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Social amoebae mating types do not invest unequally in sexual offspring

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Running Title: Sexual investment in *D. discoideum*

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Abstract

Unequal investment by different sexes in their progeny is common and includes differential investment in the zygote and differential care of the young. The social amoeba *Dictyostelium discoideum* has a sexual stage in which isogamous cells of any two of the three mating types fuse to form a zygote which then attracts hundreds of other cells to the macrocyst. The latter cells are cannibalized and so make no genetic contribution to reproduction. Previous literature suggests that this sacrifice may be induced in cells of one mating type by cells of another, resulting in a higher than expected production of macrocysts when the inducing type is rare and a giving a reproductive advantage to this social cheat. We tested this hypothesis in 8 trios of field-collected clones of each of the three *D. discoideum* mating types by measuring macrocyst production at different pairwise frequencies. We found evidence that supported differential contribution in only two of the twenty-four clone pairs, so this pattern is rare and clone-specific. In general, we did not reject the hypothesis that the mating types contribute cells relative to their proportion in the population. We also found a significant quadratic relationship between partner frequency and macrocyst production, suggesting that when one clone is rare, macrocyst production is limited by partner availability. We were also unable to replicate previous findings that macrocyst production could be induced in the absence of a compatible mating partner. Overall, mating type-specific differential investment during sex is unlikely in microbial eukaryotes like *D. discoideum*.

Key Words: mating type, isogamy, sexual selection, social amoeba, amoebozoa, sexual cannibalism, altruism

Introduction

Trivers (1972) defines parental investment as "any investment by the parent in an individual offspring that increases the offspring's chance of survival (and hence reproductive success) at the cost to the parent's ability to invest in other offspring". Understanding differences in these investments during reproduction has been crucial to understanding the evolution of sexual roles in eukaryotes (Trivers 1972). One of the most commonly recognized examples of dramatic differences in investment is anisogamy, or the production of tiny sperm by males compared to the production of comparatively huge eggs by females. These differences in parental investment evolved primarily due to tradeoffs between gamete number and gamete size (Parker et al. 1972; Birkhead et al. 2008; Claw and Swanson 2012). Another familiar instance of differential parental investment is nutrient provisioning, especially to the zygote. In many species, nutrients are provided to the embryo by the mother, either directly, for example through a placenta, or indirectly through the production of a nutrient-rich yolk (Callard and Ho 1987; Guraya 1989; Valle 1993). Other examples of sexual dimorphism in parental investment include maternal lactation in mammals, male pregnancy in seahorses and pipefishes, and sex-biased nest building in both vertebrates and invertebrates (Clutton-Brock 1991; Royle et al. 2012).

Though common in larger organisms, in microbial eukaryotes, differences in parental investment are likely to be rare. Microbes tend to show no signs of disruptive selection for different sexual roles. Gametes are generally identical in form and mass, allowing species to frequently express more than two mating types (Parker et al. 1972; Lehtonen et al. 2016). Still, evidence for dissimilarities between microbial mating types suggests that investment can vary even in these species. For example, during gametogenesis the malaria parasite *Plasmodium falciparum* changes to form morphologically and biochemically distinct male and female

gametocytes (Dixon et al. 2008). The transition to multicellularity among microbes also correlates with transitions in parental investment. In the Volvocine algae, increased gamete differentiation evolved with increasing vegetative complexity (Hiraide et al. 2013; Nozaki et al. 2014; Herron 2016). Unicellular genera like *Chlamydomonas* are isogamous, reproducing through the fusion of gametes identical in size. Interestingly, colony-forming genera like *Volvox* produce two types of sexual gametes that differ in size and structure.

The cellular slime mold *Dictyostelium discoideum* offers an exciting system for investigating the potential for differential contribution during reproduction in a microbial system. This eukaryote, which is normally haploid and unicellular, shares many of the traits of species that show no evidence for disruptive selection in gamete size. In *D. discoideum*, there are three self-incompatible mating types (Type I, Type II and Type III) that are identical in size and distinguishable only by a unique set of genes at a single genetic locus (Bloomfield et al. 2010; Douglas et al. 2016). However, the product of a single mating, termed a macrocyst, is formed through a uniquely social process in which the nutrients required for the survival and development of the zygote come from cannibalized cells that could be contributed by either parent. Though difficult to observe in nature, evidence for high rates of recombination suggests that sex in *Dictyostelium* occurs fairly frequently (Flowers et al. 2010). It occurs under environmental conditions that differ from those required for asexual growth and development, primarily darkness, excess moisture and an absence of phosphates (Nickerson and Raper 1973). Initially, two haploid cells of differing mating types fuse to form a diploid zygote, called a giant cell (Saga et al. 1983). This giant cell attracts surrounding amoebae by secreting large quantities of the chemoattractant, cyclic adenosine monophosphate (cAMP) (O'Day 1979; Abe et al. 1984). As many of these attracted peripheral cells begin to get consumed by the giant cell through

phagocytosis, the rest seal their fate by producing a cellulose wall that permanently joins them with the giant cell in a structure called a precyst (Blaskovics and Raper 1957; Filosa and Dengler 1972; Erdos et al. 1973a). As two more cellulose walls get formed around what will become a mature macrocyst, the rest of the peripheral cells are also cannibalized through phagocytosis by the giant cell.

Under conditions conducive for sex, hundreds of *D. discoideum* amoebae get phagocytized for each new zygote. Since it is most likely that there are only the two parental clones close enough together to contribute to the same macrocyst (Gilbert et al. 2007; smith et al. 2016), we can ask questions about conflict between the two partners at this stage. Analogous to yolk production, the peripheral cells contribute materially, but not genetically, to the success of haploid sexual offspring that hatch out from the macrocyst (Okada et al. 1986; Filosa and Dengler 1972; Nickerson and Raper 1973). However, unique to *D. discoideum* and other dictyostelids, this contribution is a form of cellular sacrifice or altruism. This phenomenon is familiar in another context in *Dictyostelium*. For decades, *D. discoideum* has been a model organism for social evolution because, in the asexual social cycle, starved amoebae aggregate, attracted again to cAMP but under different environmental conditions than during the sexual cycle, to form a fruiting body that is composed of a spherical ball of spore cells held up by a stalk of dead cells (Kessin 2001; Strassmann and Queller 2011). Because there is potentially a large cost to participating in either macrocyst or fruiting body formation, clones can be exploited, or cheated, if they contribute more than their partner to the respective sacrificed cells in either process.

While a number of examples of cheating to fruiting body formation have been observed in *D. discoideum* (described in Strassmann and Queller 2011), differential contribution to

112 macrocyst production has been reported between the two clones most commonly studied, NC4
113 and V12 (O'Day and Lewis 1975; MacHac and Bonner 1975; Lewis and O'Day 1977; Bozzone
114 and Bonner 1982). In these studies, V12, a Type II clone, invested disproportionately more to
115 macrocyst formation by contributing most or all of the phagocytized peripheral cells. This
116 behavior was thought to be induced in V12 by a diffusible pheromone that was produced by cells
117 of the Type I clone NC4 and could affect V12 even in the absence of NC4 cells. This
118 phenomenon was not limited to *D. discoideum,* with other species also showing signs of
119 inducible macrocyst production (Lewis and O'Day 1976; Lewis and O'Day 1979). However,
120 subsequent studies have called into question the claim by these early studies that the diffusible
121 pheromone could induce macrocyst formation in the physical absence of a sexually compatible
122 mate because they were unable to replicate the original findings (Wallace 1977; Bozzone and
123 Bonner 1982). These original studies were also limited to single representatives of mating types,
124 so the generality of their findings to other *D. discoideum* clones is unknown. There could be
125 dominance effects between clones that average out between mating types as a whole. Regardless
126 of the potential flaws of the early studies, the suggestion that mating types play separate roles in
127 macrocyst production still remains a part of the current understanding of how *D. discoideum* and
128 other *Dictyostelium* cells of different mating types interact (reviewed in O'Day and Keszei 2012
129 and Bloomfield 2013).

130 Our study investigates this potential for unequal investment in macrocyst production by
131 each of the three mating types in *D. discoideum*. We also test whether induction of one mating
132 type by another, potentially by the diffusible pheromone discussed previously, might be an
133 underlying mechanism. We propose that the behavior most likely to be influenced or cheated
134 during macrocyst production is how many phagocytized peripheral cells a given clone

contributes. Since it is difficult to measure who contributes because the cells get cannibalized, we will instead use the signature of unequal investment previously observed for V12 and NC4: fewer macrocysts when the heavily investing clone is rare (Bozzone & Bonner 1982). We tested for expected consequences in terms of macrocyst numbers based on three hypotheses for how peripheral cells are contributed (illustrated in Fig. 1): (a) that peripheral cells are contributed in proportion to the frequency of each partner, (b) that they are contributed equally, resulting in fewer macrocysts being produced when either partner is rare and (c) that one partner potentially cheats another by contributing disproportionately fewer than its fair share, resulting in a higher production of macrocysts when that partner is rare. Also, because *D. discoideum* has more than two mating types and no Type III clones have ever been evaluated for levels of investment during macrocyst production, we assessed whether a mating hierarchy exists such that contribution to reproduction differs depending on which mating types are present in a pairing.

Materials and Methods

Clones

We tested our ability to measure differential macrocyst production by comparing macrocyst production between clones NC4 and V12, the focal pair in the literature on macrocyst induction in *D. discoideum* (O'Day and Lewis 1975; MacHac and Bonner 1975; Keith E. Lewis and O'Day 1977; Bozzone and Bonner 1982). We obtained these clones from the Dictyostelium Stock Center (http://dictybase.org/StockCenter/StockCenter.html; Fey et al. 2013). Because a number of strains labeled as either NC4 or V12 have been deposited over the years, we selected five unique pairs to test for differential macrocyst production after initially checking for compatibility (Table S1, S5). We also chose to test our methods on *D. discoideum* clones

158 WS205 and IR1 because we previously observed macrocyst production when WS205 was rare
159 and IR1 was common, but not the reverse, suggesting WS205 may induce macrocyst production
160 in IR1 (unpublished data). WS205 is a Type I wild clone and IR1 is a Type II clone that has
161 been highly selected in the lab (to grow on axenic or bacteria-free medium) that still contains all
162 Type II mating type genes. These clones were also obtained from the Dicty Stock Center.
163 Clones were grown from frozen stock on nutrient agar plates using *Klebsiella pneumoniae*, also
164 from the stock center, as the bacterial food source.

165 We also tested pairwise macrocyst production among trios of previously collected *D.*
166 *discoideum* clones each from the same geographic area. We focused on three locations as the
167 populations for this study: Houston, TX (29° 46' N, 95° 27' W), Little Butts Gap trail in North
168 Carolina (35°46' N, 82°20' W), and near Mt. Lake Biological Station, VA (37°21' N, 80° 31'
169 W). Clones collected from within each of these areas, including many of the clones used in this
170 study, have been shown to share more similar DNA sequences than clones collected between
171 these areas, suggesting that these clones are more likely to interact (Douglas et al. 2011; Douglas
172 et al. 2016). We only selected wild clones that were compatible (i.e. produced macrocysts) with
173 each of the other two clones in a given trio. We tested 60 clones for mating compatibility. Of
174 the compatible trios, we tested for pairwise macrocyst production among 24 clones in total (8
175 clones each of the three mating types), from three geographic populations: 3 trios from Houston,
176 TX, 3 trios from Little Butts Gap trail in North Carolina, and 2 trios from near Mt. Lake
177 Biological Station, VA (Table S1). The mating types of each of the clones used in this study
178 were either previously identified or identified using the techniques from Douglas et al. (2016).
179

180 *Assay to measure differential macrocyst production among previously studied clones*

The relative contributions of two mating types to the macrocyst are difficult to assess directly. However, measuring macrocyst production at varying partner frequencies has been shown to be an excellent indicator of differential contribution (Bozzone and Bonner 1982). To test that our methods could identify differential macrocyst production, an indication of differential contribution to peripheral cells similar to the type described in previous literature, we compared macrocyst production between *D. discoideum* clones NC4 & V12 and also between WS205 & IR1, at five starting population frequencies (99:1, 90:10, 50:50, 10:90 and 1:99). We performed two replicates. We also tested for macrocyst production when each clone was plated alone to ensure that macrocysts were not being formed through selfing.

We performed all of our experiments in 24-well plates with 1 mL of equal parts Lactose-Peptone agar (LP: 0.1% lactose, 0.1% peptone, 1.5% agar) and Bonner's salt solution (SS: 0.06% NaCl, 0.03% $CaCl_2$, 0.075% KCl). To each well, we added a total of 5 x 10^3 *D. discoideum* spores with 10 µL of OD 2.0 A_{600} *K. pneumoniae* as food. We sealed each plate with black electrical tape to maintain humidity inside and then stored them in a dark incubator at 22°C for one week to ensure the completion of all macrocyst production. We then counted the number of macrocysts in each well using an inverted microscope.

Predicted outcomes of different hypotheses

Fig. 2 shows how we would expect macrocyst production to vary by population composition based on three hypotheses for how each mating type contributes to the cannibalized peripheral cells and will be used for comparison with the actual results. This figure reflects only our expectations when two mating types are mixed, because no macrocysts are produced when cells of only one mating type are present. In Fig. 2A we show the prediction for proportional

fairness, in which each mating type contributes a number of cells to be consumed by the zygote that is directly proportional to the number of cells of that mating type in the population. In this scenario, our null hypothesis, there is potentially no limitation on macrocysts since cells are sacrificed at rates relative to their own frequency and thus, maximum macrocyst production is possible across all ratios. In Fig. 2B, we show the prediction for absolute fairness, in which each mating type, having already contributed equally to the production of the diploid zygote, refuses to pay more than its share of peripheral cells. Since the rarer mating type will be depleted first, in this first alternative hypothesis, macrocyst production is then proportional to the number of cells of the rarer type, with very few macrocysts being produced when one type is rare (10%) and even fewer when one type is very rare (1%). Unfairness, or cheating, our second alternative hypothesis, is shown in Fig. 2C. Here one partner builds most of the macrocyst and the other partner (X) parasitizes it. Thus when X is rare, many macrocysts get made but when it is common, few get made. This figure most closely resembles the proposed differential contribution to peripheral cells from the literature (O'Day and Lewis 1975; MacHac and Bonner 1975). Partner X would gain a reproductive advantage by contributing disproportionately less to the cannibalized peripheral cells.

Diffusion chambers

To test for induced macrocyst production without physical contact between the cells or the ensuing sexual reproduction, we set up diffusion chambers modeled after the experiment described by Lewis and O'Day (1977). The purpose of these chambers is to grow clones separately, but still allow for the exchange of volatile compounds (illustrated in Fig. 3). The original study found that, when two plates of NC4 cells were grown separately, but housed

together in a diffusion chamber with one plate of V12 cells, macrocysts formed only in the plate of V12 cells, likely through induced selfing. They also found that the reciprocal design (two plates of V12 cells and one of NC4) produced no macrocysts. To test for this pattern in our study, we conducted these experiments on the pairs of clones used to test our methods for identifying differential macrocyst production (NC4 & V12 [also used by Lewis and O'Day(1977)] and WS205 & IR1). We also tested one trio from the larger experiment (V315B1, V331B1 and V341C2). We placed three small 30 x 10 mm Petri plates in one 100 x 15 mm Petri plate. We filled the small plates with 6 mL of equal parts LP agar and SS buffer and added 2.5 x 10^4 *Dictyostelium* spores with *K. pneumoniae* as food. For each pair of clones tested, A and B, we added spores to the three small plates in the following five combinations: (1) two clone A and one B, (2) two clone B and one A, (3) three clone A, (4) three clone B, and (5) one clone A, one clone B and one with both clones to verify that macrocysts can be made in our conditions. We sealed the lid of the large plate with black electrical tape and stored them in a dark incubator at 22°C for at least one week. We then checked for the presence of macrocysts using an inverted microscope.

Assay to measure differential macrocyst production among wild clones and across all three mating types

To investigate differential macrocyst production in wild *D. discoideum* clones, we compared pairwise macrocyst production among eight trios of *D. discoideum* clones, each containing one representative of each mating type. The same five starting population frequencies (and self-compatibility controls) were tested as in the experiment on pairs of previously studied

clones, but each clone was tested separately against the two other clones in the trio. We performed one replicate for each trio of clones.

Identical to the paired experiment, we performed all of our experiments in 24-well plates with 1 mL of equal parts LP agar and SS buffer. To each well, we added *D. discoideum* spores with food bacteria. We sealed each plate with black electrical tape to maintain humidity inside and then stored them in a dark incubator for one week to ensure the completion of all macrocyst production. We then counted the number of macrocysts in each well using an inverted microscope.

Viability assessment of non-aggregated cells

We also tested whether cells not contributing to macrocysts were viable in a subset of the wild clones used in this study. We used similar techniques to those described above to produce macrocysts. One week after plating the initial spores (a sufficient amount of time for macrocysts to form), we washed the entire contents of a well through a sieve made with 20 µm mesh to separate macrocysts from any remaining amoebae. We divided the macrocyst-free wash onto multiple nutrient agar plates with food bacteria to limit the total amount of liquid on a given plate and stored the plates in the light. Since these conditions are conducive for fruiting body formation (after growth and starvation), not macrocyst formation, we monitored for the presence of fruiting bodies within the week following plating. We also tested for the viability of the cells not contributing to macrocysts after being exposed to harsh environmental conditions. After macrocysts were produced in each plate, we froze the plates for 2-4 weeks at -20°C. We then removed them from the freezer, allowed them to thaw, and then used the methods as described already to test for viability.

Statistical analyses

Statistical analyses were performed using R software (version 3.2.2.) (R Core Team, 2015). We applied separate linear mixed-effects models to the data from crosses between NC4 and V12 and between WS205 and IR1 using R package "nlme" (Pinheiro, et al. 2016). We looked at how the initial percent of the predicted inducer affected macrocyst production. We treated percent inducer as the fixed effect (excluding 0% and 100%). We compared models that included only the linear term for percent inducer to models that also included the quadratic term and chose the linear model based on AIC and BIC scores. We used Type III tests to estimate the significance of the fixed effect. Because the data were not normally distributed, we square root transformed the data, which then passed the Shapiro-Wilk test of normality. Bonferroni correction was used to adjust for multiple comparisons. We report the corrected p-values. All statistical tests were performed on the transformed data but for visual presentation of the data, we show the original, untransformed data. Also for visual presentation, best-fit regression curves were calculated on the original data.

We applied similar methods to analyze macrocyst production between pairs formed all ways among the trios of wild clones. We again applied linear mixed-effects modeling to analyze how macrocyst production is affected by the frequency of a given partner (Type I in Type I x Type II, Type I in Type I x Type III, and Type II in Type II x Type III). We treated frequency as a fixed effect (again excluding 0% and 100%). We again compared a linear regression model to a quadratic regression model and also compared models that included geographic population as a fixed effect. Based on AIC and BIC scores, the quadratic model that only assessed a frequency effect fit the data best. We cube root transformed the data to normalize them.

Results

Disproportionate contribution to macrocyst production is clone-specific

When paired with their respective partners, macrocysts were produced at all population frequencies of NC4 and V12 and WS205 and IR1, respectively. Both between NC4 and V12 and between WS205 and IR1, we found a significant linear relationship between macrocyst production and the initial frequency of NC4 or WS205, respectively (NC4xV12: $F_{1,19}$=29.40, $p<0.0001$; WS205xIR1: $F_{1,7}$=414.98, $p<0.0001$, Fig. 4). However, the best-fit regression curve indicated that the direction of the effect differed between the two pairings, with increased frequency of the Type I clone correlating with increased macrocyst production in one pair but a decreased macrocyst production in the other. We found that an increased frequency of NC4 had a significant positive linear effect on macrocyst production, while increasing the frequency of WS205 had a significant negative linear effect on macrocyst production. These results most closely resemble our hypothesis (described in more detail in the "Materials and Methods" section) that one mating type cheats another during macrocyst production (hypothesis C, Fig. 2) but they go in opposite directions with respect to mating type.

Physical contact is required for macrocyst production

When plated alone, NC4, V12, WS205 and IR1 each were unable to produce macrocysts, consistent with their classification as self-incompatible strains. From the diffusion chambers, we found no evidence of induced macrocyst production without the possibility of sexual cell fusion. We set up four diffusion chambers each with the following combinations: two NC4 and one V12, two V12 and one NC4, and one NC4, one V12 and one with both NC4 and V12. We set up two

diffusion chambers each with the following combinations: three NC4 and three V12. While macrocysts were produced in all four of the small plates inoculated with both NC4 and V12 clones, no other cultures produced macrocysts. We did the same experiment with WS205 and IR1 and again found that macrocysts were produced in the small plates inoculated with both WS205 and IR1, but not in any other plates.

In clones we collected from wild populations, disproportionate contribution to macrocyst production is rare

Surprisingly, when testing for mating compatibility, we encountered pairs of clones that together produced no macrocysts even though they exhibited different mating types at the mating type locus (Table S2-S4). Of the 24 wild clones we tested, none showed evidence of macrocyst production when plated alone, but all produced macrocysts at the other pairwise population frequencies (Fig. 5). We found a significant quadratic relationship between the initial frequency of a given partner and macrocyst production in each of the three mating type pairings (Type I x Type II: $F_{2,30}=9.84$, $p<0.0001$; Type I x Type III: $F_{2,30}=14.28$, $p<0.0001$; Type II x Type III: $F_{2,30}=8.80$, $p=0.001$). Because we found clone-specific linear relationships in crosses between NC4 and V12 and WS205 and IR1, respectively, we also calculated best-fit linear regressions for each of the wild clone pairings (Fig. S1). Though additional replicates would be necessary to make more definite conclusions, we found some interesting patterns. We found significant linear relationships between only two Type I x Type III North Carolina pairs (Type I NC60.2 x Type III NC75.2: $p=0.05$; Type I NC105.1 x Type III NC61.1: $p=0.007$). The rest showed no significant linear or quadratic relationships, similar to what we would have expected if contribution to macrocyst production followed our null hypothesis (hypothesis A, Fig. 2).

Amoebae that avoid or are left out of aggregations are viable

We plated the contents of the wells in which macrocysts were produced (minus the macrocysts) and found that, within a week, fruiting bodies were produced. This result was consistent across mating pairs and across treatments (with and without freezing). This suggests that viable amoebae remained that either avoided or were left out of aggregations that ultimately matured into macrocysts.

Discussion

Dictyostelium discoideum offers an unusual and interesting model for investigating differential parental investment during reproduction. Like many other systems, nutrients to the reproductive zygote are provided by the parents, although the mechanism in *Dictyostelium* is unique. Differential contribution to these nutrients is common in nature, with primarily maternal investment dominating. Until now, however, it was unclear in *D. discoideum* if nutritional contribution to the zygote was uniparental or biparental. In this study, we show not only that sexual investment in *D. discoideum* is biparental, but also that it is somewhat dependent on the frequency of a given partner in the population rather than its mating type.

Evidence suggesting that one partner disproportionately contributed to macrocyst production by providing more of the cannibalized peripheral cells was introduced by O'Day and Lewis (1975) and independently verified with the same clone pair in the same year by MacHac and Bonner (1975). Since then, the possibility of differential macrocyst induction by *D. discoideum* mating types has persisted in the literature. Nonetheless, because these prior studies primarily focused on a single pair of clones, representing only two of the three *D. discoideum*

mating types, we expanded our investigation to include not only all three mating types, but also multiple representatives of each of these three mating types. We tested eight independent sets of wild *D. discoideum* clones, each containing representatives of all three mating types, and found little evidence for the hypothesis C pattern that would reflect investment primarily by one partner (Fig. 5). Instead, we found an overall quadratic relationship between frequency of partner and macrocyst production where more macrocysts were produced when both partners were equal and fewer at the more uneven frequencies. A quadratic effect suggests that these findings are similar to what we predicted in hypothesis B (Fig. 2), in which we hypothesized that if each partner contributes the same number of sacrificed peripheral cells during the formation of macrocysts, macrocyst production will be limited by the number of cells of the rarer type. This was a surprising result, as it conjures up the possibility of the seemingly unlikely scenario in which aggregation of one cell type ceases at some threshold X, while aggregation of the other continues. Another possibility would be that cells are attracted to the zygote at differing rates, depending on their density. It implies either that the peripheral cells can actively avoid aggregation or that the giant cell can actively pursue some cells over others based on the population composition of the aggregate surrounding the giant cell.

Still, as improbable as it may seem, the possibility of this is not completely unfounded. Evidence for active preference mechanisms in *D. discoideum* have been identified both in the sexual cycle and the social cycle. Giant cells have been shown to preferentially phagocytize cells of their own species over cells from other slime mold species (Lewis and O'Day 1986). During the social cycle, amoebae can actively sort based on clone identity and a matching pair of highly-polymorphic recognition genes, producing highly related fruiting bodies (discussed in Strassmann 2016). *Dictyostelium* cells are also able to determine neighboring cell density

through quorum sensing mechanisms (Loomis 2014). Since each mating type contributes equally to the formation of the giant cell through the fusion of morphologically identical gametes (Saga et al. 1983; Douglas et al. 2016), the giant cell is equally related to the respective clonemates of each parent cell.

However, though we find evidence for a pattern suggesting macrocyst production with equal contribution to peripheral cells, we are still skeptical of this hypothesis. First, it was unclear from previous studies if giant cells preferentially consume some *D. discoideum* cells more than others, or just recognize species. Furthermore, as the giant cells in our experiment are equally related to all of the surrounding cells, it is unlikely that they would have evolved to preferentially attract one type over another. In nature, giant cells are also likely to encounter this high level of relatedness based on what is known about the population structure of amoebae in nature (Fortunato et al. 2003; Gilbert et al. 2007). Our doubts that peripheral cells are equally contributed were further supported by looking at the relationship between partner frequency and macrocyst production at the level of the individual clone pair. Though additional replicates should be assessed to confirm these findings, in 22 of the 24 pairings, we found nonsignificant relationships between frequency of partner and macrocyst production, with the other two showing linear relationships. Since there were no individual pair quadratic effects, even though there are collective ones, the power must be fairly low for the individual effects, quadratic or linear. Evidence for nonsignificant relationships between frequency of partner and macrocyst production suggest a pattern most similar to our prediction in hypothesis A (Fig. 2).

We found little evidence for our disproportionate investment hypothesis based on macrocyst number. However, macrocyst size, which we did not measure, can also affect investment, so it is worth considering possible effects of this on our findings. We interpreted

410 frequencies showing low macrocyst numbers as reflecting low investment by one of the partners
411 (hypothesis c, Fig. 2C), but if these smaller numbers of macrocysts were fully compensated by
412 larger macrocyst size, the actual pattern of investment would be constant over frequencies, as in
413 hypothesis a. We believe this is unlikely based on our visual impression that macrocyst size
414 differences were not nearly large enough to fully compensate for some of the macrocyst number
415 differences. But even if they were, this would shift an apparent hypothesis c macrocyst number
416 pattern to a hypothesis a (Fig. 2A) investment pattern. Thus our main finding that hypothesis c
417 patterns are rare is conservative.

418 We predict that lower macrocyst production at more extreme frequencies may instead be
419 due to underlying population structure, such that when compatible mating types no longer come
420 in contact, zygote production ceases. Though spores were mixed initially, once amoebae hatched
421 from these spores and subsequently divided as they consumed the provided bacteria, patches of
422 identical individuals are likely to occur. Evidence for this type of structured growth in *D.*
423 *discoideum* has been shown in asexual development (Buttery et al. 2012; smith et al. 2016).
424 These patterns may be even stronger in the wet conditions required for macrocyst production as
425 amoebae move much slower in liquid than on solid substrates (Van Haastert 2011). At low
426 frequencies of one clone, there will be large uniclonal patches where there is no possibility of
427 zygote formation. Under these conditions, low macrocyst numbers would result from lack of
428 partners for zygote formation, rather than from willingness or unwillingness to invest in
429 macrocysts. In other words, our results might reflect the proportional investment hypothesis a
430 but with zygote limitation at extreme frequencies. This is somewhat supported by our data, since
431 if we exclude the two extreme frequencies from our modeling, the quadratic effect is no longer

significant. Artificially manipulating population structure in future mating experiments would further elucidate this theory.

A critical assumption of our hypotheses B and C, where cells are posited to be adaptively withheld from macrocysts, is that these withheld cells can have an alternative pathway to success. In our experiments, macrocyst production never fully exhausted the available cell population regardless of partner ratios. In every pairing that produced macrocysts, we observed free living amoebae that seemingly avoided or were excluded from participating in the sexual process. In addition to possible effects of population structure, avoiding aggregation could be a strategy to avoid contributing to the peripheral cells if another option is possible. In the asexual life cycle, non-aggregating cells that do not participate in fruiting body formation can colonize remaining nutrients in the environment (Dubravcic et al. 2014; Tarnita et al. 2015). This observation was important for our understanding of altruism in *D. discoideum*, as clones that were labeled "losers" for producing relatively fewer spores when mixed with other genotypes, could in reality be following an alternative strategy of producing more non-aggregating cells. In our experiments, non-aggregating cells had no advantage over aggregating cells as the subsequent lab environment was unsuitable for continued growth. However, we showed that these cells are viable if provided with food even weeks (if frozen) after macrocysts have been formed. In nature, nutrients can reestablish and failure to participate in macrocyst formation may not be an evolutionary dead end.

Evidence that cells are likely to be phagocytized relative to their frequency in the population, rather than their mating type identity, provides further insight into how the zygote giant cell feeds. As described earlier, mating in *D. discoideum* begins with the production of the giant cell, a fusion product of two cells that differ in mating type. This giant cell then produces

large quantities of the chemoattractant, cAMP, attracting surrounding cells. Though evidence for preferential feeding exists, it is unclear if the giant cell differentiates between conspecifics (Lewis and O'Day 1986). In wild clones, this does not appear to be the case. Instead, our results suggest that the giant cell acts as more of an opportunistic feeder, consuming whatever conspecific amoebae are attracted to it. Since our pairwise mating design guaranteed that giant cells would be equally related to all of their potential "victims", we cannot draw conclusions on whether giant cells attract unrelated *D. discoideum* cells more or less than cells identical to the two that fused originally.

Though we present here robust evidence against the generality of strongly differential parental investment between the mating types among wild *D. discoideum* clones, we also showed that disproportionate contribution to macrocyst production can happen between two clones. Significant linear relationships between four sets of clones, including the originally discussed NC4 and V12, suggest that though not universal, uneven investment may occur during the sexual cycle. Interestingly, the direction of unfairness that we found between Type I NC4 and Type II V12 is opposite of what was previously observed. Instead of finding evidence that NC4 cheats V12, we found that when V12 was rare, more macrocysts were produced than when NC4 was rare. This suggests that in our conditions, V12 gained the reproductive advantage assuming, as noted above, that cells it does not invest when common are able to survive and reproduce. This pattern was consistent across all five strains of this clone pair. This surprising finding could indicate a hint of plasticity in the inducing trait, such that unknown, and therefore uncontrollable, environmental factors impact how clones interact during the sexual cycle.

Our data clearly show that varying the availability of compatible partners impacts macrocyst production, but our understanding of sexual compatibility in *D. discoideum* remains

478 incomplete. Even when we paired clones whose mating types were known to be compatible, we
479 observed unexplainable incompatibility, suggesting that the current mating type classification
480 and understanding of environmental or chemical triggers for sex may be incomplete (Table S2-
481 5). This pattern reinforces previous claims that mating compatibility can be variable across
482 clones, with some clones producing no macrocysts at all (Erdos et al. 1973b). Further
483 investigation into these patterns could reveal additional insight into when and how social
484 amoebae mate.

485 Early studies proposed that disproportionate contribution to macrocyst production,
486 comparable to what we observed in just a few clone pairs, was induced by a diffusible hormone
487 that could even make otherwise self-incompatible clones undergo homothallic mating (Lewis
488 and O'Day 1975; MacHac and Bonner 1975). Since we were unable to induce macrocyst
489 production in this way, we conclude that both clones are required to produce macrocysts, likely
490 due to an inability to self. This agrees with other studies that were also unable to recreate this
491 induced selfing (Erdos et al. 1973b; Wallace 1977; Bozzone and Bonner 1982). Required
492 heterothallic mating supports our hypothesis that the linear patterns reflect cheating. The cheater
493 can gain a reproductive advantage if more macrocysts are produced when it is rare by
494 contributing the same number of cells as its partner to the reproductive zygote, but at a relatively
495 lower cost by contributing disproportionately fewer cells to be cannibalized.

496 Overall, our findings contribute further evidence that mating type-specific differential
497 investment during sex is unlikely or rare in microbial eukaryotes. Our results complement
498 previous findings that reproduction in *D. discoideum* is isogamous, involving gametes identical
499 in size and form (Douglas et al., 2016). They also fit with the assumption that evolved
500 differences between sexes are correlated with vegetative complexity (Knowlton 1974; Bell

1978). Though *D. discoideum* aggregates into a multicellular structure during its social and sexual cycles, most of its life is spent as a unicellular amoeba. In addition to being indistinguishable in appearance, the three *D. discoideum* sexes are also indistinguishable in their investment to nutrient provisioning during macrocyst production. This differs from what would be expected if the peripheral cell contribution was more analogous to yolk production or other primarily maternal investments. In general, the cost of mating (i.e. sacrificed peripheral cells) is distributed fairly (i.e. proportionate to frequency) between two mating partners in *D. discoideum*. However, we also provide evidence for cheating between individual pairs. This suggests that, though not dictated by mating type, social conflict similar to that described in asexual fruiting body formation is also a factor during macrocyst production.

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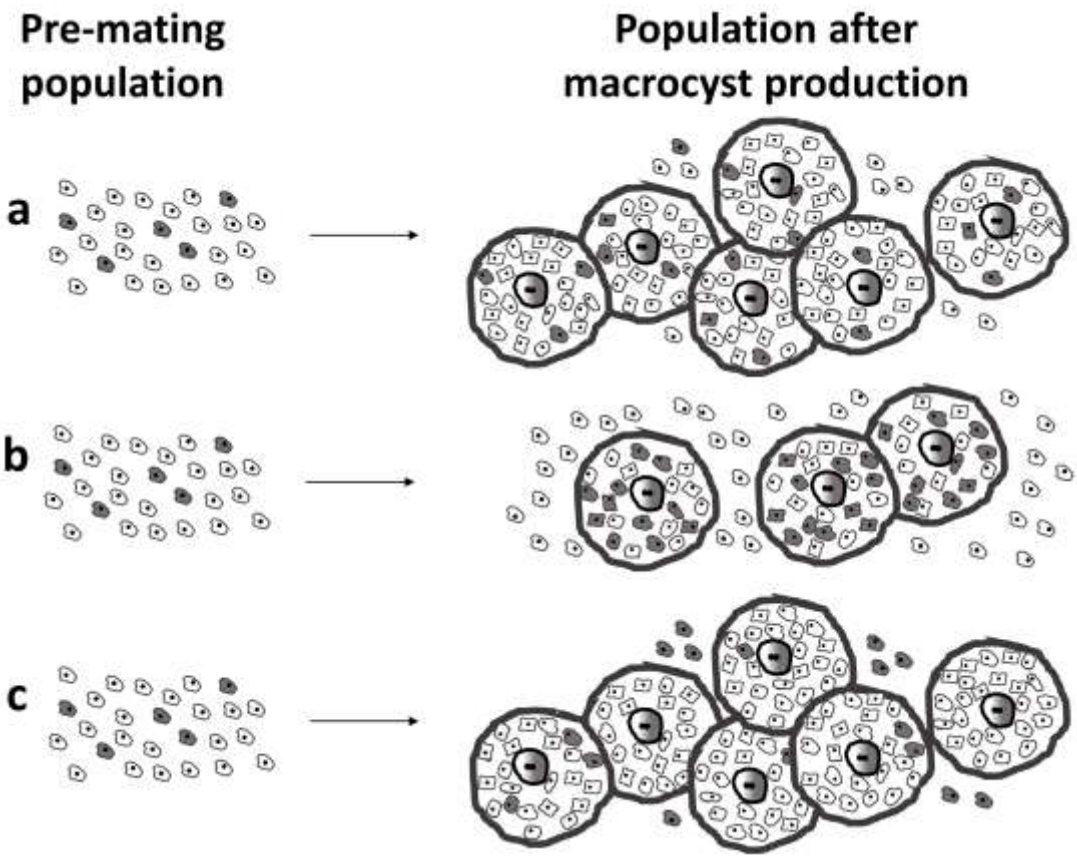


Figure 1. Alternative strategies for contributions to cannibalized peripheral cells in *Dictyostelium discoideum*. Shown are illustrations of populations of cells before macrocyst production followed by these same populations after macrocyst production. At the center of each macrocyst is a zygote formed from the fusion of one grey cell and one white cell. Here we only show scenarios where one partner is rare, represented by grey cells and the other is common, represented by white cells. In a), peripheral cells are contributed by each partner relative to its frequency in the population. In b), each partner contributes exactly the same number of peripheral cells as its mate in each macrocyst. In c), one partner induces the other to contribute disproportionately more peripheral cells, while it contributes few to no peripheral cells. In this case, the grey cells represent cells of a mating type that induces overcontribution of peripheral cells by its partner, while the white cells represent cells of a mating type that responds to this induction.

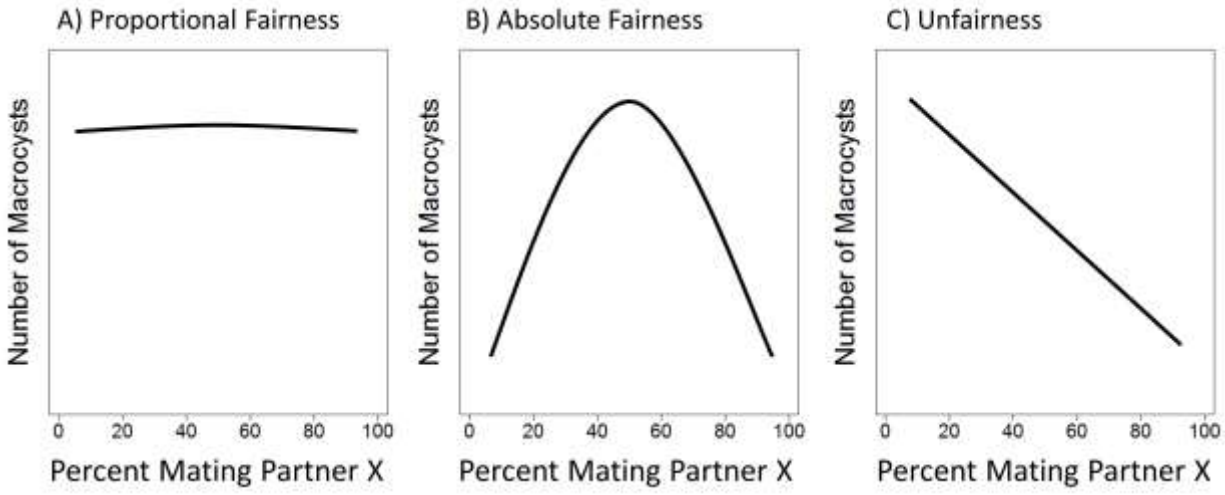


Figure 2. Predicted outcomes of different hypotheses across all mixture frequencies. Macrocyst production may reflect A) proportional contribution to peripheral cells such that a given partner contributes a number of cells relative to their frequency in the population (proportional fairness or no withholding of investment; Fig. 1, part a), B) equal contribution to peripheral cells such that each partner contributes the same number of cells (absolute fairness; each partner, when common, withholds investment like the white cells in Fig. 1, part b), or C) differential contribution to peripheral cells such that one partner contributes disproportionately fewer cells (cheating; cheater, when common, withholds investment like white cells in Fig. 1, part b; when rare, acts like the grey cells in Fig. 1, part c.).

Diffusion Chamber Combinations

Plate A	Plate B	Plate C	Expected Outcome
NC4	NC4	V12	Macrocysts in Plate C
V12	V12	NC4	No macrocysts
NC4	NC4	NC4	No macrocysts
V12	V12	V12	No macrocysts
NC4	V12	NC4 + V12	Macrocysts in Plate C

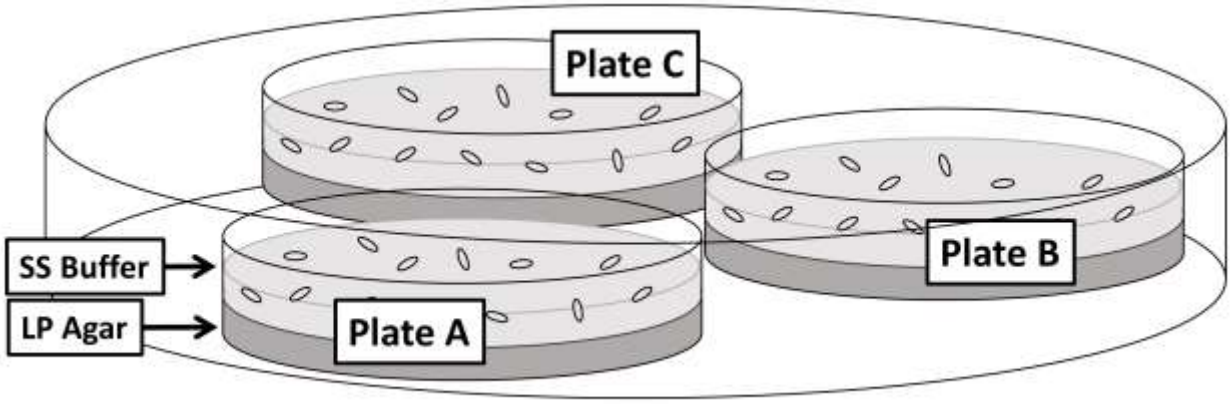


Figure 3. An example of a diffusion chamber between NC4 and V12 with the combinations of clones to be tested and the expected outcomes for each combination. This diffusion chamber is a replicate of the one described in Lewis and O'Day (1977). Based on their findings, two chambers of NC4 should induce macrocyst production in V12. Though not in the original study, the combination that includes a plate with both clones was added as a control to ensure that the overall design did not inhibit macrocyst production.

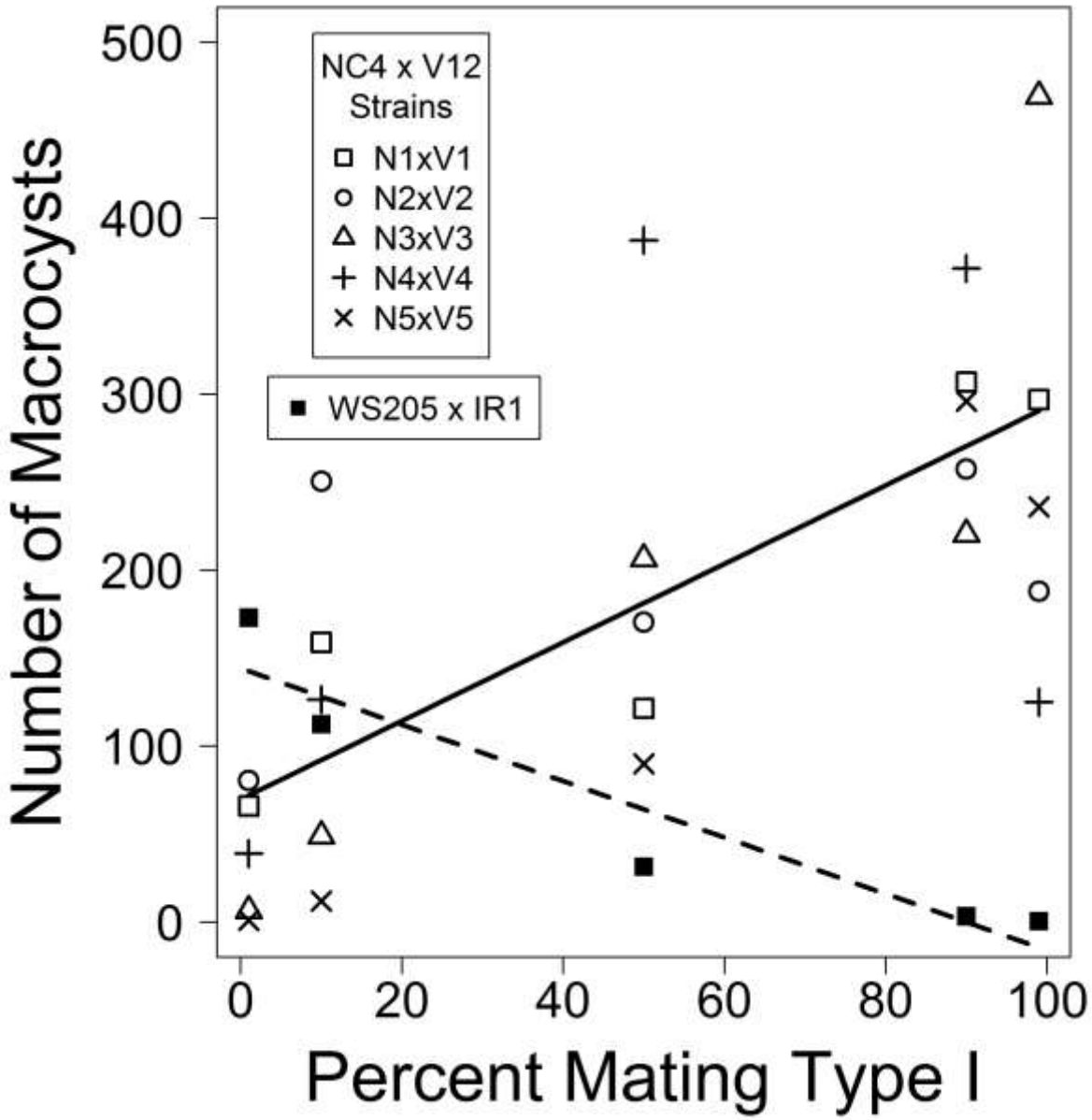
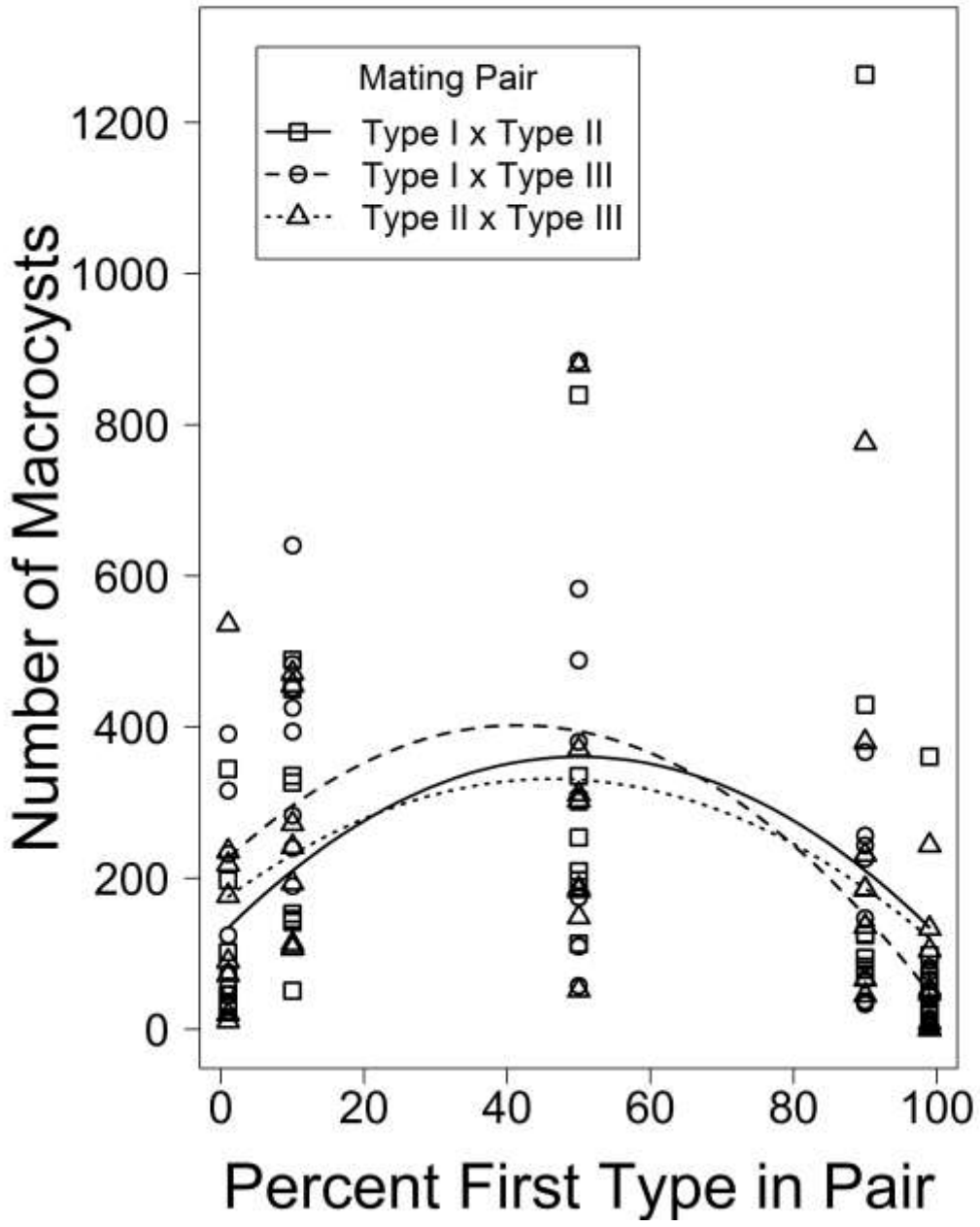


Figure 4. Type I WS205 induces macrocyst production in Type II IR1, and Type II V12 induces macrocyst production in Type I NC4. Figure shows the number of macrocysts produced at five starting frequencies of either WS205 or NC4 (both mating type I) (1%, 10%, 50%, 90% and 99%) with the reciprocal frequency of IR1 or V12, respectively. Symbols represent macrocyst production between the five strains of clone pair NC4 and V12 and the one strain of clone pair WS205 and IR1. Best-fit regression line is solid for overall NC4 x V12 and dashed for WS205 x IR1.



680

681 **Figure 5.** Fewer macrocysts are formed when either mating type in a pairing is very rare.

682 Symbols represent macrocyst production between individual clone pairs. Lines represent best-fit

683 regression curve for each mating type overall.

684