1 **Sex determination in the wild: a field application of Loop-Mediated Isothermal**
2 **Amplification successfully determines sex across three raptor species**

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16 **Running title:** Sex determination of birds in the field

17

18 **Abstract**

19 PCR-based methods are the most common technique for sex determination of birds.
20 Although these methods are fast, easy and accurate, they still require special facilities
21 that preclude their application outdoors. Consequently, there is a time lag between
22 sampling and obtaining results that impedes researchers to take decisions *in situ* and in
23 real time considering individuals' sex. We present an outdoor technique for sex
24 determination of birds based on the amplification of the duplicated sex-chromosome-
25 specific gene Chromo-Helicase-DNA binding protein using a Loop-Mediated
26 Isothermal Amplification (LAMP). We tested our method on Griffon Vulture (*Gyps*
27 *fulvus*), Egyptian Vulture (*Neophron percnopterus*) and Black Kite (*Milvus migrans*)
28 (family *Accipitridae*). We introduce the first fieldwork procedure for sex determination
29 of animals in the wild, successfully applied to raptor species of three different
30 subfamilies using the same specific LAMP primers. This molecular technique can be
31 deployed directly in sampling areas since it only needs a voltage inverter to adapt a
32 thermo-block to a car lighter and results can be obtained by the unaided eye based on
33 colour change within the reaction tubes. Primers and reagents are prepared in advance
34 to facilitate their storage at room temperature. We provide detailed guidelines how to
35 implement this procedure, which is simpler (no electrophoresis required), cheaper and
36 faster (results in ca. 90 minutes) than PCR-based laboratory methods. Our successful
37 cross-species application across three different raptor subfamilies posits our set of
38 markers as a promising tool for molecular sexing of other raptor families and our field
39 protocol extensible to all bird species.

40

41 **Introduction**

42 Accurate sex determination is critical in wildlife management, captivity breeding
43 programs and studies on behaviour, ecology and evolution. It is especially challenging
44 in monomorphic bird species (i.e. species with no phenotypic differentiation between
45 males and females) and nestling/juveniles or when samples are obtained without
46 handling individuals (e.g. non-invasive sampling). Sex determination in birds relied
47 originally on observational studies and palpation or expensive and complicated
48 surgeries, hormones analyses or endoscopies. However, these techniques were mostly
49 inaccurate, posed a threat for life or required expensive and complex facilities that
50 hamper their extension. These issues have been partially solved by molecular
51 techniques using karyotypes and, more recently, amplifying the duplicated sex-
52 chromosome-specific gene Chromo-Helicase-DNA binding protein (CHD) located on
53 the sexual W and Z chromosomes (CHD-W and CHD-Z, respectively) (Fridolfsson &
54 Ellegren 1999). These PCR-based techniques are nowadays widely applied across non-
55 ratite birds due to their relatively easy diagnosis by running an electrophoresis on an
56 agarose gel. A visual examination of these gels generally shows a single band,
57 corresponding to the double copy of the CHD-Z fragment and diagnostic of males, or a
58 double band, corresponding to the CHD-Z and CHD-W copies differing in fragment
59 sizes and characteristic of females (e.g. Griffiths *et al.* 1998; Fridolfsson & Ellegren
60 1999). A digestion with restriction enzymes is also needed in specific cases when CHD-
61 Z and CHD-W do not differ in fragment size (reviewed in Morinha *et al.* 2012;
62 Vucicevic *et al.* 2013). This easy and accurate technique based on PCR (but see
63 Robertson & Gemmell 2006) has been widely applied not only for molecular sexing to
64 fresh tissue samples (e.g. blood) but also to non-invasive samples such as museum bird
65 specimens (Bantock *et al.* 2008), feathers (Horvath *et al.* 2005) or unincubated eggs

66 (Aslam *et al.* 2012). However, this technique urges for special and expensive equipment
67 for thermal cycling and electrophoresis and entails specialized laboratories away from
68 the study sites. Researchers and wildlife managers would therefore benefit from an
69 accurate, portable and inexpensive molecular technique for sex determination that can
70 be operated in the field and yield results in relatively short time.

71 *Loop-mediated isothermal amplification (LAMP)*

72 The loop-mediated isothermal amplification (LAMP) (Notomi *et al.* 2000) uses *Bst*, a
73 DNA polymerase that contains a 5'-3' polymerase activity and owns a high strand
74 displacement activity that allows an auto-cycling strand displacement DNA synthesis.
75 In other words: no thermal cycling is needed. Two pairs of primers that recognize six
76 different regions produce a final product of stem–loop DNAs with several inverted
77 repeats of the target in the same strand. These products can be stained by using turbidity
78 (Mori *et al.* 2001), pH-sensitive dyes (Tanner *et al.* 2015) or any metal indicator
79 (Tomita *et al.* 2008) and make results easily checked by unaided eye. Because of its
80 high-specificity and sensitivity to the target region, isothermal conditions and easy
81 detection, LAMP appeared as a promising tool in molecular techniques that has been
82 already applied in medicine (Nyan *et al.* 2014), microbiology (Fukuta *et al.* 2014) and
83 parasitology (Abbasi *et al.* 2010) (among others) and applied in poultry or species with
84 some commercial interest (Hsu *et al.* 2011; Chan *et al.* 2012; Kim *et al.* 2015). As far as
85 we know, this technique has not been applied in ecology and evolution despite LAMP
86 reactions can be run under field conditions.

87 *A study case: movement ecology of Griffon Vultures in the Middle East*

88 The population of Griffon Vulture (*Gyps fulvus,* Hablizl 1783) in the Middle East has
89 dramatically decreased in the last decades mainly because of poisoning and human

90 disturbance. The Israeli Nature and Parks Authority in collaboration with the Hebrew
91 University of Jerusalem started an intensive monitoring program to understand foraging
92 and behavioural ecology of this species. It was shown how long-range forays (i.e.
93 relatively short-term movements in which individuals depart from their regular foraging
94 area, travel to remote locations and return to the original core area) were biased towards
95 females after deploying high-resolution global positioning system and accelerometer
96 tags (GPS-ACC tags) on adult birds. These long-range forays likely represent failed
97 breeding attempts (Spiegel *et al.* 2015), but further studies focusing only in female birds
98 are needed to disentangle among the causes and consequences of these movements for
99 the population persistence. In this example, a large number of vultures are captured in a
100 walk-in traps in a single monitoring/sampling/tagging effort, but only some of them (e.g.
101 a tenth) are equipped with expensive GPS-ACC tags (Spiegel *et al.* 2013). A molecular
102 technique for sex determination that could be fully applicable under field conditions
103 would be, therefore, desirable to deploy GPS-ACC tags preferably in female birds while
104 minimizing handling time of all captured birds.

105 In this study we introduce a molecular approach based on LAMP for sex determination
106 of Griffon Vultures in 90 minutes (see BOX 1). Female-specific primers were designed
107 to amplify a CHD fragment located in the W-chromosome and results were compared to
108 those obtained under standard lab conditions as a reference. We developed a fully
109 operational field technique for Griffon Vultures, using vacuum-dried primers and
110 stabilizers to preserve enzyme activity. Furthermore, we successfully applied these
111 primer sets also to Egyptian Vulture (*Neophron percnopterus*, Linnaeus 1758) and
112 Black Kite (*Milvus migrans*, Boddaert 1783) and proved for the first time the utility of
113 LAMP for sex determination in birds across species.

114

115 **Material and Methods**

116 We extracted DNA from fresh blood samples stored in absolute ethanol of four females
117 and four males of Griffon Vultures, Egyptian Vultures and Black Kites using a NaOH
118 based extraction protocol (Truett *et al.* 2000). We chose this protocol because it is
119 simple and fast hence can also be implemented in the field. First, we run one PCR per
120 sample under standard lab conditions for sex determination to be compared to our
121 LAMP-based protocol using 1x BioTaq^{TM} buffer, $MgCl_2$ 3mM, dNTPs 0.2 mM, 2550F
122 and 2718R primers 0.2 μM (Fridolfsson & Ellegren 1999) and 0.5 units of Taq
123 polymerase added to 2 μL of 1:100 dilution of DNA in a final volume of 25 μL.
124 Cycling conditions were as follows: 94ºC for 2 minutes, 55ºC for 30 seconds and 72ºC
125 for 1 minute, followed by 35 cycles of 92ºC/30sec, 50ºC/30sec and 72ºC/45 sec and a
126 final extension step of 5 minutes at 72ºC. Five microliters of PCR products of males of
127 the three raptor species (CHD-Z) were then cleaned from excess of primers and
128 nucleotides using 2 μL of an enzymatic mixture of Antarctic phosphatase and
129 *Escherichia coli* exonuclease I incubated at 37ºC during 45 minutes followed by
130 80ºC/15 minutes for enzymes inactivation. Sequences were analysed for forward and
131 reverse directions in an Applied Biosystems 3130 Genetic Analyzer using the same
132 primers for amplification in the Applied Biosystems BigDye Terminator Cycle
133 Sequencing Kit v. 1.1. Forward and reverse sequences for each PCR product were
134 edited and assembled using using Geneious 8.0.5 (http://www.geneious.com) (Kearse *et*
135 *al.* 2012) and uploaded into GenBank database (accession numbers KU563739-
136 KU563741)

137 *Sex determination by LAMP*

138 Two sets of primers are required for sex determination (table 1). ACCIW targets CHD-
139 W (specific of females) and positive LAMP reactions will be characteristic of females.
140 ACCIZ targets CHD-Z and it is used as a positive control for DNA quality and/or to
141 monitor LAMP reaction. Consequently, only females will amplify CHD-W (ACCIW+)
142 and CHD-Z (ACCIZ+) and males will be ACCIW- confirmed only after rejecting any
143 failure during LAMP reaction by amplifying CHD-Z (ACCIZ+, positive control).
144 Otherwise (ACCIZ-) repetition of LAMP reactions will be required to ensure a correct
145 sex determination.

146 *LAMP primer design*

147 We designed two specific primer sets of forward and backward external primers
148 (F3/B3) and forward and backward internal primers (FIP/BIP) (figure 1) specific to
149 Griffon Vultures based on a sequence of CHD-W (GenBank accession number:
150 EU430640) and CHD-Z based on the three studied species (GenBank accession
151 numbers: KU563739- KU563741, this study) (table 1). Primer selection for ACCIW
152 required preliminary assays of primer design and optimization in experimental
153 laboratory conditions following recommendations summarized in Tomita *et al.* (2008).
154 Primers for ACCIZ were designed using Primer Explorer V4 software (Eiken Chemical
155 Co., Ltd., Japan; http://primerexplorer.jp/e/). We prepared a primer-mix for each marker
156 (ACCIW and ACCIZ) and eight reactions containing vacuum-dried primers in a final
157 concentration of 1.6 μM of internal (FIP/BIP) and 0.2 μM of external (F3/B3) primers
158 and stored them at room temperature.

159 *LAMP reactions*

160 We prepared a ready-mix for eight reactions as in Hamburger *et al.* (2013) to preserve
161 enzyme activity at room temperature and make it portable and effective for field

162 conditions. This mix was composed by 1x enzyme buffer, dNTP 0.4mM, betaine 1M,
163 2% sucrose (used as stabilizer) and 8 units of Bst DNA polymerase (New England
164 Biolabs) per reaction. The ready-mix can be stored for months at room temperature
165 (Hamburger *et al.,* 2013) and although we did not evaluate it specifically, we observed
166 amplifications days after their preparation. Prior to LAMP, we rehydrated the primer-
167 mix with the same volume of molecular biology grade H_2O than before being vacuum-
168 dried and transferred its whole content to the microtube containing the ready-mix.
169 Finally, 23µL of this mix were pipetted to PCR-tubes and 2 µL of the 1:100 diluted
170 DNA were added. LAMP reactions were incubated between 45 to 80 minutes and
171 temperatures ranging between 55 to 69 ºC (table 1). LAMP-amplified products were
172 detected by running a 2.5% agarose gel electrophoresis. We stained LAMP reactions
173 with 5 µL of 1:50 diluted Sybr Green I Nucleic Acid Stain (Life Technologies) to allow
174 an easy diagnosis of LAMP reactions by the unaided eye. This reagent changes the
175 colour of the content within PCR microtubes from orange to yellow-green due to its
176 interaction with residuals of magnesium pyrophosphate generated during DNA
177 synthesis in LAMP (Mori *et al.* 2001). We also irradiated LAMP reactions with an UV
178 portable lamp to detect yellow fluorescence in positive reactions.

179

180 **Results**

181 *Optimization of LAMP reactions in Griffon Vultures*

182 The combination of different reaction times (45, 60 and 80 minutes) and temperatures
183 (55 ºC, 57 ºC, 59 ºC, 61 ºC, 63 ºC, 65 ºC, 67 ºC and 69 ºC) showed that ACCIW primers
184 fully discriminated females from males when LAMP reactions were performed at 64 ºC
185 for 80 minutes (table 1), as shown by the characteristic laddered pattern in the agarose

186 gel (figure 3). A different combination of time and temperature yielded unspecific
187 amplifications in males (false positives) or lack of amplification in females (false
188 negatives) (results not shown). On the other hand, ACCIZ showed a similar laddered
189 pattern in all individuals after incubation at 59ºC during 80 minutes in both females and
190 males. These results discard false negatives in ACCIW and therefore support male
191 determination in those samples that did not amplify with the female-specific set of
192 primers. Visualization of amplification products was possible at daylight when SYBR
193 Green I was added within the microtubes (ACCIW: females in yellow and males in
194 orange; ACCIZ: all individuals in yellow) (figure 4). The irradiation with 320 nm UV
195 light stressed these results and only positive reactions irradiated fluorescence (figures
196 4C and 4D).

197 *Cross-species amplification of LAMP primers*

198 The two primer sets (ACCIW and ACCIZ) also amplified the targeted regions in
199 Egyptian Vulture and Black Kite (table 1). The three studied raptor species needed 59
200 ºC to amplify ACCIZ, although Griffon Vultures and Black Kites required longer
201 incubation times (80 minutes) than Egyptian Vultures (60 minutes) to ensure positive
202 reactions in all samples. ACCIW, on the other hand, amplified CHD-W fragments of
203 females of Egyptian Vultures at 63 ºC/60 minutes and Black Kites at 67 ºC/80 minutes.

204

205 **Discussion**

206 We have developed a simple and portable method for molecular sex determination of
207 three raptor species of the family *Accipitridae* based on Loop Mediated Isothermal
208 Amplification (LAMP). This procedure shows two main advantages over classical
209 PCR-based sex determinations. First, it can be easily performed in the field because

210 DNA extraction (10 minutes) and two LAMP reactions (maximum 80 minutes in total)
211 only need a water bath or thermo-block for incubation at a single temperature. All
212 LAMP reagents (vacuum dried primers and a ready-mix including stabilizers) can be
213 stored at room temperature for months (Hamburger et al. 2013). This is an advantage
214 not only for fieldwork, but also facilitates enormously the shipment of reagents (no cool
215 boxes nor dry ice are needed). Second, sex is determined with the unaided eye
216 according to change of colour within the reaction tubes caused by the interaction of a
217 fluorescent label and the pyrophosphate residuals produced during LAMP. We believe
218 our work unties the indivisible link between molecular sex determination and fully
219 equipped laboratories and allows for the first time sex determination of individuals
220 (fledging to adults) in sampling areas located far away from wild populations.

221 *The lab in the field*

222 Logistic is one of the major issues to make a protocol applicable to field conditions.
223 Using thermo-blocks instead of thermo-cyclers cheapens and facilitates enormously the
224 procedure. However, reagents and primers used for biochemical reactions usually need
225 special storage conditions such as freezing or cooling that complicate their shipment
226 and delivery. The primer-mix (dehydrated primers) and ready-mix (reagents with
227 stabilizers) prepared prior to their shipment and delivery allow long-term storage at
228 room temperature and prevent their degradation. Although freeze-drying (i.e.
229 lyophilisation) is widely accepted as the preferred technique for achieving long term
230 storage of biological materials and oligonucleotides (Day & Stacey 2007), we chose
231 vacuum-dried primers over lyophilized because i) they were kept safe at room
232 temperature and ii) involved lower costs (vacuum driers). We also tested different
233 concentrations of sucrose to stabilize the ready-mix reagents and keep them at room
234 temperature and found that concentrations up to 2% worked optimally in the three tested

235 species. This concentration kept reagents at room temperature at least for seven days
236 and did not decreased the efficiency of LAMP reactions. We observed inhibition of
237 LAMP reactions as sucrose concentration increased above 8% of the reaction volume
238 (data not shown) in close agreement with the only and previous work optimizing
239 sucrose in LAMP reactions (Hamburger *et al.* 2013).

240 We recommend an initial effort to find an optimal DNA extraction protocol and dilution
241 of DNA template to decrease the proportion of inhibitors per reaction, especially if non-
242 invasive samples (e.g. feathers, faeces…) are used. We followed a hotshot NaOH
243 protocol for DNA extraction (Truett *et al.* 2000) from blood samples because it was
244 simple and fast (10 mins / 100ºC in NaOH 100 mM) so it can be easily performed in
245 field conditions. However, a 10 to 100 fold dilutions were needed not only to reduce the
246 amount of DNA, but also to decrease the proportion of inhibitors therein. We applied
247 our protocol to 23 individuals of Griffon Vulture (8 females and 15 males) in our lab to
248 evaluate the efficiency and accuracy of the method and correctly determined the sex of
249 20 individuals (87%) using a 1:100 dilution of template. The three samples left were
250 correctly assigned when DNA templates were diluted to 1:10. These results stress the
251 need to standardize the procedure with equal concentrations of DNA to minimize
252 inhibitions of LAMP reactions that bias sex determination. Sensitivity analyses of DNA
253 concentrations in LAMP show the high efficiency at very low DNA concentrations (e.g.
254 femtograms: 10^{-15} g), way below the expected concentration from DNA extractions
255 from nucleated red blood cells (e.g. Poon *et al.* 2006; Bonizzoni *et al.* 2009; Hamburger
256 *et al.* 2013). As far as we know, this is the first study testing outdoor conditions for
257 LAMP, despite it has always been cited as molecular technique that could be easily
258 taken to the field or, at most, taken to laboratories in hospitals with limited resources.

259 This protocol relies on the amplification of the female-specific CHD region located in
260 the W chromosome (ACCIW) and the homologous region in the Z chromosome present
261 in both males and females (ACCIZ). We recommend these two LAMP reactions in
262 parallel with a negative control (i.e. free-template LAMP reactions) so ACCIW- and
263 ACCIW+ reactions can be distinguished from false negatives and positives
264 (respectively). False positives are among the most common flaws in LAMP reactions.
265 This type I error is usually explained by cross-sample contaminations (LAMP is 10 to
266 100 times more sensitive than PCR) (Le *et al.* 2012) or background amplification (i.e.
267 amplification in template-free reactions due to primer dimers) (Kimura *et al.* 2011;
268 Wang *et al.* 2015). However, although false positives were found while optimizing the
269 technique, we discarded these two explanations because we observed no amplification
270 in template-free reactions.

271 LAMP is a relatively novel molecular technique for DNA amplification widely applied
272 in medicine (Poon *et al.* 2006; Nyan *et al.* 2014; Fernández-Soto *et al.* 2014) and
273 parasitology (Abbasi *et al.* 2010; Salant *et al.* 2012; Hamburger *et al.* 2013). It does not
274 require any molecular background nor experience, only a few days of training
275 (Hamburger *et al.* 2013; Cuadros *et al.* 2015). However, despite this great potential, the
276 application of LAMP in life and environmental sciences has been focused in species
277 with commercial interest (Hsu *et al.* 2011, 2012; Abdulmawjood *et al.* 2014; Kim *et al.*
278 2015). We believe that the proposed technique can be highly instrumental to studies in
279 ecology, behaviour and evolution, as well as for conservation projects. For instance,
280 LAMP has a great potential for species determination from faecal samples (especially
281 challenging for elusive mammals) or to distinguish between sibling species that cannot
282 easily be distinguished morphologically. LAMP has been applied for sex determination
283 in rock pigeons (*Columba livia*) but, although suggested, it was not tested on other bird

284 species within the *Columbidae* family (Chan *et al.* 2012) nor set up for field conditions.
285 Chan et al. (2012) designed primers for sex determination based on CHD and for
286 positive control based on a fragment of the mitochondrial 18S ribosomal RNA gene.
287 This marker choice may overestimate the number of individuals assigned as males when
288 the sexual marker fails because mitochondrial genomes are by several orders of
289 magnitude than nuclear genomes and this may generate amplification bias favouring
290 mitochondrial genomes.

291 *Conclusions and perspectives*

292 We have optimized a LAMP-based protocol for sex determination suitable for fieldwork
293 for three raptor species belonging to three different subfamilies within *Accipitridae.*
294 This is a relatively large family with more than 300 species with an important role as
295 top predators essentially in all terrestrial ecosystems around the world. Our successful
296 LAMP application of the same primer sets across species of three different raptor
297 subfamilies is a promising fully operational tool for molecular sexing of raptors in field
298 conditions. To our knowledge, this is not only the first fully operational field
299 application of the LAMP sex-determination technique, but also the first demonstration
300 of the utility of this approach beyond the single-species level. Furthermore, this novel,
301 portable and accurate molecular technique for sex determination is simpler (it does not
302 require electrophoresis) and cheaper than PCR-based methods (Hamburger *et al.* 2013;
303 Pooja *et al.* 2014), it can provide results in less than 90 minutes and be applied during
304 regular fieldwork conditions. Moreover, the potential of the methodology described
305 here goes beyond its application to raptors (*Accipitridae*) and aims to be extended to
306 higher taxonomic levels within *Aves* based on the homology and relative well conserved
307 region of the widely used CHD gene for sex determination across taxa (Griffiths *et al.*
308 1998; Vucicevic *et al.* 2013). Our work opens a new tool kit for ecologists that has

remained almost unknown despite its extensive use in other disciplines (e.g. Nyan *et al.* 2014; Fukuta *et al.* 2014; Abbasi *et al.* 2010). To facilitate adoption of this tool, we provide a “know how” guide to apply LAMP to projects where species identification or sex determination is needed in real time and *in situ*. Future work in this direction will facilitate enormously the work of wildlife managers and researchers as well as for poultry and exotic bird breeders with important conservationist, economic and commercial benefits for these collectives.

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333 **REFERENCES**

334 Abbasi, I., King, C.H., Muchiri, E.M. & Hamburger, J. (2010). Detection of
335 Schistosoma mansoni and Schistosoma haematobium DNA by loop-mediated
336 isothermal amplification: identification of infected snails from early prepatency.
337 *The American journal of tropical medicine and hygiene*, **83**, 427–32.

338 Abdulmawjood, A., Grabowski, N., Fohler, S., Kittler, S., Nagengast, H. & Klein, G.
339 (2014). Development of loop-mediated isothermal amplification (LAMP) assay for
340 rapid and sensitive identification of ostrich meat. *PloS one*, **9**, e100717.

341 Aslam, M.A., Hulst, M., Hoving-Bolink, R.A.H., de Wit, A.A.C., Smits, M.A. &
342 Woelders, H. (2012). A reliable method for sexing unincubated bird eggs for
343 studying primary sex ratio. *Molecular ecology resources*, **12**, 421–7.

344 Bantock, T.M., Prys-Jones, R.P. & Lee, P.L.M. (2008). New and improved molecular
345 sexing methods for museum bird specimens. *Molecular ecology resources*, **8**, 519–
346 28.

347 Bonizzoni, M., Afrane, Y. & Yan, G. (2009). Loop-mediated isothermal amplification
348 (LAMP) for rapid identification of Anopheles gambiae and Anopheles arabiensis
349 mosquitoes. *The American journal of tropical medicine and hygiene*, **81**, 1030–4.

350 Chan, K.-W., Liu, P.-C., Yang, W.-C., Kuo, J., Chang, C.L.-T. & Wang, C.-Y. (2012).
351 A novel loop-mediated isothermal amplification approach for sex identification of
352 Columbidae birds. *Theriogenology*, **78**, 1329–38.

353 Cuadros, J., Pérez-Tanoira, R., Prieto-Pérez, L., Martin-Martin, I., Berzosa, P.,
354 González, V., Tisiano, G., Balcha, S., Ramos, J.M. & Górgolas, M. (2015). Field
355 Evaluation of Malaria Microscopy, Rapid Malaria Tests and Loop-Mediated
356 Isothermal Amplification in a Rural Hospital in South Western Ethiopia. *PloS one*,
357 **10**, e0142842.

358 Day, J.G. & Stacey, G.N. (2007). *Cryopreservation and Freeze-Drying Protocols*.
359 Humana Press Inc, Totowa, New Jersey.

360 Fernández-Soto, P., Gandasegui Arahuetes, J., Sánchez Hernández, A., López Abán, J.,
361 Vicente Santiago, B. & Muro, A. (2014). A loop-mediated isothermal
362 amplification (LAMP) assay for early detection of Schistosoma mansoni in stool
363 samples: a diagnostic approach in a murine model. *PLoS neglected tropical*
364 *diseases*, **8**, e3126.

365 Fridolfsson, A.-K. & Ellegren, H. (1999). A simple and universal method for molecular
366 sexing of non-ratite birds. *Journal of Avian Biology*, **30**, 116–121.

367 Fukuta, S., Takahashi, R., Kuroyanagi, S., Ishiguro, Y., Miyake, N., Nagai, H., Suzuki,
368 H., Tsuji, T., Hashizume, F., Watanabe, H. & Kageyama, K. (2014). Development
369 of loop-mediated isothermal amplification assay for the detection of Pythium
370 myriotylum. *Letters in applied microbiology*, **59**, 49–57.

371 Griffiths, R., Double, M.C., Orr, K. & Dawson, R.J.G. (1998). A DNA test to sex most
372 birds. *Molecular Ecology*, **7**, 1071–1075.

373 Hamburger, J., Abbasi, I., Kariuki, C., Wanjala, A., Mzungu, E., Mungai, P., Muchiri, E.
374 & King, C.H. (2013). Evaluation of loop-mediated isothermal amplification
375 suitable for molecular monitoring of schistosome-infected snails in field

376 laboratories. *The American journal of tropical medicine and hygiene*, **88**, 344–51.

377 Horvath, M., Martínez-Cruz, B., Kalmar, L., Negro, J. & Godoy, J.A. (2005). An
378 overlooked DNA source for non- invasive genetic analysis in birds. *Journal of*
379 *Avian Biology*, **1**, 84–88.

380 Hsu, T.-H., Adiputra, Y.T., Ohta, H. & Gwo, J.-C. (2011). Species and sex
381 identification of Formosa landlocked salmon using loop-mediated isothermal
382 amplification. *Molecular ecology resources*, **11**, 802–7.

383 Hsu, T.-H., Gwo, J.-C. & Lin, K.-H. (2012). Rapid sex identification of papaya (Carica
384 papaya) using multiplex loop-mediated isothermal amplification (mLAMP). *Planta*,
385 **236**, 1239–46.

386 Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton,
387 S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P. &
388 Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop
389 software platform for the organization and analysis of sequence data.
390 *Bioinformatics (Oxford, England)*, **28**, 1647–9.

391 Kim, S.W., Choi, J.S., Sharma, N., Ko, Y.G., Do, Y.J., Byun, Mi., Seong, H.H., Park,
392 S.B. & Jeong, D.K. (2015). A novel approach for determination of chicken sexing
393 at an early stage of development by using loop-mediated isothermal amplification
394 method. *Turkish Journal of Veterinary and Animal Sciences*, **39**, 583–588.

395 Kimura, Y., de Hoon, M.J.L., Aoki, S., Ishizu, Y., Kawai, Y., Kogo, Y., Daub, C.O.,
396 Lezhava, A., Arner, E. & Hayashizaki, Y. (2011). Optimization of turn-back
397 primers in isothermal amplification. *Nucleic acids research*, **39**, e59.

398 Le, T.H., Nguyen, N.T.B., Truong, N.H. & De, N. Van. (2012). Development of
399 mitochondrial loop-mediated isothermal amplification for detection of the small
400 liver fluke Opisthorchis viverrini (Opisthorchiidae; Trematoda; Platyhelminthes).
401 *Journal of clinical microbiology*, **50**, 1178–84.

402 Mori, Y., Nagamine, K., Tomita, N. & Notomi, T. (2001). Detection of loop-mediated
403 isothermal amplification reaction by turbidity derived from magnesium
404 pyrophosphate formation. *Biochemical and biophysical research communications*,
405 **289**, 150–4.

406 Morinha, F., Cabral, J.A. & Bastos, E. (2012). Molecular sexing of birds: A
407 comparative review of polymerase chain reaction (PCR)-based methods.
408 *Theriogenology*, **78**, 703–714.

409 Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. &
410 Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic acids*
411 *research*, **28**, E63.

412 Nyan, D.-C., Ulitzky, L.E., Cehan, N., Williamson, P., Winkelman, V., Rios, M. &
413 Taylor, D.R. (2014). Rapid Detection of Hepatitis B Virus in Blood Plasma by a
414 Specific and Sensitive Loop-Mediated Isothermal Amplification Assay. *Clinical*
415 *infectious diseases : an official publication of the Infectious Diseases Society of*
416 *America*, **59**, 16–23.

417 Pooja, S., Sudesh, D., Poonam, K., Joginder, S.D. & Suresh, K.G. (2014). Loop-
418 mediated isothermal amplification (LAMP) based detection of bacteria: A Review.
419 *African Journal of Biotechnology*, **13**, 1920–1928.

420 Poon, L.L.M., Wong, B.W.Y., Ma, E.H.T., Chan, K.H., Chow, L.M.C., Abeyewickreme,
421 W., Tangpukdee, N., Yuen, K.Y., Guan, Y., Looareesuwan, S. & Peiris, J.. M.
422 (2006). Sensitive and Inexpensive Molecular Test for Falciparum Malaria:
423 Detecting Plasmodium falciparum DNA Directly from Heat-Treated Blood by
424 Loop-Mediated Isothermal Amplification. *Clinical chemistry*, **52**, 300–3.

425 Robertson, B.C. & Gemmell, N.J. (2006). PCR-based sexing in conservation biology:
426 Wrong answers from an accurate methodology? *Conservation Genetics*, **7**, 267–
427 271.

428 Salant, H., Abbasi, I. & Hamburger, J. (2012). The development of a loop-mediated
429 isothermal amplification method (LAMP) for Echinococcus granulosis
430 coprodetection. *The American journal of tropical medicine and hygiene*, **87**, 883–7.

431 Spiegel, O., Harel, R., Centeno-Cuadros, A., Hatzofe, O., Getz, W.M. & Nathan, R.
432 (2015). Moving beyond curve-fitting: using complementary data to assess
433 alternative explanations for long movements of three vulture species. *The*
434 *American Naturalist*, **185**, E44–E54.

435 Spiegel, O., Harel, R., Getz, W.M. & Nathan, R. (2013). Mixed strategies of griffon
436 vultures' (Gyps fulvus) response to food deprivation lead to a hump-shaped
437 movement pattern. *Movement Ecology*, **1**, 5.

438 Tanner, N.A., Zhang, Y. & Evans Jr, T.C. (2015). Visual detection of isothermal nucleic
439 acid amplification using pH-sensitive dyes. *BioTechniques*, **58**, 59–68.

440 Tomita, N., Mori, Y., Kanda, H. & Notomi, T. (2008). Loop-mediated isothermal
441 amplification (LAMP) of gene sequences and simple visual detection of products.
442 *Nature protocols*, **3**, 877–882.

443 Truett, G.E., Heeger, P., Mynatt, R.L., Truett, A.A., Walker, J.A. & Warman, M.L.
444 (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium
445 hydroxide and tris (HotSHOT). *BioTechniques*, **29**, 52, 54.

446 Vucicevic, M., Stevanov-Pavlovic, M., Stevanovic, J., Bosnjak, J., Gajic, B., Aleksic, N.
447 & Stanimirovic, Z. (2013). Sex determination in 58 bird species and evaluation of
448 CHD gene as a universal molecular marker in bird sexing. *Zoo biology*, **32**, 269–76.

449 Wang, D.-G., Brewster, J.D., Paul, M. & Tomasula, P.M. (2015). Two methods for
450 increased specificity and sensitivity in loop-mediated isothermal amplification.
451 *Molecules*, **20**, 6048–59.

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453

454 **DATA ACCESIBILITY**

455 ACCIZ sequences specific of Griffon vulture, Black Kite and Egyptian vulture obtained
456 in this study for LAMP primers design are deposited in GenBank (accession numbers:
457 KU563739, KU563740 and KU563741, respectively). The alignment of these
458 sequences is available at Dryad Project as doi:10.5061/dryad.4kr93.

459

460 **AUTHOR CONTRIBUTION**

461 A.C-C. conceived the study and A.C-C., R.N. and I.A. planned and designed the
462 experiments. A.C-C. performed the laboratory work and drafted the manuscript and
463 A.C-C. and R.N. edited the manuscript. All authors reviewed the final draft of the
464 manuscript.

465

466

467 **BOX 1.** *Sex determination by LAMP: a workflow*

468 **At the laboratory.** Primers and reagents are prepared in advance in a laboratory (see
469 text). These mixes do not require special storage conditions whereas the primer mix
470 needs to be rehydrated before being added to the ready mix.

471 **In the car.** LAMP needs a thermo-block or water bath for incubation at a single
472 temperature. Under field conditions, we suggest to use voltage inverters plugged in car
473 lighters as the portable unit of power supply required for incubation.

474 **At the field** (after sampling) (see also figure 2). **Step 1**: DNA extraction using NaOH.
475 We recommend dilutions with H_2O to avoid inhibitions during LAMP reactions. **Step 2**:
476 two reactions per sample are required to ensure correct sex determination: ACCIZ
477 (positive control) and ACCIW (sex determination) (see table 1 for specific temperature
478 and time conditions). **Step 3**: addition of Sybr-Green and evaluation of colour change
479 within the reaction tubes. **Step 4**: interpretation of the two LAMP reactions per sample
480 and sex determination.

481

482 **Table 1**. LAMP primers to amplify CHD-W and CHD-Z. F3 = forward external primer; B3 = backwards external primer; FIP = forward internal
483 primer composed by F1c and F2 primers connected by **TTTT** (bold); BIP = backward internal primer composed by B1c and B2 primers
484 connected by **TTTT** (bold).

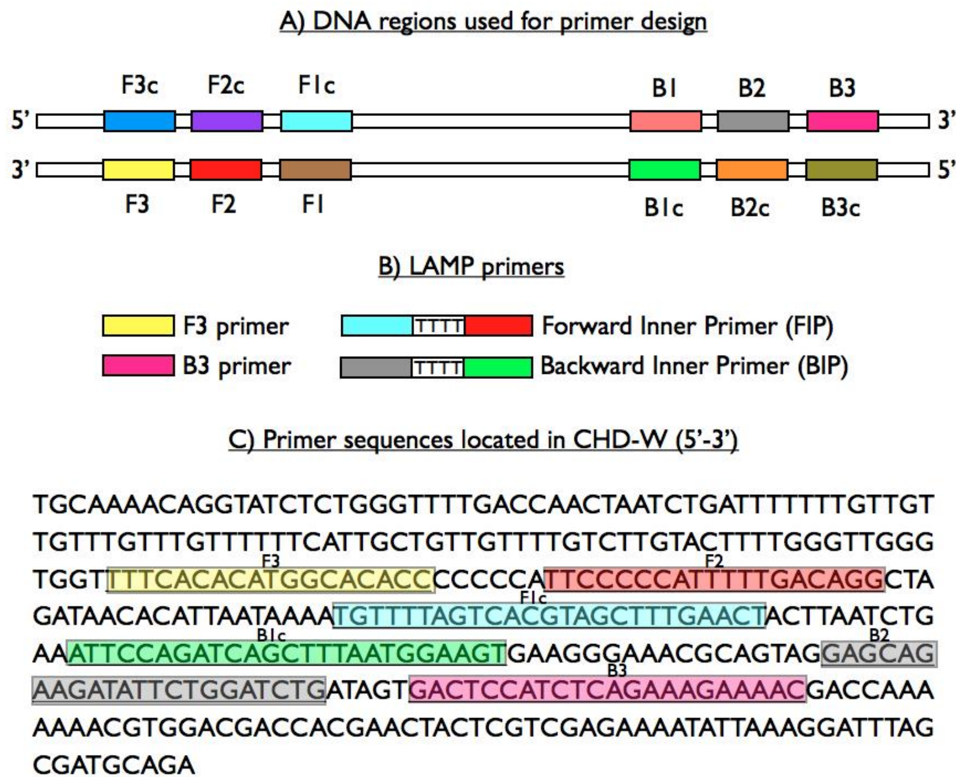
485

486

Primer set	**Primer**	**Sequence (5'-3')**	**Temperature (ºC) / Time (minutes)** *Gyps fulvus*	*Neophron percnopterus*	*Milvus migrans*
ACCIW	F3	TTTCACACATGGCACACC	64º/80’	63º/60’	67º/80’
	B3	GTTTTCTTTCTGAGATGGAGTC			
	FIP	AGTTCAAAGCTACGTGACTAAAACA**TTTT**TTCCCCCATTTTTGACAGG			
	BIP	ATTCCAGATCAGCTTTAATGGAAGT**TTTT**CAGATCCAGAATATCTTCTGCTC			
ACCIZ	F3	AMCAGCTGATATTGGAAGG	59º/80’	59º/60’	59º/80’
	B3	TTTCTTTASTYTGAGGGTGA			
	FIP	GGCAACYTGCTTTMRCTGTYG**TTTT**ACCTCTGGMTATSGTCTTG			
	BIP	CCAGGTGGCTTYTGAATGTCA**TTTT**GCRCTGGAACAAGTTGTC			

487

488 **Figure 1.** Schematic illustration of primer design for LAMP of ACCIW. A) Six
489 different regions (forward: F1, F2 and F3; backward: B1, B2 and B3) and their
490 complementary regions (forward: F1c, F2c and F3c; backward: B1c, B2c and B3c) are
491 located on target DNA. B) Two outer (F3 and B3) and two inner primers (FIP and BIP)
492 are used in each LAMP reaction. FIP (BIP) is composed by the F1c (B1c) sequence and
493 the F2 (B2) sequence joined by a T-linker. C) Location of the F3/B3 and FIP/BIP
494 primers along the CHD-W sequence. **(Note to the Editorial Board: colour online**
495 **only**)



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498 **Figure 2.** Workflow for LAMP-based sex determination. **(Note to the Editorial**
499 **Board: colour online only)**

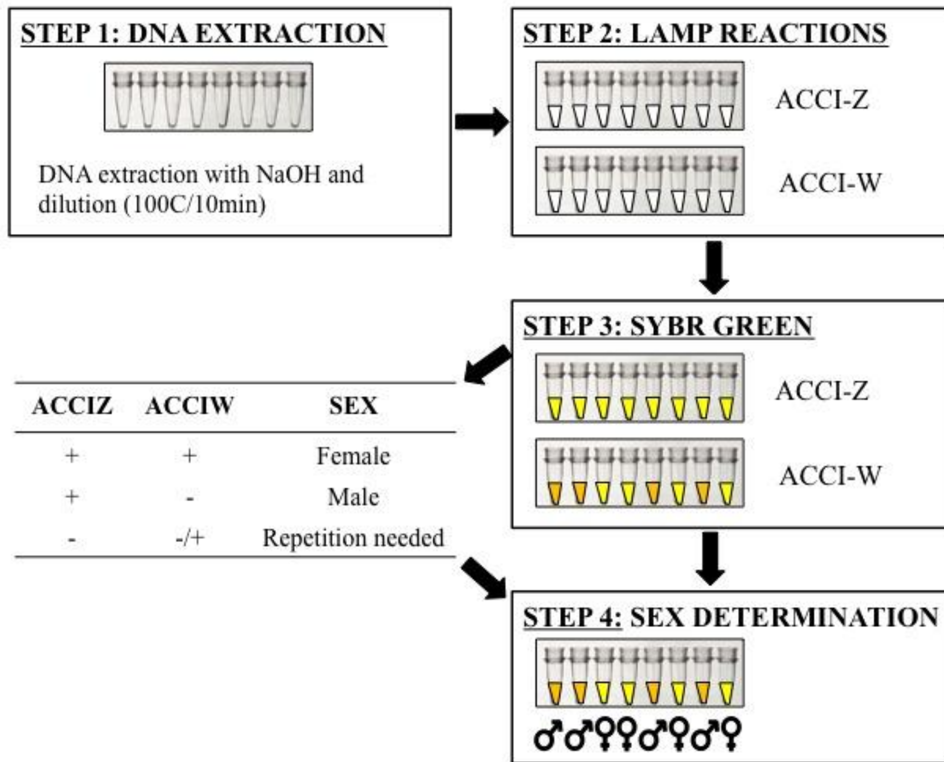

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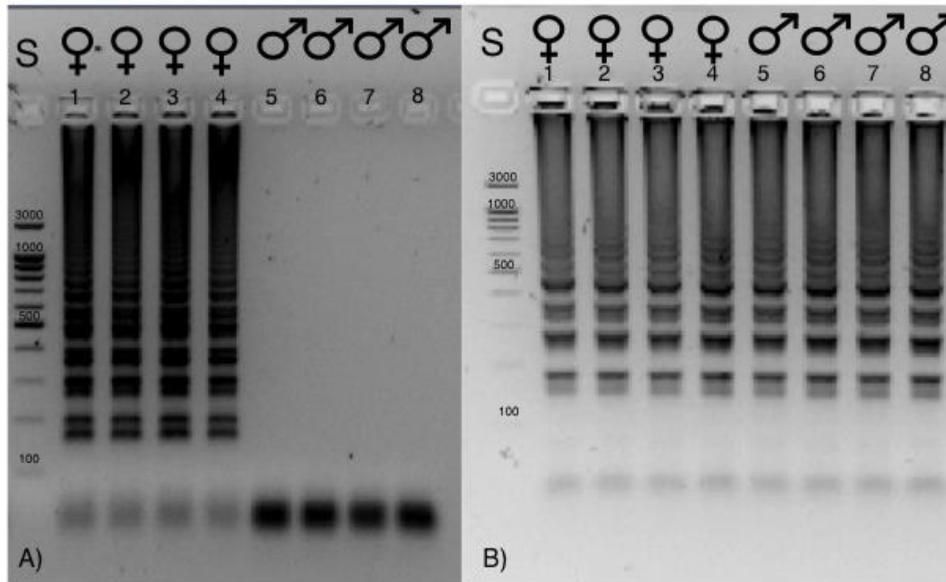
Figure 3. Agarose gel showing LAMP results tested in Griffon Vultures (*Gyps fulvus*). Primers sets ACCIW (A) and ACCIZ (B). S: 100 bp size standard.



507 **Figure 4.** Visual detection of LAMP products using the set of primers ACCIW and
508 ACCIZ. The colour of the reaction mix changed to yellow-green when LAMP reaction
509 was positive and remained orange when there was no amplification after adding SYBR
510 Green I. These colours can be observed with daylight (A and B) and after irradiating
511 with a portable UV lamp at 320 nm. Samples 1 to 8 are the same as in figure 3. **(Note to
512 the Editorial Board: colour online only)**

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