Masculinization of captive females of *Chelonoidis carbonaria* (Testudinidae)

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Abstract: Morphological, behavioral and reproductive abnormalities were found in 19 adult females of *Chelonoidis carbonaria* living in Brazilian Zoos located south of the Tropic of Capricorn. These abnormal traits were first detected by: a- slight concave plastrons and relatively long tails; b- large differentiation of the clitoris into structures similar to a penis; c- egg retention and egg hypercalcification; d- vocalizations similar to those emitted by males and, in some cases, participation in incomplete combat events with males. Testosterone and estradiol analyses of the plasma of "normal" (n=9) and abnormal (n=6) females revealed higher levels of testosterone in the latter (30-250 ng/dl). Most of the individuals studied were collected in northern and northeastern Brazil, during the hatching period, and donated to zoos as juveniles. Two possible explanations for the origin of these abnormalities are considered: a- they were generated in natural habitats by low or high temperatures during embryo development; b- they were produced by endocrine imbalance, induced by the lower mean temperatures to which most individuals were exposed during post natal development in southern latitudes.
Key words: arrhenoidy, heterosexual structures, intersexuality, pseudohermaphroditism, Testudinidae

Resumen: Masculinización de hembras cautivas de *Chelonoidis carbonaria*.- Se detectaron una serie características morfológicas, comportamentales y reproductivas anormales en 19 hembras adultas de *Chelonoidis carbonaria* cautivas en zoos brasileños situados al sur del Trópico de Capricornio. Estas características anormales fueron inicialmente detectadas por: a- la presencia de plastrones ligeramente cóncavos y colas relativamente largas; b- gran diferenciación del clítoris, adquiriendo estructuras similares a la del pene; c- retención de huevos e hipercalcificación de los mismos; d- vocalizaciones similares a las emitidas por machos y, en algunos casos, participación en secuencias incompletas de combate con machos. Análisis de testosterona y estradiol del plasma de hembras "normales" (n=9) y anormales (n=6) revelaron niveles más elevados de testostenona en las últimas (de 30 a 250 ng/dl). La mayoría de los individuos cautivos estudiados fueron capturados en el norte y nordeste de Brasil, cuando estaban recién eclosionados, y donados a los zoos en fase juvenil. Están siendo consideradas dos explicaciones posibles sobre el origen de estas anomalías en cautividad: a- que se hubieran generado en hábitats naturales, debido a temperaturas bajas o muy elevadas durante la fase de desarrollo embrionario; b- que se hubieran producido por desequilibrios endócrinos, inducidos por las temperaturas más bajas a las que muchos de estos individuos han sido expuestos durante su desarrollo post embrionario en las latitudes sureñas.
Palabras clave: arrenoidía, estructuras heterosexuales, intersexualidad, pseudohermafroditismo, Testudinidae

INTRODUCTION

Most of the turtle species and apparently all the crocodilians have no recognizable heteromorphic sex chromosomes (see BULL, 1980). In these animals sexual differentiation is influenced by the temperatures in which eggs are exposed during incubation and this process is mediated by the endocrine system (PIEAU, 1972; BULL, 1980; PIEAU *et al.*, 1984, 1998, 1999; YNTEMA & MROSOVSKY, 1982).

Nevertheless, male and female individuals frequently retain residual characteristics that are not typical of their sex (FOX, 1977). Thus, endocrine imbalances may affect ontogenetic development. When endocrine disruptions occur in the hatchling and/or juvenile phase, they can alter the normal development of the

urogenital systems inducing the appearence of "abnormal" traits and pseudohermaphroditic individuals (FOX, 1977). In poikilotherm tetrapods, heterosexual structures have been most commonly described in turtles and lizards (see FORBES, 1964; FOX, 1977). Usually these abnormal structures are considered as "non- functional", but in some extreme cases "hermaphrodites" (i.e., specimens of turtles that simultaneously produce oocytes and spermatozoa) have also been described (HANSEN, 1943; FORBES, 1964).

Here we describe abnormal behavioral, morphological and reproductive traits in captive females of *Chelonoidis carbonaria* and relate these abnormalities with endocrine dysfunctions. The possibility that endocrine disruptions in captive individuals may be produced by inadequate climatic conditions is discussed.

MATERIAL AND METHODS

Between 1979 and 1998, morphological and behavioral traits of adult captive individuals *Chelonoidis carbonaria* (=*Geochelone carbonaria*, Testudinidae) were studied in the Zoo of Ribeirão Preto (see GUIX *et al.*, 1989), located 250 km north of the Tropic of Capricorn, and compared with behavioral traits of individuals living in the Zoo of São Paulo and Taboão da Serra, both located just below (20 km south) the Tropic of Capricorn. Behavioral traits of adult individuals living in captivity in localities far away from the Tropic of Capricorn were also compared. These areas were the Zoo of Sapucaia do Sul, located in the State of Rio Grande do Sul, 720 km south of the Tropic of Capricorn and the cities of Petrolina (State of Pernambuco) and Juazeiro (State of Bahia), both located 1,570 km north of the Tropic of Capricorn (Figure 1).

Blood samples of 28 adult individuals (13 males and 15 females) living in the Zoo of São Paulo were extracted, and the levels of testosterone and estradiol in the plasma were measured (see methods in COOPER & FERGUSON, 1972; COOPER & CREWS, 1988; JACOBSON, 1993). The cloaca of all females were examined (by injecting xilocaine into the peripheral musculature) and its clitoris or pseudopenis was described and measured.

Females that presented signals of egg retention in the oviduct were radiographed and oxytocin was injected to induce laying. When fe-

Figure 1.- Regions of South America characterized by tropical (in horizontal lines) and subtropical (in vertical lines) type of climate, according to MOPU (1990). Localities where captive individuals of *Chelonoidis carbonaria* were studied (from north to south): Petrolina (PE), Juazeiro (JU), Ribeirão Preto (RP), São Paulo (SP), Taboão da Serra (TS) and Sapucaia do Sul (SS). The transversal line represents the Tropic of Capricorn.
Figura 1.- Regiones de Sudamérica caracterizadas por climas tropical (lineas horizontales) y subtropical (lineas verticales), de acuerdo con MOPU (1990). Localidades donde individuos cautivos de *Chelonoidis carbonaria* fueron estudiados (de norte a sur): Petrolina (PE), Juazeiro (JU), Ribeirão Preto (RP), São Paulo (SP), Taboão da Serra (TS) y Sapucaia do Sul (SS). La linea transversal representa el Trópico de Capricornio.

males did not respond to oxytocin, eggs were extracted surgically, by opening a window on the plastron and suturing it with artificial resins.

RESULTS

Morphological, behavioral and reproductive abnormalities were found in 19 adult females of *Chelonoidis carbonaria* living in Brazilian Zoos located south of the Tropic of Capricorn: Zoos of São Paulo and Taboão da Serra, in the State of São Paulo and the Zoo of Sapucaia do Sul, in the State of Rio Grande do Sul (see location in Figure 1). All these abnormal traits related with reproductive disfunctions were found on individuals living outside the natural distribution of the species (Figure 2).

The abnormal traits were first detected by: a- slight concave plastrons and relatively long tails; b- large differentiation of the clitoris into structures similar to a penis; c- egg retention in the oviduct and egg hypercalcification; d- vocalizations similar to those emitted by males and, in some cases, participation in incomplete combat events with males.

Figure 2.- Distribution of *Chelonoidis carbonaria*, corrected from Pritchard (1979) and Pritchard & Trebbau (1984) according to habitat requirements of the species (mainly relief). Points close to the Tropic of Capricorn (transversal line) represent localities of disjunct populations situated between Salta and Formosa Provinces, northern Argentina (Cei, 1993).
Figura 2.- Distribución de *Chelonoidis carbonaria*, corregida de Pritchard (1979) y Pritchard & Trebbau (1984) de acuerdo con los requerimientos de hábitat de la especie (mayoritariamente tipo relieve). Los puntos cercanos al Trópico de Capricornio (linea transversal) representan localidades de poblaciones disjuntas situadas entre las Provincias de Salta y Formosa, norte de Argentina (Cei, 1993).

Morphological traits

The inspection of the cloaca of females that presented behavioral abnormalities and egg retention revealed large development of the clitoris, forming structures similar to a penis (Figure 3). These structures were variable in size and degree of differentiation: some females had a small and little differentiated pseudopenis and others had a pseudopenis up to 55 mm long with differentiated longitudinal groove and glans, and the presence of corpora cavernosa (Figure 3, Table 1). Male penis dimensions after copulation are around 85-117 mm long (with a glan of 30 mm) and 21-25 mm width.

Of the 16 pseudohermaphroditic females of the Zoo of São Paulo, seven had slighty concave plastrons. Eleven had longer tails than normal females of the same size (two of them had tails as long as males of the same sizes). Four had anal-marginals apertures too small to pass an egg (including one female that was found with an egg stuck between the anals and marginals). Three of the abnormal females, which died during the study, had apparently well developed ovaries and oviducts.

Behavioral abnormalities

Five pseudohermaphroditic females of the Zoo of São Paulo were seen participating in

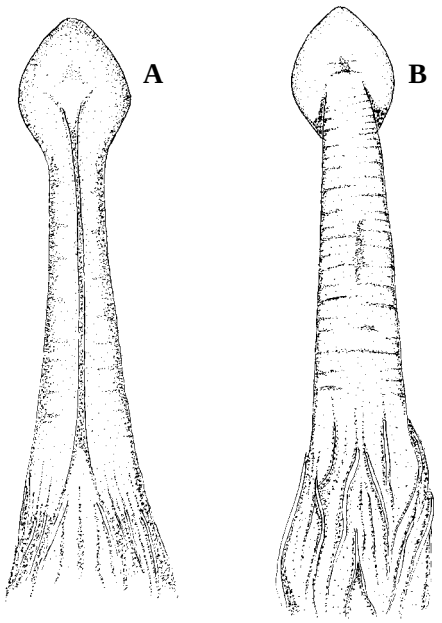

Figure 3.- Pseudopenis of a captive female of *Chelonoidis carbonaria* living in the Zoo of São Paulo, southeastern Brazil. A. Dorsal view; B. Ventral view. Note the differentiation of a structure similar to a glan and a longitudinal groove. Scale bar = 10 mm.
Figura 3.- Pseudopene de una hembra cautiva de *Chelonoidis carbonaria* en el Zoológico de São Paulo, sureste de Brasil. A. vista dorsal; B. vista ventral. Véase la diferenciación de una estructura similar a una glande y un canal longitudinal. Escala = 10 mm.

incomplete combat sequences with males of the same species (Figure 4). In the Zoo of Sapucaia do Sul, two females were also observed pushing males frontally or in the flank. These types of behaviors were never observed in females living in the Zoo of Ribeirão Preto (north of the Tropic of Capricorn) during a four year behavioral study (cf. GUIX *et al.*, 1989).

When hand-catched, nine (56%) of the pseudohermaphroditic females of the Zoo of São Paulo (n = 16) emited vocalizations similar to males. Three of these females were also observed vocalizing spontaneously when in presence of adult individuals of the same species (both males and females).

Reproductive dysfunctions

Seven females (37%; n=19) classified as pseudohermaphrodites (six living in the Zoo of São Paulo and one in the Zoo of Taboão da Serra) revealed egg retention in the oviduct and four of these females also had egg hypercalcification. Initial symptoms of egg retention in the oviduct were edema in the anterior and posterior legs. In three of these females, paralysis in the posterior legs were also observed.

No evident abnormal trait related with reproductive dysfunctions were detected in captive males or females living north of the Tropic of Capricorn (e.g. in the Zoo of Ribeirão Preto, and in captive individuals maintained as pets by people in Juazeiro (State of Bahia) and Petrolina (State of Pernambuco).

Hormonal analysis

Testosterone and estradiol analyses of the plasma of "normal" (n = 9) and pseudohermaphroditic (n = 6) females revealed higher levels of testosterone in the latter (30-250 ng/dl). Significant differences were found in the median testosterone values of "normal" and pseudohermaphroditic females (Mann-Whitney Test, $p < 0.002$). Two pseudohermaphroditic females also had high values of estradiol (910 and 954 pg/ml) (Table 2).

Three of 13 males living in the Zoo of São Paulo, in which plasma testosterone and estradiol were analysed, had hormonal levels that differed so much of other males; eg. low levels of testosterone (27 ng/dl) or high levels of estradiol (537 and 542 pg/ml) (Table 2). Mean testosterone values in males (n = 9) was 350 ng/dl and in pseudohermaphrotic females (n = 6) was 102 ng/dl.

DISCUSSION

Functionally reproductive females of several species of testudines living in their natural habitats may have well developed clitoris

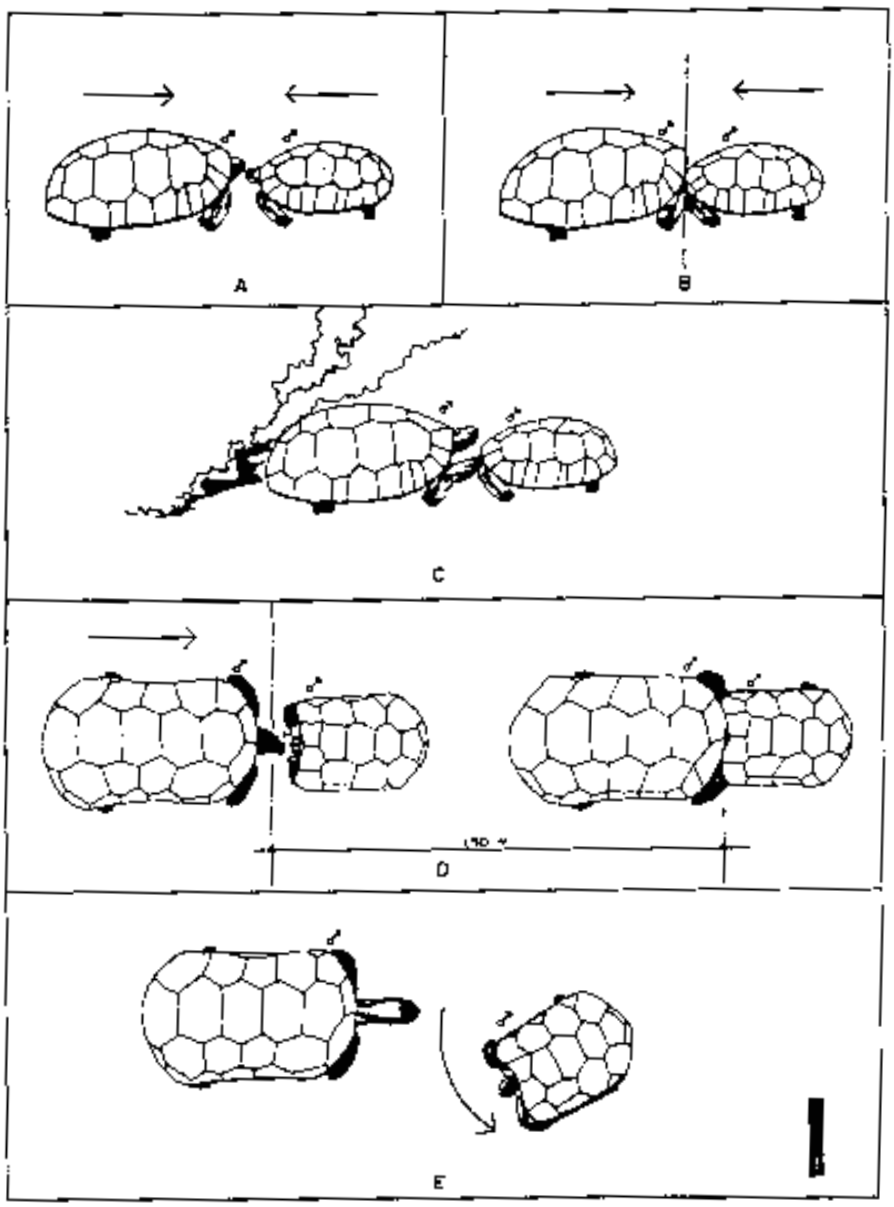

Figure 4.- Sequence of agonistic behavior (combat) between adult males of *Chelonoidis carbonaria* according to Guix *et al.* (1989). Individuals: **A.** strike each other frontally with the shell; **B.** push and **C.** bite each other; **D.** One individual displaces the other by pushing it; **E.** The displaced individual turns round and leave the combat area. Pseudohermaphroditic females sometimes participate in events **A**, **B** and **E** with males.
Figura 4.- Secuencia de comportamiento agonístico (combate) entre machos adultos de *Chelonoidis carbonaria* de acuerdo con Guix *et al.* (1989). Los individuos: **A.** Se golpean frontalmente con el caparazón; **B.** Se empujan; **C.** Se muerden; **D.** Uno de los individuos desplaza el otro empujándolo; **E.** El individuo desplazado se gira y abandona el área de combate. Las hembras pseudohermafrodíticas por veces participan en los eventos de combate **A**, **B** y **E** contra machos.

Table 1: Abnormal morphological, behavioral and reproductive traits in 16 adult females of *Chelonoidis carbonaria* found in the Zoo of São Paulo, southeastern Brazil. - = not detected.
Tabla 1: Rasgos morfológicos, comportamentales y reproductivos en 16 hembras adultas de *Chelonoidis carbonaria* del Zoológico de São Paulo, sureste de Brasil. - = no detectado.

Code	Pseudopenis (length: mm)	Vocalization like male	Egg retention
1	yes (42)	yes	yes
2	yes (38)	-	yes
3	yes (26)	-	yes
4	yes (17)	-	-
5	yes (50)	yes	yes
6	yes (21)	yes	yes
7	yes (19)	-	-
8	yes (25)	yes	-
9	yes (24)	-	-
10	yes (25)	-	-
11	yes (15)	-	-
12	yes (9)	-	-
13	yes (30)	-	yes
14	yes (55)	yes	yes
15	yes (25)	yes	-
16	yes (20)	-	-

(GUIBÉ, 1970). Nevertheless, in some abnormal females of *C. carbonaria* living in zoos of southern and southeastern Brazil (which we call *pseudohermaphrodites*), had well developed and differentiated pseudopenis.

Although endocrine parameters among turtles are considered to be highly variable (see LICHT, 1982), ten of the 28 individuals in which plasma was analysed, could be considered as hormonistically imbalanced. The results of these hormonal analysis should not be considered as conclusive, but suggest that hormonal imbalances could be related to some of the abnormal traits observed in females previously categorisized as pseudohermaphrodites.

The natural distribution of the species is closely coincident with the distribution of tropical type of climate in South America (compare Figures 1 and 2). Moreover, all the captive individuals with abnormal traits related with the reproduction were found in Zoos located in Brazilian regions domined by subtropical type of climates. In South America, the tropical climate is characterized by high mean temperatures (mean temperatures of the cooler months are always above 18^0 C), and the absence of frost. The South American subtropical climate has lower mean temperatures in relation to the tropical ones (the climate of this region is highly influenced by the cold air masses that are originated in the South Pole) and frost can occur during the austral winter (NIMER, 1979; MOPU, 1990).

Most of the individuals found in captivity south of the Tropic of Capricorn were collected in northern and northeastern Brazil, during the hatching period, transported to South and Southeastern Brazil and donated to zoos as juveniles. Two possible explanations for the origin of these abnormalities are considered: a- they were generated in natural habitats by low or high temperatures (including high variations in the temperatures) during embryo de-

Table 2: Values of testosterone and estradiol in the plasma of 28 adult individuals of *Chelonoidis carbonaria* living in the Zoo of São Paulo, southeastern Brazil. (—) = not measured. * females with abnormal traits (see numbers in table 1).
Tabla 2: Valores de testosterona y estradiol en el plasma de 28 individuos adultos de *Chelonoidis carbonaria* del Zoológico de São Paulo, sureste de Brasil. (—) = no medido. * hembras con rasgos anormales (véase numeración en la tabla 1).

Sex	Code (if abnormal)	Testosterone (ng/dl)	Estradiol (pg/ml)
MALES		464	36
		486	382
		(—)	277
		450	44
		281	542
		(—)	388
		332	344
		27	<4.0
		231	9.3
		458	24
		424	64
		(—)	121
		(—)	537
FEMALES	5*	<10	357
		<10	138
		13	<4.0
		<10	89
		<10	187
		250	23
		<10	142
		<10	30
		17	129
		<10	140
	12*	30	320
	13*	35	344
	6*	77	910
	14*	145	954
	2*	72	296

velopment or temperatures of egg incubation close to the pivotal thermal limit (see PIEAU, 1975; PIEAU *et al.*, 1998, 1999); b- they were produced by endocrine imbalance, induced by the lower mean temperatures to which most individuals were exposed during post natal development in southern latitudes.

During the turtle embryonic growth, in general cooler temperatures induce the gonadal differentiation of individuals in males and warmer temperatures in females (see PIEAU *et al.*, 1998; WIBBELS *et al.*, 1998). If hatchlings and juveniles *C. carbonaria* are thermal sensitive (and not only the embryos), cooler temperatures may

have accentuated male secondary sex characteristics in prior morphological females.

Considering the explanation "b" the high differences in the morphological abnormalities (and, in some cases, in the hormonal levels of testosterone and estradiol) found in the captive individuals located in South and Southeastern Brazil may be both the result of the time of exposures to alien climate conditions of these regions and the gradient of low temperatures in the latitude interval.

Differences between clitoris and pseudopenis are not clear in adult females of *C. carbonaria* living in zoos south of the Tropic of Capricorn, because these structures seem to be extremes of a morphological gradient. Nevertheless, without examination of a larger number of females living in areas located north of the Tropic of Capricorn ("control" females), it is difficult to classify a morphological trait as normal or abnormal (C. PIEAU, pers. comm.).

The populations of *Chelonoidis carbonaria* have experienced a high decline in natural areas, especially those located in northeastern Brazil (J.C. GUIX, pers. obs.). Collecting of adults for meat or juveniles for pet trade is a common practice in northeastern and north Brazil. Reproductive programs developed in captivity with *C. carbonaria* and other Testudine species should be priorized in areas located inside its natural distribution. Morever, it is suggested that areas located far away its natural distribution (especially those domined by lower mean temperatures) should be discarted for reproductive programs.

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