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Associative learning in male rusty crayfish (Orconectes rusticus): conditioned behavioral response to an egg cue from walleye (Sander vitreus)

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Associative learning in male rusty crayfish (Orconectes rusticus): conditioned behavioural response to an egg cue from walleye (Sander vitreus)


Abstract: Chemical communication governs a diversity of life processes in aquatic organisms. Crayfish use chemoreception during reproduction, social hierarchy formation, predation avoidance, and resource localization. Fish eggs release recognizable chemoattractants for vertebrate predators of eggs that can motivate crayfish to engage in egg predation as well. We hypothesized that male rusty crayfish (Orconectes rusticus (Girard, 1852)) from a lake free of walleye (Sander vitreus (Mitchill, 1818)) would not possess an innate recognition of a walleye egg cue. However, if conditioned by employing a single 2 h paired stimulus exposure (known food cue + egg cue), then male rusty crayfish would be attracted to the same egg cue upon subsequent exposure. Using a Y-maze behavioural arena we discovered that once conditioned, crayfish took significantly less time to choose the arm containing the egg cue alone relative to a control. Our study suggests that male rusty crayfish exhibit second-order conditioning through associative learning, allowing them to quickly and easily learn to identify novel odour stimuli from fish eggs under laboratory conditions.

Résumé : La communication chimique contrôle une diversité de processus vitaux chez les organismes aquatiques. Les écrevisses utilisent la chimioréception durant la reproduction, la formation de la hiérarchie sociale, l’évitement des prédateurs et la recherche des ressources. Les œufs de poissons émettent des composés chimiques d’attraction perceptibles par les prédateurs d’œufs, qui peuvent aussi inciter les écrevisses à procéder à la prédation des œufs. Nous émettons l’hypothèse selon laquelle un mâle de l’écrevisse à taches rouges (Orconectes rusticus (Girard, 1852)) provenant d’un lac sans doré jaune (Sander vitreus (Mitchill, 1818)) ne possède pas de reconnaissance innée du signal d’un œuf de doré. Cependant, une fois conditionné par une seule expositions de 2 h à des stimulus appariés (soit un signal connu de nourriture + un signal d’œuf), le mâle d’écrevisse à taches rouges devrait être attiré par ce même signal de l’œuf lors d’une exposition subséquente. Dans une arène de comportement avec un labyrinthe en Y, l’écrevisse, une fois conditionnée, prend significativement moins de temps à choisir la branche qui contient uniquement le signal de l’œuf par comparaison à un témoin. Notre étude laisse croire que le mâle d’écrevisse à taches rouges affiche un conditionnement de second ordre par apprentissage par association, ce qui lui permet d’apprendre rapidement et facilement à identifier de nouveaux stimulus olfactifs émis par les œufs de poissons dans des conditions de laboratoire.

[Traduit par la Rédaction]

Introduction

Chemoreception and chemical stimuli provide crucial sensory cues to a diverse array of aquatic taxa (Carr 1988; Rittschof 1992; Hara 1994; Kats and Dill 1998; Krieger and Breer 1999). This chemical information governs many aspects of intra- and inter-species communications affecting such behaviours as prey location and feeding; predator avoidance; kin, mate, or territory recognition; reproduction; and social hierarchy formation (Foster 1985; Lima and Dill 1990; Stebbing et al. 2003a, 2003b; Bronte et al. 2002; Simon and Moore 2007; Aquiloni and Gherardi 2010). This is especially true in low-light, benthic environments where inhabiting organisms rely on chemical information in the absence of other sensory cues or where resources must be located from afar (Mackie 1973; Derby and Atema 1982, 1988; Tierney and Atema 1988; Steele et al. 1999). Superior behavioural plasticity relative to native species allows aquatic invasive species to improve survival and facilitate territory adoption in new and unfamiliar ecosystems (Hazlett et al. 2003).

Aquatic invasive species cause acute and pervasive biodiversity consequences in the ecosystems that they invade (Strayer 2010). Canada is currently facing several invasive freshwater and saltwater crustaceans, including the European green crab (Carcinus maenas (L., 1758)) and rusty crayfish (Orconectes rusticus (Girard, 1852)) (DFO 2010). Crayfish are ubiquitous members of lentic and lotic freshwater invertebrate communities, accounting for 40%–60% of the total zoo-
benthic biomass (Momot et al. 1978; Momot 1995; Dorn and Mittelbach 1999; Dorn and Wojdak 2004). Though generally considered opportunistic, polytrophic, generalist predators (Hobbs 1993), crayfish are far from being indiscriminate omnivores. They are more accurately characterized as preferential carnivores that, in searching for and consuming animal protein, demonstrate incidental detritivorous and herbivorous feeding tendencies (Momot 1995). Through their rapacious foraging habits, crayfish have a capacity to modify their habitat that may in turn exact an ecological impact with potentially far reaching trophic cascades (Flint and Goldman 1975; Chambers et al. 1990). Rusty crayfish, native to the Ohio River basin (Momot et al. 1978), has heavily colonized surrounding waters in the United States (Olden et al. 2006; Bobeldyk and Lamberti 2008) and is now considered invasive in the boreal aquatic ecosystems of northern Ontario (Momot et al. 1988; Wilson et al. 2004; Lake Simcoe Science Advisory Committee 2008; Phillips et al. 2009; Phillips 2010). Invasive crayfish species like rusty crayfish often demonstrate a high degree of adaptability to new environments owing to their aggressive behaviour, superior memory, adaptability to new food resources, higher metabolic rate and hence appetite, sensitivity to a broader range of chemical alarm signals, and superior behavioural plasticity relative to native crayfish species (Jones and Momot 1983; Hazlett 2000; Hazlett et al. 2002, 2003). In addition, female rusty crayfish are able to lay eggs at much lower temperatures relative to congeners, giving them a seasonal population growth advantage (Momot 1966; Aiken 1968; Berrill and Arsenault 1982). Collectively, these attributes confer upon rusty crayfish many survival advantages and may help explain consequent native species displacement, as well as successful, ongoing, range expansion. Moreover, the shallow waters preferred by crayfish like rusty crayfish are commonly used by fish species as spawning grounds where fish eggs and young are particularly vulnerable to predation (Dorn and Wojdak 2004).

Crayfish like rusty crayfish have been implicated in the decline of fish populations through egg predation and destruction of macrophyte beds that serve as nest sites, spawning grounds, and juvenile fish nurseries (Horns and Magnuson 1981; Chambers et al. 1990; Dorn and Mittelbach 1999; Dorn and Wojdak 2004; Jonas et al. 2005; Ellrott et al. 2007). Through active foraging, crayfish have been noted to modify the structure and composition of the littoral zone to the detriment of macrophyte, macroinvertebrate, and ultimately, sympatric fish communities (Chambers et al. 1990; Olsen et al. 1991; Phillips et al. 2009). Fitzsimons et al. (2002) estimated that consumption of lake trout (Salvelinus namaycush (Walbaum in Artedi, 1792)) eggs by crayfish (genus Orconectes Cope, 1872) for a standardized 30-day period after the date of peak spawning, ranged from 0 to 65 eggs consumed/m². This represented as much as 82% of the potential egg abundance at eight established spawning reefs in Lake Ontario. Moreover, in areas of low egg abundance (<100 eggs/m²), characteristic of 5 of the 8 spawning sites observed, crayfish and freshwater sculpin (genus Cottus L., 1758) densities were sufficiently high to cause nearly 100% mortality in lake trout eggs. Thus, scatter spawning species such as walleye (Sander vitreus (Mitchill, 1818)) and lake trout (Scott and Crossman 1973) that favour littoral spawning sites risk high egg mortality when living sympatrically with rusty crayfish. While some studies suggest that fish eggs release chemotactic agents that are enticing to vertebrate predators of eggs (Dittman et al. 1998; Mirza and Chivers 2002; Fitzsimons et al. 2002, 2006), their usage by invertebrate predators of eggs like crayfish remains less understood. Perhaps the same “info-chemicals” that motivate vertebrate predators of fish eggs also motivate crayfish to prey upon fish eggs. Identifying which adaptive behaviours drive invertebrate predators of fish eggs will help promote a better understanding of the more subtle means by which rusty crayfish, and aquatic invasive species in general, exert an ecological impact.

There are various learning mechanisms by which organisms can use past experience to modify behaviour. Associative learning is the process by which an organism forms an association by simultaneously experiencing two sensory stimuli so that subsequently experiencing one helps to then recall the other (Dickinson 1980). This process is well documented in relation to recognition of novel predators (Hazlett and Schoolmaster 1998; Hazlett 2003, 2007) and adoption of new food source (Hazlett 1994a) in both crayfish genera Orconectes and Cambarus Erichson, 1846. Rusty crayfish specifically are capable of second-order conditioning wherein new stimuli become associated with a formerly neutral stimulus to produce behaviours such as predator avoidance (Hazlett 2007). Moreover, Hazlett (2007) demonstrated that second-order conditioning occurred in rusty crayfish following one pairing. The continuous generation of neural pathways in crustaceans may account for the cellular basis underlying novel olfactory associations that promote behavioural plasticity (Schmidt and Mellon 2010).

The following study was designed to measure the conditioned behavioural response of rusty crayfish to unfamiliar chemical stimuli. We hypothesized that a population of male rusty crayfish, with decades of existence without walleye, would possess no innate recognition to the smell of walleye eggs. However, once associated with an established food source through a single paired-stimulus conditioning event, these same crayfish would be attracted to the same egg cue upon subsequent exposure. While many existing studies have looked at various crayfish behavioural responses to various single or paired chemical stimuli in terms of change in posture (Hazlett 1994b), grooming, or nonlocomotory movement (Hazlett and Schoolmaster 1998), this study sought to characterize behavioural response in terms of choice, speed of choice as a measure of interest, and appeal of test stimuli (Adams and Moore 2003).

Materials and methods
Crayfish collection and holding
Male rusty crayfish were collected from Pounsford Lake (48°29′N, 88°46.34′W) in Sleeping Giant Provincial Park, Ontario, Canada, in spring of 2010. Female crayfish are generally egg-laden at this time of year and not actively foraging; hence, only males were caught and retained for study. Male rusty crayfish alone have also been used successfully in chemosensory behavioural research (Adams and Moore 2003; Acquistapace et al. 2004). To control for variability in size class and life history in our experimental population, a ran-
A static Y-maze arena (74 cm long × 39 cm wide; Fig. 1) was used to test the behavioural response of 20 rusty crayfish to a walleye egg cue before and after a paired-stimulus conditioning event. A corrugated plastic barrier running two thirds of the arena length provided chemical separation of the stimulus and control arms. A clear barrier, perforated to allow chemical exchange, separated an acclimation zone from the two arms of the Y-maze. Stimuli and controls were randomly assigned to either the left or the right delivery arm for each trial and observers were not aware which side contained the stimulus. All experimental systems were visually isolated and observers recorded behaviour through a small (4 cm × 10 cm) horizontal viewing window. Trials were conducted by first filling the arena with 10 L of holding temperature-matched, dechlorinated Thunder Bay municipal water. A randomly selected crayfish was carefully delivered into the acclimation zone and left for 20 min to adjust to maze conditions. Following acclimation, 20 mL of both stimulus and controls were gently delivered to each opposing end of the delivery arms using a 50 mL syringe connected to airline tubing and a 9 cm Pasteur pipette, operated remotely from behind a visual barrier. Preliminary trials using commercial food colouring revealed that aqueous mixtures diffused to the acclimation chamber gate within 5 min and provided a visibly consistent concentration gradient for the duration of the observation period. Following a 5 min stimulus delivery period, the perforated barrier was gently raised sufficient to allow the crayfish to pass through, and behavioural endpoints were recorded for an 8 min observation period. Behavioural endpoints were chosen in keeping with Adams and Moore...
(2003), including first choice, final position, time in the stimulus versus control arms, and latency to first-arm choice. For a given trial, first choice was defined as the first arm (stimulus or control) that crayfish entered, whereas final position was defined as the final location occupied by the crayfish (stimulus, control, or acclimation chamber) following the 8 min observation period. Y-mazes were thoroughly washed using a mild detergent (Sparkleen, Fisherbrand) solution and rinsed with dechlorinated water following each trial set to ensure no stimulus contamination would confound subsequent trials. Trials were conducted between 0900 and 1900 during June and July of 2010.

Walleye egg cue

A fish egg cue was produced following Mirza and Chivers (2002) to approximate odour signals given off by walleye eggs from spawning grounds. Minor methodological modifications were employed to address standardization and chemical variability from single male–female pairings during artificial fertilization. Fish-egg stimulus was produced in conjunction with the annual walleye spawning activities of the Atikokan Sportsman’s Conservation Club (Atikokan, Ontario, Canada). Gametes used for spawning were from wild fish, and fertilized eggs came from an artificial fertilization using the roe of five females and the milt from three males. Eggs were not “muddled” during the process of fertilization to provide for as unadulterated a stimulus as possible. Forty-five grams of freshly fertilized eggs were left to water harden in 2 L of dechlorinated water for 3 h. After 3 h, the water-hardened eggs were transferred to 1 L of fresh dechlorinated tap water and left to soak for 30 min. Eggs were then removed and an additional 4 L of dechlorinated water were added to bring the final volume up to 5 L. This 5 L of walleye egg cue stock solution was brought back to the laboratory and frozen (−20 °C) in 100 mL aliquots. A dechlorinated water control was treated, produced, and frozen in exactly the same way as the egg cue but without the egg soak.

Food cue

Food cue preliminary trials using the same Y-maze assay were conducted to ensure that we could measure a behavioural response in our experimental population. A food cue was prepared by homogenizing 10 g of commercial trout pellet food in 500 mL dechlorinated water. The homogenate was stirred for 20 min and then filtered through coarse filter wool to remove any particulate matter. Food cue was produced and used daily for behavioural trials. During preliminary trials, crayfish spent significantly more time in the food cue arm when paired with a dechlorinated water control (paired t test; $t_{28} = 2.13, P = 0.042$). This suggested an established recognition of, and attraction to, dissolved components from the trout food as a result of feeding experience (Tierney and Atema 1988).

Conditioning procedure

Conditioning procedures were modeled on the works of
Hazlett et al. (2002, 2003) designed to invoke associative learning processes. Thus, pre- and post-behavioural response to walleye egg cue could be compared. Crayfish were conditioned together for 2 h using a paired-stimuli exposure (walleye egg cue + food cue) in a 100 L tank of dechlorinated water (20 °C). Each crayfish was visually and mechanically isolated in its flowerpot enclosure and the tank containing all flowerpot enclosures was provided supplemental aeration to facilitate mixing. Once introduced into the tank, crayfish were left to acclimate for 1 h. Following acclimation, 200 mL of each paired stimulus (walleye egg cue + food cue) was slowly added to the tank. Ninety minutes later, a second 200 mL aliquot of each stimulus was added in the same manner and the crayfish were left for another 30 min. Following conditioning, animals were returned to their holding tanks. Behavioural trials were conducted 48 h following conditioning.

**Analysis**

A paired $t$ test or Wilcoxon’s signed rank test was used to compare mean time crayfish spent in the stimulus versus control arms, whereas a two-sample $t$ test or a Wilcoxon’s rank sum test with continuity correction was used to evaluate differences in mean latency to initial arm selection, depending on normality of the data. A $\chi^2$ test was used to compare first-arm choice and last position within the Y-maze following the observation period with a respective 50:50 and 33:33:33 expectation owing to random chance alone. For all analyses, results were considered statistically significant when $P < 0.05$ and all tests had statistical power of 80% or greater whenever significance was declared. Standard scores were calculated for all variables resulting in one trial being removed from each of the pre- and post-conditioning data sets prior to analysis. All statistical analyses were conducted in R version 2.10.1 (R Development Core Team 2009).

**Results**

As expected, crayfish showed no response to the walleye egg cue prior to conditioning according to any measured endpoints (first choice or last position, time in the stimulus versus control arms, and latency to first arm choice; $P > 0.05$; Figs. 2, 3, 4). The Pounsford Lake population of rusty crayfish has no life-history experience with olfactory cues from walleye. Hence, chemical stimuli associated with walleye life processes are not likely to provide behaviourally relevant information to the extant crayfish population. Once conditioned, however, the same crayfish took significantly less time to choose the walleye egg cue arm than they took to choose the control arm ($t_{[17]} = 2.57, P = 0.02$; Fig. 4). Following conditioning, crayfish took, on average, 45 s to choose the stimulus arm and over 2 min to choose the control arm. Other behavioural responses did not vary significantly between the stimulus and the control arms owing to a high degree of variability in the measured endpoints (first choice, final position, time in the stimulus versus control arms; $P > 0.05$; Figs. 2, 3).

**Discussion**

While compelling, there was a high degree of variability in the data. This allowed a response characterization according to only one of the employed behavioural endpoints. Recent research by Gardiner and Atema (2010) suggests that bilateral time differences in stimulus detection trumpp odour concentration during orienting activities, because odour plumes show chaotic intermittency in the aquatic environment. Hence, over the course of the observation period, as mixing progressed at the interface of the arms and acclimation zone of the Y-maze, observed crayfish may have been progressively less able to rely on directional stimulus signals, thereby obfuscating a clear response pattern on all behavioural endpoints. Nonetheless, the positive chemotactic response observed after conditioning, but absent before conditioning,
suggests that the treatment was likely the source of the change in behaviour.

Results from this study confirm our hypothesis regarding the behavioural response of conditioned male rusty crayfish to a walleye egg cue. Male rusty crayfish can quickly and easily form a learned association between a novel odour stimulus (walleye egg cue) and a known food cue. Once formed, this learned association results in attraction. By extension, once an association is formed between olfactory and gustatory stimulation through walleye egg contact, likely during random foraging, male rusty crayfish should readily respond with attraction to egg cues from walleye spawning grounds under field conditions. These results are consistent with Hazlett (1994a) who successfully conditioned rusty crayfish to produce a feeding response to the novel food odour of zebra mussel (Dreissena polymorpha (Pallas, 1771)). However, while Hazlett (1994a) proposed that the mechanism of learning involves the formation of an association between food odour and food taste, our results suggest that association of olfactory stimuli alone is sufficient for associative learning to take place. Our findings complement those of Hazlett (2007) by demonstrating that second-order conditioning can play a role not only in predator avoidance but during new resource acquisition as well. Furthermore, we also observed that a single paired-stimulus exposure was sufficient to allow this association to occur. The facility with which rusty crayfish can learn new chemical signals in its environment highlights the adaptive benefit of associative learning seen in this study. Being an omnivorous, polytrophic forager, invasive crayfish like rusty crayfish must be able to quickly adapt to changing resources over spatial and temporal gradients. While foraging in new environments, crayfish are exposed to numerous unfamiliar chemical signals. The learned association between an established food cue and an unfamiliar (potential) food stimulus described in this study is only one possible mechanism by which rusty crayfish form associations that allow them to capitalize on novel resources. The capacity for associative learning confers an advantage to the individual (or species) that possesses this ability by allowing them to adapt to changes in its environment crucial to its survival in contrast to the individual (or species) that does not possess this ability. Should rusty crayfish come to prey upon walleye eggs, a learned association could occur that would increase the predisposition of rusty crayfish for any potential chemically mediated homing to walleye spawning grounds.

This result has clear implications for recruitment or rehabilitation of walleye and other important sport fish in lakes invaded by rusty crayfish. Pounsford Lake is unlikely to contain any extant populations of predator-guild species following failed Ontario Ministry of Natural Resources (OMNR) introductions of largemouth bass (Micropterus salmoides (Lacepède, 1802)) in the early 1950s (Momot et al. 1988) and of walleye around 1964 (Werner 1983).¹ The most recent account of walleye is from 1982 when two were caught during OMNR sampling activities (Werner 1983).¹ However, today, only a minimal population of largemouth bass remains (C.D. Weisbord and D.T. Callaghan, personal observation). Many other lakes in northern Ontario, currently under colonization, do, however, contain native populations of walleye (Rosenberg et al. 2010). Our study suggests that within these lakes, a single exposure event could suffice for rusty crayfish to associate walleye eggs with food. Once formed, this association should persist (Hazlett et al. 2002) and additional encounters would reinforce this association (Dickinson 1980). However, learned associations may attenuate within 3 and 6 weeks (Hazlett et al. 2002). It would be interesting to see to what degree repeated exposures would prolong such an association.

Future studies might explore biochemical analysis to elucidate and characterize the putative attractive components released by fish eggs. In addition, determining whether rusty crayfish are similarly amenable to associating fish egg cues from other valued game species (e.g., salmonids) with food would serve to further expand the range of ecological impact exerted by such aquatic invasive species. Nonetheless, this is the first study to explore the role of associative learning in egg predation and provides evidence to the use of chemical cues by invertebrate predators of fish eggs. It joins an ever-growing body of knowledge surrounding the intricate mechanisms by which aquatic invasive species interact with new ecosystems.

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