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DataDot^{DNA}: an alternative marking system for tortoises of genus *Testudo*

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Abstract. It was analyzed the effectiveness of one method of individual and unique marking, alternative to the application of microchip, on 17 specimens of *Testudo hermanni* through DataDot^{DNA} technology. This technology has proven to be an effective system of marking of *Testudo spp.*, answering the need for unambiguous identification of individuals. Further advantages are the easy application and reading, the long-term resistance as well as the difficulties of possible fraudulent tampering.

Keywords. *Testudo hermanni*, *Testudo graeca*, *Testudo marginata*, DataDot^{DNA}, marking.

The species *Testudo hermanni* Gmelin, 1789, *Testudo graeca* Linnaeus, 1758 and *Testudo marginata* Schoepff, 1792 are enclosed in Annex A to Council Regulation (EC) No 338/97 and subsequent amendments and revisions. Council Regulation (EC) No 338/97 deals with the protection of species of wild fauna and flora by regulating the trade therein, harmonising the implementation of the "Washington Convention on International Trade in Endangered Species of Wild Fauna and Flora" at European level.

The legal obligation to mark all the living individuals of species included in Annex A has been introduced to control the pet trade, in accordance with the law 7 February 1992 No 150 and the mentioned Council Regulation. The animals should be univocally identified by microchip ISO 11784:1996 (E) and 11785:1996 (E) compliant. Due to the commonly used microchip dimensions (mm 12 x 2.12), and in order to guarantee the animals welfare, the CITES Management Authority (in Italy the Ministry for Environment and Territory and Sea) provided, for individuals under the age of five years, the possibility to use a photographic identification archive, instead.

The recent commercial availability of smaller microchip (mm 7 x 1.25) allowed the marking procedure of individuals within their first year of age, namely when their carapace reached the length of 5 cm. However, microchip inoculation is an invasive action that entails intrinsic risks, which are common to all the surgical procedures, but more hazardous when performed on small and very young animals. Hence, in order to minimise all the risk factors, it has been tested the possible implementation of the DataDot^{DNA} technology for marking *Testudo hermanni*, which growth rate in the first years is lower than those of the other two species *T. graeca* and *T. marginata*.

Microdot identification technology has been applied for several years in Australia, United Kingdom, Italy and many other countries, to the prevention of car theft or to guarantee the authenticity of art and collector objects. More recently, it has been employed both to oppose the oyster theft (Honan, 2007; Sydney Morning Herald, 2007), and in entomological researches, to investigate the orchid pollination by wasps (Whitehead and Peakall, 2013). This technology is based on polymer discs (diameter one millimetre or less) assembled with a unique laser

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impressed alphanumeric code. Discs are secured on the involved surface using different kinds of adhesive.

The objectives of our research were: i) prove that DataDot^{DNA} identification technology is an efficient alternative, or complementary marking method for *Testudo* tortoises; ii) test its resistance to the removal by means of natural agents (rubbing or other), or by illicit management (removal and reuse of disks); iii) verify that all the procedures were in compliance with the animal welfare requirements, and in particular that they do not affect the individual growth rate.

Seventeen individuals of unspecified gender of *Testudo hermanni*, between 1 and 3 years old, were divided into two homogeneous groups (A: experimental; B: control), constituted by 9 and 8 individuals, respectively. During the trial period, the two groups were placed in large monitored enclosures, in semi-natural conditions, ambient temperature and same food supply.

An identification form was drawn up for each subject. The main biometric measures (carapace length, width and height; plastron length; body mass) were recorded and filled in the form at the beginning of the trial. The shell of each subject of group A was cleaned and dried before placing the DataDot^{DNA} technology id system. The identification system was composed by a 9 characters alphanumeric code, impressed in specular alternating rows on discs of 0.5 mm of diameter (Fig. 1). Discs were secured with three different adhesives based on: i) aqueous solution of dipropylene-glycol-monomethylether (DGM); ii) methyl cyanoacrylate (CM); iii) n-butyl acetate (NB). The DGM based adhesive has UV fluorescence characteristics, which allow for easier detection of discs glued on the shell. If necessary, the fluorescent pigment (CIBA Uvitex NFW Optical Brightener) could be easily included in the composition of the other two kinds of glue, following the manufacturing instruction for component ratios. For each subject, three polyester discs were separately suspended in each of the adhesive solutions and placed, with the aid of a small pad, on one vertebral and two coastal scutes. After the drying time, which ranged from 5 to 20 minutes (quicker for CM, longer for NB), the readability of the discs was tested with both a portable optical microscope (100×), and a portable digital microscope (27-108×) (Fig. 2). At the end of these operations, the animals were brought back to their enclosures.

Biometric measurements were taken monthly during the trial period, according to the hibernation periods, for a total of 10 records for 13 individuals, and 11 records for the remaining 4. During these surveys, the conditions and readability of each disc were checked, as well as the presence of localised or generic growth aberrations of the shell, or on-going pathological conditions. Since the trial period started before for 4 tortoises - born between 2008 and 2009, and equally distributed in experimental and control groups - and later for the others (born in 2010

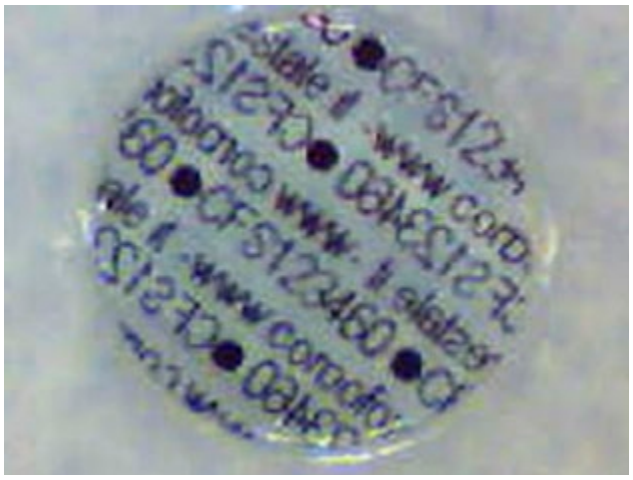

Fig. 1. Microdot disc of 0.5 mm in diameter.

Fig. 2. Experimental phases: placement (upper) and reading test (lower) of microdot discs.

and 2011), an interval of 15 months, included one winter latency period, has been taken into consideration for each animal. The differences between the last and the first measurement, over such interval of time, were recorded for each biometric parameter (Table 1, 2).

ANOVA for repeated measures cannot be applied to analyse two groups of paired data (Huck and McLean, 1975). Feasible alternatives are: i) *t*-test for independent groups on the differences between last and first measurements (Hopkins, 1997), or ii) analysis of covariance (ANCOVA) with the experimental and control groups as independent variable, the last measurement as dependent variable and the first one as covariate. In these cases, the latter is considered the most powerful test (Huck and McLean, 1975). Covariate permits to remove the influence of differences of the starting measurement within and between groups.

Neither abnormal development of carapace or plastron, nor local or systemic pathologies were found, in any subject, during the experimental period. The biometric data recorded were congruent with the age of any single tortoise, and the growth rates were in line with literature data (Cheylan, 1981).

All the alphanumeric code reading tests had positive result, for all three adhesives employed, but some discs were lost by three tortoises. Reading was reasonably easy with the portable optic microscope at 100×, while with the digital one at 108× it was pretty hard, due to difficulties to focus a bended surface, like the carapace. The only relevant reading problem was caused by discs applied on carapace black spots, because they were transparent with the code impressed in black.

ANCOVA gave not significant differences, at $\alpha = 0.05$, between the mean value of all biometric measures (Table 3). Therefore, we can confidently conclude that DataDot^{DNA} marking technology did not affect the growth of subjects included in group A.

DataDot^{DNA} technology proved to be an efficient marking system for tortoises of *Testudo* genus, alternative, or complementary, to other currently adopted techniques (Stubbs et al., 1984; Guyot and Clobert, 1997), at least for tortoises raised in controlled conditions. This methodol-

Table 1. Experimental group (A). First measurements and, in brackets, differences between last and first measurements. Start date: date of marking and first measurement; Lc: carapace length; Wc: carapace width; Hc: carapace height; Lp: plastron length; Mb: body mass.

DATADOT	Birth year	Start date (dd-mm-yyyy)	Lc (mm)	Wc (mm)	Hc (mm)	Lp (mm)	**Mb (g)**
CFS 001018	2011	27-03-2012	37.5 (7.5)	33 (4)	18 (4)	32 (3)	10 (8)
CFS 001009	2011	27-03-2012	45 (11)	39 (6)	24 (7)	39 (8)	16 (20)
CFS 001004	2011	27-03-2012	39 (12)	34 (8)	21 (7)	33 (9)	14.5 (14.5)
CFS 001000	2011	27-03-2012	42 (13)	37 (9)	21 (7)	37 (9)	15.9 (16.1)
CFS 001007	2011	27-03-2012	40 (12)	37 (7)	22 (6)	35 (9)	14 (15)
CFS 001012	2011	27-03-2012	40 (9)	36 (3)	20 (5)	34 (6)	13.9 (8.1)
CFS 001019	2011	27-03-2012	37 (8)	33 (4)	18 (5)	31 (6)	9 (9)
CFS 001002	2009	9-07-2011	45 (28)	37 (21)	23 (16.5)	38 (22)	15 (63)
CFS 001020	2008	9-07-2011	51 (26)	45 (19)	28 (19)	42 (20)	36 (68)

Table 2. Control group (B). First measurements and, in brackets, differences between last and first measurements. Start date: date of first measurement; Lc: carapace length; Wc: carapace width; Hc: carapace height; Lp: plastron length; Mb: body mass.

Id	Birth year	Start date (dd-mm-yyyy)	Lc (mm)	Wc (mm)	Hc (mm)	Lp (mm)	Mb **(g)**
CITESPE-270312-18	2011	27-03-2012	42 (9)	36 (5)	20 (6.5)	34 (7)	13 (13)
CITESPE-270312-19	2010	27-03-2012	47 (12.5)	39 (10)	23 (8)	40 (10)	19.3 (19.7)
CITESPE-270312-20	2011	27-03-2012	37 (12)	34 (8)	21 (8)	32 (6)	11.7 (13.3)
CITESPE-270312-24	2011	27-03-2012	39 (14)	35 (10.5)	20 (9)	34 (9)	11.9 (16.1)
CITESPE-270312-25	2011	27-03-2012	42 (5)	36 (5)	21 (4.5)	36 (4)	13 (7)
CITESPE-270312-35	2011	27-03-2012	39 (11)	37 (11)	20 (8)	34 (7)	12 (15)
PLLMRA-090711-6	2008	9-07-2011	57 (21)	48 (15)	31 (17)	47 (17.5)	36 (58)
PLLMRA-090711-7	2008	9-07-2011	52 (20.5)	41 (16)	28 (14.5)	42 (18)	25 (49)

Table 3. Means and standard deviations of the biometric measures within the experimental (A) and control (B) groups. Lc: carapace length; Wc: carapace width; Hc: carapace height; Lp: plastron length; Mb: body mass.

Group		Lc	Wc	Hc	Lp	Mb
A	Average	14.05	9.00	8.50	10.22	24.63
	Standard Dev.	7.59	6.56	5.38	6.44	23.55
B	Average	13.12	10.06	9.44	9.81	23.89
	Standard Dev.	5.43	4.07	4.18	5.22	18.77
ANCOVA	F_{df}, P	$F_{1,14} = 1.59$, P = 0.23	$F_{1,14} = 0.01$, P = 0.93	$F_{1,14} = 0.15$, P = 0.70	$F_{1,14} = 1.28$, P = 0.28	$F_{1,14} = 0.50$, P = 0.49

ogy meets the current regulation requirements in terms of unambiguous identification of individuals, durability on the marked animal, ease placement and reading and difficulty of possible illicit tampering of the mark.

Long-term durability tests of microdot discs on *Testudo* individuals are currently in progress, as one of the objectives of our further research. However, the following tests on durability were carried out by an independent laboratory of Melbourne (Australia): accelerated aging treatments (high and low air temperature, high humidity) and removal tests (high pressure cold water/detergent cleaner test, high pressure hot water cleaner test). Those experiments gave permanence rates, in the range of 90-100% of the marking, for over 20 years (Johnson, 2010). The current (2013) price, excluding VAT, for a kit of 500 microdots (0.5 mm) is about 15 Euros.

The only remarkable limit of this method is the impossibility to read discs placed on black surfaces. However, this problem could be solved by laser micro engraving the code on different substrata, like ceramic. This new technique - 0.2 mm micro-tags, up to 15 alphanumeric characters, better readability - has been recently developed by DataDot Technologies Ltd. (Australia), and should be tested in the field, as well.

Moreover, the fraudulent removal of discs is possible, as is of micro-chips, but their reuse appear to be very hard. In fact, the removal process damages the discs, which get included into the adhesive layer used to fix them. Readability of discs was negatively affected by the thickness of the adhesive layer. The deterrent factors against illicit removal of marks are supported by: i) the dimensions of the polyester discs: 0.3 mm with 7 characters/row code, 0.5 mm with 11 characters/row code or 1.0 mm with 21 characters/row code. The smaller the disc, the more difficult will be their visual detection; ii) the quantity of discs, with the same code, that could be placed on each animal: the more the discs placed, the harder will be their complete removal. Hence, the rational could be using several small discs (0.3 mm), fixed on different parts of the shell. This should be a strong deterrent against illicit marking removal.

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