State of California

Memorandum

To: Mr. Robert R. Treanor
Executive Director
Fish and Game Commission

Date: November 25, 2003

From: **SONKE MASTRUP, Acting Director**
DEPARTMENT OF FISH AND GAME

Subject: Agenda Item for December 5, 2003 Commission Meeting, Re: Yorktown Technologies request to exempt fluorescent-transgenic zebrafish from Section 671, Title 14.

The Department's evaluation of Yorktown Technologies' request to exempt fluorescent-transgenic zebrafish from the restricted species list in Section 671, Title 14, is enclosed. Based on a unanimous conclusion from a broad spectrum of qualified scientists that transgenic zebrafish do not appear to represent a significantly greater risk to the environment than wild (non-transgenic) zebrafish, the Department is recommending approval of the exemption request.

If you have any questions or need additional information on this matter, please contact Ed Pert, Chief, Fisheries Programs Branch, by telephone at (916) 445-3616.

Enclosures

cc: Ed Pert, Chief
Fisheries Programs Branch

Evaluation of Request to Exempt Transgenic-fluorescent Zebrafish from Restricted Species Permit Requirements

Issue Statement

In May 2003, regulations became effective that identified all transgenic aquatic animals as restricted species, thus requiring a permit for their possession, maintenance, transportation, or importation. In addition, prior to issuing permits for transgenic aquatic animals, the Fish and Game Commission (Commission) is required to provide notice to the public and discuss the California Department of Fish and Game's (Department) recommendation at a regularly scheduled Commission meeting.

Yorktown Technologies, L.P., headquartered in Austin, Texas, has requested that the Commission exempt transgenic fluorescent zebrafish from the restricted species category of transgenic aquatic animals as identified in Section 671 of Title 14, California Code of Regulations. The purpose of the exemption is to allow Yorktown Technologies to legally provide fluorescent zebrafish to the aquarium and pet industry in California. Current regulations prohibit the possession of any transgenic aquatic animal without a permit. And, current regulations provide for permits only where transgenic animals are contained in secure facilities. Thus transgenic aquatic animals cannot be distributed to the public.

Background

Zebrafish (*Brachydanio rerio*, also known as *Danio rerio*) are tropical cyprinids (minnows) native to Asia, chiefly in Pakistan, India, Nepal, and Bangladesh. In the United States, they are popular as aquarium pets, and are used extensively as research animals in laboratory conditions. Although they have been available to the public and research institutions for over 50 years, no known zebrafish populations have become established in California waters (Dill and Cordone, 1997). Because of tropical temperature requirements, and the demonstrated inability to establish self-sustaining populations in waters of California, the risk that zebrafish pose to existing aquatic communities in California appears to be very low. The concern of the Department of Fish and Game (Department) is whether genetically modified or transgenic zebrafish pose any significantly greater risk to California's fish and wildlife communities than non-transgenic zebrafish.

Yorktown Technologies is requesting an exemption for red and green fluorescent genetically modified zebrafish. The green fluorescent protein (GFP) was originally extracted from a jellyfish (*Aequorea victoria*), and the red fluorescent protein (RFP) originated from (*Discosoma* sp.), an Indo Pacific relative of sea anemones and coral. DNA constructs were made by inserting a fast muscle-specific promoter gene into red and green fluorescent protein genes. These constructs were then microinjected into zebrafish embryos at the one- or two-cell stage, and the resulting fluorescent zebrafish were bred using standard breeding methods to create stable lines that exhibit the fluorescent traits. Fluorescent proteins are expressed in the skeletal muscle, and are readily visible under normal daylight. The fluorescent color is inherited by subsequent generations.

Determination of Risk to Aquatic Ecosystems

The overarching concern for the Department was to determine if transgenic-fluorescent zebrafish pose a significantly greater risk to the environment than wild zebrafish. Specifically, we were concerned about changes in temperature sensitivity, and other factors that may cause the transgenic zebrafish to be more viable than wild zebrafish, if or when they escaped from home aquaria. In addition, concerns were expressed regarding potential toxicity or allergen issues associated with fluorescent-transgenic zebrafish.

Unpublished temperature-sensitivity data were submitted to the Commission by Yorktown Technologies that indicated significantly increased sensitivity in the red strain and slightly increased sensitivity (not statistically significant) in the green strain. Increased temperature sensitivity indicates a lower tolerance to cooler temperatures.

In support of their exemption request, Yorktown Technologies submitted letters and information from several leading scientists working in the field of transgenic research. All of these scientists expressed the general opinion that fluorescent-transgenic zebrafish, as described by Yorktown Technologies, posed no greater risk to the environment than wild-type, or non-transgenic zebrafish.

The issue of potential toxicity of fluorescent proteins and possible allergic effects if transgenic fluorescent genes were consumed by other animals was also addressed. According to information submitted by Dr. Andrew Cubitt on behalf of Yorktown Technologies:

a. Fluorescent proteins occur naturally and are widely dispersed in marine ecosystems with no known harmful effects throughout the food chain.
b. Fluorescent proteins have been widely used in many cell types and organisms, and no adverse health affects have been reported.
c. Fluorescent proteins do not share any similarity in amino acid sequence to known allergens.
d. GFP fed orally to rats has not demonstrated any allergic reaction or digestive tract irritability.

Based on this information, it is highly unlikely that transgenic fluorescent zebrafish represent a toxicological or allergen risk, if allowed to escape to an ecosystem.

The opinions of eminent scientists in the field of biotechnology and transgenic fishes that were solicited by Yorktown Technologies and documented in the proposal are credible and respected by the Department. However, the Department sought the assistance of other scientists to evaluate the information provided to the Commission. Geneticists from the University of California, the Department's Wildlife Forensic Laboratory, and other Department biologists reviewed and evaluated the information submitted by Yorktown Technologies. All of the scientists solicited by the Department concurred with the opinions documented in the Yorktown Technologies proposal.

Recommendation

Based on a unanimous conclusion from a broad spectrum of qualified scientists that transgenic zebrafish do not appear to represent a significantly greater risk to the environment than wild (non-transgenic) zebrafish, an exemption is recommended for the specific fluorescent zebrafish requested by Yorktown Technologies.

Even though the environmental risk is considered low for fluorescent zebrafish, some concerns and suggestions were noted by the Department's evaluators. These are:

1. The exemption should be specific for each organism, protein, and genetic construct to prevent potential future concerns regarding a blanket exemption of fluorescent zebrafish made using different proteins or techniques.
2. Labeling of the product as "transgenic" or "genetically modified" should be required.
3. The exemption will not initiate a relaxation of current regulatory requirements.
4. In the event threats to the environment emerge as a result of new scientific information, the Department will re-evaluate the environmental risk and recommend appropriate regulatory changes.

Written comments by each evaluator for the Department are attached. Proposed regulatory language follows on next page.

Dill, W. A. and A.J. Cordone. 1997. History and status of introduced fishes in California, 1871-1996. Calif. Dept. Fish and Game, Fish Bulletin 178.

Regulatory Language Options

Subsection (b)(11) of Section 671 is amended to read:

Alternative No. 1

(11) Transgenic Aquatic Animals
Includes freshwater and marine fishes, invertebrates, crustaceans, mollusks, amphibians, and reptiles (D), excluding the following species of transgenic animals which have been determined to pose no significant threat to the native fish and wildlife of the state and are therefore permitted:

Fluorescent Transgenic Zebrafish, a genetically altered form of Zebra danio, *Brachydanio (=Danio) rerio*, produced for use in the aquarium trade using the method originally described in W. O. Patent No. WO0049150, application date August 24, 2000. The following terms and conditions apply to this exemption: written proof of the origin of the transgenic fish identified with W.O. Patent No. WO0049150 shall be in the immediate possession of any carrier of the fish, and the origin and transgenic (genetically modified) nature of the fish shall be clearly posted at the time of sale or transfer of the fish to any member of the public.

Alternative No. 2

(11) Transgenic Aquatic Animals
Includes freshwater and marine fishes, invertebrates, crustaceans, mollusks, amphibians, and reptiles (D), excluding the following species of transgenic animals which have been determined to pose no significant threat to the native fish and wildlife of the state and are therefore permitted:

Fluorescent Transgenic Zebrafish, a genetically altered form of Zebra danio, *Brachydanio (=Danio) rerio*, produced for use in the aquarium trade using the method originally described in W. O. Patent No. WO0049150, application date August 24, 2000. This exemption is specific to green fluorescent protein (GFP) originally extracted from a jellyfish (*Aequorea victoria*), or the red fluorescent protein (RFP) originating from (*Discosoma* sp.), an IndoPacific relative of sea anemones and coral. The following terms and conditions apply to this exemption: written proof of the origin of the transgenic fish identified with W.O. Patent No. WO0049150 shall be in the immediate possession of any carrier of the fish, and the origin and transgenic (genetically modified) nature of the fish shall be clearly posted at the time of sale or transfer of the fish to any member of the public.

Note: Alternative No. 2 is similar to Alternative No. 1 but No. 2 includes a brief clarifying description of the fluorescent colors in the exemption: "This exemption is specific to green fluorescent protein (GFP) originally extracted from a jellyfish (*Aequorea victoria*), or the red fluorescent protein (RFP) originating from (*Discosoma* sp), an IndoPacific relative of sea anemones and coral."

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Dr. Edmund Pert, Chief
Fisheries Program Branch
Department of Fish and Game
1416 Ninth Street
Sacramento, CA 95814

Thursday October 16, 2003

Dear Dr. Pert,

I have examined the materials provided by Yorktown Technologies regarding the exemption of fluorescent zebrafish from the transgenic species restriction. The documentation that accompanied this application was comprehensive and appropriately directed to some of the foremost authorities qualified to talk on any potential ecological risks posed by transgenic fluorescent zebrafish. A consistent theme mentioned in all these testimonials was that transgenic zebrafish are no more likely to harm the environment than wild type zebrafish as their coloration would not expected to provide a selective advantage. Further there have been no documented cases of the establishment of a zebrafish colony in California outside of the aquarium environment. As such there does not appear to be a concern regarding the establishment of transgenic zebrafish in the waters of the state of California.

One concern I have is the absence of specifics regarding which actual proteins will be covered by this exemption, and also the methodology that was used to make the transgenic fish. The request is for exemption of "fluorescent zebrafish". I would like to know exactly what proteins are covered under this request – presumably some of the better known and widely used proteins such as the green fluorescent protein (GFP), but what other specific proteins ? As you may know in the evaluation of transgenic crops, each transgenic event is independently evaluated based on the genetic elements present in the transgenic organism, and the characteristics of the introduced protein that is being expressed. Given that it feasible that these aquarium fish may be eaten by pets, rodents or even toddlers, I think some documentation of the actual proteins being covered by the exemption, and the source of each protein is appropriate as a part of the application package. This may also prevent the future "grandfathered-exemption" of fluorescent zebrafish carrying a hypothetical toxic fluorescent protein. I think it would also be appropriate to request the general details of the genetic elements present in each exempted line. There are a variety of methods for creating transgenic fish that may or may not involve the use of antibiotic selection markers, viral vectors, and/or promoters. This information is important to determine whether these additional considerations (e.g. if the fish were also expressing an antibiotic resistance protein) would alter the decision regarding exemption. My suggestion would be to document which specific proteins and genetic constructs are covered by the exemption so as to prevent potential future concerns regarding a blanket exemption for ornamental "fluorescent zebrafish" made using different proteins or techniques.

Sincerely,

Alison Van Eenennaam

Alison Van Eenennaam, Ph.D.
Animal Biotechnology and Genomics Cooperative Extension Specialist

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October 15, 2003

Edmund Pert, Chief
Fisheries Programs Branch
Department of Fish and Game
1416 Ninth Street
Sacramento, CA 95814

Dear Ed:

I have read through the materials you provided regarding the application by Yorktown Technologies for an exemption from the regulations regarding the granting of a permit to import, transport, or possess transgenic aquatic animals in California, in this case fluorescent zebra fish.

You have already received input from some very qualified fisheries geneticists and biologists (e.g., Hallerman, Essner, Hackett, Muir, Cubitt) regarding the risk factors associated with permitting these fish to be used in the aquaculture trade in California. I concur with the conclusions of these scientists that prior research and experience suggests that (1) these fish will be released into the environment repeatedly, (2) they will probably not become established except in rare warm water environments where no predators are present, (3) there is almost no risk of the the spread of the trasnsgene into other fish (or any other organism), and (4) there will be a demand for this product in the trade. In other words, there is little downside to granting this exemption.

My only concern as a fish geneticist is where this relaxation of the regulations may lead. My students and I work on the conservation of many native California fishes. We currently work with the Department of Fish and Game to determine the extent of introgression of genes from transplanted rainbow trout into the gene pools of golden and redband trout (native California salmonids). The damage from this introgression is extensive. It would truly be unfortunate if somewhere down the line one of the kinds of transgenic fish introduced into California was a salmonid.

Sincerely,

Bernie May

Bernie May, PhD, GVL Director

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Review of Request for Zebrafish Transgenic Permit Exemption

Michael Lacy
California Department of Fish and Game
Native Anadromous Fish and Watershed Branch
October 17, 2003

I was asked to review a petition from Yorktown Technologies, LP, asking the California Fish and Game Commission to amend Section 671, Title 14, CCR, to exempt transgenic fluorescent zebrafish [*Brachydanio (=Danio) rerio*] from the restricted species list. Under regulation, the FGC may grant an exception to the regulation requiring a transgenic fish (restricted species) permit if it is determined that doing so shall not pose a significant risk to the waters or wildlife of the state.

This review addresses three questions presented in the request letter (I've either copied these directly or paraphrased): 1) Does the insertion of fluorescent protein genes into zebrafish increase the risk of zebrafish becoming established in California waters? 2) Do the documents supplied in support of the petition form a solid scientific basis for decision making? 3) What is my professional opinion on the likelihood of risks associated with the escape of these transgenic zebrafish due to their being made available for sale as pets.

A common way to evaluate risk associated with GMOs may look something like this (modified from Australian Government Department of Health and Aging 2002[1]):

Risk Factor	Probability of occurrence	Addressed by Applicant
Is the GMO harmful to other organisms?	Low	Yes
Could the GMO have ecosystem-level effects?	Low	Yes
Can the GMO transfer genetic material to any naturally occurring organism?	Low	Yes
Might the GMO spread or persist in the receiving environment?	Low	Yes
Will the GMO have a selective advantage over other similar organisms in the receiving environment?	Low	Yes
Will the GMO be toxic, allergenic, or pathogenic to other organisms	Low	Yes

Because it appears that transgenic fluorescent zebrafish will not likely establish a self sustaining population in the wild, many of the issues associated with risk assessment are not applicable. I suggest that the Department prepare a systematic risk assessment plan something like the one above that we can use for future evaluations.

Question 1: Does the insertion of fluorescent protein genes into zebrafish increase the risk of zebrafish becoming established in California waters?

We have very limited information with which to answer this question. However, for the reasons cited in the supporting documents, it seems unlikely that insertion of these protein genes will increase the risk of zebrafish becoming established in California waters.

- The likelihood that an exotic animal will become established is directly related to the similarity of the natural and receiving environments. Zebrafish are native tropical environments in the Ganges

[1] Australian Government Department of Health and Aging. 2002. Risk assessment and risk management plan. Agronomic assessment of transgenic sugarcane with reporter genes. Available at www.health.gov.au/ogtr/pdf/ir/dir019finalrarmp.ndf

River system in India, Nepal, and Bangladesh. They also have been reported from Pakistan (Indus River system?) and Sri Lanka. Their native environmental conditions are not similar to those in California, so, on that basis, it seems unlikely that they would be able to thrive here.

- Temperature sensitivity data support the contention that fluorescent lines have lower low temperature tolerance than wild-type. Both transgenic and wild-type lines seem to exhibit temperature stress behavioral indications starting at about 64° F. The break between lines occurs at about 55° F, where the fluorescent fish start to perform poorly in comparison. It should be noted that there are plenty of waterways in California where the temperature is regularly above 64° F, but I don't think any stay this warm all year long. Zebrafish would require warm water refugia to survive the periods of cooler temperatures. It is unlikely that these conditions could be found naturally. In any case, the preliminary data suggest that the fluorescent fish are less cold tolerant than the wild-type fish. Therefore, they don't appear to be more of a risk in this regard. Also, Dill and Cordone (1997[2]) stated in their review of introduced fishes that temperature requirements alone would preclude establishment of wild-type zebrafish in California.
- Survival data suggest that fluorescent zebrafish either are no different or are inferior to wild-type. This suggests that the risk associated with fluorescent strains is equivalent or less than wild-type (but, see below).
- It is commonly thought that brightly colored tropical fish cannot survive in California. Although I don't know anything about the ecological relationships in fish communities where zebrafish naturally live, I feel safe in assuming that they do have predators, but that their naturally bright coloration is not a negative fitness character there. (Alternatively, selection against bright coloration due to predation could be countered or even reversed by selection for bright coloration in intraspecific competition, mate competition, or mate choice.) Basically though, I'm suspicious about the argument that they are so brightly colored that any that escapee would be eaten immediately by predators. Obviously this doesn't happen where they are native. It would be nice to know why not. For example, bright coloration in animals is often aposematic. Is this true in zebrafish? Are they poisonous or noxious?
- However, more relevant is the fact that a very large number of wild-type zebrafish have been sold in the US for years with a very low rate of natural establishment. This is strongly suggestive that risk of natural establishment is low for wild-type zebrafish. This large influx of fish is essentially a natural experiment that can probably be relied upon to estimate risk if the transgenic fish are similar in fitness characters to the wild type fish that have been imported for years. It seems unlikely to me that a fluorescent fish would be more fit than a non-fluorescent one.

Question 2: Do the documents supplied in support of the petition form a solid scientific basis for decision making?

Mostly, I think that the information submitted in support of the petition do support the assertion that transgenic fluorescent zebrafish do not pose a greater risk to the environment or public health than non-transgenic zebrafish. However, it is important to note that only one of the supporting documents is from a peer reviewed journal, and some of the results reported in that study are not as rigorous as I would like to see for this evaluation. I have the following comments:

- Gong et al. (2003[3]) is used in several places to support the assertion that there is no difference in survival and reproduction between transgenic fluorescent and wild type zebrafish. While this study is a useful beginning for evaluation of this question, it does not address it directly from our point of view because the study was done in a rudimentary way in a laboratory, not a natural system. It could be argued that most of the important selective factors that we are interested in were not allowed to operate in this lab study. In general this part of the study is not very

[2] Dill, W.A., and A.J. Cordone. 1997. History and status of introduced fishes in California, 1871-1996. California Department of Fish and Game, Fish Bulletin 178.

[3] Gong, Z, H. Wan, T.L. Tay, H. Wang, M. Chen, and T. Yan. 2003. Development of transgenic fish for ornamental and bioreactor by strong expression of fluorescent proteins in the skeletal muscle. Biochemical and Biophysical Research Communications 308: 58-63.

convincing ecologically; it doesn't tell us whether transgenic zebrafish compare to wild-type in their potential to survive or establish a self-sustaining population in California waters. Perhaps it is unfair to hold geneticists to a high standard of ecological research, but when the results are presented and used in to support ecological risk analysis, it is important to do so. The survival rate study design was not very sophisticated: basically some fish were held together in an aquarium where they grew to adulthood. The original number of each type was compared to the number after they became adults. The aquarium likely did not represent a limiting environment in any way, and competition was likely not an issue since food was probably not limited (although in the author's letter to the proponent, it seems that some of this manipulation might have been done). Therefore, this survival study really did not test anything other than the inherent survivorship of each group. It showed that there were no inherently lethal attributes acting between hatching and adulthood in any of the fish tested. This is of limited use to us for our risk analysis, and should not be used to support the assertion that survival would be the same in the wild. Also, the authors did not subject the data to statistical analysis.

- Analysis of transgenic zebrafish relative to Muir's Categories (juvenile viability, adult viability, age at sexual maturity, fecundity, fertility, mating success) presented in the attached letter (Gong, personal communication to the proponent) supports the assertion that the transgenic fish do not pose any more risk, probably less, than wild-type. However, the fact that these results are not published or fleshed out in the letter should be taken into consideration when weighing this as evidence.

Question 3: What is my professional opinion on the likelihood of risks associated with the escape of these transgenic zebrafish due to their being made available for sale as pets.

On balance, I think that the risks associated with exemption of transgenic zebrafish are very low. Zebrafish are not endemic to California, and years of importing large numbers of these fish have failed to result in any obvious persistent and self-sustaining populations. Preliminary data suggest that fluorescent zebrafish are more temperature sensitive than wild-type zebrafish. Temperature sensitivity alone seems to adequately limit their invasive abilities. Fluorescent zebrafish also don't seem to have any fitness enhancing characters that would affect their ability to establish themselves in the State. The proteins are naturally occurring and don't appear to have any toxic or allergenic properties. The probability of zebrafish, either transgenic fluorescent or wild-type, establishing a self sustaining exotic population in California appears remote.

I suggest that, if an exception to regulation is made, that it is very specific to the modifications evaluated in this package. If any other genetic modifications are made, they should be similarly evaluated for fitness enhancing effects.

I also suggest that the Fish and Game Commission guard against unintended consequences by adding a provision to revisit this exemption if more information reveals additional or unevaluated risk. The data and analyses used to support this exemption should be made part of the permanent record. Publication and peer review of these results should be encouraged.

Qinqin Liu

Oct. 15, 2003

Review comments regarding information related to transgenic fish

In general, documents from Yorktown Technologies provide a broad range of information and references from several highly qualified experts to address important issues related to certain fluorescent transgenic zebra fish. Risk assessments from these documents support the following points:

1) No population of zebra fish has been established within U.S. (USGS fact sheet)
2) Limited research has not indicated any significant risks related to food and environmental safety.

However, other important issues need to be addressed, including:

1). No research information no any field research to compare fluorescent zebra fish with wild type regarding six factors used to evaluate environmental risk (open questions by Dr. Muir), and only the preliminary laboratory research (Dr. Gong) suggests the possibility that these specific fluorescent zebra fish may not increase the risk of the fish becoming established in California waters. But wild type zebra fish have been found in a CA waterway before, and we still do not know what would happen if the fluorescent zebra fish occur in natural conditions. Further risk management may be necessary at this point. See Dr. Hallerman's suggestions such as market triploid or monosex stocks.

2) Regarding the question of ethics, labeling issues need to be addressed in order for consumers have a choice to purchase or not purchase transgenic zebra fish (See Dr. Hackett letter).

3). Need to address more issues on safety assessment of fluorescent protein, such as the impacts related to physiological ecology and food chain (e.g., any site effects related to physiological changes; Dr. Cubitt did not provide specific information on his assessment).

4). Need more study related to temperature sensitivity test with experimental design and statistics since this is an important issue (Dr. Essner's study did not provide this information).