A novel disease affecting the predatory mite *Phytoseiulus persimilis* (Acari, Phytoseiidae): 2. disease transmission by adult females

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Abstract Adult female *Phytoseiulus persimilis* Athias-Henriot (Acari, Phytoseiidae) of one of our laboratory populations (=NR-population), show the following set of symptoms: predators shrink several days after mating, cease egg production and die several days after shrinking, show a lower degree of attraction to herbivore-induced plant volatiles and a shorter choice time in olfactometer tests, have the tendency to leave a prey patch with ample food, may carry excretory crystals in the legs, may cease prey consumption, and have a lower excretion rate. We hypothesized earlier that this characteristic syndrome, called non-responding (=NR-) syndrome, is caused by a pathogen infecting *P. persimilis*. To further support this hypothesis we here study several transmission modes of the factor causing the NR-syndrome. In all tests we measured size, short-term fecundity, mortality, predator position, response to plant odors and crystal location, thus including 6 of the 9 symptoms known yet. No evidence was found for vertical transmission from parent to offspring. Eggs from symptomatic females of the NR-population mated by males of the NR-population gave rise to normal-sized, well performing predators, when they had been surface sterilized or transferred to a new leaf. However, such eggs gave rise to shrunken females (17%) when left on the leaf where they had been laid. In the latter case transmission via products deposited on the leaf by the mothers was possible. We therefore tested several modes of horizontal transmission by exposing females of a commercial population that never showed the NR-syndrome (=R1-population) to products related to the symptomatic NR-population. No evidence was found for transmission via food or via squashed adult females. However, symptoms were induced in adult females of the R1-population after a 3-day exposure to a live adult female of the NR-population (incubation period=3–7 days, fraction shrunken females=53%) and after a 1-day exposure to feces and debris collected from such females (incubation period=2–4 days, fraction shrunken females=65%). Contact with live females and feces of the R1-population did not induce the syndrome. These results clearly indicate that the NR-syndrome is a contagious phenomenon and that the factor inducing the syndrome is transmitted horizontally among and between generations via feces and debris deposited by symptomatic females. The results are discussed in the context of mite pathology and biological control.

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Introduction

Adult female *Phytoseiulus persimilis* Athias-Henriot (Acari, Phytoseiidae) of one of our laboratory populations (=NR-population), show the following set of symptoms: predators shrink several days after mating, cease egg production immediately after shrinking, die several days after shrinking, show a lower degree of attraction to herbivore-induced plant volatiles (=HIPV) and a shorter choice time in olfactometertests, show the tendency to leave a prey patch with ample food, may carry excretory crystals in the legs, may cease prey consumption and have a lower excretion rate (Dicke et al. 2000; Schütte et al. 1995, 2006). The syndrome is called non-responding (NR) syndrome because the first symptom recorded was the reduced attraction to HIPV (Dicke et al. 2000; Schütte et al. 1995). It is expected that predators showing this NR-syndrome will be less successful predators of spider mites than non-symptomatic predators. *P. persimilis* has become a biological control agent of large economical importance (van Lenteren et al. 1997; Garthwaite 2000) and is used in research on predator-prey relations in several research groups (see for reviews Dicke et al. 1998; Sabelis & Dicke 1985; Sabelis et al. 1999). Therefore, research aimed at curing of populations that show the NR-syndrome and sustaining of populations with a normal performance in laboratories and industrial rearings is important from a fundamental and applied perspective.

   We have previously demonstrated that the lower degree of attraction to HIPV and the higher mortality are contagious phenomena (Schütte et al. 1998) and that the lower degree of attraction to HIPV is present in at least one commercial population of *P. persimilis* (Dicke et al. 2000). These findings led to the hypothesis that the behavioral change may represent a symptom of an infectious disease.

   Several pathogens and potential pathogens have been described for phytoseiid mites (see for reviews: van der Geest et al. 2000; Bjørnson and Schütte, 2003; Schütte et al. 2005; Hoy and Jeyaprakash 2005). Until now only five microorganisms detected in *P. persimilis* have been studied in more detail: the rickettsia *Rickettsiella phytoseiuli* (Šut'áková 1991) and *Wolbachia* sp. (Breeuwer and Jacobs 1996), the microsporidium *Microsporidium phytoseiuli* (Bjørnson 1998; Bjørnson et al. 1996; Bjørnson and Keddie, 1999, 2001) and two non-identified species (Bjørnson and Keddie 2000). Only for *M. phytoseiuli* clear pathological effects have been reported including negative effects on fecundity, longevity, predation rate and progeny sex ratio (Bjørnson and Keddie, 1999). However, it is unlikely that *M. phytoseiuli* or other microsporidia cause the characteristics of the NR-population, as microsporidia have never been detected in individuals of this population (S. Bjørnson and E. Beerling, personal communication; C. Schütte, unpublished data). It is thus most likely that a novel disease is manifested in our laboratory population. In the present case, where no potential pathogens could be detected in microscopic studies, knowledge about the main transmission mode of the NR-syndrome may be an important step towards pathogen isolation. Moreover, information on the mode(s) of transmission is indispensable for the development of techniques for disease cure and prevention.

   Pathogen transmission may be vertical or horizontal. Vertical transmission is defined as direct pathogen transfer from a parent organism to its offspring (Andreadis 1987).

Transmission from father to offspring (paternal-mediated) is not very common. Pathogens may be transferred to offspring via infected sperm or via veneral transfer during mating to the female with subsequent transfer to the eggs (Andreadis 1987). Transmission from mother to offspring via the egg (maternal-mediated) is an important mode of transmission of many viruses and protozoa and may be the principal mean of transmission (Andreadis 1987; Bjørnson and Keddie 2001). Pathogens may be present on the egg surface (transovum transmission) or inside the egg (transovarial transmission).

Horizontal disease transmission is defined as pathogen transfer from individual to individual but not directly from parent to offspring. This can occur among and between generations and between different host species (Andreadis 1987). In horizontal transmission pathogens may gain entrance into the host by passing the integument (many fungi and nematode species) or by entering body openings (all pathogen groups). Infection through the mouth (per os) is the most common way of entrance by insect pathogens under natural conditions (Andreadis 1987). Consumption of contaminated food, feces or conspecifics may cause infections and thus horizontal transmission.

We have investigated whether parent predators from the NR-population of *P. persimilis* may transfer the NR-syndrome directly to their offspring (vertical transmission), and whether the NR-syndrome is induced in non-symptomatic female predators of the R1-population after exposure to products related to the NR-population (horizontal transmission). To include as many symptoms as possible we have applied the bioassay developed by Schütte et al. (2006), in which 6 of the 9 symptoms of the NR-syndrome known can be measured simultaneously.

Materials and methods

Cultures

Plants and herbivores

Lima bean plants (*Phaseolus lunatus* L.) were reared in a greenhouse at 20–25°C (L16:D8). The herbivorous two-spotted spider mite, *Tetranychus urticae* Koch, was reared on whole bean plants under the same conditions.

Predator populations

The *non-responding* population (*NR*) originated from a commercial mass producer and has been reared in our laboratory for many years in a semi-open rearing system. Detached Lima bean leaves infested with spider mites were placed on a plastic platform in a caged water basin at 20–25°C (L16:D8). Fresh leaves were added every 2–3 days. Old leaves were removed weekly.

The *responding* population (*R1*) originated from another commercial mass producer and was cultured in a closed rearing system. Detached Lima bean leaves infested with spider mites were placed in Parafilm-sealed plastic Petri dishes (diameter=9 cm) in a climate chamber at 23±1°C (L16:D8). In each dish 4 gravid females were kept for egg production during 48 h after which the females were removed. New leaves infested with spider mites were added every 2–3 days. After 1 week, when offspring had become mature, gravid females were transferred to new Petri dishes to initiate a new generation or they were used in experiments. Predators thus were reared in distinct generations. At least 15 dishes were prepared per generation.

Different rearing systems were used for two reasons: (1) the responding population looses its characteristics when reared in a semi-open rearing system in our laboratory (Dicke et al. 2000; for discussion see Schütte 2006); (2) the non-responding population dies out when reared for several generations in a closed rearing system (C. Schütte, unpublished data; for discussion see Schütte 2006).

Pre-experimental rearing

To eliminate the effect of different rearing systems in our experiments both predator populations were kept in the closed rearing system described above for at least one generation prior to experiments. Twenty dishes were prepared per population. As female predators from the R1-population laid more eggs than females from the NR-population (Schütte et al. 1995, 2006), several eggs were eliminated from dishes of the R1-population to obtain similar egg densities for both populations (ca. 10 eggs per dish).

Pre-infection rearing of the R1-population

To minimize genetic variation and variation due to accidental contamination of rearing dishes we reared sisters of comparable age, mated by a brother, which then were equally distributed over control and treatment in experiment 2.3 and 2.4. Twenty-five mated female predators of the R1-population were placed individually on a spider mite-infested leaf disc (ø 2.5 cm) in a plastic Petri dish (ø 5.5 cm). After 24 h the females were removed. Dishes with a dead predator or less than 3 eggs were eliminated. The eggs of the remaining dishes were transferred to the underside of a prey-infested leaf in a new dish, as female predators prefer the underside for egg deposition. After 4 days new food was added to each dish. After 8 days each Petri dish contained the adult offspring of 1 mother. Each Petri dish contained at least one male, which had inseminated its sisters, because in a batch of 4–5 eggs, which is the daily egg production of a healthy female, the first egg produced is usually a male (Amano and Chant 1978).

Hygienic measures

All equipment used to handle predators and prey-infested leaves, like brushes and forceps, was sterilized in 0.5% sodium hypochlorite (NaClO) solution prior to use, after which they were rinsed with water several times.

Predator eggs were surface-sterilized by dipping them in a 0.5% sodium hypochlorite solution for 30 s. Subsequently they were dipped 3 times in sterilized water for 30 s each. Eggs were shortly dried on tissue paper and transferred to the underside of prey-infested bean leaves.

General bioassay set-up and symptom assessment

The following method was applied in each experiment. Adult female predators were kept individually on a spider mite-infested leaf disc (ø 2.5 cm) in a plastic Petri dish (ø 5.5 cm) placed in a climate chamber at $23 \pm 1°C$. Leaf discs cut from one leaf were evenly distributed over treatments. Size, mortality, fecundity and predator position, were assessed daily. The response to HIPV and the location of excretory crystals were determined once on the last experimental day. The person measuring the parameters did not know to which

population or treatment group the predators belonged. For a detailed description of symptom assessment see Schütte et al. (2006).

Experimental treatments

Experiment 1: Vertical transmission (see Fig. 1)

Here we tested whether parent predators from the NR-population can transmit the NR-syndrome to their offspring. Normal-sized mated females from the NR-population were collected for egg production from the semi-open predator rearing. Egg collection was done in the same way as for the pre-infection rearing. To eliminate effects of secondary microorganisms, which may colonize dead individuals, eggs were only collected from dishes carrying a live predator. To estimate the infection of the mothers, we checked whether they became symptomatic. This experiment consisted of two replicates, with 71 and 79 eggs, respectively, collected from 73 female predators. Eggs were evenly distributed over the following three treatments:

(a)   Eggs were surface-sterilized with sodium hypochlorite solution and transferred to the underside of a prey-infested leaf disc (viable microbes may only be present inside the egg=*transovarial transmission*).

(b)   Eggs were transferred to the underside of a new prey-infested leaf disc that had not been in contact with a predatory mite (viable microbes may be present inside the egg and/or on the egg surface=*transovum transmission*+transovarial transmission).

(c)   Eggs were left at the place where they had been deposited (viable microbes may be present inside the egg, on the egg surface and/or in products left on the leaf by the mother=*horizontal transmission*+transovum transmission+transovarial transmission).
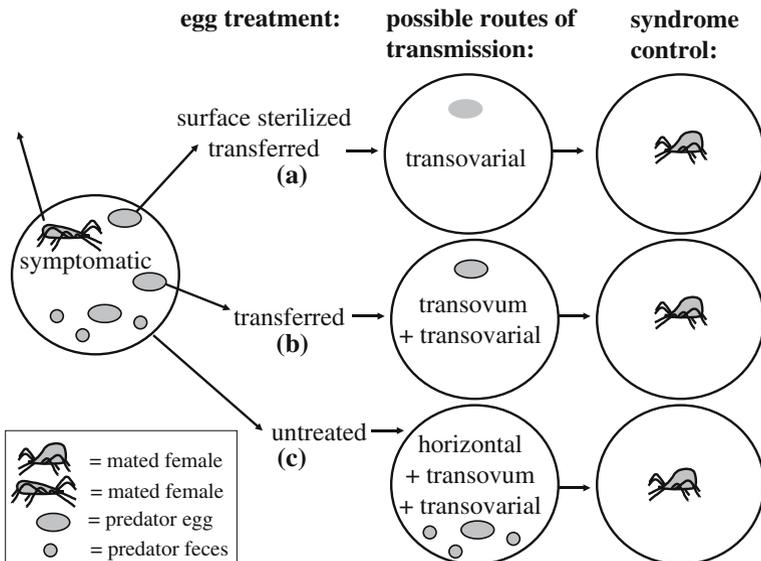

Fig. 1 Schematic representation of experimental set-up to investigate the involvement of vertical transmission (experiment 1)

The number of individuals not recovered for treatment a, b and c, was 4, 2 and 1, respectively during the egg stage and 3, 0 and 3, respectively during juvenile development. Juveniles were transferred to a fresh prey-infested leaf after 3 days. After 6 days all offspring had become adult and females and males belonging to the same treatment group were allowed to mate. A single copulation is sufficient for a female to reach maximum egg production (Schulten 1985). Because the sex ratio of *P. persimilis* is female biased, males had to mate more than once. The duration of mating was observed to be sure that it was not interrupted early. A complete mating takes about 150 min (Schulten 1985). After mating, size, mortality, fecundity and position of the female predators were recorded daily. The number of females lost during handling was 2, 0, and 1 for treatments a, b and c, respectively. When the post-oviposition period had lasted for at least 5 days for all predators, surviving predators were tested in the Y-tube olfactometer and crystal position was determined (see Schütte et al. 2006 for methodology). During a period of 30 days, symptoms were measured for 27, 28 and 30 females for treatments a, b and c, respectively.

To test if the NR-syndrome appeared in the second generation, eggs were collected near the end of the oviposition period (=day 17 after mating). The number of eggs collected was 75, 75 and 61 for treatments a, b and c, respectively. These eggs were reared individually in a Petri dish rearing and from the age of 8 days the size of mated females was checked daily. When the females were 14 days old the following parameters were determined: number of eggs/female alive during 2 days, response to HIPV and crystal location (see Schütte et al. 2006 for methodology). This experiment consisted of two replicates, with 15–18 mated females per treatment. In this way we tested 34, 31 and 33 second-generation females for treatments a, b and c, respectively.

Experiment 2: Horizontal transmission (see Fig. 2)

Four modes of horizontal transmission were tested. In all cases mated female predators from the R1-population were exposed to items related to mated females from the NR-population. Symptoms were recorded during or after exposure.

Experiment 2.1: Horizontal transmission via food   We tested whether consumption of prey mites from our laboratory rearing may induce the syndrome in predators from the R1-population. Mated adult female predators of unknown age were randomly taken from a batch of the R1-population directly after shipment from the producer and distributed over two groups. Predators were kept either on spider mite-infested leaves originating from the same commercial producer who delivered the R1-population (=control) or on spider mite-infested leaves originating from our laboratory (=treatment). As the quantities of food delivered by the commercial producer were a limiting factor we transferred predators to new leaf discs every second day. Eggs laid during the first day were eliminated. Parameters were measured for a period of 6 days. Three replicates were run, each with 10 predators per treatment.

Experiment 2.2: Horizontal transmission via squashed female predators   Next, we tested whether the presence of squashed female predators from the NR-population can induce the syndrome in predators of the R1-population. Experimental set-up and replicate number were the same as in experiment 2.1. Mated adult female predators of the R1-population were distributed over two groups. Predators of both groups were kept on spider
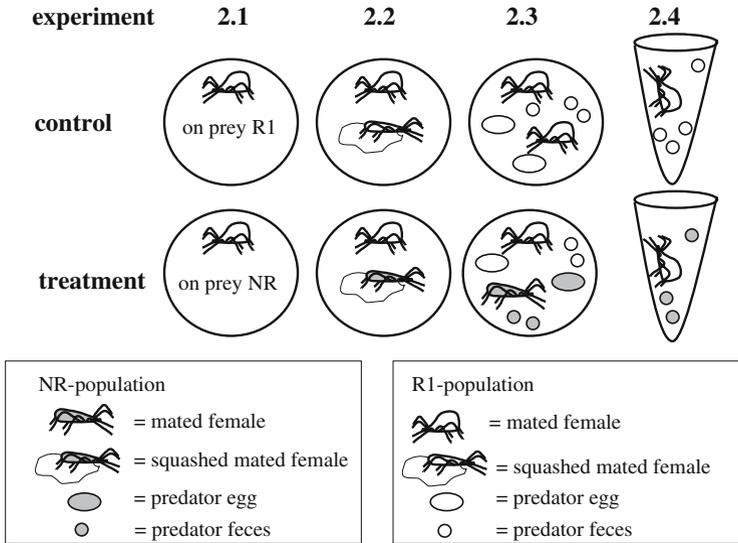
Fig. 2 Schematic representation of experimental set-up to investigate the involvement of horizontal transmission (experiment 2)

mite-infested leaf discs originating from the commercial producer who also delivered the R1-population. Leaf discs either carried two squashed female predators of the R1-population (=control) or two squashed female predators of the NR-population (=treatment). The squashed females were collected from newly delivered material of the R1-population and from the open rearing of the NR-population.

Experiment 2.3: Horizontal transmission via live female predators Tested was whether the presence of a live adult female of the NR-population may induce the NR-syndrome in predators of the R1-population. Mated adult female predators (age=7 days) from the pre-infection rearing of the R1-population were distributed over two groups. Predators of both groups were kept on spider mite-infested leaf discs originating from the spider mite rearing of our laboratory. A leaf disc carried either an additional live female (age=9–11 days) from the R1-population (=control) or an additional live female (age=9–11 days) from the NR-population (treatment). In order to distinguish these females from the other female present in the dish, they were marked with a small spot of water-soluble ink. The marked females from both populations originated from a pre-experimental rearing. After three days the marked females were removed. The unmarked females were transferred individually to new Petri dishes and NR-syndrome parameters were measured for a period of 6 days. Two replicates with 20 predators each were run.

Experiment 2.4: Horizontal transmission via feces and debris of live female predators Finally, we tested whether feces and debris excreted by predators from the NR-population can induce the NR-syndrome in predators from the R1-population. Mated adult female *P. persimilis* are of minute size (length of the body ca. 0.45 mm; Gaede 1992) and they excrete only liquid feces, which strongly adheres to the surface after evaporation.

Therefore it was not possible to collect feces without debris left on the surface by the excreting predator. This debris may, for example, consist of particles that attached to the legs at other places, or of material excreted during oviposition.

Feces and debris were collected by keeping four adult female predators (age=9–11 days) originating from a pre-experimental rearing in a plastic Eppendorf vial (volume=1.5 ml) together with a small piece of wet cotton wool (ambient temperature 23± 1°C). Per replicate 15 vials were prepared for the NR- and the R1-population. After 24 h the predators and their eggs were removed. Vials carrying dead predators were excluded from the experiment.

Adult female predators (age=7 days) from the pre-infection rearing of the R1-population were distributed over two groups. Four predators were either kept in an Eppendorf vial carrying feces and debris from the R1-population (=control) or in a vial carrying feces and debris from the non-responding (NR) population (=treatment) (ambient temperature 23±1°C). After 24 h the predators were transferred individually to new Petri dishes. In this experiment the NR-syndrome parameters were measured for a period of only 3 days in order to prevent an excess loss of predators through death. The number of replicates and predators was the same as in experiment 2.3.

Statistics

We used the Mann–Whitney U test or the paired t-test to test numerical data. A contingency table test was used for categorical data. The data from the replicates were pooled, because no relevant differences were present between the replicates.

Results

Vertical disease transmission

Characteristics of mothers

The 73 female predators from the NR-population whose eggs were used for this experiment clearly showed the NR-syndrome that has previously been described by Schütte et al. (2006). Ninety-three percent of the mothers shrank and only 48% were present on the leaf at the moment of egg collection. Moreover, they laid only 2.1±1.1 eggs during one day. Seventy-four percent of the females chose the odor of prey-infested plants and 15% carried crystals in their legs.

Characteristics of juvenile and adult offspring prior to mating

Less than 10% of the offspring was lost during development due to non-hatching of eggs and juvenile mortality (Table 1). This was true for all three treatments. The overall female sex ratio was 78%. As only 5 males developed from eggs that had been transferred to a new leaf, these males had to mate more times than those of the other two treatments (Table 1).

Characteristics of mated females

The syndrome was not transmitted vertically via the egg. Only normal-sized, well-performing females developed from eggs that were surface-sterilized or transferred to another leaf whereas untreated eggs gave rise to dorso-ventrally flattened females (17%). These

Table 1 Pre-mating characteristics of predators reared from eggs collected from the NR-population

	Eggs sterilized (51)	Eggs transferred (47)	Eggs untreated (52)
% Eggs not hatched	2 (1 out of 47)	0 (out of 45)	0 (out of 51)
% Dead juveniles	5 (2 out of 43)	2 (1 out of 45)	8 (4 out of 48)
% Females	71 (29 out of 41)	89 (39 out of 44)	73 (32 out of 44)
# Matings/♂ (average)	2.4	5.6	2.6
	29 Mated females	28 Mated females[a]	31 Mated females[b]

Eggs were transferred to another leaf after surface sterilization (=eggs sterilized), transferred to another leaf (=eggs transferred) or left at the place where the mother had laid them (=eggs untreated). Numbers in parentheses represent actual predator numbers

[a]Impossible to accomplish mating of all females during 24 h, because of a limited number of males

[b]1 Female escaped prior to mating

females shrank 2–6 days after mating and died 4–8 days later. Females that did not shrink during that period remained normal-sized until the end of the experiment. Even when they entered the post-oviposition period, i.e. when they stopped carrying eggs, they could be distinguished from dorso-ventrally flattened individuals. In the following part, data recorded from females originating from untreated eggs are presented separately for small and normal-sized females.

The age-specific oviposition curves (Fig. 3) of the normal-sized females of the three treatments were very similar and resembled the form of a trapezoid. After mating the age-specific oviposition rate rapidly increased. On the 3rd day after mating it reached a peak of 4.6, 4.8 and 4.9 eggs per female per day for predators reared from sterilized eggs, transferred eggs and untreated eggs respectively (defined as peak rate of oviposition by Sabelis and Janssen 1994). The end of the plateau phase was reached on the 13th to 14th day whereafter it decreased to zero at the end of the reproductive life on the 24th to 25th day. However, the curve of the shrunken females originating from untreated eggs is totally different, having a triangular shape (Fig. 3). The oviposition rate of these females steeply rose to a top of 3 eggs per female at the 2nd day after mating. Subsequently, it steeply decreased to zero at the end of the reproductive life, which was reached on the 7th day already. The last shrunken female already died on the 11th day after mating. During their short life, shrunken females laid an average of only 7.8±7.4 eggs and only 2 of the 5 females were found on the leaf during more than half of the time. As all shrunken females died early, crystal location and the attraction to HIPV were not determined for this group.

The normal-sized females of all three treatments performed better than their shrunken mothers and sisters. Mortality of normal-sized females up to day 30 after mating was low and fecundity was high. The mean number of deposited eggs was 60, 67.9 and 71.9 for normal-sized females of the three treatments, respectively (Table 2) compared to only 8 eggs of the shrunken females. Mortality was highest and fecundity was lowest in normal-sized females reared from sterilized eggs, but not significantly different from females reared from transferred eggs (mortality: $P=0.3$; fecundity: $P=0.65$, paired t-test). No differences were found between normal-sized females of the three treatments concerning foraging behavior. All normal-sized females were recorded on the leaf during more than half of the observations (Table 2). Although the females tested in the olfactometer for attraction to HIPV were 37 days old and had laid no eggs for at least 5 days, they were still attracted to the odor of prey-infested plants (Table 2). The attraction to HIPV was lowest for females reared from untreated eggs, but not significantly different from females reared
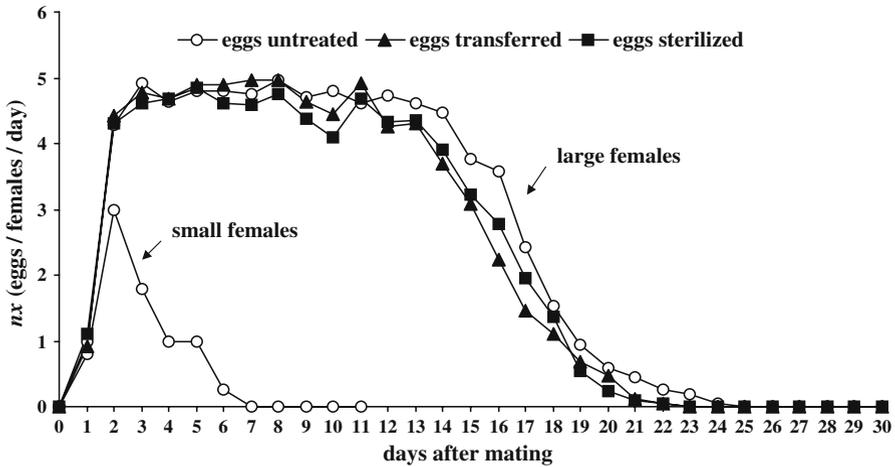
Fig. 3 Age-specific oviposition *n(x)* (number of eggs per living female of age class *x*) of normal-sized and shrunken *Phytoseiulus persimilis* reared from eggs collected from the NR-population. Eggs were transferred to another leaf after surface sterilization (=eggs sterilized), transferred to another leaf (=eggs transferred) or left at the place where the mother had laid them (=eggs untreated). Day 0 is the 7th day of development and mating took place on this day

Table 2 Characteristics of mated *normal-sized* female *P. persimilis* reared from eggs collected from the non-responding population

	Eggs sterilized (*N*=27)	Eggs transferred (*N*=28)	Eggs untreated (*N*=25)
% Dead ♀♀	22 (6 out of 27) a	11 (3 out of 28) a X	12 (3 out of 25) X
# Eggs/♀ / 30 days (average±SD)	60.0±24.7 (27) a	67.9±13.7 (28) a X	71.9±15 (25) X
% ♀♀>Half of time on leaf	100 (27)	100 (28)	100 (25)
% ♀♀ To plant odors (HIPV)	88 (14 out of 16) a	76 (16 out of 21) a X	67 (10 out of 15) X
% ♀♀ With crystals in legs	0 (out of 21)	0 (out of 24)	0 (out of 20)

Eggs were surface-sterilized and transferred to another leaf (=eggs sterilized), transferred to another leaf (=eggs transferred) or left at the place where the mother had laid them (=eggs untreated). Numbers in parentheses represent actual predator numbers. Values in the same row carrying different letters are significantly different (paired *t*-test for numerical data, 2 by 2 contingency table test for categorical data, 2 comparisons: column 1 with 2, column 2 with 3, $\alpha=0.025$)

from transferred eggs (*P*=0.71). None of these old normal-sized females carried crystals in the legs (Table 2).

Characteristics of second generation females

Eggs were collected from normal-sized females from all 3 treatments on the 17th day after mating and reared until adulthood. We did not find any evidence of syndrome occurrence in the second generation, as none of the females grown from these eggs turned small up to the age of 14 days after mating and none of these females carried crystals in the legs at that age. The mean number of eggs laid on day 13 plus 14 was 8.6±2.7, 8.9±2.3 and 8.0±2.7 for treatments a, b and c, respectively (*P*≥0.16, paired *t*-tests). Moreover, females of all three

treatments showed a strong attraction to HIPV. The percentage of females choosing the odor of prey-infested plants was 90% ($N=30$), 93% ($N=28$) and 85% ($N=26$) for treatments a, b and c, respectively ($P \geq 0.50$).

Horizontal disease transmission

Transmission via food and squashed females

No symptoms were induced in female predators from the R1-population when fed with prey-mites reared in our laboratory (Table 3a). The same is true for predators from the R1-population when exposed to squashed female predators from the NR-population (Table 3b). The data for all NR-syndrome parameters of the control predators as well as treated predators were very similar to the data obtained for predators from the R1-population in earlier experiments (Schütte et al. 2006).

Transmission via live females

All but one of the symptoms tested were induced in female predators from the R1-population after a three day-exposure to female predators from the NR-population (Table 3c). The data for size, mortality, fecundity and predator position of treated predators were very similar to data obtained for predators of the NR-population during earlier studies (Schütte et al. 2006). None of the control predators shrank or died during the experimental time, whereas about half of the treated predators shrank 3–7 days after the first contact with a symptomatic female and died ($P<0.001$, for both parameters). Females, which did not shrink during this period, remained normal-sized and alive until the end of the experiment. Treated predators laid significantly fewer eggs than control predators ($P<0.001$, Mann–Whitney U test) and significantly fewer treated females were found on the leaf in more than half of the observations ($P<0.001$). No significant difference was found between treated and control predators concerning their response to HIPV ($P=0.83$). However, due to high mortality in the treated predators, only 1 shrunken individual was present among the 15 treatment females making a choice. None of the live control predators carried crystals in the legs whereas a quarter of the live treatment predators did so ($P=0.015$).

Transmission via feces and debris

All but one of the symptoms tested were induced in predators from the R1-population after a 24 h contact with feces and debris released by female predators from the NR-population (Table 3d). The first shrunken females appeared 2 days after contact. Two days later 65% of the treated predators had shrunk, whereas all control predators were still normal-sized ($P<0.001$). Treated predators laid significantly fewer eggs during this period than control predators ($P<0.001$, Mann–Whitney U test). The fraction of females that resided on the leaf at the last parameter-assessment day was 85% for the control predators versus only 58% for treated predators ($P=0.017$). Treated predators also showed a weaker attraction to HIPV than the control predators, differences being marginally insignificant ($P=0.06$). None of the control predators carried crystals in the legs compared to 35% of the treatment predators ($P<0.001$).

Table 3 Symptoms of mated female *P. persimilis* of the R1-population

(a) FOOD	CONTROL (*N*=27)	TREATMENT (*N*=29)	*P**
Predator size			
% Small ♀♀	0 (out of 27)	0 (out of 29)	
Predator mortality			
% Dead ♀♀	26 (7 out of 27)	7 (2 out of 29)	0.11
Predator fecundity			
# Eggs/♀/6 days (average±SD)	21.8±6.1 (27)	21.9±3.8 (29)	0.99
Predator behavior in olfactometer			
% ♀♀ To plant odors (HIPV)	94 (15 out of 16)	85 (23 out of 27)	0.70
Predator position within dish			
% ♀♀ > Half of time on leaf	96 (26 out of 27)	83 (24 out of 29)	0.23
Crystal location within predator			
% ♀♀ With crystals in legs	0 (out of 19)	0 (out of 26)	
(b) SQUASH	CONTROL (*N*=25)	TREATMENT (*N*=24)	*P**
Predator size			
% Small ♀♀	0 (out of 25)	4.2 (1 out of 24)	0.98
Predator mortality			
% Dead ♀♀	16 (4 out of 25)	8.3 (2 out of 24)	0.70
Predator fecundity			
# Eggs/♀/6 days (average±SD)	21.6±4.7 (25)	29.9±5.7 (24)	0.27
Predator behavior in olfactometer			
% ♀♀ To plant odors (HIPV)	88 (14 out of 16)	82 (14 out of 17)	1.0
Predator position within dish			
% ♀♀ > Half of time on leaf	92 (23 out of 25)	92 (22 out of 24)	1.0
Crystal location within predator			
% ♀♀ With crystals in legs	5 (1 out of 20)	0 (out of 18)	1.0
(c) ALIVE	CONTROL (*N*=37)	TREATMENT (*N*=38)	*P***
Predator size			
% Small ♀♀	0 (out of 37)	53 (20 out of 38)	<0.001
Predator mortality			
% Dead ♀♀	0 (out of 37)	47 (18 out of 38)	<0.001
Predator fecundity			
# Eggs/♀/6 days (average±SD)	25.9±1.5 (37)	11.8±10.8 (38)	<0.001
Predator behavior in olfactometer			
% ♀♀ To plant odors (HIPV)	81 (25 out of 31)	73 (11 out of 15)	0.83
Predator position within dish			
% ♀♀ > Half of time on leaf	95 (35 out of 37)	50 (19 out of 38)	<0.001
Crystal location within predator			
% ♀♀ With crystals in legs	0 (out of 27)	25 (4 out of 16)	0.015
(d) FECES	CONTROL (*N*=39)	TREATMENT (*N*=43)	*P***
Predator size			
% Small ♀♀	0 (out of 39)	65 (28 out of 43)	<0.001
Predator mortality			
% Dead ♀♀	0 (out of 39)	0 (out of 43)	
Predator fecundity			
# Eggs/♀/3 days (average±SD)	11.9±1.9 (39)	7.3±3.4 (43)	<0.001
Predator behavior in olfactometer			
% ♀♀ To plant odors (HIPV)	88 (22 out of 25)	68 (25 out of 37)	0.06
Crystal location within predator			
% ♀♀ With crystals in legs	0 (out of 35)	35 (14 out of 40)	<0.001

Females were incubated (a) on prey-infested leaves originating either from the commercial producer of the R1-population (=control) or from our laboratory (=treatment), (b) together with 2 squashed adult female predators of the R1-population (=control) or the NR-population (=treatment), (c) together with 1 live adult female of the R1-population (=control) or the NR-population (=treatment) during 3 days and (d) on feces and debris of adult female predators of the R1-population (=control) or of the NR-population (=treatment) during 1 day. Numbers in parentheses represent actual predator numbers. For explanation of symptoms see Schütte et al. (2006)

*Paired *t*-test for numerical data, 2 by 2 contingency table test for categorical data

**Mann–Whitney *U* test for numerical data, 2 by 2 contingency table test for categorical data

Discussion

Vertical transmission

No evidence was found for maternal- or paternal-mediated vertical transmission. Eggs laid by symptomatic females of the NR-population mated by males of the NR-population gave rise to normal-sized well-performing females, which produced non-symptomatic offspring themselves, when eggs were surface sterilized or transferred to an uncontaminated substrate. This is in accordance with earlier studies, where 11 isofemale lines could be started from the NR-population, by transferring eggs to an uncontaminated bean leaf. Predators from these lines showed a stronger attraction to HIPV than predators from the NR-population during the same period (Dicke et al. 2000).

In contrast, Bjørnson and Keddie (2001) found 100% vertical transmission of the microsporidian pathogen *M. phytoseiuli* in *P. persimilis*. All progeny that hatched from surface-sterilized eggs were infected. Male predators did not contribute to microsporidian infection of their progeny. In another study, surface sterilization of eggs collected from a poorly performing commercial population of *P. persimilis* had a positive effect on short-term survival and oviposition (Steiner and Bjørnson 1996). Short-term performance was best in predators originating from eggs rinsed in water, compared to eggs washed with formaldehyde or tetracycline hydrochloride.

Horizontal transmission via residues of the mother

In the present study transmission from mother to offspring was only achieved horizontally via products left on the leaf by the mother. Five females shrank 2–6 days after mating and died 4–8 days later. Infection of these females must have taken place during the larval or protonymphal stage, as contact with products left by the mother was only possible during the first 3 days of the experiment. This is remarkable, because larvae of *P. persimilis* do not feed at all and only move small distances (Schausberger and Croft 1999b; Nagelkerke 1993). Such a phenomenon, i.e. when larvae or nymphs acquire benign or sublethal infections and survive to adulthood wherein they may transmit the pathogen and/or become symptomatic, is called transstadial transmission. This may allow pathogens to multiply to higher numbers in older individuals (Andreadis 1987).

Horizontal transmission via food and squashed females

Consumption of *T. urticae* spider mites that had been reared in our department did not induce the NR-syndrome in females of the R1-population. Hence, the syndrome-inducing factor is not present in the food source of the NR-population. These results are in accordance with earlier studies where non-symptomatic predators could be reared with spider mite prey from the mass rearing in our laboratory (Schütte et al. 1998; Dicke et al. 2000). Spores of the microsporidian pathogen *M. phytoseiuli* have also never been detected in *T. urticae*, not even in colonies that were fed to a *P. persimilis* population that was 100% infected with the microsporidian pathogen. In contrast, *Wolbachia* may be present in *T. urticae* as well as in *P. persimilis* (Breeuwer and Jacobs 1996).

No evidence was found for transmission via females that were squashed on the leaf surface, although transmission of the behavioral change via females that had died has been reported earlier (Schütte et al. 1998). In the latter case, however, the leaf did not carry only an individual that had died, but also products such as feces that had been left on the leaf

prior to death. It is possible that body fluid of diseased predators is not infectious, or that it does not stay infectious for a long time. Another possible explanation is predator avoidance behavior: female *P. persimilis* may avoid dead conspecifics and body fluids of conspecifics, whereas they do not avoid feces of conspecifics. Avoidance of dead conspecifics has been reported for the American cockroach *Periplaneta americana*, which is repelled by intact and ruptured corpses of conspecifics (Rollo et al. 1995). Moreover, female *T. urticae* avoid places with artificially damaged conspecifics (eggs or dead adults) (Grostal and Dicke 1999).

Horizontal transmission via live females and feces

Female predators from the R1-population showed the NR-syndrome following a 3-day exposure to a live conspecific of the NR-population and after a 1-day exposure to feces and debris of such females; transmission rates were 53% and 65%, respectively. These results support our hypothesis that the NR-syndrome is caused by a disease. Excretion of infective pathogen stages in feces is characteristic of many bacterial, viral and protozoan pathogens that infect the digestive tract of insects (Andreadis 1987). Bjørnson and Keddie (2001) report that horizontal transmission of *M. phytoseiuli* in *P. persimilis* is rather low (14%). It only occurred when immature *P. persimilis* developed while in contact with infected immature and adult predators during at least 5 days. Horizontal transmission did neither occur when uninfected adult female predators were placed on leaf surfaces previously contaminated by infected predators or by application of microsporidian spores, nor when those predators were exposed to infected female predators during 48 h (Bjørnson and Keddie 2001). We are aware of one other report of horizontal disease transmission via feces in mites. Pathogen transfer via defecation has been reported for the herbivorous mite *P. citri* infected with a non-occluded virus (Reed et al. 1975).

Pathogen uptake

Horizontally transmitted insect pathogens may gain entrance to their hosts through natural body openings or through the integument (Andreadis 1987). The majority of horizontally transmitted insect pathogens enter their host through the mouth (per os). It has been argued earlier that infection of predatory mites through the oral route is unlikely unless the prey is infected, as their mouthparts consist of several sharp stylets that puncture the prey (Bjørnson and Keddie 2001).

   We think that per os infection might be possible even if prey is not infected:

(1)   In situations where prey is scarce *cannibalism* of infected conspecifics could be a source of infection, as *P. persimilis* is known to feed on conspecifics (see for a review Schausberger 2003). Interestingly Schausberger and Croft (2001) have demonstrated the presence of kin discrimination for cannibalistic females of *P. persimilis*. In dual choice tests female predators discriminate between related and unrelated conspecific larvae and preferentially prey upon unrelated larvae. This was true for laboratory-reared, commercially mass-reared and field-collected females. A possible reason for kin recognition may be disease avoidance, as genetic similarity between predator and prey poses a greater risk to acquire deleterious pathogens, because of selection for host specificity among pathogens (Pfennig et al. 1998). However, *P. persimilis* does not discriminate between con- and heterospecific predatory mites as prey (Schausberger and Croft 1999a), which contradicts the hypothesis of disease avoidance. It

would thus be interesting to study cannibalistic behavior of predators from our NR-population.

(2) As *P. persimilis* may *drink from water* droplets (Gaede 1992) contaminated water droplets may as well be a source of oral infection.

(3) Moreover, female *P. persimilis* may *share food with other individuals*, i.e. different individuals feed on the same prey item, when predator density is high (Yao and Chant 1990), which creates possibilities for pathogen transfer via shared food.

(4) Another possible mechanism of pathogen uptake that might be important in the natural situation is the *grooming of body parts*, which had been previously in contact with feces and debris deposited by diseased predators. Adult female *P. persimilis* use their chemoreceptor-carrying pedipalps, to drum individual prey or the walking substrate (Dicke et al. 1991). This behavior may lead to contamination with the infectious agent, which could gain oral entrance through subsequent pedipalp cleaning.

In the closed Petri dishes and Eppendorf vials used for the present transmission experiments, condense water was always present due to the high relative humidity in the closed system. It is thus possible that predators drank from feces-contaminated condense water. Horizontal transmission in Eppendorf vials was not recorded when no water was added (C. Schütte, unpublished data). Grooming of contaminated parts of the body may also be a valid explanation in the present experiments. However, cannibalism and food sharing does not hold as possible explanations in the set-up in which non-symptomatic predators were exposed to feces and debris of infected predators.

In this context reports should be mentioned of insects and mites that are attracted to conspecific feces (Carlson et al. 2000; Grenacher, et al. 2001). Adult female *P. persimilis* are attracted to conspecifics (Janssen et al. 1997), but we are not aware of any study where the role of predator feces in attraction has been studied. It could be possible that *P. persimilis* uses feces as information source for the presence of conspecific individuals. Under natural conditions *P. persimilis* shows a clustered distribution and lives in aggregations of conspecifics. As each individual deposits large amounts of fecal material (Schütte et al. 2006), feces would be an excellent cue for the presence of conspecifics in a prey patch shared by conspecifics.

Pathogen release into the environment

Bjørnson and Keddie (2001) observed numerous microsporidian spores of *M. phytoseiuli* in smear preparations of fecal pellets of infected predators examined by light microscopy, whereas no spores were detected on leaf surfaces or predator feces when examined by SEM. Predator feces appeared as intact aggregates of dumbbell-shaped crystals (Bjørnson and Keddie 2001). Hence the authors thought it unlikely that spores are liberated from intact fecal pellets onto leaf surfaces. However, in that study dissected bean leaves were air-dried prior to SEM observation. In contrast, humidity may be near saturation in the region close to the leaf surface (=boundary layer) when a leaf is attached to the plant (Gaede 1992). It may thus be possible that feces are diluted and fecal components are liberated onto the leaf surface in such conditions and that they may be picked up by predatory mites in the way described above. In such a case ambient humidity and temperature, air velocity, light regime, plant condition and plant characteristics (including leaf size, leaf shape, leaf position in plant, leaf thickness, leaf surface) could influence disease

transmission, as these factors have a direct impact on the humidity within the boundary layer of the leaf (Gaede 1992).

Virulence and transmission

Insect pathogens may be divided into two groups according to their virulence and transmission (Myers and Rothman 1995): (1) highly virulent pathogens kill their hosts and are transmitted horizontally via the release of environmentally resistant, infectious particles (nuclear polyhedral viruses, bacteria, fungi). Epizootics can destabilize host populations. (2) Benign pathogens reduce the vigor of infected hosts and can be transmitted vertically between generations without destabilizing host populations (many protozoa, some viruses).

The present case has the characteristics of the first group: symptomatic female predators stop egg laying shortly after mating and die several days after reproduction ceases (Schütte et al. 2006). They release feces carrying the infectious agent that stays viable when released into the environment. Moreover, epizootics may in certain cases destabilize host populations and this may lead to eradication of the population (Dicke et al. 2000; C. Schütte, unpublished data). In such systems predator behavior is crucial for pathogen transmission (Andreadis 1987). It may therefore be expected that behavioral changes cause changes in pathogen transmission. Whether the reported behavioral changes benefit the pathogen by ensuring high transmission rates or the host by minimizing disease transmission to conspecifics, will be an interesting topic for further study.

In contrast to the disease reported here, the disease caused by *M. phytoseiuli* has the characteristics of the second group: this pathogen causes less severe reductions in fecundity, longevity, prey consumption and female offspring (Bjørnson and Keddie 1999). Pathogen transmission is mainly vertical and disease prevalence may stay at a low level in infected colonies over a long period (Bjørnson and Keddie 2001). In this system, where maternal vertical transmission is 100% and horizontal transmission is low, predator behavior will have less effect on disease transmission.

Biological control

The results of the present study clearly demonstrate that the NR-syndrome is a contagious phenomenon. It may be expected that the syndrome will be efficiently transferred among and between generations as soon as it has entered a population. Care should be taken to avoid contact of such a population with other populations of *P. persimilis*. The best diagnostic symptoms for the presence of the NR-syndrome are size of adult females and the presence of crystals within the legs. A population is most probably showing the NR-syndrome when the following requirements are met: (1) numerous dorso-ventrally flattened females are present, (2) these flattened females do not become normal sized after offering ample food and a male conspecific and die several days after shrinking (3) live adult females carrying birefringent crystals in the legs are present. It remains to be detected whether commercial populations exhibit the novel disease described in this paper.

Only normal-sized well-performing females showing normal foraging behavior were obtained from surface-sterilized eggs and eggs transferred to an uncontaminated place. Moreover, these females produced non-symptomatic offspring themselves. Egg sterilization did not have great negative effects on the progeny in terms of egg hatchability, fecundity and mortality. Net fecundity was 60±25 eggs for females grown from sterilized eggs and 68±14 eggs for females grown from transferred eggs respectively. These numbers lay within the range of data reported for *P. persimilis* (40–80 eggs/female, reviewed by

Sabelis and Janssen 1994). Transfer and/or surface sterilization of eggs are thus promising methods of curing the present disease. However, after sterilization eggs have to be transferred to an uncontaminated environment. This may be a bottleneck for many commercial producers of *P. persimilis*, as rearing space and facilities are often scarce. Sterilization methods that may lead to non-contaminated rearing facilities are therefore needed in the future.

Conclusions

The NR-syndrome is transmitted horizontally from mother to offspring and between females by feces and debris. These findings strongly support our hypothesis that a pathogen induces the syndrome. The ultimate proof of this hypothesis will be to satisfy Koch's postulates (Lacey 1997). Elucidation of feces as the main transmission mode offers new perspectives for pathogen isolation, which is a prerequisite for addressing Koch's postulates. By now, we obtained a bacterial isolate from adult female *P. persimilis* of the NR-population that did induce the non-responding syndrome in non-symptomatic adult female predators (Schütte 2006). This novel pathogen has recently been identified and described as *Acaricomes phytoseiuli* gen. nov., sp. nov. (Pukall et al. 2006). Isolation and identification of the putative pathogen is of great interest for commercial producers of biological control agents and for laboratories working with phytoseiid mites, because of its negative effects on both life history and behavior.

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