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Review

Assessment of possible ecological risks and hazards of transgenic fish with implications for other sexually reproducing organisms

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Abstract

Transgenic technology is developing rapidly; however, consumers and environmentalists remain wary of its safety for use in agriculture. Research is needed to ensure the safe use of transgenic technology and thus increase consumer confidence. This goal is best accomplished by using a thorough, unbiased examination of risks associated with agricultural biotechnology. In this paper, we review discussion on risk and extend our approach to predict risk. We also distinguish between the risk and hazard of transgenic organisms in natural environments. We define transgene risk as the probability a transgene will spread into natural conspecific populations and define hazard as the probability of species extinction, displacement, or ecosystem disruption given that the transgene has spread. Our methods primarily address risk relative to two types of hazards: extinction which has a high hazard, and invasion which has an unknown level of hazard, similar to that of an introduced exotic species. Our method of risk assessment is unique in that we concentrate on the six major fitness components of an organism's life cycle to determine if transgenic individuals differ in survival or reproductive capacity from wild type. Our approach then combines estimates of the net fitness parameters into a mathematical model to determine the fate of the transgene and the affected wild population. We also review aspects of fish ecology and behavior that contribute to risk and examine combinations of net fitness parameters which can lead to invasion and extinction hazards. We describe three new ways that a transgene could result in an extinction hazard: (1) when the transgene increases male mating success but reduces daily adult viability, (2) when the transgene increases adult viability but reduces male fertility, and (3) when the transgene increases both male mating success and adult viability but reduces male fertility. The last scenario is predicted to cause rapid extinction, thus it poses an extreme risk. Although we limit our discussion to aquacultural applications, our methods can easily be adapted to other sexually reproducing organisms with suitable adjustments of terminology.

Introduction

Transgenic technology is rapidly developing as techniques for transforming fish and other organisms improve. Current limitations of this technology lie in construct development. These limitations include: stability, position independence, regulation, tissue for expression, and equally important, which genes to insert. Our information base on all of these aspects is improving rapidly as genome characterizations of humans and other organisms become available. Soon gene constructs will be possible for nearly any use and species. However, even if the biological limitations of transgenic technology are overcome, the problem of public acceptance persists.

Consumers and environmentalists remain wary of the safety of biotechnology in agriculture. Research is needed to increase consumer confidence and to alleviate this concern. The first step in this process is to develop a risk assessment methodology that is agreed upon by scientists. If scientists cannot agree on a unified methodology for testing, the public will reject transgenic technology on the basis of uncertainty. This goal can best be accomplished using a thorough,

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unbiased examination of risks and hazards associated with agricultural biotechnology.

In this paper, we review discussion of possible hazards of transgenic fish and extend our approach to predict risk (Muir & Howard, 1999, 2001a,b). Our method of risk assessment is unique in that we concentrate on the six major components of an organism's life cycle to determine if transgenic individuals differ in survival or reproductive capacity from wild type. The specific mechanisms (e.g., physiological, behavioral, immunological) that underlie an observed difference in any net fitness component need not be identified to assess risk, thus saving considerable time and expense. Our approach then combines estimates of the net fitness parameters into a mathematical model to determine the fate of the transgene and the affected wild population.

Potential ecological risks and hazards

The escape or introduction of transgenic fish into natural communities is a major ecological concern (Tiedje et al., 1989; Kapuscinski & Hallerman, 1990, 1991; Hallerman & Kapuscinski, 1992). For risk assessment it is important to distinguish between 'risk' and 'hazard'. A hazard is a defined harm resulting from exposure to a material which may bring about the harm, i.e. a hazardous material. Risk of the hazard is defined as the likelihood of the hazard being realized, and is the product of two probabilities: the probability of exposure, P(E), and the probability of the hazard resulting given the exposure has occurred, P(H/E), i.e. Risk = P(E) x P(H/E). Thus for example, to compute expected risk of death (or other hazard) to children due to consumption of lead paint, the probability a child will consume lead paint (the exposure) must be determined as well as the probability of death (or other hazard) given the paint is consumed. The product of these two probabilities is then summed over all children in the population to obtain an estimate of total risk. We define transgene hazard as species extinction, displacement, or ecosystem disruption and transgene exposure as the probability a transgene will spread into a natural populations. We define transgene risk as the probability of exposure times the probability the hazard will result given exposure has occurred.

To show lack of risk from transgenic fish (or other transgenic organisms) either the exposure or hazard given exposure must be zero; that is, $P(E) = 0$ or $P(H/E) = 0$. For what follows we do not attempt to predict all possible hazards. Our methods primarily address risk as related to two types of hazard: those which might cause the local extinction of their own species (extinction hazard) (Muir & Howard, 1999), or those which might displace or disrupt other species (invasion hazard) (Muir & Howard, 2001b).

An extinction hazard could result because of opposing pleiotropic effects of the transgene on net fitness components, one of which drives the gene into the population, such as mating success, while the other reduces the number of offspring surviving to sexual maturity, such as juvenile viability, and eventually drives the local population to extinction. Although the hazard is apparent with an extinction risk, the entire hazard to the community affected depends on the consequences of the loss of the species, and may not be known for decades or centuries.

Invasion hazards could result because transgenic individuals possess some novel or enhanced fitness advantage over their wild-type conspecifics that causes them to be a better competitor, predator, or less attainable prey. In this regard, they are similar to introduced species (Regal, 1986; Tiedje et al., 1989). Hindsight tells up that when an exotic species expands into a territory, they can cause harm by displacing species and causing ecosystem disruption (Drake & Mooney, 1986; Lodge, 1993; Bright, 1996). The local extinction of a conspecific population could also have a disruptive effect on other species in a community such as releasing competing species from resource competition or prey species from predation; additionally, the survival of predatory species that depend on the eliminated species could be threatened. Thus, both extinction and invasion hazards could have a cascading impact on other species in the community with an unpredictable level of hazard. However, there are some cases where the introduction of an exotic species has been beneficial (or at least produces no harm), such as the European honey bee. Therefore, the only way to ensure that there is no harm to the environment is to release only those transgenic organisms whose fitness is such that the gene will not spread, that is $P(E) = 0$, in which case the hazard, P(H/E), is irrelevant because the transgene is lost from the population.

Unfortunately, for an exotic organism it is almost impossible to determine either the risk of species spread or hazard to the environment before introduction because of the nearly infinite number of direct and indirect biotic interactions that occur in nature. Fortunately in this regard, transgenic organisms are more similar to subspecies than to true exotic spe-

cies because transgenics usually can mate with their non-GM relatives. This distinction does not necessarily reduce the possible environmental impact (hazard) but does allow a more accurate assessment of the possible exposure (gene spread) because the non-GM relatives provide a standard with which to compare GM organisms.

We suggest that extinction and invasion hazards result from the pleiotropic action of transgenes on different fitness components of individuals and disagree with the argument that transgenic fish pose little environmental hazard because they typically have some viability disadvantage (e.g., Knibb, 1997; Dunham, 1999). The latter claim focuses on a single fitness component (viability) and thus ignores critical interactions between different fitness components. In particular, pleiotropic effects are of concern when transgenes enhance some fitness component(s) and disrupt others.

Initially, we examined the risk of gene spread associated with a transgene that enhanced mating success and reduced juvenile offspring viability (Muir & Howard, 1999). We modeled a situation in which a few transgenic individuals entered a large wild-type population. Our deterministic equations showed that a transgene introduced into such a population could spread rapidly as a result of enhanced mating advantage resulting in high risk, but the reduced viability of transgenic offspring could cause the eventual local extinction of both wild-type and transgenic genotypes (extinction hazard). Evolutionary biologists have long recognized that mating advantages due to sexual selection could result in species extinction (Lande, 1981). Transgenes that increase mating success at the expense of the viability of both male and female offspring should cause population extinction even more rapidly than that expected from sexual selection on naturally occurring genes because the latter primarily influence secondary sexual traits in males, hence only affect viability of sons. Results of Devlin et al. (2001) provide support that transgenic fish have the potential to produce an extinction hazard. They showed that GH-transgenic rainbow trout were both larger at sexual maturity and lower in viability than their wild-type sibs. Although the mating success of transgenic males relative to wild-type males is presently unknown in rainbow trout, large body size is known to enhance male mating success in many salmonid species (Jones, 1959; Schroder, 1982; Jarvi, 1990; Groot & Margolis, 1991).

More recently, we explored model predictions when transgenes reduce viability at the juvenile stage but provide an advantage either by accelerating the age at sexual maturity or by increasing female fecundity (Muir & Howard, 2001b). We evaluated estimates of fitness components that we obtained experimentally using hGH-transgenic Japanese medaka (*Oryzias latipes*) (transgenic fish had a 30% reduction in juvenile viability, a 12.5% reduction in age at sexual maturity advantage, and a 29% increase in female fecundity relative to wild type). Our model predicted that advantages in both age at sexual maturity and fecundity are sufficient to overcome the viability disadvantage produced by the transgene producing a risk of an invasion hazard.

As in our earlier work, we found that predictions based on only one fitness component were simplistic and greatly misleading (Muir & Howard, 2001b). Transgenes either go to fixation or are lost depending on whether they provide a fitness advantage or disadvantage, respectively. More realistic predictions are obtained when the effect of transgenes on multiple fitness components are considered, not only because genes (including transgenes) are often pleiotropic in their phenotypic effect, but also because the outcome of such pleiotropy on net fitness is the primary determinant of gene spread (exposure) and the potential for hazard. Thus, any attempt to predict the risk associated with a transgene based on a single parameter (Maclean & Laight, 2000) is seriously flawed.

At issue is how to assess the likelihood (risk) of extinction and invasion hazards. To accomplish this goal, a thorough evaluation of how fitness components of transgenic individuals compare to those of wild-type individuals is needed. Current standards do not specify which criteria are critical to determine environmental risk. For example, Guillen et al. (1999) report experiments 'performed to evaluate the behavior of transgenic tilapia in comparison to wild tilapia as a way to assess the environmental impact of introducing transgenic tilapia into Cuban aquaculture'. Based on feeding motivation, social dominance status, and adaptation to seawater, they concluded that under 'the conditions found in Cuba, (there are) no environmental implications for the introduction of this transgenic tilapia line...'. Thus, based only on a few factors that might indirectly influence survival and perhaps mating success, they conclude that GH-transgenic tilapia are safe for commercial production. These shortcomings dictate the need for a more system-wide, integrative approach to predict risk.

A critical prerequisite for an introduced transgenic organism to pose a long-term ecological hazard is that

the initially rare transgene must spread in a natural population; thus, predicting (or monitoring) changes in transgene frequency in a population is required. Thus, to assess exposure (not hazard) to transgene spread, it is only necessary to determine the conditions under which a transgene will increase in frequency. This conclusion assumes that the native population is not already in danger of extinction. For small populations, stochastic events and introduction of maladapted genes could endanger the population further.

Alternatively, an ecological hazard can occur when transgenic organisms do not escape and spread, but are artificially maintained and reintroduced into the environment periodically such as with farming operations. In this case, natural selection does not regulate transgene frequency, rather forced crossings and other breeding schemes maintain the transgene. A controversial example of such a hazard is the effect of a pesticide transgene in maize on insect populations (Losey et al., 1999; Hansen & Obrycki, 2000; but see Wraight et al., 2000). Assuming the transgene does not enter into natural populations, the potential ecological hazard is known, that is if a non-target, lepidopteran larvae consumes Bt maize they will perish. However, the exposure is unknown, that is to what extent do non-target organisms consume Bt maize or pollen. The work of Losey et al. (1999) only established the hazard; scientists and the media confused their results with risk which requires knowledge of both the exposure and the hazard.

There are two mutually exclusive outcomes for the introduction of transgenic organisms into nature: the frequency of the transgene will decline through time and eventually be eliminated from the population (no exposure), or the transgene will increase in frequency (high exposure) and use result in either extinction or invasion hazards. Determination of exposure is a two-step process: identification and estimation of critical fitness components for alternative genotypes, followed by development of a model that incorporates these estimates and predicts change in transgene frequency.

Net fitness component approach

Elsewhere, we present a comprehensive method to determine environmental risk of transgenic fish that we call the 'net fitness approach' (Muir & Howard, 2001a,b). This approach is based on six net fitness components or critical control points that influence gene spread: juvenile viability, adult viability, age at sexual maturity, female fecundity, male fertility, and mating advantage. A net fitness approach was initially advocated by Prout (1971a) who showed that the entire life cycle needs to be studied to determine overall fitness; incomplete information would result in erroneous population predictions. Prout (1971a) also emphasized that it is not necessary to study every aspect of the life cycle of an organism to determine overall fitness. Instead, aspects of the life cycle could be grouped into a small number of net fitness components.

Prout (1971a) suggested three net fitness components corresponding to sexual, fecundity, and zygotic (juvenile viability) selection. The various processes that influence each fitness component (e.g., predator avoidance, disease resistance, feeding efficiency, swimming speed, social dominance, and foraging ability for the viability fitness component) need not be demonstrated, as only the 'bottom line' of their effect is important. Likewise, factors that influence mating success such as mate attraction, nest site acquisition and protection, and mate competition, could all be subsumed under the sexual fitness component.

Prout (1971b) later showed that estimates of fitness components could be incorporated into a recurrence equation to predict mutant allele frequencies in experimental populations of fruit flies (*Drosophila melanogaster*). We (Muir & Howard, 2001a,b) extended Prout's (1971a,b) methods to include male fertility, age at sexual maturity, and split zygotic viability into juvenile viability (survival to sexual maturity) and adult viability (i.e., longevity, thus the number of breeding episodes possible in a lifetime). We provided methods to estimate these parameters, and developed a recurrence equation to predict population dynamics and transgene frequency trajectories. We have also conducted sensitivity analyses to examine the relative effect of each net fitness component on the predicted rate of spread of a transgene (Muir & Howard, 2001b).

Extinction and invasion hazards resulting from opposing pleiotropic effects of the transgene on specific fitness components

In this paper, we further explore how the effects of transgenes on multiple fitness components might influence transgene spread (exposure) and thus extinction and invasion hazards. In particular, we consider the consequences of transgene effects on fitness components that were not observed in our experiments on

Japanese medaka, but are possible in other sexually reproducing organisms. We examine these hypothetical situations because the range of new transgenic organisms is unlimited, and it is constructive for those developing such organisms to be able to anticipate how they could pose a hazard. First, we consider consequences when transgenic males have a mating advantage but the viability disadvantage occurs only in the adult stage, and compare these results to those obtained when the viability disadvantage occurs only in the juvenile stage. We then examine consequences when transgenes *increase* adult viability but reduce fertility of transgenic male offspring. Lastly, we consider the consequences when a transgene increases adult viability and mating success but reduces male fertility.

Model predictions

The model is presented in detail elsewhere (Muir & Howard, 2001b). For the wild-type population, we use estimates of the six fitness components from medaka (Muir & Howard, 2001b). The adult and juvenile viabilities are adjusted to produce a stable population size, that is the number of replacements needed to offset mortality was in balance. Parameter values of the different fitness components for wild-type individuals were: juvenile daily viability = 93.1%, adult daily viability = 95.0%, female fecundity = 8.8 eggs/spawn, male fertility = 1.0, age at sexual maturity = 64 days, and mating advantage = 1.0. To assess the potential for transgene spread, we assigned transgenic individuals a wide range of parameter values for two or three fitness components at a time while holding the others equal to those of wild type.

All results are presented graphically (Figures 1–4) in which two fitness components are plotted against each other and areas corresponding to fitness component combinations that result in risk (with possible extinction or invasion hazard), and minimal risk (transgene elimination) are identified. Within the area in which extinction hazard is predicted, we provide a series of contours that indicate how many generations are required for extinction to occur, thereby indicating the severity of hazard.

Transgenes increase male mating success but reduce juvenile viability: Trojan Gene I

The range of daily viabilities that we considered for transgenic juveniles was between 80.0 and 93.1%

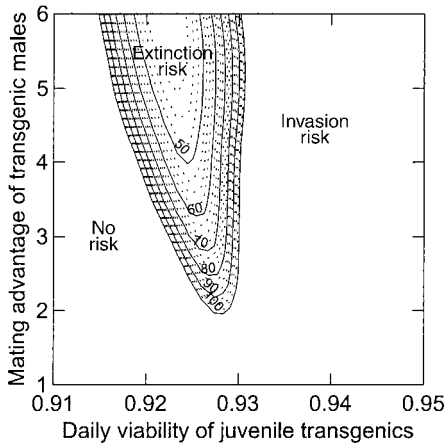

Figure 1. Extinction and invasion risks as a result of antagonistic pleiotropic effects when transgenes increase male mating advantage and reduce juvenile viability. For comparison, juvenile viability for wild type equals 0.913.

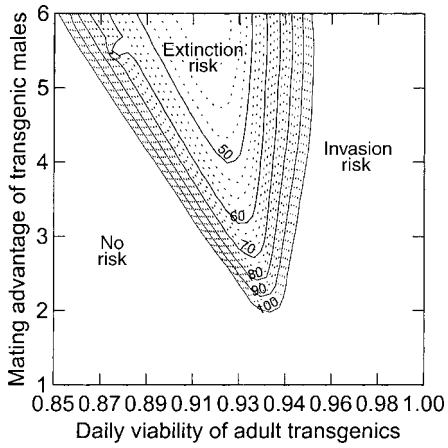

Figure 2. Extinction and invasion risks as a result of antagonistic pleiotropic effects when transgenes increase male mating advantage and reduce adult viability. For comparison, adult viability for wild type equals 0.95.

(corresponding to between 0.1 and 10.3 in 1,000 individuals surviving from birth to sexual maturity, respectively). The relative mating advantages for transgenic males ranged from 1.0 to 6.0 times that of wild type males.

Daily viabilities that could produce an extinction hazard ranged between 91.5 and 93.0% (Figure 1), which corresponds to between 3 and 9.6 in 1,000 fry surviving to sexual maturity, respectively. We previously reported this type of hazard as a Trojan Gene effect (Muir & Howard, 1999) because the transgene appears to have a desirable commercial effect but has the potential to bring about the destruction of the population. If transgenic males

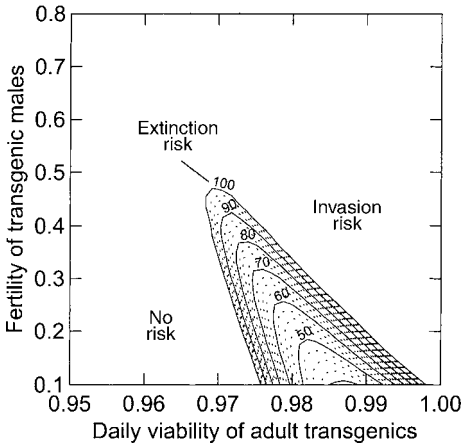

Figure 3. Extinction and invasion risks as a result of antagonistic pleiotropic effects when transgenes decrease male fertility and increase adult viability. For comparison, adult viability for wild type equals 0.95.

have a three-fold relative mating advantage, an extinction hazard is predicted if transgenic offspring have a daily juvenile viability between 92.2 and 92.8% which corresponds to between a 20 and 52% reduction in transgenic fry reaching sexual maturity compared to wild type. Extinction hazards were predicted to occur in less than 50 generations if transgenic males had a mating advantage greater than four times that of wild type males and transgenic juveniles had a daily viability between 92.0 and 92.6% (Figure 1).

Invasion hazards occurred when viability was greater than that of wild type (> 93.1%) and the rate of spread is accelerated by a mating advantage. In general, a mating advantage can overcome a relatively wide range of viability disadvantages. If the viability disadvantage is moderate, an extinction hazard is predicted; with no viability disadvantage an invasion hazard is predicted.

Transgenes increase male mating success but reduce daily adult viability: Trojan Gene II

Although we only observed a reduction in juvenile viability in transgenic medaka, a viability reduction specific to adults might occur in other fish species (e.g., if transgenic adults are slower swimmers than wild-type adults; Farrell et al., 1997) and cannot escape predators or successfully return to their spawning grounds.

The 95.0% daily viability of wild-type adults results in 37 out of 1,000 individuals surviving for 64 days beyond the age of sexual maturity. If transgenic males possess a mating advantage, an extinction hazard is predicted if transgenic adults have a daily viability of between 84.0 and 94.0% (Figure 2), which corresponds to between 0.01 and 19 in 1,000 adults surviving, respectively, or a 99.6 and 51% reduction in the number of transgenic individuals living for 64 days beyond sexual maturation relative to wild-type individuals, respectively.

If transgenic males have a five-fold mating advantage relative to wild-type males, the range of daily adult viabilities that produce an extinction hazard in 50 or fewer generations (89.9–93.0%, corresponding to between 1 and 10 in 1,000 surviving; Figure 2) is four times greater than that predicted when transgenic and wild-type offspring differ only in juvenile viability (92.0–92.6%, corresponding to between 5 and 7 in 1000 surviving; Figure 1), indicating that there is a greater extinction hazard if the transgene reduces adult viability rather than juvenile viability. In the latter case, selection acts more quickly to remove the transgene from the population, thereby reducing the possibility of an extinction event.

In contrast, an invasion hazard is more likely if the transgene *improves* juvenile viability rather than adult viability, this result occurs because juvenile viability is already much lower than that of adults for wild-type individuals (93.1% compared to 95.0%) and an improvement in juvenile viability affects more individuals because juveniles make up the majority of the population.

Transgenes increase adult viability but reduce male fertility: Trojan Gene III

Although we found no empirical support for a fertility decline in male medaka, Rahman and Maclean (1999) report that 'low sperm production was observed in all transgenic lines of tilapia, and in some cases this can lead to complete absence of sperm production'. Increased viability of transgenic adults could occur if the transgene increases immune function or confers resistance to some disease or pathogen.

The effect of increased adult viability is most easily visualized in terms of average age of the adult population. In Muir and Howard (2001b), we assume a viability model of the form

$$N_a = v^a N_0,$$

where a is age, v is adult viability, and N_0 is the population size at sexual maturity; thus, the average age A of the adult population equals

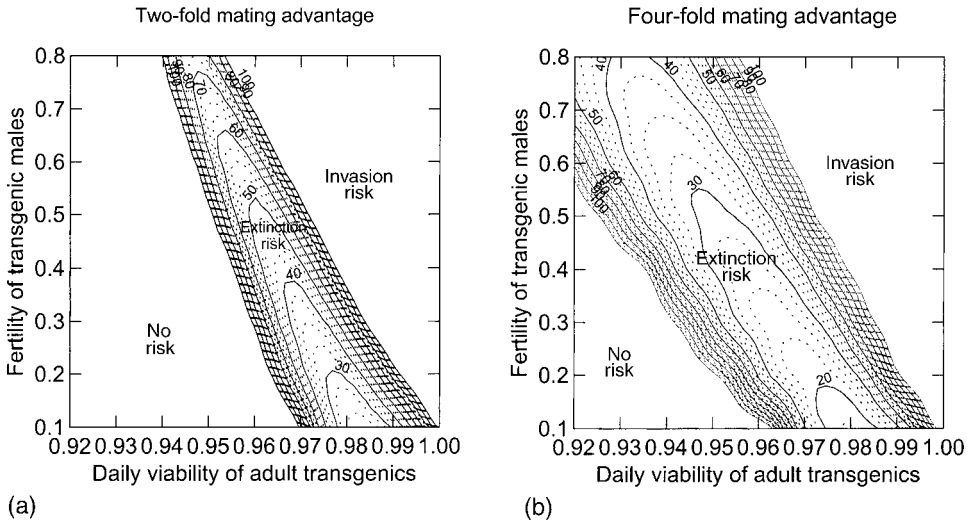

Figure 4. Extinction and invasion risks as a result of antagonistic pleiotropic effects when transgenes decrease male fertility and increase both male mating success and adult viability. For comparison, adult viability for wild type equals 0.95. (a) transgenic males have a two-fold mating advantage; (b) transgenic males have a four-fold mating advantage.

$$A = \frac{\int_{a=65}^{a=\infty} a v^{(a-64)}}{\int_{a=65}^{a=\infty} v^{a}}.$$

Which is simply the average number of age classes from sexual maturity (64 days of age) through the oldest age class, weighted by the number of individuals in each age class, over the total number of individuals. The average age of adult wild-type medaka with a daily viability of 95.0% is 84.1 days.

An extinction hazard is predicted if the transgene increases, rather than reduces, the viability of adult transgenics, if the transgene also reduces the fertility of transgenic males relative to wild-type males (Figure 3). It is important to note that, in Figure 3, the daily viability of wild-type adults equals 95.0%; thus, the viability of transgenic adults exceeds that of wild-type adults to the right of the origin. Such an extinction hazard is somewhat counterintuitive and results because the increased viability of adult transgenics increases the average age of transgenic adults relative to wild-type adults in the population, thereby providing a longer period for reproductive activity for transgenic males; however, this activity produces few offspring because of their low fertility. For example, if transgenic males have only 10% of the fertility of wild-type males, they waste most of the reproductive effort of their mate, but the offspring they do produce will have a much higher adult viability (97.4–99.5%) than wild-type offspring; as a result, the average age of transgenic offspring will be between 102 and 725 days of age and transgenic sons would obtain 20–863% more matings than wild-type males. In addition, transgenic females also have increased survival relative to wild-type females, but have no fertility disadvantage; half of their offspring will be male, however, and those sons that possess the transgene will have reduced fertility.

Thus, as the transgene increases in frequency in a natural population, transgenic adults begin to outnumber wild-type adults; while the differential representation of adult transgenics should increase with age, the few transgenic individuals that are alive at very old age classes comprise just a small fraction of the population. Thus, the spread of the transgene is not the result of an extended life span of transgenics per se, but because there are more transgenics alive to reproduce in the younger age classes than wild-type individuals. The consequences of mating differ between transgenic males and females, however. Female transgenics obtain only an advantage from the transgene (increased viability), no disadvantage; therefore, reproduction by female transgenics is a potent force increasing the frequency of the transgene. In contrast, male transgenics sire few offspring as a result of mating due to their low fertility. As the frequency of transgene increases, more and more males in the population will be transgenic and population productivity will dwindle due to their low fertility.

The extinction hazard produced by the interaction of male fertility and adult viability fitness components

is similar to that obtained using the sterile male technique for biological control of pest insects such as the screw worm (Whitten & Foster, 1975). However, in the latter program males are completely sterile and must be reintroduced repeatedly to cause extinction. In effect, the viability of sterile males is near 1.0 (due to repeated introduction) while male fertility is 0%. For this to occur with transgenes, fertility of transgenic males must be greater than 0% and adult viability of transgenic offspring must exceed that of wild type for the gene to spread in the population; however, the low fertility of transgenic males leads to the population demise. Our model predicts that the lower the fertility of males (20% or less), the more severe the hazard of population extinction (Figure 3) provided that the adult viability of transgenics is high (greater than 98%). Such population extinction as a result of the antagonistic pleiotropic effects of transgenes on viability and fertility represents a new class of Trojan Genes which suggests that attempts to reduce transgenic male fertility (Rahman & Mclean, 1999) that do not result in complete male sterility may increase hazard rather than reduce it.

Transgenes increase daily adult viability and male mating success but reduce male fertility: Trojan Gene IV

If the transgene increases growth rate and subsequent adult size, male mating success could be enhanced as a result of a size-dependent mating advantage in mate competition or mate choice, and survival ability could be enhanced if the larger transgenic adults incur less predation. Alternatively, if the transgene confers increased resistance to some pathogen (rather than an effect on growth rate), adult viability would be enhanced. Such enhancement of male health due to pathogen resistance could also affect the degree of elaboration of 'condition-dependent' male secondary sexual characters (such as pigment intensity) resulting in greater male attractiveness to females (Hamilton & Zuk, 1982; Andersson, 1986, 1994; Pomiankowski & Nee, 1991). Such condition-dependent indicators of male health have been proposed as a general mechanism for the evolution of exaggerated male characters (Hamilton & Zuk, 1982) and have received recent experimental support in a variety of taxa (Milinski & Baker, 1990; Hill, 1991; Andersson, 1994; Rintamaki et al., 2000, but see Grether, 2000). Thus, in this second scenario transgenes have no direct influence on male sexual characters, but influence their elaboration indirectly by increasing health. In either case, adult viability and mating success is increased by the transgene.

A three-way interaction among male fertility, mating success and offspring viability fitness components due to transgenes has the potential to produce extreme extinction hazard for three reasons. First, even a relatively weak (two-fold) mating advantage of transgenic males can result in population extinction (Figure 4(a)), particularly when male fertility is severely affected. In this situation, the enhanced mating success of transgenic sons greatly augments the effect of transgenic daughter reproduction to increase the frequency of the transgene in the population. Second, the range of viabilities producing a population extinction event increases as transgenic mating advantages increase (Figure 4(a), (b)). In fact, with a four-fold mating advantage, an extinction effect is predicted even if transgenic offspring have a viability disadvantage as adults (Figure 4(b)). Population extinction is predicted to occur in less than 40 generations if transgenic males have a four-fold mating advantage but suffer a 50% decline in fertility and their offspring have a 94.4% daily viability rather than the 95% daily viability of offspring sired by wild-type males (Figure 4(b)). Lastly, population extinction is predicted to occur much more rapidly with such a three-way interaction than with any combination of interacting fitness components examined thus far (Figure 4). At the extreme, if transgenic male fertility is 10% that of wild-type males but transgenic males have a four-fold mating advantage, population extinction is predicted to occur in 20 generations or less when their adult transgenic offspring have a daily viability between 97.4 and 98.6% (Figure 4(b)).

Discussion

Hazards of transgenic technology should be considered relative to current accepted practices, that is conventional breeding programs and domestication. However, there is a major difference between the product of selective breeding and transgenesis. While selective breeding is based on polygenic inheritance whereby the result is the cumulative effect of many (perhaps hundreds) of genes each with small effect (Lynch & Walsh, 1998); in contrast, transgenesis involves one gene with a major effect. An important consequence of this difference is the pleiotropic effects of the genes involved. In the selective breeding process, all the correlated traits that support enhanced

growth and reproduction, such as skeletal and vascular systems, are also selected; this is not the case in the production of transgenics.

Because of imbalances in the background genotype, transgenic organisms are often at a distinct viability disadvantage relative to a selectively bred, domesticated fish. Secondly, and more important, the Trojan Gene effect (extinction hazard) predicted by Muir and Howard (1999) is unlikely to result from selective breeding. The Trojan Gene effect is the result of a negative genetic correlation between mating success and viability. With a polygenic response, as with domesticated fish, such a genetic correlation would have to result from the sum of genes with positive and negative pleiotropic effects. Natural selection can 'break' such a genetic correlation by favoring those fish with both high viability and high mating success. With a transgene, the correlation cannot be broken, because it is the same gene product affecting both traits. Thus, the two methods of genetic modification are not substantially equivalent, although they may be governmentally regulated as such (Regulation, 1995). See Millstone et al. (1999) for further discussion of issues related to substantial equivalence.

Hazard should also be considered relative to naturally occurring mutations. There are naturally occurring mutants for size, such as dwarfism and gigantism in several species which have not caused any apparent ecological harm. However, such mutations usually reduce (or increase) body size by at most 50–100% and certainly not in the range produced in some species by transgenesis. Devlin et al. (2001) produced transgenic rainbow trout that were on average 1,700% larger than wild-type trout at sexual maturity. With such an extreme size differential, transgenic and wild-type individuals may not even recognize each other as conspecifics much less be physically capable of crossing. As a result, a successful establishment of transgenic rainbow trout in nature may well represent the instant formation of a new species or evolutionary line, which may produce a significant environmental hazard.

Furthermore, with the advent of new tools for genetic engineering through 'molecular breeding by DNA shuffling' (Crameri et al., 1998; Soong et al., 2000; Tobin et al., 2000), entire functional chromosome segments can be recombined. With molecular breeding, the mutations are a result of recombination between similar genes of different species, that is between functional homologs. The resultant recombinants are cloned and selected while in cell lines for gene expression by use of a test assay. In this way, mutations are not a random process but directed evolution producing functional genes with products far outside the norm of what is possible with natural mutations.

Several biological attributes of fish contribute to potential for risk using transgenic technology. These include high dispersal ability with low probability of recapture when out in nature, high reproductive potential, and a reasonable chance of successful establishment in nature because their relatively short history of domestication retains most of their 'wild phenotype'. In addition, insertion of growth hormone gene constructs in fish is of concern because accelerated growth (particularly in combination with attainment of large adult size) has many pleiotropic effects. For example, as reported in a recent report by the Royal Society of Canada (2001),

'Growth hormone constructs in salmonids have been shown to influence smoltification (Saunders et al., 1998), swimming ability (Farrell et al., 1997), gill irrigation (Devlin et al., 1995a,b), feeding rates (Abrahams & Sutterlin, 1999; Devlin et al., 1999), risk-avoidance behaviour (Abrahams & Sutterlin, 1999), disease resistance (Devlin et al., 1995a,b), body morphometry (Devlin et al., 1995a,b), pituitary gland structure (Mori & Devlin 1999), life span (Devlin et al., 1995a,b), and larval developmental rate (Devlin et al., 1995b) These phenotypic changes to morphology, physiology, and behaviour could theoretically have both positive and negative effects on fitness'.

Our approach is to consider these manifold phenotypic outcomes in the context of the six net fitness components that they affect. Such an approach is not only more tractable but also goes directly to the bottom line: can transgenes spread if introduced into nature and what might be their effect?

An estimation of the dispersal potential of transgenic fish can be obtained by considering the accidental escape of farmed fish into nature. During the 1990s, escaped farm fishes were estimated to comprise 20–40% of the salmon recorded over large areas of the North Atlantic (Hansen et al., 1999) and more than 80% of the salmon in some Norwegian spawning populations (Lund et al., 1991; Fiske & Lund, 1999). During 1994–1998, an average of 43,863 Atlantic salmon escaped into British Columbia waters (Noakes et al., 2000) and an estimated 32,000–86,000 farmed Atlantic salmon escaped from netpens between January and September 2000 (Mickelburgh, 2000; Sullivan, 2000). Farmed Atlantic salmon can spawn

successfully in natural habitats (Webb et al., 1991; Gross, 2000; Volpe et al., 2000), and in one Canadian river, the number of farmed fish returning to spawn during 1992–1999 was 2–8 times that of wild salmon (Carr et al., 1997; Whoriskey, 2000). Studies of farmed fish could also portent some of the effects of accidental introductions on wild populations. After a large-scale experiment on the lifetime success and interactions of farmed and wild Atlantic salmon, Fleming et al. (2000) concluded that annual invasions of farmed fish could reduce population productivity, disrupt local adaptation, and reduce the genetic diversity of wild salmon populations. Consequences of an invasion of transgenic fish on a natural population are unknown. The large number of pleiotropic phenotypic effects of transgenes (particularly growth hormone transgene constructs) create the potential for even more devastating effects than farmed fish, and the optimistic view that a supposed reduced overall fitness of transgenics in nature would result in little or no effect if introduced is untested.

In our study, we address risk as related to two types of hazards: extinction hazard when the pleiotropic effects on fitness components cause the transgene to spread in a natural population and lead to a local extinction event, and an invasion hazard when the positive effects of transgenes on some fitness components cause the transgene to spread but the negative fitness effects are insufficient to cause population extinction. In the latter case, the wild population will be transformed into a transgenic population which may then differ in its ecological relationships with other species in the community that may lead to the extinction of other species. In contrast, if transgenes reduce some fitness components sufficiently, then an introduced transgene may be lost from the population even if it confers some advantage to another fitness component, resulting in no risk provided that the wild population is sufficiently large.

We used a range of parameter values when modeling each set of fitness components for two reasons. First, this generalizes our predictions to different species and different transgene constructs. Second, even the same construct in the same species may produce a unique risk (Chen et al.,1994). Such variation can result from position effects of transgene insertion, copy number of transgenes inserted, and the genetic background of the wild-type line used in transgenesis. Any interaction between the genetic background of the population receiving a transgene on various fitness components could increase uncertainty about the type of hazard and magnitude of risk. That is, although fitness components can be assessed for the original strain of fish made transgenic, if these fish are introduced into a population with a different genetic background, the resultant fitness effects due to the transgene may be quite different. For example, if a transgenic line is produced that has no measurable viability reduction in the laboratory, the spread of the transgene into a wild population of a different stock may greatly alter viability of hybrids. Indeed, the genetic background could be a key to potential risk. The report of the Royal Canadian Society (2001) notes that highly domesticated plants (e.g., corn, soybeans) rarely become weeds in natural settings, because 'cultivated species have been genetically crippled through intense artificial selection'. Thus, they conclude that the more wild (i.e., less domesticated) a stock is, the greater the environmental risk when using that stock to make transgenics. This result is of particular ecological concern for GH-transgenic salmonids where the transgene has a much greater effect in a wild-type background than cultivated lines (Devlin et al., 2001).

In an earlier paper, we predicted that a transgenic release could effect a local population extinction event if transgenes increased success in the mating advantage fitness component but reduced offspring viability (specifically at the juvenile stage) for a broad array of parameter values for each of the two fitness components (Muir & Howard, 1999). We now refine the viability effects into a daily scale to conform to the biology of our main study organism, Japanese medaka (but which could be on any appropriate temporal scale for other fish species), consider viability reduction that occurs either exclusively at the adult stage or the juvenile stage, and model the consequences of transgenes that enhance adult viability but reduce male fertility. We also obtain model predictions when transgenes affect three fitness components simultaneously: when they increase adult viability and enhance male mating success but reduce male fertility.

Selection against transgenes that reduce juvenile viability substantially should be intense. For example, if the daily viability of juvenile transgenics is 91% (as opposed to the 93.1% daily viability of wild-type juveniles) even a six-fold mating advantage is insufficient to maintain the transgene in the population (Figure 1). Daily viabilities between 91.5 and 93% could result in an extinction hazard provided that transgenes can be maintained by an appropriately large male mating advantage (Figure 1). Mortality

differences between farmed Atlantic salmon and their wild-type counterparts are more common during the juvenile stage (McGinnity et al., 1997; Einum & Fleming, 2000; Fleming et al., 2000), and early viability of transgenic juvenile rainbow trout may be very low (Devlin et al., 2001) and strain specific. If mortality differences between wild and transgenic fish occurred primarily at the adult stage rather than the juvenile stage, however, a larger range of adult viabilities due to the transgene could be maintained in a population given an appropriately high male mating advantage (Figure 2).

The effect of GH-transgenes on a male mating advantage in species such as salmonids is complicated by the employment of alternative mating tactics by males. In many salmonids, small precociously mature males employ a 'sneak' mating tactic to fertilize eggs (Gross, 1985, 1996). In Atlantic salmon sneaking males weigh only 0.15% of the average body mass of the dominant anadromous males that aggressively defend nesting females (Gage et al., 1996), and sneaking males obtain a fertilization success that is disproportionate for their body size (Hutchings & Myers, 1988). Individual sneaking males may fertilize 26% of the eggs when in competition with a dominant male, and groups of 12 sneaking males may fertilize up to 40% of the eggs (Thomaz et al., 1997). Although small body size provides an advantage in matings by sneakers (Gross, 1985), such selection is balanced by selection favoring larger body size in males that adopt this mating tactic. In coho salmon larger fry are more likely to adopt the sneak phenotype than smaller fry (Bilton, 1980), and in both Atlantic and masu salmon larger sneaking males have greater reproductive success (Thomaz et al., 1997; Koseki & Maekawa, 2000).

Devlin et al. (1995a) found that GH transgenic coho salmon undergo precocious parr-smolt transformation approximately 6 months in advance of their non-transgenic siblings. Two critical questions must be answered for such species: do GH-transgenes predispose a male for one alternative mating tactic or the other, and what is the effect of male body size on mating success given that a transgenic male adopts either strategy? Gross (1985) shows that faster growing wild-type male coho salmon mature precociously and adopt the sneaking mating tactic. Will the same be true for the growth-accelerated transgenic males? Alternatively, if transgenic males tend to develop into dominant males rather than sneaks, studies on several salmonid species indicate that their large body size should confer a mating advantage (Jones, 1959; Schroder, 1982; Jarvi, 1990; Groot & Margolis, 1991; see also review by Muir & Howard, 2001a).

If transgenes increase offspring viability rather than reduce it (e.g., by conferring disease resistance or cold tolerance; Maclean & Laight, 2000), then such a fitness component advantage would increase the frequency of the transgene in the population. If these transgenes also reduce male fertility, as has been reported for GH-transgenic tilapia (Rahman & Maclean, 1999), our model predicts that an extinction hazard is possible, particularly if male fertility is severely reduced; alternatively, an invasion hazard may occur if male fertility is less severely affected (Figure 3). Extinction hazards predicted by our model in this case parallel the use of sterile males to eradicate pest insects (Whitten & Foster, 1975).

If transgenes increase offspring viability, reduce male fertility but increase male mating success, the model predicts a strong potential for extinction hazard (Figure 4), such hazard is still predicted even if transgenes confer no viability advantage provided the mating advantage of transgenic males is sufficiently high (Figure 4(b)).

Our modeling of transgenic risk based on six net fitness components and their potential interactions is superior to simple one-factor models (Maclean & Laight, 2000) for at least two reasons. First, transgenes, particularly growth hormone constructs, have been demonstrated to have multiple pleiotropic effects as discussed above. Simple models ignore this biological complexity. Second, some pleiotropic effects influence fitness components in a positive way, others in a negative way. That is, there is a statistical interaction between the pleiotropic effects of transgenes and different fitness components. As in any analysis involving multiple factors, if there is a significant interaction between factors, the main effect of each factor is ignored. Thus, concentrating on one factor, such as viability, as the main fitness factor to predict risk is incorrect. Furthermore, support for the predictions of our model when transgenes influence mating success and viability were obtained recently with an independently derived model (Hedrick, 2001).

Limitations of the model

The model ignores some biological complexity, such as genotype-environment interactions (GxE). Indeed, it would be erroneous to conclude that an organism tested in one environment would be safe in environ-

ments for which it has not been tested Methods of estimation and interpretation of GxE were discussed by Muir et al. (1992). A GxE interaction results if relative fitness components change depending on the environment in which they are measured. These interactions can be minor as a result of change in scale (relative fitness components remain above or below one but to different degrees), or major as a result of re-rankings of genotypes (relative fitness components change from great than to less than one, or vice versa).

To some extent the effect of these interactions on predictions of risk can be examined by sensitivity analysis whereby a range of values are used in the model, similar to the graphs we presented. Alternatively, such interactions could be determined by measuring the fitness components of transgenic and wild-type genotypes in several macro environments by varying such factors as temperature, salinity, predators, and food availability. If the prediction of risk remains relatively constant across the set of net fitness parameters from diverse environmental conditions (or are relatively insensitive to changes in parameters), the predictions are robust with clear indications of risk or lack thereof. Problems will remain if predictions differ depending on environmental conditions, in which case the transgene could eventually end up in an environment that will result in risk and containment is recommended.

Of course, no model can contain all the complexity of nature and hence all models are limited. The approach we have presented represents a best guess. However, we feel predictions for lack of risk are easy to establish and robust, that is if a transgenic organism is not predicted to become established in the relatively benign conditions established in the laboratory, it has even less of a chance in nature's less hospitable conditions. In contrast, when the model predicts risk, it is possible that no risk would actually result. The approach can therefore be used to establish when a transgenic organism is clearly not a risk, but the reverse is not necessarily true.

Extensions

Although we limited our discussion to aquacultural applications, the methods are general and can easily be adapted to sexually reproducing organisms other than fish with suitable adjustments of terminology. In plants, juvenile and adult viability, fecundity, fertility, and age at sexual maturity are all defined and measured as in animals. The primary difference in plants is the definition and measurement of mating success. Mating in plants is the successful transfer of pollen from the male part of a plant to the female part of a plant, of the same or related species. Transfer of pollen may be passive and carried by wind currents, or active and mediated by insects or other pollinating agents. In either case, the transgene may affect dispersal distance by changing aerodynamic properties of the pollen or its attractiveness to pollinators. Thus, risk assessment experiments in plants can be conducted in a similar manner to those with animals (Muir & Howard, 2001 a, b).

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