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ARTICLE

Predation and Food Limitation Influence Fitness Traits of Growth-Enhanced Transgenic and Wild-Type Fish

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Abstract

Genetically engineered fish are nearing commercialization for aquaculture. If transgenic fish escape from farms, they could encounter and interbreed with natural populations. Relative advantages of transgenic fish for fitness-related traits could drive population processes like gene flow, but fitness trait estimates will best inform risk assessments if measured under environmental conditions that are relevant to natural environments. Wild-type and growth-enhanced transgenic Japanese medakas *Oryzias latipes* of different genetic backgrounds were compared for the following fitness traits: fecundity, fertility, survival to sexual maturity, age at sexual maturity, and mating advantage. Because food availability and predation have been shown to alter the life histories of growth-enhanced transgenic fish, we measured traits under four different environments: (1) high food availability and no predation, (2) high food availability with simulated predation, (3) low food availability and no predation, and (4) low food availability with simulated predation. We found that regardless of environment, there was no clear fitness trend by genotype: transgenic females were more fecund than wild-type females, wild-type males obtained more matings than transgenic males, and offspring of transgenic × wild-type crosses had higher survival to sexual maturity than offspring of two wild-type parents, possibly due to heterosis. Regardless of genotype, females produced more eggs in environments with high food availability and matured fastest under conditions of high food availability and no predation. Our results imply that population-level processes like gene flow will be difficult to predict from measurements of one or two fitness traits taken on fish in a single environment. Therefore, gene flow risk assessments should be based on estimates of fitness traits that span the entire life cycle, incorporate relevant environmental variation, and compare the transgenic fish strain with a wild-type strain of relevant genetic background.

A number of varieties of genetically engineered fish are being developed for aquaculture production around the world (Nam et al. 2007). In many conventional aquaculture systems, escapes of large numbers of farmed fish are common (NRC 2004). If transgenic fish were to escape from farms, they could encounter and interbreed with wild fish of the same species, leading to transgene introgression, or transgene flow to the wild population. The need to assess the potential impacts of genetically engineered organisms on biodiversity was recently identified among the top research priorities for conservation biology (Sutherland et al. 2009). Assessing the risk of gene flow from transgenic fish to wild populations is a particular challenge to this kind of research (Devlin et al. 2006; Kapuscinski et al. 2007; NRC 2008). In the USA, the Food and Drug Administration is nearing a decision on commercialization of transgenic Atlantic salmon *Salmo salar*, the first genetically engineered animal that would be commercially farmed for human food (FDA 2010).

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Measuring fitness trait values for transgenic and wild-type fish is one way to predict the consequences of gene flow from transgenic fish into a wild population. Laboratory studies have found differences in fitness-related traits of transgenic and wild-type fish, such as a mating advantage for growth-enhanced transgenic male fish (Howard et al. 2004). However, it is important to take such measurements in an environment that is relevant to the conditions that fish might encounter in the wild (Devlin et al. 2007). Previous experiments comparing the fitness trait values of wild-type and transgenic fish have been carried out under relatively sterile laboratory conditions (Muir and Howard 1999, 2001; Bessey et al. 2004; Howard et al. 2004). It is likely that fitness phenotypes would be affected by limiting factors in the environment (Stearns 1989; Devlin et al. 2006), but research to indicate how the relative fitness of transgenic fish might be altered by different environments is lacking. Predation and food availability are two environmental variables that have demonstrated effects on the fitness of growth-enhanced transgenic fish.

Predation has been shown to drive changes in fitness-related traits of prey species. Giant rivulus *Rivulus hartii* had lower fecundity and suppressed adult growth rate in streams invaded by the piscivorous trahira *Hoplias malabaricus* (Fraser and Gilliam 1992). Another species, the striped killifish *Fundulus majalis*, experienced lower growth rates when exposed to predatory sand seatrout *Cynoscion arenarius* (Woodley and Peterson 2003). Even chemical cues from brook trout *Salvelinus fontinalis* caused mayflies *Baetis bicaudatus* to exhibit reduced fecundity but earlier maturation at a smaller size (Peckarsky et al. 2002). Growth-enhanced transgenic channel catfish *Ictalurus punctatus* were less able to avoid predators than were wild-type conspecifics (Dunham et al. 1999).

Fish are capable of expressing flexible growth in response to food availability (Sebens 1987; Jobling 1995). Growth-enhanced transgenic fish may have different phenotypic and behavioral responses to food availability because of their higher feeding motivation compared with wild-type fish (Devlin et al. 1999). Compared with wild-type siblings, growth-enhanced transgenic coho salmon *Oncorhynchus kisutch* more readily attacked prey items and were more likely to make repeated strikes (Sundström et al. 2004a). When subjected to low food availability, populations of coho salmon with growth-enhanced transgenic individuals present among them experienced population crashes or extinctions (Devlin et al. 2004), indicating that food availability could have populationwide consequences.

The combined effects of food limitation and predation are especially interesting because prey species must choose between foraging for food and increasing their risk of predation. This trade-off has been demonstrated theoretically (Houston et al. 1993; Lima 1998) and experimentally (Werner et al. 1983; Werner and Hall 1988). The addition of growth hormone in fish tends to elevate metabolic demands (e.g., Jönsson et al. 1996), increasing the motivation of growth-enhanced fish to forage in spite of predation risk.

Juvenile rainbow trout *Oncorhynchus mykiss* injected with growth hormone were more willing to eat in the presence of a model heron predator (Jönsson et al. 1996). In the presence of a piscine predator, growth-enhanced transgenic Atlantic salmon fed longer and ate more than did unmodified Atlantic salmon (Abrahams and Sutterlin 1999). Growth-enhanced transgenic coho salmon were more susceptible to predation than wild-type coho salmon, especially when food was limiting (Sundström et al. 2004b).

We measured fitness trait values of growth-enhanced transgenic and wild-type fish in environments with different levels of food availability and simulated predation. We compared the fecundity, fertility, survival to maturity, age at sexual maturity, and mating advantage of each genotype across the various environments.

METHODS

Model population.—The Japanese medaka *Oryzias latipes* is a small fish that is native to a variety of aquatic environments in Southeast Asia (Hirshfield 1980). Japanese medakas begin to reproduce at around 2 months of age and can mate daily for an entire spawning season (Uematsu 1990; Grant et al. 1995b). Mating activity and spawning usually take place shortly after dawn in the wild or within about 1 h of the beginning of the photoperiod in captivity (Hirshfield 1980; Wittbrodt et al. 2002; Shima and Mitani 2004). Partly because the Japanese medaka can survive and reproduce readily under laboratory conditions (Briggs and Egami 1959; Yamamoto 1975), it is a common model species in vertebrate biology. We used a line of growth-enhanced transgenic Japanese medakas (MtsGH-67; produced by W. Muir, Purdue University, West Lafayette, Indiana) engineered with an all-salmonid gene construct (Devlin et al. 1994) consisting of a rainbow trout metallothionein-B promoter (Chan and Devlin 1993) and a sockeye salmon *Oncorhynchus nerka* full-length, type-1 growth hormone gene (Devlin 1993). The transgenic fish were derived from founder Japanese medakas that the Muir laboratory obtained from Japan (Jiménez 2000) and subsequently backcrossed (Kruer et al. 2002). We also used a line of wild-type Japanese medakas that was descended from wild fish captured in Japan (this line was obtained from Pacific Aquatics, Chatsworth, California). We confirmed expression of the transgene with reverse-transcriptase polymerase chain reaction (RT-PCR); and tested for inheritance of the transgene among offspring with PCR using salmon growth hormone and beta-actin primers that directed amplification of the transgene and a control gene sequence, respectively (Pennington et al. 2010).

Environmental treatments: food availability and predation.—We tested two environmental factors with two levels each: food availability (high and low) and simulated predation (absent and present). We applied these factors to our experiments in a completely crossed design, resulting in four environmental treatments. The environment with high food availability

TABLE 1. Assignment of aquaria to food availability and simulated predation treatments, and stocking of transgenic (T) or wild-type (W) female and male Japanese medakas within different environments. Also noted (X) is whether an aquarium was used for a given fitness measurement experiment; aquaria that were used to acclimate fish before mating advantage experiments are denoted with an "a." Aquarium identifying numbers 1–24 are provided for reference.

Food availability	Simulated predation	Female genotype	Male genotype	Fecundity and fertility (four 10-d trials)	Survival to maturity and age at sexual maturity	Mating advantage	Aquarium identifier
High	Absent	T	T		X	a	19
		T	W	X	X		22
		W	T	X	X		4
		W	T	X	X		21
		W	W	X	X	X	3
		W	W		X	X	13
High	Present	T	T	X	X	a	10
		T	T	X	X	a	12
		T	W	X	X		5
		T	W		X	X	7
		W	T		X	a	24
		W	W	X	X	a	14
Low	Absent	T	T		X	X	1
		T	W		X	a	11
		T	W	X	X		15
		W	T	X	X		6
		W	T	X	X	a	23
		W	W	X	X	a	20
Low	Present	T	T	X	X	a	9
		T	T		X		17
		T	W	X	X		8
		W	T		X	a	18
		W	W	X	X	X	2
		W	W	X	X	a	16

and no predation was most similar to a standard laboratory or culture environment in which fish are well fed and free from natural predators. At the other extreme, the environment with low food availability and simulated predation was most similar to a natural environment, where food may be more limiting and where a risk of predation often exists. We also tested two intermediate environments: one with high food availability and simulated predation, and one with low food availability but no predation. We randomly assigned each of 24 experimental aquaria to one of these four treatments, and each aquarium remained assigned to the same environmental treatment for the duration of all experiments (Table 1). We then assigned each aquarium to a genotype treatment; each female–male genotype combination was represented within each of the four environmental treatments at least once, and each genotype combination (transgenic–transgenic, transgenic–wild-type, wild-type–transgenic, and wild-type–wild-type, where the female genotype is given first and the male genotype second) was repeated exactly six times over all 24 aquaria. All 24 aquaria were used to measure survival to maturity and age at sexual maturity. To balance the genotype and environment treatments, we used a subset of the aquaria for fertility, fecundity, and mating advantage experiments (Table 1).

We calculated feeding rates for high- and low-food-availability treatments by using the mass and number of fish in each aquarium. Fish that were assigned to the high-food-availability treatment were fed 10% of their total mass in flake food (Ocean Star International Marine Laboratory, Burlingame, California) each day; this ration was divided evenly between two feedings separated by at least 1 h but not by more than 8 h. This feeding rate was essentially feeding to excess but limited the amount of uneaten food. Fish in the low-food-availability treatment were fed once daily at a rate of 3% of their total mass per day. This feeding rate is intermediate between the two lowest feeding rates used for Japanese medakas in a previous study (Hirshfield 1980). Our preliminary experiments indicated that the low feeding rate was sufficient to maintain fish in reproductive condition, which was a necessity for most of our experiments. We also fed a suspension of 24-h-old live nauplii of brine shrimp *Artemia* spp. to high-food-availability

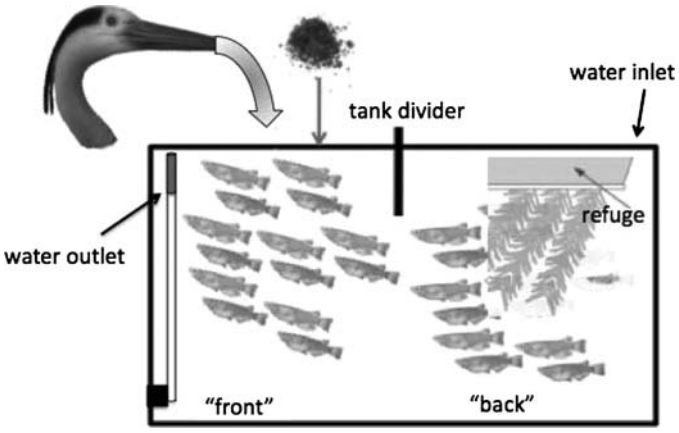

FIGURE 1. Configuration of each aquarium used in experiments with Japanese medakas. The simulated predation treatment involved the presence of a great blue heron decoy (see Methods).

treatments at a rate of one drop per adult fish in the aquarium, while the low-food-availability treatments received one drop of brine shrimp per two adult fish.

We simulated predation by using the head of a great blue heron *Ardea herodias* decoy (Sport Plast Decoy Company, Italy). Wading birds are common predators of small fishes in the rice paddy environments that Japanese medakas inhabit (Maeda 2001; Iguchi and Kitano 2008). We exposed fish in simulated predation treatments to the simulated predator concurrently with feeding flake food in the front half of the tank (Figure 1). For 5 min, approximately the time it takes for the fish to eat most of the flake food and before the food begins to sink to the tank bottom, we held the decoy's head just above the water, striking the head below the water's surface 10–20 times during a simulation. All aquaria were covered with an opaque fabric on the front to prevent fish from seeing the great blue heron's operator. We operated the predator with movements to mimic the "stand-and-stalk" hunting behavior of a great blue heron (Kushlan 1976), similar to the motion used in experiments by Jönsson et al. (1996).

Experimental environment.—We used twenty-four 113.5-L glass aquaria connected by a hybrid recirculating system; filtered well water flowed in at a replacement rate of approximately 20% of system volume per day. All aquaria were contained inside an insulated, photoperiod-controlled chamber. We maintained a photoperiod of 16 h light : 8 h dark and a water temperature of 26°C to promote mating behavior (Shima and Mitani 2004). We separated each aquarium latitudinally into front and back sections with a styrofoam tank divider that interrupted the water's surface (Figure 1). We provided a fry refugium consisting of a floating mesh basket with artificial spawning grass in the back half of each aquarium. Containment procedures, conducted in accordance with a protocol approved by the University of Minnesota's Institutional Biosafety Committee, were used to ensure that the transgenic Japanese medakas were securely confined in the laboratory.

Data analysis: general.—We carried out all analyses in the R statistical package (R Development Core Team 2008). We used linear models for continuous response variables and generalized linear binomial models for responses that could be characterized as success or failure. We included blocking factors, such as trial, where applicable. We tested for significance of genotype, environmental treatment, and genotype × environment interaction terms. We applied post hoc multiple comparison tests (Tukey's honestly significant difference [HSD] test) to investigate differences in means between genotype or environment treatments (multcomp package was used to apply Tukey's HSD tests to binomial models; Hothorn et al. 2008). We produced statistical graphs in R (including error bars coded with the gplots package; Warnes 2009). Unless otherwise stated, we report fitness trait values in the text as means ± SDs. For analyses, we considered P-values less than 0.05 to be significant.

Fecundity and fertility: experimental design.—We measured fecundity and fertility daily during four trials of 10 d each, separated by an acclimation period of at least 10 d. We randomly selected eight 10–12-month-old Japanese medakas of each sex and genotype for these experiments. We arranged one male and one female fish in each of 16 experimental aquaria in homogeneous and heterogeneous crosses according to each tank's assigned genotype treatment (Table 1), and no single fish was paired with the same mate more than once during the four trials. Fish in predation treatments were exposed to the simulated predator twice during each 10-d test period. We weighed all fish before each trial began, and to avoid additional handling we assumed that weight remained constant during the trial. If a fish died in the middle of an experimental trial, we replaced it with a fish of the same sex and genotype and noted the weight of the replacement fish. We collected and counted eggs each day and then maintained those eggs in a flowing-water incubator for 24 h. The next day, 24-h-old eggs that were successfully developing were counted under a dissecting scope.

Fecundity: data analysis.—We analyzed fecundity by using linear models, where the response was the total number of eggs produced by a female during a 10-d trial. Trial was retained in all models as a blocking factor, but we did not include female weight as a covariate because it was significantly correlated with female genotype. We tested the importance of female genotype, environmental treatment, and genotype × environment interaction by use of type II analysis of variance (ANOVA) to account for unbalanced data. We excluded data from females that died during a trial.

Fertility: data analysis.—We analyzed fertility with binomial generalized linear models where fertile eggs were "successes" and infertile but intact eggs were "failures." We retained trial as a blocking factor in all models. We used F-tests to compare models with and without terms of interest: male genotype, environmental treatment, and genotype × environment interaction.

We removed data from analysis if the female produced no eggs over a 10-d trial or if the male was replaced in the middle of a trial. We excluded one additional datum with unusually low

fecundity and fertility that was identified as an outlier by model checking procedures, including residuals versus fitted values and Cook's distance to test the influence of a data point.

Survival to maturity and age at sexual maturity: experimental design.—We measured survival to maturity and age at sexual maturity for Japanese medaka fry. To produce offspring for these measurements, we randomly assigned a pair of 6-month-old Japanese medakas to each of the 24 test aquaria according to the assigned female–male genotype combinations (Table 1). We allowed these fish to acclimate to their environments for 10 d, during which fish in simulated predation treatments were exposed to the predator two times. After the acclimation period, we collected eggs from all females and placed each clutch of eggs in a recirculating-water incubator (∼27°C) with 3-mg/L methylene blue (Kordon LLC, Hayward, California) to discourage fungal growth. We collected eggs from all tanks on the first 2 d after the acclimation period was complete; for 2 d thereafter, we continued to collect eggs from several tanks that were producing small numbers of eggs. We counted the number of fertile eggs 24 h after the eggs were placed in the incubator.

When fry hatched, we counted them; fry were then transferred to 10-cm-diameter, mesh-bottom polyvinyl chloride baskets suspended inside all 24 aquaria. Fry in all aquaria were given dry, powdered Artificial Plankton Rotifer (APR; Ocean Star International) twice daily in excess. At 10 d posthatch (DPH), we equalized the number of fry in each basket to 30 and released those randomly selected fry into the aquarium. After 10 DPH, we introduced different foods and began to feed the low- and high-food-availability aquaria at different rates. From 10 to 27 DPH, we fed 0.033 g of Artificial Plankton Rotifer suspended in water twice daily to low-food-availability aquaria. We fed brine shrimp nauplii to low-food-availability aquaria as follows: one drop from 10 to 36 DPH, two drops from 37 to 63 DPH, and three drops after 64 DPH. At 21 DPH, we began to administer finely ground flake food (Ocean Star International) at 1.5 g/d to low-food-availability aquaria. For aquaria in the high-food-availability treatment, we fed twice as much of all types of food during the aforementioned periods. We censused fry at 20, 33, 48, and 64 DPH. Beginning with the 33-DPH census, we also weighed the total mass of all fish in each tank and adjusted flake food feeding rates accordingly. Between 30 DPH and the onset of sexual maturity, the aquaria in the predation-present treatment were subjected to simulated predation five times.

Beginning on 43 DPH, we searched for females bearing eggs. When we observed a sexually mature female, we removed the female from the aquarium, removed and counted her first clutch of eggs, and weighed the female. To determine the genotype of mature females with at least one transgenic parent, we took a small sample of caudal fin tissue and analyzed the tissue with PCR. Eggs from the first three females to mature in each aquarium were held for 24 h, and we checked the eggs for fertility to confirm that males in the aquarium were also sexually mature.

Survival to maturity and age at sexual maturity: data analysis.—Survival to sexual maturity was measured in two stages: the number of fertile eggs that hatched (hatchability) and the number of fry that survived from 10 DPH to maturity (juvenile viability). Because fry were too small to genotype before sexual maturity without incurring high mortality, we used their parental genotypes as explanatory variables in models for hatchability and juvenile viability. We used binomial generalized linear models to compare the number of eggs that successfully hatched with the number of fertile eggs that did not hatch from the different parental genotypes and in the different environments. We also analyzed juvenile viability data with binomial models in which fish surviving from 10 to 48 DPH were considered successes and fry that did not survive were considered failures. To test the effect of parental genotype and environment on overall survival to maturity, we created a linear model for the product of the proportion of fertile eggs hatched and the proportion of fry surviving to sexual maturity. We transformed the response with an arcsine square-root transformation to improve normality, and we used type II ANOVA to test genotype, environment, and interaction terms.

We analyzed age at sexual maturity by using linear models. To maximize balance in our data, we included only the first through ninth females to mature in each tank. We transformed the number of days to sexual maturity with a natural logarithm to satisfy normality assumptions. Weight was excluded from models of age at sexual maturity because it was highly correlated with environmental treatment. We used type II ANOVA to compare models in which genotype of the sexually mature fish, environmental treatment, and the genotype × environment interaction were explanatory variables.

Data from six fish that PCR failed to identify as transgenic or wild-type were excluded from the age-at-sexual-maturity analysis. We also excluded one fish that was the only female to reach sexual maturity in aquarium 21. Data from aquarium 17 were excluded from the analyses of survival to sexual maturity and age at sexual maturity because the adult pair of fish in tank 17 produced no offspring.

Mating advantage: experimental design.—We measured mating advantage by observing the number of successful matings obtained with a wild-type female by a wild-type male when competing with a transgenic male. We housed fish in groups separated by genotype and sex (wild-type females, wild-type males, and transgenic males) and each environmental treatment (i.e., 12 holding aquaria total) and allowed them to acclimate to the environmental conditions for 10 d before the start of the first mating advantage trial (Table 1). We simulated predation five times during the acclimation period. One day before an experimental trial, we randomly selected four wild-type females, four wild-type males, and four transgenic males (i.e., one individual of each type from each environment). We took photographs of the selected fish for identification, and we weighed and measured standard lengths for all fish.

We observed mating advantage trials in four aquaria, each assigned to a different environmental treatment (Table 1). We carried out four trials of 4 d each, and 3-d acclimation periods occurred between trials. Fish were not used in more than one

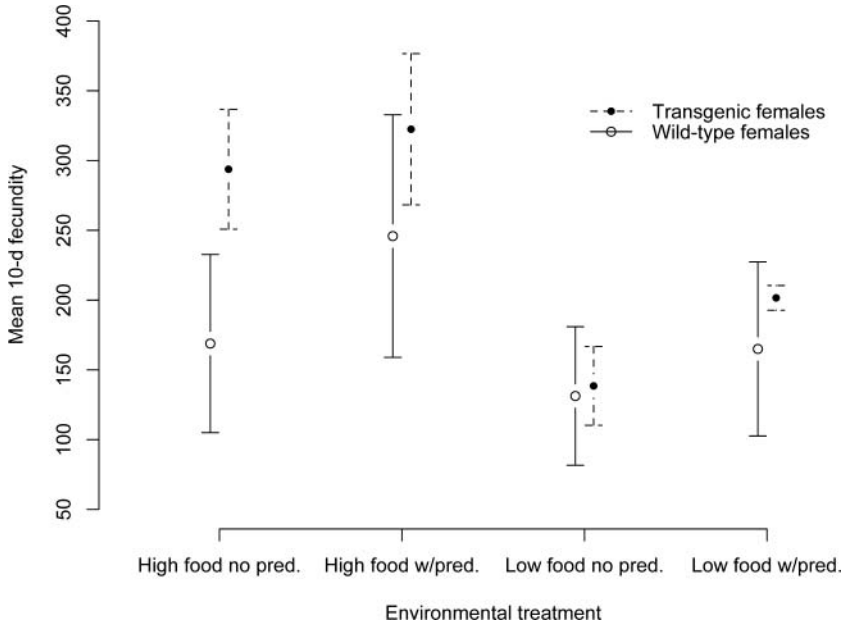

FIGURE 2. Mean (±SE) 10-d fecundity values for transgenic and wild-type female Japanese medakas in each environmental treatment (food availability = low or high; simulated predation = present or absent).

mating advantage trial. At night, we placed the males in separate baskets inside the aquarium to prevent mating before the observation period began in the morning. Each morning of a mating advantage observation, we arrived before the lights came on and released the males from their baskets. We observed the males competing for the female in real time and noted which male was mating with the female when she released eggs. Based on our experiments and on previous studies of Japanese medaka behavior (e.g., Grant et al. 1995a; Howard et al. 2004), we knew that it was possible for both males to mate with the female at the same time, and we noted these "trio matings" separately. We also recorded the number of minutes to a successful mating.

Mating advantage: data analysis.—To test the odds of wild-type males obtaining matings instead of transgenic males, we created binomial generalized linear models in which wild-type matings were "successes" and transgenic matings were "failures." We excluded trio matings from these analyses and fitted only an intercept term. In addition, we produced a separate model for each environmental treatment. For all environments, we created a second binomial model in which trio matings were divided equally between each male genotype (wild-type and transgenic) in turn. We also compared the time to successful mating for different mating types.

RESULTS

Fecundity

On average, the females in our experiment produced more than 20 eggs/d (10-d total = 209.6 ± 128.2 eggs); individual 10-d fecundities ranged from 0 to 593 eggs. The genotype × environment interaction was not significant (type II ANOVA: $F = 1.056$; df = 3, 46; $P = 0.347$), so the interaction term was dropped from the model. Using only main effects, we found that environmental treatment ($F = 6.387$; df = 3, 49; $P = 0.001$) and female genotype ($F = 5.606$; df = 1, 49; $P = 0.022$) were both significant model terms.

Ten-day egg production totals were higher for transgenic females (240.1 ± 135.8 eggs) than for wild-type females (180.1 ± 115.1 eggs; Tukey's HSD: adjusted $P = 0.023$). Transgenic females (0.340 ± 0.082 g) were heavier than wild-type females (0.282 ± 0.117 g; Tukey's HSD: adjusted $P = 0.036$). Egg production overall was higher in the environment with high food availability and simulated predation than in environments with low food availability (Tukey's HSD, low food availability without predation: adjusted $P = 0.001$; low food availability with predation: adjusted $P = 0.026$; Figure 2). Examining only the food availability factor, high food availability was positively associated with egg production (Tukey's HSD: adjusted $P < 0.001$). Presence of simulated predation was not associated with egg production (Tukey's HSD: adjusted $P = 0.136$).

Fertility

Across all environments, male fertility (proportion of eggs that were fertile) in our experiments was high (Figure 3) and did not differ between male genotypes (wild-type: 0.97 ± 0.09; transgenic: 0.96 ± 0.09). While fertility ranged from 0.62 to 1.00 (wild-type males) or from 0.63 to 1.00 (transgenic males), most fertility rates were greater than 0.95 (26 of 28 wild-type males; 23 of 28 transgenic males).

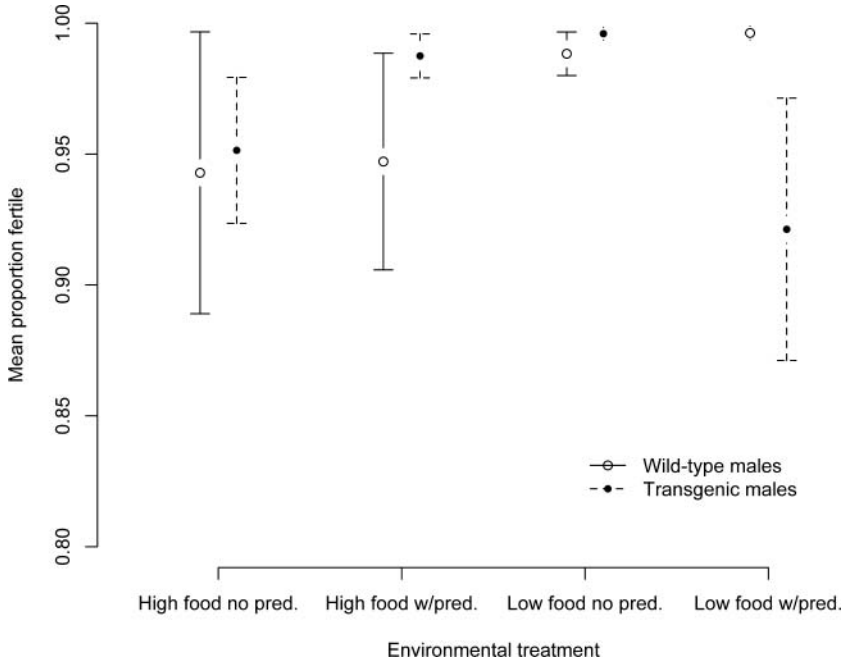

FIGURE 3. Mean (±SE) proportion of eggs fertilized by transgenic and wild-type male Japanese medakas in each environmental treatment (food availability = low or high; simulated predation = present or absent).

In binomial models of fertility, the genotype × environment interaction was significant ($F = 3.512$; df = 3, 45; $P = 0.023$). However, multiple comparison tests did not reveal differences between genotype–environment combinations (Tukey's HSD: all adjusted $P > 0.579$). Alone, neither genotype nor environment had a significant effect on fertility (genotype: $F = 1.731$, df = 1, 48, $P = 0.195$; environment: $F = 0.691$, df = 3, 48, $P = 0.562$).

Survival to Maturity

Across all treatments and parental genotypes, the proportion of fertile eggs that hatched was 0.90 ± 0.08 and the proportion of hatched fry that survived from 10 to 48 DPH was 0.84 ± 0.16. In binomial models describing hatch proportion, the genotype × environment interaction was not significant ($F = 1.121$; df = 9, 22; $P = 0.451$). Without the interaction term, neither parental genotype combination ($F = 2.573$; df = 3, 22; $P = 0.090$) nor environmental treatment ($F = 2.105$; df = 3, 22; $P = 0.140$) was significant.

As expected, juvenile viability decreased as environments became less favorable, from 0.90 ± 0.08 in the environment with high food and no predation to 0.76 ± 0.13 for fish subjected to low food availability and simulated predation. In binomial models of juvenile viability, the genotype × environment interaction term was not significant ($F = 0.733$; df = 9, 22; $P = 0.675$). When the interaction term was dropped, the parental genotype combination was significant ($F = 5.654$; df = 3, 22; $P = 0.008$), but the environmental treatment was not significant ($F = 1.206$; df = 3, 22; $P = 0.339$). Multiple comparison tests revealed that fry with one transgenic and one wild-type parent (i.e., transgenic–wild-type or wild-type–transgenic crosses) had higher survival rates than fry of wild-type–wild-type crosses (Tukey's HSD: both adjusted $P < 0.007$).

Calculating the proportion of fertile eggs hatched times the proportion of fry surviving from 10 DPH to sexual maturity attempts to capture the overall proportion of fry that survive from fertilization to maturity (Figure 4). In this linear model, the parental genotype × environment interaction was not significant (type II ANOVA: $F = 2.316$; df = 9, 22; $P = 0.140$). Without the interaction term, environmental treatment was not significant ($F = 1.713$; df = 3, 22; $P = 0.204$), but parental cross was significant ($F = 7.861$; df = 3, 22; $P = 0.002$). As with juvenile viability alone, the rate of survival to maturity for offspring of wild-type–transgenic and transgenic–wild-type crosses was greater than that of offspring from wild-type–wild-type crosses (Tukey's HSD: adjusted $P = 0.001$ and 0.020, respectively).

Age at Sexual Maturity

We detected the first sexually mature female at 47 DPH: a transgenic female in the environment with high food availability and simulated predation. The first female to reach maturity in a low-food-availability treatment was a transgenic female in the environment combining low food availability and simulated predation; this female reached maturity at 51 DPH. All clutches of eggs that we examined were fertile, indicating that males in all aquaria matured before or at the same time as females.

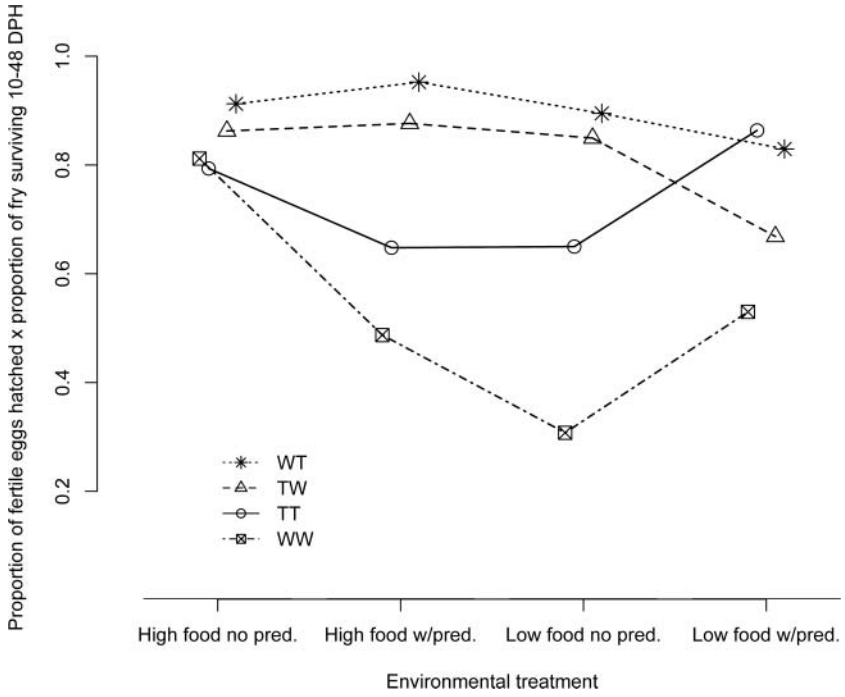

FIGURE 4. Proportion of fertile eggs that hatched times the proportion of Japanese medaka fry surviving from 10 to 48 d posthatch (DPH) in each environmental treatment (food availability = low or high; simulated predation = present or absent). Points are values for each parental genotype combination (transgenic [T] and wild-type [W]; in each two-letter code, maternal genotype is the first letter and paternal genotype is the second); lines connect the points for ease of interpretation.

The genotype × environment interaction was not significant (type II ANOVA: $F = 0.174$; df = 3, 177; $P = 0.914$). In a model with only main effects, environment was highly significant ($F = 35.078$; df = 3, 180; $P < 0.001$), but genotype was not ($F = 0.952$; df = 1, 180; $P = 0.331$). Fish in the environment with high food availability and simulated predation matured sooner than fish in all other environments (Tukey's HSD: all adjusted $P < 0.011$; Figure 5). Fish in the environment with high food availability and predation matured sooner than those in both environments subjected to low food availability (Tukey's HSD: both adjusted $P < 0.001$). Maturation time was not different between fish in the two low-food-availability environments, regardless of whether the fish were exposed to the simulated predator (Tukey's HSD: adjusted $P = 0.599$).

Transgenic females (0.164 ± 0.045 g) were the same size as wild-type females (0.173 ± 0.052 g) when they reached maturity (Tukey's HSD: adjusted $P = 0.156$). There was also no difference in the size of the first clutch produced by wild-type or transgenic females (transgenic: 5.552 ± 3.495 eggs; wild-type: 6.100 ± 4.884 eggs; Tukey's HSD: adjusted $P = 0.352$). However, weight and clutch size were both higher in the environment with high food availability and simulated predation. The first clutch size in that environment (8.000 ± 4.609 eggs) was larger than the first clutch size in all other environments (high food availability and no predation: 5.000 ± 2.884 eggs; low food availability and no predation: 5.085 ± 4.318 eggs; low food availability and simulated predation: 4.476 ± 2.597 eggs; Tukey's HSD: all adjusted $P < 0.001$). Females in the environment with high food availability and predation (0.198 ± 0.039 g) were larger than the mature females in all other environments (high food availability and no predation: 0.167 ± 0.029 g; low food availability and no predation: 0.151 ± 0.051 g; low food availability and simulated predation: 0.147 ± 0.050 g; Tukey's HSD: all adjusted $P < 0.001$). When the environmental treatment was broken into its constituent factors, both the presence of predation and high food availability had positive effects on the weight of females at sexual maturity (Tukey's HSD: adjusted $P = 0.018$ and $P < 0.001$, respectively).

Mating Advantage

At least one male successfully mated with the female in each tank for 4 d during all test periods. Of the 64 observed matings, 36 were by wild-type males alone, 20 were by transgenic males alone, and eight were trio matings in which both the transgenic and wild-type males participated. The transgenic males (0.420 ± 0.056 g) used in our mating advantage experiments were larger on average than the wild-type males (0.305 ± 0.043 g; Tukey's HSD: adjusted $P < 0.001$). In matings involving one male, the wild-type and transgenic males managed to copulate with the female relatively quickly (wild-type: 10.167 ± 8.433 min; transgenic: 7.150 ± 7.155 min) after the trial began, whereas trio matings took longer to achieve (28.500 ± 19.950 min; Tukey's HSD, transgenic versus trio or wild-type versus trio: both adjusted $P < 0.001$; wild-type versus transgenic: $P = 0.537$).

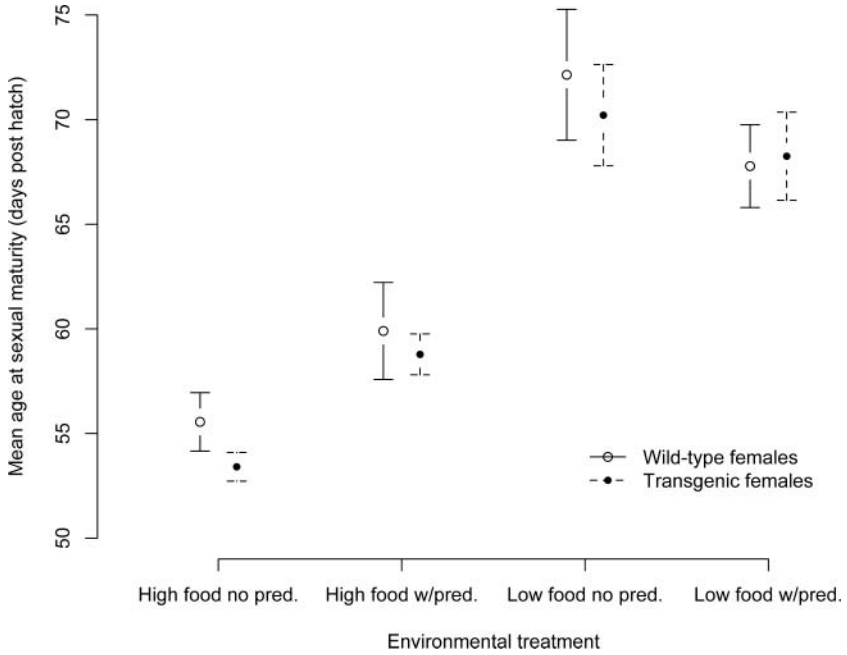

FIGURE 5. Mean (±SE) age at sexual maturity for transgenic and wild-type female offspring of Japanese medakas in each environmental treatment (food availability = low or high; simulated predation = present or absent).

Binomial models fitted with only an intercept term found that across all environmental treatments, wild-type males alone were successful more often than transgenic males alone (intercept = 0.588, $z = 2.108$, df = 15, $P = 0.035$). When we divided trio matings evenly between transgenic and wild-type males, the wild-type advantage remained (intercept = 0.511, $z = 1.978$, df = 15, $P = 0.048$). We created separate models for each environmental treatment with trio matings excluded and found that wild-type males had a mating advantage in environments with high food availability and simulated predation (intercept = 1.792, $z = 2.346$, df = 3, $P = 0.019$) and with low food availability and no predation

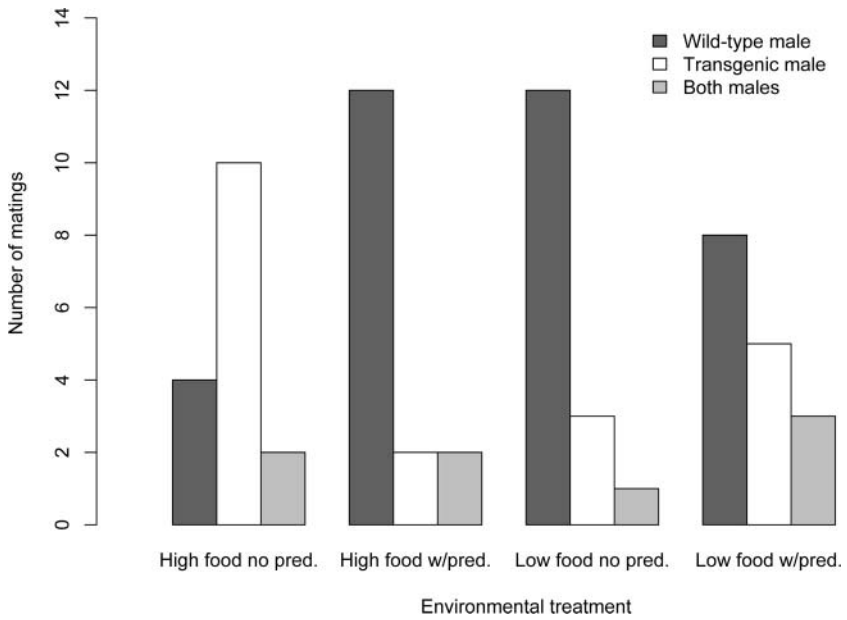

FIGURE 6. Number of matings obtained by wild-type males, transgenic males, or both males (i.e., trio matings) with wild-type female Japanese medakas in each environment (food availability = low or high; simulated predation = present or absent).

(intercept = 1.386, $z = 2.148$, df = 3, $P = 0.032$; Figure 6). While transgenic males achieved more matings in the environment with high food availability and no predation, the advantage was not significant (intercept = –0.916, $z = –1.549$, df = 3, $P = 0.121$). Neither male genotype had a significant advantage in the other extreme environment with low food availability and simulated predation (intercept = 0.470, $z = 0.824$, df = 3, $P = 0.410$).

DISCUSSION

The relative fitness trait values of growth-enhanced transgenic Japanese medakas were influenced by both genotype and environment and not always in expected ways. For example, fecundity was highest in the environment with high food availability and a simulated predator. We found differences in fitness trait values for both genotypes in different environments (Table 2). Although fecundity and age at sexual maturity were greater in the two environments with high food availability, there was no overall trend with regard to genotype; for example, transgenic females were more fecund than wild-type females, but wild-type males obtained more matings than transgenic males. Such mixed results imply that population-level processes like gene flow will be difficult to predict from measurements of one or two fitness traits taken on fish in laboratory environments. Therefore, risk assessment of transgene flow requires estimates of fitness traits that span the entire life cycle.

Fecundity

Transgenic females produced more eggs than wild-type females over 10 d, which could be related to the phenotypic effect of the transgene: transgenic females were larger on average than wild-type females, and larger Japanese medakas have been found to produce larger clutch sizes (Teather et al. 2000). Because we did not weigh clutches, we do not know whether transgenic females were heavier due only to somatic growth or because they also produced relatively heavier clutches of eggs compared with wild-type females; however, previous work did not find a relationship between female size and egg size in Japanese medakas (Teather et al. 2000). Muir and Howard (2001) found that growth-enhanced transgenic females produced more eggs than wild-type females. Although both genotypes in our study exhibited a positive correlation between fecundity and weight, the weight × genotype interaction was not significant (type II ANOVA: $F = 1.013$; df = 1, 47; $P = 0.319$), indicating that the effect of weight did not differ between transgenic and wild-type females.

The increase in fecundity in high-food-availability treatments could be attributable to the known relationship between size and fecundity in fish (e.g., Bagenal 1978; Blueweiss et al. 1978; Jobling 1995). Compared with female Japanese medakas in aquaria with low food availability, females in high-food-availability treatments gained 0.099 g more weight on average (Tukey's HSD: adjusted $P < 0.001$). While our experiments were performed with only 2 fish/tank, a previous study of Japanese medakas competing for food found that growth and fecundity were highest among females that were able to gain the most access to a limited food resource (Bryant and Grant 1995).

Predation treatment alone did not result in a significant effect on the fecundity of female Japanese medakas. Although we expected fecundity to decrease in simulated predation treatments based on prior research, we did not observe a negative relationship between fecundity and predation (Figure 2). Female mayflies *B. bicaudatus* exposed to the threat of predation had decreased fecundity relative to those in predator-free streams (Peckarsky et al. 2002). When giant rivulus were exposed to a predation threat in an experimental stream, their total egg production decreased by about half, and more fish failed to produce eggs when exposed to a predation threat (Fraser and Gilliam 1992). Contrary to Fraser and Gilliam's (1992) results, we found that simulated predation was positively related to females producing at least one egg on any given day (binomial model: $F = 17.192$; df = 1, 1,322; $P < 0.001$).

Fertility

The high fertility rates that we observed are similar to results of previous studies of male fertility in Japanese medakas (Muir and Howard 2001; control groups in Nakayama et al. 2004). For wild-type males, fertility increased from the environment of high food availability without predation through the two intermediate environments and was highest in the environment of low food availability with predation (Figure 3), whereas fertility of transgenic males increased only across the first three aforementioned environments and then dropped off in the environment with low food availability and predation. It was difficult to detect differences in fertility between treatments with multiple comparison tests because of the high number of comparisons. Our finding of a significant genotype × environment interaction term in our model may be driven by the genotype-rank change in the environment with low food availability and simulated predation, in which wild-type males outperformed transgenic males, unlike all other environments, where transgenic males had higher fertility (Figure 3).

Survival to Maturity

Overall survival from fertile egg to maturity was dependent on parental genotype. Muir and Howard (2001) measured viability from fertile egg to 3-d-old fry and found the greatest reduction in survival during this period was among offspring of two transgenic parents. In our combined analysis of survival to maturity, offspring from wild-type–wild-type crosses were at an even greater survival disadvantage than fish from transgenic–transgenic crosses (Figure 4; Table 2). Because our transgenic and wild-type fish came from different genetic backgrounds, we may have observed higher survival among heterogeneous crosses due to the simple effect of heterosis, or "hybrid vigor" (Falconer 1989).

TABLE 2. Summary of fitness trait measurement experiments with Japanese medakas. Different letters in superscript indicate differences ($P < 0.05$) between transgenic (T) and wild-type (W) individuals or between environmental treatments (Tukey's honestly significant difference tests, except for mating advantage). Genotype for survival to sexual maturity is parental genotype combination with female genotype listed first (see Methods). Environmental treatments are abbreviated as follows: A = high food availability and no predation; B = high food availability with simulated predation; C = low food availability and no predation; and D = low food availability with simulated predation (NA = not applicable).

Fitness trait	Overall genotype effect	Overall environment effect
Fecundity	$T^z > W^y$	$B^z > A^{z,y} > D^y > C^y$
Fertility	$W^z > T^z$	$C^z > B^z > D^z > A^z$
Survival to maturity	$WT^z > TW^{z,y} > TT^{y,x} > WW^x$	$A^z > B^z > C^z > D^z$
Age at sexual maturity	$T^z < W^z$	$A^z < B^y < C^x < D^x$
Mating advantage	$W^z > T^y$	NA

Age at Sexual Maturity

As expected, fish in the environment with high food availability and no predation matured soonest. The addition of simulated predation to the high-food-availability treatment increased the age at maturation. In the low-food-availability environments, however, there was no difference between environments without and with predation. The increased weight and clutch size in the environment with high food availability and predation could be related to the delayed maturation observed in that treatment compared with the environment having high food availability and no predation. Although these fish had a greater opportunity to grow larger due to the greater food availability, their maturation may have been forestalled by the threat of predation. Even though fish in both of the low-food-availability environments matured 10 d later on average than fish in the environment with high food availability and predation, they were smaller.

Predation pressure has been shown to alter the age at sexual maturity in fish. Male guppies *Poecilia reticulata* in environments with predators matured at a smaller size than their counterparts in predator-free populations (Reznick and Endler 1982). In their home environments, bluegills *Lepomis macrochirus* in a population exposed to predation on juveniles matured later and at a larger size than bluegills in a population where mainly adults were subject to predation (Belk 1995). However, when the bluegills from the two populations were raised together in a common garden experiment, they matured at approximately the same age. Like our study, this indicates that age at maturity may be influenced more by environmental factors than by genotype.

When organisms are forced by their environment to grow more slowly, age at sexual maturity might be affected in a number of different ways. Slower-growing organisms might "(1) mature later at a smaller size, (2) mature later at the same size, (3) mature later at a larger size, (4) mature earlier at a smaller size, or (5) mature at the same age at a smaller size" (Stearns and Koella 1986:894). Generalizations 1, 2, and 4 are supported by fish research reviewed in the same paper. Our results correspond most closely to generalization 1 or 2: females in both environments with low food availability, presumably subject to slower growth because of their lower rations, matured an average of 10 d later than females in the environment with high food availability and predation. Females in low-food-availability environments were smaller and produced smaller first clutch sizes than females in the high-food-availability environment with simulated predation but were not different in these respects from the early maturing females in the environment with high food availability and no predation. Earlier maturation can lead to greater fitness by increasing an organism's reproductive lifetime (e.g., Stearns 1992). Therefore, our results suggest that Japanese medakas of either genotype may express earlier ages at maturity in certain environments, thereby gaining a fitness advantage.

Mating Advantage

Overall, wild-type male Japanese medakas were most successful in obtaining matings with a wild-type female Japanese medaka. This advantage persisted in spite of the larger size of transgenic males compared with their wild-type competitors. Our results contradict the common assumption that larger males tend to obtain more matings with females (e.g., Andersson 1994) and experiments that have found a mating advantage of larger male fish over smaller males (e.g., Bisazza and Marconato 1988). Laboratory experiments with Japanese medakas under low-stress environmental conditions also found that larger males (Howard et al. 1998) and larger, growth-enhanced transgenic males (Howard et al. 2004) had a mating advantage over smaller and/or wild-type competitors. In previous studies of Japanese medaka mating advantage (Howard et al. 1998, 2004), larger males of each genotype competed with males derived from the same genetic background (the Purdue orange-red strain). Our contrary results with respect to weight and mating advantage could be due to differences in the genetic background of our wild-type Japanese medakas (e.g., Kapuscinski et al. 2007). Though the transgenic advantage in the environment with high food availability and no predation was not significant, it is the only environment in which transgenic males attained more matings than wild-type males. This may be because the strain from which our transgenic fish were derived had been kept in captivity longer than the wild-type strain, making it possible that the transgenic fish in our study had more time to adapt to accomplishing matings in an environment with ample food and no predation.

The minority of matings observed in our experiments involved both males participating in a trio mating, but the fact that these matings took about three times longer than a single-male mating indicates that competition between males may have been greatest in these cases. In fact, the weight advantage of transgenic males in trio matings was less than the weight advantage of transgenic males that obtained matings alone (Tukey's HSD: adjusted $P = 0.016$). In other words, a mating advantage trial with two males of similar size might be more likely to end with a trio mating. The likelihood of a second male joining a mating has been found to increase with female body size in fish (Marconato and Shapiro 1996). In our study, female weight was negatively related to the occurrence of trio matings, but the relationship was not significant.

Wild-type males obtained more matings than transgenic males in two environments: one with low food availability and no predation, and one with high food availability and predation. For fish species like the Japanese medaka, in which males actively court the female, there is an energetic cost to courtship. One might then expect that the larger, transgenic males would have an advantage in low-food-availability conditions, but our results ran counter to this assumption. Female Japanese medakas can refuse the attentions of a courting male by enacting a "heads-up" display (Uematsu 1990; Grant and Green 1996). Our experiments were not designed to test the effect of female choice. However, previous research on fish has found decreased female choosiness with regard to male size (Forsgren 1992) and coloration (Godin and Briggs 1996) when fish are exposed to a predator.

Neither wild-type males nor transgenic males had a significant advantage in the most "extreme" environments (i.e., high food availability without predation and low food availability with predation). Previous mating advantage tests that have studied Japanese medakas under low-stress laboratory conditions (akin to our high-food-availability, no-predation environment) have found that transgenic males dominate their wild-type competitors (Howard et al. 2004); our contradictory result may be due to the different genetic backgrounds of our wild-type and transgenic fish. Our results for the most stressful environment suggest that although a food-limited or predation-present environment might allow smaller wild-type males to gain a mating advantage, the existence of both low food availability and simulated predation in a single environment may be acting on both male genotypes and on the females in ways that erase a clear advantage conferred by size or genotype. Further research on relative mating advantage of transgenic and wild-type genotypes from different genetic backgrounds and in different environments may be warranted as models have suggested that the risk of invasion by transgenes is very sensitive to mating advantage (Muir and Howard 1999; Aikio et al. 2008).

Limitations and Future Research

Because we were not comparing strains with a shared genetic background, we cannot conclusively attribute differences in fitness measurements between transgenic and wild-type fish to the transgene alone. However, our choice of unrelated strains was intentional because transgenic fish that might escape into wild conspecific populations are likely to be derived from domesticated strains or stocks with very different genetic backgrounds than the affected wild populations.

We found a significant genotype × environment interaction for only one fitness trait: male fertility. Compared with other studies, particularly those using growth-enhanced transgenic Pacific salmon *Oncorhynchus* spp. (e.g., Devlin et al. 2004; Sundström et al. 2007; Lõhmus et al. 2010), genotype × environment interactions played a relatively small role in our results. We did not design our study to test genotype × environment interactions because our objective was to test each fitness trait in isolation and because a methodology such as a common garden experiment poses logistical challenges to separating fertility from male mating success. Also, because of the limited replication of a given genotype × environment combination in our experimental design, we were less likely to find significant differences between these treatments.

Regardless, these results may indicate that it is unwise to draw broad conclusions about the importance of genotype × environment interactions across species, strains, and studies using different environments. Finally, we note that future research of this type ought to carefully consider the relevant environment for a given transgenic trait; for example, a disease-resistant transgenic fish is likely to respond differently to a disease-ridden environment than its unmodified conspecific, while food availability may be a less-important environmental condition in that case.

Future risk assessment experiments should work to improve methods to incorporate other relevant environmental factors into confined laboratory environments. Furthermore, it is essential to combine analysis of data collected on both the fish populations and ecosystems in question with quantitative methods of uncertainty analysis (Devlin et al. 2007; Hayes et al. 2007; Kapuscinski et al. 2007).

Conclusion

Our study indicates the importance of measuring trait values under relevant environmental conditions that take into account the phenotypic effect of the transgene. Our results show that risk assessments should incorporate ecosystem- and transgene-specific environmental factors into estimates of fitness that could inform predictions of gene flow from transgenic fish to wild relatives.

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REFERENCES

Abrahams, M. V., and A. Sutterlin. 1999. The foraging and antipredator behavior of growth-enhanced transgenic Atlantic salmon. Animal Behaviour 58:933–942.

Aikio, S., K. R. Valosaari, and V. Kaitala. 2008. Mating preference in the invasion of growth enhanced fish. Oikos 117:406–414.

Andersson, M. 1994. Sexual selection. Princeton University Press, Princeton, New Jersey.

Bagenal, T. B. 1978. Aspects of fish fecundity. Pages 75–101 *in* S. D. Gerking, editor. Ecology of freshwater fish production. Wiley, New York.

Belk, M. 1995. Variation in growth and age at maturity in bluegill sunfish: genetic or environmental effects? Journal of Fish Biology 47:237–247.

Bessey, C., R. H. Devlin, N. R. Liley, and C. A. Biagi. 2004. Reproductive performance of growth-enhanced transgenic coho salmon. Transactions of the American Fisheries Society 133:1205–1220.

Bisazza, A., and A. Marconato. 1988. Female mate choice, male-male competition and parental care in the river bullhead, *Cottus gobio* L. (Pisces, Cottidae). Animal Behaviour 36:1352–1360.

Blueweiss, L., H. Fox, V. Kudzma, D. Nakashima, R. Peters, and S. Sams. 1978. Relationships between body size and some life history parameters. Oecologia (Heidelberg) 37:257–272.

Briggs, J. C., and N. Egami. 1959. The medaka (*Oryzias latipes*): a commentary and a bibliography. Journal of the Fisheries Research Board of Canada 16:363–380.

Bryant, M. J., and J. W. A. Grant. 1995. Resource defence, monopolization and variation of fitness in groups of female Japanese medaka depend on the synchrony of food arrival. Animal Behaviour 49:1469–1479.

Chan, W.-K., and R. H. Devlin. 1993. Polymerase chain reaction amplification and functional characterization of sockeye salmon histone H3, metallothionein-B, and protamine promoters. Molecular Marine Biology and Biotechnology 2:308–318.

Devlin, R. H. 1993. Sequence of sockeye salmon type 1 and 2 growth hormone genes and the relationship of rainbow trout with Atlantic and Pacific salmon. Canadian Journal of Fisheries and Aquatic Sciences 50:1738–1748.

Devlin, R. H., M. D'Andrade, M. Uh, and C. A. Biagi. 2004. Population effects of growth hormone transgenic coho salmon depend on food availability and genotype by environment interactions. Proceedings of the National Academy of Sciences of the USA 101:9303–9308.

Devlin, R. H., J. I. Johnsson, D. E. Smailus, C. A. Biagi, E. Jönsson, and B. T. Björnsson. 1999. Increased ability to compete for food by growth hormone-transgenic coho salmon *Oncorhynchus kisutch* (Walbaum). Aquaculture Research 30:479–482.

Devlin, R. H., L. F. Sundström, J. I. Johnsson, I. A. Fleming, K. R. Hayes, W. O. Ojwang, C. Bambaradeniya, and M. Zakaraia-Ismail. 2007. Assessing ecological effects of transgenic fish prior to entry into nature. Pages 151–187 in A. R. Kapuscinski, K. R. Hayes, S. Li, and G. Dana, editors. Environmental risk assessment of genetically modified organisms, volume 3: methodologies for transgenic fish. CAB International, Oxfordshire, UK.

Devlin, R. H., L. F. Sundström, and W. M. Muir. 2006. Interface of biotechnology and ecology for environmental risk assessments of transgenic fish. Trends in Biotechnology 24:89–97.

Devlin, R. H., T. Y. Yesaki, C. A. Biagi, E. M. Donaldson, P. Swanson, and W.-K. Chan. 1994. Extraordinary salmon growth. Nature (London) 371:209–210.

Dunham, R. A., C. Chitmanat, A. Nichols, B. Argue, D. A. Powers, and T. T. Chen. 1999. Predator avoidance of transgenic channel catfish containing salmonid growth hormone genes. Marine Biotechnology 1:545–551.

Falconer, D. S. 1989. Introduction to quantitative genetics. Longman Group, New York.

FDA (Food and Drug Administration). 2010. Veterinary Medicine Advisory Committee; notice of meeting. Federal Register 75:165(26 August 2010):52605.

Forsgren, E. 1992. Predation risk affects mate choice in a gobiid fish. American Naturalist 140:1041–1049.

Fraser, D. F., and J. F. Gilliam. 1992. Nonlethal impacts of predator invasion: facultative suppression of growth and reproduction. Ecology (Washington, D.C.) 73:959–970.

Godin, J.-G. J., and S. E. Briggs. 1996. Female mate choice under predation risk in the guppy. Animal Behaviour 51:117–130.

Grant, J. W. A., M. J. Bryant, and C. E. Soos. 1995a. Operational sex ratio, mediated by synchrony of female arrival, alters the variance of male mating success in Japanese medaka. Animal Behaviour 49:367–375.

Grant, J. W. A., P. C. Casey, M. J. Bryant, and A. Shahsavarani. 1995b. Mate choice by male Japanese medaka (Pisces, Oryziidae). Animal Behavior 50:1425–1428.

Grant, J. W. A., and L. D. Green. 1996. Mate copying versus preference for actively courting males by female Japanese medaka (*Oryzias latipes*). Behavioral Ecology 7:165–167.

Hayes, K. R., H. M. Regan, and M. A. Burgman. 2007. Introduction to the concepts and methods of uncertainty analysis. Pages 188–208 *in* A. R. Kapuscinski, K. R. Hayes, S. Li, and G. Dana, editors. Environmental risk assessment of genetically modified organisms, volume 3: methodologies for transgenic fish. CAB International, Oxfordshire, UK.

Hirshfield, M. F. 1980. An experimental analysis of reproductive effort and cost in the Japanese medaka, *Oryzias latipes*. Ecology (Washington, D.C.) 61:282–292.

Hothorn, T., F. Bretz, and P. Westfall. 2008. Simultaneous inference in general parametric models. Biometrical Journal 50:346–363.

Houston, A. I., J. M. McNamara, and J. M. C. Hutchinson. 1993. General results concerning the trade-off between gaining energy and avoiding predation. Philosophical Transactions of the Royal Society of London B: Biological Sciences 341:375–397.

Howard, R. D., J. A. DeWoody, and W. M. Muir. 2004. Transgenic male mating advantage provides opportunity for trojan gene effect in a fish. Proceedings of the National Academy of Sciences of the USA 101:2934–2938.

Howard, R. D., R. S. Martens, S. A. Innis, J. M. Drnevich, and J. Hale. 1998. Mate choice and mate competition influence male body size in Japanese medaka. Animal Behaviour 55:1151–1163.

Iguchi, K., and S. Kitano. 2008. Local specialists among endangered populations of medaka, *Oryzias latipes*, harboring in fragmented patches. Environmental Biology of Fishes 81:267–276.

Jiménez, L. V. 2000. Estimation of fitness components in transgenic Japanese medaka to assess environmental risk in genetically modified organisms. Doctoral dissertation. Purdue University, West Lafayette, Indiana.

Jobling, M. 1995. Environmental biology of fishes. Chapman and Hall, London.

Jönsson, E., J. I. Johnsson, and B. T. Björnsson. 1996. Growth hormone increases predation exposure of rainbow trout. Proceedings of the Royal Society of London B 263:647–651.

Kapuscinski, A. R., J. J. Hard, K. M. Paulson, R. Neira, A. Ponniah, W. Kamonrat, W. Mwanja, I. A. Fleming, J. Gallardo, R. H. Devlin, and J. Trisak. 2007. Approaches to assessing gene flow. Pages 112–150 *in* A. R. Kapuscinski, K. R. Hayes, S. Li, and G. Dana, editors. Environmental risk assessment of genetically modified organisms, volume 3: methodologies for transgenic fish. CAB International, Oxfordshire, UK.

Kruer, T. L., S. L. Peck, H. A. Hostetler, R. H. Devlin, and W. M. Muir. 2002. Efficacy of the salmon metallothionein promoter driving expression of the Pacific salmon growth hormone gene (pOnMTGH1) for growth promotion in Japanese medaka (*Oryzias latipes*). Transgenic Research 11:83.

Kushlan, J. 1976. Feeding behavior of North American herons. Auk 93:86–94.

Lima, S. L. 1998. Nonlethal effects in the ecology of predator-prey interactions. BioScience 48:25–34.

Lõhmus, M., L. Sundström, M. Björklund, and R. Devlin. 2010. Genotype-temperature interaction in the regulation of development, growth, and morphometrics in wild-type, and growth-hormone transgenic coho salmon. PLoS ONE 5:e9980.

Maeda, T. 2001. Patterns of bird abundance and habitat use in rice fields of the Kanto plain, central Japan. Ecological Research 16:569–585.

Marconato, A., and D. Y. Shapiro. 1996. Sperm allocation, sperm production and fertilization rates in the bucktooth parrotfish. Animal Behaviour 52:971–980.

Muir, W. M., and R. D. Howard. 1999. Possible ecological risks of transgenic organism release when transgenes affect mating success: sexual selection and the trojan gene hypothesis. Proceedings of the National Academy of Sciences of the USA 96:13853–13856.

Muir, W. M., and R. D. Howard. 2001. Fitness components and ecological risk of transgenic release: a model using Japanese medaka (*Oryzias latipes*). American Naturalist 158:1–16.

Nakayama, K., Y. Oshima, T. Yamaguchi, Y. Tsuruda, I. Kang, M. Kobayashi, N. Imada, and T. Honjo. 2004. Fertilization success and sexual behavior in male medaka, *Oryzias latipes*, exposed to tributyltin. Chemosphere 55:1331–1337.

Nam, Y. K., N. Maclean, C. Fu, T. J. Pandian, and M. R. R. Eguia. 2007. Development of transgenic fish: scientific background. Pages 61–94 *in* A. R. Kapuscinski, K. R. Hayes, S. Li, and G. Dana, editors. Environmental risk assessment of genetically modified organisms, volume 3: methodologies for transgenic fish. CAB International, Oxfordshire, UK.

NRC (National Research Council). 2004. Biological confinement of genetically engineered organisms. National Academic Press, Washington, D.C.

NRC (National Research Council). 2008. Genetically engineered organisms, wildlife, and habitat: a workshop summary. National Academy of Sciences, Washington, D.C.

Peckarsky, B. L., A. R. McIntosh, B. W. Taylor, and J. Dahl. 2002. Predator chemicals induce changes in mayfly life history traits: a whole-stream manipulation. Ecology (Washington, D.C.) 83:612–618.

Pennington, K. M., A. R. Kapuscinski, M. S. Morton, A. M. Cooper, and L. M. Miller. 2010. Full life-cycle assessment of gene flow consistent with fitness differences in transgenic and wild-type Japanese medaka fish (*Oryzias latipes*). Environmental Biosafety Research 9:41–57.

R Development Core Team. 2008. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.

Reznick, D., and J. A. Endler. 1982. The impact of predation on life history evolution in Trinidadian guppies (*Poecilia reticulata*). Evolution 36:160–177.

Sebens, K. 1987. The ecology of indeterminate growth in animals. Annual Review of Ecology and Systematics 18:371–407.

Shima, A., and H. Mitani. 2004. Medaka as a research organism: past, present and future. Mechanisms of Development 121:599–604.

Stearns, S. 1989. The evolutionary significance of phenotypic plasticity. BioScience 39:436–445.

Stearns, S. C. 1992. The evolution of life histories. Oxford University Press, New York.

Stearns, S., and J. Koella. 1986. The evolution of phenotypic plasticity in life-history traits: predictions of reaction norms for age and size at maturity. Evolution 40:893–913.

Sundström, L. F., M. Lõhmus, R. H. Devlin, J. I. Johnsson, C. A. Biagi, and T. Bohlin. 2004a. Feeding on profitable and unprofitable prey: comparing behaviour of growth-enhanced transgenic and normal coho salmon (*Oncorhynchus kisutch*). Ethology 110:381–396.

Sundström, L. F., M. Lõhmus, J. I. Johnsson, and R. H. Devlin. 2004b. Growth hormone transgenic salmon pay for growth potential with increased predation mortality. The Proceedings of the Royal Society of London B 271:S350–S352.

Sundström, L. F., M. Lõhmus, W. E. Tymchuk, and R. H. Devlin. 2007. Gene-environment interactions influence ecological consequences of transgenic animals. Proceedings of the National Academy of Sciences of the USA 104:3889.

Sutherland, W. J., W. M. Adams, R. B. Aronson, R. Aveling, T. M. Blackburn, S. Broad, G. Ceballos, I. M. Cote, R. M. Cowling, and G. A. B. Da Fonseca. 2009. One hundred questions of importance to the conservation of global biological diversity. Conservation Biology 23:557–567.

Teather, K. L., J. Boswell, and M. A. Gray. 2000. Early life-history parameters of Japanese medaka (*Oryzias latipes*). Copeia 2000:813–818.

Uematsu, K. 1990. An analysis of sufficient stimuli for the oviposition in the medaka *Oryzias latipes*. Journal of the Faculty of Applied Biological Science, Hiroshima University 29:109–116.

Warnes, G. R. 2009. Gplots: various R programming tools for plotting data—R package version 2.7.2. Available: cran.bic.nus.edu.sg/web/packages/gplots/index.html. (April 2010).

Werner, E. E., J. F. Gilliam, D. J. Hall, and G. G. Mittelbach. 1983. An experimental test of the effects of predation risk on habitat use in fish. Ecology (Washington, D.C.) 64:1540–1548.

Werner, E. E., and D. J. Hall. 1988. Ontogenetic habitat shifts in bluegill: the foraging rate-predation risk trade-off. Ecology (Washington, D.C.) 69:1352–1366.

Wittbrodt, J., A. Shima, and M. Schartl. 2002. Medaka-a model organism from the Far East. Nature Reviews Genetics 3:53–64.

Woodley, C. M., and M. S. Peterson. 2003. Measuring responses to simulated predation threat using behavioral and physiological metrics: the role of aquatic vegetation. Oecologia (Heidelberg) 136:155–160.

Yamamoto, T., editor. 1975. Medaka (killifish): biology and strains. Keigaku, Tokyo.