



Magnitude of the US trade in amphibians and presence of *Batrachochytrium dendrobatidis* and ranavirus infection in imported North American bullfrogs (*Rana catesbeiana*)

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ARTICLE INFO

Article history:

Received 14 October 2008

Received in revised form 31 January 2009

Accepted 8 February 2009

Available online 5 April 2009

Keywords:

Chytridiomycosis

Batrachochytrium dendrobatidis

Wildlife trade

Amphibian declines

Ranavirus

ABSTRACT

Amphibians are globally threatened by anthropogenic habitat loss, the wildlife trade and emerging diseases. Previous authors have hypothesized that the spread of the amphibian disease chytridiomycosis (*Batrachochytrium dendrobatidis*) and amphibian ranaviruses are associated with the international trade in live amphibians. The North American bullfrog (*Rana catesbeiana*) is thought to be a carrier of these pathogens, is globally traded as a live commodity, and is sold live in US markets. We obtained importation data for all live amphibians, and parts thereof, into three major US ports of entry (Los Angeles, San Francisco and New York) from 2000 to 2005. Importation of live amphibians into these ports totaled almost 28 million individuals over this 6-year period. We collected samples from freshly-imported market frogs and found infection with both pathogens in all three cities and all seasons, with an overall infection prevalence of 62% (306/493) and 8.5% (50/588) for *B. dendrobatidis* and ranaviruses, respectively, by PCR. This study definitively identifies these two important pathogens in recently imported live market frogs and suggests that the amphibian trade can contribute to introductions of these pathogens into new regions. It provides support for the recent listing of *B. dendrobatidis* and ranaviral diseases by the OIE, and provides evidence for measures to be taken to eradicate these pathogens from the trade.

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1. Introduction

In recent years, two diseases (chytridiomycosis, caused by the zoospore fungus *Batrachochytrium dendrobatidis*, and ranaviral disease) have been identified as threats to amphibian populations (Daszak et al., 2003; Green et al., 2002). Outbreaks of chytridiomycosis are often associated with high mortality rates in adult anurans, and the disease has been implicated in a series of declines and extinction events globally (Berger et al., 1998; Lips et al., 2005; Mendelson et al., 2006; Schloegel et al., 2006). Ranaviral disease is known to cause amphibian mortality, often with large die-

offs (Cunningham et al., 1996), but its capacity to cause sustained declines is unknown (Daszak et al., 2003).

Chytridiomycosis and amphibian ranaviral disease are classed as emerging infectious diseases due to their recent spread into new regions and, in the case of *B. dendrobatidis*, its significant impact on amphibian populations in recent times (Daszak et al., 2003). There is increasing evidence that anthropogenic trade in, or the introduction of, amphibians is responsible for the recent spread of these diseases, with papers reporting the presence of *B. dendrobatidis* in the pet trade, zoo animals, introduced species and the laboratory animal trade, among others (Daszak et al., 2003). Similarly, phylogenetic evidence strongly suggests that the trade in US salamanders as fish bait, and the introduction of species from the USA to the UK, resulted in the geographic expansion of ranaviruses (Hyatt et al., 2000; Storer et al., 2007).

The earliest identified infection by *B. dendrobatidis* was of an African clawed frog (*Xenopus laevis*) museum specimen dated 1938 (Weldon et al., 2004). This species appears to be resistant to the onset of disease when infected by *B. dendrobatidis*, leading

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to speculation that the widespread distribution of *X. laevis* in the 1940s and 1950s for human pregnancy testing disseminated the pathogen to differing regions of the world (Weldon et al., 2004). There is a discrepancy in the overlap of *X. laevis* distributions and the nature of *B. dendrobatidis* outbreaks, however, suggesting additional hosts must play a role in the spread of this disease (Rachowicz et al., 2005).

The North American bullfrog (*Rana catesbeiana*) is another potential carrier of *B. dendrobatidis*, exhibiting no clinical signs when experimentally or naturally-infected with the fungus (Daszak et al., 2004). This species is farmed for the international food and pet trades, and large numbers are imported into the USA annually for human consumption (Schlaepfer et al., 2005). Previous studies have established the presence of *B. dendrobatidis* in farmed and escaped bullfrogs in South America (Hanselmann et al., 2004), and in other regions (Garner et al., 2006). The international trade in live amphibians is also implicated in the spread of ranaviruses. Ranaviruses isolated from pig frogs (*Rana grylio*) farmed in China (RGV-9506, RGV-9807 and RGV-9808) are genetically similar to Frog virus 3 (FV3), originally isolated from *R. pipiens* in North America (Zhang et al., 2001). The pig frog is native to North America and was exported to China specifically for the frog farming industry (Chinchar, 2002; Zhang et al., 2001). Evidence also suggests that ranaviruses linked to seasonal mortalities in amphibians in the western US may have spread throughout the region via the trade in salamanders as bait for freshwater fishing (Jancovich et al., 2005; Picco and Collins, 2008). This and other evidence prompted the World Organization for Animal Health (OIE – Office International des Epizooties) to list *B. dendrobatidis* and ranaviral diseases of amphibians as ‘notifiable’ (www.oie.int/eng/maladies/en_classification2009.htm?e1d7, 2009, accessed 29 January).

Despite interest in the origins of these pathogens, there are no published reports on the presence of *B. dendrobatidis* or ranaviruses in Ranid frogs imported into the USA, nor accurate data on the volume and geographical pattern of these trades. To assess overall trends in the amphibian trade in the USA, and their potential to spread these diseases, we analyzed data collected by the US Fish and Wildlife Service from three major US ports of entry over a six-year period (2000–2005) and tested live amphibians sold for human consumption in each of these three cities for *B. dendrobatidis* and ranaviruses.

2. Methods

2.1. Importation data

Using the Freedom of Information Act (FOIA), a request was submitted to the US Fish and Wildlife Service (USFWS) Law Enforcement Management Information Systems (LEMIS) division for records of all imports and exports of live amphibians and amphibian parts from January 1, 2000 to December 31, 2005 for the ports of Los Angeles, New York and San Francisco.

The USFWS assigns a control number to each shipment crossing USA borders. A shipment may contain more than one species, in which case the database will contain multiple lines of entry for one particular shipment, with each line representing a distinct species. For our analyses, each line of data is referred to as a distinct shipment. The LEMIS database identifies the contents of each shipment to the species or genus level. We combined all *Rana* spp. shipments with *R. catesbeiana* shipments and refer to the combined numbers as Ranids. Similarly, *X. laevis* and *Xenopus* spp. codes are collectively referred to as Xenopus.

For each shipment, the database also contains information related to shipment date, country of origin, country of import/export, port of entry, quantity, description (indicating live animal or ani-

mal parts and derivatives), source population, and purpose. Wildlife source codes indicate whether the animal was taken from the wild, originated from a ranching operation, bred in captivity, and more. For our analyses, all shipments whose source population did not come from the wild were grouped as originating from captive populations, excluding those whose origins were unknown. Purpose codes identified whether the shipment was commercial, intended for a zoological institution, for scientific purposes, personal, for biomedical research or educational, among others.

Shipment size was recorded in one of four unit measures: number of individuals, kilograms, pounds, or gallons. More than 99% of all shipments recorded in units of kilograms, pounds, or gallons were identified as Ranid shipments. Therefore, all live shipments recorded in a weighted measure were converted to number of individuals based on the average weight of adult *R. catesbeiana* sampled at each of the markets (0.4 kg, $n = 200$). All shipments denoted as SKI (skins) and SPE (scientific specimens including blood, tissue and histological preparations) were excluded from our analyses.

2.2. Sample collection

We obtained samples from shops selling live amphibians for human consumption in three major US cities: 1 in Los Angeles, California (LA); 13 in New York, New York (NY); and 4 in San Francisco, California (SF). The market frogs were on display in large plastic bins or trash cans, typically containing water (Fig. 1). The number of frogs per container was variable and could be as few as 10 or >50.

The animals were purchased from the shops, killed by the shop owners (we were informed by shop owners in California that the sale of live frogs is illegal in that state, but calls to contacts at the appropriate authorities have been unable to verify this statement) and transported to the laboratory post mortem on ice. We requested each animal be placed in a separate bag for transportation to prevent any contamination that could result from the mixing of blood from one individual to the next. Due to language restrictions, however, there were several occasions where the animals were bagged collectively. To prevent additional cross-contamination of residual DNA, a fresh pair of gloves was used for each frog and equipment was sterilized with ethanol and subsequently flamed between specimens. Each individual was swabbed along the ventral surface and between the digits (i.e. webbing) using Medical Wire and Equipment Company's MW100 dry swab.



Fig. 1. Live *Rana catesbeiana* on sale for human consumption from a shop in New York, NY.

The swabs were analyzed using quantitative PCR to detect *B. dendrobatidis* infection (Boyle et al., 2004). Samples were run in triplicate and those that returned a low number of zoospore equivalents in only one or two wells were defined as indeterminate and were not used in our calculations of infection prevalence. A small piece of the liver was subsequently excised and placed in 70% ethanol for processing for ranavirus infection at Arizona State University. A salt-extraction protocol (Sambrook and Russell, 2001) was used to extract DNA. Each sample was screened for ranavirus infection using PCR analysis of the major capsid protein for ranaviruses (MCP) (Mao et al., 1996). To examine whether frogs showed pathological changes consistent with clinical signs of chytridiomycosis and to standardize our results between histology and PCR, toe clips were taken from a subset of individuals ($n = 74$) and placed in 70% ethanol. Toe clips were later fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin for histological analysis of *B. dendrobatidis*. Twenty-five fields of view (using a 40 \times objective lens) were examined, each containing keratinized skin for each frog. This part of the study was conducted blinded.

To confirm the identification of the market-purchased frogs, we sequenced a portion of the 12S rRNA gene for Ranid frogs. One individual from each sampling effort at all shops was included to identify the animals to the species level. Using the DNA extracted from the liver for viral strain identification (see above), we amplified a 410-bp segment of the 12S rRNA gene using PCR with the FS01/R16 primers (Sumida et al., 1998). Following PCR, we sequenced this region using an ABI 3730 DNA Analyzer at the DNA Laboratory in the School of Life Sciences at Arizona State University. All sequences were compared to Genbank[®] sequences using BLAST in order to determine the species-level identification of the samples (Altschul et al., 1990).

We used a generalized linear model with a logit link and binomial error to determine whether there was variation in the probability of a frog being infected between shop, city and season. The four seasons were divided into three month intervals as follows: spring was defined as March–May; summer as July–August; fall as September–November; and winter as December–February. We also tested whether there was any evidence that infection with *B. dendrobatidis* or ranavirus influenced the probability of being infected with the other agent.

3. Results

3.1. Overall Trends in the Amphibian Trade for Human Consumption

Between January 1st 2000 and December 31st 2005, 27,960,623 individuals (no.) and 7,082,700.05 kilograms (kg) of amphibians (live animals, animal parts and derivatives) were imported into the ports of LA, NY and SF (LA: 12,340,968 no. and 5,882,370.72 kg; NY: 8,172,665 no. and 259,251.12 kg; SF: 7,446,990 no. and 941,078.21 kg). Thus, on average, 4.66 million individuals and 1.18 million kg were imported per annum during this 6-year period. Los Angeles recorded a total of 12,126 amphibian shipments comprising 89 identified genera and 148 species; New York recorded 3,984 amphibian shipments of 40 genera and 57 species; and San Francisco recorded 743 amphibian shipments of 42 genera and 53 species. Only 5% of amphibian imports were classified in broad taxonomic terms. Ninety-five percent were classified to genus and 76% were given species-specific coding.

3.2. Imports of animal parts and derivatives

There were 813 shipments denoted as animal parts or derivatives (including frog legs) totaling 322,951 parts and weighing

5,958,108.23 kg (LA: 315,605 parts and 5,864,010.72 kg; NY: 522 parts and 35,066 kg; SF: 6,824 parts and 59,031.51 kg). Frog leg shipments comprised 56.5% (460/813) of these imports. Amphibian meat shipments were primarily imported from China, Vietnam, Taiwan and Indonesia.

3.3. Imports of Live amphibians

Over the six-year period, 30,449,152 live individuals (an average of 5.07 million per annum) were imported into LA, NY and SF combined during this six-year period. Imports of Ranids constituted 56.6% (17,677,265 individuals) of shipments, while *Xenopus* accounted for 1.3% (402,950 individuals). More than 99% of all imports were intended for commercial purposes (Fig. 2). Taiwan, Brazil, Ecuador and China exported the greatest number of amphibians (Fig. 3).

3.3.1. Los Angeles

Live shipments into LA totaled 12,071,263 individuals. Both Ranid and *Xenopus* shipments comprised less than 1% of live amphibian imports (103,293 and 2,343 individuals, respectively). The top species imports for LA, encompassing 70.6% of all live shipments, were *Hymenochirus curtipes* (37.3%), *Bombina orientalis* (18.1%) and *Cynops orientalis* (15.2%). These species are typically imported for the pet trade. Additionally, the data indicate that approximately half (55.4%) of all live shipments (all species) were sourced from wild populations and approximately half (44.3%) from captive populations, with the remainder of unknown origin. The majority of Ranid imports originated from wild populations (89.3%). All amphibian shipments were imported in greatest numbers from Hong Kong, China and Singapore (32.8%, 24.7% and 15.6%, respectively). The top exporter for Ranids, however, was Taiwan (42.9%).

3.3.2. New York

Live shipments totaled 8,732,606 individuals. Ranids constituted 83.1% (7,257,780 individuals) of all live, amphibian imports; *Xenopus* were calculated at 4.6% (399,542 individuals). All live imports into NY were sourced mainly from captive populations (74.0% all species; 81.8% Ranids). The top countries exporting amphibians to NY were Brazil (29.6% all amphibians; 35.6% Ranids), Ecuador (26.1% all amphibians, 31.2% Ranids) and Taiwan (24.0% all amphibians, 28.7% Ranids).

3.3.3. San Francisco

Live shipments totaled 9,645,282.75 individuals. The species composition of anuran imports through SF almost completely comprised Ranids (99.0% or 9,549,978 individuals). *Xenopus* imports were minimal with 1,065 individuals (less than .001%). The number of shipments (all species and Ranids) originating from captive populations was 73.5% and 73.5%, respectively. Amphibian shipments into SF were primarily imported from Taiwan (90.3%) and China (8.7%).

3.4. Sample collection

A total of 560 swabs and 588 liver samples were obtained between July, 2006 and October, 2007. All sequence analyses identified the market-purchased frogs as *R. catesbeiana*. Histological analysis of 74 toeclips showed no evidence of *B. dendrobatidis* in 74 *R. catesbeiana* individuals (PCR indicated that 29.7% or 22/74 of those individuals were positive for *B. dendrobatidis* and 9.4% or 7/74 were indeterminate).

PCR analyses of all swabs revealed that 62% (306/493) of animals were positive for *B. dendrobatidis* infection (13.5% or 67/493 of the swabs were indeterminate for *B. dendrobatidis*) and 7.9% (50/633) were positive for ranavirus infection (Table 1). We

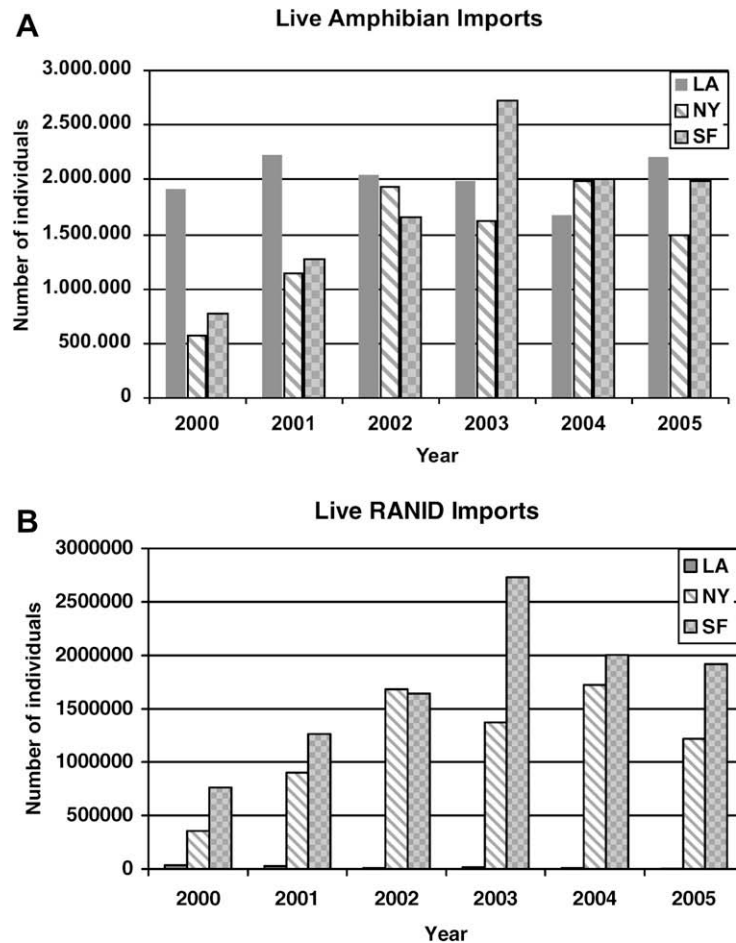


Fig. 2. (A) Annual imports of all live amphibians into Los Angeles, New York and San Francisco from 2000 to 2005 and (B) annual imports of all live *Rana catesbeiana* and unclassified *Rana* spp. for Los Angeles, New York and San Francisco from 2000 to 2005.

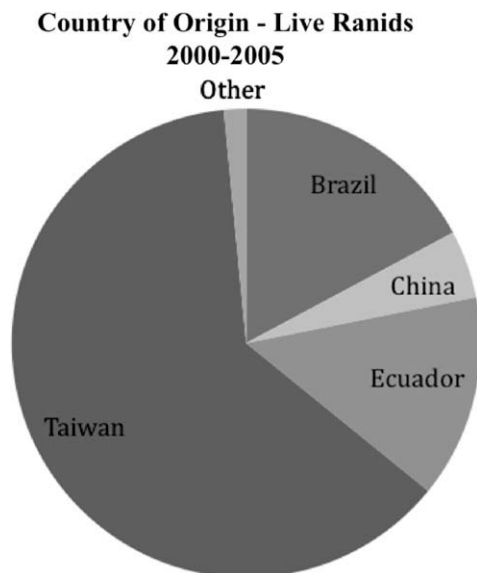


Fig. 3. Country of origin of all live *Rana catesbeiana* and unclassified *Rana* spp. for Los Angeles, New York and San Francisco from 2000 to 2005.

identified three different strains of ranaviruses in the market frogs in our sample, and these strains appeared most similar to FV3, RGV 9806 and RGV 9808 via BLAST search on Genbank. The ranavirus

strains appear to be grouped by port of entry. All ranavirus positive samples isolated from NY shops were FV3-like in their strain ($n = 19$), samples isolated from SF shops were both RGV9808-like ($n = 10$) and RGV9806-like ($n = 4$), and all positive samples isolated from LA shops were RGV9806-like ($n = 12$). This may be a representation of the stocks from which the frogs were obtained. However, we were unable to definitively identify the sequence of four isolates.

We found significant seasonal and among-city variation in the likelihood of an animal testing positive for *B. dendrobatidis* (GLM with logit link and binomial error: City, $df = 2$; $\chi^2 = 74.2$; $p < 0.001$; Season, $df = 2$; $\chi^2 = 34.1$; $p < 0.001$), with a higher probability of infection in NY than either SF or LA (p 's < 0.001) with no difference between SF and LA ($p = 0.49$). There was also significant variability between shops within both SF and NY (SF: $\chi^2 = 25.7$, $df = 3$, $p < 0.001$; NY: $\chi^2 = 109.8$, $df = 12$, $p < 0.001$). The probability of animals testing positive for *B. dendrobatidis* was highest in the US winter, followed by summer and then spring (all $p < 0.001$).

Similarly, there was significant variability seasonally and among cities in the probability of ranavirus infection (GLM with logit link and binomial error: City, $df = 2$; $\chi^2 = 18.8$; $p < 0.001$; Season, $df = 2$; $\chi^2 = 10.9$; $p = 0.012$). In contrast to the results for *B. dendrobatidis*, frogs from LA had a higher probability of having ranavirus infection than both NY ($p < 0.001$) and SF ($p = 0.02$). Our analyses also showed significant variability in ranavirus among shops within SF (SF: $\chi^2 = 9.3$, $df = 2$, $p = 0.01$). Sample size and prevalence were too low in NY shops to analyze. Frogs collected in spring had the highest probability of ranavirus infection

Table 1
Number of individuals infected with *B. dendrobatidis* and ranavirus collected between July 2005 and October 2006 broken down by city and shop.

Date	Shop ID	<i>B. dendrobatidis</i> % infected (N)	Ranavirus % infected (N)
July-2005	SF1	100% (6)	–
	SF2	0% (5)	–
	LA1	10% (20)	–
December-2005	NY1	6% (17)	0% (20)
	NY2	100% (22)	0% (20)
January-2006	NY3	79% (14)	0% (20)
	NY4	43% (14)	21% (24)
February-2006	NY1	100% (8)	25% (8)
	NY2	100% (19)	0% (20)
	SF2	72% (18)	0% (19)
	SF3	17% (18)	0% (20)
	SF4	88% (17)	0% (20)
	LA1	78% (18)	0% (20)
March-2006	NY4	25% (16)	15% (20)
	NY3	83% (18)	0% (20)
April-2006	NY2	33% (18)	25% (20)
	NY3	79% (19)	0% (20)
May-2006	NY4	17% (18)	0% (20)
June-2006	NY5	100% (15)	10% (20)
	NY6	100% (19)	5% (20)
	NY7	94% (17)	–
	NY8	95% (19)	0% (20)
	NY9	84% (19)	0% (20)
	SF3	5% (19)	60% (20)
	SF4	0% (20)	35% (20)
	LA1	100% (19)	0% (20)
	July-2006	NY13	73% (15)
	NY14	22% (9)	0% (17)
	NY15	41% (17)	0% (19)
August-2006	NY 16	95% (20)	0% (20)
October-2006	SF2	–	0% (20)
	SF3	–	20% (20)
	SF4	–	25% (20)
	LA1	–	10% (20)
Total		62% (493)	8.5% (588)

followed by those collected in summer, fall and winter, with significant differences between frogs collected in spring and summer and those collected in winter (p 's < 0.05). We found no correlation between infection with *B. dendrobatidis* and infection with ranaviruses, when controlling for season and city ($p > 0.25$). We were unable to determine whether bagging technique had any effect on the outcome of infection for *B. dendrobatidis* or ranaviruses because bagging technique and shop were confounded (no one shop had data for both bagging methods).

4. Discussion

We have demonstrated a large and growing international trade in live amphibians into the USA, and provided the first definitive evidence that this international trade involves individuals infected with pathogens responsible for two significant diseases: amphibian chytridiomycosis and ranaviral disease.

We show that an average of almost 5.1 million Ranid frogs per year including live animals and their parts and derivatives were imported during this period into three US major ports of entry. This represented more than half of all amphibian imports into the three ports for the years 2000–2005. During the same period, more than 400,000 live *Xenopus* individuals were imported into the USA through these ports. These data suggest that, if infection prevalences are similar between the two groups, Ranids pose a far greater current risk for the transport of amphibian disease through the

wildlife trade. However, our data only cover a six-year time period beginning in 2000, and it is highly likely that *Xenopus* spp. represented a higher proportion of amphibian imports in prior years, particularly when pregnancy testing using live *X. laevis* was first set up in the USA. Unfortunately, historical data on the numbers of *Xenopus* spp. and Ranid species imported prior to the onset of large-scale amphibian declines in the USA (1970s) appear to be unavailable (import records older than 5 years are destroyed by the USFWS). Therefore, the involvement of these species in the initial introduction of *B. dendrobatidis* into the USA is difficult to ascertain.

The market for live Ranids in LA was found to be minute when compared to NY and SF. Despite extensive searches over 2 weeks, we found only one shop selling live frogs for human consumption in LA, while numerous locations were found in both NY and SF, using the same search intensity and methods. Additionally, the data revealed differences in the species composition of imports. The species representing the highest proportion of imports into LA were identified as non-Ranid amphibians and were typically intended for the pet trade, while Ranid imports comprised the majority of shipments into NY and SF. Imports into LA were primarily sourced from wild populations, while those into NY and SF were more likely to be captive bred. The discrepancy may be accounted for by the fact that NY and SF import more Ranids reared for human consumption per annum, suggesting that amphibians intended for the pet trade are more likely to be taken from wild populations than animals traded as a food commodity.

This study shows definitively that live bullfrogs sold in three major US cities are infected with both *B. dendrobatidis* and ranaviruses. When questioned, most shop owners reported China or Taiwan as the origin of the frogs, and stated that these animals were kept together following collection from customs. This makes it unlikely that they had contact with North American frog populations. We therefore believe that our findings confirm that *B. dendrobatidis* and ranaviruses are imported into the USA via the bullfrog trade, even though we did not test them directly upon initial entry into the USA, which would require permission from the importers. Previous studies have shown that farmed amphibians are capable of harboring both *B. dendrobatidis* and ranavirus infections (Mazzoni et al., 2003; Zhang et al., 2001). Furthermore, while the presence of *B. dendrobatidis* has not been confirmed in either China or Taiwan, recent preliminary predictions indicate that these countries do contain suitable climatic regimes for the growth of this pathogen (Ron, 2005). Additional sampling in these regions is necessary to determine at which point along the trade route market frogs are exposed to these pathogens. Sampling at key points in the trade route will also allow us to assess changes in pathogen prevalence which could fluctuate along with animal density, environmental conditions and stress levels. Whether animals are kept in moist or dry conditions (and the length of time those conditions persist during transportation), and the co-mingling of animals from different shipments/origins in the markets, could significantly impact transmission among individuals.

It appears that the wildlife trade *per se* does not amplify the prevalence of these pathogens in bullfrogs. The prevalence of *B. dendrobatidis* we report in imported bullfrogs is not significantly higher than those reported from wild bullfrogs; e.g. 64% in *R. catesbeiana* tadpoles at the Savannah River Site, Georgia, USA (Peterson et al., 2007) and 14–100% prevalence reported from populations sampled in other countries (Garner et al., 2006). Furthermore, the prevalence in frog farms is likely to be high due to amphibian rearing at high densities; e.g. 96% prevalence in Venezuela (Hanselmann et al., 2004). However, none of these studies measured the intensity of infection (number of pathogen life stages per individual), and it is possible that housing at high density throughout the trade process, coupled with nutritional and other stressors,

could increase the intensity and pathogenicity of infections. The low prevalence on histological examination of US market frogs compared to PCR suggests that *R. catesbeiana* present in the trade may harbor a low level of infection, however, and supports previous papers that demonstrate PCR as a much more sensitive test for *B. dendrobatidis* infection (Hyatt et al., 2007; Kriger et al., 2006). This is not surprising considering the relative resistance of this species to the onset of disease following infection, and the light infections noted in experimentally-infected individuals (Daszak et al., 2004). However, previous work on naturally-infected, introduced bullfrogs shows that relatively intense infections can occur in some individuals when examined histologically (Hanselmann et al., 2004). This may be important to the conservation impact of the trade, and also to the economic impact these pathogens may have on amphibian farmers and traders – an important factor in setting international policy.

Our data indicate that there was strong seasonal variation in the prevalence of both pathogens examined within frogs, with elevated prevalence of *B. dendrobatidis* in the US winter, and elevated prevalence of ranaviruses in the US spring. Growth of *B. dendrobatidis* is known to be temperature dependent *in vitro*, with more rapid growth of colonies in cooler conditions (Piotrowski et al., 2004). A previous study examining the presence of *B. dendrobatidis* in the Northeastern US found that the number of infected specimens decreased in the summer months with rising temperatures and increased as the weather cooled (Longcore et al., 2007). Furthermore, prevalence of *B. dendrobatidis* infection prevalence in the stony creek frog (*Litoria wilcoxii*) from Australia was found to be highest in the winter and early spring (Kriger and Hero, 2007). Similarly, Rojas et al. (2005) found that ranavirus-infected salamanders (*Ambystoma tigrinum* virus) had higher levels of infection when exposed at lower temperatures. While our data suggest season is important in the risk these trade routes represent for pathogen introduction, further work is required to identify whether prevalence fluctuations are due to environmental conditions in the market, in the countries of origin and/or transport, or are due to other factors. This will include obtaining information on the timing of each step in the trade route.

The conservation significance of our findings depends on two poorly understood factors. First, there is little information on the likelihood of either *B. dendrobatidis* or ranaviral disease being transmitted from imported to wild amphibians. Plausible scenarios for this include the release of zoospore-contaminated water from bullfrog containers into drains (and thence to wetlands), the release of live bullfrogs, or inappropriate disposal of amphibian skin, which is removed prior to cooking. The release of live bullfrogs would obviously require the purchase of live individuals and, while shop owners in California insist this is not possible, we have negotiated the purchase of live frogs (with the argument that the animals were required for research purposes) on two separate occasions. Additionally, there is no impediment to the purchase of live bullfrogs in the state of New York. Secondly, the known distribution of *B. dendrobatidis* includes regions throughout the North American continent (www.spatalepidemiology.net/bd, 2009, accessed 3 January) and ranaviruses have been previously reported from North American amphibians. Accordingly, the amphibian trade may not increase the geographic distribution of these two pathogens. However, it may introduce new strains of these pathogens as previous studies have illustrated important differences in pathogenicity among isolates/strains (Berger et al., 2005; Retallick and Miera, 2007). It is therefore imperative that the possible introduction of alien isolates/strains, either through escaped animals or water wastes, be minimized in order to limit any damaging effects.

We identified Asia, particularly Taiwan and China, as the region from which most live imports originated. Previous studies have detected *B. dendrobatidis* and ranaviruses in a number of captive pop-

ulations throughout Asia, though data on the extent of their true distribution is minimal (Une et al., 2008; Zhang et al., 2001). Taiwan and China each actively participate in the production of the North American bullfrog, and a recent study by Hou et al. (2006) identified the establishment of feral *R. catesbeiana* in Taiwan. The risk to Taiwan's native amphibians could be significant if these animals harbor *B. dendrobatidis* and ranavirus infections. Given the large numbers of Ranids imported from this region of the world, and the confirmed presence of *B. dendrobatidis* and ranaviruses in market frogs, extensive sampling of trade and wild anuran populations in these countries should be conducted (Daszak et al., 1999, 2004).

Our findings that traded amphibians are frequently infected with *B. dendrobatidis* and ranavirus should be taken into account when considering any additional expansion of the amphibian trade into regions that have been classified as free from these amphibian pathogens, or where the presence of these diseases has not yet been assessed. Surveys in Madagascar, for instance, indicate that *B. dendrobatidis* has not yet invaded this island nation (Weldon et al., 2008). Madagascar boasts a rich biodiversity of amphibian fauna with high endemism (Andreaone et al., 2008). Should the amphibian chytrid fungus be introduced into this region, the consequences could be especially devastating.

The continued global decline of amphibian populations highlights the need to determine the means through which *B. dendrobatidis* and ranaviruses are spread to new geographic regions. Our study not only identifies the presence of amphibian pathogens in live markets in three US cities, but illustrates the extent to which live amphibians are imported across US borders each year. Our results therefore support the hypothesis that the trade in frogs for human consumption could be a major contributor to the continued spread of these pathogens. They are of particular importance given the recent listing of *B. dendrobatidis* and ranaviral diseases of amphibians as 'notifiable' to the World Organization for Animal Health (OIE – Office International des Epizooties) (www.oie.int/eng/maladies/en_classification2009.htm?e1d7, 2009, accessed 29 January). Our analysis provides support for this listing by demonstrating these diseases within the international trade network. It also suggests that these pathogens may become an economic hindrance to the trade, which could be used to drive research and policy towards removing the source of these pathogens and instituting a disease-free network to the benefit of both traders and conservationists.

Acknowledgements

This study was funded by The New York Community Trust, The Eppley Foundation, the National Science Foundation (IRCEB Grant DEB-02133851; NSF/NIH Ecology of Infectious Diseases Grant EF-0622391) and core funding to the Consortium for Conservation Medicine from the V. Kann Rasmussen Foundation. We would also like to thank Christine Schneider, Kari L. Schmidt and Nikkita Patel for assistance in sample collections and Gary Townsend at the US Fish and Wildlife Service for data acquisition and support. Also thanks to Dr. James P. Collins, Dr. Cheryl Briggs, Dr. O. Roger Anderson, Dr. David Porter, and Dr. Victoria Vazquez for use of laboratory resources.

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