Video Article

Who is Who? Non-invasive Methods to Individually Sex and Mark Altricial Chicks

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Abstract

Many experiments require early determination of offspring's sex as well as early marking of newborns for individual recognition. According to animal welfare guidelines, non-invasive techniques should be preferred whenever applicable. In our group, we work on different species of song birds in the lab and in the field, and we successfully apply non-invasive methods to sex and individually mark chicks. This paper presents a comprehensive non-invasive tool-box. Sexing birds prior to the expression of secondary sexual traits requires the collection of DNA-bearing material for PCR. We established a quick and easy method to sex birds of any age (post hatching) by extracting DNA from buccal swabs. Results can be obtained within 3 hours. For individual marking chick's down feathers are trimmed in specific patterns allowing fast identification within the hatching order. This set of methods is easily applicable in a standard equipped lab and especially suitable for working in the field as no special equipment is required for sampling and storage. Handling of chicks is minimized and marking and sexing techniques are non-invasive thereby supporting the RRR-principle of animal welfare guidelines.

Video Link

The video component of this article can be found at http://www.jove.com/video/51429/

Introduction

Individual recognition, sexing and genotyping are fundamental prerequisites in a variety of experimental studies. Obtaining DNA bearing material and marking subjects unambiguously (even at an early age) should have minimal impact on physiology, behavior, and survival. Whenever possible, invasive procedures should be avoided according to the RRR principle[1].

Non-invasive methods are not only beneficial for the animal but also might improve the obtained data as animals are less affected by the treatments.

In birds, DNA sexing can be performed on a number of non-invasively obtainable materials as droppings[2], feathers[3,4] or buccal swabs[3,5,9]. Regardless of subject´s condition and age buccal swabs are the method of choice for avian sexing, because they are easy to perform, rarely fail and handling is short.

So far, DNA from buccal swabs was either extracted with commercially available kits[3,6] or time consuming standard DNA extraction protocols[3,6-8]. Kits are not only rather expensive, but their protocols can impose challenges for field work. Some procedural details, *e.g.* drying and incubation of samples, are not practical in the field. Especially in a setting where experimental protocols require sex dependent treatment from as early as a few minutes post hatching, there is urge for a quick, non-invasive, reliable and easy method to obtain results.

Across the avian taxa a considerable toolbox for marking individuals has been developed[10]. The wide array of available techniques accounts for the variety of research objectives, species and budgets. However, marking small nestlings has confronted researchers with additional challenges. In some species (*e.g.* passerines) chicks are too small to apply leg bands and require alternative methods, which do not alter parent-offspring behavior. As awareness and interest in improving animal welfare and techniques in field and laboratory studies is growing, the use of non-invasive techniques is strongly encouraged and preferred.

This protocol provides a non-invasive, quick, easily recognizable and persistent method to individually mark very young nestlings before applying leg bands is feasible. This marking method is introduced on one of the most important avian laboratory model species, the Zebra Finch (*Taeniopygia guttata*)[11-13]. The protocol complies with all of the previously published objectives for individual marking techniques[10] and has been already successfully applied[14,15].

Protocol

All procedures were performed in compliance with the German law for animal protection (TierSchG).

1. Preparation of Reagents and Consumables

1. Prepare a 5% (w/w) Chelex-100 solution in molecular grade water. Prepare aliquots of 200 µl in standard 1.5 ml reaction tubes. As the Chelex resin precipitates fast from the suspension it is necessary to re-homogenize the suspension constantly during the preparation of the aliquots. It's advisable to prepare 50 ml or more in one batch and keep the suspension homogenized using a magnetic stirrer. The Chelex solution can be stored for years at ambient conditions.
2. Cut pieces of Whatman paper with a width smaller than the beak width of the bird to be tested. The length of the paper depends on the method to take the sample:
    1. Using forceps, the piece should be long enough to enable sample collection without the forceps being inserted into the beak of the bird. As a rough estimate, for a beak length of 0.5 cm a piece of paper with the length of 1 cm should be fine.
    2. If not using forceps, the piece must be longer, but the stiffness of the piece should still allow sampling of epithelial cells.
3. Prepare one aliquot of PCR premix for each sample to be analyzed. For a PCR in a total volume of 25 µl the mix contains 0.18 mM dNTPs, 2 mM $MgCl_2$, 70 mM Tris-HCl, 17.25 mM ammonium sulfate , 0.1% Tween-20, 0.32 µM P2 primer (TCTGCATCGCTAAATCCTTT), 0.32 µM P8 primer (CTCCCAAGGATGAGRAAYTG)[16] and 2 units Taq polymerase. Homemade Taq polymerase[17] can be used. To allow addition of the maximum amount of DNA-solution, a 6 µl premix can be prepared and stored at -20 °C for several weeks.

2. Sample Collection

Wearing gloves is not essential for avian sex determination as the PCR primers cannot anneal to human DNA. For other genotyping purposes it might be advisable.

1. Capture the bird of interest and gently hold it in one hand *e.g.* with the 'ringer's grip'[18]. Make sure not to squeeze the bird.
2. Hold a piece of Whatman paper with a forceps and swipe it several times across the inside of the cheeks, the tongue and the choana. Usually samples are obtained within less than 30 seconds.
    1. Adult animals: depending on the species it might be difficult to get the bird to open its beak. Usually touching the side of the beak and applying gentle pressure will work.
    2. Nestlings: Sampling from hatchlings or nestlings is especially easy using this technique, as their begging behavior provides easy access to the inside of the beak. It might even be possible to obtain the sample without handling them at all, as they readily beg *e.g.* when their nestbox is opened.
3. Immediately store the paper in a prepared reaction tube containing 200 µl of the 5% (w/w) Chelex-100 solution.

If you also intend/need to mark the chicks, do so now as described in section 6.

3. Storage of Samples Before DNA Extraction

1. If working in the lab, store samples at -20 °C. If this is not possible or difficult (*e.g.* in the field) store samples at ambient temperatures.
2. Samples can be stored for more than 3 years at temperatures ranging from room temperature to -20 °C.

4. DNA Extraction

1. If the sample is frozen, thaw it at room temperature.
2. Ensure that the piece of paper is on the bottom of the tube submerged in the Chelex-100 solution.
3. Incubate samples for 15 min at 56 °C.
4. Vortex briefly and spin down the content of the tube.
5. Incubate the samples for 8 min at 100 °C.
6. Spin the samples at 15,000 x g for 3 min.
7. Use the supernatant for subsequent genotyping.

5. Molecular Sex Determination

1. Prepare one PCR tube with 6 µl premix per sample plus two additional tubes for the negative (mandatory) and positive control (optional). For routine sex determination include a sample from the last successful sexing as a positive control.
2. Add 19 µl of the supernatant from the DNA extraction to the PCR premix. Make sure to pipette from the surface of the solution to avoid carryover of Chelex beads. This is crucial, as Chelex is a cation exchanger and thus severely inhibits all enzymatic reactions.
3. Run the following PCR program: Incubation at 94 °C for 3 min, 45 cycles each with a 30 sec melting step 94 °C, followed by a 30 sec annealing step at 55 °C, followed by a 45 sec elongation step at 72 °C. In a final chase off step, the reactions are incubated for 5 min at 72 °C.
4. Prepare a 2% standard TBE or TAE agarose gel to separate the PCR products.
5. Run the agarose gel with appropriate voltage settings.
6. Visualize the bands under UV light and take a picture. Make sure the two bands in the samples originating from females are well separated.

7. Distinguish male from female samples by the presence of one (male) or two (female) bands.

6. Marking Nestlings

1. Check nests for newly hatched chicks on a schedule appropriate for the species/study (*e.g.* daily checks in the mornings are advised as most chicks hatch then).
2. Cut the down feathers of each chick within a nest at a different characteristic location. In zebra finches tufts of downs grow at four characteristic and distinct areas across the nestling's body (**Figure 4A**). For each chick cut one area (**Figure 4B**). If there are more than four chicks within one nest, the unique four 'haircuts' can be combined.

For zebra finches: Apply leg bands at ten days post hatching when down feathers become hard to detect and nestling size allows ringing.

Representative Results

Buccal swabs can be used to obtain DNA for sex determination in a variety of small birds

Samples were collected from Zebra Finches (*Taeniopygia guttata,* 99 individuals, age 0 days - 5 years), Canaries (*Serinus canaria*), Bengalese Finches (*Lonchura striata*), Nightingales (*Luscinia megarhynchos*), Great Tits (*Parus major*) and Blackbirds (*Turdus merula*) (for all other species: sample size 1-3, age unknown) (**Figure 1**).

For samples taken from Zebra Finches, the success rate for the PCRs was 100%. The PCR result always matched the sex determined by prior (in adults) or later (in nestlings) phenotypic inspection. The intensity of bands did not differ systematically between nestlings or adults indicating that similar amounts of DNA-bearing material are gained from all ages.

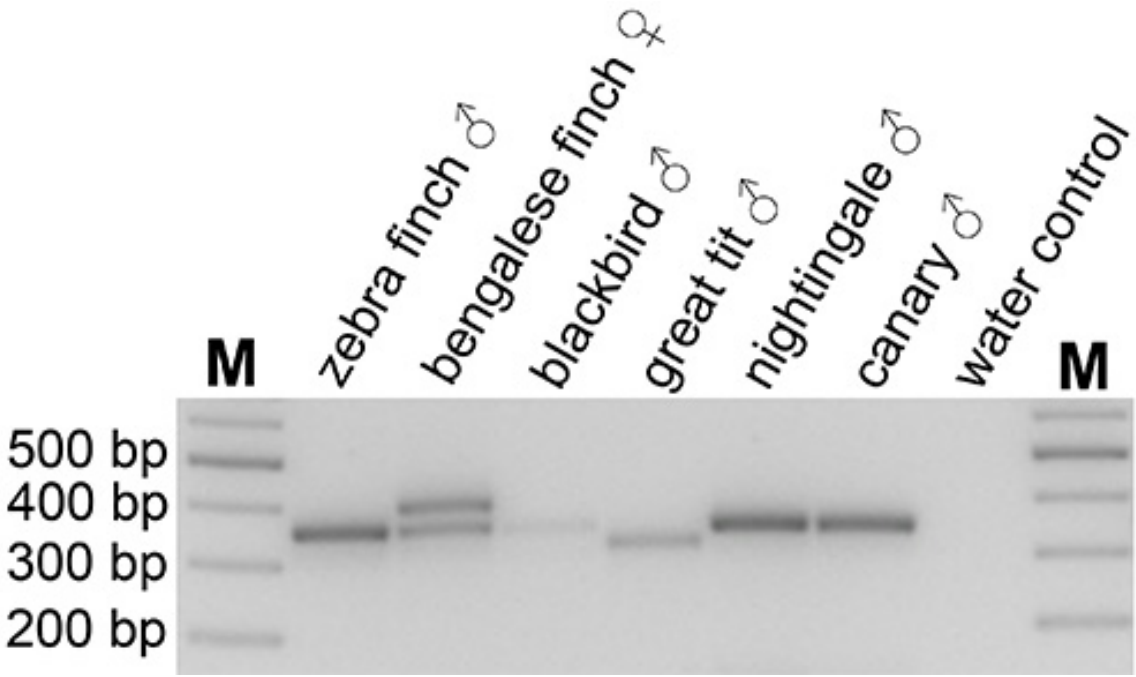


Figure 1. Molecular sexing. Examples of sexing results from buccal swabs obtained from various avian species. 2% agarose gel stained with ethidium bromide. From left to right: size marker, Zebra Finch male, Bengalese Finch female, Blackbird male, Great Tit male, Nightingale male, Canary male, water control, size marker.

It is important to prevent Chelex carryover contamination in the PCRs

To demonstrate the effect of Chelex resin contamination in a PCR reaction, we included a small amount of Chelex beads in a PCR reaction (**Figure 2**). As can be seen in **Figure 2A**, carryover contamination of Chelex beads prevents the amplification of DNA which is further illustrated by the lack of primer dimer formation in the contaminated sample (**Figure 2B**). In **Figure 2A** lane 2 we used the same sample for the PCR without carrying over Chelex. Usually contaminated samples are easily recognizable by dark spots in the pockets of the agarose gel (**Figure 2B**).

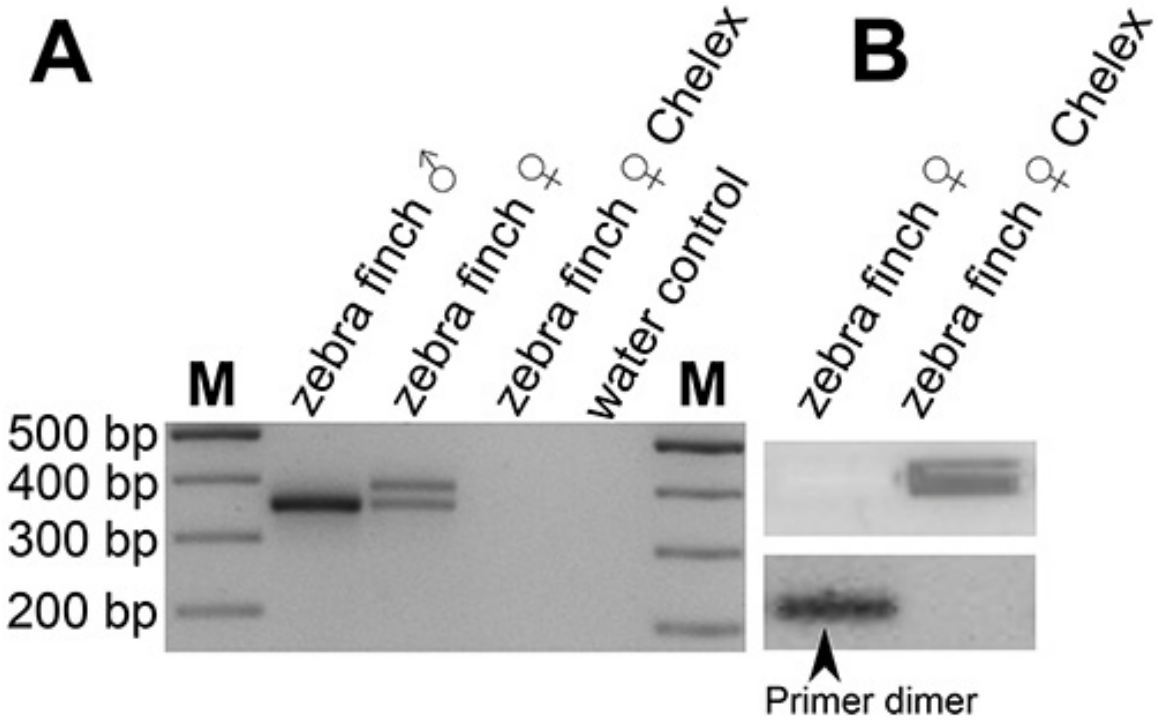
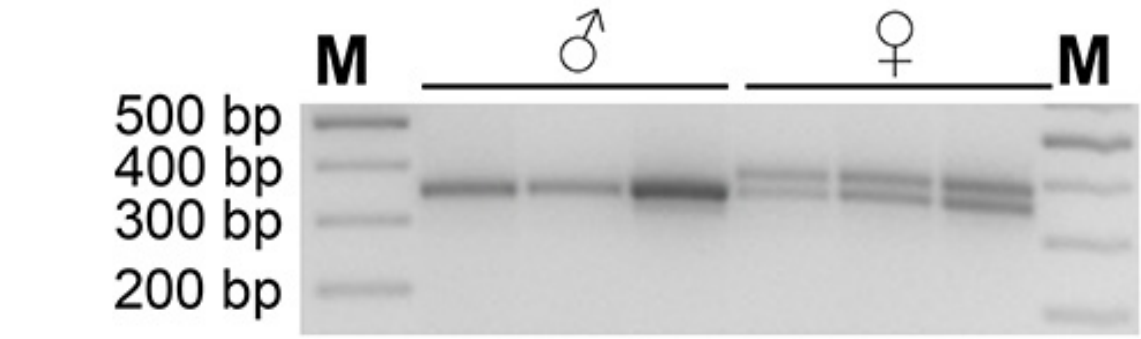


Figure 2. Chelex contamination. A) Chelex carryover into the PCR reaction inhibits the amplification of DNA. From left to right: size marker, Zebra Finch male, Zebra Finch female, same sample with intentional carryover of Chelex, water control, size marker. **B)** Chelex beads are readily visible as dark staining in the pocket of the gel (left without Chelex, right with Chelex. The severe inhibition of the PCR reaction in the presence of Chelex is also illustrated by the missing primer dimer formation in the contaminated sample.

Samples can be stored at room temperature before DNA extraction for more than three years

In order to investigate whether successful extraction can still be performed after long storage, samples were taken repeatedly from the same bird and stored in the lab at ambient temperatures for maximally three years. The samples were stored close to a window with natural exposure to light and heat from the sun. After three years DNA was extracted and the sexing PCR performed. The result matched the one performed three years earlier (right after sampling) and did not indicate any effect of storage (**Figure 3**).



Storage before extraction	1 day	1 day	3 years	1 day	1 day	3 years
Temperature	-20°C	-20°C	RT	-20°C	-20°C	RT
Storage after extraction	3 years	3 years	1 day	3 years	3 years	1 day
Temperature	-20°C	4°C	4°C	-20°C	4°C	4°C

Figure 3. Chelex allows long-term storage of samples before and after DNA-extraction. Samples of two animals (male left, female right) were stored under different conditions as indicated in the table. Signals were obtained successfully from all samples.

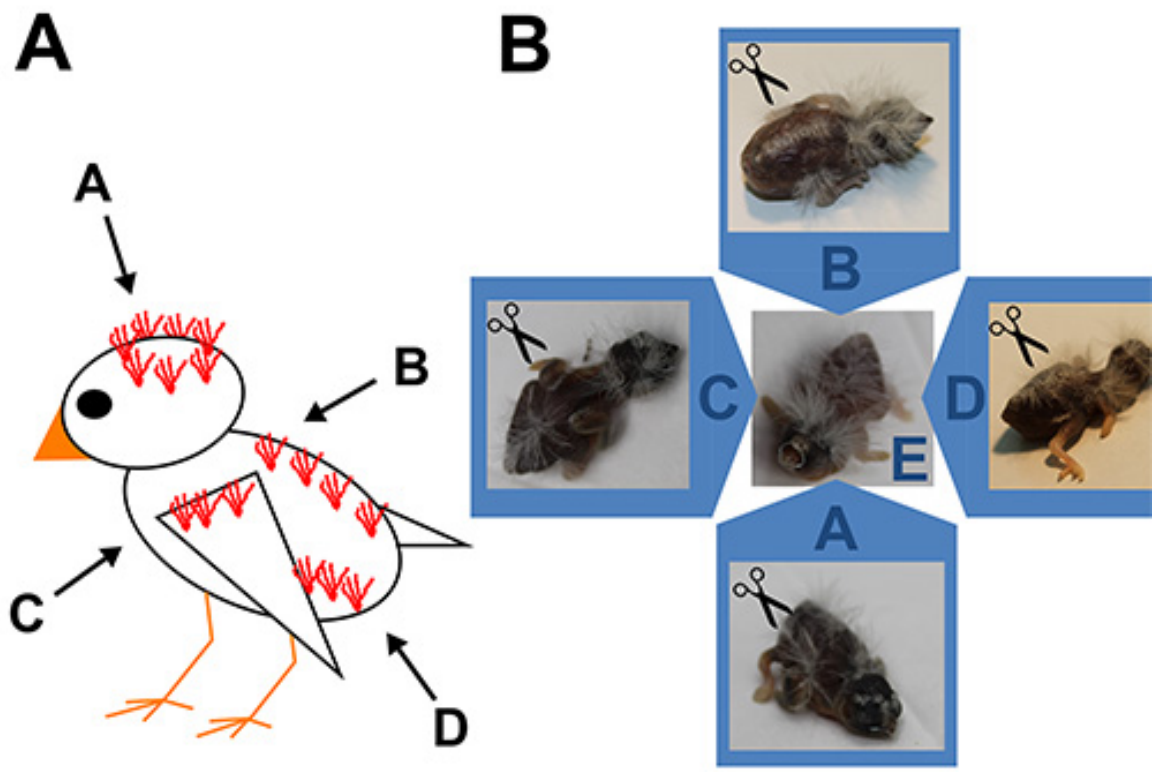


Figure 4. Marking of chicks. A) Schematic representation and nomenclature of the four distinct areas of down feather growth in zebra finch nestlings: A = head, B = back, C = wings (both), D = flanks (both). **B)** Example pictures of nestlings which either received one of the respective 'haircuts' (A-D) or no 'haircut' (E).

Extracted DNA can be stored for more than three years at 4 °C

Zebra finch samples from the first experiment were stored for three years in a standard laboratory fridge at 4 °C and successfully retested. All samples yielded the same PCR results as directly after DNA extraction (**Figure 3**).

Hatchlings can be marked by trimming their down feathers to enable individual recognition from hatching on

Marking individual nestlings right after hatching by cutting their down feathers made them easily recognizable (**Figures 4, 5, 6**). Down feathers have a fluffy appearance as their barbs are not joined together to form a smooth vane. They can still be recognized at posthatch day 10 on the tips of the developing feathers, which by then cover the body (**Figure 5**). Their absence at distinct locations makes recognition easy, sometimes even without handling the nestlings (**Figure 6**). Applying these so called `haircuts' is an elegant technique, which successfully fills the gap for the time from hatching until the body size of nestling zebra finches makes the application of leg bands feasible.

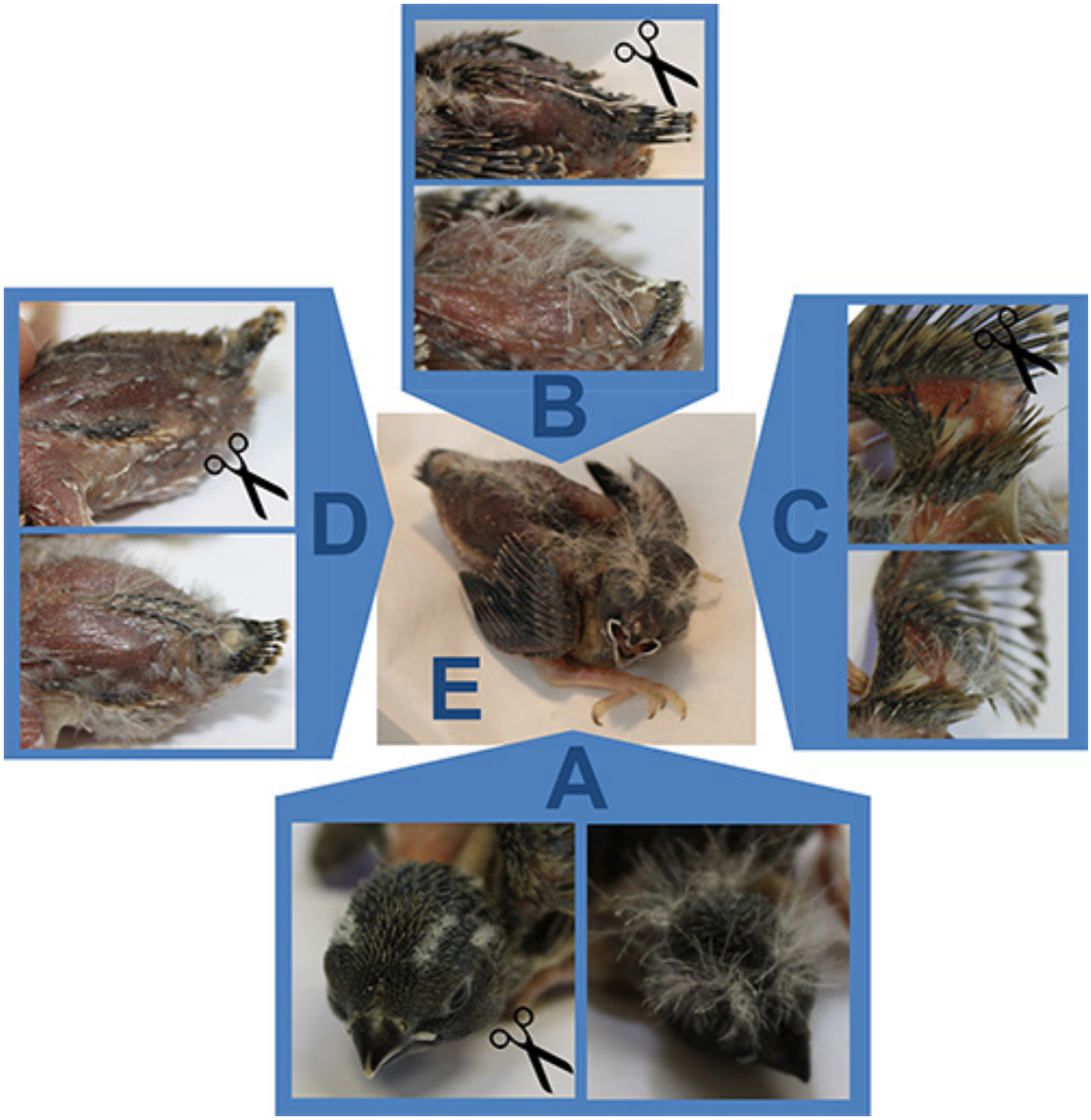


Figure 5. Marked Zebra Finch nestlings at post hatch day 10. Pictures show nestlings either with (scissors) or without (arrow) markings. An arrow points to the location of interest according to the respective nomenclature of down feather 'haircuts': **A** = head, **B** = back, **C** = wings, **D** = flanks.

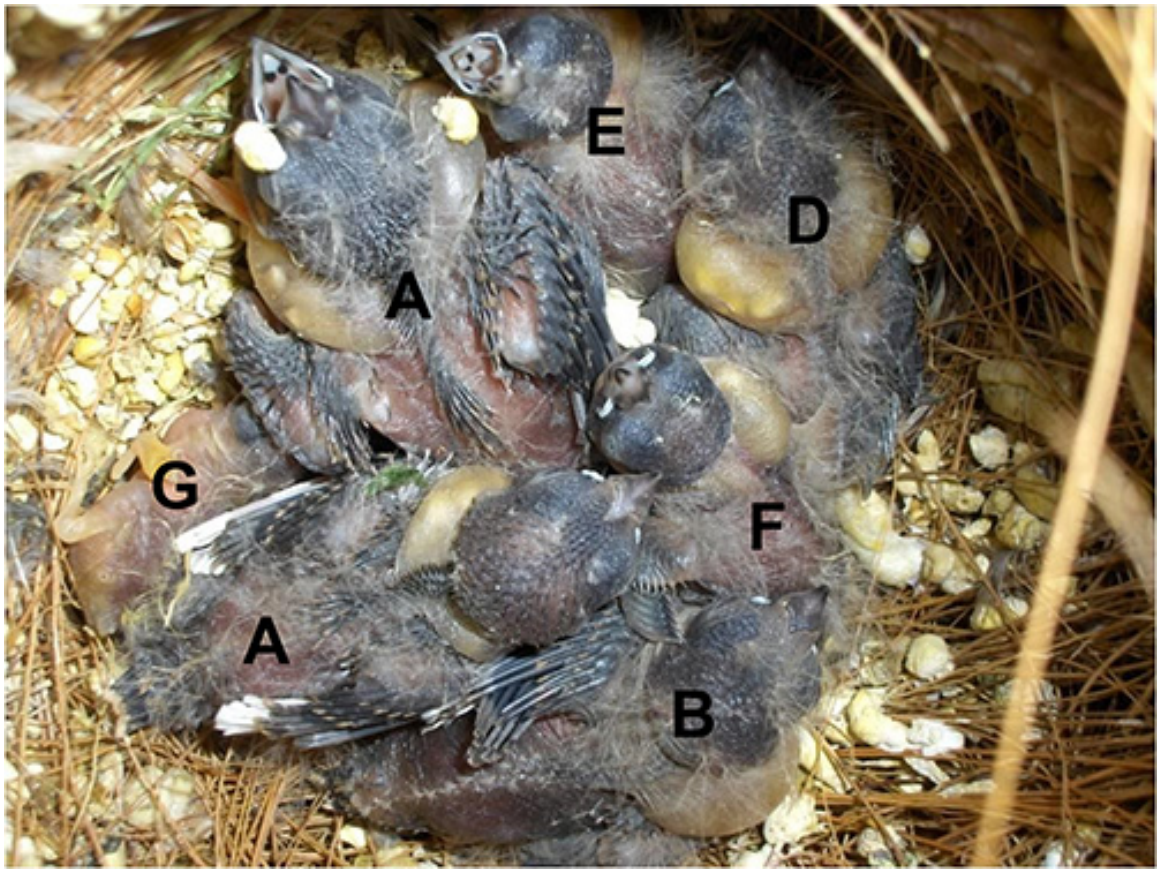


Figure 6. Recognition of marked nestlings (mean age = 10 days) within the natal nest according to the nomenclature. A = head, easy to spot due to his prominent down feathers, which are only missing on the head. **B** = back, down feathers visible at all locations but on the back. **C** = wings, needs an attentive observer as both wings are missing down feathers, but nestling´s posture makes the down feathers from the head cover the right wing. **D** = flanks, can only be recognized by the process of elimination as its flanks cannot be seen. It can be reliably recognized as D as it shows clear down feather growth at the head, the back and its wings. **E** = a combination of 'haircuts' head (A) and back (B), is easy to distinguish as down feathers on the head and back are missing. **F** = a combination of 'haircuts' A (head) and D (flanks), which can only be differentiated through eliminating other options as its head marking is obvious and no other unidentified nestling exhibits a head marking. **G** = did not receive any markings and is a lot smaller than its siblings as it was the last to hatch. Additionally G is a good example for a nestling which does not express down feathers at all locations.

Discussion

Sexing from buccal swabs using Chelex showed an extremely high success rate. Cutting hatchling's down feathers enabled differentiation between nestlings until leg banding was possible.

Chelex-DNA extraction from buccal swabs yielded enough DNA to successfully perform molecular sexing. Sex determination was 100% correct as validated by sexually dimorphic plumage. The success rate reported here is markedly higher than the rate reported by two previous studies[7,9]. The DNA extraction with Chelex minimizes pipetting and precipitation steps in which material can be lost. However, one of the studies validating our protocol in a different species (*Apus apus*) still achieved a lower success rates of 89%[9] even though this was already markedly higher than the 82% reported in Arima *et al.*[7].

What could be the cause? The most crucial part is to attentively follow the protocol, including the part for how to take samples (gently swipe tissue several times in the mouth) and to prevent carry-over of Chelex beads into the PCR reaction. As Chelex is a cation exchanger it severely inhibits the PCR reaction preventing amplification in contaminated samples. Contaminated samples can be easily recognized by beads visible in the pockets of agarose gels and the lack of primer dimers.

Less likely reasons for failure to successfully sex samples could also be due to species differences or the use of a different technology to detect the band difference in the female samples.

Cutting the down feathers of hatchling zebra finches is a safe way to individually mark subjects. These 'haircuts' were easy to recognize until nestlings were old enough to receive numbered leg bands (*e.g.* at day 10 in zebra finches). This marking technique may be readily adjusted to meet different experimental demands; *e.g.* for video recordings markings can be limited to the head of chicks. Combinations of cuts at two positions can be applied if nests are larger than five nestlings. Keeping a predefined sequence in applying 'haircuts' (*e.g.* the first hatchling in a nest always receives 'haircut A', the second always receives 'haircut B' etc.) allows fast recognition within the hatching order throughout the

nestling phase. This enables quick identification, which can reduce or even prevent handling of chicks. In zebra finches, lack of downs at one of the four locations can occur due to natural fluctuations. Thus, it may not always be possible to stick to a predefined marking sequence.

Even for someone inexperienced in handling nestlings it is a quickly to learn, safe and easy marking technique to cut the down feathers. However, as the innate gaping response of nestlings includes head movements, some people find it more difficult to place markings on the head. Error prone identification relies on precise markings. It is of eminent importance to thoroughly cut the entire down feathers at a given spot, as imperfect markings/ single left-out down feathers may later confuse identity.

This set of methods enables extremely fast, easy, non-invasive, reliable and low-cost, sex determination of birds and their non-invasive, quick, safe and easily recognizable marking within minutes after hatching.

Disclosures

The authors have nothing to disclose.

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