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RESEARCH ARTICLE

A test of the haploid susceptibility hypothesis using a species with naturally occurring variation in ploidy

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Abstract The haploid susceptibility hypothesis (HSH) was proposed as an explanation for how behavioral roles in haplodiploid social systems evolved. It posits that haploid males are more susceptible to disease than diploid females due to decreased genetic variability at key disease resistance loci. The resulting decreased immunocompetence is hypothesized to have played a role in the evolution of social behavior by limiting the behavioral repertoire haploids perform. Here, we test this hypothesis in a study system that separates ploidy from behavioral sex roles: *Polistes dominulus*, a social wasp, has colonies with naturally occurring diploid males. We report results from two immune function assays—hemolymph phenoloxidase activity and encapsulation response—performed on haploid males, diploid males, and diploid females. Our data suggest that ploidy is not a significant contributor to immune function in *P. dominulus*; thus, our data do not support the HSH for the evolution of behavioral roles. Instead, our data indicate that time of emergence is the best predictor of immune function in *Polistes*. We speculate that seasonal trends result from seasonal differences in pathogens and parasites.

Keywords Phenoloxidase · Encapsulation · Invertebrate immunology · Ecological immunology · Diploid male · Hymenoptera

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Introduction

Eusociality, a social system characterized by overlap of generations, cooperative brood care, and reproductive division of labor, is both widespread and complex in the aculeate Hymenoptera (Wilson, 1971). Eusociality in Hymenoptera is distinguished by sexual dimorphism in behavioral roles, with entirely female worker castes and reproductively specialized males. Hamilton (1964) proposed that workers are exclusively female due to the haplodiploid genetic system of the Hymenoptera, which results in higher relatedness between sisters than between other kin associations, thus increasing the inclusive fitness benefit for females to be workers.

However, the relatedness asymmetry explanation for the behavioral differences between male and female social Hymenopterans has been challenged by alternatives (Strassmann and Queller, 2007), among them a suggestion that sexual dimorphism in behavioral roles evolved due to inherent differences in susceptibility to disease (O'Donnell and Beshers, 2004). It is thought that heterozygosity in disease resistance loci improves immune response to pathogens; haploid males cannot be heterozygous, and are thus proposed to be more susceptible to disease than diploid females. This increase in susceptibility would render males less likely to engage in typical worker tasks due to the increased probability of, for example, infecting brood.

Prior investigations of immunocompetence between haploids and diploids suggest haploids are more susceptible to disease (van Zon et al., 1964; Moret and Schmid-Hempel, 2001; Santillán-Galicia et al., 2002; Gerloff et al., 2003; Vainio et al., 2004). However, tests of the haploid susceptibility hypothesis (HSH) have proved inconclusive, most likely due to difficulties with confounding factors relating to sex and caste (hypothesis supported: Baer et al., 2005; Baer

and Schmid-Hempel, 2006; Huth-Schwarz et al., 2012; hypothesis not supported: Ruiz-González and Brown, 2006; Rutrecht and Brown, 2008). Baer and colleagues (2005) pointed out that immune response is energetically costly and that males may be investing in reproduction at the expense of immunocompetence; thus, the behavioral differences between males and females are themselves a confound for the effects of ploidy.

These confounding factors are not insurmountable. *Polistes dominulus* is an invasive wasp in North America. Invasive populations are genetically diverse due to multiple introductions, with heterozygosity comparable to that in their native range (Johnson and Starks, 2004; Liebert et al., 2006). In addition, in the North American range, nests of *P. dominulus* are susceptible to brood parasitoids (Madden et al., 2010). All of these characteristics make *P. dominulus* a suitable study system for the genetics of immunology. However, this organism provides a unique opportunity to test the HSH because it produces both haploid and diploid males (Liebert et al., 2004, 2005), thus allowing us to disentangle sex from the analysis.

Ploidy and sex are thought to be determined in *P. dominulus* via single-locus complementary sex determination (CSD), whereby hemizygous individuals are male and heterozygous individuals are female (Tsuchida et al., 2002). Occasionally, homozygous individuals are produced that result in the male phenotype (see Liebert et al., 2004, 2005). Diploid males are often produced early in the colony cycle during the worker phase, alongside early haploid males, which helps mitigate the caste issue in a system where season of eclosion influences role ('worker' versus 'reproductive') (Mead et al., 1990). The presence of functional triploid offspring provides evidence that diploid males occupy the same behavioral role as early haploid males, copulating with reproductive females and fertilizing their eggs (Liebert et al., 2004, 2005).

The frequency of naturally occurring diploid males, and the apparently normal behavior of these males, allows for a unique opportunity to test the HSH by disentangling the effect of ploidy from behavior. In this paper, we use measures of immunocompetence to compare disease susceptibility between haploid males, diploid males, and diploid females in *P. dominulus*.

Methods

Immunocompetence is the ability of an organism to mount an immune response (Owens and Wilson, 1999). The degree of immunocompetence is a strong indicator of the degree and effectiveness of the host's ability to thwart pathogens and parasites (Pye, 1974; Carton and David, 1983; Leonard et al., 1985; Ochiai and Ashida, 1988; Paskewitz and Riehle, 1994; Gorman et al., 1996; Washburn et al., 1996; Ashida and Brey, 1997; Doums and Schmid-Hempel, 2000; Siva-Jothy, 2000; Kraaijeveld et al., 2001a, b; Trudeau et al., 2001; Wilson et al., 2001; Beck and Strand, 2007). We measured the immunocompetence of individual wasps using two complementary tests of cellular and humoral immune function: hemolymph phenoloxidase activity and encapsulation response to a novel foreign body.

Specimen collection

Between 2007 and 2009, and from sites across Massachusetts, southern New Hampshire, and southeastern New York, we collected 106 *P. dominulus* nests and 411 individuals at fixed time points during the beginning and end of the colony cycle. Nests were identified visually for newly emerging *P. dominulus* wasps. Each nest was immediately transported to the International Social Insect Research Facility (ISIRF) at Tufts University, Medford, MA. To control for unknown effects on immune function, foundresses were removed from the nest because of their unknown pathogen exposure history. All pupae were allowed to eclose as adults and tested with our two assays at simultaneous time intervals, to standardize age among individuals. Caste was standardized by time of collection, as behavioral role in this species is determined by the colony stage at eclosion.

A range of 1–30 individual *P. dominulus* adults eclosed per colony (mean $\pm$ SD $= 3.9 \pm 4.6$ wasps). Each new adult was fed a diet of 50 % sucrose solution and waxworm larvae ad libitum. The encapsulation assay occurred 24 h post-eclosion. The wasps were then allowed another 24-h period for hardening and darkening of the cuticle before they were frozen and used for genetic and phenoloxidase assays. This step enabled us to control for the form of the phenoloxidase enzyme (PO) we assayed during tests of immune function (hemolymph PO and not wound PO or granular PO, which are both used in cuticular development; see Ashida and Brey, 1997). No parasitism or outward sign of disease was noted on any wasp or nest included in this study. Of note, not all wasps collected were included in the genetic, encapsulation, and PO assays due to a variety of factors, including survival, hemolymph quantity and quality, and grooming out implants.

Immunology

Encapsulation response (ER)

Twenty-four hours post-eclosion, we performed a standard encapsulation response assay as a direct measurement of each wasp's ability to neutralize a foreign body using methods developed by König and Schmid-Hempel (1995), Wilson-Rich and colleagues (2008), and Wilson-Rich and

Starks (2010). We elicited an ER by inserting a nylon 'pseudoparasite' (Cox-Foster and Stehr, 1994) between each wasp's abdominal sternites to mimic the behavior of Strepsipteran endoparasites within the genus *Xenos*. *Xenos* are parasites of *Polistes* that protrude through the intersegmental membrane and reduce host fitness (Beani, 2006). We then removed the pseudoparasite and used digital imaging to quantify melanin deposition on the nylon monofilament.

Hemolymph collection

Hemolymph was collected 48 h after eclosion by first ice anesthetizing individual wasps and then piercing the abdomen using sterile technique. The resulting drop of liquid was collected, and any fluid which appeared yellow or brown was discarded to avoid gastric or other non-hemolymph fluid. One microliter of hemolymph was collected and transferred to a microcentrifuge tube (0.5 ml, BD Falcon) containing nine microliters of sterile phosphate-buffered saline and frozen until later PO analyses.

Phenoloxidase activity

We assayed PO activity using slightly modified methods originally developed by Wilson-Rich and coworkers (Wilson-Rich and Starks, 2010). To control for variation in hydration state of individuals, hemolymph protein concentration was controlled at 0.02 mg/ml and added in varying volumes to the reaction mixture (Parkinson and Weaver, 1999). The enzymatic substrate L-dopa, a tyrosine derivative, was then added to each solution to reach a final concentration of 0.03 M. Next, melanin production was recorded using a Bio-Rad Benchmark Plus microplate spectrophotometer and Microplate Manager software (Bio-Rad, version 5.2.1). PO activity was quantified by recording the change in sample absorbance at 492 nm every 30 s for 9 min (the linear phase of reaction; pers. obs.). Lastly, PO activity was quantified as the slope of the linear phase of reaction (Rolff and Siva-Jothy, 2002).

Genetics

The ploidy status of each individual was determined using microsatellite genetic analysis following the methods described by Johnson and Starks (2004), and Liebert and colleagues (2004, 2005). All analyses were conducted in the Starks Laboratory in the Dana Laboratories at Tufts University, Medford, MA. DNA was extracted from 344 frozen adult wasps. Female individuals were genotyped twice at three loci (Pdom1, Pdom25, and Pdom117) (primers developed by Henshaw, 2000). All males were genotyped across 8 loci (Pdom1, Pdom2, Pdom25, Pdom93, Pdom117, Pdom121, Pdom122, and Pdom127b) to reduce the probability of a polyploid male being homozygous at a particular microsatellite site. An individual was determined to be diploid (or triploid) if it displayed multiple alleles at any particular microsatellite loci. Males that were polyploid at only one locus were re-analyzed at this locus to prevent misidentification.

Statistics

We created linear mixed models with phenoloxidase activity and encapsulation response as dependent variables; sex, season (spring/early summer or late summer/fall), ploidy (haploid or diploid), and their interactions as fixed effects; and year and colony of origin (to remove any possible pseudoreplication) as random effects. Phenoloxidase activity levels were log-transformed for normality prior to analysis. All statistical tests were run with the computer program SPSS for Windows (v. 20).

Results

Genetics

Genetic analysis of up to eight microsatellite loci revealed the presence of 240 diploid females, 4 triploid females, 60 haploid males, 37 diploid males, and 3 triploid males. Triploids were discarded for this analysis.

Immunology

Encapsulation response

We analyzed data from 103 wasps taken from 45 colonies (mean ± SD = 2.33 ± 1.46 wasps per nest). Our analysis included 74 wasps collected in spring and 31 in the fall. Our sampled wasps consisted of 77 females, 16 haploid males, and 10 diploid males. All fixed effects and interactions were non-significant ($p > 0.05$; Table 1) except for season ($F = 66.672$, $df = 50$, $p < 0.001$; Figs. 1, 3).

Phenoloxidase (PO) activity

We analyzed data from 117 wasps taken from 42 colonies (mean ± SD = 2.86 ± 2.05 wasps per nest). Our analysis included 80 wasps collected in spring and 37 in the fall. Our sampled wasps consisted of 80 females, 20 haploid males, and 17 diploid males. All fixed effects and interactions were non-significant ($p > 0.05$; Table 1) except for season ($F = 4.195$, $df = 67$, $p = 0.044$; Figs. 2, 4).

Table 1 A summary of results for two linear mixed models evaluating the significance of three fixed effects—season, sex, and ploidy—and two random effects, year and colony of origin (not shown)

	Season			Sex			Ploidy		
	F	*df*	*p*	*F*	*df*	*p*	*F*	*df*	*p*
Phenoloxidase activity	66.672	50	**<0.001**	0.076	36	0.783	1.366	103	0.245
Encapsulation response	4.195	67	**0.044**	0.532	75	0.468	0.016	82	0.898

p values below the 0.05 level of significance are indicated in bold

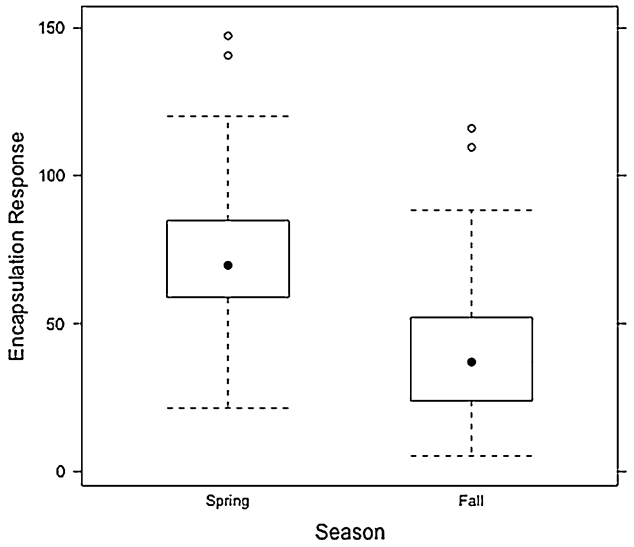

Fig. 1 A comparison of encapsulation response, induced by a nylon pseudoparasite, between early season and late season *Polistes dominulus* wasps. Encapsulation response is quantified as optical density, a measure of melanin deposition on the pseudoparasite. Encapsulation response was significantly higher in early season wasps ($p < 0.05$). *Error bars* are ±2 SE

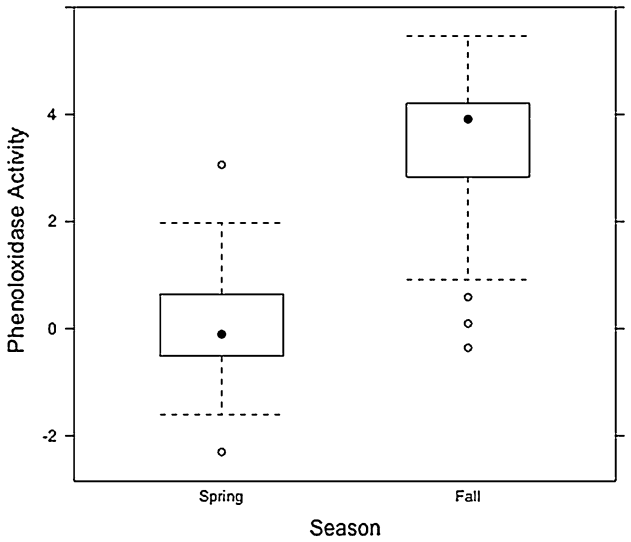

Fig. 2 A comparison of phenoloxidase activity, extracted from hemolymph, between early season and late season *Polistes dominulus* wasps. Phenoloxidase activity is quantified by 492 nm light absorbance in a spectrophotometer. Phenoloxidase activity was significantly higher in late season wasps ($p < 0.001$). *Error bars* are ± 2 SE

Discussion

Overall, our results do not support the haploid susceptibility hypothesis (HSH). Ploidy had no effect on either indicator of immunocompetence we measured (Figs. 3, 4). Seasonal factors have clearer influences on the immunocompetence of *Polistes* wasps. We found a robust temporal effect in both direct immune measures, albeit in different directions (Figs. 1, 2). The humoral (cell-free) assay, hemolymph PO activity, increased from early season to late season (Fig. 2). Encapsulation showed the opposite movement, decreasing from early to late season (Fig. 1).

Together, these findings suggest a trade-off in immune defense from cellular to humoral mechanisms. Pathogen pressure for these invasive populations of *P. dominulus* in the northeast United States are poorly documented; however, we speculate that this shift likely reflects the type of parasites that infect these hosts during different seasons of the year. Although tempting to suggest *Xenos vesparum* as a driving force, data do not support that assertion.

Xenos vesparum is a parasite that bores through wasp membranes, and infests *Polistes* primarily in the spring (Beani, 2006; Hughes et al., 2003; Manfredini et al., 2007, 2010). However, the parasite infects larvae and thus cannot explain the higher encapsulation response of adults in that season. It is possible that hibernating wasps are at heightened risk for disease, accounting for the increase in phenoloxidase activity in the fall. However, an alternative hypothesis is that decreased ER in late season wasps compared to early season wasps could reflect the natural aging process of *Polistes.* Much work on this topic remains to be explored.

It appears that time of year plays the most important role in predicting immunocompetence in *P. dominulus.* These results may not be surprising given the primitively eusocial nature of *P. dominulus*, where time of year is a major pre-

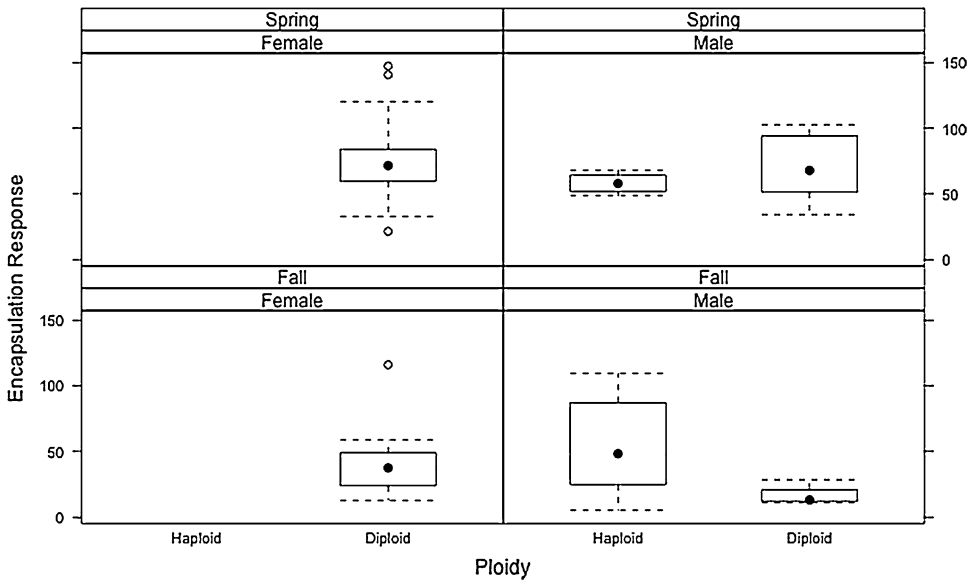

Fig. 3 A comparative boxplot of encapsulation response between ploidy levels across sex and season. Encapsulation response was stimulated by a pseudoparasite and quantified by optical density, a measure of melanin deposition on the pseudoparasite. Season was the only significant effect ($p < 0.05$)

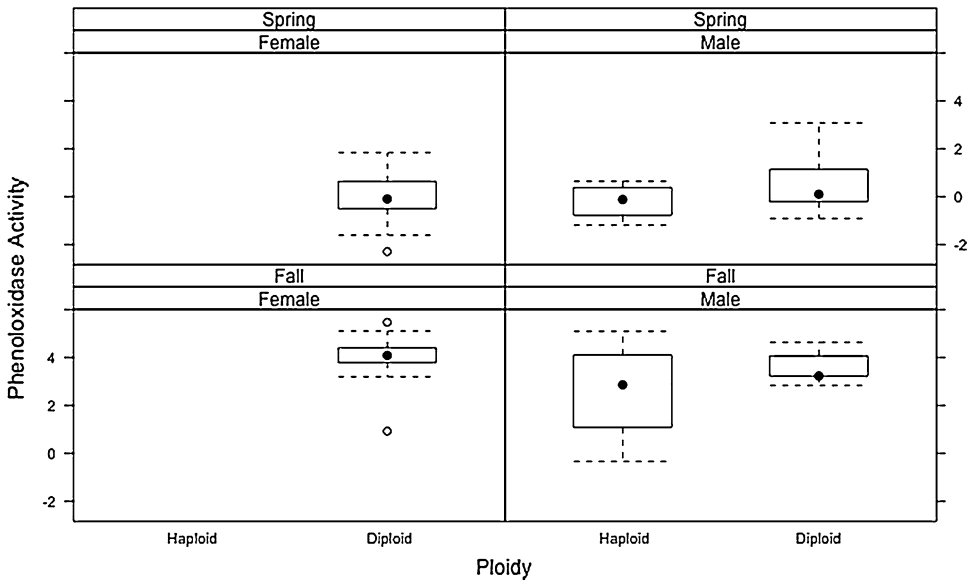

Fig. 4 A comparative boxplot of phenoloxidase activity between ploidy levels across sex and season. Phenoloxidase activity is quantified by 492 nm light absorbance in a spectrophotometer. Season was the only significant effect ($p < 0.001$)

dictor of behavioral role (Mead et al., 1990). The life history of *P. dominulus* involves one or more reproductively dominant female foundresses who start a new nest in the springtime. These females mated the previous fall, from which they lay fertilized eggs to produce worker females. During this early season, workers typically do not mate but instead assist the foundress(es) in various tasks such as nest building, brood care, and foraging (Reeve et al., 1998).

Later in the season, reproductives (both female and male) emerge, while the workers continue to focus on their colony rearing tasks. The reproductives mate, and in general only late season females overwinter, while all other individuals die (see Reeve et al., 1998; Starks, 2001). Our data show that the individuals with the longest lifespan, and with the greatest reproductive potential, have the strongest humoral immune activity. They are also potentially put at risk of disease during diapause, when wasps from different colonies overwinter together (Dapporto et al., 2004). A parallel can be drawn from honeybee workers, where the oldest individuals have the highest PO activity, putatively because they are more likely to be in contact with pathogens (Wilson-Rich et al., 2008, 2009).

This study advances our understanding of haploid susceptibility because we used multiple measures of immunocompetence and included naturally occurring variation in ploidy in our sample. As such, our approach enabled a strong test of the HSH. In a prior report where the immunocompetence of diploid males was compared to haploid

males and diploid females, Gerloff and colleagues (2003), who found highest to lowest immunocompetence in females, haploid males, and diploid males respectively, relied on just one immune measure (ER). Relying on one method constricts the lens to only one defense capability (see Adamo, 2004), and may have generally contributed to the ambiguity of prior studies of the HSH.

Future studies should determine the behavioral role of diploid males (and early haploid males) within the parameters of the colony cycle, continue to incorporate multiple approaches when investigating immunocompetence, and identify specific seasonal pathogen and parasite pressures. Results reported here indicate that temporal factors play an important role in the degree to which individuals can thwart pathogens. In particular, time of emergence—a proxy for caste—is the single best predictor of immune function in *P. dominulus*.

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