

**Confounding effects on speciation: Hybrid
vigor in *Daphnia pulex* x *D. pulicaria***

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BIOSCI 35502: Practicum in Field Biology

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2012

ABSTRACT

Speciation has become an exciting topic in recent years, as scientists seek to discover natural selection at work in the present, and attempt to illuminate past evolutionary divergences that structure the species we see today. One model of this separation is the ecological speciation model, where organisms adapt to exploit different ecological niches and eventually are severed genetically. However, one confounding effect of this severance may be hybrid vigor – a case in some species where their hybrid shows higher fitness than either of its parent species. *Daphnia*, a freshwater zooplankton and model species with a central role in global aquatic food webs, can produce hybrids that occur naturally, and have been observed to completely dominate vernal ponds relative to their parent species. Here, we examined *Daphnia pulex*, *D. pulicaria*, and their hybrids in an experimental test for the presence of hybrid vigor in fitness aspects of body size and growth rate across replicate ponds of varying light, algal, and predatory abundances in an effort to tease out factors influencing hybrid vigor in these species. Over the course of our study, hybrids displayed increased growth rate relative to the native pond-dwelling *D. pulex*, and larger body size than either of its parent species. Our findings may hold implications underlying complexities of speciation occurrences.

INTRODUCTION

Evolutionary ecologists have long researched the effects of environmental interactions on organism genotype and phenotype. In recent years, an exciting shift has

appeared exploring the implications of genetic and phenotypic variation on community structure (Bolnick et al. 2011). With increased anthropogenic effects on organisms such as habitat destruction and fragmentation, one implication of significant species variation is that of speciation, or the gradual severance of genetic ties among two or more populations of one species. Understanding of the underlying mechanisms of speciation may not only reveal driving factors of present and future species evolution, but more importantly those of past species, allowing us a clearer picture of how our current species came to be. Intraspecific variation can also have ecological repercussions (Post et al. 2008), but these lie largely unexplored at present.

One theory that ties speciation with ecological impacts is that of hybrid vigor. Hybrid vigor, or heterosis, proposes that when two parent species impart their genes to a hybrid, the genetic recombination may leave their hybrid more successful than either of its parent species in terms of overall fitness (i.e. biomass, speed of development, or other characteristics; Birchler et al. 2010). Plant and animal breeders have utilized this concept for many years, obtaining desirable traits by mating two different pure-bred lines. Heterosis has been studied extensively in these contexts, and more recently in the context of conservation practices (Fitzpatrick and Shaffer 2007), but natural hybrids have been less researched (but see Rieseberg 2003, Rieseberg 2007 for work in wild sunflower systems). However, natural hybrids pose an interesting question: What drives speciation in the presence of hybrid vigor? We believe the answer to this may lie in differential successes of hybrids between specific environments, that hybrids only express increased fitness under certain conditions. We aim to tease apart some of the conditions that may drive or hinder hybrid vigor.

Achieving understanding of the complex link between genes and ecosystems is best accomplished through the use of model organisms: ones with substantial, known ecological roles and available genomes (Miner et al. 2012). *Daphnia*, a freshwater zooplankton, is an organism with critical ecological importance across the globe as a primary grazer of phytoplankton, and serves as ideal prey for planktivorous fish and other aquatic secondary consumers. The genome of *D. pulex* has also been sequenced (Colbourne et al. 2011) and its biology well-studied, both for genetic variation and phenotypic plasticity (Cerny and Hebert 1999). Most *Daphnia* reproduce via cyclical parthenogenesis: females typically reproduce asexually, but produce males and reproduce sexually during periods of environmental stress, such as the drying up of a temporary pond (Cáceres 1997; Innes 1997; Cáceres and Tessier 2004). These sexual eggs are encased in durable structures called ephippium.

Daphnia pulex and *D. pulicaria* serve as fitting models of evolutionary divergence. The two species are morphologically almost identical, with the lactate dehydrogenase (LDH) allozyme locus conventionally serving as the indicator of species designation (Cerny and Hebert 1999). Often the only means of discernment in the field is their ecological separation; in many places, such as our study site in the Upper Peninsula of Michigan, *D. pulex* reside exclusively in vernal (temporary) ponds, whereas *D. pulicaria* abide in permanent lakes. This may be an indication of ecological speciation, where two species evolve to occupy different ecological niches, interrupting gene flow (Pfrender et al. 2000)

An intriguing aspect of these species, and the stimulus of this research, is their ability to hybridize, and the implications thereof. These hybrids have been documented

to occur in natural environments (Pantel et al. 2011), which would suggest a parapatric speciation process between *D. pulicaria* and *D. pulex*. These hybrids tend to appear in vernal ponds, but mechanisms for their hybridization across environments remain untested (Heier and Dudycha 2009). However, while not physically separated, the two parent species have been genetically disconnected, as evidenced by their hybrid's obligate parthenogenesis in natural environments (Lynch 2008, but see Heier and Dudycha 2009). Long-term co-occurrences between assemblages of *D. pulex*, *D. pulicaria*, and hybrids are fairly rare (Hebert 1993), with one species tending to dominate (though other *Daphnia* species may also be present).

That hybrids are able to not only inhabit ponds, but sometimes dominate over their parent species, shows the potential hybrid vigor present in their F1 genotype. Yet, the fact that this phenomenon is restricted to *some* ponds suggests that there are other variable effects at work in the *Daphnia* dominance structure. Our goal here is to test for increased fitness in hybrids, and tease out some of these effects that may lend competitive ability. We tested for fitness effects (body size, growth rate) on *Daphnia* populations across three treatments: fish predator presence, algal abundance, and increased light source. Our hypothesis is that *D. pulex* x *D. pulicaria* hybrids will display increased body size and growth rate relative to *D. pulex* and *D. pulicaria*. We also predict these fitness differences to vary by treatment type. These findings may reveal clues to gene interaction between these diverging species, and clarify the relationship between speciation and hybrid vigor.

METHODS

Study Site – Research was conducted at the University of Notre Dame Environmental Research Center (UNDERC), which lies on the border between Wisconsin and the Upper Peninsula of Michigan (N 46° 13.630', W 89° 31.432').

Organism Sample – *D. pulex* and *D. pulicaria* were obtained from habitats on UNDERC property (Pond 5 and Bay Lake, respectively), using an integrated tow. *D. pulicaria* were separated from other lake *Daphnia* under microscope, and *D. pulex* was the only *Daphnia* present in the sampled pond. *D. pulex x pulicaria* hybrid clones were used from near the Kellogg Biological Station near Kalamazoo, MI. Sampled organisms were then stored in culture and fed harvested algae (collected from a local pond and frozen to kill cohort organisms) once every 3-4 days.

Containment Construction – Four 91-gallon steel cattle tanks of varying diameters (77.5cm-93.5cm) were used as pond mesocosms, painted and sealed to prevent zinc coating from leaching into the water. Within each mesocosm were placed twelve cages where *Daphnia* were held. These cages were constructed from empty 2L soda bottles (rinsed, rinsed with 70% ethanol solution, and then allowed 24 hours to soak in lake water). Each bottle was drilled with two side holes of 3 inch diameter, and holes of 7/8 inch diameter were drilled into the bottom and the bottle cap. These holes were covered with 80µm mesh and sealed with hot glue and PVC cement. This mesh was found to be large enough to allow water and nutrient flow through the bottles, while preventing escape of the smallest *Daphnia* (Qualitative observation, Leibold and West 1993). Bottles were then let to sit in their respective mesocosms for 24 hours. Water

levels in each tank were maintained at a level 1-2 inches below the bottle cap to allow air flow through the cap mesh and into each bottle (leaving total tank water volumes ranging from 100-170L).

Mesocosm Setup and Maintenance – Tanks were set under the following conditions:

- One (“Light”) was set out of the shade of trees and an adjacent building to allow increased light levels relative to the other three treatments
- A second (“Fish”) was stocked with planktivorous fish, bluegill (*Lepomis macrochirus*) and pumpkinseed (*Lepomis gibbosus*), to inundate the mesocosm with fish kairomones. The fish were not additionally fed or aerated, so as to not add unwanted factors to the tank
- In another (“Algae”), 1 gallon of algae (collected from a local pond) was added as an increased food source, and allowed to grow and thrive in the mesocosm
- The last (“Control”) was left unaltered

Each mesocosm contained 12 bottles, with four replicates of each species, strung around the edge for stability, and to equalize any edge effects from the tank. Six adult *Daphnia* were transferred to each bottle via pipette at the start of the experiment, and allowed to grow and reproduce for 20 days. Every 2-3 days, bottles were lifted from the water and allowed to drain halfway, then replaced, to encourage flow through the mesh and cycle the available water. Three bluegill were initially added to the Fish mesocosm, and two were replaced after a week (due to mortality) with one pumpkinseed. After

initial algae introduction to the Algae mesocosm, it was allowed to grow uninhibited, and no more was added.

Final Sampling – Bottles were emptied through another 80µm mesh screen, and the filtered *Daphnia* collected for analysis. *Daphnia* were counted under a microscope, and final count per bottle recorded. Mean body length was measured by collecting a subsample of each bottle until SE of the Mean was <15% (Sampling protocol, Pfrender 2000) and measured (from crown to spine tip) using LAS EZ computer imaging software and microscope. Population growth rate, r , was used as an indicator of fitness (Lampert and Trubetskova 1996), and determined using the following equation:

$$N = N_0 e^{r\Delta t}$$

where N is equal to the final population count, N_0 is equal to the initial population size (6), Δt is equal to the change in time (20 days), and r is the growth rate (Rigler and Downing 1984). Mean growth rate and body length were recorded for each species-treatment and analyzed using a two-way ANOVA on SYSTAT 13 biostatistical analysis software (SYSTAT Software, Chicago, IL)

RESULTS

Body Length – The two-way ANOVA for mean body length showed a significant difference in body length across species ($F_{(2,36)} = 0.770$, $p = 0.007$) (Figure 1a). A Tukey's Post-Hoc analysis demonstrated hybrids to have significantly longer body length than both *D. pulex* ($p = 0.024$) and *D. pulicaria* ($p = 0.010$), while there was no

significant difference in length between *D. pulex* and *D. pulicaria* ($p = 0.939$). Our analysis showed no significant difference across treatments ($F_{(3,36)} = 1.569$, $p = 0.214$) (Figure 1b) and no interaction between species and treatment type ($F_{(6,36)} = 1.067$, $p = 0.400$) (Figure 1c).

Growth Rate – The two-way ANOVA for mean growth rate indicated that growth rate significantly differed across species ($F_{(2,35)} = 5.302$, $p = .010$) (Figure 2a). A Tukey's Post-Hoc analysis showed *D. pulex* to have a significantly slower growth rate than both *D. pulicaria* ($p = 0.019$) and hybrids ($p = 0.021$), while hybrids and *D. pulicaria* did not significantly differ ($p = 0.999$). Also, mean growth rate was shown to differ across treatment types ($F_{(3,35)} = 5.948$, $p = 0.002$) (Figure 2b). A Tukey's Post-Hoc analysis showed growth rate to be higher in the Fish mesocosm relative to both the Control ($p = 0.001$) and Algae ($p = 0.042$) treatments, but not relative to the Light treatment ($p = 0.080$). All other interactions were non-significant ($p > 0.050$). There was no significant interaction between species and treatment ($F_{(6,35)} = 0.352$, $p = 0.904$) (Figure 2c).

DISCUSSION

Our results found significant differences between fitness factors of *D. pulex*, *D. pulicaria*, and *D. pulex* x *D. pulicaria* hybrids. Hybrids were shown to be larger than their parent species (Figure 1a). This alone may be indicative of competitive potential, as body size is strongly correlated to fitness in certain organisms and environments

(Post and Palkovacs 2008). Hybrids also displayed increased growth rate compared to *D. pulex*, the native vernal pond dwellers in this area.

Our analysis showed no significant interaction between treatment type and body length or growth rate (Figures 1c and 2c). This is not unexpected, however, as our study period (20 days) may not have been large enough to capture environmental effects on genotype or phenotype. In this time period, the original *Daphnia* would still be part of the final sample, and maternal effects may still linger in even the youngest generation sampled. Another possibility of the lack of treatment effect may be that the factors were not extreme enough, or did not test factors that substantially alter growth rates or body size. Further studies might include variables not tested here: nitrogen/phosphorous levels, dissolved oxygen/carbon, or other factors, especially ones that may be evidenced in the environments of naturally occurring hybrid populations.

One factor of growth rate and body length not shown in our analysis is a difference in life history strategies and energy investments between the three *Daphnia* species studied. An important asymmetry is the allocation of energy to sexual eggs. These ephippia represent an adaptive shift to invest not only in sexuality, but dormancy, as a reproduction strategy. In our study, only *D. pulex* produced ephippia. *D. pulicaria* are capable of producing ephippia, but may require different conditions than our tanks provided to do so. Hybrids, being obligate asexuals, are only capable of producing clonal ephippia (Dudycha 2009). However, while sexual ephippia represent an adaptation to current conditions and potential for favorable genetic recombinants, hybrid ephippia represent a retreat until favorable conditions return. This effect may be evident in our data concerning slower *D. pulex* growth rates (Figure 2a). *D. pulex* may have

sacrificed energy toward growth rate in favor of producing ephippia, which are slow to hatch and were not counted as part of the final population count (variability in hatching success discredit ephippia count as a definite population addition). *D. pulicaria* and hybrids, however, were able to invest completely in a much faster asexual cloning process. Thus, differences in life history strategies and energy investment add complexity to dominance structure in vernal ponds, possibly confounding tests of this kind and make side-by-side comparison studies difficult to pursue.

While *D. pulicaria* and hybrids showed similar increased growth rates, if an ecological speciation model of these species holds true, *D. pulicaria* may be constrained to lake environments, leaving vernal pond competition to *D. pulex* and the hybrids (of course, hybrid appearance whatsoever would require at least some colonization by *D. pulicaria*). While these underlying mechanisms of hybridization remain unidentified, some aspects of hybrid vigor are evident and may hold implications for the speciation of these *Daphnia*. If hybrids truly are generally more fit than either parent species, we could see a merging of *D. pulex* and *D. pulicaria*. This implies the potential of a sort of bidirectional speciation process, where genetic separation of species is in flux, and evolution is more dependent upon environment than directionality. The converse of this would be that hybrids can only naturally dominate habitats of specific qualities, and that they remain as a lingering leftover of niche specialization of the two parent species. Future research that encompasses longer time periods, or more direct competition between *Daphnia* species, may be able to better clarify this complex relationship between speciation and hybrid vigor.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor Ben Clifford for his mentorship, flexibility, and continued curiosity in this project. I also thank Pat Leary for his assistance in obtaining and rearing local *Daphnia*, and with project construction. Special thanks to Luke DeGroote and Maggie Wisniewska for their statistical expertise and guidance as Teaching Assistants. Thanks to the CASCADE team for their assistance with chlorophyll sampling, Nick Anderson and Chelsea Guyure for mesocosm setup, and Matt Ileski and Ashley O'Reilly for *Daphnia* identification guidance. Finally, I thank the directors of the UNDERC program Gary Belovsky and Michael Cramer for this opportunity, and the Hank family for their continued funding of undergraduate research such as this.

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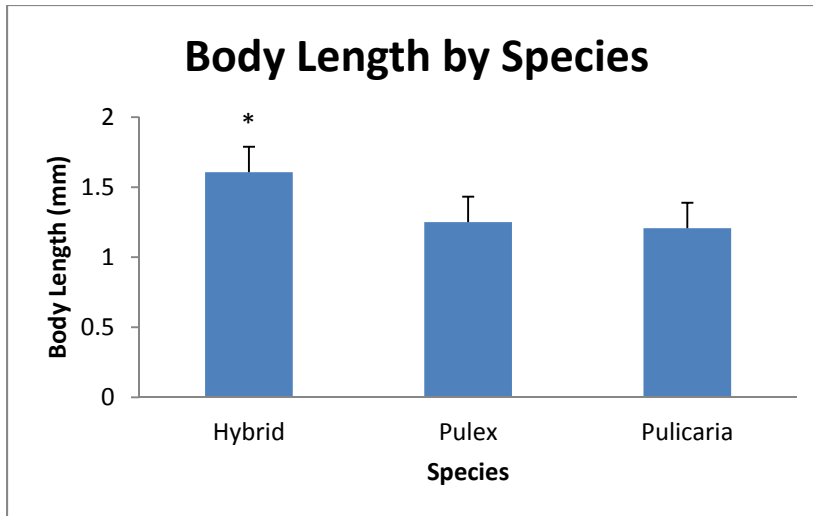


Figure 1a Average body length in mm across *Daphnia* species. Error Bars represent 95% Confidence Intervals of ANOVA test

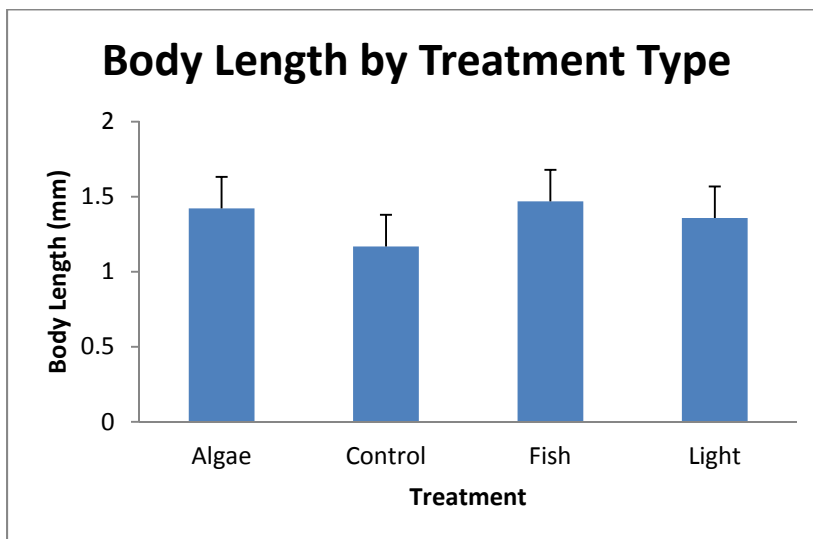


Figure 1b Average body length in mm across treatment types. Error Bars represent 95% Confidence Intervals of ANOVA test

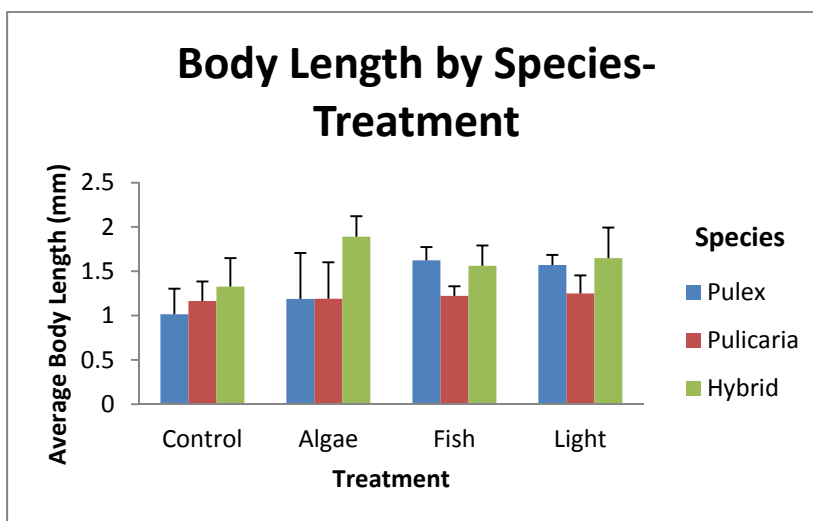


Figure 1c Average body length in mm across species-treatments. Error Bars represent 95% Confidence Intervals of two-way ANOVA test

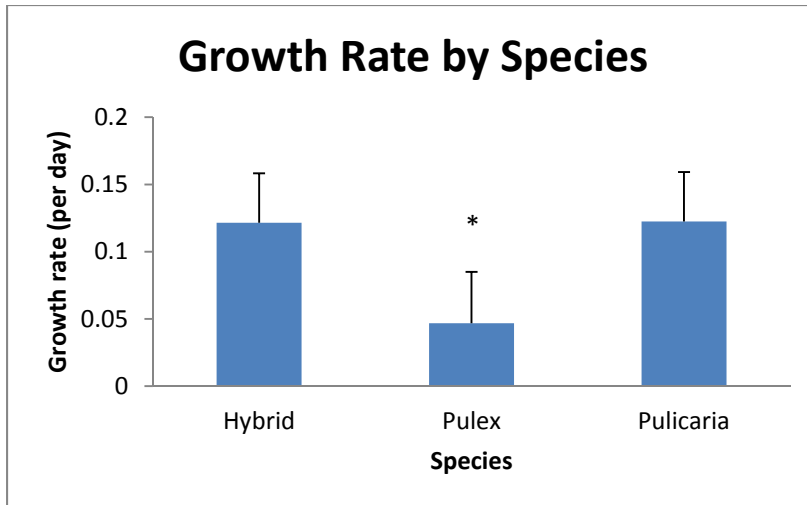


Figure 2a Average growth rate, r , per day across *Daphnia* species. Error Bars represent 95% Confidence Intervals of ANOVA test

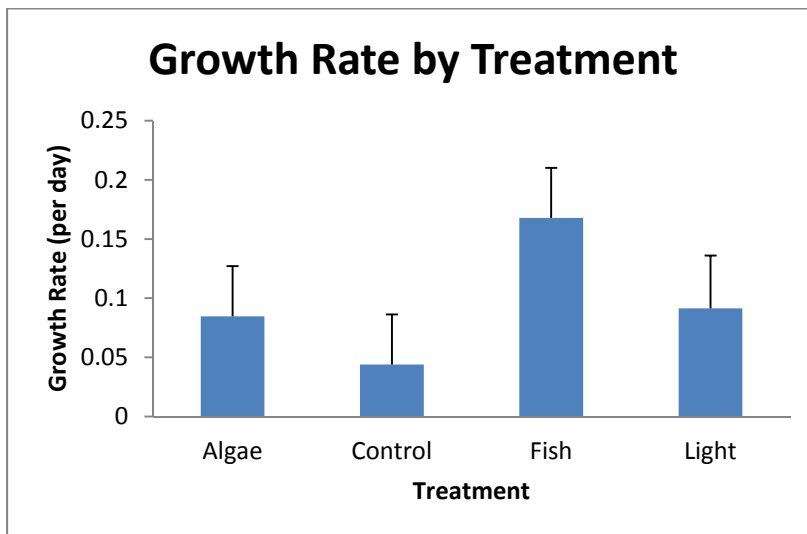


Figure 2b Average growth rate, r , per day across treatment types. Error Bars represent 95% Confidence Intervals of ANOVA test

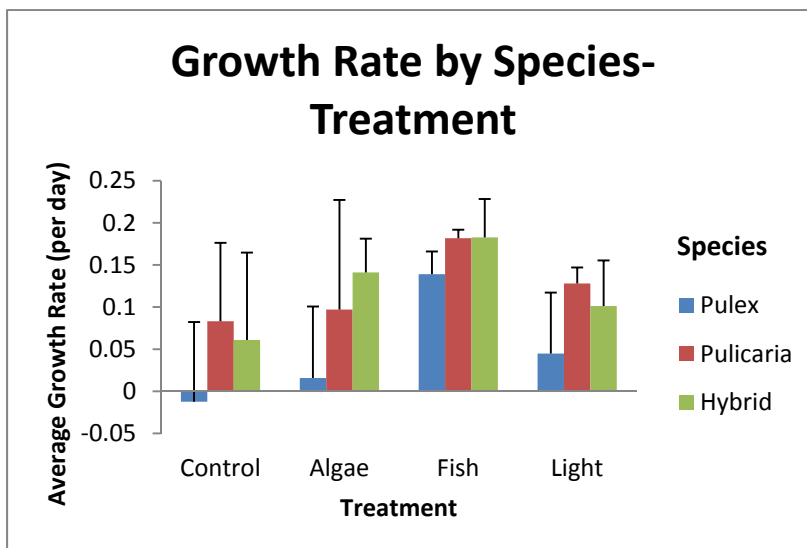


Figure 2c Average growth rate, r , per day across species-treatments. Error Bars represent 95% Confidence Intervals of two-way ANOVA test