European Journal of Soil Biology 69 (2015) 52–56

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

Growth rate and survival of terrestrial isopods is related to possibility to acquire symbionts

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ARTICLE INFO

Article history:
Received 12 November 2014
Received in revised form
13 May 2015
Accepted 13 May 2015
Available online 17 May 2015
Handling editor: S. Schrader

Keywords:
Symbiont transmission
Nutrition
Cellulose
Terrestrial isopods
Woodlice

ABSTRACT

The acquisition and maintenance of symbiont-host associations is considered an important prerequisite for the successful colonisation of land by animals because symbionts allowed the hosts to dwell on low-quality food sources. Digestive tract symbionts are suggested to either enhance digestive efficiency of cellulose or supply the host with nutrients otherwise limited in the food. Terrestrial isopods are the most successful land colonisers among the Crustacea, and although symbiotic bacteria have been identified in digestive tract, the mechanism of transmission and the nutritional role of the symbionts are at present poorly understood.

We used a novel *in vitro* approach to isolate and culture juveniles of *Porcellio scaber* at a time when only the vertical mode of symbiont acquisition could have occurred, which allowed us to follow juvenile growth and to simulate alternative modes of symbiont acquisition through feeding manipulations. Thus, we experimentally obtained groups of juveniles that could have acquired symbionts only through vertical transfer (mother-offspring), or additionally, through horizontal (through faeces or contact with conspecifics) and environmental (through leaves) transfer. We quantified survival and growth rates over two months for the different experimental acquisition modes, and significantly different growth rates were observed ($p < 0.001$). Growth of juveniles suggests that first, symbiont inoculation is mediated through horizontal and environmental transfer and second, the symbionts may in fact serve as a source for fatty acids and vitamins. The growth rates further question that vertical transfer occurs in woodlice. Although survival did not differ significantly between different acquisition modes ($p = 0.051$), juveniles supplemented with potential sources of symbionts showed a tendency towards increased survival. The successful invasion of land may thus have been facilitated through the uptake of symbionts from the surroundings.

1. Introduction

According to the original definition by Anton de Bary [1], symbiosis can be described as the association (temporal or spatial) between individuals who do not belong to the same species, independent of the effects on organisms involved, be they negative (parasitism), neutral (commensalism) or positive (mutualism). In animals, host-symbiont associations include transient gut passengers that pass through the digestive tract with ingested food, permanent gut residents, gardening of fungi, and surface-associated and/or intracellular symbionts [2]. The acquisition of novel biological properties via symbiosis played an important role in adaptation, evolution and diversification of animals, e.g., allowing the host to explore new ecological niches [3]. Symbiotic associations are widespread in diverse taxa in both aquatic and terrestrial environments [2,4,5]. In the largest animal group on Earth, insects, up to half of the species are estimated to harbour obligatory symbionts [2]. Protection against pathogens, improved growth and survival, and provisioning of limiting nutrients are examples of the beneficial effects of symbionts [5–8]. Nutritional symbiosis appears to be common in terrestrial arthropods that feed on diets scarce in vitamins, sterols, and essential amino acids or on diets rich in cellulose and lignin [7,9–13]. However, the modes of symbiont acquisition and their potential nutritional contributions are at present not understood.

In general, the establishment of the symbiont-host relationship can be facilitated through three transmission mechanisms:

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http://dx.doi.org/10.1016/j.ejsobi.2015.05.003
1164-5563/

vertically transmitted symbionts are passed from mother to progeny by infecting milk glands, eggs or embryos [14], horizontally transmitted symbionts may be acquired through feeding on infected corpses, faeces, exuviae or through physical contact between conspecifics [14,15], and environmentally transmitted symbionts are acquired through contact with or uptake of any matter [14,16,17]. The present understanding of the mechanisms that mediate symbiont transmission is based on empirical evidence available from only a few model systems. Vertical transmission appears as the predominant mechanisms for symbiont acquisition in insects [2,18], and environmental transmission is described for species living in aqueous environment [4,17]. The horizontal transmission of microbial species through faecal-oral contact in ruminant herbivores has received the most attention [19]. Thus, the transmission mode can be considered to be habitat and taxa specific, but overall, the understanding has to be qualified as poor.

The suborder Oniscidea (Crustacea) is composed of more than 3500 terrestrial species, and their association with symbiotic bacteria has been confirmed only in seven species [20]. The microbial contribution to cellulose and lignin digestion has hereby received the most attention [3,6,11,21]. Symbionts are assumed to improve the ability of the host to digest a terrestrial diet by providing necessary enzymes or essential nutrients such as fatty acids and vitamins [22]. The mode of microbe transmission in terrestrial isopods is generally assumed to be environmental [23], however due to the methodological approach this contribution cannot fully exclude the horizontal (through contact with mother) mode of transfer. We experimentally excluded and/or facilitated symbiont transmission by dissecting embryo stages from the marsupium and subsequent *in vitro* culturing of the juveniles with different food supplements with the goal to determine the mode of symbiont acquisition and the beneficial effects of symbionts in the terrestrial isopod species *Porcellio scaber*. Different sources for symbiont inoculation were provided to juveniles through nutritional supplements to an artificial standard diet to test for the vertical (mother-offspring), horizontal (through faeces or contact with conspecifics) or environmental (through leaves) mode of transfer. Based on the assumption that symbionts positively affect growth and survival, we predicted: i) no difference in growth and survival if vertical transmission occurs; ii) difference in growth and survival if vertical transfer does not occur, and iii) dietary supplements of fatty acids and vitamins offset the lack of symbionts by improving isopod growth.

2. Materials and methods

2.1. Collection of animals

Specimens of woodlice (*P. scaber)* were collected in spring 2013 in Kraków, Poland. The isopods were maintained in plastic boxes (52 mm × 48 mm Ø, 100 ml) under a constant temperature of 20 °C and a photoperiod of 16L:8 D. The progeny of 30 gravid females were used for the experiment, summing up to a total of 830 dissected mancas.

2.2. Mimicking conditions inside marsupium

The embryos were removed from the marsupium (= motherly brood pouch) at the later stage of gravidity (stage 3, [24]) when they could not have encountered symbionts through horizontal or environmental transfer, and thus only vertical transfer could have occurred. The marsupial development is characterised by Haeckel's 'ontogeny recapitulates phylogeny' because individuals inside the brood pouch experience the change from the water to land conditions one week before hatching. During this period, the marsupial fluid is absorbed, individuals start to move and they switch from aquatic respiration to air breathing by using pleopodal lungs. To mimic such conditions *in vitro*, the approach used by Surbida and Wright [25], who transferred embryos of the isopod species *Armadillidium vulgare* to fresh 12 × 5 ml well-plates and cultivated them in culture saline, was modified. Because this method did not allow individuals to freely move out from the water and thus follow the ontogenic development after hatching, a funnel made out of filter paper was attached to each well (volume of 1 ml, water level reached to approximately half of the funnel height). The novel funnel-approach allowed mancas to crawl out of the liquid bottom of the funnel and onto the funnel part above the liquid surface to survive and to breath with lungs (marsupial manca stage, [26]). We used a normal filter paper and did not use any additional measures to sterilise it, assuming that all individuals experience the same contamination conditions during this period.

2.3. Diet manipulation

The day when individuals crawled freely inside the wells was defined as the time of hatching (one to two weeks after dissection). A total of 267 juveniles moved from the liquid phase into the air phase and subsequently 'hatched' *in vitro* (32% survival of all cultured individuals). After hatching, the body mass of juveniles was determined to the nearest 0.001 mg (Mettler Toledo XP26, Greifensee, Switzerland), and one or two juveniles were subsequently kept in individual boxes (52 mm × 48 mm Ø, 100 ml) containing wet sand (Grudzeń Las, glass sand, class III) and an autoclaved piece of clay pot, which served as a shelter and also a feeding place. The juveniles of each clutch were split equally and randomly assigned to five experimental diet groups. The individuals in all diet groups were offered *ad libitum* 'minimum diet' [27] modified after [22]; the details on diet composition and diet preparation are presented in Appendix A1. The food provided to group A contained only this minimum diet (10–15 mg), while group B was supplemented with fatty acids and vitamins (0.4–0.6 mg), group C was supplemented with a tiny piece of alder leaf (0.2–0.4 mg), group D1 was supplemented with a single faecal pellet of an adult conspecific (0.1–0.3 mg), and group D2 was supplemented with the gut (including hepatopancreas) of a freshly killed adult (0.4–0.6 mg). The tiny amounts of supplements were used to facilitate possible symbiont inoculation without serving as significant source of extra nutrients. The supplements and the diets were provided only every 7th day in the form of a fresh artificial diet pellet placed on the clay pot; the supplements for groups C and D were placed at least 1 cm from the food pellet to avoid contamination of the artificial diet. The artificial diet was always produced fresh (agar was sprinkled to the boiling water and additional ingredients were added while keeping the fluid warm; for details see Appendix A1). Because all experimental groups faced the same risk of contamination, we expected that any growth or survival differences between groups must stem from the supplement added to the diet.

2.4. Statistical analyses

Growth rates were determined by weighing each individual at hatching (initial body mass) and after four and eight weeks. All data were tested for normality of distribution and homogeneity of variance prior to analyses. To examine the effect of diet on growth rates, repeated-measures ANOVA and Bonferroni *post-hoc* tests were used with initial body mass as a covariate, diet, time points (day of hatching, four weeks and eight weeks), the interaction term between the two factors, and number of juveniles per box as explanatory variables. Because juveniles were assigned to