Chapter 1

Introduction
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A note on Biological Control as a unifying theme

This thesis may seem unconventional in featuring two major topics, polyploidy and host specificity. Polyploidy is the condition of having more than the normal number of chromosome sets; it is the focus of chapters 2-4. Host specificity describes the range of species a parasitoid or parasite can use as a food source and developmental environment. Chapter 5 of this thesis provides new genetic insights for this trait. While there is clear distinction in theme for these two branches of research, they originated from a single initial objective: to advance the theoretical and practical knowledge of biological control.

Biological control is the practice of using organisms to control agricultural pests (van den Bosch, 1971). It is often a cheaper and arguably less environmentally damaging alternative to chemical pesticides and current Genetically Modified Organisms (GMOs) (Bale, van Lenteren, & Bigler, 2008; Barratt et al., 2018). This makes it an important option to growing global challenges such as stable food production (Bale et al., 2008). While the field of biocontrol has been broadly successful (Stiling & Cornelissen, 2005; Messing & Wright, 2006), there are negative aspects to its use. For example, a biocontrol agent is unsuccessful if it fails to control a pest adequately, or (from an economical point of view) if a captive commercial population cannot be established (Hoddle, 2006; Messing & Wright, 2006; van Lenteren, 2012). Furthermore, non-target effects (species other than the target pests being harmed) (Howarth, 1991; Lockwood, 1996; Louda et al., 2003; Wright et al., 2005) have previously driven native species to a sharp decline or extinction (Howarth, 1983; Simberloff & Stiling, 1996; Louda et al., 2003). Any evidence of non-target effects disqualify otherwise effective biocontrol agents from use for legal and ethical reasons (van Lenteren et al., 2006a,b). Genetics-based methods have long been suggested for improving biological control in terms of pest control efficacy, industrial production, and environmental safety (Hoy, 1986; Rosenberg & Hoy, 1988; Hopper, Roush, & Powell, 1993). However, understanding the genetic basis for successful biocontrol is in its early stages. Research linking specific genes and genetic mechanisms to significant traits is still very limited, particularly for insects (Roderick & Navajas, 2003).

A major genetic factor influencing biocontrol traits is polyploidy. Polyploidy causes major phenotypic changes, both in spontaneous mutants and for long term evolutionary trajectories (Comai, 2005). It has a specific relevance to parasitoid wasps, the most widely utilized and economically important class of biological control agent (Hassell & Waage, 1982; Strand & Obrycki, 1996; van Lenteren, Roskam, & Timmer, 1997; van Lenteren, 2012). In a number of hymenopteran
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Polyploidy represents a significant extinction risk for small and/or captive populations breeding (Zayed & Packer, 2005; Hein, Poethke, & Dorn, 2009; Fauvergue et al., 2012; Faria et al., 2016; Zaviezo et al., 2018). This phenomenon is tied to a well-known hymenopteran sex determination mechanism, complementary sex determination (CSD). In CSD, individuals that are heterozygous or hemizygous for a CSD locus or loci develop into reproductive females or males, respectively, but homozygotes are sterile males. As CSD allelic diversity is lost through bottlenecks or inbreeding, which is common for captive populations used for biological control breeding, sterile male incidence rises, and the population eventually dies out (Stouthamer, Luck, & Werren, 1992). Ironically, while this has contributed to polyploidy being generally characterized as a biological control impediment, most parasitoid wasps actually do not have CSD (Figure 1).

Inbreeding does not cause polyploidy in non-CSD species, although it can arise through different means, and very little is known about its effects. Therefore there is actually a gap of knowledge on how polyploidy impacts many species in the group with the most biocontrol potential.

The polyploid work of this thesis began as an investigation on how polyploidy effects non-CSD parasitoid wasps in a biocontrol context. As there was no existing evidence for polyploidy being detrimental to non-CSD parasitoid wasps, an initial hypothesis included the suggestion that it could be advantageous in some ways. Chapter 2 is a study on the baseline biology of a long-maintained, inbred polyploid line of a non-CSD parasitoid species, which had been used in sex determination research for decades but had not been evaluated specifically for its polyploid biology. It concluded that there are a few biocontrol detriments of non-CSD polyploidy, but that these effects may be modulated through outbreeding. Chapter 3 was intended to be a follow-up study to investigate this outbreeding hypothesis through generation of neopolyploids in a genetically variable background. However, surprising results that were highly divergent from all other hymenopteran polyploidy studies resulted in change of direction to investigate the mechanisms of polyploidization itself, a ubiquitous and in many aspects paradoxical evolutionary driver. Chapter 4 continued this research in a way that bridged the practical and theoretical by examining the biocontrol traits of induced polyploidy in a non-CSD species and the implications for application.

While a majority of this thesis deals with polyploidy in biological control and evolutionary contexts, a final research chapter addresses one of the most controversial aspects of biological control practice: host specificity and its relationship to non-target effects. Although it was previously preferable for a biological control agent to have a generalist strategy (to be broadly effective against multiple pests) (van den Bosch, 1971; Symondson, Sunderland, & Greenstone,
this approach has precipitated declines for a large number of benign or native species (Howarth, 1991; McEvoy, 1996; Louda et al., 2003). Disastrous non-target effects of the past have precipitated a legal and ethical requirement for biocontrol agents to be highly specialized and be assessed for risk pre-release (Bigler, Babendreier, & Kuhlmann, 2006). This translates to a loss of many potential agents that may be highly effective against a pest because they cannot be used if they exhibit any non-target effects (Louda et al., 2003; van Lenteren et al., 2006b).

A means to manipulate a biocontrol agent to be more host-specific is thus highly desirable. The key may be genetic in nature. There are many instances of closely related insect taxa that nevertheless contrast in their host preference. In several cases, an underlying responsible genetic mechanism has been identified (e.g. Gardiner et al., 2008; McBride et al., 2014; Keesey, Knaden, & Hansson, 2015; Auer et al., 2020). In parasitoid wasps, the first system for which a genetic link resulting in different host preference phenotypes has been identified is the genus Nasonia (Desjardins et al., 2010). However, the putative host specificity region is a formidable 16 Mb in length, and has numerous coding genes. The identity, function, and location of the exact gene(s) responsible are therefore unknown. Further study of this region has been complicated (and possibly stalled) by the need for highly regional, highly seasonal hosts to properly assay host specificity phenotypes (Desjardins et al., 2010). Chapter 5 adds new insights to our understanding of this region by narrowing the location of the gene(s) to a 4.1 Mb region that is enriched in odorant receptors, using a newly designed assay with easily accessible hosts.

All work of this thesis was conducted in the parasitoid wasp genus Nasonia. Polyploid work was exclusively in the most extensively studied species, Nasonia vitripennis, whereas host specificity work used both N. vitripennis as a generalist representative and Nasonia giraulti as a specialist representative. This introduction will thus first introduce Nasonia biology before delving into the subjects of polyploidy and host specificity underpinning this thesis.

Nasonia as a Biological Model

Nasonia is a genus of gregarious parasitoids of blowfly pupae with four described species: vitripennis, longicornis, giraulti, and oneida (Whiting, 1967; Darling & Werren, 1990; Raychoudhury et al., 2010; Werren et al., 2010). The first is a cosmopolitan species occurring worldwide, and the last three have distributions limited to North America (Darling & Werren, 1990; Desjardins et al., 2010; Raychoudhury et al., 2010). Populations of N. giraulti and N. oneida exist with some overlap in the Northeast, and N. longicornis is restricted to the Northwest (Lynch, 2015). All Nasonia species are commonly associated with birds’ nests, where they host on bird blowfly pupa feeding on the blood of nestlings (Werren & Loehlin, 2009a).

Nasonia has been used for studies in genetics, development, and behavior since the 1950s (Whiting, 1967; Lynch, 2015). This is due to the relative ease of rearing large cultures of wasps in a laboratory setting, large family sizes, and the ability to inbreed Nasonia to produce healthy
isogenic lines (Beukeboom & Desplan, 2003; Pultz & Leaf, 2003; Werren & Loehlin, 2009b; Werren et al., 2010; van de Zande et al., 2014) (Figure 2). Fertile hybrids can also be created within the Nasonia genus so long as strains are cured of their Wolbachia bacteria first, which normally act as a species barrier. This approach has been useful for delineating the genetic basis for the considerable interspecific variation in morphology and behavior (Werren et al., 2010). Such hybrid crosses have for example been useful in linking genetic loci to phenotype through introgression studies, with a specific gene region from one species to another inducing a phenotypic change (Weston, Qureshi, & Werren, 1999; Desjardins et al., 2010; Loehlin et al., 2010b; Hoedjes et al., 2014).

Like all hymenopterans, Nasonia has a haplodiploid sex determination system. Unfertilized haploid eggs develop into males and fertilized diploid eggs develop into females (Whiting, 1967; Verhulst, 2010). Mated females produce female-biased (80-90%) broods (Lynch, 2015), and virgin females produce all-male broods (Pultz & Leaf, 2003). The life cycle of Nasonia is similar for all species. Adult females arrest the development of hosts by injecting venom through the ovipositor. They then lay eggs that hatch in 24 hours. Larvae undergo four instars while feeding on the host before pupating approximately 8 days after oviposition. They eclose and emerge as mature wasps about 6 days later. This 14-16 day egg-to-adult development schedule occurs with rearing conditions of long day period and 25-28°C temperatures, but a reduction or increase of temperature can lengthen and shorten development times, respectively (Pultz & Leaf, 2003) (Figure 2).

For all species, males emerge earlier than females to maximize their mating competitiveness (Moynihan & Shuker, 2011). Newly emerged males release pheromones to attract virgin females (Ruther et al., 2007, 2010). They then court females by mounting them and probing their antennae in a series of head nods. Females signal receptivity by lowering their
antennae, which immediately prompts males to establish genital contact. After mating, males
display post-copulatory behavior, which works in conjunction with the virgin-attracting
pheromones to discourage females from mating again (Beukeboom, 1994; Ruther et al., 2007,
2010). Females mate soon after emerging and store sperm in the spermatheca to use throughout
their lifetime (Chirault et al., 2015, 2016; Ferree et al., 2019). They then disperse to new host
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Females also can induce their offspring to enter diapause, a period of temporary
developmental arrest (Saunders, 1966; Pultz & Leaf, 2003). Diapause is a strategy used by many
insects to survive seasonal cold, e.g. overwintering. For *N. vitripennis*, annual timing of diapause
brood production (the switch point) in natural populations varies according to regional
photoperiod (Paolucci, van de Zande, & Beukeboom, 2013). However laboratory strains produce
diapause broods when mothers are reared in dark and cool conditions to simulate seasonal
change (Pultz & Leaf, 2003; Werren & Loehlin, 2009b). These diapause broods must be kept in cold
storage (4°C and complete darkness) for at least 2 months, but can then be stored for up to 1.5
years (Werren & Loehlin, 2009b; Paolucci et al., 2013). They resume regular development when
exposed to room temperature (Pultz & Leaf, 2003).

While *Nasonia* has been used as biological model for over half a century (Whiting, 1967;
Werren et al., 2010; Lynch, 2015), advanced genetic resources have more recently become
available, facilitating genome-wide analyses linking loci to function, studies on gene expression,
and their regulation (e.g. Werren et al., 2010; Pannebakker et al., 2013; Sackton, Werren, & Clark,
2013; Wang et al., 2013; Ferree et al., 2015). Specifically, the genomes of three species, *N.
vitripennis*, *N. longicornis* and *N. giraulti*, have been fully sequenced for a decade (Werren et al.,
2010), and there are many tools for investigating gene function through null phenotypes
generated with RNAi knockdown (Lynch & Desplan, 2006) and CRISPR knockout (Li et al., 2017).
These properties have made *Nasonia* highly amenable for studies of polyploidy and host
specificity, both in the past and for this thesis.

**Polyploidy**

The majority of this thesis explores the short term and long term evolutionary effects of
polyploidy. Polyploidy is the condition of having more than the number of homologous
chromosomes sets (Comai, 2005). Polyploidy can either arise within a species (autopolyploidy), usually originating from unreduced gametes (Leitch & Leitch, 2008) or in a hybridization event of two species (allopolyploidy) (Comai, 2005). Polyploidy is prevalent in plants, as an estimated 57-70% (or possibly 100%) of angiosperm species (Otto, 2007) and almost all important domesticated crop species (Leitch & Leitch, 2008) are polyploid. In contrast, polyploidy has only been recorded in about 200 species of vertebrates (mostly fish and amphibians) and insects, although many more examples are known in other invertebrates (Comai, 2005; Otto, 2007).

As plant polyploidy is far more common and commercially relevant than animal polyploidy, it is not surprising that it is far more extensively studied (Mable, 2003, 2004; Comai, 2005; Choleva & Janko, 2013; Wertheim, Beukeboom, & van de Zande, 2013). These studies have been facilitated by readily available means to artificially induce plant polyploidy (Ranney, 2006). While stable polyploidy occurs frequently in some animal groups, generally polyploid animals (particularly those with an odd ploidy number) are infertile (Mable, 2004; Mable, Alexandrou, & Taylor, 2011). These lines cannot be maintained, so opportunities to study the effects of animal polyploidy extending beyond a single generation are limited.

Independent of the logistic difficulties, animal polyploidy being under-researched is also due to it being in many ways a catastrophically deleterious condition. In sexually reproductive species, it is associated with sterility, negative epigenetic interactions, and problems of unbalanced chromosome segregation during cell division, which produces aneuploid cells (Mable, 2004; Wertheim et al., 2013). Accordingly, it has long been believed that that animal polyploidy is a rare event, and that animal polyploid evolution is defined primarily by stabilization of the polyploid state. For these reasons, polyploidy has long been considered an evolutionary dead end, and insignificant to long-term animal evolutionary trajectories (Muller, 1925; Stebbens, 1950, 1971; Wagner, 1970).

Recently there has been a paradigm shift recognizing polyploidy as a major evolutionary driver. It is in the ancestry of most eukaryotic branches in the form of whole genome duplications followed by subsequent re-diploidization, including many insect orders (Li et al., 2018) and all vertebrata (Ohno, 1970, 1999) (Figure 3). The prevalence of polyploidy in the Tree of Life corresponds to its apparent ability to drive speciation, confer resistance to abiotic stress, and enable evolution of more complex gene networks through neo- and subfunctionalization of additional gene copies (Comai, 2005; Choleva & Janko, 2013; Wertheim et al., 2013; Mable et al., 2018). There has been an ensuing flurry of calls to address the long-delayed question, how are the extreme problems of animal polyploidization modulated as downstream advantage emerges (Mable, 2003, 2004; Comai, 2005; Soltis, Soltis, & Buggs, 2010; Madlung, 2013; Wertheim et al., 2013; Spoelhof, Soltis, & Soltis, 2017; Baduel et al., 2018)? The following reviews why polyploidy is immediately harmful, how it can ultimately be evolutionarily advantageous, and our current lack of knowledge on how these two states are bridged.
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Figure 3. Polyploidy prevalence throughout Eukaryota. Red x2 indicate an ancient whole genome duplication (WGD) event (frequently followed by re-diploidization), notably two WGDs at the base of vertebrata. Numbers estimate species within a group (figure taken from http://ohnologs.curie.fr/, compiled using the references within; accessed 16 September 2019).

The detriments of polyploidization

The immediate detriments of polyploidization are 1) nuclear and cellular enlargement, 2) aneuploid cells from irregular mitosis and meiosis and 3) negative epigenetic instability from non-scalar modifications of gene regulation (reviewed by Comai, 2005). For the first, an increase in genomic content increases the surface area of the nucleus, but not in a locked ratio with volume. For example, when genomic content and nuclear volume doubles, surface area only increases by a factor of 1.6. This perturbs the stoichiometry of interactions between chromatin and nuclear membrane proteins and cell structure proteins (Comai, 2005). This problem of cell geometry may also lower metabolic rates, slowing development time and decreasing adult size (Choleva & Janko, 2013).

Cell division in polyploids is complicated by the formation of abnormal spindles that result in aneuploids, cells with irregular chromosome numbers. This can cause meiotic lethality, and since aneuploid gametes vary in fertility, there may be degrees of polyploid sterility (Comai, 2005). Aneuploids may also lead to epigenetic instability through dosage imbalance of gene expression regulatory factors, changes in imprinting patterns, and exposure of unpaired chromatin regions to abnormal epigenetic effects during meiosis (Osborn et al., 2003; Comai, 2005; Choleva & Janko, 2013).

Outside of aneuploid effects, it might be expected that relative gene expression patterns would persist in polyploids, as every gene of the genome has been copied. However, many genes are subject to regulatory factors that do not scale proportionately with ploidy. While not well-
studied, these deviations are presumably deleterious in neopolyploids because parental expression patterns were optimized under selection (Comai, 2005). These negative epigenetic interactions may be particularly pronounced in species with separate sexes (Wertheim et al., 2013). Dioecious species have dosage compensation mechanisms to balance gene expression in the heterogametic sex with the degenerate chromosome, and these can be disrupted by polyploidization (Wertheim et al., 2013). Dioecy being more common in animals than plants may partially account for the large number of successful plant polyploid species versus the few successful animal ones (Muller, 1925; Orr, 1990; Mable, 2004; Wertheim et al., 2013).

**The evolutionary advantages and applications of polyploidy**

Despite the challenges of polyploidy, genome duplication events can be and have been advantageous to many evolutionary lineages, and can frequently be stabilized (Wertheim et al., 2013). Research on gene ancestry indicates that following polyploidization, a neopolyploid species typically undergoes rapid diplloidization, or the process of inactivating or modifying duplicate genes, reducing redundancy (Comai, 2005). However, having multiple gene copies expands the organism’s evolutionary toolbox. One gene copy may retain an essential function, for example, while the other may be altered in order to provide an additional function that enhances the organism’s fitness (Wertheim et al., 2013; Mable et al., 2018). Multiple gene copies can mask deleterious recessive genes while the population is undergoing a bottleneck. This theoretically assists the population’s survival until there are enough individuals for deleterious alleles to be selected out (Comai, 2005).

The epigenetic modifications discussed in the previous section may also not be wholly disadvantageous, particularly when there are pathways in place to restore transcriptomic balance (Adams et al., 2003; Wertheim et al., 2013). Interestingly, there is evidence that this process does not target whole haplomes; silenced gene copies can vary by tissue type (Pala, Coelho, & Schartl, 2008). This additional level of epigenetic variation could represent another source of evolutionary potential. For example, it is possible that polyploidy provides an advantage in environments of high abiotic stress. There is a greater incidence of polyploid plants in the arctic, and polyploid amphibians and fish in temperate freshwater ecosystems (which have greater seasonal variation than oceans) (Comai, 2005; Wertheim et al., 2013).

Polyploidization is generally a gentler process for plants than animals (Muller, 1925; Mable, 2003, 2004; Wertheim et al., 2013). Agriculture has greatly benefited from this greater tractability of plants to polyploidy. Polyploidy is often preferentially bred or induced in many domesticated plants to stabilize a hybrid, increase size, or to introduce sterility to better control propagation (Ranney, 2006). Since the 1930s, new plant polyploids have been reliably generated with mitotic inhibitors such as colchicine (Ranney, 2006). However, equivalent technologies do not exist for animals, and there have been very few applications for animal polyploidy thus far. An exception is in aquaculture, where fish and shellfish are intentionally produced to increase size, improve taste, and induce sterility to prevent interbreeding with natural populations (Rasmussen & Morrissey,
This may be because animal polyploidy generally results from cell division errors that are lethal or ultimately result in sterility, which limits its use in animal breeding (Fankhauser, 1945).

“The missing bridge in polyploid evolution”

A major enigma in polyploid evolutionary theory is how a highly deleterious condition that greatly heightens the likelihood of extinction is actually co-opted into a major evolutionary advantage in what has been described as a high risk, high reward strategy (Baduel et al., 2018). Little is known about the mechanisms by which barriers to polyploid establishment are overcome (Escudero et al., 2014; Baduel et al., 2018). Chief among these barriers are, larger cell size; disturbed epigenetics, with extreme consequence for dosage mechanisms in sexual species; and sterility due to improper meiotic alignment (reviewed by Comai, 2005; Baduel et al., 2018). How are these problems modulated, both immediately following polyploidization and over time?

Polyploidy is often positively related to size, but this mainly holds for plants, whereas in animals the results are inconsistent. Some groups appear to have larger body sizes (e.g. rotifers, molluscs; Walsh & Zhang, 1992; Guo & Allen, 1994; Piferrer et al., 2009; Zhou & Gui, 2017) but others do not (e.g. amphibians and fish (Fankhauser, 1945; Legatt & Iwama, 2003). There is some evidence that vertebrate polyploids undergo cell number reduction to compensate for larger cells (Cavalier-Smith, 1978; Hessen, Daufresne, & Leinaas, 2013), but whether this also applies to invertebrates, implying a general animal polyploid adaptation, has been ambiguous (Fankhauser, 1945). Thus the link between ploidy level and cell number and size is still unclear, but it is not an inconsequential matter. Unregulated, the larger size of polyploid cells can have severe consequences ranging from imbalanced surface area to volume stoichiometry of cells and organs (Olmo, 1983; Conlon & Raff, 1999; Kondorosi, Roudier, & Gendreau, 2000) to gigantism beyond an organism’s biophysical limits (Fankhauser, 1945; Guo & Allen, 1994).

How a neopolyploid organism copes with a sudden increase in genomic material is one of the biggest questions of polyploid biology (Yoo et al., 2014; Baduel et al., 2018). It has been intuitively assumed that gene product is simply multiplied by the appropriate copy number to maintain stoichiometry, but there have been almost no studies to support this (Coate & Doyle, 2010, 2015; Visger et al., 2019). Accordingly, there is very little understanding on how gene expression is regulated in polyploids, and whether it scales to ploidy level, sex, or organ function (Visger et al., 2019). In addition, dosage compensation mechanisms vary between organismal groups (e.g. mammals versus birds versus insects, reviewed by Ercan, 2014). However, a polyploidization obstacle that might be more stringent for some taxa than others is developmental constraint such as the mode of sex determination (Muller, 1925; Orr, 1990) or dosage compensation (Wertheim et al., 2013). Accordingly, the mechanism of sex determination has been proposed as one possible reason for why polyploidy is rarer in animals than in plants (Muller, 1925; Orr, 1990; Mable, 2004). In sexual animals with heterogametic sex determination systems numerical alterations of sex chromosomes may be detrimental due to disruption of dosage...
compensation. There is little understanding on how the apparent transcriptomic shock could be overcome.

The downstream benefits of polyploidy are manifold, so arguably it would be advantageous for all lineages to undergo polyploidization if they have the means to bypass its disadvantages. Despite an apparent evolutionary advantage, it is unknown why polyploidy is prevalent in some groups and virtually absent in others. The invertebrates are far more enriched in both ancestral and neopolyploid species than vertebrates (Otto & Whitton, 2000; Comai, 2005; Otto, 2007) (Figure 3), but existing animal polyploid research has been heavily biased towards fish and amphibians (Fankhauser, 1945; Mable, 2003; Bogart & Bi, 2013). This is possibly due to their popularity as models, but this translates to a striking dearth of knowledge of polyploid evolution in the taxa for which it is most common. It has repeatedly been suggested that variation in polyploid mechanisms must impact the severity of these phenotypes and thus determine an organism’s likelihood of success versus extinction. However, a lack of empirical evidence and the few experimental means to generate it has been concurrently emphasized (van de Peer, Maere, & Meyer, 2009; Soltis et al., 2010; Choleva & Janko, 2013; Madlung, 2013; Spoelhof et al., 2017).

Polyploidy in Hymenoptera

Hymenopteran polyploidy is atypical in several ways. In all other animals, normal (non-polyploid) individuals are diploid, and polyploids have an aberrant higher ploidy level. As hymenopterans (the bees, wasps, ants, and sawflies) have haplodiploid sex determination, polyploid hymenopterans are diploid males and triploid or tetraploid females (higher level males and females have not yet been observed). Hymenoptera is far more prone to polyploidy than most animal groups, with neopolyploids being observed in over 80 phylogenetically diverse species (van Wilgenburg, Driessen, & Beukeboom, 2006; Heimpel & de Boer, 2008; Harpur, Sobhani, & Zayed, 2013). Therefore, the Hymenoptera are uniquely suited for advancing knowledge of animal polyploidy: different ploidy levels being normal to the sexes may be exploited to understand mechanisms of intraspecific ploidy variation, and the abundance of species for which neopolyploids exist can be used to study how higher ploidy impacts sex determination and dosage (Muller, 1925; Wertheim et al., 2013). For example, it is not clear whether dosage compensation mechanisms exist in haplodiploids (Rasch, Cassidy, & King, 1977; Aron et al., 2005; Wertheim et al., 2013; Glastad et al., 2014), but studying this across ploidy and sex can lead to broader inference on animal polyploid evolution.

Most of what is known about hymenopteran polyploidy is linked to complementary sex determination (CSD), the best known and most widely studied form of hymenopteran sex determination. In CSD species, individuals that are hemizygous for a CSD locus or loci develop into haploid males, heterozygotes develop into diploid females, and homozygotes develop into diploid males. While it has been previously suggested that animal polyploidy has incurred less interest than plant polyploidy because it has less applied significance (Fankhauser, 1945), CSD-linked polyploidy is an exceptional case. Diploid males of nearly all CSD species are sterile (Cowan &
Stahlhut, 2004). When csd allelic diversity is lost, e.g. from small initial sampling, drift, or inbreeding (all common phenomena in breeding for biocontrol or captive breeding of endangered insects) more and more diploid males arise. Females, which are usually monoandrous, do not discriminate against them in favor of fertile haploid males (Harpur et al., 2013), so the population becomes smaller and inevitably goes extinct. This “diploid male vortex” has been a focal point of hymenopteran polyploidy, with particular emphasis on its negative applied implications (Zayed & Packer, 2005; Hein et al., 2009; Fauvergue et al., 2015; Faria et al., 2016; Zaviezo et al., 2018) (Figure 4).

Nevertheless, on an individual level, hymenopteran individuals seem to tolerate neopolyploidization better than many animal groups, although the reasons why are unknown. This makes hymenopterans highly suitable for studying mechanisms by which archetypal polyploid disadvantages are mitigated. However, most hymenopterans are still sterile, and a system that can be bred for continuously is needed to study how polyploidy can persist and benefit a lineage.

**Polyploid Nasonia**

Most of the parasitoid wasps, the group most broadly used in biological control, have a non-CSD sex determination mechanism (Beukeboom, Kamping, & van de Zande, 2007), but polyploidy has been hardly studied for these species. For non-CSD species, polyploidy does not arise through inbreeding, but not much else is known about their polyploid phenotypes.

The only species for which a non-CSD mechanism (maternal epigenetic genomic imprinting sex determination, or MEGISD) has been well defined and polyploids have been studied is the chalcid wasp *N. vitripennis*. In the 1950s, spontaneous triploids and tetraploid mutants appeared in Whiting’s *N. vitripennis* stocks (Whiting, 1960) and are, unusually, reproductively competent polyploids. A derived Whiting Polyploid Line (WPL) has since been maintained as a research resource. From this line, it is known that polyploid females lay many eggs, but most are aneuploid and shrivel (Whiting, 1960; Beukeboom & Kamping, 2006). Viable eggs fertilized by haploid males produce diploid (fertile) or triploid (low fecundity) daughters. Unfertilized viable eggs develop into fertile haploid and diploid sons (with haploid and diploid sperm, respectively) (Whiting, 1960). A low percentage of triploid females (~2%) also occasionally produce females and gynandromorphs from diploid unfertilized eggs (Beukeboom & Kamping, 2006).
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Polyploid *N. vitripennis* present a rare system to study animal polyploidy in a multi-generational context. They have previously been primarily used to investigate mechanisms of the haplodiploid sex determination system (Dobson & Tanouye, 1998; Beukeboom & Kamping, 2006; Beukeboom *et al.*, 2007b; van de Zande & Verhulst, 2014). They have been instrumental in determining how parental chromosome origin and ploidy number influences gender development and viability. A recent study also used polyploids to study dosage and dominance effects on hybrid incompatibility (Koevoets *et al.*, 2012). However, polyploid biology *Nasonia* has not been well studied despite evidence that their life history differs from euploids in several major ways.

For example, as previously mentioned, triploid females have lower fecundity because of aneuploid eggs, although diploid males are highly fertile and produce large numbers viable offspring (Whiting, 1960). Also, triploid females and diploid males are generally larger than their diploid and haploid counterparts, and diploid males exhibit abnormalities of body pattern (Whiting, 1960). However, these differences are not always pronounced enough to reliably distinguish polyploids from euploids (Whiting, 1960; Beukeboom & Kamping, 2006), which is why eye color markers are used in WPL maintenance.

Besides this WPL line, which has been maintained in an inbred state for decades, it is also possible to generate *Nasonia* polyploids *de-novo* in any background by knocking down sex determination genes of *Nasonia*’s MEGISD pathway. In MEGISD, several genes are essential to feminization: *transformer* (*tra*), *transformer*-2, and *womanizer* (Verhulst, 2010; Verhulst *et al.*, 2013; Geuverink *et al.*, 2017). Female pupae that are injected with double stranded RNA for any of these genes and are then mated with normal males as adults produce diploid offspring that are male instead of female because the feminization pathway was deactivated. Diploid males are then crossed with diploid females to create triploid females, and a new polyploid line that can be stably bred is founded (Figure 5). Together with the WPL, *Nasonia* is ideal for posing the questions, what are the effects of polyploidy in non-CSD hymenopterans? How do traits in a long-standing polyploid line differ from neopolyploid lines, and what are the consequences of polyploidization mode? What are the immediate effects of polyploidization, and what can we infer about polyploid evolution from them?

![Figure 5. Components of the *Nasonia* MEGISD system. RNAi knockdown of targets in bold deactivates the feminization pathway and generates diploid males. These diploid males produce triploid daughters, and are used to establish new polyploid lines. Circled *tra* was targeted in Chapters 3 and 4 of this thesis (figure adapted from Geuverink 2017).](image-url)
Host specificity

The second topic of this thesis is host specificity. While the unintended ecological effects of biocontrol have been a major concern in the field since the 1980s (e.g., Howarth, 1983, 1991; Lockwood, 1996), biocontrol-based solutions are an important pest control method in agriculture. Although agricultural pests are still widely suppressed using pesticides or genetically modified organisms (e.g. transgenic crops with *Bacillus thuringiensis* toxin genes), these are often cited as expensive to purchase or develop, and harmful to human and environmental health (Roderick & Navajas, 2003; Bale *et al.*, 2008; Hertz-Picciotto *et al.*, 2018). Therefore, it is crucial that one of the biggest stumbling blocks to wider biocontrol implementation, the ecological risk of non-target effects, is resolved.

Non-target effects on native species vary in degree from not affecting the population density of the accidental target (Barratt *et al.*, 2010), to directly causing a species’ extinction (Howarth, 1991; Lockwood, 1996; Messing & Wright, 2006). It can be difficult to interpret how great of an ecological risk non-target effects pose overall. For example, two contrasting views on non-target effects have arisen in the case of Hawaii. In the Hawaiian Islands, biological control is commonly used because of a large agricultural industry and an abundance of introduced pests, but its use is controversial because of its perceived threat to a high diversity of endemic plant and animal species (Messing & Wright, 2006). Funasaki *et al.*, (1988) claimed that a vast majority of biocontrol programs have protected crops for over a century without any interactions with native species. Others rejected this view and held biocontrol directly responsible for the decline or extinction or endemic species such the koa bug, multiple moths, and Oahu tree snails (Howarth, 1991; Hadfield *et al.*, 1993; Henneman & Memmott, 2001). In response, some authors have indicated that significant non-target effects in Hawaii and worldwide are actually incredibly rare based on existing data (Messing & Wright, 2006; Bale *et al.*, 2008). Nonetheless, non-target effects are often at the crux of environmental objections or legal obstacles to biological control programs (Howarth, 1991; Lockwood, 1996; McEvoy, 1996; Louda *et al.*, 2003). Ensuring the prevention of non-target effects is a necessity for future biocontrol programs (Thomas & Willis, 1998; Louda *et al.*, 2003; Barratt *et al.*, 2010).

*Host specificity in Nasonia*

High host specificity, or the tendency of parasitoids to use only one or a few species as hosts (McEvoy, 1996), is a critical trait for candidate biological control agents because it limits the likelihood of non-target effects. The term host preference may suggest a more nuanced definition implying favoring a host species out of multiple viable options, but in this thesis the term host preference and host specificity are used interchangeably. Notably, a previous study identified a putative host specificity region that was introgressed from *N. giraulti* into *N. vitripennis*, switching the latter from a generalist host to a specialist host (Desjardins *et al.*, 2010).
The generalist species *N. vitripennis* can complete its life cycle successfully on many species of blowfly across multiple genera, and does so naturally across its broad Holarctic range (Desjardins *et al.*, 2010; Rivers & Losinger, 2014). In contrast, all other *Nasonia* species, despite being readily adapted to a number of factitious hosts in artificial conditions, strongly prefer to host on *Protocalliphora* flies in the wild where they co-occur in parts of North America (Darling & Werren, 1990; Desjardins *et al.*, 2010). The males of *N. vitripennis*, the most phylogenetically basal species (Loehlin, Enders, & Werren, 2010a), have highly reduced wings and are flightless. The male wings of all other species are closer in size to those of large-winged, mobile females (Loehlin *et al.*, 2010a). A study that introgressed the wing size QTL *wing size 1* (*ws1*) from *N. giraulti* into *N. vitripennis* inadvertently lead to discovery of a proximate gene region controlling host specificity. The *ws1* introgression line had difficulty parasitizing *Sarcophaga bullata* hosts used for general rearing (Weston *et al.*, 1999). In a follow up study with a line deliberately bred to target host specificity *bbbwg* (named for its physical markers from *N. giraulti*, black eyes (bk) and big wings (bg)), a single *N. giraulti* gene region was capable of producing a specialist phenotype (preference for *Protocalliphora* sp.) in an genomic background that was otherwise entirely *N. vitripennis* (Figure 6).

**Figure 6.** The *Nasonia* host specificity region *bbbwg*. The dominant specialist (*N. giraulti*) allele of a putative *Nasonia* host specificity gene(s) host preference 1 (*hp1*) is sufficient to induce a specialist host specificity phenotype in an otherwise generalist (*N. vitripennis*) background. It exists somewhere in *A*) close to the centromere of chromosome 4 (shown here with additional phenotypic markers) in between *ws1* and *bk576* (in red), in a **B**) 11-16 MB region. Creation of the *bbbwg* introgression line isolated this region from *N. giraulti* in the pe333 “peach” *N. vitripennis* line background. Phenotypic markers from *N. giraulti, wing size 1* (*ws1g*) and *black body* (*bkg*) allow for tracking inheritance of the specialist *bbbwg* region. The region is maintained heterozygously because in the original study, homozygous females failed to sting and oviposit hosts. To maintain the line, *C*) peach-eyed, large winged heterozygous females produce male haploids carrying the specialist *bbbwg* with large wings from *ws1g* and white eyes from *bkg* (due to an epistatic interaction with *N. vitripennis pe333* on chromosome 1). These males are crossed back to heterozygous females to recoup the heterozygous female and restart the breeding cycle.
This bkbw_g region was estimated to be 11-16MB in size. Due to its location close to the centromere of chromosome 4, a region of low recombination (Niehuis et al., 2010; Desjardins et al., 2013), it was difficult to narrow the region further. It was proposed that the host preference gene hp1 exists somewhere in this region, but its identity, function, and exact location were not delineated. Complicating further study, assays depended on the availability of both a generalist host choice (in the original study, Sarcophaga bullata) and the preferred host of the specialist (Protocalliphora spp.) to demonstrate a phenotypic change. The latter host however is challenging to work with because it must be collected during the limited bird nest season from a specific geographic region in North America. Furthermore, extensive work is needed to acquire even a low number of hosts (Werren and Desjardins, personal comm.) We combined a newly developed assay exclusively using the commercially available factitious host Calliphora sp. with further genetic investigation of bkbw_g to provide new insights on hp1.

Thesis overview

Part of this PhD research was conducted under the EU funded Marie Curie Initial Training Network (ITN), Breeding Invertebrates for Biological control (BINGO). This project began as one of thirteen biological control-themed projects across seven European countries hosted at academic, industrial, and NGO partners. This ITN was formed in recognition that biological control breeding and practice with established populations must be improved in light of international trade law limiting the import of new biocontrol agent candidates (Cock et al., 2010; Lommen, de Jong, & Pannebakker, 2017; Deplazes-Zemp et al., 2018; Kruitwagen, Beukeboom, & Wertheim, 2018). The root inspiration of biological control is apparent in much of the work of this thesis, even as the polyploid work also branched into the more fundamental realm of evolutionary theory.

In Chapter 2 I assay the baseline biology of the decades old, inbred Whiting Polyploid Line (WPL) of N. vitripennis as the first perspective of polyploid effects in a non-CSD hymenopteran species. This was done partially in the context of assessing the downstream impacts on biocontrol breeding and practice. I found that WPL polyploids resemble non-polyploids for many traits, having similar lifespans, first-mate fecundity and offspring sex ratio, male mate competition ability, and size. However, I also found some significant defects in the polyploids including previously unobserved fecundity reduction in diploid males and highly truncated parasitization ability in triploid females. A single generation of outbreeding appears to mitigate some of these detriments, suggesting that inbreeding, while it does not cause polyploidy in itself such as in CSD species, may exacerbate some of its disadvantages.

In Chapter 3 I continue polyploid research in the WPL but also a neopolyploid line generated with RNAi knockdown of tra in the genetically variable lab N. vitripennis population HVRx (the tra knockdown line, or tKDL). Surprisingly, the results of this study diverged strongly from those of Chapter 2. Although diploid males of the tKDL background are highly fecund like WPL males, they were severely handicapped against haploid males in competitions for female mates. This phenotype persisted for several generations, indicating that it is a heritable polyploid
phenotype and not a single generation RNAi effect. This is the first instance of a diploid male being strongly discriminated against by female mates for any hymenopteran species. Triploid tKDL females had up to 10 times more offspring than their WPL counterparts, suggesting a means to circumvent the stereotypical aneuploidy of polyploidization that acts as one of the strongest barriers to the establishment of polyploid lineages. Cell reduction mechanisms were also observed in WPL as a rare observation this adaptation against gigantism in an invertebrate, but they were absent in the tKDL background. Furthermore, contradicting several existing studies on haplodiploid dosage compensation, sex-linked dosage mechanisms were observed as absolute expression of housekeeping genes ef1α and ak3 is conserved by sex and does not scale to ploidy. This study is the first evidence for the oft-proposed but previously never empirically tested hypothesis that multiple routes to polyploidization correspond to a gradient in polyploid phenotypes, and thus, determine the likelihood of extinction versus evolutionary success. Factoring the additional potential of tra2 KD and wom KD polyploid lines, N. vitripennis represents the best-suited system so far for understanding why polyploidy factored so heavily as an evolutionary force across Animalia and Eukaryota.

In Chapter 4 I continue to study tKDL from the viewpoint of investigating the impacts of polyploidy on the traits important to the biocontrol performance of non-CSD species, the most prevalent class of biocontrol agent. Body size and lifespan was greater for male polyploid than non-polyploids, but the opposite was observed for females. However, in sperm depletion assays in which males were provided a series of female mates, diploids have reduced sperm count relative to haploids. This difference in fecundity was not detectable in single crosses, indicating the need for more extensive assays to uncover fitness costs in so-called fertile diploid hymenopteran males. Also, although triploid tKDL females had much higher fecundity than WPL triploid females, this did not rescue poor parasitization. This suggests that offspring number is not an important factor in host killing, and that non-reproductive deficiency underlies the weak parasitization ability of triploid females (possibly venom attenuation). Additionally, tra knockdown females have increased production of diapause offspring, an effect that is partially inherited in a subsequent generation. This may have implications for developing biological control strains that are more amenable to long-term storage.

In Chapter 5 I switch to the genetics of host specificity. I first developed a new host specificity assay that did not require the hard-to-obtain Protocalliphora sp. specialist hosts of the original study. Specifically, testing with purebred lines, I found that N. vitripennis as a generalist has a higher parasitization rate on commercially purchased European host species Calliphora sp. than the specialist N. giraulti. A parasitization rate approximating that of N. vitripennis was considered a generalist phenotype, and the same for N. giraulti for a specialist phenotype. Next, a number of bkbw8 recombinant males were generated, representing individuals that all had a reduced bkbw8 region, but with some retaining the hp1 and others losing it. These males were then mated to purebred N. vitripennis and N. giraulti and their female offspring assayed for host specificity with the new assay. The males were then scored for a panel of 13 indels distributed
across bkbw_9 with each having different banding patterns for *N. giraulti* (G) and *N. vitripennis* (V) alleles. The indel genotypes of each female were cross-referenced to their parasitisation data, and it was found that only indel 9 showed a significant difference in phenotype for VV versus VG and GG phenotypes. The gene controlling host specificity, *hp1*, was thus narrowed to a 4.1MB region we call bkbw_9. Bioinformatics analyses of the annotated *N. vitripennis* genome recovered N=294 genes in bkbw_9 that could be *hp1*, but most notably N=21 odorant receptor genes, which in other (non-parasitoid) systems have been implicated in host specificity. Furthermore, bkbw_9 is the most odorant receptor enriched region in the *Nasonia* genome, suggesting that they are the functional basis for this trait.

Chapter 6 is the conclusion of this thesis, summing the biological control and evolutionary theory implications of the polyploid chapters. It expands on the many directions this research can take, including identifying specific mechanisms of polyploid adaptation across Eukaryota that allowed polyploidy to be such an important factor in evolution despite its apparent disadvantages. I also propose means to continue the identification process for the parasitoid host specificity gene *hp1*.