Frontiers in Ecology and the Environment

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Experimental harvesting of fish populations drives genetically based shifts in body size and maturation

Serinde J van Wijk[1], Martin I Taylor[1,2], Simon Creer[1], Christine Dreyer[3], Fernanda M Rodrigues[1,4], Indar W Ramnarine[5], Cock van Oosterhout[6], and Gary R Carvalho[1*]

Size-selective harvesting in commercial fisheries can induce rapid changes in biological traits. While experimental and wild harvested populations often exhibit clear shifts in body size and maturation associated with fishing pressure, the relative contributions of genetic and environmental factors to these shifts remain uncertain and have been much debated. To date, observations of so-called fisheries-induced evolution (FIE) have been based solely on phenotypic measures, such as size data. Genetic data are hitherto lacking. Here, we quantify genetic versus environmental change in response to size-selective harvesting for small and large body size in guppies (*Poecilia reticulata*) across three generations of selection. We document for the first time significant changes at individual genetic loci, some of which have previously been associated with body size. In contrast, variation at neutral microsatellite markers was unaffected by selection, providing direct genetic evidence for rapid evolution induced by size-selective harvesting. These findings demonstrate FIE in an experimental system, with major implications for the sustainability of harvested populations, as well as impacts on size-structured communities and ecosystem processes. These findings highlight the need for scientists and managers to reconsider the capacity of harvested stocks to adapt to, and recover from, harvesting and predation.

Human-induced changes in phenotypic traits have been reported in an increasing number of species (Palumbi 2001; Darimont *et al.* 2009) and may have particularly undesirable consequences (eg marked reductions in body size and fecundity, as well as localized depletions or even extinctions) in harvested species (Jackson 2001; Coltman *et al.* 2003; Jørgensen *et al.* 2007; Garcia *et al.* 2012). Phenotypic shifts driven by harvesting are typically much greater than those resulting from natural or anthropogenic stressors (Darimont *et al.* 2009). There is a need to explore and predict the consequences of size-selective harvesting at both the species (Reznick *et al.* 1997; Darimont *et al.* 2009) and ecosystem (Palkovacs *et al.* 2012) levels. Mounting evidence indicates that commercial harvesting, and size-selective fishing in particular, may lead to changes in the life histories of exploited species, including effects on body size, growth, and maturation (Jørgensen *et al.* 2007; Sharpe and Hendry 2009), and that these phenotypic effects can occur over relatively short time periods (Darimont *et al.* 2009). Despite the wealth of experimental

(eg Conover and Munch 2002) and field-based (Jørgensen *et al.* 2007) observations, it remains unclear whether observed changes in harvested phenotypes are due to plasticity from environmental variation or are associated with genetic changes arising from natural selection (Kuparinen and Merilä 2007; Garcia *et al.* 2012). Specifically, it has not yet been determined whether life-history shifts arise as a direct result of size-selective harvesting or are also driven by co-varying environmental factors, such as changes in ecosystem productivity or population density (Brander 2007). It is therefore crucial to try to identify a heritable genetic basis for harvest-induced shifts in life-history traits under controlled conditions. Genetic, as opposed to phenotypic, changes may result in long-lasting reductions in harvest yield (Stokes and Law 2000; Enberg *et al.* 2009; Salinas *et al.* 2012) as well as an increased risk of stock collapse (Árnason *et al.* 2009) with concomitant ecosystem-wide effects (Palkovacs *et al.* 2012), thereby compromising the multi-billion-dollar global fishing industry.

Discriminating between phenotypic plasticity and genetic change in exploited fish has proven difficult given the challenges involved in estimating quantitative genetic parameters in the wild (Law 2007) and the paucity of life-history and genetic data that predates commercial harvesting (Jackson 2001). These issues have hindered the identification of causal relationships between changes in selection pressures and responses at both the phenotypic and genetic level in wild populations. Until now, most evidence supporting fisheries-induced evolution (FIE) is based on phenotypic data and

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indirect measurements of evolutionary change (Jørgensen *et al.* 2007). Although correlations between size shifts and genetic change have been detected (Jakobsdóttir *et al.* 2011), to date there has been no direct molecular evidence in support of a genetic basis for phenotypic shifts. Furthermore, few studies have tested whether the intensity of selection imposed by harvesting is comparable to the magnitude of phenotypic changes observed (Swain *et al.* 2007; Nusslé *et al.* 2011), or estimated the proportion of phenotypic trait variance explained by the genotypes (the heritability, h^2, of traits) in wild populations (Law 2007).

Selection experiments, in which replicate populations are exposed to different harvesting regimes under controlled conditions, provide a powerful approach for exploring the genetic changes that can occur after size-selective harvesting (Fuller *et al.* 2005). Previous research has shown that rapid phenotypic shifts take place in experimental fish populations over just a few generations of intense harvesting selection (Conover and Munch 2002). However, parental fish in selection lines in these studies were wild-caught. Environmental heterogeneity and consequential variation in female body condition (so-called maternal effects) could therefore have affected offspring size and induced size differences among them. Likewise, simultaneous molecular genetic approaches investigating genetic change associated with shifts in size and maturation have not been undertaken.

Here, we test whether size-selective harvesting induces detectable changes at genetic loci over short time scales using artificially selected lines of the Trinidadian guppy, *Poecilia reticulata*. This species was chosen for its amenability to experimental manipulations and its well documented evolution of life-history traits in the wild (Reznick *et al.* 1990).

■ Materials and methods

Experimental design and rearing protocol

A sample of 90 male and 90 female guppies (F_0) was collected from the Lower Tacarigua River on the Caribbean island of Trinidad (10°38'49.5"N, 61°22'47.2"W) in March 2008 and transferred to the aquarium facility at Bangor University (UK); immediately thereafter, experimental fish breeding commenced.

After two generations of random breeding (F_1–F_2) to standardize growing conditions and environments for all fish and their parents (to remove possible maternal effects), we carried out size-selective harvesting on the subsequent three generations (F_3–F_5). Only adult male body size was selected for, because males exhibit determinate growth (ie they stop growing after maturation). Females, on the other hand, continue to grow throughout their lifespan. It was thus possible to remove any confounding influence of variation in the age distribution of differently selected lines. Moreover, the use of random

females reduced the likelihood of inbreeding depression, because of the reduced likelihood of similarly aged and/or related females being used. Fifty males (20%) were selected for each of the experimental lines (the 20% smallest males in the "S-lines", the 20% largest males in the "L-lines", and 50 random males in the control line) in generations F_3–F_5. These males were allowed to mate with 75 randomly selected females from within each line. At the end of each generation, tissue samples were taken from all male fish, placed directly in 100% ethanol, and stored at room temperature until DNA extraction. Maturation age (Age_{mat}) and size ($Size_{mat}$) were measured for 50 individual males from each line in the final (F_6) generation (see WebPanel 1 for details). Once all F_6 males had reached maturity for at least 30 days, the experiment was terminated. An overview of the experimental design is provided in WebFigure 1.

Phenotypic measurements and analyses

We measured the standard length (SL) of each fish, recorded in millimeters, by photographing individual fish in a Petri dish using a millimeter scale on a light table. Pictures were analyzed manually through Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). We analyzed phenotypic measurements using PASW Statistics 18.0.3 (SPSS, Quarry Bay, Hong Kong) and a nested analysis of variance (ANOVA; within generations) and regression analysis (over generations). When only two generations were compared, randomization tests were used.

Realized heritability (h^2) was estimated by way of the breeder's equation (Falconer and Mackay 1996) $R = h^2S$, which is a measure of the proportion of phenotypic variance explained by genotypes and is calculated from the cumulative response to selection R over the cumulative selection differential S over generations with selection. The evolutionary rate of male SL was calculated both in darwins and haldanes, two commonly used measures of the rate of trait change over years (darwins) and generations (haldanes), and calculated via the change in SL observed over three generations of selection (F_3–F_6) with selection intensity $i = 0.2$ (see WebPanel 1).

Genotyping

To assess overall levels of genetic diversity in selection lines, we quantified diversity at eight neutral microsatellite markers in each generation. Analysis of 1236 males yielded 9875 genotypes. In addition, all males in the F_0, F_2, and the final two generations (F_5 and F_6) were genotyped for 17 candidate loci (genes chosen based on known biological, physiological, or functional relevance). Candidate genes were obtained from three different sources. First, three markers were used that previously were shown to be closely linked to a continuously varying trait (the so-called quantitative trait locus, or QTL) on
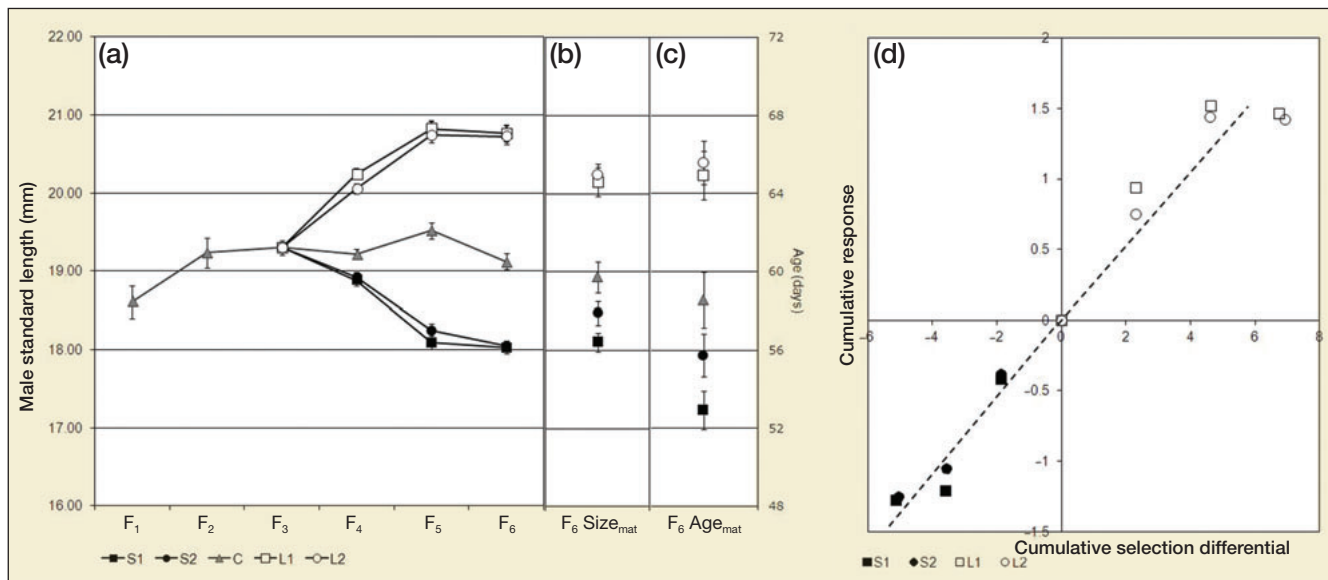
Figure 1. *Trends in male standard length (SL) for selection lines and the control in captive breeding generations F_1–F_6 (a) and the correlated response in (b) Size$_{mat}$ and (c) Age$_{mat}$ in F_6 males. Open circles/squares represent replicate lines selected for large SL (L1, L2), solid circles/squares for small SL (S1, S2), and solid triangles for the control line (C). Error bars indicate standard error of the mean. Fish used for estimating Size$_{mat}$ and Age$_{mat}$ were reared on different food levels to those in the main experiment. Size$_{mat}$ in (b) is therefore not directly proportional to SL in (a). The cumulative response to selection plotted against the cumulative selection differential appears in (d). The linear regression through these data (dashed line) is an estimate of heritability. For large SL: slope = 0.199, r^2 = 0.808, $F_{1,5}$ = 20.974, P = 0.006. For small SL: slope = 0.269, r^2 = 0.933, $F_{1,5}$ = 69.487, P = 0.000.*

the sex or Y chromosome, which influences male SL (M9, M30, and M987), as well as a marker on an autosome (M1046) from the same study (Tripathi *et al.* 2009). For M30 and M1046, the same single nucleotide polymorphism (SNP) as used by Tripathi *et al.* (2009) was polymorphic in our lines. An additional SNP was genotyped for M1046 (M1046–2) and M9 (M9–403). Insertion/deletions (indels) were analyzed for M9 and M987 (M9–indel and M987–indel). The M9–indel consisted of a three base-pair (bp) insert, and the insert in M987–indel had three different sizes: 9, 10, and 11 bp. Second, on the basis of knowledge of gene function in growth- and maturation-related pathways in other (fish) species, we designed 10 primer pairs for five genes obtained from the GenBank National Center for Biotechnology Information nucleotide collection for *P reticulata*. Finally, we analyzed a microsatellite locus (Pr39) that showed significant Hardy–Weinberg deviations, indicating a non-random distribution of genotype frequencies, alongside other candidate markers. In total, 17 candidate polymorphisms were genotyped for all breeding male fish in generations F_0, F_2, and F_5, and for the males used to estimate Size$_{mat}$ and Age$_{mat}$ in generation F_6 (713 individuals in total; WebTable 1).

Genetic analysis

We tested for evidence of selection at individual loci using a suite of complementary approaches: (1) analysis of molecular variance (AMOVA), (2) deviations from Hardy–Weinberg equilibrium (HWE), (3) patterns of allelic differ-

entiation, (4) outlier analysis, (5) individual-based modeling (IBM), and (6) association analysis. Details of individual analyses can be found in WebPanel 1.

■ Results

Phenotypic response to selection

Male SL was significantly greater in F_1 than in wild-caught males (mean ± standard deviation [in millimeters]: 18.90 ± 1.27 and 16.72 ± 1.61, n = 69 and 49, respectively, two-sample randomization test, P < 0.000). SL did not change over generations F_1–F_3 without selection (Figure 1a, linear regression on F_1–F_3: r^2 = 0.003, $F_{1,841}$ = 2.289, P = 0.131), suggesting that environmental variation in body size was successfully standardized in the first two generations. Mean male SL significantly increased from 19.30 mm to 20.75 mm (7.5%) in L-lines and decreased to 18.05 mm (6.5%) in S-lines over selected generations (linear regression on F_3–F_6: S1: *b* = –0.495, r^2 = 0.130, $F_{1,1158}$ = 172.948, P = 0.000; S2: *b* = –0.463, r^2 = 0.113, $F_{1,1144}$ = 145.471, P = 0.000; L1: *b* = 0.616, r^2 = 0.151, $F_{1,1142}$ = 203.001, P = 0.000; L2: *b* = 0.614, r^2 = 0.133, $F_{1,1103}$ = 169.456, P = 0.000). In contrast, SL did not change significantly over generations in the control line (linear regression on F_3–F_6 in the control: *b* = 0.007, r^2 = 0.000, $F_{1,1145}$ = 0.030, P = 0.863). Significant divergence of male SL was observed between, but not within, treatments (nested ANOVA; P = 0.010, P = 0.003, and P = 0.000 for generations F_4, F_5, and F_6, respectively; Figure 1a; WebTable 2). Size$_{mat}$ and
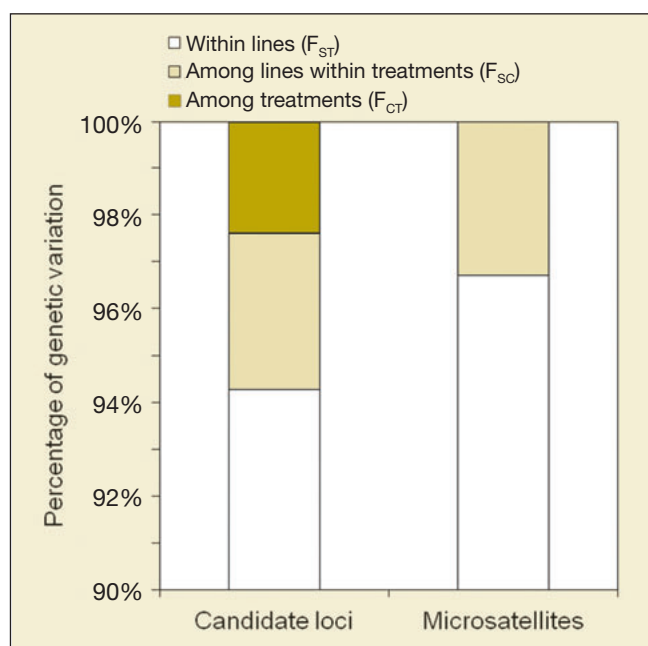
Figure 2. *Results from an analysis of molecular variance for 17 candidate loci and eight neutral microsatellites in generation F_6. Molecular variance is partitioned among treatments (F_{CT}, dark brown), among lines within treatments (F_{SC}, light brown), and within lines (F_{ST}, white), showing a significant among-treatment component for candidate genes only. See WebPanel 1 and WebTable 5 for accompanying statistics.*

Age_{mat} in F_6 males were also significantly different between treatments ($P = 0.038$ for Age_{mat} and $P = 0.022$ for $Size_{mat}$; Figure 1, b and c; WebTable 2). No selection response was observed in females (WebFigure 2), suggesting that maternal effects did not drive the strong response to selection we observed.

Assuming that the genetic basis for male body size was fully linked to the Y chromosome, we observed a conservative, minimal estimate of $h^2 = 0.269 \pm 0.032$ (mean $\pm$ standard error of the mean) for S-lines and $h^2 = 0.199 \pm 0.043$ for L-lines (Figure 1d). Although some autosomal control of body size in guppies is expected, we did not specifically examine its extent with our experiments. We opted for a conservative estimate of heritability because strong Y-linkage of male SL is supported elsewhere (Reznick *et al.* 1997), and our own observations indicate a lack of selection response in females (WebFigure 2).

Response at molecular genetic markers

We used eight microsatellite markers to estimate neutral genetic variation in each line of each generation. All microsatellite loci were amplified through the use of polymerase chain reaction, and there was no evidence of null or non-amplifying alleles or any significant dependence among loci after correcting for multiple tests. Allelic richness (A_R) was significantly higher in the F_0–F_1 generations ($n = 16$) than in the captive-bred generations F_2–F_6 ($n = 128$, two-sample randomization test, $P =$

0.032). However, we found no evidence that genetic erosion occurred as a result of selection. No trends in A_R over captive-bred generations were observed either in the control line over generations F_2–F_6 (logarithmic regression over all loci, $b = -2.357$, $r^2 = 0.055$, $F_{1,38} = 2.212$, $P = 0.145$) or in selected lines over generations F_3–F_6 ($P = 0.524$, 0.570, 0.576, and 0.453 for S1, S2, L1, and L2, respectively).

In contrast, AMOVA revealed significant treatment-level variation (F_{CT}) for candidate loci but not for neutral microsatellites, suggesting a non-random distribution of genetic variance at candidate loci only (Figure 2). We further identified evidence of selection at four out of 17 candidate loci (*Pr39, M9–403, M30,* and *M987*) using a suite of complementary approaches; significant deviations from HWE (WebTable 3) in selection lines were observed for locus *Pr39* in the L2 (F_5 and F_6) and the L1 (F_6) and for *M987–indel* in the L2 (F_5 and F_6). Such deviations from neutral expectations suggest that selection may affect allelic differentiation at these loci. Genetic differentiation between treatments observed at seven out of the 17 candidate loci provides further evidence of a genetic response to selection. Additionally, no candidate loci differed significantly between both L-lines (details summarized in WebTable 4). Conversely, none of the neutral microsatellite loci showed this treatment-specific pattern. Locus *Pr39* demonstrated a particularly marked divergence in allele frequencies of a single allele ("174") between large- and small-selected lines (WebFigure 3). These test results confirm the significance of patterns of allele frequency divergence between treatments (Web-Figure 4) for *M9–403, Pr39, Prol1, M30,* and *TBC1*. Outlier analyses that rely on the fdist method (a modeling approach designed to identify loci under selection, based on the divergence between populations/lines and the level of variation for each marker; Beaumont and Nichols 1996) revealed that *M30, M987–indel, M9–403,* and *Pr39* were significant outliers and are candidates for divergent selection (WebFigure 5). IBM simulations, which incorporated the specific breeding regime of our experimental lines, confirmed FDist analysis and identified *M30, M987–indel, M9–403,* and *Pr39* as outliers from neutral distributions (WebFigure 6). For one locus, *TBC1*, only the control line appeared to be an outlier in the IBM, but this was not confirmed by fdist. Moreover, using both outlier analysis and IBM, we observed no significant deviations for the eight putatively neutral microsatellites (WebFigures 5 and 6).

Significant associations between individual genotypes and phenotypes (WebFigure 7) were observed for nine of the candidate loci in WebTable 1, four of which (*M30, M987–indel, M9–403,* and *Pr39*) support results from other analyses, indicating a genetic response to size-selective harvesting (Table 1). Three of these loci (*M30, M9–403,* and *M987*) have previously been mapped to the proximal region of the Y chromosome. They are associated with a QTL for SL (Tripathi *et al.* 2009) and support

a sex-specific response to selection, as observed here. Although linked to the same QTL, these loci are not pooled into a single marker class because dynamics of allele frequencies over generations (see Web-Figure 4) indicate some independence between them. For three loci (*M1046*, *Prol2*, and *GH2–60*), a significant association between male SL and genotype was observed; however, no deviations from neutrality or treatment-specific differentiation were detected in other analyses (Table 1). These loci therefore likely contributed little to the selection response in our experimental lines.

Figure 3. *Two individuals of* P reticulata *from generation F_6, selected for small and large body size over three generations.*

■ Discussion

We have demonstrated a rapid phenotypic (Figure 3) and genetic (Table 1) response to selection as a result of size-selective harvesting. We controlled the environmental variation found in natural systems and used an experimental approach to simulate the nature of FIE in the wild, eliminating potentially confounding factors as putative drivers of directional phenotypic change. Importantly, by looking at genetic variation at candidate loci identified by previous mapping of genetic markers and quantitative traits (QTL mapping: Tripathi *et al.* 2009), our study transcends ordinary correlational studies.

In commercially exploited stocks, longer generation times and environmental stochasticity may impede a genetic response to selection. However, given the heritability estimates of life-history traits in the wild (Law 2000, 2007) as compared with those observed here, and the correspondingly substantial selective fishing mortalities in a majority of exploited populations (Reznick *et al.* 1997; Jørgensen *et al.* 2007), our conclusions provide insights into the potential responses in naturally harvested fish populations.
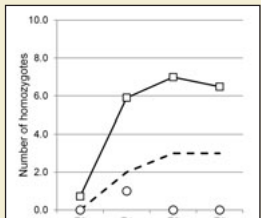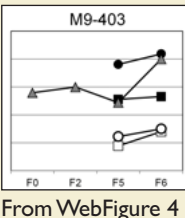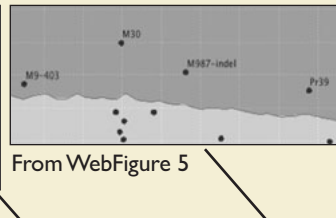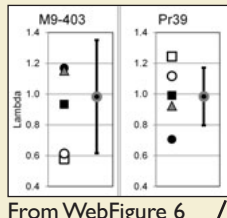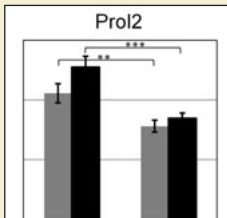
Our phenotypic data indicate an evolutionary rate for male SL of 50–55 kilodarwins or 0.3 haldanes; this is two to five times as great as those observed for natural guppy populations (Reznick *et al.* 1997; Reznick and Ghalambor 2005) and less than five times the mean rate observed for commercially harvested species (Darimont *et al.* 2009). Further estimates are provided in recent work by Devine *et al.* (2012), who explored trait evolution in over 20 fish stocks and found rates of change in maturation of up to 153 kilodarwins and –2.2 to 0.9 haldanes, values similar in order of magnitude to those observed here. The greatest rates of change were observed in the most heavily exploited stocks, with a reduced rate of evolutionary change when fishing moratoria were imposed. Over the past 40 years, declines in body length of as much as 20%

have been reported in populations of exploited fish (Shackell *et al.* 2009); a decrease in predation on guppies in the wild has resulted in 11–20% larger body sizes over 18 generations (Reznick *et al.* 1990; Reznick and Ghalambor 2005). Thus, although the selection intensity imposed in the current study was severe, the response observed (±7% SL over three generations) is comparable in magnitude to that seen in nature, and shows that rapid phenotypic shifts can be accompanied by identifiable genetic change. This supports the existence of FIE in the wild. We realize that the strong Y-linked response observed here, and in a species with determinate growth, is not directly comparable to many commercially harvested fish species due to differences in the genetic control of body size. However, while caution is often required in extrapolating evidence from the laboratory to the wild, the parallel life-history changes observed in our experiments and in wild scenarios, which also coincide with predicted life-history shifts to size-selective harvesting (Law 2000), strengthen the case for extending our discovery of genetic change to harvested species in fisheries.

Our evidence for genetic change by size-selective harvesting is of two types: (1) empirical molecular data, collected in parallel with phenotypic data, both at candidate markers identified by QTL mapping (Tripathi *et al.* 2009) and associated with SL (WebFigure 7), and at neutral markers used to test for effects of inbreeding; and (2) IBM and outlier tests that provided confidence intervals of expected divergence under neutral expectations, and thus allowed for independent verification of those markers that show selection effects (WebFigures 5 and 6). Through the use of this combined approach, our study provides direct support for the hypothesis of FIE over just three generations of selection.

Our findings have major implications for the sustainability of exploited resources because the rates of, and potential for, recovery of life-history traits differ funda-

Table 1. Summary of analyses performed on candidate loci

From WebFigure 3   From WebFigure 4   From WebFigure 5   From WebFigure 6   From WebFigure 7

	HWE	Genotypic differentiation	LOSITAN (fdist)	IBM	Association analysis
Rationale	Significant deviations from HWE	Significant genotypic differentiation between lines from different treatments, as well as a treatment-specific pattern of allelic divergence over generations	Significant outliers from neutral CI	Significant outliers from neutral CI	Significant associations between genotype and male SL
Location	WebFigure 3; WebTable 3	WebFigures 3–4; WebTable 4	WebFigure 5	WebFigure 6	WebFigure 7
Marker					
Myo	X (F_2)				
GH2–60					*
M30		X	X (99%)	X	X
TBC1		X (control line)		X (control line)	X
M1046					*
Pro1		X			X
Prol2					*
M9–403		X	X (95%)	X	X
M987–indel	X (L2 F_5 and F_6)		X (99%)	X (L2)	X
Pr39	X (L2 F_5, L1, L2 F_5 and F_6)	X	X (99%)	X	X

Notes: Support for selection at individual loci (listed on the left) is highlighted for the different analyses performed. An "X" in a column indicates that the particular analysis provides support for selection at the respective locus. None of the analyses suggested selection operating at markers *GH1, GH2–74, GH2–165, GH2–211, SF1, M1046–2*, and *M9–indel*. Loci highlighted in dark brown are ones for which the majority of analyses suggested selection is acting. Loci marked with an asterisk are those at which significant SL–genotype associations were observed but no other analysis indicated selection resulting from different harvesting selection. CI = confidence intervals. Details of individual analyses can be found in Figure 2, WebFigures 3–7, and WebTables 3–5. Exemplary sections of the respective WebFigures are provided.

mentally for genetic and phenotypically plastic change (Stokes and Law 2000). For example, results of some studies suggest that the genetic effects of exploitation on growth-related traits will be slow or impossible to reverse (Enberg *et al.* 2009). While some experimental investigations in fish provide evidence to the contrary (Conover *et al.* 2009; Salinas *et al.* 2012), trait values in these experiments did not completely return to pre-exploitation levels. Heritability of traits, effective population size, and selection differentials are all likely to determine response rates to harvesting and the degree of subsequent recovery. Nevertheless, it remains important to elucidate the impacts of such factors under a range of experimental and natural harvesting scenarios.

In addition to the projected effects of life-history evolution on sustainability and recovery rates of exploited populations, evidence suggests that shifts in community size structure can impact ecosystem processes (Palkovacs *et al.* 2012). Because aquatic food webs are highly size-structured, anthropogenically driven trait changes can lead to

effects across trophic levels. Such "trophic cascades" may act independently of changes in density and biomass of top predators (Shackell *et al.* 2009). We acknowledge that impacts of size-selectivity can occur whether the underlying responses are entirely environmentally driven or genetically based. However, the key point in relation to resource management is that the extent of genetic changes associated with phenotypic shifts will affect the persistence of perturbations in size structure, thereby affecting the ability to generate predictive estimates of response.

Although all fishery models typically represent quantitative effects of fishing in terms of population abundance and biomass, not all biomass is equal: significant but more subtle changes associated with a reduction in biomass occur as a result of truncated age and size structure, reducing reproductive potential (Murawski *et al.* 2001) and increasing vulnerability to environmental perturbations (Ottersen *et al.* 2006). Thus, it is the coincidence of genetically based long-term shifts in size structure with reduced opportunities to recover even after a fishing moratorium

(Jørgensen *et al.* 2007) that can constrain subsequent resilience and persistence in changing environments.

To our knowledge, the current work provides the first empirical demonstration that size-selective harvesting can drive genetically based shifts in traits that determine growth and reproductive outputs over the course of just a few generations. Such rapid evolutionary change requires a reconsideration of adaptation to, and recovery from, size-selective harvesting or predation. Finally, our data show that monitoring variation at neutral genetic markers – a practice common in conservation genetics studies – may not capture the loss of adaptive genetic variation induced by directional harvesting. To successfully manage harvested resources, we argue that it is imperative to apply population genomic approaches (Rusello *et al.* 2012) to detect and mitigate detrimental genetic effects of harvesting selection. Collectively, our findings highlight the importance of developing high-density genetic maps and genomic tools to assess the evolutionary impact of selective harvesting in the wild (Hemmer-Hansen *et al.* 2011).

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WebPanel 1. Materials and methods

Experimental design and rearing protocol

Fish were cared for in accordance with Bangor University guidelines and kept at constant cycles of 12 hours of light and 12 hours of darkness in identical 120-L aquaria (60 cm × 50 cm × 40 cm) divided over two levels and with a continuous flow-through system. Adult fish were kept in densities of less than one individual per liter. All breeding and rearing tanks were distributed randomly between the two levels, had a coral sand substrate and artificial vegetation, and were kept at constant water temperatures between 24.5–25.2˚C and pH levels of 7.2–7.5.

Fish were fed ad libitum live brine shrimp (*Artemia salina*) nauplii daily in the afternoon, except during some weekends and holidays, when they were fed commercial flake food. Tanks were inspected for fry daily over a period of 60 days. Any fry found were immediately transferred to separate tanks, where they were reared in densities of up to 2.08 fry per liter. When the fry reached 21 days of age or older, they were checked daily for maturity, and maturing individuals were moved to an identical separate, single-sex, 120-L aquarium at densities of up to 1.25 individuals per liter. Males and females were separated well before reaching sexual maturity and kept in single-sex tanks until selection was performed. When all males had been mature for at least 30 days (a period sufficient for male guppies to reach their maximum size), individuals that were to be used for breeding the next generation were selected, photographed, and measured for standard length (SL).

In generation F_1, 50 females were kept for breeding and mated to 50 randomly selected males. In generation F_2, 375 females were mated to 250 randomly selected males to produce F_3, which was used to set up five selection lines. From the 550 mature F_3 males, 50 males were randomly chosen, measured, and designated to the control line (C). The remaining 500 mature males were all measured; the 20% extreme percentiles were retained and randomly divided over two selection lines each, resulting in four groups of 50 males each, two of which consisted of the smallest 20% of males (S1 and S2) and two lines of the largest 20% of males (L1 and L2). Each group of selected F_3 males was mated to 75 randomly selected F_3 females and left to mate freely for 30 days to produce the next generation.

The same procedure was used to produce the F_4 and F_5 generations; in each generation, 50 males (20%) and 75 females were selected from 250 males and 250 females per line. For the F_6 generation, a random fraction of 100 fry per line was taken to estimate maturation age and size (see below) and approximately 100 males and 100 females were reared in the main experiment as in previous generations. Once all F_6 males had been mature for at least 30 days, the experiment was terminated.

Estimation of maturation age and size in F_6 males

From the F_6 fry, up to 12 newborns (depending on the number of fry observed) were taken at random daily and placed in 4-L plastic jars. Up to six fry born on the same day were placed in each jar. All jars were aerated and kept in a controlled temperature-environment at 25˚C. Fry were fed ad libitum brine shrimp nauplii daily and a full water change was performed once per week. At this point, each jar was moved to a randomly chosen location within the controlled temperature room, in order to randomize any effects of microclimate on maturation. Maturity status was confirmed daily by visual inspection of all fish over 14 days of age. Female fish (recognizable by black pigment speckling in the anal area) were removed from the experiment. Male fish (recognizable by the onset of gonopodium development) were kept and reared to maturity. A maximum of five males were reared in any one jar. Upon reaching sexual maturity, as indicated by the presence of a fleshy hood extending beyond the gonopodium tip (Houde 1997), the male was measured. A tissue sample was taken from each male before it was removed from the experiment.

Repeatability of phenotypic measurements

Repeatability of measurements was high (mean ± standard deviation [SD]: 0.95 ± 0.050) and calculated using a set of 50 males, each of which was photographed three times, following Lynch and Walsh (1998): $\Sigma 1$–50 $(1-[\sigma_k^2/\sigma_t^2])/50$, in which σ_k is the SD between repeated measures of the kth fish and σ_t is the SD over all measurements.

Evolutionary rate

Rate in 1×10^3 darwins was calculated as: $(\ln X_2 - \ln X_1)/-\Delta_t$, with X_2 the mean SL of the F_6 and X_1 of the F_3 and time (t) in years. Rate in haldanes was calculated as: $([X_2/S_p] - [X_1/S_p]) g^{-1}$, with S_p being the pooled SD $([n_1 - 1]S_1 + [n_2 - 1]S_2) /([n_1 - 1] + [n_2 - 1])$ and g the number of generations (3) following Hendry and Kinnison (1999).

DNA extraction

Genomic DNA was extracted using hexadecyltrimethylammonium bromide (CTAB), according to the following protocol: a small amount of tissue was incubated overnight at 60˚C in 350 μl 2% CTAB buffer (100 mM Tris-HCl pH 8.0; 1.4 M NaCl; 20 mM EDTA pH 8.0; 2% CTAB; 0.2% 2-mercaptoethanol) with 20.0 μl proteinase K solution (QIAGEN). For the extraction, 300 μl of choloroform:isoamyl alcohol (24:1) was added, mixed for 5 minutes with an automated rotator, and then centrifuged at 13 000 rotations per minute (rpm) for 5 minutes. The supernatant was transferred to a new 1.50-ml tube and the extraction step repeated, after which 660 μl 100% ethanol and 30.0 μl 3 M sodium acetate at pH 4.8 were added to the retained supernatant. This solution was mixed for 3 minutes, left to stand for 10 minutes, and then centrifuged at 13 000 rpm for 10 minutes, after which the supernatant was discarded and 500 μl 70% ethanol was added to the pellet. This was centrifuged for 5 minutes to wash the pellet, and the supernatant was discarded again. Pellets were left to dry at 37˚C and resuspended in 100.0 μl H$_2$O. DNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and a working solution diluted to a volume of 50.0 μl at 10.00 ng per μl and stored at 4˚C. The remaining DNA was kept at extraction concentration and stored at –20˚C.

Microsatellite genotyping

Male fish were genotyped at nine microsatellite loci: *Pr39, Pr92* (Becher *et al*. 2002), *Pret32, Pret69, Pret77* (Watanabe *et al*. 2003), *Hull70–2* (van Oosterhout *et al*. 2006), *G82, G102*, and *G289* (Shen *et al*. 2007). Microsatellite marker products were

continued

WebPanel 1. – *continued*

obtained using a QIAGEN Multiplex polymerase chain reaction (PCR) kit in two 10.00 μl reactions: a reaction at 53˚C annealing temperature (AT) for *Pret69, Pret77, Pr39, Hull70-2*, and *G102*, and at 58˚C AT for *Pret32, Pr92, G82*, and *G289*. The following reaction mix was used: 6.00 μl Multiplex mix, 1.00 μl Q solution, 1.00 μl H_2O, 1.00 μl DNA at 10.00 ng per μl, and 1.00 μl primer mix (containing a mix of equal volumes of forward primers at 1.00 μM and reverse primers and dye-labeled universal primers at 10.00 μM concentrations). PCR products were obtained using a Tetrad2 Peltier thermal cycler (BIO-RAD) and the following program: 95˚C for 15 minutes; 94˚C for 30 seconds, AT for 90 seconds, 72˚C for 90 seconds, for 12 cycles; 94˚C for 30 seconds, 50˚C for 90 seconds, 72˚C for 90 seconds, for 30 cycles; 60˚C for 30 minutes.

Forward primers were extended with an 8-bp tail, complementary to a FAM/NED/VIC/PET fluorescent dye-labeled universal primer following Schuelke (2000) and amplified using a QIAGEN Multiplex PCR kit. PCR products were resolved on a 3130xl Genetic Analyzer (Applied Biosystems) using GeneScan LIZR500 as an internal size standard and genotyped using GeneMapper 4.0 (Applied Biosystems).

SNP genotyping
Primer3 (Rozen and Skaletsky 2000) was used to design a total of 10 primer pairs for five genes obtained from the GenBank NCBI nucleotide collection for *P reticulata*, based on knowledge of gene function in growth- and maturation-related pathways in other fish species. Primer sequences are provided in WebTable 1. Single nucleotide polymorphisms (SNPs) were identified by sequencing pooled samples of the smallest/largest 10 individuals in the F_6 generation for each individual marker. Indel genotyping was done by incorporating the two indels into the microsatellite multiplex panel. SNP genotyping was performed by KBioscience (www.kbioscience.co.uk).

Genotyping failure was low (<2%) and no significant BLAST hits were obtained for our primer sequences on GenBank, other than for the gene they were designed for, making chimeric PCR amplification (Bradley and Hillis 1997) and erroneous genotypes resulting from non-specificity of primers unlikely.

Genetic analyses
We verified the absence of null alleles at microsatellite loci using MICRO-CHECKER 2.2.3 (van Oosterhout *et al*. 2004) and tested for genotypic linkage disequilibrium (LD) between microsatellites and candidate loci separately, using a log-likelihood ratio statistic for each pair of loci in Genepop 4.0.10 (Raymond and Rousset 1995; Rousset 2008). LD was tested in the wild-caught (F_0) fish only because (artificial) selection is predicted to increase LD irrespective of the genomic distance between marker loci (Falconer and Mackay 1996) and could affect LD test results. Allelic richness (A_R; El Mousadik and Petit 1996) was calculated per microsatellite locus based on a minimum sample size of n = 32 (F_1), using FSTAT 2.9.3.2 (Goudet 1995). Significant differences in A_R between treatments (within generations) were tested using nested ANOVA in PASW Statistics 18.0.3. To test for significant changes over generations, we used regression analysis (PASW) or, when only two populations were compared, randomization tests with 100 000 randomizations in Rundom Pro 3.14 (Jadwiszczak 2009).

Conformity to Hardy–Weinberg equilibrium (HWE) was tested at each locus in each population and for each generation independently, using the Hardy–Weinberg probability test in Genepop with 10 000 batches and 10 000 iterations per batch. Results were corrected for by performing multiple tests using Benjamini-Hochberg (B-H) corrections in the program SGoF+ (Carvajal-Rodríguez *et al*. 2009).

Using Fisher's method in GenePop, we performed a log-likelihood-based test for genotypic differentiation at each candidate locus and between all pairs of populations in generations F_5 and F_6 (Option 3, suboption 4, 10 000 batches and 10 000 iterations per batch). B-H corrections were applied to correct for performing multiple tests.

Arlequin 3.5.1.2 (Excoffier *et al*. 2005) was used to carry out analyses of molecular variance (AMOVAs) on neutral microsatellites and candidate loci separately in generation F_6, using the following hierarchical levels: (1) within lines (F_{ST}), (2) within treatments-among replicate lines (F_{SC}), and (3) among treatments (F_{CT}).

The FDist method (Beaumont and Nichols 1996), as implemented in the software LOSITAN – Selection Workbench (Antao *et al*. 2008), was used to estimate outlier loci from the entire dataset of 25 markers (microsatellites and candidate loci) in the F_5 and F_6 generations, using 50 000 simulations, an infinite alleles model, and a forced neutral mean F_{ST}.

An individual-based model (IBM) that specifically incorporated the population dynamics and breeding regime of our experimental lines was written in Minitab to simulate the changes in expected heterozygosity (expressed in lambda: $λ = H_SF_6/H_SF_2$) over three generations. The model was used to verify outlier analysis using the FDist method. It utilized the allele frequency data from generation F_2 (prior to selection) as initial allele frequencies and assumed all alleles were inherited neutrally. For each locus, mean simulated $λ$ ± 99% confidence intervals (CIs) with Bonferroni corrections (3.61 × SD) were calculated and compared to observed $λ$ values. In generations and lines where a locus was not in HWE (*Pr39* in L1 and L2 in the F_6 and *M987–indel* in the L2 in the F_6), $λ$ from the actual data was calculated as changes in observed heterozygosity rather than H_e ($λ = H_OF_6/H_OF_2$). Loci falling above or below the CI were highlighted as possible candidates under directional selection. The model code is available from the authors upon request.

Association analysis
Using the online software SNPStats (Solé *et al*. 2006), we tested for associations between male SL and genotype for each candidate locus in the control line independently (combined F_2, F_5, and F_6) and in a global test over all lines in generations F_2, F_5, and F_6. For *M987–indel* and *Pr39*, for which more than two alleles were present in the data, SNPStats was used to test for associations of SL with the absence/presence of an insert (irrespective of its size) for *M987–indel*, and the absence/presence of allele "174" at *Pr39*. Five different inheritance models were considered: co-dominance, dominance, overdominance, recessive, and a log-additive model. Interactions between loci were not considered. The most likely model of inheritance was inferred based on the lowest *P* value and lowest value for the Akaike Information Criterion (AIC) in the analysis on the control line.

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Author contributions

GRC and MIT conceived the study. GRC, MIT, CvO, and SC contributed to experimental design, analyses, and writing, and supervised SJvW. CvO wrote the IBM. SJvW conducted the experiments, laboratory work, analyses, and writing. CD provided QTL markers, advice, and contributed to the writing. IWR contributed to fish sampling and transport logistics. FMR assisted with the experiments and molecular data collection.
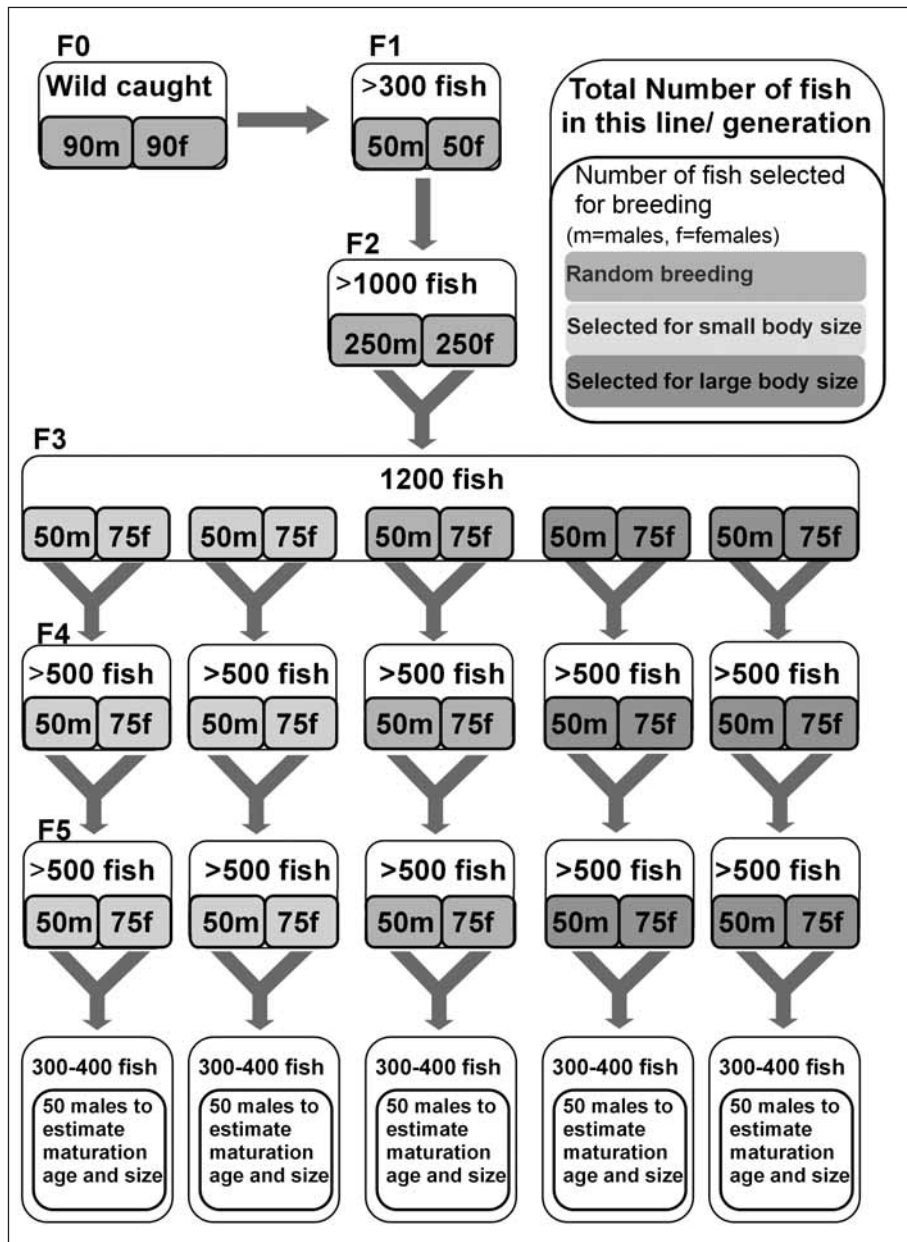
WebFigure 1. *Schematic representation of selection experiments. Numbers of fish selected and used for breeding in each of the generations F_0–F_6 are marked in gray, the different shades indicating the different treatments: light gray for small-selected lines, dark gray for large-selected lines, and intermediate shading for random breeding generations.*
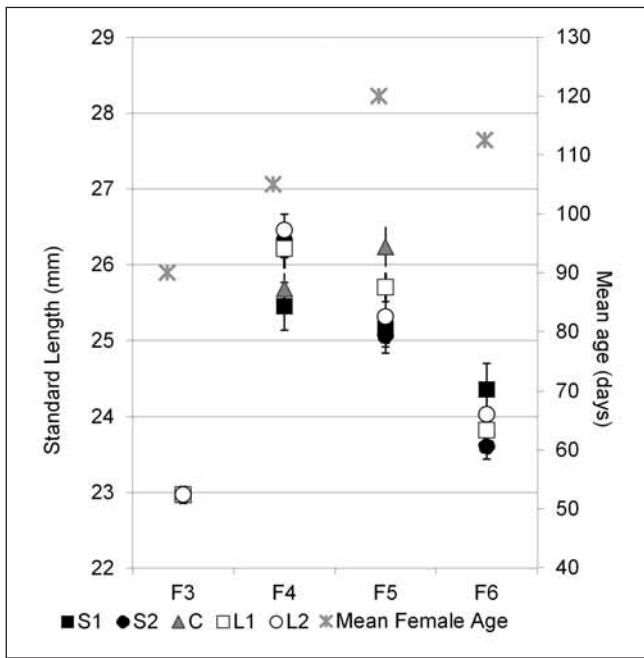
WebFigure 2. *Standard length (SL) and mean age of female guppies over generations F_3–F_6. SL of females in generations F_3–F_6 for selection lines and control line, plotted with mean age of females per generation (ж). Gray circles and squares represent replicate lines selected for large SL, dark circles and squares represent small SL, and the control line is given by closed triangles. Female size and mean age were significantly correlated (Pearson's correlation = 0.315, P = 0.000) explaining variation in female SL over generations by differences in generation times. Error bars represent standard error of the mean.*
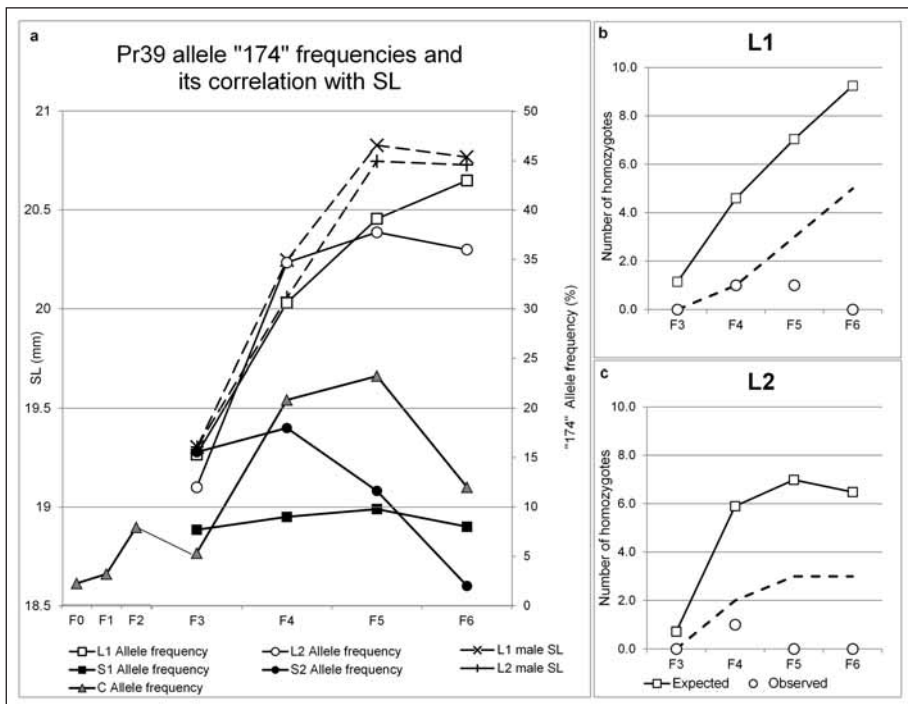
WebFigure 3. Pr39 *allele "174" frequencies and its correlation with SL. (a) Allele frequencies of allele "174" of microsatellite locus* Pr39 *(right axis) in the control (gray triangles) over generations F_0–F_6 and of selection lines in the F_3–F_6. S-lines are represented by black squares (S1) and circles (S2). L-lines are represented by white squares (L1) and circles (L2). The correlation of allele frequency with male SL (left axis) in L-lines is also shown (dashed lines, x L1, + L2). Allele "174" is associated with increased SL (Pearson's correlation $r = 0.953$, P = 0.000) and shows highly significant homozygote deficiencies in the L1 and L2 lines in generations F_4–F_6 (b and c). Dashed lines in (b) and (c) represent the cumulative binomial distribution of homozygosity probability for allele "174", based on the allele frequency. Any observed number of homozygotes below the line has a probability less than 0.05.*
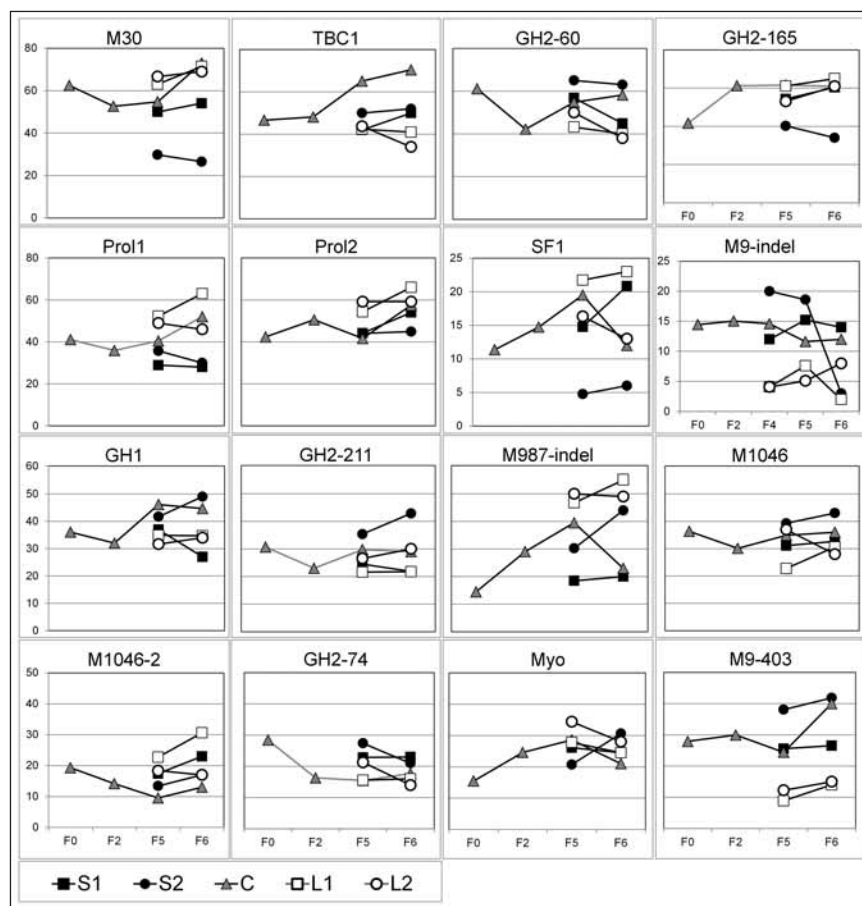
WebFigure 4. *Changes in allele frequency of candidate loci over generations F_0, F_2, F_5, and F_6. For SNP markers, minor allele frequencies are shown. For M987–indel, the frequency of the short allele (ie the absence of an insert) is shown, for M9–indel the insert frequency, which was also genotyped for the F_4.*
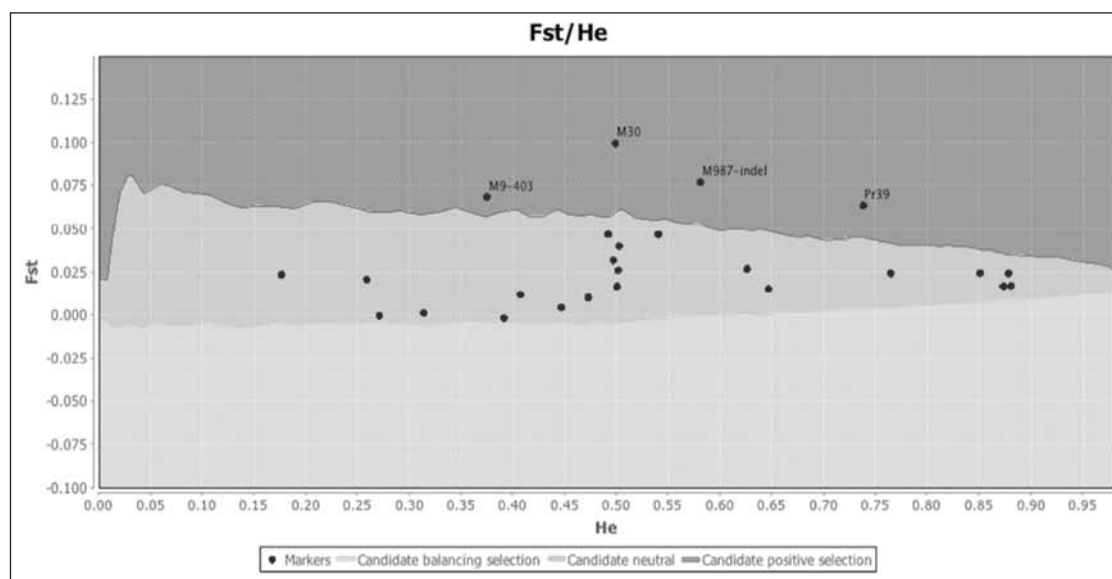
WebFigure 5. *Outlier analysis using the FDist method. Results from outlier analysis using the program LOSITAN (Beaumont and Nichols 1996; Antao et al. 2008) indicate M9–403, M30, M987–indel, and Pr39 as significant outliers. M30, M987–indel, and Pr39 were significant at the 99% CI level (P = 0.001, 0.003, and 0.003, respectively; P = 0.031 for M9–403). Generations F_5 and F_6 were both included in this analysis to increase the number of populations per treatment.*
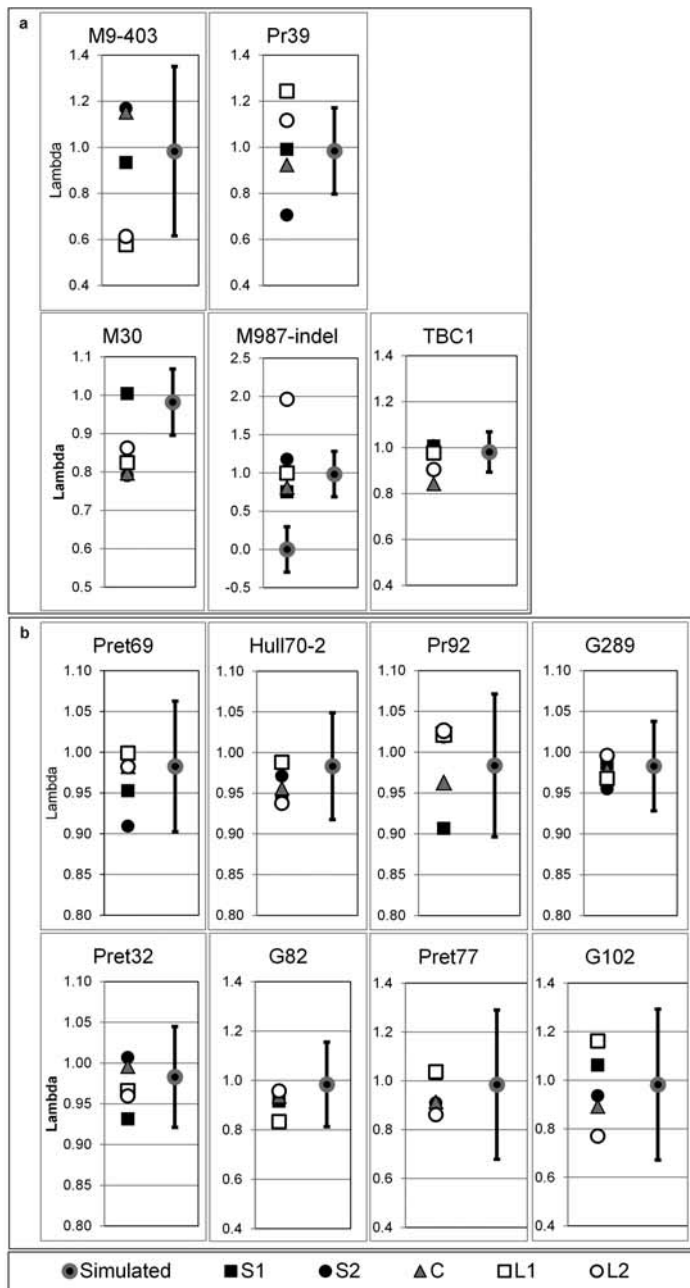
WebFigure 6. *Results of individual-based model (IBM) simulations showing locus-specific changes in heterozygosity ($\lambda = H_S[F_6]/H_S[F_2]$) compared to locus-specific simulated λ. The results confirm the four outliers identified by FDist analysis (M9–403, Pr39, M30, and M987–indel (a); and reveal no changes in λ greater than expected under neutrality at neutral microsatellites (b). The IBM incorporated observed allele frequencies and the specific breeding regime of our experiment. Confidence intervals for simulations were calculated using a Bonferroni correction as 3.61 times the SD over 1000 simulations. For Pr39 in the L1 and L2 and M987–indel in the L2, λ was calculated using the observed heterozygosity H_O rather than H_S because of HW deviations in the F_6 for these loci (WebTable 3).*
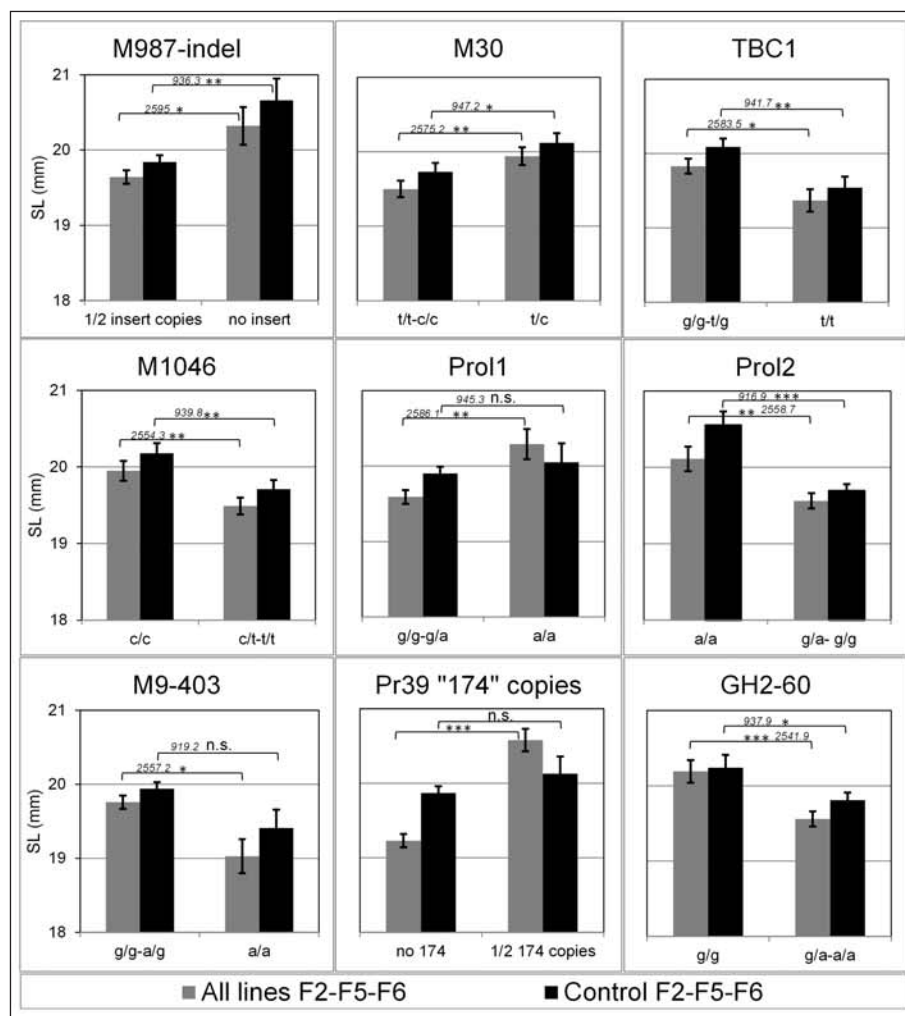
WebFigure 7. *Associations between male SL and genotype at candidate loci. Genotype (x axis) associations are shown with SL in millimeters (y axis) over all data (gray bars) and within the control line (black bars) for all males in generations F_2, F_5, and F_6. Error bars present standard error of the mean. Loci showing differences in size between homozygotes and heterozygotes indicate that overdominance is the most likely model of inheritance. Those where one homozygote genotype is associated with a different SL than found in the heterozygote and the other homozygous genotype, represent recessive/dominant inheritance. P values are indicated by asterisks above the comparisons (*P < 0.05, **P < 0.01, ***P < 0.001). AIC values are in italics. For locus Pr39, too few "174" homozygotes (only two in L-lines) were observed to test which is the most likely inheritance model. For loci M9–403, Pr39, and Pro11, the association was not significant in the control line independently (P = 0.095, 0.22, and 0.55, respectively), which is likely the result of low minor allele frequencies and consequentially skewed genotypic frequencies (8%, 21%, and 16%, respectively). Associations between male SL and genotype that were significant in the control line independently represent especially strong support for the genetic differences between lines at these marker loci being unlikely due to drift but partly explaining the observed differences in SL between treatments.*

WebTable 1. Candidate genes for standard length in male *P reticulata* investigated in the present study

Search term	*P. reticulata* sequence obtained / putative function	GenBank Accession Number	Sequence	SNP Marker(s)	Primer Sequence
M009	Rab interacting lysosomal protein-like 1	ES375672	cDNA	M9-indel: M9-403:	F: CTCATTCTGTGCTTCAACCTG R: GAAATGGCCTCCAGTATCTCC F: AATCGTTTCCACAGGAGGTG R: AGTCGCTAAATGCTGGTGGT
M380	Medaka Chrom. 15, in scaffold91 contig45820	FH889280	BAC genomic	---	
M987	Unknown	FH893254	BAC genomic	M987-indel:	F: AGCGGTCATGCACTAACAAG R: ACATCTGCTCTGCCACACAA
M155	Splicing factor, arginine/serine-rich	ES385036	cDNA	---	
M030	Fructose-1,6-bisphosphatase 1 [Danio rerio]	ES382008	cDNA	M30:	F: TTGGTAGACCGAGACGTGAAG R: TCAGCCTTGACATGAGTTACG
M061	Ectodermal-neural cortex 1 ENC1	ES379217	cDNA	---	
M1046	Melanocortin 4 Receptor	FJ236224	mRNA, partial cds	M1046, M1046-2:	F: GCTGGAGAACATCCTKGTG R: GGAACATGTGGACGTAGAG
M1078	Insulin growth factor 1	FJ236241	intron	---	
Steroidogenic factor	SF1 nuclear receptor 5A1 steroidogenic factor	FJ236230	mRNA, partial cds	SF1:	F: GAAGAGCTGTGTCCGGTGTG R: CGCACGTGTACCTCTTGTTG
Insulin growth factor	Insulin growth factor 2	DQ337476	mRNA, complete cds	---	
IGF Binding protein	IGF-Binding Protein 1	FJ236240	mRNA, partial cds	---	
Growth hormone	Growth hormone	U63805	partial cds	GH1: GH2-60, GH2-74, GH2-165, GH2-211:	F: TTTTGACCCAGGAGCATTGT R: GGAGGTTTGAGGCGTCAGTA F: CTGCAGAAATACGCCACTCA R: ACAATGCTCCTGGGTCAAAA
Growth hormone	putative GH-regulated TBC protein (Mus musculus)	ES386819	cDNA	TBC1:	F: TGCGGCTGTATTGATAAAAT R: CAACGAAAACTGAGCTAGGA
Heat Shock Protein	Similar to Heat-shock protein beta-1	ES385844	cDNA	---	
Myostatin	similar to myostatin; isoform II	ES380902	cDNA	Myo:	F: TAAAAGCCTGGACAGACCAA R: ACTCACATCTCGGGTTCACT
Pit-I	similar to PIT54 (Gallus gallus)	ES377286	cDNA	---	
Transcription factor	Transcription factor JunB	EF408832	cDNA, partial cds	---	
Prolactin	similar to prolactin 1	ES374674	cDNA	Prol1, Prol2:	F: TCATCACAGTGGTGTACCTG R: AGCGAAGGACTTTCAAGAAG
Pr39	Unknown	AF467903	Microsatellite	Pr39:	F: GGTAAGGACTGATGAATAGCTTG R: TTAGGGCCGTGTCTTTTG

Notes: The top box lists markers (*M9* to *M1078*), obtained from Tripathi *et al.* (2009). In the middle box, candidate genes obtained from a *P reticulata*-specific search on GenBank are listed, and the bottom box contains microsatellite locus *Pr39*. Query term, putative functions of obtained markers, GenBank association numbers, and DNA type of sequences are given. If SNPs were successfully developed for the marker/gene in question, SNP marker names are provided in the final column.

WebTable 2. ANOVA table for nested analysis of variance in standard length

		F4	F5	F6		
		SL	SL	SL	Size mat	Age mat
Within treatments	d.f.	2, 1211	2, 1230	2, 336	2, 228	2, 228
	F	1.041	1.037	0.008	1.337	1.429
	P	0.354	0.355	0.992	0.265	0.242
Among treatments	d.f.	2, 2.092	2, 1.985	2, 2.800	2, 1.895	2, 1.902
	F	87.016	412.277	14307.362	51.284	28.574
	P	0.010	0.003	0.000	0.022	0.038
n	S1	244	244	130	46	46
	S2	245	245	114	45	45
	C	232	249	124	50	50
	L1	248	249	105	48	48
	L2	247	248	68	44	44

Notes: Variance between lines is nested within treatments. Analyses were done independently on generations F_4, F_5, and F_6. The table shows degrees of freedom (d), F and P values for each analysis, as well as the sample size (*n*) per line and generation. Because of the variation in *n* in the F_6, ANOVA on SL was done on a random subset of 68 individuals of each line (the minimum sample size of line L2), eliminating problems resulting from an otherwise unbalanced design.

WebTable 3. Observed (Ho) and expected (Hs) heterozygosity values for candidate loci in generations F_0, F_2, F_5, and F_6

		M1046	M1046-2	M30	GH1	GH2-60	GH2-74	GH2-165	GH2-211	Myo	Prol1	Prol2	TBC1	SF1	M9-403	M9-indel	M987-indel	Pr39
F0	Ho	0.636	0.295	0.477	0.395	0.467	0.386	0.500	0.386	0.214	0.511	0.457	0.400	0.182	0.279	0.244	0.356	0.696
	Hs	0.466	0.316	0.474	0.467	0.481	0.412	0.492	0.431	0.265	0.489	0.494	0.505	0.204	0.409	0.250	0.374	0.676
F2	Ho	0.462	0.251	0.487	0.464	0.500	0.273	0.505	0.385	0.276	0.463	0.468	0.527	0.274	0.467	0.255	0.489	0.788
	Hs	0.422	0.244	0.500	0.437	0.489	0.274	0.476	0.355	**_0.372*_**	0.461	0.501	0.501	0.252	0.421	0.255	0.548	0.758
F5-S1	Ho	0.356	0.304	0.378	0.609	0.682	0.370	0.522	0.400	0.261	0.489	0.279	0.568	0.205	0.244	0.304	0.413	0.739
	Hs	0.434	0.290	0.507	0.470	0.494	0.356	0.501	0.373	0.391	0.415	0.502	0.492	0.255	0.386	0.260	0.356	0.726
F5-S2	Ho	0.500	0.268	0.548	0.595	0.550	0.452	0.561	0.561	0.317	0.571	0.548	0.512	0.095	0.476	0.372	0.581	0.698
	Hs	0.483	0.235	0.422	0.491	0.460	0.402	0.486	0.462	0.333	0.463	0.498	0.506	0.092	0.477	0.306	0.607	0.636
F5-C	Ho	0.512	0.143	0.667	0.564	0.619	0.262	0.535	0.452	0.381	0.619	0.500	0.512	0.341	0.349	0.233	0.721	0.814
	Hs	0.459	0.175	0.499	0.503	0.500	0.265	0.478	0.423	0.413	0.486	0.492	0.459	0.318	0.374	0.208	0.557	0.706
F5-L1	Ho	0.273	0.152	0.609	0.419	0.500	0.311	0.378	0.341	0.378	0.467	0.565	0.674	0.391	0.133	0.152	0.804	0.870
	Hs	0.356	0.246	0.470	0.460	0.496	0.265	0.482	0.342	0.406	0.505	0.501	0.492	0.343	0.164	0.142	0.590	0.732
F5-L2	Ho	0.478	0.367	0.583	0.510	0.660	0.383	0.646	0.490	0.438	0.479	0.408	0.458	0.286	0.204	0.102	**_0.918_**	**_0.837_**
	Hs	0.471	0.302	0.448	0.436	0.504	0.338	0.502	0.393	0.456	0.505	0.489	0.498	0.276	0.217	0.098	**_0.621***_**	**_0.709*_**
F6-S1	Ho	0.531	0.420	0.510	0.420	0.531	0.380	0.500	0.354	0.204	0.240	0.400	0.560	0.417	0.449	0.280	0.460	0.720
	Hs	0.443	0.357	0.502	0.398	0.500	0.358	0.483	0.345	0.375	0.409	0.503	0.504	0.332	0.393	0.243	0.412	0.751
F6-S2	Ho	0.580	0.300	0.327	0.500	0.340	0.340	0.520	0.490	0.367	0.280	0.449	0.400	0.120	0.469	0.060	0.780	0.660
	Hs	0.494	0.285	0.395	0.505	0.472	0.335	0.453	0.495	0.430	0.426	0.500	0.505	0.114	0.492	0.059	0.645	0.535
F6-C	Ho	0.440	0.180	0.420	0.596	0.551	0.271	0.500	0.500	0.300	0.469	0.468	0.347	0.240	0.480	0.240	0.500	0.700
	Hs	0.466	0.229	0.398	0.499	0.491	0.295	0.480	0.415	0.336	0.505	0.494	0.422	0.213	0.485	0.213	0.443	0.700
F6-L1	Ho	0.408	0.327	0.490	0.490	0.560	0.320	0.620	0.391	0.327	0.500	0.400	0.500	0.380	0.280	0.040	0.680	**_0.980_**
	Hs	0.429	0.303	0.412	0.457	0.484	0.271	0.458	0.343	0.374	0.471	0.454	0.489	0.358	0.243	0.040	0.547	**_0.710***_**
F6-L2	Ho	0.480	0.300	0.500	0.480	0.520	0.280	0.420	0.440	0.400	0.480	0.367	0.480	0.220	0.260	0.160	**_0.960_**	**_0.880_**
	Hs	0.407	0.285	0.431	0.453	0.476	0.243	0.481	0.424	0.407	0.502	0.489	0.453	0.229	0.258	0.149	**_0.565***_**	**_0.696*_**

Notes: Significant deviations from HWE after the Benjamini-Hochberg correction were observed for *Pr39* in the L2 (F_5 and F_6) and the L1 (F_6), for *M987–indel* in the L2 (F_5 and F_6), and for *Myo* in the F_2; these are shown underlined in bold (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

WebTable 4. Significant Benjamini-Hochberg corrected *P* values for pairwise tests of genotypic differentiation, as obtained from a log-likelihood based exact test

	F5 Locus	F5 P	F6 Locus	F6 P
L-L				
S-S			GH1	0.0126
			GH2-165	0.0018
			GH2-211	0.0134
			SF1	0.0076
	M30	0.0413	M30	0.0022
	M987-indel	0.0016	M987-indel	0.0000
	Pr39	0.0083	Pr39	0.0000
S1-L1	Prol1	0.0126	Prol1	0.0001
			M9-indel	0.0109
	M987-indel	0.0001	M987-indel	0.0000
	Pr39	0.0000	Pr39	0.0000
S1-L2	Prol1	0.0280		
	M987-indel	0.0000	M987-indel	0.0000
	Pr39	0.0002	Pr39	0.0000
S2-L1	GH2-60	0.0206	GH2-60	0.0147
	GH2-165	0.0484	GH2-165	0.0000
			GH2-211	0.0109
			Prol1	0.0004
			Prol2	0.0320
	SF1	0.0042	SF1	0.0058
	M9-403	0.0001	M9-403	0.0002
	M30	0.0000	M30	0.0000
	M987-indel	0.0022	M987-indel	0.0000
	Pr39	0.0000	Pr39	0.0000
S2-L2			GH2-60	0.0083
			GH2-165	0.0022
	M9-indel	0.0145		
	M9-403	0.0010	M9-403	0.0007
	M30	0.0000	M30	0.0000
	M987-indel	0.0058	M987-indel	0.0030
	Pr39	0.0019	Pr39	0.0000

	F5 Locus	F5 P	F6 Locus	F6 P
C-S1			GH1	0.0321
			Prol1	0.0147
	TBC1	0.0084	TBC1	0.0225
			M30	0.0321
	M987-indel	0.0009		
			Pr39	0.0010
C-S2	GH2-165	0.0253		
			Prol1	0.0276
	SF1	0.0175		
	M30	0.0018	M30	0.0000
			M987-indel	0.0000
			Pr39	0.0002
C-L1			GH2-60	0.0413
	TBC1	0.0048	TBC1	0.0009
			M9-indel	0.0326
	M9-403	0.0492	M9-403	0.0007
	M987-indel	0.0210	M987-indel	0.0000
	Pr39	0.0021	Pr39	0.0000
C-L2			GH2-60	0.0225
	TBC1	0.0245	TBC1	0.0000
			M9-403	0.0015
	M987-indel	0.0083	M987-indel	0.0015
			Pr39	0.0035

Notes: Shown are significant B-H corrected *P* values for the putative candidate loci for each possible population pair within each generation. Tests between S- and L-lines are shown in gray, within-treatment comparisons and comparisons of selection lines with the control line in white.

WebTable 5. AMOVA of eight microsatellite loci (Msat) and 17 candidate markers (Cand) for selection in the F_6 generation, showing significant among treatment variation for candidate loci only

Source of variation	Sum of squares		Variance component		Percentage of variation	
	Msat	Cand	Msat	Cand	Msat	Cand
Among treatments (Va, Fct)	21.35	60.38	-0.014	0.090	0.000	2.405*
Among lines within treatments (Vb, Fsc)	25.99	31.66	0.100	0.124	3.308***	3.307***
Within lines (Vc, Fst)	1458.99	1733.48	2.949	3.533	97.166***	94.287***
Total	1506.33	1825.52	3.035	3.747		

Notes: Significant components of variation are indicated by asterisks (* $P = 0.036$, *** $P = 0.000$).