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Research Article

Feeding preferences and digestion of talitrids in a Bay of Fundy, Canada salt marsh

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Abstract

The function of detritivores in salt marsh ecology still presents many unknowns. We experimentally investigate the food preferences of two talitrids found in Bay of Fundy salt marshes: *Orchestia grillus* (Bosc, 1802) (=*Speziorchestia grillus* Bosc, 1802) and *Platorchestia exter* Myers & Lowry, 2023 Food preferences were found to depend on (1) the availability of marsh plant litter at the supralittoral level where the talitrids live; (2) the degree of physical/microbiological decay of the plants present in marsh litter; (3) the talitrid ecotype that is doing the consuming. Our food preference experiments with different plant foods in culture conditions confirm that *O. grillus* is a palustral specialist limited to a single plant food class that contains structural lignocelluloses. *Platorchestia exter* is a wrack generalist able to acclimate in experimental cultures to a range of decaying plant foods including those containing structural celluloses (Phaeophyta) and lignocelluloses (Angiosperms). These results confirm the applicability of the generalist/specialist continuum within the Talitridae. Cellulolytic microbiological culture methods applied to dissected gut parts of *O. grillus* are consistent with the hypothesis that the midgut is where endosymbiotic enzyme activity occurs. The digestive enzymes originate from both resident hepatopancreatic and ingested microbes.

Key words: salt marsh plant decay, *Orchestia grillus* (Bosc 1802) (BOLD AAD1236), *Platorchestia exter* Myers & Lowry 2023, talitrid specialist and generalist ecotypes, digestion, coprophagy

Introduction

Salt marshes are common ecological features of the northeast, Atlantic coast of North America from Newfoundland in the north (~50°N) to Florida in the south (~25°N). The salt marsh macroecology of these systems is well-known (Roberts and Robertson 1986; Vernberg 1993) and in Atlantic Canada they are mostly formed in river deltas with marsh sediments supplied from river runoff. Bay of Fundy salt marshes differ because they are formed in submergent coastal lands often remote from major sources of river runoff. Bay of Fundy tidal ranges are some of the highest in the world and marsh accretion is provided by marine sediments carried by tidal and wind-wave action (Roberts and Robertson 1986). The importance of salt marshes in the near-shore marine/estuarine environment is emphasized in the review by Vernberg (1993).

This study aimed to increase the knowledge of the role of talitrids (Amphipoda, Talitridae) in salt marsh ecology by determining their diet preferences. Our approach to the experimental study of feeding and digestion is focused on the species. The ecological classification to which a chosen talitrid species belongs, based on the primary ecotope occupied (Wildish 2017) and the generalist/specialist concept (Wildish and Radulovici 2019; Wildish 2024), is extremely helpful in designing feeding/digestion experiments.This is because it leads directly to hypotheses about how digestion works in a particular talitrid species. During plant tissue decay microbes attack the plant epidermis releasing defensive chemicals, whose original purpose was to deter herbivores (Tugwell and Branch 1989), but which may still be able to deter detritivorous talitrids. The microflora of the hepatopancreas of a wrack generalist, *Orchestia montagui* Audouin, 1826, contains a unique endosymbiotic, bacterial consortium believed to be involved in lignocellulose degradation (Russini et al. 2021). Since the endosymbiotic bacteria are absent in food or from the immediate environment, the question is: how does the hepatopancreas of the new talitrid recruits receive the endosymbiotic bacteria? One possibility is for the juvenile talitrid to consume the feces of adult talitrids (coprophagy), thereby becoming infected with spores from the "correct" species of endosymbiotic bacteria.

The study site consisted of a small salt marsh within the town of St. Andrews, New Brunswick, included within Pagan Point Nature Preserve. Pagan Point salt marsh is located at the mouth of the Bay of Fundy, in Passamaquoddy Bay, at ~45° North and experiences a cold temperate climate (Wildish et al. 2023). Freshwater input to the salt marsh is minimal, involving localized runoff in a few small streams.

Only two talitrid species (Crustacea, Amphipoda, Talitridae) were found within Pagan Point salt marsh: *Orchestia grillus* (Bosc, 1802) = *Speziorchestia grillus* (Bosc, 1802) a palustral specialist and the wrack generalist *Platorchestia exter* Myers & Lowry, 2023. Another palustral specialist, *Uhlorchestia spartinophila* Bousfield & Heard, 1986, has been recorded in Maine (Bousfield and Heard 1986), to the south of Pagan Point. Extensive field sampling in Pagan Point salt marsh during this study failed to find *U. spartinophila*.

The molecular genetic findings of Radulovici (2012) suggest that *O. grillus* in salt marshes all along the North America Atlantic and Gulf of Mexico coasts exists as a species complex with up to seven species. The accepted name for this complex is *Speziorchestia grillus* in the World Register of Marine Species (WoRMS) and is controversial. To ensure that our experimental subject from Pagan Point salt marsh can be accurately identified when compared with future studies we have resorted to molecular genetic methods to define it.

The three research questions addressed in this study were to:

– Determine the food preferences of *O. grillus* and *P. exter* from Pagan Point salt marsh. Follow-on investigations from this were to test for the presence of defensive chemicals in the plants fed (by boiling them) or presence of aerobic microorganisms on plants surfaces (by measuring the DO uptake from plant surfaces), acting either as deterrents or stimulants to talitrid feeding.
– Investigate the digestion in *O. grillus* with cellulolytic bacterial counting methods.
– Determine if the resident endosymbiotic, lignin-decomposing bacteria lodged in the hepatopancreas could be transferred from adults to juveniles in the feces by experimentally testing for coprophagy in *O. grillus*.

Materials and methods

Plant list for Pagan Point salt marsh

A flowering plant list for Pagan Point salt marsh is provided to identify the potential range of plants available for consumption by herbivorous and detritivorous animals in the marsh. Flowering plants were named according to the Database of Vascular Plants of Canada (VASCAN) at (https://data.canadensys.net/vascan/search). Plant associations within the salt marsh were identified by the dominant plant species.

Genetic identification of *Orchestia grillus*

The current taxonomic name for *O. grillus* in the World Register of Marine Species (WoRMS) is *Speziorchestia grillus* (Bosc, 1802) and is controversial. Because of the nomenclature difficulties outlined in Supplementary Data #1 we have identified our palustral specialist experimental subject genetically, thus *O. grillus* (Bosc, 1802) (BOLDAAD1236). The other talitrid from the salt marsh studied, *Platorchestia exter* Myers & Lowry, 2023 (formerly *P. platensis*), can be recognized by the morphological characters for *P. platensis* given in Bousfield (1973). Voucher specimens have been deposited in the Canadian Museum of Nature, Ottawa.

Genomic DNA was extracted from two individuals from the Pagan Point salt marsh and analysed for the CO1 gene (see Method in Supplemental Data #1).

Collection and culture of test talitrids

A survey of the salt marsh was made to determine where in the marsh the palustral talitrids were present. Collections of talitrids for experimental purposes were made from the salt marsh beginning in June 2023, when the perennial marsh grasses, sedges, and rushes were just starting to re-grow. Samples were collected for the food preference experiments from June to September 2024. Near the supralittoral of the southern end of the marsh a small area was cleared of plant debris around a few live *Carex* sedges and the stationary, hiding talitrids picked up between finger and thumb. The primary ecotope of *P. exter* is drifted wrack on shingle/sand beaches and it is sometimes transported over the shingle bank during storms and deposited on the marsh. *Platorchestia exter* was collected from recent wrack from the beach at Pagan Point, by shaking the wrack into plastic bags. The last collection of *P. exter* was made on 7 October 2024.

In the laboratory, talitrids for long-term culture were placed in plastic, lidded culture boxes in a controlled environment cabinet. The bottom of the culture boxes was lined with 1 μm filtered seawater-dampened cotton cheesecloth and excess food, either decaying *Carex* leaves or freshly collected *Fucus* wrack added with the latter renewed if it became slimy. A temperature logger recorded the ambient air temperature every 10 min in the environment cabinet, the seawater had a PSU = 29–31, and the photoperiod was set to 16:8 h light:dark.

Freshly captured talitrids obtained the day before, or after laboratory culture and intended for feeding preference experiments, were placed in lidded plastic boxes containing 5 cm depth of local, aerated, 1 μm filtered seawater at room temperature. All food sources had been removed from the seawater tank and the talitrids were allowed to empty their guts during a 24 h period prior to the experiments.

Food preference experiments

The common plant in the highest part of the supralittoral saltmarsh where the talitrids were sampled was *Carex palaecea* Schreber ex Whal., with *Fucus vesiculosus* (Linnaeus, 1753) occasionally present after onshore storms which occur with high tides, hereinafter referred to as *Carex* and *Fucus*, respectively. Four decay stages were identified by the appearance of each plant (Fig. 1).

The shingle/cobble, rocky shore at Pagan Point has a few large boulders in the upper mid-littoral with growing colonies of *F. vesiculosus* attached to it. Living green/yellow thalli (F1) were collected here (Fig. 1). In the lower littoral and sublittoral, *Ascophyllum nodosum* (L) Le Jolis, 1862 grows attached to rocks. The wrack bank near the salt marsh consisted mostly of *A. nodosum* and *Fucus*, although other macroalgae, flowering plant remains, and driftwood were also present in smaller amounts. Brown, recently dead *Fucus* (F2) was collected from the most recent wrack banks which were still damp. Two higher wrack banks present in

Fig. 1. Decay stages of the sedge (*Carex paleacea*). C1: green, fresh; C2: yellow, dry; C3: yellow/brown, dry; C4: brown, wet, and the bladderwrack (*Fucus vesiculosus*); F1: yellow/green, fresh; F2: brown, wet; F3: brown/black/orange; F4: black, fungal. Visual stages used for feeding preference experiments.

June/August 2024 on the shingle bank were remnants of past spring tides. From the lowest of these, we collected F3 and from the highest F4.

Collection of plant material from the salt marsh was made with scissors from green leaves of actively growing *Carex* plants ~50 cm tall from June to October (C1) close to the shingle bank at the southern end of the marsh. The subtending leaves at the base of each plant which had yellowed were cut out for C2. During the winter, after the *Carex* above-ground plants die, the dead plants are laid flat on the marsh substrate. We selected dry, yellow–brown stems and leaves that were still elevated above the substrate for C3. Eventually, the decaying plant comes into intimate contact with the marsh substrate with evidence of wetness and decay (leaf disintegration, talitrid fecal pellets and soil present) was selected as C4. Resident palustral talitrids were active and had made shallow holes or tunnels in the plant litter which helped incorporate the decaying C4 leaf from the previous year into the sediment.

Both talitrids were tested against the food that was most readily available to them in the salt marsh, that is C4. Freshly collected samples were used immediately by cutting out 0.8 cm diameter discs (with a paper punch) from the *Carex* leaf (if the leaf was < 0.8 cm wide or too crumbly scissors were used to cut an 0.8 cm leaf section) and *Fucus* thallus. The cut-out discs were used to derive wet:dry weight relationships for each food and directly in food preference tests.

Petri dish cultures for food preference tests were prepared by adding a 4 cm square of doubled cotton cheesecloth wetted with local 1 μm filtered seawater (5–7.5 mL per dish). A seawater-wetted cotton ball was included for talitrid shelter and source of moisture. Two wet-weighed discs of the paired test foods were added to each Petri dish. Freshly collected or cultured talitrids of both species were used. After allowing them to empty their guts in aerated seawater for 24 h a single talitrid was placed in each Petri dish. Transfer from the seawater tank to the Petri dish was by capturing a swimming talitrid on a piece of gauze held by forceps, placing both on the damp cheesecloth, and closing the lid rapidly to prevent escape. During the 96 h long Petri dish experiments additions of 2.5 mL of local seawater were added as needed to replace that lost by evaporation. Each treatment was replicated from 9 to 10 times. The endpoint was the difference between the initial and final dry weight of the food discs. Four test series were run in 2024 as follows:

1. Freshly collected *P. exter* from natural wrack (June 28 to July 2; September 17–21)
2. Freshly collected *O. grillus* from natural *Carex* litter (July 22–26; September 10–14)
3. Cultured *P. exter* experimentally acclimated to *Carex* litter (July 4–8; October 14–18)
4. Cultured *O. grillus* experimentally acclimated to *Fucus* wrack (July 9–13; September 30 to October 4)

The acclimation period in culture for #3 above was 6 or 15 days and for #4 it was 6 or 16 days.

A second series of feeding preference experiments was initiated to reduce both the microbes, and the defensive chemicals present in C1 and F1 by boiling (Faller and Fialho 2009) 8 mm diameter discs in tap water for up to 30 min with gentle stirring. After cooling the disks were stirred at lab temperature in local, filtered seawater for 30 min, then left to dry on filter paper. As in the first series two pre-weighed discs of each type were added to each Petri dish before adding further seawater (up to 7.5 mL) and one talitrid. The following replicated experiments were completed: C1 (fresh):C1 (boiled) and C1 (boiled):C4 (fresh) with *O. grillus* as the subject. With *P. exter* as the subject we also tested F1 (fresh): F1 (boiled) and F1 (boiled): F2 (fresh). Each of the experiments lasted for 96 h.

Biological measurement of aerobic microbial decay on plant surfaces

Aerobic decomposition on plant surfaces was measured by oxygen depletion from seawater in a sealed vial (Hargrave 1972; Hargrave and Phillips 1977). Dissolved oxygen as percent DO was measured with a fibre optic probe placed on an Optrode sensor glued to the wall of a 25 mL glass vial. Temperature-equilibrated, aerated seawater which had been filtered (0.22 μm) and autoclaved was added to each vial followed by an appropriate number of plant discs (determined from preliminary tests). The vial was then closed so that air bubbles were excluded with a plastic screw cap. The vials were placed on an orbital shaker which was stopped to make the readings. Before the DO reading the vial was inverted so the plant discs traversed the length of the vial, to ensure mixing. Dissolved oxygen levels were recorded by Fibox 3 software (PreSens, Precision Sensing GmbH, Am Biopark 11, Regensburg, Germany). Four to six measurements were made during a ~2–4 h experimental period at recorded times so that the oxygen depletion never fell below 50% of the initial reading. For each oxygen depletion experiment scatterplots were prepared in MS Excel, with y = dissolved oxygen, as percent DO, and x = sampling time in minutes. Experimental runs where DO depletion was > 50% or $R^2 < 0.5$ were discarded. From the fitted regression line of this relationship, the negative slope value was used to calculate the oxygen uptake rate as mg O_2/L/min (Wildish and Robinson 2018). Photosynthetic oxygen production during the measurements was limited by running all experiments in darkened conditions. After determining the surface area of each side of a disc from $2\pi r^2$, calculations were made to convert the data to mg/hr per g dry weight or mg/hr per cm^2 of plant surface area.

Quantitative cellulolytic bacterial counts in food, guts, and fecal pellets of *O. grillus*

Dissections were made on adult *O. grillus* of body length from 15 to 25 mm. Because most of the specimens of *P. exter* we examined were < 15 mm it was not possible to dissect the gut.

Talitrids were euthanized by immersion in 50 °C seawater for 10 s. Each animal was placed in a Petri dish on their right side and viewed through a binocular microscope (magnification × 16). A mid-dorsal incision exposed the gut which was removed from the esophagus to the anus. Excised guts were separated into anterior and posterior parts by an incision in the middle of the midgut, taking care not to damage the anterior hepatopancreas. Gut parts were kept in a drop of autoclaved seawater until all necessary material was gathered and were then placed in pre-weighed Eppendorf tubes for weighing. Excess water was removed by blotting before transferring. No special precautions during dissection were made to maintain sterile conditions. Wet-to-dry weight relationships were established so that mass comparisons of the gut and common food materials could be based on dry weights. Wet/dry weight relationships were determined for the anterior and posterior parts of the whole gut (inclusive of anterior hepatopancreatic caecae in the former and posterior caecae in the latter), decaying *Carex* leaves (C4) and wrack (small fronds of dead *Fucus*, F2) cut into small pieces. Gut and food materials were weighed wet, dried overnight (24 h) at 100 °C and reweighed.

The method described by Delalibera et al. (2007) for isolating cellulose-degrading bacteria in a liquid medium enriched by adding a Whatman No. 1 filter paper was adapted. Each sample tissue (anterior/posterior gut) was homogenized in a known volume of 0.9% basal saline solution with a tissue lyser (VWR, Mini Bead Mill; speed 3 for 30 s) with 2 mm diameter ceramic beads added to the tube. To obtain microbes from the food sources (*Carex* and *Fucus* wrack), 1 g of each was transferred to a sterile 15 mL falcon tube with 6 mL of 0.9% basal saline solution. The sample was then vortexed for 30 s to resuspend microbes. For the homogenized samples (food and guts) a series of sterile 2 mL test tubes were prepared, each containing 0.7 mL of basal saline. A known volume of homogenate was added to the initial dilution tube. The tube was mixed by vortexing. A dilution series was prepared from this initial sample and 100 μL of each dilution was pipetted onto a Congo-red carboxymethyl cellulose (CMC) agar plate (2 replicates) and incubated at 37 °C for 5 days, aiming for 30–300 colony range counts. The number of colony-forming units (CFUs) was determined on each plate and the CFU/mL was calculated as follows: (colony count) * (dilution)/volume plated. After incubation, the plates were removed from the incubator and kept at room temperature (20 °C) thereafter. CFU counts were finalized on day 18 after starting the cultures.

To examine the effect of food treatments on cellulolytic microbial activity in the anterior and posterior gut we prepared nine Petri dish cultures, each with 5 animals/dish. Each Petri dish had one Whatman No. 1 filter paper wetted with 2–5 mL of 1 μm filtered natural seawater (PSU = 30). Three food treatments were tested: fasted, *Carex* (C4) only and *Fucus* (F2) only (N = 3/treatment). The plants were freshly collected and placed in each dish so the talitrids had shelter. For the fasted treatment without food, an additional filter paper was cut up, folded, and seawater-wetted so that the talitrids had shelter. Five *O. grillus* > 15 mm in body length were selected from stock cultures (either C4 or F2 wrack) and added to each dish. Petri dishes were placed in the environment cabinet at ~20 °C and 12 h light periodicity. After 7 days the five talitrids in each Petri dish were euthanized and dissected (as above). Following dissection, the wet weights of the anterior and posterior gut were determined by combining the five gut

sections from each test dish in their respective pre-weighed microcentrifuge tubes. Sample wet weights of C4 and F2 removed from the Petri dishes were weighed after being placed in 15 mL Falcon tubes. From the wet weight of the tissue, dry weight was determined from previously determined wet/dry linear regressions. For each of the plated cultures, a cellulolytic fungal, and bacterial count was determined based on dry weight tissue and was compared to the counts obtained from the respective food items.

Comparing microbes within food and feces

Dry weights of fecal pellets were determined by collecting individual pellets with a fine artist's brush, moistened with seawater. Fecal pellets were obtained from seawater-dampened Whatman No. 1 filter paper left overnight (24 h) on the culture floor of a stock culture unit containing *O. grillus* and aged *Carex* (C4) as food. Fecal pellets were transferred individually to an aluminum weighing dish until a range of numbers (15–60 fecal pellets) had accumulated. Wet and dry weights were determined accurate to 0.01 mg, before and after drying in an oven at 100 °C for 24 h.

Fecal pellets < 24 h old were collected, weighed wet, and used for microbial testing as follows: fecal pellets were collected on clean, seawater-moistened Whatman No.1 filter papers from the culture boxes. Fifty brown fecal pellets (from talitrids eating C4) and 50 white pellets (from talitrids eating Whatman No. 1 filter paper) produced by the talitrids overnight were wet weighed separately and converted to dry weight from previously determined wet/dry regression relationships. The fecal pellets were suspended in 700 μL of 0.9% saline and then transferred to a microcentrifuge tube. Ceramic beads were added, and the sample was homogenized in a tissue lyser. A dilution series of homogenate was prepared with saline solutions and 100 μL of aliquots spread on cellulose culture plates (as above) in sterile conditions on an ethanol cleaned bench under a flame. Plates were incubated at 37 °C, monitored for growth over a period of 12 days, and colonies were counted as described above. The counts were compared with plant tissues (C4 and F2) prepared for cellulolytic counting as above.

Direct test of coprophagy

We determined whether *O. grillus* would eat fresh fecal pellets produced by others of its kind in culture. Fecal pellets < 24 h old were collected from established cultures, weighed wet and used for microbial testing as follows: fecal pellets were collected on clean, seawater-moistened Whatman No.1 filter papers from the culture boxes. Fifty brown fecal pellets (from talitrids eating C4) and 50 white pellets (from talitrids eating Whatman No. 1 filter paper) produced by the talitrids overnight were wet-weighed and converted to dry weights from wet/dry weight regressions. Ten numbered Petri dish cultures were prepared as described above. One individual, adult *O. grillus* was added to each Petri dish together with a seawater-wetted cotton ball. After fasting for 3 days, three < 24 h old fecal pellets were placed on a small piece of Whatman No. 1 filter paper and added to each Petri dish culture. There were only enough white fecal pellets for 3 Petri dish treatments, so 7 treatments were supplied with normal brown fecal pellets. Observations were made over the next 6 days to see when the fecal pellets were consumed.

Statistical analysis

Statistical analysis and plots were made with software R and RStudio (R version 4.4.1; R Core Team 2024). In the food choice experiments, to analyse the preference between C4 and the other test materials offered to the talitrid, a mixed linear model was applied on every treatment (total of 8 mixed linear models for *O. grillus*–wild caught, and the same for *O. grillus–Fucus* acclimated, for *P. exter*–wild caught, and for *P. exter–Carex* acclimated) using "Test Material" as a factor. Because the experiments were carried out in both June–July and September–October, the random factor "season" was included to account for seasonal changes in food quality and possible changes in talitrid feeding and digestive physiology.

For microbial numbers, an Analysis of variance (ANOVA) was used to test for differences between food and gut or anterior and posterior portions of the gut. The data's normality and homoscedasticity ANOVA assumptions were analyzed with a Shapiro–Wilk and a Bartlett test, respectively. Because the microbial numbers were not normally distributed based on CFU/mL/mg dry weight values for different feeding regimes (food versus gut; anterior versus posterior gut) in *O. grillus*, a non-parametric Kruskal–Wallis test was used. The significance threshold was set as 0.05.

Time-response analysis of the coprophagy experiment was performed with the package "drc" (Ritz et al. 2015) applying the three-parameters Weibull function, with calculations of the ET50 values (time necessary to eat half of the fecal pellets provided).

Results

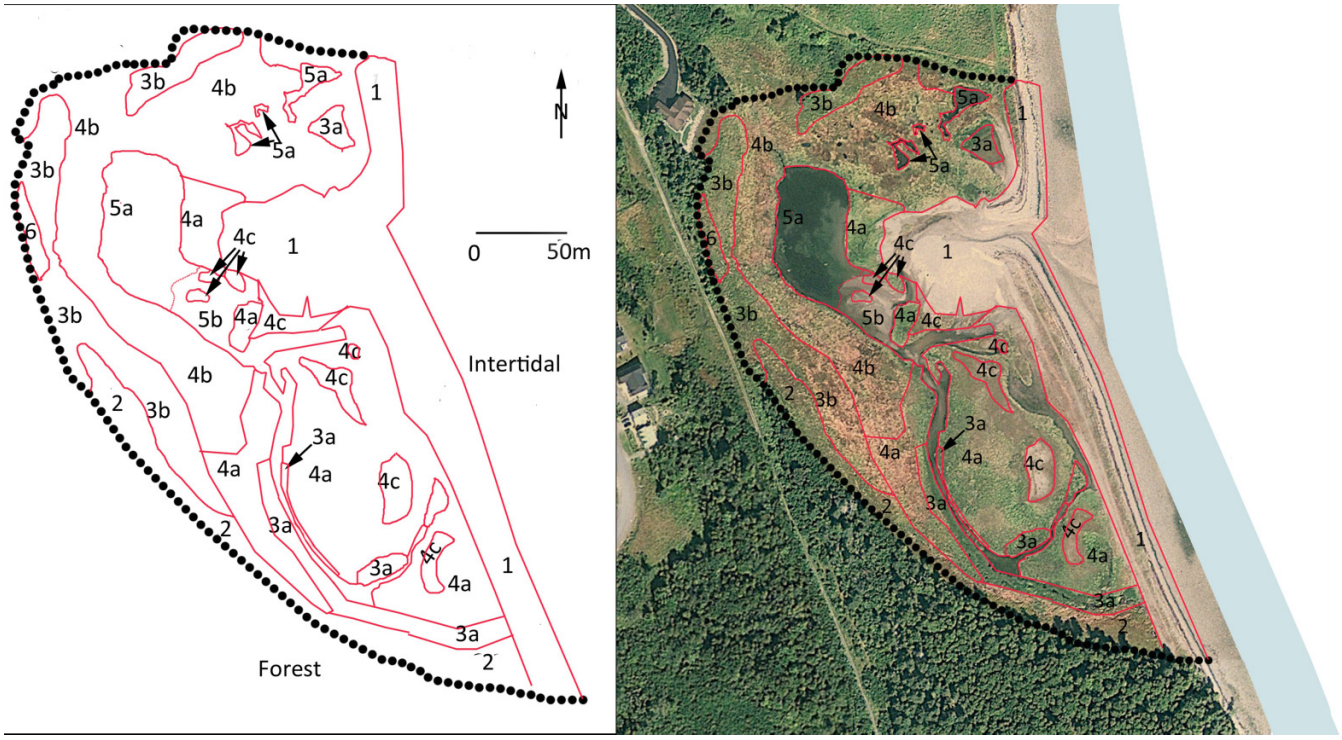
Pagan Point salt marsh

Pagan Point Nature Preserve, St. Andrews, New Brunswick, Canada, includes a small salt marsh (5.5 ha) and surrounding woodland. The salt marsh is part of the Passamaquoddy Bay coastline at the mouth of the Bay of Fundy, Canada. The sampling location for *O. grillus* was at the southern end of the marsh at 45.07676°N, 67.03884°W. The marsh is protected by a shingle bar on the seaward side of the marsh with one drainage channel (dry in the summer) cutting through the shingle bar (Fig. 2). During winter storms, the shingle bar moves landwards, and wrack and driftwood are sometimes thrown over it and onto the marsh.

Six flowering plant associations were recognized within the salt marsh:

1. Gravel bar
2. *Carex*–dominated upper salt marsh
3. *Juncus*/*Sporobula alterniflorus* (Losiel.) P. M. Peterson & Saarela–dominated salt marsh
4. Grass–dominated lower salt marsh (around pond margins)
5. Salt marsh ponds
6. Cattail marsh

Fig. 2. Outline map of Pagan Point salt marsh. Based on satellite imagery (imagery Data 9 August 2024 © Google Earth, right panel). Numbers refer to plant associations given in the text.



A flowering plant list for all species is in Supplementary Data #2 with the occurrence of each species within the plant associations indicated.

Palustral talitrids were found throughout the salt marsh near the recent highest spring tides extreme high water springs (EHWS), that are around the marsh periphery. They were not linked to a particular plant association and were present near recent spring tide drift. Thus, in the small cattail marsh (#6, Fig. 2) palustral talitrids utilized driftwood as cover to hide under. At the southern end of the marsh, the three areas labelled 4C (Fig. 2) are raised islands of shingle formed by seawater incursion and with a sparse plant cover of sea plantain (*Plantago maritima* L.) and samphire (*Salicornia europaea* L.). A narrow zone at recent EHWS levels contained sparse populations of palustral talitrids sheltering under fist-sized stones.

Genetic identification of *Orchestia grillus*

The two new cytochrome C oxidase subunit 1 (COI) sequences from the study population are like samples in the BOLD database from Maryland (39°N), Maine (43–45°N), and New Brunswick (45–48°N), with a maximum pairwise distance among sequences from these locations of 1.1% (Fig. 3). This indicates that the study population falls into haplogroup 4 of Radulovici (2012) with a barcode index number (BIN) of AAD1236. The average difference between haplogroup 4 and haplogroup 3 (BIN AAI1254) from South Carolina is 4.2%.

In searching within the BOLD database, the most similar COI sequence to *O. grillus* was *Atlantorchestoidea brasiliensis* (Dana 1853). The average distance between *O. grillus* (BIN AAD1236) and *A. brasiliensis* was 19.9%. Distances were greater between *O. grillus* (BIN AAD1236) and *Orchestia gammarellus* (Pallas, 1766) (23.7%), *Speziorchestia stephenseni* (Cecchini, 1928) (24.5%), and *Talitrus saltator* (Montagu, 1808) (26.1%), the type species of the genera *O. grillus* had previously been placed in by different authors.

Further research using nuclear genes is underway to determine whether haplogroups 3 and 4 of Radulovici (2012) are different species, and if *O. grillus* is referable to a new genus or belongs to an existing one. Until that research is completed, we will refer our study population to the older established name *Orchestia grillus*.

Food preferences of natural talitrid populations

From a wild caught population of *O. grillus* from the marsh (Fig. 4A) C4 is preferred over C1 and C2 in both runs of the experiments, with the test between C4: C3 not significantly different, as also in the control (C4: C4). *Orchestia grillus* populations feeding on *Carex* in the wild prefer C4 over *Fucus* (F1, F2, and F3) showing no significant preference in the C4: F4 test.

From wild caught populations of *P. exter* (Fig. 4B) when *Carex* is offered as food, C4 is preferred over C1, C2, and C3 and no preference is shown in the control (C4: C4). If both *Fucus* and *Carex* are offered in the experimental tests *P. exter* prefers C4 over F1, but no preference is shown between C4: F2, C4: F3, and C4: F4.

The data for this and all the following experiments can be found in the online Data File.

Fig. 3. Neighbour-joining tree of COI sequences from Atlantic populations of *Orchestia grillus* (BOLDAAD1236). SG1 and SG2 are new sequences from this study. The scale bar is Kimura 2-parameter distance.

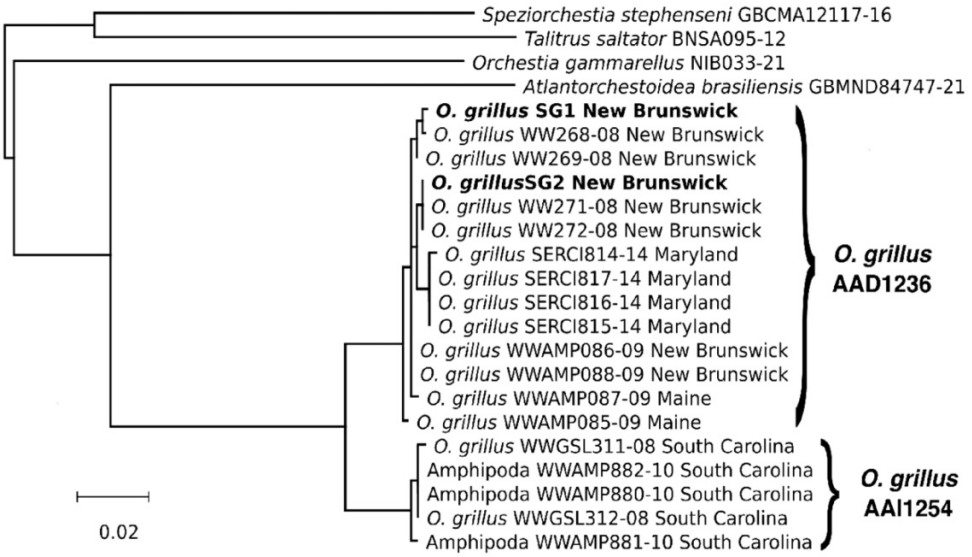


Fig. 4. Pairwise comparisons of food preferences shown by: (A) *Orchestia grillus* (BOLDAAD1236) and (B) *Platorchestia exter*. Results as mean ± standard error. Light grey bars represent C4; dark grey bars represent the other test material (see the *x*-axis for details). *N*: number of replicates. "*": $p < 0.05$, "**": $p < 0.01$, "***": $p < 0.001$; "ns": not significant.

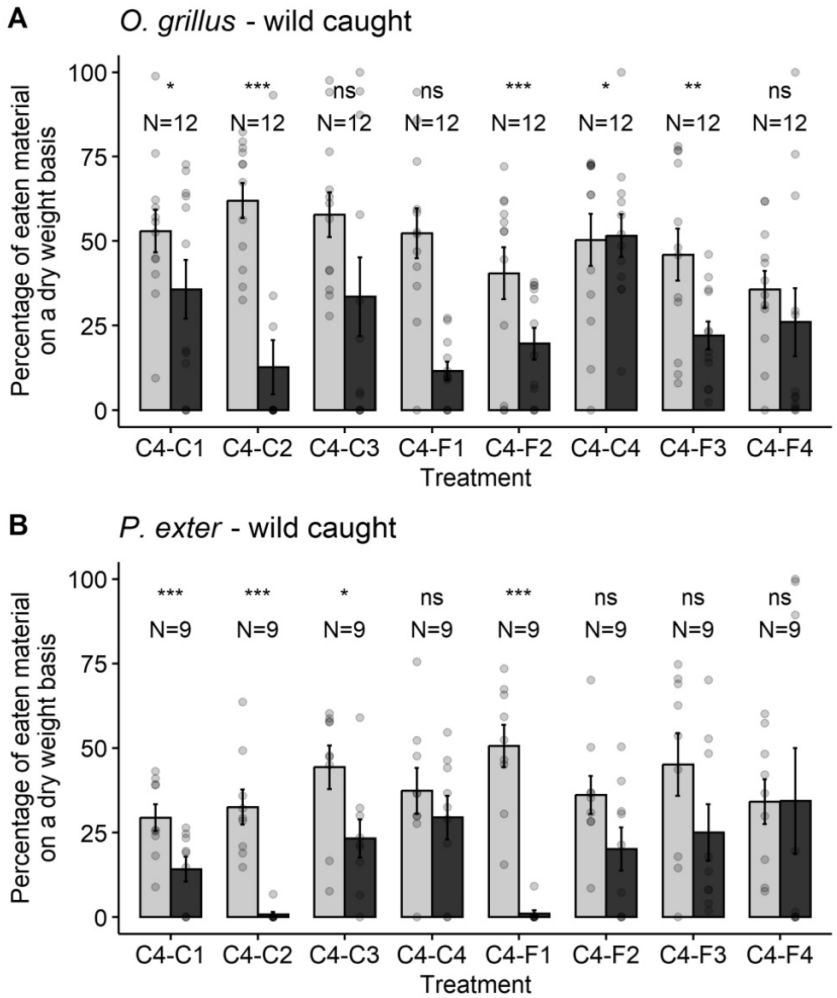
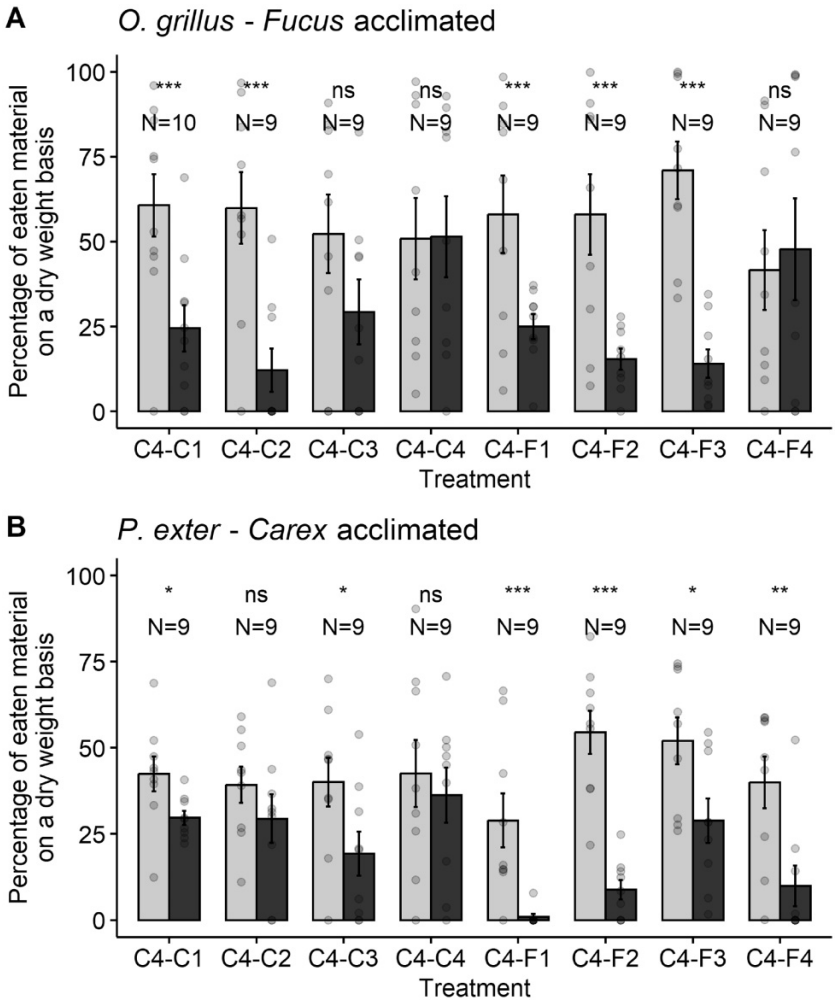


Fig. 5. Pairwise comparisons of food preferences after reversing food offered in culture: (A) *Carex* to *Fucus* for *Orchestia grillus* (BOLD AAD1236), and (B) *Fucus* to *Carex* for *Platorchestia exter*. Results depicted as in Fig. 4.



Food preferences after experimental acclimation to a different food class

Following *Fucus* acclimation for > 6 days *O. grillus* in the *Carex* food treatment produces the same results as in a wild caught population. After *Fucus* acclimation C4 is still preferred over F1, F2, and F3, with F4 not preferred over C4 (Fig. 5A). These results suggest that *O. grillus* cannot be acclimated to a *Fucus* diet.

Results for *P. exter* after acclimation to *Carex* (Fig. 5B) suggest that if *Carex* is the only food offered C4 is slightly preferred over C1, C2, and C3 (either ns or $p < 0.05$) with the control without preference. Contrary to the wild caught experiments with this species, *P. exter* showed significant preference in all treatments after acclimation with C4, suggesting that *P. exter* had been acclimated to prefer *Carex*.

Boiling of plant tissues in C1 and F1

For *O. grillus* boiled *Carex* (C1) is rejected as food with only very small amounts eaten. This resulted in significant differences in food consumption between C1:C1B and C1B:C4 (Fig. 6).

For *P. exter* in both treatments the mean percentage of plant discs eaten was low in all cases (<25%) without evidence of significant difference between boiled and either fresh or F2.

The amount of fresh C1, C4, and F2 eaten in this trial was not significantly different from the amount of the same material eaten by wild caught animals in the food preference trial described above. However, fresh F1 was eaten significantly (Kruskal–Wallis test; chi-squared = 5.828, df = 1, $p = 0.016$) more during this trial (where *P. exter* had to choose between F1 and boiled F1) than during the food preference trial with wild caught *P. exter* described above (where individuals had fresh F1 or C4 as choices).

Aerobic microbial activity of plant surfaces

For *Carex* the leaf surface microbial activity is less in C3 and C4 than other stages. On a dry weight basis (Fig. 7A) there is less aerobic activity in C3 and C4, when compared to C1 and C2. On a surface area basis (Fig. 7B) this difference largely disappears.

For *Fucus* comparing the aerobic microbial activity on both a dry weight and surface area basis suggests that the F2 decay

Fig. 6. Feeding by *Orchestia grillus* (BOLD AAD1236) and *Platorchestia exter*, on fresh and boiled C1 and F1. Results as mean ± standard error. *N*: number of replicates. "****": $p < 0.0001$; ns': not significant.

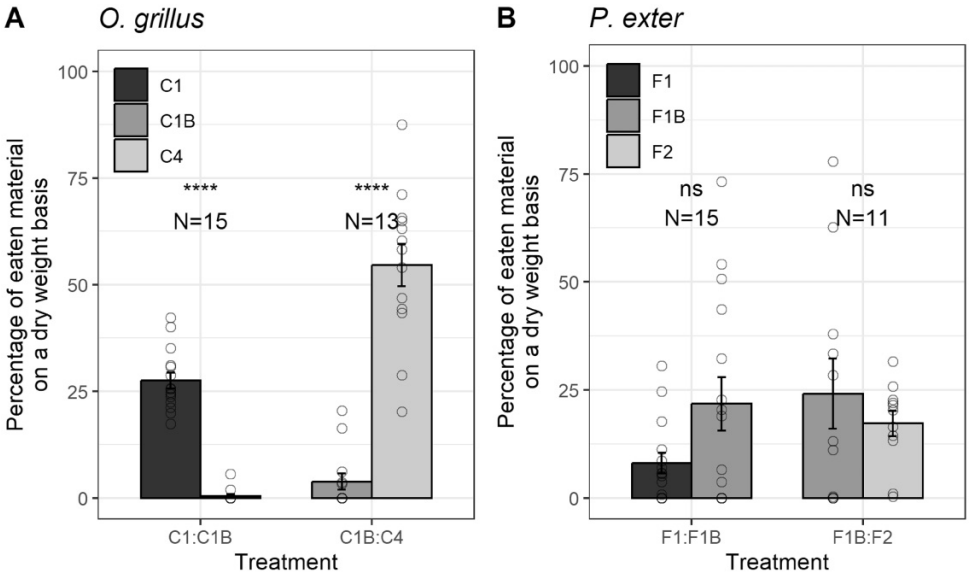
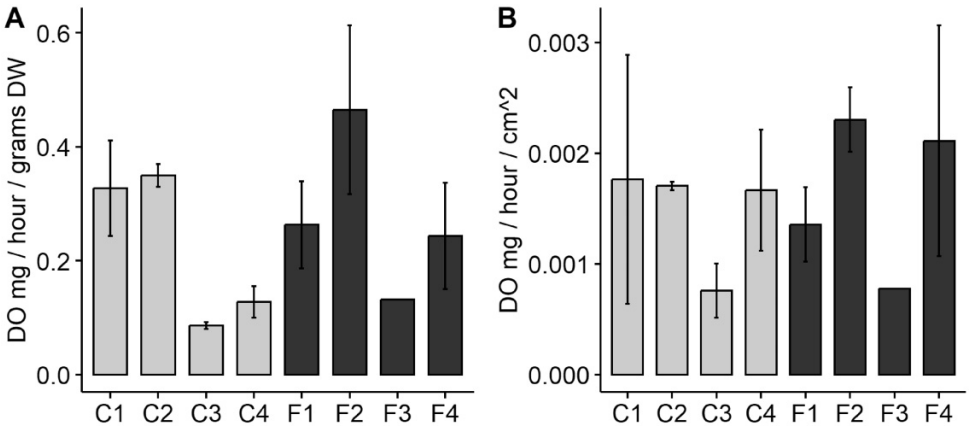


Fig. 7. Microbial aerobic activity on plant surfaces of *Carex* (C1, C2, C3, C4) and *Fucus* (F1, F2, F3, F4) decay stages. Results expressed on (A) dry weight basis, or (B) surface area basis. Results as mean ± standard deviation.



stage is always the greatest (although on a surface area basis, there is no difference between F2 and F4).

Cellulolytic microbes in food, gut, and fecal pellets of *O. grillus*

The average ± standard deviation temperature during culture and experiments in the controlled environment cabinet was 21.05 ± 0.83 °C. The regressions between dry weight (*y*) on wet weight (*x*) of the various compartments (gut parts, food sources, and fecal pellets) are available in the online Data File.

From the CMC agar plates, we were able to differentiate bacterial and fungal colonies (as described in Wildish et al. 2022). Bacterial colonies were small, round, and dark coloured, whilst fungal colonies were larger, white growths. The anterior and posterior gut parts were combined and averaged to compare the microbial counts of the whole gut with those of the other compartments. For bacterial counts (Fig. 8), there was significantly more CFU in the gut than in the food eaten for both *Carex* (chi-squared = 3.857, df = 1, *p*-value < 0.05) and *Fucus* (chi-squared = 3.857, df = 1, *p*-value < 0.05). For fungal counts, there was no significant difference between the food eaten (*Carex)* and combined gut CFU's after feeding on it (chi-squared = 1.191, df = 1, *p*-value = 0.275). When *Fucus* was eaten, there was significantly more fungal CFU in the combined gut than in the food offered (chi-squared = 3.857, df = 1, *p*-value < 0.05).

From separate experimental cultures ($N = 5$) fed *Carex* only, *Fucus* only, and fasted for 7 days the following results comparing the anterior and posterior parts of the gut were obtained. For bacterial counts (Fig. 9) the only case showing significantly more cellulolytic bacterial growth in the gut was in those cultures that were fed *Carex* only (chi-squared = 3.857, df = 1, *p*-value < 0.05). For *Fucus* only (chi-squared = 0.429, df = 1, *p*-value = 0.513) and the fasted treat-

Fig. 8. CFU/mL/mg following 7 days culture on Agar plates selective for cellulolytic bacteria in *Orchestia grillus* (BOLD AAD1236) whole gut fed either aged *Carex* or *Fucus*. Results as mean ± standard error. "*": $p < 0.05$, $N = 3$. CFU, colony-forming unit.

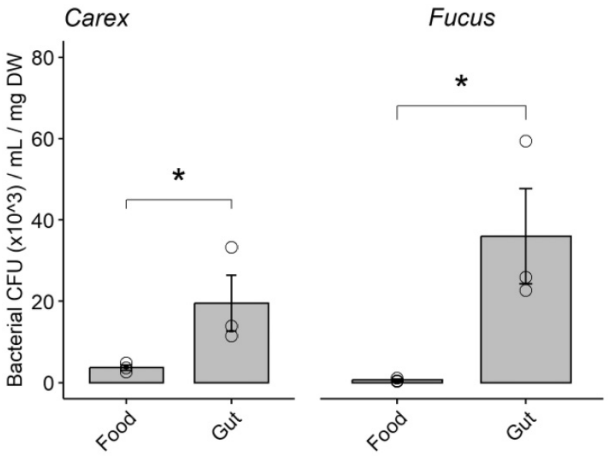
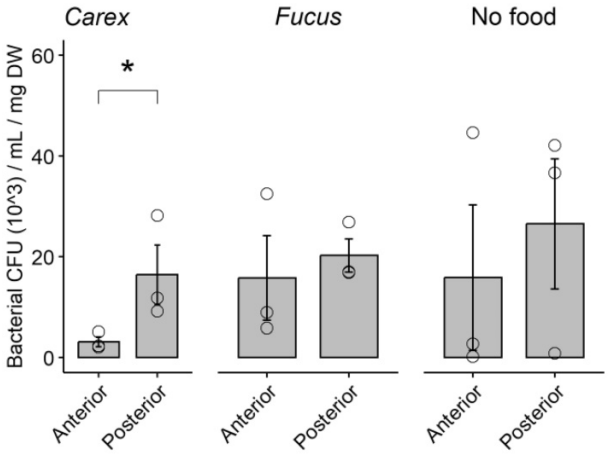


Fig. 9. CFU/mL/mg following 7 days culture on Agar plates selective for cellulolytic bacteria in *Orchestia grillus* (BOLD AAD1236), anterior or posterior gut fed on three different diet regimes. Whatman No. 1 filter paper was eaten during the "No food" treatment. Results as mean ± standard error. "*": $p < 0.05$, $N = 3$. CFU, colony-forming unit.



ment (chi-squared = 0.048, df = 1, *p*-value = 0.827) there was no difference between anterior and posterior gut bacterial CFU counts. For fungal counts, there was no significant difference between the anterior and posterior parts of the gut. Statistical results were *Carex* only (chi-squared = 1.1905, df = 1, *p*-value = 0.275), *Fucus* only (chi-squared = 0.048, df = 1, *p*-value = 0.827) and fasted treatment (chi-squared = 0.067, df = 1, *p*-value = 0.796). In the fasted treatment we found that *O. grillus* readily ate the Whatman No. 1 filter paper used to line the Petri dish, and this resulted in white fecal pellets.

Microbial counts in food and fecal pellets

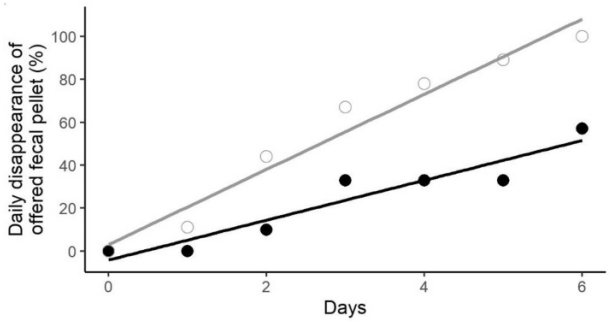
The average dry weight of a single fecal pellet of *O. grillus* was 58.7 μg. A comparison of the microbial counts from fecal

Table 1. Comparing the cellulolytic content of foods fed to *Orchestia grillus* (BOLDAAD1236) with the fecal pellets produced, as colony-forming unit (CFU) (counts/mL/mg dry weight).

Category	Type	CFU type	Mean	SD	*N*
Food	*Carex*	B	3.7	1.1	3
		F	0.1	0.1	3
	Fucus	B	0.6	0.4	3
		F	0.1	0.1	3
Fecal pellets	Brown feces	B	40×10^3		1
		F	0.2×10^3		1
	White feces	B	1.8×10^3		1
		F	0.01×10^3		1

Note: Fecal pellets were collected from experimental cultures on day 7 supplied either with aged *Carex* (C4) or *Fucus* (F2) both giving brown feces, or Whatman No.1 filter paper giving white feces. B = bacterial, F = fungal.

Fig. 10. Percentage of fecal pellets consumed by *Orchestia grillus* (BOLD AAD1236). Filled circles—brown fecal pellets (feeding on C4), open circles—white fecal pellets (feeding on Whatman No. 1 filter paper).



pellets produced by *O. grillus* feeding on *Carex* and moistened Whatman No. 1 filter paper is shown in Table 1 (and the online Data File). We were unable to replicate the fecal pellet microbiology test, so it was not possible to apply a statistical test of the difference between food and fecal pellet microbial counts. However, Table 1 shows that the bacterial CFU is ten times greater in fecal pellets than in the aged *Carex* that they ate.

Coprophagy test with *O. grillus*

After 6 days of observation, all nine white pellets had disappeared, whilst 9 of 21 brown pellets remained. The plot in Fig. 10 shows that white fecal pellets are consumed at a faster rate (Linear regression, White $y = 318\ 3.1 + 18x$, $R = 0.98$, $p < 0.001$) than brown fecal pellets (Linear regression, Brown $y = 4.1 + 9.3x$, $R = 0.95$, $p = 0.001$). The disappearance was caused by the consumption of pellets by the single talitrid in each Petri dish. The ET50s were determined as approximately 3 days for white pellets and 9 days for brown pellets. All 10 *O. grillus* survived the Petri dish experiment and were found sheltering under the damp cotton ball. Three individuals supplied with brown pellets molted as evidenced by the molt skin present next to the newly molted individual. Within

24 h the molt skin had been eaten by the same individual that produced it. Animals readily ate the filter paper on which the fecal pellets were supported, as evidenced by serrations at the edges of the filter paper. One adult female supplied with brown pellets dropped juveniles and all survived to the end of the experiment.

Discussion

Based on the studies reported herein and a review of previous work we summarize current findings as follows.

Food preferences

Food preferences within the Talitridae depend on (1) the availability of a given plant source within the leaf litter/wrack, (2) the degree of decomposition of the preferred plant, and (3) the talitrid ecotype that is doing the consuming.

(1) Along a zoogeographic gradient throughout a given talitrid's range the dominant salt marsh grass plants vary. Thus, for *O. grillus* at Pagan Point salt marsh, the dominant plant at the appropriate tidal level is *Carex palaecea* (present study), in Sippewissett salt marsh, Massachusetts, it is *Sporobolus patens* Swallen (Reitsma et al. 1982) and in Flax Pond salt marsh, New York, it is *Sporobolus alterniflorus* (Lopez et al. 1977). In all cases decomposing species of the dominant marsh grass present at an appropriate tidal height (near EHWS) was the preferred food of *O. grillus*. Within Pagan Point salt marsh, field observations suggested that decomposing angiosperms other than *Carex palaecea* could also be used as food. We conclude that the preferred food plant is not fixed and that, at a different location, a palustral talitrid can physiologically acclimate to different salt marsh plants. This agrees with Pennings et al. (2000) working with two west-coast North American talitrids. It provides a possible explanation as to why *O. grillus* from two different salt marshes on the east coast of North America preferred other salt marsh plants: *Sporobolus* versus *Carex* (Rietsma et al. 1982; this study). The zoogeographic range of *O. grillus* from the Gulf of Mexico–Florida–Newfoundland (Bousfield 1973) may include genetically distinct populations (Radulovicii 2012). It must be determined if food preferences are/or are not genetically controlled in future studies.

The food availability concept also encompasses a small spatial scale. Thus, *O. grillus* is unable to reach the growing parts of C1, as it does not climb the salt marsh grass stem and so cannot sample green leaves. *Orchestia grillus* can reach C2, which are the yellow subtending leaves at the base of the plant stem, but our results suggest that it does not do so and prefers C4 over C2. Because of the usual dryness of C3 in the field *O. grillus* does not consume it, although because it was wet during experiments it was readily eaten. Similarly for *P. exter*, it cannot reach F1 (live *Fucus vesiculosus*) which grows on rocks at mid-littoral level and this talitrid is limited to the supralittoral.

(2) The degree of physical and microbiological decay of the seagrass plant determines when a given species of angiosperm or brown macroalga is preferred as food by both palustral and wrack generalist ecotypes (Pennings et al. 2000; this study). Thus, for *O. grillus* in Pagan Point salt marsh, the preferred food is *Carex* sp. in the later stages of decomposition (C3, C4) when the facultatively anaerobic microbial flora (bacteria, fungi, and protozoa) have climaxed. For *P. exter* the preferred food plant is *Fucus* sp. at an earlier stage of decomposition, but when the surface aerobic microflora are maximal (F2). This result corroborates the findings of Pennings et al. (2000) with two West Coast talitrids, *Traskorchestia traskiana* (Stimpson, 1857) (wrack generalist) and *Megalorchestia californiana* Brandt, 1851 (psammophile). They found significant differences between fresh and aged plants for all five species of brown macroalgae (Phaeophyceae) tested with aged (wrack) samples being preferred over freshly collected, living ones.

(3) Of the two species included in our study, one was a wrack generalist (*P. exter*) and the other a palustral specialist (*O. grillus*). Experimental tests herein showed that *P. exter* could be physiologically acclimated to prefer the later stages of decomposing *Carex* (C4) over the later stages of *Fucus* (F2, F3, F4). This ability ensures that the wrack generalist can occupy more ecotopes than the specialist. *Platorchestia exter* has also been experimentally shown (Wildish and Robinson 2018) to acclimate to driftwood as a diet. Wrack generalists such as *O. gammarellus* can live in European salt marshes (Hernandez et al. 2021) as a secondary ecotope, suggesting that it too can acclimate to a diet rich in structural lignocelluloses. We propose that physiological switching (=acclimation) in *P. exter* (and perhaps other wrack generalists) is controlled by a cellular turn on/off switch (epigenesis; see Jablonka and Lamb 2014). The turn on/off switch independent of a direct genetic mechanism is a hypothesis worth investigating in future research. Experimental attempts to acclimate *O. grillus* to *Fucus* (F2) failed, consistent with the singular primary angiosperm ecotope occupied by this specialist (Wildish 2024). These findings allow us to predict that the talitrids studied by Pennings et al. (2000) including *T. traskiana* can consume and digest both Phaeophyceae and angiosperm leaf litter, whereas *M. californiana* is limited to Phaeophyceae.

Besides the major ones listed above, the other factors studied by Pennings et al. (2000) were of local influence, inclusive of pH, plant toughness, and presence/absence of chemical defensive substances. We observed during salt marsh field collecting, that if the spring tide wrack banks dry out in hot weather on the Bay of Fundy intertidal side of the shingle bank the resident *P. exter* abandons the older drying F3 and F4 *Fucus* and migrates down shore to the most recently arrived and still damp wrack bank. The environmental trigger for this behavioural response is the relative humidity levels within the wrack bank which can fall below the survivability range in hot weather (Moore and Francis 1985). This observation shows that talitrid behavioural responses not directly concerned with digestive physiology may influence the latter.

Food preference experiments suggest that defensive chemicals (polyphenols) in living plants of *Fucus* are much reduced soon after death (F2) by abrasion and microbial activity. Tugwell and Branch (1989) have shown in South African kelps (Phaeophyceae) that most of the polyphenols are limited to the cell monolayer (meristoderm) covering the thallus. Our result (Fig. 7) shows that surface microbial activity is greatest in F2 which is consistent with the meristoderm layer being ruptured by microbial activity with consequent losses of polyphenol during F2. In living plants of *Carex* the chemical defensive compounds if present are unknown although likely to be similar to those in *Sporobolus* sp., where glucosides of hydroxy aromatic acids have been identified (Valiela et al. 1979). In our study, the defensive chemicals in *Carex* sp. were likely lost by the decomposition stage C3. However, although it may be lying flat in the salt marsh in C3, it hasn't yet encountered the soil and consequently remains dry and unavailable to *O. grillus*. When it touches the soil, it becomes moistened with seawater and salt marsh soil microbes and then develops into C4.

The boiling method used leads to different feeding responses by the two talitrid species in our study in responding to fresh-boiled *Carex* or *Fucus* (Fig. 6) which can be explained by the different digestion mechanisms available to each talitrid. *Orchestia grillus* utilizes digestion method 2 (see below) and is unable to digest C1B because some of the full suite of lignocellulose digesting enzymes that are taken in with food are removed by boiling. Normally the enzymes are present in ingested live microbes which are needed for complete lignocellulose decomposition in the midgut. In *P. exter* utilizing digestion method 4 (see below) the absence of microbes in boiled *Fucus* does not deter feeding. This is because the native digestive enzymes produced in the hepatopancreas can handle either boiled or natural forms of *Fucus* because it does not depend on ingested microbes. The boiling of plant tissue method does not allow us to distinguish between chemical defense compounds as deterrents, microbial content of leaf surfaces as attractants or the influence of chemical/physical changes associated with heat treatment on plant palatability. Thus, in our results (Fig. 7) with *P. exter* offered F2 the aerobic bacterial count on thallus surfaces is maximal suggesting an attractive effect, although we cannot exclude a reduction of chemical defensive compounds effect.

The independent, mechanical processes that are necessary before C3 and C4 decay stages of salt marsh plants appear include flattening by strong winds, inundation with HW springtide seawater and in Pagan Point marsh deer which rest (hidden) among the *Carex* stems.

Talitrid digestion

Considering hypotheses for talitrid digestion Wildish et al. (2022) proposed that psammophiles primarily use native gastric enzymes (method 1), palustral talitrids primarily use microbial symbiosis within the gut (method 2), whereas wrack generalists utilize both methods 1 and 2 combined (as method 4).

Our preliminary findings with microbiological culture methods in *O. grillus* show that there are significantly more cellulolytic, aerobic bacteria in the posterior (mid/hindgut) than the anterior gut and in the gut than in ingested food. Fecal pellets too have a high content of cellulolytic bacteria. Previously published molecular genetic results provide a possible explanation for these findings. Thus, Abdelrhaman et al. (2017) utilized them to study the gut microbiota of five species of Mediterranean talitrids, showing that *Orchestia montagui* consuming *Posidonia* wrack had the highest proportion of microbial genes coding for lignocellulose degradation in the gut. Using similar molecular genetic methods Russini et al. (2021) in *O. montagui* and Henandez et al. (2021) in salt marsh-acclimated *O. gammarellus* demonstrated that these talitrids also carried lignocellulose degrading, symbiotic bacteria in the hepatopancreas. The lignocellulose degrading symbionts were unique to the talitrid hepatopancreas and have proved to be un-culturable by standard methods (Russini et al. 2021). If these results apply to the talitrids in our study, the anterior half of the gut (with attached hepatopancreas containing endosymbiotic, anaerobic, lignocellulose-degrading bacteria) would have a lower count with our aerobic, cellulolytic bacterial method because our culture method did not measure lignocellulose degrading bacteria.

Our studies have confirmed that coprophagy occurs in *O. grillus* and that this could be the way that rare, endosymbiotic, lignocellulose degrading bacteria are transferred between individuals, notably to juveniles on entering the population.

We hypothesize that in both palustral specialists and wrack generalists' enzymatic activity occurs in the midgut from endosymbiotic microbial sources originating in the hepatopancreas and from free microbes taken in with the food. Wrack generalists differ from palustral specialists because they possess the ability to produce native cellulolytic enzymes within the hepatopancreas (Wildish and Poole 1970). When they are consuming a lignocellulose-rich diet native enzyme production is reduced and transferred to endosymbiotic microbes, resulting in a saving in energy costs to the animal (Wildish et al. 2022). When they are consuming a cellulose-rich diet (Phaeophyceae) native enzyme production is increased.

Conclusions

The experimental pairwise food preference tests demonstrated that C4 is the preferred *Carex* decay stage for *O. grillus*, and F2 is the preferred *Fucus* decay stage for *P. exter*.

Experimental culture for at least 6 days after reversing the preferred food sources above proved that *O. grillus* cannot be acclimated to prefer *Fucus* (F2), whereas *P. exter* can be acclimated to prefer *Carex* (C4).

These results highlight *O. grillus* as a specialist feeder and *P. exter* as a generalist feeder. Attempts to untangle the possible multiple causes (presence of chemical defenses, presence of increased microbial activity and chemical/physical condition of plant foods) for the food preferences established above were unsuccessful, reflecting the intricate mechanisms involved in talitrid food preferences and digestion.

Results of microbial culture methods applied to the digestive system of *O. grillus* showed that the greatest cellulolytic activity occurs in the posterior half of the gut, i.e., the midgut. This is consistent with the hypothesis that a complex suite

of lignocellulose degrading enzymes is involved in digestion. In *O. grillus* digestion involves endosymbiotic microbes resident in the hepatopancreas combining with microbes taken in with the food to provide a complete digestion of lignocelluloses.

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Data availability

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The authors declare there are no competing interests

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Supplementary material

Supplementary data are available with the article at https://doi.org/10.1139/cjz-2025-0009.

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