayfish *Pacifastacus leniusculus* (Dana, 1852)) over three different exposure times (one, three and six hours). We aimed to assess which juvenile crayfish species are more prone to predation and the role of exposure time to the predator in this interaction. We hypothesised that juveniles of all tested crayfish species are suitable prey for the three-spined stickleback and that increased exposure to the predator increases the likelihood of predation. In the second experiment, we assessed the predation of three size groups of marbled crayfish juveniles by adult three-spined sticklebacks during a single exposure time (three hours). We hypothesised size-dependent predation rates for this latter experiment, with none or little predation on the largest size group.

Material and Methods

Experimental animals and husbandry

Three-spined sticklebacks were captured from a small pond near Bavorov, Czech Republic (49°07'34.5" N, 14°03'48.4" E), using a small fishing net. Crayfish are absent from this site, thus providing crayfish-naïve individuals. The fish were transferred to a common aquarium (medium size), where they were housed and fed *ad libitum* with defrosted chironomid larvae until the beginning of the experiments.

Marbled crayfish were obtained from our indoor laboratory culture kept at the experimental facility of the Research Institute of Fish Culture and Hydrobiology in Vodňany, University of South Bohemia in České Budějovice (49°09'14.8" N, 14°10'08.7" E). Ovigerous females of the signal crayfish and spiny-cheek crayfish were collected from the Křesanov brook near Vimperk (49°03'35.3" N, 13°45'33.4" E) and the River Vltava in České Budějovice (48°58'35.2" N, 14°28'01.1" E), respectively. Each female was housed separately within enclosed drinking water systems, ensuring they were protected against direct and indirect exposure to fish predators and their odours. Crayfish were fed *ad libitum* daily with defrosted chironomid larvae and grated carrots.

Only crayfish juveniles with intact appendages (pereiopods and chelae) and fully hardened

exoskeletons (intermoult stage) were used for the experiments. They were sorted by species in separated recirculating water systems under a light:dark regime of 14:10, at a temperature of 18 °C, and oxygen concentration > 8 mg l⁻¹ for acclimatization to experimental conditions.

Experimental setup

The standard length (SL, from the tip of the snout to the base of the caudal peduncle) of each experimental fish was measured with a ruler to the nearest 1 mm. Similar-sized three-spined stickleback adults (SL range = 40-50 mm, considered as adults based on the findings of Baker et al. (2008)) were starved for 24 hours before each trial to standardize their hunger level. They were placed individually into the experimental arenas (10 l black plastic buckets) one hour before prey were added to allow for acclimatization. All experimental crayfish juveniles were weighed using a digital precision scale (Kern 572-35, Kern and Sohn, Germany).

The arenas were filled with 7 l of dechlorinated tap water, maintained at 18 °C, and dissolved O₂ exceeding 8 mg l⁻¹ throughout the experiments. The light regime was a natural ambient of 500 lux. The arenas were enriched with plastic plants to provide a refuge for predators and prey (Gebauer et al. 2019). The position of each arena in the experimental room was randomised before the start of each experiment to eliminate potential external biases. At the end of the experiments, we counted the number of crayfish juveniles that survived, were eaten, killed, or had died, based on the methodology of Veselý et al. (2019). Specifically, we defined as survived those crayfish juveniles that remained alive and intact, without missing claws or walking legs, killed as those that did not survive and exhibited missing appendages, and dead as those without apparent physical injury, and eaten as the difference between the original number of individuals and the sum of the above-described categories. After every trial, the water was changed to remove odour cues that might have affected subsequent trials. For each tested group, a control group was also assigned to monitor the background mortality of juvenile crayfish without predators.

Experiment 1: predation on early juveniles of different crayfish species over time

For this experiment, we used three-spined sticklebacks with SL = 44.7 ± 2.8 mm (mean ± SD). After predator acclimatization, five naïve early juveniles of either marbled, spiny-cheek or signal crayfish were

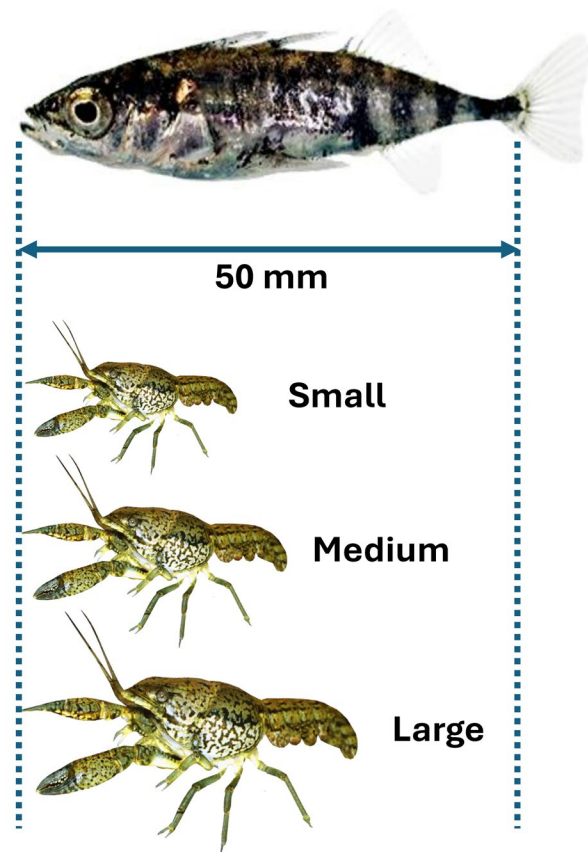


Fig. 1. A schematic comparison of the sizes of the three-spined stickleback as a predator and upper ranges of the three size groups of marbled crayfish prey according to the actual scale in Experiment 2. The weights of the individual groups were Small (W = 20-80 mg), Medium (W = 100-200 mg), and Large (W = 250-350 mg). Note that crayfish sizes were proportionally smaller in Experiment 1.

assigned to three different exposure times (one, three and six hours). Their average weight per individual derived from a bulk weighing of 100 animals was 11.28 mg for the marbled crayfish, 12.52 mg for the spiny-cheek crayfish (Stage 3 juveniles at the onset of independence for both species), and 22.39 mg for the signal crayfish (Stage 2 juveniles at the onset of independence; Kouba et al. 2021). The number of replicates in tested groups varied according to the number of available crayfish juveniles. We had 30 replicates for marbled crayfish, 17 for spiny-cheek crayfish, and 13 for signal crayfish. These numbers were consistent for each species across exposure times.

Experiment 2: predation on three size groups of marbled crayfish juveniles

For this experiment, we used three-spined sticklebacks with SL = 48.0 ± 1.9 mm. We divided the marbled crayfish juveniles into three size groups. We attempted to visually select abundant groups of similar-sized juvenile crayfish available in our laboratory stock, providing a sufficient size range

of tested groups. These were further adjusted by removing the smallest and largest individuals, making the test groups as consistent as possible. The resulting weight (W) classes were as follows: Small (W = 20-80 mg), Medium (W = 100-200 mg), and Large (W = 250-350 mg), as illustrated in Fig. 1. After predator acclimatization, five naïve juveniles of marbled crayfish from either size group were released into the arena. The experimental time was fixed at three hours according to the outcome of the previous experiment (predatory interactions were limited after this time). For each group, 30 replicates were completed.

Data analysis

We considered four ordinal response variables ordered as follows: eaten = dead (yes), killed (yes), eaten (yes); killed = dead (yes), killed (yes), eaten (no); dead = dead (yes), killed (no), eaten (no); survived = dead (no), killed (no), eaten (no). For the first experiment, the differences in the response variables among crayfish species and the effects of fish SL and exposure time (both used as numerical variables) were tested using a Cumulative Link (Mixed) Model – CL(M)M by Likelihood Ratio Test (LRT) (Christensen & Christensen 2015). We also tested the significance of first- and second-order interactions of crayfish species, fish SL, and exposure time by the same method for comparing models with different complexity. All numerical explanatory variables were centred before modelling and testing interactions. The arena identity and the time of the trials were also tested as random effects on the intercept.

For the second experiment, data were analysed using the same approach, with crayfish size and fish SL as predictors. All statistical analyses were performed in R version 4.1.3 (R Core Team 2022). Raw data and R script are available in Table S1 and Appendix S1.

Results

Experiment 1: predation on early juveniles of different crayfish species over time

We observed no dead juvenile crayfish for all control groups, suggesting limited intra-specific interactions. Therefore, we excluded these results from further comparisons between tested groups. Adult three-spined sticklebacks consumed early juveniles of all experimental crayfish species (Fig. 2). Significant differences between crayfish species in the ordinal response variables (eaten, survived, killed, and dead) were detected ($\chi^2_2 = 143.26$, $P < 0.001$), as well as

two significant additive relationships between these responses and fish SL with exposure time ($\chi^2_1 = 16.40$, $P < 0.001$ and $\chi^2_1 = 16.11$, $P < 0.001$, respectively; Fig. 2). However, first- or second-order interactions of the given fixed-effect predictors were not significant ($P \geq 0.161$). The arena identity was a highly significant random-effect factor ($P < 0.001$), while the specific time of trials had only a marginal effect ($P = 0.054$) on the CLMM fit. Thus, the latter was omitted in the final model.

The probability of being eaten increased, and survival decreased with increasing fish SL and longer exposure time, with the same magnitude in all three crayfish species. The bigger signal crayfish juveniles showed significantly greater resistance to predation compared to the marbled and spiny-cheek crayfish, which did not differ from each other (considering the 95% confidence intervals – CIs; Fig. 2). Nevertheless, signal crayfish juveniles suffered significantly higher mortality with increasing fish SL and longer exposure time by either being killed or dying compared to the other two species (Fig. 3).

Experiment 2: predation on three size groups of marbled crayfish juveniles

We observed no dead juvenile crayfish for all control groups, suggesting limited intra-specific interactions. Therefore, we excluded these results from further comparisons between tested groups. Significant differences between the size groups (small, medium, and large) of marbled crayfish juveniles in the ordinal response variables were detected after three hours of exposure to three-spined stickleback ($\chi^2_2 = 81.87$, $P < 0.001$). Nevertheless, fish SL and the interaction between fish SL and crayfish size group had no significant effects ($\chi^2_1 = 0.01$, $P = 0.913$ and $\chi^2_2 = 1.48$, $P = 0.478$, respectively). The variance in stickleback SL was two times lower than in Experiment 1 (3.70 and 7.61, respectively). None of the two tested random-effect factors improved the CLM significantly ($P = 1.00$). Significant differences ($P < 0.05$) were detected between each size group (tested by Tukey *post-hoc* test); however, not within each ordinal response variable (according to 95% CIs; Fig. 4). Large marbled crayfish juveniles showed more than 95% mean survival probability (with the range of 95% CIs 0.91-0.98%) and the lowest probability of being eaten (only 0.02% with the range of 95% CIs 0.01-0.05%; Fig. 4). Simultaneously, differences between large and medium juveniles within the killed and dead groups as response variables were not significant ($P > 0.05$ according to 95% CIs; Fig. 4).

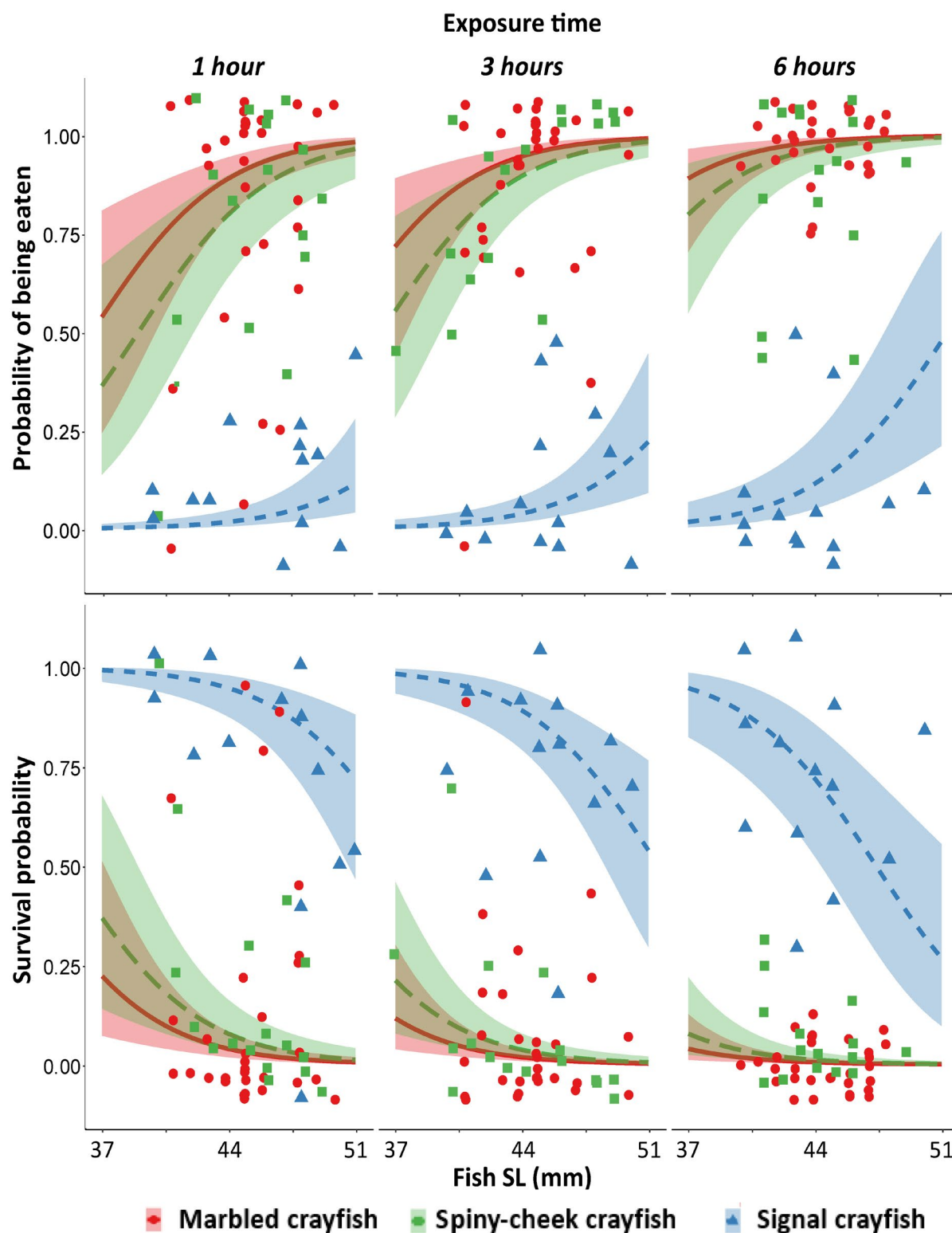


Fig. 2. Logistic regression using Cumulative Link Mixed Models (CLMM) showing differences between early juveniles of tested species – marbled crayfish *Procambarus virginalis* (red), spiny-cheek crayfish *Faxonius limosus* (green), and signal crayfish *Pacifastacus leniusculus* (blue) in the probability of consumptive mortality (upper half) caused by adults of the three-spined stickleback *Gasterosteus aculeatus* in the experimental arenas across the time (one, three and six hours). Survival probability (lower half) was fitted with the same regression model. Five juveniles of each tested crayfish were constantly exposed to one three-spined stickleback. Dot symbols (scattered by a jitter function) represent individual probabilities for each observation, and shaded areas represent 95% confidence regions. Overlaps of these regions indicate non-significant differences ($P > 0.05$).

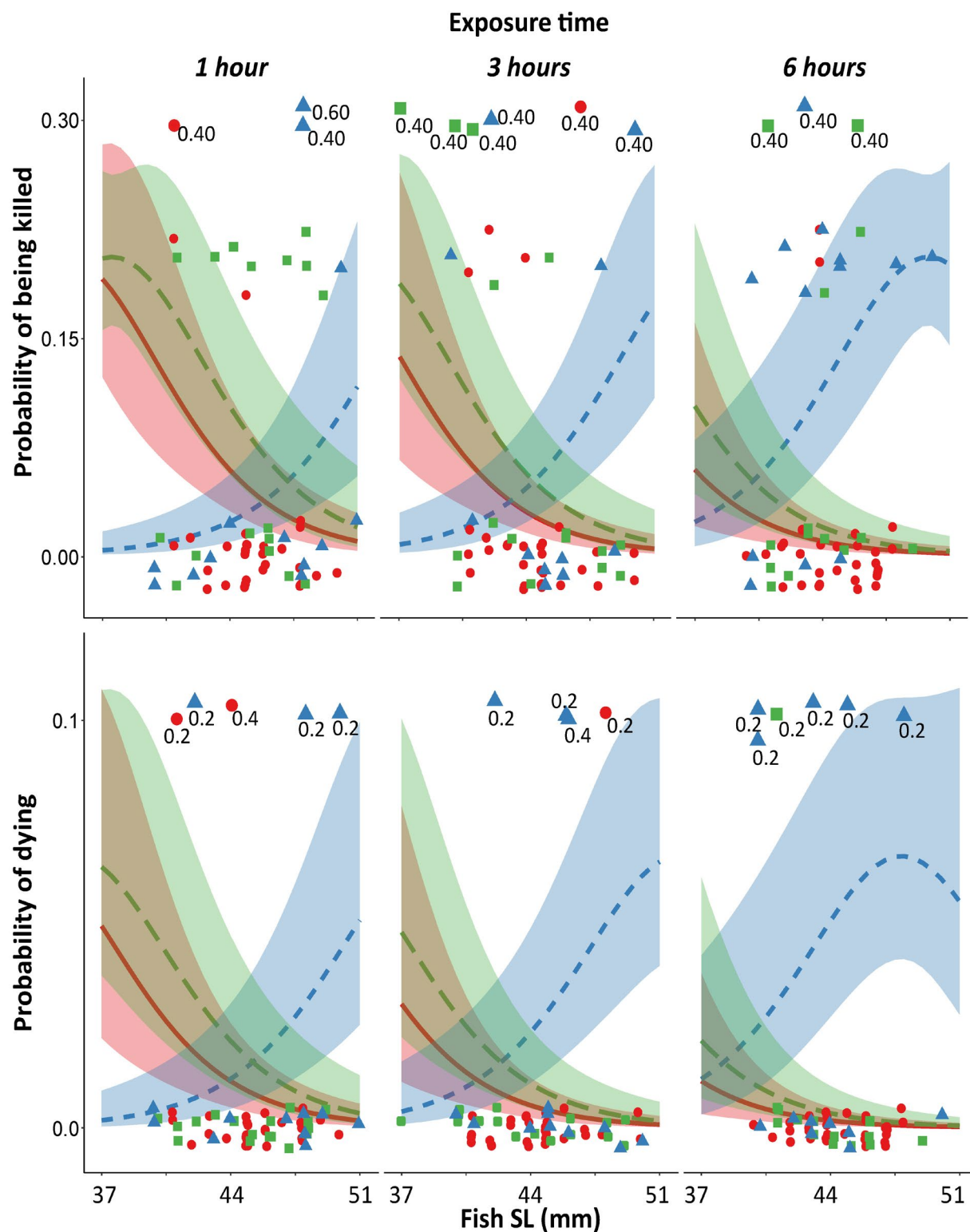


Fig. 3. Logistic regression using Cumulative Link Mixed Models (CLMM) showing differences between early juveniles of tested species – marbled crayfish *Procambarus virginalis* (red), spiny-cheek crayfish *Faxonius limosus* (green), and signal crayfish *Pacifastacus leniusculus* (blue) in the probability of specific mortality (upper half) caused by adults of the three-spined stickleback *Gasterosteus aculeatus* in the experimental arenas across the time (one, three and six hours). The mortality probability (killed or dead individuals; lower half) was fitted by the same regression model. Five juveniles of each tested crayfish were constantly exposed to one three-spined stickleback. Dot symbols (displaced with a 'jitter' function) represent individual probabilities for each observation, and shaded areas represent 95% confidence regions. The enlarged dot symbols with their specific high probability values are displayed in the line of the highest values of y-axes for better visualization of the relationships. Overlaps of confidence regions indicate non-significant differences ($P > 0.05$).

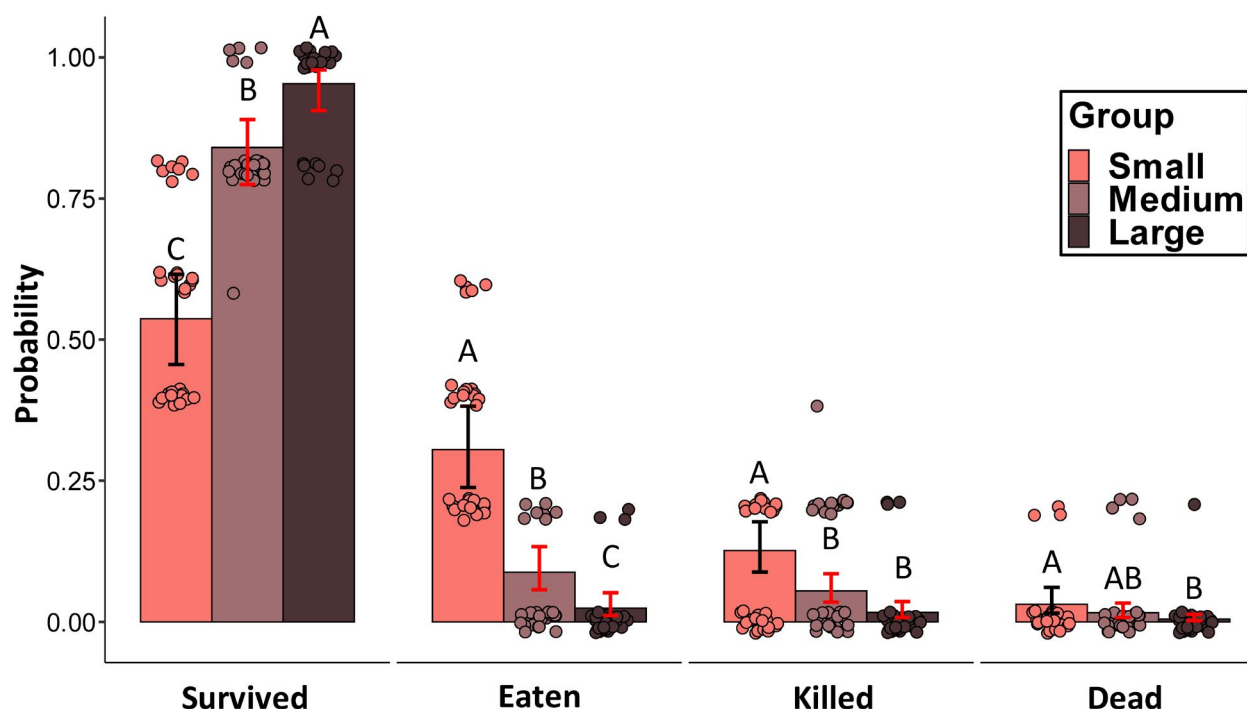


Fig. 4. Logistic regression using Cumulative Link Models – CLM showing differences between juveniles of marbled crayfish *Procamburus virginalis* in three size groups (small, medium, and large) in the probability of survived, eaten, killed, and dead in the presence of the three-spined stickleback *Gasterosteus aculeatus* in the experimental arenas for three hours. Five juveniles of each tested crayfish size group were exposed to one three-spined stickleback. Bars represent estimated probabilities, and ‘whiskers’ represent 95% confidence intervals. Overlaps of lower/upper 95% confidence intervals indicate non-significant differences between estimates ($P > 0.05$). Black-contoured circles represent the proportion of juveniles in arenas for each given ordinal response on the x-axis.

Discussion

Experiment 1: predation on early juveniles of different crayfish species over time

In this study, we assessed the predation of adult three-spined sticklebacks on juveniles of three invasive crayfish: the marbled, spiny-cheek, and signal crayfish. We found that marbled and spiny-cheek crayfish early juveniles were more susceptible to predation than signal crayfish juveniles.

Notably, no dead individuals were observed among marbled and spiny-cheek crayfish throughout the six-hour experimental period compared to signal crayfish. This finding implies that the highest predation rate was directed towards marbled crayfish and spiny-cheek crayfish rather than signal crayfish juveniles. We found that there was an increase in the probability of being eaten or killed as exposure time increased. Most predatory interactions occurred within the first hour of exposure. Then, predator digestion and eventual further consumption would play a much larger role (further discussed below). However, there was no increased likelihood of predation on the signal crayfish juveniles over time, likely due to the satiation of the three-spined stickleback after the

initial attack(s), during which almost twice as much biomass (considering the average weights of juveniles) was consumed compared to the other two crayfish species. Additionally, this lower predation may indicate a learned behaviour from failed attacks, with predators waiting for a better opportunity to ambush prey. This finding is supported by the significantly higher percentage of killed signal crayfish juveniles compared to the other two crayfish species.

Despite suffering the lowest predation rate and thus the lowest probability of being eaten, juvenile signal crayfish had a significantly higher probability of being killed or dying than the other crayfish species. This result suggests that, despite the rapid growth potential of marbled crayfish (Kouba et al. 2021), the signal crayfish’s robust carapace and larger chelae provide a distinct advantage against a fish predator (Kouba et al. 2011).

With the increasing length of the fish predator, the probability of consumption of all crayfish species juveniles increased, as well as the likelihood of being attacked (the killed group) in the signal crayfish. On the other hand, for marbled and spiny-cheek crayfish juveniles, the probability of being killed decreased



with increasing fish length, as larger predators can readily swallow smaller prey whole. Therefore, the relative size of juvenile crayfish and the fish predator played a significant role in the predatory interactions (Dörner & Wagner 2003, Aquiloni et al. 2010).

Differences in digestibility of the crayfish carapace, which is less digestible in signal crayfish than in the other two species, can also affect predation rates by three-spined sticklebacks. A similar effect has been found for planktonic crustaceans (Sutela & Huusko 2000). For instance, the less digestible hard parts of the shell of *Bosmina* sp. remained in the three-spined sticklebacks' digestive tract for a longer time, and a preference due to better digestibility has been found for the genus *Daphnia*, besides its larger size (Bretzel et al. 2021).

Despite similar sizes of juvenile marbled crayfish and spiny-cheek crayfish, the latter could represent a more challenging prey for fish predators, due to the more robust claws and cephalothorax carrying protective spines (Hossain et al. 2019). This observation is supported by the results of Linzmaier et al. (2018), who showed that marbled crayfish frequently exhibited freezing behaviour in response to a simulated threat, while spiny-cheek crayfish responded offensively or defensively.

Experiment 2: predation on three size groups of marbled crayfish juveniles

The follow-up experiment revealed that three-spined sticklebacks were even capable of preying on large marbled crayfish juveniles, with probabilities of up to 40% with juvenile crayfish in the small-size group, but also consuming individuals from the largest size group, though at a lower rate. In a previous study, marbled crayfish juveniles served as prey for the round goby (Roje et al. 2021), which possesses a considerably larger mouth gape than the three-spined stickleback in our experiments. Therefore, our results showed a considerable capability of three-spined stickleback to prey upon juvenile crayfish of similar size compared to the study by Roje et al. (2021), highlighting the differential predatory efficiency between these two invasive predators concerning their respective sizes and weights (with three-spined stickleback being considerably slimmer).

We detected significantly different survival probabilities between all juvenile marbled crayfish size groups, with the highest survival probability in the large and the lowest in the small group.

Large marbled crayfish juveniles avoided predation by three-spined sticklebacks after three hours of exposure, probably due to satiation in the fish predator. Handling smaller prey is probably also easier for the predator, aligning with the results of Experiment 1. Predators feeding on medium and large prey could have been satiated with only a few individuals or even parts of them. This idea is supported by findings that stomach capacity is crucial for prey selection and consumption in three-spined sticklebacks (Gill & Hart 1994), which can tear prey into smaller fragments before consumption (Beukema 1968, Allen & Wootton 1984, Gill & Hart 1994). Although we focused on individual predation only, it is expected that the predatory success of three-spined sticklebacks will be further enhanced by their schooling behaviour, which facilitates easier prey detection and coordinated attacks, eventually allowing them to target more demanding prey items in natural settings (Harcourt et al. 2009; pers. observ.).

Possible predation on native European crayfish species

The size at the time of first independence, which occurs in the second developmental stage in native European crayfish species (Astacidae; Kawai & Kouba 2022), is a critical factor in assessing their potential predation by the three-spined stickleback. Independent juveniles of the most widespread European astacid – the stone crayfish *Austropotamobius torrentium* (von Paula Schrank, 1803), weighing around 28 mg, and especially the noble crayfish *Astacus astacus* (Linnaeus, 1758) and the narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823), both commonly exceeding 35 mg – are bigger than the largest juveniles in Experiment 1, including the signal crayfish. However, intraspecific variance can also be substantial (cf. Kanta 2007 and Kouba et al. 2010). Despite this, juveniles of all species fell within the ingestible range of the three-spined stickleback, as even marbled crayfish from the largest size group were consumed during Experiment 2.

Considering the current distribution of European crayfish species (Kouba et al. 2014) and the three-spined stickleback (Mäkinen et al. 2006), we anticipate possible distributional overlaps. Despite not frequently co-occurring in their respective distributions, populations of the noble and narrow-clawed crayfish, and native or non-native populations of three-spined stickleback potentially overlap across a significant portion of Europe (Mäkinen et al. 2006, Kouba et al. 2014, Veselý et al. 2021). In

some countries, such as Poland (Kopij & Szymczyk 2024) and Hungary (Weiperth et al. 2020, 2022), syntropies have already been documented. The stone crayfish usually occurs in habitats unsuitable for the three-spined stickleback. Nevertheless, these two species have documented co-occurrences in streams connected to Lake Constance in southern Germany (Renz & Breithaupt 2000). It is, therefore, reasonable to expect that some populations of stone crayfish inhabiting tributaries of lakes, dams, or ponds may face a threat from the expansion of the three-spined stickleback into these tributaries.

Conclusions

We found that early juveniles of all tested crayfish species were suitable prey for adult three-spined sticklebacks, with signal crayfish juveniles being significantly less susceptible to predation than marbled and spiny-cheek crayfish. The expected relationship between predator exposure time and the number of crayfish that were eaten, killed or died was approved. In addition we observed a significant relationship between predation probability and three-spined stickleback size. Our results also demonstrated that three-spined sticklebacks can successfully prey on relatively large crayfish despite their small size, potentially posing a threat to both non-native

and native crayfish species at invaded localities. Understanding these mechanisms is crucial for evaluating potential cascading effects on freshwater ecosystems where invasive fish and crayfish coexist and contributes to a broader understanding of fish and crayfish interactions in the context of biological invasions.

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Author Contributions

M. Musil: conceptualization, methodology, validation and formal analysis, investigation, resources, data curation; M. Let: validation and formal analysis, visualization, data curation, software; M. Riebel: conceptualization, validation and formal analysis, investigation, data curation; P. Balzani: validation and formal analysis; A. Kouba: conceptualization, methodology, resources, funding acquisition. All authors reviewed, edited, and revised the manuscript.

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Supplementary online material

Table S1. Raw data from Experiment 1 and Experiment 2 (<https://www.ivb.cz/wp-content/uploads/JVB-vol.-73-2024-Musil-M.-et-al.-Table-S1-1.xlsx>).

Appendix S1. R Script on statistical analyses of data from Experiment 1 and Experiment 2 (<https://www.ivb.cz/wp-content/uploads/JVB-vol.-73-2024-MusilM.-et-al.-Appendix-S1-1.pdf>).