Pond Turtles Deseases Monitoring Project: epidemiology of syntopic populations of *Emys orbicularis* and *Trachemys scripta* in the Po Plane

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Summary
During first five years of our Pond Turtle Disease Monitoring Project groups of *Trachemys scripta elegans* introduced in artificial and natural ponds in the Po Plane (North Italy) were examined for an epidemiological survey of infectious agents. From 2001 onwards we began a survey of some syntopic populations with *Emys orbicularis*. All analyses were done at the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna in Brescia. Observation, isolation and identification of micro-organisms have been accomplished using standard procedures. We present results of the analysis of 312 samples collected from *T.s.elegans* and 100 from *E.orbicularis*. 86% of *T.s.e.* and 96% of *E.o.* had positive results for bacteriological analysis; 48,1% of these samples for *T.s.e.* and 68,7% for *E.o.* were recognized as specific Chelonian infectious agents (as *Aeromonas hydrophila*, *Citrobacter freundii*, *Pseudomonas* spp. and *Edwarsiella tarda*). Most of other recognized microbic organism live in waters and ground normally and are patogenous opportunists that penetrate carapax micro lesions.

Key words: *Emys orbicularis*, *Trachemys scripta*, epidemiology, north Italy.

Introduction
The casual release of thousands of individuals of *Trachemys scripta elegans* in all kinds of natural and artificial waters since 1970 has created the largest known phenomenon of species introduction involving a reptile. Almost all countries around the Mediterranean Rim, Saudi Arabia, South-East Asia, South Africa and North America have been affected by this colonisation, which in certain cases has been followed by a process of acclimatisation and breeding success. Hence widespread concern has arisen over the possible negative impact on autochthonous fauna and in particular on other native chelonian species. It has been shown that, in places where individuals from the colonising species are constantly being released, important concentrations may be created which can compete negatively with pre-existing concentrations of native pond turtles, especially when the syntopic species are already at risk from threats of human origin (as is the case in Europe with *Emys orbicularis* and *Mauremys leprosa*). In Italy, in just over 15 years the growing trade in *Trachemys* spp. – together with the resulting phenomena of animals being released into the wild once their owners grow tired of their pets – has made these allochthonous pond turtles far more common and better known than the European pond turtle *Emys orbicularis* (*E.o.*). The latter species is either extinct or on the brink of extinction throughout Northern Italy, as a result of a number of causes derived from the now irreversible alteration of the wetland areas of the Po Valley and Venetian Plain; its decline in regions such as Piedmont, Liguria, Lombardy and parts of the Veneto began well before the arrival of *Trachemys scripta elegans* (*T.s.e.*) (Ferri, 1995; Semenzato *et al*., 1998). Today, as a number of authorities plan reintroduction or population growth schemes for *E.o.*, especially in protected areas along northern rivers (Ferri, 1999, 2000, 2002a, b), there is a increasing need for studies on the impact of allochthonous on the vitality and survival capabilities of the surviving populations.

Since 1994, as part of the ARCADIA/*Trachemys* Project (Ferri *et al*., 1999a), data have been collected on inter-specific interaction between *T.s.e.* and *E.o.*. Nine years of counts and observations have confirmed the high numbers and widespread distribution of the allochthonous species as compared to the rare and highly local *Emys orbicularis*, as also the low or non-existent level of ecological interaction with autochthonous fauna in the areas of release (Agosta & Parolini, 1999; Bruekers & van der Keijlen, 1999; Parolini, 2002; Soccini, 2002; Soccini & Ferri, 2003). Since 1997, in cooperation with the Brescia laboratories of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZS) in Brescia, a scheme is under way to monitor the state of *T.s.e.* populations in artificial and natural waters, approved by the Conservation Committee of the *Societas Herpetologica Italica* (*S.H.I.*) late in 1998 (Ferri *et al*., 1999b; Ferri & Soccini, 2001). The results of activity are presented here.

Materials and Methods

Objectives

The "Pond Turtles Diseases Monitoring Project" was launched with the following objectives:

- to perform a nation-wide census of the main introduced populations (above 10 individuals) of *T.s.e*. using and distributing a special report form;
- to highlight possible instances of negative interaction linked to the presence of *T.s.e.* in potential habitats for *E.o.*, intensifying and promoting ecological studies at localities ascertained as syntopic for the two species;
- to set up a data base on the diseases and involved micro-organisms known to occur in Italian Chelonians (through consultation of all available scientific publications);
- to identify the causes of the various diseases occurring in large percentages in individuals of *T.s.e.* found in the wild;
- to develop a standardised diagnostic procedure for recognition of the various pathogenic micro-organisms;
- to draw up an operational protocol aimed at identification, isolation and registration of the various viral, bacterial and parasitic pathogens, enabling optimum monitoring of the health of pond turtles.
- The last three objectives form the basis of the cooperation with the IZS in Brescia (Nieddu *et al*., 1999).

Study areas

In the first phase (1999-2000) health monitoring was carried out on groups of *Trachemys scripta elegans* released in ponds in urban and suburban areas in the provinces of Milan (6 localities) and Brescia (2 localities). Subsequently (since 2001) syntopic sites for *E.o.* and *T.s.e.* were ascertained, together with other sites where only the European pond turtle is present. A list of these localities and of estimated numbers of turtles present, counted *in situ* during 2002, is given in table 1 (Ferri, 2002; Soccini, 2002). As regards *E.o*., individuals from the Cameri breeding centre were also monitored.

Laboratory tests

The procedures and techniques used for observation, isolation and identification of micro-organisms in the various samples studied were those commonly used in routine practice in the Departments of Diagnostics, Specialised Bacteriology, Microbiology and Electro-Microscopy at the IZS in Brescia (Nieddu, 2000). Bacteriological, virological and parasitological tests were performed.

Sampling methods
Samples for Diseases Monitoring were taken in accordance with the operational protocol worked out at the IZS in Brescia in concert with the Centro Studi Arcadia:-
appropriate and non-harmful techniques of capture, to be carried out with minimum handling and constraint of turtles, where possible in conditions of asepsis, so as to prevent any contamination;
- use of sterile swabs with culture medium for the various materials to be examined;
- immediate forwarding of diagnostic samples to the laboratory; in cases where this is impossible, samples to be preserved at +4°C awaiting shipment, which in all cases must take place as early as possible. The study was carried out on:
- samples of water taken from the fish-farm tanks or other waters in which the captured individuals lived;
- faecal samples from individuals living in captivity;
- cloacal swabs from animals captured or handed in by persons;
- carcasses of animals which died in natural conditions.

Results
The state of health of pond turtles
Before beginning discussion of the results from laboratory analyses, it is important to recapitulate the environmental situation at the sites selected for pond turtle disease monitoring. Most of these sites are characterised by serious degradation of water quality: water in advanced stage of eutrophication and very limited transparency, because of limited in/outflow and circulation and a direct or indirect link with polluted streams or rivers (f.e. LAGMON; FONBIN DUCOS1). Chemical analyses carried out during monitoring revealed the presence of high levels of nitrates, phosphates and "Fe". State of water at the other sites is comparatively fair, thanks to constant in/outflow or the presence of artificial sources (f.e. LAGARC, FONNOV, DUCOS2, LAGBS), broad and deep expanses of water (CAVFA) or rich aquatic vegetation (ARETOR, VILSOR).

Action taken
T.s.e.: 312 cloacal swabs and 15 carcasses examined in Sites without presence of *E.o.*; 37 cloacal swabs in Sites with sintopic population of *E.o.* (Tabs 2, 4, 5)
E.o.: 64 cloacal swabs in a breeding centre; 36 cloacal swabs in Sites with sintopic population of *T.s.e.* (Tabs 2, 6, 7). Autopsies on 15 carcasses of *T.s.e.* which had died in natural conditions highlighted malnutrition in all cases, with no food present in either the stomach or the whole of the intestinal tract and a more or less general state of anaemia, very probably due to such deficiencies. Anatomical-pathological examinations revealed a high incidence of pulmonary lesions (N=7), superior to both enteric (N=4) and hepatic (N=4). 50% of the carcasses showed *Septicemic Cutaneous Ulcerative Disease* (SCUD) lesions.
The results of the bacteriological tests performed are given in table 3, while tables 4 and 5 show the typified microbial species of greater interest and their frequency of isolation.

Discussion
Although the virological and parasitological examinations always gave negative results, the bacteriological tests yielded interesting data which attest to the validity of the Project and provide good reason for it to be continued.
There is a high percentage of positive samples (N=398 88.64%) as compared to the total of samples examined (N=449), and in a number of cases micro-organisms were isolated which are of interest given their role as specific pathogens for Chelonians. A number of authors have identified *Citrobacter freundii* (5.73% positivity, 20 samples out

of 349 in *T.s.e.)* as the principal micro-organism involved in SCUD: in animals affected by the disease. Secondary infection bacteria of the genus *Serratia* (0.85% positivity, 3 s.o./349 in *T.s.e*., 1.00% positivity, 1 samples out of 100 in *E.o.*), become established on pre-existing traumatic lesions and by their lytic action permit the entry of *C.freundii* at deep levels, inducing symptomatic patterns characterised by crateral ulcers in the necrotic tissue, hepatic necrosis and, in certain cases where the lesions invade the deeper layers of the shell, the death of the animal through septicemia (Frye, 1977). *Aeromonas hydrophila* (14.89% positivity, 52 s.o./349 in *T.s.e*. and 37.00% positivity, 37 s.o./100 in *E.o.*) also appears to be responsible for numerous deaths linked to phenomena of anorexia and septicemia (MAS *Motile Aeromonas Septicemia*) in pond turtles (Reichenbach *et al*. 1990). *Aeromonas* infections are caused by bacteria wich are present in the water all of the time; usually when pond turtles get sick with an *Aeromonas* infection, something has happened to make them susceptible to bacterial invasion. Common causes are poor water quality and overcrowding. Frequently elimination of the underlying stress factor may be sufficient to resolve the disease outbreak.
The pathogenic role of *Pseudomonas* spp. (0.57% positivity, 2 s.o./349 in *T.s.e*. and 2.00% positivity, 2 s.o./100 in *E.o.*) remains uncertain, although it does appear to create problems for animals subject to thermal stress; the most valid hypothesis, in the opinion of several authors, is that this bacteria provokes anorexia and blepharo-conjunctivitis (lesions noted in 4 *T.s.e*, from DUCOS1 site), (Holt *et al*. 1984). Another micro-organism responsible for latent intestinal infections is *Edwardsiella tarda* (0.57% positivity, 2 s.o/349 in *T.s.e.*), which occurs as an opportunist pathogen in various reptiles (Quinn *et al*., 1994).
So far as the other bacterial species highlighted are concerned (Tabs 4-7), since they are commonly present in the gastro-enteric apparatus as commensals the extent of their involvement as agents of infection must remain open to question. Furthermore, in some samples bacterial examination revealed the presence of micro-organisms of interest in terms of their potential impact on public health, at least in severely debilitated individuals: *Aeromonas*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Proteus* e *Serratia, Salmonella* spp..
In reality it is still a matter of debate whether such pond turtles represent a genuine public health risk or should instead be considered as signal fauna in relation to the state of degradation of urban and suburban waters.It is not by coincidence that the highest percentage of positivity (to the typified microbial forms) was found in samples from individuals of *T.s.e*. collected at the sites where the water is in the most seriously degraded state (f.e. LAGMON, FONBIN and DUCOS1), which are also the sites with the highest number of dead or dying individuals.
There is also a significant relation between the origin of the animals and the presence of pathological alteration which may be ascribed to SCUD: observations carried out over the nine years of management of the collection points of the ARCADIA/*Trachemys* Project and in the various localities investigated show that only 7% of turtles are affected at the time they are released; this percentage rises to 47% at sites in the province of Milan where environmental conditions are best and to 97% where they are worst, while the percentage is very low at sites in the province of Brescia (2%) and falls virtually to zero in the province of Modena.
The results also highlight the difficulties faced by exotic turtles in attempting to adapt after their release into the wild, and their low rate of survival. Animals which are used to living in captivity in aqua-terrariums, fed by their owners on a daily diet composed entirely of dried crustaceans, find themselves left to their own devices in highly stressful environments with little or no food supply. In recaptured individuals of *T.s.e.* (N= 68), 66.1% showed obvious signs of weight loss, 11.7% appeared stable and only 22% had apparently gained in weight.

One surprising and puzzling aspect (without dragging pond bottoms, the disappearance of individuals cannot be explained with any certainty) was the fall in numbers of *T.s.e.* in the studied sites after the winter season: almost 50% of individuals in artificial and urban sites in Milan (LAGPAL) and Brescia (DUCOS1), but also high (10-20%) in semi-natural sites (FONBIN, FONARC).
The phenomenon of casual release of *Trachemys scripta elegans* has slowed only slightly over the past five years, partly as a result of the provisions of EU Regulation No. 2551 of 15.12.1997, but commercial prospects are opening up for other subspecies (*T.s.troosti* and *T.s.scripta*) and other genera (*Pseudemys* and *Graptemys*). Only a definitive ban on trade of all chelonians will bring a rapid solution to the problems created by the presence of allochthonous turtles in Italy and Europe.

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Tab. 1. Localities studied and estimated numbers of *Trachemys scripta elegans* (*T.s.e.*) and *Emys orbicularis* (*E.o.)* present at each site with year of first report of *T.s.e*.

site code	Locality	type of wetland	Town or city and Province	#. *E.o.*	#. *T.s.e*	year of first report of *T.s.e.*
LAGBS	Bosco in Città (Public Gardens)	regenerated artificial lake	Milan MI	0	approx. 75	1992
CAVFA	disused "Cabassi" sand pits in Via delle Forze Armate	regenerated artificial lake	Milan MI	0	approx. 150	1992
LAGMON	Villa Reale in the Gardens of the Monza Public Park	artificial urban ponds	Monza MI	0	approx. 150	1989
FONBIN	Fontanile dei Frati, Binasco, MI	former phreatic spring	Binasco MI	0	approx. 70	1984
FONARC	Fontanile Laghetto del Parco Arcadia (pond in public park)	former phreatic spring	Bareggio MI	0	approx. 150	1992
FONNOV	Fontanile Nuovo di Bareggio	phreatic spring	Bareggio MI	0	<10	1992
DUCOS1	Ducos Urban Park	artificial urban pond	Brescia BS	0	approx. 100	1985
DUCOS2	Ducos/San Polo Urban Park	artificial urban pond	Brescia BS	0	approx. 120	1992
ARETOR	Torrazzuolo di Nonantola ecological regeneration area	regenerated irrigated basin	Nonantola MO	approx. 50	0	/
VILSOR	Villa Sorra Gardens	regenerated artificial lakes	Castelfranco Emilia MO	approx. 150	approx. 250	1990

Tab. 2. Samples used for laboratory test at IZS in Brescia (1999-2000). (1) Sites only with *T.s.e.*; (2) Site with syntopic population of *E.o.* and *T.s.e.*.

Site code for place of origin of sample	Carcasses		Cloacal swabs	
	T.s.e.	*E.o.*	*T.s.e.*	*E.o.*
LAGBC (1)	/	/	12	/
CAVFA (1)	/	/	18	/
LAGMON (1)	4	/	14	/
FONBIN (1)	6	/	9	/
FONARC (1)	3	/	30	/
DUCOS1 (1)	2	/	49	/
DUCOS2 (1)	/	/	137	/
VILSOR (2)	/	/	37	36
from captivity	/	/	43	/
from Breeding centre	/	/	/	64
total	15	/	349	100

Tab. 3. Data on samples tested (Ferri & Soccini, 2001; Nieddu, 2000; Cadeo, 2003). "Various Microbial Flora" is used here to mean: *Citrobacter braakii, Klebsiella* spp., *Morganella* spp., *Proteus* spp. etc.

material examined	total nr. of samples	negative samples		samples positive for Various Microbial Flora	typified microbial species of interest (nr. of isolations)
Trachemys scripta elegans					
Cloacal swabs	349	46	13.18%	57	266
Carcasses	15	3	20%	4	8
Emys orbicularis					
Cloacal swabs	100	4	4%	11	91

Tab. 4. Typification results for microbial species of interest regarding *T.s.e.* in no-syntopic Sites with *E.o.* (Ferri & Soccini, 2001; Nieddu, 2000; Cadeo, 2003). Total of samples tested: *cloacal swabs* N=312, *carcasses* N=15.

Bacterial strain isolated	Source of isolation	nr. of isolations	% out of total # of samples
Aeromonas spp.	cloacal swab	12	4.48
Aeromonas caviae	cloacal swab	2	0.75
Aeromonas sobria	cloacal swab	62	23.13
	intestine (15)	3	20.00
Aeromonas hydrophila	cloacal swab	31	11.57
Edwarsiella tarda	cloacal swab	2	0.75
Salmonella thompson	cloacal swab	1	0.37
Salmonella typhimurium	cloacal swab	1	0.37
Salmonella arizonae	cloacal swab	3	1.12
Steptococcus spp.	cloacal swab	2	0.75
Pseudomonas diminuita	cloacal swab	2	0.75
Pseudomonas spp.	liver (15)	1	6.67
	lung (15)	1	6.67
Providencia stuardi	cloacal swab	13	4.85
Escherichia coli	cloacal swab	50	18.66
	skin (9)	1	11.11
	intestine/faeces (15)	4	26.67
	liver (15)	1	6.67
Serratia odorifera	cloacal swab	1	0.37
Serratia liquefaciens	cloacal swab	2	0.75
	uterus (3)	1	33.33
Citrobacter freundii	cloacal swab	20	7.46
Pasteurella haemolitica	lung (15)	1	6.67
Yersinia kristensenii	cloacal swab	1	0.37
Hafnia alvei	cloacal swab	3	1.12
	intestine (15)	1	6.67
Clostridium perfrigens	cloacal swab	4	1.49
Enterobacter agglomerans	cloacal swab	4	1.49
Enterobacter cloacae	cloacal swab	2	0.75

Tab. 5. Typification results for microbial species of interest regarding *T.s.e.* in syntopic sites with *E.o.* (Ferri & Soccini, 2001; Nieddu, 2000; Cadeo, 2003). Total of samples tested: *cloacal swabs* N= 37.

Bacterial strain isolated	Source of isolation	nr. of isolations	% out of total # of samples
Aeromonas sobria	cloacal swab	25	45.45
Aeromonas hydrophila	cloacal swab	21	38.18
Escherichia coli	cloacal swab	2	3.64

Tab. 6. Typification results for microbial species of interest regarding *E.o.* in no-syntopic sites with *T.s.e.* (Ferri & Soccini, 2001; Nieddu, 2000; Cadeo, 2003). Total of samples tested: *cloacal swabs* N=64.

Bacterial strain isolated	Source of isolation	nr. of isolations	% out of total # of samples
Aeromonas sobria	cloacal swab	12	19.35
Aeromonas hydrophila	cloacal swab	32	51.61
Pseudomonas diminuta	cloacal swab	2	3.23
Escherichia coli	cloacal swab	13	20.97
Enterobacter spp.	cloacal swab	1	1.61

Tab. 7. Typification results for microbial species of interest regarding *E.o.* in syntopic sites with *T.s.e.* (Ferri & Soccini, 2001; Nieddu, 2000; Cadeo, 2003). Total of samples tested: *cloacal swabs* N=36.

Bacterial strain isolated	Source of isolation	nr. of isolations	% out of total # of samples
Aeromonas sobria	cloacal swab	19	47.50
Aeromonas hydrophila	cloacal swab	5	12.50
Escherichia coli	cloacal swab	5	12.50
Enterobacter agglomerans	cloacal swab	1	2.50
Serratia liquefascens	cloacal swab	1	2.50