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Hotspot of tadpole abnormality in suburban south-west Florida

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A high concentration of Cuban treefrog (*Osteopilus septentrionalis*) tadpoles displaying morphological abnormalities was discovered in an untreated swimming pool in Bonita Springs, Florida. This find initiated a 4-year survey (2012-2015) of surrounding roadside drainage ditches that had been treated with insecticide for mosquito control. The study was extended to the populations of Ave Maria, Florida, and Everglades National Park. The core data set of 36,550 tadpoles from the swimming pool and ditches contained 25,136 abnormal tadpoles, an abnormality average of 68.8 %, well above the 5 % minimum definition for a hotspot. The frogs from Ave Maria and the Everglades National park were 0 % abnormal. The type of tadpole abnormality differed between the suburban treated roadside drainage ditches versus the untreated swimming pool, although the same abnormalities were found in both the suburban treated and untreated water. In the untreated swimming pool, 70.1 % of tadpoles displayed abnormalities such as bent tails, abnormal limbs, and disfigured or absent mouthparts. Larvae in the untreated swimming pool metamorphosed en masse despite abnormalities. The high frequency of abnormal metamorph abnormalities found were: kyphosis, scoliosis, microcephaly, and forelimb abnormalities. In the treated roadside drainage ditches, Gosner stage 16-25 tadpoles could not undergo metamorphosis and experienced mass mortality. The abnormalities found at Gosner stage 16 of the embryo were in the head and body. Tadpoles at Gosner stages 19-25 failed to develop gills, were bloated, had growth retardation, and mouthpart abnormalities. The older Gosner stage 25-41 tadpole populations in the ditches showed bloating, lumps, emaciation, and growth retardation. A brief synopsis of *O. septentrionalis* treefrog biology is also given, including breeding congregations, average 8 hour time to hatching, and 19 days metamorphosis.

Keywords: anurans, *Osteopilus septentrionalis*, tadpoles, normal development, abnormality

INTRODUCTION

Amphibians are globally threatened, with 32.5 % categorised as endangered and 43.3 % species declining (Stuart et al., 2004, 2008). The global regions with high concentrations of decreasing amphibians have been identified as Mexico, Central America, the Northern Andes, Brazil's Atlantic forest, western Africa and Madagascar, India, south-east Asia, Indonesia and the Philippines (Ceballos et al., 2017).

In the USA, many common anuran species have been disappearing (Fisher & Shaffer, 1996; Fisher et al., 2012; Ceballos et al., 2017). A National United States Fish and Wildlife Service (USFWS) frog survey covering field seasons in 2000-2009 reported that all Florida anuran species were healthy and at a normal, low abnormality rate of 2.0 % (Reeves et al., 2013). USFWS reported that there were only three abnormality hotspots nationwide, located in the Mississippi River Valley, California, and Alaska. The three hotspots were said to be a local problem (Reeves et al., 2013). Since then, in 2018, the same lab produced another report with survey data from 2012-2016 showing that the three nationwide abnormality hotspots had changed to 96 hotspots in the USA, located in the north-east, south-east, and western regions of the USA. Furthermore, the newly identified hotspots had 5 %-25 % abnormalities and were associated with the presence of oil and gas wells at sites, as well as a history of pesticide application-parasite infection (Haas et al., 2018).

Since 1995 when malformed frogs were discovered in south central Minnesota, USA, it has been world-wide practice to mainly survey specimens at the end of metamorphosis (USFWS, 2018; Reeves et al., 2013), or for one particular abnormality (Sanchez-Domene et al., 2018). Only recently, sampling throughout the whole metamorphic period coincided with an increase in reported frequency of hotspots in the USA (Haas et al., 2018). However, data is lacking about malformations across the whole larval period or when mass death occur, which will allow us to assess if there is a larval stage that is more susceptible to chemical effects.

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The Cuban treefrog *Osteopilus septentrionalis* (Dumeril & Bibron, 1841) is a large hylid treefrog from West Indies. In Florida it is considered to be a very common, invasive, robust species that consumes smaller native frogs (Meshaka, 2001). The IUCN considers *O. septentrionalis* a species of least concern. The frog was first reported in 1931 from Key West, Monroe County, Florida (Barbour, 1931) and has rapidly colonised most of Florida (Meshaka, 2001,2011). *Osteopilus septentrionalis* populations in Florida were most recently surveyed in 2013 and reported to be healthy (Johnson, 2013). However, after finding malformed tadpoles of *O. septentrionalis* during a survey in suburban South-western Florida in 2012, we decided to carry out extensive population monitoring (2012-2015) in order to determine the extent of these abnormalities. Abnormal tadpoles occurred in a heavy insecticidal spray zone, which does include the spraying of a petroleum distillate spreader to control mosquitoes.

To recognise abnormal patterns during larval development in a wild population, biologists need to establish a baseline that gives clear descriptions of normal morphology and normal development timeframes. To do this, several developmental staging tables have been proposed, with that of Nieuwkoop & Faber (1994) for *Xenopus laevis* and that of Gosner (1960) for the Gulf Coast toad *Incillus valliceps* as the most commonly used. Unfortunately, a survey of staging tables among anurans has indicated that the volume of information published represents less than 1.5 % of the diversity of current amphibians, with several families in which most aspects of their ontogenies are unknown (Fabrezi et al., 2017). Therefore, we first characterised the normal development of *O. septentrionalis* and then subsequently described numerous developmental malformations and associated abnormality prevalences for multiple populations of *O. septentrionalis* in Florida, USA. Finally, we discuss the use of *O. septentrionalis* as a sentinel species to detect negative anthropogenetic effects on wild fauna in Florida.

METHODS

All tadpoles examined in this survey were collected from the field between 2012 and 2015. All the animals were assessed for abnormalities on site at the time of sampling. The only rearing performed was of the collected abnormal GS 42 stage tadpoles from the untreated H artificial pond site to visualise metamorph and adult frog abnormality. There were 36,550 tadpoles examined in 240 collections from 11 sites located in suburban Bonita Springs, Florida, with additional unsprayed zone sites added in 2013 to the survey from Golden Gate Naples and the Everglades National Park (see Supplementary Materials Fig. S1). The frequency of abnormality was calculated by the total of abnormal frogs collected divided by the total number of frogs collected.

There were three types of sites in the suburban area of South-western Florida: The insecticide treated roadside drainage ditch (see Supplementary Materials Fig. S2 A- E), the untreated artificial pond (see Supplementary Materials Fig. S2 F), and containers (see Supplementary Materials Fig. 2 G, J). The ephemeral roadside drainage ditch sites lining the streets averaged 1.83 m x 1.22 m in size with 254 mm maximum rainwater depth at mid-pool. They were treated continually with insecticide. The artificial pond was untreated with insecticide and comprised a permanent body of water filled with rainwater. The dimensions of this pond were 3.048 m x 4.572 m with a depth of 1.22 m. The "H yard" container sites (see Supplementary Materials Fig S2. G, J) were outdoor empty plastic containers naturally-filled with rainwater, and then left undisturbed. These containers held a maximum of four litres. The control site types, U.S. Wildlife Refuges and Golden Gate Naples rural, were outside the mosquito control insecticidal zones (see Supplementary Materials Fig S2. H, I, K, L) and ephemeral puddles of rainwater were similar in both size and site longevity to suburban sites. Exceptionally, the ENP site located inside the park was permanent (see Supplementary Materials Fig. S2L).

The abnormality assessment and population tracking commenced when the completely dry field sites became filled with rainwater, and continued for as long as the pools remained full of rainwater. A field site sampling visit entailed the capturing and counting of aquatic larvae from generally 2-3 net sweeps at each site location. All specimens were counted, assessed then photographed on site in the collection pan. Observations were recorded with a P510 Nikon digital camera with automatic date, time and GPS. The field data was organised by site and collection date. All animals were euthanised following international ethical procedures. All Everglades National Park frogs (Study # EVER-00493) including 1432 individuals were returned to the park and archived at the South Florida Collections Management Center.

RESULTS

The field oviposition site for *Osteopilus septentrionalis* were ephemeral roadside drainage ditches located in a suburban neighbourhood that during the hurricane season (April-November) had filled with rainwater. The ditches were all temporary bodies of water lasting from between one day to a maximum of 4 weeks (before drying up). In many cases, the roadside drainage ditch was an unstable habitat simply because it did not provide the amphibian enough time to metamorphose.

Each year between 4AM and 7AM during periods of heavy rainfall, more than 100 *O. septentrionalis* congregated for breeding for three days. Both males and females turned bright yellow during this period. The amplexant pair travelled the length of the pool, dropping partial clutches.

Time Frame for Normal Development of Larval Stages to Metamorphosis (Fig. 1 A, Table 1).

The tracking of an entire in-situ population from eggs being deposited to mass metamorphosis was observed at the untreated H artificial pond site. The time taken for a normal population of *O. septentrionalis* to develop

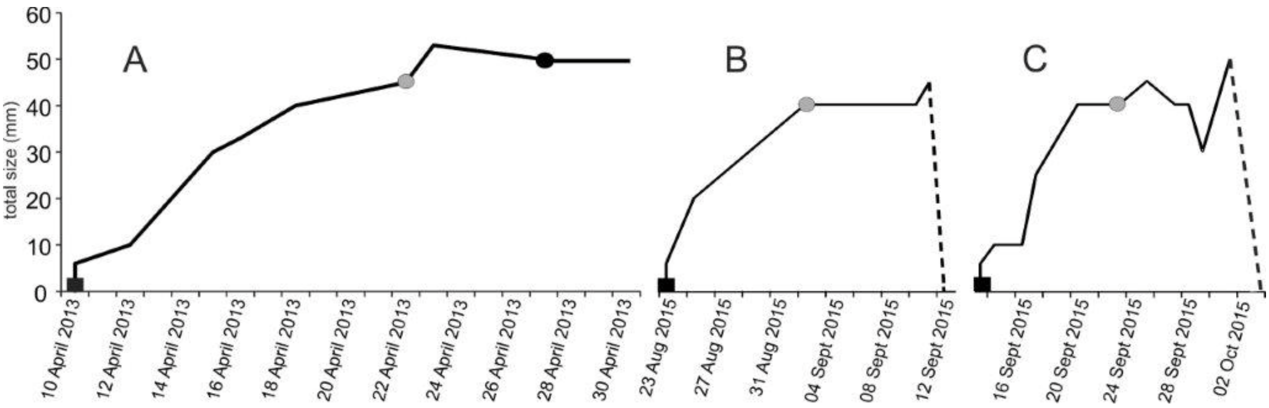

Figure 1. Survivorship Curves of three field populations in treated vs untreated water (see Table 1). (A) H artificial pond, untreated pool water. H artificial pond had normal growth with a maximum TL size of 53mm with 12th day hind limb eruption (grey circle), 17th day forelimb protrusion (black circle) and a 19-day mass metamorphosis. (B) RP1, treated pool water. (C) RP2, treated pool water. RP1, RP2 field populations had abnormal growth with a maximum size of 45mm for RP1 and 50mm for RP2. On the 15th day RP2 and on the 19th day RP1 had mass death (dotted line) of the population following hind limb eruption on the 10th day for RP2 and 12th day for RP1. Black square indicates eggs deposition.

Table 1. Aquatic tadpole length in mm (TL) and stage (eggs to metamorphosis) in days for three field site populations in treated RP1,RP2 (right) vs untreated water H artificial pond (left).

H artificial pond date	H artificial pond size (TL) Stage	RP1 ditch pool date	RP1 Size (TL) Stage	RP2 ditch pool date	RP2 Size (TL) Stage
10 April 2013	eggs	23 August 2015	eggs	12 September 2015	eggs 6 mm
10 April 2013	6 mm 8hr hatch	23 August 2015	6mm 8hr hatch	13 September 2015	new eggs+6 mm hatch
12 April 2013	10 mm	25 August 2015		14 September 2015	10 mm
15 April 2013	30 mm	2 September 2015		15 September 2015	
16 April 2013	33 mm	5 September 2015		16 September 2015	10 mm
18 April 2013	40 mm	10 September 2015	40mm dead	17 September 2015	
22 April 2013	45 mm hind limbs	11 September 2015	45mm dead	18 September 2015	30 mm
23 April 2013	50-53mm 1met	12 September 2015	100 % dead	20 September 2015	40 mm
27 April 2013	50 mm forearms			23 September 2015	hind limb mass dth
28 April 2013	1met			25 September 2015	45 mm
29 April 2013	mass met			27 September 2015	45 mm
30 April 2013	mass met			28 September 2015	40 mm
01 May 2013	mass metamorphosis			29 September 2015	30 mm dead
				1 October 2015	50 mm dead
				3 October 2015	100 % dead

from egg to metamorph in clean water was 19 days. The hind limbs appeared in 12 days, forearms protruded in 17 days, with mass metamorphosis in 19 days. Normal development is extensively described in Supplementary Materials. The GS 42 tadpoles with a total length of 45 mm rapidly went into tail atrophy to develop into a SVL 16-18 mm GS 46 metamorph of brown, cream or olive green coloration, with or without a white lateral line. The whole series metamorphosed in synchrony and were of almost identical size (+/- 2mm).

Tadpole Malformations

Malformations are named and described by the developmental stages during which they occurred. Most developmental malformations were lethal to the entire clutch/population, but had survivors. The abnormal field populations produced abnormal clutches whose tadpoles developed in synchrony, but died upon reaching a certain developmental stage.

Time frame for Abnormal Development of Larval Stages to Metamorphosis (Fig. 1B, C Table 1).

By the 10th to 12th day, in those field populations subject to insecticidal spray, the abnormal tadpoles were either dead, or had been eaten. When the hind limbs appeared, a mass die off began, and the field populations never achieved GS 42. By the 19th day, when the population should be mass metamorphosing, instead mass mortality was observed (Fig. 1B, C).

Stage 16 malformation (Fig. 2): there were numerous malformations that occurred between 10 and 13 hrs. The GS 16 embryo prematurely formed a GS 18 tail bud, and the head formed head tubercles where the lateral eyes should normally have been located (Fig. 2C). Other GS 16 abnormality types were observed with premature distinction of the head and body regions, without any

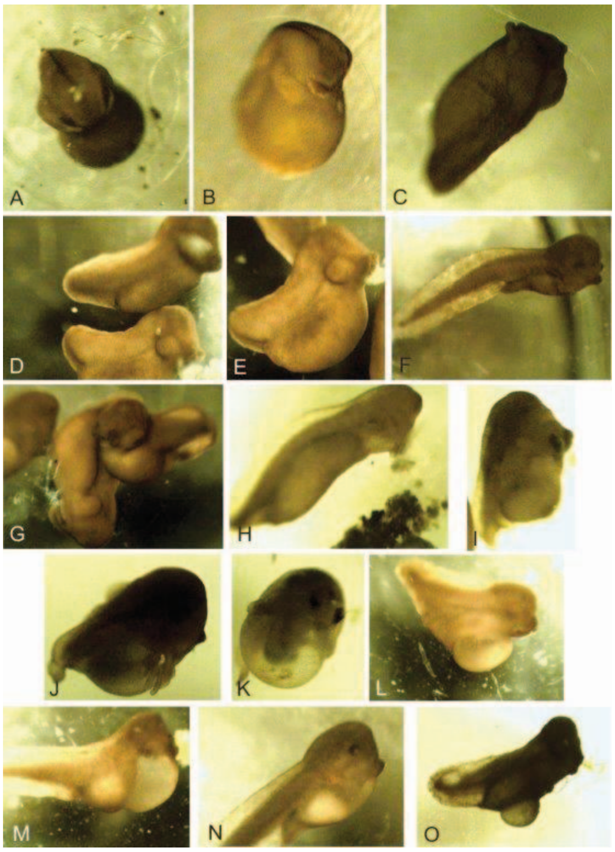

Figure 2. GS 16-GS 23 abnormal morphology roadside drainage ditch and H container. (A) GS 16 dorsal view of the enlarged head, gill buds flared, misshapen olfactory pits. (B) GS 16 dorsal view of the mouth precursor cleft. (C) GS 16 dorsal view of the tail bud, and head tubercles. (D) GS 19 lateral view of gill bud, tail fail to develop. (E) GS 19 lateral view of the gill bud, tail fail to develop. (F) GS 19 lateral view. (G) GS 19 lateral view of the tail. Body fails to lengthen, irregular shape. (H) GS 19 lateral view of the mouth with tubercles. (I)-(K) GS 19 same animal lateral view (I) 10 hrs, (J) 23 hrs. (K) 72 hrs progression of bloat. (L) GS 19 bloat lateral view. (M) GS 19 bloat lateral view. (N) GS 19 lateral view. (O) GS 19 bloat lump lateral view.

evidence of formation into a correct body or neural tube elongation. The body appeared rounded and the head region appeared abnormally enlarged and elongated. One irregular type had an asymmetrically enlarged head angled to the right (Fig. 2B). Another abnormal GS 16 type had an abnormally large elongated head with oversized, flared gill buds (Fig. 2A, B), abnormally large misshapen olfactory pits (Fig. 2A, B) with the olfactory pits incorrectly located at the stomadeum (Fig. 2B). A deep cleft formed the precursor of the mouth (Fig. 2B). The hypophyseal cleft was enlarged, and of an abnormal shape (Fig. 2A).

Stage 19 malformation (Fig. 2): malformation occurred immediately after hatching. At 24 hrs, the entire clutch typically lay motionless at the bottom, in dead carcass piles (Fig. 2D). The gill buds and tail fail to develop (Fig. 2D, E). In another GS 19 mass die off, the gill buds also failed to develop, however the tail elongates normally, with a clear dorsal and ventral tail fin (Fig. 2F). In

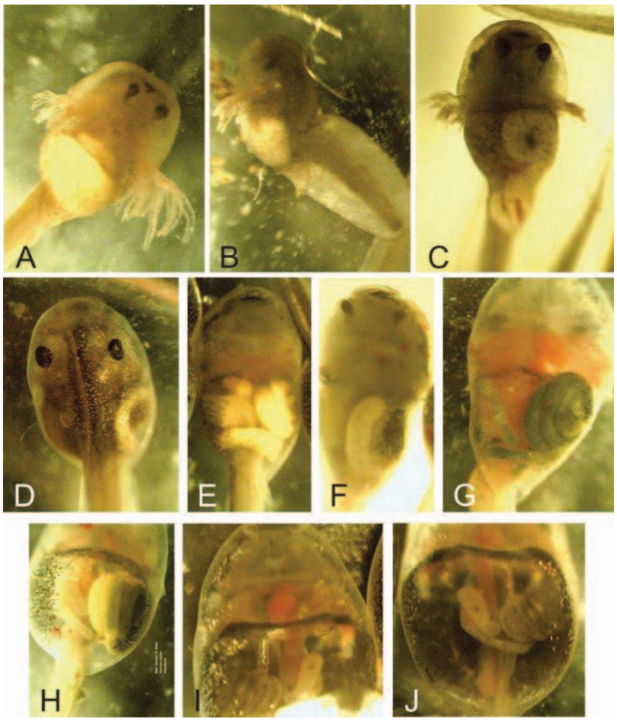

Figure 3. GS 23-25 abnormal morphology roadside drainage ditch and H container. (A) GS 23, ventral view, asymmetrical gills. (B) GS 23, lateral view, body not lengthen. (C) GS 23 intestinal location abnormality. (D) GS 25 dorsal view, intestinal location abnormality and GS 25 intestinal location abnormality. (E) GS 25, ventral view, intestinal location abnormality. (F) GS 25 intestinal location abnormality. (G) GS 25 intestinal location abnormality. (H) GS 25 organ abnormality. (I) GS 25 organ abnormality. (J) GS 25 organ abnormality.

some cases, either the tail, the body (or both structures) failed to lengthen, and were irregularly shaped (Fig. 2G). In some specimens, the mouth appeared abnormal with tubercles (Fig. 2F, H). Bloat in the body cavity was found at 23 hrs, and rapidly worsened with age to cause mortality by 72 hrs (Fig. 2I-K). Most malformations occurred in combinations with other malformations (Fig. 2H, I, M, N, O).

Stage 23 malformation (Fig. 3): the incidence of the GS 23 malformation was rare. The hatch most often could successfully internalise the gills. If the GS 23 could not internalise the gills, it was unable to lengthen its body, had asymmetrical gills, or it showed signs of intestinal abnormality (Fig. 3A-C).

Stage 25 malformation (Fig. 3-4): intestinal abnormalities included incomplete formation, malrotation, or either oversized or growth retarded organ. The location of the small and large intestine became abnormal (Fig. 3D-G). The abnormal growth of the intestine displaced the location of the other organs which were also abnormally formed in size and shapes (Fig. 3H-J). All intestinal irregularities appeared to be lethal, as all field tadpoles with intestinal malformation had a rapid, synchronous death. Another malformation found at the GS 25 was a normal intestine accompanied by a body size total length (TL) retardation (Fig. 4C) of 6 mm, compared with a normal total size of 15-20 mm.

The majority of the clutch ceased growth and remained

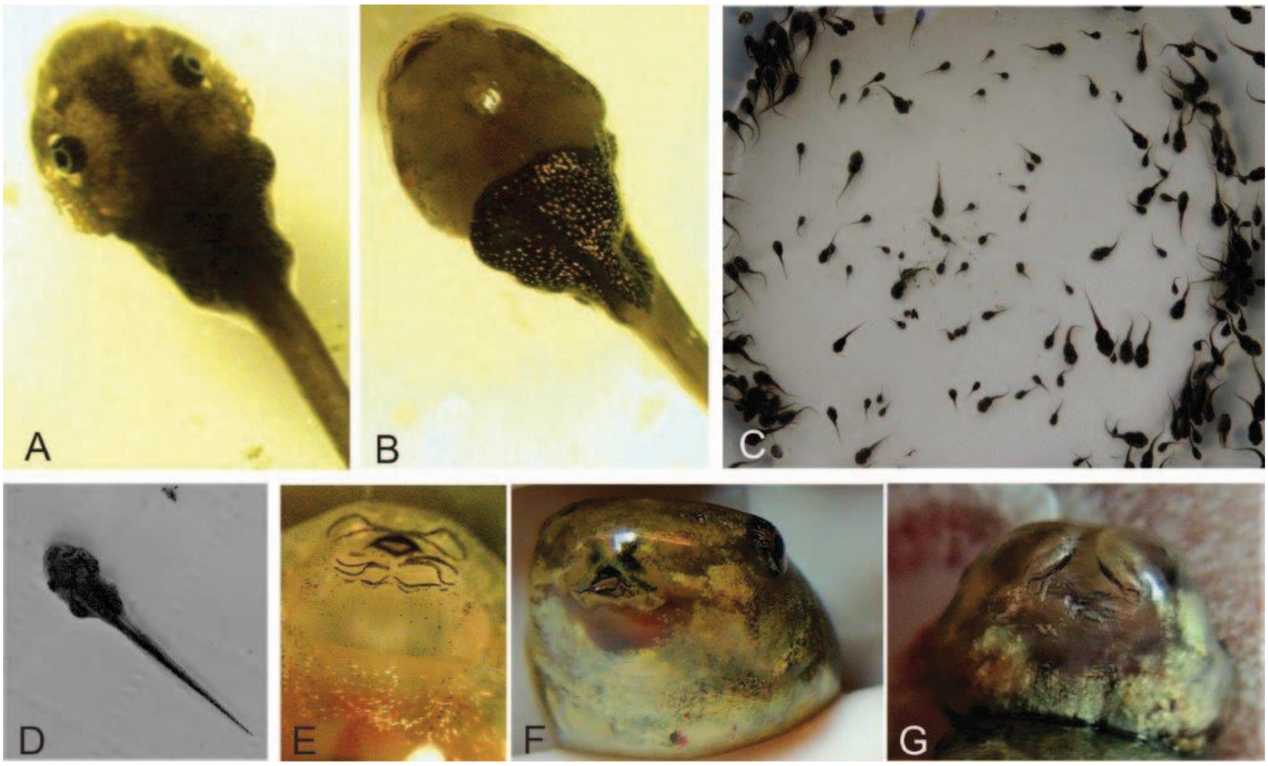

Figure 4. GS 25 abnormality roadside drainage ditch and H container. (A) GS 25, dorsal view, growth retardation, emaciation, lumps. (B) GS 25, ventral view, growth retardation, emaciation, lumps, epidermal blackening. (C) GS 25 one clutch all GS 25 growth retarded and normal growth. (D) GS 25 normal growth, emaciated, lumps. (E) GS 25 disfigured upper and lower labial tooth rows. (F) GS 25 disfigured lower labial tooth rows. (G) GS 25 cleft mouth.

at a 6 mm body size. Clutches with this severe TL body size growth retardation were observed alive, swimming, feeding and fully alert, while excreting waste through the vent tube, which had become blackened and dry (Fig. 4A, B). The field growth retarded clutches each day became more and more emaciated, and often developed lumps (Fig. 4A). The normal growers also developed lumps and became emaciated (Fig. 4D). The entire clutch soon underwent mass die off in synchrony between 2-4 weeks. The time taken for death from the emaciation and wasting away varied depending on the clutch. In some cases, the growth retardation malformation was not combined with the black shrivel epidermal blackening. There were always a few individuals in a clutch that grew to normal size. The growth-retarded 6 mm tadpoles were a valuable food source for the larger normal 15 mm clutch mates, which cannibalised their smaller siblings. The GS 25 mouthparts found more often were abnormal (Fig. 4B, E-G). The lower and upper labial tooth rows found disfigured (Fig. 4E, F), missing (Fig. 4B, F), or all missing. A rare cleft mouthpart was found (Fig. 4G).

Stage 41 malformation (Fig. 5): bloat, a common malformation, frequently occurred at the stage when tadpoles were at least 40 mm, and had fully formed hind limbs. There were two types of pronounced bloat: 1) Tadpoles turned black with the trunk and head bloated, and often lopsided (Fig 5A). The necropsied larvae revealed black bloat to be a form of forearm amelia caused from multiple organ system dysfunction (MODS). The animals typically near death were in the advanced stage of the bloat, with the forearms presumably unable to erupt because the internal organs visibly discernible through the transparent skin appeared blackened. The death of the field population from black bloat was always in synchrony, affecting almost all tadpoles (Fig. 1B, C). 2) The specimen was not black, but bloated in the gastro-intestinal region, which is discernible earlier in GS 19 (Fig. 2I-K, Fig. 5B). These bloated tadpoles with transparent skin were often the color of its internal fluid. The intensity of the red subcutaneous fluid colouration was dependent upon the concentration of the spray in the water of the site; the more concentrated and red-orange the spray water, the more red-orange the tadpoles appeared. The maximum fluid build-up in the stomach region resulted in lethality. Other abnormal red skin conditions were a red stained ventral epidermis, again the colour of the habitat water (Fig. 5E). A collected rare albino (Fig. 5D), a rare subcutaneous red anophthalmia (Fig. 5C) and a rare red patched tail with an internal gas bubble (Fig. 5F).

Stage 42-46 malformation (Fig. 6, 7; Table 2): the H artificial pond produced malformed metamorphs. Tadpoles grew to an abnormally gigantic GS 42 total size of 60 mm producing giant 21-23 mm SVL froglets. The same population commonly metamorphosed both oversized 21-23 mm and normal size 12-20 mm GS 46 (Fig. 6A). Normal brown, lime green intermingled with red-orange, yellow and rarely albino tadpoles and metamorphs were found in a single population. Some almost or recently metamorphosed kyphosis-emaciated and scoliosis frogs often showed abnormal colouration at metamorphosis (Fig. 6B-C). Another abnormality included one or both right and left forearm amelia (Fig. 6E-G). The tadpoles with both forearms amelia (Fig. 6E, G) had open wounds from failure of the slits in the body cavity to close. The larvae

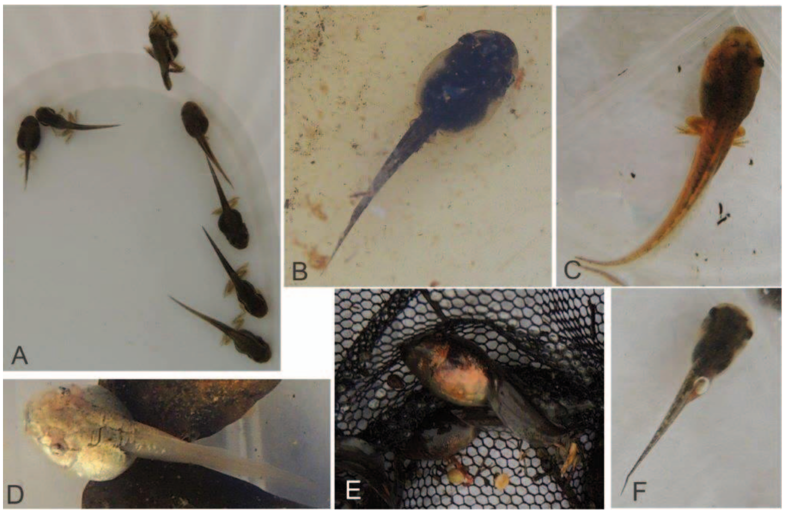

Figure 5. GS 25- GS 41 roadside drainage ditch. (A) GS 40-GS 42 bloat black. (B) GS 36 bloat clear with red subcutaneous fluid. (C) GS 39 one eye anophthalmia, red subcutaneous fluid (D) GS 25 albino. (E) GS 25 epidermal red discoloration. (F) GS 25 trapped bubble in tail.

unable to sprout both forearms had a strange swimming behaviour they swam on their back (Fig. 6G). Some tadpoles spent an abnormally extended period of time with their opercular sac extended without the forearm rupturing out (Fig. 6H). All post-metamorphic specimens from the site captured as GS 45 or GS 46 demonstrated spontaneous twitching of the limbs as older metamorphs neared adulthood.

Stage 46 malformation (Fig. 6-7; Table 2): Tail scoliosis (Fig. 6H-J) was a high frequency malformation found only at the untreated swimming pool in years 2012-2013 which produced both scoliosis or normal spine GS 46 metamorphose (Fig. 6I, J). The scoliosis tail appeared on the 5th day GS 25. There were different types of metamorph scoliosis (i.e., curvature of the spine) combined with or without total length growth retardation. The lethal scoliosis type occurred when the spine curved around the stomach with or without hind limb displacement (Fig 6I). In the common scoliosis cases (Fig. 6J) field captured GS 42 reared to GS 46 achieved a normal adult body size with sexual maturity evident in both genders, despite the formation of a large horn on the spine. The backbone rise with spine horn rapidly grew in height immediately after metamorphosis. Other abnormalities found at the untreated pool site were bloat (Fig. 6N), right forearm amelia (Fig. 6F), microcephaly-emaciation (lethal) (Fig. 6M), tumours on the tail (Fig. 6K) and black skin lesions (Fig, 6O).

The research effort resulted in 36,550 *O. septentrionalis* tadpoles examined in the field from 11 sites and found 25,136 abnormal ones, which is an abnormality incidence of 68.77 % (Fig. 8; Table 3). All sites in town had a high abnormality incidence regardless of treated or untreated water. The lowest abnormality average of 11.11 % occurred at site "CL" located on the outskirts of town, surrounded by forest (see Supplementary Materials Fig. S2E). The highest abnormality percentages came from the inner-city sites: HC 99.85 %, H container 98.9 %, H 75.29 %, H artificial pond 70.11 %, RP 57.36 % and RF 32.96 % (see Supplementary Materials Fig. S2A-E, G, J). The four non-spray control sites located inside the ENP Park and

Figure 6. GS 42-GS 46 H artificial pond abnormality. (A) GS 46, recently absorbed tail day 1 metamorphose. normal size 16mm (left), giant 21-23mm (right). (B) GS 46 kyphosis, emaciated, abnormal coloration, lethal. (C) GS 42 albino scoliosis. (D) GS 43, GS 45 lime green scoliosis giant (right) and brown normal size kyphosis (left). (E) GS 41 both forearm amelia with open wounds does not swim on back, lethal. (F) GS 42 right forearm amelia. (G) GS 42 both forearm amelia swims on back lethal. (H) GS 42 scoliosis, opercular sac abnormality. (I) GS 46 scoliosis hind limb displacement, growth retardation, lethal. (J) GS 46 scoliosis raised backbone with horn. (K) GS 41 tumor on tail. (L) GS 43 left hind limb amelia. (M) GS 46 kyphosis,emaciated, lethal. (N) GS 46 bloat clear, lethal. (O) GS 42 black spot on head.

Table 2. Individual animal count for each abnormality type collected at the H artificial pond (untreated) field site. Tadpole and metamorph abnormality type with count.

Tadpole	Scoliosis	Forearm Amelia	Skin black Spot	Forearm Amelia Swim on Back	Bloat	Tail Tumour	Forelimb dev.	Others (Kphosis)	Normal
Total Count	66	5	12	28	2	6	7	3	55
Metamorph	Kyphosis	Scoliosis	Amelia	Microcephaly	Bloat	Black Spot			Normal
Total Count	51	21	4	37	26	12			69

Table 3. Individual animal count totals for each field site. The % abnormality prevalence (left) and survival to metamorphosis (metamorph count) (right).

Abnormal	Normal	% AB	Total Animal	Metamorph	Total Animal	% Metamorph	Raw number
RP				RP met	RP total		
5799	4311	57.36 %	10110	1	10110	0.01 %	9.89E-05
H				H met	H total		
524	172	75.29 %	696	84	696	12.07 %	0.120689655
HC				HC met	HC total		
684	1	99.85 %	685	3	685	0.44 %	0.004379562
H pond				H pond met	H pond total		
129	55	70.11 %	184	1762	3459	50.94 %	0.509395779
H containers				H container met	H container total		
17608	196	98.90 %	17804	13	17804	0.07 %	0.000730173
Cl				CL met	CL total		
156	1248	11.11 %	1404	11	1404	0.78 %	0.007834758
RF				RF met	RF total		
117	238	32.96 %	355	4	355	1.13 %	0.011267606
ENP/Ave Maria				ENP/AM met	ENP/AM total		
0	2037	0.00 %	2037	0	2037	0.00 %	0

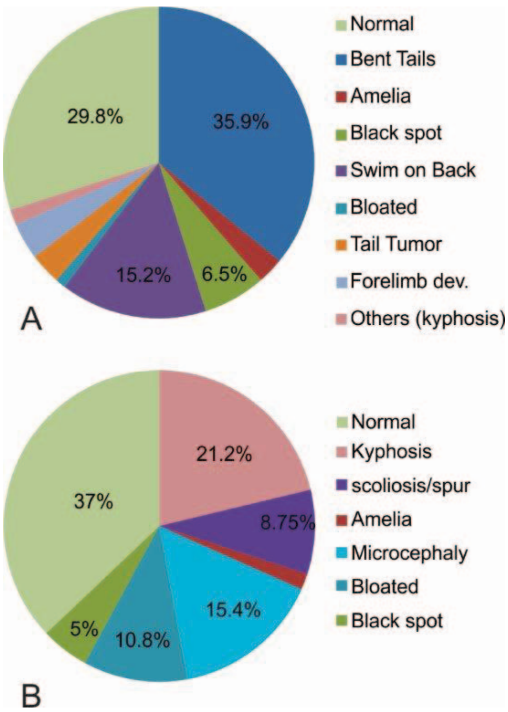

Figure 7. Pie Chart Graph of H artificial pond field site tadpole and metamorph abnormality types and proportions of each abnormality type (see Table 2). (A) Total tadpole abnormalities and proportion of each type. (B) Total metamorph abnormalities and proportion of each abnormality type.

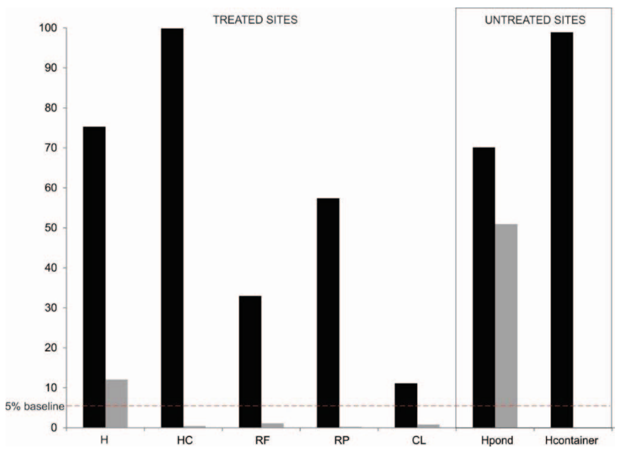

Figure 8. Bar Chart percentages of abnormal frogs (abnormality frequency) and percentages of number of metamorphs (survival to metamorphosis) for each in town field site H, HC, RF, RP, CL, H pond, H container for the field seasons 2012-2015 (see Table 3). The four control sites ENP (2 locations) & Ave Maria (2 locations) located in untreated insecticidal spray zones were not included in the Bar Chart having 0 % abnormality. The H container site represents single clutch data.

Ave Maria community (see Supplementary Materials Fig. S2H, I, K, L) had no tadpole field abnormalities. A total of 1878 metamorphs were found at 8 out of the 11 sites, with almost all survival-to-metamorphosis from the untreated H artificial pond site (see Supplementary Materials Fig. S2F) in which 50.94 % had successfully mass metamorphosed into frogs. The insecticide treated sites (see Supplementary Materials Fig. S2A-E) had no more than 12.07 % survival to metamorphosis.

DISCUSSION

To know the entire biology of the frog population might mean a massive task for most scientists, considering many frogs are data deficient in biological data (Nori et al., 2018). This study couples *Osteopilus septentrionalis* embryonic and larval staging with a field study of a hotspot of frog abnormality. To identify a hotspot, it is necessary to know the entire biology and normal morphology for all developmental stages for the frog or of all frog species that frequent the site. The examination of eggs, embryos and all earliest developmental stages must be examined to find abnormality as it was this case in SW Florida.

Larval development in *O. septentrionalis* follows the standard table of Gosner (1960) for the Gulf Coast toad, *Incilius valliceps*, and extensive to most Orton's (1953) type-IV larvae. The duration of the larval period for the population studied here (around 19 days) is quite short compared with other populations and species described so far (e.g., Altig & McDiarmid, 2015). In fact, a previous description of larval growth of this species refers to larval growth as rapid as less than one month to the end of metamorphosis, at about 15-16 mm SVL (Meshaka, 1993, 2001). This data implies a fast rate of development and growth in the population under study here. Later in the lifecycle, by stage 42, specimens have attained their metamorphic size similar to most anuran species (e.g., Hall et al., 1997; Fabrezi, 2011). Also, significant post-metamorphic growth is needed to reach the minimum adult size in males (27 mm SVL) and females (45.0 mm SVL) (Meshaka, 2001). The metamorphic period, between stages 42 and 46, lasts 7 days which is the average for many species (e.g., Downie et al., 2004).

This study extends the geographic range and type of anuran abnormalities found in the Unites States to SW Florida (see USFWS, 2018). According to Duellet (2000) and Lannoo (2008) a hotspot area is any area with a 5 % or higher abnormality prevalence. By this definition, this SW Florida hotspot is unique in that it is the first report of a hotspot located in a suburban area in Florida with an unusually high 68.77 % amphibian abnormality prevalence. The overall nationwide amphibian abnormality frequency average was low at 2 % abnormal, with three hotspots having a maximum abnormality frequency of 40 % (Reeves et al., 2013). An additional survey work reporting national hotspots maximum frequency increased in number from 3 to 96 hotspots with a maximum hotspot frequency of 25 % (Haas et al., 2018).

The USFWS survey (Reeves et al., 2013) differs from the SW Florida hotspot survey in site type. The National survey sampled frog populations in National park wetlands without any anthropogenic disturbance. We sampled an anthropogenic disturbance site in a suburban area. USFWS found skeletal, eye abnormality and limb abnormalities. The limb abnormality portion of the national lab findings, which was over half of their findings, resulted an abnormality to be expected considering wetland trematode populations are known to naturally exist (Sessions & Ruth, 1990; Johnson et al., 1999, 2004). Since there were no trematodes in the suburban roadside ditch, we did not find most of the limb abnormalities reported by USFWS.

An abnormality we did find with considerably high prevalence, that USFWS found also in the park wetland, was the backbone abnormality scoliosis frog. At the SW Florida hotspot we found an 8.75 % prevalence of scoliosis while the national laboratory found a 12.6 % national frequency. SW Florida had 8.75 % prevalence of scoliosis in metamorphs with a 35.9 % larval prevalence (bent tail). This reduction in metamorph abnormality frequency (compared to a high of 35.9 % in larvae) could be attributed to the lethal types of scoliosis identified in this study and found only in the metamorph. The eye anophthalmia abnormality for both projects (national and local) was at background level frequency. The abnormalities presented by USFWS were scarce in type with some high frequency abnormalities. Our suburban hotspot exhibited numerous types of abnormality, very high frequency abnormalities are to be expected in consideration of the fact that we are dealing with a chemical agent with damage to the embryo in the very stage when the frog forms the head, body and tail.

It has been reported that mass malformations in amphibians correlate with global population declines (Alroy 2015; Whitfield et al., 2016). The known natural agents causing mass malformations said to be infective in nature include the microbial agent ranavirus (Teacher et al., 2010; Earl & Gray 2014), chytrid fungus *Batrachochytrium dendrobatidis* (Muths et al., 2003; Vredenberg et al., 2010) and a parasitic trematode agent (Johnson et al., 2002).

Malformations have also been linked to exposure to chemical agents in the field, and in the laboratory. Chemical agents are known to have direct effects on developing systems whereas infective agents such as trematodes do not (Lannoo, 2001). There are many experimental studies which demonstrate that chemical agents can disrupt normal embryonic development and induce congenital malformations (e.g., Osano et al., 2002; Hu et al., 2015). Lowcock (1997) demonstrated that abnormal genetic profiles were more frequent in green frog populations that had been exposed to pesticides. Epigenetic malformations occur when genes and gene expression are normal, but some deviation from expected environmental circumstances occurs at the time when genes are being exposed to cause the abnormality (Lannoo, 2001). Congenital malformations are defined as damage to the primary DNA sequence genome, either overall, or at a specific locus, or of the organ(s), tissue(s), or cell(s) resulting in the alteration of the developmental field (Hennekam et al., 2013).

Genomic analysis on abnormal *O. septentrionalis* to look for genomic damage in order to classify the hotspot frog abnormality as congenital has not been performed. However, we do have data to hypothesize that our abnormalities are congenital, and inherited. The abnormality occurring at the hotspot in this study is at the embryonic stage during neural tube formation and early enough in development to indicate altered genes and gene loci. We also have found the same frog abnormalities in both the treated and untreated water on a repeat annual basis, which indicates the adults have been exposed to a chemical agent that is affecting their reproductive output. The reproductive organs are abnormal (Pratt Anzaldua, pers. obs). The types of gonadal abnormality found are classified as congenital in humans and associated with an increased gene copy number of the VAMP7 gene (Tannour-Louet et al., 2014).

A chemical-parasite agent combination has been recently proposed by USFWS as the leading cause for skeletal and eye abnormalities during metamorphosis (Haas et al., 2018). The recent USFWS (2018) report (Haas et al., 2018) claimed that the increase in hotspots nation-wide is attributable to discovered oil well contamination at refuge sites. Interestingly, Florida larvicidal insecticides contain a petroleum distillate spreader. However, the abnormality found exclusively in the treated roadside ditch pool was black bloat, not scoliosis backbone abnormality, and not limb abnormalities, simply because the tadpoles could not even emerge their limbs to metamorph. In the treated water contaminated with the petroleum oil spreader, there was severe bloat which worsened with the increase in concentration and freshness of the spray. Conversely, skeletal scoliosis abnormality was not found in the treated water, so it is unlikely that scoliosis can be attributed to the petroleum oil. It is more likely that the bloat we see at the SW Florida hotspot is an abnormality likely attributable to oil poisoning because bloat is a symptom of oil poisoning in cattle exposed to oil fields (Osweiler, 2018).

Three studies support our hypothesis that a chemical agent or a combination of chemical agents is responsible for causing the Florida hotspot frog abnormalities. The same field tadpole mouthpart abnormalities comprising partial to complete absence of keratinisation of lower and upper tooth rows found in SW Florida suburban were recently found in SE Brazil (Navarro-Loano et al., 2018). The qPCR analysis of abnormal tadpoles detected no evidence of the fungal pathogen *Batrachochytrium dendrobatidis* (Bd). In the laboratory, Sayim (2008) subjected GS 21 Marsh frog (*Rana ridibunda*) tadpoles to the organophosphate pesticide Malathion then photographed and described the same abnormalities as we describe: abnormal gut coiling, general edema, swimming on backs, and twitching. Bullfrog tadpoles in a constructed wetland located in Georgia which had received treated wastewater (Ruiz et al., 2010) were found with open wounds, due to failure of the slits to close after forelimb emergence, and tadpole scoliosis abnormalities.

It has been only recently recognised in herpetology the lack of studies assessing amphibian malformations and their causes in hotspot biodiversity areas (Sanchez-Domene et al., 2018). There have been even fewer number of surveys for abnormal amphibians conducted in the suburban heavy mosquito control insecticidal spray zone areas. We have shown that the frequency of the frog abnormality in one small region in suburban Florida is alarmingly high at 68.77 % which may well indicate that all amphibians in Florida may be in particular trouble, when one considers that all of Florida has been subjected to the same pesticides. As such, this publication could be used as a tool for the identification of other malformation hotspots in Florida using *O. septentrionalis* as a sentinel species.

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REFERENCES

Alroy, J. (2015). Current extinction rates of reptiles and amphibians. *Proceedings of the National Academy of Sciences of the United States of America* 42, 13003-13008.

Altig, R. & McDiarmid, R.W. (2015). *Handbook of larval amphibians of the United States and Canada*. Cornell University Press 1-376.

Barbour, T. (1931). Another introduced frog in North America. *Copeia* 1931, 140.

Ceballos, G., Ehrlich, P. & Dirzo, R. (2017). Biological annihilation via the ongoing sixth mass extinction signaled by vertebrate population losses and declines. *Proceedings of the National Academy of Sciences of the United States of America* 30, E6089-E6096.

Downie, J. R., Bryce, R. & Smith, J. (2004). Metamorphic duration: an under-studied variable in frog life histories. *Biological Journal of the Linnean Society* 83, 261-272.

Duellet, M. (2000). Amphibian deformities: Current state of knowledge. *Ecotoxicology of Amphibians and Reptiles*. Pensacola: SETAC Press 617-646.

Dumeril, A. & Bibron, G. (1841). Erpetologie general, ou, Histire naturelle complete des reptiles. 8, Roret, Paris.

Earl, J.E. & Gray, M.J. (2014). Introduction of ranavirus to isolated wood frog populations could cause local extinction. *Ecohealth* 4, 581-592.

Fabrezi, M. (2011). Heterochrony in growth and development in Anurans from the Chaco of South America. *Evolutionary Biology* 38, 9128-9135.

Fabrezi, M., Quinzio, S., Cruz, J. Chuliver, M., Abdala, V., Ponssa, M., & Goldberg, J. (2017). Forms, size and time in ontogeny of Amphibians and Reptiles. *Cuadernos de Herpetologia* 31, 103-126.

Fisher R.N. & Shaffer, H.B. (1996). The decline of amphibians in California's Great Central Valley. *Conservation Biology* 10, 1387–1397.

Fisher, M. C., Henk, D.A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., & Gurr, S. J. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484, 186-94.

Gosner, K. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16, 183-190.

Haas, S.E., Reeves, M.K., Pinkney, A.E. & Johnson, P. (2018). Continental-extant patterns in amphibian malformations linked to parasites, chemical contaminants, and their interactions. *Global Change Biology* 24, e275-e288.

Hall, J., Larsen, J. & Fitzner, R. (1997). *Postembryonic ontogeny* of the spadefoot toad, *Scaphiopus intermontanus* (Anura: Pelobatide): external morphology. *Herpetological Monographs* 11, 124-178.

Hennekam, R.C., Biesecker, L.G., Allanson, J.E., Hall, J.G., Opitz, JM., Temple, IK. & Carey, J.C. (2013). Elements of morphology: general terms for congenital anomalies. *American Journal of Medical Genetics Nov 161A* 11, 2726-33.

Hu, L., Zhu, J., Rotchell, J.M., Wu, L., Gao, J., Shi, H. (2015). Use of the enhanced frog embryo teratogenesis assay- *Xenopus* (FETAX) to determine chemically-induced phenotypic effects. *Science of the Total Environment* 508, 258-265.

Johnson, P., Lunde, K., Ritchie, E., Launer, A. (1999). The effect of trematode infection on amphibian limb development and survivorship. *Science* 284, 5415, 802-804.

Johnson, P., Lunde, K.,Thurman, E., Richie, E., Way, D., Sutherland, J., Kapfer, T., Frest, J., Bowerman, J., Blaustein, A. (2002). Parasite (*Ribeiroia ondatrae*) infection linked to amphibian malformations in the Western United States. *Ecological Monographs* 72, 151-168.

Johnson, P. & Chase, J. (2004). Parasites in the food web: linking amphibian malformations and aquatic eutrophication. *Ecology Letters* 7, 521-526.

Johnson, S. (2013). The cuban treefrog (*Osteopilus septentrionalis*) in Florida. UF/IFAS document is #WEC 218. Google Scholar.

Lannoo, M.J. (2001) What amphibian malformations tell us about causes [abs.]: *Society for Integrative and Comparative Biology Annual Meeting*, Chicago,Ill.,January 3-7:172.

Lannoo, M.J. (2008). *Malformed frogs: The Collapse of Aquatic Ecosystems*. Berkeley: University of California Press 1-270.

Lowcock, L.A., Sharbel T., Bonin, J., Ouellet M., Rodrigue, J., DesGranges, J. (1997). Flow cytometric assay for in vivo effects of pesticides in green frogs (*Rana clamitans*). *Aquatic Toxicology* 38, 241-255.

Meshaka, W.E. Jr. (1993). Hurricane Andrew and the colonization of five invading species in southern Florida. *Florida Scientist* 56, 193-201

Meshaka, W.E. Jr. (2001). *The cuban treefrog in Florida: life history of a successful colonizing species*. University Press of Florida. Gainesville, FL. 191 p.

Meshaka, W.E. Jr. (2011). A runway train in the making: the exotic amphibians, reptiles, turtles, and crocodilians of Florida. *Herpetological Conservation and Biology* 6 (Monograph 1), 1-101.

Muths, E., Corn, P., Pessier A. & Green, D.E. (2003). Evidence of disease related amphibian decline in Colorado. *Biological Conservation* 110, 357-365.

Navarro-Lozano, A., Sanchez-Domene, D., Rossa-Feres, D., Bosch, J. & Sawaya, R. (2018). Are oral deformaties in tadpoles accurate indicators of anuran chytridiomycosis? *PLOS ONE* 13, e0190955.

Nieuwkoop, P.D. & Faber J. (1994). Normal Table of *Xenopus laevis* (Daudin): a systematical and chronological survey of the development from fertilized egg till the end of metamorphosis. New York: Garland Pub 252 p.

Nori, J., Villalobos, F. & Loyola, R. (2018). Global priority areas for amphibian research. *Journal of Biogeography* 45, 2588-2587.

Osano, O., Oladimeji, A.A., Kraak, M.H. & Admiral, W. (2002). Teratogenic effects of amitraz, 2,4-dimethylaniline, and paraquat on developing frog (*Xenopus*) embryos. *Archives Environmental Contamination Toxicology* 43, 42-49.

Osweiler, G. (2018). Overview of Petroleum Product Poisoning. Merck Veterinary Manual.com.

Reeves, M., Medley, K., Pinkney, A., Holyoak, M., Johnson, P. & Lannoo, M.J. (2013). Localized hotspots drive continental geography of abnormal amphibians on U.S. wildlife refuges. *PLOS ONE* 8 e:77467. Doi: 10.1371/journal.pone.0077467, 1-14.

Ruiz, A., Maerz, J., Davis, A., Keel, K., Ferreira, A., Conroy, M., Morris, L. & Fisk, A. (2010). Patterns of development and abnormalities among tadpoles in a constructed wetland receiving treated wastewater. *Envionmental Science and Technology* 44, 4862-4868.

Sanchez-Domene, D., Lozano-Navarro, A., Acayaba, R., Picheli, K., Montagner, C., Rossa-Feres, D., Silva, F. & Almeida, E. (2018). Eye malformation baseline in *Scinax fuscovarius* larvae populations that inhabit agroecosystem ponds in southern Brazil. *Amphibia-Reptilia* 10.1163/15685381-20181038.

Sayim, F. (2008). Acute Toxic Effects of Malathion on the 21st Stage Larvae of the Marsh Frog. *Turkey Journal of Zoology* 32, 99-106.

Sessions, S.K. & Ruth, S.B. (1990). Explanation for naturally occurring supernumerary limbs in amphibians. *Journal of Experimental Zoology* 254, 38-47.

Stuart, S.N., Chanson, J., Cox, N., Young, B., Rodrigues, A., Fischman, D. & Waller, R. (2004). Status and Trends of Amphibian Declines and Extinctions Worldwide. *Science* 306, 1783-1786.

Stuart, S.N. (2008). *Threatened Amphibians of the World*. Lynx Editions; 1st edition 1-776.

Tannour-Louet, M., Han, S. & Louet,, J.F. (2014). Increased gene copy number of the vesicle SNARE VAMP7 disrupts human male urogenital development through altered action. *Nature Medicine* 20, 715-724.

Teacher, A., Cunningham A. & Garner T. (2010). Assessing the long-term impact of Ranavirus infection in wild common frog populations. *Animal Conservation* 13, 514-522.

Vredenberg, V.T., Knapp, R., Tunstall, T. & Briggs, C. (2010). Dynamics of an emerging disease large-scale amphibian population extinctions. *Proceedings of the National Academy of Sciences of the United States of America* 21, 9689-9694.

Whitfield, S., Lips, K. & Donnelly, M. (2016). Amphibian Decline and Conservation in Central America. Copeia 104, 351-379.

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