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Differential Selection of Lateral Plates in the Threespine Stickleback

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in

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by

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The Thesis of Laura Varey is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

For Dad

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Thanks to Dr. Rennison for walking me through all of this and being endlessly patient with me, taking a chance on me in the lab, and for supporting me and teaching me so much. Thanks to my committee members Professor Shurin and Professor Kohn for agreeing to be on my committee and for giving me feedback and support during our committee meetings. To the Rennison Lab for all of your support, feedback, and for listening to the same presentation about a thousand times – you guys are the best and I wish you all the best of luck in your future endeavors.

Thanks to Mom and Max for keeping it real. To Melissa and Bethannie for keeping me sane and listening to me whine and for being the best friends a girl could ask for.

ABSTRACT OF THE THESIS

Differential Selection on Lateral Plates of the Threespine Stickleback

by

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Professor Diana Rennison, Chair

Frequently the source of selection driving divergence of putatively adaptive traits is unknown. Even in cases where traits or genes have been studied for decades, we often don't know what agents of selection are involved. Manipulative experiments provide an opportunity to link phenotypic and genotypic shifts with a causative agent. In order to explore the role of predation in the divergence of lateral plates of the threespine stickleback, I used data generated from a manipulative experiment where a toothed and gape limited predator was manipulated. I sought to estimate natural selection on lateral plate number and area, to see if there was evidence of an effect of predation. I also used existing genomic data and quantitative trait mapping data to determine if there was a genetic basis for the two plate phenotypes. Tracking shifts in plate phenotypes over one generation of selection revealed evidence that lateral plate number was generally selected against, yet greater plate area was favored in the presence of predators. This indicates that the plate area is potentially a key trait that can aid in the protection of stickleback and is under selection by toothed predators.

Several genetic regions on chromosomes 7, 11, 20, and 21 were identified that explained variance in the two lateral plate traits.

INTRODUCTION

Differences in ecological factors between habitats are well known to drive evolutionary divergence (Benton 2009) and can lead to the generation of new species (Schluter 2000). For example, in Darwin's finches, differences in resource use have been shown to lead to the diversification of beaks (Boag and Grant 1981). Over the past few decades, many similar studies have found evidence suggesting a given genotype or phenotype is adaptive (reviewed by Barrett and Hoekstra 2011). However, relatively few studies have been able to identify the direct mechanisms responsible for differences in fitness. In general, we lack knowledge of the environmental factors that are important sources of natural selection and have little idea of how they contribute to diversification among populations. Estimates of natural selection from manipulative experiments provide a rare opportunity to attribute phenotypic (and genotypic) shifts with the causative agent of selection (Rennison *et al.* 2019). Such experiments isolate the contribution of a particular agent of selection and can help explain patterns of evolution in nature (Barrett and Hoekstra 2011).

The threespine stickleback (*Gasterosteus aculeatus*), is a small fish found throughout coastal regions of the Northern hemisphere (Bell and Foster 1994). Stickleback are an ideal organism for studying the process of adaptive evolution using experimentation as they exhibit rapid evolution (Kitano *et al.* 2008; Lescak *et al.* 2015), and can be reared in the lab or kept in artificial ponds (Bell and Foster 1994; Barrett *et al.* 2008; Arnegard *et al.* 2014; Rennison *et al.* 2019). There is also a sequenced and annotated genome (Jones *et al.* 2012), which allows characterization of the genetic basis for the adaptation, meaning that phenotypic change can potentially be connected to a genomic basis.

Threespine stickleback have existed in marine habitats for millions of years. However, 10 – 12,000 years ago, after the last ice age, marine stickleback colonized newly formed freshwater lakes and streams (Bell and Foster 1994; Taylor and McPhail 1999) – within these freshwater habitats fish diverged from their marine ancestors in many aspects of their ecology, behavior, and morphology (Bell and Foster 1994). The variety of freshwater habitats colonized also generated considerable trait diversity among freshwater populations (Bell and Foster 1994). Several sources of selection are thought to be important in the generation of this diversity. These include abiotic factors such as mineral availability or temperature (Heuts 1947; Giles 1983; Marchinko and Schluter 2007), and biotic factors, like resource competition (Schluter 1994) or predation (Hagen and Gilbertson 1973a; Marchinko 2009; Gross 1978; Moodie 1972; Reimchen 2000; Reimchen 1992; Vamosi and Schluter 2004). Yet, there are few cases in which the specific selective factor has been definitively identified for a putatively adaptive phenotype or genotype.

One of the key phenotypic differences between marine and freshwater stickleback is in their bony armor, which consists of a series of up to 32 lateral plates that run along the flank of the fish, as well as three dorsal and two pelvic spines (Bell and Foster 1994). This armor has been hypothesized to help in predation avoidance and protect against injury (Reimchen 1992; Reimchen 2000). Interestingly, freshwater populations generally have fewer lateral plates and shorter spines (Reimchen 1992) than their marine ancestors – while marine stickleback typically have 32 plates, freshwater fish often have between 0 and 9 plates (Bell and Foster 1994; Hagen and Gilbertson 1972). Armor phenotypes are also highly variable among freshwater populations. For example, sympatric benthic and limnetic freshwater ecotypes of stickleback, which have evolved in several lakes in British Columbia, Canada (McPhail 1984; McPhail 1993; Gow *et al*. 2008), repeatedly differ in their armor morphology. The limnetic species is more armored,

having more lateral plates (5 – 9 plates) and longer spines compared to benthics (0 – 4 plates) (McPhail 1993). There is also evidence that lateral plate volume and surface area, which are distinct from plate number, have been repeatedly reduced in freshwater populations (Wiig *et al*. 2016).

Differential predation between habitats (marine vs fresh or pelagic vs littoral) is thought to be a major drive of diversification of lateral plates (Gross 1978; Reimchen 1995; Vamosi 2002). This is because the primary function of lateral plates is hypothesized to be interference with the pharyngeal jaw of a toothed predator, which increases handling time by the predator and increases chance of escape (Reimchen 2000). Lateral plates are also thought to support the dorsal and pelvic spines by "buttressing" them, which in theory would prevent compression from a predator during an attack (Reimchen 1983). Due to the internal microstructure of lateral plates, they are able to resist penetration from a toothed predator (Song *et al.* 2010). Field studies seem to support this anti-predation role of lateral plates, as stickleback with a greater number of plates appear to have higher survival rates when exposed to toothed predators (Reimchen 1992). There is also evidence of decreased injury in higher plated stickleback, as plates are thought to protect internal organs (Reimchen 1992). Interestingly, predation by insects may directly select against lateral plates (Marchinko 2009), although results have been conflicting between studies (Zeller *et al.* 2012). Thus, a difference in predation regimes between habitat types (e.g. littoral vs pelagic), has been suggested to generate the differences in armor (Foster *et al.* 1988; Vamosi 2002; Vamosi and Schluter 2002; Reimchen 1995). Support for predation as a mechanism for selection on lateral plating comes largely from observational field studies (Reimchen 1992; Reimchen 1995; Hagen and Gilbertson 1973a; Kitano *et al.* 2008). Unfortunately, observational work does not allow for estimation of the direct contribution of predation to patterns of armor evolution.

Furthermore, covariance between abiotic factors and predator assemblies in many habitats makes it difficult to exclude the contributions of non-predatory agents to observed patterns. Thus, we still do not know the degree to which vertebrate predation directly contributes to patterns of lateral plate evolution.

Sympatric benthic and limnetic stickleback provide a promising opportunity to investigate whether predation is a key selective agent contribution to variation in lateral plating. This is because these two ecotypes live within the same waterbody, so their exposure to abiotic factors (salinity, temperature, mineral availability, pH, etc.) should be similar. However, benthic and limnetic stickleback have different feeding habits and habitat usage, and as a result are thought to experience different predation regimes (Vamosi and Schluter 2004). Specifically, benthic stickleback feed on invertebrates and limnetics feed on plankton (Schluter 1994). While feeding on these different resources, the two ecotypes occupy different regions of the lake – benthics tend to be in the vegetated littoral region, while limnetics tend to be more in the open water or beside deep rocky cliffs. Different predators are thought to dominate these two habitats, with benthics exposed primarily to invertebrate predation (by dragonfly larvae and backswimmers) and limnetics exposed to vertebrate predation (by diving birds or cutthroat trout) (Bell and Foster 1994). This differential exposure to vertebrate predation has been hypothesized to underly the difference in lateral plating (Vamosi and Schluter 2004). It is thought that invertebrate predators may utilize armor in order to grasp and manipulate stickleback, so fish with less armor may be better able to escape from predatory attacks (Vamosi and Schluter 2004). On the other hand, since limnetics experience predation from toothed and avian predators, armor plating could protect vital organs during an attack, thus increasing the chance of survival from failed predation attempts (Vamosi and Schluter 2004). Previous work has shown that limnetics

and benthics have highest survival rates in their preferred habitats, suggesting adaption to their local predators (Vamosi 2002).

Lateral plate number has been shown to evolve rapidly (Bell *et al.* 2004), with phenotypic shifts occurring in as little as one generation (Kristjansson 2005; Barrett *et al.* 2008) and phenotypic changes in lateral plating have been shown to be highly heritable (Hagen 1973; Hagen and Gilbertson 1973b; Hansson *et al.* 2016). In fact, the reduction in lateral plate number in freshwater stickleback has been shown to be due to changes in the *Ectodysplasin (Eda)* gene (Colosimo *et al.* 2005). *Eda* is involved in lateral plate formation and most freshwater populations have fixed the low-plate allele, which reduces the number of plates from the standard 32 found in marine populations to around 9 plates (Colosimo *et al.* 2005). Interestingly, despite differences in their lateral plate morphology, benthic and limnetic stickleback both have the fixed *Eda* low-plate allele, therefore the difference in genetic loci other than *Eda* must be the source of variation in these ecotypes.

To explore the contribution of differential predation to divergent evolution of lateral plate phenotypes, I analyzed phenotypic data resulting from a manipulative predation experiment (Rennison *et al.* 2019). By comparing fish before and after one generation of selection I sought to (1) test whether predation by a toothed and gape limited predator (cutthroat trout) led to natural selection on lateral plate phenotypes, (2) determine whether the pattern of selection differed between lateral plate number and lateral plate area, and (3) characterize the genetic basis of these two plate phenotypes. Based on the hypothesized role of lateral plates in predation avoidance and protection against injury through the buttressing of spines (Reimchen 1992), I predicted that both lateral plate number and total plate area would increase in the presence of predators, relative to the control treatment.

METHODS

Pond Experiment

The initial experiment was conducted by Dr. Diana Rennison between May 2012 and September 2013 at the experimental pond facility at the University of British Columbia in Vancouver, Canada. For full experimental details, see Rennison *et al.* 2019. Briefly, male limnetic and female benthic threespine stickleback from Paxton Lake, British Columbia, Canada, were crossed to create four hybrid F_1 families. Each of these F_1 families was split into a pair of the eight experimental ponds. The F_1 fish bred naturally to produce the F_2 generation. In September 2012, a sample of 50 fish per pond was collected from the F_2 generation. Cutthroat trout were then added to one of the paired ponds becoming a "predation treatment pond" while the other pond served as a "control pond." The F_2 generation was then sampled in January 2013 and April 2013, with 50 fish sampled per pond. In the spring of 2013, the F_2 generation bred naturally, producing the F_3 generation. A final sample of 50 fish per pond was taken in September 2013.

Phenotyping of Lateral Plate Traits

Each fish specimen was fixed in formalin and stained with Alizarin red to highlight calcified structures. Stained fish were photographed under standardized lighting conditions alongside a ruler, which was used to calibrate scale. The number of lateral plates was counted on the left side of each fish. Linear measurements were taken from the photographs using the software program, ImageJ (Rasband 2018), with each plate measured in the vertical and horizontal dimensions to estimate the area of each plate. Total plate area was estimated as the

sum of individual plate areas. Standard length of each fish was measured from snout tip to caudal peduncle.

Data Analysis

All statistical analyses were performed using the program R, version 4.1.2 (R Core Team 2021). Standard length was found to strongly correlate with lateral plate area ($r = 0.742967$, $p < 2.2e\text{-}16$, Figure S1). Standard length was also strongly correlated with lateral plate number ($r = 0.24$, $p < 2.2e\text{-}16$, Figure S2). To remove this confounding effect of body size, a size correction was performed for both traits. Size correction was done by correcting individual measurements to the mean standard length (3.48 cm) of the samples in the experiment using the equation:

$$Y_i = X_i - \beta(L_i - \overline{L})$$

where Y_i is the size-adjusted trait value, X_i is the original trait value, β is the regression coefficient of non-adjusted trait values, L_i is the standard length and $\overline{L}$ is the mean average length of the sample. This was done separately for both plate area and plate number, at all time points. All subsequent analyses were done using these size corrected measurements.

To estimate selection between the relevant time points within the F_2 generation (i.e. September 2012 to January 2013 and January 2013 to April 2013) for each of the lateral plate traits, the following equation was used:

$$s' = (\overline{X}_{after} - \overline{X}_{before})/\delta_{pooled}$$

where s' is the selection intensity, $\overline{X}_{after}$ is the mean phenotype of each pond after selection, $\overline{X}_{before}$ is the mean before selection, and δ_{pooled} is the pooled sample variance.

Treatment effect within a generation was calculated using:

$$\Delta s' = s'_{predation} - s'_{control}$$

where $s'_{predation}$ is the selection intensity for the predation ponds, $s'_{control}$ is the selection intensity for control ponds, and $\Delta s'$ is the treatment effect.

The evolutionary response of each plate trait between generations (i.e. September 2012 and September 2013), was estimated using the following equation:

$$h = (\overline{Z}_{after} - \overline{Z}_{before})/\hat{\sigma}_{pooled}$$

where h is the evolutionary response, $\overline{Z}_{after}$ is the mean phenotype of the F_3 generation (post-selection), $\overline{Z}_{before}$ is the mean phenotype of the F_2 generation (pre-selection), and $\hat{\sigma}_{pooled}$ is the square root of the pooled sample variance.

The treatment effect of each plate trait was estimated within a family using the equation:

$$\Delta h = h_p - h_c$$

where Δh is the treatment effect, h_p is the evolutionary response of the predation pond, and h_c is the evolutionary response of the control pond. The statistical significance of the mean selection intensity, mean evolutionary response, and treatment effects were determined using a t-test (null of zero) with ponds (n = 8) or paired ponds (n = 4) as replicates depending on the test.

Analysis of correlated characters

To disentangle the selective response due to direct and indirect selection as a result of the correlation between the two plate traits, selection gradients were estimated (Lande and Arnold 1983) using the following equation:

$$\hat{\beta} = P^{-1}[\bar{x}_{after} - \bar{x}_{before}]$$

where $\hat{\beta}$ is the vector of estimated selection coefficients, P is the matrix of variances and covariances of the traits before selection, $\bar{x}_{before}$ is the phenotype trait scores before selection, and $\bar{x}_{after}$ is the phenotype trait scores after selection.

QTL Analysis

For full details of the sequencing and genotyping of samples, see the methods of Rennison *et al.* 2019. Briefly, to estimate genotypes of individuals, DNA was extracted from fin clips using a standard phenol-chloroform extraction protocol. From the September 2012 F_2 and the September 2013 F_3 cohorts, 50 individuals were sequenced per pond (800 individuals total). The F_1 parents and pure benthic and limnetic grandparents were also sequenced. DNA libraries were prepared using the *Pstl* enzyme following the genotyping by sequencing method of Elshire *et al*. 2011, and sequenced on an Illumina HiSeq platform. Single nucleotide variants were identified using a standard, reference-based bioinformatics pipeline. MasterBayes R package was used to construct a pedigree (Hadfield 2012) and a genetic map was building using JoinMap (Ooijen and Voorrips 2002).

2243 diagnostic SNP markers were used for the quantitative trait locus (QTL) mapping. Genotypes were coded as A for benthic alleles, B for limnetic alleles, and AB for heterozygotes. QTL analysis of the lateral plate traits was done using Haley-Knott regression the R/qtl software package (Broman and Wu 2013). In the QTL analysis, sex and F_1 family were set as covariates. The percent variance explained (PVE) was calculated for each candidate using the equation:

$$PVE = 1 - (10^{(-2*(\frac{LOD}{n}))}$$

where LOD is the estimated LOD score and *n* is the sample size.

RESULTS

Response to selection within generation

When considering selection within the first generation, young of the year fish (juveniles) (collected September 2012) were first compared to sub-adults (collected January 2013). Across this period, regardless of treatment, there was significant selection for increased plate area (mean selection intensity (s') = 0.26 ± 0.11, $t_7 = 2.45$, $p = 0.04$; Figure 1A; Table 1), in contrast, plate number was found to decrease in seven of the eight experimental ponds, although the shift was not statistically significant. Interestingly, when comparing the pattern of selection between the control and predation treatments, plate area tended to have a greater increase in predation ponds relative to paired control ponds. This was reflected by a positive (but non-significant) treatment effect (mean treatment effect (Δs') = 0.39 ± 0.14, $t_3 = 2.73$, $p = 0.07$; Figure 1B, Table 1). During the same time period, there was selection against plate number, with plate number decreasing in 7 of the 8 ponds; although the average strength of selection was not significantly different from zero (Figure 1A; Table 1). When comparing paired predation and control ponds, there was no significant difference in the pattern of selection, resulting in a non-significant treatment effect (Figure 1B; Table 1).

In the second period, the sub-adults (collected January 2013) were compared to reproductive adults (collected April 2013). During this period, when ignoring treatment, there was no consistent pattern of selection (s') across the eight pond replicates for plate number (Figure 1C, Table 1). There was a trend of selection against plate area seen for seven of the eight ponds, however this was not statistically significant (Figure 1C, Table 1). When paired predation and control ponds were compared, there appeared to be a trend of weaker selection against plate area in predation ponds relative to controls (three of four ponds); however, this treatment effect

was not significant (Figure 1D; Table 1). For plate number, there was no consistent or significant treatment effect (Figure 1D; Table 1).

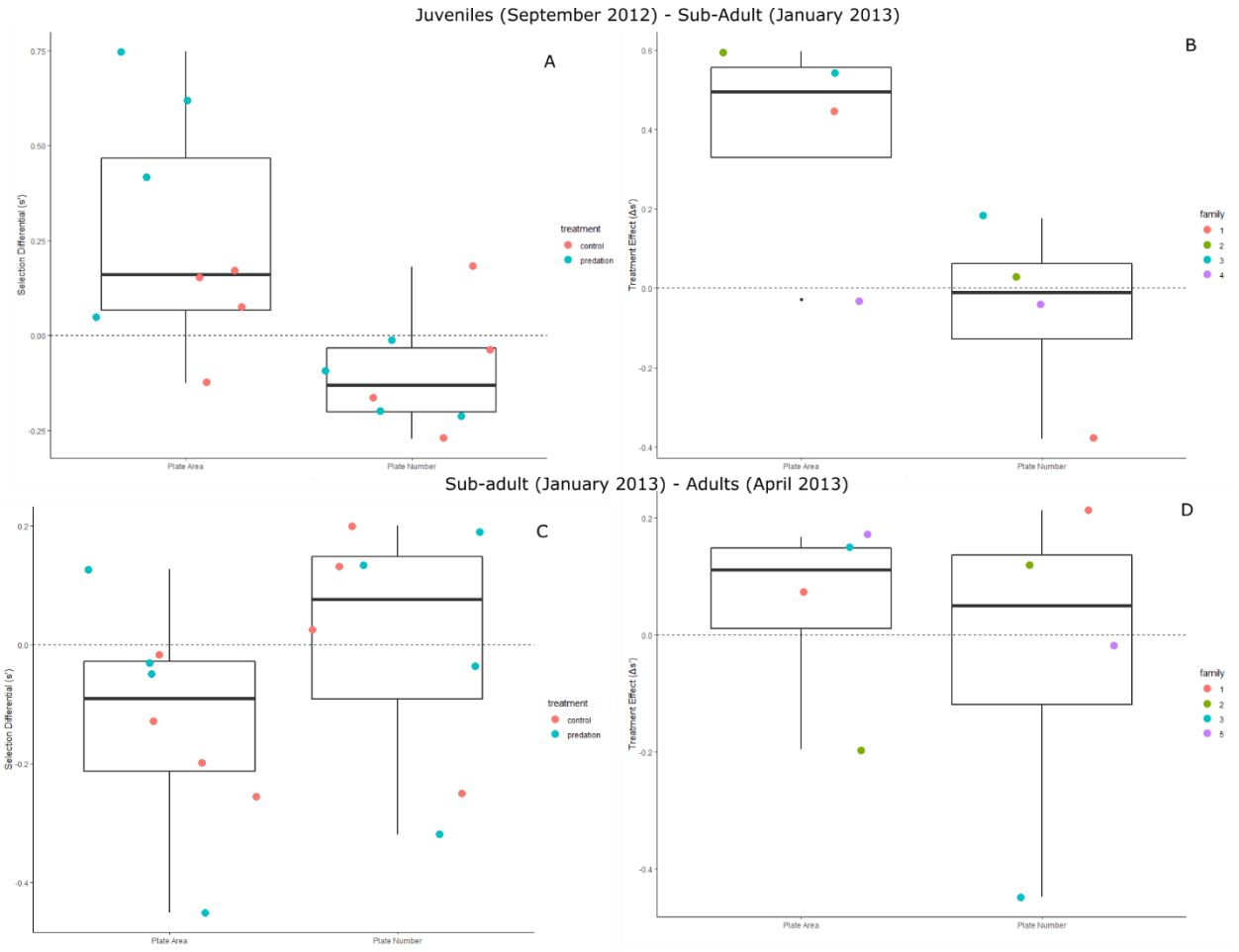

Figure 1: (A) selection intensity between and (B) treatment effect between September 2012 and January 2013. (C) Selection intensity between and (D) treatment effect between January 2013 and April 2013. In panels A and C red indicates control ponds and blue indicates predation ponds. In panels B and D families are indicated by different colors.

Table 1: Selection intensity for plate number and plate area at each time point. Significant values ($p < 0.05$) are indicated in bold

Comparison	Estimate	Trait	Mean	Standard Error	p-value	df	t
Juvenile – Sub adult	Overall Selection (s')	Plate number	-0.102	0.05	0.08	7	-2.004
		Plate area	**0.26**	**0.107**	**0.044**	**7**	**2.449**
	Treatment Effect (Δs')	Plate number	-0.056	0.117	0.663	3	-0.477
		Plate area	0.390	0.143	0.072	3	2.730
Sub adult – Adult	Overall Selection (s')	Plate number	0.009	0.070	0.90	7	0.1214
		Plate area	-0.12	0.062	0.083	7	-2.019
	Treatment Effect (Δs')	Plate number	-0.033	0.14	0.833	3	-0.229
		Plate area	0.049	0.083	0.601	3	0.583

Evolutionary response between generations

When comparing the pattern of evolution between the F_2 generation in September 2012 and F_3 generation in September 2013, there was a significant evolutionary response (*h*) for plate number (mean evolutionary response (*h*) = -0.20 ± 0.04, $t_7 = -5.03$, $p = 0.002$; Figure 2A; Table 2); in all eight ponds, regardless of treatment, plate number decreased between the first and second generation. However, there was no difference in the pattern of evolution of plate number between paired predation and control ponds, resulting in a non-significant treatment effect (Figure 2B; Table 2). Across all ponds, there was also not a significant evolutionary response for plate area (Figure 2A; Table 2). However, in contrast, this was due to the fact that in predation ponds there was consistent selection for increased plate area, and in control ponds there was selection against plate area. This produced a significant treatment effect for plate area (mean

treatment effect (Δh) = 0.45 ± 0.06, t_3 = 7.80, p = 0.004; Figure 2B; Table 2) and a pattern of divergent evolution between treatments.

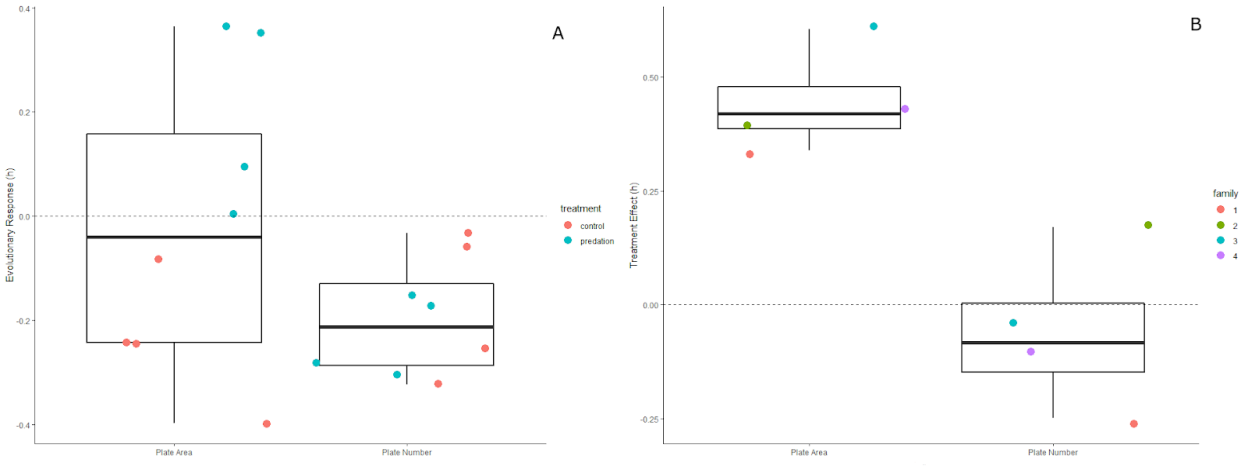

Figure 2: (A) Evolutionary response (h) and (B) treatment effect (Δh) between the F_2 and F_3 generations. In panel A red indicates control ponds and blue indicates predation ponds. In panel B families are indicated by different colors.

Table 2: Evolutionary response and treatment effect for plate number and plate area between generations. Significant values (p < 0.05) are indicated in bold

Estimate	Trait	Mean	Standard Error	p-value	df	t
Overall evolutionary response (h)	Plate number	**-0.198**	**0.039**	**0.002**	**7**	**-5.030**
	Plate area	-0.019	0.099	0.851	7	-0.195
Treatment Effect (Δh)	Plate number	-0.061	0.087	0.533	3	-0.703
	Plate area	**0.446**	**0.057**	**0.004**	**3**	**7.801**

Analysis of selection on correlated characters

After size correction, lateral plate number and lateral plate area were found to be strongly but not completely correlated (r = 0.58, p << 0.001; Figure S4; Table S1).

When this correlation between the two lateral plate traits was taken into account, the patterns of selection remained: a divergent pattern of selection was still seen between treatments for lateral plate area resulting in no overall pattern, and in contrast, lateral plate number was disfavored across all ponds. Across all eight ponds, the mean selection gradient for plate area was not significantly different from zero (mean selection gradient (B) = 0.15 ± 0.24, t_7 = 0.61, p = 0.558) (Figure 3A; Table 3). For plate number, there was a significant reduction in plating between generations (mean selection gradient (B) = -0.35 ± 0.17, t_7 = -2.01, p = 0.008) (Figure 3A; Table 3). When comparing the selection gradients between paired treatment and control ponds there was a significant treatment effect for both traits although the direction of the effect differed: area (mean treatment effect (ΔB) = 1.07 ± 0.15, t_3 = 7.37, p = 0.005; Figure 3B; Table 3) and plate number (mean treatment effect (ΔB) = -0.76 ± 0.18, t_3 = -4.15, p = 0.03; Figure 3B; Table 3).

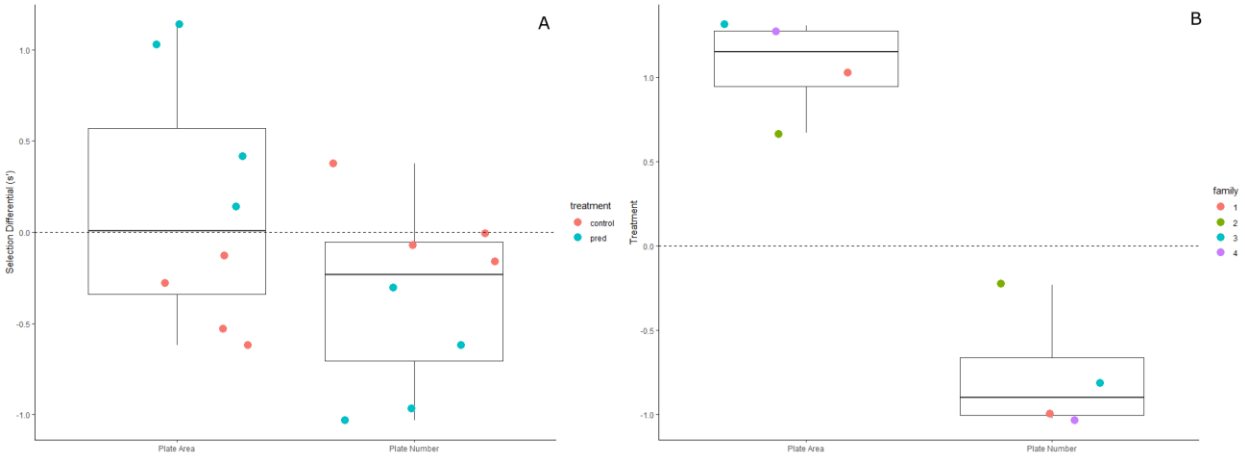

Figure 3: Lande-Arnold analysis of plate traits. (A) Selection gradient (B) and (B) treatment effect on the selection gradient (ΔB). In panel A red indicates control ponds and blue predation ponds. In panel B families are indicated by different colors.

Table 3: Between generation estimates of selection gradients and associated treatment effect. Significant values ($p < 0.05$) are indicated in bold

Estimate	Trait	p-value	Mean Selection Estimate	Standard Error	Df	T
Selection gradient (B)	Plate area	0.5575	0.146	0.237	7	0.616
	Plate number	0.08397	-0.348	0.173	7	-2.013
Treatment effect (Δh)	Plate area	**0.005174**	**1.071**	**0.145**	**3**	**7.365**
	Plate number	0.0253	-0.763	0.184	3	-4.153

QTL Mapping

QTL analysis incorporating all four F_1 families revealed several candidate regions for both lateral plate traits on chromosomes 7, 11.2, 20, and 21 (Figure 4; Table 4). For both traits, the regions explaining the most variance were on chromosome 7: the significant peak on chromosome 7 for plate number had a LOD of 18.45 and explained 19.27% of the trait variance (Figure 4; Table 4). The significant peak for lateral plate area on chromosome 7 had a LOD of 10.99 and explained 11.97% of the trait variance (Figure 4; Table 4). Chromosomes 11.2 and 21 explained less variance for each trait (4.74% for number, 7.88% for area, and 4.40% for number, respectively) (Table 4). Including sex as a covariate in the analysis did not qualitatively change the results (Figure 5; Table 6; see figures S9-S12 for individual families). Individual QTL analysis for each F_1 family revealed several additional candidate peaks (Figures S5-S8; Table 4). Peaks on chromosome 7 were shared among three of the F_1 families. New peaks not identified in the all-family analysis were found on chromosomes 7, 13, and 20. In the all-family analysis, the candidate loci for both traits on chromosomes 7, 11.2, and 20 had dominance deviation

coefficients greater than 0 (Table 5). This indicates that heterozygous individuals (AB) tended to have phenotypic values more similar to those of individuals with a homozygous limnetic genotype (BB) than a phenotype that is intermediate between the two homozygotes. The candidate locus on chromosome 21 had a value of essentially zero (Table 5), indicating a perfectly intermediate phenotype for heterozygous individuals. In the individual family analysis, all dominance deviation estimates were again greater than zero (Table 5).

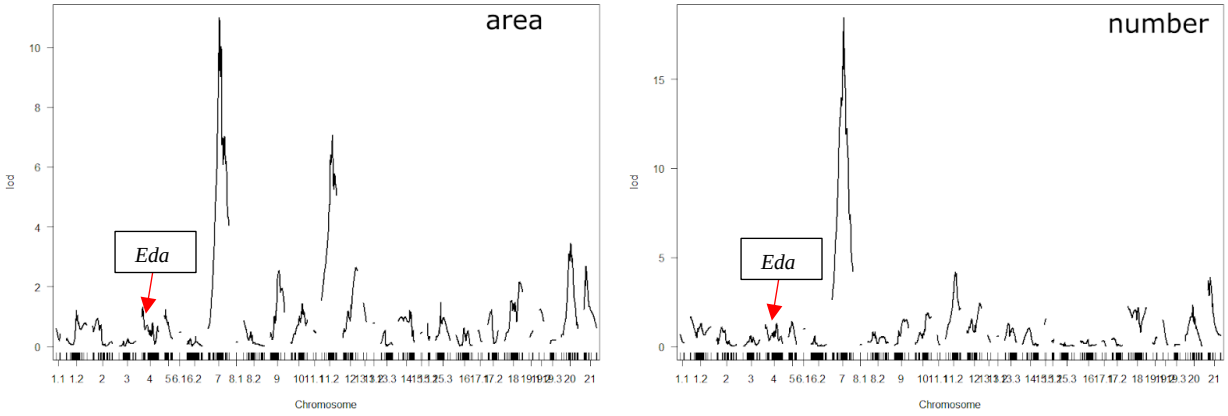

Figure 4: LOD score for all families. Area LOD score is significant at chromosome 7, chromosome 11.2, and chromosome 20. Number LOD score is significant at chromosome 7, chromosome 11.2, and chromosome 21. *Eda* is highlighted on chromosome 4 for reference.

Table 4: Candidate loci for lateral plate traits from QTL mapping. Significant p-values ($p < 0.05$) are in bold.

Lateral Plate Trait	Linkage Group	Position	LOD Score	PVE	p-value	Family	Sample Size
Number	7	47.9	18.45	19.27	**<0.001**	all	397
Number	11.2	40.7	4.19	4.74	**0.012**	all	397
Number	21	29.8	3.88	4.40	**0.024**	all	397
Area	7	47	10.99	11.97	**<0.001**	all	397
Area	11.2	45.2	7.08	7.88	**0.001**	all	397
Number	13.3	42.6	2.97	12.90	0.168	Family 1	99
Area	21	23.9	2.45	10.77	0.376	Family 1	99

Table 4 continued:

Number	7	46.2	6.56	26.07	**<0.001**	Family 2	100
Area	7	46.2	3.74	15.82	**0.034**	Family 2	100
Number	7	44.8	7.62	29.84	**<0.001**	Family 3	99
Area	7	53.2	5.47	22.47	**0.002**	Family 3	99
Area	20	32.9	4.01	17.02	**0.036**	Family 3	99
Number	7	48	3.96	16.82	**0.016**	Family 4	99
Area	7	62.2	4.83	20.12	**0.001**	Family 4	99

Table 5: Dominance coefficients

Lateral Plate Trait	Family	Linkage Group	Position	Additive effect	Standard error	t	Dominance Deviation	Standard Error	t
Number	all	7	47.9	0.6023	0.067	9041	0.331	0.095	3.491
Area	all	7	47	0.587	0.067	8.727	0.287	0.0975	2.947
Number	all	11.2	40.7	0.295	0.076	3.901	0.2113	0.107	1.983
Area	all	11.2	45.2	0.322	0.076	4.217	0.135	0.109	1.245
Area	all	20	54	0.124	0.083	1.498	0.241	0.114	2.108
Number	all	21	29.8	0.336	0.078	4.297	-0.0078	0.108	-0.072
Number	Family 1	13.3	42.6	0.094	0.099	0.946	0.539	0.147	3.663
Area	Family 1	21	23.9	0.240	0.117	2.05	0.095	0.155	0.613
Number	Family 2	7	46.2	0.730	0.137	5.342	0.369	0.203	1.818
Number	Family 3	7	44.8	0.782	0.1307	5.984	0.307	0.189	1.631
Area	Family 3	7	53.2	0.728	0.130	5.615	0.332	0.194	1.722
Area	Family 3	20	30.9	0.37	0.283	1.305	0.560	0.352	1.592
Area	Family 3	21	37.9	0.384	0.146	2.632	0.718	0.261	3.484
Number	Family 4	7	48	0.567	0.141	4.012	0.333	0.193	1.724
Area	Family 4	7	62.2	0.526	0.169	3.12	0.4121	0.253	1.629

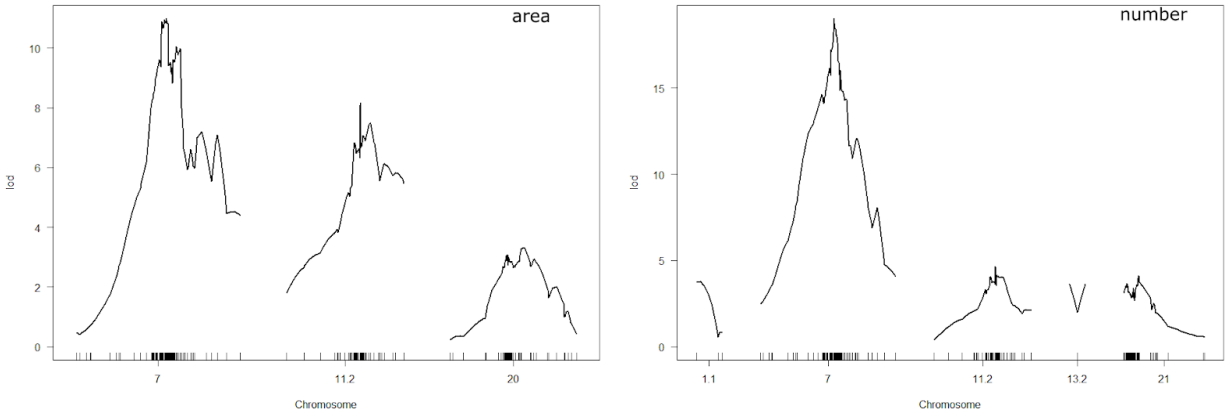

Figure 5: Significant QTL for lateral plate area and number with sex as a covariate for all families.

Table 6: Sex covariate LOD scores

Lateral Plate Trait	Linkage Group	Position	LOD Score	Family
Number	1.1	2.5	3.77	all
Number	7	47.9	19.02	all
Number	11.2	40.0	4.66	all
Number	13.2	2.8	3.63	all
Number	21	30.4	4.11	all
Area	7	48.5	10.99	all
Area	11.2	40.0	8.17	all
Area	20	55.9	3.32	all
Number	13.3	42.6	3.14	Family 1
Number	18	0.0	3.57	Family 1
Area	4	40.2	2.03	Family 1
Area	7	72.5	2.20	Family 1
Area	11.2	36.6	2.77	Family 1
Area	21	23.9	2.45	Family 1
Number	7	46.2	6.47	Family 2
Number	9	68.5	3.17	Family 2
Area	7	46.2	3.72	Family 2

Table 6 Continued

Area	10	40.8	3.54	Family 2
Area	11.2	40.0	4.03	Family 2
Number	7	44.8	7.51	Family 3
Number	21	42.7	3.43	Family 3
Area	7	54.9	6.11	Family 3
Area	9	78.6	4.10	Family 3
Area	20	20.9	7.09	Family 3
Number	7	48	4.22	Family 4
Area	7	60	4.08	Family 4

DISCUSSION

There are several hypotheses about which selective factors lead to the reduction of armor in freshwater. One key hypothesis is that lower levels of phosphorous (Hood *et al*. 2005) and calcium in freshwater (Boersma *et al*. 2008) have led to armor reduction (Heuts 1947; Giles 1983; Klepaker *et al*. 2016), as these minerals are important for bone production in fishes (Hendrixson *et al.* 2007). Despite the correlations between mineral availability and armor seen in the field (e.g., Klepaker *et al.* 2016), experimental evidence has not provided much support for this hypothesis (Archambeault *et al.* 2020). Salinity (Gelmond 2009; Hansson *et al.* 2016) and acidity have also been hypothesized to have an effect on stickleback armor (Begum *et al.* 2022). Yet, again, most of the work implicating these abiotic factors from armor reduction has been observational, and experimental support for these hypotheses has been mixed (e.g. Hansson *et al*. 2016). Reduced predation within freshwater environments, due to lower diversity of piscivorous fish, has also been hypothesized to lead to armor reduction (Gross 1978). Yet, results of previous experimental work have been variable (Reimchen 2000; Vamosi 2002; Vamosi and Schluter 2002) and observational field studies have uncontrolled variables (Reimchen 1992; Moodie 1972; Leinonen *et al*. 2011). Here, using a manipulative experiment, we find evidence of direct selection by cutthroat trout on lateral plate area, but not plate number.

We looked at changes in lateral plate morphology across several time periods. In the juvenile to sub-adult period we found evidence of selection for increased lateral plate area and decreased plate number regardless of treatment. Although there is a hint that there was stronger selection for plate area in the presence of vertebrate predation, there was no significant difference in the pattern of evolution between treatments. This suggests that vertebrate predation is not a key selective agent during this life stage. These results are similar to those seen for spine

traits in the same experiment (Rennison *et al.* 2019). In juveniles, dorsal and pelvic spines were selected against in both the predation and control treatments. Rennison *et al*., hypothesized that this was due to increased invertebrate predation during this life stage. This is because invertebrates tend to grapple the spines of the stickleback (Reimchen 1980; Marchinko 2009), therefore reduced armor in this stage of life would potentially be more beneficial in escaping invertebrate predation. The pattern of increased lateral plate area suggests that invertebrate predators do not constrain the evolution of this trait in the same way. The differential pattern of selection on the two plate traits may suggest that plate number and area are perhaps functionally different.

During the sub-adult to adult life stage the pattern of selection changed, with lateral plate number increasing and area decreasing on average, although the selection differentials were not significantly different from zero for either trait. This suggests that in general there is not strong selection on plate traits during this period. Here we again saw that greater areas were favored in the trout treatment ponds; although the result was not significant it does suggest that again plate number and area are possibly functionally different.

Between generations there was a strong pattern of divergent selection with strong selection for plate area in the presence of trout and selection against plate area in the absence of trout. This suggests that lateral plate area is adaptive when trout are present, and thus may provide some protection against predation increasing survival and/or reproductive output. Interestingly, plate number was selected against regardless of treatment. This conflicts with previous findings where an increase in plate number appeared to be selected for, however, this is likely due to the fact that these previous findings were based mostly from observational studies and did not control for covariant factors. Another explanation is that many of these studies

surveyed populations that had a greater number of plates (Reimchen 1992; Reimchen 2000; Leinonen *et al*. 2011; Hagen and Gilbertson 1973a; Kitano *et al*. 2008). In addition, it could be that bird predation or other toothed fishes are the key selective agent on lateral plate number. In this experiment abiotic factors such as water chemistry, nutrient availability, refuge availability, etc. were all controlled. Thus, the results of this study support the hypothesis that trout predation is a selective agent on lateral plates, at least for area.

Previous work has largely focused on lateral plate number, with many studies reporting that lateral plate number tends to increase in the presence of predators (Hagen and Gilbertson 1973a; Reimchen 2000; Reimchen 1992). The finding of differential selection on lateral plate area, but not lateral plate number, suggests that plate area, perhaps, is a more important component of defense against toothed predators. This could be due to the buttressing effect hypothesized by Reimchen (1983). Reimchen suggested that the anterior lateral plates help to protect the body from compression by "buttressing" the dorsal and pelvic spines. Large plates, with a greater area, that span from the dorsal spine apparatus to the pelvic girdle and contact other plates would provide greater buttressing of spines than small plates. Since plate number and area are only moderately correlated ($r = 0.58$) it is possible to have 9 plates (the full limnetic complement) but a small plate area if plates are narrow and short. Such a plate configuration would likely provide little buttressing.

Lateral plate variation between marine and freshwater populations of stickleback has been previously shown to largely map to the *Eda* locus (Colosimo *et al.* 2005) which is involved in lateral plate formation. Benthic and limnetic fish have the same *Eda* allele (the low-plated form) which means that the genetic loci responsible for the difference in their plating must be in regions other than *Eda*. QTL mapping of these benthic-limnetic F_2 hybrids identified several

candidate regions found that explain variance in lateral plate area and/or plate number. These include regions on chromosomes 7, 11, 20, and 21. Chromosome 7 has been previously associated with lateral plate number in benthics and limnetics (Arnegard *et al.* 2014; Conte *et al.* 2015) and in marine x freshwater crosses (Glazer *et al.* 2015). Co-mapping of lateral plate number and plate width (a component of plate area) has also been found for the *Eda* locus (Colosimo *et al*. 2004). Chromosome 11 has been previously associated with plate height divergence in lake and stream populations (Berner *et al*. 2014) and plate number in benthic and limnetic crosses (Arnegard *et al.* 2014). Chromosome 20 has been found to be associated with plate width and plate height in marine and freshwater populations (Colosimo *et al*. 2004; Indjeian *et al*. 2016). Chromsome 21 has been previously implicated in lateral plate number in benthic x marine crosses (Erickson *et al.* 2016; Wark *et al.* 2012; Colosimo *et al.* 2004), as well as benthic x limnetic crosses (Peichel *et al*. 2001) and Atlantic freshwater and marine populations (Liu *et al*. 2014). The QTL results support previous work that suggests benthic and limnetic fish both have the low *Eda* allele, as none of the candidates fall on chromosome 4.

Lateral plate number and area are moderately correlated, and map to many of the same genomic regions. However, it appears their pattern of evolution in response to selection are largely uncorrelated. The Lande-Arnold analysis, which takes trait correlation into account, indicated that the selection observed for lateral plate area and plate number were largely a product of direct selection. Thus, neither trait is evolving due to a correlated response between the two plate traits. However, these traits are also likely correlated to unmeasured (non-plate) traits, which could also be influencing the observed pattern of evolution. Future work incorporating additional armor traits could be particularly illuminating in disentangling the mechanisms generating the observed pattern of selection.

Between generations, there was strong selection plate area in the presence of trout, while plate number was selected against regardless of treatment. This suggests that predation is a selective agent for lateral plate traits, at least for area. Our findings also provide some support Reimchen's (1983) buttressing hypothesis and suggest that plate area is potentially a more important component of defense against toothed predators than just plate number. In addition, our findings support previous work that suggests that benthic and limnetic fish both have the low *Eda* allele and that other genetic loci are involved in the difference in plating in these ecotypes.

APPENDIX: SUPPLEMENTARY FIGURES AND TABLES

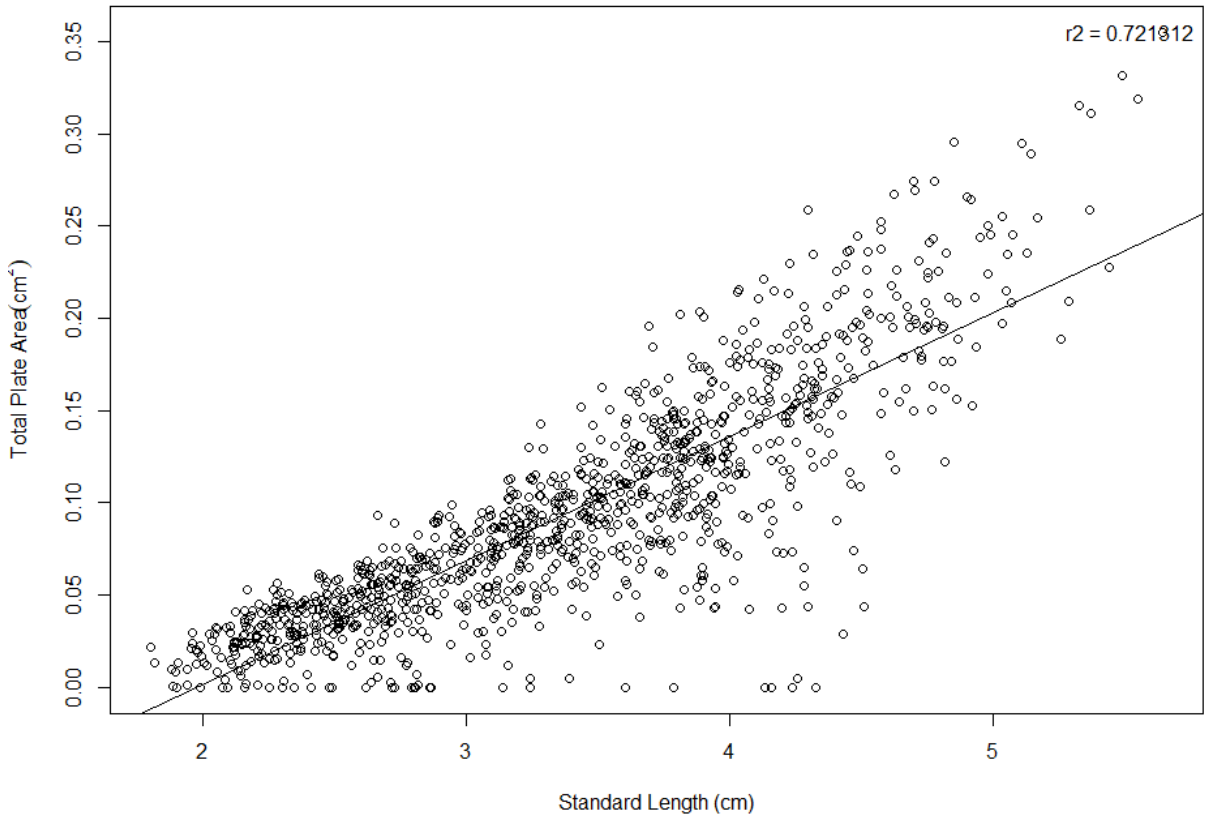

Figure S1: Correlation between standard length and total plate area without size correction

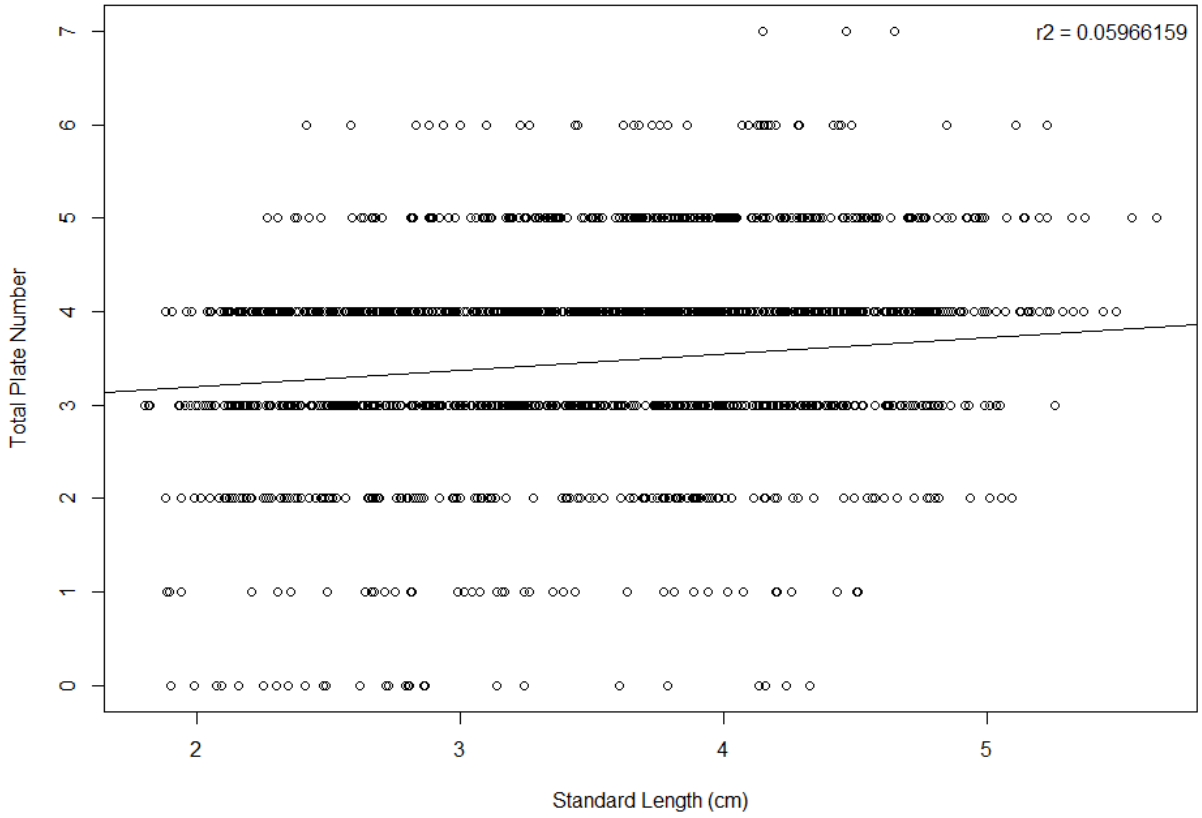

Figure S2: Correlation between standard length and total plate number without size correction

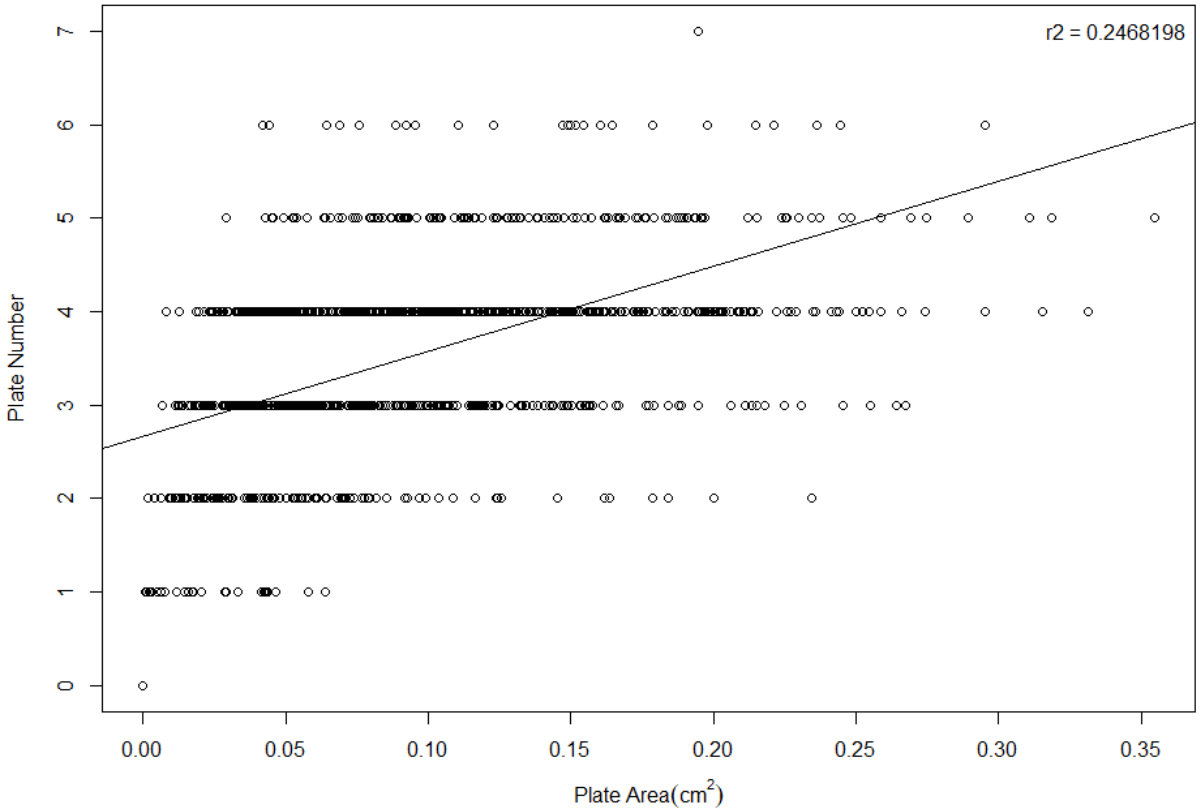

Figure S3: Correlation between plate area and total plate number without size correction

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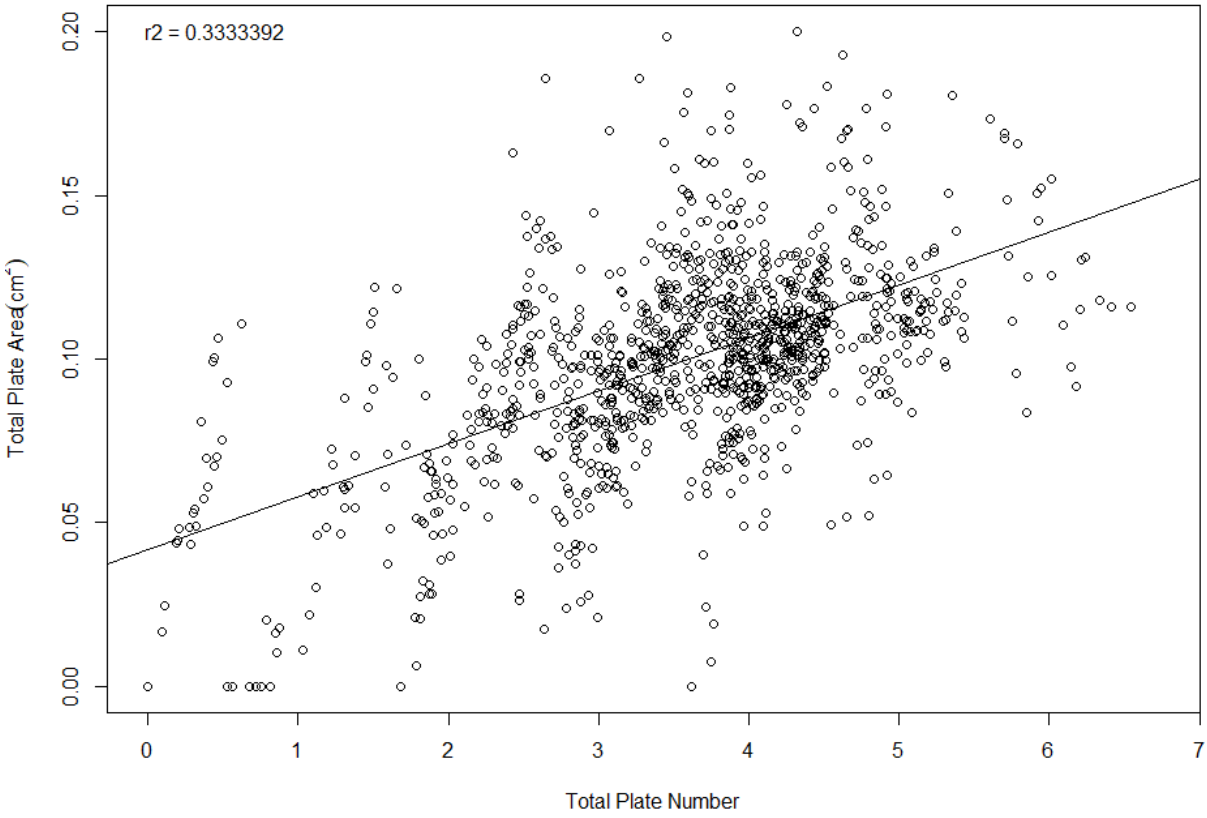

Figure S4: Correlation between plate area and total plate number after size correction

Table S1: Correlation coefficients between body length and lateral plate traits. All pairwise correlations were significant ($p << 0.001$).

Lateral Plate Trait	Correlation Coefficient (r)
Standard length and Plate number	0.24
Standard length and Plate area	0.85
Plate number and Plate area	0.58

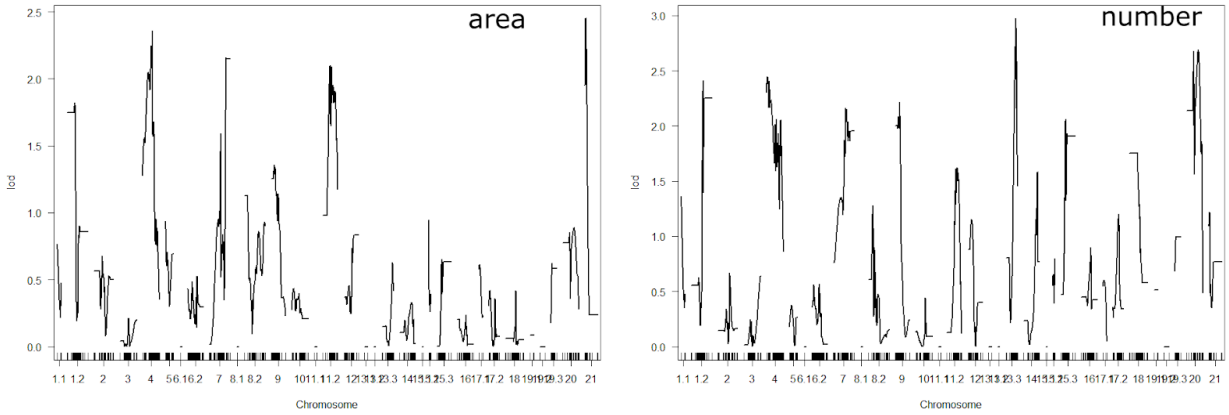

Figure S5: LOD score for Family 1

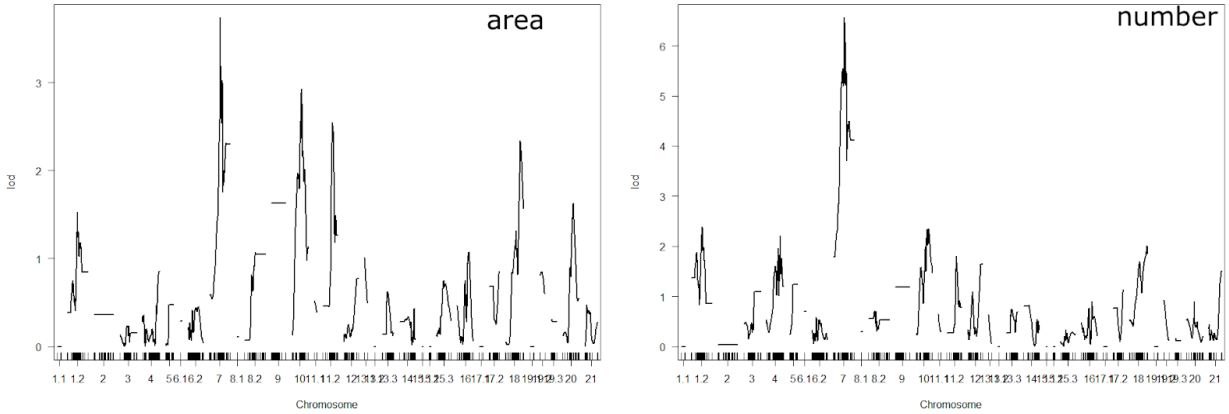

Figure S6: LOD score for Family 2.

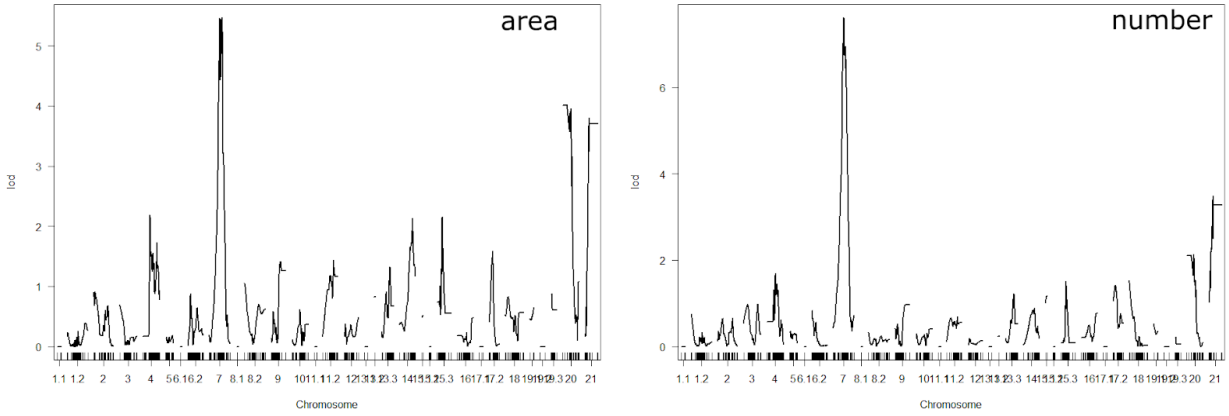

Figure S7: LOD score for Family 3.

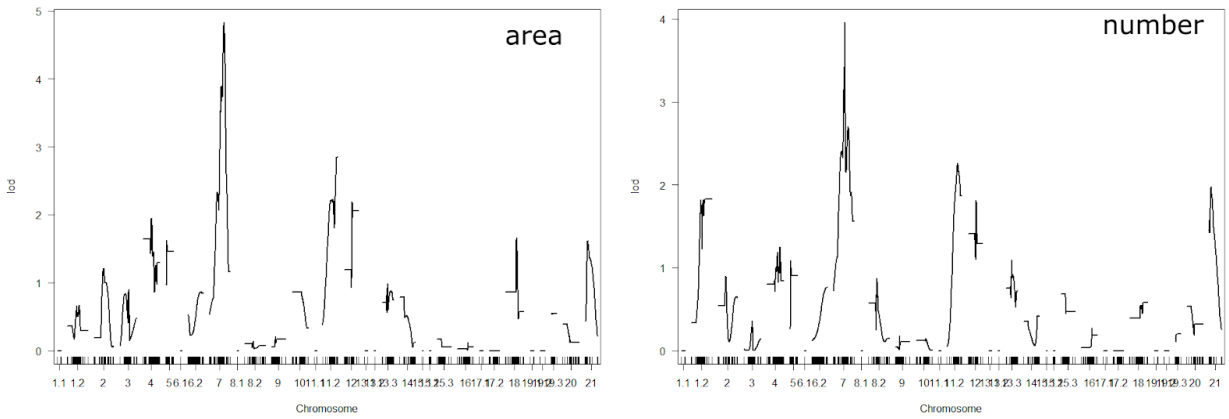

Figure S8: LOD score for Family 4.

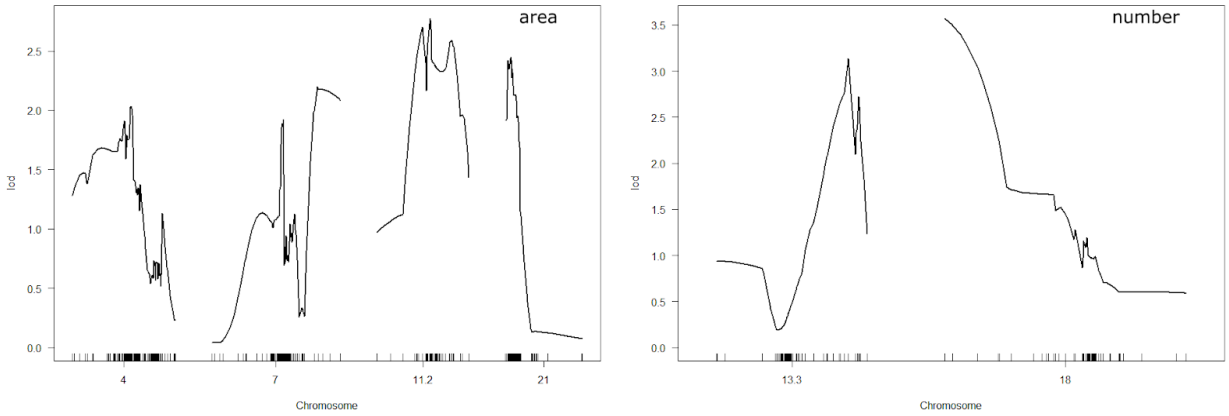

Figure S9: Sex covariate for Family 1

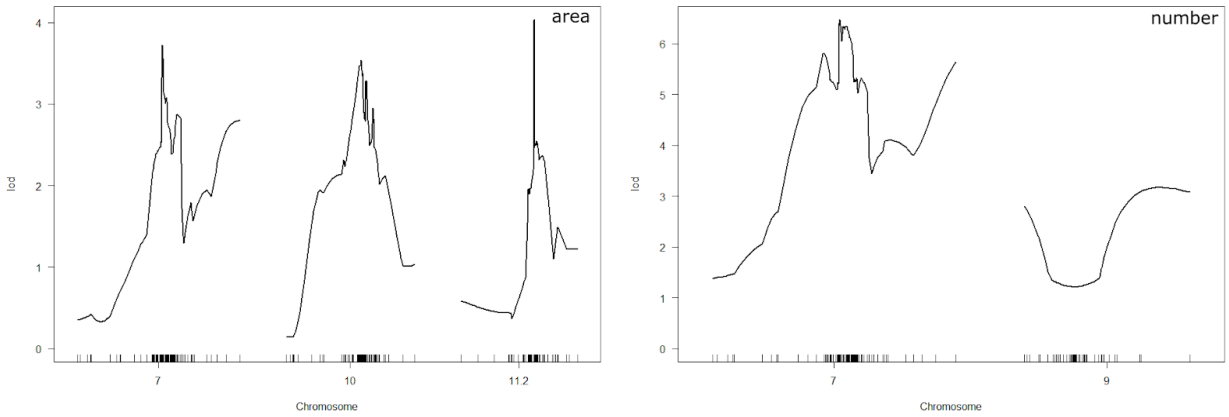

Figure S10: Sex covariate for Family 2.

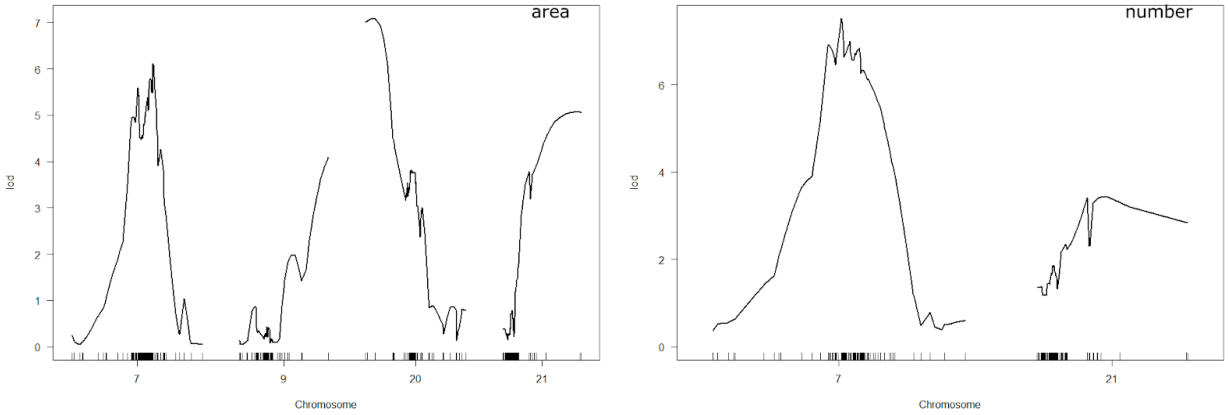

Figure S11: Sex covariate for Family 3.

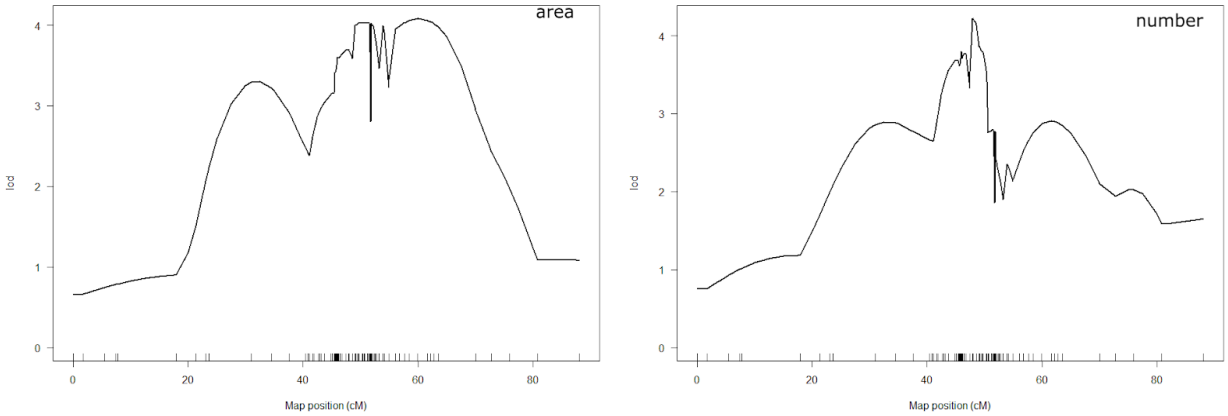

Figure S12: Sex covariate for Family 4

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