Hindawi Publishing Corporation
International Journal of Zoology
Volume 2011, Article ID 294394, 5 pages
doi:10.1155/2011/294394

Research Article

Dietary Preference of *Gammarus pulex* and *Asellus aquaticus* during a Laboratory Breeding Programme for Ecotoxicological Studies

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Received 29 March 2011; Accepted 1 June 2011

Academic Editor: Chris Lloyd Mills

An investigation was undertaken to establish if *Gammarus pulex* and *Asellus aquaticus* preferred a diet of unconditioned, artificially or naturally conditioned alder leaves (*Alnus glutinosa*). Standardised, 24 hour *ex situ* feeding assays were undertaken with both species to determine their food preference. The results showed that *A. aquaticus* ate more leaf material compared to *G. pulex* (Z 23.909, *P* 0.001) when exposed to all three test variables. Also, both *G. pulex* and *A. aquaticus* demonstrated a preference for naturally conditioned leaves compared to the other two variables, with unconditioned leaves proving the least popular food option for both macroinvertebrates (Z 18.803, $P < 0.001$). However, both species ate varying amounts of all the leaf treatments (Z 136.399, $P < 0.001$). Subsequently, the author outlined a feeding methodology for natural alder leaf conditioning that could be used during a laboratory breeding programme.

1. Introduction

What is the best diet for *Gammarus pulex* and *Asellus aquaticus* during a laboratory breeding programme and/or ecotoxicological study? Over the years, scientists have used a variety of nutritional supplements to feed macroinvertebrates during breeding programmes and experiments, including dog food [1], baby, and fish food [2]. If the macroinvertebrates were being bred for ecotoxicological studies (or as test subjects within bioassays) they need to be representative of wild specimens, and it is well documented that a test, animals response could be affected by their past history, diet, life stage, disease and so forth [3, 4]. Therefore, by feeding the animals with an unnatural diet, which may not contain the appropriate nutritional requirements, they could display a false negative/positive response during a test. Most workers, however, have gone down the more traditional route of using detritus to feed detritivores [4].

The role of allochthonous organic matter (e.g., leaves, wood) in streams and rivers has been extensively documented [5]. Freshly fallen leaves and other plant detritus that enter the water are rapidly colonized by microorganisms, a process referred to as conditioning [6]. There is considerable experimental evidence that shredders fed on detritus show preferences for and survive better on substrata that has been previously colonized by fungi, for example, Bueler [7]. It has been assumed that microbial colonization improves the nutritional quality of detritus through fungi having a differential ability to eliminate plant allelochemicals [8], fungal synthesis of micronutrients, production of mycotoxins [9], and/or the ability of detritivores to utilize acquired fungal enzymes [10]. Graca et al. [9] also demonstrated that *G. pulex* and *A. aquaticus* both discriminated between fungal mycelia and either fungally colonized or uncolonized leaf material. However, whereas *A. aquaticus* fed by scraping the leaf surface, thereby, selectively ingesting fungal mycelia, *G. pulex* nibbled the leaf, consuming both fungal and leaf matrix.

The food quality of detritus has been defined in terms of chemical (e.g., nitrogen and lignin), physical (e.g., resistance), and biological (e.g., microbial biomass) parameters. High-quality food has a low C : N ratio, low lignin content,

low resistance, and high microbial biomass [10]; therefore, alder would be described as a high-quality food. When *G. pulex* have been offered the choice between alder (*Alnus glutinosa*), beech (*Fagus sylvatica*), oak (*Quercus robur*), elm (*Ulmus glabra*), ash (*Fraxinus excelsior*), and willow (*Salix caprea*), the alder leaves were ingested at a much faster rate [11].

In contrast, Willoughby and Sutcliffe [1] found that the best diet for *G. pulex* was a mixture of conditioned elm and oak leaves. On this diet, the animals had a growth rate of approximately 150 μg day^{-1} at 10°C in specimens of less than 16 mg body weight. In larger specimens, the rate apparently increased to about 350 μg day^{-1}. However, workers such as Nilsson [11] found that, at 15°C, an average of 1928.7 calories were produced from alder leaves g^{-1} day^{-1}, which is considerably greater than other leaves, for example, beech (197.6 calories were produced from beech leaves g^{-1} day^{-1}). The growth rate for Nilsson's smaller *G. pulex* specimens, which were fed on alder leaves was similar to the rate of 130.8 μg day^{-1} at 15°C obtained by Willoughby and Sutcliffe [1] with a diet of oak and elm.

Researchers have previously used artificial [12] and natural [4, 13] methods to condition leaf material. The aim of this paper is to establish if the macroinvertebrates *G. pulex* and *A. aquaticus* prefer a diet of artificially or naturally conditioned alder leaves by undertaking *ex situ* feeding assays. In addition, the animals preference for conditioned and unconditioned leaf material will be assessed.

2. Materials and Methods

The *G. pulex* and *A. aquaticus* used in this study were obtained from a standardised laboratory breeding programme. The breeding programme's founder population originated from an unpolluted river source. Animals were captured, transported to the laboratory, and maintained under standardised conditions. The specimens were allowed to randomly copulate and the subsequent F^1, F^2, F^3 generations, and so forth were used for experimental purposes [4].

G. pulex (12–15 mg dry mass) and *A. aquaticus* (7–10 mg dry mass) males were used in the experiments. 24 hours prior to the test, 300 *A. aquaticus* were removed from the culturing tank and divided equally between 30, 500 mL sterile plastic pots (with screw lids), which contained 500 mL of deionised water. The animals were maintained under oxygen-depleting conditions without nutritional supplements at 15°C. For 16 hours per day, the animals were illuminated with a fluorescent light (with a specification for freshwater invertebrates), to simulate on a small scale the macroinvertebrates natural climatic conditions. The glow mimicked the thermal warmth and daytime illumination obtained from the sun radiation. The same procedure was also undertaken with 300 *G. pulex*. Bloor et al. [3] previously showed that in a deionised water test media (without aeration) both *G. pulex* and *A. aquaticus* could survive for several weeks without mortalities.

Alder leaves (*Alnus glutinosa*) were collected during the autumn fall (from Hillier's Arboretum, Romsey, UK), air dried, and stored in refuge bags (in a dry location) until required. The leaf material was cut into 1800 squares (length 2.0 cm and width 2.0 cm). 600 squares (1.16 g) were placed in 500 mL of enriched water (Table 1), inoculated with a standard amount of *Cladosporium* fungus (fungi : leaves, 1 : 20) and incubated for 10 days [12]. 600 squares (1.16 g) were soaked in 500 mL of river water containing 0.50 g of decaying detritus for 10 days (river water and detritus were collected from the River Itchen, Southampton, UK). The remaining squares were saturated in 500 mL of deionised water for 10 days.

Table 1: "Enriched" water recipe. 5 mL of each stock solution was mixed and made up to one litre with deionised water (extracted from [12]).

Stock solution	g L^{-1}
$CaCl_2 \cdot H_2O$	58.80
$MgSO_4 \cdot 7H_2O$	24.65
$NaHCO_3$	12.95
KCl	1.15

The 1800 squares were then air dried for 24 hours and weighed. 120 squares from each treatment were put into the separate 500 mL test pots containing *A. aquaticus* and fed to the animals (the deionised water was not changed, and aeration was not applied); therefore, each test was replicated 10 times. After 24 hours, the squares were removed, air dried (for 24 hours), and reweighed. The amount of consumed detritus was then calculated by subtracting the final leaf weight from the conditioned weight. The same investigation was then repeated with *G. pulex*.

The data was analysed using PASW 18 statistical software. Initially, the Kolmogorov-Smirnov test was used to determine normality ($P > 0.05$). As the data was normally distributed, a parametric paired *t*-test was applied to establish if there was a significant difference between the initial and final weights of the leaves ($P < 0.05$). Finally, a general linear model was undertaken to investigate which leaf type was preferred by *G. pulex* and *A. aquaticus*.

3. Results

Application of Kolmogorov-Smirnov test indicated that there was no departure from normal distribution ($P > 0.05$) for the *G. pulex* unconditioned leaves (Z 0.160, P 0.757), natural conditioned leaves (Z 0.211, P 0.385), or artificially conditioned leaves (Z 0.151, P 0.411). The *A. aquaticus* data was also normally distributed (unconditioned leaves (Z 0.195, P 0.574), natural conditioned leaves (Z 0.163, P 0.621), or artificially conditioned leaves (Z 0.184, P 0.199)).

This enabled application of the parametric paired *t*-test ($P < 0.05$), which showed that there was a significant difference between the initial and final weight of unconditioned leaves (Z 8.157, $P < 0.001$), natural conditioned leaves (Z 34.259, $P < 0.001$), and artificially conditioned leaves (Z 9.918, $P < 0.001$) for *G. pulex* and also *A. aquaticus* (unconditioned leaves (Z 11.420, $P < 0.001$), natural conditioned leaves (Z 66.002, P 0.001), and artificially conditioned leaves (Z 35.146, $P < 0.001$)).

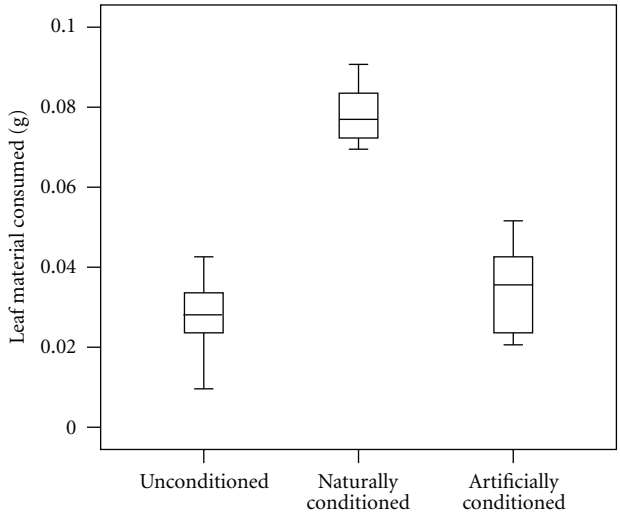

Figure 1: Box and whisker plot of leaf material consumed for each treatment (g) by *G. pulex*. The figure illustrates that during 24 hour feeding assays, *G. pulex* consumed a greater proportion of naturally conditioned leaf material, compared to artificially conditioned and unconditioned ($n = 10$ replicated tests for each treatment).

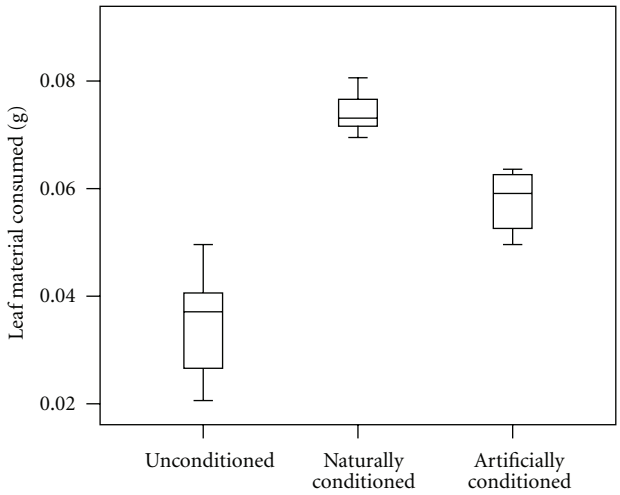

Figure 2: Box and whisker plot of leaf material consumed for each treatment (g) by *A. aquaticus*. The figure illustrates that during 24-hour feeding assays, *A. aquaticus* consumed a greater proportion of naturally conditioned leaf material, compared to the artificially conditioned and unconditioned ($n = 10$ replicated tests for each treatment).

Finally, a general linear model demonstrated that there was a significant difference between the amount of leaf material consumed by *G. pulex* and *A. aquaticus* (Z 23.909, P 0.001), the type of leaf treatment consumed (Z 18.803, $P < 0.001$), and the amount each species consumed of each leaf type (Z 136.399, $P < 0.001$). Figures 1 and 2 illustrate that *G. pulex* and *A. aquaticus* consumed the leaf material in the order of naturally conditioned > artificially conditioned > unconditioned.

4. Discussion

Bacteria and fungi are important components of the detritivore diet [1], *G. pulex* and *A. aquaticus* both discriminated between fungal mycelia and either fungally colonized or uncolonized leaf material [9], which was illustrated by this study. The results clearly demonstrate that both species of macroinvertebrates preferred a diet of conditioned leaf material over unconditioned leaves, with natural conditioning being the favoured conditioning option. On comparing the initial and final weights of the natural and artificially conditioned leaf material, it can be concluded that natural conditioning produced heavier and noticeably softer leaves, which could be attributed to the colonization of microorganisms. Research has demonstrated that *A. aquaticus* feed, by scraping the leaf surface, thereby, selectively ingesting fungal mycelia, which would explain why these animals preferred the naturally conditioned leaves [9]. In contrast, *G. pulex* nibbles the leaf, consuming both fungal and leaf matrix [9]. As such, it might have been expected that the

G. pulex would not discriminate between the natural and artificial leaves, but the results of this study showed that natural conditioning was the diet choice for both species.

Studies have shown that *G. pulex* and *A. aquaticus* would grow to sexual maturity and reproduce on a diet of decaying leaves [4]. Few studies have measured or compared the rates of growth on different diets, but some authors have claimed that decaying leaves with rich flora of bacteria and fungi are more palatable and support faster growth of *G. pulex* than leaves without microorganisms [14].

However, workers, such as Graca et al., [9] demonstrated that although the growth of *A. aquaticus* was reduced when unconditioned leaves were provided, leaf conditioning does not influence *G. pulex* growth. This is because *G. pulex* has the ability to compensate for a low-energy uptake by reducing its energy expenditure. The mechanism behind this principle remains unclear but is probably linked to a decline in activity [15]. Whatever the mechanism, the outcome of this difference in response is that reduction in food quality has a greater impact on the energy balance of *A. aquaticus* than that of *G. pulex*, resulting in less energy being available. *G. pulex* may also resort to cannibalism in experimental situations when insufficient/inappropriate nutritional supplements are available [16], which could hinder a laboratory breeding programme.

When establishing a laboratory breeding programme for ecotoxicological studies, it is important that the animals are maintained in standardised and repeatable conditions. The animals need to remain stress-free or their toxicological response could be manipulated [4]. The animals diet is an important factor in maintaining a healthy and stress-free population, and consequently, it is important to keep the animals in the most natural environment as possible. By providing a diet that mimics their natural food source and contains the appropriate nutritional requirements for growth and reproduction, the animals would be representative of wild stocks during ecotoxicological studies. Therefore, the author would suggest that naturally conditioned alder leaves are an excellent diet choice for *G. pulex* and *A. aquaticus* populations within a laboratory breeding programme. The presented research supports the use of the feeding methodology outlined in Bloor [4], in order to breed and maintain healthy populations of both macroinvertebrates during a breeding programme.

Bloor [4] discussed that abscised alder leaves (*Alnus glutinosa*) should be collected during the autumn fall (from one tree), air dried, and stored. As such, the food source would be standardised as all the leaves were collected from the same tree on the same day. 10 L of river water and a handful of organic detritus should be collected from an unpolluted source and transferred to the laboratory in a lidded plastic container. On return to the laboratory, the water and detritus should be poured into a 15 L plastic box (the box should not be sealed with a lid). Handfuls of the precollected alder leaves should be submerged in the water and mixed with the precollected organic detritus (no precise measurements), which would inoculate the alder leaves with bacteria and fungus. The leaves should be conditioned for at least 10 days. After that time and when required, leaves should be extracted from the box and placed in the aquariums (excess liquid should be squeezed from the leaves to reduce the level of organic enrichment applied to the water). Additional air-dried leaves should then be immersed in the conditioning box to replace the utilised ones.

The leaves should be liberally scattered in the culture and rearing aquariums, to fulfil the animals nutritional requirements and replaced at regular intervals (enough leaves to cover the aquarium floor to a depth of approximately 50 mm). The juveniles should, however, be supplied with conditioned alder leaves for shelter and grazing but also fed upon adult faeces that should be syringed from the culture aquariums (when required), until the animals can feed entirely upon conditioned leaves (after about 25 days).

5. Conclusions

In summary, when undertaking a laboratory breeding programme with *G. pulex* and *A. aquaticus,* naturally conditioned alder leaves would be the recommended food source. As such, a feeding methodology was outlined that could be utilised during a breeding programme. The author would recommend that a priority for future research would be to investigate if the diet/health of laboratory populations of *G. pulex* and *A. aquaticus* could be improved by feeding a mixed diet.

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