Temperature stress increases hybrid incompatibilities in the parasitic wasp genus *Nasonia*  

T. KOEVOETS, L. VAN DE ZANDE & L. W. BEUKEBOOM  
Evolutionary Genetics, Centre for Ecological and Evolutionary Studies, University of Groningen, Groningen, The Netherlands

**Keywords:**  
cytonuclear interaction;  
haplodiploidy;  
hybrid incompatibilities;  
temperature stress;  
transmission ratio distortion loci.

**Abstract**  
Hybrid incompatibilities, measured as mortality and sterility, are caused by the disruption of gene interactions. They are important post-zygotic isolation barriers to species hybridization, and much effort is put into the discovery of the genes underlying these incompatibilities. In hybridization studies of the haplodiploid parasitic wasp genus *Nasonia*, genetic incompatibilities have been shown to affect mortality and sterility. The genomic regions associated with mortality have been found to depend on the cytotype of the hybrids and thus suggest cytonuclear incompatibilities. As environmental conditions can affect gene expression and gene interaction, we here investigate the effect of developmental temperature on sterility and mortality in *Nasonia* hybrids. Results show that extreme temperatures strongly affect both hybrid sterility (mainly spermatogenic failure) and mortality. Molecular mapping revealed that extreme temperatures increase transmission ratio distortion of parental alleles at incompatible loci, and thus, cryptic incompatible loci surface under temperature stress that remain undiscovered under standard temperatures. Our results underline the sensitivity of hybrid incompatibilities to environmental factors and the effects of unstable epistasis.

**Introduction**  
Questions about the evolutionary processes that drive speciation have raised much controversy among biologists since Darwin’s ‘The Origin of Species’. Even the scope of reproductive isolation is under debate (Wu, 2001; Feder & Nosil, 2010; Butlin *et al.*, 2011). Where the concept of reproductive isolation relates to individuals (and thus complete genomes), a genomic view of speciation considers the isolation of specific differentiated genes in the genome. These drive the process of speciation as genomic regions linked to the genes under selection start diverging and cause differentiation of more and more loci. One key aspect of post-zygotic reproductive isolation is the lowered fitness of hybrid offspring, which is referred to as hybrid incompatibility and is usually measured as hybrid mortality and/or sterility. Hybrid incompatibilities are believed to be caused by the disrupted interaction between two or more genes that have diverged in different species, because selection acts on the interaction between genes within a species but not between species. These negative epistatic gene interactions are known as Dobzhansky–Muller interactions (see Coyne & Orr, 1998; Turelli & Orr, 2000; Bordenstein & Drapeau, 2001 for more details). The effects of hybrid incompatibilities can be numerous (e.g. mortality in different stages of development, behavioural sterility and spermatogenic sterility), as the genes that cause the incompatibilities can be of various functions and genomic locations (Coyne & Orr, 2004). Therefore, the types of hybrid incompatibilities can be both nuclear–nuclear (including autosomal–sex chromosomal) and cytoplasmic–nuclear (i.e. cytonuclear). Much effort has been directed towards identifying the genes underlying genic incompatibilities, referred to as ‘speciation genes’ (reviewed by Presgraves, 2010). Even though no general pattern in the types of genes or processes involved has emerged, it seems that the genes underlying the incompatibilities often bear marks of adaptive evolution, which is counterintuitive for a maladaptive trait like hybrid incompatibility. The signs of adaptive evolution, however, are likely a response to...
the preceding neutral and perhaps deleterious genomic changes that are expected to occur as ‘genomes are intrinsically unstable’ and these genomes need to respond to mutations and invasions by pathogens and genetic elements (Presgraves, 2010).

The haplodiploid (diploid females and haploid males) wasp genus *Nasonia* is an emerging model system for evolutionary genetics and the genetics of speciation, due to its short generation time, haplodiploid genetics and sequenced genomes (Werren et al., 2010). The genus consists of four species (*N. vitripennis, N. longicornis, N. giraulti* and *N. oneida*), which are reproductively isolated by *Wolbachia* infections, but show various levels of pre- and post-zygotic reproductive isolation when cured from *Wolbachia*. When hybridizing either of the two closely related species *N. longicornis* or *N. giraulti* to the more distant *N. vitripennis*, F1 hybrid females are viable and fertile, whereas genic incompatibilities in F2 hybrid males lead to significant mortality (Breeuwer & Werren, 1995; Koevoets et al., 2011) and sterility (Clark et al., 2010; Koevoets et al., 2011).

The genetic basis of hybrid mortality in *Nasonia* has been studied by genetic mapping approaches (Niehuis et al., 2008; Koevoets et al., 2011). The results showed that *Nasonia* hybrids suffer from mortality induced by nuclear genomic regions (referred to as transmission ratio distortion loci, or TRDLs) that differ between the reciprocal hybrid crosses and are less severe when the cytophore is *N. vitripennis*. This asymmetry and the selective advantage of the allele that matches the cytoplasm suggest an effect of the cytoplasm in causing hybrid mortality, with the disruption of oxidative phosphorylation (OXPHOS) as a promising candidate process (Ellison et al., 2008). As *Nasonia* hybridizations seem to affect the interaction between nuclear and mitochondrial genes, the question was raised whether temperature stress would amplify this disruption as mitochondria are presumed to adapt to the thermal environment (Ballard & Whitlock, 2004).

Although the effect of environmental conditions on gene interactions has been discussed previously (Bordenstein & Drapeau, 2001) and the interaction between temperature and cytoplasm has been shown to affect incompatibilities in hybrids (Willett & Burton, 2003; Demuth & Wade, 2007; Arnyvist et al., 2010), no studies have evaluated the effect of developmental temperature on the genomic regions involved in genic incompatibilities. However, if environmental conditions play a large role in the strength of incompatibilities and the discovery of the underlying genes, then knowledge of such effects is crucial when comparing across hybrid incompatibility studies and inferring evolutionary processes that take place under fluctuating natural conditions.

Our research focuses on the detection of incompatibilities in F2 haploid male hybrids of *N. vitripennis* and *N. longicornis*. We expose F3 offspring to different developmental temperatures and measure the effects on sterility and mortality. By determining the recovery rate of parental alleles of 32 microsatellite markers, we test whether temperature stress increases the number of loci that cause mortality, or causes a stronger allelic distortion at the same genomic regions across temperatures.

**Materials and methods**

The strains that were used for this experiment are AsymC for *N. vitripennis* (origin: Leiden, the Netherlands) and IV-$\frac{1}{2}$S for *N. longicornis* (origin: UT, USA), which have been cultured in the laboratory for several years and are identical to those used by Koevoets et al. (2011). The strains were cultured under constant light, at 25 °C and reared on *Calliphora* sp. fly pupae as hosts.

Figure S1 gives an overview of the experimental set-up. The experiment was designed after Koevoets et al. (2011) to measure hybrid incompatibility in F2 males. Because of haplodiploid reproduction, F1 (diploid) hybrid females are bred as virgins to produce F2 hybrid males that are haploid and recombinant. We measured a number of fitness-related traits in F2 hybrid and pure species males that developed at three different temperatures (i.e. 15, 25 and 31 °C). These temperatures were chosen because pure species *Nasonia* are able to develop within this temperature range (Grassberger & Frank, 2003) and pilot studies have shown that hybrid incompatibilities are increased at the extremes of this range.

**Crosses**

Virgin male (+24 h old) and female wasps (+72 h old and kept on hosts for feeding and to initiate egg-laying) were set up in four different crosses (one $\frac{3}{4}$ x one $\frac{1}{2}$): *N. vitripennis × N. vitripennis, N. longicornis × N. longicornis, N. longicornis × N. vitripennis and N. vitripennis × N. longicornis*. These crosses are referred to as VV[V], LL[L], LV[V] and VL[L], respectively; they refer to a diploid nonrecombined genome for F1 mothers and a haploid recombinant genome for F2 males. F1 females were collected as virgins $\pm$72 h prior to eclosion (i.e. before shedding the pupal skin) and kept in plastic tubes in groups of $\pm$15 females. Upon eclosion, females were kept on hosts for 48 h prior to the experiment to feed and initiate egg-laying.

**Egg-to-adult survival and development**

As egg-counting requires dissection of the fly host, it leads to high levels of mortality. Therefore, egg-to-adult survival is determined indirectly by comparing the number of eclosing wasps with the number of eggs from a control group of the same cross. F1 virgin females were randomly divided over four groups (see Table S1 for sample sizes): (i) egg-counting or development at (ii) 15 °C, (iii) 25 °C and (iv) 31 °C. Females from all groups were transferred to individual plastic tubes and received two hosts for 24 h for three consecutive days.
opening the hosts, F2 male offspring were counted in four
until ±72 h prior to eclosion of the first adults. Upon
groups 2–4 were incubated at their specific temperature
(2011), only males from the LL[L] cross were tested with
individually to a virgin female. Following Koevoets
et al. (2011), and the normal distribution was used to
test for an effect of temperature and cross-type on
development disruption using generalized linear models
(GLM) in R. For this, the total offspring of a female was
used rather than the average over all parasitized hosts.

Sterility
Twenty-four hours after eclosion, male courtship behav-
ior was scored for 10 min by introducing a male
individually to a virgin female. Following Koevoets et al.
(2011), only males from the LL[L] cross were tested with
N. longicornis virgin females, and all other males were
tested with N. vitripennis virgin females. Seven behav-
ioral categories were distinguished (Table S2): no interest,
interest, mounting, display, short copulation, only cop-
ulation and full copulation (including post-copulatory
behaviour). These behaviours are successive, and pure
species males normally transit from the first to the last
category with high probabilities (Koevoets et al., 2011).
Mated females were isolated for 24 h and subsequently
provided with three hosts for 48 h to test for the
production of female offspring (showing F2 male sper-
matogenic fertility). Males were stored at −20 °C for DNA
analysis.

Male sterility was determined in three ways: behavio-
ural, spermatogenic and overall sterility. Behavioural
sterility was measured as a male’s transition probability,
i.e. the frequency of males that perform a specific
behaviour in category a that also perform the behaviour
in category a + 1 (after Clark et al., 2010). Differences in
transitions from one category to the next between
developmental temperatures were tested with 2 x 3 $\chi^2$
tests for every cross after Bonferroni correction. When a
significant difference was found, transitions were tested in
a pairwise fashion (2 x 2 contingency tables) to
determine at which temperature male behaviour dif-
fered. Furthermore, for every cross and developmental
temperature, the proportion of males that performed a
specific behaviour was determined ± standard error
($\text{SE} = \sqrt{[p(1-p) \times n]}$). An overall mea-
sure of behavioural sterility was obtained from the
percentage of tested males that was able to induce female
receptivity and progressed to copulation.

Spermatogenic sterility was determined as the per-
centage of males that copulated with a female, but did
not father any (female) offspring. This type of sterility
could only be calculated for mated males, which resulted
in small sample sizes in some categories.

The overall sterility of a cross was determined by
combining information on behavioural and sperma-
togenic sterility, estimated as the percentage of tested males
that did not produce any (female) offspring.

$\chi^2$ tests on proportions were used to test whether the
level of sterility depends on the developmental tem-
perature. When true, a Tukey-type multiple comparison was
made to test which crosses differed significantly (Zar,
1999).

Genotyping
F2 hybrid male DNA was extracted using a high-
throughput protocol adjusted from Hoarau et al. (2007)
and Whitlock et al. (2008) (see Protocol S1 for details).
Microsatellite markers were amplified using the Qiagen
(Hilden, Germany) multiplex PCR kit according to
manufacturer’s recommendations (PCR profile: 15 min
at 95 °C, followed by 30 cycles of 30 s at 94 °C, 1.5 min
at TA and 1 min at 72 °C, followed by 45 min at 72 °C).
The F2 hybrid adults were genotyped using multiplex sets 1–5 from Koevoets et al. (2011), with a total of 32 microsatellite markers (see Koevoets et al., 2011 for primer information). All reactions were performed in 5-µL volumes (including 1 µL working DNA, see Protocol S1) using Applied Biosystems Veriti or Applied Biosystems 9700 thermocyclers. Fragments were diluted 400 times, separated on the Applied Biosystems 3730 DNA Analyzer and analysed using GeneMapper v4.0 (Applied Biosystems).

Transmission ratio distortion loci

The segregation bias for all markers in hybrid male adults was tested with χ2 tests (d.f. = 1) against the expected segregation of 1 : 1 of parental N. vitripennis and N. longicornis alleles, corrected for continuity (Yates) and multiple testing (sequential Bonferroni). We refrained from testing control embryonic samples, because F2 multiple testing (sequential Bonferroni). We refrained from testing control embryonic samples, because F2 hybrid male embryos from crosses between N. vitripennis and N. longicornis lack segregation bias for all markers used in this experiment (Koevoets et al., 2011). The recombination frequency was determined per pair of adjacent markers, and the effects of cross-type (LV[V] or VL[L]) and temperature (15, 25 and 31°C) were tested with an ANOVA with cross and temperature as fixed factors on the arcsine-root-transformed recombination frequencies (α = 0.05, using SPSS 16.0 Armonk, NY, USA). No significant effect of cross, temperature or their interaction on the recombination frequencies was found (ANOVA, cross F1,156 = 0.002, P = 0.963, temperature F2,156 = 0.131, P = 0.878, cross*temperature F2,156 = 0.136, P = 0.873).

Results

A number of fitness-related traits were measured in F2 haploid hybrid males of N. vitripennis and N. longicornis at three developmental temperatures (15, 25 and 31°C) and compared with those measured in pure species males.

Development rate (until eclosion)

The eclosion was scored for those wasps collected as pupae ±11 days after oviposition. Development rate increased with temperature in all crosses. At 25°C (control), the two pure species start eclosing 13 days after oviposition and the last eclosing males follow soon after the first (average ± σ: 13.13 ± 0.47 for pure N. vitripennis and 13.54 ± 0.69 for pure N. longicornis). For the hybrids, eclosion overlaps with the pure species but is spread over a larger time frame (14.85 ± 0.99 for LV[V] and 15.47 ± 1.25 for VL[L]). At 31°C (high), a similar pattern is seen, but the development rate is faster and the window of eclosion is smaller (10.08 ± 0.29, 10.27 ± 0.46, 11.51 ± 0.81, 11.69 ± 0.82 for VV[V], LL[L], LV[V] and VL[L], respectively). At 15°C (low), the development rate is retarded so much that pure N. longicornis start eclosing after the last pure N. vitripennis males have eclosed (45.36 ± 1.46 for pure N. vitripennis and 59.67 ± 2.49 for pure N. longicornis), with intermediate rates for the hybrids and a larger window (54.05 ± 7.48 for LV[V] and 55.11 ± 7.14 for VL[L]). Overall, hybrid progenies show longer and more variable development rates than pure species progenies. For many hybrids, the development is comparable to the pure species, but for other hybrids, the development is greatly retarded. Hybrid offspring never eclosed before pure species offspring.

Disrupted development

As development is retarded in a large fraction of hybrids, counting the offspring at the moment that the fastest developers are in their final pupal instars results in high frequencies of incomplete development (Table 1). In pure species, the proportion of immature stages is low for all temperatures, but larger for N. longicornis than for N. vitripennis. The proportion of adults at 25°C (eclosed wasps collected in their final pupal instar) in the two reciprocal hybrid crosses is lower than that of the pure species (0.88 for N. vitripennis cytoplasm and 0.82 for N. longicornis cytoplasm) and decreases under extreme temperatures. GLM analysis, however, showed no effect of temperature on the proportion of adults in a clutch (P = 0.453). When testing the extreme temperatures separately (15°C vs. 25°C and 31°C vs. 25°C), cross-type and the interaction of the two factors do have a significant effect on the proportion of adults in a clutch (P = 0.006 for 15°C vs. 25°C and P = 0.025 for 31°C vs. 25°C). Comparing the number of adults from the two experiments [(i) after eclosion in the egg-to-adult survival experiment and (ii) after extended development to measure emergence from the host puparium] shows no differences (t-tests, see Table 1). This indicates that incompletely developed wasps encountered in the egg-to-adult survival experiment are not likely to finish their development into adults when left to develop as in the emergence experiment.

Egg-to-adult survival

The egg-to-adult survival of pure and hybrid males was determined by comparing the number of eggs oviposited in subsets of hosts with the number of eclosing adults from the remaining hosts that developed at three different temperatures until ±2 h prior to eclosion of the first adults. We found that the applied temperatures had no effect on the survival of pure species males (N. vitripennis F2,56 = 0.89 and N. longicornis F2,56 = 0.27), but that extreme developmental temperatures lowered the survival of hybrid males considerably (Fig. 1, hybrid with N. vitripennis cytoplasm F2,56 = 13.49, P < 0.0001.
and hybrid with *N. longicornis* cytoplasm *F*<sub>2.5</sub> = 39.11, *P* < 0.0001). Hybrid survival did not differ between 15 and 31 °C for both reciprocal crosses (Tukey-Kramer test).

### Emergence

Pure species males that developed at 25 °C emerged from their host in almost all cases (Fig. 2). When lowering the developmental temperature to 15 °C, the number of eclosed adults that remained inside the host increased (more for pure *N. longicornis*). Raising the developmental temperature to 31 °C decreased the total number of pure *N. vitripennis* offspring, but this was not due to arrested development, indicated by low frequencies of immature stages. Early mortality likely caused this decrease in offspring number, because individuals that die early during development (e.g. during embryogenesis) cannot

### Table 1

<table>
<thead>
<tr>
<th>Cross</th>
<th>Temperature</th>
<th>N</th>
<th>Adults</th>
<th>Larvae</th>
<th>Prepupae</th>
<th>Diapause</th>
<th>P-value <em>t</em>-test on adults from the two experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>V[V]</td>
<td>15</td>
<td>20</td>
<td>31.98 (0.98)</td>
<td>0.05 (0.00)</td>
<td>0.15 (0.01)</td>
<td>0.35 (0.01)</td>
<td>5 36.70 0.308</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>20</td>
<td>36.60 (0.98)</td>
<td>0.00 (0.00)</td>
<td>0.13 (0.00)</td>
<td>0.58 (0.01)</td>
<td>5 40.25 0.420</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>19</td>
<td>36.92 (0.99)</td>
<td>0.16 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.24 (0.01)</td>
<td>5 30.00 0.483</td>
</tr>
<tr>
<td>LV[V]</td>
<td>15</td>
<td>20</td>
<td>8.55 (0.69)</td>
<td>0.13 (0.01)</td>
<td>1.96 (0.16)</td>
<td>1.63 (0.14)</td>
<td>5 7.68 0.582</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>20</td>
<td>18.50 (0.68)</td>
<td>0.15 (0.01)</td>
<td>1.43 (0.07)</td>
<td>0.63 (0.04)</td>
<td>5 23.42 0.225</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>20</td>
<td>11.00 (0.60)</td>
<td>0.43 (0.02)</td>
<td>5.93 (0.31)</td>
<td>1.20 (0.06)</td>
<td>5 9.37 0.198</td>
</tr>
<tr>
<td>VL[L]</td>
<td>15</td>
<td>19</td>
<td>7.24 (0.53)</td>
<td>1.18 (0.08)</td>
<td>1.87 (0.15)</td>
<td>3.58 (0.24)</td>
<td>4 3.58 0.012</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>20</td>
<td>22.70 (0.82)</td>
<td>0.70 (0.03)</td>
<td>2.50 (0.09)</td>
<td>1.85 (0.07)</td>
<td>5 23.95 0.655</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>20</td>
<td>7.40 (0.41)</td>
<td>2.00 (0.11)</td>
<td>6.53 (0.42)</td>
<td>0.90 (0.05)</td>
<td>5 6.36 0.519</td>
</tr>
<tr>
<td>LL[L]</td>
<td>15</td>
<td>18</td>
<td>18.44 (0.99)</td>
<td>0.00 (0.00)</td>
<td>0.06 (0.00)</td>
<td>0.06 (0.00)</td>
<td>3 22.42 0.305</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>18</td>
<td>17.55 (0.92)</td>
<td>0.32 (0.02)</td>
<td>0.00 (0.00)</td>
<td>0.97 (0.06)</td>
<td>3 20.42 0.612</td>
</tr>
</tbody>
</table>

*Dead offspring not shown, due to uncertainties in counting dead offspring.
†No other developmental stages shown, as they are only found in very low frequency. All eclosed adults pooled (both emerged and nonemerged).
be detected. For pure *N. longicornis*, raising the developmental temperature to 31 °C increased only the fraction of adults that remained inside the host. Extreme temperatures are detrimental to both types of hybrids. None of the hybrid adults emerged from the host at 15 and 31 °C, and the proportion of undeveloped wasps increased greatly. GLM analysis showed that both temperature (*P* = 0.005) and cross (*P* < 0.0001) have an effect on the ratio of emerging adults, but that there is no temperature*cross interaction (*P* = 0.063).

**Sterility**

**Courtship behaviour**

Pure *N. vitripennis* males performed normal courtship behaviour at all developmental temperatures (Fig. 3a), which is illustrated by the equal transition probabilities between the behavioural categories (Table S3). Pure *N. longicornis* show behavioural defects at all temperatures (Fig. 3b). Although striking for a pure species, this is likely a result of the difficulty with which *N. longicornis* females become receptive (they are still the better choice as partners for *N. longicornis* males as *N. vitripennis* females discriminate strongly against *N. longicornis* males). The two types of hybrid males show high frequencies of aberrant courtship behaviour (Fig. 3c,d), as the proportion of males with a full copulation is very small for all developmental temperatures (Table 2). In general, low developmental temperature in hybrids causes fewer males to mount and copulate. For hybrids with *N. vitripennis* cytoplasm, extreme developmental temperature increased behavioural sterility significantly (Table 2). Hybrids with *N. longicornis* cytoplasm developing at 25 °C already suffered from high levels of behavioural sterility and extreme temperatures did not aggravate this. Furthermore, cytotype had a small effect on the courtship behaviour, because the two types of hybrid males did not differ in courtship behaviour ($\chi^2$ association tests of LV[V] vs. VL[L] behaviour for the three different temperatures only showed significant differences for ‘mounting’ of males developed at 31 °C, data not shown). When developed under high or low extreme temperatures, both types of hybrids were less successful at mounting the females, whereas low developmental temperature leads to fewer copulations of hybrids with *N. vitripennis* cytoplasm and less frequent displays of courtship behaviour in hybrids with *N. longicornis* cytoplasm (Table S3). In general, hybrids developed under extreme temperatures mated less with females, and hybrids with *N. vitripennis* cytoplasm perform slightly better than hybrids with *N. longicornis* cytoplasm. This asymmetry has been established

---

**Fig. 3** (a–d) Male courtship behaviour patterns in pure species and hybrid males. For every cross-type (a–d), male courtship behaviour is given at three developmental temperatures as the proportion of all tested males that performed a particular behaviour (±SE).
Developmental temperature increases spermatogenic failure when mated with a female. The overall trend is that none of these males successfully mated with *N. vitripennis* with F2 hybrid male embryos of extreme temperatures induce (near) complete sterility. The small sample sizes of all groups, see Table 2) and (significant effect of temperature only for LV[V] due to different alleles.

### Table 2

Different types of sterility measured in pure species and hybrid males. The effect of temperature was tested per cross-type using a $\chi^2$ test on proportions. When temperature had an effect, a Tukey-type multiple comparison was made to test which temperatures were significantly different per cross-type, indicated by the different letters.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Temperature (°C)</th>
<th>Behavioural sterility</th>
<th>Spermatogenic sterility</th>
<th>Total sterility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total tested</td>
<td>Total mated</td>
<td>% Mated</td>
<td>Total mated</td>
</tr>
<tr>
<td>V[V]</td>
<td>15</td>
<td>155 122</td>
<td>78.7*</td>
<td>122 109</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>46 32</td>
<td>69.6*</td>
<td>32 32</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>28 21</td>
<td>80.8*</td>
<td>21 17</td>
</tr>
<tr>
<td>L[V]</td>
<td>15</td>
<td>181 7</td>
<td>3.9*</td>
<td>7 2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>163 36</td>
<td>22.1*</td>
<td>36 23</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>77 7</td>
<td>9.1*</td>
<td>7 0</td>
</tr>
<tr>
<td>V[L]</td>
<td>15</td>
<td>150 5</td>
<td>3.3*</td>
<td>5 2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>167 11</td>
<td>6.6*</td>
<td>11 6</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>70 0</td>
<td>0.0*</td>
<td>0 0</td>
</tr>
<tr>
<td>L[L]</td>
<td>15</td>
<td>114 54</td>
<td>47.4*</td>
<td>54 50</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>58 18</td>
<td>31.0*</td>
<td>18 18</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>36 5</td>
<td>13.9*</td>
<td>5 5</td>
</tr>
</tbody>
</table>

Previously (Clark et al., 2010; Koevoets et al., 2011) but has not received much attention.

**Spermatogenic sterility**

For all copulated males, the spermatogenic sterility was measured by scoring the daughter production by their mates (Table 2). Both types of pure species males from all temperatures were highly fertile. The two types of hybrid males showed high spermatogenic sterility when developing at 25 °C, and this level increased at extreme developmental temperatures, in particular for hybrids with *N. vitripennis* cytoplasm. Spermatogenic sterility of hybrids with *N. longicornis* cytoplasm from 31 °C could not be determined, because none of these males successfully mated with a female. The overall trend is that developmental temperature increases spermatogenic failure in hybrids.

**Overall sterility**

Information on male behavioural and spermatogenic sterility was combined into an overall sterility measure that reflects the proportion of tested males that yielded no offspring (Table 2). Pure *N. vitripennis* males are largely fertile at all developmental temperatures, but pure *N. longicornis* males show moderate levels of overall sterility (mostly due to behavioural problems). Both types of hybrid males show high levels of hybrid sterility: only hybrids developing at 25 °C produce some offspring (significant effect of temperature only for LV[V] due to the small sample sizes of all groups, see Table 2) and extreme temperatures induce (near) complete sterility.

**Transmission ratio distortion loci**

F2 hybrid male embryos of *N. vitripennis* and *N. longicornis* show Mendelian inheritance of alleles (Koevoets et al., 2011). Both types of adult hybrids tested here, however, show marker transmission distortions towards the cytotype of the hybrid. In addition, the distortion of the loci depends on the developmental temperature (Fig. 4).

Hybrids with *N. vitripennis* cytoplasm were distorted for a single locus on chromosome 5 (distortions indicated by boxes left of each chromosome in Fig. 4) when developed at 25 °C (dark grey boxes). These regions are comparable to those found in previous research (Koevoets et al., 2011). At low temperature (light grey boxes), this locus expanded to the complete 5th chromosome (with the exception of marker Nv125). In addition, transmission of all markers on chromosome 1 became distorted, as well as the central parts of chromosomes 2 and 3. High developmental temperature (black boxes) also increased the number of distorted loci compared with 25 °C but fewer than at low temperature: a single marker on chromosome 1, the central region of chromosome 2, and a single marker on chromosome 5. In all cases, the distortion was towards the *N. vitripennis* cytotype (Fig. 4). The regions distorted under low developmental temperature encompass the distorted regions of 25 and 31 °C, but cover larger genomic regions.

For hybrids with *N. longicornis* cytoplasm (distortions indicated by boxes right of each chromosome in Fig. 4), the central part of chromosome 4 was distorted at 25 °C, which is in agreement with Koevoets et al. (2011). At low temperature, this distortion expanded towards the top of chromosome 4 and a large part of chromosome 5. For hybrids developing under high temperature, chromosome 2 was distorted for two distal markers, chromosome 3 for a single marker, a large part of chromosome 4 and the complete 5th chromosome. In all cases (except chromosome 2), the distortion was towards the *N. longicornis* cytotype (Fig. 4). The regions distorted under high developmental temperature encompass the distorted regions of 15 and 25 °C, but cover much larger genomic regions.
As described earlier, extreme developmental tempera-
tures expanded the distorted regions and additional
regions became distorted comparing the regions from
the control to the extreme temperatures. For the loci that
were distorted under all three developmental tempera-
tures, it was determined whether the strength of the
distortion changed with temperature using $\chi^2$ 
association tests. In hybrids with *N. vitripennis*

cytoplasm, only

![Fig. 4 Segregation distortion in hybrid males with Nasonia vitripennis or Nasonia longicornis cytoplasm developing at three different temperatures. The five linkage groups denote the five Nasonia chromosomes, with map distance (in Haldane cM) on the left and marker number on the right. The distorted regions identified in hybrids with *N. vitripennis* cytoplasm are located on the left of each chromosome (next to the cM distance), and the distorted regions identified in hybrids with *N. longicornis* cytoplasm are located right of each chromosome (next to the marker names). The three different shades of bars show the recovery bias at the three developmental temperatures (light grey for 15 °C, dark grey for 25 °C and black for 31 °C) and encompass those markers that significantly departed from 1 : 1 based on $\chi^2$ test with $\alpha = 0.05$ and sequential Bonferroni correction. The dark arrows indicate oxidative phosphorylation genes that differ between the species (Gibson et al., 2010), and the grey arrows indicate the location of γDNA polymerase. All distorted regions are distorted towards the maternal allele that matches the cytotype (except *). *The two markers that are distorted at this chromosomal location are distorted towards *N. vitripennis* alleles rather than towards *N. longicornis* alleles. **The distortion does not include marker Nv125, but includes unmapped Nv324, which is in close proximity to Nv125 (see Koevoets et al., 2011).
marker Nv322 was distorted at all three developmental temperatures. The percentage of *N. vitripennis* (V) alleles varied from 66.2% to 76.7% (Table 3) and did not differ between the three temperatures ($\chi^2 = 4.23, P = 0.121$). Hybrids with *N. longicornis* cytoplasm had three loci that were distorted at all developmental temperatures (Nv323, Nv114 and Nv309). The overall percentage of *N. vitripennis* alleles for these markers varied from 19.8% to 32.6% (Table 3), and extreme temperatures increased the distortion towards the allele that matches the cytotype (in this case *N. longicornis*).

**Discussion**

We tested the effect of high and low temperature on development, mortality and sterility of pure and hybrid males of *N. vitripennis* and *N. longicornis*. We found that the applied temperatures did not affect pure species, but that development, mortality and sterility of hybrids were greatly affected under extreme developmental temperatures. Furthermore, the genomic regions associated with hybrid mortality (TRDLs) increased in size and number under extreme developmental temperatures.

**Incompatibilities in *Nasonia* hybrids**

F2 hybrid males of *N. vitripennis* and *N. longicornis* cultured under standard conditions show reproductive perturbations, mortality, abnormalities of courtship behaviour and spermatogenic failure. When developing under extreme temperatures, the effects of these incompatibilities increase to complete reproductive isolation. None of the few surviving hybrids succeeded to emerge from the host at extreme temperatures. Although manual opening of hosts allowed hybrid males to interact with females, behavioural abnormalities and spermatogenic failure prevented the formation of F3 hybrid offspring. Importantly, all *Nasonia* species are reproductively isolated by various independent *Wolbachia* infections that prevent the formation of F1 female hybrids. As prezygotic isolation by *Wolbachia* has been shown to precede hybrid incompatibilities in the process of speciation in *Nasonia* (Bordenstein *et al.*, 2001), the incompatibilities identified in this study are likely the result of independent divergence of the genes underlying the incompatibilities. Whereas the evolution of hybrid incompatibilities is usually considered to be maladaptive (Presgraves, 2010), we can assume that the accumulation of hybrid incompatibilities within the *Nasonia* genus is the result of neutral or adaptive evolution within species, as there is no selection against the evolution of hybrid incompatibilities in natural *Nasonia* populations. This casts a new light on the evolution of hybrid incompatibilities as it shows that neutral or adaptive divergence within species can lead to the disruption of gene interactions between species. This will drive the process of speciation even when prezygotic reproductive barriers are present between the species. Thus, the genomic incompatibilities identified here are the result rather than the cause of speciation. It shows that Dobzhansky–Muller interactions are not always involved in a genic view of speciation, although we do not question their involvement in genic speciation of other genera (where postzygotic isolation occurs on specific genes that drive the isolation of complete genomes).

**Genomic regions associated with hybrid mortality (TRDLs)**

The genomic regions that deviate from equal transmission from the F1 mother to the adult F2 male offspring are indicative of selection on specific genotypes in these regions in F2 males, as embryonic samples do not show such deviations (Koevoets *et al.*, 2011). This selection pressure results in genotype-specific mortality, and screening the genotypes of the adult offspring with molecular markers can identify the genomic regions associated with inducing hybrid mortality.

Under standard culturing temperature (25 °C), F2 haploid hybrid *Nasonia* males show transmission ratios of maternal alleles that deviate from the expected ratio of 1 : 1 for several nuclear genomic regions (TRDLs) (Niehuis *et al.*, 2008; Koevoets *et al.*, 2011). This is suggestive of a role of the cytoplasm in inducing mortality, as these genomic regions differ between reciprocal crosses and are always biased towards the maternally transmitted allele that matches the cytotype. Molecular mapping in the current study revealed that under temperature stress, the distorted genomic regions become larger, the number of distorted regions increases and the regions become more strongly distorted. This strong environmental effect on the identification of TRDLs explains the slight discrepancy between the results from 25 °C of this study and of Koevoets *et al.* (2011), which were both performed under identical laboratory conditions but are, nonetheless, subject to minor fluctuations such as host quality. This underlines the caution that should be taken when interpreting the results of a single laboratory study and

---

**Table 3** Distortion of recovered alleles, given in percentage *Nasonia vitripennis* alleles (V). Only those markers that were significantly distorted at all three temperatures are given. The P-values indicate whether there is a significant effect of temperature on the recovery bias of parental alleles (L : V) ($\chi^2$ tests, d.f. = 2). Significant P-values are indicated in boldface.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Marker</th>
<th>%V (°C)</th>
<th>15</th>
<th>25</th>
<th>31</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>L[V]</td>
<td>Nv322</td>
<td>76.7</td>
<td>66.2</td>
<td>67.6</td>
<td></td>
<td>0.121</td>
</tr>
<tr>
<td>V[L]</td>
<td>Nv323</td>
<td>18.2</td>
<td>32.7</td>
<td>22.9</td>
<td></td>
<td>0.014</td>
</tr>
<tr>
<td>V[L]</td>
<td>Nv114</td>
<td>19.4</td>
<td>32.9</td>
<td>19.6</td>
<td></td>
<td>0.007</td>
</tr>
<tr>
<td>V[L]</td>
<td>Nv309</td>
<td>23.7</td>
<td>32.2</td>
<td>16.9</td>
<td></td>
<td>0.008</td>
</tr>
<tr>
<td>V[L]</td>
<td>Overall</td>
<td>20.4</td>
<td>32.6</td>
<td>19.8</td>
<td></td>
<td>0.015</td>
</tr>
</tbody>
</table>
extrapolating the results to other genera and even natural conditions.

The current study shows that the expression of hybrid incompatibilities depends strongly on environmental conditions such as temperature. Possible explanations on how this is effectuated range from changes in gene expression under stress to weak homeostasis of hybrid offspring and the susceptibility of the biological processes associated with the disrupted gene interaction to thermal fluctuations. Many organisms, including bacteria, plants, nematodes, hexapods and vertebrates, have been shown to respond to stress by altering gene-expression levels (i.e. Liu et al., 2001; Stintzi, 2003; Lo et al., 2006; Zou et al., 2009; Nota et al., 2010; Shin et al., 2011). Genes are either up- or down-regulated to cope with stressful conditions such as elevated temperature. Temperature is expected to be a major stressor in ectotherms, as the internal temperature of these organisms fluctuates with the environment. In a transcriptome analysis of springtails subjected to heat stress, Nota et al. (2010) not only found the expected response of heat shock proteins, but also increased the expression of genes associated with oxidative stress. They ascribed this to increased OXPHOS activity, as also reported for Drosophila (Sorensen et al., 2005) and a Caribbean coral (Desalvo et al., 2008). Thus, gene-expression profiles appear to be sensitive to fluctuations in temperature and genes involved in stress responses are expected to be rapidly evolving to adapt to fluctuating natural conditions (Zou et al., 2009). This makes temperature-responsive genes more prone to be involved in hybrid incompatibilities, which explains why more distorted genomic regions (additional to the gene interactions disrupted under optimal conditions) are identified when screening for incompatibilities under extreme developmental temperatures.

A second explanation for the effect of temperature on the genomic regions involved in Nasonia hybrid mortality is strong genotype-by-environment (G × E) interactions and overall weak homeostasis of hybrids. As all parameters (development, mortality and sterility) of hybrid incompatibility measured in this study are intensified by the temperature process responsible for hybrid mortality in Nasonia are susceptible to thermal fluctuations. The asymmetry of the incompatibilities of reciprocal hybrids, the different genomic regions involved in the mortality of reciprocal hybrids and the effect of the distorted genomic regions towards the cytotype (Breeuwer & Werren, 1995; Nielsen et al., 2008; Koevoets et al., 2011) suggest a strong impact of the cytoplasm on the induction of incompatibilities in Nasonia hybrids. A promising candidate biological process involved in these cytonuclear incompatibilities is OXPHOS. During OXPHOS, nuclear and mitochondrial encoded proteins interact to form clusters that ensure the production of ATP (reviewed by Gershoni et al., 2009). Ellison et al. (2008) found that Nasonia hybrids have low oxidative potential, indicating a role of OXPHOS in inducing Nasonia hybrid mortality. Most distorted genomic regions identified here encompass at least one OXPHOS gene that codes for a protein that differs between N. vitripennis and N. longicornis (black arrows in Fig. 4, after Gibson et al., 2010). The OXPHOS process is likely susceptible to temperature fluctuations as mitochondria adapt to the thermal environment (Ballard & Whitlock, 2004). Interpopulation hybrids of marine copepods were found to be affected in their oxidative potential compared with pure population individuals, and the interaction between temperature and cytoplasm in inducing incompatibilities was demonstrated both in vitro (Rawson & Burton, 2002) and in vivo (Willett & Burton, 2003). Similarly, interpopulation hybrids of Tribolium beetles were affected by the interaction between temperature and cytoplasm in their clutch sizes (Demuth & Wade, 2007) and metabolic rates (Arnvist et al., 2010). We have taken a first step in unravelling the genetic basis of the effect of temperature on cytonuclear interactions and opened the way for additional research on this topic.

Although there is compelling evidence for the role of OXPHOS in Nasonia hybrid incompatibilities, other processes such as mitochondrial replication, transcription and translation (Woodson & Chory, 2008) could also lead to cytonuclear incompatibilities. For instance, the location of the γ DNA polymerase genes involved in mitochondrial replication (indicated in Fig. 4 by grey arrows) overlaps with OXPHOS genes, which obstructs the disentanglement of their independent effect. More complicated incompatibilities are also a possible explanation for the effect of the cytoplasm in hybridization, such as higher-order interactions between several nuclear genes and the cytoplasm (cyto-nucleo-nucleo interactions) or alterations in epigenetic modifications. Although Koevoets et al. (2011) found no disrupted cyto-nucleo-nucleo interactions under standard conditions, the temperature stress in this experiment could induce such higher-order incompatibilities. Research on epigenetic modifications in Nasonia is still limited, but its role has been suggested in Nasonia sex determination (Verhulst et al., 2010) and
DNA methylation of several genes has been identified in *Nasonia* (Park et al., 2011).

Currently, the various explanations for how temperature stress increases hybrid mortality levels and how this affects the genomic regions involved cannot be distinguished. Additional research into the genetic basis of hybrid incompatibilities, including gene identification and gene-expression profiling, is required to resolve this issue.

**Effects of temperature stress on courtship behaviour and spermatogenic failure**

Courtship behaviour of pure species was largely unaffected by developmental temperature. Both types of hybrids that developed at 25 °C showed great disruptions in their courtship behaviour, leading to low copulation frequencies. Temperature stress induced nearly complete behavioural sterility of hybrid males. Spermatogenic failure of the few copulating hybrid males completed the post-zygotic reproductive isolation between *N. vitripennis* and *N. longicornis* when hybrids developed under extreme temperatures. We thus conclude that developmental temperature affects hybrid sterility. Similar to the effects of temperature on the induction of hybrid mortality, the effect of temperature on various sterility parameters can be attributed to different genetic mechanisms.

First, temperature stress might have an effect on the differential expression of courtship genes, and the interaction of these differentially expressed genes could be disrupted in hybrids. The expression of courtship genes in *Drosophila* has been found to be subject to differential expression with male age (Ruedi & Hughes, 2009) and temperature affects the courtship behaviour of wolf spiders (Davis, 1989). However, still little is known about the effect of temperature on the expression profiles of courtship genes and how susceptible such temperature-specific gene networks are to perturbations. Studies on the differential expression of genes involved in spermatogenesis are equally limited, although sperm cell degeneration and differential gene expression after heat shock therapy of mouse testes suggest temperature sensitivity of spermatogenic failure (Rockett et al., 2001). Thus, the differential expression of courtship and spermatogenesis genes might be affected by temperature stress, which could induce hybrid sterility under extreme temperatures that is not expressed under standard temperatures.

Second, the observed effects of temperature stress on the disturbance of hybrid courtship behaviour could also be linked to weak homoeostasis and low metabolism of hybrid wasps, which is intensified by temperature stress. Metabolism of hybrids has been found to be affected by the interaction between temperature and cytoplasm (Arnqvist et al., 2010), but no studies to date have linked this decrease in metabolism to a lowered ability to reproduce. It is likely that hybrid individuals with limited energy supply are not able to perform correct courtship behaviour and thus cannot proceed to copulation. Whether the effect of temperature stress on courtship behaviour and spermatogenic failure found in the current study is caused by disrupted gene interactions between differentially expressed nuclear courtship genes or by general weak homoeostasis of the hybrids remains unresolved. Further studies should focus on the role of temperature-specific gene networks of courtship genes during the process of speciation.

Asymmetrical incompatibilities between the reciprocal hybrid *Nasonia* crosses (Niehuis et al., 2008; Koevoets et al., 2011 and the current study) and previous research on *Nasonia* OXPHOS protein complexes (Ellison et al., 2008) suggest that disruptions of cytonuclear interactions are the main cause of hybrid incompatibilities in *Nasonia*. This is in contrast with most diploid species in which nuclear–nuclear interactions appear to dominate among genes leading to hybrid incompatibilities (reviewed by Presgraves, 2010). Cytonuclear incompatibilities may be a specific feature of male hybrids of haplodiploid species, as they appear rarely in *F*1 diploid hybrids (but see Willett, 2011). This could be due to higher detection ability in haploids, assuming that such incompatibilities are largely recessive (supported by the lack of such incompatibilities in *F*1 diploid hybrid females of haplodiploids). More studies in a larger range of organisms are needed to resolve this issue.

To summarize, hybrid incompatibilities such as developmental perturbations, mortality levels and sterility levels were found to be highly amplified by temperature stress. Although it remains unresolved whether changes in gene expression, *G* × *E* interactions or the effect of temperature on cytonuclear incompatibilities is the cause of the results found in the current study, it is evident that homoeostasis in hybrid individuals is disrupted and that small perturbations lead to more incompatibilities between genes. *G* × *E* interactions in disrupting epistasis in hybrids have not received much attention apart from the marine copepod *T. californicus* (reviewed by Willett, 2011). Here, we provide the first genome-wide screen of the effect of temperature on the regions associated with inducing hybrid mortality. These genomic regions increased in size and number with temperature stress, which is in line with the increased mortality and sterility levels found under temperature stress. Although elucidation of the underlying genetics awaits further study, our results have large implications for the identification of speciation genes. The effects of speciation genes are likely intensified under fluctuating natural conditions, and thus more, and perhaps different, genes are expected to function as reproductive isolation barriers in nature than identified under controlled laboratory conditions.

**Acknowledgments**

We are grateful to S. Ferber for his help with hosting the wasps and behavioural observations, M. Krikke, E. Kuil
and E. Rosendaal for a pilot study on temperature-dependent mortality in *Nasonia* and S. Pérez Vila for advice on GLM analysis. We also thank Corneel Vermeulen and two anonymous reviewers for valuable comments on the manuscript and analyses. This work has been made possible by grant ALW 816.01.004 and Pioneer grant ALW 833.02.003 of the Netherlands Organisation for Scientific Research to LWB.

**References**


**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Set-up of the temperature-dependent incompatibilities experiment.

**Table S1** Sample sizes of F1 mothers for which the offspring production was measured (egg-to-adult survival and emergence experiments).

**Table S2** Categories of courtship behaviour distinguished to test for F2 male sterility.

**Table S3** Transition probabilities between behavioural categories during courtship.

**Protocol S1** DNA extraction protocol Koevoets, van de Zande and Beukeboom. Temperature stress increases hybrid incompatibilities in the parasitic wasp genus *Nasonia*.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

Received 18 August 2011; revised 24 October 2011; accepted 24 October 2011