Inversion frequencies and phenotypic effects are modulated by the environment

Emma Berdan[1], Hanna Rosenquist[2], Keith Larson[3], Maren Wellenreuther[4,*]

[1]Department of Marine Sciences, University of Gothenburg, Gothenburg, Sweden

[2] MEMEG, Ecology Building, Lund University, Lund, Sweden

[3]Department of Ecology and Environmental Sciences, Climate Impacts Research Centre, Umeå University, Umeå, Sweden

[4]The New Zealand Institute for Plant & Food Research Ltd, Auckland, New Zealand

*Corresponding author: Maren Wellenreuther, The New Zealand Institute for Plant & Food Research Limited, Box 5114, Port Nelson, Nelson 7043, New Zealand.

tel.: +64 3 989 7658; fax: +64 3 546 7049; e-mail: maren.wellenreuther@plantandfood.co.nz

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Abstract

Understanding how environmental variation drives phenotypic diversification within species is a major objective in evolutionary biology. The seaweed fly *Coelopa frigida* provides an excellent model for the study of genetically driven phenotypes because it carries an α/β inversion polymorphism that affects body size. *Coelopa frigida* inhabits highly variable beds of decomposing seaweed on the coast in Scandinavia thus providing a suitable test ground to investigate the genetic effects of substrate on both the frequency of the inversion (directional selection) and on the phenotype (genotype x environment effects). Here we use a reciprocal transplant experiment to test the effect of the α/β inversion on body size traits and development time across four suitable natural breeding substrates from the clinal distribution. We show that while development time is unaffected by GxE effects, both the frequency of the inversion and the relative phenotypic effects of the inversion on body size differ between population x substrate combinations. This indicates that the environment modulates the fitness as well as the phenotypic effects of the inversion karyotypes. It further suggests that the inversion may have accumulated qualitatively different mutations in different populations that interact with the environment. Together our results are consistent with the idea that the inversion in *C. frigida* likely evolves via a combination of local mutation, GxE effects, and differential fitness of inversion karyotypes in heterogeneous environments.

Keywords: Coelopa frigida, inversion polymorphism, adaptation, GxE interactions, frequency effects

Introduction

One of the central goals of evolutionary biology is to understand how organisms cope with environmental heterogeneity (Savolainen *et al*, 2013). Phenotypic plasticity can solve this problem but the phenotypic response to variation in an environmental variable (i.e. phenotypic plasticity) may vary among species as well as among genotypes of the same species. This is commonly referred to as a Genotype-by-Environment interaction (GxE, Falconer, 1990; Lynch and Walsh, 1998; Via and Lande, 1985). Abundant experimental data and theoretical work now provide strong support for the idea that the relative performance of different genotypes across environmental gradients may be involved in the maintenance of phenotypic plasticity and genetic variation as well as the evolution of fitness related phenotypes (Carreira *et al*, 2006; Ungerer *et al*, 2003).

The study of environmental heterogeneity has been championed by work on geographic patterns along clines, with classic examples coming from genetic clines in flies almost a century ago (Dobzhansky, 1948; Sturtevant, 1921). Other examples include the genetic underpinnings of traits involved in adaptation to high altitude (Storz, 2002) and pigmentation (Hoekstra and Coyne, 2007). Clines hold a large attraction as natural Darwinian laboratories because they allow researchers to quantify the gradual effects of changing environments on

genotype, phenotype, and the interaction between them (Endler, 1977). These studies have shown that while many environmental tolerance traits that vary clinally are quantitative and polygenic (Wellenreuther and Hansson, 2016), they can also be located within chromosomal segments that segregate as a single locus (Tigano and Friesen, 2016). If environmentally adaptive variants are chromosomally linked, either because they are found on chromosomal inversions or are in areas of low recombination, their selective coefficients act in an additive manner and they may be less likely to be lost via genetic drift and more likely to contribute to rapid adaptation (Kirkpatrick and Barton, 2006). In the extreme case, complex adaptations may be accomplished through the fixation of linked sets of adaptive variants (Yeaman, 2013), often called supergenes, a case in which polygenic adaptation may be more accurately modeled with single-locus models (Schwander *et al*, 2014). Good examples for such single-locus supergene adaptations come from *Drosophila melanogaster* where inversion polymorphisms produce latitudinally varying phenotypes for traits including heat and cold tolerance and body size (Reinhardt *et al*, 2014; Schrider *et al*, 2016).

Chromosomal inversion polymorphisms are being identified at a fast rate from an increasing number of populations of organisms, e.g. insects, plants, bacteria and humans, and are commonly linked to trait and fitness variation (Hoffmann and Rieseberg, 2008; Kirkpatrick and Barton, 2006). Inversions are particularly common in flies, and an intriguing mystery is why there are huge differences in levels of polymorphism and rates of fixation for inversions between closely related species and even between chromosomes in *Drosophila* spp. (Kirkpatrick,

88 2010). The seaweed fly *Coelopa frigida* (family Coelopidae) and its sister species
89 *C. nebularum* have a large chromosomal α/β inversion polymorphism on
90 chromosome I encompassing about 10% of the genome and containing ~100
91 genes (Aziz, 1975). The inversion polymorphism is known to affect a wide range
92 of life history and reproductive traits, such as development time (Day and
93 Buckley, 1980), body size (Butlin *et al*, 1982a), longevity and female fecundity
94 (Butlin and Day, 1984a; Butlin *et al*, 1982c), female resistance to male mating
95 attempts (Gilburn and Day, 1994; Gilburn *et al*, 1992) and also male's ability to
96 overcome female resistance (Butlin *et al*, 1982a).

97 The most striking of these phenotypic traits is a three-fold size difference in
98 males: male homokaryotypes for the inversion type α are much larger (~9mm)
99 than β- homokaryotypes (~3mm), whereas the heterokaryotype αβ is
100 intermediate in size (~6mm) (Wellenreuther pers. obs., Butlin and Day, 1984b).
101 Female size is not significantly affected by karyotype (Butlin and Day, 1984b).
102 Maintenance of the polymorphism is secured by strong heterosis (Collins, 1978),
103 since αβ-heterokaryotypes have higher fitness than either homokaryotype (Butlin
104 and Day, 1984a; Butlin and Day, 1989). The heterokaryotype αβ has a higher
105 viability during development than either homokaryotype, both in experiments
106 (Butlin and Day, 1984a; Leggett *et al*, 1996) and in the field, as indicated by
107 heterokaryotype excess (Butlin *et al*, 1982c; Day *et al*, 1983; Day *et al*, 1982b;
108 Gilburn and Day, 1994). Heterokaryotype excess increases under interspecific
109 competition with the congener *C. pilipes* and under intense intraspecific
110 competition (Butlin and Day, 1984a; Leggett *et al*, 1996). The effect is

111 disproportionally strong in males, especially decreasing the frequency of αα-
112 homokaryotypes (Butlin and Day, 1984a), possibly indicating lower viability of
113 males of this karyotype. However, inversion frequencies also form a cline from
114 the North Sea to the Baltic Sea, with the α inversion becoming more rare towards
115 the Baltic Sea (Day *et al*, 1983).

116 Here we explore if the environment modulates inversion frequencies and
117 phenotypic effects in *C. frigida*. For this, we study *C. frigida* populations in
118 Scandinavia that show variation in karyotype frequencies (Day *et al*, 1983).
119 Specifically, we test the possible effects of the α/β inversion polymorphism on
120 body traits and development time across four suitable natural breeding
121 substrates using a fully balanced reciprocal transplant experiment. We also
122 elucidate the relative fitness of the inversion karyotypes on these varying
123 substrates by examining the frequencies of the three karyotypes in different
124 environments.

125 Materials and methods

126 Study species and sites

127 *Coelopa frigida* belongs to the group of acalyptrate flies which exclusively forage
128 on decomposing seaweed (Cullen *et al*, 1987) and is found all along the
129 seashores of Scandinavia (Mcalpine, 1991). This species plays a vital role in
130 coastal environment health by accelerating the decomposition of algae, allowing
131 for faster release of nutrients (Cullen *et al*, 1987), and are an important food
132 source for predatory coastal invertebrates and seabirds (Fuller, 2003; Summers

133 *et al*, 1990). *Coelopa frigida* has 5 chromosomes with inversions occuring on 3 of
134 them (Aziz, 1975). The largest of these is the α/β which occurs on chromosome I.
135 The α/β inversion occurs at stable frequencies in all populations so far studied in
136 Northern Europe (Butlin *et al*, 1982a), although there may be seasonal (Butlin,
137 1983) and clinal variation (Day *et al*, 1983).

138 Inversion frequencies of *C. frigida* form a cline from the North Sea to the Baltic
139 Sea, with the α inversion becoming more rare towards the Baltic Sea (Day *et al*,
140 1983). Such clines are classic signs of selection and this cline is associated with
141 a decrease in salinity and tidal range and associated changes in the composition
142 of the wrackbed (i.e. the species of algae present). As salinity decreases from
143 the North Sea to the Skagerrak and Kattegat regions (Figure 1) there is a
144 corresponding decrease of marine species of brown seaweeds such as *Fucus*
145 *spp*. and *Laminaria spp.* moving into the Baltic Sea, *Zostera* eelgrass and red
146 algae gradually displace the brown seaweeds. We selected four populations
147 (Kämpinge, Kåseberga, Mölle and Steninge) from the southern part of the cline
148 that differ in tidal range, salinity, and wrackbed composition (Figure 1, Table 1).

149 **Reciprocal transplant experiment**

150 We conducted a fully balanced reciprocal transplant experiment involving all four
151 locations, where each fly location was allowed to grow on its own wrackbed
152 substrate and the substrates of the remaining three locations. For each location,
153 fly larvae and wrackbed substrate were collected between February and May
154 2014. The wrackbed substrate was placed in labelled plastic bags and then

155 frozen to ensure that no viable eggs or larvae remained. Fly larvae from were
156 reared on 150g of their own substrate in aerated plastic containers until a
157 puparium was formed, and then kept in individual Eppendorf tubes (to ensure
158 virginity) containing a small piece of cotton soaked in 5% sucrose solution. When
159 a fly eclosed, the Eppendorf was moved to the fridge to slow down development
160 time. Over a maximum of ten days, adult flies were collected until a minimum of
161 25 couples could be set up per location. Eggs were collected from 14-15 mating
162 pairs per population and subsequently mixed to populate three replicates of each
163 population x substrate combination with 30 eggs each (4 populations x4
164 substrates x 3 replicates, each one with 30 eggs, total eggs for the experiment:
165 1440). Replicate pots were kept at 25° C with a 12/12 hour light:dark cycle and
166 each pot (190x110x70 mm) was aerated and contained 150 g of representative
167 seaweed. Pots were checked daily and all eclosing flies were sexed and then
168 stored at -20° C on the same day.

169 **Morphological measurements**

170 Morphological measurements were carried out on adult flies only. All flies were
171 weighed (Sartorius Quintix 124-1S microbalance) to the nearest 0.0001 grams.
172 The thorax width of each fly was measured with a digital calliper (Cocraft Digital
173 Vemier Caliper) at the widest place and to the nearest 0.01 mm. This
174 measurement was repeated twice and the average was used. Then, the left wing
175 and right middle leg were removed (if one side was damaged the other side was
176 used). The wing and leg were placed under a thin glass slide and placed under
177 the microscope and a picture was the taken using a Nikon Digital Sight DS-Fi2

178 mounted on a Nikon SM2800 microscope set to a magnification of two power.
179 The resulting image was labelled with an individual identifier. Measurements
180 were conducted blind to location and substrate type to avoid bias.

181 **Molecular determination of inversion types**

182 In *C. frigida* the two most common alcohol dehydrogenase (Adh) allozymes, Adh-
183 B and Adh-D are in complete linkage disequilibrium with the α and β forms of the
184 inversion (Day *et al*, 1982a). Gel electrophoresis was used to visualise the
185 alcohol-dehydrogenase allozymes associated with the two inversion types and
186 this allowed for straightforward genotyping of the inversion types. Starch gels
187 (12%) were prepared by combining 24.5 g potato starch (Sigma-Aldrich, S4251)
188 with 90 ml TEB-buffer, which was then shaken in a 500 ml Buchner flask until
189 dissolved. Heated water (90 ml dH_2O) was added to the Buchner flask, and
190 shaken until homogenized. The Buchner flask was then heated in the microwave
191 at intervals of 5-10 seconds, and shaken in between to evenly distribute the heat.
192 The heating continued until the mixture was boiling, after which the flask was
193 closed with a rubber plug and de-gassed using a tap-based vacuum pump until
194 the mixture was without visible air bubbles. It was poured onto an 18.5x10 cm
195 glass plate with a plastic frame and left to set for 30 minutes, after which it was
196 left in a $4^{O}C$ refrigerator overnight.

197 Fly samples were taken from storage in -20° C freezer and kept on ice during
198 sample preparation. Porcelain plates with 12 wells were placed on ice packs. On
199 each well, a fly was placed together with a small amount of carborundum powder

200 (Fisher Chemical, #C192-500) and one drop of cold dH_2O. The flies were then
201 homogenized using a glass rod. Once homogenized, 3x4 mm filter paper wicks
202 were placed in the wells to soak up the homogenate. The wicks were then placed
203 in the starch gel.

204 Starch gels were put in the electrophoresis chamber, which was connected to a
205 power supply (Consort E863). The two chambers of the electrophoresis machine
206 were filled with TEB-buffer. Two cloth wicks were soaked in TEB-buffer and
207 placed so that they touched evenly along each of the exposed sides of the gel,
208 while still connected to the buffer. An ice pack was placed on top of the gel and
209 the electrophoresis (350 V, 50mA) took 1.5 hours.

210 After electrophoresis, the gel was cut horizontally with a metal thread and only
211 the bottom half was used for staining. The agar overlay used for staining
212 consisted of 20 ml of 2% agar, 10 ml HCl-Tris buffer(pH 8.6), 6 ml propan-2-ol,
213 1.5 ml 3-(4,5-dimethylthiazolyl-2)-2,5- diphenyltetrazolium bromide (MTT), 1 ml
214 Phenazine methosulphate (PMS), and 10 ml Nicotinamide-adenine dinucleotide
215 (NAD). The stain was mixed and poured onto the cut surface of the gel. The
216 covered box with the gel was then carefully put in a dark heating cabinet at 37° C
217 for 40 minutes, or until the banding pattern was clear and then photographed.

218 **Statistical analysis**

219 We analyzed weight and development time (in days) in R (R Development Core
220 Team, 2015) using general linear models. Weight was highly correlated with all
221 other morphological characters (Supplemental table S1) so we only analyzed

222 weight data and development time in a statistical framework. Weight data was
223 normally distributed and so was analyzed using a Gaussian distribution.
224 Development time is essentially count data and right skewed, so we analyzed the
225 data using a Poisson distribution. For both data sets we considered individual
226 flies to be our unit of replication and used the lme4 package in R (Bates *et al*,
227 2014) to construct a model with all factors (population, substrate, sex, and
228 karyotype) and all two and three way interactions. We also included pot
229 (replicate) nested within population*substrate as a random factor. We ran the
230 'Anova' function in the R package car (Fox and Weisberg, 2011) on the full
231 models and then examined the F-values for each term. Starting with a model with
232 only an intercept we built up a series of nested models adding a single main
233 effect, two or three way interaction at a time by decreasing F-value (i.e. starting
234 with the term with the highest F-value). We then used the 'Anova' function with
235 the χ^2 distribution to compare the models in a nested fashion (i.e. each model is
236 only compared to the one above it) and used a χ^2 test to determine if they were
237 significantly different. We selected the model with the lowest AIC whose nested
238 models were not significantly better.

239 To get an overview of our results we also visualized the morphological
240 measurements with a Principal Components Analysis (PCA). The PCA was run
241 on the four highly correlated morphological traits: weight, thorax width, leg and
242 wing length using the 'prcomp' function in R (R Development Core Team, 2015).

243 Visual inspection of our morphological data indicated that males were more
244 variable than females. We formally tested for differences in variability in a

245 statistical framework using Krishnamoorthy and Lee's (2014) modified signed-
246 likelihood ratio test (MS-LRT) implemented in the R package cvequality (Marwick
247 and Krishnamoorthy, 2016). We used a Bonferroni correction to account for
248 multiple testing (Bland and Altman, 1995).

249 We further examined the relative proportions of the three karyotypes (αα, αβ, and
250 ββ) in all population x substrate combinations. For these analyses we used pot
251 as our unit of replication. We tested for differences in two ways. First, we tested
252 for karyotype differences using a χ^2 test on a 3 karyotypes x 16 population-
253 substrate combination matrix. Second, we used a Dirichlet regression model in
254 the R package DirichletReg (Maier, 2014). The Dirichlet regression model allows
255 us to analyze karyotypes (variables with a bounded interval that sum up to a
256 constant - in our case frequencies of karyotypes in a population) exhibiting
257 skewness and heteroscedasticity, without having to transform the data (Maier,
258 2014). We used a nested approach starting with a simple model and then adding
259 factors. Models were tested against the one above them in a nested fashion
260 using the 'Anova' function in the R package car (Fox and Weisberg, 2011) to
261 determine the best model.

262 Results

263 Phenotypic data

264 We had different sample sizes for different treatment combinations (see figure
265 legends for Figures 3 and 4) due to differential survival of the three karyotype
266 categories. Three of the 96 combinations had zero flies (ex: αα Kämpinge

267 females in Kämpinge) but we do not expect that this unduly influenced our results
268 as we did not investigate any 4 way interactions in any of our statistical models
269 and thus did not examine these three combinations directly. All morphological
270 characteristics were highly and significantly correlated with each other
271 (Supplementary Table S1). To get an overall view of the variation in
272 morphological traits we conducted a PCA using all four morphological variables
273 (wing length, leg length, thorax width, and weight). The first principal component
274 explained 93.5% of the variance and the second principal component explained
275 6.5% of the variance (Figure 2).

276 We only examined weight, as this morphological trait is a good proxy of the other
277 morphological measurements (Pearson correlation coefficients with other traits
278 varied from 0.78-0.89, Supplemental Table S1) while still being an easily
279 tractable and understandable measurement. Weight was not strongly correlated
280 with development time (Pearson's product-moment correlation: 0.10) and
281 therefore development time was examined separately. F-values from the full
282 models indicated that effect sizes were much larger for weight than development
283 time (Table S2). The best model for weight included all main effects, the two-way
284 interactions: sex*karyotype, population*sex, population*karyotype,
285 population*substrate, substrate*karyotype, and the three-way interaction:
286 location*sex*karyotype. Comparing the best model with the next most simplified
287 model gave a χ^2 value of 43.3 (P = 0.00073). The best model for development
288 time included the main effects and the two-way interaction karyotype*sex.
289 Comparing the best model with the next most simplified model gave a χ^2 value of

290 8.6 (*P* = 0.0135). The best models for both weight and development time
291 included all main effects and a subset of interactions (Table 2). However, a GxE
292 interaction (as evidenced by a population x substrate interaction) was only
293 present for morphological characteristics, but not for development time.

294 For all measured traits, the Krishnamoorthy and Lee's (2014) modified signed-
295 likelihood ratio test showed that males had significantly more phenotypic
296 variation than females (Table 3). This variation could be partially accounted for
297 by karyotype. Previous work has shown that males are on average bigger than
298 females and that within male size differences are primarily driven by the αβ
299 inversion system: αα males are the biggest followed by αβ and ββ (Butlin *et al*,
300 1982c). This held true in our male data set for all morphological traits (Figure 2)
301 but the magnitude of these differences depended on both population and
302 substrate type (Figure 3, Supplementary Figures 2-5). Although the relative sizes
303 and size differences of the karyotypes differed between combinations of location
304 and substrate, the pattern of both leg length and wing length, thorax width, and
305 weight (Figure 3, Supplementary Figures 2-5) in relation to karyotype and sex
306 was clear and consistent for males, but much less pronounced for females. For
307 example, while the morphological PCA groups of the different male karyotypes
308 formed distinct yet overlapping clusters, a clustering of female karyotypes was
309 absent (Figure 2). Likewise, across treatments (combination of population and
310 substrate), αα-males had the largest mean size, followed by αβ-males and ββ-
311 males in all different population x substrate combinations (Figure 3). For females,

312 less than half of the combinations of population and substrate followed the same
313 pattern.

314 Development time did not follow the same pattern as observed for morphology
315 (Figure 4). While αα-males generally took longer to develop, this was not always
316 the case for every combination of population and substrate. For example, ββ flies
317 from Steninge, raised on Kämpinge substrate took longer to develop than any
318 other flies from Steninge. For females, MS-LRT demonstrated that there was
319 considerably less variation between karyotypes compared to males and patterns
320 varied substantially between populations and substrates.

321 **Inversion types**

322 With the exception of Steninge, frequencies of the karyotypes rarely matched
323 Hardy-Weinberg expectations based on previously estimated allele frequencies
324 (Figure 5). The frequencies of αα, αβ, and ββ were significantly different across
325 the Population x Substrate combinations (χ^2 = 71.5, df=30, P < 0.0001).
326 Generally αβ was the most frequently observed karyotype, but ββ could be just
327 as frequent or more frequent depending on the substrate. In ¾ of the populations
328 (all except Steninge) ββ was dominant in at least one substrate and that was
329 usually the substrate from Mölle.

330 We used Dirichlet regression to see which parameters best predicted the
331 karyotype frequencies. With this model we were not able to examine F-values so
332 we compared all possible models with the 'Anova' function from the R package
333 car (Fox and Weisberg, 2011). The best model was population + substrate +

334 population*substrate. Comparing this with the next most simplified model gave a
335 χ^2 value of 73.99 (P = 0.0002).

336 Discussion

337 Our results demonstrate that the environment modulates both the frequencies of
338 the karyotypes as well as their phenotypic effects in *C. frigida*. The presence of a
339 significant GxE term for both of these indicates that there is population specific
340 variation in larval fitness of the different inversion types as well as in the
341 phenotypic effects of the inversion. The same pool of eggs was used to seed
342 every substrate treatment per population so the neutral expectation (without
343 sampling bias) would be similar karyotype frequencies across substrates.
344 Instead, we found strong differences in karyotype frequencies between
345 substrates with some trends being consistent across multiple populations (Figure
346 5). While it is possible that these results are due simply to sampling bias, it is
347 unlikely that sampling bias would produce consistent trends. Furthermore, this
348 result confirms previous research showing that the substrate composition has a
349 strong selective effect on the inversion (Butlin *et al*, 1982b; Day *et al*, 1983;
350 Edward and Gilburn, 2013). It is likely that population specific variation has
351 accumulated in the inversion, as not all trends were consistent across
352 populations. For example, ββ was the most common karyotype on Mölle wrack
353 for every population except Steninge (Figure 5). Instead, in Steninge, frequencies
354 were often close to Hardy-Weinberg expectations and little variance was seen

355 between substrate combinations compared to other populations, possibly
356 because heterosis was stronger in Steninge than in the other populations.

357 Our results also indicate that inversion-related size effects in *C. frigida* are
358 affected by the environment, particularly in males as females show little to no
359 effect of karyotype on size. Male fly inversion phenotypes from the same
360 population could vary two-fold for weight when grown on the same substrate (e.g.
361 Steninge population on Mölle substrate), and three-fold when comparing male
362 inversion phenotypes across all four growing substrates (e.g. Steninge, Figure 3).
363 Male size has a high heritability with virtually all the variance in size being
364 attributable to the chromosomal inversion system (Wilcockson *et al*, 1995). In all
365 of our treatment combinations, environmental effects never swamped the effect
366 of the inversion. Male αα were always the biggest, followed by αβ and ββ, which
367 is consistent with previous work (e.g. Butlin *et al*, 1982a). Population and
368 substrate changed the magnitude of these differences as well as size averages
369 but never the pattern of the inversion size effects on males. In contrast to the
370 strong environment and inversion effects on male size, the inversion exhibited
371 negligible size effects in females. Specifically, our data show much lower
372 between inversion variation in size and considerably less variation across
373 environments for females. A similar difference in effect size between males and
374 females has also been found in a common garden study on *C. frigida* grown on
375 artificially mixed substrates containing in excess either the seaweed genus
376 *Laminaria* or *Fucus* (Edward and Gilburn, 2013). The study by Edward and
377 Gilburn (2013) also found that flies grown on an excess of *Laminaria* showed

378 greater among inversion size differences compared to *Fucus*, suggesting that the
379 type of seaweed itself, or the associated microbiome of the seaweed, causes
380 phenotypic differences in size. The wrackbed composition differs markedly along
381 the populations studied, as a consequence of the declining salinity from the North
382 Sea to the Baltic Sea (see Figure 1 and 2 in Wellenreuther *et al*, 2017), and have
383 likely been major selective environmental factors in modulating *C. frigida*
384 populations. Development time, another trait known to be affected by inversion
385 type, also showed significantly higher variation across male karyotypes, but
386 exhibited less extreme environmentally induced effects compared to size, and
387 consequently no significant GxE effects. These results together with previous
388 studies (Butlin and Day, 1984a; Day *et al*, 1980) underscore that the inversion
389 related phenotype effects in *C. frigida* are significantly affected by sex, with male
390 phenotypes being more labile compared to female phenotypes.

391 Interactions between genotypes and the environment are commonly observed in
392 quantitative traits (Gupta and Lewontin, 1982), particularly those associated with
393 fitness (e.g. Fanara *et al*, 2006), and are thought to provide a potent force for
394 maintaining genetic variation (Fernández Iriarte and Hasson, 2000; Gillespie and
395 Turelli, 1989; Hedrick, 1986). The habitat used by *C. frigida* is heterogeneous
396 and the resulting local population structure is similar to Levene's migration model
397 of subdivided populations, namely by a single pool of individuals that mate at
398 random and breed on discrete and ephemeral resources. Environmental
399 fluctuations in seaweed substrate composition likely favors the persistence of
400 alternative rearrangements (i.e. the α/β inversion) containing sets of co-adapted

401 alleles via balancing selection (Carroll *et al*, 2001; Filchak *et al*, 2000; Weinig,
402 2000), with each set being optimal under different conditions (Dobzhansky,
403 1970). This hypothesis may account for the so called “supergene” idea
404 (Schwander *et al*, 2014) such as those determining mating types found in
405 heterostylous plants and wing mimicry polymorphisms found in *Heliconius*
406 butterflies (Thompson and Jiggins, 2014). Balancing selection resulting from
407 seasonal and stochastic temporal and spatial wrackbed changes may thus be a
408 significant contributor to maintaining the fitness related inversion variation in size.
409 Other examples for selection on fitness trade-offs come from introduced apple
410 *Malus pumila* and native hawthorn *Crataegus* spp. hosts which has led to
411 divergence in phenology and thermal physiology in the true fruit fly *Rhagoletis*
412 *pomonella* (Filchak *et al*, 2000).

413 Balancing selection has also been implicated in the maintenance of other
414 inversion polymorphisms, but whether balancing selection can maintain genetic
415 polymorphisms over long time scales is still debated (Bernatchez, 2016).
416 Theoretical studies indicate that balancing selection may primarily be a short-
417 term process because the genetic load that it creates then has a negative
418 feedback that hinders the long-term maintenance of adaptive alleles (Fijarczyk
419 and Babik, 2015). Furthermore, rapid environmental changes may cause
420 dramatic allele frequency shifts that could cause the loss of formerly stable
421 polymorphisms (Fijarczyk and Babik, 2015). Despite this, recent studies have
422 provided some evidence that balancing selection can operate even over longer
423 time scales. Lindtke et al. (2017) investigated the inversion related cryptic colour

polymorphism in *Timema cristinae* walking stick insects using a comprehensive dataset of natural populations and population genetic analyses. They were able to demonstrate that inversion colour variation has likely been maintained over extended time periods by balancing selection, which has previously been thought to be only possible over short time frames.

In conclusion, our results indicate that the inversion in *C. frigida* likely evolves via a combination of local mutations, GxE effects, and differential fitness of karyotypes in heterogeneous environments. Future work should apply high-resolution genome-wide approaches that target the regions inside the inversion to identify the true genic targets of balancing varying selection. This approach would also elucidate which genes inside the inversion are linked to these large size effects in males, and how the environment has shape the differentiation between these genes along gradients.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Data Archiving

All raw phenotypic data and karyotype frequencies will be deposited on Dryad upon acceptance.

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Tables

Table 1. Spatial coordinates (WGS 1984 DD), sea surface salinity (PSU), inversion frequency, and wrackbed composition of the study sites.

	Coordinates (WGS84, DD)		Sea surface salinity (PSU)[a]		Inversion frequency[b]	Wrackbed composition			
	Latitude	Longitude	Mean annual	Annual SD	Frequency of the α inversion	Percentage *Fucus spp.*	Percentage Kelp[c]	Percentage red algae[d]	Percentage eelgrass[e]
Steninge	56.77	12.63	15.79	16.62	0.49	50	20	30	0
Mölle	56.28	12.49	15.38	16.88	0.42	30	30	30	10
Kämpinge	55.4	12.98	12.82	11.74	0.35	30	0	30	40
Kåseberga	55.38	14.06	9.91	5.4	0.34	30	0	50	20

[a] Data source: Sbrocco & Barber (2013), accessed on 1 November 2016.
[b] Day et al. (1983).
[c] *Saccharina* and *Laminaria spp.*
[d] *Furcellaria lumbricalis*
[e] Zostera marina

Table 2. Best models for adult weight and development time. An X indicates that a main effect or interaction was present in the best model.

	Main Effects				2-way interactions						3-way interactions
	Karyotype	Population	Sex	Substrate	Karyotype:Population	Karyotype:Sex	Karyotype:Substrate	Population:Sex	Population:Substrate	Sex:Substrate	Karyotype:Population:Substrate
Weight	x	x	x	x	x	x	x	x	x		x
Development Time	x	x	x	x		x					

Table 3. Results from Krishnamoorthy and Lee's (2014) modified signed-likelihood ratio test (MS-LRT) looking to see if the coefficient of variation is different between males and females. Bolded p-values are significant at the P< 0.0001 level after a Bonferroni correction.

Trait	MS-LRT Statistic	P-value
Weight	79.22	**0**
Thorax Width	83.79	**0**
Leg Length	61.64	**4.11E-15**
Wing Length	118.05	**0**
Development Time	22.09	**2.61E-06**

Titles and legends to figures

Figure 1: Map of *C. frigida* fly populations used in this study. Base map from d-maps (http://d-maps.com/carte.php?num_car=23102).

Figure 2: Principal Components Analysis (PCA) of four morphological measurements (thorax width, weight, wing length and middle leg tibia length) of adult flies from the reciprocal transplant experiment. Only the first two PCs are shown. Flies are categorized by sex (males-squares, females-circles) and karyotype (black - αα, grey - αβ, and white-ββ).

Figure 3: Boxplot of weight across populations (listed at Top), substrate (listed on x-axis), sex (indicated by top (male) and bottom (female) charts), and karyotype (indicated by color, black - αα, grey - αβ, and white-ββ). Outliers are not shown for clarity. N for each population x sex combination (from left to right for each karyotype and substrate as listed in the figure): Kämpinge Male: 1, 9, 4, 3, 20, 15, 3, 13, 19, 1, 21, 6, Kåseberga Male: 2, 11, 6, 10, 19, 5, 5, 9, 10, 2, 9, 6, Mölle Male: 1, 6, 8, 10, 14, 13, 6, 12, 13, 2, 6, 10, Steninge Male: 0, 3, 2, 7, 17, 7, 5, 15, 5, 7, 16, 14, Kämpinge Female: 0, 5, 4, 1, 12, 11, 0, 13, 18, 3, 17, 9, Kåseberga Female: 2, 3, 6, 7, 24, 11, 4, 12, 18, 5, 13, 5, Mölle Female: 4, 10, 4, 4, 9, 4, 6, 6, 5, 4, 3, 8, Steninge Female: 2, 5, 4, 8, 21, 8, 8, 19, 6, 7, 21, 7.

Figure 4: Boxplot of development time across populations (listed at Top), substrate (listed on x-axis), sex (indicated by top (male) and bottom (female) charts), and karyotype (indicated by color, black - αα, grey - αβ, and white-ββ). Outliers are not shown for clarity. N for each population x sex combination (from left to right for each karyotype and substrate as listed in the figure): Kämpinge Male: 1, 9, 4, 3, 20, 15, 3, 13, 19, 1, 21, 6, Kåseberga Male: 2, 11, 6, 10, 19, 5, 5, 9, 10, 2, 9, 6, Mölle Male: 1, 6, 8, 10, 14, 13, 6, 12, 13, 2, 6, 10, Steninge Male: 0, 3, 2, 7, 17, 7, 5, 15, 5, 7, 16, 14, Kämpinge Female: 0, 5, 4, 1, 12, 11, 0, 13, 18, 3, 17, 9, Kåseberga Female: 2, 3, 6, 7, 24, 11, 4, 12, 18, 5, 13, 5, Mölle Female: 4, 10, 4, 4, 9, 4, 6, 6, 5, 4, 3, 8, Steninge Female: 2, 5, 4, 8, 21, 8, 8, 19, 6, 7, 21, 7.

Figure 5: Box plot of frequencies of different karyotypes (black - αα, grey - αβ, and white-ββ) across populations (listed at the top of each graph) and substrates (listed on the x-axis). Dashed lines indicate HWE expectations (solid - αα, dashed - αβ, and dotted-ββ) based on Day et al (1983) data.

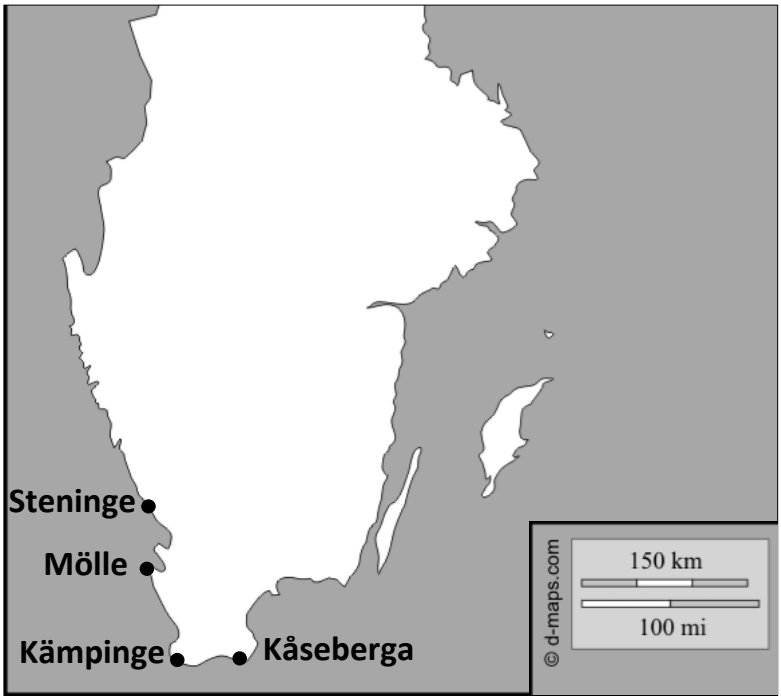


Figure 1

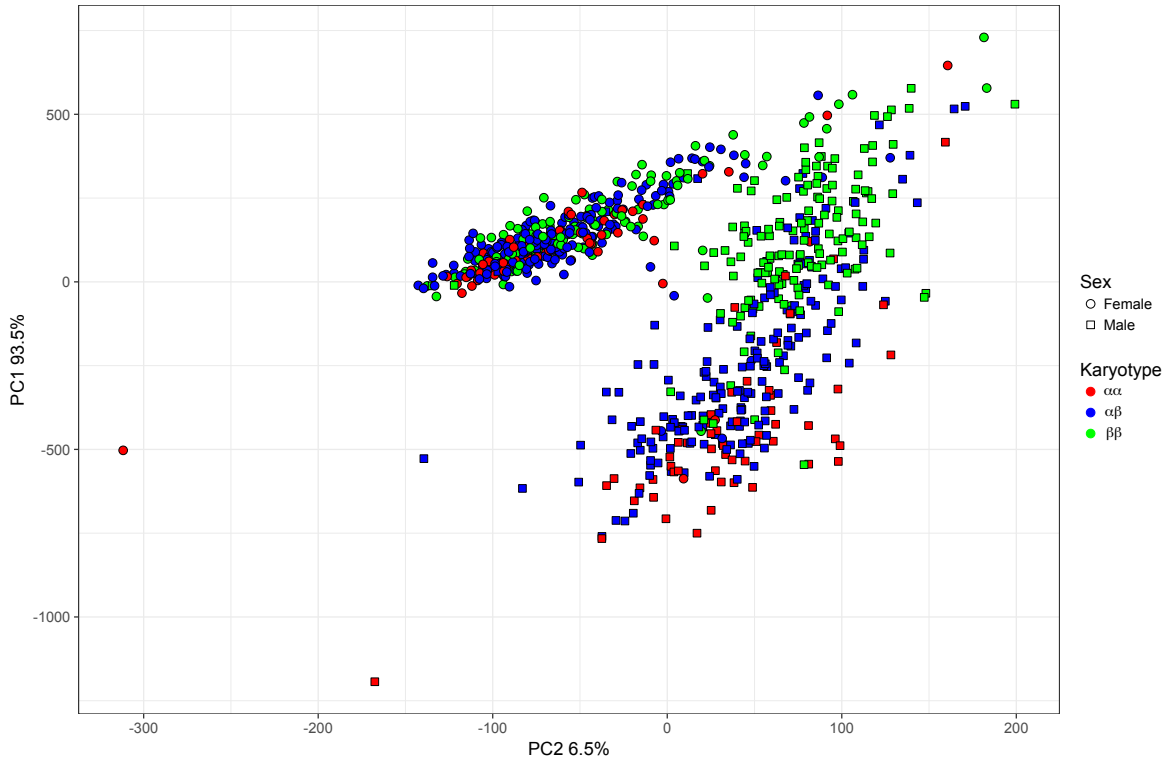

500
0
-500
-1000
PC1 93.5%
-300
-200
-100
0
100
200
PC2 6.5%
Sex
Female
Male
Karyotype
αα
αβ
ββ

Figure 2

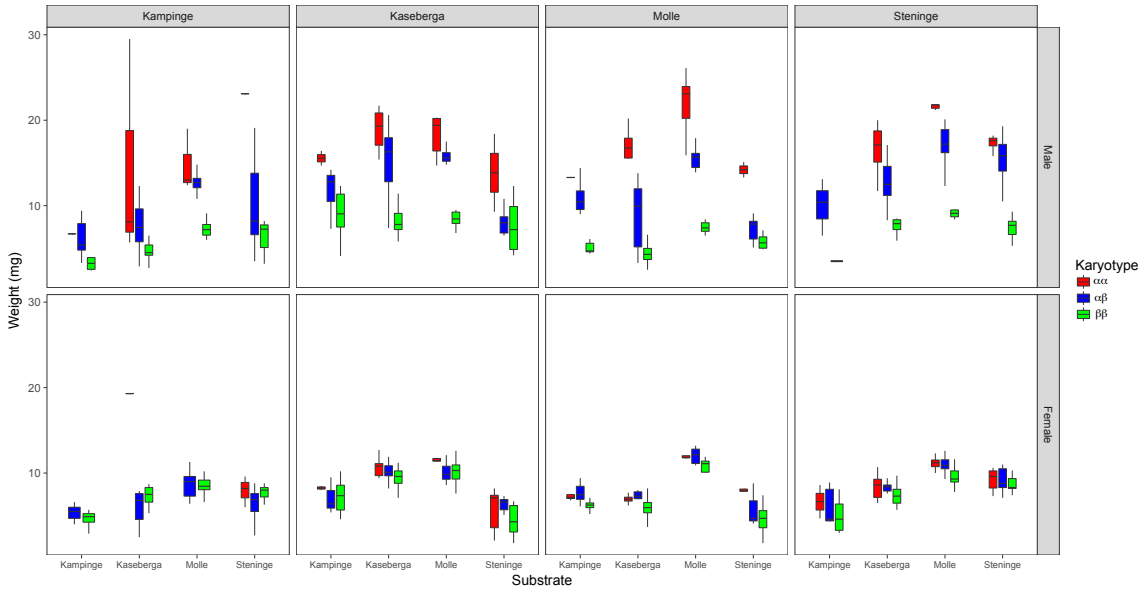


Figure 3

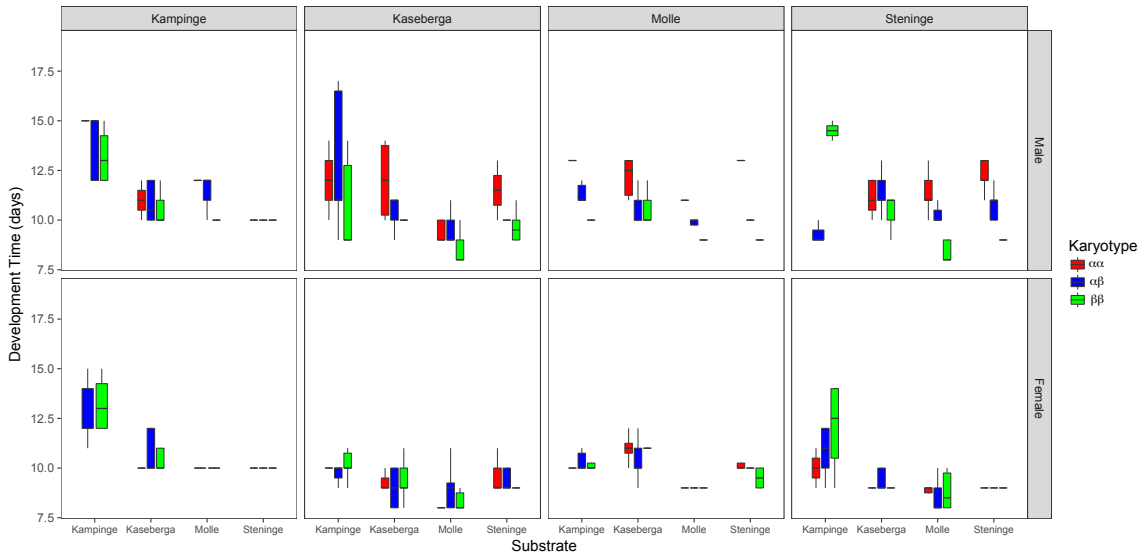
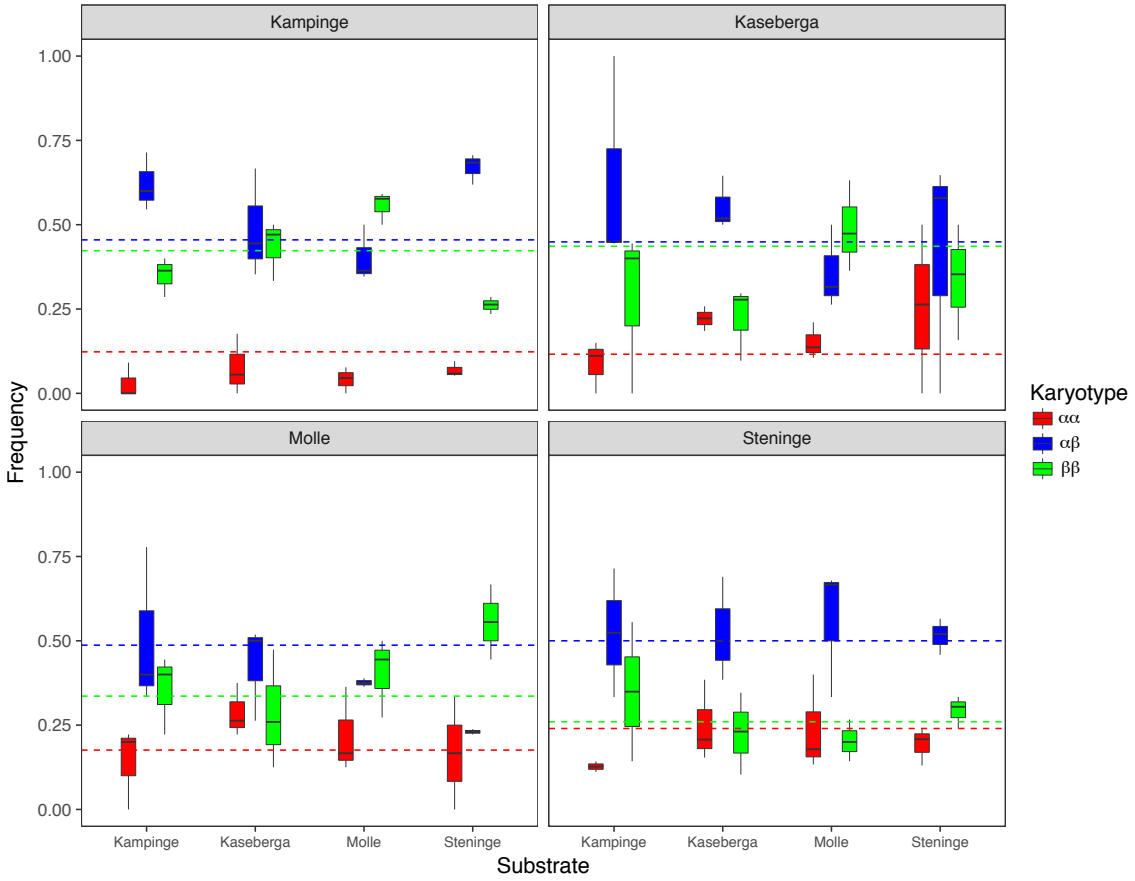


Figure 4


Kampinge
Kaseberga
Molle
Steninge
Frequency
1.00
0.75
0.50
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Kampinge
Kaseberga
Molle
Steninge
Substrate
Karyotype
αα
αβ
ββ

Figure 5

Supplemental Material

Table S1. Pearson correlations of morphological factors.

	Adult Weight	Thorax Width	Wing Length	Leg Length
Weight	1	0.8306678	0.8850752	0.7786367
Thorax Width	0.8306678	1	0.8452453	0.7628986
Wing length	0.8850752	0.8452453	1	0.865498
Leg Length	0.7786367	0.7628986	0.865498	1

Table S2. ANOVA tables for the full models adult weight (A) and development time (B). Bolded terms are included in the final models.

A.

Term	DF	Sum of Squares	Mean Square	F-Value
Population	**3**	**201.08**	**67.03**	**13.6683**
Substrate	**3**	**327.74**	**109.25**	**22.2781**
Sex	**1**	**1559.27**	**1559.27**	**317.9702**
Karyotype	**2**	**2589.74**	**1294.87**	**264.0532**
Population:Substrate	**9**	**185.65**	**20.63**	**4.2065**
Population:Sex	**3**	**422.75**	**140.92**	**28.7363**
Population:Karyotype	**6**	**228.57**	**38.09**	**7.7683**
Substrate:Sex	3	18.59	6.2	1.264
Substrate:Karyotype	**6**	**116.87**	**19.48**	**3.9719**
Sex:Karyotype	**2**	**1406.79**	**703.4**	**143.4383**
Population:Substrate:Sex	9	64.31	7.15	1.4572
Population:Substrate:Karyotype	**18**	**220.67**	**12.26**	**2.5**
Population:Sex:Karyotype	6	36.49	6.08	1.2401
Substrate:Sex:Karyotype	6	53.67	8.95	1.8242

B.

Term	DF	Sum of Squares	Mean Square	F-Value
Population	**3**	**11.039**	**3.6796**	**3.6796**
Substrate	**3**	**35.277**	**11.759**	**11.759**
Sex	**1**	**20.778**	**20.7776**	**20.7776**
Karyotype	**2**	**6.958**	**3.4792**	**3.4792**
Population:Substrate	9	9.476	1.0528	1.0528
Population:Sex	3	6.46	2.1533	2.1533
Population:Karyotype	6	0.461	0.0769	0.0769
Substrate:Sex	3	0.598	0.1994	0.1994
Substrate:Karyotype	6	1.639	0.2732	0.2732
Sex:Karyotype	**2**	**6.725**	**3.3625**	**3.3625**
Population:Substrate:Sex	9	3.591	0.399	0.399
Population:Substrate:Karyotype	18	4.861	0.2701	0.2701
Population:Sex:Karyotype	6	0.263	0.0438	0.0438
Substrate:Sex:Karyotype	6	0.534	0.0889	0.0889

Figure S2: Boxplot of thorax width across populations (listed at Top), substrate (listed on x-axis), sex (indicated by top (male) and bottom (female) charts), and karyotype (indicated by color, red - αα, blue - αβ, and green-ββ). Outliers are not shown for clarity.

Figure S3: Boxplot of leg length across populations (listed at Top), substrate (listed on x-axis), sex (indicated by top (male) and bottom (female) charts), and karyotype (indicated by color, red - αα, blue - αβ, and green-ββ). Outliers are not shown for clarity.

Figure S4: Boxplot of wing length across populations (listed at Top), substrate (listed on x-axis), sex (indicated by top (male) and bottom (female) charts), and karyotype (indicated by color, red - αα, blue - αβ, and green-ββ). Outliers are not shown for clarity.

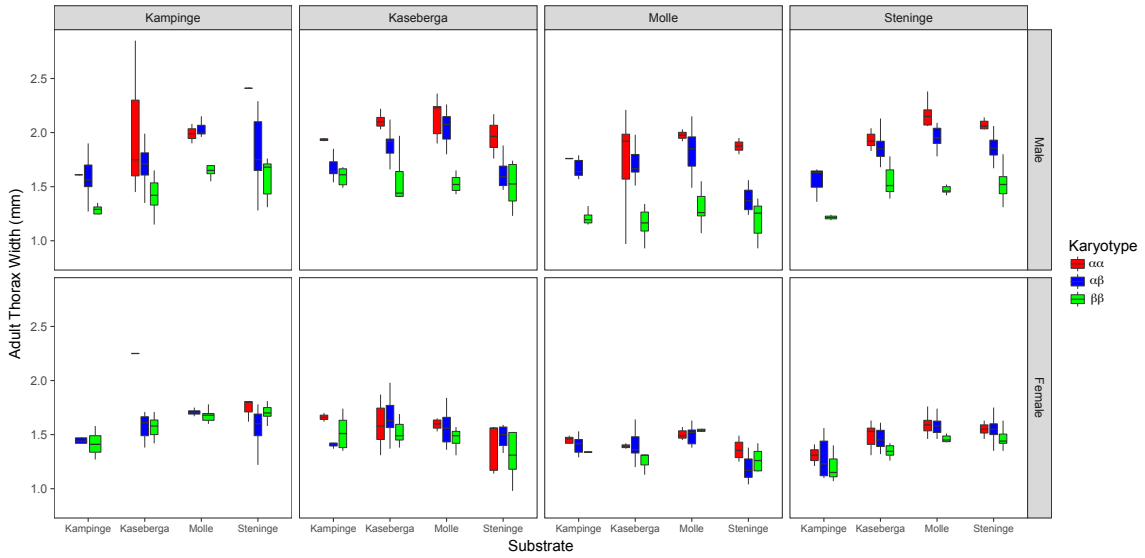

Figure S2

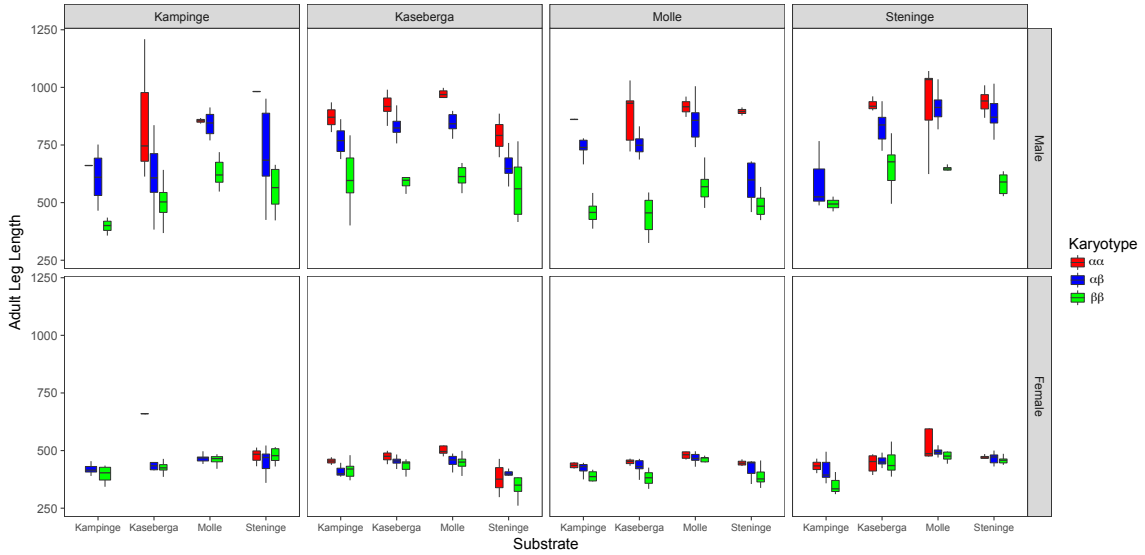

Figure S3

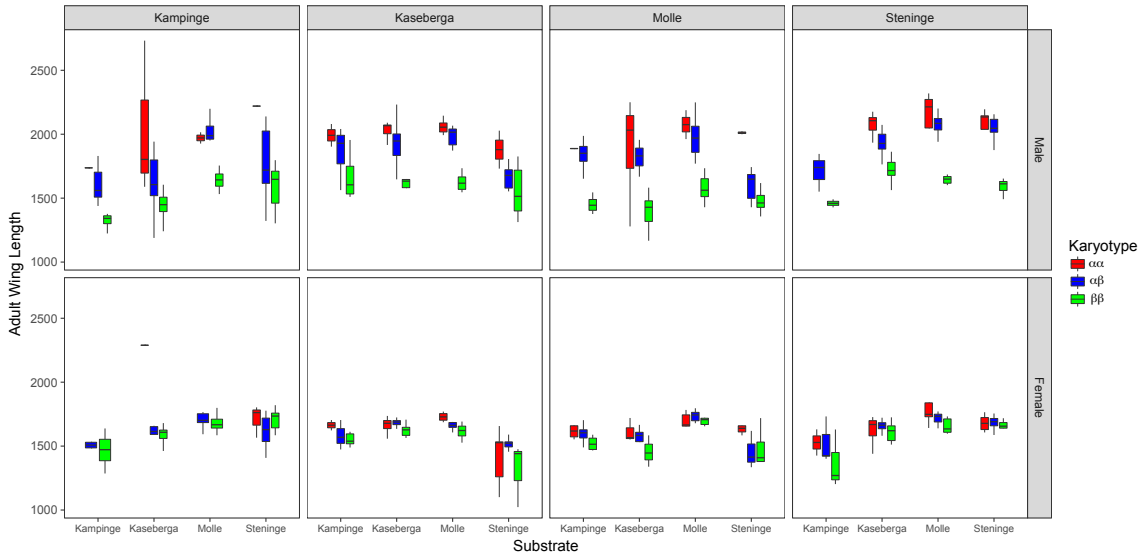
Kampinge
Kaseberga
Molle
Steninge
Male
Female
Adult Wing Length
2500
2000
1500
1000
Substrate
Karyotype
αα
αβ
ββ

Figure S4