Fitness Components and Ecological Risk of Transgenic Release: A Model Using Japanese Medaka (*Oryzias latipes*)

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ABSTRACT: Any release of transgenic organisms into nature is a concern because ecological relationships between genetically engineered organisms and other organisms (including their wild-type conspecifics) are unknown. To address this concern, we developed a method to evaluate risk in which we input estimates of fitness parameters from a founder population into a recurrence model to predict changes in transgene frequency after a simulated transgenic release. With this method, we grouped various aspects of an organism's life cycle into six net fitness components: juvenile viability, adult viability, age at sexual maturity, female fecundity, male fertility, and mating advantage. We estimated these components for wild-type and transgenic individuals using the fish, Japanese medaka (*Oryzias latipes*). We generalized our model's predictions using various combinations of fitness component values in addition to our experimentally derived estimates. Our model predicted that, for a wide range of parameter values, transgenes could spread in populations despite high juvenile viability costs if transgenes also have sufficiently high positive effects on other fitness components. Sensitivity analyses indicated that transgene effects on age at sexual maturity should have the greatest impact on transgene frequency, followed by juvenile viability, mating advantage, female fecundity, and male fertility, with changes in adult viability, resulting in the least impact.

Keywords: fish, fitness components, genetically modified organisms, life history, risk assessment, transgenics.

Transgenic technology has attracted commercial and scientific interest in both aquaculture and sport fishery. Fast-growing fish are particularly desirable, and DNA sequences for growth hormone (GH) genes and cDNAs have been well characterized. Devlin et al. (1994*a*, 1994*b*, 1995*a*, 1995*b*) and Du et al. (1992) have produced transgenic salmonids: coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*), cutthroat trout *Oncorhynchus clarki*), and chinook salmon (*Oncorhynchus tshawytscha*), the juveniles of which are, on average, 10–15 times larger than nontransgenic controls. With the same or a similar construct, transgenic juvenile Arctic charr are 14-fold larger than controls (Pitkanen et al. 1999). At present, it is unclear whether such growth enhancements extend to adults in all salmonid species; however, Devlin et al. (2001) have recently shown that transgenic rainbow trout are significantly larger at sexual maturity than wild-type controls. Also, transgenic tilapia for GH are threefold larger than controls as both juveniles and adults when grown under the same conditions (Rahman and Maclean 1999). Several other species have also been transformed with GH, producing growth enhancements up to 100% relative to wild-type fish (reviewed by Fletcher and Davies 1991; Houdebine and Chourrout 1991; Pandian and Marian 1994; Chen et al. 1995; Hew et al. 1995; Iyengar et al. 1996; Devlin 1997; Sin 1997; Sin et al. 1997; Maclean 1998; Pitkanen et al. 1999).

The anthropogenic introduction of any exotic organisms into natural communities is a serious ecological concern because exotics could adversely affect communities in many ways, including eliminating populations of other species (Mooney and Drake 1986; Lodge 1993; Bright 1996). The release of transgenic organisms into natural environments, however, poses additional ecological risks because, although transgenic individuals retain most of the characteristics of their wild-type counterparts, they may also possess some novel advantage. As a consequence, transgenic organisms might threaten the survival of wild-type conspecifics as well as other species in a community (Tiedje et al. 1989; Kapuscinski and Hallerman 1990, 1991; Devlin and Donaldson 1992; Hallerman and Kapuscinski 1992). Escape of domesticated fish, whether transgenic or not, into feral populations might also adversely affect wild-type populations by introducing alleles that are poorly adapted to natural environments. If the wild population

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is sufficiently large, these alleles should eventually be eliminated by natural selection; however, stochastic events could fix the alleles in small populations. We consider here only the case in which the affected natural population is large and not declining, thereby allowing time for natural selection to operate and to limit stochastic events. In such a situation, a necessary prerequisite for ecological risk associated with the introduction of a transgenic organism is that the transgene can spread in a natural population when rare. We recognize that risk could also result for other reasons after the release (or escape) of transgenic (or domesticated) fish. For example, released fish could introduce diseases or compete with native species for limited resources, causing population declines. If introduced males are sterile but still mate with wild females, the reproductive effort of these females is wasted. An increase in transgene frequency in a natural population is often assumed to be unlikely because transgenic organisms typically have some viability disadvantage (e.g., Knibb 1997). In our study, we evaluate this assumption by incorporating data on life-history differences between transgenic and wild-type conspecifics into a fitness-component model that predicts the fate of the transgene after the simulated release of a few transgenic individuals.

Levin et al. (1987) suggest that the potential impact of releasing transgenic animals into natural communities could be addressed by prior collection of appropriate performance data on transgenic animals reared in confinement. Our approach is to incorporate experimentally derived estimates of net fitness components of transgenic and wild-type individuals into a general model to predict how the separate and combined effects of these components could influence the fate of a wild-type population that experienced an introduction of fertile transgenic individuals. We used the small cyprinodont fish, Japanese medaka (*Oryzias latipes*) to obtain the basic life-history data. Transgenic fish were derived from our wild-type stock by inserting a human growth hormone gene construct. We thus used medaka as a model organism to examine the possible impact of transgenes on net fitness parameters and to parameterize a mathematical model that predicts changes in transgene frequency. Our model addresses theoretical concerns of a population-level effect resulting from the introduction of a genetic novelty that can influence several fitness parameters simultaneously.

Our model follows the spread (or loss) of the transgene when population growth is unconstrained; we have explored predicted consequences when population growth is constrained, and the results we obtained were qualitatively similar to those reported here. We emphasize that predictions regarding the outcome of releasing a particular transgenic stock of any one species must be placed in the more general context of a range of possible fitness component estimates. Even in the same species, different transgenic lines are likely to vary in fitness even if the same transgene construct is used because of differences in copy number and insertion sites of the transgene. To take such variation into account as well as to make our model generally applicable to any organism and transgene construct, we utilize a range of parameter values for all fitness components in our model. The range of values we use encompasses the particular fitness component estimates that we obtained experimentally. Using a similar approach, Muir and Howard (1999) examined the population consequences when transgenes simultaneously influence two net fitness components (mating advantage and viability reduction).

We first present our model, the procedures we used to produce transgenic fish, then empirical estimates of net fitness parameters for our particular stocks of transgenic and wild-type medaka. Finally, we provide the model's predictions of transgene fate, both for transgenic medaka, and then, more generally, for any transgenic introduction.

The Model

The major means by which natural selection can alter the frequency of a transgene can be reduced to six distinct mechanisms. We refer to the following as "net fitness components" because each may include any number of subcomponents: juvenile viability (probability of survival to sexual maturity), adult viability (survival after sexual maturity), age at sexual maturity, female fecundity (clutch size), male fertility (male fertilization success), and mating advantage (e.g., Lande 1982; Arnold and Wade 1984; Spiess 1989; Roughgarden 1996). Of these components, juvenile viability is usually considered to have the greatest effect on overall fitness (e.g., Clutton-Brock 1988). However, all components can have a strong influence on the rate of increase of a genotype. Increased growth rate resulting from a growth hormone transgene can enhance fitness in several ways, including size-related advantages in foraging (Abrahams and Sutterlin 1999; Devlin et al. 1999) or predator avoidance (but see Abrahams and Sutterlin 1999) and earlier attainment of sexual maturity. Increased adult viability, male fertility, or female fecundity as a result of accelerated growth can also increase the contribution of a genotype to the next generation. Last, if accelerated growth results in larger adult body size, transgenic individuals could obtain a mating success advantage, which would contribute significantly to fitness (Bundgaard and Christiansen 1972; Muir and Howard 1999).

The independent effects of differential female fecundity and male fertility selection on fitness is difficult to determine analytically because the number of progeny produced by a mated pair results from the combined contribution

of both parental types, which are not necessarily predictable from the genotype of each parent considered alone (Hartl and Clark 1997). Thus, in general, there are as many parameters to estimate as there are distinct types of mating pairs, and analytical results are not possible except for special cases, although behavior of such systems can still be examined by simulation (Ewens 1979; Clark and Feldman 1986). Overall male fertility and female fecundity can be written as the product of parameters of the parents (i.e., the parental genotypes have independent impacts on fertility and fecundity, as we assume). Nagylaki (1987) shows that components related to fertility or fecundity selection are similar to components of viability selection. However, differential fecundity or fertility can oppose viability selection and maintain a polymorphism even if there is directional selection (Hartl and Clark 1997). Other interactions between fitness components can also result in conflicting effects on overall fitness. For example, high female fecundity may jeopardize the survival of each offspring when resources or parental ability to provide food are limited. Thus, it is important to examine interactions of components because results cannot always be anticipated when only isolated components are considered.

We first develop a general model with six net fitness parameters that predicts the fate of a transgene when introduced into a wild-type population; we then discuss relevant assumptions. The model will be limited here to one locus with two alleles and complete dominance of the transgene, thereby producing three possible genotypes but only two phenotypes. The number of parameters in the model is considered to be the minimum necessary to approximate the potential for a transgene to spread. The recurrence equation for the expected frequency of genotypes in the $t + 1$ time step is the product of six factors: the probabilities of the various genotypes mating, the number of eggs produced by each female genotype (female fecundity), the probability that the eggs will be fertilized by the sperm of each male genotype (male fertility), the probability that an embryo will be a specific genotype given its parental genotypes and the probability that the fry will survive to the next time step or that the parents will survive to the next time step.

The model begins with an arbitrary population composed of N^f_{ja} females and $N^m_{j'a}$ males of genotype j or j' (j or $j' = 1$ [AA], 2 [Aa], and 3 [aa]) in the ath age class. The frequency with which mating occurs and offspring are produced determines the number of ages that need to be monitored. For a species such as medaka, which mates and produces offspring every day after attaining sexual maturity, time (t) is measured in days. The age at sexual maturity for the jth genotype is s_j. The maximum longevity of any genotype is d. Consequently, the population size at time t is

$$N_t = \sum_{j=1}^{3}\sum_{a=1}^{d} N^f_{ja} + \sum_{j'=1}^{3}\sum_{a=1}^{d} N^m_{j'a}.$$

Note that estimation of d is not necessary and simply denotes that sums are taken over all age classes until the last age class dies off. To determine gene frequency changes, it is necessary to ascertain the number of sexually mature individuals of each genotype and sex. The total number of sexually mature females in the population is

$$N^f = \sum_{j=1}^{3}\sum_{a=s_j}^{d} N^f_{ja}.$$

The relative frequency of sexually mature females of genotype j at time t is

$$G^f_{jt} = \frac{\sum_{a=s_j}^{d} N^f_{ja}}{N^f},$$

with the relative frequency of sexually mature males being given by the same equation with m replacing f.

To present our complete model, we include treatment of differential mating advantages with respect to genotype; the effect of this component on transgene spread is presented elsewhere (Muir and Howard 1999). Let f_j be the relative mating advantage of the jth female genotype. Let m_j be the relative mating advantage of the jth male genotype. If mating is independent of genotype, the relative frequency of mating combinations (female genotype j with male genotype j') is

$$U_{jj'} = \left(\frac{f_j G^f_{jt}}{\sum_{j=1}^{j=3} f_j G^f_{jt}}\right)\left(\frac{m_{j'} G^m_{j't}}{\sum_{j'=1}^{j'=3} m_{j'} G^m_{j't}}\right).$$

For the case of nonindependence of mating types, such as frequency-dependent mating success or assortative mating, affinity factors ($A_{jj'}$) for each mating type must be estimated (Muir and Howard 2001) and the above modified as

$$U_{jj'} = \frac{G^m_j G^f_{j'} A_{jj'}}{\sum_j \sum_{j'} G^m_j G^f_{j'} A_{jj'}}.$$

To obtain the expected number of offspring for each genotype, we first define c_j as the female fecundity (clutch size) of the jth female genotype, r_j as the male fertility of the jth male genotype, $M_{ijj'}$ as the expected frequency of the ith genotype at birth among offspring from a mating between genotypes j and j', v_j as juvenile viability (prob-

ability of survival to next time step before sexual maturity of genotype j. The value is assumed constant to sexual maturity. This assumption could be relaxed by estimating survival at each time step), and u_j is defined as adult viability (probability of survival to next time step after sexual maturity of genotype j; the value is assumed constant for the remainder of the lifetime, and this assumption could be relaxed by estimating survival at each time step).

Assuming an equal sex ratio at birth, the expected number of offspring of the ith genotype for females in the first age class from all matings is

$$N_{i1}^f = \frac{N^f\left[\sum_{j=1}^{3}\sum_{j'=1}^{3}(U_{jj'}r_{j'}c_jM_{ijj'})\right]}{2},$$

where the term in the summation provides the expected number of offspring per female of each mating pair. The number of male offspring is given by the same equation with m replacing f. These individuals compose the first age class. All other juvenile ages advance to the next class after reducing numbers of each genotype by v_j. Similarly, all adult ages advance to the next class after reducing the number of each genotype by u_j and the oldest age class of each genotype dies off. The number of females in all age classes of genotype j at time $t + 1$ adjusted for such mortality is

$$N_j^f = \left(v_j\sum_{a=1}^{s_j-1}N_{ja}^f + u_j\sum_{a=s_j}^{d}N_{ja}^f\right),$$

with the number of males being given by the same equation with m replacing f. If juvenile and adult viabilities differ at each time step a as v_{ja} and u_{ja}, the equation would simply be modified by moving the term within the summation sign as

$$N_j^f = \left(\sum_{a=1}^{s_j-1}v_{ja}N_{ja}^f + \sum_{a=s_j}^{d}u_{ja}N_{ja}^f\right).$$

The equation with constant juvenile and adult viabilities effects a two-leg, log-linear reduction in population size as a function of age because, from the recurrence equation, the juvenile portion of the above equation at age a is

$$N_a = vN_{a-1}$$

or, starting at a population size of N_0,

$$N_a = v^aN_0$$

and, after taking natural logs of both sides,

$$\log_e(N_a) = \log_e(N_0) + a\log_e(v).$$

Similarly, the adult portion of the curve is

$$\log_e(N_a) = \log_e(N_s) + (a - s_j)\log_e(u).$$

Thus, the relationship between population size and age is log linear but with different slopes in the pre- and post-maturity stages. The age at sexual maturity of the jth genotype (s_j) also determines the relative duration of juvenile and adult mortality. Medaka, as most fish, have a Type III survivorship curve (after Deevey 1947), which is described by a negative exponential equation. The negative exponential survivorship curve is also log linear (Roff 1992). Therefore, the iterative equations described above and a Type III survival curve describe the same process. The population size at time $t + 1$ is

$$N_{t+1} = \sum_{j=1}^{3}(N_j^f + N_j^m).$$

The relative frequency of the jth genotype at time $t + 1$ is

$$G_{j,\,t+1} = \frac{N_j^f + N_j^m}{N_{t+1}},$$

and the frequencies of the two alleles at time $t + 1$ are

$$p_{1,\,t+1} = G_{1,\,t+1} + \frac{1}{2}G_{2,\,t+1},$$

$$p_{2,\,t+1} = 1 - p_{1,\,t+1}.$$

To implement this model and to predict the consequences of releasing a few transgenic individuals into a large wild-type population, we developed a computer program to keep constant accounting of numbers and genotypes of all life stages through time. Effects of the transgene on juvenile and adult viability, age at sexual maturity, female fecundity, and male fertility were considered relative to wild-type controls.

We examined model predictions in three ways. First, as an example, we estimated a number of net fitness components on a population of fish (Japanese medaka) transgenic for human growth hormone (TR) and its wild-type (WT) counterpart. These data were then used to parameterize the recurrence model and to predict changes in transgene frequency. Second, we generalized our results

by extending the model's predictions using various combinations of the fitness components. Finally, we performed sensitivity analyses to determine the relative impact of each parameter on the spread of transgenes.

In the model, the wild-type population initially consisted of 60,000 fish with an equal sex ratio and one genotype (+/+) into which we "introduced" 30 male and 30 female adult (i.e., 56-d-old), homozygous, transgenic fish. We then ascertained whether transgene frequency increased or decreased. An increase in transgene frequency in the population could only result if the viability reduction of transgenics was more than offset by enhancements in other net fitness parameters relative to wild-type individuals.

Material and Methods

The Model Organism

Medaka are common freshwater cyprinodont fish native to Japan, Korea, Taiwan, and China, where they inhabit both fresh and brackish water (Yamamoto 1975*a*). Males and females do not exceed 40 mm in length (Yamamoto 1975*b*). Females may live for as long as 3 yr and males for 4 yr under laboratory conditions (Yamamoto 1975*b*), but most adults do not live much beyond 1 yr in nature (Aida 1921; Egami et al. 1988). Several color varieties of medaka exist (Takeuchi 1975).

Female medaka may breed daily throughout the breeding season. In nature, ovulation occurs several hours before dawn; most mating activity takes place at dawn and lasts for about an hour (Egami and Nambu 1961). When reared in the laboratory under a constant photoperiod, mating begins after "lights on" and continues for about an hour. Fertilization is external. Eggs remain attached to the female's vent by filaments until the female brushes them off onto vegetation (Kamito 1928).

Production of Transgenic Fish

Vielkind (1992) demonstrated that medaka was an ideal organism to study both transient and stable transgenic systems. The stability and segregation pattern of transgenes were demonstrated by Kinoshita et al. (1996), who produced a line of medaka that transmitted an active chloramphenicol acetyltransferase (CAT) gene to all offspring for six generations in a normal Mendelian fashion. We used the Purdue orange-red strain of medaka to produce our transgenic founder line. This strain was established from a large, heterogeneous, randomly mating population of medaka that was homozygous for the mutant color orange-red. Offspring from the founder line were then crossed with WT individuals from Japan. Medaka were reared using infusoria as an initial food source, followed by trout rations of AP100, trout mash, and number 1 trout starter as individuals grew. Adults were fed Tetra Min flake food. Brood stock were kept in aquaria containing algae and fed artemia three times a day before and during breeding. Water temperature was held at a constant 25°C under a 14 : 10 photoperiod (light to dark). Fish were reared and tested in a closed recirculating water system consisting of 144 40-L tanks with undergravel discharge of water to a biofilter, which was then pumped back into the tanks by overtank outlets. Water was recirculated at the rate of 3 L/min/tank.

A construct containing the salmon growth hormone promoter (psGH) and the human growth hormone gene (hGH) was supplied to us by Dr. Geoffrey Waldbieser (USDA-ARS, Catfish Genetics Research Unit, Stoneville, Miss. 38776). The construct was a fusion product containing 660 bp of the 5′ regulatory DNA from the Atlantic salmon (*Salmo salar*) growth hormone gene fused with the coding sequence from the human growth hormone gene. *Escherichia coli* were transformed with the psGH-hGH construct using methods described by Sambrook et al. (1989) and were grown overnight in a tube containing Luria-Bertani (LB) media. Plasmid DNA was isolated, digested with the restriction enzymes *Xba I* and *Eco* RI, purified, and diluted to 10 ng/μL.

The transgenic founder line was produced using the cytoplasmic microinjection technique of Ozato et al. (1992). Eggs were collected from females, and the chorionic filaments were trimmed from them using surgical scissors and a pair of fine-point tweezers within 90 min of fertilization. The single-celled eggs were then microinjected (600–800 hpa with a backpressure of 400 hpa) with approximately 200 pg of transgene DNA in 20 pL of injection buffer (filter sterilized 1M Tris/EDTA, pH 7.5). The microinjected eggs were incubated in a hatching tank in the presence of methylene blue to prevent fungus growth.

Polymerase chain reaction (PCR) was used as a first assay to identify the transgenic founder and subsequently to test progeny for germ line transmission or mRNA expression. A simple protocol for rapid PCR sampling based on the method of Higuchi (1989) was used. For this protocol, small pieces of fin tissue were immersed in 10 mM NaOH and boiled for 5 min. Ten microliters of that solution was added to a standard PCR reaction that contained 20 pmol of each 30-mer PCR primers, 10 mM Tris (pH 8.3), 25mM KCl, 2.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Promega, Madison, Wis.) in a total volume of 50 μL. The primer sequences were in exons 2 and 4 of the hGH gene and amplified a 661-bp fragment. These primers worked well at an annealing temperature of 55°C and did not crossreact with endogenous medaka GH. Poly-

merase chain reactions were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide.

We injected 842 eggs with the psGH-hGH construct, of which 716 fry (85%) were alive by first feeding. Ten psGH-hGH somatic transgenic fish were identified by PCR amplification. To test for germ line transmission, each positive fish was backcrossed to the WT strain to produce the BC_1 generation. The BC_1 fry were weighed at 6 wk of age and tested for the psGH-hGH construct using the PCR method described above. The confirmed positive fish of the BC_1 generation all came from one founder and resulted in our sole germ line transgenic fish. These BC_1 offspring were backcrossed again to WT by mating each transgenic female to one WT male and each transgenic male to two WT females, thereby producing eight BC_2 families.

We delayed testing the BC_2 fish until they were 10 wk of age (2 wk after sexual maturity) because fin clips taken from 6-wk-old BC_1 individuals resulted in high mortality. Offspring from each BC_2 family were then weighed and the presence of the psGH-hGH construct determined by the PCR method described above.

Genomic integration was confirmed and copy number determined in the BC_2 progeny by Southern blot analysis (Southern 1975). High molecular weight DNA was isolated from two transgenic and one nontransgenic medaka according to the methods of Jowett (1986) and digested with the restriction enzymes EcoRI or BamHI. Plasmid DNA (psGH-hGH) was digested with EcoRI to be used as a quantification control. DNA fragments were separated by electrophoresis and transferred to a nylon membrane and covalently bound to the membrane. Approximately 50 ng of psGH-hGH probe was then labeled with ^{32}P. The probe was then hybridized to genomic DNA for identification of the target sequences. Nucleic acid hybridization protocols and washes followed the methods of Strauss (1993). Autoradiography was performed by placing the membrane in a metal cassette with film for 2 d. The film was then developed using standard procedures. Densitometry analysis of the 2.8 Kb fragment from the Eco-RI digest of transgenic fish and comparison with known concentrations of psGH-hGH indicated an approximate copy number of two to three in the two fish tested. The BamHI digests were consistent with head to tail insertions of the construct.

Expression of the transgene in muscle was confirmed by reverse transcriptase (RT)-PCR analysis. Muscle tissue was isolated and pooled from three mature BC_1 fish heterozygous for the transgene. Expression was determined by the guanidinium isothiocyanate/CsCl method of Chirqwin et al. (1979) as modified for the tabletop ultracentrifuge by Reddy et al. (1990). The preparations required a 1-h centrifugation for cDNA-quality total RNA isolation. Complementary DNA was synthesized from 1 μg of total RNA using random hexanucleotides and MMLV reverse transcriptase according to manufacturer's directions. The cDNA was added directly to a PCR reaction to verify mRNA expression.

Phenotypic Effect of the Transgene on Growth at Different Ages

Before collecting fitness component data, it was critical to determine whether the transgene influenced growth independent of the background genotype of the founder. To estimate growth rate, we backcrossed eight pairs of BC_3 heterozygous transgenic fish with wild type in separate aquaria to obtain BC_4 progeny; however, only four of the eight pairs produced eggs consistently on a daily basis for use in experiments. We collected eggs daily from each pair during a 2-wk period and placed them into separate hatching cups. Upon hatching, 100 fry from each pair were divided into five aquaria (each containing 20 fry). All fry within a tank differed by no more than 2 d of age. All fry were fed once a day. At 2-wk intervals, all individuals in one tank from each pair were killed, weighed using a Mettler Toledo balance (AG204), and fin clipped for PCR identification of transgene presence. This procedure was repeated every 2 wk for 10 wk.

Estimation of the Juvenile Viability (v_{ja}) *Fitness Component*

We used fish from the BC_4 generation to obtain data on all net fitness components. To assess juvenile viability, we conducted a 2 × 2 factorial experiment, with TR versus WT male parents as the first factor and TR versus WT female parents as the second factor. We used 10 mated pairs for each combination and collected all eggs from each female 2 h after lights on, then returned the female to her tank. Eggs were counted and placed in individual hatching cups in a hatchery supplied with constant flowing recirculating water. Twenty-four hours later, the eggs were examined under a dissecting microscope and classified as fertile or infertile based on presence or absence of embryo development. We collected eggs from each pair for a period of 10 d, obtaining a total of 1,910 fertile eggs. Three days later, we estimated juvenile viability as the percentage of 3-d-old fry that emerged from fertile eggs. These estimates obviously do not take into account all aspects of viability before sexual maturity, such as survival from cannibalism, but merely serve as an example. Ideally, juvenile viability should be measured just before sexual maturity. For the purposes of this example, we assumed that the mortality difference between TR and WT fish during the first 3 d of life were proportional to that just before sexual maturity.

To ascertain the importance of an additional source of mortality on juveniles, we conducted two additional tests

to examine how growth rate might affect juvenile survival from cannibalism. In the first test, we examined cannibalism of fry by adults. Two sexually mature females, producing an average of 20 eggs/d, were placed with a sexually mature male into a large 1,200-L tank filled with heavy plant cover. These fish were fed flake food twice daily to satiation. The tank was monitored for 100 d for surviving fry. In the second test, we examined the effect of fry age on cannibalism rate. We put 100 8-d-old, wild-type fry and three adults into a 1,200-L aquarium with heavy plant cover for 1 wk. Surviving fry were noted, and the procedure was repeated using 9-d-old fry and so forth up to 21-d-old fry.

The maximum likelihood estimate of cumulative juvenile viability during these 3 d of life for transgenic and wild-type offspring was found by maximizing the following likelihood function:

$$L = \prod_{i=1}^{4} \binom{N_i}{A_i} (V_i)^{A_i} (1 - V_i)^{(N_i - A_i)},$$

where N_i is the number of fertile eggs of the ith mating-type combination, A_i is the number of juvenile fish surviving from the ith mating-type combination, and V_i is the expected overall proportion of progeny surviving from the ith mating-type combination (table 1), which is the viability of each genotype of offspring times the expected Mendelian proportion of each genotype given the genotypes of the parents.

Cumulative juvenile viabilities (v_j') were converted to per day viabilities (v_j) between consecutive census time periods (a_{t+1} and a_t) by assuming a log-linear reduction in daily viability between time periods sampled using the following equation:

$$v_j = \exp\left[\frac{\log_e(v_j')}{a_{t+1} - a_t}\right].$$

In our case, $a_{t+1} - a_t = 3$ d.

Estimation of the Adult Viability (u_{ja}) *Fitness Component*

The daily reduction in adult survival of each genotype (u_{ja}) should be measured in as natural an environment as possible until all fish die. We assumed a log-linear reduction in daily viability and only observed enough time periods to establish a trend. We observed survival of 10 mated pairs of each genotype for 100 d past sexual maturity. The cumulative survival at the termination of the experiment of each genotype (u_j') was related to the daily reduction in survival (u_j) by the following equation:

Table 1: Offspring viabilities for each mating-type combination

Mating-type combination (i)	Expected proportion surviving (V_i)	Observed number surviving (A_i)
WT♂ × WT♀	v_1'	A_1
WT♂ × TR♀	$(1/2)v_1' + (1/2)v_2'$	A_2
TR♂ × WT♀	$(1/2)v_1' + (1/2)v_2'$	A_3
TR♂ × TR♀	$(1/4)v_1' + (3/4)v_2'$	A_4

Note: The parameters v_1' and v_2' are the cumulative juvenile viabilities of WT and TR fish, respectively; v_1' and v_2' were found by maximizing the likelihood L.

$$u_j = \exp\left[\frac{\log_e(u_j')}{a_{t+1} - a_t}\right].$$

In our example, $a_{t+1} - a_t = 100$ d.

Estimation of Age at Sexual Maturity (s_j) *Fitness Component*

We estimated age at sexual maturity for TR females by first considering growth rate differences between WT and TR fish before sexual maturation. We then ascertained the average body mass and age at first clutch production for 40 WT females. Using both sets of data, we estimated the age of sexual maturity for TR females.

Estimation of the Female Fecundity (c_j) *Fitness Component*

Twenty-week old fish were used in a 2 × 2 factorial experiment, in which 20 TR and 20 WT males were paired with 20 TR and 20 WT females in all possible combinations resulting in 10 matings per combination. Weight of each parent was recorded. Eggs were collected and counted from five sequential spawns.

Estimation of the Male Fertility (r_j) *Fitness Component*

The ability of the male genotype to fertilize eggs was examined by a simple completely randomized design experiment in which 10 transgenic males and 10 wild-type males were randomly single-pair mated with wild-type females in separate 40-L tanks for 8 d. The first three egg masses produced by each female in that 8-d period were collected and incubated in a hatching tank in the presence of methylene blue to prevent fungus growth. Twenty-four hours later, the eggs were examined under a dissecting

microscope and classified as fertile or infertile based on presence or absence of embryo development.

Sensitivity Analyses

We conducted sensitivity analyses to examine the relative effect of each net fitness component on the predicted rate of spread of the transgene. For this analysis, we obtained the partial derivative of the change in transgene frequency per change in each component. Our empirical estimates of juvenile viability and adult viability, age at sexual maturity, female fecundity, and male fertility, of the nontransgenic population were used as the point in hyperspace at which to take the derivative. To linearize the function around the point, we assumed an initial gene frequency of 0.5 for the transgene. The derivative was calculated as the observed change in transgene frequency after 40 generations per 10% change in a specific fitness component, holding all other components constant.

Results

Phenotypic Effect of the Transgene on Growth at Different Ages

At 6 wk of age, the confirmed transgenic BC_1 progeny had an average weight of 66.9 ± 8.3 mg ($\bar{X}$ ± SE; $n = 6$) compared to 54.4 ± 2.0 mg ($n = 138$) for their nontransgenic full and half siblings. This 22.9% difference was not significant, however, because of the low number of BC_1 transgenic fish detected. The low number of TR progeny most likely resulted because the founder's gonadal tissue was mosaic for the transgene. The transgene was observed in each family of the BC_2 generation, indicating a stable integration of the transgene into the genome. Individuals differed significantly in average body weight among the eight families ($F = 6.38$, df = 7,376, $P < .001$; ANOVA), and transgenic individuals were heavier, on average, than nontransgenic individuals: 191 ± 5 mg and 167 ± 4 mg, respectively ($F = 8.10$, df = 1,376, $P = .005$). Since, on average, transgenic individuals were heavier than wild-type individuals, there was little question that the sGH construct was expressed and affected growth. Relative growth of the BC_2 generation (defined as the difference in average weight between transgenic and wild-type individuals divided by the average weight of wild-type individuals) was highly variable across families, ranging from a low of −4% to 76%. Because all fish were descendants from one founder, differences in body weight among families reflect both sampling error and the impact of genetic background on transgene expression. Higher levels of growth hormone expression appear to be detrimental because the medaka family with the highest growth rate (76%) showed abnormalities of the head and spine and died before they could reproduce. Similar results were observed in Chinook salmon transgenic for GH (Devlin et al. 1995*a*).

In the BC_4 generation, the TR line had a distinct early growth advantage over WT, peaking at 4 wk of age with a 39% size advantage (fig. 1). At 8 wk of age, the size advantage of TR individuals had disappeared. At 10 wk of age, TR and WT fish were nearly identical in weight (140.0 ± 2.7 mg vs. 140.9 ± 2.7 mg); both of them were significantly ($P < .01$) less than the average weight of similarly aged individuals of the previous generation, indicating that uncontrolled environmental factors, such as water quality, may have stunted growth in both lines.

Zhu (1992) also observed that carp and loach transformed with human GH showed marked increase in variation in growth compared to controls. In crucian carp, some transgenic fish growth rates were less than that of controls. Transgenic common carp were, on average, only 9.4% heavier than controls but the largest transgenic was 1.4 times heavier than the largest control. In crucian carp, the average difference was 78%, but the largest transgenic was 2.1 times larger than the largest control. Similar to our results, Lu et al. (1992) showed that the mouse metallothionein (mMT) or chicken actin promoters fused to the same hGH gene used in our study could increase the size of transgenic medaka between 20% and 60% relative to controls. The range in body size in our experiment was

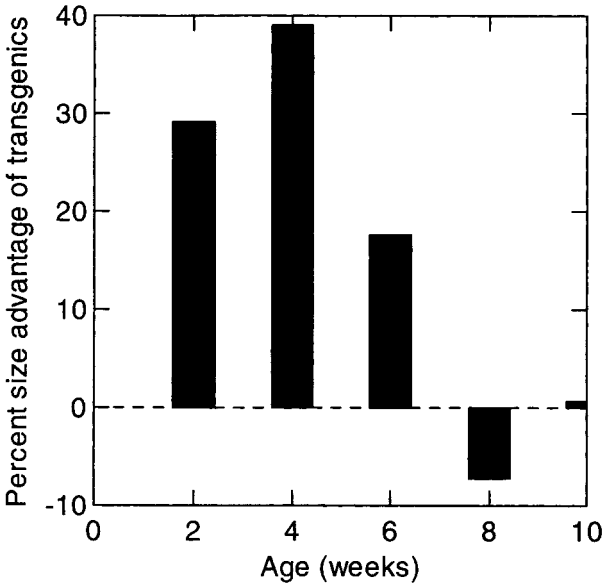

Figure 1: Size advantage of transgenic juveniles of the BC_4 generation relative to wild-type juveniles expressed as relative body size [(transgenic body weight/wild-type body weight) − 1] as a function of age. Forty TR and WT fish were measured for each age group.

Table 2: Percentage of survival of fertile eggs to 3 d of age from crosses with transgenic (TR) and wild-type (WT) medaka

Mating	Total number of fertile eggs	Number surviving
WT♂ × WT♀	204	49.5
WT♂ × TR♀	482	44.8
TR♂ × WT♀	202	47.5
TR♂ × TR♀	593	38.4

much greater than that of Lu et al. (1992), indicating that the background genotype has significant impact on expression of the transgene. Chen et al. (1994) also observed great variability among families with respect to degree of transgene expression.

Juvenile Viability (v_j') *Fitness Component*

Based on the cumulative percentage of survival of young to 3 d of age (table 2), the maximum likelihood estimates of cumulative juvenile viability were $v_1' = 0.52$ and $v_2' = 0.36$ for wild-type and transgenic fry, respectively, giving a relative juvenile viability fitness of 0.70. The per day viabilities were

$$v_1 = \exp\left[\frac{\log_e(0.52)}{3}\right] = 0.80,$$

$$v_2 = \exp\left[\frac{\log_e(0.36)}{3}\right] = 0.71.$$

These estimates of daily juvenile viability underestimated survivorship at the end of the juvenile period. In four samples of 50 wild-type fry that we reared, 25.5% ± 6.08% survived to sexual maturity, yielding a daily viability of 0.9755; we used this value for v_1 to obtain predictions from the model below rather than 0.80. Similar estimates for daily viability of transgenic juveniles were not obtainable because this would require killing the fry to identify their genotype; thus, we assume that transgenic juvenile viability was 70% of wild type. Our estimates of juvenile viability did not include mortality from predation or cannibalism, as neither predators nor adult medaka co-occurred with the developing young. In the two cannibalism tests using WT medaka, we observed extremely high mortality, particularly on young, small fry. In one test, only one fry survived in a 120-L tank containing two adult females and one adult male during 100 d despite continuous breeding. Such high mortality is most probably an artifact of confinement despite the presence of heavy plant cover for refuge. When groups of 100 fry ranging in age from 8-21 d old were added each week to the same tank containing three adults, no fry younger than 19 d old survived, and only 3% of the 19-d-old fry survived. In contrast, 50% of the 21-d-old fry survived. These results indicated that even a slight increase in growth rate could reduce mortality from cannibalism.

Adult Viability (u_j) *Fitness Component*

We found no differences in adult viability between TR and WT. For each of the 20 individuals of each genotype followed for 100 d after sexual maturity, we observed two deaths. Thus, both u_1' and u_2' equaled 0.90. The estimate of per day adult viability of both genotypes is

$$u_1 = u_2 = \exp\left[\frac{\log_e(0.90)}{100}\right] = 0.9989.$$

Age at Sexual Maturity (s_j) *Fitness Component*

The growth rate of TR fish from 2 to 6 wk of age was 16% greater than that of WT (2.62 mg/d vs. 3.04 mg/d). WT females produced their initial clutch at a body mass of 121 ± 26 mg at an average age of 56 d. We therefore assumed WT fish would reach sexual maturity 16% later (or about 7 d later) than TR and set age at sexual maturity of TR (s_2) to 49 d and WT (s_1) to 56 d. Data from Jiménez (2000) supported this prediction. Using fish from the BC_5 generation and directly observing age at first egg production, she found that TR females attained sexual maturity 6 d earlier than WT.

Female Fecundity (c_j) *Fitness Component*

Transgenic females produced larger clutches than WT females did (table 3). Transgenic females produced spawns of $c_2 = 11.4 \pm 1.0$ ($\bar{X} \pm$ SE) eggs/d compared to $c_1 = 8.8 \pm 1.0$ for WT. Although TR females (414 ± 9 mg) and WT females (387 ± 11 mg) were similar in body weight ($t = 0.86$, df = 38, $P = .39$), TR females produced sig-

Table 3: Female fecundity for different mating combinations of transgenic (TR) and wild-type (WT) medaka

Mating	Total number of eggs laid over 5 d	Eggs/female/d
WT♂ × WT♀	441	8.8 ± 1.2
WT♂ × TR♀	505	10.1 ± 1.2
TR♂ × WT♀	458	9.2 ± 1.2
TR♂ × TR♀	633	12.7 ± 1.2

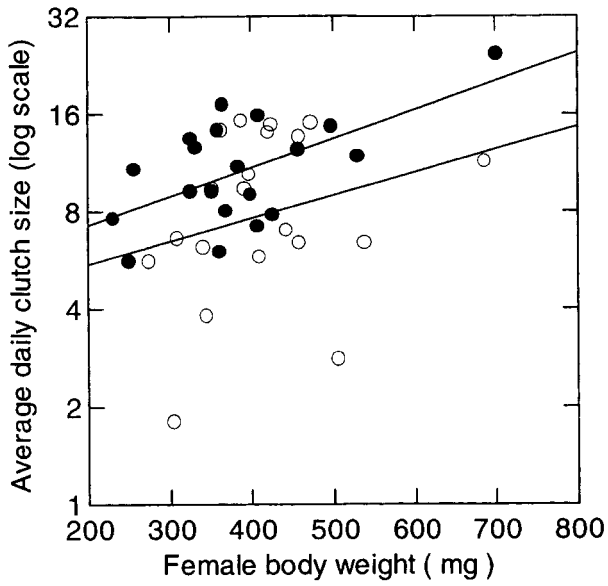

Figure 2: Average daily clutch size (on a log scale), based on five sequential spawns for each female as a function of body weight. *Filled circles*: TR females; *open circles*: WT females.

nificantly larger clutches than WT females did, on average (after log transformation to meet the assumption of normality: $t = 1.99$, df $= 38$, $P = .05$). The slope of the relationship between clutch size (log transformed) and body weight did not differ between the two types of females (ANCOVA: $F = 0.07$, df $= 1, 36$, $P = .79$); however, the intercept of the relationship did differ between female types ($F = 5.96$, df $= 1, 36$, $P = .02$; fig. 2). Thus, the average spawn size of TR females was 29% greater than that of similarly sized WT females. Male genotype (TR or WT) had no effect on clutch size, either as a main effect (two-way ANOVA: $F = 0.24$, df $= 1, 35$, $P = .63$) or in interaction with female genotype ($F = 0.45$, df $= 1, 35$, $P = .51$)

Male Fertility (r_j) *Fitness Component*

Fertilization success of transgenic males ($r_2 = 95.3\% \pm 3.5\%$) and wild-type males ($r_1 = 97.1\% \pm 3.1\%$) did not differ significantly ($t = -1.24$, $P = .23$; average of three clutches/male for 10 crosses of each male type with WT females).

Model Predictions

Based on the data obtained in the above experiments, for WT fish we set juvenile and adult viabilities to $v_1 = 0.9755$ and $u_1 = 0.9989$, respectively, female fecundity to $c_1 = 8.8$ eggs/d, average age at sexual maturity to $s_1 = 56$ d posthatch. Transgenic fitness parameters were initially set equal to those of WT and then varied one at a time or in combination. The range of values assigned to the TR fitness parameters included those estimated for TR in the above experiments.

If the transgene only conferred a negative or positive effect on a single fitness component, the transgene would either be lost or go to fixation, respectively. In contrast, when the transgene provided both an age at sexual maturity advantage and a juvenile viability disadvantage, the model predicted that transgene frequency should increase across a broad range of parameter values (fig. 3). Given the 7-d reduction in age to sexual maturity (a 1.125 advantage) that we observed in transgenic medaka, the model predicts that the transgene should increase in frequency with transgenic offspring juvenile viabilities as low as 60% relative to wild type. Thus, with the observed 70% relative viability of juvenile transgenic medaka, the model indicates that the transgene should spread after introduction. Based on our experimental estimates of viability disadvantage and reduced age to sexual maturity, the model predicts that the transgene frequency should rise to over 34% in 40 generations.

A female fecundity advantage of nearly twofold would

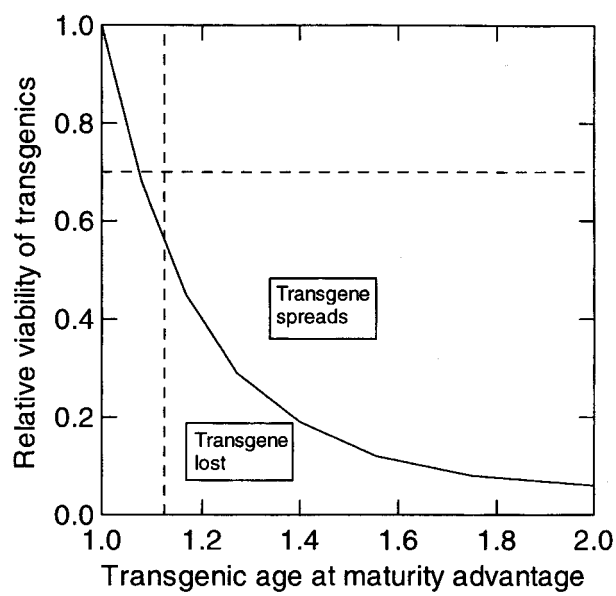

Figure 3: Influence of transgene age at sexual maturity advantage (s_2/s_1) and juvenile viability disadvantage (v_2'/v_1') on transgene frequency when transgenic individuals are introduced into a wild-type population. Above the curve, the transgene is predicted to spread in the population; below the curve, the transgene is predicted to be lost. Dashed lines indicate experimental results obtained for medaka in this study.

be required to counter the relative TR viability of 70% that we observed in our experiment (fig. 4). As a result, our model predicts that the female fecundity advantage we observed in transgenic medaka females would be insufficient to offset the observed reduction in transgenic offspring viability.

Based on experimental data, sexual maturity, female fecundity, and juvenile viability should all be important factors in determining the fate of the transgene if introduced into a natural population of medaka. We did not include an impact of the transgene on adult viability or male fertility because there is no evidence of its effect on either component. Despite a 70% relative viability of TR, the combination of a 12.5% reduction in age at sexual maturity, combined with a 29% female fecundity advantage, is expected to produce an even more rapid rate of increase in transgene frequency than that predicted with just a reduced age at sexual maturity and juvenile viability disadvantage. The model predicts that the transgene frequency is expected to exceed 63% in 40 generations.

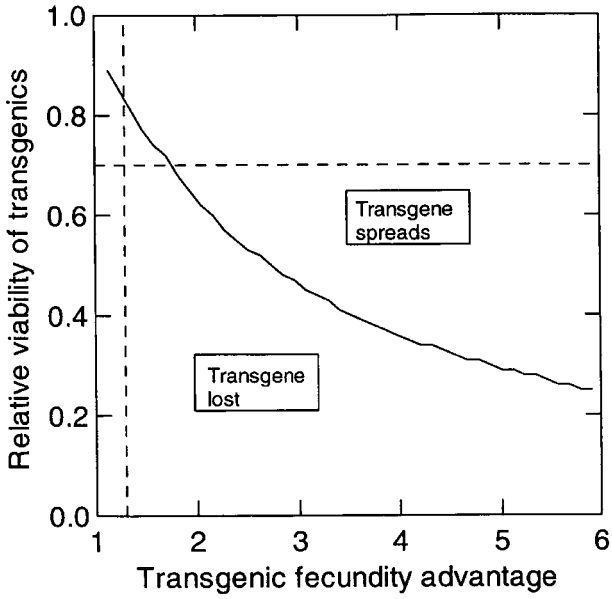

Figure 4: Influence of transgene female fecundity advantage (c_2/c_1) and juvenile viability disadvantage (v'_2/v'_1) on transgene frequency when transgenic individuals are introduced into a wild-type population. Above the curve, the transgene is predicted to spread in the population; below the curve, the transgene is predicted to be lost. Dashed lines indicate experimental results obtained for medaka in this study.

Sensitivity Analysis

The sensitivity analysis indicated that age at sexual maturity was by far the most important variable affecting the spread of the transgene, followed by juvenile viability (see table 4). Male fertility, female fecundity, and mating success had similar but smaller effects, and adult viability had the least impact.

Discussion

The large-scale and unexpected disruption of ecological communities resulting from the accidental anthropogenic release of such exotic organisms as gypsy moths, lampreys, and zebra mussels in North America poses a major problem in environmental biology. An ongoing debate exists concerning the degree to which the release of a transgenic organism into nature might produce a similar ecological hazard. Concern over the possible ecological impact of a transgenic release have been voiced by numerous authors (e.g., Regal 1987; Tiedje et el. 1989; Kapuscinski and Hallerman 1990; Hallerman and Kapuscinski 1995); however, others maintain that such risks are minimal. For example, Knibb (1997, p. 59) recently stated that "laboratory induced allele frequency/genotype changes and novel alleles or genes have a negligible probability of being selectively favored in wild populations under natural selection, and accordingly, without sustained large-scale releases, have little potential for ecological impact."

Transgenic organisms are presumed to pose little risk to natural communities because genetically engineered organisms typically have reduced viability relative to their natural counterparts. Our investigation challenges this claim. According to our model, the release of growth-enhanced transgenic fish into nature may pose an environmental risk even if they have a reduced viability as a result of counterbalancing advantages in another fitness component. Such differential effects are likely to occur when transgenes affect growth rate and body size because body size is a critical determinant of variation in almost every aspect of survival and reproduction (Roff 1986). For example, larger body size often enhances competitive ability (Magnuson 1962), female fecundity (Gall and Gross 1978), and mating success (Andersson 1994). The interaction of these fitness components may not only increase frequency of the transgene, but also genetic load as a result of the viability cost of the transgene. In the extreme, such genetic load could result in the extinction of wild-type populations (Muir and Howard 1999). Whether or not the spread of a transgenic organism constitutes a serious environmental threat remains an open question, however, and is likely to vary on a case-by-case basis.

Our experiments on medaka have shown that transgenic young have reduced juvenile viability relative to wild-type; however, surviving transgenic hatchlings grow at a faster rate than wild type until about the age of sexual maturity. Zhu (1992) also reported that growth enhancement of

Table 4: Predicted change in transgene frequency after 40 generations when each fitness component is changed by 10%

Fitness parameter	Base parameter estimate	Base parameter estimate with 10% change	Frequency of transgene after 40 generations (A)	Percentage of change in outcome (sensitivity) $(A - .5)/.5$
Juvenile viability	.9755[a]	.9737[b]	.21	58%
Adult viability	.9989[c]	.9979[d]	.47	6%
Relative male fertility (%)	100	110	.58	16%
Relative male mating success (%)	100	110	.58	16%
Female fecundity (eggs/clutch)	8.8	9.68	.58	16%
Age at sexual maturity (d)	56	50	.86	72%

Note: Starting frequency of the transgene in this sensitivity analysis was set to 50%, and base parameter estimates equal values estimated for wild-type medaka.

[a] 25% survival to sexual maturity.

[b] 22.5% survival to sexual maturity.

[c] 90% survival 100 d past sexual maturity.

[d] 81% survival 100 d past sexual maturity.

transgenic medaka was much greater in the fry to juvenile stages than in the adult stage. Such a growth advantage could translate into a survival advantage. As observed in other studies (e.g., Kamito 1928), a major source of early mortality in medaka is cannibalism by adults. Our tests for cannibalism most likely yielded overestimates of natural intensities of cannibalism as a result of confining adults and offspring in a relatively small enclosure; however, the tests did reveal that fry mortality could be reduced after fry reached a sufficient size to escape predation from gape-limited adults. Thus, the faster growth rate of transgenic medaka young is expected to increase their early survival relative to wild type. The prediction of improved early survival assumes, however, that other sources of predation are similar between transgenic and wild-type medaka. This assumption may not hold for all species. For example, Jonsson et al. (1996) found that enhanced growth of rainbow trout with GH implants made them more susceptible to aerial predators because of associated changes in feeding behavior. Similarly, Abrahams and Sutterlin (1999) found that increased foraging rates of Atlantic salmon results in greater risk of predation.

Based on our estimates of per day juvenile viability during their first 3 d of life, about one in a million and one in a billion WT and TR medaka should survive to sexual maturity, respectively. Clearly, our assumption of a log-linear juvenile survival curve was not met, as we typically observed that ~25% of the WT fry survive to adulthood ($v_1 = 0.9755$). Thus, per day mortality before 3 d of age must be considerably higher than that between 3 d of age and 56 d of age (sexual maturity). A similar pattern of differential mortality was demonstrated in mackerel (Sette 1943), in which the mortality rate during transition from larval to postlarval stages was three to five times higher than that during the postlarval but preadult stage.

Although juvenile viability itself does not fit a single parameter survival curve as we assumed, the relative survivorship of TR to WT individuals at the end of the juvenile period is more critical than the exact shape of the survivorship curve. The reason is straightforward: offspring do not begin to contribute directly to the next generation until they mature sexually. The relative survivorship of TR to WT adults should also be more important than the precise shape of the survivorship curve, except for species in which mating success increases with age independent of body size. In this case, the model would need to be modified to account for increases in mating potential with age.

The greater per day viability of adults compared to juveniles that we observed with medaka is similar to that reported for mackerel (Sette 1943). Sette estimated that the total survival from fry to adults was ~0.04%, resulting in a per day survival rate of 95.80%; in contrast, he estimated that yearly survival of adults was 10%–50%, resulting in a per day adult survival rate between 99.81% and 99.97%.

Body size also has a strong effect on the age of sexual maturity in fish (Roff 1986); thus, an increased growth rate of transgenic fish may allow them to reach sexual maturity at an earlier age than their wild-type conspecifics. Devlin et al. (1994*b*) reported that some transgenic coho salmon matured precociously at 2 yr of age rather than the normal 3 yr of age, a 33% reduction in age to sexual maturity.

Model predictions based on effects of single fitness components are simple and straightforward with the exact tra-

jectories of the transgene dependent on the degree of transgene disadvantage or advantage. If a transgene only reduces viability, the gene will be eliminated from the population. Thus, for example, holding all other effects constant, our model predicts that transgenic rainbow trout should be eliminated from a wild population because of their greater susceptibility to predation rather than posing a risk to population survival, as suggested by Jonsson et al. (1996). If the transgene simply reduces age at sexual maturity or increases female fecundity or mating success, the transgene should increase in frequency.

In contrast, the combined effects of multiple fitness components have less simplistic consequences and reveal the false sense of security that transgenic viability disadvantages will preclude a transgene from spreading after it is introduced into a natural population. An age-at-sexual-maturity advantage, even more modest than the 1.125 advantage that we detected in TR medaka, is expected to offset the observed 30% reduction in viability of transgenics; thus, holding other factors constant, the transgene is predicted to spread. In contrast, the 29% female fecundity advantage of TR observed in our experiments is expected to be insufficient to counter the viability disadvantage, illustrating the overall weaker effect of female fecundity effects than age at maturity effects on transgene spread in medaka (table 4). Of course, if a female fecundity advantage occurs along with an age at maturity advantage, our model predicts that viability disadvantages are offset even further, causing a more rapid spread of the transgene. It is important to realize that the effect of transgene spread on the dynamics of the natural population (as well as on other species in the community) is uncertain in these cases. The only situation in which our model predicts population extinction is when transgenes increase mating success and simultaneously reduce offspring viability (Muir and Howard 1999).

Assessing the ecological risk of a transgene release is complex not only because a number of fitness components may be involved but also because multiple outcomes are possible for any transgene. Each gene construct used to transform each species or even the same construct in different fish of the same species may produce a unique risk (Chen et al. 1994). Several reasons underlie such variable outcomes, including alternative insertion sites and copy number of the transgene, genetic regulatory mechanisms, the effect of transgenes on growth rate, and the scale and frequency of their introduction into natural populations (Hallerman and Kapuscinski 1990, 1995). Furthermore, the fitness effects may be sensitive to environmental conditions. For example, the relative fitness of the transgene may depend on the type and diversity of predators present. The complexity of the problem should not deter further study, however. Conducting thorough investigations on all fitness components under near natural environmental conditions provides our only hope of assessing the risk of transgene spread. Given the demonstrated adverse effects of exotic introductions of nontransgenic organisms (Bright 1996; Richter et al. 1997), we need to proceed with caution before introducing transgenic organisms into nature. We offer our model as a general methodology to regulatory agencies to use in assessing risk in other transgenic organisms.

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