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Development of a non-amphibious amphibian - an interview with a coquí

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ABSTRACT Development without a free-living tadpole is common among Ibero American frogs. The most derived condition is direct development where the tadpole has been eliminated, and the most investigated direct developing frog is *Eleutherodactylus coqui*. To provide a different point-of-view, an imaginary interview with a coqui is conducted. Opinions are offered on invasive species, developmental features that are surprisingly conserved, and novelty in germ layer specification.

KEY WORDS: *metamorphosis, amniote evolution, invasive species, EvoDevo, fate map, yolk*

Introduction

The Ibero American animals that have contributed the most to investigations of embryonic development are undoubtedly the frogs. *Gastrotheca riobambae* and *Eleutherodactylus coqui* have been most frequently involved (Elinson and del Pino 2012), and *Lepidobatrachus laevis* (Bloom *et al.,* 2013; Amin *et al.,* 2015) has attracted attention recently. There are many others, whose interesting life histories promise to provide unique perspectives (Araújo *et al.,* 2016; Fabrezi *et al.,* 2010, 2016; Goldberg and Vera Candioti 2015; Goldberg *et al.,* 2012; Grosso *et al.,* 2019).

The reason these Ibero American frogs have been attractive to developmental biologists is that they often do not fit the amphibian paradigm of aquatic eggs developing into feeding, herbivorous tadpoles and metamorphosing into carnivorous, usually terrestrial adults. That paradigm arose because much of Western science developed in the temperate zones of Europe and North America, and in those areas, all frogs show that pattern of ontogeny. Had science arisen in Ibero America, there would be a very different paradigm for amphibian development. In fact, these animals probably would not have even been called amphibians, as many Ibero American frogs do not lay aquatic eggs or have tadpoles living in water.

Rather than laying thousands of small eggs (less than 1.8 mm diameter), Ibero-American frogs of the genera *Gastrotheca*, *Eleutherodactylus*, and related ones lay small numbers of large, greater than 3 mm diameter eggs (Fig.1A). This heavy investment makes these large eggs individually valuable, so to protect them, the Ibero-American frogs have evolved methods of parental care. In *Rhinoderma darwinii*, the fertilized eggs are swallowed by the male, who incubates them in his vocal sac. With *Pipa pipa*, the eggs stick to the female's back. The back skin then swells up to surround the eggs in individual chambers, where they develop. In *Gastrotheca* spp, the eggs are shoved into a pouch on the female's back. In these and other cases, the egg develops into a tadpole, which is non-feeding for part or all of its development. The offspring are released from their parent either as large tadpoles or as small frogs.

In contrast to these modes, the extremely speciose group of Terraranan frogs has direct development (Fig 1). There is no tadpole. The large eggs, frequently brooded by their father, develop directly to tiny frogs (Fig. 2). The best studied frog of this type is *Eleutherodactylus coqui*, and I will now take this opportunity to interview one of these frogs.

As an *Eleutherodactylus coqui*, can you tell us a little about yourself?

Sure, you can call me coquí. I am from Puerto Rico, and everyone knows me there. My name is on coffee, glue, night clubs, and many other things, and really, I am a national symbol of the island. I am even on a United States quarter (Fig. 2)! When you grow up in Puerto Rico, you go to sleep with my singing as your lullaby. When Puerto Ricans move off the island, they often feel something is missing, which they cannot place. They remember when they hear my song.

There is a myth that I would like to dispel at the start, and that is if a coquí leaves Puerto Rico, it will die of a broken heart. That is not true. This warning may have been directed at the humans themselves to encourage them to stay on the island.

Abbreviations used in this paper: TS stage, Townsend Stewart stage.

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Since you brought up emigration, I would like to ask a politically sensitive question. There is a lot of concern currently about refugees, illegal aliens, undocumented immigrants, and invading species. As the coquies have now emigrated from Puerto Rico to southern California and Hawaii, what is your opinion of the reception that they have received?

I have no opinion about how humans treat other humans, but the way humans have dealt with immigrant coquies is insane. On the one hand, humans give a lot of lip service to the problem of disappearing amphibians. A number of frog species have gone extinct and others are threatened, due undoubtedly to environmental disruptions caused by humans. On my own island of Puerto Rico, two species of 16, related to us, have disappeared in the last 40 years.

One solution for amphibians is to emigrate around the world, so if a disaster occurs at one locale, we can survive in another. But what happens when we try to immigrate? People in California complain of the noise from our beautiful singing. Is that noise really worse than the traffic and the horns?

More troubling is the situation in Hawaii. The Hawaiian government is engaged in active coquí genocide (Fig. 3). They have slaughtered thousands of us by spraying citric acid through the forests. These criminal actions have apparently worked on some islands, but most notably not on Maui or the Big Island of Hawaii. The deep, jungle valleys in Maui have given us sanctuary, and we are thriving on the Big Island. There are such wonderful retreats for us there in the holes in the volcanic rock, so it is hard to imagine that they can wipe us out.

We have been on the Big Island for 30 years, so a whole generation of humans has grown up, hearing us singing in the night. We are no longer an alien species to most people, so I predict that like many immigrants, we will end up being accepted.

Thank you for your frank comments. Now on to science. Beginning around 1990, a number of labs realized that you could serve as a model species for studies of EvoDevo. Do you think coqui will continue as a model for this kind of research?

That is really hard to say. Fashions ebb and flow in science. Development of other Eleutherodactylids was studied intensively for several decades by Lynn and Hughes with a focus on metamorphosis and neural development (Lynn 1942; Lynn and Peadon 1955; Hughes 1962, 1966; Hughes and Edgar 1972). Direct development in frogs was then largely ignored for 20 years until the Evo Devo investigations began.

I have to admit that we present certain limitations for the investigator. First, even in the lab, we decide when to mate and lay eggs. No one has figured out hormonal or environmental regimes to get embryos on demand. Second, we only produce about 30 embryos at a time, so we do not provide the unlimited numbers that frogs like *Xenopus* do. The investigator has to be ready to work on our schedule, not theirs.

I am interested in your comments about the difficulties in obtaining coquí embryos. These difficulties are worse for most of the other interesting Ibero-American frogs, and in many cases, the embryos are only known through the chance finding of a clutch in the wild. Are these difficulties worth the effort?

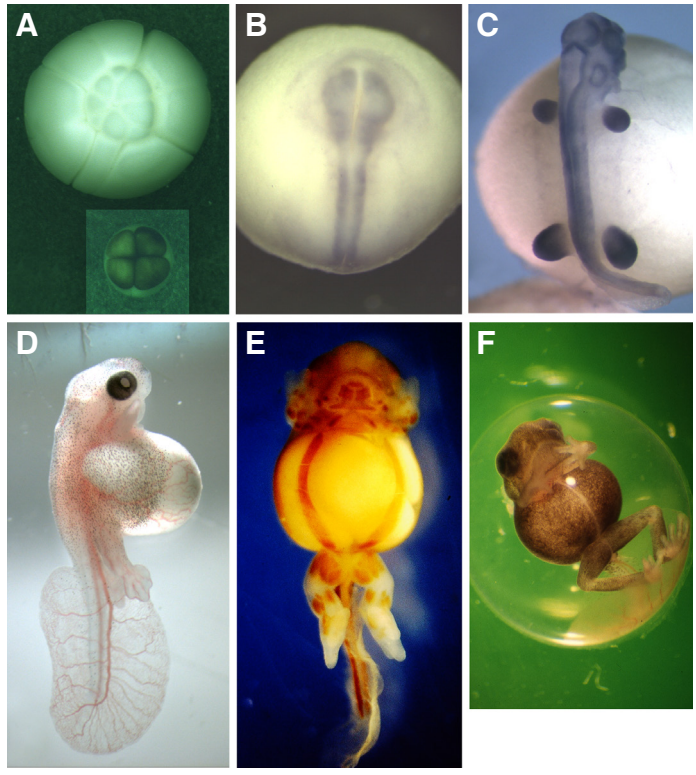

Fig. 1. *Eleutherodactylus coqui* development. (A) *Animal pole views of a 16-cell* E. coqui *embryo (top) and an 8 cell* Xenopus laevis *embryo (bottom). The embryos were photographed together to illustrate the size difference. (From Ninomiya* et al.*, 2001.)* **(B)** *TS stage 3 (neurula) and* **(C)** *TS stage 5 embryos stained* in situ *for cyclin (ccnd1) expression. (From Nath* et al.*, 2013.)* **(D)** *TS stage 10, removed from its jelly capsule allowing its vascularized tail to unfurl. The pigmented body wall encompasses half of the yolk mass.* **(E)** *TS stage 11 embryo stained for skeletal muscle (12.101 antibody). Ventral view showing muscle bands on either side of the unencompassed yolk mass.* **(F)** *TS stage 12 within the fertilization envelope.*

There is a distinct advantage that counteracts the difficulties. Examination of the embryos, derived from large eggs and/or raised in the unusual environments inside a frog, is a win-win proposition. The investigator might hope to discover something novel, but finding conserved elements under these unusual circumstances is also meaningful.

Can you give some examples of "meaningful" conserved elements?

There are many, so I will confine myself to three. The first element is obvious. Despite the large egg size of any of these Ibero-American frogs, cleavage is complete (holoblastic) (Fig. 1A) as in all other amphibians and in the sister group lungfish. That constraint was broken at least once in an unknown way with the evolution of the amniotes, the reptiles, birds, and mammals. Experimentation with coquí embryos provides ways to envision how the constraint may have been broken. When cell division of the vegetal yolky area of our eggs was partially inhibited, gastrulation and formation of a complete body axis still occurred (Buchholz *et al.,* 2007). Our embryos can accommodate some non-cellularized yolk, a potential pre-condition for the evolution of an amniote-like egg.

Fig. 2 (left). Coquí on a coin. *As part of its commemorative park series, the United States issued a quarter in 2012 for El Yunque National Forest in Puerto Rico. The quarter depicts an adult coquí, but just-hatched coquí froglets are only slightly larger than the engraved image.*

Fig. 3 (right). Hawaiian anti-coquí poster.

The second conserved element is the formation of primordial germ cells. In all frogs investigated, primordial germ cells are determined by germ plasm, which is initially localized to the vegetal surface of the egg. The vegetal surface in our large eggs is more than 3mm from the egg nucleus, a very far distance, so one might predict that if we have germ plasm, it would be localized closer to the animal pole. That is not the case. Localization of the germ plasm at the vegetal surface is conserved (Elinson *et al.,* 2011). In retrospect, this should not have been a surprise. During gastrulation in all frogs, the vegetal surface ends up as the floor of the archenteron. From there, the primordial germ cells have a relatively short migration up the dorsal mesentery to the genital ridge.

The third "meaningful" conserved element is thyroid hormone induced metamorphosis. Our development looks so continuous to a frog morphology, with early limb buds and frog-like eyes and jaw (Fig. 1, 2), that it was reasonable to think that metamorphosis itself had been abandoned. Despite the precocious appearance of frog-like features, we need thyroid hormone stimulation to complete our development (Callery and Elinson 2000). We express thyroid hormone receptors, deiodinases, and other molecules in the thyroid axis in patterns similar to other frogs (Callery and Elinson 2000; Laslo *et al.,* 2019).

It is worth mentioning that we have thyroid hormone receptor RNAs and thyroid hormone in our oocytes (Callery and Elinson 2000; Laslo *et al.,* 2019). This has led to the speculation that there are thyroid hormone mediated events, before our embryos form a thyroid gland (Callery *et al.,* 2001; Laslo *et al.,* 2019), but no one has figured out how to test this hypothesis.

One of the striking features of your development is the early formation of large limb buds and the continuous development of frog legs. Is this a novelty in your development?

It is a novelty morphologically compared to frogs with tadpoles, but novelty has not been found molecularly. Limb gene expression is like other vertebrates (Fang and Elinson 1996; Hanken *et al.,* 2001; Elinson *et al.,* 2008; Kerney and Hanken 2008; Sabo *et al.,* 2009; Gross *et al.,* 2011; Nath *et al.,* 2013a,b; Laslo *et al.,* 2019).

Wait a minute. Certainly there are differences in the expression of some genes in the limbs of coquí compared to other animals (Kerney and Hanken 2008; Gross *et al.*, 2011). Are you ignoring those?

I do not want to dismiss that data, but the importance of any of those differences, particularly as they relate to direct development, is not known. On the other hand, the recent finding that our digits are sculpted by interdigital cell death, as in amniotes, provides a fascinating insight into this evolutionary change (Cordeiro *et al.,* 2019).

In spite of all of the data, there is presently no explanation for our ability to form large limb buds precociously. Our limb buds are huge compared to those in a tadpole (Elinson 1994; Nath *et al.,* 2013b). What are the molecular and cellular bases for that morphology? There are several genes which can serve as start-

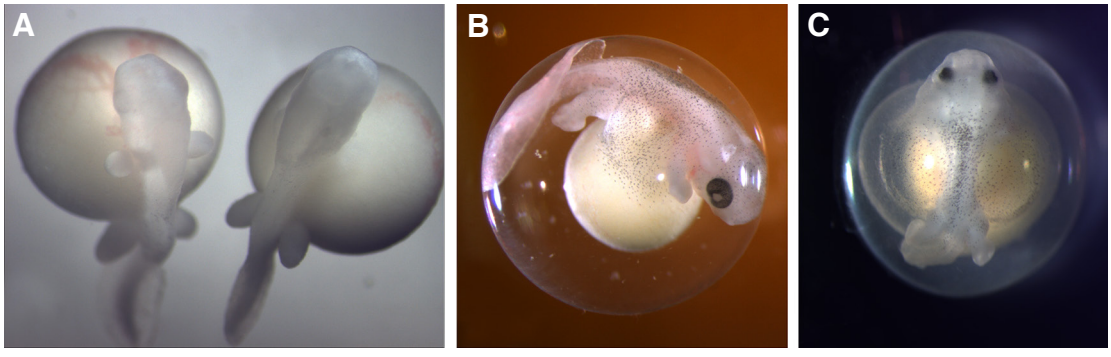

Fig. 4. Citral inhibition of coquí forelimbs. (A) *The TS stage 6 embryo on the left has both forelimbs and hindlimbs. The embryo on the right was treated with citral and lacks forelimbs.* **(B)** *Normal TS stage 8 embryo within its fertilization envelope.* **(C)** *Citral treated embryo has hindlimbs but lacks forelimbs. It is swollen with fluid. (From Lee and Elinson 2008).*

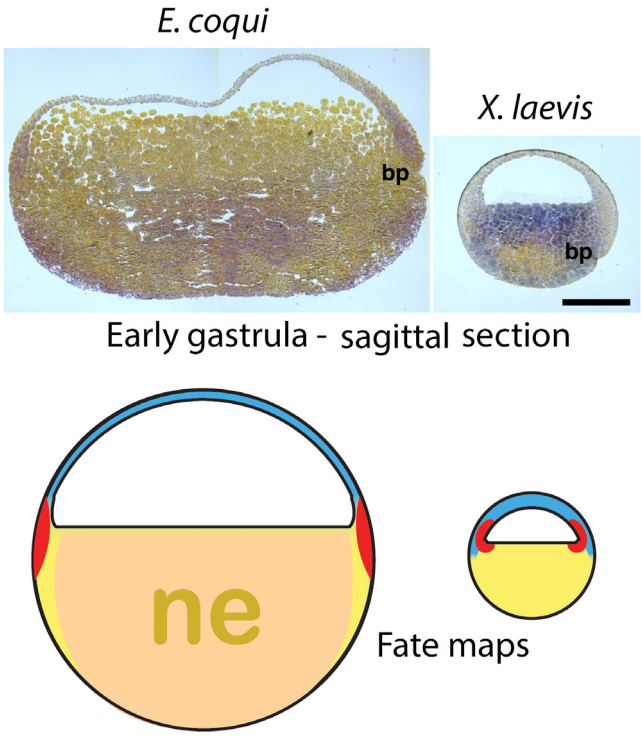

Fig. 5. Gastrula fate maps. (Top) *Sagittal sections of early gastrulae of* E. coqui *and* X. laevis. *Dorsal lip of the blastopore (bp). Scale bar: 500μm. (From Ninomiya* et al., *2001).* **(Bottom)** *Fate maps of* E. coqui *and* X. laevis. *The ectoderm (blue), mesoderm (red), and definitive endoderm (yellow) in* E. coqui *are more animal and peripheral than in* X. laevis *due to the large central mass of nutritional endoderm (ne, pale orange). The cells of the nutritional endoderm are discarded as their yolk is used up. (From Elinson and del Pino 2012).*

ing points for this question. The cell cycle genes, cyclin D2 and N-myc, are expressed in broad domains, anticipating the formation of the hindlimb bud (Nath *et al.,* 2013b). These expression domains may represent the limb field, and manipulating these domains would be a way to explore the allocation of cells to the large limb buds.

Another gene of interest is EcRaldh2, expressed in the presumptive forelimb bud region (Elinson *et al.,* 2008). EcRaldh2 codes for retinaldehyde dehydrogenase, one of the enzymes in the pathway that converts Vitamin A to retinoic acid. A striking, but for me sad, syndrome sometimes appears in embryos of females kept for many months in a lab. The embryos lack forelimbs and swell up with fluid. This forelimb-edematous syndrome can be replicated by treatment with citral, an inhibitor of retinoic acid synthesis (Fig. 4) (Lee and Elinson 2008). This result indicates that we need Vitamin A in our diet and that EcRaldh2 expression is necessary for forelimb formation as in other vertebrates.

The anatomy and morphology of your embryo is so different from those of tadpole-producing species; yet, nothing you have said so far gives much insight into the cellular or molecular basis for the differences. The tadpole is considered basal in frog phylogeny, with multiple independent origins of direct development (Duellman and Trueb 1986; Thibaudeau and Altig 1999). Is there any novelty that helps us understand how direct development has arisen in evolution?

At the risk of being accused of toadying up to my interviewer, a significant novelty is the change in germ layer specification leading to a novel tissue, the nutritional endoderm (Fig. 5) (Buchholz *et al.,* 2007). In the model system of *Xenopus laevis*, the fate map of the early embryo places mesoderm equatorially between the more animal ectoderm and the more vegetal endoderm. A key regulator of this germ layer formation is the transcription factor VegT, whose RNA is localized to the vegetal surface of the egg. Embryos of other frogs have similar fate maps, and *Rana pipiens* VegT RNA shares a similar vegetal localization (Nath *et al.,* 2005).

In contrast, our mesoderm and definitive endoderm form at the periphery of the embryo, closer to the animal pole (Fig. 5) (Ninomiya *et al.,* 2001; Buchholz *et al.,* 2007). VegT RNA correspondingly is not localized to the vegetal surface but is in the animal region (Beckham *et al.,* 2003). I used the phrase "definitive endoderm" for the endoderm that forms the digestive tract and endodermal organs, because the great mass of large vegetal cells does not form organs. They make up a novel tissue, the nutritional endoderm (Fig. 6) (Buchholz *et al.,* 2007). Much like the yolk sac in birds, these cells provide nutrition to the embryo and then disappear from our froglet when the yolk is depleted.

Let me mention another novel feature associated with the nutritional endoderm. The prospective nutritional endoderm is surrounded by ectoderm and mesoderm during gastrulation, as in all frogs. Later in our development however, the nutritional endoderm is secondarily surrounded by the body wall, consisting of pigmented ectoderm and mesoderm (Fig. 1D) (Elinson and Fang 1998). The developing rectus abdominis muscles on

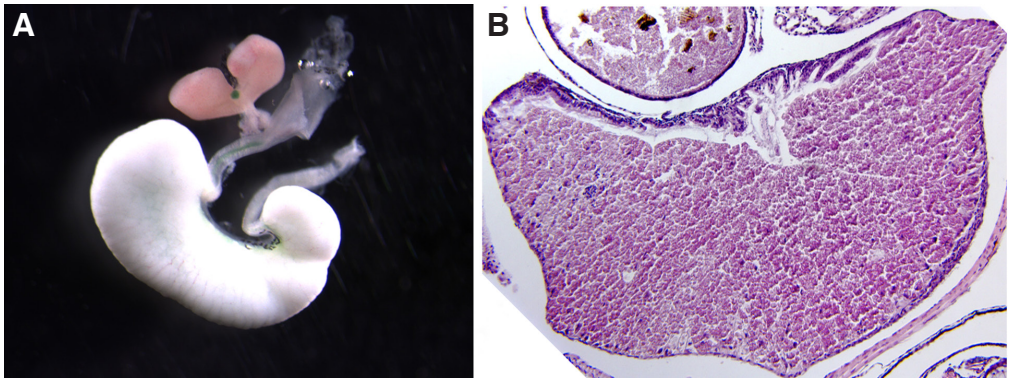

Fig. 6. Nutritional endoderm. (A) *Digestive tract isolated from a hatched froglet (post TS stage 15). The pink liver lobes are attached to the translucent intestine at the base of the stomach. The white mass is the nutritional endoderm which is attached to the intestine.* **(B)** *A section through the nutritional endoderm reveals intestinal tissue along the upper surface. The rest of the yolk-rich nutritional endoderm is bounded by a thin epithelium. (From Buchholz* et al., *2007).*

the right and left sides are at the leading edges (Fig. 1E), and this muscle fuses at the ventral midline. This surrounding of the yolk mass is reminiscent of the formation of the yolk sac in birds, although it is not analogous.

How unusual! Are there other molecular differences, beyond the distribution of VegT RNA, that are known for nutritional endoderm?

Yes. Although RNAs and proteins of several signal transduction systems are present in the prospective nutritional endoderm (Karadge and Elinson 2013; Chatterjee and Elinson 2014), this mass of large, yolky vegetal cells lacks mesoderm-inducing activity at late blastula and early gastrula stages (Ninomiya *et al.,* 2001). Although some activity arises later (Karadge and Elinson 2013), the lack of activity contrasts with its strong presence in the geographically similar vegetal cells of *Xenopus laevis*. There is some nuclear localization of Smad2 and Smad4, parts of the nodal signaling pathway, albeit at lower levels than in the definitive endoderm (Chatterjee and Elinson 2014). Notably, transcription is repressed throughout the nutritional endoderm (Chatterjee and Elinson 2014).

This global transcription repression should be enough to prevent development into specific endodermal tissues, but there are some genes that escape this repression. There is an up-regulation of thyroid hormone receptor β in the nutritional endoderm, close to the time of hatching. This up-regulation is important, since the utilization of the yolk in the nutritional endoderm depends on thyroid hormone (Singamsetty and Elinson 2010).

Do you think this cellular nutritional endoderm was a step in the evolution of yolk sac that allowed the first amniote to have its eggs develop on land?

That is an interesting hypothesis, but it would be presumptuous to claim it is true. It is unlikely that biologists would ever be able to test hypotheses like this, so is this really in the realm of science? On the other hand, the amniote egg is one of the great evolutionary innovations. It led to invasion of the land by vertebrates, and it is fun to speculate. One speculation is prompted by the recent discovery that the yolk in snake and lizard eventually becomes incorporated into cells, unlike the yolk in chicken (Elinson and Stewart 2014; Elinson *et al.,* 2014). An initial evolutionary event would be that a tissue like the nutritional endoderm is invaded by blood vessels. The association of yolk-rich endodermal cells with blood vessels would lead to the spaghetti-like mass, characteristic of the snake yolk sac. Such a morphology should enhance the transport of yolk metabolites into the body of the growing embryo. Only secondarily would cellularization of the yolk be lost, giving rise to the liquid yolk in birds that everyone is familiar with.

Finally, what do you expect for the future of investigations on your development?

I expect that the next major use of coquí for developmental studies will come when the coquí genome is known. There have been a number of attempts at this, but as far as I know, none have been successful. I keep hoping that some rich Boricua will fund the coqui genome project as part of the island's patrimony. Once the genome is available, many questions, such as what the earliest molecular events are in deviating from the tadpole process of indirect development, can be easily approached.

Fig. 7. Biosketch of Dr. Richard (Rick) Elinson. *Professor of Biology at the University of Toronto, Canada, and Duquesne University, Pittsburgh, USA. His lab made important discoveries on amphibian early development, including the transient array of parallel microtubules involved in antero-dorsal polarization and the activity of lithium to respecify embryo polarity. Rick collaborated with scientists in Argentina, Ecuador, and Puerto Rico. He was a pioneer in using the direct developing frog* Eleutherodactylus coqui *for evo-devo investigations as well as for public outreach. One of his volunteer activities in retirement is at Sagamore Hill, the home of Teddy Roosevelt, a president who had tremendous impact, both good and bad, on Ibero America.*

Thank you for your comments. Is there anything further you would like to add?

I was trying to think of something profound, but I prefer simply to urge your readers to enjoy our beautiful singing. There are many clips on YouTube. Co-quí! Co-quí! Co-quí qui qui qui!

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