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CONTROL OF CHEMICALLY STIMULATED FEEDING BEHAVIOR IN SAND FIDDLER CRABS *Uca pugilator*: EVIDENCE FOR HEMOLYMPH FEEDING INHIBITORY FACTOR

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Abstract—*Uca pugilator*, the sand fiddler crab, is a deposit-feeder. It feeds on exposed sand flats as the tide recedes. Feeding movements are evoked by stimulation of chemoreceptors on the dactyls. Previous studies have demonstrated that a proteinaceous factor associated with the sinus gland region inhibits chemically-stimulated feeding behavior. Here, that work is extended to show the existence of a similar inhibitory factor present in the hemolymph of fed crabs in the laboratory and in the hemolymph of crabs returning from feeding in the field. The factor is not detected in fasted laboratory crabs or in field crabs walking from burrow areas to feeding sites. Injection of glucose results in inhibition of feeding activity in intact crabs. Injection of glucose, but not galactose, stimulates feeding in eyestalk-ablated crabs. We suggest that neural responsiveness is stimulated by glucose, and that elevated glucose in intact crabs results in release of feeding inhibitory factor from the sinus gland. Release of feeding inhibitory factor into the blood enables crabs to return to burrow areas without stopping when they encounter food-laden sediments.

Key Words—*Uca pugilator*, crustacean feeding, endocrine factors, feeding behavior, daily movements, feeding ecology.

INTRODUCTION

The needs of organisms, and consequently their behavior, can be ordered into a hierarchy (c.f., Maslow, 1970), a prioritization of activities that has, through

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evolutionary time, proven successful. For example, organisms meet energetic needs and then perform activities that use energy to ensure survival and reproduction. We hypothesize that in crabs, behavioral prioritization can be hormonally controlled. That is, hormones play a direct role in determining the behavioral hierarchy and time budgeting of daily activities. This concept is supported for crustaceans by two reports in the literature (Gleeson et al., 1987; Sears et al., 1991). For the sand fiddler crab (*Uca pugilator* Bosc), daily activities include feeding (Robertson et al., 1980, 1981), burrow building and maintenance, sexual displays, and (at appropriate lunar phases) mating (Crane, 1975; Salmon and Hyatt, 1983). Here we provide evidence that hormones modulate chemosensory behavior in sand fiddlers.

Crab and shrimp eyestalks contain a proteinaceous factor that modulates chemically-stimulated feeding behavior in *U. pugilator* (Sears et al., 1991). This feeding inhibitory factor (FIF) is associated with the eyestalk (X-organ, sinus gland complex), a secretory neuroendocrine organ that has a major role in the regulation of moulting, ovulation, ion balance, and hemolymph glucose levels (for review see Beltz, 1988). FIF-like activity can be found in fed intact crabs and in intact crabs injected with glucose. Crabs captured while walking from burrow areas to feeding sites and fasted intact crabs do not contain detectable amounts of FIF activity in their hemolymph. Crabs returning from feeding, and fed intact crabs, contain high levels of FIF in their hemolymph. Eyestalk-ablated crabs are also devoid of FIF activity and do not produce FIF-like activity when fed or injected with glucose. The molecular size of circulating FIF is similar to that of FIF isolated from eyestalks.

Here, we show that the circulating levels of FIF are related to the behavioral and satiation state of the crabs.

METHODS AND EXPERIMENTS

Eyestalk-Ablated Crabs

Eyestalks were ablated with forceps. At least 12 hr elapsed after ablation and before testing (Rittschof and Buswell, 1989). This interval was sufficient for depletion of eyestalk hormones as indicated by chromatophore state (Brown, 1940). Eyestalk-ablated crabs were held overnight in finger bowls containing several mm of seawater at 23°C. Bowls were rinsed and seawater was replaced 15 min prior to feeding assays.

Assays of Feeding Inhibition

For assays, intact and eyestalk-ablated crabs were transferred to 20-cm finger bowls containing 20 ml of 0.0625 M glucose (the threshold concentration for a detectable response) in 100 kd filtered seawater. Feeding responses were

observed directly. Feeding assays tested glucose-stimulated feeding responses (Sears et al., 1991). Seawater (34–37 ppt), filtered to remove particles >100 kd, was used to make solutions for feeding assays in which 10–30 crabs were tested in groups of five and were observed for 30 sec. A positive feeding response was recorded for each crab that moved the minor chela at least twice in a feeding motion.

Hemolymph Glucose Assays

Hemolymph glucose concentrations were assayed enzymatically using the deproteinated blood protocol from a commercially available glucose oxidase reagent kit (Sigma Chemical, St. Louis, MO #510-DA). Hemolymph glucose levels were determined on intact, eyestalk-ablated, and ablated crabs injected with glucose 1 hr beforehand in isosmotic (900 mOsM/1) 100 kd filtered seawater.

Effects of Increased Hemolymph Glucose on Feeding Responses of Intact and Ablated Crabs

The effect of elevated glucose levels on feeding responses was determined using 30 intact and 30 eyestalk-ablated crabs. Each crab was injected with 25 μl of 4 g/l (100 μg) glucose in saline solution. Based on previous glucose assays, this amount of glucose was sufficient to bring the hemolymph glucose concentration of a 2.1 g ablated crab to levels (10 mg/dl) found in intact crabs. Feeding assays were conducted 1 hr after injection.

Effects of Increased Hemolymph Glucose or Galactose on Feeding in Ablated Crabs

Glucose can be metabolized directly by neural tissue, but galactose cannot (Lehninger, 1978). The effects of elevated glucose and galactose in eyestalk-ablated crabs were compared. Thirty ablated crabs were injected with saline, 25 μl of 4 g/l or 40 g/l glucose or galactose solution. Based on previous glucose assays, these concentrations were sufficient to bring hemolymph hexose concentrations to 10 mg/dl and 100 mg/dl, respectively. Sixty minutes after injection, feeding responses were measured and stimulation of feeding was calculated:

$$\frac{\text{Final \% response} - \text{Initial \% response}}{\text{Initial \% response}}$$

FIF Titres of Hemolymph from Unfed and Fed Crabs

FIF titres in fiddler crab hemolymph were estimated by injecting hemolymph into eyestalk-ablated crabs 1 hr prior to bioassay. A 25 μl volume of hemolymph was drawn from the sinus within the large cheliped of a male crab, using a Hamilton syringe. This sample of hemolymph was immediately injected into an eyestalk-ablated crab (technique described in Sears et al., 1991). Eyestalkless crabs were assayed immediately before injecting samples and then 1 hr after injection. Materials tested for inhibition of feeding activity were diluted with isosmotic (900 mOsM/l) 100 kd filtered seawater and 25 μl volumes injected into crab blood sinuses. Injection was either into the blood sinus within the large cheliped of eyestalkless males, or into the sinus at the proximal joint of the fifth walking leg (both sexes). Preliminary tests indicated equivalent response times for the two injection methods.

Hemolymph titres of FIF with respect to feeding were determined in both laboratory and field situations. In the laboratory, intact crabs were maintained for 3 days with either an unlimited supply of fish flake food (Tetramin) and cat food (Friskies Ocean Fish Flavor), or fasted in finger bowls containing 100 kd filtered seawater. Hemolymph was drawn from 30 crabs in each condition, injected into eyestalk-ablated crabs, and the injected crabs bioassayed for feeding inhibition.

In the field, 25 μl samples of hemolymph were taken from crabs on the way down the beach to feed as the tide ebbed, or 2 hr after low tide as crabs traveled back to their burrows after feeding. Samples were from crabs located at Bird Shoal, Beaufort, North Carolina, USA. Hemolymph from each sample of crabs was immediately injected into eyestalk-ablated crabs which had been brought into the field. Crabs were tested in the field in the feeding bioassay immediately before injection and in the field 1 hr after injection. When not in use, ablated crabs were stored in buckets containing 1 cm of seawater, covered with towels. Ambient temperature was 37°C.

Size Estimation of FIF from Hemolymph

Hemolymph containing FIF activity was subjected to size fractionation to estimate the molecular size of circulating FIF. Cascade pressure dialysis was used to estimate the molecular size of hemolymph FIF. A total of 2 ml of hemolymph was drawn in the field from 100 intact male crabs as they were returning to their burrows after feeding (approximately 2 hr after low tide). The pooled hemolymph was diluted as it was collected into 20 ml of isosmotic 100 kd filtered seawater stored on ice until it was returned to the laboratory.

In the laboratory, the diluted hemolymph was centrifuged at 10,000 rpm for 10 min to remove particulates. The supernatant was then separated into $<5{,}000$ d and $>5{,}000$ d size fractions by ultrafiltration through a 5000 d mem-

brane (Amicon YM series). The >5000 d fraction residue was washed twice with isosmotic saline and brought back to the original 20 ml volume. Both the filtrate and the residue were lyophilized and returned to 2 ml volumes. FIF titres in the two size fractions of hemolymph from fed crabs was determined. For assays, 25 μl of <5000 d and >5000 d size fractions of hemolymph were injected into 30 eyestalk-ablated crabs. Feeding responses were measured and percent inhibition of feeding was calculated.

Statistical Analysis

Levels of feeding responsiveness under different conditions were compared by determining a Z statistic for the difference between two proportions (Walpole, 1974). Significance was set at the $p < 0.05$ level.

RESULTS

Effects on Feeding of Elevated Hemolymph Glucose Concentrations in Intact and Ablated Crabs

Injection of glucose inhibited feeding in intact crabs but stimulated feeding in eyestalk-ablated crabs (Figure 1). A 10 mg/dl increase in hemolymph glucose concentration resulted in 45% inhibition of feeding by intact crabs ($Z = 3.15$, $P < 0.001$) compared to 112% stimulation in ablated crabs ($Z = 10.6$, $P < 0.001$).

Effects of Increased Glucose or Galactose on Feeding of Eyestalk-ablated Crabs

Glucose stimulated feeding to a much greater extent than galactose in ablated crabs (Figure 2). Hemolymph concentrations of glucose $\geq$ 10 mg/dl resulted in 100% stimulation of feeding ($Z = 10.6$, $P < 0.001$). In contrast, injection of 10 and 100 mg/dl galactose resulted in 17% and 22% stimulation, respectively, which failed to differ significantly from control (saline injected) crabs.

Effects of Hemolymph Taken from Unfed and Fed Crabs

Injection of 25 μl of hemolymph drawn from intact fed crabs in both the laboratory and the field significantly inhibited feeding ($Z = 7.46$, $P < 0.001$) in eyestalk-ablated crabs (Figure 3). Injection of hemolymph from fasted (unfed) laboratory crabs stimulated feeding ($Z = 1.79$, $P < 0.05$), while hemolymph from crabs moving toward feeding areas (field) had no effect.

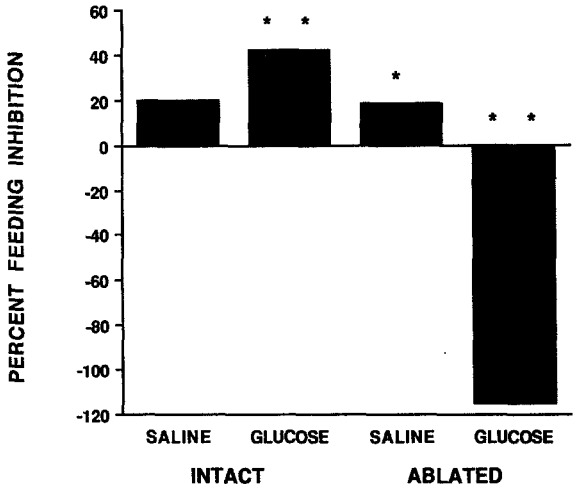

Fig. 1. Change in feeding activity of intact and eyestalk-ablated crabs following injection of saline or glucose. Feeding activity for each group of crabs was determined prior to injection. One hour after injection feeding activity was again assayed. Injection of glucose significantly inhibited feeding in intact crabs while injection of saline did not. Eyestalk-ablated crabs injected with saline were inhibited from feeding, but when injected with glucose, feeding activity increased (negative inhibition is stimulation of feeding). * = $p < 0.05$; ** = $p < 0.01$.

Comparison of Hemolymph Size Fractions

Feeding inhibition activity was found in the >5000 d fraction of hemolymph drawn from satiated intact crabs. Injection of the >5000 d fraction resulted in 80% inhibition of feeding responses by eyestalk-ablated crabs ($Z = 5.58$, $P < 0.001$), whereas the <5000 d fraction resulted in 0% inhibition.

Summary of Injection Experiments

Inhibition of feeding responses and corresponding eyestalk equivalents for injection experiments are summarized in Table 1. Levels of FIF were undetectable in ablated crabs injected with saline or with hemolymph from fasted or crabs in the field that were on their way to feeding areas.

DISCUSSION

Feeding inhibition might be the result of depressed glucose levels due to the absence of crustacean hyperglycemic hormone (CHH) (Kleinholz et al., 1967). Injection of glucose into eyestalk-ablated crabs stimulates feeding

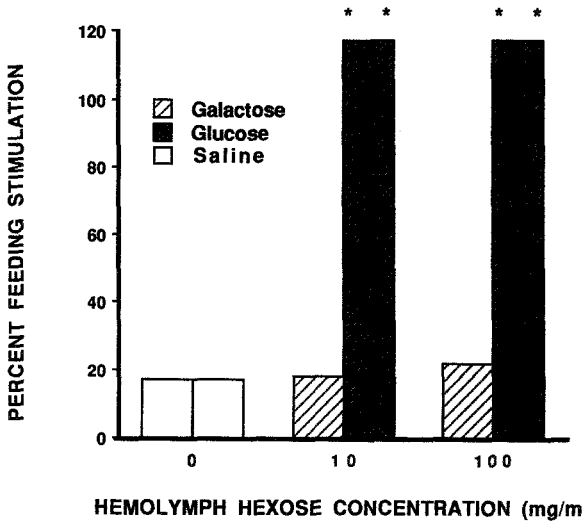

Fig. 2. Feeding activity after injection of hexose sugars into eyestalk ablated crabs. After determination of feeding activity, galactose or glucose was injected into eyestalk-ablated crabs. One hour later, crabs were assayed a second time. Glucose dramatically stimulated feeding. Galactose had no effect. ** = $p < 0.01$.

responsiveness to glucose. This result eliminates the possibility that feeding inhibition is mediated indirectly by CHH through elevation of glucose levels.

Since injection of glucose stimulates feeding in ablated crabs, we suggest it acts directly on the nervous system. The nervous system is an obligate glucose user (Lehninger, 1978). Ablated crabs which lack CHH have depressed glucose levels (Kleinholz et al., 1967). After injection of glucose, the nervous system may work more efficiently, thereby increasing feeding responsiveness. Thus, for ablated crabs, the more they eat, the more they are stimulated to feed. A common dietary sugar, galactose, cannot be used directly by nervous tissue. Injection of galactose resulted in only slight stimulation. This supports the hypothesis that glucose is acting directly on the nervous system.

Glucose injections result in inhibition of feeding in intact animals, but not in ablated animals. This suggests that the sinus gland secretes FIF in response to elevated hemolymph glucose levels. Injection of hemolymph from satiated, but not from hungry crabs, inhibits feeding in ablated crabs. The biological activity found in the hemolymph is similar in molecular size to FIF isolated from the eyestalk (Sears et al., 1991). These results suggest that after the crabs have fed for some time, increased glucose levels stimulate the release of FIF. FIF modulates and finally halts chemically-stimulated feeding responses.

We have constructed an algorithm of fiddler crab responsiveness. As fid-

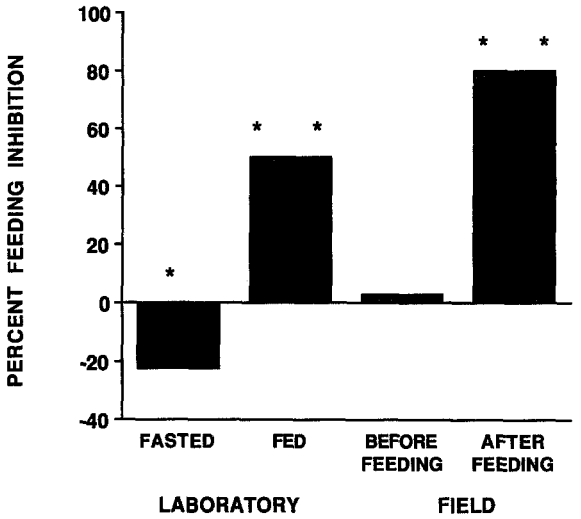

Fig. 3. Feeding activity of crabs following injection of hemolymph from crabs sampled in the laboratory and in the field (on their way to and returning from feeding areas). Hemolymph from unfed (fasted) crabs in the laboratory stimulated feeding in eyestalk-ablated crabs. Hemolymph from fed crabs from either the laboratory of the field dramatically inhibited feeding in ablated crabs. * = $p < 0.05$; ** = $p < 0.01$.

dler crabs feed, hemolymph glucose levels rise. Elevated glucose triggers the release of FIF from the sinus gland into the hemolymph. Circulating FIF acts on the central or peripheral nervous system to decrease responsiveness to external chemical stimuli, first restricting feeding to rich patches of food and finally halting feeding entirely. Once feeding has stopped, crabs return to burrow areas and continue with other activities. There are times when crabs are food-limited and forced to stop feeding by the tides (Salmon and Hyatt, 1983). It would be informative to titer FIF levels at these times. If normal FIF titres decline with fasting at a rate comparable to injections of FIF (Sears et al., 1991), then inhibitory effects of FIF could be expected to diminish prior to the time of the next declining tide.

Male fiddler crabs spend considerable time performing elaborate mating displays (Salmon and Hyatt, 1983). In addition, fiddler crabs are at risk of predation when they are away from their burrows on the sand flat feeding (see Salmon and Hyatt, 1983, p. 41). They must build burrows for predator avoidance and for use as incubation chambers for gravid females. By halting feeding once nutritional and energetic needs are met, FIF plays an important role in budgeting time crabs spend on non-feeding activities.

TABLE 1. SUMMARY OF INHIBITION OF FEEDING RESPONSES AND APPROXIMATE CORRESPONDING EYESTALK EQUIVALENTS OF FIF IN EYESTALK-ABLATED CRABS OF 0.1 ml HEMOLYMPH VOLUME AFTER INJECTION WITH SALINE, HEXOSES, OR HEMOLYMPH. NEGATIVE INHIBITION VALUES INDICATE STIMULATION

Material injected	Percent inhibition	FIF eyestalk equivalents per crab
Lab		
Saline	17	0
Glucose	112	0
Galactose	−22	0
Unfed hemolymph	−22	0
Fed hemolymph	43	0.32
< 5000 d fed hemolymph	0	0
> 5000 d	80	1.2
Field		
Unfed hemolymph	0	0
Fed	80	1.2

There are a variety of criteria for determining whether a specific factor is a hormone (Beltz, 1988): (1) presence in a neurosecretory organ: FIF has been localized to the region of the X-organ sinus gland complex in shrimp eyestalks. Immunocytochemistry would be required to demonstrate the existence of FIF in this neurosecretory organ. (2) Release into the circulation: FIF-like biological activity has been demonstrated in both the laboratory and the field in the hemolymph of fed, but not of unfed crabs. This circulating biological activity has the same approximate molecular size as FIF from the sinus gland region. Purification and characterization of FIF should establish whether or not eyestalk and hemolymph factors are actually the same molecules. (3) Effectors that respond to the factor at its circulating concentration: crabs show dramatic alteration in chemically-stimulated behavior when injected with FIF from eyestalk extracts or from hemolymph of fed crabs. Donor crabs with different FIF titres exhibit different behaviors. Eyestalk-ablated crabs do not respond by producing FIF activity as do intact crabs. (4) Sufficient stability in the hemolymph: although FIF is less solvent-, heat-, and trypsin-stable than many other sinus gland hormones (Sears et al., 1991), FIF is sufficiently stable in the hemolymph to be detected for changes in its titre before and after feeding. These results support the hypothesis that FIF is a hormone from the sinus gland. Its function appears to be to regulate behavior by modulating chemically-stimulated feeding.

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