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**COURTSHIP DISPLAYS OF INTROGRESSED,  
INTERSPECIFIC HYBRID NASONIA MALES:  
FURTHER INVESTIGATIONS INTO  
THE ‘GRANDFATHER EFFECT’**

by

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**Summary**

Previously, we investigated courtship behaviour of bidirectional, interspecific hybrid males of two species of *Nasonia* (Beukeboom & van den Assem, 2001). Characteristics of the displays were intermediate between those of the parental species, but at the same time were biased towards the paternal side. Due to the haplodiploid sex determination system of the Hymenoptera, the nearest male parent of the haploid (hence, fatherless) *Nasonia* males are their grandfather. Therefore, we have called this bias the ‘grandfather effect’. In the present paper, we investigate one of the possible causes of the ‘grandfather effect’: nucleo-cytoplasmic interaction. In interspecific hybrids, the paternally donated nuclear genes must operate in an ‘alien’ environment: the maternal (heterospecific) cytoplasm. Adverse effects may prevail in this situation, and result in a biased gene transmission (although to the maternal side). With introgression techniques and subsequent hybridisation of introgressed lines, we constructed male progeny in which paternally contributed nuclear genes are conspecific with the maternal cytoplasm. Courtship of these males provide a test of the interaction hypothesis. Because our results show a persistence of the ‘grandfather effect’, a simple nucleo-cytoplasmic interaction seems an unlikely explanation of the phenomenon.

*Keywords:* *Nasonia*, courtship, interspecific hybrids, introgression, nucleo-cytoplasmic interaction, genomic imprinting.

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## Introduction

The behaviour of interspecific hybrids provides promising openings for the study of speciation and the maintenance of species integrity. As part of a research project on components of the behavioural repertoire and their underlying genetics, we investigated courtship and mating behaviour of interspecific hybrids of the pteromalid wasp *Nasonia* (Beukeboom & van den Assem, 2001). Thus far, very few such behaviour studies have been carried out and almost all of them involve *Drosophila* species pairs (Coyne & Charlesworth, 1997; Boake *et al.*, 1998; Ishii *et al.*, 2001; Williams *et al.*, 2001).

*Nasonia* (parasites of blowfly pupae) provide excellent opportunities for behaviour-genetic studies. Three species are known: the cosmopolitan *N. vitripennis*, and two others with restricted distributions, *N. longicornis* and *N. giraulti*, in respectively the Western and Eastern United States (Darling & Werren, 1990). The species can be differentiated on behavioural, morphological, and genetic characters. Their courtship behaviour has been studied in detail (overview in van den Assem, 1996), genetic mapping techniques are being developed (Gadau *et al.*, 1999), and interspecific hybrids can be constructed by crossing *Wolbachia*-free strains (see section on Material, and Breeuwer & Werren, 1990). As all Hymenoptera, *Nasonia* has haplodiploid sex determination, meaning that males develop from non-fertilised, haploid eggs and females from fertilised, diploid eggs. The haploid condition is very useful for genetic analysis because effects of dominance and recessivity are non-existent.

Courtship behaviour of bidirectional *vitripennis* × *longicornis* hybrids was the topic of previous investigations (Beukeboom & van den Assem, 2001). As expected, most components of hybrid displays were intermediate between those of the parental species. However, at the same time, they showed an unexpected, significant bias towards characteristics of the paternal line. We have referred to this phenomenon as the ‘grandfather effect’ (grandfather because being haploid, male Hymenoptera are fatherless but do have a grandfather). A satisfactory explanation of the phenomenon was not on hand, but two sets of hypotheses were considered. One possible explanation is that in the progeny of hybrid mothers there is a bias in the recovery of genotypes, either due to complex nucleo-cytoplasmic interaction or to a form of meiotic drive. The alternative explanation envisages a bias in

gene expression rather than in transmission as the cause of the behavioural bias (hence, an epigenetic factor).

Nucleo-cytoplasmic interaction refers to a bias in recovery of hybrid genotypes in the zygote, due to lethal interactions between nuclear genes of one species and cytoplasmic genes of the other (Breeuwer & Werren, 1995). However, such interactions typically result in a higher recovery rate of maternal rather than paternal nuclear genes. The reason is that the maternal genes are better adapted to the maternally inherited, hence conspecific, cytoplasm. In contrast, the paternal genes end up in an unfamiliar cytoplasm, which may lead to detrimental effects. Introgressed hybrid lines can provide material for a test of this hypothesis. By repeated backcrosses between females of an interspecific hybrid line and pure-line males, the nuclear genome of one species can be replaced by that of the other. Hence, in the hybrid progeny obtained from crosses with introgressed lines, the nuclear genes of paternal descent and the maternally derived cytoplasmic genes can be made conspecific. Therefore, we expect an absence of the grandfather effect in such crosses if it were the result of nucleo-cytoplasmic interaction. Persistence of the effect would rule out this kind of interaction as a valid explanation. Here we present the results of observations on courtship displays of two types of introgressed males, and of the hybrids that resulted from crossing pure-species males with introgressed females.

## Material and methods

### *Lines*

*Nasonia* are 2–3 mm long parasites of blowfly pupae; they are easily cultured under laboratory conditions. *Wolbachia* bacteria play a role in the reproductive isolation of *Nasonia* species (Breeuwer & Werren, 1990, 1995; Bordenstein *et al.*, 2001). *Wolbachia*-free, so-called 'cured', lines — a prerequisite for successful interspecific crosses — can be established in the laboratory by feeding antibiotics. Our material are cured lines of *N. vitripennis* strain AsymC HS, derived from strain Leiden LabII (labelled V[V], where V refers to the type of nuclear genome and [V] to the type of cytoplasm) and of *N. longicornis* strain IV7 R2, derived from strain IV7 (labelled L[L], as above). The same strains were used in our previous study (Beukeboom & van den Assem, 2001). Crosses of pure-line males and females yield F<sub>1</sub> interspecific hybrid daughters and F<sub>2</sub> interspecific hybrid sons (male V × female L yield VL[L] offspring; male L × female V yield LV[V] offspring). Repeated backcrosses of hybrid females with pure-line males (V male × VL[L] female and L male × LV[V] female produce introgressed lines V[L] and L[V] respectively (Fig. 1a)). The share of the paternally derived nuclear genome increases asymptotically with consecutive generations, and approaches — in theory — 100% in the 10<sup>th</sup> generation (Breeuwer & Werren, 1995). For the final test,

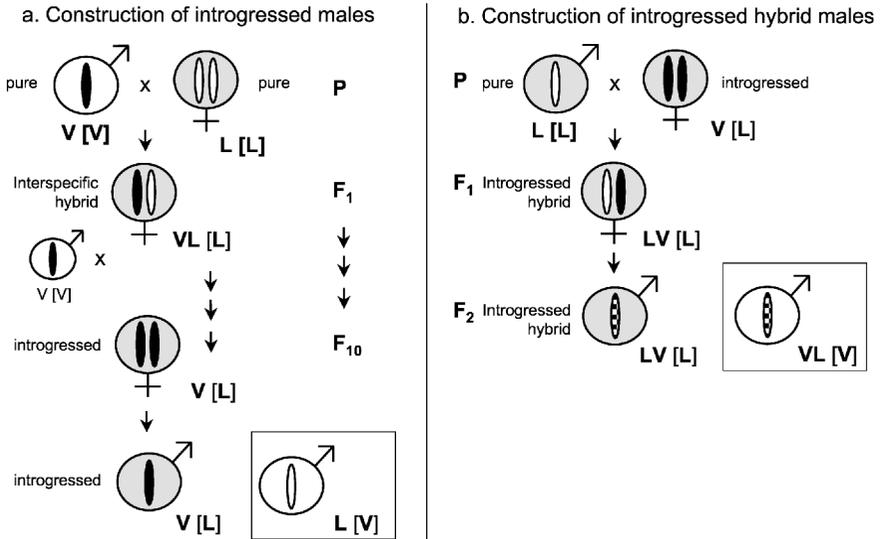


Fig. 1. Crosses of *Nasonia vitripennis* ( $V[V]$ ) and *Nasonia longicornis* ( $L[L]$ ) were used to generate interspecific hybrids. In the notation of hybrid genomes the first letter refers to the paternal contribution, the second to the maternal contribution (in the figure black for  $V$ , white for  $L$ ). The letter in square brackets refers to the type of cytoplasm. a. *N. vitripennis* males × *N. longicornis* females produced  $F_1$  hybrid daughters with a  $VL$  genome in the  $L$  cytoplasm, hence: ( $VL[L]$ ). Hybrid daughters were backcrossed over 10 generations to produce an introgressed line, in which a  $V$  nuclear genome was introduced into the  $L$  cytoplasm, hence: ( $V[L]$ ). The resulting male type of reciprocal introgression is shown in the box. Courtship of introgressed males was observed. b. Introgressed females were crossed with pure-line males to produce introgressed hybrid females. These females were propagated as virgins to produce  $F_2$  introgressed hybrid sons. Courtship of these introgressed males was used for a test of the nucleo-cytoplasmic interaction hypothesis. The type of male resulting from the reciprocal cross is shown in the box.

10<sup>th</sup> generation introgressed females were crossed with pure-line males (male  $V$  × female  $L[V]$ , and male  $L$  with female  $V[L]$ , to produce  $VL[V]$  and  $LV[L]$  offspring (Fig. 1b)). Thus, the courtship behaviour of males of four types (pure, interspecific hybrid, introgressed, and introgressed interspecific hybrid) is available for comparison.

In spite of the confirmed *Wolbachia*-free status of our *Nasonia* lines, not all crosses did equally well. In particular, those required to yield  $LV[L]$  introgressed interspecific hybrids frequently failed. This is likely due to nuclear-cytoplasmic interaction, which is more severe in the combination of a  $V$  nuclear genome and an  $L$  cytoplasm than in the reverse combination. In addition, several hybrid individuals appeared handicapped because of morphological malformation or impaired motor ability. Manifestly handicapped males were never included in a test.

### *Observations and procedures*

Procedures were similar to those in Beukeboom & van den Assem (2001). Courtship was observed with a dissection microscope at 10 $\times$ . Illumination was provided by glass-fibre optics; the ambient temperature was *ca* 22°C. Couples were confined to glass tubes (60 mm high, diameter 10 mm), closed off by a plug of cotton wool. A male had spent a period of at least 12 hrs in isolation in his tube of residence before a female was introduced. We recorded the courtship performance of males that mounted on the female within 10 min of her introduction. Durations were measured with digital timers; frequencies of species-specific display components were counted and scored as they happened. Couples involved a test male with a non-receptive pure-species female V[V]. The type of female is no longer a factor once the male is courting; males will even court female dummies, in the same way as they do living females (van den Assem & Jachmann, 1982). Two observers took part in most observations. However, this does not mean that all items could be quantified for all couples. Sometimes we simply missed an item, or the position of a courting couple prevented us from having a full view of the entire sequence. In addition, not all courtship bouts yielded data for every component analysed. This explains discrepancies in sample size.

Normally, a female stops moving once the male has arrived at the stereotype courtship position (on top of the female's dorsum, with the forefeet placed on her compound eyes, Fig. 2) and she will persist in this condition for the duration of the display. Introgressed hybrid males in particular often failed to immobilise a female momentarily, or keep her so, which interrupted the display for at least some time. In extreme cases, the female dislodged the male with hindleg strokes, or she ran away from underneath. Obviously, persistently mobile females interfere with proper courtship performance. Such cases were discarded.

Several aspects of male courtship production (Barrass, 1960b; van den Assem *et al.*, 1984) and female attractiveness (van den Assem, 1986, 1996) change with age and experience. Therefore, test individuals were standardised at 2-4 days post-emergence. Males were inexperienced. Females were mated prior to the observations, to switch off receptivity so that they would not cut short a display with a mating signal (*i.e.* a change in the position of her antennae that makes the male stop courting and back up to copulate). Following observations, males were deep-frozen and stored for genetic analysis. Females were discarded.

### *Discriminative components of courtship behaviour*

A search for the specific origin of biases in the display behaviour of hybrid males must focus on discriminative features, *i.e.* elements that are present in one species and absent in the other, or show significant quantitative differences (in numbers or duration) between species (van den Assem & Werren, 1994).

*Nasonia* displays consist of a repetition of similar but not fully identical cycles (Fig. 2). All cycles include a cluster of various movements (series of head noddings with synchronous mouthpart extrusion are most conspicuous) and various pauses. Difference in number of head nods in the first and second cycle is a basic discriminative feature of both species; in V the second cycle typically contains fewer headnods than the first, in L numbers stay on the same level or increase slightly (Beukeboom & van den Assem, 2001). Duration of cycles, incidence of forefeet rubbing and 'minus' nods are additional, discriminative features of V and L courtship behaviour. Forefeet rubbing refers to pronounced side to side movements over the female's eyes with, in alternation, the left and right foretarsus. 'Minus' nods are solitary

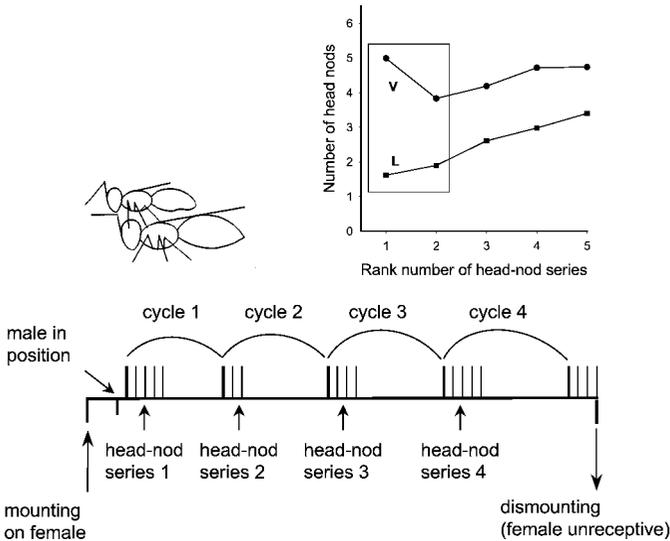


Fig. 2. Schematic representation of the cyclical nature of *Nasonia* display behaviour. Clusters of display movements alternate with pauses (bottom figure). Series of head noddings are conspicuous elements of every cluster. Vertical lines represent separate nods; first nods are drawn in bold face. Mouthparts are extruded in synchrony with nodding. First nods coincide with the release of a receptivity provoking pheromone. The interval between the first nod of two consecutive series is used as a measure of the cycle's duration. The male's stereotyped courtship position is illustrated top left. Mean number of headnods in the first five cycles are shown for *N. vitripennis* (V) and *N. longicornis* (L) in the top right graph. The box indicates the species specific decrease respectively increase in number of head nods of series 1 and 2.

head nods that occur during the pauses in between head-nod series and do not coincide with mouthpart extrusion. Both are always present in L and always absent in V. In hybrids, the amplitude of forefeet rubs and 'minus' nods, if present at all may vary considerably. We have only accepted full-fledged movements in the analyses.

We have considered one further item: the duration of the latency period, *i.e.* the period between introduction of the female and mounting of the male on that female (called 'in-on' period in Beukeboom & van den Assem, 2001). Not strictly part of a display, it is a function of the mobility of both partners, the quality of the female's chemical cues and the quality of the male's receptors. Because the type of female was constant in all tests, and males were screened for motor ability, observed differences are likely due to receptor quality of males in the first place.

For further background information about *Nasonia* courtship, see Barrass (1960a), van den Assem (1975, 1986), van den Assem *et al.* (1980), Jachmann & van den Assem (1996) for *N. vitripennis*; van den Assem & Werren (1994) for *N. longicornis*; Beukeboom & van den Assem (2001) for *vitripennis-longicornis* hybrids.

*Statistics*

Data were analysed with non-parametric statistics using SPSS 10 and STATISTICA for Windows. Probabilities of  $p < 0.05$  were accepted as indicators of significant differences between samples.

**Results**

Qualitative observations on introgressed males (V[L] and L[V]) are in line with the results that were obtained earlier with *vitripennis* × *longicornis* hybrids (Beukeboom & van den Assem, 2001). The introgressed hybrid males (VL[V] and LV[L]), however, were more variable, and some produced ‘unstable’, or even ‘chaotic’ sequences.

Duration of the latency period of males that did court within the 10-min period is shown in Table 1. The data are arranged in two groups: paternal contribution to the nuclear genome V or L, ignoring the type of cytoplasm. Within these groups, the four male types (pure-line, interspecific hybrid, introgressed, and introgressed hybrid) are entered in that order from top down. Except for the pure-line males, those with paternally donated V were quicker to mount than L-line counterparts. Performances deteriorated from pure-line males, over interspecific hybrids and introgressed males, to introgressed hybrid males; the latter being significantly slower in mounting than the pure lines.

Courtship features are compared in Table 2. Again, we have arranged the data according to the kind of paternal contribution to the genome (V or L),

TABLE 1. *Duration of the latency period*

Male type		Sample size	% on <1 min	% on 1-3 min	% on >3 min
Pure line	V[V]	60	92	8	0
Interspecific hybrid	VL[L]	40	77	23	0
Introgressed line	V[L]	39	31	56	13
Introgressed interspecific hybrid	VL[V]	26	15	39	46
Pure line	L[L]	59	86	12	2
Interspecific hybrid	LV[V]	61	64	20	16
Introgressed line	L[V]	34	17	33	50
Introgressed interspecific hybrid	LV[L]	24	4	38	58

See Appendix for statistics.

TABLE 2. Comparison of discriminate features in four types of *Nasonia* males, each type with either V or L as the paternally contributed genome. Median for duration of 1<sup>st</sup> cycle, average  $\pm$  SE for number of nodes in 1<sup>st</sup> series and number nodes series 2 minus series 1, percentage of feet rubbing and 'minus' nodes. Sample size in brackets

Paternal donor of nuclear genome is V	Male type	Duration 1 <sup>st</sup> cycle (secs)	Number nodes		Number nodes series 2 minus series 1		Proportion forefeet rubbing	Proportion 'minus' nodes
			1 <sup>st</sup> series	Number nodes	series 2 minus series 1	Number nodes		
Paternal donor of nuclear genome is V	Pure line	7 (55)	4.95 $\pm$ 0.16 (59)	-0.98 $\pm$ 0.15 (50)	0 (60)	0 (60)	0 (60)	
	Interspecific hybrid	VL[L]	4.44 $\pm$ 0.21 (39)	-1.10 $\pm$ 0.29 (21)	0 (20)	0 (20)	5 (20)	
	Introgressed line	V[L] <sup>4)</sup>	2.69 $\pm$ 0.21 (39)	-0.59 $\pm$ 0.20 (39)	3 (30)	0 (31)	0 (31)	
	Introgressed interspecific hybrid	VL[V]	3.42 $\pm$ 0.41 (24)	-0.39 $\pm$ 0.33 (23)	23 (22)	8 (24)	8 (24)	
Paternal donor of nuclear genome is L	Pure line	13 (55)	1.58 $\pm$ 0.15 (55)	0.40 $\pm$ 0.10 (52)	100 (55)	100 (55)	100 (55)	
	Interspecific hybrid	LV[V]	2.22 $\pm$ 0.14 (76)	0.21 $\pm$ 0.12 (73)	22 (76)	29 (73)	29 (73)	
	Introgressed line	L[V] <sup>2)</sup>	1.94 $\pm$ 0.21 (34)	0.59 $\pm$ 0.24 (34)	63 (32)	40 (31)	40 (31)	
	Introgressed interspecific hybrid	LV[L]	1.91 $\pm$ 0.25 (22)	0.73 $\pm$ 0.27 (22)	55 (22)	36 (22)	36 (22)	

1) paternal and 2) maternal parental line of introgressed interspecific hybrid VL[V].

3) paternal and 4) maternal parental line of introgressed interspecific hybrid LV[L].

See Table 4 and Appendix for statistics.

TABLE 3. *Statistical support for the effectiveness of introgression. Behavioural features of introgressed males are compared to those of pure-line males*

	Duration 1 <sup>st</sup> cycle (secs)	Number nods 1 <sup>st</sup> series	Number nods series 2 minus series 1	Proportion forefeet rubbing	Proportion 'minus' nods
V[L] vs V[V]	*	***	NS	NS	NS
vs L[L]	***	***	***	***	***
L[V] vs L[L]	*	*	NS	***	***
vs V[V]	***	***	***	***	***

NS = not significant, \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , see Appendix for details of statistics.

ignoring the nature of the cytoplasmic genes. Displays of pure-line V and L males differ significantly in a number of characters, in agreement with previous experiments. The data on interspecific hybrid offspring bring out the dominant effect of the paternal-line's share on the expression of certain courtship features (Beukeboom & van den Assem, 2001). In particular, this is true for the difference in head nods in series 1 and 2, which is a basic distinctive feature of the structure of V and L displays.

Table 2 shows that all types with paternal V nuclear genes are more or less V-like, and those with L are L-like, no matter the kind of associated cytoplasmic genes. There is no overlap in the mean values of the entire V and L groups. The displays of introgressed males (V[L] and L[V]) resemble those of the pure species with corresponding nuclear genome. Although some of the V[L] characteristics differ significantly from V[V] (Tables 2 and 3), all of them are significantly different from L[L]. Similarly, L[V] males are closer to L[L] than to V[V]. The data indicate that introgression has been effective, but not complete (see Discussion).

The results of the introgressed interspecific hybrids are of particular interest. The introgressed hybrid lines were constructed by crossing pure-line males with introgressed females; hence, VL[V] were obtained from V  $\times$  L[V] crosses and LV[L] from L  $\times$  V[L] crosses (Fig. 1). The expectation is that the introgressed hybrids are intermediate between the parental lines, and, in case of a grandfather effect, show a bias towards paternal features. Results are presented in Tables 2 and 4. LV[L] and VL[V] are significantly different from one another for three (or four, feet rubbing is a borderline case) of the

TABLE 4. *Statistical support for the grandfather effect. Behavioural features of both types of introgressed interspecific hybrid males are compared among themselves and with those of their parental lines*

	Duration 1 <sup>st</sup> cycle (secs)	Number nods 1 <sup>st</sup> series	Number nods series 2 minus series 1	Proportion forefeet rubbing	Proportion 'minus' nods
VL[V] vs LV[L]	NS	*	*	NS	*
VL[V] vs V[V] vs L[V]	*** NS	NS **	NS *	** **	NS *
LV[L] vs L[L] vs V[L]	*** **	NS *	NS ***	*** ***	*** **

NS = not significant, \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , see Appendix for details of statistics.

five courtship components analysed. These results bring out the dominant contribution of the grandpaternal nuclear genes in the bias towards courtship features. This is particularly evident for the difference in number of head nods in series 1 and 2. Numbers 2 minus 1 are negative for V and positive for L (Table 2). The statistical comparisons bring out that VL[V] males show more resemblance to the paternal line (V[V]) than to the maternal line (L[V]). Similarly, LV[L] males show a bias towards the paternal L[L] behaviour (Table 4).

## Discussion

An analysis of interspecific-hybrid courtship presents an opportunity to investigate the genetics underlying the components of the displays. In an earlier study, we investigated the courtship behaviour of bidirectional hybrids between *Nasonia vitripennis* and *N. longicornis*. As expected, courtship of hybrid males appeared intermediate between the parental species. In addition, and unexpectedly, we also found a significant bias towards the species that had acted as the paternal donor (Beukeboom & van den Assem, 2001). We have referred to this phenomenon as the 'grandfather effect'. A ready explanation was not on hand but we presented two hypotheses that might be relevant, one based on a biased recovery of paternal genes due to nucleo-cytoplasmic interaction or to meiotic drive, the other suggesting a bias in the

*expression* of paternally donated genes rather than in their *transmission*. In the present study we tested the nucleo-cytoplasmic interaction-hypothesis. We constructed two so-called introgressed *Nasonia* lines in which the nuclear genome of one species was introduced into the cytoplasm of the other. Such lines can be obtained through repeated backcrosses. Theoretically, backcrossing over 10 generations would suffice to produce an almost completely introgressed line (*i.e.*  $1 - (0.5^{10}) = 0.999$ ).

Displays of most introgressed males were of the normal *Nasonia* type. Both lines (L[V] and V[L]) resembled the paternal species' behaviour, an indication that introgression had been successful. However, some remnants of the maternal species' behaviour were also evident (*e.g.* presence of feet rubbing in V[L]). This kind of retention could be due to nucleo-cytoplasmic interaction, where cytoplasmic genes directly affect courtship behaviour. Alternatively, the theoretically expected degree of introgression may not have been reached, due to retention of some nuclear genes of the maternal donor in the introgressed line. Indeed, evidence provided by a preliminary screen test with genetic markers suggests that some genomic regions of the paternal species are incompatible with the other species' cytoplasm (Beukeboom, unpublished results). Previous studies of *N. vitripennis* × *giraulti* hybrids did produce evidence of negative effects on viability as a result of nucleo-cytoplasmic incompatibility (Breeuwer & Werren, 1993) and hybrid breakdown (Breeuwer & Werren, 1995). Therefore, the females used for the construction of the introgressed interspecific hybrids were not completely V (or L), and the resulting introgressed hybrid sons may have carried more of the paternal-type genome than was contributed by the grandfather. Nevertheless, the comparisons with males of pure-lines (paternal donors) and with males of introgressed lines (maternal donors) point to the presence of a grandfather effect.

Selected crosses of pure-species males and introgressed females (*i.e.* V males × L[V] females and L males × V[L] females) make it possible to circumvent the effects of nucleo-cytoplasmic interactions that might originate from 'heterospecificity' of paternal nuclear genome and maternal cytoplasm. Since the paternal-line genes come to reside in conspecific cytoplasm in these crosses, a classical nucleo-cytoplasmic interaction (*sensu* Breeuwer & Werren, 1995) becomes unlikely as an explanation for the grandfather effect. Another, though rather improbable, genetic explanation would require that the paternal genes were transmitted by hybrid females at

a higher rate than the genes of maternal origin ('meiotic drive'). However, this needs to be independent of the cytoplasm and requires that the paternally derived genomes take precedence over the maternal counterparts.

Our alternative hypothesis for the explanation of the grandfather effect supposes a stronger expression of paternally donated genes in hybrid males, based on some epigenetic phenomenon. It would require that males activate the genetic substrate underlying courtship behaviour in one way or another, and that this imprint 'survives' transmission through the hybrid daughters (see Beukeboom & van den Assem, 2001 for further discussion). Previously, effects of genomic imprinting have been suggested in studies on sex determination in *Nasonia* (Beukeboom, 1995; Dobson & Tanouye, 1999). Homologous genes of paternal and maternal origin seem to act differently in inducing maleness or femaleness. Presently we do not know whether the grandfather effect is a biologically relevant phenomenon or merely an artefact of hybridisation. Before speculating about any biological significance, we must show that this effect can also be traced in intraspecific crosses.

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## Appendix

### *Details of statistical analyses*

Table 1: Tests for differences in latency periods. V[L] vs V[V]: Chi-square tests;  $\chi^2 = 37.3$ ,  $p < 0.001$ ; VL[V] vs V[V]:  $\chi^2 = 57.1$ ,  $p < 0.001$ ; VL[V] vs V[L]:  $\chi^2 = 9.2$ ,  $p = 0.012$ ; L[V] vs L[L]:  $\chi^2 = 52.0$ ,  $p < 0.001$ ; LV[L] vs L[L]:  $\chi^2 = 45.9$ ,  $p < 0.001$ ; LV[L] vs L[V]:  $\chi^2 = 0.129$ , NS. If 2 cells had expected count less than 5, two adjacent cells were pooled.

Table 3: Tests for effectiveness of introgression (introgressed males versus pure line males). V[L] vs V[V]: Mann-Whitney *U* tests; duration of 1<sup>st</sup> cycles,  $z = -2.069$ ,  $p = 0.039$ ;

number nuds in 1<sup>st</sup> series,  $z = -6.874$ ,  $p < 0.001$ ; number nuds series 2 minus series 1, NS; Fisher-Exact tests; numbers of feet rubbing, NS; 'minus' nuds, NS V[L] vs L[L]: MWU; duration of 1<sup>st</sup> cycles,  $z = -7.306$ ,  $p < 0.001$ ; number nuds in 1<sup>st</sup> series,  $z = -4.646$ ,  $p < 0.001$ ; number nuds series 2 minus series 1,  $z = -4.158$ ,  $p < 0.001$ ; Fisher-Exact tests; numbers of feet rubbing,  $p < 0.001$ ; 'minus' nuds,  $p < 0.001$ . L[V] vs L[L]: MWU; duration of 1<sup>st</sup> cycles,  $z = -2.007$ ,  $p = 0.045$ ; number nuds in 1<sup>st</sup> series,  $z = -2.547$ ,  $p = 0.011$ ; number nuds series 2 minus series 1, NS; Fisher-Exact tests; numbers of feet rubbing,  $p < 0.001$ ; 'minus' nuds,  $p < 0.001$ . L[V] vs V[V]: MWU; duration of 1<sup>st</sup> cycles,  $z = -6.716$ ,  $p < 0.001$ ; number nuds in 1<sup>st</sup> series,  $z = -9.632$ ,  $p < 0.001$ ; number nuds series 2 minus series 1,  $z = -5.757$ ,  $p < 0.001$ ; Fisher-Exact tests; numbers of feet rubbing,  $p < 0.001$ ; 'minus' nuds,  $p < 0.001$ .

Table 4: Tests for the grandfather effect (introgressed interspecific hybrid males versus introgressed line males). VL[V] vs V[V]: Mann-Whitney  $U$  tests; duration of 1<sup>st</sup> cycles,  $z = -3.947$ ,  $p < 0.001$ ; number nuds in 1<sup>st</sup> series,  $z = -3.221$ ,  $p = 0.001$ ; number nuds series 2 minus series 1,  $z = -2.269$ ,  $p = 0.023$ ; Fisher-Exact tests; numbers of feet rubbing,  $p < 0.001$ ; 'minus' nuds, NS VL[V] vs L[V]: MWU; duration of 1<sup>st</sup> cycles, NS; number nuds in 1<sup>st</sup> series,  $z = -2.690$ ,  $p = 0.007$  number nuds series 2 minus series 1,  $z = -2.059$ ,  $p = 0.039$ ; Fisher-Exact tests; numbers of feet rubbing,  $p = 0.056$ ; 'minus' nuds,  $p = 0.013$ . LV[L] vs L[L]: MWU; duration of 1<sup>st</sup> cycles,  $z = -4.508$ ,  $p < 0.001$ ; number nuds in 1<sup>st</sup> series, NS; number nuds series 2 minus series 1, NS; Fisher-Exact tests; numbers of feet rubbing,  $p < 0.001$ ; 'minus' nuds,  $p < 0.001$ . LV[L] vs V[L]: MWU; duration of 1<sup>st</sup> cycles,  $z = -3.199$ ,  $p = 0.001$ ; number nuds in 1<sup>st</sup> series,  $z = -2.352$ ,  $p = 0.019$ ; number nuds series 2 minus series 1,  $z = -3.585$ ,  $p < 0.001$ ; Fisher-Exact tests; numbers of feet rubbing,  $p < 0.001$ ; 'minus' nuds,  $p = 0.004$ . VL[V] vs LV[L]: MWU; duration of 1<sup>st</sup> cycles, NS; number nuds in 1<sup>st</sup> series,  $z = -2.533$ ,  $p = 0.011$ ; number nuds series 2 minus series 1,  $z = -2.415$ ,  $p = 0.016$ ; Fisher-Exact tests; numbers of feet rubbing,  $p = 0.062$  'minus' nuds,  $p < 0.032$ .

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