Parallel patterns of development between independent cases of hybrid seed inviability in *Mimulus*

Jenn M. Coughlan[1,2], John H. Willis[1]

1. *Biological Sciences, Duke University 25 Science Drive, Durham NC*
2. *Biology Department, University of North Carolina, Chapel Hill*

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Running headline: *Parental conflict and hybrid seed inviability in Mimulus*

Summary

Rationale: The evolutionary drivers of many reproductive barriers are unknown. Hybrid seed inviability (HSI) is a common barrier in angiosperms. We test the idea that conflict between maternal and paternal interests in resource allocation to developing offspring (i.e. parental conflict) drives HSI in two independent incidences of HSI.

Methods: We quantified HSI between *M. guttatus* and two clades of *M. decorus* with oppositely asymmetric HSI. In the northern, weaker-conflict clade, seeds are inviabile when *M. decorus* is the mother, while in the southern, stronger-conflict clade, inviability occurs when *M. decorus* is the father. We quantified whole-seed development for hybrids and parents to assess whether reciprocal F1 seeds show parent-of-origin dependent growth phenotypes, as predicted by conflict.

Key Results: Crosses between *M. guttatus* and both clades of *M. decorus* show parent-of-origin effects on reciprocal F1 seed development. Inviable hybrid seeds exhibit paternal-excess phenotypes, wherein endosperm is large and chaotic. In contrast, viable hybrid seeds show maternal-excess phenotypes, wherein endosperm development is precocious.

Main Conclusions: We find strong parent-of-origin effects on development in reciprocal F1s that are repeated across independent incidences of HSI in *Mimulus.* These patterns suggest that parental conflict may play a strong role in shaping the evolution of HSI.

Introduction

The repeatability of speciation is a contentious topic in evolutionary biology. Much pre-zygotic reproductive isolation can be attributed to adaptations to the abiotic or biotic environment (Coyne & Orr 2004), and the repeatability of extrinsic reproductive isolation may be directly related to the repeatability of adaptation (i.e. Rundle *et al.* 2000; Rolán-Alvarez *et al.* 2004; Stankowski 2013; Soria-Carrasco *et al.* 2014; Stuart *et al.* 2017). Yet, intrinsic post-zygotic reproductive isolation also often manifests as canonical hybrid phenotypes (i.e. abdominal ablation in hybrid *Drosophila* species pairs- Orr *et al.* 1997; Gavin-Smyth & Matute 2013; CMS hybrid sterility phenotypes in plants- reviewed in Hanson & Bentolila 2004; Chase 2007; Rieseberg & Blackman 2010; hybrid necrosis in multiple plant and fungi species pairs- reviewed in Bomblies & Weigel 2007), and much less is known about the evolutionary mechanisms that cause this repeatability at the phenotypic level. Moreover, in many systems it is unclear whether these common and repeated incompatibility phenotypes are developmentally similar, and whether this can inform our understanding of the drivers of hybrid incompatibilities. A classic hybrid incompatibility phenotype in plants is that of hybrid seed inviability (HSI).

Proper seed development is achieved through the effective coordination of three tissues with differing genetic compositions: the maternally derived seed coat, the developing embryo, and a nutritive tissue that sustains the embryo until germination- the endosperm. In diploid species, seed development begins with a double fertilization event; one in which the ovule is fertilized and the developing embryo is formed, while the other involves the fertilization of a diploid central cell that gives rise to a triploid endosperm tissue. The endosperm is thus comprised of 2:1 maternal:paternal genetic composition. This ratio is essential for appropriate seed development, because many genes exhibit parent-of-origin specific expression in the endosperm (i.e. imprinting; Köhler & Weinhofer-Molisch 2010). In *Arabidopsis*, this is caused primarily by DNA methylation and histone methylation (Köhler *et al.* 2012), the later of which are controlled by the Fertilization Independent Seed- Polycomb Group Complex (FIS-PcG complex; Köhler *et al.* 2003; Köhler *et al.* 2005; Gehring *et al.* 2006; Makarevich *et al.* 2006; Köhler &

74 Makarevic 2006; Gehring *et al.* 2009; Hsieh *et al.* 2009; Wolff *et al.* 2011; Waters *et al.*
75 2011; Derkacheva *et al.* 2016). Indeed, imprinting in plants is thought to have evolved as
76 a mechanism to decrease conflict over paternal and maternal interests in nutritive tissue
77 growth (i.e. parental conflict; Trivers 1974; Haig & Westoby 1989).
78 Parental conflict is the hypothesis that maternal and paternal interests in resources
79 allocation to offspring differ in non-monogamous systems (Trivers 1974; Haig &
80 Westoby 1989). As maternity is guaranteed, selection should favor equal distribution of
81 resources from mothers to offspring, but when paternity is variable, selection should
82 favor the evolution of paternally derived alleles which increase resource expenditure on
83 developing offspring (Trivers 1974; Haig & Westoby 1989). If populations have evolved
84 differentially in this co-evolutionary arms race, then mismatches between resource-
85 restrictive maternal alleles and resource-acquiring paternal alleles in hybrids can lead to
86 developmental defects, and ultimately, death. One prediction of parental conflict is that
87 reciprocal F1s should show significantly different developmental trajectories, wherein
88 strong-conflict populations should produce growth-restrictive phenotypes when the
89 maternal contributor, and growth-excessive phenotypes when the paternal contributor in
90 crosses with weaker conflict populations.
91 In *Arabidopsis* hybrid seed inviability in interploidy crosses, maternal-restrictive
92 and paternal-excess phenotypes are observed (Scott *et al.* 1998). Inviability in this case
93 arises because of de-repression of normally imprinted genes, particularly that of *ADM,*
94 *SUVH7, PEG2*, and *PEG9*, which are all associated with carbohydrate sequestration and
95 cellularization in normally developing seeds (Kradofler *et al.* 2013; Wolff *et al.* 2015).
96 Overexpression of these alleles causes excessive growth and improper cellularization of
97 the endosperm associated with paternal excess, while maternal excess is often associated
98 with precocious endosperm development (Scott *et al.* 1998; reviewed in Lafon-Placette &
99 Köhler 2016). These defects in the endosperm are causative to hybrid inviability; both
100 embryo rescues and dosage rescue experiments strongly suggest that in these interploidy
101 crosses, hybrid seed inviability is due specifically to inappropriate development and
102 growth of the endosperm, rather than incompatibilities arising in the embryo itself

103 (Bushell *et al.* 2003; Josefsson *et al.* 2006; Dilkes *et al.* 2008; Erilova *et al.* 2009;
104 Kradolfer *et al.* 2013).
105 While hybrid seed inviability is quite common in diploid species pairs (Tiffin *et*
106 *al.* 2001), far fewer studies have determined the mechanistic underpinnings of hybrid
107 seed inviability among diploids. In plants with nuclear endosperm development, such as
108 *Arabidopsis* and *Capsella,* hybrid seed inviability mimic interploidy crosses with a
109 delayed or premature endosperm cellularization, depending on the maternal and paternal
110 donor in the cross, and endosperm defects are intrinsically linked with hybrid seed
111 inviability (Joseffson *et al.* 2006; Burkart-Waco *et al.* 2012; Rebernig *et al.* 2015; Lafon-
112 Placette *et al.* 2017l Lafon-Placette *et al.* 2018). In plants with cellular endosperm
113 development (i.e. *Solanum* and *Mimulus*), hybrid seed inviability is much less explored,
114 although there is some evidence to suggest that inappropriate replication of endosperm is
115 at least partially causative of hybrid seed inviability in both *M. nudatus* and *M. guttatus*
116 inviable hybrid seeds (Oneal *et al.* 2016), and in wild tomato varieties (Lafon-Placette *et*
117 *al.* 2016; Roth *et al.* 2017).
118 We previously reported a wide diversity of hybrid seed barriers between *M.*
119 *guttatus* and its morphological variant, *M. decorus* which confer relatively strong
120 reproductive isolation (Coughlan *et al.* 2018). We found that *M. decorus* is in fact
121 multiple biological species; a northern clade exhibits strong asymmetric hybrid seed
122 inviability when it is the maternal donor in crosses to *M. guttatus*, a southern clade that
123 exhibits strong asymmetric hybrid seed inviability when it is the paternal donor in crosses
124 to *M. guttatus,* and at least one tetraploid taxon that exhibits variable levels of
125 reproductive isolation via hybrid seed inviability. Here, we test whether hybrid seed
126 inviability between different diploid accessions of *M. decorus* and *M. guttatus* are
127 associated with similar developmental defects, whether these defects are endosperm
128 related, and whether they are consistent with paternal excess/maternal restriction,
129 hypothesized by parental conflict. In addition, we assess the level of developmental
130 parallelism of inviable and viable hybrid seeds between diploid species pairs that have
131 strongly asymmetric hybrid seed inviability, but in the opposite direction.
132

133 **Methods**

134 *Growth conditions and plant crossing*

135 Based on previous results, *Mimulus decorus* exhibits two distinct crossing
136 patterns: the northern clade of *M. decorus* produces inviable seeds when they are the
137 maternal plant in crosses with *M. guttatus*, while for the southern clade produces inviable
138 seeds when they are the paternal plant in crosses with *M. guttatus*. To determine the
139 extent of developmental parallelism, we chose a representative diploid population from
140 each crossing type of *M. decorus* (northern clade: *IMP*; southern clade: *Odell Creek*) to
141 cross with an annual population of *M. guttatus*- Iron Mountain (*IM*). For each population,
142 we planted seed from 3-4 maternal families or inbred lines. For all plants, seeds were
143 sprinkled onto moist Fafard 4-P soil, left to stratify at 4°C for 1 week, then moved to the
144 Duke greenhouses. Plants were transferred within the first 3 days after germination to one
145 plant per 2" pot, then were grown in long days (18h days, 21C day/ 18C nights).

146

147 *Crossing Assays*

148 Plants were emasculated and crossed reciprocally, as well as self pollinated to
149 yield an average of 6 crosses for each pairwise combination of maternal families between
150 IM and Odell Creek, and IM and IMP (IMxOdell range: 2-13 replicate crosses, IMxIMP:
151 2-10 replicate crosses for a total of 298 crosses). Fruits were allowed to develop until ripe
152 but before dehiscence, then seeds for each cross were counted and categorized as inviable
153 or viable based on morphology for each fruit.

154

155 *Microscopy and measuring growth*

156 We sought to determine the extent of developmental parallelism across hybrid
157 seeds which show opposite patterns of inviability between these two populations of *M.*
158 *decorus.* We next crossed plants, as in above, but collected fruits at 4,6,8,10 and 14 Days

159 After Pollination (DAP). Fruits were collected and immediately placed in FAA fixative.
160 Plants were processed in a similar manner to White & Turner (2012). In brief, after at
161 least 24 hours in fixative, fruits were gradually dehydrated in a Tetrabutyl-Alcohol
162 dehydration series, then mounted in paraffin wax with ~5% gum elemi resin to increase
163 hardness. Paraffin mounted specimens were then sliced to 8 micron ribbons and mounted
164 onto slides. We performed a staining series using Safranin-O and Fast-Green, which stain
165 for nucleic acids and carbohydrates, respectively. Cover slips were then adhered for
166 permanent storage.

167 We visualized and photographed at least three developing seeds from a single
168 fruit for each cross type and time point combination (40 unique combinations, 120 seeds
169 measured). At least 25 endosperm cell widths were measured per seed using ImageJ
170 (Scheider *et al.* 2012).

171

172 *Embryo Rescues*

173 To assess whether hybrid seed inviability was due largely to inappropriate
174 development of the endosperm tissue, we also performed embryo rescue assays on both
175 IMxOdell seeds and IMPxIM seeds, which would normally be inviable if left to develop
176 fully in the seed pod. For this, we crossed IM reciprocally to both IMP and Odell creek,
177 as well as performed self fertilizations for IMP, Odell Creek, and IM. Fruits were left to
178 mature for 7 or 9 days. Based on our developmental time series, embryos appear to die
179 between 10-14 DAP for both sets of crosses. We plated whole developing seeds for each
180 cross type on a sucrose rich growth media (1/2 MS salts, 0.7% agar, 4% sucrose, adjusted
181 to pH 5.9) at 7 and 9 DAP. Plates were cold stratified for 2 days, then placed in the Duke
182 Greenhouses (18h days, 21C day/ 18C nights).

183

184 *Statistical Analyses*

185 Cross incompatibilities were determined with a linear mixed model using the
186 package *lme4* and a type III ANOVA using *car* (Bates *et al.* 2015; Fox & Weisberg
187 2011) in the statistical interface R. All analyses were completed separately for IMxOdell
188 and IMxIMP crosses. For both total seed number, and proportion viable seed, we treated
189 cross type as a fixed factor and replicate maternal and paternal lines as random effects
190 (i.e. *Proportion viable seed ~ Cross Type + (1|maternal replicate) + (1|paternal*
191 *replicate)* or *total seed count ~ Cross Type + (1|maternal replicate) + (1|paternal*
192 *replicate)*). Pairwise T-tests with Holm correction for multiple testing were performed to
193 determine which cross types varied significantly from one another. Differences in
194 developmental trajectories were also assessed using a linear mixed model, as above. In
195 this case, cross type, DAP, and their interaction were the fixed effects and the seed
196 replicate was treated as a random effect.

197

198 **Results**

199 *Post-Mating Reproductive Isolation*

200 IMP and Odell Creek show consistent patterns of strong, and asymmetric hybrid
201 seed inviability when crossed with individuals from Iron Mountain, but in opposing
202 directions (Figure 1; Odell Creek: X^2= 4598, df=3, p<0.0001; IMP: X^2= 2969, df=3,
203 p<0.0001). IMP produces entirely inviable seeds when the maternal donor in these
204 crosses, while Odell Creek produces entirely inviable seeds when the paternal donor (and
205 some inviable seeds when the maternal donor; Figure 1). Inviable hybrid seeds appear
206 larger, disc-like, and shriveled, while viable hybrid seeds appear smaller overall (similar
207 to Coughlan *et al.* 2018).

There is some evidence of pollen-pistil incompatibilities between annual *M. guttatus* and Odell Creek (Odell Creek: X^2= 49.33, *df*=3, *p*<0.0001; Figure S1), wherein IM lines set less seed when pollinated by Odell Creek than any other cross type. We find no evidence of pollen-pistil interactions between IM and IMP, although we note that IMP has substantially larger ovaries and produces more seeds, regardless of the pollen donor (X^2= 23.7, *df*=3, *p*<0.0001; Figure S1).

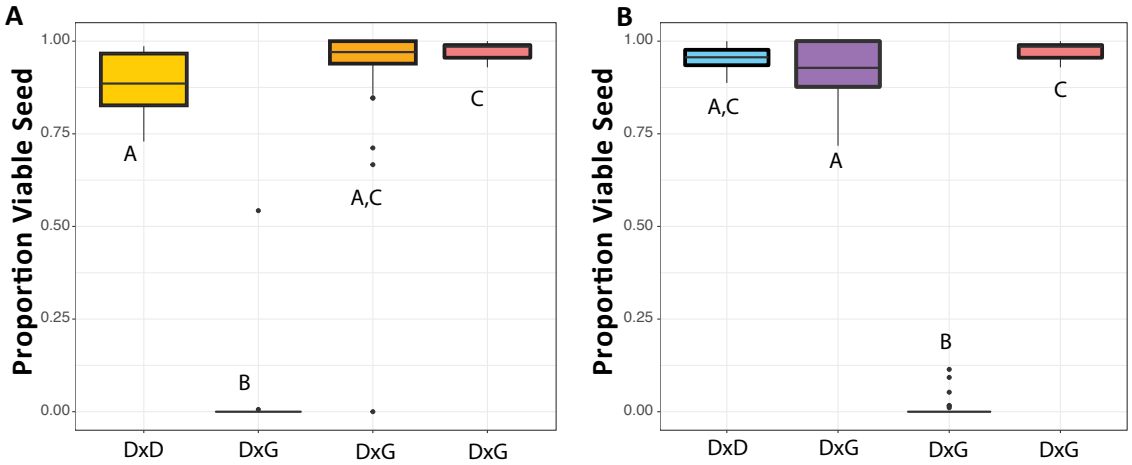

Figure 1: Proportion viable seed between three lines of *M. guttatus* (IM) and (A) four maternal families of northern *M. decorus* (IMP) and (B) three maternal families of southern *M. decorus* (Odell Creek). Maternal parent is listed first.

Developmental Trajectories

The three parental species surveyed here display similar seed development. In all three parents, endosperm is tightly packed around the embryo and continues to grow until around 8-10 DAP. At this point, the endosperm is depleted, presumably to feed the growing embryo. Embryo development among the three parents is also quite similar, with IM displaying a slightly accelerated growth, relative to either Odell or IMP (i.e. they reach a heart-shaped embryo around 10 DAP, while this is not achieved in Odell or IMP until around 14 DAP; Figure 2; Figure 3). By 14 DAP the embryo comprises most of the embryo sac, with a thin layer of endosperm remaining.

228 Hybrid seeds from both the IM/IMP and IM/Odell crosses show distinct
229 developmental trajectories based on the maternal versus paternal donor of the cross, but
230 both hybrid crosses display similar seed trajectories, depending on the fate of the seed
231 (i.e. whether it will remain viable or not). In both cross types, seeds which remain viable
232 (e.g. IMxIMP and OdellxIM) display a precocious development, with several small
233 endosperm cells that are quickly degraded by the embryo (Figure 2; Figure 3) . By 14
234 DAP, the only remaining endosperm is a thin layer which surrounds the embryo (Figure
235 2; Figure 3). The overall seed size is also much smaller than either parent or the
236 reciprocal hybrid (Coughlan *et al.* 2018). In contrast, the hybrid seeds which will
237 eventually become inviable (i.e. IMPxIM and IMxOdell) exhibit a chaotic endosperm
238 growth, producing fewer, but larger and more diffuse endosperm cells (Figure 2; Figure
239 3). Embryos in this direction of the cross remain small, relative to both parents and the
240 reciprocal hybrid (Figure 2; Figure 3). By 14 DAP, most embryos were entirely degraded.
241

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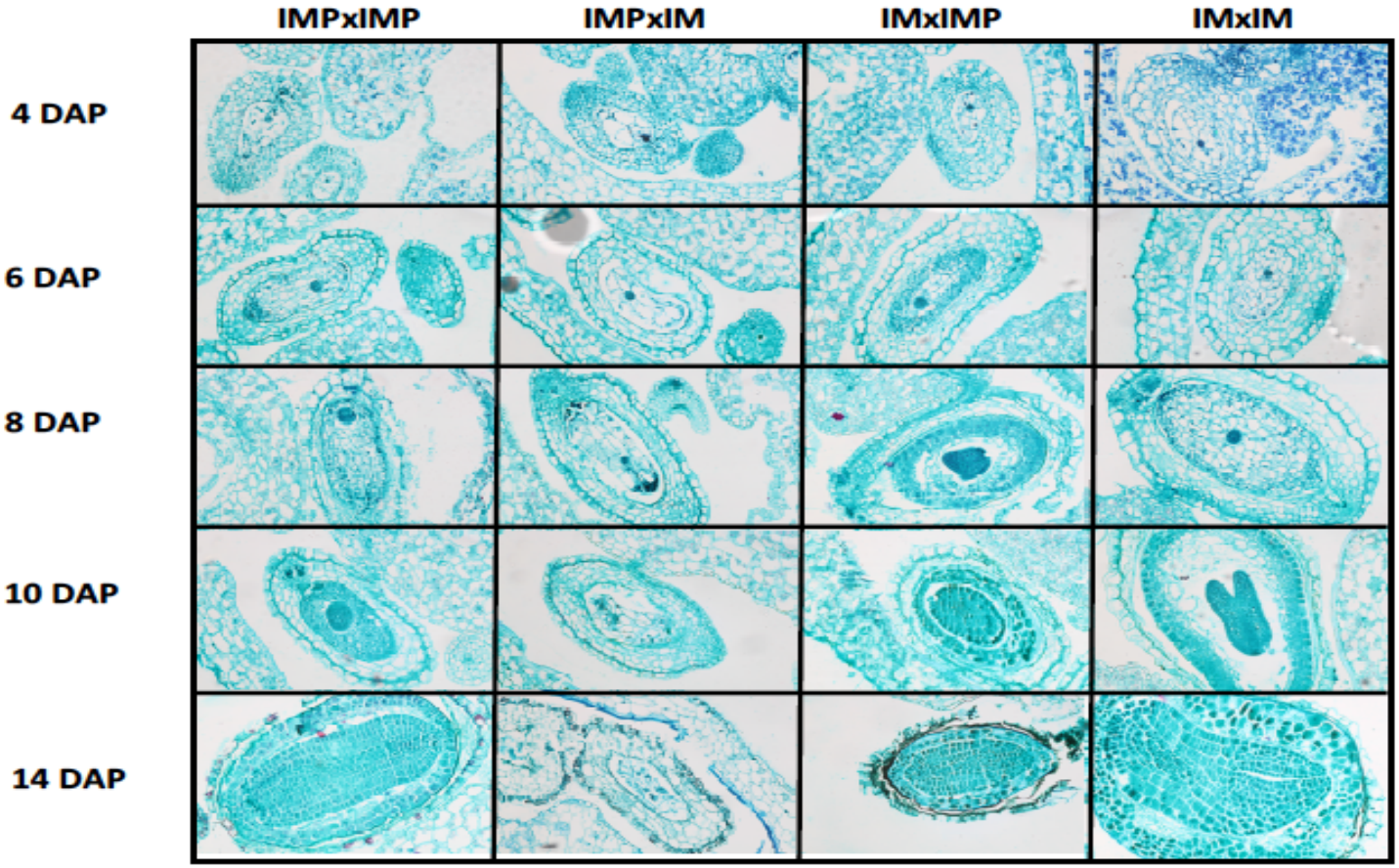

243

244 **Figure 2: Seed development of IM62 x IMP crosses at 4, 6, 8, 10, and 14 Days After Pollination (DAP). Maternal parent**

245 **is listed first.**

These patterns are recapitulated when looking simply at endosperm cell width. In both crosses, the type of cross significantly effected the endosperm cell width (Table 1; Figure 4, Figure S2). Reciprocal F1 hybrids display the most extreme endosperm cell sizes, with inviable seeds exhibiting the largest endosperm cell size, and viable seeds displaying the smallest endosperm cells. For both IMxIMP and IMxOdell Creek crosses, the relationship between cell size and cross type was dependent on DAP (although we note this relationship is not linear; significant interaction term: Table 1; Figure S2).

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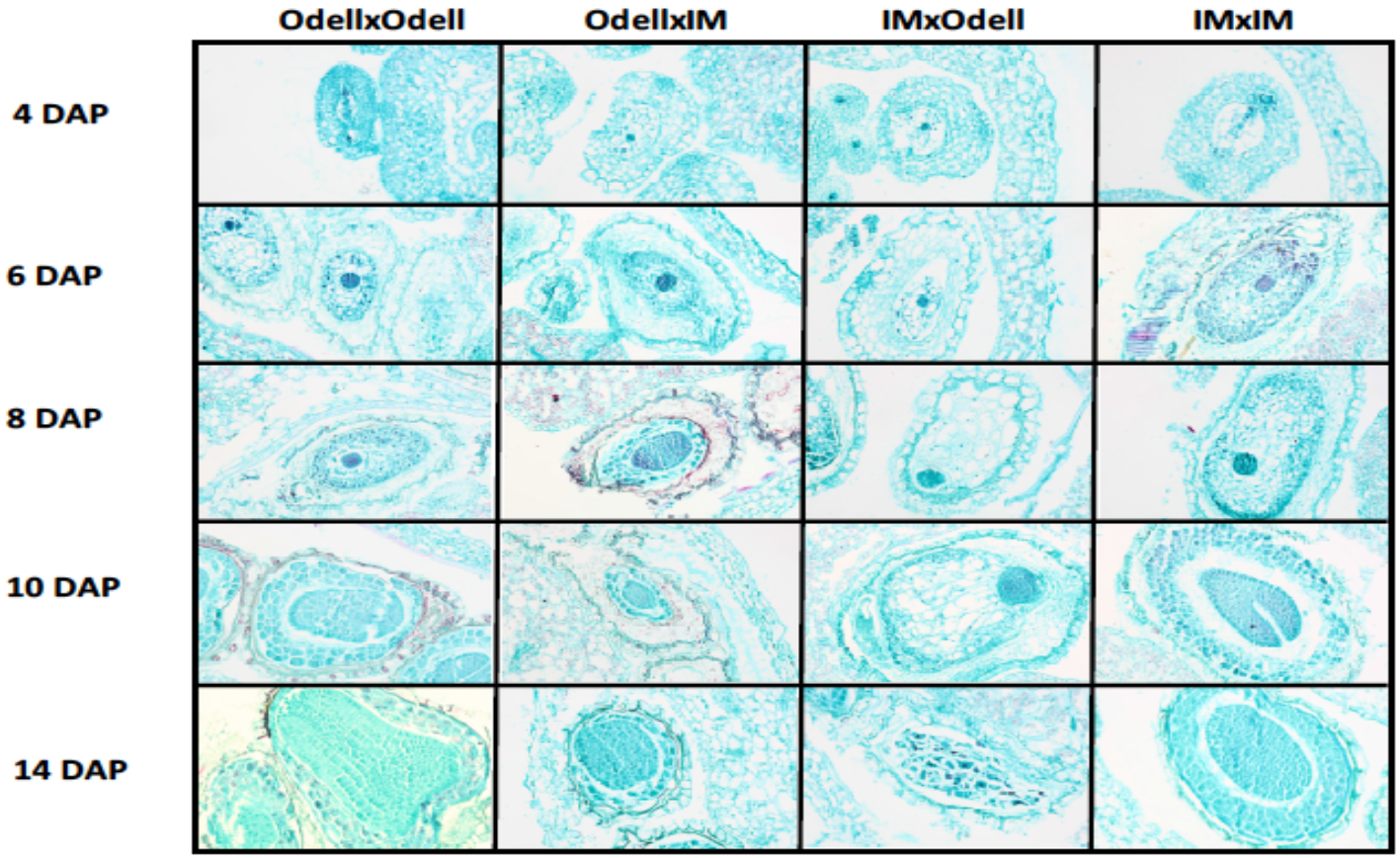

256

257 **Figure 3: Seed development of IM62 x Odell Creek crosses at 4, 6, 8, 10, and 14 Days After Pollination (DAP). Maternal**

258 **parent is listed first.**

While the two different crosses display quite similar overall patterns of development, there are some distinctions between hybrid seeds formed between IMxIMP, and IMxOdell Creek. For example, in IMPxIM crosses, the embryo remains comparatively small, and does not appear to grow past the 6 or 8 DAP. In contrast, seeds formed from a IMxOdell cross often exhibit a developing embryo up until 10 DAP, suggesting that the seed is aborted slightly later in this cross.

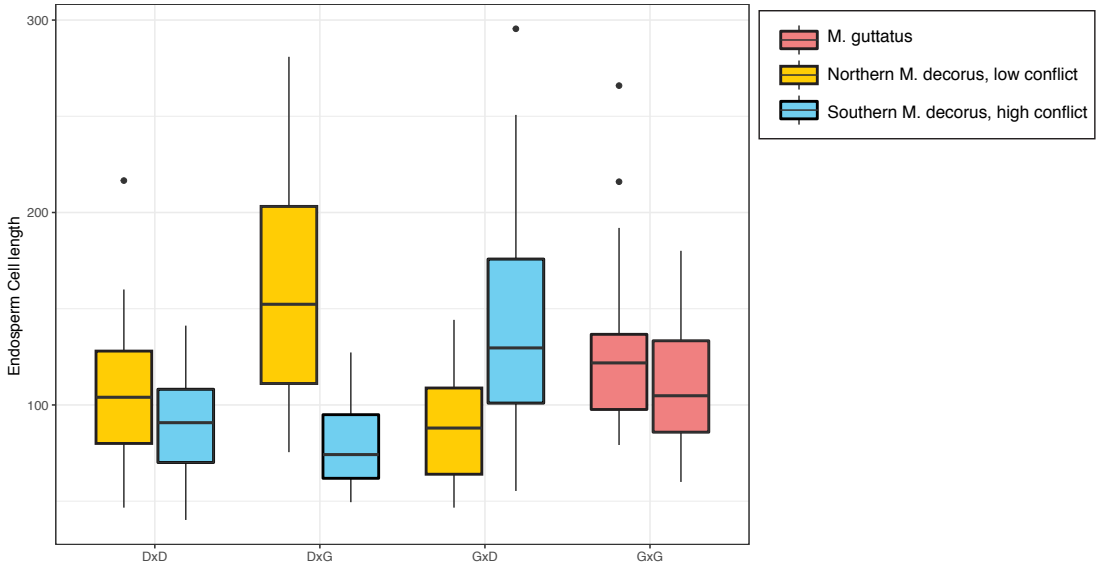

Figure 4: Cell width at 8 Days After Pollination (DAP) for each cross type (maternal parent listed first), for both *M. guttatus* crossed to IMP (yellow) and Odell Creek (blue).

Overall, we see striking parallelism between two distinct seed incompatibilities with reciprocal F1 seeds displaying either excessive and chaotic growth patterns or precocious and stunted patterns, depending on whether the seeds are eventually aborted or not. We suggest that hybrid seed inviability in both of these cases is likely a result, at least in part, of defective endosperm development which manifests as chaotic and excessive growth.

277 **Table 1: Mixed model for type III ANOVAs for seed cell size across developmental time for**
278 **IMP and Odell Creek crossed reciprocally to IM62. Cross Type= both reciprocal F1**
279 **crosses and self-fertilizations from each species treated as a separate level; treated as a**
280 **fixed effect), DAP= Days After Pollination (fixed effect). Seed replicate was treated as a**
281 **random effect.**

	IMP			Odell Creek		
	X^2	*DF*	*p*	X^2	*DF*	*p*
Intercept	295.04	1	<0.0001	123.74	1	<0.0001
Cross Type	119.848	3	<0.0001	14.92	3	0.0018
DAP	36.033	1	<0.0001	0.0029	1	0.96
Cross Type*DAP	42.583	3	<0.0001	32.95	3	<0.0001

282

283 *Embryo Rescues*

284 Embryo rescues were generally unsuccessful in these crosses. No seeds germinated when
285 collected 7 DAP. Only a small fraction of IM62 and hybrids which were viable (i.e. OdellxIM
286 and IMxIMP) germinated in the 9 DAP collections (i.e. 5-10%). Overall, this suggests that 7
287 DAP may be too premature for proper endosperm development of viable and inviable seeds to
288 occur outside of the maternal environment, while only rapidly developing seeds are able survive
289 transplants at 9 DAP. Unfortunately, determining whether hybrid seed inviability is due solely to
290 endosperm malformation is yet to be determined in this group.

291

292 **Discussion**

293 As previously reported, the northern and southern clades of *M. decorus* exhibit strong,
294 and asymmetric reproductive isolation with *M. guttatus*, but in opposite directions of the cross.
295 This observation is true for all maternal family/line combinations tested here (as well as multiple
296 populations tested in Coughlan *et al.* 2018), suggesting that HSI is not geographically restricted,
297 as other intrinsic post-zygotic barriers have been found to be (Sweigart *et al.* 2007; Case &

298 Willis 2008; Martin & Willis 2010; Sicard *et al.* 2015; Barnard-Kubow & Galloway 2017). Total
299 reproductive isolation in these crosses was ~52% for both both species pairs, and thus may
300 represent a significant barrier to reproduction. Hybrid seed inviability has been shown to be a
301 formidable barrier to reproduction in other systems (i.e. Tiffin *et al.* 2001; Lowry *et al.* 2008),
302 but the developmental causes of this incompatibility have really only been quantified for a small
303 number of diploid species pairs.

304 Here we show that hybrid seed inviability is strongly associated with developmental
305 defects in endosperm deposition. In inviable directions of the cross, endosperm is chaotically
306 distributed, and the cells are few and large, suggesting a failure to proliferate. In the viable
307 directions of the cross, seeds develop precociously, and endosperm deposition is limited, as
308 witnessed by the general lack of endosperm remaining when seeds are fully developed, as well as
309 the generally smaller size of both the cells and final seeds (Coughlan *et al.* 2018). Overall, these
310 developmental patterns fit with a paternal excess/maternal repression model of hybrid inviability,
311 wherein paternal excess is more likely to lead to seed abortion. Unfortunately, given the
312 generally low success rate of the embryo rescues, it remains unclear whether hybrid seed
313 inviability is due strictly to malformation of the endosperm, or whether genetic incompatibilities
314 in the embryo also contribute.

315 These patterns have been shown in wild species pairs of both *Arabidopsis* and *Capsella*,
316 which have a nuclear-type endosperm development (Rebernig *et al.* 2015; Lafon-Placette *et al.*
317 2017). In these crosses, as well as interploidy crosses, endosperm over-proliferation is
318 accompanied by a failure of endosperm cells to cellularize (Scott *et al.* 1998; Bushell *et al.* 2003;
319 Rebernig *et al.* 2015; Lafon-Placette *et al.* 2017), and at least in *Capsella,* paternal excess is
320 associated with inviability, but maternal excess still produces viable seed (Rebernig *et al.* 2015).
321 Initial findings from species with cellular-type endosperm development (e.g. wild tomatoes and
322 other *Mimulus* species pairs) also show endosperm defects as a major cause of hybrid inviability
323 (Roth *et al.* 2017; Oneal *et al.* 2016). However, these defects to not represent defects in rates of
324 cellularization, but rather rates of proliferation, cell size and distribution, and optical density
325 (Oneal *et al.* 2016; Roth *et al.* 2017).

326 The developmental trajectories of viable vs non-viable hybrid seeds are strikingly similar
327 between OdellxIM, and IMPxIM crosses, despite the fact that inviability occurs in the opposite
328 cross direction. This suggests that similar evolutionary pressures may indeed be causing hybrid

seed inviability within this complex, namely that of parental conflict. Despite considerable developmental parallelism, it remains to be seen how constrained the genetic targets of selection are between these species pairs. In other post-zygotic reproductive barriers, canonical hybrid incompatibilities often share similar genetic bases. For example, hybrid necrosis is often caused by interactions involving R genes (reviewed in Bomblies & Weigel 2007), and CMS almost always involves PPR genes (polcentric peptide repeat genes; reviewed in Rieseberg & Blackman 2010). Yet, whether this remains true for hybrid seed inviability remains to be seen. Indeed, one may predict that the genomic target size for influencing resource provisioning to seeds is presumably larger than the number of genes responsible for auoto-immune function or managing organelle expression, as is the case with hybrid necrosis and CMS, respectively. However, this prediction remains to be tested.

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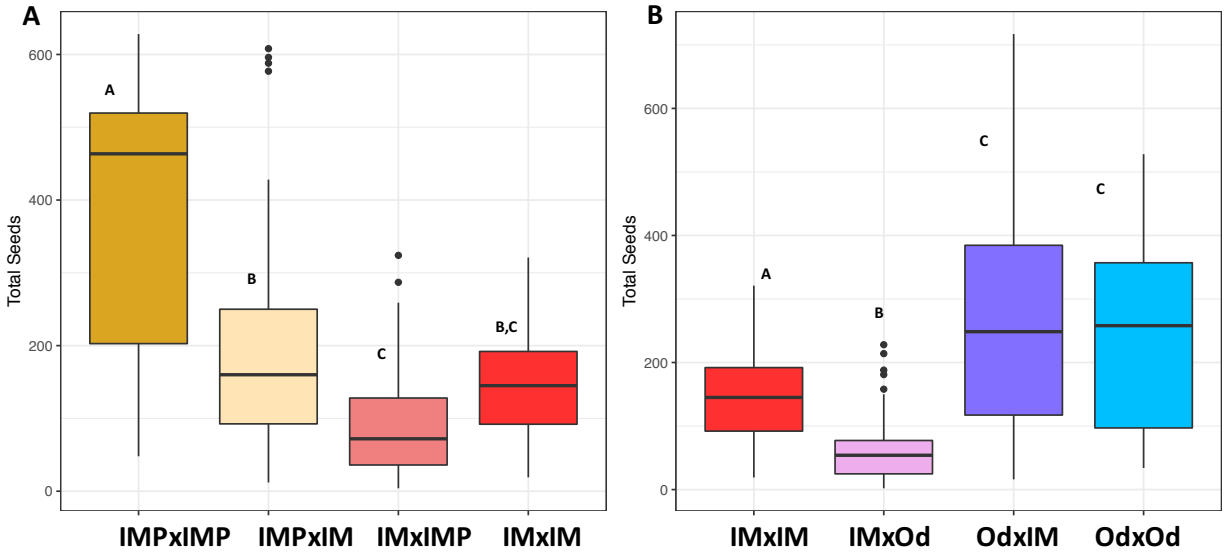
Data Availability

Data for the manuscript will be made publically available.

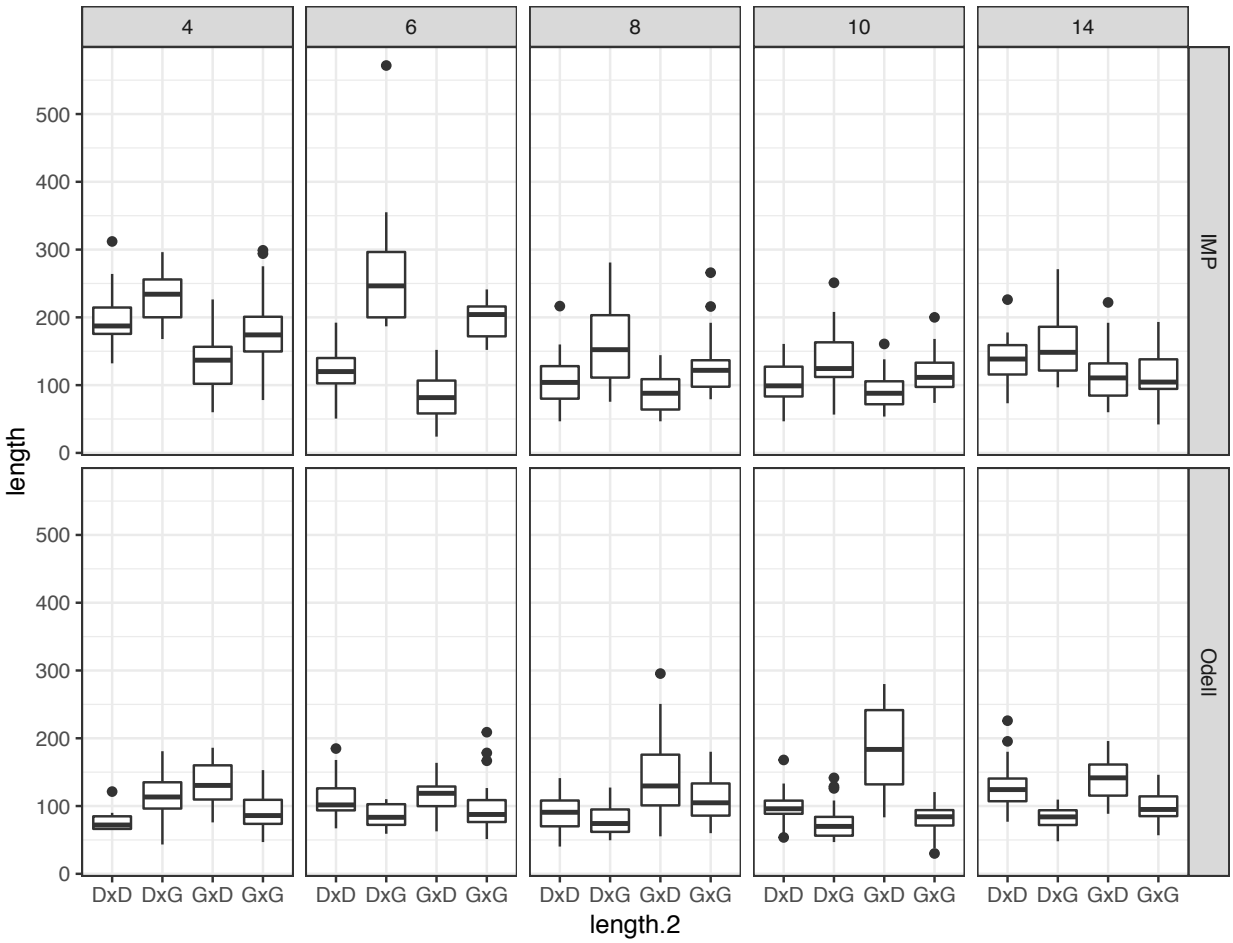
357 **Supplemental Figures**



358

359 **Figure S1: Total number of seeds in crosses for (A) three lines from IM and four maternal**
360 **families of IMP selfed and crossed reciprocally and (B) three lines of IM and three**
361 **maternal families of Odell Creek (Od) selfed and crossed reciprocally.**

362



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364 **Figure S2: Cell lengths across all cross types for 5 developmental stages for both IMxIMP**

365 **and IMxOdell crosses.**