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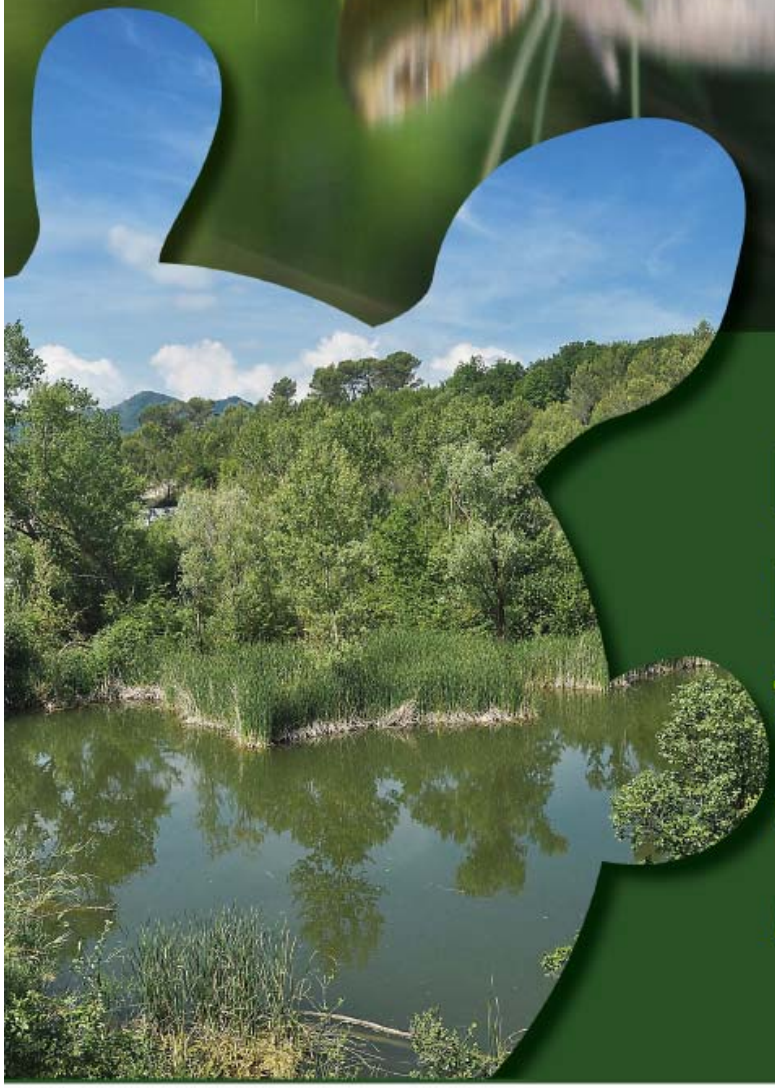
NATURA 2000

Integrated Management Plan for the conservation of *Emys orbicularis*



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LIFE URCA PRO*EMYS*

URgent **C**onservation **A**ctions **pro** ***Emys orbicularis***
in Italy and Slovenia

Work Package 2

INTEGRATED MANAGEMENT PLAN FOR THE CONSERVATION OF *EMYS ORBICULARIS*

2023

Coordinatore beneficiario

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INTEGRATED MANAGEMENT PLAN FOR THE CONSERVATION OF *EMYS ORBICULARIS*

This draft of the integrated management plan will be updated following the monitoring of its implementation during the project period

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In memory of Prof. Luigi Sala who participated with passion in the construction of LIFE URCA PRO*EMYS* but who left us too soon

Summary

THE OPINION OF ISPRA (Istituto Superiore per la Protezione e la Ricerca Ambientale) [Italian Superior Institute for Environmental Protection and Research]

In a note dated 3 June 2024, Protocol No. 0030942/2024, ISPRA expressed its favourable opinion on this Integrated Management Plan, the contents of which *"will be a useful reference for the assessments falling within ISPRA's role, and in particular for the expression of the technical opinions required by the regulatory framework".*

THE OPINION OF SLOVENIAN MINISTRY of Natural Resources and Spatial Planning

In a note dated 20 December 2024, Number: 5440-3/2022-2550-34, the Ministry *“welcomes the prepared Integrated management plan for the conservation of Emys orbicularis” and “find the plan’s content useful for implementation of Slovenian Natura 2000 Management Programme for the period 2023-2028 (PUN) regarding Emys orbicularis.” Ministry stated that as “With the implementation of the plan within the scope of the project LIFE URCA PROEMYS, the feasibility of concrete measures will be tested also in Slovenia” that “can contribute to the implementation of PUN measures for Emys orbicularis and relevant habitat types not only in Natura 2000 sites, included in the project LIFE URCA PROEMYS, but also in other Natura 2000 sites with Emys orbicularis as a qualification species”*

INTRODUCTION

The project LIFE21-NAT-IT-LIFE URCA PRO*EMYS* - **UR**gent **C**onservation **A**ctions pro *Emys orbicularis* in Italy and Slovenia (101074714/LIFE21-NAT-IT-LIFE URCA PRO*EMYS*), has been approved on 17 May 2022 within the *Programme for Environment and Climate Action* (LIFE), Call: LIFE-2021-SAP-NAT.

The main objective of LIFE21-NAT-IT-LIFE URCA PROEMYS is to improve the conservation status of *Emys orbicularis* in Italy and Slovenia, maintaining the genetic diversity of existing populations. The specific objectives are:

1. To develop an Integrated Management Plan for the protection of the Italian and Slovenian populations of native turtles, including veterinary protocols. The implementation of this task is in accordance with EU Regulation 1143/2014, the Italian Legislative Decree No. 230/2014 and the Decree of the President of the Italian Council of Ministers, the Italian Legislative Decree No. 230/2017, and the guidelines of the Italian Ministry of the Environment and Energy Security, the Italian Institute for Environmental Protection and Research (ISPRA) and the Italian Herpetological Society (SHI). The Plan will be adopted by all Natura 2000 sites involved in the project.
2. In Slovenia, the Integrated Management Plan will be implemented in cooperation with the Slovene Herpetological Society (SHS), the Centre for Cartography of Fauna and Flora (CKFF), and the Institute of the Republic of Slovenia for Nature Conservation (ZRSVN).
3. To promote a transboundary management model for *Emys orbicularis* based on the Management Plan mentioned above.
4. To improve the condition of natural habitats of *Emys orbicularis* in at least 29 selected sites.
5. To adapt and/or set up seven breeding and reproduction centres for *Emys orbicularis*.
6. To repopulate and/or reintroduce *Emys orbicularis* in at least 13 selected areas;
7. To reduce (≥ 80% of the population) and/or eradicate invasive alien turtles in at least 39 Italian and three Slovenian sites and enhance the network of alien turtle management facilities in Italy.
8. To define a long-term monitoring plan for *Emys orbicularis* populations.

9. To evaluate the implementation of the Integrated Management Plan on a regional scale in a pilot region.
10. To raise public awareness on the conservation of *Emys orbicularis* and the threat posed by the introduction of invasive species into natural habitats.

THE INTEGRATED MANAGEMENT PLAN FOR THE CONSERVATION OF *EMYS ORBICULARIS*

The lack of coordination at a national - as well as cross-border - level between the public and private parties involved in the protection of *Emys orbicularis* is one of the main issues relating to the conservation of this species. One of the objectives of LIFE is the drafting and adoption by the bodies having jurisdiction of this Integrated Management Plan for the conservation of *Emys orbicularis* which will serve to establish the principles and actions to be implemented to safeguard the species at national level and in a shared manner. The Plan is implemented by involving the Italian Institute for Environmental Protection and Research (ISPRA), the Italian Ministry of the Environment and Energy Security (MASE), the Institute of the Republic of Slovenia for Nature Conservation (ZRSVN), experts, operators, and other interested parties. The Plan includes veterinary, management and diagnostic procedures to cover all aspects of animal health and welfare, public health protection, zoonotic pathogens (EU One Health Approach). The veterinary protocol contains procedures for the management of *E. orbicularis* specimens reproduced *ex situ* and intended for release at restocking sites and *Trachemys* spp. specimens (or other non-native species) captured at eradication sites and intended for collection centres.

The protocol includes:

• Animal management, handling, and transport procedures for *E. orbicularis* and *Trachemys* spp. (or other allochthonous species).

• Procedures for marking and application of identification microchips for *E. orbicularis* and *Trachemys* spp. (or other allochthonous species).

• Veterinary monitoring and clinical testing procedures for *E. orbicularis* and *Trachemys* spp. (or other allochthonous species).

• Action procedures in the presence of pathogens detected on *E. orbicularis* and *Trachemys* spp. (or other allochthonous species).

The Plan also updates the *Disease Risk Analysis* (DRA), formulated during the LIFE EMYS project (LIFE12NAT/IT/000395) and already implemented in Liguria, and other restocking experiences in the LIFE context (LIFE09 NAT/ES/000529, LIFE14 IPE/IT/000018).

The implementation of the Plan will be monitored through an in-depth analysis for the entire duration of the project in the Emilia-Romagna region, which has been selected as a pilot region since it involves a large number of protected areas and partners in the LIFE project, including the Consorzi di Bonifica Emilia Centrale e della Burana, managers of infrastructures in a large portion of the Emilia-Romagna territory that will participate in the working tables. Furthermore, the implementation of the Plan in the Slovenian sites will make it possible to test its effectiveness in a legislative context other than the Italian context and will allow an exchange of technical best practices at the transnational coordination level on the conservation of this species.

SPECIES CARD

- ***Emys orbicularis*** (Linnaeus, 1758) – European Pond Turtle
- ***Emys trinacris*** Fritz, Fattizzo, Guicking, Tripepi, Pennisi, Lenk, Joger & Wink, 2005 – Sicilian Pond Turtle

DISTRIBUTION

Global Distribution. *E. orbicularis* is distributed from western North Africa (Morocco, Algeria, Tunisia) to southern and central Europe as far as Denmark, Poland and Lithuania; eastwards from Portugal to the Balkan Peninsula across Anatolia, Caspian Sea coasts (including northern Iran) to the Aral Sea (Sindaco & Jeremcenko, 2008; Sillero et al., 2014). *E. trinacris* is only present in Sicily (Ottonello et al., 2021a).

Italian Distribution. In Italy, the European pond turtle is present in most of the Po Valley (with greater continuity proceeding eastwards), in a fragmentary manner along the Adriatic side, from the Abruzzo to the Apulian coasts; along the Tyrrhenian coast, especially along the Tuscan and Latium coasts; and along the Ionian coast. The northernmost Tyrrhenian population is located in the Province of Massa Carrara, but there are doubts as to its origin, while the population of the Piana di Albenga (Savona) is very isolated. It is absent on the Tyrrhenian side of Campania and Calabria. Reported in Sardinia, along the northern and eastern parts of the island, absent in the south-western and western parts, and in Sicily, especially in the central-western portion.

Slovenian distribution. *Emys orbicularis* is present in varying densities throughout Slovenia, with the exception of the mountainous region. Larger populations have been found at Ljubljansko barje, on the Slovene coast, in Bela Krajina and the Sava Basin (Krofel et al., 2009, Vamberger & Kos, 2011).

Comment on the distribution map. The evident distributional gaps in many Italian regions are probably due to the scarcity of suitable habitats, while in Sardinia, Campania and Calabria to a lack of research; in Sicily it is absent in the north-eastern sector of the island (Peloritani Mountains, a large part of the Madonie Mountains, and the Termini Imerese Mountains).

There has been an improvement in reports compared to the 2006 Atlas, especially for Tuscany, Sardinia, Abruzzo and Molise regions.

Italian range limits. No evidence appears in the Italian range limits.

Disjunct populations.

Insular distribution. The two species are only present in Sardinia (*E. orbicularis*) and Sicily (*E. trinacris*) as far as the distribution on Italian islands is concerned.

Introduced populations. There are no known recently introduced populations in any part of the range.

ECOLOGY

The European pond turtle breeds in spring-summer, the female lays between 3 and 9 eggs (an average of 6; Zuffi et al., 1999), sometimes with double laying (Zuffi & Odetti, 1998), while in southern Italy pond turtles have a slightly smaller brood size (Zuffi et al., 2007); the eggs develop in about three months, with hatching between late August and October, and it is not uncommon for them to emerge from the nest the following spring. Sexual maturity is reached between 5 and 10 years of age. In Sicily, pond turtles have a slightly different brood size (around 4-5 eggs, from 2 to 8; Ottonello et al., 2021), but similar development and sexual maturity.

The trophic spectrum includes various aquatic vertebrates and invertebrates, but also aquatic vegetation (*E. orbicularis*: Ottonello et al., 2005), with a strong variation depending on the site and location (*E. trinacris*: Ottonello et al., 2017, 2021a, 2021b).

The Italian populations of *E. orbicularis* and Sicilian populations of *E. trinacris* are mainly found in two types of wetland habitats, ponds and canals (Lebboroni & Chelazzi, 1998), both in open environments and near or sometimes within wooded areas. Normally present in natural or semi-natural contexts, they also adapt to environments of artificial origin (reservoirs, reservoirs of exhausted quarries, etc.). They are also found in natural habitats at medium-high altitudes in southern Italy and Sicily characterised by the dominance of grassland or beech forests. Optimal environments are characterised by still and shallow waters with high helophytes concentrations, although in specific contexts they may be found in minor watercourses often with intermittent water flows, while, especially along the Veneto coast, they may also be found in brackish waters.

E. orbicularis is found from sea level up to over 1,500 m altitude, with a strong prevalence at low altitudes (0-200 m). The highest altitudes are only reached in the southernmost portion of the Italian range, while in the central-northern sectors it is confined to medium-low altitudes in lowland areas. *E. trinacris* is found from sea level up to 1,250 m altitude, with a clear

predominance in coastal and hilly lowland areas, except for the Nebrodi area where it is fairly common in the mountainous area.

The two species are active from mid- to end-February until November, with a greater frequency between March and mid- to end-July, with a significant decrease in the following months, especially in the warmest areas. Juveniles and sub-adults have a comparable period of activity, while newborn turtles are visible from mid-summer until the end of the active season.

In the coastal areas of Sicily, *E. trinacris* does not experience a prolonged winter dormancy period (Lo Valvo et al., 2008), while at higher altitudes the period of inactivity runs from mid-October to April (Lo Valvo et al., 2014).

This species tends to avoid humans and areas with human presence.

CONSERVATION

Frequency and population size. Regarding the known populations, the species' distribution varies greatly, with average distribution areas, while in other parts of the Italian range it is localised (e.g. Sardinia and Calabria). It is most easily observed in flat areas, while in hilly or mountainous areas it is more localised. Population densities are very variable, ranging from around 240-250 specimens/ha (Liuzzo et al., 2021; Ottonello et al., 2021a) to around 184-147 specimens/ha (Zuffi et al., 2020) up to 3-10 specimens/ha (Mazzotti et al., 2007; Seglie, 2015).

Known or perceived decline/increase of populations at regional level. In some areas monitored with some constancy [last 30 years, e.g. Bosco Mesola (Ravenna), Tenuta di San Rossore (Pisa), Parco regionale della Maremma (Grosseto)] the presence and densities are good and no significant declines have been observed. Overall, a picture of stability for the two species emerges, which however follows a period of decline caused by the destruction, alteration and fragmentation of wetlands and, not least, the introduction of allochthonous species. As long-lived species, the mere presence of adult specimens in isolated areas is no guarantee of a satisfactory conservation status.

A. TYPES OF INTERVENTION AND CONSERVATION MEASURES

HABITAT RESTORATION

The negative trend of *Emys orbicularis* in Europe is related to various threats, including the loss and/or transformation of suitable habitats (Ficheux et al., 2014); it is therefore essential to ensure the good status of habitats, through their protection and restoration, especially where 'core' populations persist, which can also be reinforced through restocking activities (Canessa et al., 2016).

From the experience of LIFE EMYS (LIFE12 NAT/IT/000395) and from references in specific literature (e.g. Ficetola et al., 2004; Ficheux et al., 2014; Canessa et al., 2016), an increase of *E. orbicularis*, varying, depending on the sites, between 5 % and 25 %, can be estimated following environmental restoration interventions.

Before addressing the concept of "*habitat restoration*", it is useful to recall some definitions, starting with that of "habitat".

A.1 HABITAT

Zoologists and botanists, when collecting specimens of living beings in order to classify them, used to indicate on the labels of the Museums where the specimens were kept, also the entry "habitat" ("it inhabits...", in Latin), i.e. a summary description of the characteristics of the places where the specimen was found (e.g. "habitat: coniferous forest"); by extension, the term has come to indicate the prevailing environment where the set of individuals of the various species dwells and can be collected... habitat is thus the set of biotic and physical factors that together make up the characteristics of the place where an animal lives (Malcevschi, 1991[1]).

The Habitats Directive, 43/92/EEC, takes up some of the original meaning and extends it, defining natural habitats as *"terrestrial or aquatic areas which are distinguished by their geographical, abiotic and biotic characteristics and which are entirely natural or semi-natural*".

The types of habitats included in the Directive basically refer to vegetation associations, which can generally be traced back to a more detailed physiognomic-structural or phytosociological

[1] Malcevschi S., 1991 – *Qualità ed impatto ambientale. Teoria e strumenti della valutazione d'impatto*. Etas libri

nomenclature. The Directive recognises natural habitats of Community interest, listed in Annex 1, which:

i) Are in danger of disappearing in their natural range; or
ii) Have a reduced natural range as a result of their regression or because their area is inherently restricted; or
iii) Are outstanding examples of characteristics typical of one or more of the following five biogeographical regions: Alpine, Atlantic, Continental, Macaronesian, and Mediterranean.

Finally, the Directive then distinguishes the habitat of a species as the environment defined by specific abiotic and biotic factors in which the species lives during one of the phases of its biological cycle.

A.2 HABITATS of *Emys orbicularis*

Italian and Slovenian populations are mainly found in two main types of wetland habitats: the first consists of ponds, pools, marshes, wetlands (e.g. Sacca del Bardello, Ravenna, Sečoveljske Soline, Draga pri Igu), with open reed beds (e.g. Valle delle Canne, Ravenna; Po River Delta) and with abundant aquatic vegetation. This habitat type consists of one or more natural lentic water bodies, either in open areas (e.g. Valle delle Canne, Ravenna) or in areas of mature woodland (e.g. Monte Rufeno, Pollino, Castel Porziano). The second habitat type can be traced to "canals" (e.g. Bosco Mesola, Camp Darby, Uccellina) (Lebboroni e Chelazzi, 1999), characterised by watercourses (Zuffi, 1987) and artificial drainage canals (Ljubljansko barje), generally in open areas or with riparian woodland and with still or weakly flowing water. It is also possible to find *Emys orbicularis* in man-made environments such as reservoirs, exhausted quarry basins (e.g. Alfonsine, Ravenna; the Valloni in Villanova d'Albenga), rubble, water springs, and rice-field areas. Especially in the two largest islands they may also be found in intermittent water streams. Outside water, specimens live in urban areas, cultivated fields, lowland and hygrophilous woods (e.g. Punte Alberete, Ravenna) and coastal pine forests (e.g. Pineta di Classe and Pineta di San Vitale, Ravenna)" (Sindaco et al., 2006[2]).

Below is the list of habitats of Community interest (Dir. 43/92/EEC) that are the subject of the habitat restoration interventions envisaged by Life URCA PROEMYS, which are a significant sample of the habitats of *Emys orbicularis* in Italy and Slovenia:

[2] Sindaco R., Doria G., Razzetti E. & Bernini E., 2006 - *Atlante degli Anfibi e dei Rettili d'Italia/Atlas of Italian Amphibians and Reptiles.* Societas Herpetologica Italica. Ed. Polistampa, Florence, page 792

- Mediterranean salt meadows (*Juncetalia maritimi*) (1410);
- Mediterranean and thermo-Atlantic halophilous scrubs (*Sarcocornetiea fruticosi*) (1420);
- Calcareous fens with Cladium mariscus and Caricion davallianae (7210);
- Pseudo-steppe with grasses and annuals of *Thero-Brachypodietea* (6220);
- Natural eutrophic lakes with *Magnopotamion* or *Hydrocharition* (3150);
- Mediterranean temporary ponds (3170);
- Water courses of plain to montane levels with the *Ranunculion fluitantis* and *Callitricho-Batrachion vegetation* (3260);
- Estuaries (1130);
- Coastal lagoons (1150);
- Wooded dunes with *Pinus* spp. (2270);
- *Tilio-Acerion* forests of slopes, screes and ravines (9180);
- Arborescent matorral with *Laurus nobilis* (5230);
- Petrifying springs with Tufa formation (7220);
- Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior* (91E0);
- Eastern white oak woods (91AA);
- Fixed coastal dunes with herbaceous vegetation (2130);
- Coastal dunes with *Juniperus* spp. (2250);
- Semi-natural dry grasslands and scrubland facies on calcareous substrates (6210);

A.3 HABITAT RESTORATION ACTIONS

Habitat restoration can be included in the broader concept of renaturation, *understood as the set of interventions and actions aimed at restoring the environmental characteristics and ecological functionality of an ecosystem in relation to its potential conditions, determined by its geographical location, climate, geological and geomorphological characteristics of the site and its past natural history*[3]**.** Renaturation can be pushed as far as restoring the pre-existing natural conditions of an area, as it can be carried out according to intermediate or specific objectives (e.g. restoration of flooding capacity; reduction of runoff velocity; recovery of self-purification capacity; preservation of particularly valuable species). Habitat restoration interventions are therefore renaturation actions that can be "targeted" at restoring

[3] In "Appendix. Definitions" Page 11 of: Basin Authority of the Po River. Parma – *Guidelines for the definition of interventions as per art. 36 of the PAI regulations. Technical and procedural guidelines for the design and assessment of renaturation interventions*. Attached to the resolution No. 8/2006 dated 5 April 2006

favourable conditions for specific biocenoses or particular species, such as *Emys orbicularis* in the case of this plan.

Among the main types of interventions, included in various LIFE projects (LIFE 12 NAT/IT/000395, LIFE 21 NAT IT- URCA PROEMYS, LIFE 14 IPE IT 018 - GESTIRE 2020), there are:

1. The creation of new water bodies;
2. The restoration, extension, and diversification of wetlands;
3. The creation of barriers (fences) to prevent intrusions by predators and ungulates;
4. Small auxiliary hydraulic works for water control aimed at guaranteeing the presence of appropriate water quantity.

Especially in the case of wetland construction and restoration works, a careful evaluation of the best areas and periods in which to carry out the works must be carried out, in order to interfere as little as possible with local biodiversity.

In the case of construction or restoration of ponds, please note the following:

- Newly built ponds outside existing wetlands:

- Preliminary verification, if the area is adjacent to ponds with populations of *E. orbicularis*, potentially suitable or already used for nesting;
- Verification that the worksite does not interfere with nesting areas of *E. orbicularis* if the areas are close to ponds with populations of *E. orbicularis*; in which case work must be carried out in April/May;
- Verification of the absence of interference with habitat types according to Dir. 92/43/EEC or species habitats;

- Restored ponds in existing wetlands with *E. orbicularis* present in the area:

- Deepening and remodelling work is to be carried out between August and September, preferably in August (limited impact on avifauna and herpetofauna);
- Excavations should be carried out gradually, with expert supervision to ensure that there are no animals in activity. Good practice may be, for example, to work first on one side and then move to the opposite side;
- It is advisable to put down any material removed, if not immediately put back in place to resurface the banks, for at least one night on site so that any animals (invertebrates, etc.) have a chance to go back to the water;

- Before constructing/restoring connections between ponds or with the surface network, check that no allochthonous/undesirable species (e.g. fish) are present and make sure that there are systems in place to protect the new environments against these species;
- In the case of any extension reduction of reed beds, aquatic and bank vegetation, etc., carry out activities in August/September, leaving areas covered by vegetation and only opening up clearings/channels between the vegetation.

Obviously, the periods indicated may vary depending on the latitude and altitude of the intervention site.

In general, **earth moving** may be carried out with mechanical means (e.g. excavators) and appropriate equipment depending on the sector regulations and authorisation procedures to be followed. **Thermal regulation points** (basking) can easily be created with natural materials (e.g. logs with large branches) but, in particular situations, also with artificial floating items. Special care must be paid to **water level management** in order to guarantee a suitable habitat for turtles in all seasons. It is advisable to **protect known nesting sites** with fences, permanent barriers or repellents to prevent nest predation or disturbance, especially due to earth movement activities by wild boars.

The main types of intervention are schematically illustrated below. A cost estimate has not been included because it varies too much from region to region and between Italy and Slovenia. It is advisable to consult regional price lists for costs.

A.3.1 CREATION OF NEW WATER BODIES

Purpose: To create and maintain new aquatic habitats suitable for the life of *Emys orbicularis*.

Other species concerned: Amphibians in general; other small autochthonous aquatic fauna.

Location: The location of a new water catchment unit must take into account the natural expansion possibilities of the species for which the intervention is planned. The European pond turtle is used to periodically moving along waterways departing from the population habitat area. This may lead to the colonisation of new sites or to a risk of no-return abandonment of the area of origin or loss of individuals due to the threats.

The flooded area, the way it is filled in, the depth, and the type of waterproofing are characteristics that depend on the different project contexts; in general, waterproofing sheets, or layers of clay or bentonite are used; in various situations it has been found that landfill sheets are more resistant (e.g. than clay) (Regional Park Gessi Bolognesi).

Notes of caution: It is important that the maximum depth of a new water catchment is reached by means of sloping banks and at least on the larger sides with very gentle slopes; that the perimeter is as jagged as possible and that the profile is asymmetric. In order to ensure a sufficient water supply to these reservoirs through surface water or rainfall runoff, a new reservoir must be built in a depression or at or near the base of pre-existing rainwater runoff, or a direct supply from an irrigation point, an artesian well, or collection tanks. F

The creation of a new water catchment, intended for the use and establishment of a vital pond turtle core, if appropriately set up and managed, is not incompatible with human presence (if controlled, marginal and marked at least with a regulatory sign posted in the area), but at least in the most critical periods, such as reproductive periods, it should be discouraged. Obviously, the establishment of a visual barrier on the visitors' walkway and a camouflaged observation point (like a birdwatching hut) can avoid any risk of impact on particularly sensitive chelonians. In the WWF protected area Stagni di Casale (Vicenza), excavations were carried out to create small wetlands - 1.2 to 15 m deep. The excavations were carried out with the intention of intercepting the (particularly shallow) water table and guaranteeing the stable presence of a water level connected to it. Periods of drought, however, have over time led to the emptying of the small reservoirs in the summer months, entailing both disadvantages (difficulties for the survival of hydrophytes) and advantages (the prolonged drought drives away alien species such as the Louisiana crayfish *Procambarus clarkii* and cancels out any colonisation of *Pseudorasbora parva*

and other alien fish). In any case, the protected area still has year-round flooded ponds nearby. So it is advisable to create two separate but connected bodies of water, one temporary and one permanent, to allow control of water levels but also the movement of alien species.

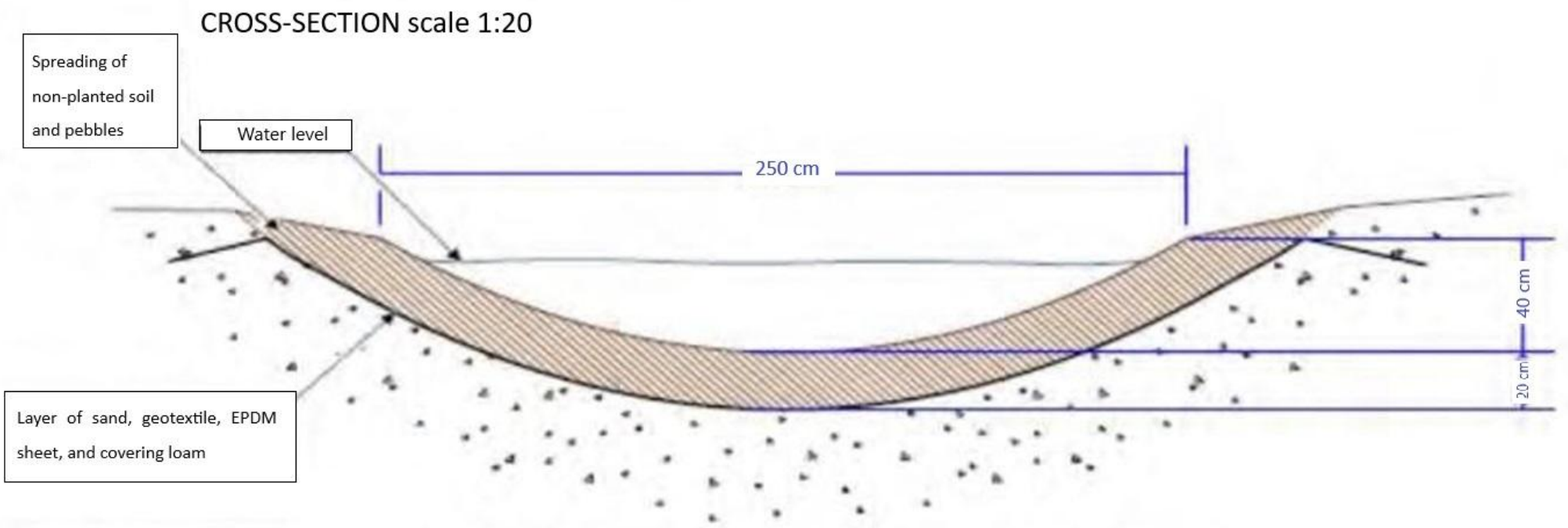
Maintenance: The lack of maintenance in the years following the creation would quickly render the new water collection almost useless. Sealing the bottom to ensure effective waterproofing is crucial.

Status indicators: The time for which water is maintained in the new pool is the main indicator for assessing the effectiveness of the intervention; for *Emys orbicularis* the permanence of water should be guaranteed all year round (for amphibians, for example, this condition is not mandatory). In the vast majority of the known Italian populations, overwintering takes place in water, on the bottom of the inhabited basin; breeding always in water in the spring months; feeding always in water in both juveniles and adults during the entire active phase, from March to October, with winter activities in Central-Southern Italy on warmer days.

Description of the action: The new water catchment should have a multifunctional use: for the needs of *Emys orbicularis* and other species including the freshwater invertebrate fauna (primarily Odonates, Hydroadephaga Coleoptera and native Gastropod Molluscs that are all undergoing a general collapse). In addition, with due care, the new water catchment can be included in an awareness-raising and nature education programme.

Operational steps: In general, this is the succession of activities required for the creation of a small water catchment (but proportionally - in terms of size - also applicable to a large body of water):

1. The excavation with a small bulldozer of the main bed for water catchment, taking care to set aside the most superficial layer of soil removed for the subsequent renaturation of the banks. Average excavation depth: 80-120 cm, maximum depth 150-200 cm; for an area 20-30 m long by 10-20 m wide;
2. The removal of all materials from the bottom and sides of the excavation that may have asperities that in the long term may cause damage to the geotextiles and the waterproofing panel placed on top; the removal and drastic cutting of roots that grow from trees and/or shrubs too close to the excavation (to avoid this, it would be best to locate the excavation at a distance of at least three to four metres from the trees and/or shrubs); roots that may extend over time and damage the waterproofing layer;
3. Pressing and thrashing of the bottom soil;
4. Spreading of a 20-30 cm layer of sandy loam over the entire excavation area;
5. Laying of a small-meshed galvanised net (approx. 2 cm per side) to prevent voles and moles from entering from the bottom;
6. Laying of geotextile sheeting over the entire surface, taking care that it is well arranged up to the limit of the excavated cavity;
7. Laying of EPDM waterproof sheeting (high-performance rubber sheeting with appropriate thickness and resistance for the project) to cover the entire surface; the part exceeding the excavated cavity will be buried in a specific perimeter furrow; large flat stones will be placed on top of this furrow, followed by soil and/or large clods from the previous excavation;
8. The arrangement of the banks in such a way that they and the water inside are accessible to small animals (1/3 steep bank and 2/3 bank with small natural steps or with gradual raising of the bottom). The maximum depth should be towards the shadier side (in the absence of vegetation in the vicinity - over 2 metres to prevent roots from damaging the waterproofing - a few shrubs will be planted);
9. The flooding of the catchment up to its maximum level;
10. The perimeter positioning of an anti-intrusion and safety barrier (such as a fence and in the case of the presence of wild boars also with galvanised mesh supported by stakes, with autoclave treatment, and partially buried, 10 cm side squares);



Overall diagram for the construction of a new pond.

11. Planting of herbaceous species around the banks suitable for the formation of a hygrophilous riparian vegetation belt (only sedges should be used, as cattail and reeds would be invasive and have a deep rooting capacity);
12. Annual environmental monitoring and activation of long-term monitoring;
13. The installation of a notice board with an information panel illustrating the species concerned and the purpose of the intervention;
14. Annual routine maintenance carried out with volunteer event-days.

Monitoring after implementation: Two after-work checks (at the end of March and the end of May of the first year) and then constant monitoring over the years to assess attendance, a count of the adults and the biological activity of the species.

Expected outcomes: Stable presence of a viable population of *Emys orbicularis*.

A.3.2 CREATION OF SEMI-NATURAL STRUCTURES AS TEMPORARY AND/OR PERMANENT SHELTERS FOR *Emys orbicularis* AND SMALL NATIVE FAUNA

Purpose: To create shelter points to reduce threats to reptiles and amphibians in the early stages of life. Useful shelters for summering and overwintering periods of breeding adults and as hiding places.

Notes: The presence of predators of *Emys orbicularis* obliges careful consideration of the location of these structures. Accessible locations are also recommended for monitoring. Anti-intrusion barriers can also be provided around the shelters against wild and domestic ungulates. The area must not be accessible to water (neither from rainfall nor from rising groundwater levels).

The photo sequence below shows the steps involved in constructing a temporary shelter structure.

Description:

Semi-buried shelter (temporary - overwintering): Excavation with a depth and width of 50 cm, length 100 cm. In succession, a large mass of wood shavings and dry leaves are placed in the excavation, all natural; then large pieces of cut branches, overlaid with others of lesser thickness and green faggots; the whole is then covered with other branches to create a mound. To be placed in strategic points for the fauna concerned.

Underground shelter (for overwintering): Excavation with a depth and width of 50 cm, length 100 cm, height of stones on the outside, at least 50 cm. In succession, pieces of large cut branches, large sharp-edged stones, green faggots are placed in the excavation. To be placed at strategic points for the fauna concerned.

Underground (fireproof) shelter: Excavation with a depth and width of 100 cm, length 150 cm, height of stones on the outside, at least 50 cm. In succession, large pieces of large cut branches are placed in the excavation, overlapping to leave wide space for animals to enter; then a layer of green bundles on which flat stones of various sizes are laid. To be placed in strategic points for the fauna concerned.

Maintenance: All these structures have a lifespan of about two years, after which they need partial or total reconstruction.

Monitoring: After construction, two after-work checks (at the end of March and at the end of October of the first year) and then monitoring to understand whether functionality is ensured and the urgency or otherwise of rehabilitation maintenance.

Expected results: To encourage the use by *Emys orbicularis* as a shelter.

A.3.3 CONSTRUCTION OF AUXILIARY HYDRAULIC WORKS FOR WATER CONTROL (HYDRAULIC ADJUSTMENT SYSTEMS)

Purpose: To maintain aquatic habitats suitable for *Emys orbicularis*.

Other species concerned: Amphibians in general; other small native freshwater fauna.

Notes: Control of water availability within semi-natural or artificial water catchments is often essential to maintain suitable habitats for aquatic fauna. In addition to adequate waterproofing, manual or mechanical manhole covers may be required to regulate water outflow. These structures must always be accessible for management operations and routine maintenance. It is important to assess the possible risk to other species (they may become trapped) and possibly make small arrangements to allow trapped fauna to escape.

Maintenance: It is necessary to ensure constant maintenance of the structures.

A.3.4 CASE STUDY: RESTORATION OF A FORMER MINING SITE AND CREATION OF A SUITABLE HABITAT FOR TURTLES IN SLOVENIA

Purpose: To lower the existing overhanging banks and reduce their slope. Remove any vegetation that prevents turtles from finding suitable places for basking.

Description and method of intervention: Widening and lowering the existing mine tailings banks with a small excavator, as well as reducing the slope towards the water and creating earth ramps to facilitate access. Excess vegetation was removed manually and by mechanical means.

Location of intervention: Rudnik-Korea 45.479046°N+ 13.619268°E

Outcomes: The embankments were lowered and the slope was reduced, and the turtles could access the water more easily. Existing soil from the area was also added to the embankments to allow the turtles to nest. The removal of overgrown vegetation has provided the turtles with more space for basking.

Monitoring after construction: Regular monthly monitoring of the site.

Contact person of the action: Iztok Škornik, 0038651261350, mail: iztok.skornik@soline.si

A.3.5 CONSTRUCTION OF BARRIERS (FENCES) TO PREVENT THE INTRUSION OF PREDATORS OR UNGULATES

Purpose: To restore, improve, maintain aquatic habitats suitable for the life of *Emys orbicularis*.

Other species concerned: Amphibians in general; other small autochthonous freshwater fauna.

Mode of action: Creation of a safety barrier to prevent invasions by wild (wild boar) and domestic ungulates. Effective anti-intrusion materials will be used to delimit a small enclosure for the safe living of young turtles. The structure consists of wooden perimeter posts, placed every 3 metres, on which a metal mesh is placed. The following are required to build this structure:

- Wooden poles 1.50 m high and 8-10 cm in diameter;
- Sheets (portions) of metal mesh, 2 m high, 2 m long, with 4 mm wire, 10 x 10 cm mesh. Each portion is cut into two 1m x 2m strips.

The posts will be driven into the ground to a depth of at least 50 cm, without the use of concrete. Each sheet of metal mesh is anchored to the posts of the structure and driven 20-30 cm into the ground.

The structure can be reinforced externally, in the upper part (at a height of approximately 1.5 metres) and in the lower part (at a height of approximately 30 cm) with two electrifiable metal cables. For this part, the following are required: steel or aluminium-magnesium alloy cable; screw insulators for wooden posts for the cable; one 3 Joule electrification unit; one 25-Watt solar panel, including its fixing brackets; one 80 Amp rechargeable battery; double-sided electric fence signalling plates; one grounding post; one lightning rod for electric fences.

A.3.6 CREATION OF A BARRIER TO PREVENT PREDATION/DESTRUCTION OF NESTS

In some situations, it may be necessary to protect nests with a wire mesh structure measuring 19x19 mm, 100 cm high (30 buried and 70 above ground). The temporary structure must be removed when the eggs hatch (this requires constant monitoring of the area). It consists of wooden perimeter posts (1.20 m high and 4-8 cm in diameter), positioned 1 m apart, the net buried for at least 30 cm and placed around the nesting area; for greater security, it is advisable to cover it with a similar net to prevent intrusion from above.

After-work monitoring: An after-work check followed by constant monitoring to assess functionality and the need for maintenance.

Expected outcomes: Effective defence against predators.

Temporary protection systems for pond turtle nests (source https://www.reddit.com/r/turtles/comments/14baq3u/eastern_painted_turtle_nest_protection/?rdt=33871 and Riley & Litzgus, 2013

A.4 PROCEDURES

Environmental restoration works, especially if of a certain size, with earth movements and transformation of the state of the sites, require suitable procedures to be carried out.

Although they are generally natural recovery operations, implemented with low-impact naturalistic engineering works and aimed at improving the quality of the landscape and environment, it is necessary to consider the difficulty of framing similar works within the normal planning and authorisation framework envisaged by the various Authorities, and the consequent need for a preliminary check of the authorisations not only envisaged, but even beforehand requested by the offices in charge within the various sectoral Bodies responsible for governing the territory (water sector, soil protection, landscape, Protected Areas, etc.).

It is therefore indispensable, before activating any intervention, to carry out a preliminary feasibility analysis that allows on the one hand to verify the consistency with existing planning instruments, especially with respect to constraints, and on the other hand to consequently activate the involvement of the Bodies and Offices in the decision-making process. This makes it possible to immediately have as exhaustive a list as possible of the authorisations, clearances and opinions required to proceed with the interventions.

In the case, for example, of works that envisage a transformation of the site, such as the digging of a pond or planting of a certain extent, it is possible to envisage a procedural process as follows:

A.4.1 VERIFICATION OF OWNERSHIP OF THE AREA

The status of private or public property obviously requires different procedures. Particularly in the case of private owners, it is useful to stipulate an agreement that guarantees the maintenance of the work in the long term and allows the appropriate authorisation requests to be made with a simple power of attorney to activate the construction sites. Once the power of attorney has been obtained, it is possible to proceed as described below for works in public areas.

In the case of public areas, it is necessary to communicate the start of work to the relevant municipality (generally a CILA[4] is enough in Italy), attaching a project summary and the

[4] Comunicazione di Inizio Lavori Asseverata (Notification of Commencement of Works)

appropriate authorisations that the municipality must acquire or that the proponent has already acquired. Each municipality has its own internal procedures for submitting applications with costs that may vary from municipality to municipality. If the intervention falls within a state-owned area, it is necessary to check what type of state property is involved and what is required to act and donate permanent works (in some cases the state property authorities ask for a notice and must 'accept' to receive the work).

A.4.2 ENVIRONMENTAL CONSTRAINTS

If the intervention falls within Natura 2000 Sites, it is necessary to ask the Managing Authority for an assessment of the compatibility of the intervention with the indications provided in the site-specific Conservation Measures and in the possible Management Plan (Italian Presidential Decree 357/97 and subsequent amendments and additions). Even though these interventions are aimed at the conservation of a species of European Union interest, there could in fact be specific critical points that make them incompatible with the management tools in force (e.g. the intervention falls in an area affected by a habitat in Annex I of Dir. 92/43/EEC for which the Conservation Measures and Management Plans envisage maintenance or improvement). For this it is necessary to use the tool of Impact Screening. Each Italian Region has now transposed into its own legislation the indications of the National Guidelines for Impact Assessment (VIncA)[5] – Habitats Directive 92/43/EEC Article 6, paragraphs 3 and 4 issued with the Agreement of 28 December 2019, pursuant to Article 8, paragraph 6, of Law No. 131 dated 5 June 2003, between the Italian Government, the Italian Regions and the Autonomous Provinces of Trento and Bolzano. All the Regions (and the Managing Authorities accordingly) have adapted the specific regulations in the context of VIncA by adopting the Incidence Screening tool and model.
Since these are interventions in favour of the preservation of species and habitats, the Screening of Incidence can simply require a "verification of correspondence of pre-assessed proposals" made available by the individual regions (if the regions have not yet fulfilled this obligation, it will still be possible to proceed with a classic Screening of Incidence verification), and a project summary is required. In these cases, the process ends with the Managing Authority's opinion.

[5] "National Guidelines for Impact Assessment (VIncA) Directive 92/43/EEC "Habitat" art. 6, paragraphs 3 and 4" Italian Official Journal G.U. 28-12-2019, General Series - No. 303

If the intervention falls outside Natura 2000 Areas, but within Protected Areas, it is necessary to check the individual planning instruments of the Protected Areas to ascertain the need for the authorisation of the Protected Area's Managing Body, which may in any case be requested together with the CILA.

A.4.3 LANDSCAPE CONSTRAINT

When work is carried out on existing wetlands that require maintenance operations (e.g. draining, removal of sediments and material, cutting of vegetation in the waterbed) in areas subject to landscape regulations pursuant to the Italian Legislative Decree No. 42 dated 22 January 2004 and subsequent amendments and integrations, it is usually not necessary to acquire a landscape authorisation, but it is still good practice to consult the body having jurisdiction. In the case of building new basins, especially if equipped with hydraulic artefacts to regulate water levels, it is instead necessary to carry out the procedure to obtain the landscape authorisation, which, depending on the type of intervention, may be simplified or ordinary. It is therefore advisable to first carry out a check on the landscape constraint, which can be done, for example, by consulting the website http://vincoliinrete.beniculturali.it/VincoliInRete/vir/utente/login. In the presence of constraints of this type, it is likely that it will be necessary to file an application with the bodies having jurisdiction (e.g. Parks, Municipalities, etc.), subsequently acquiring any building permits (in Italy, SCIA[6], CILA, etc.).

A.4.4 HYDRAULIC CONSTRAINT

If the intervention falls within the river public domain (within the active riverbed or within the buffer strips identified by the individual River Basin Plans), it is necessary to apply for hydraulic clearance, to which a project summary and some fundamental elements must be attached, such as the destination of waste materials (e.g. vegetation clippings) and excavated rocks and soil. Particularly for works in areas with hydraulic constraints, but also for excavations in other types of land, it is important to carefully evaluate the volumes excavated and the type of soil in order to identify a destination that is compatible with the regulations in force. For example, it is advisable

[6] Segnalazione Certificata di Inizio Attività (Certified Declaration of Start of Activity)

to provide in the design phase for the relocation of rocks and soil in situ (e.g. for remodelling the banks of ponds), limiting the relocation or disposal of soil, also for cost reasons. Hydraulic clearance can generally be requested from regional or provincial soil protection offices.

A.4.5 PRELIMINARY ASSESSMENT FOR THE PRESENCE OF RESIDUAL WAR EXPLOSIVES RISK

Pursuant to Law No. 177 dated 1st October 2012 "Amendments and additions to the Italian Legislative Decree No. 81/2008", it is mandatory for projects involving excavations to carry out a preliminary assessment of the presence of residual war explosive risk, which may or may not lead to an effective clearance of explosive remnants of war. This obligation concerns the project developer and should be assessed especially for those areas where there is evidence of critical events in World War II (bombings and similar activities) or recent findings of explosive items in the target territory. This obligation implies the identification of a specialised company and additional costs, which on average amount to approximately € 3,000-4,000.

Lastly, it should be remembered that all applications submitted to the Public Administration for authorisation requests entail costs: office costs, tax stamps, and in some cases fixed costs linked to the type of application. These costs vary from authority to authority, but can rise on average up to 200 and 300 Euros.

Regarding the compatibility of the interventions with the current state of the sites and populations of *Emys orbicularis* and other species of heritage interest and subject to protection, it is worth remembering that works affecting areas of naturalistic importance could unintentionally interfere with other components of local biodiversity (habitats and species).

For this reason, as part of the preliminary feasibility of the intervention/work, knowledge of the project areas must be inventoried, taking care to carefully choose the best and most compatible sites and timing (see Paragraph A.3).

A.4.6 STANDARD DATA FORM

It is also advisable to check whether the Standard Data Form of the Natura 2000 Site in which you are working indicates the presence of *Emys orbicularis*, because it may happen that in some situations the species has not been included.

Following the interventions, if the species is not already included in the specific Standard Data Form, it is necessary for the Managing Authority and the reference Region to start the updating process with the inclusion of *Emys orbicularis* and the relevant data on the conservation status and abundance of the populations.

A.4.7 REGULATIONS IN FAVOUR OF RENATURATION

The need to restore the environment has become a global priority and the United Nations has called for 2021-2030 to be the UN Decade on Ecosystem Restoration[7], dedicated to protecting and reviving ecosystems worldwide for the benefit of people and nature. There are several European Strategies aimed at environmental protection and restoration (European Biodiversity Strategy[8], Farm to Fork[9]…) or Directives that already provide specific objectives for nature protection and restoration, starting with the Habitats Directive 92/43/EEC on the conservation of natural and semi-natural habitats and of wild fauna and flora.

The Water Framework Directive (2000/60/EC) has the main objective of achieving 'good ecological status' of water bodies by 2027. Considering that a large part of aquatic ecosystems is not in good condition, there is a need for widespread rehabilitation of freshwater ecosystems. In Italy, this is possible by promoting 'integrated interventions for the improvement of the ecological status of watercourses and the protection of ecosystems and biodiversity (from Article 7, paragraph 2, of Decree-Law No. 133 dated 12 September 2014, converted, with amendments, by Law No. 164 dated 11 November 2014), which should be implemented by the Italian regions. In this way, habitats for *Emys orbicularis* could also be specifically restored.

In the new Common Agricultural Policy (CAP), there are specific objectives to contribute to climate change mitigation and adaptation (Specific Objective 4) and to pursue environmental objectives for the protection of natural resources and biodiversity (Specific Objectives 5 and 6, respectively).

[7] https://www.decadeonrestoration.org/about-un-decade

[8] https://www.europarl.europa.eu/news/it/headlines/society/20200519STO79422/tutelare-la-biodiversita-in-europa-la-strategia-dell-ue?at_campaign=20234-Green&at_medium=Google_Ads&at_platform=Search&at_creation=DSA&at_goal=TR_G&at_audience=&at_topic=Biodiversity&gclid=EAIaIQobChMIhNybpLv3_gIVivZ3Ch1J-Q1qEAAYASAAEgIq4PD_BwE

[9] https://www.europarl.europa.eu/italy/it/succede-al-pe/strategia-farm-to-fork-e-politica-agricola-comune-nella-prossima-programmazione-ue-2021-2027

The Italian National Strategic Plan of the CAP 2023-2027 has been received at the regional level, e.g. in Lombardy, through the 'Complement for Rural Development of the National Strategic Plan of the CAP 2023-2027 of the Lombardy Region' and offers significant opportunities for the implementation of habitat restoration actions. There are non-productive investments for the "construction and/or restoration of the functionality of ecological water-related infrastructures (e.g. wetlands, fountains, ponds and other watering structures)"; for the "restoration or planting of vegetation in the river beds (macrophytes) and on the banks (riparian strips) in the minor water courses, such as the restoration and/or planting of aquatic and riparian vegetation or other ecological upgrading interventions". There is the possibility to build irrigation and reclamation infrastructures or to provide for the "extraordinary maintenance, by the irrigation bodies, of the artificial network of the lowlands for irrigation and reclamation purposes and related facilities, in order to maintain or create the provision of ecosystem services linked to aquatic ecosystems"; there can also be active management interventions of ecological infrastructures that provide for an annual payment per hectare for those who engage in the management of ecological infrastructures such as, for example, wetlands.

In addition, habitat restoration actions can be promoted by participating in European programmes such as the Horizon[10], which receive 100% financial support for their costs, or the LIFE programmes, which allow co-financing between 60 and 75 per cent.

Finally, there are multilateral funds (e.g. Green Climate Fund - GFC)[11] or the Adaptation Fund [12]), multilateral development banks (e.g. Asian Development Bank[13]), debt-for-nature swaps[14], technical and financial cooperation activities, foundations and philanthropy.

At the Italian national level, there are various possibilities with the use of national public resources but also from private entities such as banks, foundations (e.g. Fondazione Cariplo, Fondazione con il Sud, CariVerona), insurance companies[15], private companies (corporate social responsibility).

[10] https://www.catalyze-group.com/horizon-europe-2023/?utm_campaign=Horizon%20Europe&utm_term=Horizon%20Europe%20funding&gclid=EAIaIQobChMImP-Qj4r3_gIVWPl3Ch2wnwk6EAAYASAAEgKQ7_D_BwE

[11] https://www.greenclimate.fund/

[12] https://www.adaptation-fund.org/

[13] https://www.adb.org/

[14] https://en.wikipedia.org/wiki/Debt-for-nature_swap

[15] https://www.swissre.com/our-business/public-sector-solutions/our-solutions/nature-based-solutions.htm

B. POPULATION RESTOCKING - GENETICS

B.1 INTRODUCTION

During the last decade, population genetics and the development of new DNA investigation methodologies integrated with ecological and demographic studies have found wide application in multidisciplinary contexts, such as conservation biology, directed towards the development of wildlife management and conservation plans for wild animal populations. The study of the genetic structure of populations makes it possible, in fact, to identify units that are sufficiently distinct to be considered separate management units, to determine the population of origin of single specimens and the degrees of kinship, all in order to provide information necessary for the development of reintroduction plans or genetic and demographic reinforcement.

Portions of nuclear and mitochondrial DNA are used for this purpose. For nuclear DNA, the class of genetic markers is represented by units of repeated nuclear genomic sequences, called microsatellites, whose degree of polymorphism is determined by the difference in the number of repeats observed between specimens. The small size of microsatellites (100-500 nucleotides) allows them to be identified, by means of the polymerase chain reaction (PCR), from minute amounts of DNA. The process of characterising the level of genetic variability at microsatellite loci is commonly referred to as DNA fingerprinting, because it is capable of unequivocally distinguishing, except for homozygotic twins, one individual from another. Microsatellites are used to define kinship relationships and to determine an individual's population of origin by means of population structure analysis and assignment tests.

A further level of definition can be achieved through the analysis of nuclear DNA sequence data obtained by next-generation sequencing techniques. The analysis of these data makes it possible to identify tens if not hundreds of thousands of genetic variations throughout the genome of the individuals examined. Genetic variations related to a single position along the DNA sequence are called single-nucleotide polymorphisms (SNPs). These variations consist of the substitution of one of the four nitrogenous bases for another at a specific position in the genome. SNPs are the most common type of genetic variation, are found throughout the genome and are responsible for much of the genetic diversity within a population. These genetic markers have become a valuable tool in various fields of genetics and genomics, including population genetics and forensic biology.

Mitochondrial DNA is, in most cases, present in single copies in every eukaryotic cell, in vertebrates it is transmitted maternally, it is characterised by a high rate of evolution, mainly due to the lack of mechanisms that correct errors made during DNA replication, and it is practically

free of recombination phenomena. The characterisation of the sequence of coding and non-coding regions of mitochondrial DNA provides information on the maternal lineages which, coupled with fingerprinting and SNPs results, can increase the degree of resolution of the genetic investigation. A first level of genetic assignment, for example, is based on the comparison of specific mitochondrial DNA sequences between turtles that make up natural populations and individuals of unknown origin. This comparison concerns the determination of the degree of similarity in terms of inter-individual nucleotide base composition and is used for macro-scale assignments, such as whether a specimen belongs to the populations on the Tyrrhenian rather than the Adriatic side of the Italian peninsula.

B.2 OBJECTIVES OF GENETIC ANALYSES

In planning actions for the demographic reinforcement of natural populations of *Emys orbicularis* or the reintroduction of individuals in areas where the species has disappeared but the environmental conditions suitable for its survival and reproduction have been re-established, the genetic analyses, in compliance with the prescriptions dictated by the IUCN, are based on the following points:

a) Genotyping of natural populations by characterising allelic variability at microsatellite loci and/or SNPs and comparison of mitochondrial DNA sequences for the definition of the level of intraspecific genetic differentiation as a basic tool for repopulation management;
b) Genotyping and assignment to the respective natural populations of origin of the specimens kept in the recovery and breeding centres, including potential breeders being there (or kept in the future) through analyses of genetic markers used to study natural populations;
c) Identification of individuals with low kinship ties for the formation of breeding pairs or triplets (one male and two females) so as to minimise inbreeding and preserve the genetic variability of the breeding stock. The breeding stock should also come from the same source population to avoid a possible reduction in offspring fitness due to outbreedingdepression caused by the loss of specific gene interactions of individuals adapted to certain environmental conditions;
d) Genotyping and paternity testing of offspring, to ensure that, despite the possibility of sperm retention in females, these are actually derived from fertilisation between individuals chosen as breeding stock.

The genetic characterisation of the Italian populations of *E. orbicularis* includes 29 mainland and peninsular Italian populations, 4 populations in Sardinia and 6 in Sicily for *E. trinacris* (Fig. 1). The populations in Sicily are considered among the baseline populations even though they belong to *E. trinacris*, so as to detect a possible insular origin of individuals kept in captivity.

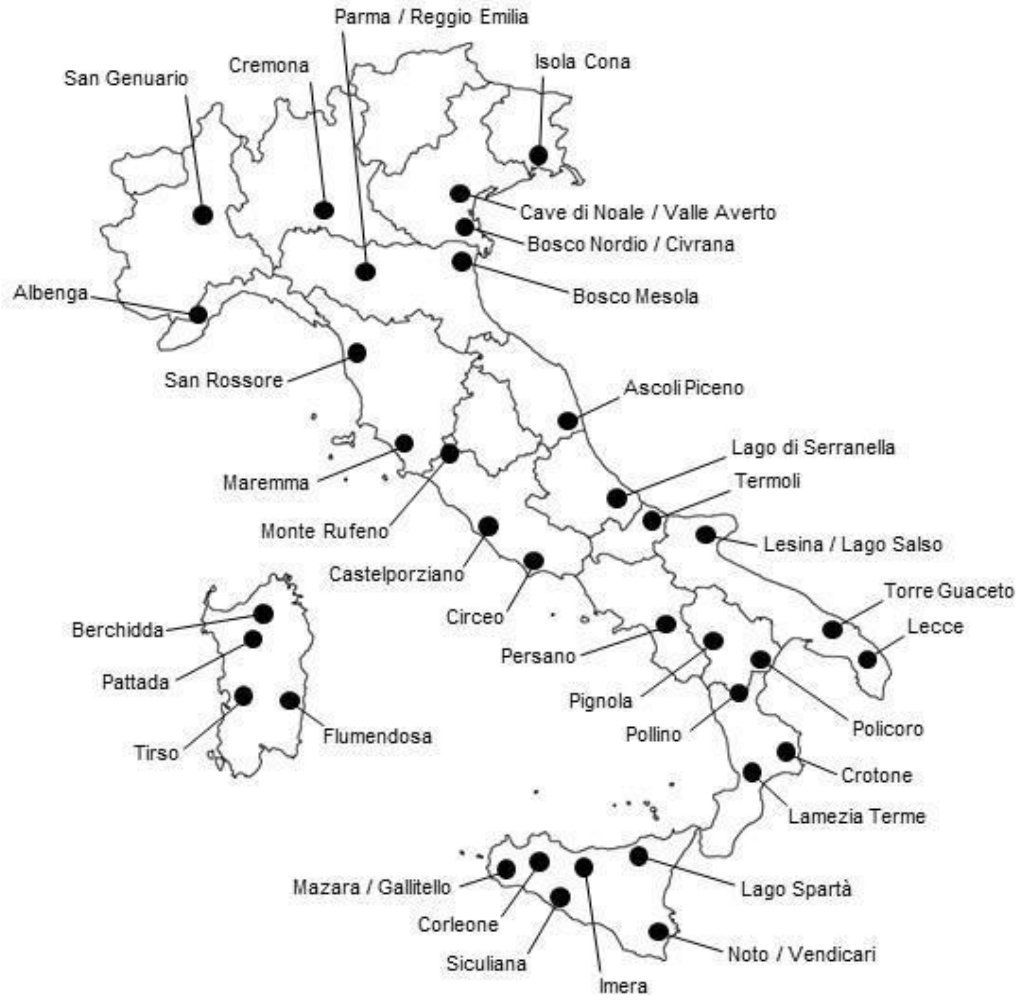


Figure 1. Map of the main natural Italian populations of *E. orbicularis* and *E. trinacris* (Sicily) with known genetic structure, used to determine the areas of origin of individuals kept in captivity.

B.3 SAMPLING

The genetic characterisation of *E. orbicularis* specimens is carried out from purified DNA from blood samples.

For each individual, depending on size, approximately 0.05 cc (juvenile specimens) or 0.1 cc (medium-sized and adult specimens) of blood is taken from the subcarapacial venous plexus using

a 1 ml syringe and a disposable 23Gx1" or 26Gx½" needle for adult and immature specimens respectively (Fig. 2). A 30Gx5/16" needle is instead used for blood sampling from young specimens. Blood samples are temporarily stored at room temperature in a 2.0 ml cryogenic vial containing 0.8 ml of a lysis buffer (0.1M Tris, 0.1M EDTA, 0.2M NaCl, 1% sodium dodecyl sulphate, pH 8.0). Storage of blood samples at room temperature requires conditions that inhibit nuclease activity. A high salt concentration reduces the activity of nucleases and a similar result is obtained with a high concentration of EDTA. Nucleases are enzymes dependent on divalent cations. EDTA chelates divalent metal cations such as Mg^{2+}, thus weakening ionic bonds. EDTA is also used as an anticoagulant, as it complexes plasma Ca^{2+} and prevents blood clotting. Sodium dodecyl sulphate (SDS) is a strong detergent that degrades the lipid components of cell and nuclear membranes, allowing nucleic acids to be mixed with EDTA. The samples are then stored permanently at – 80°C.

Figure 2. Collection of a blood sample from the subcarapacial venous plexus of an adult *Emys orbicularis*.

B.4 GENETIC ANALYSES

B.4.1 DNA Purification

The DNA is purified from blood samples using a standard protocol based on disruption of cytoplasmic membranes, removal of protein contaminants with organic solvents and subsequent isolation of high molecular weight nucleic acids in the presence of salt and ethanol. DNA

purification is performed from 50-100 µl of sample by disaggregation of cytoplasmic components and protein dissociation with protease *K* in the presence of 0.7 ml of a lysis buffer containing 100 mM Tris, 5 mM EDTA, 10 mM NaCl, 0.5% SDS, pH 8.0 for approximately 8 h at 37 °C on a stirrer. The DNA is subsequently separated from the protein components by mixing the digestion product with phenol-chloroform-isoamyl alcohol (24:24:1). Centrifuging the mixture at 13,000 rpm produces two phases: a lower organic phenolic phase containing the protein components and an upper less dense aqueous phase containing the nucleic acids. After centrifugation, the upper aqueous phase is removed and the process is repeated with chloroform-isoamyl alcohol (24:1). After the second separation process, the supernatant is mixed with 2.5 volumes of 99.9 % ethanol to concentrate the nucleic acids. The nucleic acid precipitate is then sedimented by centrifugation, the ethanol is removed and the precipitate is resuspended in a holding buffer containing 100 mM Tris-HCl; 10 mM EDTA; pH 8.0. EDTA chelates heavy metal ions commonly required for DNAase activity, while the use of a basic pH minimises deamidation. The DNA is then stored at -80 °C.

An alternative method of DNA purification involves binding and subsequent elution of the DNA on a silica membrane. Lysis is achieved by incubating the sample in a proprietary solution containing SDS and proteinase K at 70°C for 15 minutes. The sample is then placed on a silica membrane in a microcentrifuge column, which is in turn placed in a collection tube. Optimal conditions for DNA binding to the silica matrix are achieved by the addition of chaotropic salts and ethanol to the lysate, which is then centrifuged to remove protein contaminants and cellular detritus through the matrix into the collection tube. The silica membrane is subsequently washed twice by centrifugation with two different buffers. In each step, the resulting centrifugation liquid is discarded. DNA binding to the matrix is reversible and specific for nucleic acids. The DNA is eluted under low ionic strength conditions in the presence of a slightly alkaline elution buffer by centrifugation into a new collection tube. The DNA is then stored at - 80 °C.

B.4.2 Characteristics of Mitochondrial DNA as a Molecular Marker

Mitochondrial DNA (mtDNA) is the genome of mitochondria, structures within cells that convert energy from food into a form that cells can use for metabolic processes. Vertebrate mitochondrial DNA is a circular double-stranded molecule of approximately 16,000-18,000 nucleotide base pairs (bp). The two strands of mtDNA are distinguished by their nucleotide content: the guanine-rich strand is called the heavy strand, while the cytosine-rich strand is called the light strand. The

mtDNA also includes an important non-coding region (the control region) that is responsible for replication and transcription of the mitochondrial genome.

In sexual reproduction, mitochondria are generally inherited through the maternal route. The mitochondria present in the tail of the sperm are usually destroyed by the egg cell after fertilisation or otherwise eliminated with the loss of the sperm tail during fertilisation. Therefore, while nuclear DNA has biparental and diploid inheritance, mtDNA is haploid and is inherited asexually, predominantly, if not completely, through the maternal lineage. Studying the differentiation of mtDNA is therefore equivalent to studying the female population. This, combined with its haploid characteristic, makes the effective population size of this genome four times smaller than that of the nuclear genome. Consequently, the rate of differentiation due to genetic drift is particularly rapid in the mitochondrial genome when the overall population size is small. Furthermore, unlike nuclear DNA, which is inherited from both parents and in which genes are subject to the process of recombination, there is no major variation in mtDNA from parent to offspring. Although recombination also takes place for mtDNA, this occurs predominantly within the same mitochondrion. For this reason and because of the low effective population size and relatively high mutation rate, mtDNA is extremely useful for tracing matrilineal lineage and is used to assess genetic and evolutionary relationships between specimens, groups, populations at both intra- and interspecific levels. Mitochondrial DNA analysis is generally carried out by examining sequence variations of coding regions that do not involve amino acid substitution or non-coding intergenic sequences, such as the control region, that are not subject to selective constraints and thus have a higher mutation rate. This makes it possible to assess the degree of genetic differentiation between groups that have diversified as recently as populations within a species.

The mtDNA control region, in particular, is an area of the mitochondrial genome consisting of non-coding DNA. It contains the replication origin of the heavy strand and the transcription origin for both strands, and is the most polymorphic sequence in the mitochondrial genome, with varying degrees of polymorphism concentrated in hypervariable regions. The mtDNA of *E. orbicularis* is approximately 16,770 base pairs (bp) long and contains all 33 genes typically found in other species of the superfamily Testudinoidea (Lourenco et al. 2012) and has a control region approximately 1,000 bp long. The characterisation and comparison of the sequences of the mtDNA control region provide an appropriate and rapid approach for the analysis of the intraspecific differentiation of *E. orbicularis* populations along the Italian peninsula and for the assessment of the population of origin of captive and captive-bred specimens.

The control region of mtDNA is first amplified by polymerase chain reaction (see § 4.3), and the nucleotide sequence of the amplification products is subsequently characterised by chain termination reaction (see § 4.4). The cyclic sequencing products are then resolved by capillary electrophoresis in an automatic nucleic acid analyser (§ 4.5).

B.4.3 Polymerase Chain Reaction (PCR) for Amplification of a Partial Portion of the Control Region of Mitochondrial DNA

Polymerase Chain Reaction (PCR) is a technique that allows the multiplication (amplification) of nucleic acid fragments the initial and terminal nucleotide sequences of which are known. Amplification by PCR allows the amount of genetic material required for subsequent applications to be obtained very quickly. In practice, a specific step in cell reproduction is reconstructed in vitro, i.e. the reconstitution (synthesis) of a complete (double-helix) DNA segment from a single-helix strand. The in-vitro amplification process takes place via Taq polymerase, a DNA polymerase that is stable up to about 95 ˚C (originating from the bacterium *Thermus aquaticus*, which lives at high temperatures). Taq polymerase synthesises a new chain using free deoxynucleotides, starting with a single-helix strand bound to a DNA half-helix consisting of 10-30 deoxynucleotides called a primer. Primers are synthesised in vitro and have species-specific sequences to match the upstream (forward primer) and downstream (reverse primer) sequences of the portion of DNA to be characterised. PCR thus consists of a first step of denaturing the DNA double helix, a second step in which the primers bind to the complementary sequences of the denatured hemihelices (annealing), and a third step of polymerising the complementary hemihelices to the denatured hemihelices to form two new DNA double helices from the starting double helix. This process is repeated about 35 times so that at each amplification cycle the number of DNA double helices is doubled and the DNA is amplified exponentially.

PCR requires a number of reagents including: 1) the DNA containing the region to be amplified, 2) two primers with sequences complementary to the sequences upstream and downstream of the portion of DNA to be amplified respectively, 3) Taq polymerase, 4) deoxynucleoside triphosphate (dNTPs) that will form the new DNA strand, 5) a buffer solution that provides a suitable chemical environment for optimal DNA polymerase stability and activity, and 6) divalent cations (Mg^{2+}), a cofactor for Taq polymerase activity. The cations also help stabilise the two strands after denaturation and are added to the reaction in the form of $MgCl_2$.

The control region of the mitochondrial DNA of *E. orbicularis* is amplified using the light (forward) strand primer DES1 (5' - GCATTCATCTATTTTCCGTTAGCA - 3') and the heavy (reverse) strand primer DES2 (5' - GGATTTAGGGGTTTGACGAGAAT - 3'). The light-strand primer DES1 and the heavy-strand primer DES2 bind at position 15.967 and position 16.627, respectively, in the mitochondrial DNA control region of *E. orbicularis* (Fig. 3). The binding positions of the primers refer to the 3' base position on the mitochondrial genome sequence of *E. orbicularis* published by Lourenco et al. (2012). PCR is performed in a total volume of 10 µl with approximately 10 ηg of total DNA, 1X buffer solution, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer and 0.5 units of Taq DNA polymerase. The thermal profiles consist of an initial denaturation step of 5 minutes at 95 °C, followed by 35 cycles of 30 seconds at 95 °C, 30 seconds at 52 °C, and 1 minute at 72 °C, with a final polymerisation step of 10 minutes at 72 °C. The PCR produces a 707 bp sequence amplicon of the mitochondrial DNA control region.

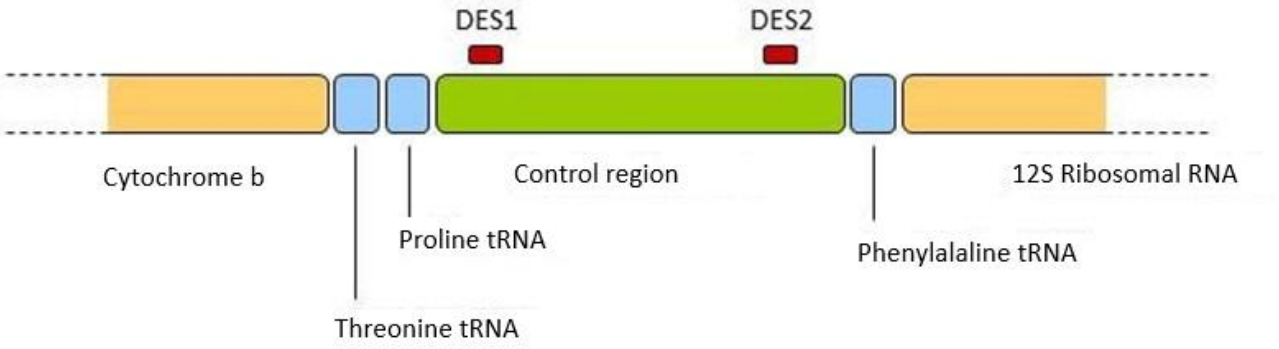


Figure 3. Attachment positions of DES1 and DES2 primers for the amplification of a partial portion of the control region of mitochondrial DNA relative to other contiguous genes (see text for further details).

B.4.4 Chain Termination Reaction or Cyclic Sequencing of PCR Products

The chain termination method is conceptually similar to PCR in that it utilises the ability of DNA polymerase to synthesise a complementary copy of a DNA strand. The synthesis of the strand complementary to the mould half-helix takes place from only one of the two primers used in § 4.3. DNA polymerase polymerises the new semi-helix by adding nucleotides via phosphodiester bonds between the 3'-hydroxyl group of the last added nucleotide and the 5' α-phosphate of the next dNTP. The synthesis reaction is terminated by the incorporation of a modified nucleotide that does not allow subsequent dinucleotide incorporation (hence the name chain termination). The modified dinucleotides that terminate the reaction are the 2',3'-dideoxynucleosides 5'-triphosphates (ddNTPs), which differ from conventional dNTPs in that they lack a hydroxyl residue

at the 3' position of deoxyribose. Once incorporated by DNA polymerase, the absence of a 3'-hydroxyl residue prevents the formation of a phosphodiester bond with the next dNTP. The reaction products are thus a series of oligonucleotide chains the length of which is determined by the distance between the primer termination and the termination sites. Using the four different ddNTPs each labelled with a different coloured fluorophore, groups of oligonucleotides terminating at the positions occupied by each A, C, G or T are generated and subsequently resolved by capillary electrophoresis (see § 4.5). Each reaction contains DNA polymerase, template, primers, buffer, all four dNTPs and four ddNTPs, each labelled with a dye (fluorochrome) of a different colour.

The chain termination reaction is performed through 25 cycles of denaturation, annealing of a specific single-stranded primer, and polymerisation in the presence of Taq DNA polymerase. Although similar to PCR, in cyclic sequencing, amplification occurs on a single strand (using only one primer per reaction) and is therefore linear. PCR amplification, on the other hand, is exponential as the two strands are synthesised simultaneously. The chain termination reaction for sequence characterisation of the mtDNA control region of *E. orbicularis* amplified according to the method described in § 4.3 is performed in a total volume of 10 µl with approximately 20 ηg of PCR product, 0.5 µl of Terminator Ready Reaction Mix (containing polymerase, dNTPs, fluorochrome-labelled ddNTPS and ions), 1X buffer solution and 3.2 µM primer. The thermal profiles consist of an initial denaturation step of 3 minutes at 95 °C, followed by 25 cycles of 10 seconds at 95 °C, 5 seconds at 50 °C and 4 minutes at 60 °C. Once the reactions are complete, the cyclic sequencing products are precipitated in isopropanol, which is then evaporated, resuspended in deionised formamide (Hi-Di) and resolved by capillary electrophoresis. Two cyclic sequencing reactions are carried out for each PCR product. The first sequencing reaction uses the DES1 primer, while the DES2 primer is added to the second reaction. This is necessary because the chain-termination reaction produces a series of oligonucleotides, each labelled with a different colour fluorescence depending on the final dideoxynucleotide, which during their resolution by capillary electrophoresis do not allow reliable nucleotide base definition for lengths greater than about 600 bases from the primer (see also § 4.5). The DNA sequence obtained with the DES1 primer is inverse and complementary to the sequence obtained using the DES2 primer. The decrease in signal that is observed at the end of the reaction from the DES1 primer is therefore compensated for by the strong signal that is obtained from the reaction from the DES2 primer and vice versa (Fig. 4).

B.4.5 Resolution of Cyclic Sequencing Products by Capillary Electrophoresis

The capillary electrophoresis reaction of the chain-termination products takes place, for each sample, within a capillary into which a polymer is injected to form the new medium through which the electrophoretic run develops. Capillary electrophoresis takes place inside an automatic nucleic acid analyser. The automatic sequencer consists of two units: an electrophoretic chamber equipped with a fixed laser and an external computer connected to the chamber, equipped with software capable of analysing the fluorescence emission. There is also a detection chamber, where each capillary comes into close proximity to the laser. In this section, fluorochromes of chain-termination products migrating by electrophoresis to the anode are excited by the laser and emit fluorescence. The DNA fragments separated by electrophoresis arrive at different times in the capillary zone exposed to the laser, resulting in the excitation of the fragment's fluorochrome, which emits fluorescence that is directed through a series of lenses to a spectrograph that separates the wavelengths and directs them to a Charged-Coupled-Device (CCD) camera equipped with different filters depending on the fluorochromes to be analysed. This camera analyses the emission spectrum and converts the light signal into a digital signal that is recorded on the computer associated with the machine. The data obtained from the signals emitted during capillary electrophoresis for allelic characterisation and processed by appropriate software are then represented as graphical lines with the position of each peak on the x-axis representing a chain-terminating product of different length.

Specifically, products terminating with a ddATP are represented by a green signal; a blue signal indicates termination with ddCTP; black signals represent fragments terminated with ddGTP; and red signals indicate oligonucleotides terminated with ddTTP. The computer associated with the automatic nucleic acid analyser assembles the DNA sequence according to the colour and order in which the cyclic sequencing products pass in front of the laser. The raw data obtained from capillary electrophoresis are subsequently transformed into electropherograms (Fig. 4).

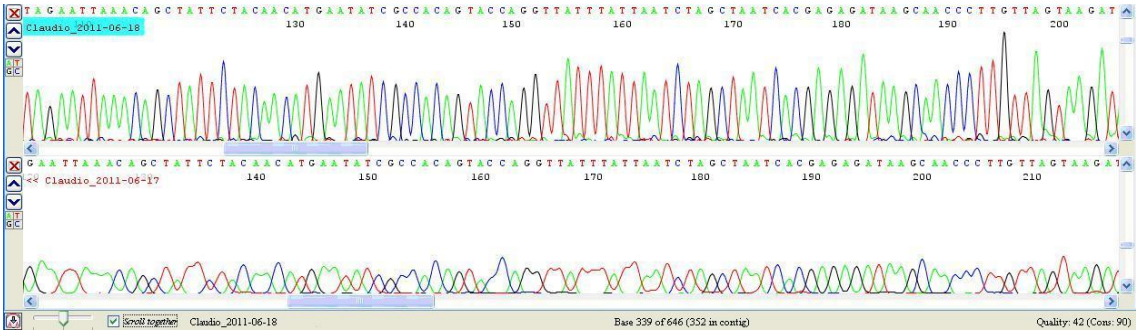


Figure 4. Electropherograms of a portion of the mtDNA control region of *E. orbicularis*. The low-quality signal of the terminal part of the sequence obtained from the DES2 primer (bottom) is compensated for by the high-quality signal of the sequence obtained using the DES1 primer (top). The same but inverse situation is proposed at the end of the sequence obtained with DES1 to obtain a consensus sequence of 659 bp for each specimen.

For each specimen, the four groups of cyclic sequencing products, each characterised by a different colour, are resolved by electrophoresis in a single capillary. For each turtle, the sequencing products obtained from the DES1 primer are separated in one capillary, while a second capillary electrophoresis reaction distinguishes the products obtained from the DES2 primer. The reverse complement of one of the two sequences is then aligned to the complementary sequence to create a consensus sequence for the control region of each turtle. The consensus sequence corresponds to a total of 659 nucleotides of the mtDNA control region after deletion of sequences corresponding to the primers and some sub-optimal signal regions.

B.4.6 Characteristics of Microsatellite Loci as Molecular Markers

A second class of molecular markers particularly useful for determining the degree of intraspecific genetic variability and specifically for assigning specimens of unknown origin to the most genetically similar population are a particular type of Variable Numbers of Tandem Repeats (VNTRs) of nuclear DNA called Simple Sequence Polymorphisms or microsatellites.

Microsatellites are repeats of short genomic sequences, consisting on average of 2 to 6 nucleotides, such as (TG)n or (AAT)n. Microsatellites represent highly polymorphic genetic markers and are found distributed in the nuclear genome of eukaryotes and some prokaryotes. Characterisation of allelic differences at the intra- and inter-specimen level is due to variation in the number of repeats of these nucleotide sequences. The utility of microsatellites in population genetics lies in the relatively small size of the loci. A sequence consisting on average of 40 dinucleotide repeats corresponds to a microsatellite locus of 80 nucleotides. A portion of the genome of this size can be easily amplified by PCR for a final product of 100-300 nucleotides, if we consider the portion of the final product that includes the primer sequences. It is therefore

evident that a study of inter-specimen allelic variation based on microsatellite loci can be performed even on degraded DNA samples where the probability of having a disrupted microsatellite locus is very low. Moreover, such dimensions allow the detection of very fine allelic differences down to a single nucleotide inter-specimen difference by capillary electrophoresis. They are also inherited by the Mendelian pathway and thus represent an ideal genetic marker for kinship analyses and fine demographic investigations, at the specimen population level and for genetic assignment tests.

The two forms of a specimen's microsatellite locus, called alleles, can be of the same length (same number of repeats) or different length (different number of repeats). In the first case, a turtle is homozygous for that particular microsatellite locus, in the second case the animal is heterozygous. The characterisation of the two allelic forms at a particular microsatellite locus provides the genotype of a specimen for that locus. The number of alleles then varies in a population for a particular locus, up to a maximum number of 2xN alleles, where N is the number of specimens that make up a population. Since the microsatellite repeat unit is generally less than 6 nucleotide bases, the entire microsatellite sequence rarely exceeds 300 bp. The microsatellite loci are amplified by PCR (see § 4.3.1) and differences in allele size between specimens of even one single repeat unit can be detected.

Genetic profiling of the nuclear DNA of *E. orbicularis* is based on the determination of allelic diversity at 14 microsatellite loci, 8 of which have been characterised by Ciofi et al. (2009), specifically Emys1, Emys2, Emys4, Emys5, Emys6, Emys7, Emys8, and Emys11, and 6 defined by Pedall et al. (2009), specifically msEo2, msEo21, msEo22a, msEo25, msEo29, and msEo32.

The genotyping procedure involves marking the forward primers of each microsatellite locus with a fluorochrome, which is required for the identification of the PCR amplification product by the laser system of the automatic nucleic acid analyser (§ 4.5) during the capillary electrophoresis process. Four different coloured fluorochromes are used for the allelic characterisation analysis. For each sample, four microsatellite loci can thus be analysed simultaneously with forward primers labelled with fluorochromes of different spectral lengths.

The amplification reaction is performed using forward primers labelled with FAM, HEX, NED, PET fluorescence. For each microsatellite locus, PCR is conducted in a total volume of 10 µl containing approximately 10 ng DNA, 1X buffer solution, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of forward and reverse primers, and 0.5 units of Taq DNA polymerase. The thermal profiles consist of an initial denaturation step at 94 ˚C for 5 minutes, followed by 35 cycles of 40 s at 94 °C, annealing

for 40 s at the primer-specific temperature, and polymerisation for 90 s at 72 °C, with a final polymerisation step of 5 minutes at 72 °C.

The products of the PCR (i.e. the alleles of each microsatellite locus) are identified by fluorescence detection as the alleles, during the capillary electrophoresis process, run in front of the laser of the automatic nucleic acid analyser. The fluorescent signal is processed and transformed into a signal that results in a peak of intensity proportional to the amount of amplified product.

Two alleles of the same length (homozygous specimens) or different lengths (heterozygous specimens) are detected for each locus. All microsatellite alleles for a locus will be identified by fluorescence of the same colour and will differ depending on the number of nucleotide repeats. Alleles of different microsatellite loci will differ depending on the fluorochrome used to mark the forward primers. The data obtained from the signals emitted during capillary electrophoresis for the characterisation of the microsatellite loci are represented by one or two peaks respectively if the specimen is homozygous or heterozygous for that particular locus (Fig. 5).

For each turtle, the PCR products of four loci with different fluorescence can be analysed simultaneously in the same capillary. In each capillary, a pool of 16 single-stranded fragments of known length from 35 bp to 500 bp, labelled with a different fluorochrome from those used to mark the forward primers of the microsatellites, is inserted together with the PCR products. These size standards are necessary for the subsequent definition of the allelic size of each microsatellite locus for each specimen. In this way, four microsatellite loci can be analysed simultaneously for each specimen.

The allele size for each microsatellite locus is determined by comparison to fragments of known length of the size standard using the GENEMAPPER (ABI) software. For each capillary, the software compares the electrophoretic run time of the alleles of each microsatellite locus with the time from the start of the electrophoretic reaction to the time each fragment of known length arrives in front of the laser. The allele sizes are therefore relative to the size standard. Figure 5 shows the genotypes of four specimens for three microsatellite loci labelled with the HEX, NED and FAM fluorochromes, respectively. The allele size is indicated for each specimen. Unlabelled peaks in the figure are PCR artefacts.

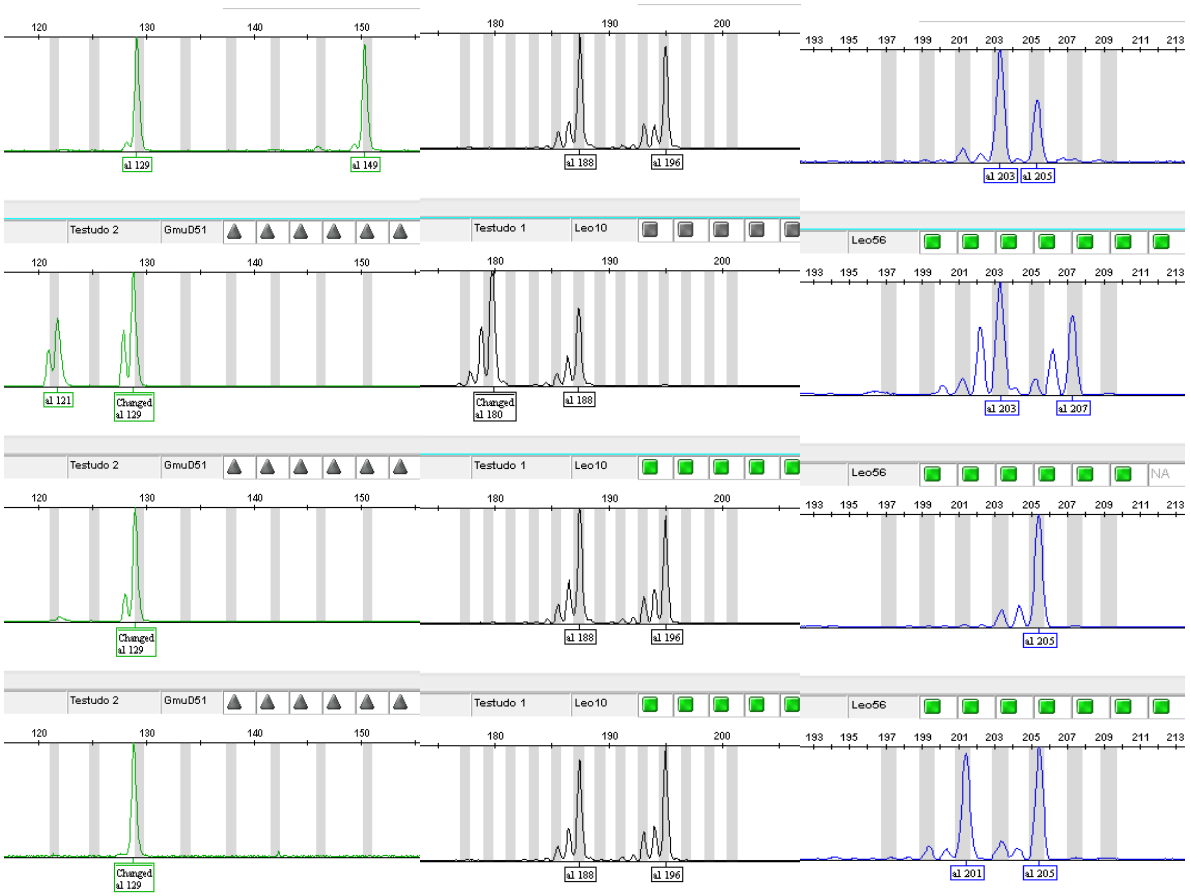


Figure 5. Resolution by capillary electrophoresis of the genotypes of four specimens for three microsatellite loci labelled with different fluorochromes: HEX (green), NED (black), and FAM (blue). The peaks of lower intensity upstream of the alleles are PCR artefacts.

The allele sizes determined for each locus and specimen are subsequently transferred to a calculation table as shown in Fig. 6.

B.4.7 Characteristics of Single Nucleotide Polymorphisms (SNPs) as Molecular Markers

Next-generation sequencing technologies make it possible to massively produce information on a large part of the genetic material of a species, even for several samples simultaneously. Analysis of these data makes it possible to identify hundreds of thousands of genetic variations throughout the genome of the specimens examined. Genetic variations related to a single position along the DNA sequence are called single-nucleotide polymorphisms (SNPs). These variations consist of the substitution of one of the four nitrogenous bases for another at a specific position in the genome. SNPs are the most common type of genetic variation, are found throughout the genome and are responsible for much of the genetic diversity within a population. These genetic markers have

become a valuable tool in various fields of genetics and genomics, including population genetics and forensic biology.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	SAMPLING SITE	LOCATION	Sample	Emys1-FAM		Emys2-HEX		Emys4-FAM		Emys5-H/F		Emys6-NED		Emys7-NED		Emys8-FAM		Emys11-HEX	
14	LIGURIA	Albenga	EAL029	117	117	176	176	98	98	110	110	157	157	127	127	127	127	87	87
15	LIGURIA	Albenga	EAL030	117	121	176	186	98	108	110	110	157	157	127	127	127	127	95	103
16	LIGURIA	Albenga	EAL031	117	153	176	186	108	108	110	110	157	179	127	127	127	127	87	87
17	LIGURIA	Albenga	EAL033	117	117	176	176	98	108	110	112	155	157	127	127	127	127	87	87
18	LIGURIA	Albenga	EAL036	121	121	178	186	108	108	110	110	145	157	127	127	127	141	87	87
19	LIGURIA	Albenga	EAL037	117	121	176	186	98	108	110	110	157	157	127	127	127	127	87	103
20	LIGURIA	Albenga	EAL047	125	153	176	186	108	108	110	110	155	157	127	127	99	127	87	87
21	LIGURIA	Albenga	EAL049	117	153	186	186	108	108	110	110	157	179	127	127	99	99	87	87
22	LIGURIA	Albenga	EAL081	117	125	176	186	108	108	110	110	155	179	127	127	127	129	87	87
23	LIGURIA	Albenga	EAL083	117	153	176	176	108	108	110	110	155	179	127	127	99	99	87	87
24	LIGURIA	Albenga	EAL093	117	117	176	186	108	108	110	110	179	179	127	127	99	99	87	87
25	LIGURIA	Albenga	EAL100	117	121	176	186	108	108	110	110	145	157	127	135	99	127	87	87
26	LIGURIA	Albenga	EAL101	117	121	176	186	108	108	110	110	145	145	127	135	127	141	87	87
27	LIGURIA	Albenga	EAL102	137	137	174	174	96	96	106	136	157	157	129	131	135	141	97	117
28	LIGURIA	Albenga	EAL103	133	143	162	188	96	96	110	128	157	173	129	129	131	145	91	107
29	PIEMONTE	San Genuario	EPI001	125	163	178	178	94	108	110	110	151	167	127	139	93	145	87	95
30	PIEMONTE	San Genuario	EPI002	163	163	178	194	108	108	110	110	145	151	127	127	99	141	87	87
31	PIEMONTE	San Genuario	EPI003	145	163	176	176	108	108	100	110	151	155	127	127	99	141	87	95
32	PIEMONTE	San Genuario	EPI004	125	163	176	178	94	108	108	110	155	155	127	127	141	145	87	87
33	PIEMONTE	San Genuario	EPI005	125	151	176	194	94	108	110	110	145	145	127	151	93	99	87	95
34	PIEMONTE	San Genuario	EPI006	159	161	176	178	94	108	96	110	145	151	127	127	141	141	87	95
35	PIEMONTE	San Genuario	EPI007	125	125	176	178	108	108	96	110	145	151	139	139	141	141	87	95
36	PIEMONTE	San Genuario	EPI008	125	163	176	176	108	108	110	110	151	157	127	127	99	145	87	95
37	PIEMONTE	San Genuario	EPI009	125	153	176	178	94	108	96	110	145	155	127	139	141	145	87	87
38	PIEMONTE	San Genuario	EPI010	125	145	176	178	94	108	100	110	145	151	127	127	99	141	87	95
39	PIEMONTE	San Genuario	EPI011	125	145	176	176	108	108	106	136	145	151	127	139	141	141	87	95
40	PIEMONTE	San Genuario	EPI012	125	125	176	178	94	94	110	110	155	167	127	127	141	145	87	87
41	PIEMONTE	San Genuario	EPI013	125	163	176	178	108	108	110	110	145	151	127	141	99	137	87	87
42	PIEMONTE	San Genuario	EPI014	125	157	176	178	94	108	110	110	151	167	127	151	141	141	87	95
43	PIEMONTE	San Genuario	EPI015	125	163	176	176	94	108	96	110	151	157	127	127	141	145	87	95
44	PIEMONTE	San Genuario	EPI016	125	163	176	176	108	108	110	110	151	157	127	127	99	145	95	107
45	PIEMONTE	San Genuario	EPI017	125	153	176	178	94	108	96	110	145	155	139	139	145	145	87	87

Figure 6. Example of a calculation table showing, for each turtle under study, the region and place of sampling, the sample name and the allele size for 8 of the 14 loci on which statistical analyses are subsequently performed.

One application of SNPs is in assignment tests, used to determine the likely population of origin of a specimen based on its genetic profile. Assignment tests involve comparing a specimen's SNP profile with the SNP profiles of reference populations to estimate the probability that the specimen belongs to each population. In particular, the presence or absence of certain SNP alleles can be used to infer a specimen's ancestral origin or population of origin. The process of using SNPs in an assignment test typically involves the following steps:

1. SNP data collection: The first step is to obtain the SNP profile from the specimen to be tested. This can be done by massively sequencing the DNA of the specimen under test. The data produced are then used to genotype the specimen by identifying the specific alleles at the various SNP loci.
2. Collection of reference population data: To conduct the assignment test, a comprehensive database of SNP profiles from the various reference populations of *E. orbicularis* defined in

Fig. 1 is required. These reference populations represent the diversity of genetic variation in different geographical regions of the species' range.

3. Data analysis: The SNP profile of the specimen is compared with data from the reference population. Bayesian statistical methods or likelihood tests are commonly used to estimate the probability that the specimen's genetic profile belongs to each of the reference populations. Based on the analysis, the assignment test provides probabilities or scores for the specimen's membership in each reference population. The population with the highest probability is considered the most likely source of ancestry for the specimen (see § 5.4).

It is important to note that even assignment tests using SNPs, as well as those based on microsatellites, are not absolute determinants of a specimen's ancestry or origin. They provide statistical probabilities based on the available data, and uncertainties arise from the complex nature of the dynamics of the populations under analysis and genetic drift. Moreover, these tests are most accurate when the reference populations closely correspond to the true ancestral populations of the specimen examined.

B.5 STATISTICAL ANALYSES

B.5.1 Mitochondrial DNA Diversity Analysis

Sequence variations in the control region of mitochondrial DNA are determined by the number of haplotypes and nucleotide segregating sites. The number of haplotypes refers to the number of sequences that differ by one or more nucleotides. Turtles that show the same sequence at the control region have the same haplotype. If we consider a set of aligned DNA sequences and compare each homologous nucleotide site between these sequences, the sites where the sequences differ in the nucleotide base are called segregating sites. The number of segregating sites gives an indication of the degree of DNA sequence variation in a sample.

A first parameter for quantifying the diversity of the mtDNA control region in a population is haplotype or gene diversity (*h*), i.e. the probability that two randomly selected haplotypes in a group of specimens are different (see formula in Nei, 1987). A second important measure of mitochondrial DNA polymorphism is the average number of nucleotide differences between all pairs of sequences. This parameter, weighted by the number of nucleotide sites in the sequence, is the average number of differences per site between all sequence pairs, or nucleotide diversity π (see formula in Nei, 1987).

B.5.2 Mitochondrial DNA Divergence Analysis

Estimation of the genetic distance between sequences in the mtDNA control region is performed by assessing the number of sites and the percentage of sites for which two haplotypes differ (pairwise difference and percentage difference, respectively). A series of corrections based on the percentage of nucleotide differences between the DNA sequences are generally used for multiple substitutions per nucleotide site (Jukes & Cantor, 1969; Tajima & Nei, 1984) and different substitution rates between transitions and transversions (Kimura 2-parameters, Kimura, 1980; Tamura & Nei, 1993) with unequal nucleotide frequencies (Tamura, 1992).

The degree of genetic diversity or similarity between the sequences of the mtDNA control region of *E. orbicularis* is assessed in particular by reconstructing the phylogenetic relationships between the sequences of specimens of unknown origin and the sequences of natural reference populations. Methods used to reconstruct phylogenetic relationships include 1) techniques that operate on a matrix of genetic distances between sequences (such as the neighbour-joining method), in which each genetic distance value is an estimate of the amount of divergence between two turtles since they last shared a common ancestor, and 2) discrete methods such as maximum parsimony, which directly considers variation at each nucleotide site and identifies the tree that requires the least amount of evolutionary change, or maximum likelihood, which selects the tree (or trees) that, of all of them, is most likely to have produced the observed data (Page & Holmes, 1998; Hall, 2001; Lemey et al., 2009).

Since a phylogenetic tree is usually constructed from a limited amount of data, it is important to examine the reliability of the obtained tree. Bootstrap analysis is a simple and effective technique for checking the relative stability of groups within the phylogenetic tree (Felsenstein 2004, p. 338). The basic idea of bootstrapping is to deduce the variability of an unknown distribution from which data have been extracted by resampling the data. In bootstrap resampling, the *n* nucleotide sites of a sequence are chosen randomly and are used to form a new set of DNA sequences, which is used to construct another tree. The topology of this tree is then compared with that of the original tree. Each inner branch of the original tree that provides the same partition of sequences as the simulated tree is given the value 1 (identity value), while the other inner branches are given the value 0. This process is repeated several hundred times and the percentage of times each inner branch of the original tree receives the identity value 1 is calculated. This is the bootstrap confidence value (Nei & Kumar, 2000 § 9.3). Branches of the tree or groups with confidence values greater than 75% are considered reliable, and the haplotype of a turtle of unknown origin is

therefore considered to belong to a group of haplotypes that characterise the turtles of a given area (Lemey et al., 2009 § 5.3.1).

B.5.3 Allelic Diversity Analysis at Microsatellite Loci

Genetic variation at microsatellite loci is quantified using allele and genotype frequencies. Genotype frequencies are represented by the proportion of specimens displaying a given genotype, while the description of gene frequency involves identifying the alleles present at each microsatellite locus and calculating the proportions of the different allelic types.

The proportion of heterozygous genotypes in a group of specimens is used to calculate the observed heterozygosity (H_O) for each locus and the average values on the 14 microsatellites for each group of specimens. Using allele frequencies, the expected heterozygosity under Hardy-Weinberg equilibrium (H_E) is calculated for each locus and averaged over the 14 microsatellite loci used in the analyses (Nei, 1987; Nei & Kumar, 2000).

The genetic properties of a population are influenced from one generation to the next by several factors, including mutations, immigration and emigration, differential mating and the sex ratio. Selection due to differences in parental fertility or different survival rates of offspring can also lead to variation in gene and genotype structure between generations. Of particular importance is the variation in gene frequencies between successive generations due to the random sampling of gametes that go on to form the zygotes of the new generation. The genes that are passed from one generation to the next are a sample of the parent generation, and the smaller the number of parents, the greater the variation due to allelic sampling. This dispersive process is also known as genetic drift and is particularly relevant when we consider the loss of genetic variability in small populations or limited groups of specimens maintained under captive conditions.

In order to assess whether the genetic structure of a group of specimens is influenced by these factors, the observed heterozygosity is compared with the heterozygosity expected in a panmictic (randomly mated) population with a very large number of specimens, in the absence of selection, mutation and migration. In this situation, the frequency of genes and genotypes does not vary significantly from one generation to the next. The population is said to be in Hardy-Weinberg equilibrium and the genotype frequencies predicted by the Hardy-Weinberg principle are calculated using the allele frequencies observed in the group of specimens under study as if they were the frequencies of the previous generation. If the difference between H_O and H_E values is not statistically significant, the group of specimens represents a population in Hardy-Weinberg

equilibrium. Conversely, if the values are statistically different, one or more factors affect the demography of the population, and in particular, if H_O is smaller than H_E it is very likely that the population in question consists of too small a number of specimens with low genetic variability and therefore requires demographic reinforcement.

The X^2 *goodness-of-fit test* can be used to test for a significant difference between H_O and H_E. However, as this test sometimes leads to false rejection or false confirmation of Hardy-Weinberg proportions when sample sizes are small, Fisher's exact probability test is generally used (Guo & Thompson, 1992; Raymond & Rousset, 1995).

B.5.4 Assignment of Specimens of Unknown Origin to Genetically Similar Populations

The assignment of turtles of unknown origin to the main Italian natural populations, represented in 29 continental sites, 4 in Sardinia and 6 in Sicily for *E. trinacris* (Fig. 1), is carried out using genotypic assignment tests developed by Piry et al. (2004). The belonging of specimens to a group of certain origin is confirmed or excluded based on the characterisation of multi-locus genotypes. The genotypic assignment criteria used are described in Baudouin & Lebrun (2000) and Paetkau et al. (1995). The analysis uses a Monte Carlo algorithm to calculate, for each specimen, the probability of belonging to a given reference population. The algorithm in question, based on the work of Paetkau et al. (2004), creates, on the basis of the observed multi-locus genotypes, a new population of the same size as the real one and calculates the probability of assigning specimens of unknown origin to the simulated population. The operation is repeated for 10,000 iterations. The assignment probabilities of specimens of unknown origin to the real population are compared with the values obtained from the simulations to obtain statistical significance of the probability (greater than 5%) of the observed assignment.

A second method of assigning multi-locus genotypes of specimens of unknown origin to the most genetically similar population is based on a Bayesian clustering modelling analysis developed by Pritchard et al. (2000). The method considers an unknown number of *K* populations characterised by a set of allele frequencies for each locus and attempts to probabilistically assign specimens on the basis of their genotypes to the populations, or jointly to two or more populations if their genotypes indicate a mixed origin. The programme estimates the most appropriate number (*K*) of populations needed to interpret the observed genotypes (posterior probability of *K*, or the probability of observing genotypes *X* given a certain *K*), and then provides the proportion of a

turtle's genome that comes from a certain population (cluster). The value of K with the maximum posterior probability is then used as preliminary information to estimate the probability that a specimen belongs to a certain cluster.

B.5.5 Kinship Analysis

The definition of captive breeding plans presupposes knowledge of the natural source populations of specimen breeding stock, determined by means of population structure analysis and genotypic assignment tests (§ 5.4). Having established the origin of the specimens, captive breeding aimed at reintroduction or demographic reinforcement aims to maintain genetic diversity and prevent inbreeding depression (i.e. breeding between inbred specimens). Inbreeding depression is a problem caused by physiological and/or morphological defects due to deleterious genetic mutations that are likely to occur mainly in small populations in which only mating between related specimens occurs. Thus, pairs or triplets of specimens (e.g. one male and two females) are selected that come from the same natural population but show a low degree of relatedness. In this way, the genetic diversity of the offspring is maximised and the risk of both inbreeding and outcrossing depression minimised. The latter factor defines a reduction in offspring fitness due to mating between specimens from different regions and the consequent loss in the offspring of specific gene interactions of specimens adapted to certain environmental conditions.

The degree of inbreeding within a population or group of specimens can be determined by calculating a coefficient of inbreeding or an index of identity. Since two closely related specimens are more likely to share identical alleles by descent than two unrelated specimens, r_{xy} = twice the probability that a randomly chosen allele from a specimen *x* is identical by descent to an allele also randomly chosen from a specimen *y* can be defined. Since closely related specimens are more likely to produce homozygous offspring, the effects of inbreeding as the proportion of homozygous loci (for identical alleles by offspring) can alternatively be measured in the offspring (Belkhir et al., 2002).

Once the pairs or triplets of specimens have been determined for mating and following the production of eggs by the female, the kinship determination between the possible parents and the offspring is carried out to exclude fertilisation of the eggs by sperm from previous matings. Kinship is determined, for each microsatellite, by identifying the presence of the alleles of each offspring in the parents. Since microsatellites are genetic markers of biparental inheritance, the two alleles

present in the offspring must be found one in the father's genotype and one in the mother's genotype respectively.

For example, if two parents are heterozygous for the alleles 240,244 (the father) and 244,248 (the mother) respectively, and two offsprings show heterozygous 240,244 and 244,248 genotypes, for the first offspring the 240 allele is necessarily inherited from the father and the 244 from the mother, while the second heterozygous offspring 244,248 will have the 248 allele inherited from the mother and therefore the 244 from the father (Fig. 7).

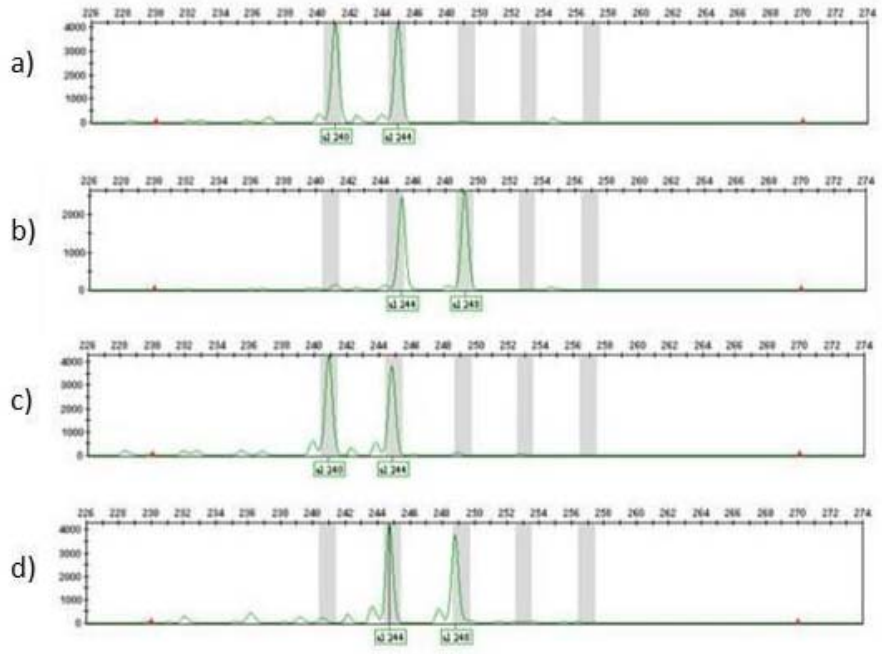


Figure 7. Genotypes for a single microsatellite locus of two parents (a, b) and their offspring (c, d).

Kinship reconstruction is performed using multi-locus genotypes and in the case of *E. orbicularis* by comparing alleles at 14 microsatellite loci between parents and offspring.

A simple approach to kinship analysis is based on an exclusion process. The genotypes of the candidate parents are compared with the genotype of the offspring (taking into account the genotype of the other parent, if available). Possible parents are excluded as such if there is a mismatch at one or more loci. The presence or absence of paternal or maternal alleles is also used to identify multiple paternity events. Figure 8 shows the multi-locus genotypes of two *E. orbicularis* females (in red) and those of their respective offspring. The Emys3 locus shows, for both females, the contribution of 3 male alleles to the offspring and thus evidence of polyandry.

With few candidate parents and highly polymorphic loci, this process should usually leave only one candidate parent unexcluded for both sexes. However, in less favourable circumstances, it is

common for multiple candidate parents to remain non-excluded. In this case, the exclusion approach is inappropriate because it is not possible to identify which non-excluded candidate parent is the actual parent. Even in the case of multiple paternity linked to the choice of triads (two males and one female) rather than pairs, the number of previous, unmonitored mating may be such that it leads to a decrease in the level of definition.

Animale #	Nido	Emys1		Emys2		Emys3		Emys4		Emys5		Emys6		Emys7		Emys8		Emys11	
EMR044	DAM A	117	117	182	186	107	107	100	110	116	124	153	153	123	123	127	163	103	105
EMR176	NIDO 044	117	117	186	186	107	107	100	110	116	126	153	173	123	123	127	157	103	105
EMR177	NIDO 044	117	159	180	182	107	131	100	100	124	124	153	153	123	137	157	163	95	105
EMR178	NIDO 044	117	117	180	186	107	135	110	110	114	116	153	153	123	137	127	157	103	103
EMR049	DAM A	117	117	180	206	107	123	100	110	114	114	153	153	123	123	127	159	103	103
EMR169	NIDO 049	117	159	180	182	107	131	100	110	114	118	149	153	123	137	127	159	103	103
EMR170	NIDO 049	117	159	180	182	107	131	100	110	114	114	149	153	123	137	127	157	103	103
EMR171	NIDO 049	117	117	180	206	113	123	110	110	114	126	153	173	123	123	157	159	103	103
EMR172	NIDO 049	117	117	206	206	123	135	100	100	114	114	153	153	123	123	157	159	103	103

Figure 8. Multi-locus genotypes of two females (in red) and their respective offspring (in black). Following a comparison based on the biparental inheritance of the alleles, the Emys3 locus shows the presence, in both depositions, of 3 alleles of paternal origin and thus shows multiple paternity in both cases.
From the figure above: Animale = Animal - Nido = Nest

In these cases, a likelihood estimate is used, the aim of which is to find the parameters that are most likely to have produced the observed data, e.g. the probability that a multi-locus genotype matches the parent's genotype given the allele distributions in the offspring's genotype.

In particular, the likelihood that, for example, a given male is the true father (Likelihood of paternity) is calculated. This value is then compared with the likelihood that any male in the population is the true father, and the logarithm of the likelihood ratio (LOD - Log-Likelihood ratio) is calculated. A value of LOD = 0 implies that the putative father has the same probability of being the father of the offspring as a randomly selected male. A positive LOD value implies that it is more likely that the alleged father is the true father of the offspring than any random male. Negative LOD values can occur if the putative father and offspring share a set of alleles that are often found within the population. Most commonly, negative LOD values indicate that the genotype of an alleged parent does not match that of the offspring at one or more loci.

In the case of a breeding pair and the presence, in the offspring, of multi-locus genotypes of two or more males with a positive and relatively high LOD value, a second parameter is calculated, i.e.

the difference between the LOD value of the most probable parent and the LOD value of the second most probable parent: $\Delta = LOD_1 - LOD_2$. Subsequently, simulated genotypes are generated for a relatively high number of offspring (e.g. 10,000). Each of these genotypes are used to determine the value of Δ for the likely parents, and thus produce a distribution of Δ values resulting from the simulations, against which the actual Δ value is compared (Marshall et al., 1998).

C. BREEDING AND MAINTENANCE CENTRES OF *EMYS ORBICULARIS* IN LIFE URCA PRO*EMYS*

Respect for the welfare of the animals is a crucial requirement of the LIFE URCA PRO*EMYS* project, both for ethical reasons and to achieve the project objectives; poor welfare conditions can in fact encourage disease, increase mortality and compromise reproductive success.

Some minimum requirements for facilities used for breeding and maintaining turtles *ex situ* are outlined below.

C.1 CHARACTERISTICS OF REPRODUCTION AND BREEDING CENTRES (MINIMUM REQUIREMENTS)

In order to ensure the welfare of the animals, all breeding and rearing centres must meet minimum standards of maintenance and management. The centres must have an outdoor enclosure, with semi-natural environmental conditions, and an indoor enclosure, with controlled environmental conditions.

La Bora breeding centre (San Giovanni in Persiceto, Bologna).

Outdoor Enclosure

The outdoor enclosure will have to house the breeding individuals and must therefore be subject to the natural cycles of the seasons, both in terms of photoperiod and air and water temperature. Below are the minimum requirements:

- The outdoor enclosure should consist of a minimum of three tanks: one for breeding individuals, one for immatures, and one for animals under treatment. The tanks should be at least 70 cm deep to avoid excessive temperature peaks (positive or negative) in the summer and winter seasons.
- The tanks should be equipped with water recirculation and purification systems, with mechanical and/or phyto-purification filters and/or a regular water change system.
- The tanks should accommodate a number of animals compatible with the size of the tanks, avoiding overcrowding situations that could cause stress to the animals and inadequate water quality. The ratio can be considered suitable when each adult individual has 2 square metres of surface area; for sub-adult turtles and juveniles aged 1-3 years the minimum surface area required drops to 1 square metre and 0.5 square metre respectively.
- The tanks should provide suitable areas for basking (exposure to the sun), egg-laying and hibernation, taking into account the number of specimens housed; it is important for the welfare of the animals that they also have submerged, dimly lit areas created by riparian vegetation and floating objects (e.g. pieces of cork bark).
- The structures should provide adequate fencing and wire mesh covering to prevent escape of turtles and entry of predators.
- The structures should be equipped with a video surveillance system.

Indoor Enclosure

- The indoor enclosure shall house new-born individuals (less than one year old) under controlled environmental conditions. Keeping them in controlled conditions will avoid the first winter hibernation, thus significantly decreasing mortality in the first year of life and increasing the growth rate. After the first winter season, at about one year of age, immature turtles can be transferred to the outdoor enclosure to continue development in semi-natural conditions before release (which will occur at around two years of age).
- The indoor enclosure should consist of at least one tank to house the newborns, of sufficient size to avoid overcrowding and ensure the welfare of the animals. The size can

be considered suitable when each individual has a minimum volume of 15 litres, with a water depth between 15 and 20 centimetres.

- The water temperature should be maintained in a range between 25 and 28°C to ensure that the turtles are active and feed regularly even during the cold season.
- The enclosure should be equipped with a filtration system that is adequate for the volume of water and the number of turtles housed; alternatively, sufficient water exchange must be ensured to maintain a suitable quality of the breeding environment.
- The indoor enclosure should be equipped with infrared and UVB lighting, with a photoperiod similar to the natural one, and areas for basking and thermoregulation.
- It is important that the tank is set up with plenty of submerged vegetation (even synthetic plants), which can serve as shelter and a foothold to easily reach the surface.
- The indoor enclosure should also include an artificial incubator, which can be used to improve the reproductive success of the turtles and/or to determine the sex of the offspring (by adjusting the incubation temperature of the eggs).
- If the centre has difficulty in setting up an indoor enclosure, it may rely on another public or private breeding centre that is willing to temporarily (at least for the first year of life) accommodate new hatchlings in its facilities.

D. SUITABILITY CRITERIA FOR THE IDENTIFICATION OF RESTOCKING AREAS OF *E. ORBICULARIS* FROM CAPTIVITY

It is important to select suitable sites for restocking and/or reintroduction actions. These sites, characterised by habitat/microhabitat, must guarantee the survival, reproduction and increase of populations of *E. orbicularis* over the long term within the areas (Protected Areas, Natura 2000 Areas, etc.) involved in these actions, providing for minimum maintenance over the long term. The selection is based on some binding criteria and some other 'outline' criteria that allow the site's characteristics to be better defined and possible improvement actions to be identified. The following sheet summarises the criteria required for a suitability assessment. The result of filling in the form should allow the site to be declared "suitable" (high suitability), "potentially suitable" (medium suitability) if some improvement actions or measures are taken, or "unsuitable" (low suitability) if there is not even room for improvement.

The criteria take into consideration the location, the management of the area, the context in which it is located, the habitat for *Emys orbicularis*, threats and any previous (or ongoing) conservation actions.

D.1 SUITABILITY CRITERIA SHEET FOR THE IDENTIFICATION OF AREAS SUITABLE FOR RESTOCKING OF *E. ORBICULARIS* FROM CAPTIVITY

GENERAL DATA

Area: (name of protected area/Natura 2000, reserve etc.) _____
Extension of the area[16] (ha)_____
Extension of the species habitat[17] (ha) _____
Region_____ **Municipality (Province)** _____ **Location** _____
Managing body of the area _____
Reference population of *E. orbicularis*: Southern ___ Adriatic___ Tyrrhenian _ __
Nearest *E. orbicularis* breeding centre __ ___ ___ ____ ____ __ ___ ___ __KM____

Wetland within a protected area (Nature Park, Reserve, Oasis, Special Conservation Area, Special Protection Area, etc.)	YES			NO
Availability of the area	Suitable[18]	Potentially suitable		Not suitable
SPATIAL CONTEXT				
Connectivity (water matrix). Means the connection with other external viable populations or habitats suitable for colonisation	Suitable[19]	Potentially suitable[20]	Not suitable[21]	Do not know
Land use within 500 m of the wetland	**Suitable** Natural or semi-natural (prevalence of areas not used by man or with extensive agro-sylvo-pastoral activities)		**Not Suitable** Human presence (prevalence of artificial areas or areas used for intensive agriculture)	
HABITAT				
Heterogeneity within the species habitat	**High** (e.g. presence of permanent and temporary wetlands,	**Medium** (e.g. clear dominance of one type over another)		**Low** (e.g. presence of only one type)

[16] This means the entire extension of the protected area or site.
[17] Indicate the extension of the area used by turtles, which is generally smaller than the extension mentioned in the previous point.
[18] "Suitable" means if the area is owned by the body that manages the reintroduction or restocking actions, or if there is a convention/agreement with an appropriate subject to follow these activities; "Potentially suitable" means when there is the possibility of acquiring or signing a convention or agreement for its use; "Not suitable" when there are no guarantees to manage restocking adequately.
[19] Presence of canals and/or watercourses crossing the wetland that connect to other viable populations or habitats suitable for colonisation.
[20] Presence of obstacles/barriers that can be removed or bypassed, allowing connection to other viable populations or habitats suitable for colonisation.
[21] Presence of obstacles or infrastructure that prevent connectivity by isolating the area and cannot be removed or bypassed.

	canals, lakes, ponds)			
Slope of the banks	**Low** (mostly <25°)	**Medium** (mostly between 25° and 45°)	**High** (mostly >45°)	
Water speed	Lentic waters	Slightly lotic waters	Lotic waters	
Shallow water areas (max. 2 metres)	>30% of the body of water	<30% of the body of water		
Vegetation (helophyte cover)	>60% of the banks	between 30 and 60% of the banks	<30% of the banks	
Presence of basking sites (thermoregulation)	**High** (widespread presence of logs emerging from the water and hydrophytic/helophytic vegetation)	**Medium** (presence of logs emerging from water and/or hydrophytic/helophytic vegetation)	**Low** (low presence of logs emerging from water and hydrophytic/helophytic vegetation)	
Presence of suitable nesting sites within 100 m of the wetland	**Suitable** (>30% of land occupied by herbaceous/shrub vegetation)	**Potentially suitable** – bring to at least 30%	**Not Suitable**	
THREATS				
Presence of allochthonous turtles	**Suitable**[22]	**Potentially suitable**[23]	**Not suitable**[24]	**Don't know**
Presence of predatory alien fish species	**Suitable**[25]	**Potentially suitable**[26]	**Not suitable**[27]	**Don't know**
Alien fish species present				
Other alien species dangerous to *Emys*				
Presence of heronries in or near the wetland (< 5 km)	**YES** (abundant presence of nests and diversity of species)	**YES** (low presence of nests and species diversity)	**None**	
Presence of disturbing human activities in the wetland	**Suitable** (none)	**Potentially suitable** (there are some, but they can be eliminated or regulated)	**Not suitable** (no disturbing human activities can be restricted)	
Disturbing human activities *(entry of the specific pressure and threat code in the Pressures and Threats Annex is optional)*	A. Agriculture _ _ _ Code _ _ B. Forestry _ _ Code _ _ C. Mining, quarrying and energy production _ _ Code _ _ D. Infrastructure for energy production and transport _ _ Code _ _ E. Urbanisation, residential and commercial development _ _ Code _ _ F. Biological resources excluding agriculture and forestry _ __ _ _ _ Code _ _			

[22] No or minimal presence of alien swamp turtles and/or they are controlled by the managing body.

[23] Presence of alien swamp turtles that can be removed.

[24] Abundant presence of non-removable allochthonous swamp turtles.

[25] Not present or insignificant presence.

[26] Presence of predatory allochthonous ichthyofauna that can be contained or removed.

[27] Presence of predatory allochthonous ichthyofauna that cannot be contained or removed.

	G. Human intrusion and disturbance _ _ Code _ _ H. Pollution _ _ Code _ _ I. Other invasive or problematic species and genes _ _ Code _ _ J. Changes in natural systems _ _ Code _ _ K. Natural biotic and abiotic processes (excluding catastrophes) _ _ Code _ _ L. Geological events, natural disasters _ _ Code _ _ M. Climate change _ _ Code_ _ XO Threats or pressures from outside the Member State _ _ Code _ _ XE Threats or pressures originating outside the EU _ _ Code _ _

PREVIOUS CONSERVATION ACTIONS			
Is *E. orbicularis* present in the area?	YES (currently or historically, please specify)	NO	Don't know
The population is:	viable	Made up of few specimens	Don't know
Has *E. orbicularis* been reintroduced?	YES	NO	Don't know
If yes when and how many	Year and No.	Year and No.	Year and No.
Were restocking actions carried out?	YES	NO	Don't know
If yes when and how many (at least enter the last three restocking operations)	Year and No.	Year and No.	Year of last restocking and No.
Origin of the re-introduced *Emys* (specify 'don't know' if the origin is unknown)			
Results	Good[28]	Insufficient[29]	Don't know

NOTES
DATE___ ___ ___ COMPLETED BY First and last name _____ _____ _____ _____ _____ E-mail _____ Telephone _____

[28] Significant population increase observed

[29] More or less stable population and insignificant or no increase

D.2 PRELIMINARY LIST OF NATURA 2000 AREAS FOR RESTOCKING IN LIFE URCA PRO*EMYS*

The following is a preliminary list and was drawn up based on indications obtained from the Managing Authorities and other individuals and entities with a direct role in the management of the Areas.

ABRUZZO:

IT7140215 “Lago di Serranella e Colline di Guarenna”

BASILICATA:

IT9220055 “Bosco Pantano di Policoro e Costa ionica Foce Sinni” *

IT9220080 “Costa Ionica Foce Agri”

EMILIA-ROMAGNA:

IT4040015 “Valle di Gruppo”

IT4030007 “Fontanili di Corte Valle Re”

IT4040007 “Salse di Nirano”

IT4030011 “Casse di espansione del Secchia”

IT4020001 “Boschi di Carrega”

IT4020025 “Parma Morta”

IT4020006 “Monte Prinzera”

IT4090002 “Torriana, Montebello, Fiume Marecchia”

IT4020017 “Aree delle risorgive di Viarolo, Bacini di Torrile, Fascia golenale del Po”

IT4050019 “La Bora”

IT4040009 “Manzolino”

IT4050025 “Biotopi e ripristini ambientali di Crevalcore”

IT4050031 “Cassa di espansione del torrente Samoggia”

IT4050023 “Biotopi e ripristini ambientali di Budrio e Minerbio”

LIGURIA:

IT1324909 “Torrenti Arroscia e Centa”

IT1324896 “Lerrone – Valloni”

MARCHE:

IT5310022 “Fiume Metauro da Piano di Zucca alla foce”

LAZIO

IT6030023 “Macchia Grande di Focene e Macchia dello Stagneto”

PIEMONTE:

IT1120007 “Palude di San Genuario”

IT1180005 “Ghiaia Grande”

IT1180028 “Fiume Po - tratto vercellese alessandrino”

E. CONTROL AND/OR ERADICATION OF ALIEN TURTLE SPECIES

E.1 INTRODUCTION AND CONTEXT ANALYSIS

Invasive Alien Species (IAS) are considered one of the main direct drivers of biodiversity loss after habitat destruction (http://www.cbd.int/invasive/). It has also been estimated that invasive alien species could cost the world economy up to 5% of global GDP (Pimental et al., 2005). The cost to EU Member States, mainly in terms of management or direct economic impacts rather than ecological consequences, could reach approximately 12 billion Euros per year (https://environment.ec.europa.eu/topics/nature-and-biodiversity/invasive-alien-species_en).

Pets have been kept and transported by humans for millennia, and currently half of European households keep pets, with species belonging to a wide range of taxa (including mammals, birds, reptiles, amphibians, fish and aquatic and terrestrial invertebrates).

However, the constant increase in international trade has led to an escalation of biological invasions worldwide, with no clear signs of stabilisation in the near future (Hulme, 2009; Seebens et al., 2015), leading to a worsening of the conservation status at the scale of biogeographic regions of species and habitats, already compromised by a wide range of impacts attributable to human activities, as well as endangering the few environments still intact.

From an ecological point of view, the costs may concern various criticalities caused to native species and habitats. The introduction of elements outside their native geographic range and their potential spread affect not only native biodiversity and ecosystem services through, for example, competitive substitution (Bertolino et al., 2014) or hybridisation with native taxa (Beukema et al, 2015; van Riemsdijk et al., 2020), pathogen transmission (Kilpatrick et al., 2010; Mihalca et al., 2017) and food web modification (David et al., 2017), but also on human health (Pauwels et al., 2015; Schindler et al., 2015) and the economy (Kettunen et al., 2009; Lovell et al., 2006). Management of invasive alien species translates into prevention, eradication and control, the success of which relies primarily on early detection of incipient populations (Eyre et al., 2021).

Also for reptiles, the exotic pet trade is the most significant cause of the introduction of alien taxa worldwide (Kraus, 2009), and the highest densities of exotic reptiles in Europe have been recorded in aquatic habitats (Pysek et al., 2010). Freshwater turtles in particular are very popular in the pet trade and are very frequently abandoned or released into the wild, where they may naturalise after a variable period depending on the species and territory (Masin et al., 2014; Perry et al.,

2007). Although turtles are considered a relatively species-poor order, they have the highest number of introduction occurrences among reptiles (Kraus, 2009): at least 61 species of chelonians have been recorded in the global pet trade (Gong et al., 2009), and at least 17 alien turtles have been reported in Europe in the wild, including 13 freshwater turtles (Kopecky et al., 2013; Kalaentzis et al., 2023). 'Hotspots' of allochthonous chelonians have been reported in Italy with around ten species present syntopically, several of which are already successfully reproducing (Ferri et al., 2020, 2021; Di Blasio et al., 2021).

Among freshwater turtles, the subspecies and hybrids of the Pond slider *Trachemys scripta* (Thunberg in Schoepff, 1792) are historically very famous. Turtles belonging to this group were traded on a global scale (Ballasina, 1995; Kraus, 2009) and were usually sold in their juvenile stages, facilitating their release into the wild once they had grown up and could no longer be managed in households (Crescente et al., 2014; Foglini et al., 2017).

Trachemys ssp. poses a significant threat to native freshwater turtles, with which it competes for food sources, basking and nesting sites (Polo-Cavia et al., 2010; 2014). *T. scripta elegans* (Wied, 1838) is, in particular, according to the Global Invasive Species Database (GISD), one of the most damaging IAS in the world (GISD—Global Invasive Species Database, 2023) and in 2016 was listed as an IAS of EU interest (EU Regulation 2016/1141). It has been the most traded subspecies of freshwater turtle since the 1950s and until the ban of its introduction in the EU in 1997 (EU Regulation 338/1997; EU Regulation 349/2003). So far, spawning populations have been reported in Spain (Pleguezuelos et al., 2003), Italy (Ficetola et al., 2003), France (Cadi et al., 2004), Portugal (Martins et al., 2018) and possibly also in Greece (Bruekers et al., 2006; Tzoras et al., 2018), Serbia (Đordević et al., 2015), and Slovenia (Standfuss et al., 2016). Following the European import ban on *T. s. elegans* in 1997, the interest of the pet trade shifted not only to other subspecies and hybrids of *T. scripta*, but also to other turtle species (Scalera, 2007, Ficetola et al., 2012).

Apalone, *Graptemys*, *Kinosternon*, *Mauremys*, *Pelodiscus*, *Pelomedusa*, *Pseudemys* and *Sternotherus* are just a few examples among the most traded genera, due to a very affordable retail price per juvenile specimen (Masin et al., 2014; Foglini, 2021). On the other hand, some species, such as *Chelydra serpentina* (Linnaeus, 1758) and *Macrochelys temminckii* (Troost in Harlan, 1835) are banned in some EU countries (Italy and Germany) as they are considered 'dangerous species' (Masin et al., 2014).

In Italy, Art. 12 of Presidential Decree No. 357 dated 8 September 1997 and subsequent amendments states that "*The reintroduction, introduction and repopulation in the wild of non-*

native species and populations are prohibited." This principle is reiterated and better defined by the Decree of the Ministry of the Environment and Protection of Land and Sea of 2 April 2020, which clarifies the "*Criteria for the reintroduction and repopulation of native species in Annex D of Presidential Decree No. 357 of 8 September 1997, and for the introduction of non-native species and populations.*" As of today, therefore, there is a ban on the deliberate introduction of non-native species on Italian territory, including turtle species that are commercially available and freely sold. Following a specific risk analysis carried out at European level, with Regulation No. 1143/2014, which came into force on 1st January 2015, the European Union has organically established rules to prevent, minimise and mitigate the negative effects on biodiversity caused by the introduction and spread, both deliberate and accidental, of invasive alien species within the Union. Of these, the only freshwater turtle species listed in the Regulation is *T. scripta*, including its subspecies and hybrids. On 30 January 2018, the Legislative Decree 230 dated 15 December 2017 was published to adapt national legislation to the provisions of Regulation 1143/2014, which entered into force on 14 February 2018. The list of species subject to the indications of the Regulation is updated periodically, and to date *T. scripta* remains the only turtle species included. In Slovenia, rules on alien species are included in the Nature Conservation Act (National Official Journal No. 96/04 – amended official text, 61/06 – ZDru-1, 8/10 – ZSKZ-B, 46/14, 21/18 – ZNOrg, 31/18 82/20, 3/22 – ZDeb, 105/22 – ZZNŠPP and 18/23 – ZDU-1O). This law defines alien animal and plant species and contains provisions on standards and requirements concerning the intentional introduction of alien species and the breeding of alien animals. However, Slovenia does not yet have measures in place to reduce and eliminate the negative impacts of settled ITV.

The recognition as an invasive alien species of EU interest determines prohibitions but also obligations for EU Member States, which, following the amendment and adaptation of their legislation, must implement all appropriate strategies to counteract the spread of the species in the wild and preserve ecosystems and native species. In Italy, *T. scripta* is currently present throughout the entire territory, including the major islands. The species is most widespread in northern Italy (Ferri & Soccini, 2008), but also in central Italy, with particular reference to Tuscany and Latium (Ferri et al., 2019), while distribution in southern Italy and the islands is more localised and punctiform.

In 2022, the Italian Ministry of Ecological Transition (now Ministry of the Environment and Energy Security) in collaboration with ISPRA drew up a specific "*National Management Plan for the American pond slider (Trachemys scripta)*" in accordance with the requirements of Reg.

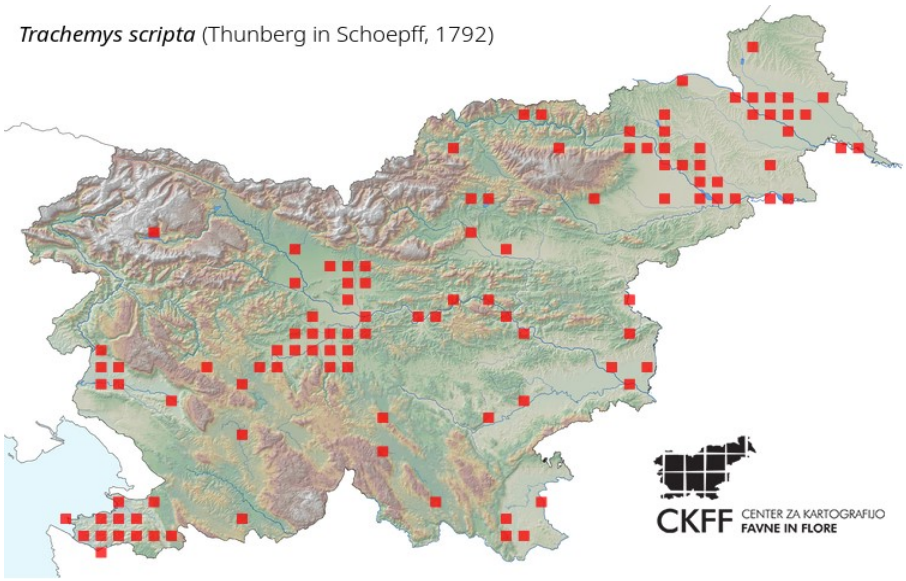
1143/2014. The Plan defines objectives and strategies for the prevention of introduction and expansion and management of *T. scripta* in the national territory.

As a result, *T. scripta* is the priority alien freshwater turtle species on which the planning of actions and strategies is necessary and required, at a national but above all at a regional and local level, for its containment and where possible eradication in order to preserve the native populations of *Emys orbicularis* and *Emys trinacris*.

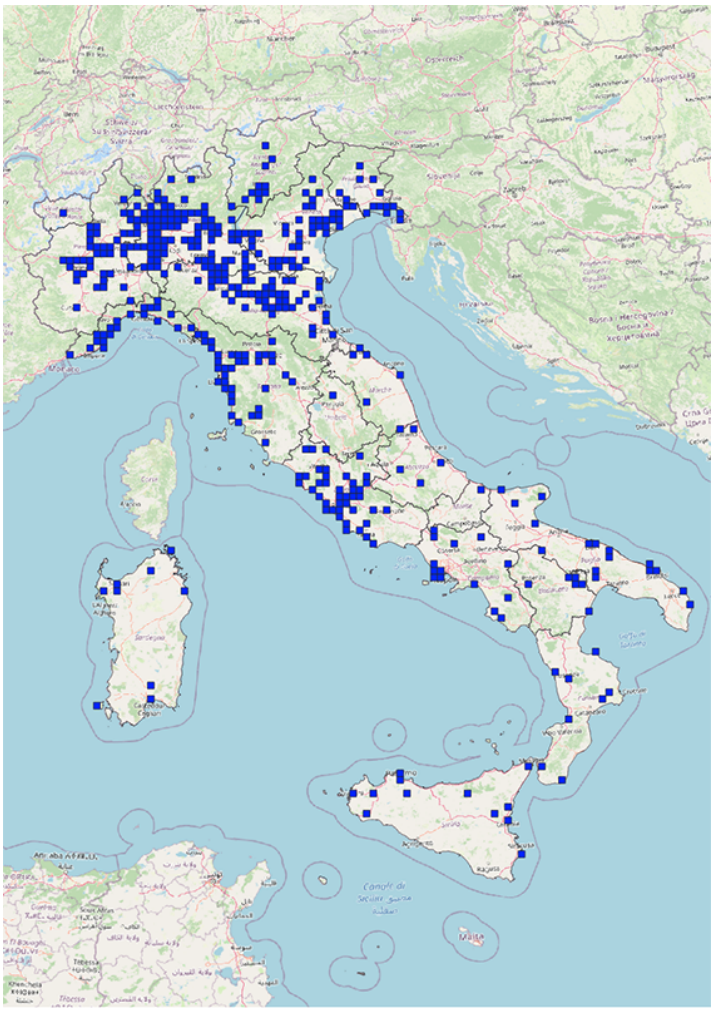
According to the national *Trachemys* plan (page 18), in the case of widespread occurrence in the wild, control can be carried out by euthanasia killing or transfer to permanent (regional) holding centres. However, as part of the overall management of the species, the National Plan emphasises that priority should be given to euthanasic suppression of specimens caught in the wild, and precisely to ensure that individuals can entrust their pets to centres.

In Slovenia the species is widespread and colonises mainly ponds, former gravel pits, oxbows, lakes, but also rivers (e.g. Ljubljanica, Vipava, Krka) and streams. Most data on its occurrence come from Ljubljana, Nova Gorica, the coastal area and a larger area around Maribor and Murska Sobota. The species successfully breeds in Primorska, the Vipava Valley, and parts of central Slovenia.

In 2018, the Institute of the Republic of Slovenia for Nature Conservation prepared guidelines for the management of invasive exotic species widespread in Slovenia, and for *Trachemys scripta*, including the description of the species, its status in Slovenia, introduction routes and recommendations for management measures (preventive measures, control in the wild and handling of specimens).



Distribution of Trachemys scripta in Slovenia
(source: CKFF, 2023. Karta razširjenost Trachemys scripta. Center za kartografijo favne in flore (stanje: 1.12.2023)).



Distribution of *Trachemys scripta* in Italy
Source: (Carnevali et al., 2021)

Finally, the opening up of markets to new species of freshwater turtles has already led to the introduction of other species of turtles that are allochthonous to the wild and potentially invasive (Ficetola et al., 2007; Ficetola, 2009; authors pers. comm.), with effects on which suitable assessments and analyses have not yet been carried out, but which may potentially cause damage to native biodiversity. Therefore, while focusing on the species of EU interest, i.e. *T. scripta*, it is considered essential that the same strategy is adopted for all alien freshwater turtle species transiting on Italian and Slovenian territory, as a precautionary principle. The Slovenian guidelines for the management of *Trachemys scripta* also stresses that it is important to make buyers of exotic turtles that are not yet invasive and still available on the market aware of the longevity and proper care of freshwater turtles in order to prevent their release into the wild.

E.2 RELEVANT LEGISLATION

The following is a list of the main reference regulations and plans regarding the management of alien species for the Italian territory, including invasive alien species (IAS).

E.2.1 EU Regulations

Regulation	Subject Matter
EU Regulation No. 1143/2014 dated 22 October 2014	Provisions to prevent and manage the introduction and spread of invasive alien species
EU Implementing Regulation 2016/1141 dated 13 July 2016	Adoption of a list of invasive alien species of EU relevance pursuant to EU Regulation No. 1143/2014 of the European Parliament and of the Council
EU Implementing Regulation 2017/1454 dated 10 August 2017	Specification of the technical formats for reporting by the Member States pursuant to Regulation No. 1143/2014 of the European Parliament and of the Council
EU Implementing Regulation 2022/1203 dated 12 July 2022	Amendment of EU Implementing Regulation 2016/1141 to update the list of invasive alien species of EU relevance

E.2.2 National Legislations

ITALY

Presidential Decree No. 357 dated 8 September 1997	Regulation implementing Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora Issues concerning allochthonous species: Art. 2 Definitions: *r) Introduction: The introduction of an animal or plant specimen into a territory outside its natural range*. Art. 12 Paragraph 3: *Reintroduction, introduction and repopulation in the wild of non-native species and populations are prohibited.*
Presidential Decree No. 102 dated 5 July 2019	Regulation containing further amendments to Article 12 of Presidential Decree No. 357 dated 8 September 1997 on the implementation of Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora Issues concerning alien species: Art. 1 Definitions: *r-bis) Release: Any action of introduction, reintroduction and repopulation of specimens of non-native species and populations*. Article 2 Amendments to Article 12 of Presidential Decree No. 357 dated 8 September 1997: 1. *The Ministry of the Environment and Protection of the Land and Sea, having consulted the Ministry of Agricultural, Food, Forestry and Tourism Policies, the Ministry of Health, and the Permanent Conference for Relations between the State, the Regions and the Autonomous Provinces of Trento and Bolzano, after receiving the opinion of the Council of the National System referred to in Article 13, paragraph 2, of Law 28 June 2016, No. 132, shall adopt by its own decree the criteria for the reintroduction and repopulation of the native species referred to in Annex D, as well as for the introduction of non-native species and populations referred to in paragraph 3, in compliance with the purposes of this Regulation and the health and welfare of the species.* 3. *The release of non-indigenous species and populations into the wild is prohibited, except as provided for in paragraph 4. This prohibition also*

	applies to species and populations indigenous to Italian territory when their introduction affects portions of territory outside their natural range, according to the criteria set out in paragraph 1. *4. At the request of the regions, the autonomous provinces of Trento and Bolzano or the management bodies of the national protected areas, the introduction into the wild of the non-native species and populations referred to in paragraph 3 may be authorised for justified reasons of overriding public interest, related to environmental, economic, social and cultural needs, and in any case in such a way that no harm is caused to the natural habitats in their natural range or to the local wild fauna and flora.*
Presidential Decree No. 230 of 15 December 2017	Adaptation of national legislation to the provisions of EU Regulation No. 1143/2014 of the European Parliament and of the Council dated 22 October 2014, laying down provisions to prevent and manage the introduction and spread of invasive alien species Topics related to alien species: Art. 1 Purpose: *1. This Decree lays down measures for the adaptation of national legislation to the provisions of EU Regulation No. 1143 of the European Parliament and of the Council dated 22 October 2014, hereinafter Regulation, with particular reference to:* *a) The official controls necessary to prevent the deliberate introduction of invasive alien species of EU relevance;* *b) The issuing of permits provided for in Articles 8 and 9 of the Regulation;* *c) The establishment of the national surveillance system provided for in Article 14 of the Regulation;* *d) The management measures for the eradication, population control or containment of populations of invasive alien species of Union, transnational or national importance;* *e) The discipline of sanctions for the violation of the provisions of the Regulation and of this Decree.*
DD Ministry of Ecological Transition 16 March 2022	Identification of roles and tasks within the Surveillance System of Specimens of Invasive Alien Species of Union and National Importance and definition of the Guidelines for the setting of regional monitoring systems and programmes, pursuant to Article 18 of Legislative Decree No. 230 dated 15 December 2017 Issues concerning alien species: *Art. 2 Roles and tasks within the Surveillance System* *Art. 3 Monitoring of invasive alien species of EU and national importance still absent from the territory of the region or autonomous province* *Art. 4 Monitoring of invasive alien species of EU and national importance already present in the regional territory or in the autonomous province* *Art. 5 Data collection, validation and transmission*
Annex I to the DD dated 16 March 2022	"Guidelines for the drafting of monitoring programmes for invasive alien species of EU or national relevance", states among other things: *For the monitoring of exotic species of EU relevance, the Regions and Autonomous Provinces make use of a specific structure capable of organising monitoring activities and providing for the centralised collection and analysis of information. The structure is composed of personnel trained and competent in the field of invasive alien species and the planning of monitoring activities.* *In order to optimise resources, the monitoring of exotic species of EU relevance makes use, whenever possible, of the monitoring structures and networks that already exist on the territory, such as those in charge of implementing Article 11 of Directive 92/43/EEC ("Habitats Directive"), Article 8 of Directive 2000/60/EC ("Water Directive") and Article 11 of Directive 2008/56/EC (Marine Strategy Framework Directive).*
DL 5 August 2022 No. 135	Provisions for the implementation of EU Regulation 2016/429 of the European Parliament and of the Council of 9 March 2016 on trade, import, conservation

	of animals of wild and exotic fauna and training for animal handlers and professionals, also with a view to reducing the risk of outbreaks of zoonoses, as well as the introduction of criminal provisions aimed at punishing illegal trade in protected species, pursuant to Article 14, paragraph 2, letters a), b), n), o), p) and q), of Law No. 53 dated 22 April 2021. Issues concerning alien species: Article 1 Scope and definitions: *c) Alien or exotic or alien species: a species that does not belong to the native fauna or flora of a given geographical area, but has arrived there through direct intentional or accidental human intervention;* *e) Establishment: Premises and facilities of any kind or, in the case of open-air breeding, any room or place where animals or germinal material are kept, on a temporary or permanent basis, excluding dwellings in which pet animals are kept, surgeries or veterinary clinics, as referred to in Article 4, Item 27) of EU Regulation 2016/429;* Article 3 Prohibitions concerning live specimens of wild and exotic species and their hybrids: *1) Without prejudice to the provisions of paragraph 2, it is prohibited for any person to import, possess, trade and reproduce live animals of wild and exotic species taken from their natural environment as well as hybrids between specimens of the aforementioned species and other wild species or domestic forms taken from their natural environment.* *2) Notwithstanding the provisions of Legislative Decree No. 230 dated 15 December 2017 and Article 4, the prohibition referred to in paragraph 1 shall not apply:* *e) To establishments authorised pursuant to Articles 8 and 10 of Legislative Decree No. 230 dated 15 December 2017.* Article 4 Dangerous species to health, public safety, or biodiversity: *1. Notwithstanding the provisions of Article 3, it is prohibited for anyone to keep live animals of wild species, including those born and bred in captivity, that constitute a danger to health and public safety or to biodiversity, as well as hybrids between specimens of the aforementioned species and other wild species or domestic forms and their successive generations.* *3. The prohibition referred to in paragraph 1 shall not apply to keepers of animals used in projects, plans as well as in reintroduction or repopulation activities authorised in accordance with the legislation in force and to the following establishments:* *e) Establishments referred to in Articles 8 and 9 of EU Regulation No. 1143/2014, and Legislative Decree No. 230 dated 15 December 2017, authorised, if necessary, by the Ministry of Ecological Transition also for the holding of specimens of the species referred to in Paragraph 1, in agreement with the local healthcare unit (ASL);* *4) Centres for the holding of animals of invasive alien species activated by the regions pursuant to Legislative Decree No. 230 dated 15 December 2017, only in the case of specimens of species included in the list of invasive alien species of EU relevance in the list of invasive alien species of national relevance.*
DM 28 September 2022	Decree approving the National Management Plan of the American Pond slider Issues related to alien species: National Management Plan of the American pond slider (*Trachemys scripta*) https://www.mase.gov.it/pagina/piano-di-gestione-nazionale-della-testuggine-palustre-americanab
Guidelines for the proper keeping of pets belonging to invasive exotic species (Legislative Decree 230/2017, art. 27, paragraph 4)	Guidelines for the proper keeping of pets belonging to invasive exotic species (Legislative Decree 230/2017, art. 27, paragraph 4) Appendix for the Pond slider, *Trachemys scripta*

SLOVENIA

Nature Conservation Act (ZON - UPB2, Official Journal No. 96/2004)	This Act establishes measures for the conservation of biodiversity and establishes a system for the general protection of natural values in order to contribute to the goal of nature conservation. Relevant issues of alien species: - Defines alien animal species (Art. 11, paragraph 21), and alien plant species (art. 11, paragraph 21). - Ban on the introduction of alien animal and plant species. It is possible to derogate from this ban if, following a positive assessment of the risk to nature, a special permit is issued by the competent Ministry for the introduction of alien species (Article 17). -
Rules on the assessment of risk to nature and on the authorisation	These Rules specify the conditions and methods for risk assessment prior to the introduction or repopulation of non-native wild plants and animals in the wild or the breeding of alien wild animals. Relevant issues of alien species: Introductory Provisions (Section 1), Risk Assessment (Section 2), Authorisation (Section 3), Transitional and Final Provisions (Section 4).
Rules on wild animal species that do not require a permit for captive breeding	This Rules determine the wild species of allochthonous and autochthonous animals that are not required to obtain a permit for captive breeding because they do not threaten autochthonous species.
Decree on Special Protection Areas (Natura 2000)	This decree establishes the Natura 2000 Special Protection Areas, security objectives in these areas and conservation policies to maintain or achieve a favourable conservation status for wild animal and plant species, their habitats and the conservation of habitat types. Relevant issues of alien species: - Establishes conservation policies, including “non-native species of animals and plants and genetically modified organisms shall not be introduced into protected areas”
Decree on the Ljubljansko barje Landscape Park	This regulation defines the areas of the landscape park, protected and restricted areas, management rules, protection systems, management and control methods, financing and other practices related to achieving the objectives of the landscape park as well as guidelines for the development of the landscape park and how they are to be implemented. Relevant issues of alien species: - In particular, the introduction of non-native plant and animal species into the nature park is not allowed (Art. 10, paragraph 9). - The nature conservation task to be performed in the landscape park is also the prevention of the spread of alien and particularly invasive plant and animal species (Article 18, Paragraph 5).

E.3 *TRACHEMYS SCRIPTA* (Thunberg in Schoepff, 1792)

A turtle allochthonous to Europe of medium-large size, with an oval, flattened carapace, greenish-brown in colour and crossed by yellow stripes. The plastron is yellow with dark spots, while the head shows red, yellow or orange lines depending on the subspecies (*T. s. elegans*, *T. s. scripta*) or possible hybrid form (formerly *T. s. troosti*).

Characters of the two subspecies of *Trachemys scripta*

Characters of the hybrid forms unambiguously attributed to *T. s. troosti.* This subspecies has recently been refuted based on genetic analyses (Parham et al., 2020) and reduced to the status of a hybrid between the two subspecies *T. s. scripta* and *T. s. elegans*.

Sexual dimorphism is given by body size: females are larger than males that have a longer tail that is broader at the base and significantly longer front leg nails:

Females

- Larger than males (1.15 to 1.55 times): up to 30 cm (max. 35). The maximum size officially known to date in Italy was recorded in a female of *T. s. elegans*: 28.3 x 20.0 cm, respectively carapace length and width (Di Tizio & Di Cerbo, 2011).
- Weight: up to 3 kg (record of almost 5 kg)
- Hind leg nails generally more developed
- Sexual maturity around 3-5 years (carapace length: approx. 18 cm)

Males

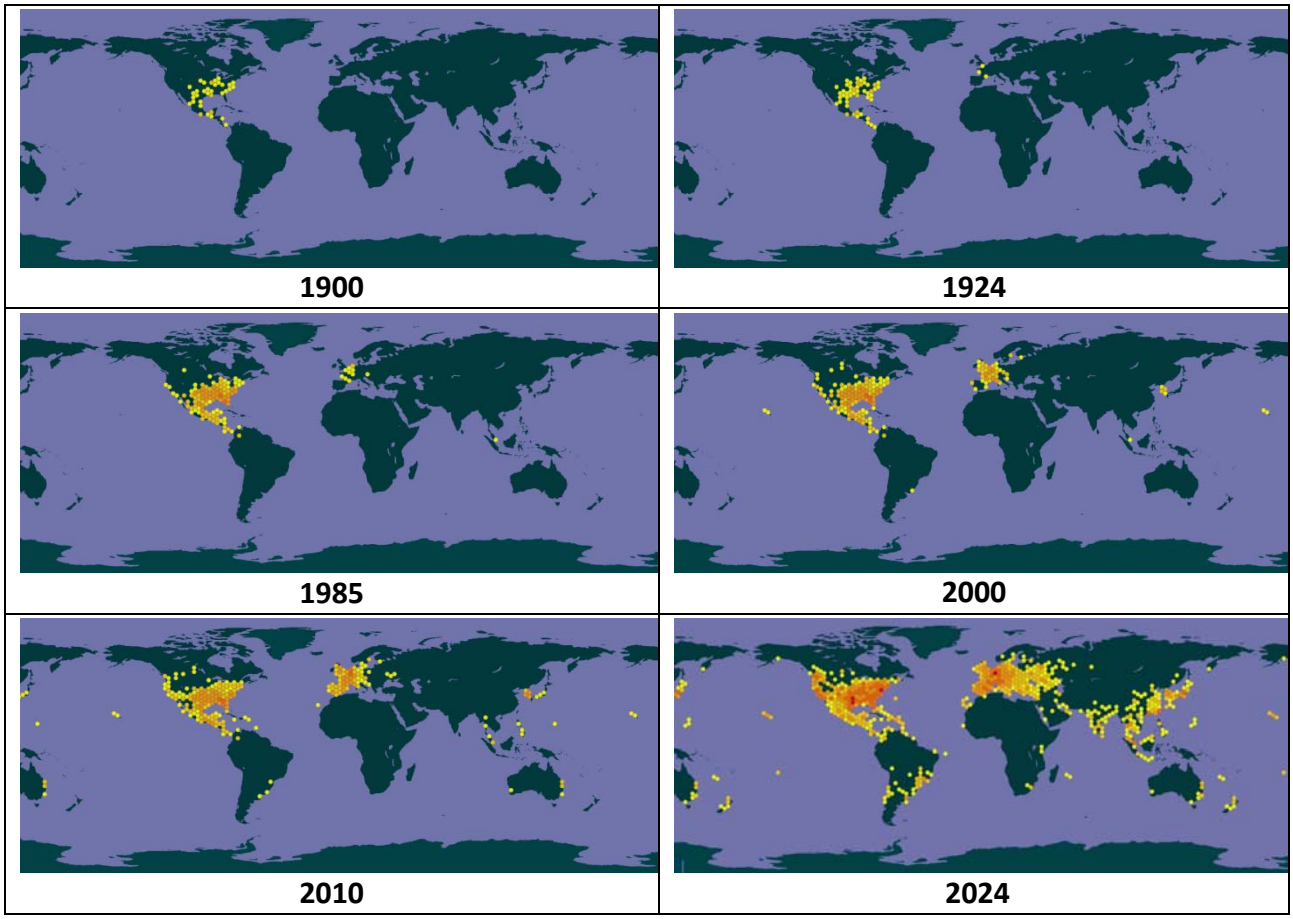
- Slightly more concave plastron
- Greater distance between plastron attachment and cloaca
- Tail longer and thicker at the attachment
- Nails of the 2nd, 3rd and 4th toe of the forelegs almost always longer
- Sexual maturity: 2-3 years (carapace length: approx. 10-12 cm)

Mating occurs from March-April to October, mainly at the bottom of water bodies. In their areas of origin, females may lay 10 to 30 eggs per brood several times a year. The eggs are whitish and elongated (approx. 4 cm long):

- 1-3 broods per year: between April-June - From 2 to 25 eggs per brood according to Ernst & Barbour (1989), from 6 to 16 eggs in Lombardy (Ferri & Soccini, 2003), from 1 to 16 (average 4.3 per nest) in Lake Angitola (Calabria), where nesting of the subspecies *T. scripta elegans* was studied for three years, in the period 2008-2010, with data based on 1081 eggs laid in 250 nests (Crescente et al., 2014).
- Temperature- dependent Sex Determination (TSD) (< 28 °C: males, > 29 °C: females)
- Incubation: 60-90 days
- Hatching: August-September
- Sperm retention
- Late broods: newborn spending the winter in the nest

Source: LIFE09 NAT/ES/000529 TRACHEMYS

In Italy the species usually winters at the bottom of aquatic habitats, in the mud or buried near the banks, between November and March. It is usually active, even in winter if conditions permit, with temperatures above 12-14°C. It is an omnivorous and opportunistic species, feeding on plants, fish, amphibians, bird nestlings and invertebrates.



Evolution of the expansion of the areal of *T. scripta* (GBIF 2024: https://www.gbif.org/species/2443002)

However, a predominantly carnivorous diet (52%) is found in juveniles, which changes to an essentially vegetarian or only slightly carnivorous regime (9%) in adults (Clark & Gibbons, 1969; Agosta & Parolini, 1999). The transition from one diet to the other would usually take place around the second year of age and, in particular, at the end of the summer season. The choice of adults towards a predominantly vegetarian diet is opportunistic: the search for plant material is certainly less costly, in terms of energy expenditure, than the search for mobile prey (Agosta & Parolini, 1999).

It is a species characterised by remarkable ecological plasticity, which has allowed to acclimatise without problems in many areas of the planet. Able to colonise the most diverse bodies of water, both natural and artificial: rivers, canals, small and large lakes, ponds, fountains and disused quarries. There is a real possibility of numerical expansion of the nuclei present and consequent naturalisation of the species, which has already been reported. The reproductive success of the species outside its native range is greater in countries with a Mediterranean climate, however, even where reproduction is not established, adult specimens released by man can survive for many years even in sub-optimal areas. In addition to the increase in the number of specimens, the

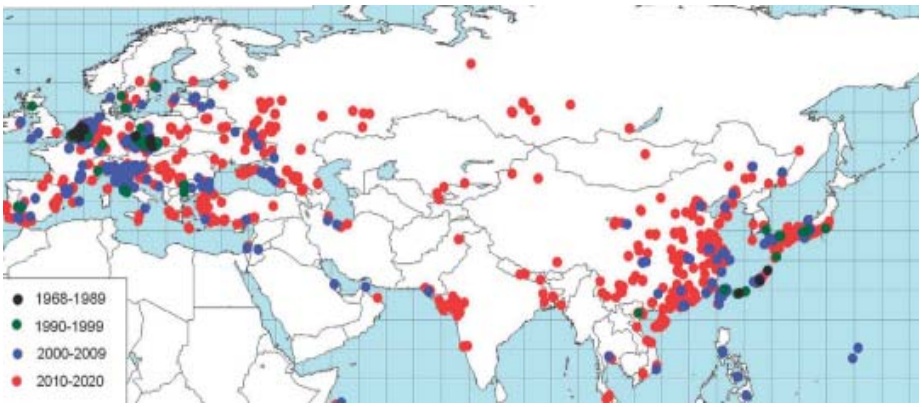
spatial expansion of the species can take place in temporarily or permanently connected wetland environments, in natural or artificial settings, or by land, thanks to the discrete motor skills of this species on land, which can make even fairly long journeys in search of new wetlands.
Export from the United States to other countries has reached millions of specimens per year (Telecky, 2001; Reed & Gibbons, 2003). Since the end of the 20th century, large-scale commercial breeding of *T. s. elegans* has also begun in China and South-East Asian countries, where it is used for human consumption (van Dijk et al., 2000).

E.4 IMPACTS AND FUTURE SCENARIOS

In captivity, *T. scripta* grows rapidly and large specimens require more space, are less attractive, aggressive and may bite. Releasing them into the nearest water body is a common way to get rid of a troublesome pet. This turtle adapts easily to outdoor conditions and can breed and establish stable populations in regions with a suitable climate (Rödder et al., 2009; Kikillus et al., 2010; Crescente et al., 2014). Once established, especially in narrow basins, it can induce a negative impact on native macrophytes and animals (Ficetola et al., 2012). Amphibians are considered particularly vulnerable because this turtle feeds on tadpoles and the presence of chemical signals released by this predator can influence the development rate and size at tadpole metamorphosis (Polo-Cavia et al., 2010; Vodrážková et al., 2020). This allochthonous species, given its larger size and higher reproductive characteristics can also successfully compete with native freshwater turtle species for food, basking sites and, in some cases, spawning grounds (Cadi & Joly, 2003; Perez-Santigosa et al., 2008; Pérez-Santigosa et al., 2011; Polo-Cavia et al., 2011; Pearson et al., 2015). Experimental studies have confirmed an increased mortality of the native European pond turtle in the presence of alien species (Cadi & Joly, 2004). Furthermore, this alien freshwater turtle is involved in the life cycles of native parasitic species, which are able to infect native freshwater turtles increasing the risk of zoonoses (Iglesias et al., 2015; Demkowska-Kutrzepa et al., 2018). It can be a carrier of pathogenic microorganisms for humans (Nagano et al., 2006; Shen et al., 2011) and in the past, prohibitions to the import of juveniles of *T. scripta elegans* from the USA have also been reiterated in Italy until the issuance of a health regulation that imposed the "Salmonella free" status of traded specimens (Soccini & Ferri, 2004).

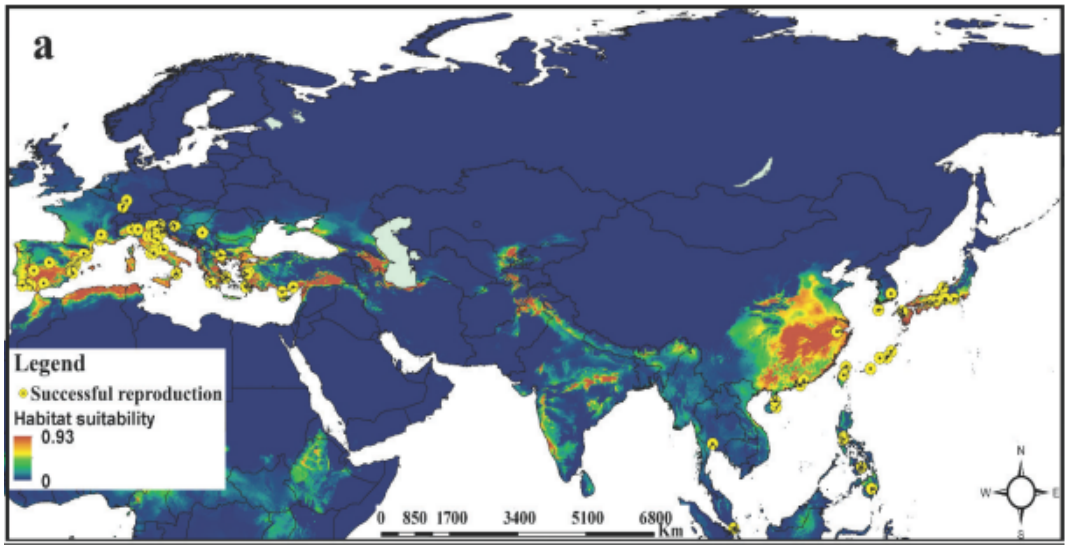
Reshetnikov et al. (2023) analysed the invasiveness characteristics of *T. scripta elegans* in different parts of Eurasia, up to a verification of its status as an 'invasive species' and future management scenarios, analysing about 7,500 data collected from verified sources.

Geographical distribution of *T. s. elegans* in Eurasia (Reshetnikov et al., 2023)

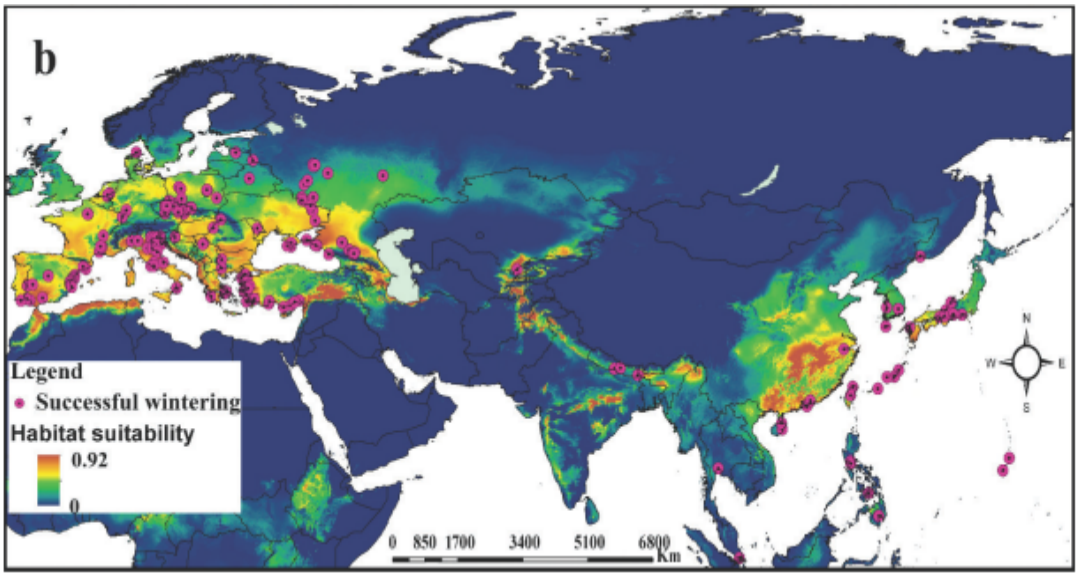


Spatial and temporal distribution of *T. s. elegans* data in Eurasia (Reshetnikov et al., 2023)

The study analyses breeding success and overwintering ability in the different localities of occurrence, confirming that breeding is more successful in Southern Europe and Asia. Wintering success in Europe, on the other hand, would be negatively correlated with latitude.



Potential range of reproduction success in *T. s. elegans* in Eurasia (Reshetnikov et al., 2023)



Potential range of overwintering success in *T. s. elegans* in Eurasia (Reshetnikov et al., 2023)

The results of the study confirm a higher density of observations in Europe and East Asia. Furthermore, the density of observations can be positively correlated with human population density (Banha et al., 2017), and release pressure (the number of release events) is often positively correlated with human population density, while the survival and reproductive success of turtles depend mainly on climatic conditions, i.e. the ecological niche. Both climatic and anthropic factors are therefore crucial for the expansion of this species, and analysing its characteristics reveals that certain factors (e.g. a larger portion of natural inhabited water bodies, a higher number of specimens per water body, overwintering success, the presence of juvenile specimens, reproductive success and the establishment of viable populations) tend to be present in coastal regions, such as the Mediterranean coast of southern Europe, the western part of

western Asia, the Pacific coast of eastern Asia and islands. Coastal regions may have milder temperature and more favourable humidity regimes, which are therefore important for the reproduction of this reptile. However, the current geographical spread of this turtle is not limited to climatically suitable areas for reproduction, as the main vector and driver of invasion is the release of domestic animals (García-Díaz et al., 2015). Although breeding is restricted to a few regions in Eurasia with relatively high summer temperatures and sufficient air humidity, naturally released specimens of *T. scripta elegans* can indeed tolerate a wide range of temperatures and air humidity near receiving water bodies.

In contrast, overwintering success is recorded in different parts of all continents, except in the northern regions of Asia, which are characterised by low winter temperatures. The winter survival of this species is linked to its specific physiological characteristics: duration of ice cover in the receiving water bodies, as the species cannot withstand more than 44-50 days of anoxic conditions in the water (Ultsch, 2006); number of days with water temperatures above 10° C in the warm season before overwintering, which can affect, for example, the lipid reserves required for long hibernation without feeding.

It would therefore be important, also when planning eradication interventions, to define the capacity to sustain the overwintering of this species in the different territories, rather than limiting its reproductive capacity, as this turtle is an excellent example of an invasive species with a wide geographical range, which does not depend on the naturalisation of its populations (=reproduction) but rather on the recruitment of new specimens linked to repeated releases that increase populations.

E.5 INTERVENTION METHODOLOGIES

The National Management Plan for *T. scripta* points out that it is not possible to identify a univocal strategy at national level, due to the considerable lack of homogeneity in the distribution and characteristics of colonised environments. However, the principle remains that in the presence of isolated and numerically reduced nuclei or new reports in ecologically relevant areas, rapid eradication must be considered a priority action, especially in cases of syntopy with native turtles.

The Plan defines a framework of regional management actions as shown in the table below.

Regione	Eradicazione	Controllo	Risposta rapida	Monitoraggio
Abruzzo	X		X	X
Basilicata	X		X	X
Bolzano	X		X	X
Calabria	X		X	X
Campania		X		X
Emilia Romagna		X		X
Friuli Venezia Giulia		X		X
Lazio		X		X
Liguria		X		X
Lombardia		X		X
Marche		X		X
Molise		X		X
Piemonte		X		X
Puglia		X		X
Sardegna	X		X	X
Sicilia	X		X	X
Toscana		X		X
Trento		X		X
Umbria		X		X
Valle d'Aosta	X		X	X
Veneto		X		X

The management strategies basically concern the control and eradication of alien turtle populations in the various regions. These actions are always accompanied by useful monitoring to verify the status of alien populations, possible criticalities in the eradication/control actions and the success of the actions undertaken.

Considering the invasiveness characteristics of the species, a prioritisation process can be envisaged that allows the urgency of a control/eradication intervention to be defined, based on, for example: intrinsic characteristics of the organism, characteristics of the environment in the area being reported that define the risks to biodiversity (e.g. the presence of native turtle populations), the possibility of its spread throughout the region and the ease of control/eradication.

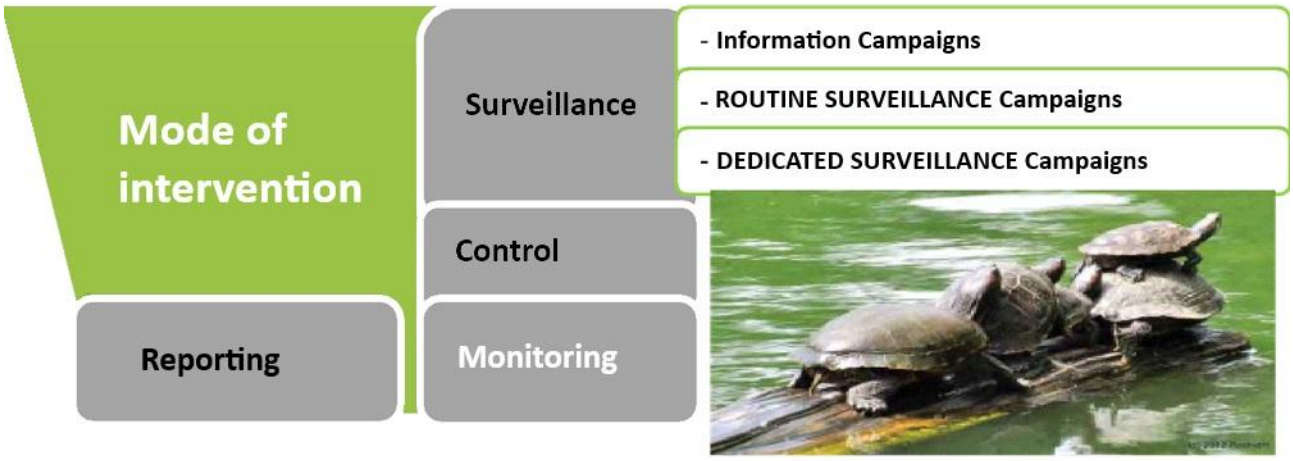
By integrating the information on the presence of alien turtles with information on, for example, the current and potential distribution of native turtles or other valuable components, the presence and territorial extension of aquatic environments of interest for the conservation of biodiversity and other biogeographical information, or with respect to the local distribution of the Natura 2000 network, it is possible to draw up a map of high-risk areas, i.e. areas of the regional territory in which prevention, early warning, eradication/control resources must be concentrated for the effective defence of biodiversity assets. These areas can be defined on the basis of data processing in a GIS environment referring to the distribution of exotic species, compared with those of the distribution of autochthonous *taxa* and with other topics, such as protected areas (Natura 2000 Network, in particular), the water network, the presence of amphibian and reptile

species of conservation interest (potential competitors or prey of alien turtles), the location near infrastructures or with heavy human presence.

Depending on the situation found and therefore the lines of action to be taken, the different areas of intervention can be categorised as follows:

- ***Prevention Areas.*** Areas with the highest risk of alien turtle arrival, where appropriate prevention mechanisms should be activated. Prevention at the destination site, therefore to be activated in different ways depending on the type of area/site, through effective surveillance and, if necessary, the equally effective application of appropriately calibrated EDRR (*Early Detection and Rapid Response*);
- ***Areas for Monitoring***. Areas where alien species are naturalised but where there are still environmental aspects such as to make it necessary to carefully monitor alien populations and host ecosystems and their native components;
- ***Containment Areas***. Sites characterised by threatened and at the same time priority environmental aspects (e.g. presence of *Emys* sp., amphibians etc.) in which, for example due to the conditions of the context, eradication is unfeasible;
- ***Areas for Eradication***. Sites characterized by priority environmental aspects (e.g. presence of *Emys* sp., amphibians, etc.) and where environmental and logistical conditions or alien population densities are such as to assume the success of an eradication campaign.

The success of interventions in favour of a native species or to combat the spread of an alien species requires the involvement of the largest possible number of Entities and local volunteer organisations related to them. In fact, active involvement makes it possible, on the one hand, to raise awareness of the issues and, on the other, to share efforts and objectives by optimising the resources, especially human resources available in the area. For example, in the case of surveillance and control activities, the greater the involvement, the greater the likelihood of making this activity effective.



E.6 SURVEILLANCE

Surveillance activity is one of the main stages in the process of controlling the spread of alien species. It should be concentrated in those areas most at risk, in particular, where there have not yet been any reports of the presence of these species and in those in the vicinity of the area of presence of the alien species. The activity can be the responsibility of the local entities in charge (e.g. Natura 2000 Network or Protected Area management bodies) and can also be supported through citizen science tools and by all users of these environments, thus achieving continuous monitoring and timely reports. It is therefore necessary to involve the supervisory personnel of the administrative bodies, Forest Carabinieri police, volunteer supervisors (Guardie Ecologiche Volontarie, etc.), as well as Agenzie Regionali per la Protezione Ambientale (ARPA) personnel, Civil Protection operators, amateur and professional fishermen, and the greatest number of people who simply happen to visit these environments. In order to make surveillance activities as effective as possible, those who are involved must be properly trained and offered a simple and effective action protocol that is easy to understand and implement.

E.7 INFORMATION AND AWARENESS CAMPAIGNS

Actions aimed at informing and raising the awareness of those who are directly and indirectly involved in this field concern in particular the bodies involved in various capacities in environmental surveillance, such as Forest Carabinieri police officers, surveillance personnel of local authorities and GEV (Guardie Ecologiche Volontarie = Volunteer Ecological Guards). Targeted

training meetings must be held for these people on exotic species and the problems connected with them, the legislation in force, and the operational intervention methods from the point of view of surveillance, monitoring, and the health precautions to be taken. Alongside targeted training courses, the production of educational/dissemination material could be envisaged. It would also be appropriate to involve them in practical activities in the field so that they can apply what they have learned in practice.

Similar activities should also be carried out for amateur or professional fishermen, involving for example the sector associations (e.g. Federazione Italiana Pesca Sportiva e Attività Subacquee - FIPSAS, etc.) as they are in close contact in many regional contexts with the environments where both autochthonous and allochthonous freshwater turtles live.

As far as occasional visitors to protected areas are concerned, it may be useful as a mean of information and awareness-raising to create information panels installed in various locations or along watercourses, as well as information brochures to be made available at the info-points of parks and reserves. All this material should also be made available online on any protected area websites or social media pages.

Finally, those who are supposed to carry out educational activities at various levels (e.g. environmental and tourist guides, etc.) should also be properly trained on these issues, both through dedicated courses and information material.

E.8 ROUTINE SURVEILLANCE

Routine surveillance activities are part of the activities carried out during normal work or voluntary work. This will involve checking for the presence of any specimens of exotic and native turtles, or the presence of dead specimens in the water or found hit by vehicles on roads near the water bodies being monitored. Fishermen may also find it useful to check the stomach contents of fish caught and retained in certain contexts, e.g. in the presence of predatory or large fish such as pike, trout perch, or sheatfish.

E.9 DEDICATED SURVEILLANCE CAMPAIGNS

Dedicated surveillance activities are linked to sites of special interest where particular conditions exist and which should be kept systematically controlled and monitored. In particular, it is important to monitor those areas close to the areas of spread of alien turtles and sites that are still free and colonised by native species only.

Surveillance is effective if it is carried out continuously and systematically, with trained and well-motivated personnel. To this end, it is useful to draw up an annual activity calendar for the various sites to be monitored, especially during the periods of greatest turtle activity depending on latitude (approximately March - October).

The monitoring activity will have to be carried out by means of one of the techniques presented here, for which the personnel in charge will have to be duly trained, as required by the regulations in force and the National Guidelines. During the field activity, both environmental data and data related to the animals captured/detected will be collected by filling in a form similar to the one in **Annex 4** herein.

The data obtained from these activities will provide a fundamental historical framework useful to determine the local expansion trend of exotic turtle species, with a special focus on *T. scripta*, as well as keeping the knowledge framework on *Emys* sp. up to date.

E.10 MONITORING

This action has a preparatory function to the control and eradication activities and to verify the population trends following the interventions, and can therefore be differentiated in *ex ante* and *ex post* monitoring.

In both cases, activities are carried out through counting (Visual Encounter Survey) from fixed observation points or along transects, as follows:

- Selection of fixed observation stations according to the type of environment, with good visibility from the banks and the possibility of observing basking animals without disturbance. In some cases, existing structures can be used for birdwatching and other activities in the monitoring areas. The number of stations/transects will be functional to cover the areas of the monitored sites, and each station/transect will be filed and georeferenced to allow a long-term monitoring plan.
- Carrying out at least three observation sessions during the turtle activity period (March to October depending on the area), noting on special forms (**Annex 3**) the area monitored, the observation station, and information such as time and weather conditions.
- Counting the maximum number of specimens that can be observed at the same time, noting on special sheets (**Annex 3**) the species, if it can be recognised unambiguously, an indication of the age class (young/adult), the sex where possible, and indications on activity (basking, etc.). Observations may also be supplemented with data on other both autochthonous and allochthonous species. To ensure monitoring consistency, it is not necessary to determine the species of non-native turtles at the specific or subspecific level, especially in order not to incur errors when using non-specialist observers. This also applies to any observations of *Emys orbicularis*, which should only be noted if the observation is made by experienced personnel and otherwise verified before consolidating the data.

Prior to the planning of monitoring, any existing species-specific information and data for the area of interest must be collected and validated, by surveying projects of various kinds (research, monitoring, citizen science, etc.) that are active or concluded.

Monitoring by means of Visual Encounter Survey does not require specific authorisations, except those necessary for access to the areas, where required.

The presence of at least one operator is required for monitoring; however, to increase the robustness of the data collected, the use of double observers with comparison of the data

collected by each is recommended. Double observers may also be useful for safety reasons due to the special types of environments where activities are carried out.

E.11 CONTROL

In the areas identified as a result of the surveillance and monitoring actions, and identified as priority areas, it is necessary to define a management strategy for alien turtles that minimises their negative impacts on biodiversity, related ecosystem services, public health and animal health, agriculture and animal farming activities, or on the economy (art. 22 of Italian Legislative Decree 230/17), through targeted and diversified control measures depending on the environmental context and the degree of spread of the species.

For this it is possible to operate with:

- **Removal** of cores/populations of the exotic species by capture with live traps in areas classified as suitable for eradication and prioritised in sympatric sites with recognised and significant presence of native freshwater turtles.
- **Containment** of cores/populations of exotic species by live trapping in areas classified as suitable for containment only because the species is so widespread as to make eradication impracticable or unfeasible, also on the basis of cost/benefit analysis.

In Italy, the activities are carried out by trained and authorised personnel at the regional level as required by the National Management Plan. Personnel are trained on intervention methods, management of captured animals, and after-capture management methods. The request for authorisation must be sent to the specific offices in charge of the regions where the activity is carried out.

In case of intervention in areas with the presence of native turtles or other species included in Annex IV of Directive 92/43/EEC, as a precautionary measure, it is also necessary to apply for a derogation authorisation for the capture and handling of species to the Ministry for the Environment and Energy Security https://www.mase.gov.it/pagina/deroghe-ai-sensi-dellart-16-della-direttiva-habitat pursuant to Art. 11 of Presidential Decree No. 357/97.

Captured specimens of alien species cannot be released, but must be retained for suppression/euthanasia or transferred to detention centres, identified by the Regions or Autonomous Provinces that have the competence to apply eradication and management measures in the area pursuant to Legislative Decree 230/17, and where they will be kept permanently in captivity.

With regard to captures, similarly to what was done for monitoring activities through direct observation, some unambiguous parameters can be summarised:

- Period: April to October or - to avoid an impact on breeding ornithological species at the intervention sites - July to October.
- Establish the trapping point according to the type of trap.
- Take GPS coordinates of the trapping point (using any smartphone/iPhone application suitable for the purpose).
- Mark the location of the trap with tape tied to vegetation or a stand on site.

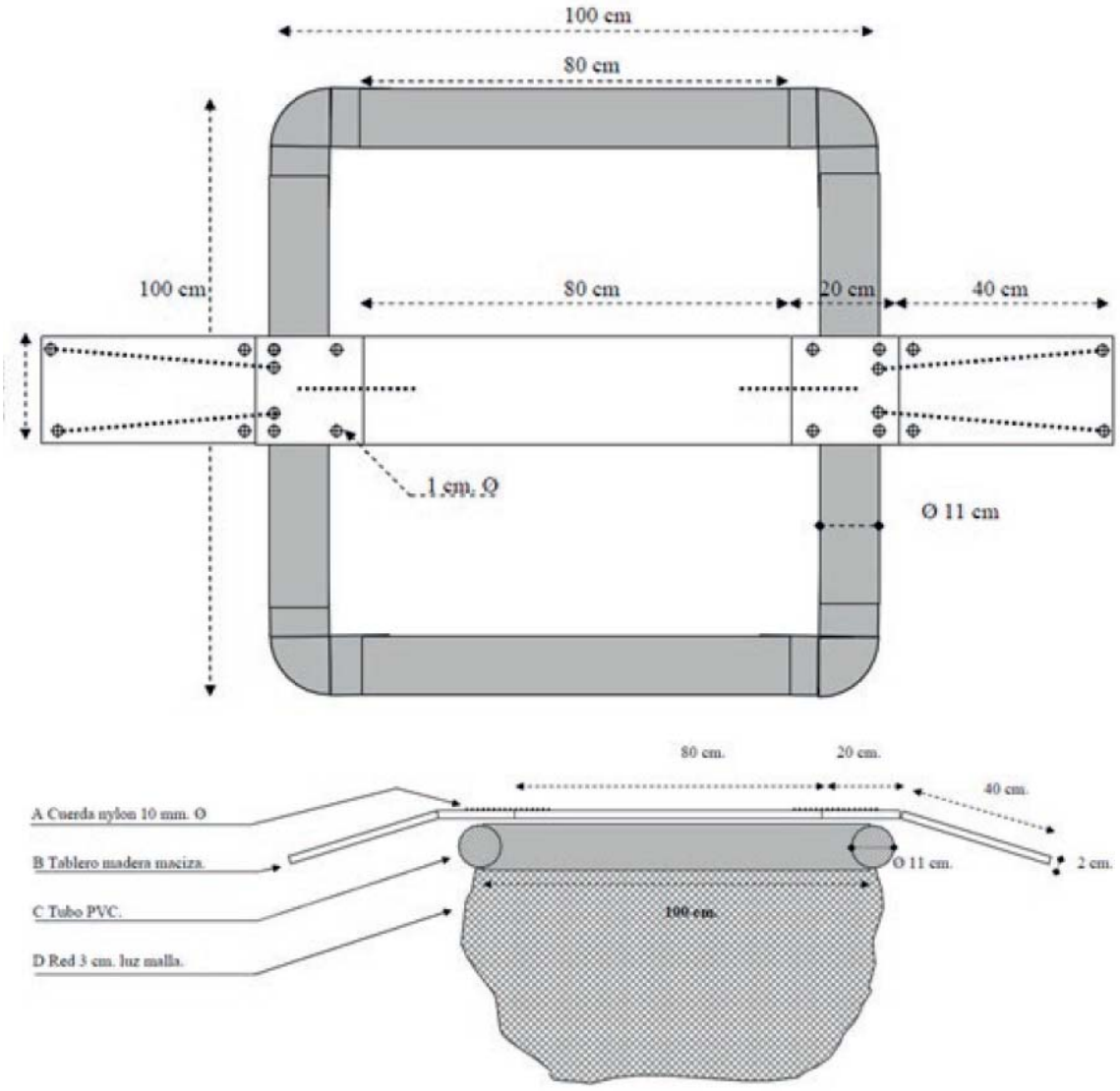
E.12 TRAPPING METHODS

There are now many experiences worldwide that have verified the functionality and efficiency of traps of different types for capturing freshwater turtles, whether native or not: Italy (LIFE Emys, 2016), Spain (Martinez-Silvestre et al., 2006; Izquierdo et al., 2010; Valdeon et al., 2010; LIFE Trachemys, 2016), Latvia (Pupins & Pupina, 2007a, 2007b), the Netherlands (Bugter et al., 2011), Brazil (Bujes, 2010), Taiwan (Chen, 2006), the USA (Lui et al., 2013), and Australia (O'Keeffe, 2009). Above all, the LIFE Trachemys tested the different types of traps and their efficiency according to the wetland case histories in which the trapping should be carried out. The most effective techniques are proposed below.

BASKING TRAPS

These traps are suitable for catching alien turtles in large water bodies, particularly in positions away from the banks. The trap consists of a central floating body with a side of at least 1 metre with a submerged net, where turtles enter through wooden ramps on all four sides. If necessary, a wooden board can be added to the centre of the trap. Experiences (e.g. Stagni di Casale, Vicenza) have shown that if bark is fixed in the centre and in the ramps in the water, for example, to better simulate a natural basking site, an increase in catches occurs. The animals access the trap for basking and fall into the net when they dive into the water.

Example of *basking traps*



Scheme of *basking traps* (Sancho Alcayde et al., 2015)

These traps have very good catchability in large water bodies with few natural basking sites, and are quite selective catching almost exclusively alien freshwater turtles.

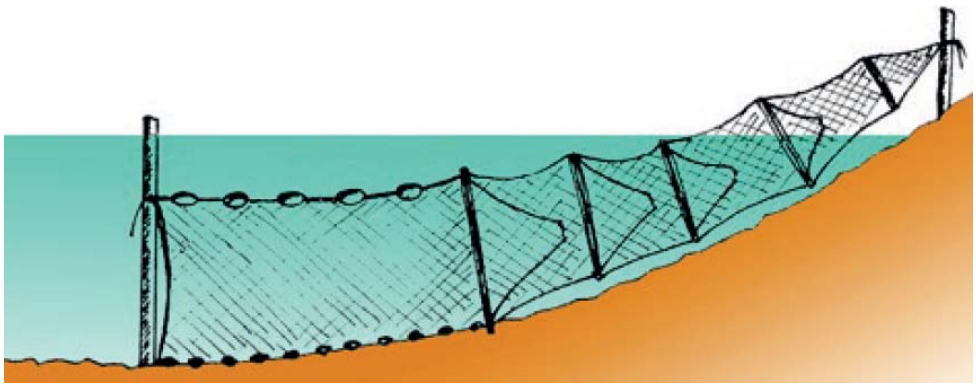
Positioning of *basking traps*

These traps have been used in several projects in many different geographical areas, with great success. However, they have some drawbacks and critical issues that may make them an unsuitable choice in some cases, such as:

- The traps have to be constructed; models exist on the market, but they are very expensive. The construction is not complicated, as construction materials are available in any building materials or plumbing shop for cheap. However, if many traps are needed, their construction can become costly in terms of time and space.
- These traps are very bulky and relatively heavy. The floating structure is fixed of at least 1 m^2, while the submerged net of at least 70 cm depth can be rigid or flexible. In any case, storage and transport require many operators and appropriate means. In areas that cannot be reached by car, transport may become difficult.
- Traps should be placed in deep water and away from existing basking sites. It is therefore necessary to have a boat to set them.
- In areas with a moderate presence of basking sites, the attractiveness of the traps may decrease significantly (see LIFE Emys).
- Constant maintenance should be carried out, as the net or flotation system can become damaged over time and lose their effectiveness.

CREELS AND SIMILAR SYSTEMS

These are common creels used in fishing for eel and other fish species mainly in lagoon areas. The creel may consist of a single catch with a single submerged decoy and the opposite end tied out of the water: the turtle enters the creel and ascends the trap to the emerged part, remaining trapped.



Single-decoy creel scheme (Sancho Alcayde et al., 2015)

A very functional variant is characterised by two single-decoy creels connected by a central net-barrier of variable length (2-3 metres). The ends of the creels are tied out of the water to poles and suitable structures on site while the decoys and the net-barrier are submerged. Therefore, the turtles encounter the net-barrier and tend to move laterally and actively enter the pots up to the emerged part, becoming trapped.

These types of creels have a high catchability (Ottonello et al., 2016), especially in correspondence with channels or items where animals must pass underwater (e.g. openings between dense reeds or at preferential exit points from the water on the banks).

They are not very selective, as they can also easily attract and capture waterfowl (moorhens, ducks, etc.), mammals (e.g. coypus), autochthonous or allochthonous amphibians and reptiles (e.g. *Natrix helvetica*, *Lithobates catesbeianus*), crayfish (e.g. typically *Procambarus clarkii* and *Orconectes limosus*), and fish. In these cases, predation or cannibalism may occur in the presence of different species or individuals.

Barrier-net and double-decoy traps

These traps are very effective for catching freshwater turtles. However, even in this case there are critical issues to consider when planning a prolonged trapping campaign, such as:

- The creels have to be purchased, and the cost can be very high (around 80/100 Euros per creel), making a trapping campaign based only on these traps too costly.
- These traps work well in areas of modest depth, no more than 1 m, to increase the possibility of intercepting animals moving along the bottom. They therefore require a preliminary analysis of the trapping sites to verify their suitability.
- Creels must be positioned so that one side is left out of the water, to allow the turtles to come out to breathe. They must therefore be tied to poles or supports present, taking care that they do not collapse or end up in the water, for example, if there are many turtles and consequently more weight.
- If the environments have an unstable bottom on which is not possible to walk safely with boots, and it is not possible to place the traps in the water, a boat is needed to set them.

MINNOW TRAPS

These are commercially available traps of different sizes and formats, characterised by a single trap body with two side decoys of different sizes. The traps are normally used for catching crustaceans and fish. They can also be used for freshwater turtles by adding one or more items that ensure their buoyancy (including recycled ones, such as plastic bottles), and the emersion of at least the upper portion to allow the captured animals to breathe. The traps can be equipped with an internal pocket, in which a bait can be placed to significantly increase capture success.

These types of traps have high capture success (Ottonello et al., 2016), especially at banks or basking sites in areas most visited by turtles.

Similarly to the already mentioned creels, they are not very selective (see above for details).

Commercially available products include a foldable net, which does not require large storage spaces and also makes it easy to transport a large number of traps to areas that cannot be reached by car.

These traps have a medium to high capture success of freshwater turtles, and it is essential to use bait (canned fish in natural form, normal commercial products such as dog/cat food, etc.) to ensure the full functionality of these traps in all types of environments. They can also be used by properly trained volunteers and placed from the shore, taking care to tie them to vegetation or other existing items.

Some critical issues related to their use:

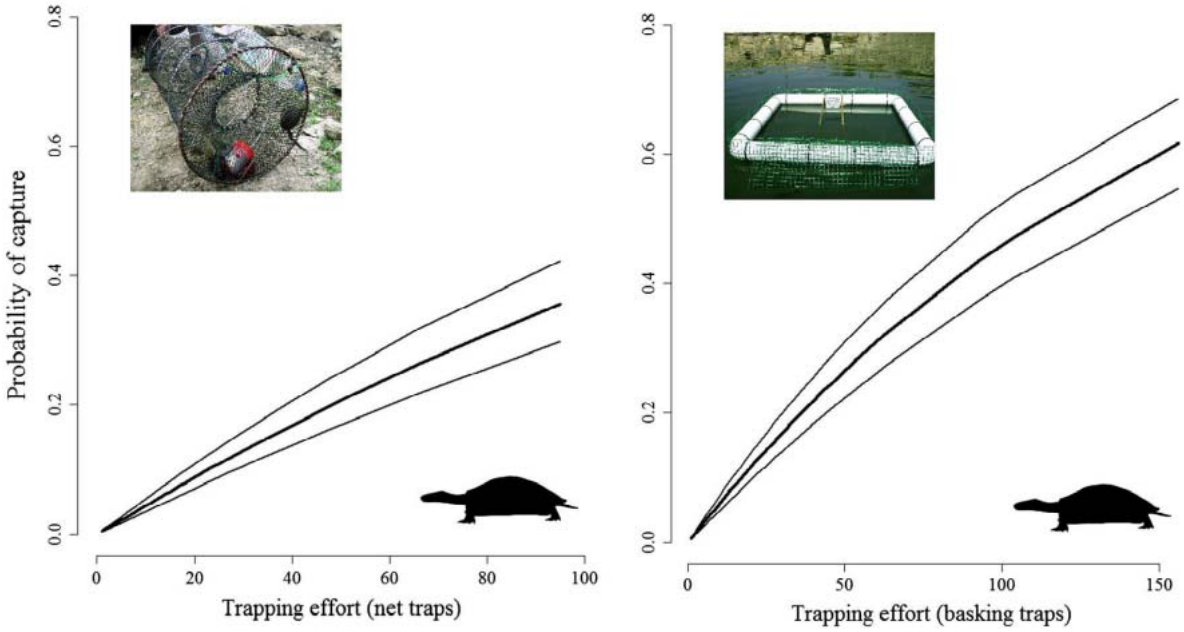
- Commercially available minnow traps can be roughly manufactured and break after a few uses in the water, especially the emerged part and the seams.
- Especially after several months in the water, the creels may become brittle and facilitate escape for captured animals.
- The creels should be placed with additional flotation systems, and they should be monitored frequently to avoid cases of death by drowning of turtles (e.g. in the presence of many animals).

Floating minnow traps

When planning a catching campaign, it is therefore necessary to assess a number of priority aspects before committing human and economic resources or to modulate them effectively:

- Preliminarily identify the sites, within a capture area, where there is the highest density of allochthonous freshwater turtles and/or the greatest impacts with native freshwater turtle populations.
- Assess the feasibility and effort required to set and control the traps in the long term (reachability by vehicles, accessibility of the banks in the presence of dense vegetation in spring-summer, need for a boat or other means to set the traps and monitor them).
- Budget available in relation to the capture effort required. In some cases, it may be unnecessary to commit resources to situations that only require containment rather than focusing on eradication in areas with a greater impact on native biodiversity.
- Availability of personnel: once the traps have been set, whichever type is chosen, constant monitoring is necessary throughout the trapping period at least twice a week, to limit deaths, damage to traps or theft. In addition, the engagement of at least two operators is advisable, to facilitate activities and for safety reasons.
- Assess the possibility of vandalism (by fishermen, conservationists, animal-right activists, or poachers).

As an example, Garcia-Diaz et al. (2017) carried out an evaluation of the effectiveness of eradication in Valencia by comparing creels/minnow traps and basking traps, among others, with slightly better success of basking traps if used for a continuous and adequate period of time.
Therefore, an assessment of the feasibility related to the contingent situations of each trapping area remains a priority.



E.12 AFTER-CAPTURE MANAGEMENT OF *TRACHEMYS* SP.

RECORDING OF CAPTURED ANIMALS

Captured alien turtles cannot be released, but included in a process either of permanent holding or suppression/euthanasia.

Firstly, for each captured animal it is necessary to:

- Determine the species (and subspecies), sex, and estimate the age class (young/adult). It is advisable to take a photo of the whole animal, the carapace, the plastron and a detail of the head for possible verification of the accuracy of the determination.
- Record some morphometric data (carapace and plastron length, and weight) useful for characterising the structure and characteristics of the alien turtle population (optional).
- Mark each animal with a permanent unique code on the marginal plates of the carapace (specimen code) and on the plates of the plastron (area code number; see below for details).
- Note any obvious diseases or anything else useful for characterising the captured animal.

All data should be recorded on a form (**Annex 4**).

In particular, for turtles entrusted to a permanent housing centre, specimen marking is necessary. As for the permanent marking (see below), two possibilities are proposed:

- Installation of a microchip, according to current regulations and assessing the technologies currently available (in accordance with Art. 66 of EU Regulation No. 865/06).
- Applying by means of an engraving a permanent unique code on the marginal plates of the carapace (specimen code) and on the plates of the plastron (area code number).

Within the framework of the LIFE URCA PRO*EMYS* working group, a cost/benefit assessment was carried out on the use or non-use of one of the previous marking methods for alien turtles.

In general, the installation of a microchip is the ideal and permanent solution for marking a turtle. However, following an evaluation with respect to the objectives and needs of the project, some critical issues emerged that must be considered when planning an eradication activity, such as:

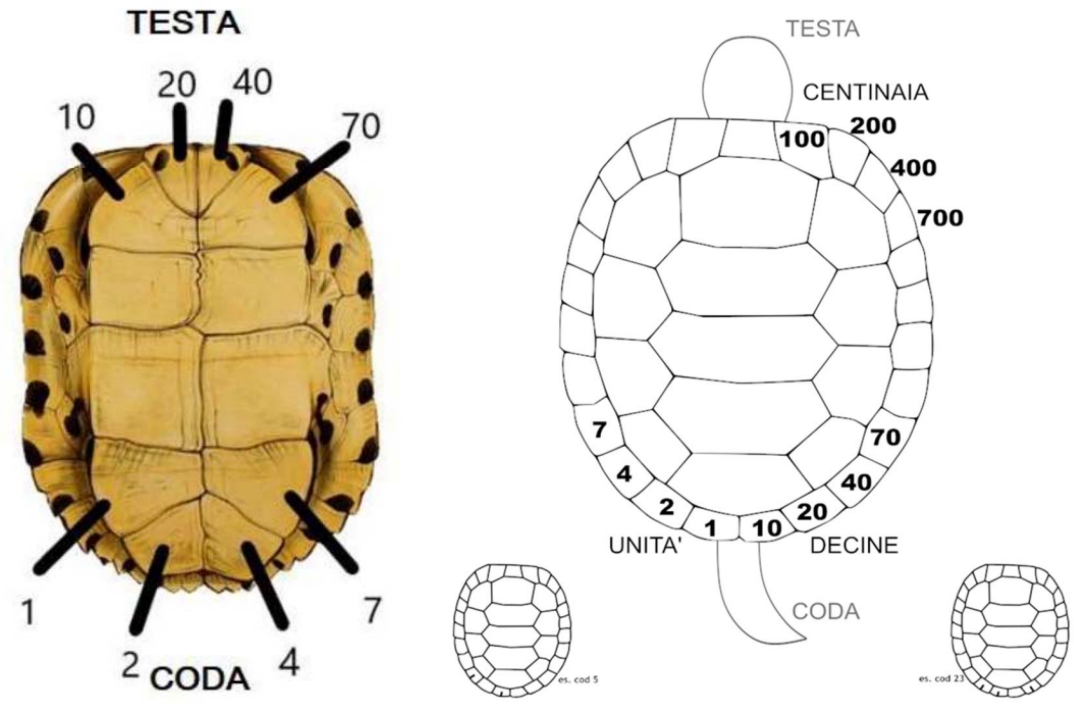
- Associated cost: the cost of a microchip and scanning system affects the budget of a project, especially if substantial numbers of turtles are expected to be captured.

- Installation: a microchip must be placed by a veterinarian, to ensure animal welfare. This implies the availability of veterinary staff at the capture areas and possibly an additional cost to be considered.
- It cannot be taken for granted that microchips can be read by all scanners on the market, so there is a possibility that the systems used will become obsolete years later. Furthermore, the microchip is not visible, so any animals caught in the wild cannot immediately be traced back to previous captures and/or escapes from housing centres.

As a result of these evaluations, the LIFE URCA PRO*EMYS* project has chosen to carry out only the marking of alien turtles by engraving on the marginal plates. However, this does not mean that the microchip option cannot be considered in projects with an adequate budget or, on the contrary, in small projects.

In the case of marking on the plates, the LIFE URCA PRO*EMYS* project proposed to make incisions with a hacksaw on the marginal plates of the plastron and carapace:

1. Area code number: marking of the plastron
2. Specimen code number: marking of the carapace



Example of engraving on *Trachemys scripta* and marking code used in LIFE URCA PRO*EMYS*
From the figure above: Testa = Head - Centinaia = Hundredths - Unità = Units - Decine = Tenths - Coda = Tail

Regarding photographs, which in some cases are essential to confirm the species determination, it is necessary to take a photo of: the whole animal, the carapace, the plastron, the head laterally, and to catalogue the photos according to the areas of capture.

Example of photographs that need be taken for the correct identification of captured animals

AFTER-CAPTURE MANAGEMENT

Pursuant to current European and national legislation, and according to the specifications in the National Management Plan, captured specimens cannot be released, but must be retained for suppression or transferred to housing centres, identified by the Regions or Autonomous Provinces that are responsible for implementing eradication and management measures locally, pursuant to the Italian Legislative Decree 230/17, where they will be kept permanently in captivity. The Plan

specifies how suppress, although implemented with methods designed to spare animals any possible suffering, could be very likely strongly opposed, especially by public opinion, sometimes even by political parties, particularly in the case of pets, despite being provided for by EU and national regulations.

The costs of control/eradication can be considerably high in some cases, inevitably subtracting funds from conservation activities for native species.

Considering that housing centres for exotic freshwater turtles are to be used primarily for specimens coming from individual owners (pets) as opposed to those captured in the wild, the Plan envisages that an objective choice will be made through a cost/benefit assessment comparing the option of suppression vs. permanent enclosure.

The cost/benefit analysis should consider costs related to the following items:

- Unit and overall cost of temporary housing (one-off purchase of housing systems, construction of any wastewater management plant, man-hours for maintenance, food, and any veterinary care).
- Unit and overall cost of transporting animals to the permanent housing facility.
- Unit and overall cost of keeping animals in the centre for at least 25 years.
- Unit and overall cost of euthanasia (veterinary fee, any equipment).
- Unit and overall cost of carcass treatment.

In case, for the same biodiversity benefits, the cost of permanent housing is disproportionate, the Plan specifies how the suppression/euthanasia option is to be performed, with an appropriate preventive information campaign.

In Slovenia there are no housing centres for exotic animals, so suppression is currently the only option contemplated.

PERMANENT HOUSING

According to the provisions of Article 27, paragraph 4, of the Italian Legislative Decree 230/2017, the Ministry in charge (formerly MATTM) published on 14 May 2018 the "*Guidelines for proper management of invasive exotic species of EU relevance kept as pets for non-commercial purposes"*, with particular reference to the risks associated with such keeping. The guidelines, developed with the support of ISPRA and the *Societas Herpetologica Italica*, are available at the following address:

https://www.mase.gov.it/sites/default/files/archivio/allegati/biodiversita/Linee_guida_animali_compagnia_specie_esotiche_invasive_appendice_Trachemys_scripta.pdf and have a specific appendix dedicated to *Trachemys scripta*. Subsequently, in the framework of LIFE ASAP), targeted recommendations were drafted by ISPRA for owners or centres keeping specimens of these species on the criteria for proper keeping also aimed at ensuring the welfare of the turtles ("*Recommendations for the proper keeping of pets belonging to invasive exotic species of EU relevance: the* Pond slider *Trachemys scripta"*, Alonzi et al., 2018; "*Plan for the control and management of exotic species of* freshwater *turtles Trachemys scripta* ssp.", Ferri, 2019).

During capture sessions, it may be necessary to temporarily keep captured individuals at capture areas before their final transfer to housing centres. This action must be planned to guarantee and optimise veterinary monitoring activities and the subsequent adequate transport of captured *Trachemys* to housing centres.

In such cases, captured turtles should be kept in an identified and equipped area with tanks or other types of temporary housing for the animals.

In this case, the following is necessary:

- Above-ground tanks of a suitable size, also purchased from commercially available models such as cargopallets or tanks for agricultural use (tubs) that prevent the animals from escaping.
- A system for loading and emptying tank water. Wastewater must flow into the sewer.
- Availability of water, even aqueduct water.
- Covering of the tanks to provide shade.
- Availability of a protected area, not freely accessible.

Examples of tanks for temporary keeping of captured animals

Example of a tank for temporary keeping of *Trachemys scripta* (LIFE12 NAT/IT/000395 Emys).

Example of a structure for temporary keeping of *Trachemys scripta* (LIFE12 NAT/IT/000395 Emys). The higher tanks are reservoirs in which the water remains at room temperature

In these temporary facilities, the animals should stay for a maximum of one week, before moving them to permanent housing centres or until suppression/euthanasia.

Regarding Permanent Housing Centres, several contributions have been produced since 2018 to establish Guidelines for proper identification of Centres and management of housed animals. Please refer to these documents for more details:

- Guidelines for the proper management of invasive alien species of EU relevance kept as non-commercial pets
  https://www.mase.gov.it/sites/default/files/archivio/allegati/biodiversita/Linee_guida_animali_compagnia_specie_esotiche_invasive_appendice_Trachemys_scripta.pdf
- Recommendations for proper keeping of pets belonging to invasive alien species of EU relevance: the Pond slider *Trachemys scripta*
  https://www.lifeasap.eu/file/LifeASAP_Raccomandazioni-Trachemys-estese.pdf
- Control and management plan of exotic species of Pond slider (*Trachemys scripta* ssp) LIFE IP GESTIRE2020
  https://www.regione.lombardia.it/wps/wcm/connect/85638e14-7506-4107-96d1-52d11f5df1bb/Piano+di+controllo+e+gestione+delle+specie+esotiche+di+testuggini+palustri+-+Azione+A7.pdf?MOD=AJPERES&CACHEID=ROOTWORKSPACE-85638e14-7506-4107-96d1-52d11f5df1bb-mM0b5g0

Below are the characteristics that permanent housing centres must have in order to be suitable for regulatory and biological requirements of the species kept:

- They must be considered as permanent controlled housing sites, i.e. permanent until the end of the natural life of the housed specimens.
- Holding facilities may also consist of tanks, fountains or ponds suitable for permanent confinement of turtles, provided they are organised in such a way as to prevent any risk of escape and prevent the animals from reproducing.
- Centres that may be connected to natural environments must be completely enclosed or fenced in, with a suitably high net buried at least 50 cm into the ground. All tanks, including those for temporary housing (quarantine), if connected to natural environments, must be constructed in such a way as to exclude any possible escape, for example by providing high concrete sides, which also serve the purpose of preventing any possible laying of eggs in the ground.
- In order to avoid reproduction (which is expressly forbidden by law), it is advisable to perform either penis amputation in males, endoscopic sterilisation in females, or destruction of the eggs as soon as they are laid, if the banks of the ponds allow spawning. Alternatively, the two sexes must be kept separately.

- Even in detention centres, euthanasia of diseased specimens is permitted in the first instance, as well as suppression of individuals caught in the wild in the event of overcrowding in the centre.

At present, not all Italian Regions and Autonomous Provinces have these facilities. The following is a non-exhaustive list, which may be updated in the future (from LIFE URCA PRO*EMYS*), including the centres in operation as of May 2023 and those in the process of being activated.

List and contacts of Permanent Housing Centres for pond sliders in Italy (May 2023)

	Region	Name	Management	For information
1	Emilia Romagna	Centre in Neviano degli Arduini	ENPA Parma	ENPA provincial office of Parma telephone +39 0521 1716793 (opening hours Thursday 5.00 to 7.00 pm and Saturday 10.00 am to 1.00 pm) and Rescue Dogs Association of Noceto telephone +39 388 6443131 e rescue.dogs@libero.it
2	Emilia Romagna	Centre "La Casa di Tarta" in Tramuschio (Mirandola, Modena)	Azienda agricola Riccò Federico	Riccò Federico: info@aziendaagricolaricco.it, telephone +39 334 9959604 (opening hours 9.00 am to 12.00 noon and 3.00 to 7.00 pm Monday to Saturday) https://www.aziendaagricolaricco.it/
3	Emilia Romagna	Centre for the Recovery of Alien Aquatic Turtles - Centro di Recupero Testuggini Acquatiche Alloctone Pianura Cesenate	Tarta Club Italia	Mirko Giorgioni (vice-president of Tarta Club Italia) centrorecupero@tartaclubitalia.it / info@tartaclubitalia.it
5	Friuli-Venezia Giulia	Regional Centre for Exotic Animal Keeping - Centro Regionale detenzione animali esotici	Friuli-Venezia Giulia Region	Monday to Saturday from 3.30 to 7.00 pm Baradel Damiano telephone numbers +39 348 4056523 or 338 4786312 or 0481 711574 https://www.regione.fvg.it/rafvg/cms/RAFVG/salute-sociale/igiene-urbana-veterinaria/FOGLIA28/
6	Lazio	LabTer of the Parco Riviera di Ulisse	Parco Riviera di Ulisse	Full to date, waiting for new spaces
7	Liguria	Bormida Centre	Liguria Region	Liguria Region
8	Lombardia	Municipality of Buccinasco, (Laghetto dei Pensionati)	Municipality	Lombardy Region
		Municipality of Brescia (two small lakes in Ducos2 Park)	Municipality	Lombardy Region
		Groane Regional Park for the two small lakes in Minoprio	Park	Lombardy Region
9	Marche	Conero Park	Conero Park	Parco Naturale del Conero info@parcodelconero.eu +39 071/9331161
10	Marche	C.R.A.S Marche - Centro di Recupero Animali Selvatici Regionale (Regional Centre for the Recovery of Wild Animals)	Province of Pesaro and Urbino	cras.pu@libero.it Monday to Friday from 7.00 a.m. to 1.30 p.m. - telephone +39 3667786451 Saturdays from 7.00 a.m. to 1.00 p.m. -

	Region	Name	Management	For information
				telephone +39 3667786451 Monday to Saturday from 2 p.m. to 6 a.m. - telephone +39 3667786450 holidays 24 hours a day - telephone +39 3667786450
11	Piemonte	Wildlife Rescue Centre - Centro Recupero Animali Selvatici di Bernezzo	Private	Telephone/Fax: +39 0171-82305 Cell phone: +39 328-5325296 E-mail: info@centrorecuperoselvatici.it https://www.centrorecuperoselvatici.it/
12	Puglia	Calimera Authorised Centre	Puglia Region/Municipality of Calimera	daily from 9.30 am to 12.30 pm +39 324 8898814 info@msns.it https://www.msns.it/centro-recupero-fauna-selvatica/
13	Toscana	C.R.A.S. WWF "L'ASSIOLO"	WWF	+39 0585/241768 Cell. Phone +39 360/234789 parcodidattico@virgilio.it; massacarrara@wwf.it centrodidatticoronchi@hotmail.it On the web: http://centrodidatticowwfronchi.wordpress.com/
14	Umbria	Centro Ittiogenico del Trasimeno di S. Arcangelo	Province of Perugia	+39 366 8309346 opening hours: 8.30 am to 1.00 pm - Monday to Friday https://www.regione.umbria.it/turismo-attivita-sportive/centri-ittiogenici
15	Veneto	Municipality of Bovolone	Municipality	Planned activation in 2025

The transport and fostering of allochthonous turtles, in particular *T. scripta*, must be carried out guaranteeing the welfare of the animals and their traceability.

For the capture and transport of *Trachemys*, no permits are needed if the activities fall within the scope of the management measures for the alien species in the region. For this, handover and receipt forms can be prepared for the animals to be filled in and signed at the handover (see attached **Annex 5**.

As far as transport is concerned, in view of the characteristics of the species in question, it is considered sufficient to transport the animals to the Centres using a normal vehicle, including vehicles provided by individual operators, excluding specialised live animal transport services, which would only result in an unsustainable increase in cost.

In this case, it is necessary to provide suitable containers, for example an igloo cooler of at least 100 litres capacity or polystyrene thermal containers perforated according to the distance to be travelled. Dampened jute bags can be used to limit the animals' injuries.

SUPPRESSION AND EUTHANASIA

The Italian Legislative Decree D. Lgs. 230/2017, in Art. 19, Early detection and rapid eradication, states in paragraph 3 that: *The Regions, the Autonomous Provinces of Trento and Bolzano and the managing bodies of the national protected areas: (b) ensure the complete and permanent elimination of the population of invasive exotic species by sparing the specimens subject to eradication avoidable pain, distress or suffering, limiting the impact on non-target species of the measures and on the environment, and giving due consideration to the protection of public and animal health, agriculture and animal farming, and the environment.*

It is important to specify that the term 'euthanasia' is used when acting on animals in poor health and suppression is to be used in cases of animals caught in the wild, such as for eradication campaigns.

However, both the suppression and euthanasia of exotic turtles must be carried out using techniques that minimise pain, distress and suffering of the animals, taking into account best practice and international guidelines (www.avma.org/KB/Policies/Documents/euthanasia.pdf). Euthanasia is a medical act and must be practised by authorised veterinarians, while suppression can be performed by appropriately trained non-medical/veterinary personnel, applying the methods specified in the "*Manual for the management of vertebrate invasive alien species of*

Union concern, incorporating animal welfare"[30] published by the EU; the presence of a veterinarian is only necessary when suppression is operated with pharmaceutical products.

In veterinary medicine, euthanasia of animals is carried out to relieve suffering and is practised by first anaesthetising the animal and then administering drugs that cause cardio-respiratory blockade. The combination of the active ingredients Embutramide + Mebezonium iodide + Tetracaine (trade name Tanax) is commonly used. However, in reptiles, due to their physiology, this drug is not very applicable.

Sodium pentobarbitone/Pentobarbitone, Morbital®, can be administered intravenously or intracoelomatically in varying doses depending on the species, ranging from 60 to 100 mg/kg body weight. In the event of difficulty in administering the euthanasic drug, other active ingredients such as Ketamine or a Tiletamine+Zolazepam combination can be administered earlier. To be certain of death in the unconscious animal, an additional physical method such as freezing is possible (Divers & Stahl, 2019). Obtaining venous access, which is the main route of administration of anaesthetic substances in chelonians, is quite complex. In the event that it is not possible to administer the drug by this route, it is possible to proceed with an intracoelomatic one.

Euthanasia is a medical act, which therefore only a veterinarian is authorised to perform. In contrast, suppression is not a medical act and can be performed by trained non-medical personnel. However, it remains the case that suppression is carried out by means of drugs, so it must be practised by veterinary surgeons who are authorised to administer these active substances.

Directive 2010/63/EU, although it is addressed to animals used for research, adds to euthanasia the suppression methods and minimum welfare requirements that must be observed. The Directive states that animals must undergo suppression with the least possible pain, suffering and distress and that suppression must be performed by a person having specific knowledge on the matter (Article 6 of Directive 2010/63/EU on the protection of animals used for scientific purposes). The Directive also lays down specific methods (Annex IV) that are acceptable for different classes of animals.

The European Union also produced in 2022 a manual for the management of vertebrate invasive alien species of Union concern, incorporating animal welfare (https://www.specieinvasive.it/8-home/125-manuale-di-vertebrates-management-and-animal-welfare). The following tables show

[30] https://easin.jrc.ec.europa.eu/easin/Document/Final-deliverables-humane/Manual_management_vertebrate_IAS_incl_welfare_medres.pdf

which methods are acceptable for IAS identified by the EU, if the criteria of this Directive were to be followed. It should be noted that not all of these techniques are applicable or practical in a field situation or when controlling large numbers, but it provides an ethical basis for the type of management specified.

This document also contains a matrix with applicable measures to suppress the 22 vertebrate species of EU relevance. It should be noted that the manual presents an assessment of the impact of each measure on animal welfare, together with information on its effectiveness for the different management objectives (rapid eradication, eradication, population control and containment), and on costs and side effects. In this specific case it refers to *T. scripta*.

Measure	Commonly integrated with	Objective and availability					Effectiveness and costs
		Un	RE	Er	Cl	Ct	
Cage traps	Shooting; Sterilisation; Immunocontraceptive vaccines by injection	A	P	A	A	A	Cage trapping provides a widely used, flexible and effective method to catch and restrain a wide variety of different species. It is a frequently used and successful method to support the management of IAS. The requirement for regular checking brings significant implications for cost and their cost-effectiveness can be low in situations where the rate of animal capture is poor. Although the method is known to be available for the target species, further details are needed on its effectiveness and costs.
Hand removal	Trapping; Passive netting; Electrofishing; Pond fencing	P	A	A	A	A	Hand removal is considered effective for eradication or population control only when combined with other removal techniques. The method includes also the destruction of eggs, nests, and hatchlings. *Trachemys scripta* can be captured by hand or through various trapping devices. Eradication could be obtained by draining a water body, removing sliders by hand, and finally filling again with water.
Physical fishing methods	Electrofishing; Hand removal; Shooting; Biocontrol; Pond draining	P	A	P	A	A	Seine nets, longline fishing, various traps, hook and line are all physical fishing methods used for *Trachemys*. For example, longline fishing can be used to help control populations of *T. scripta*. Fishing with a hook and line has been tested as a management technique for *T. scripta*. In Spain, France, Portugal and Italy, population control and eradication campaigns have successfully used various types of traps to capture specimens of *T. scripta*; fyke nets seemed to be particularly effective in canals and ditches. A particular type of trap that has also been effectively used in management campaigns of *T. scripta* are basking traps, which consist in floating enclosures with sloping sides and a basking surface, from which a mesh basket hangs suspended. In Corsica, France, the use of the trapping technique proved relatively effective in a confined and isolated area, but did not eradicate the species.

Measure	Commonly integrated with	Objective and availability					Effectiveness and costs
		Un	RE	Er	Cl	Ct	
Aquatic habitat management - Pond drying/ draining	Hand removal; Trapping; Netting; Spearing; Shooting; Electrofishing	P	P	A	P	P	Draining invaded waterbodies can be used for localised management of *T. scripta* populations. This has been successfully implemented in Australia, where a series of water bodies invaded with *T. scripta* were drained, de-silted using an excavator, filled and compacted; the muck was spread, turned and raked with a tractor, and all remaining animals were removed by hand. When a water body is drained rapidly, up to 75% of sliders will emigrate, therefore sites should be secured with fences and pitfall traps to prevent emigration.
Shooting	Traps; Judas animals; Pond draining		P	P	A	A	Shooting is widely used as a wildlife management tool. This reflects its selective nature, its ability to manage animals at a distance, and the flexibility offered by the range of weapons and applications available. Although the method is known to be available for the target species, further details are needed on its effectiveness and costs
Native predators	Many other non-lethal measures				P		The measure might include introducing predators where they have recently been absent, or enhancing the predatory activity of existing native predators. Although the method is known to be (potentially) available for the target species, further details are needed on its effectiveness and costs.
Hunting dogs (tracking/ baying)	Traps		P	P	A		Detection dogs which never hunt or come in direct contact with the IAS, are used to find egg laying places for *Trachemys* species, e.g. in Spain. In particular, the LIFE Trachemys project mentioned that the measure works for new laid nests or for hatchlings, but that older nests are missed by sniffer dogs, which reduces the effectiveness of the measure. The method seems particularly effective for early detection/rapid eradication projects. Care needs taken when undertaken in places where native turtles also nest.
Physical terrestrial barriers	Trapping; Pond drainage	P				P	In France, the measure was very effective in keeping the red-eared sliders in the studied ponds during the project duration (4 years). However, fences were temporary, therefore the long-term effectiveness (individuals can live for up to 40 years) is unknown and will depend upon regular upkeep and maintenance. The relatively high cost of fencing—both building and maintenance—means it is only appropriate for use in relatively small or specific areas.
Judas animals	Hunting dogs; Shooting; Sterilisation	U					The Judas animal technique, which is based on the use of tagged individuals to find conspecifics, in species that are known to aggregate, is considered as potentially applicable to pond sliders. The method is most cost-efficient at very low densities of the target population. When only few animals are left in a population up for eradication, Judas animals will often be the only way of finding them all. Although the method is known to be potentially available for the target species, further details are needed on its effectiveness and costs.

Availability and effectiveness of management measures for *Trachemys scripta* (A = Available; U = Under development; P= Potential)

The same document reports the available methods for suppression/euthanasia and availability assessed for *T. scripta.*

Measures to dispatch/remove once captured	
Measure	**Availability**
Freezing	A
Injection euthanasia	A
Shooting - dispatch restrained animals	A
Cervical dislocation	P
Cranial depression	P
Electrocution	P
Keeping in captivity	A
Surgical sterilisation	A
Modified atmospheres	P

Availability of suppression measures for *Trachemys scripta* after capture
Legend

- Availability =
  A – Available;
  U – Under development;
  P – Potential.
- Objective =
  Un – Unknown/other objective;
  RE – Rapid eradication;
  Er – Eradication;
  Cl – [Population] Control;
  Ct – Containment.

CARCASS TREATMENT

The Italian National Management Plan for *T. scripta* specifies that the treatment of carcasses will take place by means of their transfer to disposal facilities, in accordance with current legislation, or to laboratories for possible health analyses (e.g. competent *Istituti Zooprofilattici Sperimentali*), to check for the presence of any parasites transmissible to other animal species and to man. It is also possible to provide for the transfer of specimens to research institutes or museums that make an explicit request.

All health issues must be agreed with the health authorities responsible for the area, such as the transport, in accordance with legal parameters, of the carcasses of slaughtered animals, the identification of any temporary storage sites (freezers, etc.), the identification of suitable methods of carcass disposal (e.g. through systems linked to slaughter activities).

E.13 PERSONNEL INVOLVED

Operators involved in IAS management activities shall be duly trained and/or experienced in the various procedures involving the capture, handling and transfer to the Animal Housing Centres or to the veterinary facilities designated for suppression of animals. In fact, the National Management Plan of *T. scripta* provides that interventions must be carried out by specially trained and specifically authorised personnel by the Region, Autonomous Province or local Protected Area specifically appointed to do so. Similarly, the Slovenian Action Plan for *Trachemys scripta* suggests that field actions should be carried out by people experienced and adequately trained in capturing turtles to ensure animal welfare measures and prevent damage to native species.

Similarly, the Guidelines in DD dated 16 March 2022 No. 12 state that:

Active monitoring of invasive alien species of EU relevance must be carried out by specialised or adequately trained personnel. Staff training and refresher courses are provided by the Regions and Autonomous Provinces through a continuous training approach, made indispensable by the complexity and extreme dynamism of the topic of invasive alien species.

b) Training can be carried out through in-presence, online learning (recorded or live), or through shadowing specialised personnel during the implementation of monitoring activities. Materials that can be used as part of staff training courses are available at www.specieinvasive.it.

c) Regardless of whether they are basic training courses or refresher courses for already trained personnel, the teaching activities must be carried out by recognised experts in the field of the study and management of invasive alien species.

d) Basic training courses must be scheduled in relation to specific needs (e.g. extent of monitoring, species-specific skills); on the contrary, refresher courses must be held at least once a year.

e) The topics that must necessarily characterise basic training are the following:

- EU and national regulatory framework on invasive alien species;

- List of invasive alien species of EU and national relevance;

- Description of the presence and distribution of invasive alien species of EU and national relevance in the national and regional territory;

- Species-specific monitoring and management methods contents of national management plans;

- Main technical and scientific literature on monitoring of the species considered;

- Organisation of the regional or provincial monitoring system for invasive alien species of EU and national importance;

- *Data collection and validation methods.*

Authorisation to carry out interventions related to the management of IAS, and in particular of *T. scripta* and other alien freshwater turtle species, can be obtained by filling in a form with the minimum information concerning them:

- Framework of the project in the context of national and/or international research and conservation issues inherent to the species;
- Actions and methodologies envisaged by the project;
- List of the areas in which it is intended to operate and location of the intervention sites;
- List of personnel involved with an indication of any training and/or demonstrable experience;
- Modes of transport and destination of captured animals.

Attached (**Annex 2**) is an example of an authorisation request proposed for the Emilia-Romagna Region by LIFE URCA PRO*EMYS*.

E.14 OTHER ALIEN SPECIES OF FRESHWATER TURTLES

Many scientific studies prove, for various of the exotic freshwater turtle species traded (e.g. Ficetola et al., 2007; Ficetola et al., 2009; Ferri, 2019), a comparable or even higher invasiveness than *T. scripta*.

Some of these species are already well regulated by international conventions such as CITES or banned for decades, such as the Italian Ministerial Decree dated 19 April 1996 regarding the "List of animal species that may constitute a danger to public health and safety and whose keeping is prohibited". Despite this, there is an important illegal trade widespread in all geographical areas and a lack of sensitivity and environmental ethics in the management of these turtles in captivity.

Below is a non-exhaustive list of exotic turtles reported in Italy to date (https://www.urcaproemys.eu/testuggini-esotiche/).

- ✓ *Apalone spinifera*
- ✓ *Apalone ferox*
- ✓ *Chelydra serpentina*
- ✓ *Chelus fimbriatus*
- ✓ *Chelodina longicollis*
- ✓ *Clemmys guttata*
- ✓ *Clemmys insculpta*
- ✓ *Chrysemys picta* (*C. p. picta* e *C. p. bellii*)
- ✓ *Graptemys geographica*
- ✓ *Graptemys ouachitensis*
- ✓ *Graptemys pseudog.*(*G. p. pseudog.* e *G. p. kohni*)
- ✓ *Macrochelys temminckii*
- ✓ *Mauremys sinensis*
- ✓ *Mauremys reevesii*
- ✓ *Pelomedusa olivacea*
- ✓ *Pelomedusa subrufa*
- ✓ *Pseudemys concinna* (*P. c. concinna* e *P. c. floridana*)
- ✓ *Pseudemys nelsonii*
- ✓ *Pseudemys peninsularis*
- ✓ *Pseudemys rubriventris*
- ✓ *Pelodiscus sinensis*
- ✓ *Kinosternon subrurum*
- ✓ *Sternotherus carinatus*
- ✓ *Sternotherus odoratus*

These species have different levels of threat to native biodiversity, depending on their ecological and biological characteristics.

The Plan for the control and management of exotic species of freshwater turtles (*Trachemys scripta* ssp. and others) drawn up by the WWF within the framework of the LIFE IP GESTIRE2020 for Lombardy region describes the level of risk to biodiversity linked to the spread of some of these species, describing how invasiveness may or may not be facilitated by the embryonic development and reproduction characteristics of these animals.

In fact, all turtles lay their eggs in nests and successful development depends on the incubation temperature, i.e. the average temperature of the nesting site during the embryonic development period. In most Chelonians, embryos develop successfully at incubation temperatures in the range of 24 - 32 °C, but in many species the sex ratio of the young hatched depends on temperature (*Temperature-dependent Sex Determination*, TSD).

There are two variants, one of which is the most common, i.e. *TSD 1A*, in which mostly males are born with relatively cold incubation temperatures and mostly females with relatively warm incubation temperatures. Only incubation of eggs around an established thermal centre (typically around 29 °C) produces both sexes (MF system, males and females).

However, some turtle species have an FMF system, called *TSD II*, in which both males and females are produced around two different thermal centres (around the first, between 20 and 21 °C, and above the second, usually between 26 and 28 °C, only females are produced), while with

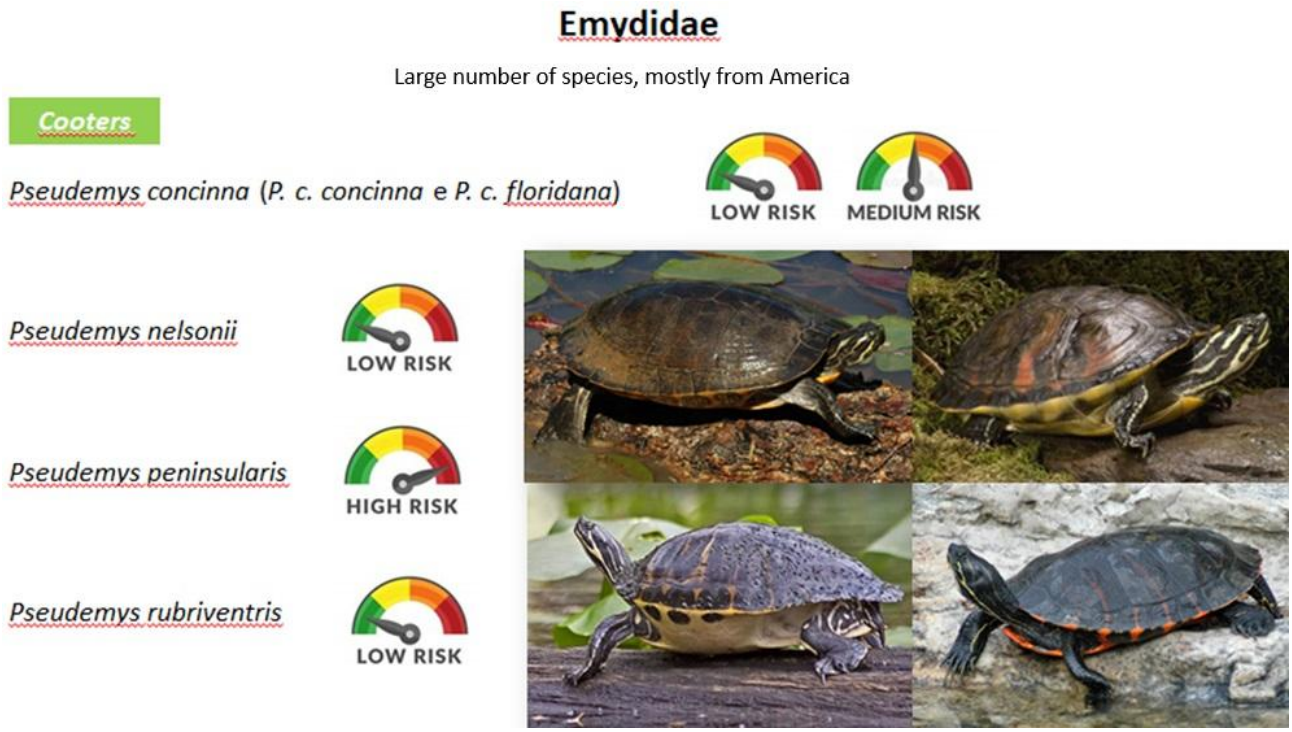
intermediate temperatures between these thermal centres only males are produced (Wyneken et al., 2008; Ferri, 2019).

For example, three species that are fairly traded and found in Europe have FMF, i.e. *Sternotherus odoratus*, *Chelydra serpentina* and *Chrysemys picta*. All these species have a particularly wide North American distribution with a considerable latitudinal range, a sign that they can endure well in temperate climates.

A minority have genotypic sex determination (GSD) and this is typical of species with distribution in relatively temperate territories.

Of the species listed above, excluding *T. scripta*, four have GSD, two have a TSD II system and only three have a confirmed TSD 1A system. For the remaining species, the system is unclear. Therefore, the majority of those species have the possibility to breed and have a correct sex-ratio at birth even in conditions of low mean temperatures, thus a fundamental step towards naturalisation in most European countries (Ferri, 2019).

Some of the other exotic freshwater turtle species potentially invasive to our territory are listed below, with an indication of the level of risk (iconographic material taken from Uetz et al., 2024).



Chrysemys picta
(C. p. picta e *C. p. bellii)*

Clemmys guttata

EN

Clemmys insculpta

Map turtles

Graptemys geographica

Graptemys pseudogeographica

Graptemys ouachitensis

Geoemydidae

Family with the largest number of species in the world: 60 species in 23 genera

Mauremys reevesii

Mauremys sinensis

Other *Mauremys* indigenous to Europe

Kinosternidae

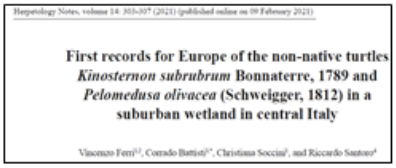
Musk turtles

Sternotherus odoratus

Sternotherus carinatus

Mud turtles

Kinosternon subrurum



Herpetology Notes, volume 14: 303-307 (2021) (published online on 09 February 2021)

First records for Europe of the non-native turtles *Kinosternon subrubrum* Bonnaterre, 1789 and *Pelomedusa olivacea* (Schweigger, 1812) in a suburban wetland in central Italy

Vincenzo Ferri[1], Corrado Battisti[2,*], Christiana Soccini[3], and Riccardo Santoro[4]

Pelomedusidae

Helmeted turtles

African Species

Pelomedusa subrufa

MEDIUM RISK

Pelomedusa olivacea

Trionychidae

Softshell turtles

Apalone ferox

MEDIUM RISK

- South-East USA
- Females up to 70 cm and 40 kg
- No TSD
- Up to 7 egg laying/year

Apalone spinifera

MEDIUM RISK

Pelodiscus sinensis

VU

HIGH RISK

- ***East and South-East Asia***
- ***Most traded chelonian: about 200 million specimens/year***
- ***Food and pet trade***

Chelydridae

Snapping turtle

Chelydra serpentina

HIGH RISK

The Italian Ministerial Decree dated 18 April 1996 includes *C. serpentina* among the **dangerous animals**

Alligator snapping turtle

Macrochelys temminckii

HIGH RISK

M. temminckii is included among the **dangerous animals** under the Italian Ministerial Decree dated 18 April 1996

Suborder **Pleurodira**
Family **Chelidae**

Chelodina longicollis

Snake turtle

✓ *Australia*

Chelus fimbriatus

Mata mata

✓ *Latin America*

F. MONITORING OF THE STATUS OF *EMYS ORBICULARIS* POPULATIONS

The Manual for monitoring of species and habitats of Community interest in Italy in relation to animal species[31] indicates the methods for monitoring *Emys orbicularis* and *Emys trinacris* for proper drafting of the Habitats Directive (92/43/EEC) Report that EU countries must prepare periodically. The LIFE URCA PRO*EMYS* has explicitly referred to what ISPRA has indicated and which is reported below. For monitoring of *Trachemys* see E.10.

F.1 MONITORING TECHNIQUES

In Italy, at sites with access to the water body, the population estimate must be carried out using the capture-mark-recapture (CMR) method, a method that requires ministerial authorisation as an exception to Presidential Decree 357/97 (and trained and qualified personnel) to be requested through a special form that can be downloaded from the MASE website https://www.mase.gov.it/pagina/deroghe-ai-sensi-dellart-16-della-direttiva-habitat. Alternatively, but less efficiently, in sites with difficult access to the water body but with good visibility of the banks even from a distance, it is possible to carry out repeated counts on either (i) known areas or (ii) along transects without the need to capture animals.

Capture-Marking-Recapture protocols are based on the principle of carrying out **trapping sessions** at **regular intervals**, at **fixed sites**, in which specimens of the species of interest are captured, marked and then released. In subsequent sessions, some of the marked specimens will be recaptured one or more times. The data collected in this way (number of captures of new specimens, number of recaptures of marked specimens, time interval between recaptures, etc.) will make it possible, by means of statistical models, to estimate the total number of specimens present in the population of the study area and other parameters of the population itself.

For the CMR method to work, it is essential that:

- **Several** trapping **sessions** are carried out correctly (minimum **three**, better 5 or 10);

[31] Lo Valvo M., Oneto F., Ottonello D., Zuffi M.A.L. (2016). In: Stoch F., Genovesi P. (Ed.): Manuali per il monitoraggio di specie e habitat di interesse comunitario (Direttiva 92/43/CEE) in Italia: specie animali. ISPRA, Serie Manuali e linee guida, 141/2016

- The points where the traps are set (**trapping sites**) are **fixed**, remaining the same between one session and the next;
- All trapped animals are **correctly marked** and the markings are read correctly in the event of recapture;
- The interval between one session and the next is as regular as possible (e.g. one session every week).

In Italy complete **session** consists of **three trap nights**: on the first day the traps are set (first trap night); on the second day the traps are checked, captured animals are measured/marked/released and the traps are reactivated in position (second trap night); on the third day the same operations are repeated (third trap night); on the fourth day the traps are checked again, captured animals are measured/marked/released and the traps are deactivated. In Slovenia, traps can be alternatively set for up to four trap nights. For the capture of European pond turtles, **double decoy creels** or **square creels** (in Slovenia) are used (see Section E10).

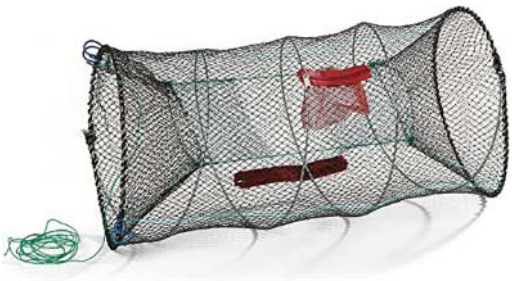
The following is a summary of the activities that Life URCA PRO*EMYS* has planned for the monitoring of *Emys* spp. and that can be replicated in other contexts:

DAY 1

- Carrying out a preliminary survey with observation through binoculars (visual census) of the presence of turtles, both target and alien species, on the banks of the bodies of water in the study area or floating in them;
- Identifying suitable sites for the placement of creels and positioning them:
  - In a water body (pond or canal) in the study area, we will place 10 open creels at a distance of approximately 50 m apart; depending on the site, slightly different distances can be chosen, as long as the range between each creel is 30-50 m. If there are no water bodies of sufficient size at the site to accommodate the 10 creels, or if there are several micro-environments suitable for the species, it is possible to divide the creels into two/maximum three different water bodies, sufficiently close together. It is advisable not to sample more than three water bodies, in order not to excessively fragment the data.
  - Each creel should be fixed by means of a nylon string to a stable support (riparian vegetation or stakes driven into the ground); inside each creel, place one/two empty plastic bottles, equal to a total volume of at least 1.5-2 l, which will act as a float; in this

way part of the creel will remain above the water level, allowing the captured animals, both of the target species and others, to breathe.

- The creels should be placed at least a couple of metres from the shore and in water at least 40-50 cm deep, preferably close to areas used by the animals for thermoregulation (basking sites), at passage points, in areas with dense aquatic or riparian vegetation. Both entrances must be completely submerged in water and well accessible to the animals, i.e. not adjacent to submerged obstacles or the shoreline. The creels can be positioned either parallel or perpendicular to the shore, depending on the micro-environmental context.
- Finally, take the GPS coordinates of each creel (georeferencing), using a GPS device or mobile phone app (e.g. OruxMaps, Locus Map, Commander Compass), and mark the position of each creel with a marker tape, with the corresponding creel number written on it.



DAY 2

- Checking the creels. The following tasks will be carried out for each creel:
  - Extract any animals caught, close the creel and bring the creel and animals to shore.

- Secure the animals in a container (e.g. a plastic box of suitable size or bucket) so that they cannot escape.
- For each specimen of the species of interest:
    - Collect biometric data
    - Mark the animal
    - Photograph the animal
    - Only for the autochthonous species *E. orbicularis*, release the animal near the capture site, placing it in a quiet spot on the shore and letting it move freely.
- Only after finishing all operations on one specimen, move on to the next. In areas with a high probability of non-native fish being caught, it is preferable to carry two or more buckets/boxes and always use one for *Emys*, the other for non-natives. It is advisable to disinfect your hands with hydroalcoholic gel between each animal and clean the containers with alcohol after each use, especially if alien species specimens are caught.

The same procedure should be repeated in subsequent capture sessions.
The optimal period for monitoring is from May to the end of June, or in any case until there is sufficient water in the pond/canal where the traps are placed and the local average temperatures do not become excessive; it is also possible to postpone the sessions to September/October, when the animals finish summering and there is a second peak of animal activity; this autumn activity is, however, less intense than the spring one and monitoring carried out in this period could result in a slight underestimation of the population density.

BIOMETRIC DATA COLLECTION

Data should be recorded on the appropriate field sheets. Use a different card for each site and for each day of each monitoring session.
For each *Emys orbicularis* specimen, the data to be collected are:

- ➔ Sex (M, F, JUV ND*) (*ND is used for non-determinable juveniles).
- ➔ Length of carapace (a) measured along the head/tail line, placing the calibre teeth in the notch between the nuchal scutes and in the notch between the supracaudal scutes.

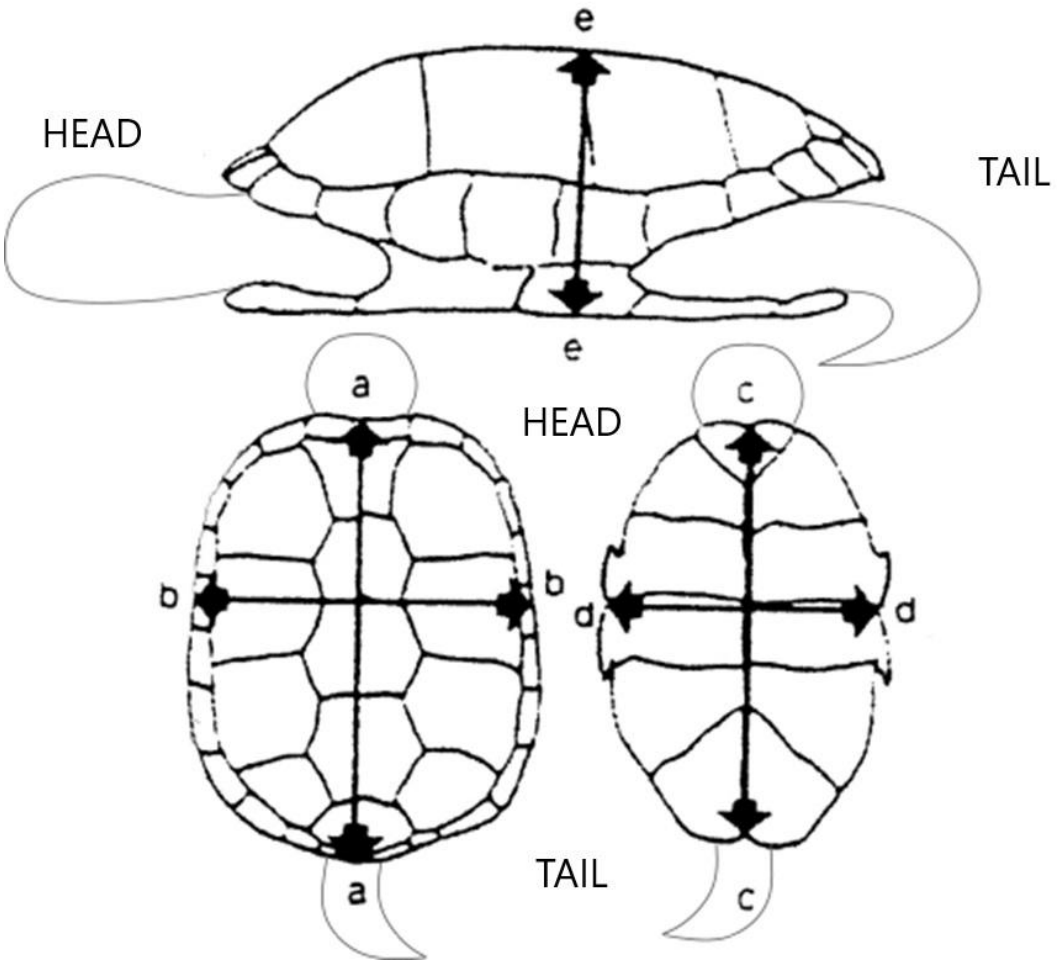
- ➔ Width of the carapace (b) measured at the suture between the third and fourth rows of plates of the plastron, positioning the animal ventrally and considering the bridge between the carapace and the plastron.
- ➔ Length of the plastron (c) measured along the head/tail line, with the animal in ventral view, placing the gauge teeth in the notch between the gular plates and in the notch between the anal plates.
- ➔ Width of the plastron (d) measured at the suture between the third and fourth row of plates of the plate, positioning the animal ventrally and excluding the bridge.
- ➔ Height of carapace (e) measured at the suture between the third and fourth row of plates of the plastron, with the animal on the side (not always measured).
- ➔ Weight (in g), using precision scales.
- ➔ Presence or absence of eggs by groin palpation (adult females only) (eggs yes/no).
    - Groin palpation is carried out by inserting the index fingers of both hands into the grooves of the animal's hind legs and feeling the abdomen; it is possible to recognise the internal presence of more or less mature eggs, which are perceived as rigid or semi-rigid ogival-shaped structures (similar to large grapes), but not to establish their number reliably.

In Slovenia, in some cases additional measurements, such as width of plastron on different places, length and width of nuchal scute, length of the tail and distance from the end of the plastron to the cloaca are taken. Also colour of the eyes, algae on shell, presence of parasites and any other peculiarity are recorded. For every specimen also age class in which it belongs is determined based on the visible growth range between the plates on the plastron and the wear of the annuli or growth rings on the carapace and pectorals on the plastron (Castant, 1988; Zug, 1991): juvenile, subadult, juvenile adult, adult, old adult.

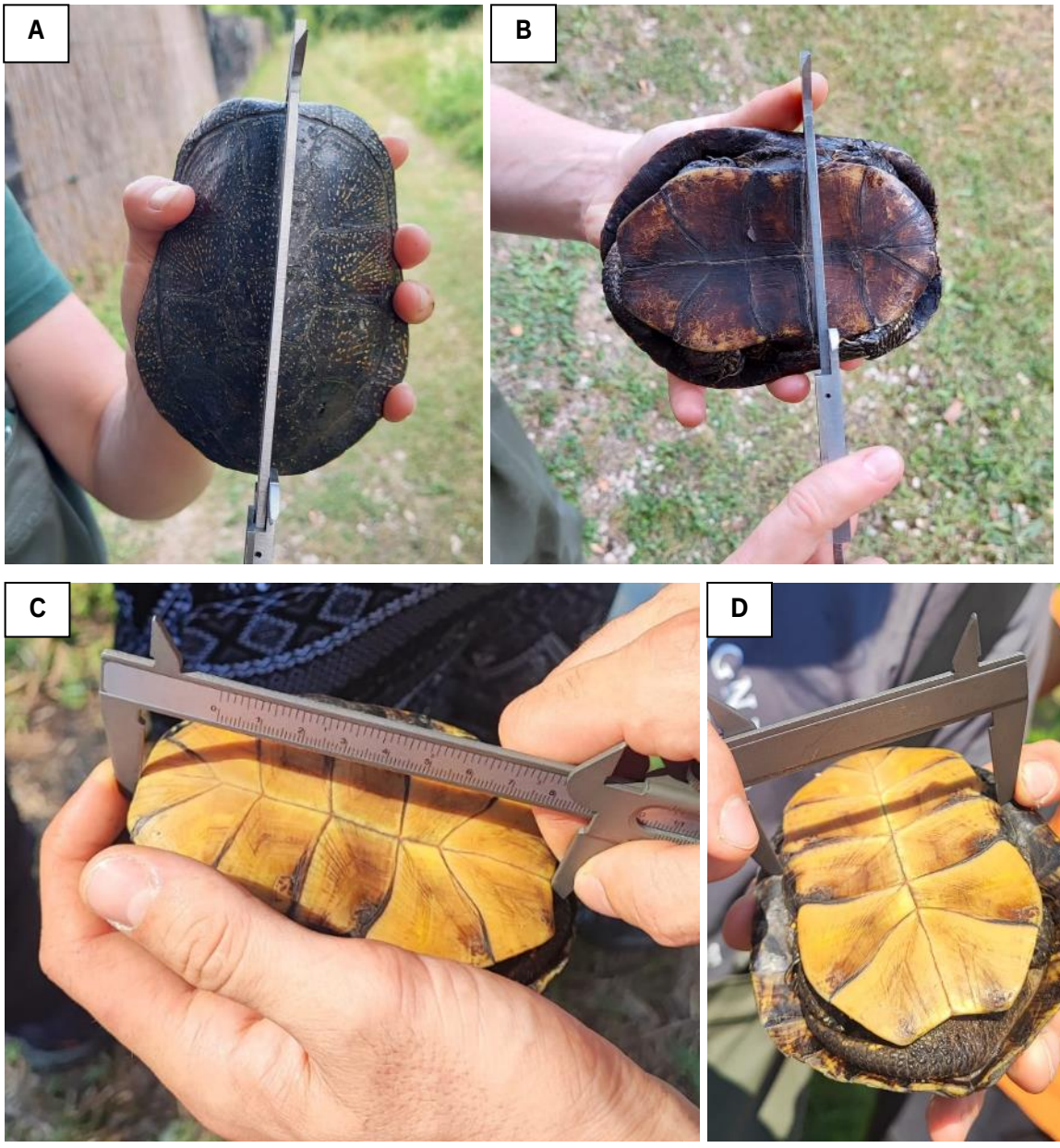
All measurements, observations and remarks are taken every first time in a year when turtle is caught. At the same time, two pictures of turtles are taken, one from ventral and one from abdominal site.

Measurements should be taken with a rigid analogue caliper, taking care to position it correctly and read the measurement correctly; they are recorded in mm.

Groin palpation of *Emys* adult female to check for eggs. Photo by J. Nardi.



Measuring points.



Measurement points for *E. orbicularis* shown during actual operation. From top to bottom: carapace length (A), carapace width (B), plastron length (C), plastron width (D), carapace height (E). Two different specimens are shown. Photo LIFE URCA PROEMYS.

Emys orbicularis measurements - field data

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SITE: _____

ID	Trap	date	sex	Carapace length	Carapace width	Plastron length	Plastron width	Carapace height	Body mass	Reproduction	New/ Recap	notes

sex: m = male; f = female; j = juvenile; h = hatchling.
Reproduction: YES/NO

– ALL MEASUREMENTS IN MILLIMETERS; BODY MASS IN GRAMS.

Example of the field form for *E. orbicularis*.

Legend of the field sheet for *Emys orbicularis*:
SITE = name of the reserve/protected area/SPA where the specimen was captured
ID = specimen tag code assigned to the specimen
Trap = trap number where the specimen was caught
date = date of the day of capture
sex = sex
Carapace length (a)
Carapace width (b)
Plastron length (c)
Plastron width (d)
Carapace height (e)
Body mass = weight of the animal
Reproduction = presence or absence of eggs (only adult females)
New/Recap = indicates whether the animal was captured for the first time or is a recapture; mark N for first capture, R for recapture
notes = any notes or peculiarities to be reported for the animal (e.g., wounds, scars, abnormal colouration, presence of SCUD).

MARKING

In turtles, marking can be carried out simply by **engraving a notch in the edge of some marginal plates with a handsaw**, according to a code explained in the dedicated sheet. Essentially, this procedure follows Stubbs et al. (1984).

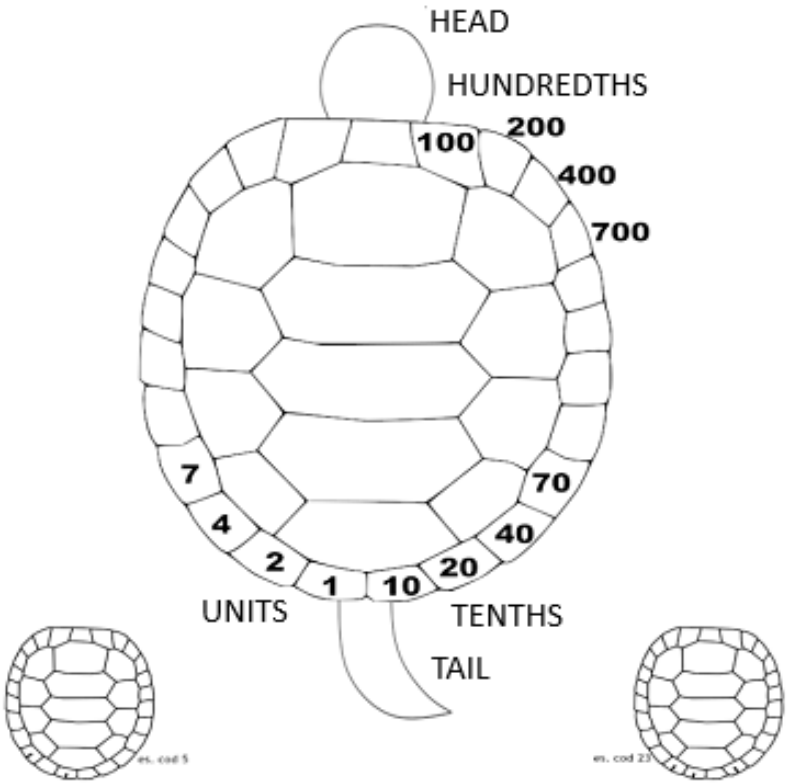
The notch should be **3-4 mm** deep, perpendicular to the edge of the plate, and remain visible for years. If correctly performed, this method is completely harmless for the animal. If the cut is too deep, there may be a very slight bleeding (stop and do not cut any further if you notice blood starting to come out while cutting with the hacksaw!), which however denotes a superficial wound that is not serious. It is advisable to mark carefully and to check the progress of the notch often to minimise the risk of going too deep.

The standard marking codes in use in the project in Italy are shown in Figures in next pages. In Slovenia, similar markings are used but with different code (Vamberger, 2012). To avoid confusion, the same code as in the previous monitoring is used. If specimens of *Emys* have already been marked in the protected area in the past, it is recommended to continue to use the same type of previous marking codes, proceeding in ascending order (to avoid possible reading errors between the 'old' and 'new' marking scheme in the case of recaptures from previous years), and to indicate the marking scheme used in the notes.

Example of correctly executed marking (on a terrestrial tortoise), dorsal and ventral view. Photo by J. Nardi.

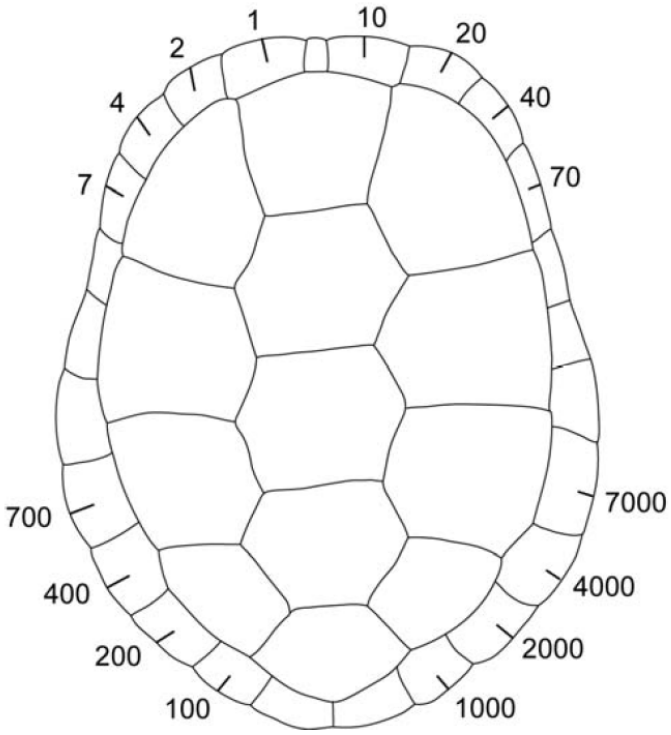
Example of a possible model of marking hacksaw (approx. 15 cm blade).

Marking being performed on an *E. orbicularis* specimen. Photo LIFE URCA PROEMYS.



Marking diagram for freshwater turtle specimens <u>used in Italy</u>, with on marginal plates.

Carapace drawing repurposed from Magwene & Socha2012.



Marking diagram for freshwater turtle specimens <u>used in Slovenia</u>, with engraving on marginal plates (adapted from Turjak & Vamberger, 2008).

Marking per specimen:

This marking should be carried out on every specimen caught, whether of *E. orbicularis* or *T. scripta* and other alien turtle species. Though in Slovenia specimens meant for euthanasia are excepted. The marginal plates (forming the edge of the carapace) are marked. In Italy, the four plates to the left of the tail, with the animal in dorsal view, are 'worth' 1, 2, 4 and 7 respectively. The four to the right 10, 20, 40, 70. The four to the right of the head 100, 200, 400, 700. Different code is used for marking turtles caught in Slovenia (figure above). The four plates to the left of the head (turtle in dorsal view), represent numbers 1, 2, 4 and 7 respectively, the four plates to the right of the head 10, 20, 40 and 70, the plates left from the tail, but with exception of first plate, represent numbers 100, 200, 400 and 700 and plates right from the tail, but with exception of first plate, represent numbers 1000, 2000, 4000 and 7000.

Each code is a number given by the sum of the values of the marked scutes; so, for example, if a specimen is marked (in Italy) on the first and third scutes to the left of the tail, its code will be "5"

(1 + 4); if a specimen is marked on the second to the right of the tail and the first and second to the left, its code will be "23" (20 + 1 + 2), etc. Codes must be assigned in ascending order for each area, so the very first animal caught in a certain area will have the code '1', the second '2', and so on. In Slovenia numbering is on a national level so two turtles do not have the same number in Slovenia.

The codes must be unique: never mark two different animals with the same code in the same area, even if caught in different sessions.

Sampling stations are identified within a suitable number of 10×10 km cells in which the species is known. For monitoring within specimen Natura 2000 areas, the application of population studies by capture/marking/recapture or alternatively the method of repeat counts is proposed. All selected sites are recorded and transects or observation/capture points mapped to allow standardised repetitions over the years. The assessment of the distribution of *E. orbicularis* at the national scale is carried out using models based on the detection of the number of 'localities' within the national 10×10 km grid.

The number of total reports in that cell is considered as a measure of sampling effort. For *Emys trinacris*, due to the small number of reports, this parameter is assessed by periodic confirmation of the species' presence in all the 10×10 km cells in which it is reported.

F.2 ESTIMATION OF THE POPULATION PARAMETER

Attempts will be made to obtain numerical estimates at a suitable number of sample sites for each biogeographical region and, at sites where the CMR method is adopted, data on sex-ratio and population structure will also be obtained.

F.3 ESTIMATION OF HABITAT QUALITY FOR THE SPECIES

The main parameters for defining the habitat quality of pond turtles are: the presence of aquatic and riparian vegetation, the presence of basking sites and suitable laying areas and, in general, shallow water depth, as well as the absence of alien competitor species, pollutant sources, the absence of roads in the vicinity of the sites, and low anthropic disturbance in general.

Operational indications: The CMR methods described above are applied to estimate absolute abundance.

Estimated workdays per year. For CMR three to five sessions/year consisting of at least three days each per site in the indicated period, possibly distributed in equidistant visits over time. For repeated counts three days/year per site within the specified period.

Minimum number of persons to be employed. At least two operators are required to carry out monitoring in the case of CMR, one in the case of counts.

Number of monitoring to be carried out over the six-year period according to Art. 17 of the Habitats Directive. Monitoring is to be repeated every two years.[32]

[32] Lo Valvo M., Oneto F., Ottonello D., Zuffi M.A.L. (2016). In: Stoch F., Genovesi P. (Ed.): Manuali per il monitoraggio di specie e habitat di interesse comunitario (Direttiva 92/43/CEE) in Italia: specie animali. ISPRA, Serie Manuali e linee guida, 141/2016

G. COMMUNICATION AND DISSEMINATION

In every nature conservation project it is essential that, in addition to concrete conservation actions, an extensive and intense communication and awareness-raising action is also carried out, which is fundamental for the success of the conservation efforts implemented and to ensure that the results are rooted in the territory and that there is a constant direct line with the local population, in order to make them participate in the progress, difficulties and successes achieved during the implementation of the actions and to encourage compatible use of the local natural resources by the local community and tourists.

For communication on the conservation of *Emys orbicularis*, messages should be adapted to a non-specialist audience, avoiding complex technical language and favouring stories and other elements (visual and otherwise) that make the message easy to understand. A special care must be put on messages concerning the management and containment of alien species (see also E.7), which must necessarily aim to get individuals to adopt virtuous behaviour, as a result of an acquired awareness and attention to the issue. It is therefore a priority to stimulate such behaviour, increasing public knowledge of alien turtles and awareness of the need and urgency of their management through well-planned and targeted communication programmes.

The general objectives to keep in mind are:

1) The raising of awareness among the public and the various stakeholders about the presence of *Emys orbicularis* in a specific area, the conservation risks it is subjected to, the impacts caused by habitat alteration/destruction and invasive alien species on its survival and how to prevent the introduction of alien species of pond turtles into the wild;

2) The active participation of local communities, aimed at the adoption of positive behaviour to limit the introduction of alien species into the wild and the collection of useful data on the presence and spread of invasive alien species;

3) The active involvement of Natura 2000 site management bodies and public authorities in the implementation of the management plan.

Below are some suggestions for effective communication/dissemination:

1. **Knowledge of the Species**: Ensure that you have a thorough knowledge of the *Emys orbicularis* pond turtle, including the threats it faces, its natural habitat and its role in the ecosystem. This information will provide a solid basis for communication.

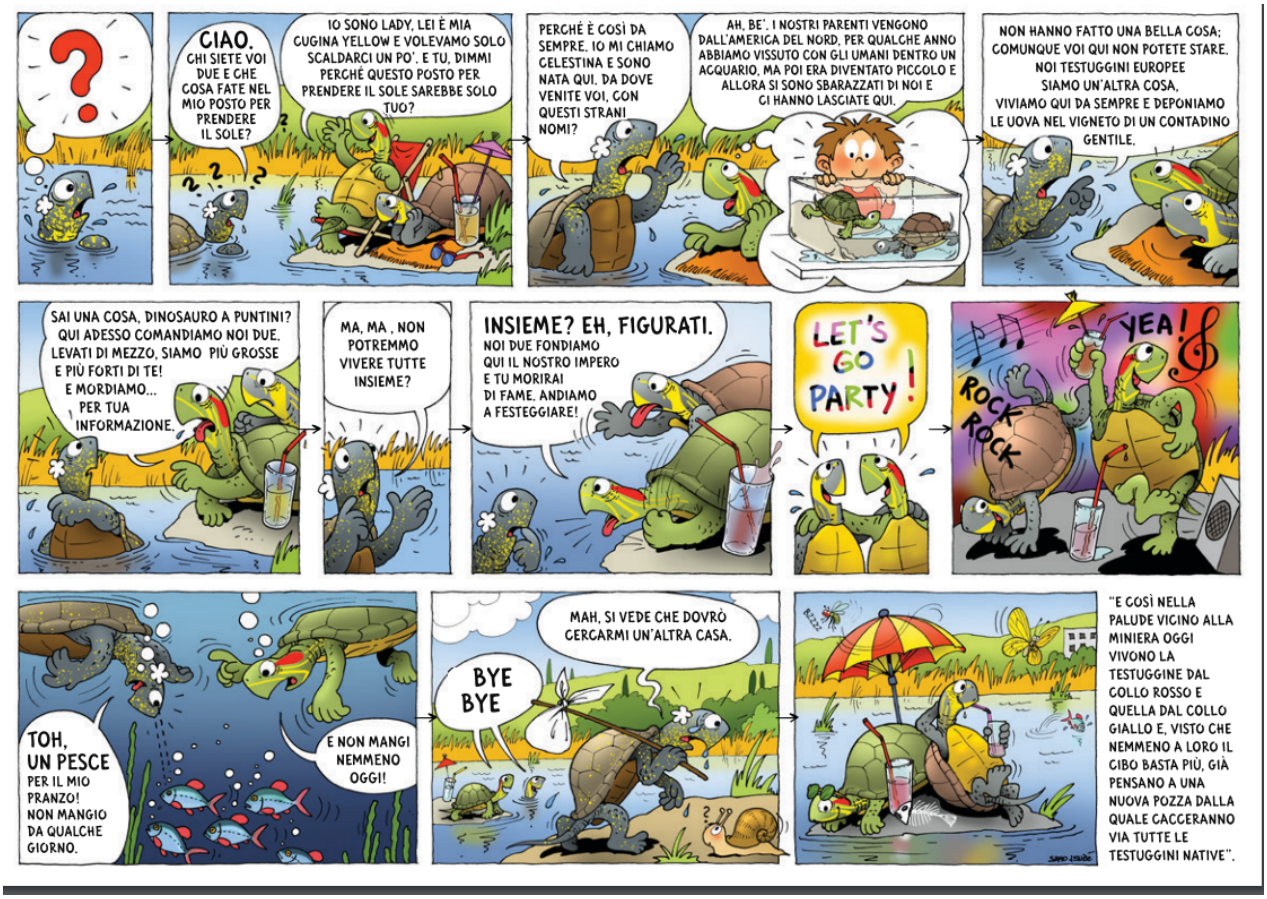
2. **Positive Communication**: Use a positive and inspiring tone in communication. Emphasise the intrinsic value of biodiversity and the key role pond turtles play in the balance of the ecosystem.
3. **Engaging Stories**: Tell engaging stories about the lives of pond turtles and their conservation successes. Personal stories and concrete examples can emotionally connect the audience to the issue.
4. **Visual Storytelling:** Use pictures and videos to communicate visually. Show the beauty and vulnerability of the pond turtle and its habitat to elicit empathy and attention.
5. **Public Involvement**: Actively involve the public in initiatives. Organise events, workshops, or educational activities that allow people to directly experience the importance of conservation.
6. **Collaborations:** Collaborate with environmental organisations, scientific institutions and other entities interested in conservation. Partnerships can increase the reach of initiatives and provide additional resources.
7. **Use of Social Media:** Use social media to reach a wider audience. Create interesting content, use relevant hashtags and engage the community through polls and discussions.
8. **Environmental Education**: Provide educational information through printed materials, seminars or presentations in schools and communities. Environmental education is key to raising awareness.
9. **Involvement of Local Communities**: Involve local communities in conservation activities. Understanding and respecting local traditions can contribute to greater adherence to the cause.
10. **Long-Term Sustainability**: Planning initiatives that have a long-term impact on pond turtle conservation. Provide and suggest concrete ways and activities for the public to contribute to the cause over time.

It is important to remember that the key to effective communication is consistency in the message and the ability to engage the public effectively. See also the LIFE URCA PRO*EMYS* website (https://www.urcaproemys.eu/materiale-educativo/)

INFORMATION TO BE DISSEMINATED AND KEY MESSAGES

Topic	Message	Target	Behaviour Change	Activities/Channels
Importance of native pond turtles for maintaining wetland biodiversity	Warning: *Emys orbicularis* and *Emys trinacris* are endangered	Tourists, visitors to protected areas students, teachers	Do not disturb the pond turtles, do not destroy their habitat	Distribution of information material, outdoor activities and guided tours, events, social media communication, websites
Effects of human activities on *Emys* spp.	Human activities can have negative effects on wetland environments, causing loss of biodiversity	Children, visitors to protected areas, general public, web users	Act responsibly, bearing in mind that our behaviour has an impact on the environment	Dissemination of videos and information materials, social media, educational events
Invasive alien species	Alien pond turtles are a major threat to the survival of *Emys* spp.	Pet traders, alien turtle owners, children, students, general public	Do not release exotic turtles into the wild. Inform customers who want to buy an exotic bog turtle of the consequences this may entail	Production and dissemination of dedicated information material, dissemination of information through social channels and specialised magazines, creation and dissemination of the list of refuge centres for alien turtles
Conservation actions need to be put in place	*Emys* spp. have an inadequate conservation status. This is in line with the fact that in the last century the species has faced a drastic decline due to the extensive draining and reclamation of wetlands	Managing bodies of Natura 2000 sites, research institutes, bodies involved in environmental management and protection	Collaboration and participation in the drafting of the integrated management plan, acquisition of the management plan	Workshops, technical meetings

EXAMPLES OF TEACHING MATERIALS



TESTUGGINE PALUSTRE EUROPEA

(Emys orbicularis)

La testuggine palustre europea è l'unica testuggine d'acqua dolce autoctona slovena. La incontriamo più frequentemente nelle Barje di Lubiana, nella Bela Krajina, nel bacino del fiume Sava e nell'Istria slovena, ma la si trova anche in altre zone della Slovenia. È elencata nella Convenzione di Berna ed è anche protetta dalla rete di siti NATURA 2000.

Vive in acque interne stagnanti e a lento corso con scarsa vegetazione ripariale. È facile notarla mentre prende il sole sulla riva, ma è molto timida e corre in acqua al minimo segnale di allarme. La gente la confonde con la testuggine palustre americana *Trachemys scripta*, più frequente e non autoctona, che è molto più aggressiva e che, tra le altre cose, scaccia continuamente la *Emys orbicolaris* dal suo habitat. Trascorre la maggior parte del tempo in acqua, dove cattura le sue prede e può vivere fino a 120 anni.

Il Parco Naturale delle saline di Sicciole ospita probabilmente la più grande popolazione slovena di questa specie. Si trova nell'area della palude d'acqua dolce nei pressi della miniera di Sicciole, nel fossato vicino all'aeroporto, nell'estuario del fiume Dragonja e alle Stojbe.

LO SAPEVATE?

- Che la testuggine palustre europea non può inghiottire il cibo senz'acqua.
- Che la testuggine palustre europea può vivere fino a 120 anni.
- Che la testuggine palustre europea è un indicatore di buone condizioni ambientali.
- Che in Slovenia vivono due sottospecie di testuggine palustre europea e nell'Istria slovena è presente solamente la sottospecie *E. o. hellenica* che può raggiungere fino a 16 cm di lunghezza.

TESTUGGINE PALUSTRE AMERICANA

(Trachemys scripta)

Delle tre sottospecie note se ne riconoscono facilmente due, ossia quella dal collo rosso (*T. s. elegans*) e quella dal collo giallo (*T. s. scripta*). La testuggine palustre americana, originaria dell'America settentrionale e centrale, è da molto tempo uno degli animali domestici preferiti. Oggi è la specie aliena di testuggine più diffusa in Slovenia che, oltre a svernare con successo se rilasciata nell'ambiente, è in grado di riprodursi. In natura la testuggine palustre americana sceglie un habitat simile a quello della testuggine palustre europea con la quale lotta con successo per accaparrarsi i posti migliori per prendere il sole e deporre le uova. Scaccia la testuggine di palude, influenza negativamente anche altre specie e, inoltre, mette in pericolo persino la salute umana trasmettendo nuove malattie. Dal 2016 è inclusa nell'elenco delle specie esotiche invasive nell'UE ed è soggetta a misure rigorose, tra le quali il divieto di vendita e il divieto di rilascio nell'ambiente. Oltre alla testuggine americana, in futuro si può prevedere la diffusione di altre specie di testuggini non autoctone.

LO SAPEVATE?

- Che sono riconosciute tre sottospecie di testuggine palustre americana, vale a dire: la testuggine dalle orecchie rosse, la testuggine dalle orecchie gialle e la testuggine dalle orecchie arancioni.
- Che la testuggine palustre americana può raggiungere anche i 60 cm di lunghezza.
- Che la testuggine palustre americana in cattività può vivere fino a 50 anni.

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ANNEX 1. VETERINARY PROTOCOL

LIFE21-NAT-IT-LIFE URCA PROEMYS

LIFE URCA PRO*EMYS*

URgent **C**onservation **A**ctions pro *Emys orbicularis* in Italy and Slovenia

VETERINARY PROTOCOL

Date of Sending	24 July 2023
Version	1
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Action Deliverable	WP2
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This document is the preliminary veterinary protocol of the LIFE URCA PRO*EMYS* project, which will be used in the management of autochthonous and allochthonous turtles. The document may be modified and updated during the course of the project on the basis of experience gained and the results of diagnostic analyses.

The document is the result of the elaboration of the text:
LIFE EMYS Ligurian Invasive Fauna Eradication pro indigenous Emys orbicularis restocking
Disease Risk Analysis report (DRA)

1 Abstract

The objective of the LIFE URCA PRO*EMYS* project is the conservation of the European pond turtle, *Emys orbicularis*, in Italy and Slovenia, through actions of removal of allochthonous turtles, environmental restoration and the release of individuals of *Emys orbicularis* reproduced *ex situ*.

Restocking actions involving the release of individuals reproduced ex situ, entail the risk of introducing into the wild, together with the released individuals, potentially dangerous pathogens that could spread in the wild population. Restocking actions must therefore include, in their planning, health procedures aimed at mitigating this risk.

At the same time, management procedures must ensure the welfare of the animals at all stages of the project and the good health of the individuals intended for release in order to optimise their survival in the restocking sites.

Starting from the Disease Risk Analysis Report elaborated during the LIFE EMYS project (Ligurian Invasive Fauna Eradication pro indigenous *Emys orbicularis* restocking - LIFE 12NAT/IT/0000395), this document illustrates the sanitary procedures that will be adopted by the LIFE URCA PRO*EMYS* project in order to minimise the sanitary risks connected to restocking actions. The document also contains some procedures for the management of allochthonous turtles captured during the reclamation activities and destined to the recovery and permanent holding centres.

The analyses foreseen by the protocol will be carried out by *Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (IZSPLV)*, which collaborated in the drafting of this document and with which a collaboration agreement has been established.

This protocol must be considered a preliminary document, which may be modified and updated during the course of the project, on the basis of the experience gained and the results of the analyses carried out.

2 Introduction

The objective of the LIFE URCA PRO*EMYS* project is the conservation of the European pond turtle, *Emys orbicularis*, on the Italian and Slovenian territory, through the removal of alien turtles (with particular reference to the turtles of the genus *Trachemys*), which compete with the autochthonous turtles in their original habitats, the environmental restoration and the release of specimens of *Emys orbicularis* reproduced *ex situ*.

Repopulation actions involving the release of *ex situ*-reproduced specimens entail the risk of introducing potentially dangerous pathogens into the wild with the released animals, which could spread to the wild population. Repopulation actions must therefore include specific health procedures in their planning to mitigate this risk. These procedures must also minimise the risk of zoonoses for operators who come into contact with the animals.

At the same time, the management procedures must ensure the welfare of the animals at all stages of the project and the good health of the specimens intended for release, in order to optimise their survival in the restocking sites.

According to the DRA (*Disease Risk Analysis*) developed during the LIFE EMYS project (LIFE 12NAT/IT/0000395, 2014-2016), in the case of freshwater testudinates, veterinary prophylaxis must prevent the spread of specific pathogens (see below), which are considered medium-high risk due to their potential to spread among wild specimens and/or among operators who come into contact with infected animals.

Starting from the DRA developed during the LIFE EMYS project, this document illustrates the sanitary procedures that will be adopted by the LIFE URCA PRO*EMYS* project in order to minimise the sanitary risks related to restocking actions. The document also contains some procedures for the management of allochthonous turtle specimens captured during the recovery activities and destined to the recovery and permanent holding centres.

The analyses foreseen by the protocol will be carried out by the *Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta* (IZSPLV) which has collaborated in the drafting of this document and with which Costa Edutainment has established a collaboration agreement.

This protocol shall be considered as a preliminary document, which may be modified and updated during the project, on the basis of the experience gained and the results of the analyses carried out.

Summary of the objectives of the Veterinary Protocol:

1. Ensure the welfare of the animals in all stages of the project.
2. Minimise the risk of introducing pathogens from the controlled environment into the wild, potentially dangerous to wild populations.
3. Ensure the good health of *Emys orbicularis* individuals destined for release to maximise their likelihood of survival in repopulation sites.
4. Minimise the risk of zoonoses to operators who come into contact with the animals.

3 Animal Welfare

Respect for the welfare of animals is a fundamental requirement of the LIFE URCA PRO*EMYS* project, both for ethical reasons and in order to achieve the project objectives; poor welfare conditions can in fact promote disease and increase mortality. Some minimum requirements for turtle breeding and keeping facilities and procedures for handling and transporting animals are outlined below.

3.1 Characteristics of breeding and rearing facilities (minimum requirements)

In order to ensure the welfare of the animals, all breeding and rearing centres must meet minimum standards of maintenance and management. Centres must have an outdoor enclosure, with semi-natural environmental conditions, and an indoor enclosure, with controlled environmental conditions.

- **Outdoor enclosure**
  - The outdoor enclosure must consist of a minimum of three tanks: one for breeding stock, one for immature animals, and one for animals undergoing treatment. The tanks should accommodate a number of animals compatible with the size of the tanks, avoiding overcrowding situations that could cause stress to the animals and not allow adequate water quality to be maintained. The ratio can be considered suitable when each adult specimen has 2 m^2 of surface area; for sub-adult turtles and juveniles of 1-3 years of age, the minimum required surface area drops to 1 m^2 and 0.5 m^2 respectively. The tanks should provide suitable areas for basking, spawning and hibernation, taking into account the number of specimens housed.
  - Facilities must provide adequate fencing and wire mesh covering to prevent escape of turtles and entry of predators.
  - The tanks should be equipped with water recirculation and purification systems, with mechanical and/or plant-purification filters and/or a periodical water change system.
  - Facilities should be equipped with a video surveillance system.
- **Indoor enclosure**
  - The indoor enclosure shall consist of at least one tank to house the newborn individuals, equipped with infrared and UVB lighting, with a photoperiod similar to the natural one.
  - The indoor enclosure shall also include an artificial incubator, which can be used to improve the reproductive success of the turtles and/or to control the sex ratio (by adjusting the incubation temperature of the eggs).

3.2 Handling of animals

Handling of animals can cause stress and should be limited as much as possible. It will still be necessary to isolate and handle animals for veterinary examinations (basic health assessment), swabbing, blood sampling, microchipping and carapace marking. Handling should always be carried out by experienced personnel, using the necessary precautions to prevent the animals from injuring themselves or injuries to the operator (see 6.1). Whenever possible, it is always preferable to carry out multiple operations on the same occasion to avoid having to isolate and handle the animal several times.

3.2.1 Basic health assessment

The basic health assessment shall be carried out by an experienced veterinarian who, on the basis of the evidence, will decide whether to proceed with further investigations. This assessment requires careful observation of the animal from various points of view, such as:

- Behaviour
- Length/weight ratio

- Carapace/plastron, skin and limb integrity
- Condition of mucous membranes
- In females, possible presence of calcified eggs (by palpation).

3.2.2 Oral swab

The oral swab will be used to search for pathogens (Herpesvirus, see 4.1, 4.2) in native turtles (*Emys orbicularis*). This must be carried out by experienced personnel (veterinarians or biologists authorised to handle animals). The animal must be contained so that it cannot injure itself or the operator (see also Chapter 6). The swab chosen must be of an appropriate size and be rubbed on the mucous membranes of the oral cavity (tongue/palate/cheek) (see ISPRA Manual).

3.2.3 Blood sampling

Blood sampling will be used to obtain blood samples for genetic analysis of native turtles (*Emys orbicularis*). Blood sampling must be carried out by experienced veterinary personnel. The maximum recommended sampling volume must not exceed 0.5 % of the animal's body weight (see ISPRA Manual).

3.2.4 Microchip placement

Microchips shall be placed by an experienced veterinarian. Microchips cannot be placed on individuals that are too young (less than about 5-6 cm in length) or in poor health (see 3.2.1) in the judgement of the veterinarian.

3.2.5 Carapace marking

Carapace marking will be used mainly on wild individuals of *Emys orbicularis* captured during monitoring activities and on non-native turtles captured during reclamation activities and destined for recovery centres. Marking on the carapace must be carried out by experienced and authorised personnel, to avoid causing injury to the animals, and cannot be practised on very young animals (minimum weight around 50 g). The marking number is assigned by making small cuts on the marginal plates of the carapace, using a code that allows up to 1,500 animals to be marked with a maximum of 8 cuts. A well-sharpened and disinfected hacksaw should be used for this purpose. The cut should affect the keratinous part of the shield and, especially in young specimens, should be shallow. After marking, the cuts shall be disinfected for prophylactic purposes.

3.3 Transport of animals

Transport can cause stress to the animals and should therefore be limited in duration as much as possible and carried out properly to minimise stress. In order to keep the turtles in optimal humidity conditions, they should be placed individually or in small groups inside sturdy wet jute bags (or other similar fabrics), which will be placed inside heat-insulating containers (e.g. igloos, polystyrene boxes). The air inside the containers will have to be changed at regular intervals.

4 Sanitary management of native turtles (*Emys orbicularis*)

The DRA drawn up during the LIFE EMYS project (see Introduction) identifies the following organisms as potential pathogens for *Emys orbicularis* at medium/high risk: *Herpesvirus*, *Mycobacterium* spp., *Entamoeba* spp., *Salmonella* spp. The same organisms are potential infectious agents for operators coming into contact with infected animals.

The veterinary protocol of LIFE URCA PRO*EMYS* therefore provides for a preliminary characterisation of the health status of each breeding centre, with reference to the above-mentioned pathogens, analysing all the individuals already kept in the different centres and the new incoming animals.

The pond turtles housed in each breeding centre must be kept in isolation, avoiding, as far as possible, direct contact with foreign animals of the same and/or different species.

4.1 Health management of *Emys orbicularis* individuals already in breeding centres (breeders) and incoming animals

For a preliminary characterisation of the health status of each breeding centre, the following tests must be carried out on all individuals already present in the various centres and on new incoming animals:

- Medical examination to assess the basic health status;
- Installation of recognition microchips;
- Faecal culture for the detection of *Salmonella* spp. in a faeces pool (consisting of a maximum of 10 individuals;
- Faecal culture for the detection of *Mycobacterium* spp. in a faeces pool (consisting of a maximum of 10 individuals);
- Faecal examination for the detection of protozoa and helminths (including *Entamoeba* spp.) in a faeces pool (consisting of a maximum of 10 individuals);
- Oral swab for the detection of *Herpesvirus*.

In the event of positive results in the screening tests, it will be necessary to implement procedures to limit the spread of pathogens within the centre as much as possible:

- Isolation of potentially infected individuals and treatment according to the instructions of the veterinarian in charge;
- Quarantine and monitoring of individuals in the same tank (only in the event of a potentially infectious disease).

4.2 Health management of *Emys orbicularis* individuals reproduced *ex situ* and intended for restocking

If the preliminary investigations described in the item above do not reveal any particular critical situations, the individuals reproduced *ex situ* in the respective centres and intended for release must undergo the following tests:

- Medical examination to assess the basic health status;
- Installation of recognition microchips;
- Faecal culture for the detection of *Salmonella* spp. in a faeces pool (consisting of a maximum of 10 individuals);
- Faecal culture for detection of *Mycobacterium* spp. in a faeces pool (consisting of a maximum of 10 individuals);
- Faecal examination for the detection of protozoa and helminths (including *Entamoeba* spp.) . ni a faeces pool (consisting of a maximum of 10 individuals);
- Oral swab for the detection of *Herpesvirus* (sampled from 20 % of individuals);

In the event of positive results in the screening tests, it will be necessary to implement procedures to limit the spread of pathogens within the centre as much as possible:

- Isolation of potentially infected individuals and treatment according to the instructions of the veterinarian in charge;
- Quarantine and monitoring of individuals in the same tank (only in the event of a potentially infectious disease). Individuals may not be released before a control test to be carried out at the end of the quarantine period.

4.3 Sanitary management of *Emys orbicularis* individuals captured during remediation and monitoring activities at restocking sites

With regard to *Emys orbicularis* individuals captured during remediation activities (capture and removal of alien terrapins) and monitoring in restocking sites, the veterinary protocol does not provide for specific analyses.
However, the following operations should be performed:

- Morphometric data collection for health assessment (Body Condition Score) and sexing;
- Marking on carapace.

4.4 Health Management of deceased *Emys orbicularis* individuals

All deceased individuals of *Emys orbicularis* shall undergo necropsy. The necropsy examination will be carried out by IZSPLV.

5 Health management of alien terrapins

This section describes the sanitary procedures for the management of alien terrapins that will be captured at restocking sites during remediation activities.

5.1 Health management of alien terrapins destined for keeping centres

With regard to alien terrapins destined for keeping centres, the veterinary protocol does not provide for specific analyses. Since the animals are destined for permanent keeping, it will be up to the rehabilitation centres themselves, together with their reference veterinarian, to carry out any health analyses according to their own prophylaxis procedures and current legislation.

Below are the management procedures included in the project:

- Medical examination to assess basic health status[33];
- Marking on the carapace, respecting the handling procedures planned (see 3.2.5) and using the identification code of the LIFE URCA PRO*EMYS* project (see **Annex 2**);
- Registration of all incoming and outgoing individuals (in case of death or transfer to another facility).

5.2 Health management of deceased alien terrapins

Deceased alien terrapins must be disposed of according to the procedures provided by the respective Local Health Authorities (incinerator).

6 Specimen protection and disinfection procedures

Since testudinates can host pathogens that are potentially infectious for operators who may come into contact with them, preventive measures must be taken, both for individual protection when handling animals and for the sanitisation of facilities.

6.1 Sanitary procedures for individual protection

The following is a list of the minimum individual protection measures required for operators who come into contact with animals:

- Handling of autochthonous and allochthonous terrapins shall always involve the use of protective gloves to prevent any injury and infection;
- In the case of animals with zoonotic diseases (even suspected ones) use, in addition to gloves, a hygienic mask to cover nose and mouth;
- At the end of every operation, always wash hands with soap and water and/or sanitising gel.

6.2 Sanitary procedures for disinfection of facilities

All objects that come into contact with the animals (autochthonous and allochthonous terrapins) must be washed and disinfected after each use with non-toxic sanitising products. This procedure applies in particular to the instruments and facilities used for the control and inspection of animals

33 The destination facility, in agreement with the reference veterinarian, may assess the need for diagnostic tests, quarantine, therapy, or euthanasia.

(scales, measuring instruments, support benches, etc.) and to containers for transport and temporary housing.

7 References

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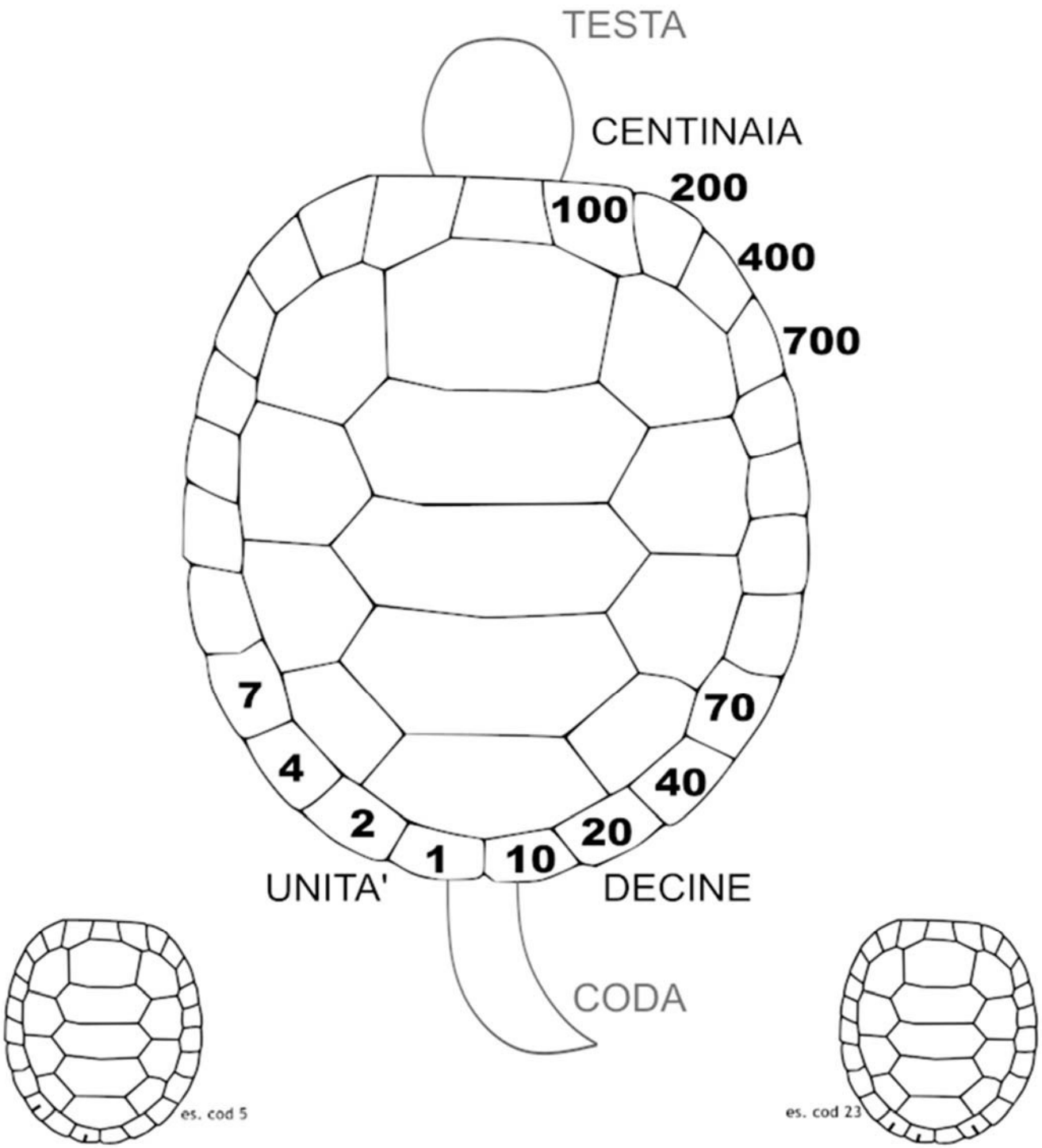
ANNEX 2. **Marking Codes**

SITE CODE MARKING
Trachemys spp. ON PLASTRON

From the figure above: Testa = Head - Coda = Tail

PROGRESSIVE SPECIMEN MARKING

Trachemys spp. ON CARAPACE



From the figure above: Testa = Head - Centinaia = Hundredths - Unità = Units - Decine = Tenths - Coda = Tail

Region	SITE	AREA	Trachemys Plastron Code
Abruzzo	IT7140215	Serranella	1
Basilicata	IT9210142	Lago Pantano di Pignola	2
Basilicata	IT9220055	Costa Ionica Foce Sinni	3
Basilicata	IT9220080	Costa Ionica Foce Agri	4
Campania	IT8050021	Medio corso del Fiume Sele - Persano	5
Emilia-Romagna	IT4020003	Torrente Stirone	6
Emilia-Romagna	IT4020006	Monte Prinzera	7
Emilia-Romagna	IT4020017	Aree delle risorgive di Viarolo, Bacini di Torrile, Fascia golenale del Po	8
Emilia-Romagna	IT4020021	Medio Taro	9
Emilia-Romagna	IT4020025	Parma Morta	10
Emilia-Romagna	IT4030007	Fontanili Corte Valle Re	11
Emilia-Romagna	IT4030011	Casse di espansione del Secchia	12
Emilia-Romagna	IT4040007	Salse di Nirano	13
Emilia-Romagna	IT4040015	Valle di Gruppo	14
Emilia-Romagna	IT4050001	Gessi Bolognesi	15
Emilia-Romagna	IT4050019	La Bora	16
Emilia-Romagna	IT4060015	Bosco della Mesola, Bosco Panfilia, Bosco di Santa Giustina, Valle Falce, La Goara	17
Emilia-Romagna	IT4070006	Pialassa dei Piomboni, Pineta di Punta Marina	18
Emilia-Romagna	IT4070011	Vena del Gesso Romagnola	19
Emilia-Romagna	IT4070021	Biotopi di Alfonsine e Fiume Reno	20
Emilia-Romagna	IT4090002	Torriana, Montebello, Fiume Marecchia	21
Lazio	IT6030023	Macchia Grande di Focene e Macchia dello Stagneto	22
Liguria	IT1324896	Lerrone - Valloni	23
Liguria	IT1324909	Torrenti Arroscia e Centa	24
Lombardia	IT2050005	Boschi della Fagiana	25
Lombardia	IT2050006	Bosco Vanzago	26
Lombardia	IT2080002	Basso Corso del Ticino	27
Marche	IT5310022	Fiume Metauro da Piano di Zucca alla foce	28
Marche	IT5340001	Litorale Porto d'Ascoli	29

Piemonte	IT1120007	Palude di San Genuario	**30**
Piemonte	IT1180005	Ghiaia Grande	**31**
Puglia	IT9140005	Torre Guaceto	**32**
Toscana	IT5170002	Selva Pisana - Cornacchiaia	**33**
Veneto	IT3220005	Ex Cave di Casale - Vicenza	**34**
Veneto	IT3250017	Cave Noale	**35**
Veneto	IT3250023	Lido di Venezia biotopi litoranei	**36**
Veneto	IT3250030	Laguna medio-inferiore di Venezia	**37**
Veneto	IT3250032	Bosco Nordio	**38**
Veneto	IT3270007	Gorghi di Trecenta	**39**

ANNEX 3. Request for Advice on the Capture and Transport of *Trachemys*

To the kind attention of:

xxxxxxxxxxxxxx

At

Region
xxxxxxxxxxxxxx
PEC

REQUEST FOR AN ADVICE ON THE IMPLEMENTATION OF CAPTURE AND TRANSPORT ACTIVITIES UNDER LEGISLATIVE DECREE D.Lgs. 230 DATED 15 DECEMBER 2017 AND ITS AMENDMENTS AND INTEGRATIONS AND "*NATIONAL PLAN FOR THE MANAGEMENT OF THE AMERICAN MARSH TURTLE (TRACHEMYS SCRIPTA)*"

Applicant _____ _____

_____

Taxpayer and VAT number _____

Address _____ _____

Telephone +39 _____*E-mail*_____ _____

Scientific Supervisor (if different from the Applicant) _____

Member of the Entity _____

Telephone +39 _____*E-mail*_____

FOREWORD

Framework of the project in the context of national and/or international research and conservation issues concerning the species

- **References to relevant national and international legislation**

E. orbicularis is a protected species, included in Annexes II and IV of the Habitats Directive 92/43/EEC, and its trade is prohibited by the Bern Convention of 1979. According to the most recent report available for Italy, (Art. 17 of the Habitats Directive report 2013-2018) *E. orbicularis* has an inadequate or poor conservation status (U1 and U2, for Slovenia and Italy, respectively) in the main biogeographical areas where it is found, and in particular in the area covered by this project ("Continental" and "Mediterranean" biogeographical area) with populations considered to be "declining". This is in line with the fact that in the last century this species has faced a drastic decline due to the extensive draining and reclamation of wetlands. The European Red List classifies *E. orbicularis* as near-threatened (NT): however, the assessment was carried out in 2004 and appears completely anachronistic compared to the collapse that the species has recorded within the European range, and as also highlighted in the Red List itself urgently needs updating (https://www.iucnredlist.org/species/7717/97292665). The Italian Red List considers the species to be endangered (EN) (http://www.iucn.it/scheda.php?id=1273952988, data from 2013),

The American marsh turtle *Trachemys scripta* (particularly the subspecies *T. s. elegans*), has been included by the IUCN (IUCN SSC Invasive Specie Specialist Group) among the 100 worst invasive species in the world (Lowe et al., 2000).

Trachemys scripta is currently found throughout Italy, including the major islands. The species is most widespread in northern Italy, but also in central Italy, with particular reference to Tuscany and Lazio, while distribution in southern Italy and the islands is more localised and punctiform. *Trachemys scripta* was immediately included in the first list of invasive alien species of EU relevance in 2016, adopted by Implementing Regulation No. 2016/1141, also transposed at national level by the Legislative Decree D.Lgs. No. 230/2017 adapting to the previously mentioned European regulation. Pursuant to Article 6 of that decree, species of EU relevance may not be adapting to the previously mentioned European regulation. Pursuant to Article 6 of that decree, species of EU relevance may not be:

- Introduced into or transited through national territory, even under customs supervision;
- Held, even in confinement, except in cases where the holding takes place in the context of management or eradication measures ordered under the aforementioned decree; bred or cultivated, even in confinement;

- Transported or caused to be transported within the national territory, except in cases where transport takes place in the context of management or eradication measures ordered under the aforementioned Decree;
- Sold or placed on the market;
- Used, given away free of charge or exchanged;
- Placed in a condition to reproduce or grow spontaneously, even in confinement;
- Released into the environment.

Exemptions to the aforementioned prohibitions (Art. 8) are only provided for authorisations enabling institutions to carry out ex situ research or conservation activities or, in relation to human health, for the scientific production and subsequent medical use of products derived from species of EU relevance.

The Legislative Decree D. Lgs 230/2017 provides in Articles 19 and 22 that the Regions, the Autonomous Provinces of Trento and Bolzano and the managing authorities of national protected areas apply the rapid eradication (Art. 19) and management (Art. 22) measures. These Authorities apply the eradication and management measures by availing themselves, if necessary, of the collaboration of other administrations, which must carry out the activities with the resources available under the legislation in force in their own budgets, or of private subjects. The Ministry is informed of the application of the measures and the results achieved in the course of the activities.

The *"National Management Plan of the American Marsh Turtle (Trachemys scripta)*" drafted by ISPRA and published in June 2022, approved by DM 370 dated 28 September 2022, provides that *"Management interventions for the exotic species will be conducted by specially trained and specifically authorised personnel by the Regions, Autonomous Province or territorially competent Protected Area."*

- **Actions envisaged by the project**

- **Methods Implemented**

Considering the above, the Project …… hereby

ASKS FOR

Advice about the carrying out of management activities of alien turtles in the SACs and sites specified for the entire duration of the project.

ANNEX 4. **Trachemys Field Data Sheet**

Trachemys measurements - field data

101074714 — **LIFE21-NAT-IT-LIFE URCA PROEMYS** — LIFE-2021-SAP-NAT

SITE: _____

Area Code	ID	Trap	date	sex	Carapace length	Carapace width	Body mass	Reproduction	notes

sex: m = male; f = female; j = juvenile; h = hatchling.
Reproduction: YES/NO

– ALL MEASUREMENTS IN MILLIMETERS; BODY MASS IN GRAMS.

ANNEX 5. Delivery Form to Housing Centres

DELIVERY FORM
of ALIEN TURTLE SPECIMEN

NAME of CENTRE _____

NAME and SURNAME of the RECEIVER _____

NAME and SURNAME of the DELIVERER _____

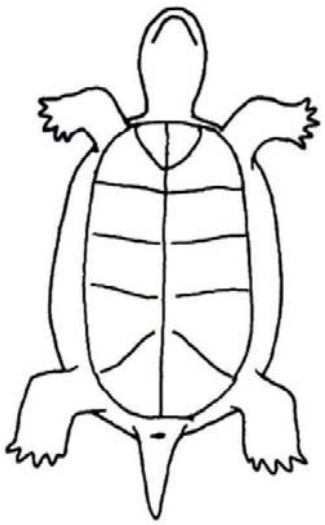
CAPTURE DATE _____ **SITE CODE N2K**_____

CAPTURE POINT COORDINATES _____

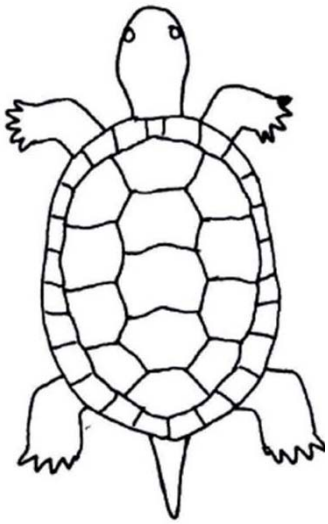
SPECIES _____ **SEX** _____

CARAPACE LENGTH (cm) _____ **CARAPACE WIDTH (cm)**_____ **WEIGHT (g)**_____

AREA CODE _____**(plastron)**



SPECIMEN CODE _____ **(carapace)**



NOTES_____
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Place _____ **Date** _____

Signature of the person delivering

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