Entomopathogenicity of *Vairimorpha* sp. (Microsporidia) in the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae)

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Abstract

The biocontrol potential of a microsporidian pathogen, *Vairimorpha* sp., obtained from field collected diamondback moth, *Plutella xylostella* Linnaeus, was assessed in a laboratory colony of this insect. The pathogenicity was quantified using a standard bioassay procedure by infecting second instar larvae *per os* with variable dose levels of the pathogen. *Vairimorpha* sp. caused 100% mortality even at a dosage of 1.5×10^3 spores per larva. The median lethal dose (LD_{50}) was 2.2×10^4 spores per larva on day 5 post-inoculation. The time taken to achieve 90–100% mortality was dose dependent and varied from 5 days with 1.5×10^6 spores per larva to 11 days with 1.5×10^3 spores per larva. The pathogen reduced the food consumption of *P. xylostella* larvae by 75%, 93% and 95% at doses 1.5×10^4, 1.5×10^5 and 1.5×10^6 spores per larva, respectively. Histological observations showed that the pathogen preferentially infected adipose tissue cells but spread to almost all tissues. The pathogen was transmitted transovarially as well as horizontally and had a marked influence on progeny performance. It was concluded that *Vairimorpha* sp. has sufficient potential to be tested in the field as a biocontrol agent for *P. xylostella*.

Introduction

The diamondback moth *Plutella xylostella* Linnaeus (Lepidoptera: Yponomeutidae), first recorded in 1746 (Harcourt, 1962) remains the most serious pest of cruciferous crops worldwide, particularly in Southeast Asia (Talekar, 1992). The extensive and regular use of all categories of insecticides has induced widespread insecticide resistance and *P. xylostella* has now become unmanageable by conventional insecticide use (Sun *et al*., 1986; Syed, 1992). These problems have provided the main impetus for development of integrated pest management (IPM) systems using endo-larval parasitoids (Ooi, 1992) or nematodes (Mason & Wright, 1997) as key components. However, establishment of the parasitoids in the ecosystem has been adversely affected by the extent of pesticide usage (Talekar *et al*., 1992) and there is a need to incorporate ecologically sound tools as alternatives to chemical insecticides. Microbial insecticides based on *Bacillus thuringiensis* (*Bt*) and the macrocyclic lactone, abamectin have been incorporated into an IPM system for *P. xylostella* (Ooi, 1992; Ibrahim & Low, 1993) but resistance to these biopesticides by field populations of *P. xylostella* has already been reported (Tabashnik, 1994; Iqbal *et al*., 1996) and the long term usefulness of these products must be in doubt for *P. xylostella* management (Wright *et al*., 1995). During the past few years, the mitosporic fungus *Beauveria bassiana* (Deuteromycotina) and *Zoophthora radicans* (Entomophthorales: Zygomycotina) have been tested against *P. xylostella* and found effective (Ibrahim & Low, 1993; Furlong *et al*., 1997) but their slow mode of action has led to criticism of their use in pest management. The potential as control agents of some viruses has also been tested for *P. xylostella* management with some

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https://doi.org/10.1017/S0007485399000231

success (Su, 1990; Padmavathamma & Veeresh, 1991; Abdul Kadir, 1992).

Populations of *P. xylostella* collected from the Cameron Highlands of Malaysia in the late 1980s and early 1990s appeared to be free of significant rearing problems due to pathogens, when set up as laboratory cultures (D.J. Wright, personal observation). However, populations imported into the UK in 1992/93 were infected with microsporidia, which led to reduction in fecundity and increased larval and pupal mortality within a few generations. Similar problems were observed in populations collected in 1994, 1995, 1996 and 1997. In 1995, evidence of widespread microsporidial infections in several regions within the Cameron Highlands was also obtained during long-term field trials on *P. xylostella*–parasitoid interactions (R.H.J. Verkerk, unpublished observation) and similar field-based observations were made in 1997 (J.M. Mason and D.J. Wright, personal observation). Ultrastructural studies (E.U. Canning, unpublished observations) have suggested that this microsporidium is a species of *Vairimorpha* (Microsporidia: Burenellidae) showing elements of disporous and octosporous sporogonies, although the octosporous sequence was not completed under the environmental conditions employed. The specific identification will be the subject of a further publication. The present research project was undertaken to assess the potential of this *Vairimorpha* sp. as a biocontrol agent for *P. xylostella* which might be used alone or included with *B. thuringiensis*, various viral formulations, fungi or nematodes into IPM for this pest.

Materials and methods

Insect cultures

Two strains of diamondback moth were used in this study: a microsporidia-free sub-population of the Rothamsted culture (ROTH strain) which had been maintained at the Imperial College laboratories at Silwood Park and a field strain collected in 1997 from Sungai Palas, Cameron Highlands, Malaysia (MAL strain) and imported with the *Vairimorpha* sp. as infected pupae and eggs. The ROTH and MAL strains were maintained separately, the former in a constant environment room at 20°C, 60 ± 5% rh under a 14:10 h light: dark cycle and the latter in a room with variable temperature (16–22°C), no humidity control and a 14:10 h light: dark cycle. Samples of larvae and pupae from both cultures were routinely examined fresh and in Giemsa-stained smears to assess their microsporidia-free and microsporidia-infected status. When used as controls in experiments, the ROTH strain was kept under MAL strain conditions.

Insect foods

Chinese cabbage leaves (*Brassica chinensis* cv. Tip top) four to six weeks old were used as larval food. Cabbage plants were grown in pots containing a standard potting mix (Levington Multipurpose Compost, Fisons®, UK) in the glasshouse. No added fertilizer or insecticide were used but the glasshouse was occasionally fumigated with nicotine to eliminate aphids from plants. Fumigated plants were not used for 96 h. For adult moths, 50% honey solution was used as food.

Tested pathogen

The microsporidian pathogen *Vairimorpha* sp. used in this study was harvested from the transovarially infected *P. xylostella* population of the MAL strain. For harvesting spores, larval cadavers were first surface treated with phosphate-buffered saline (PBS) with added penicillin (10,000 i.u ml^{-1}) and streptomycin (10,000 µg ml^{-1}) (PS) and then homogenized in PBS-PS antibiotic solution, filtered through cheese cloth, centrifuged at 1500 *g* for 10 min and finally purified by centrifugation in 50% Percoll (Sigma) at 1000 *g* for 15 min. The purified pellet was resuspended in distilled water, the spores were quantified by haemacytometer (Improved Neubauer, Weber, England) and stored at 4°C in distilled water.

Bioassay protocol

Vairimorpha spore doses in 5-µl droplets of suspension in distilled water were dispensed onto the upper surface of 5 mm diameter cabbage leaf discs and allowed to dry at room temperature. Four dose levels of 1.5×10^3, 1.5×10^4, 1.5×10^5 and 1.5×10^6 spores were tested and control insects were supplied with distilled water only. Second instar larvae (10–14 h after moulting) from the microsporidia-free ROTH strain were starved for 3–4 h before being individually allowed to feed on a single leaf disc in the wells of 24-well tissue culture plates. A piece of water-soaked filter paper (Whatman No. 1) was placed in each well and a piece of aluminium foil paper was placed underneath the lid of the plate. Only those larvae that consumed the entire leaf disc in a 24 h period or less were included in the assays. The treated larvae were placed on a fresh whole cabbage leaf in a plastic box ($28 \times 16 \times 10.5$ cm), containing dampened tissue paper at the bottom. The cut end of the cabbage leaf was covered with water-soaked cotton wool in aluminium foil. For aeration, the lids of the boxes were provided with nylon mesh-covered windows. Twenty larvae were used for each dose level with three replicates (n = 60). Food was renewed every day. The assays were carried out in an incubator at 20°C and 14:10 h light:dark cycle. Mortality of the larvae was monitored every 24 h until all had died or pupated. The cause of death was monitored by microscopic examination of fresh mounts with phase contrast microscopy or of Giemsa stained smears.

Food consumption

Newly moulted second-instar larvae of the ROTH strain, infected with *Vairimorpha* sp. at four dose levels, 0, 1.5×10^4, 1.5×10^5 and 1.5×10^6 spores as described above were individually transferred onto a single 2×2 cm square piece of cabbage leaf in a petri dish (5.5 cm diameter) humidified with damp filter paper. Before cutting the leaf squares, the cut ends of the cabbage leaves were immersed in water for 15–20 min. Ten larvae were used for each dose level and they were incubated as before. Every 24 h until the larvae had died or pupated, old leaf squares were replaced with new ones and the leaf area consumed by individual larvae was measured with the help of graph paper, to the nearest mm^2.

Tissue specificity

Second- and early third-instar larvae of the MAL strain transovarially infected with *Vairimorpha* sp. were fixed in

https://doi.org/10.1017/S0007485399000231

alcoholic Bouin's fixative, dehydrated in alcohol, embedded in paraplast wax, sectioned, stained in Giemsa and mounted in green euparal. In addition, adipose tissue, midgut, salivary glands and Malpighian tubules from transovarially infected final instar larvae were dissected out, washed briefly in phosphate buffered saline, smeared and stained in Giemsa.

Transovarial transmission

To observe the effect of transovarial transmission of *Vairimorpha* sp. on the offspring, 100 transovarially infected second instar larvae of the MAL strain (F_1 generation) were separated into four groups of 25 larvae and reared on cabbage leaves in plastic boxes as before. Larvae which pupated were allowed to emerge as adult moths. Larval mortality, pupation and adult eclosion were recorded. Additionally, 48 second instar larvae of the ROTH strain were infected *per os* at 1.5×10^2 spores per larva and those larvae which emerged as adult moths were allowed to mate and lay eggs. Larvae emerging from these eggs were examined at third- and final-instar stages for microsporidian infection either in fresh mounts or in Giemsa stained smears.

Statistical analysis

The median lethal dose (LD_{50}) for the microsporidian pathogen was calculated by probit analysis (Finney, 1971) using Mstat package programme. The data on food consumption and percent mortality were subjected to ANOVA and LSD tests. Percentage data were transformed by square root transformation before statistical analysis.

Results

Pathogenicity of Vairimorpha *sp.*

One hundred percent larval mortalities of *P. xylostella* were observed when second instar larvae were infected at all the doses of the *Vairimorpha* sp. tested except at the lowest dose (1.5×10^3 spores per larva) when 93.3% of larvae died (fig. 1). At this dose, the remaining four larvae pupated without forming cocoons on day 10 post-inoculation but none emerged as adult moths. Larval mortality began on day 3 post-inoculation at all doses tested except the lowest dose when mortality started on day 4 post-inoculation (fig. 1). Higher mortalities were always observed at higher doses on each day except on day 3 where a higher mortality occurred at a dose of 1.5×10^4 than at 1.5×10^5 (13.3% larvae vs. 3.3% larvae). All the larvae had died by six, eight and ten days post-inoculation at doses 1.5×10^6, 1.5×10^5 and 1.5×10^4, respectively (fig. 1). No mortality was observed in the controls in which all larvae had pupated by 6–7 days post-exposure. The median lethal dose (LD_{50}) for the pathogen was 2.16×10^4 spores per larva on day 5 post-inoculation. The high slope of the regression equation ($Y = 3.737 + 0.947x$) indicates that mortality was dose dependent.

Daily food consumption by P. xylostella *infected with* Vairimorpha *sp. and by control larvae*

Vairimorpha sp. significantly reduced the total food consumption per larva ($P < 0.01$). Each larva consumed a mean of 24.9 ± 2.7 mm^2, 36.1 ± 4.1 mm^2 and 123.9 ± 21.7 mm^2 of leaf when infected with doses of 1.5×10^6, 1.5×10^5 and 1.5×10^4 spores, respectively, compared to 493.4 ± 41.1 mm^2 consumed by the control larvae up to pupation (LSD = 67.5). Among the doses tested, there was no significant difference between the higher two doses but the levels of food consumption at these doses were significantly different from that consumed by larvae infected with the lowest dose ($P < 0.05$). The percent reduction of food consumption over control were 95, 93 and 75% for doses of 1.5×10^6, 1.5×10^5 and 1.5×10^4 spores, respectively.

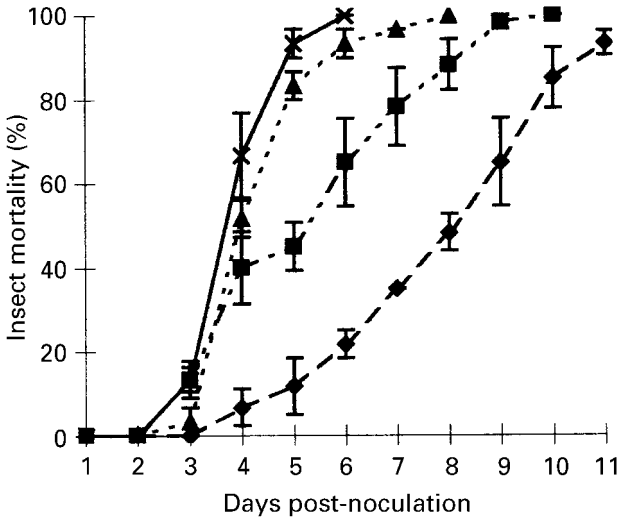

Fig. 1. Cumulative percent daily mortality of *Plutella xylostella* larvae inoculated with *Vairimorpha* sp. Larvae were inoculated with 1.5×10^3 spores (◆), 1.5×10^4 (■), 1.5×10^5 (▲) or 1.5×10^6 spores (×) at second instar. Vertical bars = S.E.

Daily food consumption by *P. xylostella* larvae infected with *Vairimorpha* sp. and by control larvae are presented in fig. 2. Analysis of data on a daily basis showed that control larvae always consumed significantly higher amounts of food ($P < 0.01$) compared with the treated larvae except on days 1 and 7 post-inoculation when there were no significant differences ($P > 0.05$). In all treatments there was a slight fall in food consumption on day 2. At the higher two doses, daily food consumption continued to decrease with time and all the larvae had died by the end of the fourth and fifth days post-inoculation. At the 1.5×10^4 dose, there was a marked increase in daily food consumption with time with the greatest quantity (51.9 ± 9.1 mm^2) consumed on day 4 post-inoculation. The food consumption declined thereafter and all larvae had died by day 9 post-inoculation; on this day the few larvae surviving after day 8 did not take any food before death. Among the controls, daily food consumption increased with time and the maximum food (153.6 ± 22.1 mm^2) was consumed on day 5. Thereafter there was a decline in the daily leaf consumption and all the larvae had successfully pupated by day 7.

Tissue specificity of Vairimorpha *sp.*

All tissues examined were infected with *Vairimorpha* sp. but the fat body carried the heaviest burden. In Giemsa stained smears, infection was observed in fat body, midgut, salivary glands and Malpighian tubules.

https://doi.org/10.1017/S0007485399000231

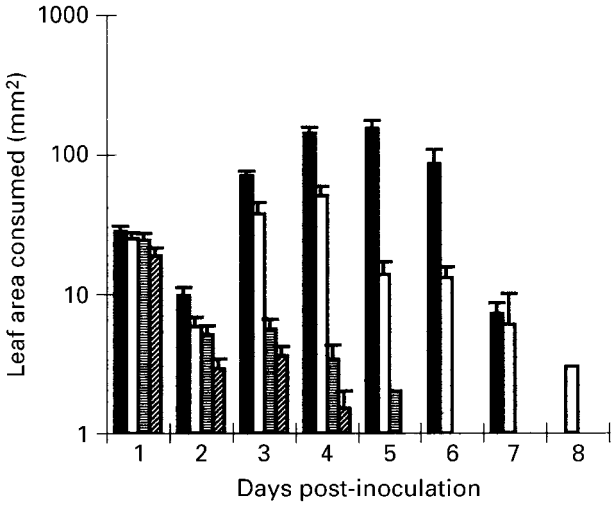

Fig. 2. Daily cabbage leaf consumption by *Plutella xylostella* larvae, uninfected (■) or infected at second instar with 1.5×10^6 (▨), 1.5×10^5 (▤) or 1.5×10^4 (□) spores of *Vairimorpha* sp. Vertical bars = S.E.

Transovarial transmission of Vairimorpha *sp.*

In the F_1 generation of the MAL strain, which were transovarially infected with *Vairimorpha* sp., there was 86% larval mortality, 14% pupation and only 12% emergence as adult moths. In the ROTH strain, third and final instar larvae which had developed from infected parents were heavily infected but no data were collected on their further progress.

Discussion

Jungen (1995) studied a microsporidian pathogen, *Vairimorpha* sp. isolated from *P. xylostella*. He found that the pathogen reduced the fecundity of infected *P. xylostella* females but had no effect on egg hatching or larval mortality. The pathogen was sustained throughout the study in the *P. xylostella* colony at a low level without destroying it but could not be transmitted horizontally. It is unlikely that the species of *Vairimorpha* used by Jungen (1995) is the same as that used in the present study as, in our experiments, the *Vairimorpha* sp. was easily transmitted from the MAL strain to the ROTH strain and induced such high mortality that the infected colony could not be maintained. The sequence of the small subunit rRNA gene, to be presented with ultra-structural data, will characterize this strain for comparison with strains available from other sources.

The *Vairimorpha* sp. obtained from a field population used in the present study was highly pathogenic when transferred to the *P. xylostella* population of the ROTH strain. The results show that susceptibility of the *P. xylostella* larvae to the pathogen was dose dependent. All the larvae had died by day 6 post-inoculation at a dose of 1.5×10^6 compared to ten days at a dose of 1.5×10^4. Even at a dose level of 1.5×10^3 the pathogen caused 100% mortality, i.e. 93.3% larval and 6.7% pupal mortality. The rapid mortalities of *P. xylostella* larvae at higher doses might be attributed to intestinal damage and bacterial septicaemia but the slower mortality at lower doses must be attributable directly to the microsporidial burdens as reported by Fuxa (1981). The speed of action of *Vairimorpha* sp. was greater than that of PxGV virus against *P. xylostella* (Abdul Kadir, 1992) and, although in Abdul Kadir's study (1992), another virus, GmNPV, acted faster at the highest dose tested, neonate larvae were used in the experiment. Had we used neonate larvae, which are likely to be less robust than the second instar larvae, the results with *Vairimorpha* sp. might have been even more impressive.

The purpose of insect control is to reduce crop losses caused by insect damage to plant tissue. This can be achieved either by killing a sufficient number of pest insects or by reducing their activity, including feeding activity. *Vairimorpha* sp. used in this study has exhibited both these attributes in *P. xylostella*. In addition to causing 100% mortality at most doses, *Vairimorpha* sp. reduced food consumption by up to 95%. A reduction in food consumption at all doses was observed even on day 2. At the higher two doses, the rate of food consumption rapidly decreased with time probably because the normal midgut epithelial cells became packed with spores, thus interfering with digestion, absorption and passage of food residue through the gut. Abdul Kadir (1992) also reported reduced damage to cabbage caused by baculovirus-infected *P. xylostella* but did not record how the damage was measured.

Early reduction in feeding by *Vairimorpha*-infected *P. xylostella* is important as it counters the frequent criticisms that biological control of insects using microbial pathogens is slow and allows the pest almost to complete its development before death. Furlong *et al.* (1997) reported that *P. xylostella* larvae infected with a fungal pathogen, *Z. radicans* consumed 44% less food compared with control larvae, as they effectively stopped feeding two days after infection. This is in accord with our observations on the effect of *Vairimorpha* sp. Reduction in food consumption due to *Nosema locustae* Canning (Microsporidia: Nosematidae) infection has also been reported by Johnson & Pavlikova (1986) in *Melanoplus sanguinipes* Fabricius (Orthoptera: Acrididae) and by Oma & Hewitt (1984) in *Melanoplus differentialis* Thomas (Orthoptera: Acrididae).

The histological observations showed that *Vairimorpha* sp. infects almost all the tissues although the fat cells were most heavily infected. This observation is in agreement with the findings of Jungen (1995) who reported that *Vairimorpha* sp. infected all organs and tissues of *P. xylostella*. However in Jungen's study (1995) there was almost no host mortality. This suggests that the multiplication rate was lower than with the *Vairimorpha* sp. from Malaysian *P. xylostella*. The species studied by Jungen (1995) also differed from our microsporidium in that it could not be transmitted horizontally. The *Vairimorpha* sp. obtained from Malaysia spread transovarially to the offspring and had a marked effect on the development of the progeny. Only 12% of transovarially infected larvae (F_1 generation) successfully emerged as adult moths. Many researchers have shown that transovarial transmission of microsporidian pathogens can cause an increase in mortality in the next generation. Siegel *et al.* (1986) reported that *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) larvae transovarially infected with *Nosema pyrausta* (Paillot) (Microsporidia: Nosematidae) experienced 36% higher mortality than uninfected larvae. Han & Watanabe (1988) observed an acute lethal infection, with most of the larvae

https://doi.org/10.1017/S0007485399000231

dying by the end of third instar, in the F_1 population of silkworms *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae) that were infected transovarially with *Nosema bombycis* Naegeli (Microsporidia: Nosematidae).

Jungen (1995) demonstrated a model for transovarial transmission of *Vairimorpha* sp. in *P. xylostella*, showing that during organogenesis vegetative stages were usually localized in central regions of the egg without interfering with development of the organs. The microsporidian pathogen then entered the gut lumen as vegetative stages, together with the remaining yolk, finally developing into spores in parallel with larval development. From the gut lumen, the infection spread to the other organs of the larvae after hatching. A different mechanism was observed in winter moth, *Operophtera brumata* (Linnaeus) (Lepidoptera: Geometridae) infected with *Orthosomella operophterae* Canning (Microsporidia: Unikaryonidae) (Canning *et al*., 1985). In this system, spore morphogenesis occurs in the yolk residue, which is consumed by the larva as it emerges from the egg. The spores are infective and are responsible for infecting the larvae after hatching. It is notable that both these systems prevent mortality to the larvae before emergence. The mechanism for transovarial transmission was not investigated in the present study but, as the larvae survived for several days after emergence, it is likely that their tissues were not infected within the egg. This would be a good survival strategy for the microsporidia as the host would survive long enough to transmit the infection horizontally and, for survivors, vertically to the next generation.

In selecting a microbial pathogen for pest management apart from high virulence, two other properties, capacity to survive and capacity to disperse, need to be considered because persistence of microbial pathogens in the ecosystem is greatly influenced by abiotic factors (Ignoffo *et al*., 1977). *Bacillus thuringiensis*, a widely used bacterial pathogen and *Vairimorpha necatrix*, a microsporidian pathogen with biocontrol potential, are often criticized for their inability to survive and disperse in the environment and are considered to have limited value for long term control programmes. Transovarial transmission guarantees transmission to the next host generation as long as a proportion of hosts survive to adulthood. Further work is required on the effect of *Vairimorpha* sp. through several generations without re-application of spores.

Results of this study shows that *Vairimorpha* sp. has considerable potential as a biocontrol agent for *P. xylostella* management. This is particularly important since these insects are difficult to control by chemical pesticides as resistance has developed towards them. The baseline data of the present laboratory findings will provide scope for testing this microsporidian pathogen in field cage trials or directly in the field. However, before releasing the *Vairimorpha* sp. in the field, its impact on other natural enemies, particularly on the endolarval parasitoids, of the insect pest needs to be investigated. It would be of interest to test the *Vairimorpha* sp. in combination with other biocontrol agents. Some of these may be antagonistic, e.g. *B. thuringiensis*, which destroys the gut wall at an early stage. This is the point of entry for *Vairimorpha* sp. during horizontal transmission. Also, interference during entry into the haemocoel between microsporidian and viral pathogens usually makes the interaction antagonistic (Nordin & Maddox, 1972; Fuxa, 1979). However, the fungal pathogen *Beauveria bassiana*, which has been isolated from *P. xylostella*, has a different portal of entry, the cuticle, and does not become intracellular. The conidiospores of *B. bassiana* germinate on the body surface, invade through the cuticle with the aid of the toxin beauvericin (Roberts, 1981) and the fungus grows profusely in the haemocoel, utilizing haemolymph rather than cells (Puzari *et al*., 1994). Thus the *Vairimorpha* sp. and *B. bassiana* could establish themselves independently in the insect body and theoretically could act synergistically.

Acknowledgements

We are pleased to acknowledge Dr Judy Mason of the Department of Biology, Imperial College of Science, Technology and Medicine for helping to establish this project and Dr D.J. Galley for use of facilities. Dr Haque is pleased to acknowledge The Royal Society for a grant, which enabled him to visit Imperial College. Insects were imported under plant health licence PHL17/2495 (11/1997) from the Ministry of Agriculture, Fisheries and Food.

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(Accepted 24 December 1998)