REPORT Task 3.1

2024

LIFE TURTLENEST

KEEP THEM SAFE

Review of the best practices-state of art methods to monitor and protect sea turtle nesting sites, to assess habitat suitability and to define nesting intensity.

TRUTLENEST PROTOCOLS

Cofinanziato dall'Unione europea

Beneficiario coordinatore

LEGAMBIENTE

Beneficiari associati

ARPAT · Beta UVIC · CESTMed · ISPRA · REGIONE BASILICATA · REGIONE CAMPANIA · REGIONE LAZIO · REGIONE PUGLIA · SAPIENZA UNIVERSITÀ DI ROMA · STAZIONE ZOOLOGICA ANTON DOHRN · UNIVERSITAT DE BARCELONA

3.3. TURTLENEST PROTOCOLS (Task 3.1)

PROTOCOL N. 1 - BEACH SURVEYS (Diurnal patrolling)

ASSOCIATED PROTOCOLS: *Nest protection [N.7] and Beach suitability assessment for nesting [Task 3.3]*

STANDARDISED DATA SHEET: Data sheet N. 1 - Beach patrolling / Database; Data sheet N. 2 – Nest and track ID.

MANPOWER:

ECONOMIC RESOURCES: €€€.

TIME/FREQUENCY: Variable depending on the monitoring scheme, possible also ad hoc.

AUTHORIZATION: No.

QUALIFIED PERSONNEL: Yes. It is recommended that people who carry out beach patrolling have completed at least the basic training on diurnal beach patrolling, with information about nest and track identification. Observers (both technical staff and volunteers) must be aware of the datasheet to collect the information and proficient with the apps eventually used to upload the data to the project database.

1. Reason

Beaches are critical habitats for breeding marine turtles, where adult females come ashore to nest during a well-defined period. Diurnal beach patrolling facilitates principally encountering the tracks left by nesting females which also permits to successfully enforce nest protection measurements and to collect relevant scientific information about the nesting event (see protocols from 1 to 11).

2. Context and relevance in Western Mediterranean

The loggerhead turtle has only recently begun to nest regularly in the Western Mediterranean Sea. Many events are still accidentally reported by tourists, and several potentially suitable locations have not yet been monitored to verify the presence of nesting females. Diurnal beach surveys are essential to count loggerhead turtle tracks, estimate nest abundance and enforce proper protection measurements in this new nesting area. It is important that nesting survey programs follow specific protocols aiming at obtaining the minimum standard data that can be compared across different locations and contribute to the monitoring of nesting trend in the Western Mediterranean Sea.

Diurnal beach patrolling is the most effective way to use the available resources and can be performed by trained volunteers without any specific authorization.

3. Equipment

Diurnal beach surveys are regularly conducted on foot and require a minimum amount of equipment. On specific occasions, these surveys may be conducted with the aid of fat e-bikes, drones, ultralight aircraft or other vehicles to reach difficult-to-access locations or cover wider extents of coastline.

In general, the observer team will need to bring:

- Data sheet (preferable digital forms to be filled in directly from the smartphone or other device)

- Camera (included in a smartphone)
- GPS (also included in a smartphone)
- Measuring tape (minimum 20 m)
- Appropriate clothing, including comfortable shoes and a hat.
- Sunscreen
- Drinking water.

4. Actions

Surveys should take place during the June to August breeding season, although the time frame can be expanded to include May and September/October to encounter early nesting events and nest hatching.
Monitoring can follow one of the three protocols:

A. Intensive monitoring [equivalent to SWOT Protocol B]: Intensive monitoring (minimum 5 times/week) throughout the nesting season (June to August)
B. Diffuse regular monitoring [equivalent to SWOT Protocol E] (minimum 2 times/week) during the nesting season peak (July)
C. Ad-hoc monitoring of a specific beach.

In general, on beaches where nesting activity has regularly occurred in the previous years, it is advisable to use **Protocol A** to assess the effective nest abundance, evaluate possible nesting trend and to use it as potential index site for nesting activity in the Western Mediterranean.

For areas that have been only occasionally used by females in the previous years or which have a high nesting habitat suitability as defined in Task T3.2, protocol B is recommended.

Protocol C is preferred in those occasions when a crawl is detected without any nest on a specific beach, and monitoring of that beach and the neighboring sites in the following 2-3 days may led to the discovery of the track with the nest laid by the same female. Likewise, it is recommended to patrol the beaches in the vicinity of a detected nest starting from 10 days after the nest was laid up to 18 days, to increase the chance of detecting another clutch laid by the same female.

Diurnal beach surveys should, ideally, be carried out at first light as (1) it reduces the chance for the track to be obscured by human or environmental actions, (2) the tracks are easier to detect with a low inclination of the sun, (3) it permits the timely relocation of a nest by authorized personnel should the procedure be necessary to mitigate the potential impact of threats, and (4) the surveyor avoids working during the excessive heat of daytime.

Diurnal beach surveys should be carried out in the middle-lower part of the beach (high tide line), to identify well the exit and entry traces of the turtle, without confusing them with other tracks that may be on the beach.

The monitored sections should be approximately 4 km or an appropriate length that can be patrolled in two hours at maximum. Longer beach sections can be surveyed with the aid of fat e-bikes. Drones and ultralight aircraft can also be additional and very useful instruments to support the monitoring of beaches (e.g., those difficult to access such as bays surrounded by cliffs or extensive areas with low nest density). **KEEP IN MIND:** The use of this equipment may require specific authorization from the competent authority and must comply with the related national regulations.

At the end of the diurnal beach survey, the observers must fill in the **Standardized Data Sheet No. 1 – Beach patrolling** to keep track of the monitoring effort and to collect the required data. Every day that a specific beach is patrolled the following minimum information are required:

- Place (name of the beach and locality to which it belongs).
- Date and time of the start of the survey.
- Name of the observers, etc.
- Climatology (wind direction and strength) and visibility conditions (sunny, cloudy, etc.), as this condition the results of the survey. Also, if the night or the previous day has rained and / or if the beach cleaning brigade has already passed.
- Effort (km covered).

When a track is found volunteers must:

- inform the nesting expert responsible for that specific area or call the EMERGENCIES PHONE NUMBER (according to the region/country).
- make a video moving slowly along the entire track and share it as quickly as possible with the nesting expert to determine if it is just an attempt or if the presence of a nest is possible and the Nest and track identification must be applied.
- Fill in the Standardized Data Sheet (see Google Form prepared to be adapted by every study area) as described in the Nest and track identification.
- If there is a real possibility of a nest, the entire track must be protected, preventing it from being accidentally erased by people's footsteps, beach cleaning machines, vehicle traffic, etc... It is very important to preserve the body pit and the nearby traces to help experts in the eventual location of the camera.

IMPORTANT: All nesting activities must be registered on the appropriate data form

Variables that may affect the result of the diurnal beach survey:

- **Observer experience:** The observer's experience can influence the accuracy of the census; therefore volunteers and beach surveyors must be properly trained before the nesting season begins.
- **Type of beach:** type of sediment (sand, gravel, pebbles, etc...) by which the beach is formed, as well as its profile, can help or complicate the process of identifying tracks by influencing tack stability in time.
- **Time (position of the sun):** the lower the angle of incidence of the sun, the better the identification of the tracks, because these will create deeper shadows that will make them more visible. Cloudy days, on the other hand, could be worst for prospecting. To eliminate this variable, it is recommended to carry out the censuses always at the same time, preferably in the early hours of the morning.
- **Wind:** the intensity, direction, and duration of the wind can determine the success of a survey, since it can totally or partially eliminate traces. The direction of the wind and the orientation of the beach will determine the degree of erosion of the traces.
- **Rain:** rain conceals the tracks and complicates their identification. That is why it is important to note if it has been raining prior to prospecting or during it.
- **Waves:** wave action can eliminate parts of the tracks or entire tracks if these are short
- **Human activity on the beach:** Human activities can mask tracks and nests.

5. Ethics

Diurnal beach patrolling raises no ethical issues and does not require a specific authorization, but associated protocols relating to interacting with turtles and nests can be conducted exclusively by authorized personnel.

6. Collaboration potential

Diurnal beach surveys are very demanding in terms of manpower and can be difficult to program over successive nesting seasons. Therefore, it is essential to establish collaboration with MPAs, NGOs and other stakeholders that may contribute with volunteers to the monitoring activity. These collaborators must be properly trained and informed on the different protocols and data form used to monitor loggerhead turtle nesting in the Western Mediterranean

7. Data collection

Data to be inserted in the beach monitoring register (T4.2) includes details on the monitored transects and monitoring effort day by day (see Google Form as example, and Excel Data Base).

8. References

- Alan F. Rees (2020). Marine Turtles in MPAs: a monitoring and management guide. Med-PAN Collection. 68 pp
- FFWCC (Florida Fish and Wildlife Conservation Commission) (2016) Marine turtle conservation handbook. https://myfwc.com/media/3133/fwc-mtconservationhandbook.pdf accessed on 1 April 2020.
- Marco, A., Abella, E., Liria, A., Martins, S., Loureiro, N. D. S., & Lopez-Jurado, L. F. (2012). Manual for the monitoring of sea turtles in the Cape Verde Islands. Cape Verdean Zoology. 3. 1-23.
- Schoreder, B., & Murphy, S. (2000). Population surveys (terrestrial and aerial) in nesting beaches. At KL Eckert, KA Bjorndal, FA Abreu-Grobois, & M. Donnelly. Research and Management Techniques for Sea Turtle Conservation, 51-63.
- SWOT Scientific Advisory Board. 2011. The State of the World's Sea Turtles (SWOT) Minimum Data Standards for Nesting Beach Monitoring, version 1.0. Handbook, 28 pp.

Standardized Data Sheet N. 1 – Beach patrolling (Required fileds)

Field	Options
Data	
Id monitored transect	
Method	on foot, e-bike, drone, airplane, other
N. of observer	
N. track identified	0, 1, 2, 3,....
N. of nest identified	0, 1, 2, 3,....
Nesting female encountered	Yes, No
Hatchlings encountered	Yes, No
Mechanical Beach Cleaning	absent, about to begin, in action, already passed
% of transect mechanical cleaned	0%, <25%, >25%<50%, >75%
Manual beach cleaning	absent, about to begin, in action, already passed
% of transect manually cleaned	0%, <25%, >25%<50%, >75%
Density of people on the beach	Low: few or no people, the possibility of identifying a track is not affected, Medium: several people, but it should still be possible to identify a track even if partially erased, HIGH: many people, the possibility of identifying a track is likely affected
Wind intensity	Absent, Low: gentle breeze not likely to erase a track, Medium: clearly perceptible wind that can partially erase a track, High: strong wind, likely to delete a track in a short time
Rain intensity	Absent, Low: light rain not likely to erase a track, Medium: rain persistent but moderate that can partially erase a track, High: heavy rain, likely to delete a track in a short time

Standardized Data Sheet No. X – Nest and track identification [X] (Required fileds)

Field.	Options
Activity ID:	Country Abbreviation [2 letters] Region Abbreviation [first 3 letters] Year [2 digits] Activity progressive numer [3 digits]. Example: IT_CAM_23_001. The activities include both crawls and nests
Municipality:	insert the municipality to which the beach belongs where the activity was registered
Region:	insert the region in which the activity was registered
Country:	insert the country in which the activity was registered
Latitude:	insert the latitude of the track/nest location in decimal degrees with 6 decimal digits
Longitude>	insert the longitude of the track/nest location in decimal degrees with 6 decimal digits
Date_Activity:	insert the date of the activity (date of egg laying or emergence of the female). If a track is detected following a turtle activity during the previous night, then use the date of the day following that night. If the date is not known leave cell blanc.
Type_Activity:	Nest, undetected nest, or crawl. Crawls are all turtle emergences that did not result in eggs being laid; undetected nests are nests that are not found when they were laid but any time after the egg laying, for example when wave action exposes the eggs of a nest or when hatchlings are found on a beach emerging from a nest
Activity_Detection:	Monitoring team, private citizen, beach operator (including beach cleaning operators, personnel of beach bars and beach establishments etc), fisherman, MPA staff; if the rapporteur cannot be assigned to one of these categories, can can select “private citizen” as default and insert a comment in the note, if necessary.
Dist_HWM_m:	enter the distance to the high water mark from the highest point of the track or the nest; the unit is meters with 2 decimal digits
Dist_VEG_m:	enter the distance from the highest point of the track or the nest to the beginning of the vegetation at the back of the beach; If the beach is limited by man-made structures take this as the reference point; the unit is meters with 2 decimal digits
Track_width_cm:	enter the width of the turtle track; unit is centimetres with 1 decimal digit
Min_Depth_Eggs_cm:	enter the minimum depth of the eggs measured from the sand surface to the first eggs that appear at the top of the egg chamber; unit is centimetres
Max_Depth_Eggs_cm:	enter the maximum depth of the egg chamber measured from the sand surface to the bottom of the egg chamber, measured either during nest relocation or final excavation; unit is centimetres

Nest_relocation:	Yes, No or N/A if the nest was not relocated or
Relocation_date:	insert the date in which the nest was relocated or insert N/A if it was not relocated or the activity was a crawl
New_Dist_HWM_m:	enter the new distance to the high water mark of the relocated nest; insert N/A if it was not relocated or the activity was a crawl
New_Latitude:	enter the new latitude of the relocated nest; insert N/A if it was not relocated or the activity was a crawl
New_Longitude:	enter the new longitude of the relocated nest; insert N/A if it was not relocated or the activity was a crawl
T_sensor_depth_cm:	insert the depth at which the temperature datalogger/sensor was planted; unit is centimetres
T_sensor_type:	insert specifications on the datalogger/sensor that you use, like the model, manufacturer, etc.
T_sensor_position:	Top clutch, Middle clutch, Bottom clutch, Sand outside the nest, N/A if no sensor was planted
Nest_Management:	None, fence, cage, fence + cage, incubator, splitting, shading, wave barrier, other; note: fence is external around the nest, cage is horizontal under the sand surface, splitting is relocating at least one half of the nest to another part of the beach or in an incubator
Anth_impact:	low = low frequency utilisation as bathing beach, medium = constant utilisation as bathing beach (during nesting and hatching period) including use of sunbeds and umbrellas, high = presence of beach establishments, bars, and/or restaurants, where structures are build or equipment (sunbeds, umbrellas) is kept on the beach, and/or the beach is delimited by roads, buildings and parking areas
Beachfront_Lighting:	0 = no artificial lighting present, 1 = indirect light visible by an observer on the beach, but not likely to present a strong attraction to nesting or hatching turtles, 2 = direct light or a visible globe, glowing element, lamp, or reflector likely to disorient turtles
Notes:	insert any additional information that you think is important to consider for this nest, e.g. predator species, or other nest management methods applied.

IMPORTANT: DO NOT LEAVE A CELL EMPTY. INSERT N/A IF THE DATA DOES NOT APPLY OR IS FOR ANY REASON NOT AVAILABLE.

PROTOCOL N. 3 - MEASURING TURTLES

ASSOCIATED PROTOCOLS: *Beach survey [1].*

HUMAN RESOURCES:

ECONOMIC RESOURCES: €

TIME/FREQUENCY: ⌛⌛, on encounter with a nesting female or during the hatching phase

AUTHORIZATION: Yes

QUALIFIED PERSONNEL: Yes. Permits are necessary for handling sea turtles. Although the measuring protocol itself is non-invasive, it does give rise to ethical considerations regarding the proper handling of the subject animals.

1. Reason

Sea turtles are measured for various purposes, utilizing a wide array of methods and tools with different levels of complexity and accuracy. On nesting beaches, females are measured to relate body size and reproductive output. Additionally, on the long term, these measurements may help to determine the minimum size at which sexual maturity occurs in the study area and to monitor the size of females nesting in a specific region.

2. Context and relevance in Western Mediterranean

Knowing the reproductive size of nesting females, as well as their reproductive status, health, and individual peculiar characteristics, is essential for improving scientific knowledge about nesting along the Spanish, Italian and French coastline.

3. Equipment

- Torch with red light
- Camera
- Flexible measuring tape

4. Actions

There are different biometric measures to characterise sea turtles, some of which are the most widely used internationally, allowing for the comparison of standards between populations.

• If the turtle is nesting, wait until it has finished the camouflaging process and is heading towards the sea.
• Once it is moving down, restrain it.
• The basic shell measurements (Fig. 19) that should always be taken are: minimum curved carapace length (CCLmin) and standard curved carapace length (CCLest), and curved carapace width (CCW). A flexible measuring tape should be used for this.
• For the minimum curved carapace length (CCLmin), measure from the anterior midpoint (nuchal scute) to the midpoint of the posterior notch between the supracaudal scutes, ensuring the tape is stretched out and taut over the central scutes.

• For the standard curved carapace length (CCLest), measure from the anterior midpoint (nuchal scute) to the tip of one of the supracaudal scutes, ensuring the tape is stretched out and taut over the central scutes.
• For the curved carapace width (CCW), measure at the widest point; there are no anatomical reference points. It is helpful to place a finger at the lower edge of the marginal scute where the measurement starts, pass the tape over the central scutes of the turtle, and finish at the edge of the marginal scutes on the opposite side.

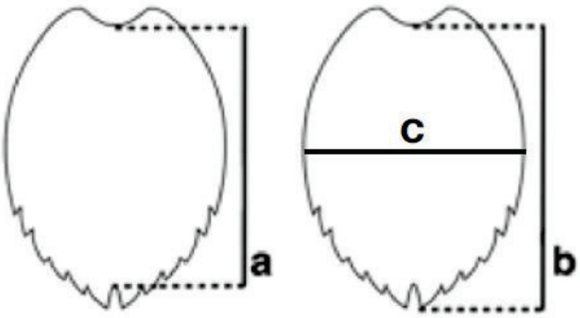

Figure 19. Anterior and posterior anatomical points for three carapace length measurements. (a) Measurement of CCLmin, (b) Measurement of CCLest, (c) Measurement of CCW. Source: Eckert et al., Research and Management Techniques for the Conservation of Sea Turtles (2000).

Optionally, to better characterise the animal, the following head measurements can also be taken, preferably using callipers (or a flexible measuring tape if unavailable): head length (HL) and head width (HW). HL is measured along the longest line of the head's length, and HW is measured along the longest line of the head's width.

5. Ethics

Authorisation to handle marine turtles is typically necessary and is pertinent to this protocol. While the protocol itself is non-invasive, it does raise ethical considerations regarding the proper treatment and handling of the animals involved.

6. Collaboration potential

No collaboration is required to carry out this protocol, although the presence of qualified personnel is necessary to handle the female.

7. Data collection

CCLnt/CCLmin and SCLn-t/SCLmin should be recorded as the main measurements to take.

8. References

- Alan F. Rees (2020). Marine Turtles in MPAs: a monitoring and management guide. MedPAN Collection. 68 pp.
- Bolten, A. B. (1991). Techniques for measuring sea turtles. In: Eckert, K. L., K. A. Bjorndal, F. A. Abreu-Grobois, and M. Donnelly (Editors). 2000 (Spanish translation). Research and Management Techniques for the Conservation of Sea Turtles. IUCN/SSC Marine Turtle Specialist Group Publication No. 4.
- Casale, P., Freggi, D., Rigoli, A., Ciccocioppo, A., & Luschi, P. (2017). Geometric morphometrics, scute patterns and biometrics of loggerhead turtles (Caretta caretta) in the central Mediterranean. Amphibia-Reptilia, 38(2), 145-156.

PROTOCOL N. 4 - BIOLOGICAL SAMPLING FOR GENETICS AND STABLE ISOTOPES

ASSOCIATED PROTOCOLS: *Nest protection [6], Headstarting [10].*

HUMAN RESOURCES: 🚹 - 🚹🚹

ECONOMIC RESOURCES: €

TIME/FREQUENCY: ⌛⌛. When encountering a nesting female, while exhumation and as part of a headstarting program.

AUTHORIZATION: Yes. The specific requirements can vary depending on the country and if sampling dead or living animals.

QUALIFIED PERSONNEL: Sampling must be performed by qualified staff, especially when sampling on live animals, and following national and regional legislation.

1. Reason

Obtaining biological samples from the nesting events can provide essential information through genetic and stable isotopes analysis that would be impossible to obtain using other techniques. However, a standardized protocol for sampling, but also for sample preservation, storage and associated data collection, is crucial to obtain reliable results.

2. Context and relevance in Western Mediterranean

In the context of a colonization process, it is important to determine the population of origin of the colonizers, the number of individuals contributing to the establishment of new rookeries and to find which nests were laid by the same nesting females in order to detect renesters and remigrants (Carreras et al. 2018, Luna-Ortiz et al 2024). Additionally, information of the foraging grounds used by the nesting females can be extracted by analyzing skin and carapace samples of nesting females and hatchlings with stable isotopes (Cardona et al 2024). As nesting events in the Western Mediterranean are generally scarce (particularly in some regions), and consequently intense monitoring is rare, sampling should be considered a priority. In order to obtain as much information of each nest, it is essential to sample the nesting female (when available) and hatchlings (dead or alive). Multiple samples would provide additional information about progenitor origins (for example 10 hatchling samples or more can inform about the presence of multiple paternity).

3. Equipment

The sampling shall be performed with properly sterilised material (single-use or flame sterilised) to avoid possible infections and contamination of the samples. It is recommended to have the following material ready in advance in order to be able to carry out the sampling outside any controlled facilities (such as a rescue center) if necessary:

- Surgical gloves
- Disinfectant and/or 70% alcohol for cleaning the sampling site

- Sterile gauze pads
- Scalpel and/or dissecting scissors
- 96% alcohol free of any additives to preserve samples
- Flame (lighter or similar) in case no single-use material is available
- Sampling tubes with a watertight nut for samples presented in liquid.
- Material for labelling samples: onion paper, hard-tipped pencils and markers
- Box for storing samples
- Biopsy punch 6 mm diameter
- Eppendorf tubes for samples without liquid.

4. Actions

In order to optimise the sampling with the minimum impact on the animals, the following decision box has been proposed, summarising the key points to consider when deciding what to sample and when to do it to accomplish the two objectives proposed in this sampling protocol. The following scheme (Figure 1) illustrates the suitable type of sampling for each situation (a-e). Details of how to carry out each type of sampling (a-e) can be found in section 3 of this document. For nest excavation, only protocols b, c and e will be used.

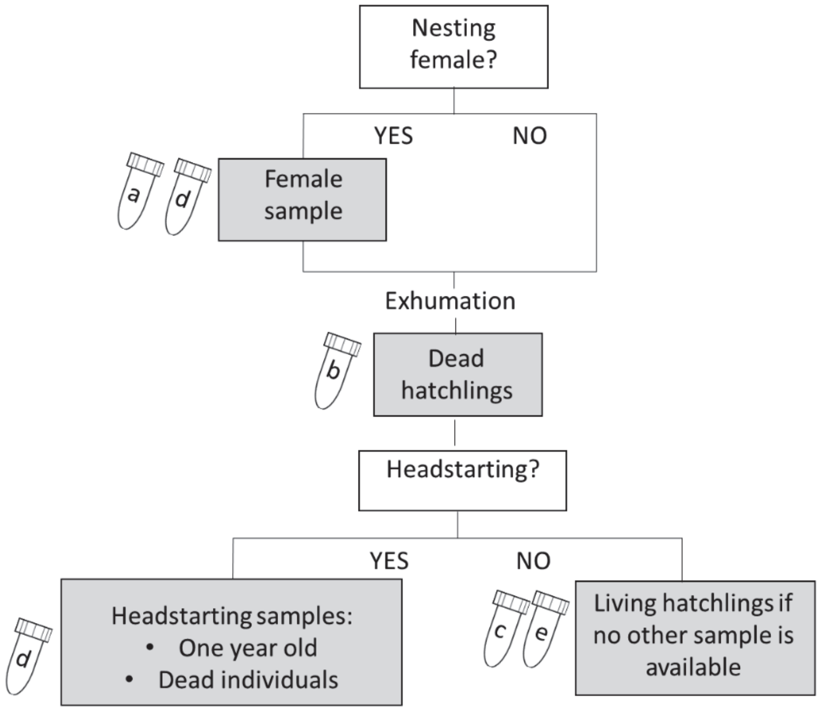

Figure 1: Sampling decision tree. Individuals to be sampled and sampling protocols to be used depending on the different conditions associated to the nesting event.

***Labelling.** It is essential that samples are properly labelled to ensure that the traceability of the data collected from the animals is not lost. Sample storage tubes must be identified with a unique code and must contain an identifying label inside (**on onion paper written with a hard-tipped pencil**). In the case of sampling hatchlings (live or dead), the information from the samples collected shall be incorporated into the TURTNENEST databases of nesting events as well as into the individual records of the specimens, for example those which enter a headstarting programme.

***Sample preservation.** Details on how to preserve and store the samples are provided in each sampling protocol. It is crucial to strictly follow the preservation protocol to guarantee good results.

*** Custody of samples.** Samples can initially be preserved at room temperature without sunlight, humidity or exposure to high temperatures, although they can ideally be stored in a fridge. Samples shall remain in the custody of the personnel who collected them and shall be sent as soon as possible to the scientific staff who will process and store them at -20ºC.

***Number of samples.** The number of samples will be determined by the specific objectives of the projects, but at least one sample per nest should be collected in order to obtain a minimum information. For the genetic samples it is recommended to sample the nesting female (if available) and for the hatchlings it is recommended to sample all dead animals and all headstarted animals. For stable isotope analysis, this includes the carapace and skin of the nesting female (if available) and at least one hatchling per nest.

Specific Sampling Protocols

Specific sampling protocols are detailed below. In order to know which protocol should be applied in each case, please refer to the decision-making schemes provided in the section 2 of this document (Figure 1). **For nest excavation, only protocols b, c and e will be used.**

a).- Skin and carapace samples from live adults: Skin samples for genetics shall be collected from live individuals only in those cases where it is not feasible to obtain a quality blood sample, either because field conditions are not ideal, or because their preservation cannot be guaranteed (e.g. immediately put them in alcohol and process them as soon as possible). Skin samples for stable isotopes should be collected even if blood samples are collected.

In the case of nesting females, they should be sampled when they start to cover the nest after laying, as there is no risk of nest abandonment after this point. Ideally, red lights should be used and avoid shining directly into the turtle's eyes at all times. **Sampling the nesting female is of great importance**. For this reason, the female should be sampled **whenever** she is found. Additionally, if there is a chance of obtaining samples from adult males or females (for instance, arriving to rescue centres), samples for genetics and isotopes could also be taken.

The **genetic skin** sampling protocol is as follows:

- Disinfect the area to be sampled, preferably an edge of an anterior fin without very large scales (Figure 2a).
- Using a properly sterilised scalpel, cut out a strip of skin approximately 0.4 cm x 1 cm (Figure 2b).
    - If a biopsy punch is available, remove the piece of skin with a 6mm punch.

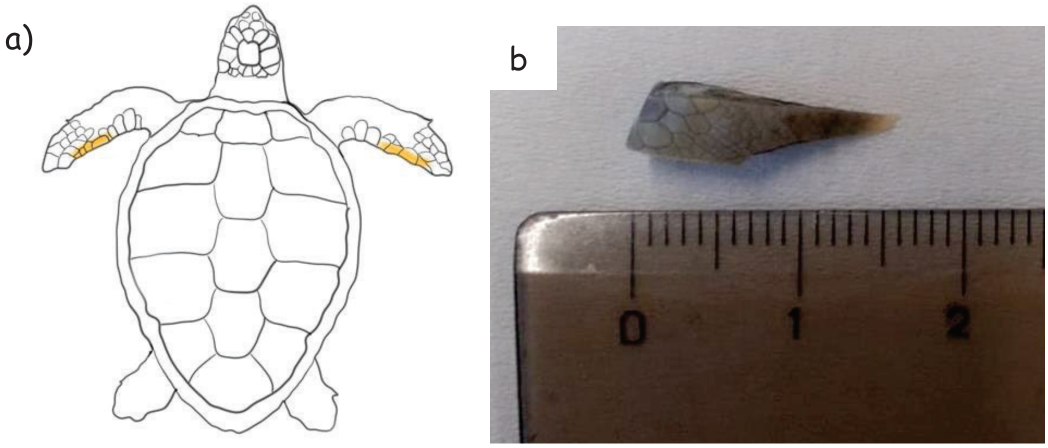

***Figure 2**: **a)** Suitable sampling area for skin genetic sampling. **b)** Example of suitable dimensions and appearance of the skin sample for genetic studies.*

- Place the sample in a properly labelled sampling tube and fill with 96% ethanol.
- Sampling should not normally cause the animal to bleed, but if it does, apply pressure with a gauze pad sterilised with disinfectant for a few minutes until the bleeding stops.
- Disinfect the sampling site.
- After 1-2 hours, remove the liquid content of the tube and replace it with fresh 96% ethanol.
- Replace with fresh 96% ethanol after 24 hours.

The **carapace** sampling protocol for **stable isotopes** is as follows:

- Clean the distal part of the 3rd lateral scute (right or left) (Figure 3) with ethanol.

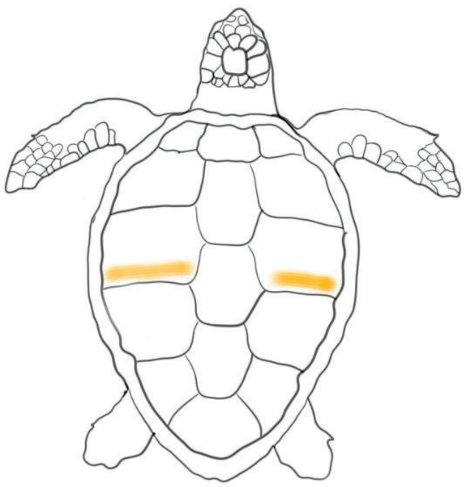

***Figure 3**: **a)** Suitable sampling area of carapace for stable isotopes.*

- Remove a sample from the carapace scute with a 6 mm diameter biopsy punch; the punch should reach the base of the scute, but the bone should not be sampled. Sometimes, the bone may bleed. Disinfect the wound.
- Store the sample in a staining cassette (Figure 4) preserving the polarity of the sample (dorsal up, ventral down).
- Freeze the sample at -20°C.

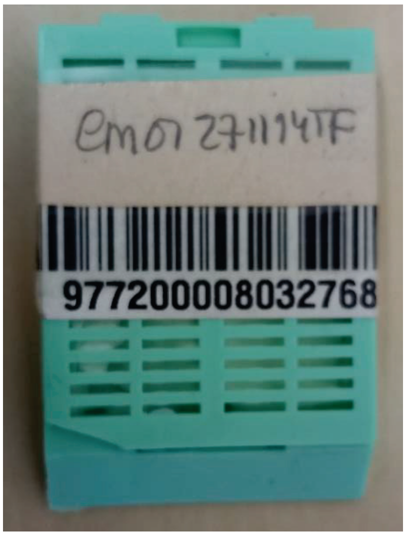

Figure 4*: Staining cassette that needs to be used for carapace samples for stable isotopes in order to preserve the polarity of the samples for stable isotopes analysis*

The **skin** sampling protocol for **stable isotopes** is as follows:

- Disinfect the base of one front flipper (Figure 5) with ethanol.

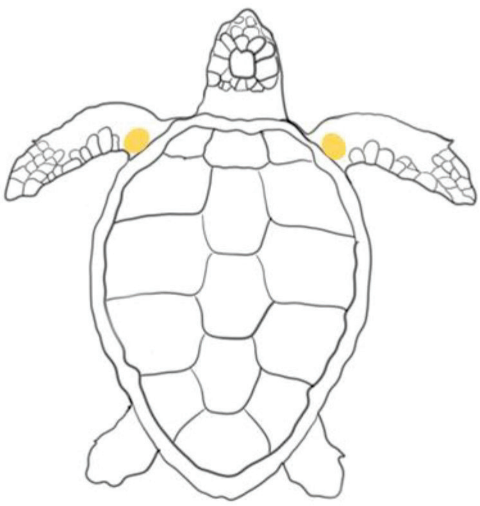

Figure 5: Suitable sampling area for skin sampling for stable isotopes.

- Remove a skin sample with a 6 mm diameter biopsy punch, without reaching the muscle. Disinfect the wound.
- Store the sample in a dry Eppendorf tube. Alternatively, the same sample collected for genetics can be used, if it is large enough.
- Freeze the sample at -20°C. Alternatively, store it in ethanol.

b).- Dead individuals:

Sampling dead hatchlings during the exhumation of a nest does not entail any risk to the animals, although the samples may be of lower quality. Therefore, all dead hatchlings should always be sampled to ensure that a reservoir of samples is available in case better quality samples cannot be obtained. If no hatchlings are found, an examination of the unsuccessful eggs in the nest shall be carried out to find embryos. This same sampling protocol shall be applied for any hatchlings that die in the case of artificial incubation of eggs.

The **genetic** sampling protocol is as follows:

- Cut a fin from the dead hatchling (Figure 6) (or the entire embryo if there are no dead hatchlings). In the case of adults, cut a generous piece of musculature trying to take a slice in the best possible condition.
- Place the sample in a properly labelled sampling tube and fill it with 96% ethanol.

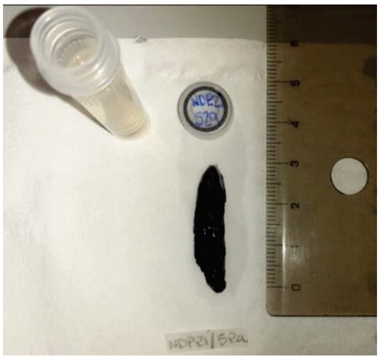

***Figure 6**: Example of a fin sampled from a dead hatchling and the corresponding labelling*

- After 1-2 hours, remove the liquid content of the tube and replace it with fresh 96% ethanol.
- Replace with fresh 96% ethanol after 24 hours.

The protocol for **stable isotopes** sampling is as follows:

- Once the sample has been collected for genetics, store the rest of the hatchling (or embryo) in a labelled tube (Figure 7), without adding any preservative. In the case of adult individuals, take another piece of muscle. Samples can be stored on plastic bags, plastic containers or aluminium foil.

- Freeze at -20°C.

***Figure 7**: Example of dead embryo stored without preservative.*

c).- Live hatchlings: The sampling of live hatchlings is the one which can potentially have the highest impact on the individual due to the small size of the individuals when born. Therefore, **live broods will only be sampled if it is strictly necessary** as detailed in the sampling scheme on the previous page (Figure 1), i.e., in case no headstarting of any offspring takes place and if no umbilical cord is available (protocol e).

The **genetic** sampling protocol is as follows:

- Disinfect the area to be sampled, preferably the posterior side of the carapace.
- Using a properly sterilised scalpel, cut out a very thin strip of shell of approximately 0.2 cm x 0.5 cm (Figure 3).

***Figure 3**: Example of a strip of live hatchling carapace.*

- Place the sample in a properly labelled sampling tube and fill with 96% ethanol.
- Sampling should not normally cause the animal to bleed, but if it does, apply pressure with a gauze pad sterilised with disinfectant for a few minutes until the bleeding stops.
- Disinfect the sampling site.
- After 1-2 hours, remove the liquid content of the tube and replace it with fresh 96% ethanol.
- Replace with fresh 96% ethanol after 24 hours.

Live hatchlings shall not be sampled for isotopes.

d).- Blood sampling. Blood samples provide optimal quality DNA if they have been properly collected and preserved and, allow to carry out the sampling under suitable conditions in order to avoid any impact to the animals sampled. **Therefore, it will be the preferred method of sampling individuals when it is feasible**. Sampling shall be carried out when the animal's sanitary conditions allow for it, and may be performed taking advantage of blood sampling for veterinary control purposes.

In the case of nesting events, blood collection from the nesting female must be performed exclusively by qualified staff following national and regional legislation. All headstarting individuals shall **always** be sampled only when the hatchlings weigh more than 300 grams. Sampling may be conducted in combination with a routine veterinary check to avoid additional stress to the animal.

- Perform a blood collection (~100-300 µl) following the veterinary protocols of the headstarting facility.
- Add 3 volumes of 96% ethanol.
- Centrifuge 1 minute at low speed (~8000rpm).
- Discard the supernatant.
- Fill the tube with fresh 96% ethanol.
- Replace with fresh 96% ethanol after 24 hours.

Headstarting individuals shall not be sampled for isotope or contaminants, because values have been modified by the diet they consume while in captivity.

e).- Umbilical cord from hatchlings. This is a very simple, non-invasive methodology for live hatchlings and involves no risk to the animal. Therefore, every umbilical cord found should always be sampled if found to ensure that a reserve of samples is available in case better quality samples cannot be obtained.

The **genetic** sampling protocol is as follows:

- Carefully collect the umbilical cord by hand, directly from the ventral area of the individual (this tissue is usually attached to the plastron of the offspring) (Figure 8 a-b).

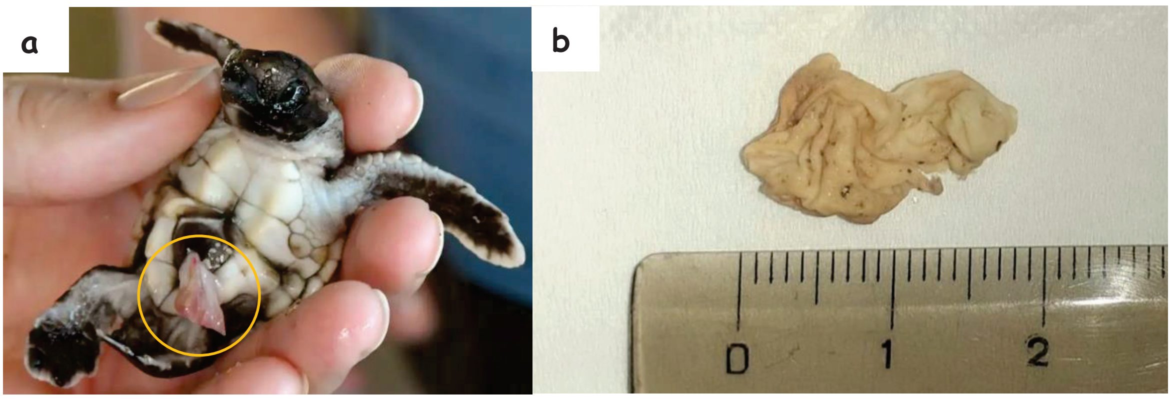

***Figure 8: a)** Newborn Sea turtle hatchling with its umbilical cord attached to the plastron. **b)** Example of a suitable sample of a whole umbilical cord.*

- Place the sample in a properly labelled sampling tube and fill it with 96% ethanol.
- After 1-2 hours, remove the liquid content of the tube and replace it with fresh 96% ethanol.
- Replace with fresh 96% ethanol after 24 hours.

5. Ethics

Sampling must be performed by qualified staff, especially when sampling on live animals, and following national and regional legislation. Permits will be needed depending on the country and if sampling living or dead individuals. Additional permits are likely to be needed to send and process the samples, especially if more than one country is involved.

6. Collaboration potential

Collaboration is fundamental among the stakeholders managing the nesting events, the rescue centers participating in headstarting programs and the scientific community performing the analysis to guarantee a correct collection and preservation of samples and metadata and to send the samples for the analysis.

7. Data collection

Each sample container must be uniquely labeled following the procedures described in the protocol to avoid the erase of the identifier. Metadata of the individual, the particular nesting event, and the nesting female (if applicable) must be unambiguously associated to the code of the sample.

8. References

- Luis Cardona, Sara Abalo-Morla, Alessandra Cani, Blanca Feliu-Tena, Nuria Izaguirre, Jesús Tomás, Eduardo J. Belda (2024) Identifying the foraging grounds of the new loggerhead turtle nesters in the western Mediterranean. *Aquatic Conserv: Mar Freshw Ecosyst.* 2024;34:e4059. https://doi.org/10.1002/aqc.4059
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PROTOCOL N. 5 - EGGS CHAMBER LOCATION

ASSOCIATED PROTOCOLS: Nest relocation.

STANDARDIZED DATA SHEET: Data sheet N. 2 / Database.

MANPOWER:

ECONOMIC RESOURCES: €

TIME/FREQUENCY: ⌛ Every time there is a nest detected.

AUTHORIZATION: Yes, from de Governement.

QUALIFIED PERSONNEL: Yes.

1. Reason

The precise location of the clutch enables the protection of the nest, monitoring of incubation, estimation of incubation duration, assessment of hatching success and sex ratio of the hatchlings, and safeguarding the emergence of the neonates.

2. Context and relevance in Western Mediterranean

Identifying clutch locations is vital in the Western Mediterranean for two reasons: 1) it is crucial for nest protection, and 2) it provides key information to determine the feasibility of establishing a nesting rookery on a particular beach or area under a colonisation scenario.

Due to the scarcity of data on the characteristics of sporadic nests in a species colonisation context, it is highly important to locate clutches and gather comprehensive information for each nest within the Western Mediterranean. Information such as clutch size, nest depth, distance from the sea, and the distance to the end of the beach may be less significant in well-studied and established rookeries compared to sporadic nests found in newly identified nesting sites. The impact of nest characteristics on the colonisation process in the Western Mediterranean remains unknown, thus necessitating further data collection.

3. Equipment

Paper, pencil, and torch or headlamp (if at night).

4. Actions

The location of clutches must always be conducted or verified by specialists in marine turtle nesting, as improper handling can damage embryos or erase interpretive markings of the track indicating the presence of a clutch.

Before proceeding with locating the nest, it is advisable to record a complete video of the track in detail, avoiding stepping on or erasing it. Additionally, it is recommended to mark the edges of the track in the area of the potential nest with stones, ropes, or sticks before starting to dig, to avoid losing reference points if the clutch is not found on the first attempt.

Efforts to find clutches should occur either immediately after egg laying or when hatchlings are seen heading towards the sea (if the nest has not been monitored previously). Once the clutch is found after laying, nest relocation should be considered if it is not in a safe location (refer to protocol 7). This action should only be performed by experts and in accordance with appropriate egg management protocols (see protocol 7). If nest relocation is deemed necessary, counting the eggs is crucial in the Western Mediterranean as it provides more accurate data on hatchling success. During this process, various measurements should be recorded (number of eggs, nest depth, distance to the sea and the end of the beach, depth to the top egg from the sand surface, beach surface characteristics—such as sand size and colour, vegetation, or presence of rocks).

Before closing the nest, a temperature data logger should also be placed to monitor nest temperature (see protocols 9 and 8). If the clutch is discovered after observing hatchlings making their way to the sea, it should be protected until it appears that all the hatchlings have emerged. Once experts determine that no more hatchlings are likely to emerge, the clutch can then be opened.

5. Ethics

Since identifying the clutch location involves modifying the incubation conditions of the eggs and is the initial step towards relocating the clutch, it should only be performed by trained and authorized personnel. The manager must determine if there are potential threats to the incubating clutch that justify changing its exact location shortly after deposition, or if confirmation of clutch deposition can be made the time of hatching.

6. Collaboration potential

No collaboration is required to implement this protocol; however, the presence of qualified personnel is essential. Moreover, sharing the collected data is beneficial within the context of collaboration for general research objectives.

7. Data collection

Important data: distance to sea, depth to the top egg from the sand surface, number of eggs, nest depth (it should be measured from the bottom of the egg chamber to the sand surface level using as a reference a stick placed horizontally in the sand surface). Data to be inserted in “Nesting Activity Database” (T4.3).

8. References

- Alan F. Rees (2020) Marine Turtles in MPAs: a monitoring and management guide. MedPAN Collection. 68 pp
- Eckert, K. L., Bjorndal, K. A., Abreu-Grobois, F. A., & Donnelly, M. (2000) Técnicas de Investigación y Manejo para la Conservación de las Tortugas Marinas. *Grupo especialista en Tortugas Marinas. Unión*

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- FFWCC (Florida Fish and Wildlife Conservation Commission) (2016) Marine turtle conservation handbook.
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PROTOCOL N. 6 - NEST PROTECTION (from predation and human activity)

ASSOCIATED PROTOCOLS: *Beach surveys [1], Nest relocation [7].*

STANDARISED DATA SHEET: Data sheet N. 2 / Database.

MANPOWER:

ECONOMIC RESOURCES: €€

TIME/FREQUENCY:

AUTHORIZATION: Yes

QUALIFIED STAFF: Supervision of specialised nesting personnel is recommended.

1. Reason

The protection of the clutch is fundamental to avoid predation of the nest by wild, feral, or domestic animals, and disturbance by human activities or vandalism. On one hand, the aim is to increase survivorship of clutches and hence recruitment to the population in a yearly cohort of hatchlings, where depredation of clutches is a proven problem. On the other hand, loggerhead turtle nests in the Western Mediterranean Sea may be laid on urban or highly touristic beaches where human activities may negatively impact on nest development, so it is necessary to apply appropriate protection measures. All nests must be protected, to a different level depending on each situation and depending on the beach, but a minimum must always be ensured.

Thus, this protocol aims at improving the survival of the nest from predation and alterations caused by anthropogenic factors.

2. Context and relevance in Western Mediterranean

One of the most widespread threats to egg survivorship, other than flooding, is depredation by mammals, such as dogs, foxes, or rats. These animals detect where the clutch is buried in the beach and dig until the eggs are exposed. Preventing predators from digging into the sand to reach the eggs, using some form of screen or barrier, is a much used and effective way of combating this threat, therefore resulting in higher hatchling recruitment.

Moreover, considering that Western Mediterranean nesting is recent, and most of the public is unaware of the presence of sea turtle nests, protection against human activities is highly recommended. In these areas, nests are often laid within public and popular bathing beaches. For example, a clutch can be destroyed by sticking a parasol in the sand, digging in the sand, stepping on the nest area repeatedly and other activities undertaken by people during the practice of games, cultural and sporting events on the beach. Vehicles, such as beach cleaning machinery can also destroy nests. Therefore, additional protection is needed to assure that people do not damage the nest.

3. Equipment

The materials used can be made of metal, plastic and wood or other natural materials. Whenever possible it is recommended to use ecofriendly, recyclable and magnetically inert materials.

Protection around the nest:

- Stakes or posts.
- Depending on the kind of fence, adequate quantity of screens or ribbons or ropes

Protection from above

- Net of appropriate dimension with a mesh size of 5-10 cm^2. If it is possible, it is highly recommended to use a net such as a soccer goal net, or similar.

Protection from predators:

- Anti-predation screen/cages. Depending on the potential predators present in the area, different type of screen/cages can be used that belong to two main categories: self-realising (minimum mesh size 5 X 10 cm) and restraining screen/cages (mesh size < 5 X 10 cm). The minimum area protected by the screen/cage must be approximately 1 $m^{2.}$

Information and warning signs.

4. Actions

As soon as the location of a nest has been verified (see Egg chamber location protocol N.6), the expert must evaluate the risks and potential threats to decide if and which type of protection should be applied. Nest protection measures should be applied as soon as possible after the nest has been detected, especially where predation and/or disturbance by intense human activities are known to occur.

Anti predation protection:

In new emerging nesting sites where no data on nest predation are available, as much information as possible on the presence of potential predators (e.g., feral dogs, foxes, wild boars, rats, and/or ghost crabs etc.) needs to be sought, so that the appropriate protection measure can be applied (e.g. cages to avoid predation by foxes). In case of doubts anti-predation measures should always be applied.

The use of non-self-releasing cages/screens is recommended only where small predators are present that could pass the 5 X 10 cm mesh (e.g., small rodents or ghost crabs). For all other types of predators, the use of self-releasing cages/screens is preferred.

- The screen of at least 1 m^2 should be placed centrally above the egg chamber and 10 cm below the sand surface. Insert a hooked stakes at the corners of the screen to hold it securely in place. Always make sure that the stakes are angled away from the egg chamber. If non-self- releasing screens are used, remember to remove them before the start of hatchling emergence.

- A cage can be either completely or partially buried around the nest. To install a cage, place it on top of the sand with the egg chamber in the centre, mark the position of the 4 cage sides and then dig trenches for each side down to 30 cm below the sand surface. Insert the cage and cover it again with the sand removed during digging. The cage can be anchored by driving stakes angling away from the egg chamber through the corners. Alternatively, the cage sides can be designed to have outward pointing flanges (15 cm long), in which case the trenches must be dug wide enough to allow the cage to be inserted. Self-releasing cages should be installed so that two opposite sides are facing the sea, and the 10 cm opening is parallel to the sand surface.

If screens or cages are installed, they must be checked regularly throughout the incubation period to ensure that they are not partially or completely removed or exposed by wind/wave action or human interference.

Protection against human threats:

Evaluate the presence and intensity of human activity at the nest site to decide whether the nest needs appropriate fencing. The size of the fenced area depends on the surrounding context but should be at least 1 m^2 and can be enlarged as required by the situation if sufficient space is available. Care must be taken to ensure that there are no elements, from the fence itself or other structures (e.g., sunbed or umbrella), that can shade the nest during any time of the day and during the entire incubation period.

- Install poles for the fenced area at a distance of at least 1 m between each. Make sure that the nest is always at the centre of the fenced area and hence, that all poles are placed at the largest possible distance to the egg chamber. The poles should be inserted at least 50 cm below the sand surface to hold them securely in place. The recommend height above sand is at least 1 m.
- Fix ribbons or ropes tightly to each pole around the entire perimeter of the area to be fenced. It is advisable to apply the rope/ribbon at two or more different heights (e.g., the 1st at 50 cm above sand and the second at 1 m).
- If there is a high probability of trespassing and intrusion of humans, use screens or nets instead of the ropes to delimit the area. Fix them tightly to each pole.
- It is also recommended (especially on urban beaches) to place a large light mesh (soccer goal net type) across the upper part of the enclosure, to prevent flying objects (balls, etc.) from entering the nest area.

Always fence the nest if an external anti-predator cage has been installed, thus, to restrict access to the nest and avoid that the cage is removed or meddled with. Pay attention to secure the ribbons/screens so that they will not give way to people who lean on the fence.

Apply information board (containing the emergence number to report any violation) and warning signs to the fence before leaving.

When nests are placed in areas with low or without human activity, it may not be necessary to apply all the above protection measures against human threats, but the protection against predation must be considered anyway. Remember to always mark the position of the nest, especially if no fence is applied.

5. Ethics

Nest protection actions may potentially tamper with the nest environment and must therefore be conducted exclusively under the supervision of experienced personnel authorise by the National Competent Authority.

6. Collaboration potential

The incubation period of the nests is usually more than 45 days, and it is advisable to work with local environmental organisations that have a network of volunteers who can help carry out adequate surveillance and protection of the nest after carrying out specific training for this purpose. It is very important to take advantage of nesting events to promote awareness and environmental education.

7. Data collection

Data to be inserted in “Nesting Activity Database” (T4.3): Nest ID, Nest_management; other related data: Nest inundation, nest predation, anthropogenic impact, beachfront lighting.

8. References

- ALan F. Rees 2020. Marine Turtles in MPAs: a monitoring and management guide. MedPAN Collection. 68pp
- Eckert, KL, Bjorndal, KA, Abreu- Grobois, FA, & Donnelly, M. (2000). Investigation and Management Techniques for the Conservation of Sea Turtles. *Sea Turtle Specialist Group. International Union for Conservation of Nature and Species Survival Commission, Publication*, (4).
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PROTOCOL N. 7 - NEST RELOCATION

ASSOCIATED PROTOCOLS: Eggs chamber location [5], Artificial Incubation [8].

STANDARDIZED DATA SHEET: Same used for recording nest data (to get data from the new nest).

MANPOWER:

ECONOMIC RESOURCES: €

TIME/FREQUENCY:

AUTHORIZATION: Yes, from the Regional Government.

QUALIFIED PERSONNEL: Yes, nesting specialist and authorized technical personnel.

1. Reason

Guarantee the survival of the nest depending on the place and date of laying, adverse weather phenomena, and even to guarantee its protection when surveillance systems are not available.

2. Context and relevance in Western Mediterranean

For Western Mediterranean, where emergence and sporadic nesting exist, is especially important to assure all nests are in a safe place, and to consider conservation actions to protect them in case of risk. Therefore, some extra special situations should be considered when trying to identify if a nest location is safe: (1) the nest to close to the High Water Mark, this should be identified with the help of locals who know the beach dynamics and can guess if a nest is likely to be inundated; (2) the nest is in the way of possible freshwater coming from inland, which can occur in case of rainfall; (3) the nest is in a place where a conflict with human activity is highly possible (presence of an establishment, passage to arrive to boats, etc); (4) the nest is made in a place which is shaded by objects, vegetation or a building. Here it is also important to consider the seasonal changes, especially at the higher latitudes of in the Western Mediterranean. The sun and temperatures will be different at the end of the season, and therefore is important to consider all the conditions you will have all over the incubation period and take conservation actions if needed (*see Artificial Incubation protocol*).

3. Equipment

Nitrile gloves, flashlight or headlamp, GPS, insulating boxes.

4. Actions

The methodology and procedure for relocating eggs must be carried out carefully and requires previous experience, since mortality of the embryos may occur induced by movement, by incorrect handling of the eggs or by not selecting the nest relocation site well (risk of flooding, inappropriate temperatures, high water table, etc.). The relocation of a nest should preferably be done 10 hours before laying, to minimize injuries caused by movement to the embryos. However, it will be taken into account that, after 4 hours from spawning, the risk of embryo death due to sudden movements or rotation increases. It is advisable to have everything necessary for the complete relocation process prepared, including the approximate location of relocation, before beginning to dig the nest and extract the eggs, to minimize the time the eggs remain off the beach.

Therefore, rotations and shaking will be avoided or minimized (always maintain the axial position of the egg) starting 4 hours after oviposition. Under no circumstances will the eggs be turned, and they will only be cleaned unless they have a lot of mud adhered to them. If cleaning is required, it will always be done without sudden movements or vibrations. The eggs must be transported all together, simulating a real nest, maintaining the oviposition order of the original nest, using the order of the eggs in the nest and using a rigid isothermal container. In case of high risk of egg survival, the eggs could be moved before the 4th day and after the 35th day, but without turning or rotating the eggs. In extreme cases of risk of embryo death (low or high temperatures, surface runoff or flooding, storms, etc.), and very exceptionally, a transfer can be carried out, extreme handling conditions to minimize any change in position or movement of the eggs.

The temperature of the beach sand at the incubation depth should never be lower or higher than 25.5 and 32.5 °C, respectively. Nests deposited on the beach starting in August, according to current thermal regime data, are susceptible to not completing their development correctly due to temperatures that are too cold after the middle of the incubation period. Likewise, on dark sand beaches, high temperatures can be detected, which can negatively affect embryonic development. If, through sand temperature monitoring data, it is detected that the incubation temperature could put the development of the eggs at risk, proven techniques will be used to increase or decrease the incubation temperature on the beach or the possibility of moving the nest to an incubator will be considered.

If the oviposition of a clutch is observed in a place with conditions that are not suitable for the incubation of the eggs, the collection of these can be carried out as the turtle is laying them. To do this, you can place a bag inside the hole and let the eggs fall inside, or gently pick them up or catch them as they fall.

- Note the depth at which the first egg is located, the total depth of the incubation chamber and its diameter.
- The depth of the nest will be used to replicate natural conditions in the new location.
- To minimize the movement of the eggs during transport, they will be placed in boxes filled with moist sand to help ensure proper support and protection.
- The new nest location should be as close as possible to the location chosen by the female to reduce the risks associated with its transport.
- It is essential that the new spawn location area be more than 1.5 m above sea level (preferably 2 m or more) completely out of reach of the water table. The nest should also be away from runoff areas or wave action, considering the risk in times of storms (it is recommended that it always be more than 25 m away from the high tide line).
- Once in the new location, a new chamber of the same depth and diameter as the previous one will be dug. The shape of the nest must simulate that of an upside-down light bulb .
- The eggs will have to be laid carefully, one by one, maintaining the order of oviposition (the lower eggs of the laying at the bottom, and the upper eggs at the top) and trying not to leave them too tight. They will be covered using the moist sand that we have dug to make the chamber, never with dry sand from the surface since this is hotter and could damage the eggs.
- To handle the clutch as little as possible, whenever possible, during the repositioning of the clutch, automatic temperature recorders will be placed to monitor the incubation temperature.

5. Ethics

Nest relocation, as nest protection, actions may potentially tamper with the nest environment and must therefore be conducted exclusively under the supervision of experienced personnel authorise by the National Competent Authority.

6. Collaboration potential

The incubation period of the nests is usually more than 45 days, and it is advisable to work with local environmental organisations that have a network of volunteers who can help carry out adequate surveillance and protection of the nest after carrying out specific training for this purpose. It is very important to take advantage of nesting events to promote awareness and environmental education.

7. Data collection

Data to be inserted in "Nesting Activity Database" (T4.3): Nest new location; Nest new mesurements; other data related.

8. References

- ALan F. Rees 2020. Marine Turtles in MPAs: a monitoring and management guide. MedPAN Collection. 68pp
- Abella, E., Marco, A., & López-Jurado, LF (2007). Success of delayed translocation of loggerhead turtle nests. *The Journal of Wildlife Management*, 71(7), 2290-2296.
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- Najwa-Sawawi, S., Azman, N.M., Rusli, M.U., Ahmad, A., Fahmi-Ahmad, M., & Fadzly, N. (2021). How deep is deep enough? Analysis of sea turtle eggs nest relocation procedure at Chagar Hutang Turtle Sanctuary. *Saudi Journal of Biological Sciences.*

PROTOCOL N. 8 - ARTIFICIAL INCUBATION AND MONITORING

ASSOCIATED PROTOCOLS: *Nest protection [6], Temperature monitoring [9], Head Starting Program [10].*

STANDARISED DATA SHEET: There is no standardised data sheet for this protocol.

MANPOWER:

ECONOMIC RESOURCES: €€€

TIME/FREQUENCY: On encountering a nest.

AUTHORIZATION: Yes

QUALIFIED STAFF: Supervision of specialised nesting personnel is recommended.

1. Reason

Artificial incubation is conservation tool that can be use in extreme cases when environmental conditions are not conductive to embryo development (i.e., late nests, sand temperature too high or too low, unsuitable substrate, etc.) to maximise hatchling production in a specific region. Given its potential impacts on the sex, size, shape, behaviour and movement ability of hatchlings, this practice should be considered as a last resort, within a much wider and articulated conservation strategy and must be always conducted under strict scientific supervision.

2. Context and relevance in Western Mediterranean

Loggerhead turtle nesting in the Western Mediterranean has drastically increased in the last couple of decades, but habitat suitability at some locations may still be low. Moreover, the nesting season is generally shorter compared to the warmer eastern basin, and egg clutches laid in the second half of August or later have a very high chance of encountering climatic conditions not suitable for the embryonic development, which may result in the complete loss of the nest. To maximize nest survival, one of the conservation tools available to conservation authorities, parks, zoos, and other management stakeholders is the ex-situ incubation of eggs followed by hatchling or juvenile release (Eckert et al. 1999). This practice typically involves removing eggs from natural nests, placing the eggs in boxes or incubators, and allowing eggs to hatch under controlled incubation conditions (Páez et al. 2015) which also allows to evaluate reproductive parameters such as fertilization rate or sex ratios. After hatching, juveniles are either released immediately, or are 'head-started', which involves raising individuals to larger body sizes before release to theoretically increase survival in the wild (Mullin 2019). Artificial incubation regimes should be performed responsibly and resemble natural incubation regimes in order to avoid degradation of quality of hatchlings released to the wild, because incubation temperatures not only affect sex ratio determination (Mrosovsky & Pieau 1991) or survival (Bobyn & Brooks 1994) but also have profound impacts on many aspects of hatchling phenotypes and performance (Sim et al. 2015, Kobayashi et al. 2018).

In those cases, where nesting is still rare and previous information on hatching success are scares, it is possible to apply an incubation diversification setting, under potentially high risky scenarios, that foreseen incubating under controlled conditions a small percentage of eggs (10%-20% if it is appropriate according to clutch size) while leaving the rest on the beach. This conservation measure should contribute to increase the hatchling production while still acquiring scientific data on the suitability of that specific site.

3. Equipment

- Incubator
- Vermiculite
- Waterproof calibrated temperature loggers (with an accuracy of ±0.2 °C)

4. Actions

Before the arrival of eggs in the laboratory, the incubators where the eggs will be placed must be checked for correct calibration. Depending on incubator characteristics, it would be essential to place the incubator in an air-conditioned room to avoid temperature fluctuations during incubation due to environmental temperature. In hot regions, it is possible to have air temperatures up to 30ºC, which can modify the desired temperature regime inside the incubator. So, the incubator should be programmed and checked to see if it reaches the desired range of incubation temperatures. For accurate data, temperature should be recorded in the incubator by waterproof temperature data loggers placed inside boxes containing moist vermiculite (water potential – 150kPa), as done for natural egg incubation. To ensure good hatching success, it is important to ensure there are no extreme temperature or moisture level deviations during the incubation.

The most adequate temperature regime of incubation would depend on the conservation strategy for individual nests but in any case, artificial incubation regimes would fall within a normal range of incubation temperatures naturally experienced by the species, ideally predicting production of a proportion of both sex hatchlings from each clutch. Data loggers should be maintained recording incubation temperature until egg hatching. Egg transportation from the beach to incubator should ideally take place within 6 hours of nesting, to minimize movement-induced injury to embryos (Boulon Jr, 1999). However, if care is taken to avoid rotation and jarring of the eggs, the practice can be undertaken within first four days of nesting and result in negligible impact on hatching success (Abella et al. 2007) (see protocol 7).

Once in the laboratory, eggs should be placed in sealed plastic boxes with small ventilation holes in their lids. Inside the boxes the eggs should be separated without contact with one another (e.g.,10 per box), and surrounded and slightly covered by moist vermiculite (water potential -150kPa). Immediately after that, every single box must be weighed in order to monitor and replace water lost during incubation. Every box and egg are identified by writing an identification code on the box. During incubation, eggs should be checked periodically. 48 hours after oviposition, it is possible to observe a white spot at the top of the eggs indicating embryo development has commenced and allowing a rough estimation of egg viability short time after oviposition. If fungal infestation is detected in any egg, it should be isolated from healthy eggs. If water loss is detected through weighing the boxes, mineral water should be added until the box recovers its initial weight. If changes in incubation temperature are suspected, temperature data logger would be launched as frequently as necessary.

It is recommended to check egg appearance once per week without taking or moving the eggs (just removing a little bit of the vermiculite to observe the top of the shell). Towards the end of incubation, boxes must be inspected daily to determine the day of pipping and day of hatching. Upon hatching, hatchlings should be isolated at 25ºC to 27ºC in jars lined with a wet paper towel for an additional 1 to 3 days to allow the carapace and plastron to take on their proper shape (Fujimoto et al. 2020). Once hatchlings have matured, they would be measured and sampled (see *Protocol 4*).

5. Ethics

Artificial incubation is an invasive technique that requires ethical justification and a permit from the relevant national authority. Incorrect egg management during artificial incubation procedures can have a harmful impact on hatching success, sex ratio and hence the recruitment of new turtles to the wild. Thus, this practice should only be carried out by trained personnel. Permits will be required to transport and manipulate the eggs.

6. Collaboration potential

Artificial incubation and monitoring can be carried out by zoos, parks, marine rescue centres, NGOs, or university teams which have appropriated equipment and trained personnel and must always be linked to a specific research program to fully investigate the results of these artificial incubation trials, the sex ratios produced, and compare these to those from natural nests.

7. Data collection

The number of eggs developed, hatching success, incubation duration until pipping and hatching, and raw temperature data from incubation temperature loggers should be processed to provide mean daily temperature during incubation. It is also important to record the degree of humidity.

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PROTOCOL N. 9 - NEST TEMPERATURE MONITORING AND MANAGEMENT

ASSOCIATED PROTOCOLS: *Egg Chamber location [5], Nest protection [6], Nest Relocation [7], Artificial Incubation [8], Head Starting [10].*

STANDARDISED DATA SHEET: There is no standardized worksheet for this protocol.

MANPOWER:

ECONOMIC RESOURCES: €€€

TIME/FREQUENCY: (all nesting season)

AUTHORIZATION: No

QUALIFIED STAFF: Supervision of specialized nesting personnel is recommended.

1. Reason

Incubation temperature is directly related to the incubation duration, hatching success, and the sex ratio of hatchlings produced on the beaches. Nest temperature monitoring in real-time permits to take quick management decisions to preserve nests at risk in the Western Mediterranean due to non-adequate incubation temperature regimes by detecting changes of nest temperature below or above the optimal range of incubation temperature (e.g. extremely cool beaches, nests laid at the end of the season, shaded nests, etc.).

Monitoring incubation temperature facilitates additional nest management actions aiming at improving the nest emergence success, as well as protecting the emerging hatchlings on their way to the sea from present threats (e.g. disorientation by artificial lighting). For some of these management actions timing is vital and hence, the date of emergence can be estimated using the recorded incubation temperatures. Temperature monitoring is also crucial for the assessment of natural primary sex ratios in beaches of current nesting areas of colonization. Knowing precise incubation temperature regimes and profiles rather than only sand beach temperatures is important as the proportion of female hatchlings produced in those areas may vary over time and space and hence colonization time estimations based only on beach sand temperatures can be excessively inaccurate.

2. Context and relevance in Western Mediterranean

Incubation temperatures of nests in the Western Mediterranean remain largely unknown and are essential for comparison with the incubation temperature regimes in the Eastern Mediterranean to identify potential differences. Understanding the natural dispersal of nesting to the Western Mediterranean is crucial for detecting changes in regional population dynamics. Accurate data on regional nest incubation temperature ranges are necessary to determine nest viability due to temperature, which is vital information for managers and decision-makers. This data also helps in estimating nest survival rates related to temperature and in predicting primary sex ratios using a simple, non-invasive method. Monitoring nest incubation temperatures can enhance hatching success by mitigating extreme incubation temperatures through proper nest management techniques, such as shading or relocating nests to artificial incubators.

Nest characteristics in the Western Mediterranean are highly varied (e.g., number of developing eggs, clutch size, nest depth), leading to differences in the amount of metabolic heating generated. Data on metabolic heating in Western Mediterranean nests could be crucial for understanding variations in nest incubation regimes beyond general beach temperatures. Additionally, it allows for more accurate sex ratio estimations and facilitates the development of population dynamics models considering climate change scenarios, not only in the Western Mediterranean but throughout the entire Mediterranean region.

3. Equipment

Waterproof calibrated temperature loggers, with an accuracy of at least ±0.5°C (e.g. HOBO Pendant MX 2201) or ±0.2°C (preferred), if higher accuracy is required as for sex ratio estimates (e.g. HOBO TidbiT MX, http://onsetcomp.com). It is recommended to use Bluetooth dataloggers, so that the temperature data can be downloaded at any time during the incubation, as required, without having to retrieve the datalogger from the nest (and consequently disturb the clutch).

4. Actions

Automatic temperature data loggers should be programmed to record temperature at least every 30 min. Data loggers (factory calibrated at purchase) should be checked for accuracy by deploying all of them simultaneously in sand or a controlled water bath for at least 48 h before and after the data collection period, to detect possible differences among the temperature recordings. Configure and activate the dataloggers before you begin digging in the sand for their deployment. If possible, delay the start of logging or set a start date and time to syncronize the start of data recording with the time that the logger is firmly deployed in its position.

Data Logger Deployment

Temperature loggers should be deployed on the first day of incubation and as soon after egg laying as possible. The position in the nest depends on the primary objective, either (a) nest management and sex ratio assessment or (b) nest management only.

a) Temperature data loggers should be deployed in the middle of the clutch. For this, it is important to know the clutch size, so that you can remove half of the eggs, place the logger and replace the eggs. If clutch size is not known, because the number of eggs were not counted during the egg laying process, then remove and count all eggs with the same precautions and procedures applied during nest relocation (see Protocol 7). A second datalogger should be buried at least at 70 cm from the nest, parallel to the shoreline and at the same depth of the first datalogger. This will serve as a control to establish clutch temperature increases due to metabolic heating considering that temperature recorded inside the clutch will become increasingly warmer than sand temperatures from the second third of incubation period onward.

b) To minimise the disturbance of the clutch, the temperature logger can be placed in the side of the nest or in the top, where eggs are briefly exposed during the process of verification of the egg chamber (see Protocol 5), and before the eggs are covered again. One logger per nest is sufficient. In alternative, if for some reason a datalogger cannot be placed inside the nest, the datalogger can be buried in the sand close to the nest (at least 70 cm distance parallel to the shoreline) at a mean depth of 40 cm.

In case of nest relocation, depending on the availability of dataloggers, they can be deployed in two or more positions in the nest, one in the middle of the clutch, one on top of the egg chamber and/or one on the bottom of the egg chamber. This would allow to monitor temperature dynamics with respect to the position in the nest, which can also be used for sex ratio assessments.

To monitor incubation temperature of a nest in case it was not possible to acquire Bluetooth loggers, optical data loggers should be deployed to monitor sand temperature close to the nest and at mean egg chamber depth. However, these data would be only a proxy of the real incubation temperatures. Data download is also less immediate, as digging and retrieving the datalogger and subsequential re-deployment is required.

Always mark the nest which contains a datalogger and record the depth and GPS position of each logger.

Temperature Monitoring

The use of Bluetooth temperature data loggers allows to monitor incubation temperature in real time and as often as required without the need to dig the logger out. This is the best option to monitor nest incubation temperature in those areas where extreme temperature regimens are suspected to negatively affect nest incubation and viability, because managers can make quick decisions on the appropriate nest management techniques to apply to protect the nest from extremely low or high temperatures. In addition, temperature data can be downloaded for inspection after particularly strong weather events (storms, washovers, heavy or prolonged periods of rainfall, etc.), to check if temperatures were affected in a way that could impact embryonic development and to decide if any action is needed to prevent possible clutch failure.

To estimate the date of hatchling emergence, temperature data should be downloaded after the 34th day of incubation and average incubation temperature of the middle third of incubation should be calculated. The middle third of incubation can be assumed to be between the 17th and the 34th day of incubation, using on a mean incubation duration of 51 days. Based on the good correlation between mean temperature during the middle third of incubation and the incubation duration, the date of emergence can be estimated. Adding the resulting days to the first day of incubation gives you the presumed date of hatchling emergence. If emerging hatchlings need to be protected, regular check for any signs of movement in the nest should be taken already two days before the presumed date of emergence.

5. Ethics

As deployment of data loggers in the middle of the nest involves altering, to some degree, the incubation condition of the eggs and clutch size sampling implies egg management, it should only be carried out by trained and authorized personnel. Further actions to protect nests against extreme temperatures, as nest translocation or installation of warming or shading equipment on the beach, should also be done by trained and authorized personnel.

6. Collaboration potential

Universities or NGOs can place temperature loggers inside nests in close partnership with the relevant authorities. However, it is necessary for them to share the temperature logger and nest data with research scientists, who can then thoroughly investigate potential incubation temperature patterns, evaluate methods to protect nests from extreme temperatures, determine sex ratios within the areas, and compare this information seasonally with data from other locations.

7. Data collection

Data to be inserted in "Nesting Activity Database" (T4.3): Nest ID, datalogger/sensor model, datalogger depth, datalogger position, and latitude and longitude of nest (GPS location). Each temperature profile should be associated with a nest and GPS coordinate.

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PROTOCOL N. 10 - HEAD STARTING PROGRAM

ASSOCIATED PROTOCOLS: Artificial Incubation [9]

STANDARDIZED DATA SHEET: Data base – *Head Starting*

MANPOWER: Minimum two for every group of 30 headstarted hatchlings

ECONOMIC RESOURCES: €€€.

TIME/FREQUENCY: . Head starting facilities must be ready at the beginning of the hatching season and be operative 7 days per week during the entire duration of the head starting program.

AUTHORIZATION: Yes.

QUALIFIED PERSONNEL: Yes. It is necessary that people who carry out head starting programs have specific training and experience in rearing post hatchling loggerhead turtles. The specialized staff may be supported by volunteers and trainees that must receive adequate prior training and operate always under the direct supervision of qualified staff. Both technical staff and volunteers must be aware of the datasheet to collect the information and proficient with the apps eventually used to upload the data to the project database.

1.Reason

Headstarting is an ex-situ conservation practice which consists in raising hatchlings in captivity until they reach a minimum size before their release to the wild, assuming that this will increase the likelihood of their survival. However, due to the long time required to reach sexual maturity, headstarting program must be associated with the adequate protection of juveniles and adult at foraging and breeding sites, in order to be effective. Beyond being a conservation tool, headstarting programs offer the opportunity to tag small juveniles to study their behaviour and dispersal during the first years of life and to conduct studies to improve our understating of this cryptic life stage.

2. Context and relevance in Western Mediterranean

The loggerhead turtle is expanding its nesting range to the Western Mediterranean and headstarting programs may support a faster stabilization of new nesting sites if adequately integrated into a wider conservation strategy which also involves the protection of juveniles and adults. Moreover, headstarting programs will allow studying movements and behaviour of early juveniles born on Western Mediterranean beaches and identify the pelagic developmental habitats utilized.

3. Equipment

Marine turtle headstarting facilities must be operative 7 days per week, all year round. It is also mandatory that they are closely linked to a scientific institute, university or research centre to maximize scientific output. Headstarting is very expensive since it required specific infrastructures, equipment and specialized staff. Therefore, it is advisable that advisable that new head starting facilities develop within marine turtle rescue centres, aquariums or marine research centres that already possess the required knowledge and technical equipment.

An headstarting facilities should possess at least: 1) Rearing tanks of appropriate volume and dimensions with respect to the number of hatchlings raised; 2) Treatment tanks to administer therapeutic treatments or isolate individuals with sign of distress or pathologies; 3) Access to natural, clean sea water, 4) Life support System

(LSS) adequate to the volume of the tanks. It is important that the rearing and the treatment tanks have separated LSS and both are isolated from the rest of the facility. In order to have a better control on water quality parameters, it is advisable to build headstarting facility as Recirculating Aquaculture System (RAS) with at least mechanical and biological filtration, Foam Fractionator, UV water treatment system, thermostatic systems. In those cases, where direct sunlight is not an option, adequate UV lighting systems must be installed; 5) Food preparation area; 6) All the equipment and the related permit to apply PIT tags to the head started individuals prior their release.

4. Actions

Hatchlings should be reared for at least 8-12 months, until they reach a minimum weight of 800 g and a curved carapace length (CCLst) 15 cm. Different approaches exist to grow sea turtle hatchlings in captivity. Some facilities use individual tanks whose size increases with turtle' age, all through the headstarting period to prevent them from injuring each other (Higgins, 2003). Other accommodate several turtles in single tank of adequate dimension that usually possess a higher water column respect to the individual container. It has been observed, in fact, that in this way hatchlings do much more physical activity, have higher stamina and generally growth better, although at a slower rate, than individually held subjects. Furthermore, when conducted at medium/low densities, the common rearing does not generate stress or aggressive behaviour (Kanghae et al., 2016; Martín, 2020; Usategui-Martín et al., 2021). In any case, stocking density must be low (Higgins 2003, Bluvias & Eckert 2010, Kanghae et al. 2016) and daily observations should be carried out to promptly identify any biting event among individuals housed in the same tank. Sick or injured turtles should be held in isolation tanks for medical treatment to reduce the risk of disease transfer and health complications.

Rearing and treatment tanks must be made of seawater resistant, non-toxic and easily cleanable materials and have smooth and rounded edge with no inside protrusions. Each pool should be equipped with one or two inflowing water faucets, a drainage hole in the upper portion and another drainage with a faucet on the bottom.

Each tank must be supplied with a continuous circulation of sea water adequately filtered and terminally treated. Rearing and treatment tanks must possess separated and independent LSS to prevent pathogens outbreak.

Water quality should be regularly monitored controlled. Salinity and pH should be kept close to the natural values of the Mediterranean Sea. Both should be adjusted preferentially by cleaning and changing water. Adding chemicals to the water to regulate the pH or remove suspended solid should be avoided.

Water temperature should never fall below 19ºC with a preferred temperature range between 22ºC and 25ºC. Thermostatic systems should be always present because temperature fluctuations could produce stress shock to the animals.

To reduce the risk of contagion each pool should have its own cleaning equipment (abrasive cloth, sponges, siphons, etc.). e.

The scientific literature provides several examples of a correct dietary plan (fresh high protein diet of varying proportion of fish, squid, vegetable, etc.) for hatchling and juvenile sea turtles (Higgins 2003, Bluvias & Eckert 2010). The feeding strategy is generally to provide approximately 7% to 10% of the turtle's body weight in grams of fresh food per day (Cambell, 1996) in 2 to 3 meals supplemented with essential vitamins and minerals. Rigorous monitoring has to be done, and weight gains and losses must be used as a guide for dietary management. Turtles should undergo periodic veterinary check-ups in order to detect physical or biochemical problems during husbandry. It is recommended to monitor (weight and length) the animals once per week.

Environmental enriched tanks are highly recommended for better rearing results. Enrichment items are excellent for tactile stimulation and natural exploration of healthy turtles. Hiding places simulating algae or floating objects are essential for hatchlings. It is mandatory to regularly disinfect the enrichment items and never move items directly from one tank to another.

Before releasing, an assessment must be conducted by a veterinarian in order to assess the turtle health status. A complete blood analysis is required. Sampling (see *Tissue Sampling* protocol), if required, must be performed few weeks before the release as well as the PIT tagging (see *PIT tagging* protocol). If available, satellite tracking equipment should be deployed to the major number of turtles as possible in order to follow them and determine their post-head starting behaviour, dispersal movements, feeding areas used, and estimate juvenile survival rates.

Once a turtle is cleared for release and tagged, the animal should return to a safe and non-polluted area at sea, ideally in the same region where the nest was found. Post hatchling release can be done from the beach or directly at sea. In the latter case, the location must be selected among those where the presence of post/hatchling and early juveniles have already been ascertained.

5. Ethics

Headstarting is an invasive conservation technique and requires ethical justification and a specific permit from the national competent Authority. Incorrect animal management during husbandry can have a deadly impact on post hatchlings survival, thus this practice should only be carried out by skilled and trained personnel.

6. Collaboration potential

Headstaring can be carried out by zoos, parks, marine turtle rescue centres, NGO or University teams which have appropriated installations, equipment, and trained personnel. However, the manager may want to share headstarting results with research scientists who can fully investigate benefits of headstarting program results regarding species and population dynamics in the Mediterranean under a colonization scenario.

7. Data collection

From hatching, rigorous monitoring of individual hatchling growth (mass and length), feeding, rearing behaviour, periodic health assessments and medical history (if it is the case), as well as release or death date of every animal should be recorded.

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