

## Do brominated natural products defend marine worms from consumers? Some do, most don't

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### Abstract

Worms and other marine invertebrates living in soft sediments commonly produce brominated natural products that have been hypothesized to function as defenses against consumers, but this hypothesis has not been tested directly. When 16 species of worms from a Georgia mud flat were fed to two sympatric fishes (*Fundulus heteroclitus*, *Leiostomus xanthurus*) and a crab (*Callinectes similis*), 15 species (94%) were palatable to all three predators. Only the hemichordate *Saccoglossus kowalevskii* was unpalatable to both fishes, but even it was readily consumed by the crab. Bioassay-guided chemical investigations demonstrated that *Saccoglossus kowalevskii* was rejected by fishes because it contained 2,3,4-tribromopyrrole at 0.2% of worm dry mass. This is the first direct test of brominated worm metabolites as defenses against sympatric consumers. The deterrence of 2,3,4-tribromopyrrole in *S. kowalevskii* may explain why densities of this worm increase 40-fold during seasons when predation is high and densities of palatable worms decline sharply. To more broadly examine the effects of brominated metabolites on worm palatability, we collected from North Carolina, Georgia, and Florida 14 species reported to produce brominated metabolites. These species were fed to sympatric consumers to assess palatability and also analyzed for brominated metabolites by gas chromatography mass spectrometry (GC/MS). Nine of the fourteen species contained brominated metabolites, but only two were unpalatable. In a final test, five additional brominated metabolites produced by marine worms were added to palatable foods at natural concentrations and at up to 20× natural concentrations. None deterred feeding at natural concentrations, one was deterrent at 5–15× natural concentration, and four had no effect at even 20× natural concentration. Thus, while one worm was defended by a brominated compound, most worms containing brominated metabolites were palatable, and brominated natural products seldom functioned as chemical defenses against consumers.

Marine invertebrates living in soft-sediment habitats such as mud flats can experience heavy predation (Peterson 1979; Quammen 1984; Wilson 1990), which could select for anticonsumer traits such as chemical defenses. As a possible example, brominated phenolics and pyrroles are commonly produced by invertebrates living in temperate mud flat or sand flat habitats, and this phenomenon is geographically and taxonomically widespread. Numerous species, particularly worms, from the Northeast Pacific (Woodin et al. 1987; Woodin et al. 1993), Northwest Atlantic (Fielman and Tar-

gett 1995; King et al. 1995; Giray and King 1997a), West-central Atlantic (Ashworth and Cormier 1967; Woodin et al. 1997; Fielman et al. 1999; Cowart et al. 2000), and Eastern Atlantic (Weber and Ernst 1978; Emrich et al. 1990; Goerke and Weber 1990; Goerke et al. 1991; Jensen et al. 1992) contain these compounds. As an indication of the commonness of this phenomenon, Fielman et al. (1999) investigated 40 species of infaunal invertebrates and found that 43% of the species contained halogenated metabolites.

These metabolites have repeatedly been identified and quantified from numerous species, but the ecological significance of most of these compounds has rarely been investigated. Several authors have predicted (Woodin et al. 1987) or provided correlative evidence (Yoon et al. 1994; Fielman and Targett 1995; Fielman et al. 1999; Cowart et al. 2000) that these brominated compounds could function as predator deterrents. For example, the highest concentration of brominated metabolites in the annelids *Notomastus lobatus* and *Thelepus extensus* and the hemichordate *Saccoglossus kowalevskii* are in body parts that are nearest to the substrate surface and, thus, at greater risk of predation from fishes or crabs (Goerke et al. 1991; Yoon et al. 1994; Fielman and Targett 1995). However, when worms that produce brominated compounds have been exposed to predators, outcomes

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have been variable. Thomas (1972) and Prezant et al. (1981) found the hemichordates *Saccoglossus otagoensis* and *S. kowalevskii* (which both produce 2,3,4-tribromopyrrole) were unpalatable to fishes, but Giray and King (1997a) found *Saccoglossus bromophenolosus* (which produces 2,4 dibromophenol) was palatable to two predatory polychaetes and a hermit crab. In none of the above examples were extracts or specific metabolites tested directly to assess the potential chemical basis of feeding patterns. Thus, there is no unambiguous evidence that brominated natural products do or do not function as chemical defenses against consumers.

Brominated compounds could have functions other than consumer defenses. One correlative study suggested that species producing brominated metabolites were using these to alter faunal abundance near their burrows (Jensen et al. 1992), but other studies could not replicate this pattern (Steward et al. 1992, 1996). Direct tests of the effects of brominated compounds on microbial respiration and assimilation also produced mixed results. King (1986, 1988) and Giray and King (1997b) found that these metabolites inhibited microbial respiration and assimilation, but Steward and Lovell (1997) and Lovell et al. (1999) did not. Evidence that these metabolites may inhibit invertebrate settlement is more consistent. Both studies that addressed this (Woodin 1985; Woodin et al. 1993) demonstrated that fewer invertebrates settled in sediments that had previously contained worms producing these metabolites. Direct tests of natural concentrations of 4-bromophenol, 2,4-dibromophenol, and 2,4,6-tribromophenol (all found in sediments containing the annelid *N. lobatus*) demonstrated that these metabolites inhibited settlement (Woodin et al. 1997).

Given the large impact of consumers on populations of soft-sediment invertebrates (e.g., Virnstein 1977; Woodin 1981; Peterson 1982) and the common occurrence of brominated metabolites among species like worms, it seems reasonable to hypothesize that predation selects for chemical defenses among soft-sediment species and that brominated metabolites could serve this function. To test this hypothesis, we examined the palatability to sympatric fishes and a crab of annelid and hemichordate worms from a Georgia mud flat. Unpalatable worms were investigated for chemical deterrence. From broader geographic areas (North Carolina and Florida), we also collected species (or their close relatives) previously reported to produce brominated compounds and assessed their palatability to generalist consumers, as well as their production of brominated metabolites. Because almost all worms producing brominated metabolites were readily eaten by all consumers, we performed a final test where we added brominated compounds known to be produced by infaunal worms to palatable foods at concentrations from 1× to 20× their natural concentration and assessed their effects on feeding. To see whether being chemically defended might allow unpalatable species to persist during periods when palatable species were suppressed by consumers, we also monitored the seasonal abundance of palatable versus unpalatable worms on a Georgia mud flat.

## Methods

Between June–August 2001 and 2002, we collected all commonly occurring worm species from a mud flat on Little

Tybee Island, Georgia (31°57'N, 80°56'W). To assess palatability, we fed these worms to common, cooccurring consumers (the fishes mummichog [*Fundulus heteroclitus*, 4–6 cm long] and spot [*Leiostomus xanthurus*, 5–8 cm long], as well as the lesser blue crab [*Callinectes similis*, 4–8 cm carapace width]) that we collected locally. Invertebrates, including worms, make up the bulk of these consumers' diets (e.g., Chao and Musick 1977; Baker-Dittus 1978; Hsueh et al. 1992).

Animals were kept individually in recirculating seawater systems in either 2.4-liter (mummichogs) or 4.7-liter (spot, lesser blue crab) containers at the Georgia Institute of Technology's marine facility on Skidaway Island, Georgia. To ensure that consumers were not feeding indiscriminately due to unusual hunger levels (Cronin and Hay 1996), consumers were fed to satiation each morning, and feeding assays started about 1 h later. Mummichogs were fed frozen brine shrimp, spot were fed brine shrimp sticks, and crabs were fed chunks of thawed squid.

We used standard feeding assays to assess worm palatability (e.g., Hay et al. 1989; Pawlik et al. 1995; Hay et al. 1998). Consumers were first offered a palatable control food (a brine shrimp for mummichogs, a brine shrimp stick for spot, and a piece of squid for crabs). If this was consumed, then that assay animal was offered a fresh worm or worm portion. Consumers that rejected the worm were offered a second control food to ensure that rejection was not due to satiation. Crabs were usually offered whole worms, but whole worms of some species were often too big for our small fishes to eat. Our previous experience with fish and crab feeding (Kicklighter 2003) suggested that consumers fed similarly on either whole worms or worm portions and that the release of body fluids from worm portions did not stimulate feeding on species that would have been avoided otherwise. As examples, for worms small enough for our fishes to handle (e.g., *Spiochaetopterus oculatus*, *Streblospio benedicti*, *Tharyx marioni*), consumption of whole worms or worm portions was always equivalent, and in assays with larger fishes and worms from other habitats, worms that were rejected when offered entire in the field were also rejected when segments were offered in the lab (Kicklighter and Hay pers. obs.).

In all assays, each consumer was offered a portion from a separate worm to ensure independence among replicates. Consumers that did not consume either the initial or second control food were excluded from consideration, but this was uncommon (rejection of the second control occurred 15 times out of 1561 portions offered). For a replicate to be included, the consumer also was required to take the worm into its mouth, ensuring an assessment of palatability as opposed to visual discrimination. However, consumers almost always tasted our offerings. These procedures resulted in sample sizes of 9–11 for each predator, for each assay. Fisher's Exact test evaluated feeding on palatable control food versus worm portions.

In feeding assays, only *S. kowalevskii* was unpalatable, suggesting it might be chemically defended against consumers. Because the brominated metabolites of *S. kowalevskii* were obviously volatile (the worm smells of halogens), we conducted our initial extraction so as to capture volatiles and

allow solvent removal without placing the most volatile metabolites under vacuum. Freshly collected *S. kowalevskii* were placed in methanol equivalent to twice their volume. Worms were then cut into small pieces with scissors; distilled water (volume equivalent to the methanol added) was added; and the vial was shaken. An equal volume of pentane was added, and the vial was shaken several times. The pentane layer was drawn off and this addition repeated twice more to ensure efficient extraction. Pentane extracts were combined, kept on ice, and concentrated under a stream of nitrogen. The methanol/water extract was filtered to remove particulates and dried on a Speedvac Concentrator. Pentane and methanol/water fractions were bioassayed separately.

For bioassays, extracts were solubilized in ethanol and transferred to a 2.0-ml microcentrifuge tube, where they were mixed with a squid-based food until evenly dispersed throughout the food. The food was made by mixing lyophilized, homogenized squid mantle, water, and 0.03 g sodium alginate per milliliter of squid paste. This paste was drawn into a 50- $\mu$ l pipette and extruded into a 0.25 mol L<sup>-1</sup> calcium chloride solution, which caused the paste to harden to the consistency of cooked pasta. The squid strand was removed from the calcium chloride and cut into small pieces that were fed to consumers. By varying the concentration of squid added to the food mix, we made foods that equaled the caloric content per volume of the worm under investigation. Caloric content of homogenized squid mantle and worms was determined by bomb calorimetry of 0.3–0.5 g dry mass of lyophilized tissue in a Parr 1425 Semimicro bomb calorimeter ( $n = 6–7$  for each species).

Treatment foods were presented to the fishes and crabs versus a palatable control that consisted of the same squid-based food and aliquot of ethanol, but without the treatment extract (methods of Lindquist and Hay 1996). For each assay, sample size was 8–15 for each predator. Fisher's Exact tests were used to assess feeding on control squid versus squid containing extract.

Because the halogenated compounds in *S. kowalevskii* were volatile and the bioactivity was diminishing with each purification procedure (e.g., deterrent effects declined as a function of the number of separation procedures required), extracts were initially added to squid at 2 $\times$ , 4 $\times$ , or 6 $\times$  natural volumetric concentration (i.e., for 2 $\times$ , the extract from 2 ml of worm was placed into 1 ml of food), depending on whether they had progressed through one, two, or three separation steps, respectively. After identifying the active metabolite, we quantified its natural concentration in the worm using high performance liquid chromatography (HPLC). We used these concentration data to evaluate the ecological relevance of our 6 $\times$  natural concentration assay and to repeat our bioassay at natural concentration for additional consumers.

Because the pentane extract was deterrent and the methanol/water was not, we further separated the pentane extract by preparative thin layer chromatography (60 Å silica gel plate, 250- $\mu$ m thickness, Whatman) using a 60:40 ratio of pentane:diethyl ether. Based on coloration and ultraviolet (UV) absorbance, six bands were collected separately in diethyl ether. Each band was dried under nitrogen and tested in feeding assays. Only one band deterred feeding. This band

was further separated by HPLC [Waters 2690 separations module coupled to a Waters 996 photodiode array detector, using an Alltech Alltima silica 5- $\mu$  column (250 mm  $\times$  10 mm)] with an 85:15 hexanes:diethyl ether mobile phase. This produced five peaks. Only one peak was deterrent. It was analyzed with a Hewlett-Packard 5890 gas chromatography (GC) coupled to a VG 70-SE mass spectrometer (J&W DB-5 column, 30 m  $\times$  0.25  $\mu$ l). The system was run in electron impact positive mode running a temperature program starting at 30°C, held for 1 min, then ramped to 300°C at 15°C min<sup>-1</sup> and held for 11 min. Following mass spectrometry, we characterized the compound by <sup>1</sup>H Nuclear Magnetic Resonance (NMR) spectroscopy (Bruker DRX, 500 MHz) in deuterated diethyl ether (Et<sub>2</sub>O-d<sub>10</sub>), in anhydrous carbon tetrachloride (CCl<sub>4</sub>), and in deuterated chloroform (CDCl<sub>3</sub>) to determine the proton chemical shift. In addition, <sup>13</sup>C NMR spectroscopy (solvent = CDCl<sub>3</sub>) was used to analyze carbon chemical shifts.

Once the deterrent compound was identified, we quantified its natural concentration in four individual worms using HPLC. Quantification in each worm was based on the mean of three replicate injections of methanol extract from each worm. Specimens for this procedure were collected in November 2002, placed in HPLC-grade methanol, and stored at -20°C for 2 weeks. After extraction, remaining worm tissues were dried to a constant mass at 60°C and concentration of the deterrent metabolite calculated as the percentage dry mass for each worm.

Several worms consumed in our feeding assays produce brominated metabolites hypothesized to serve as chemical defenses against consumers (Woodin et al. 1987; Yoon et al. 1994; Fielman and Targett 1995; Fielman et al. 1999; Cowart et al. 2000). To ensure that the specific species and individuals we used contained these metabolites, we analyzed our samples for brominated metabolites by gas chromatography mass spectrometry (GC/MS) (identifying brominated compounds by the presence of molecular ion peaks separated by 2 atomic mass units for the two isotopes of bromine). To expand our investigation to additional species and geographic areas, we also investigated other areas of Georgia, as well as Florida and North Carolina, to collect worm species (or close relatives) that were reported to produce brominated metabolites. We assessed their palatability to generalist consumers, as well as their production of brominated metabolites. Collections were made from an exposed mud flat on another part of Little Tybee Island, Georgia (31°56'N, 80°57'W), August 2002; Middlemarsh, North Carolina (34°41'N, 76°37'W), July 2002; intertidal and subtidal soft-sediment areas around Key Largo, Florida (Rodriguez Key 25°08'N, 80°25'W; Pickles Reef 24°60'N, 80°24'W; Blackwater Sound 25°08'N, 80°25'W; mile marker 110 25°11'N, 80°25'W), July–August 2002; and from exposed mud flat in Estero Bay, Bonita Springs, Florida (26°27'N, 81°56'W) and Clam Pass, Naples, Florida (26°14'N, 81°48'W), August 2002.

Many of these newly collected worms were fed to the bluehead wrasse *Thalassoma bifasciatum* as well as to several of the previously used consumers. Bluehead wrasse are generalists that feed mainly on small benthic prey (Feddern 1965) and are commonly used as model consumers in in-



vestigations of prey chemical defenses (e.g., Pawlik et al. 1995; Lindquist and Hay 1996; Kubanek et al. 2002; Pisut and Pawlik 2002). Individuals (5–7 cm long) were collected from Pickles Reef, Florida, kept individually in 2.1- to 2.4-liter containers in a recirculating seawater system, and fed a maintenance diet of frozen brine shrimp.

All Florida worms were fed to the bluehead wrasse, with *Dasybranchus lumbricoides* and *Ptychodera bahamensis* also being fed to mummichog and the lesser blue crab. *D. lumbricoides* occurs as far north as North Carolina (Day 1973), so it is sympatric with all of these consumers. *P. bahamensis* is not known from Georgia or North Carolina (Ruppert and Fox 1988), so it is sympatric with bluehead wrasse but not the other consumers. Worms collected from North Carolina and Georgia were fed to mummichogs, spot, and the lesser blue crab, with *Chaetopterus variopedatus*, *N. lobatus*, and *Balanoglossus aurantiacus* also being fed to bluehead wrasse. *C. variopedatus* and *B. aurantiacus* occur in South Florida (Kicklighter pers. obs.) and are, thus, sympatric with all of our consumers. *N. lobatus* is not known from Florida (Day 1973), so it may not overlap with bluehead wrasse. Feeding assays were as described for the worms from Georgia, with the exception that (1) the control food for bluehead wrasse was a brine shrimp, (2) feeding of North Carolina worms was conducted at the University of North Carolina at Chapel Hill's Institute of Marine Sciences, Morehead City, North Carolina, and (3) feeding of Florida worms was conducted at NOAA's National Undersea Research Center in Key Largo, Florida, or at the Georgia Institute of Technology's marine facility on Skidaway Island, Georgia. Florida specimens fed to mummichog and the lesser blue crab were frozen upon collection in Florida and thawed for feeding assays in Georgia.

Worms for GC/MS analysis of brominated metabolites were placed in HPLC-grade methanol and frozen for 1–2 weeks prior to analysis. Large worms were extracted individually; small worms were extracted as groups of 4–9 individuals ( $n = 3$ –5 independent individuals or groups for each species). Samples were analyzed by GC/MS using a Hewlett-Packard 5890 series II gas chromatograph (Econ-Cap EC-5 Alltech column 30 m  $\times$  0.25 mm internal diameter; 0.25- $\mu$ m film) with a Hewlett-Packard 5971A mass selective detector (MSD, quadrupole mass spectrometer).

Since only two of the nine worm species containing brominated compounds were unpalatable, we also investigated the palatability of specific brominated compounds produced by various worms. We acquired 4-bromophenol, 2,4-dibromophenol, 2,6-dibromophenol, 2,4,6-tribromophenol, and bromohydroquinone from Sigma-Aldrich and incorporated them into squid paste. All metabolites were tested at a natural concentration reported in the literature: 4-bromophenol (4200 ng g<sup>-1</sup> wet tissue mass from *Glycera americana* from Australia, Whitfield et al. 1999); 2,4-dibromophenol (13.4  $\mu$ mol g<sup>-1</sup> wet tissue mass from *S. bromophenolus*, King et al. 1995); 2,6-dibromophenol (2.84  $\times 10^{-6}$  g ml<sup>-1</sup> tissue from *B. aurantiacus*, Kicklighter unpubl. data); 2,4,6-tribromophenol (10.4 mg per 930 g wet tissue mass from *Balanoglossus carnosus*, Higa et al. 1980); bromohydroquinone (458 mg per 1.2 kg wet tissue mass from *Glossobalanus* sp., Higa et al. 1980). All metabolites except for 2,4-dibromo-

phenol were also tested at 5 $\times$ , 10 $\times$ , and 20 $\times$  natural connection. 2,4-Dibromophenol was also tested at 2 $\times$ , 4 $\times$ , and 5 $\times$  (bluehead wrasse), 5 $\times$ , 7 $\times$ , and 10 $\times$  (mummichog), and 5 $\times$ , 10 $\times$ , and 15 $\times$  (crab) natural concentration. Assay foods were made to mimic the caloric content volume<sup>-1</sup> of the particular species from which the metabolite had been quantified (*G. americana* from Georgia for 4-bromophenol and *B. aurantiacus* for 2,6-dibromophenol). If such data were unavailable, we used the caloric value of the most closely related species for which data were available (*S. kowalevskii* for 2,4-dibromophenol and *B. aurantiacus* for 2,4,6-tribromophenol and bromohydroquinone).

To determine whether unpalatable species persisted in seasons when palatable worms declined, 13 sediment cores (36 cm<sup>2</sup>, 30 cm deep) were taken at approximately monthly intervals between November 2001 and October 2002 from a mud flat on Little Tybee Island, Georgia (31°57'N, 80°56'W). Core location was randomized from coordinates (0.5 m apart) on a 19 m  $\times$  3 m grid, and cores were never repeatedly taken in the same location. Cores were sieved through a 1-mm mesh (thus, only adult specimens were collected), material on the sieve was placed in buffered 7% formalin and sorted under a dissecting microscope. Only whole worms and fragments containing a worm head were counted. Species that inhabit a strong tube (i.e., *Clymenella torquata*, *Diopatra cuprea*, *Owenia fusiformis*, *S. oculatus*), were excluded due to the possibility of the tube offering a structural refuge from predation that might confound assessment of palatability and susceptibility to epibenthic predators. All other palatable species (determined by our feeding assays) were pooled by core to determine the seasonal abundance of palatable mud-flat worms. Three of the sample sets deviated slightly from normality (determined by skewness and kurtosis), but variances were homogeneous (Bartlett's Test). Because analysis of variance (ANOVA) is robust with departures from normality, especially with equal sample sizes (Underwood 1997), an ANOVA was performed to evaluate changes in density among times. Tukey–Kramer post hoc analyses were used to identify significant groupings across different sampling times.

Owing to the fragility and lower density of the unpalatable worm *S. kowalevskii*, the core sampling method was ineffective. However, *S. kowalevskii* deposits a distinctive, coiled fecal cast on the sediment surface and counts of fecal casts can be used to estimate density of *Saccoglossus* spp. (e.g., Knight-Jones 1953; Gypson 1989). Thus, *S. kowalevskii* density was estimated by counting the total number of fecal casts in the 19 m  $\times$  3 m sampling grid. This is a conservative estimate, since only individuals that have deposited a fecal cast are counted, but it serves as a good approximation to the number of active individuals present. Because *S. kowalevskii* was the only unpalatable worm at this site, its abundance was contrasted with that of the pooled sample of palatable species.

## Results

Of the 16 worm species from the Little Tybee Island mud flat that we could collect in adequate numbers for feeding

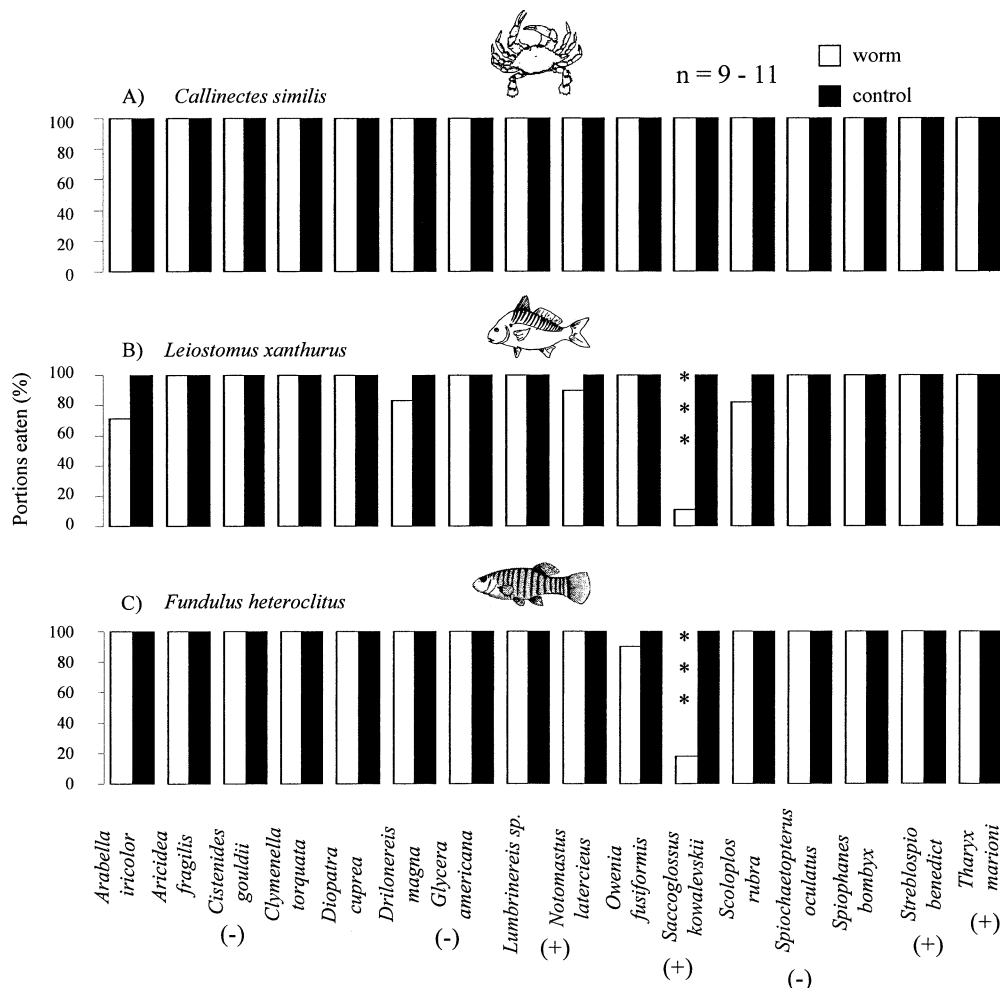


Fig. 1. Palatability assays of whole worms or worm portions to (A) the crab *C. similis*, (B) the spot *L. xanthurus*, and (C) the mummichog *F. heteroclitus*. In all cases, worm portions (white bars) and palatable control food (black bars) were fed to each consumer. Seven of the worm species we assayed had been reported to contain brominated natural products. For these seven species, we indicate a + (= brominated metabolites present) or - (= brominated metabolites absent) based on GC/MS analysis of the populations of worms we used in our feeding assays. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  by Fisher's Exact test.

assays, 15 species (94%) were palatable to all consumers, and one was unpalatable to two of our three consumers (Fig. 1). The annelids *Arabella iricolor*, *Aricidea fragilis*, *Cistenides gouldii*, *C. torquata*, *D. cuprea*, *Drilonereis magna*, *G. americana*, *Lumbrinereis sp.*, *Notomastus latercieus*, *O. fusiformis*, *Scoloplos rubra*, *S. oculatus*, *Spiophanes bombyx*, *S. benedicti*, and *T. marioni* were readily eaten by all three consumers. The hemichordate *S. kowalevskii* was unpalatable to mummichogs and spot, but was readily eaten by the lesser blue crab.

When the crude chemical extract from *Saccoglossus* was partitioned between pentane and methanol/water, the pentane partition deterred feeding by mummichogs, the methanol/water partition did not (Fig. 2A). Bioassay-guided fractionation of the pentane partition (Fig. 2B,C) indicated that a single compound (HPLC peak 5) was responsible for deterring feeding of both mummichogs and spot (Fig. 2C,D). Mass spectral analysis suggested a tribrominated, nitrogen-

containing molecule with molecular weight of 304, most likely a pyrrole. 2,3,4-Tribromopyrrole and 2,3,5-tribromopyrrole were both possible structures given the mass spectral data. The  $^{13}\text{C}$  NMR spectrum indicated four aromatic carbons, with chemical shifts 99.7, 99.9, 101.8, and 119.1. Only the carbon at 119.1 was attached to a hydrogen, determined by a Distortionless Enhancement by Polarization Transfer (DEPT)  $^{13}\text{C}$  NMR experiment. The 6.83 chemical shift for the single aromatic proton (run in deuterated chloroform [similar values when run in deuterated diethyl ether and carbon tetrachloride]) differed by 0.3–0.7 parts per million (ppm) from reported values for 2,3,4-tribromopyrrole and 2,3,5-tribromopyrrole (Gilow and Burton 1981; Emrich et al. 1990). Nevertheless, the doublet structure of the proton signal (coupling constant = 3.0 Hz), indicative of an adjacent proton on the pyrrole nitrogen, strongly suggested 2,3,4-tribromopyrrole. This agrees with previous studies that have identified 2,3,4-tribromopyrrole as the tribrominated com-

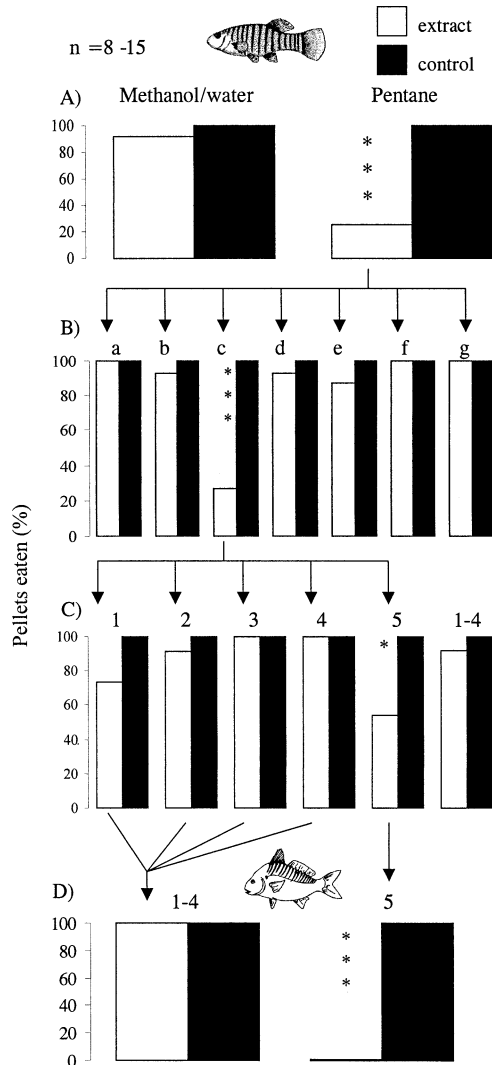


Fig. 2. Bioassay-guided fractionation of *S. kowalevskii* extracts fed to (A–C) *F. heteroclitus* or (D) *L. xanthurus*. White bars represent squid pellets containing extract. Black bars represent control pellets (treated with an equivalent aliquot of ethanol but not containing the extract or compound). To offset possible losses of volatile or unstable metabolites with each separation step, assays in (A) were run at 2 $\times$ , those in (B) at 4 $\times$ , and those in (C) at 6 $\times$  the actual yield. The 6 $\times$  concentration of peak 5 in (C) was later determined to be only 70% of the natural concentration. (A) Assays of pentane versus methanol/water partitions. (B) Assays of the seven normal phase silica fractions (a–g) separated from the pentane extract. (C) Assays of the five HPLC peaks (1–5) from the deterrent c band fraction. (D) Assays of HPLC peak 5 and of HPLC peaks 1–4, fed to *L. xanthurus*. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  by Fisher's Exact test.

compound in this species (Woodin et al. 1987; Fielman and Targett 1995; Fielman et al. 1999).

In our initial bioassay-guided separation scheme, we were concerned that each separation procedure might cause loss of active compounds that were volatile or unstable. To counter this, we tested initial extracts (Fig. 2A) at 2 $\times$  their natural yield and final partitions (Fig. 2C,D) at 6 $\times$  natural yield. This could have resulted in assays being conducted at un-

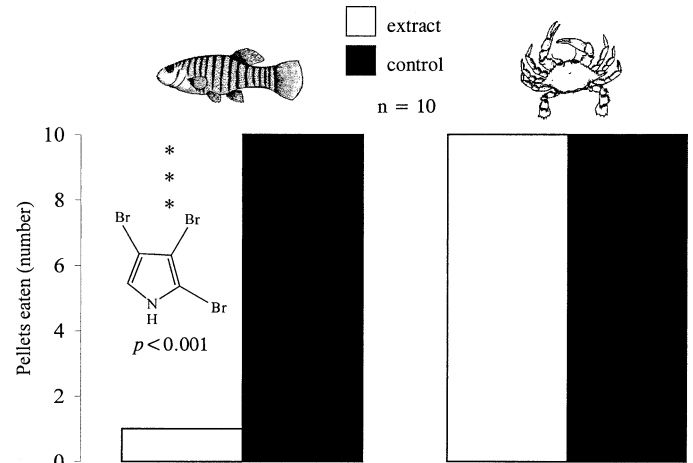


Fig. 3. Effects of 2,3,4-tribromopyrrole at the natural concentration of 0.2% dry mass when fed to the mummichog *F. heteroclitus* and the crab *C. similis*. Methods, symbols, and analysis as in Fig. 2.

naturally high concentrations. HPLC quantification of 2,3,4-tribromopyrrole in four individuals of *S. kowalevskii* and in the material we had used in our 6 $\times$  assay (Fig. 2C,D) determined that natural concentration in worms was  $0.20 \pm 0.05\%$  dry mass, while our 6 $\times$  test concentration had been only 0.14% dry mass (i.e., natural concentration was actually 40% greater than our 6 $\times$  test concentration). When 2,3,4-tribromopyrrole was added to squid food at natural concentration, mummichogs were strongly deterred but crabs were not (Fig. 3). This mirrors the feeding of these consumers on intact worm tissue (Fig. 1).

When we used GC/MS to be sure which of our test worm populations actually contained brominated metabolites, we detected brominated compounds in *B. aurantiacus*, *D. lumbricoides*, *Heteromastus* sp., *N. latercieus*, *N. lobatus*, *P. bahamensis*, *S. benedicti*, and *T. marioni* but not in *Cirriformia tentaculata*, *C. gouldii*, *C. variopedatus*, *G. americana*, *Glycera dibranchiata*, and *S. oculatus* (Fig. 1; Table 1). Of the nine species containing brominated compounds, only *P. bahamensis* and *S. kowalevskii* were unpalatable to consumers (Fig. 1; Table 1). *C. tentaculata* was also unpalatable, but it did not contain brominated metabolites; this species was defended by a family of nonbrominated pyrroles, (Kicklighter et al. 2003). For *P. bahamensis*, crude extracts and further fractions were unpalatable to the bluehead wrasse, but it is unknown whether brominated metabolites were responsible, since the activity was lost with further purifications (Kicklighter et al. 2003). This prevented identification of the active metabolite(s).

When we directly tested five brominated metabolites known to occur in several genera of soft-substrate inhabiting worms (e.g., species of *Glycera*, *Saccoglossus*, *Balanoglossus*, and *Glossobalanus*), none were unpalatable at natural concentration. When elevated to 20 $\times$  natural concentration, all three predators still readily consumed squid pellets containing 4-bromophenol, 2,6-dibromophenol, 2,4,6-tribromophenol, and bromohydroquinone (Fig. 4A–C). 2,4-Dibromophenol was deterrent at the unnaturally high concentrations

Table 1. Presence/absence of brominated metabolites and results of palatability assays for additional worm species collected from Georgia, North Carolina, and Florida. *p* values in bold are significant.

Species	Location	Brominated compounds present	Predator	<i>n</i>	Consumption (%)		<i>p</i> value
					Worm	Control	
<i>Balanoglossus aurantiacus</i>	Georgia	Yes	<i>Fundulus heteroclitus</i>	11	63.6	100.0	0.09
			<i>Leiostomus xanthurus</i>	14	92.9	100.0	>0.99
			<i>Callinectes similis</i>	9	100.0	100.0	>0.99
			<i>Thalassoma bifaciatum</i>	10	90.0	100.0	>0.99
<i>Cirriiformia tentaculata</i>	Florida	No	<i>Thalassoma bifaciatum</i>	10	0.0	100.0	< <b>0.01</b>
			<i>Fundulus heteroclitus</i>	10	90.0	100.0	>0.99
<i>Chaetopterus variopedatus</i>	North Carolina	No	<i>Leiostomus xanthurus</i>	10	90.0	100.0	>0.99
			<i>Callinectes similis</i>	10	80.0	100.0	0.47
			<i>Thalassoma bifaciatum</i>	10	80.0	100.0	0.47
			<i>Fundulus heteroclitus</i>	10	100.0	100.0	>0.99
<i>Dasybranchus lumbricoides</i>	Florida	Yes	<i>Fundulus heteroclitus</i>	10	100.0	100.0	>0.99
			<i>Callinectes similis</i>	9	88.9	100.0	>0.99
			<i>Thalassoma bifaciatum</i>	10	100.0	100.0	>0.99
<i>Glycera dibranchiata</i>	North Carolina	No	<i>Fundulus heteroclitus</i>	10	80.0	100.0	>0.99
			<i>Leiostomus xanthurus</i>	10	80.0	100.0	>0.99
			<i>Callinectes similis</i>	10	100.0	100.0	>0.99
			<i>Thalassoma bifaciatum</i>	13	76.9	100.0	0.22
<i>Heteromastus</i> sp.	Florida	Yes	<i>Fundulus heteroclitus</i>	10	80.0	100.0	0.47
			<i>Leiostomus xanthurus</i>	10	90.0	100.0	>0.99
<i>Notomastus lobatus</i>	North Carolina	Yes	<i>Callinectes similis</i>	10	100.0	100.0	>0.99
			<i>Thalassoma bifaciatum</i>	10	100.0	100.0	>0.99
			<i>Fundulus heteroclitus</i>	10	50.0	100.0	< <b>0.03</b>
			<i>Callinectes similis</i>	10	20.0	100.0	< <b>0.01</b>
<i>Ptychodera bahamensis</i>	Florida	Yes	<i>Thalassoma bifaciatum</i>	11	18.2	100.0	< <b>0.01</b>

of 5× for bluehead wrasse, 10× for mummichogs, and 15× for crabs (Fig. 4A–C).

The caloric content (calories g<sup>-1</sup>) of worms and of homogenized squid mantle was determined and converted to calories ml<sup>-1</sup>. Mean calories ml<sup>-1</sup> ± 1 standard error (SE) were as follows: *S. kowalevskii*, 1230 ± 32; *B. aurantiacus*, 459 ± 26; *P. bahamensis*, 404 ± 30; *G. americana*, 1283 ± 9; and homogenized squid mantle, 834 ± 11 calories ml<sup>-1</sup>.

When we monitored seasonal abundance of common palatable worms (11 species) versus the chemically defended worm *S. kowalevskii* on a mud flat at Little Tybee Island, Georgia, the chemically defended species reached peak abundance during summer when predator abundance is high. Palatable worms, as a group, declined by >50% during the April to June period when the unpalatable species increased by about 40-fold (Fig. 5). Palatable species that were pooled to determine patterns of abundance over time included *A. fragilis*, *A. iricolor*, *C. gouldii*, *D. magna*, *G. americana*, *Lumbrinereis* sp., *N. latercieus*, *S. benedicti*, *S. bombyx*, *T. marioni*, and *S. rubra*.

## Discussion

Although soft-sediment worms commonly produce brominated metabolites that have been hypothesized to function as consumer deterrents, when we surveyed the palatability of 16 common worms occurring in a Georgia mud flat, 15 (94%) were palatable to sympatric fishes and a crab (Fig. 1). Only the hemichordate *S. kowalevskii* was unpalatable to mummichogs and spot, but even this species was readily

eaten by the lesser blue crab. This high frequency of palatable worms suggests brominated metabolites, as a class, are not defensive, or that the worms we investigated did not contain these metabolites. To address the second possibility, worms from the Little Tybee Island mud flat that were reported to contain brominated metabolites were analyzed for the presence of brominated compounds using GC/MS. We also expanded our search to other mud flats in Georgia, as well as North Carolina and Florida, to collect additional species (or their close relatives) reported to produce brominated compounds and assessed their palatability to generalist consumers, as well as their production of brominated metabolites. Nine species contained brominated metabolites based on our GC/MS analysis (Fig. 1; Table 1). Many of these species have been reported to contain a variety of brominated compounds, including various pyrroles, phenols, and substituted alkanes (Fielman et al. 1999). Of these nine species, only two were unpalatable (Fig. 1; Table 1); *S. kowalevskii* was chemically defended by 2,3,4-tribromopyrrole (Figs. 2, 3); *P. bahamensis* was also defended chemically (based on bioassays like those shown in Fig. 2), but the deterrent metabolites were not determined (Kicklighter et al. 2003).

We also directly examined the effects of five brominated natural products (4-bromophenol, 2,4-dibromophenol, 2,6-dibromophenol, 2,4,6-tribromophenol, and bromohydroquinone) on consumer feeding. None of the five compounds were deterrent at natural concentrations reported in the literature, and four compounds remained palatable to all consumers at even 20× natural concentration (Fig. 4A–C). Only 2,4-dibromophenol was deterrent, but even this compound



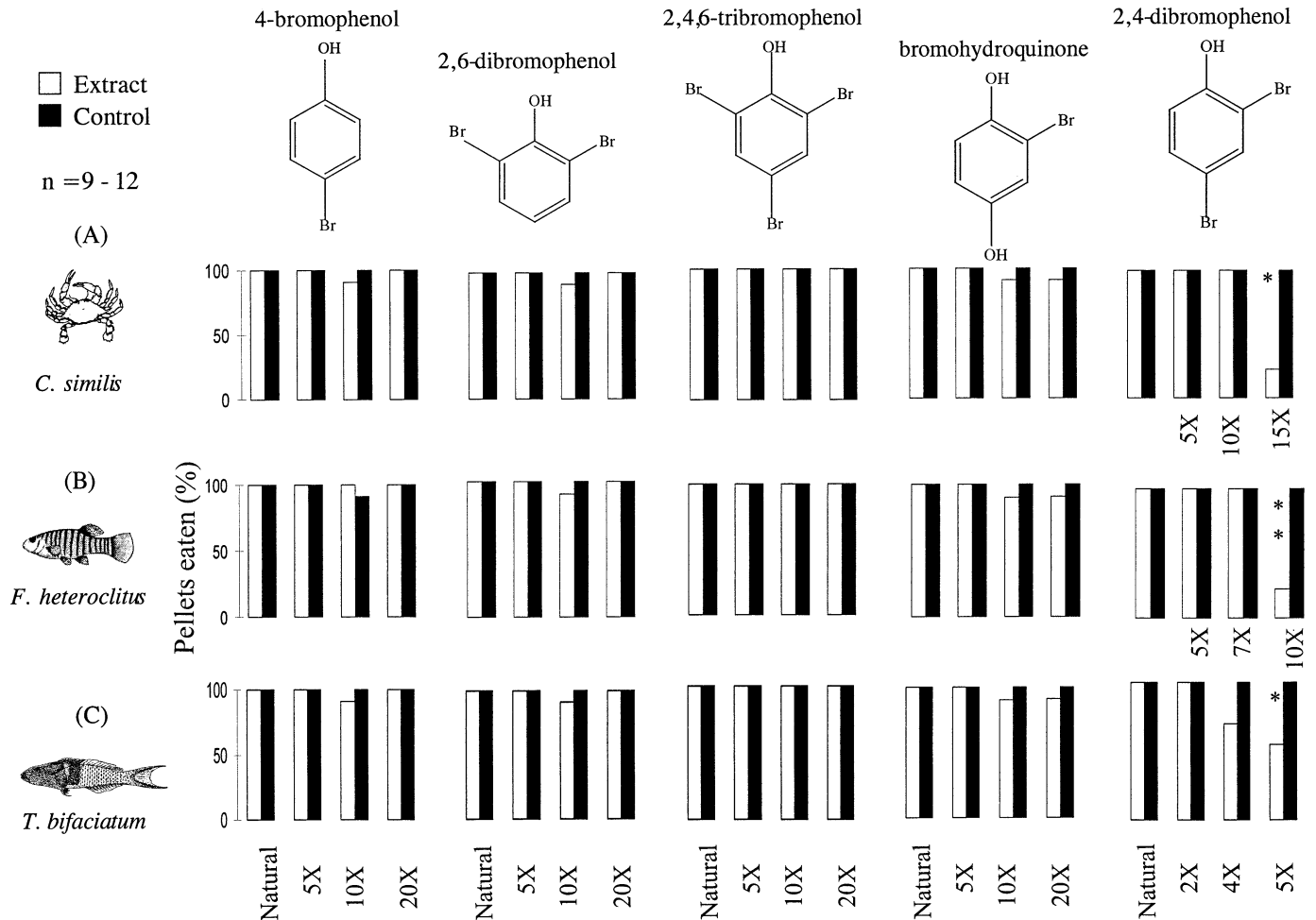


Fig. 4. Effects of five brominated natural products on feeding by (A) *C. similis*, (B) *F. heteroclitus*, and (C) *T. bifasciatum* when tested at natural, and well above natural, concentrations found in the worms. Methods, symbols, and analysis as in Fig. 2.

had to be at 5 $\times$  (for the wrasse) to 15 $\times$  (for the crab) natural concentration before consumer feeding was suppressed (Fig. 4A–C).

Worm tissues vary in their concentration of brominated metabolites (Goerke et al. 1991; Yoon et al. 1994; Fielman and Targett 1995; King et al. 1995), and in some species, body portions closest to the surface, where they may be at more risk of predation, contain elevated concentrations of compounds relative to whole body values. In these situations, natural concentrations based on whole body values could be lower than the ecologically meaningful concentrations that a consumer would encounter when attacking these most chemically rich portions of the worm. Thus, it becomes important to evaluate how our test concentrations may relate to the higher values found within certain tissues.

We could find only three investigations that reported whole body concentrations of brominated metabolites as well as concentrations for different portions of worm bodies. King et al. (1995) found for *S. bromophenolosus* that compared to whole body values, 2,4-dibromophenol was 54% greater in the proboscis and 68% less in the tail. If this worm is typical, then our findings of deterrence at 500–1,500% of natural concentration for this compound (Fig. 4) would have

no ecological meaning. However, Fielman and Targett (1995) found that the concentration of 2,3,4-tribromopyrrole in the proboscis of *S. kowalevskii* was 7.8 $\times$  higher than the mean value for the whole body, and Goerke et al. (1991) found that the five metabolites they investigated in different portions of *Thelepus* spp. could be 2–6 $\times$  higher for tentacles alone than for the whole body mean. Given presently available studies on this topic, it appears that the 10 $\times$  and 20 $\times$  concentrations we tested in Fig. 4 are likely to equal, or considerably exceed, the concentrations that a consumer would ever encounter in the field—no matter which worm portion they attacked. This suggests that 4-bromophenol, 2,6-dibromophenol, 2,4,6-tribromophenol, and bromohydroquinone are unlikely to function as chemical defenses against consumers. If 2,4-dibromophenol varies as much within some worms as other metabolites do in the worms studied by Goerke et al. (1991) and Fielman and Targett (1995), then it is conceivable that this metabolite could play a defensive role for some parts of some species when attacked by some consumers, but this is at present conjecture that has not been demonstrated to be ecologically meaningful. Overall, the brominated natural products that we evaluated in Fig. 4 were ineffective as feeding deterrents.



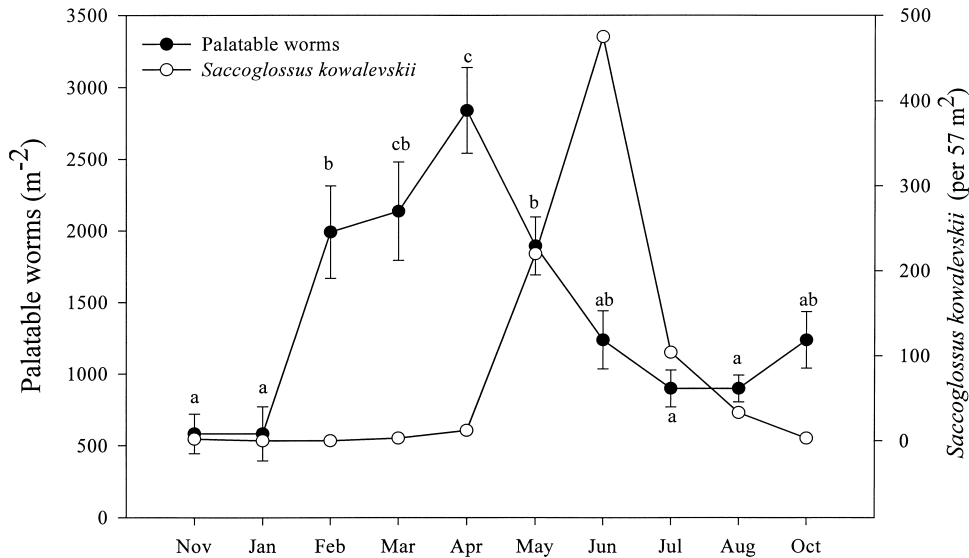


Fig. 5. Monthly densities of combined species of palatable worms ( $\text{m}^{-2}$ )  $\pm$  1 SE and the total number of *S. kowalevskii* occurring in 57  $\text{m}^2$  of mud flat at Little Tybee Island, Georgia. Letters indicate significant differences between monthly densities of palatable worms, as determined by ANOVA with Tukey–Kramer multiple comparisons test.

Our assay results for specific metabolites also correlate well with the palatability of worms known to contain many of these compounds. 4-Bromophenol, 2,4-dibromophenol, and 2,4,6-tribromophenol occur in *N. lobatus* (Yoon et al. 1994; Fielman et al. 1999), and 2,6-dibromophenol occurs in *B. aurantiacus* (Fielman et al. 1999); these species and a congener (Fig. 1) were readily consumed in our assays (Table 1). We only tested phenolic metabolites, since these were the only compounds we could readily find for purchase. It is possible that other types of brominated compounds could function as defenses. However, based on mass spectral data, Fielman et al. (1999) proposed that *S. benedicti* contained alkyl bromides and that *T. marioni* contained brominated alkylpyrroles; both of these species were palatable in all of our assays.

From our data, it is clear that one cannot discuss the ecological role of brominated metabolites produced by infaunal worms as if they have a uniform function or bioactivity. The deterrent effects of a compound are a function of the specific structure of the compound and the consumer it is tested against (Figs. 1, 4; Table 1). As examples of among-consumer variance in the effects of defensive compounds, 2,3,4-tribromopyrrole strongly deterred feeding by fishes but had no effect on lesser blue crab feeding (Figs. 2, 3), and 2,4-dibromophenol concentrations that deterred feeding by bluehead wrasse had to be doubled to deter feeding by mummichog and tripled to deter feeding by lesser blue crabs (Fig. 4). The inability to predict compound activity by structural class or by degree of halogenation is also well demonstrated by the patterns shown in Fig. 4. For example, 2,4-dibromophenol significantly deterred wrasse feeding at a concentration of 5 $\times$ , but none of the other metabolites affected feeding at concentrations of 20 $\times$ , even though they were also all brominated and based on a phenolic ring structure. For some contrasts, the pattern is even more striking. 2,6-

Dibromophenol has the same atomic mass and formula as 2,4-dibromophenol (the molecules differ only by the site of attachment of one bromine atom), yet one molecule is deterrent while the other is not. When the structure of the deterrent metabolite 2,4-dibromophenol is altered by adding a single bromine (becoming 2,4,6-tribromophenol), by removing one bromine (becoming 4-bromophenol), or by substituting an OH for one of its bromine atoms (becoming bromohydroquinone), it loses its deterrence (Fig. 4). Clearly, activity is a function of very specific structural features and not of general traits such as whether the molecule is a phenolic or is brominated. This pattern of compound-specific activity is also the case for other groups of prey species and compound types (e.g., Hay and Fenical 1988; Pawlik 1993; Hay 1996), so ecologists should refrain from assuming function based on general molecular traits such as compound class or the presence of specific atoms (such as halogens).

Of the 24 worm species investigated, at least nine contained brominated metabolites, but only two of these species (*S. kowalevskii*, *P. bahamensis*) were unpalatable (Fig. 1; Table 1). The metabolite defending *P. bahamensis* is unstable and thus unknown (Kicklighter et al. 2003). *S. kowalevskii* was defended by the brominated natural product 2,3,4-tribromopyrrole, which it produced at  $0.20 \pm 0.05\%$  of worm dry mass (Fig. 3). This metabolite strongly deterred fish, but not crab, feeding (Figs. 1–3). Fielman and Targett (1995) also quantified 2,3,4-tribromopyrrole in *S. kowalevskii* from Delaware and determined the concentration to be  $0.15 \pm 0.2\%$  dry mass with a range of 0.035–0.58% dry mass. Our mean value is about 33% higher than their mean natural concentration, but it falls within the range of values determined from their seasonal investigation of variation in concentration of 2,3,4-tribromopyrrole. Fielman and Targett (1995) cited unpublished data that 2,3,4-tribromopyrrole was deterrent to fish predators at 2% ash-free dry mass, which

is about 0.3% dry mass (based on our ash-free dry weight to dry weight conversions for *S. kowalevskii*), or 2× their average natural concentration of 2,3,4-tribromopyrrole. This concentration is about 1.5× the natural concentration of the worms we studied. In our assays, 2,3,4-tribromopyrrole was deterrent at only 0.142% dry mass (about 70% of natural concentration) (Fig. 2C–D). It may be deterrent at even lower concentrations, but we did not conduct assays to determine this.

Our data assessing the palatability of specific brominated metabolites and of the worms containing these metabolites agree with previous assays assessing the palatability of these worms. Thomas (1972) observed that *S. otagoensis* (which produces 2,3,4-tribromopyrrole) was unpalatable to fishes, while Giray and King (1997a) found that *S. bromophenolus* (which produces 2,4-dibromophenol) was palatable to invertebrate consumers. In our assays, 2,4-dibromophenol did deter feeding, at 5–15× the natural concentration reported for this compound (Fig. 4A–C), but it is unclear whether these concentrations are ever ecologically relevant. Thus, different brominated compounds, even those from closely related species and with very similar structures, may not share a common ecological function. In general, our assays suggest that brominated metabolites from infauna seldom serve as chemical defenses against consumers. They may play other important roles, but with the exception of effects on larval settlement (Woodin 1985; Woodin et al. 1993), these remain to be demonstrated.

As with seaweeds (Hay and Fenical 1988; Hay 1996) and sponges (Pawlik et al. 1995) that can persist in predator-rich environments by being chemically defended, 2,3,4-tribromopyrrole may allow *S. kowalevskii* to persist or even increase in abundance (Fig. 5) when predation pressure is high. During the April to June period when *S. kowalevskii* was increasing by about 40-fold, palatable species declined by >50% (Fig. 5). Several studies have documented that the highest density of mobile epibenthic predators (e.g., spot, blue crabs, croaker, spottail pinfish, horseshoe crabs, etc.) in inshore waters of the Southeast Atlantic occurs during late spring through early fall and that consumer densities are lowest in the winter and early spring—when predators have moved to deeper, warmer waters (e.g., Holland et al. 1980; Darcy 1985; Nelson et al. 1991). Observations at our study site agree with this pattern. Feeding pits excavated by blue crabs, horseshoe crabs, and stingrays occur much more frequently in the summer than at other times of the year (also see Woodin 1978). In addition, spot, mummichogs, and lesser blue crabs occur much less frequently in our traps and seines during the late fall through early spring (Kicklighter pers. obs.). Low densities of infaunal prey also have been correlated with the warm season peak in consumer activity by other investigators (Virnstein 1977, 1979; Holland et al. 1980), and Virnstein's studies demonstrated that polychaete species living at or near the sediment surface increased in density when epibenthic predators were excluded.

Although a few previous studies have investigated the palatability of worms (Thomas 1972; Prezant et al. 1981; Martin et al. 2000) or their crude extracts (Gaston and Slattery 2002), this and a companion study (Kicklighter et al. 2003) appear to be the first to experimentally demonstrate chemical

defenses of worms from marine soft-sediment habitats. The antifeedant properties of 2,3,4-tribromopyrrole lower the susceptibility of *S. kowalevskii* to predation by certain fishes and may allow *S. kowalevskii* to achieve high densities in the summer when predation appears greatest. Although this chemical defense is a brominated compound, this appears to be the exception rather than the norm. Out of 24 species from Georgia, North Carolina, and Florida, nine were documented to contain brominated compounds, but only two of these bromine-containing species were unpalatable. When five brominated natural products produced by marine worms were tested directly, none deterred feeding at natural concentration. Thus, although brominated secondary metabolites have commonly been hypothesized to function as predator deterrents for marine worms, it appears that they rarely have this ecological function (Fig. 3; Table 1). Some brominated compounds do act as deterrents (Figs. 2, 3), but worm resistance to consumers cannot be predicted on the basis of presence or absence of halogenated organic metabolites as a class (Figs. 1, 4; Table 1).

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