The Expanding Genetic Toolbox of the Wasp *Nasonia vitripennis* and Its Relatives

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ABSTRACT The parasitoid wasp *Nasonia* represents a genus of four species that is emerging as a powerful genetic model system that has made and will continue to make important contributions to our understanding of evolutionary biology, development, ecology, and behavior. Particularly powerful are the haplodiploid genetics of the system, which allow some of the advantages of microbial genetics to be applied to a complex multicellular eukaryote. In addition, fertile, viable hybrids can be made among the four species in the genus. This makes *Nasonia* exceptionally well suited for evolutionary genetics approaches, especially when combined with its haploid genetics and tractability in the laboratory. These features are complemented by an expanding array of genomic, transcriptomic, and functional resources, the application of which has already made *Nasonia* an important model system in such emerging fields as evolutionary developmental biology and microbiomics. This article describes the genetic and genomic advantages of *Nasonia* wasps and the resources available for their genetic analysis.

KEYWORDS *Nasonia*; genomics; haplodiploid; hybrid genetics; model organisms

NASONIA VITRIPENNIS has been a model for genetic analysis for more than half a century (Whiting 1967), and the past decade has seen a rapid increase in the number and power of its genetic toolkit. These tools, in combination with the wealth of interesting biology (Werren *et al.* 2010), make the *Nasonia* system one of the premier genetic systems among insects.

Life History of *Nasonia* Wasps

Nasonia wasps are parasitoids, meaning that they use a host for nourishment for only the larval portion of their life cycle, and the host is killed in this process (Whiting 1967). *N. vitripennis* is the most commonly used laboratory model system in the genus and is distributed worldwide in association with human populations (Whiting 1967). Three additional species in the *Nasonia* genus have been described that have much more restricted distributions in North America. *N. giraulti* and *N. oneida* are found in the Northeast, and *N. longicornis* is restricted to the Northwest (Darling and Werren 1990; Raychoudhury *et al.* 2010).

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These wasps develop fairly rapidly, with a generation time of 14 days at 25° or 10 days at 28°. Embryogenesis is also quite rapid in these wasps, with larvae emerging a little more than 24 hr after egg laying (Pultz *et al.* 2005). After larval emergence, the wasps begin to feed on the host hemolymph and undergo up to four larval instars before the onset of pupation (Pultz and Leaf 2003) (Figure 1).

Like all Hymenoptera, *Nasonia* use haplodiploid sex determination, which means that unfertilized eggs give rise to haploid males, while fertilized eggs give rise to diploid females (Whiting 1967) (Figure 1). The existence of haploid males is one of the most important features of these wasps, and the many ways that this haploidy can be exploited experimentally will be described later.

It should be noted here that although haplodiploid sex determination is ancestral and highly conserved among the Hymenoptera, the molecular basis for this process is variable. One mode, employed by honeybees and many others, is complementary sex determination (CSD) (Whiting 1943). In this mode, the presence or absence of heterozygosity at a sex-determining locus leads to female or male development, respectively. Normally, there is sufficient polymorphism in an outbred population to prevent the mating of individuals carrying the same allele, so fertilized eggs will develop as heterozygous diploid females. Unfertilized eggs are hemizygous but are effectively homozygous and develop as males. If inbreeding occurs, there is the possibility that

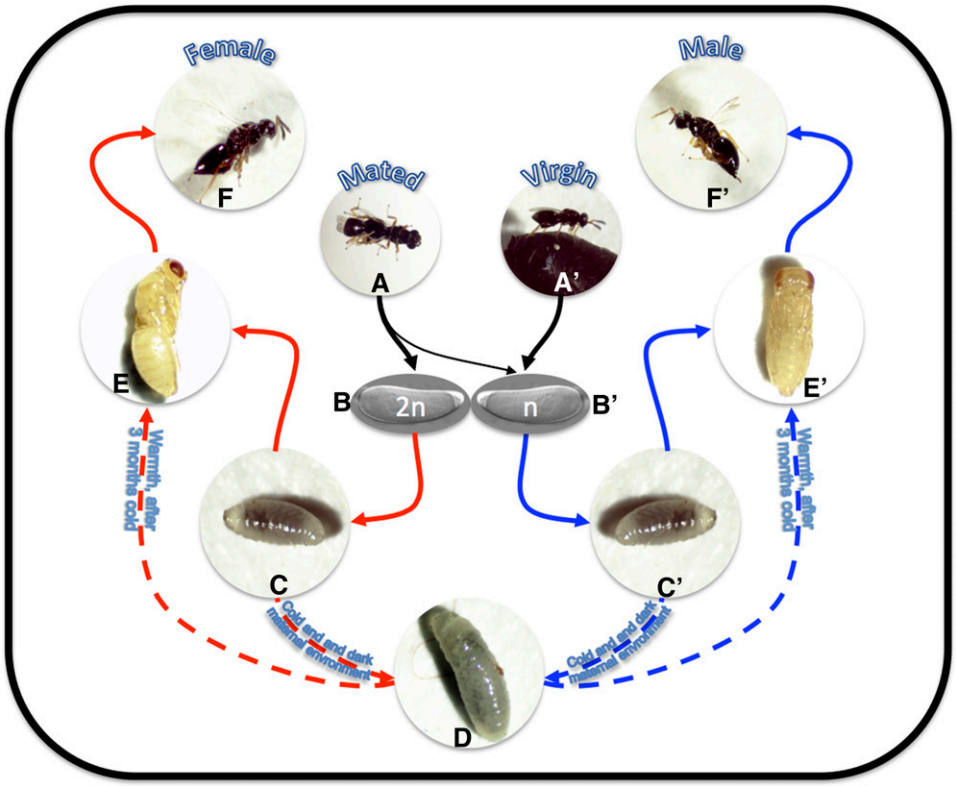

Figure 1 Life cycle of *Nasonia* wasps. Both mated (A) and virgin (A′) females will readily lay eggs. Mated females (B) will produce predominantly (80–90%) fertilized eggs, while virgins (B′) can only lay unfertilized eggs. (C and C′) Embryos will develop into legless, simplified larvae. (D) If the mother experienced cold and short-daylight environments, she is more likely to program her offspring to enter diapause, which occurs in the last larval instar. Diapause larvae can survive for more than a year and require an extended cold period to progress in development. (E and E′) As holometabolous insects, *Nasonia* undergo an extended pupal stage. (F and F′) Adults deriving from fertilized eggs will normally be diploid females, while those from unfertilized eggs will be haploid males.

a male and a female carry the same allele, and half their progeny will be nonviable diploid males (Whiting 1943).

It is clear that *Nasonia* do not use a CSD system (Dobson and Tanouye 1998). *Nasonia* can be heavily inbred (*i.e.*, brother-sister and mother-son matings) for many generations without the appearance of diploid males or any other observable defects (Skinner and Werren 1980; Dobson and Tanouye 1998). Therefore, a single-locus CSD system is excluded, and a multilocus CSD system is still highly unlikely. The currently favored hypothesis is that sex determination relies on differential imprinting on one or more loci in the genomes of the gametes (Beukeboom and van de Zande 2010; Verhulst *et al.* 2010). This hypothesis rests on dependence of "femaleness" on the presence of a functioning male chromosome set during early embryogenesis. A locus that can confer "femaleness" may be imprinted and repressed on the maternally derived chromosome but is unimprinted on the male chromosomes. Thus, only when the male genome is able to participate in development is the female form generated. The nature of the imprint and the identity of the locus imprinted are as yet unknown (Zwier *et al.* 2012; Verhulst *et al.* 2013).

Another noteworthy feature of *Nasonia* is the ability to make fertile, viable hybrids between any of the four species by curing them of otherwise incompatible *Wolbachia* infections (Breeuwer and Werren 1990). *N. vitripennis* is about 1 million years diverged from the other three species, while the latter are from 300,000 to 400,000 years diverged from one another (Werren and Loehlin 2009a) (Figure 2). This feature, in combination with all the other advantages of the system, makes *Nasonia* a premier model organism for genetic dissection of the evolutionary process that has taken place over relatively short periods of time (Werren and Loehlin 2009a).

Nasonia as a Genetic Model in the Laboratory

Nasonia are very amenable to culture in the laboratory, with hosts that are easily reared in the laboratory (Werren and Loehlin 2009b), and they are also commercially available (*Sarcophaga* pupae, Carolina Biological Supply Company, Burlington, NC). They are perfectly happy in *Drosophila* culture tubes or smaller vessels such as plastic test tubes. Recently, a sterile, host-free *in vitro* method for rearing *Nasonia* from egg to adult was developed (Brucker and Bordenstein 2012). This technique has already allowed crucial insights into how the wasp interacts with its symbiotic and commensal bacterial communities (Brucker and Bordenstein 2013). More detailed descriptions of how *Nasonia* can be maintained in the laboratory can be found elsewhere (Werren and Loehlin 2009a), and here only the most useful characteristics of these wasps for genetic experiments will be described.

A major practical advantage of *Nasonia* biology is the cold hardiness of these wasps at several life stages. Further development or maturation of the wasps can be suspended by incubation at 4° and then reinitiated if they are returned to higher temperatures within a certain time frame. The amenable stages include the embryo (up to 48 hr; J. A. Lynch, personal observation), the early (yellow) pupal stage (2–3 months), the late, fully pigmented pupa (1 month), and the

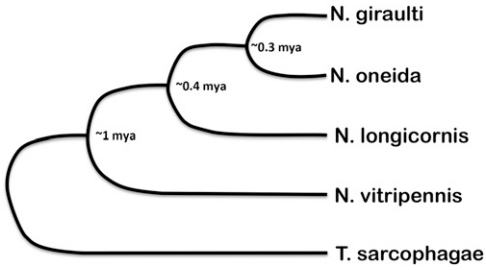

Figure 2 Phylogenetic relationships and approximated divergence times among *Nasonia* species. Adapted from Werren and Loehlin (2009a) and Werren *et al.* (2010).

adult wasp (~3 weeks). In addition, a facultative larval diapause can be induced, which can allow wasp lines to persist for a year or more with no maintenance effort (Figure 1).

The pupae of *Nasonia* show the body structure of the adult very clearly and are covered with only a thin cuticle (Figure 1). This allows very easy sexing and phenotyping of pupae. These features are quite convenient for setting up genetic crosses because selection of virgin females and mutants can be done at the investigator's leisure. In addition, the sex ratio can be manipulated in *Nasonia* to a large degree, taking advantage of haplodiploidy. All-male broods can be easily generated by allowing virgin females to parasitize hosts, while broods of up to 90% female progeny can be generated if mated females are hosted under optimal conditions of low density (Whiting 1967).

In addition to these investigatory conveniences, *Nasonia* can be used to address a wide variety of biological questions. Not only are they useful as a comparative system for more established models (*i.e.*, *Drosophila*), but they also have features that cannot be addressed in other systems. For example, *Nasonia* use DNA methylation for genomic regulation, unlike the model insect *Drosophila melanogaster*. Approximately a third of genes are methylated in *Nasonia*, and methylation occurs over gene bodies and is correlated with broad gene expression across development (Wang *et al.* 2013).

As alluded to earlier, *Nasonia* are emerging as a uniquely powerful model system for understanding both the nature and evolution of the hologenome (the genetics of how species and their associated symbiotic and commensal microorganisms interact with each other and their environment), and many of the functional tools described later in this paper can be applied to understanding the evolutionary impact of *Nasonia*'s microbiomic milieu (Bordenstein and Bordenstein 2011; Kent *et al.* 2011; Brucker and Bordenstein 2013). These tools also include the cellular and developmental genetics of pathologic interactions between *Nasonia*, its symbionts, and selfish genetic elements (Tram and Sullivan 2000; Ferree *et al.* 2008; Swim *et al.* 2012; Akbari *et al.* 2013). In addition, *Nasonia* constitute an ideal model for understanding how parasitoids manipulate their hosts using a potent cocktail of biologically active molecules contained in venom (Danneels *et al.* 2010; De Graaf *et al.* 2010; Martinson *et al.* 2014).

Finally, *Nasonia* have a great potential as a model to understand the evolution of behavior. Each species has a distinct, complex, and quantifiable courtship behavior that could be genetically dissected (Beukeboom and van den Assem 2001). Other examples include understanding the genetics of host preference (Desjardins *et al.* 2010), sex-ratio decisions (Pannebakker *et al.* 2011), memory formation and retention (Hoedjes *et al.* 2012; Hoedjes and Smid 2014; Hoedjes *et al.* 2014), and circadian rhythms (Paolucci *et al.* 2013; Bertossa *et al.* 2014).

Further descriptions of the interest of *Nasonia* biology can be found elsewhere (Beukeboom and Desplan 2003; Werren *et al.* 2010) and are outside the scope of this paper. Instead, I will focus on the tools available to leverage the inherent advantages of the *Nasonia* system.

Nasonia Genomics

Given the unique advantages of *Nasonia* genetics and the large number of questions that can be addressed with these wasps, the three described (at the time) species of the *Nasonia* genus were chosen for whole-genome sequencing. *N. vitripennis* was sequenced and assembled with 6× Sanger shotgun coverage, while *N. giraulti* and *N. longicornis* were sequenced to 1× with Sanger chemistry and to 12× with Illumina short reads (Werren *et al.* 2010) (Table 1).

The ability to make interspecies crosses was used to map contigs and scaffolds to chromosomes, which further improved the assembly of the *N. vitripennis* genome (Niehuis *et al.* 2010; Desjardins *et al.* 2013) (Table 1) and made it one of only a handful of species whose scaffolds have been mapped to chromosomes.

Currently, efforts are underway to improve the coverage and assembly of the other *Nasonia* species. For example, *N. giraulti* is being sequenced using paired-end Illumina reads in combination with long Pacific Bioscience single-molecule real-time (SMRT) reads (Table 1). In addition, *N. longicornis*, the recently described *N. oneida*, and *Trichomalopsis sarcophagae*, a representative of the sister genus to *Nasonia* (Figure 2), are being sequenced using primarily Illumina technology (Table 1). This will be especially useful in determining the polarity of evolutionary change within the *Nasonia* genus.

In concert with sequencing of the genome, major efforts to characterize and annotate the transcribed portion of the genome were undertaken (Werren *et al.* 2010). A combined approach of using ESTs and *in silico* predictions led to the production of a transcriptome annotation that included more than 24,000 genes (Gilbert 2012) (Table 1).

The sequencing of the three *Nasonia* genomes and transcriptomes allowed the production of high-resolution microarray resources, including tiling arrays covering the entire genome of *Nasonia* (Wang *et al.* 2013) and mapping arrays that allow the mapping of interspecies traits (Desjardins *et al.* 2013). Having well-annotated transcriptomes and genomes also is a great aid in applying analytical tools that

Table 1 Genomics Resources Available or in Development for *Nasonia*

Resource	Details	References
Genome sequence *N. vitripennis*	6× coverage Sanger array-aided assembly	Werren *et al.* 2010, Niehuis *et al.* 2010
Genome sequence *N. giraulti*	1× coverage Sanger, 12× short-read Illumina, 10× PacBio, 120× Illumina paired end	Werren *et al.* 2010, J. A. Lynch, Y. Kelkar, and J. Werren, in preparation
Genome sequence *N. longicornis*	1× coverage Sanger, 12× short-read Illumina in-progress hybrid assembly	Werren *et al.* 2010, J. Werren, personal communication
Genome sequence *N. oneida*	In-progress hybrid assembly	J. Werren, personal communication
Genome sequence *T. sarcophaga*	In-progress hybrid assembly	J. Werren, personal communication
Curated transcriptomes *N. vitripennis*	1.2, 2.0	http://nasoniabase.org http://arthropods.eugenes.org/EvidentialGene/
Genome database	Genome browser, BLAST, data downloads, and more	www.nasoniabase.org
RNAi target design, predicted methylation regions	Database of methylated and transcribed regions plus tool for dsRNA design	http://www.waspatlas.com/home

take advantage of next-generation sequencing techniques, for example, in using the cufflinks package for the analysis of differential expression in different developmental stages (Trapnell *et al.* 2012) or in RNA interference (RNAi) cases (Akbari *et al.* 2013; Sackton *et al.* 2013).

The *Nasonia* genome is hosted as part of the Hymenoptera Genome Database project (Munoz-Torres *et al.* 2011) (Table 1). Here the annotated genome can be browsed using JBrowse and searched with BLAST to identify genes of interest. Other online resources include a genome-wide methylation database and a tool for designing robustly active and specific double-stranded DNA (dsRNA) target sequences for RNAi experiments hosted by the Tauber Lab (Table 1).

Functional Resources

Reverse genetics

The ability to apply RNAi to knockdown gene function in *Nasonia* was a major factor in its success so far as an emerging model organism, especially in regard to developmental questions. The original method was parental RNAi, where female pupae were injected with dsRNA against a gene of interest, and then phenotypes were evaluated in the resulting progeny (Lynch and Desplan 2006). The technique has been adapted recently to other life stages, including adults, larvae, and embryos (Abdel-Latief *et al.* 2008; Werren *et al.* 2009; Rosenberg *et al.* 2014), greatly expanding the number of questions that can be addressed in *Nasonia* (Table 2). RNAi has been used to identify novel systems for embryonic axial patterning (Lynch *et al.* 2006; Ozuak *et al.* 2014), to uncover the logic of *Nasonia* sex determination (Verhulst *et al.* 2010), and to confirm the role of a species-specific enzyme in the production of a novel pheromone component (Niehuis *et al.* 2013).

Other methods for manipulating candidate gene function have been or are in the process of being developed. Morpholinos have been employed for knocking down gene function in embryos (Rosenberg *et al.* 2014), which will be especially useful in cases where RNAi has proven to be difficult. In addition, new techniques for genome editing, such as transcription activator-like effector nucleases (TALENs), zinc finger nucleases, and clustered regularly interspersed palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) (Gaj *et al.* 2013), are in principle applicable to *Nasonia* given that methods for raising injected embryos to adulthood now have been firmly established in the wasp (Rosenberg *et al.* 2014) (Table 2).

Forward genetics

The amenability of *Nasonia* to classical forward genetics techniques is one of the unique strengths of the system in comparison with other emerging model systems. *Nasonia* have a small number of chromosomes (five) and the advantage of haploid males, which greatly aid in screening for recessive mutations because phenotypes can be screened in the male progeny of F_1 females (Figure 3A). The advantages of haploidy have already been demonstrated by Mary Anne Pultz, whose screens generated and identified many interesting mutations affecting embryonic patterning (Pultz *et al.* 1999, 2000, 2005).

Resources available in *Nasonia* to aid in identification of the causative lesion in such screens include visible (eye or

Table 2 Reverse Genetic Approaches Available for *Nasonia*

Approach	Works in *Nasonia*?	References
RNA interference	Yes (parental, embryonic, larval, adult)	Lynch and Desplan 2006; Abdel-Latief *et al.* 2008; Werren *et al.* 2009; Rosenberg *et al.* 2014
Morpholinos	Yes (embryonic)	Rosenberg *et al.* 2014
Germ-line transformation	Limited success	C. Desplan, personal communication
CRISPR/Cas9	Preliminary success	J. A. Lynch, personal observation
TALENs	Yes	C. Desplan, personal communication
Zinc finger nucleases	Not tested	n/a

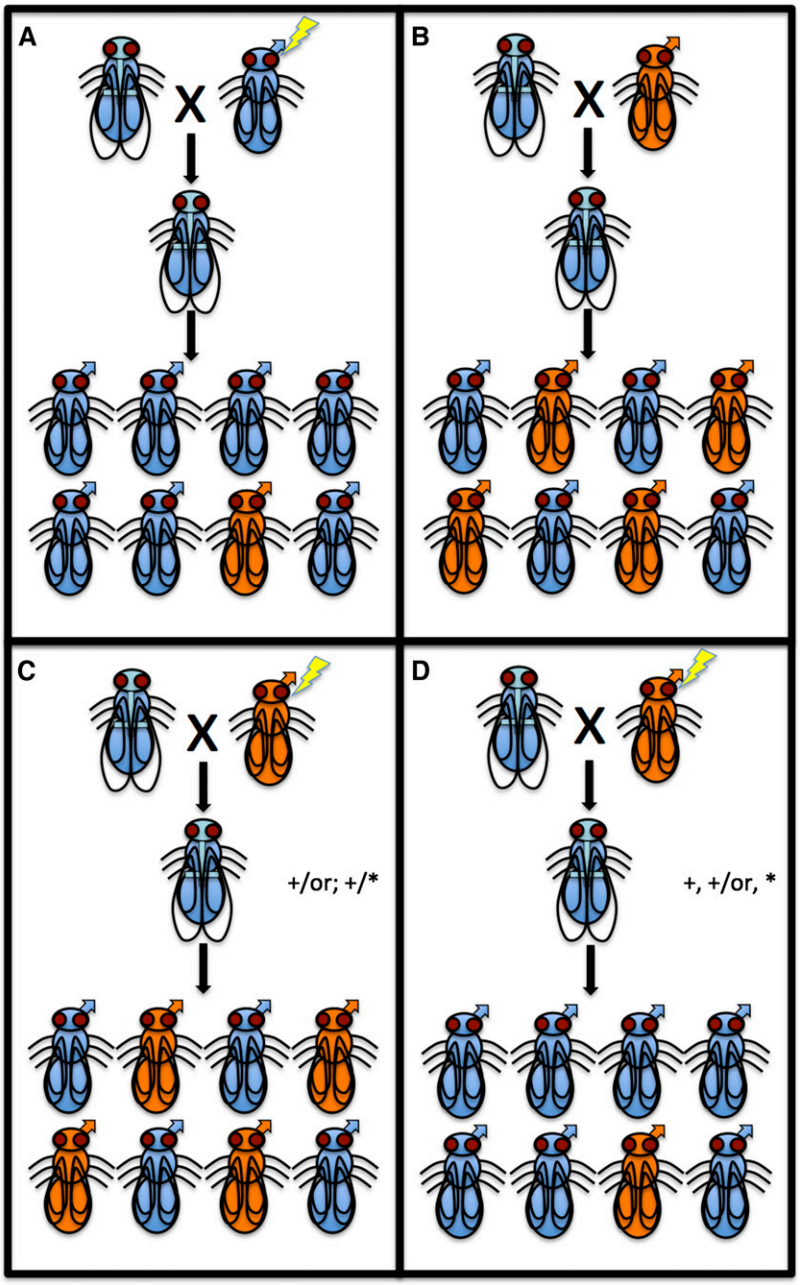

Figure 3 Haplodiploid genetics in *Nasonia*. Males are indicated by an arrow extending from the head and smaller wings. A hypothetical *orange* (*or*) mutation is used throughout the figure. (A) A mutation induced in a germ-line cell of a parental-generation male can be screened in the F_2 progeny of the male's virgin daughters. (B) Behavior of a recessive mutation in the *Nasonia* genetic system. (C) Behavior of a visible mutation and an induced lethal recessive mutation that is not linked. (D) Behavior of a visible mutation and a lethal recessive mutation that are linked. All nonrecombinants carrying the *or* mutation die, so only relatively rare recombinant *orange* offspring are observed in F_2.

body color or morphology) marker lines (at least one per chromosome)(Werren and Loehlin 2009a); molecular markers in the form of RAPDs, RFLPs, AFLPs, and SNPs; and the previously mentioned mapping arrays (Gadau *et al.* 1999; van Opijnen *et al.* 2005; Niehuis *et al.* 2010; Desjardins *et al.* 2013). In addition, a small number of novel mapping strategies were devised in the genomic era that can be leveraged in *Nasonia*. These include whole-genome resequencing techniques (Blumenstiel *et al.* 2009) and multiplexed shotgun genotyping (Andolfatto *et al.* 2011). These techniques will play an important role in overcoming the limitations of the candidate-gene approach in *Nasonia*, especially in regard to developmental biological questions.

Another useful feature of the *Nasonia* system is its high tolerance for inbreeding. Unlike other insects (including other hymenopterans), sibling:sibling and offspring:parent matings produce no ill effects in *Nasonia*. The ability to easily produce isogenic females in combination with the fact that haploid males produce sperm that are genetically identical allows the production of large populations of females with the same genotype. These "clonal" females can be used to test the role of environmental factors in any given mutant or recombinant phenotype (Velthuis *et al.* 2005; Pannebakker *et al.* 2011). Velthuis *et al.* (2005) used this tool to map loci affecting mating preferences in *N. longicornis*, while Pannebakker *et al.* (2011) used clonal recombinant lines

Table 3 Forward Genetic Approaches Applicable to *Nasonia*

Resource	Details	Example
Haplodiploidy	More rapid identification of mutations	Pultz *et al.* 1999; Pultz *et al.* 2000; Pultz *et al.* 2005
Interspecies hybrids	Detection of evolutionarily relevant variants	Loehlin and Werren 2012
Linked lethal mapping strategy	Detection of interspecies differences	Loehlin *et al.* 2010
Visible mutations	Aid in mapping and stock maintenance	Pultz *et al.* 1999; Pultz *et al.* 2000
Mapping arrays	Genome assembly detecting recombination in hybrids	Desjardins *et al.* 2013
SNP maps	Narrowing QTL regions	Niehuis *et al.* 2013
Mapping population	Highly outbred line for selection and mapping of complex traits	Van De Zande *et al.* 2014
Segmental introgression lines	Defined genomic fragments from one species in the genomic context of another	Desjardins *et al.* 2013

to map QTL associated with heritable variation in sex allocation in different *N. vitripennis* populations.

Forward genetic strategies also can be combined with the ease of generating interspecies hybrids to detect naturally occurring differences between *Nasonia* species. All the mapping resources described in the preceding paragraph can be applied to these traits. In addition, a particularly powerful method to detect recombination events taking place near any particular locus has been developed in *Nasonia*. In this method, hybrid males showing a phenotype of interest are mutagenized and then mated with wild-type females. The resulting virgin females produce male progeny that are screened for the presence of lethal mutations linked to the causative gene (assessed by a much lower proportion than expected of males showing the trait of interest). Once linkage of a lethal mutation has been detected, these females are mated with wild-type males. Male progeny of the females resulting from this cross are scored, and the survivors that show the phenotype represent recombinants between the linked lethal and causative genes. The power of this technique can be enhanced by combining it with additional linked visible markers (Figure 3C and D). This approach has been used to map the genomic bases of different aspects of interspecific wing size to the *cis*-regulatory region of the sex-determining gene *doublesex* (Loehlin *et al.* 2010) and to multiple changes in the *cis*-regulatory region of the growth factor *unpaired* (Loehlin and Werren 2012). The latter is one of the few cases where the causative loci of a morphologic difference between species have been mapped to such high resolution.

Another strategy for mapping interspecies differences also has been devised using a high-density *N. vitripennis* microarray that can detect the species origin of segments of DNA in the genome of a hybrid animal (Desjardins *et al.* 2013) (Table 3). This approach was used to map a locus involved in host preference differences between *N. giraulti* and *N. vitripennis* to a relatively small genomic region (Desjardins *et al.* 2010). This approach also led to the identification of a gene responsible for producing a species-specific evolutionarily relevant pheromone whose role was verified using RNAi (Niehuis *et al.* 2013).

An additional resource available in *Nasonia* is a set of segmental introgression lines (SILs) in which fragments of the *N. giraulti* genome, marked either visibly or genetically, were back-crossed into a *N. vitripennis* background for over eight generations and then were made homozygous (Table 3). The break points of the introgression have been estimated, and these lines serve as a crucial resource for screening for interspecies differences in any trait of interest (Desjardins *et al.* 2013).

Recently, a highly outbred line of *N. vitripennis* was established, and a protocol for maintenance of its genetic diversity was devised. This line will be useful for experimental evolution, mapping of complex traits, and population genetics, among others (Van De Zande *et al.* 2014) (Table 3).

Tools for Molecularly Characterizing and Interpreting Phenotypes

In addition to tools for generating, maintaining, and mapping mutants, a successful genetic model organism needs tools for visualizing and quantifying the activity of genes and their products. Robust techniques for *in situ* hybridization to detect

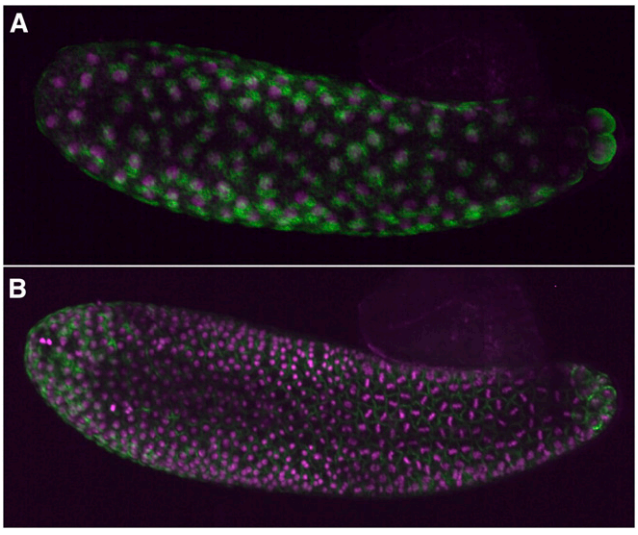

Figure 4 Live imaging in *Nasonia*. *Nasonia* embryos were injected with mRNAs encoding Histone::RFP (magenta, marking chromosomes) and Life Actin::GFP (green, marking f-actin) according to the protocols of Benton *et al.* (2013). Embryos shown are undergoing the eighth (A) and tenth (B) synchronous syncytial divisions of early embryogenesis. No dechorionation was necessary for injection or microscopy.

spatial patterns of mRNA expression have been developed and applied to both embryos and larvae. In the embryo, multiple transcripts can be detected simultaneously with fluorescence (Ozuak *et al.* 2014), and a technique to detect nascent transcripts in nuclei has been reported recently (Verhulst *et al.* 2013). High-throughput probe production and hybridization in 96-well plates has been established for *Nasonia* embryos (J. A. Lynch, in preparation), and a chamber for mass egg lay called the *Waspinator* (Buchta *et al.* 2013) will allow for mass screening of gene expression patterns in the *Nasonia* embryo, as has been done for *Drosophila* (Tomancak *et al.* 2002).

The *Nasonia* egg is relatively small and optically clear, making it an ideal system for time-lapse analysis of early developmental events. Such analyses have already been performed using differential interference contrast illumination (Buchta *et al.* 2013) and fluorescent live imaging (J. A. Lynch, personal observation) (Figure 4). The combination of live imaging with the forward and reverse genetic techniques will make *Nasonia* an even more powerful model for embryonic patterning and morphogenetic processes.

The Future

With the increasing power and decreasing cost of next-generation sequencing technologies, the possibility to robustly identify causative nucleotides underlying naturally occurring and laboratory-induced phenotypic differences will increase at an accelerating rate. The *Nasonia* species group is uniquely well poised to take advantage of these developments. It has a strong foundation of genetic and genomic resources, is the most experimentally tractable haplodiploid genetic model system, and has a still largely untapped promise in its ability to make hybrid and recombinant progeny across species. These possibilities, combined with the evolutionary questions (*i.e.*, venom, development, methylation, hologenomics, biochemistry, population genetics, and speciation, behavior, among many others) that will become increasingly experimentally tractable in the genomic era, make the *Nasonia* genus one of the most exciting emerging model systems with which to probe the deepest mysteries of biology.

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