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Establishing a Freshwater Turtle (*Emydura subglobosa*) Laboratory Line (FTLL) as a novel model species for research and education

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SUMMARY

The Jardine River turtle (*Emydura subglobosa*) was selected as a potential model species for studies on freshwater turtles and general reptile physiology. Attempts to establish a freshwater turtle laboratory line were made when an adult pair of *E. subglobosa* was received in 2016 by the Laboratory of Inland Fisheries and Aquaculture (Poznań University of Life Sciences). The first generation of offspring was obtained in 2017, and the second generation in 2023. In each generation, unrelated specimens were added to the animal cohort to avoid inbreeding. Husbandry regimes were established, and a basal diet for nutritional experiments was developed and manufactured by two methods, producing extruded feed and a gelatine-solidified variant. The establishment of the Freshwater Turtle Laboratory Line (FTLL) provides an opportunity to improve the development of husbandry techniques, increase knowledge of reptile physiology, and use laboratory-raised animals as model species for research and education.

KEY WORDS: Captive breeding, Jardine River turtle, Model organism, Reference diets

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INTRODUCTION

The aims of the use of model organisms include general physiological studies and disease research which can be extrapolated for a wide range of species. In the case of animals, most models involve terrestrial warm-blooded species such as the mouse (*Mus musculus*) and brown rat (*Rattus norvegicus*) or invertebrates such as the lesser fruit fly (*Drosophila melanogaster*) (Hedges 2002, Matthews and Vosshall 2020). For aquatic and ectotherm vertebrates, the most widely studied model is the zebrafish *Danio rerio* (Choi et al. 2021). Reptiles are rarely represented as model organisms in scientific research, which leads to a situation in which data from the wild or general observations are extrapolated to captive populations (Rawski et al. 2018a). Moreover, in the case of chelonians, their longevity, generation gaps, and poorly studied breeding biology reduce their availability for *in vivo* studies. Most reports published on turtles in captivity concern animals which are commercially obtained from the pet market or farms or are short-term captive specimens (Rawski et al. 2014, Rawski et al. 2016, Rawski et al. 2018b). The pet market as a source of animals for research has a number of disadvantages, including the frequently unknown origin and quality of the animals, which may have been traded multiple times, with untraceable history and unknown health status or inbreeding level. Moreover, in Poland, according to Resolution No. 15/2016 of the National Ethical Commission for Animal Experiments, animals from pet shops or wholesalers cannot legally be used in research procedures. Another disadvantage is that the most popular turtle species in captivity, the pond slider (*Trachemys scripta*), is recognized as an invasive alien species in the European Union by Regulation (EU) No 1143/2014 of the European Parliament and of the Council of 22 October 2014 on the prevention and management of the introduction and spread of invasive alien species, which banned its import, trade and breeding. Moreover, in the system of permits implemented by the act of 11 August 2021 on alien species (Journal of Laws 2021 item 1718), invasive alien species cannot be used in research in Poland. In the case of animals from zoo collections, the main issue with the availability of the animals is that most represent endangered species, and their use and the degree of invasiveness of experimental procedures are restricted as well. Hence there is a need for a source of specimens of known origin which are laboratory-hatched and raised. For these reasons, drawing from many years of experience with turtle studies (Rawski et al. 2014, Rawski et al. 2016, Rawski et al. 2018b), the authors aimed to verify the possibility of maintaining and breeding the Jardine River turtle (*Emydura subglobosa*) in laboratory conditions. The study had three specific objectives: 1) to establish a breeding colony of *E. subglobosa* as a potential model species and breeding stock for a Freshwater Turtle Laboratory Line (FTLL); 2) to develop maintenance and breeding methods for *E. subglobosa* in laboratory conditions; and 3) to develop practical diets for juvenile turtles applicable in nutritional studies.

E. subglobosa is a medium-sized turtle species, reaching up to 22.4 cm straight carapace length (Freeman et al. 2016) and body weight up to 1800 g (authors' observations). Its natural distribution range is Papua New Guinea and the Jardine River in Australia (IUCN 2000, Freeman et al. 2016). However, due to the Australian ban on export of native species, the captive population is derived from the Papuan turtles. The species has been popular in the pet trade since the 1970s and, due to frequent breeding and a high fecundity rate, captive populations in the USA and Europe are self-sufficient, with no need for import of wild-caught animals. Like all representatives of the side-necked turtles (Pleurodira), they hide their heads in the space under the marginal scutes near the front legs. In contrast to cryptodiran species, *E. subglobosa* is not able to retract its head inside the shell. Yellow

postorbital stripes are present on both sides of the head (Figure 1A). Pastel, hypomelanistic and albinotic colour morphs are present in captivity (Figure 1B). Carapace colouration is typically olive to dark brown, and the plastron may vary from yellow to bright red (Figure 1C and 1D).

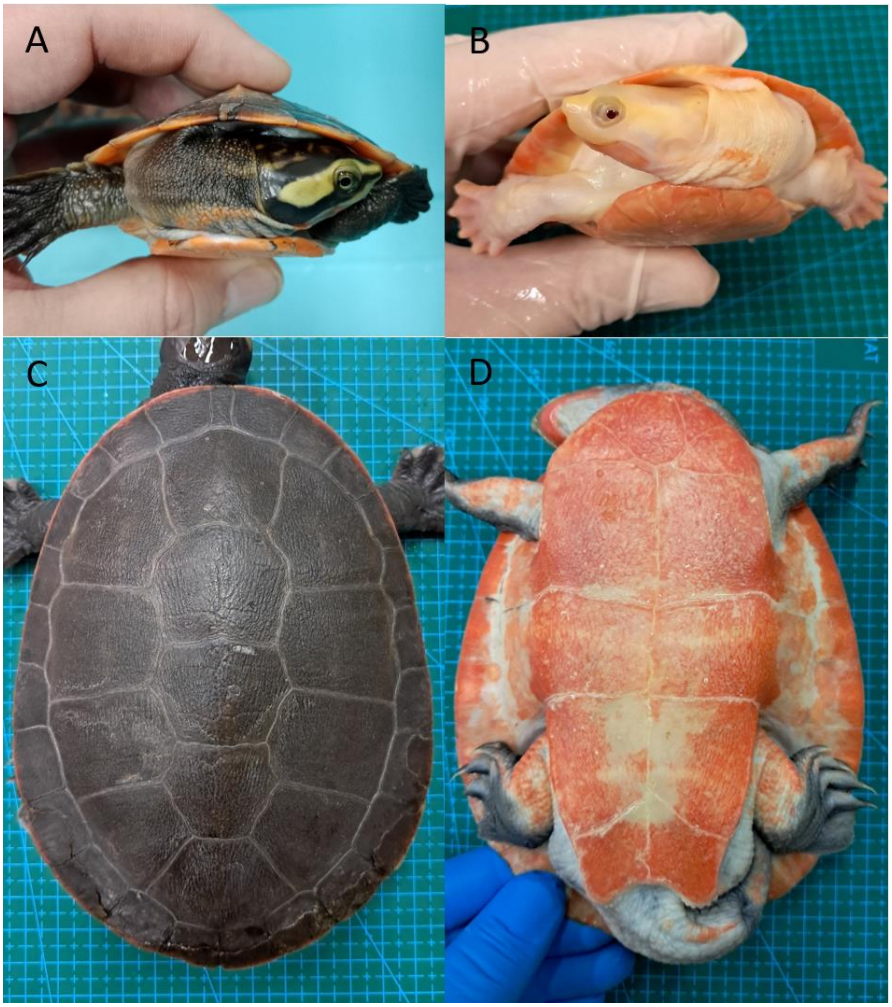

Figure 1. A) wild-type specimen of *Emydura subglobosa*; B) albinotic specimen; both individuals display features characteristic of Pleurodira: head retraction on the side and postorbital stripes behind the eye; C) carapace colouration; D) plastron colouration.

The carapace pattern (Figure 2A) consists of one cervical scute, 24 marginal scutes (12 on each side), eight costal scutes (four on each side) and five vertebral scutes. The plastron (Figure 2B) consists of one intergular scute and two (one on each side, symmetrically) gular, humeral, pectoral,

abdominal, femoral and anal scutes. The plastron and carapace are joined by a bridge in the area of the pectoral and abdominal scutes connected to the abdominal part of the marginals.

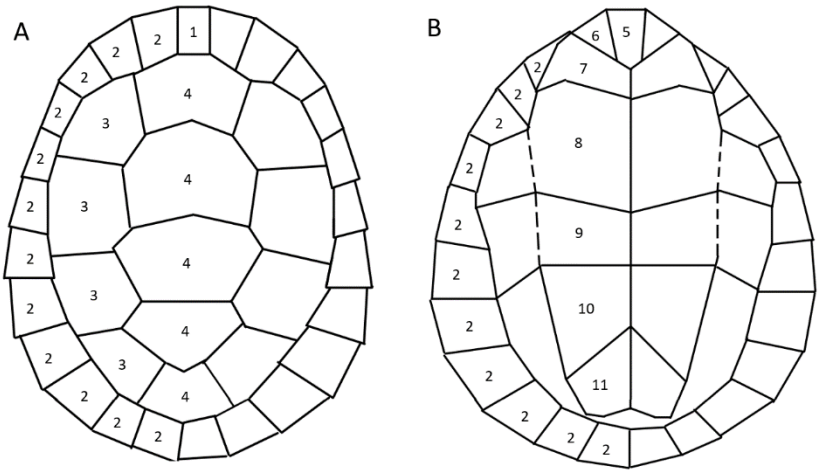

Figure 2. A) carapace scutes: 1 – cervical, 2 – marginal, 3 – costal, 4 – vertebral; B) plastron scutes: 2 – marginal, 5 – intergular, 6 – gular, 7 – humeral, 8 – pectoral, 9 – abdominal, 10 – femoral, 11 – anal.

Sexual dimorphism in *E. subglobosa* develops in the second year of life and is manifested mainly by body size – males are smaller than females, reaching up to 18.5 cm straight carapace length (Freeman et al. 2016). Males have a long tail with the cloaca located out of the range of marginal carapace scutes, while in females the tail is short, with the cloaca located near its base (Figure 3). The species is characterized by fast juvenile development, high fecundity, and easy egg incubation. Moreover, due to frequent captive breeding and a lack of trade restrictions, animals can legally be obtained for breeding stock, without using animals caught in the wild. A number of studies have already used *E. subglobosa*, mainly as a developmental and genetic model (Werneburg et al. 2009, Werneburg et al 2013, Paluh et al. 2013, Mazzoleni et al 2020, Yu 2022).

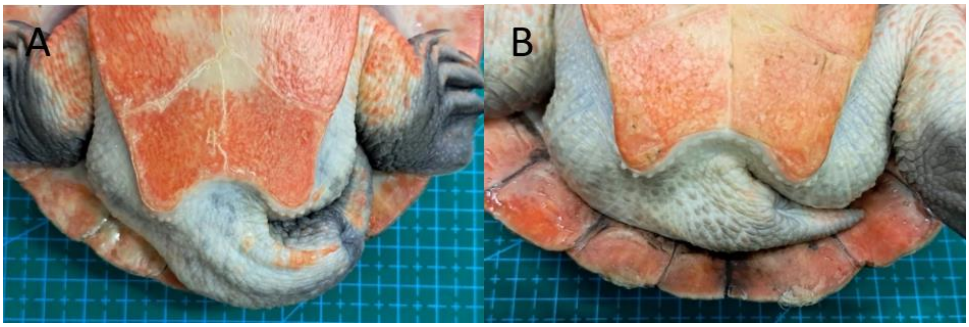

Figure 3. Sexual dimorphism in *Emydura subglobosa*: A) male; B) female.

MATERIAL AND METHODS

Legal status

Emydura subglobosa is listed by the International Union for Conservation of Nature as a least-concern species (IUCN, 2000). It is not listed in the appendices of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), so international trade of the species is not restricted among the Convention parties. In the European Union, the species is not listed in the appendices of Council Regulation (EC) No 338/97 of 9 December 1996 on the protection of species of wild fauna and flora by regulating trade therein. It is not recognized as an invasive alien species in the European Union.

Animals used to establish the Freshwater Turtle Laboratory Line (FTLL)

Two adult specimens (F0), female (ES01) and male (ES02), were received by the Laboratory of Inland Fisheries and Aquaculture, Department of Zoology, Poznań University of Life Sciences (unit allowed for experimental animal use, no. 0091) in November 2016. They had been kept long-term at the University of Zielona Góra for educational purposes, where they produced no offspring (Zawada, personal communication). The first clutch of three non-fertile eggs was obtained in May 2017, followed by the second clutch of four eggs in September, resulting in the first hatchlings after 52 days of incubation. From among the offspring from the 2019 season, three F1 females (ES03, ES04, and ES05) were selected for further breeding. To avoid inbreeding, in August 2020 a subadult male was obtained from the Wrocław Zoological Garden (ES06). In total, over 250 F1 turtles were hatched from 2017 to 2024. The first F2 clutches were obtained in 2023; juvenile specimens (ES07–ES11) were selected from five of them for further breeding. In March 2023, two juvenile albinotic specimens, ES012 (male) and ES013 (female), were obtained for future mating with F2 specimens. The Freshwater Turtle Laboratory Line currently consists of three generations, providing an opportunity for further development.

RESULTS

Husbandry protocol

Due to the lack of published protocols for maintaining a laboratory colony of freshwater turtles, a husbandry regime methodology was developed. For every new specimen introduced to the breeding group or maintained for experimental purposes, quarantine protocols were applied. First, a clinical examination was performed. The animal was not added to the existing colony for at least 90 days.

During this time it was isolated in a separate quarantine room or, if that was not possible, in a separate tank with filtration and equipment (water siphons, nets and buckets) used only for this animal, to avoid pathogen transfer. Faecal examination for parasites was performed once a month during the quarantine, and if infection was detected, treatment was applied and quarantine was extended until negative test results were obtained. For practical purposes, we classified the animals into the following groups: hatchlings – turtles younger than one month; juveniles – older than one month before reaching 10 cm of carapace length or showing signs of sexual dimorphism; subadults – larger than 10 cm; sexed specimens and adults – animals larger than 15 cm. *E. subglobosa* may be kept in groups; however, size groups should be formed in all cases. Due to their highly aquatic behaviour, the turtles were kept in aqua-terrariums with water occupying most of the surface area. Land area (covering an area equal to 25–33% of the water surface area) was also necessary, to enable natural basking and thermoregulation. The basking spots were equipped with a heat source – usually halogen lamps (35–75 W). While we recommend the use of a UVB source, *E. subglobosa* may develop well without one. Among UVB sources, 50 W metal halide flood-type lamps were the most positively assessed during our work, resulting in total UV emission in the range of 1.0 to 2.5 mW/cm^2. The water temperature was maintained at 24–26°C; where needed, thermostatic aquarium heaters were used. In the case of specimens with body weight over 250 grams, glass heaters were equipped with protective covers or replaced with plastic or metal ones to avoid the risk of breaking. In the land area, the temperature gradient was in the range of 25–31°C. Water in the hatchling tanks was changed daily in the first month, while internal filtration was used in the juvenile phase, and external canister filters were necessary for subadults and adults. In the case of adults, the number of animals was adjusted to the available space and behaviour of specimens. When aggression or dominance was observed, individuals were transferred between tanks. Pairs or harem groups were determined to be most effective in terms of breeding efficiency and welfare. Minimum tank size (water surface and depth) was adjusted to meet the requirements of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The formal requirements with practical proposals (minimal water area dimensions and filtration flow) are presented in Table 1. We concluded that glass aqua-terrariums, which are most commonly used for turtle maintenance and breeding, were the most useful for rearing hatchlings. An example of a custom-designed glass aqua-terrarium used for *E. subglobosa* is presented in Figure 4. For subadults and adults, plastic tanks were mainly used (Figure 5), due to the high durability and light weight of this material.

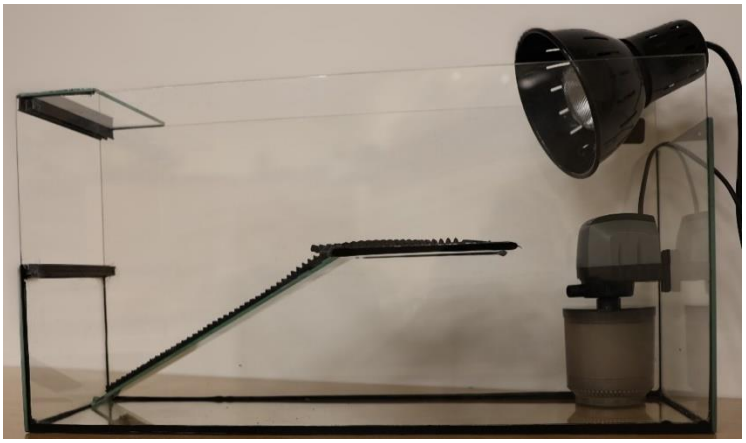

Figure 4. Glass aqua-terrarium designed for *Emydura subglobosa* hatchling maintenance (Terra-Robson, Poland), equipped with a power-head filter (Aqua Szut, Poland) and a UVB metal halide flood-type lamp (Solar Raptor, Germany).

Figure 5. Plastic aqua-terrariums with built-in nesting areas; A, D – commercially available model, made of recycled polyethylene (WaterlandTubs, USA); B, C – custom-made model, made of welded polyvinyl chloride (Dmytro Kudlaienko, Ukraine).

Table 1.
Minimum requirements for *Emydura subglobosa* husbandry according to EU Directive 2010/63/EU and practical experience-based proposals

Carapace length (cm)	Water surface (cm^2)	Water surface/ additional animal (cm^2)	Minimum water depth (cm)	Minimum tank dimensions (cm)	Minimum filtration flow (dm^3/h)
up to 5	600	100	10	30 x 20	-
5-10	1600	300	15	50 x 35	100
10-15	3500	600	20	80 x 45	500
15-20	6000	1200	30	100 x 60	1000
20-30	10,000	2000	35	140 x 75	2000
more than 30	20,000	5000	40	200 x 100	5000

Nutrition

General nutrition rules may be applied according to Rawski et al. (2018a). *E. subglobosa* is mainly a carnivorous species which accepts a wide range of live, raw and processed feeds. Hatchlings in their first days of life may not accept food due to resorption of the yolk sack. During the first month post-hatch, most specimens accepted mainly live food, i.e. bloodworms or *Artemia salina*. Extruded, pelleted and dried feeds were well accepted and utilized in the case of juveniles, subadults and adults. Examples of commercial feeds used for the maintenance of the turtles are given in Table 2.

Table 2.
Examples of commercial feeds used for *Emydura subglobosa* maintenance – ordered according to decreasing share of crude protein

	Feed brand							
Nutrient (%)	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Crude protein	52.5	43.0	42.0	40.0	35.0	32.0	31.5	25.0
Crude fat	7.0	10.0	10.0	6.0	5.0	5.0	5.0	5.0
Fibre	6.0	3.0	3.5	3.0	5.0	8.0	3.9	8.0
Ash	18.3	13.0	ND	ND	13.0	ND	5.2	ND
Calcium	5.0	2.5-2.9	2.0	ND	1.0-1.3	2.2	2.1	1.3-1.7
Phosphorus	1.2	1.0	1.0	ND	1.0	1.1	0.8	1.0

A – Serra Raffy Nature; B – Zoomed Aquatic Turtle Food – Hatchling Formula; C – Tropical Biorept Supreme Young; D – Tropical Biorept W; E – Zoomed Aquatic Turtle Food – Growth Formula; F – Tropical Biorept Supreme Adult; G – Sera Raffy Mineral; H – Zoomed Aquatic Turtle Food – Maintenance Formula; ND – no data provided by manufacturer

A Freshwater Turtle Laboratory Line Basal Diet (FTLL-BD) was developed. Its composition is given in Table 3. The FTLL-BD was prepared in two variants: extruded and gelatine-solidified. The extruded variant was produced with a single-screw warm extruder (Metalchem S-60 Gliwice, Poland) at the Experimental Station of Feed Production Technology and Aquaculture in Muchocin (Poznan

University of Life Sciences). The processing conditions were as follows: 90°C cylinder temperature in the zone of increasing pressure, 110°C cylinder temperature in the zone of high pressure, 120°C head temperature, 52 rpm screw speed, and 3 mm nozzle diameter. During postproduction, fish oil was added by vacuum coating (Rollermac BA 15 FR aut. Pomati Group S.R. L, Codogno, Italy). The gelatine-solidified FTLL-BD variant was developed using a modification of the methodology applied by Rawski et al. (2016). For each 100 g of the mixture of feed ingredients, 85 ml of water and 15 g of gelatine were added. Boiling water was used for the gelatine suspension, then cooled down to 40°C and manually mixed with the ingredients until a homogenous structure was obtained. The mixed feed was placed on Petri dishes and left at 4°C to solidify. Alternatively, agar may be used instead of gelatine. The extruded FTLL-BD variant was stored in non-transparent plastic bags until use, while the gelatine-solidified variant was stored at 4°C, or if used for longer than 3 days post-production, frozen at −20°C. The nutritional value of FTLL-BD is given in Table 4.

Table 3.
Composition of Freshwater Turtle Laboratory Line Basal Diet (FTLL-BD) used for *Emydura subglobosa*

Ingredients (g/kg)	
Fish meal[1]	300
Erythrocyte meal[2]	90
Soy protein isolate[3]	51
Wheat gluten[4]	100
Wheat meal	156
Corn starch	200
Fish oil	35
Lecithin	10
Mineral premix[5]	15
Vitamin premix[6]	1
Choline chloride	2
Carboxymethyl cellulose	20
Limestone	20
Vitamin C[7]	0.15

[1]Crude protein 64%, crude fat 12%, crude ash 8.5%; [2]spray dried, crude protein 90%; [3]crude protein 90%, crude ash 5%; [4]crude protein 75%; [5]Polfamix W, BASF Polska Ltd. Kutno, Poland; contains the following (per kg): vitamin A 1,000,000 IU, vitamin D_3 200,000 IU, vitamin E 1.5 g, vitamin K 0.2 g, vitamin B_1 0.05 g, vitamin B_2 0.4 g, vitamin B_{12} 0.001 g, nicotinic acid 2.5 g, D-calcium pantothenate 1.0 g, chloride 7.5 g, folic acid 0.1 g, methionine 150.0 g, lysine 150.0 g, Fe 2.5 g, Mn 6.5 g, Cu 0.8 g, Co 0.04 g, Zn 4.0 g, and J 0.008 g; [6]Vitazol AD_3E, BASF Polska Ltd. Kutno, Poland; contains 50,000 IU vitamin A, 5000 IU vitamin D_3, 30.0 mg vitamin E; [7]Stay C, DSM Nutritional Products Ltd., Mszczonów, Poland

Table 4.
Nutritive value of Freshwater Turtle Laboratory Line Basal Diet (FTLL-BD) used for *Emydura subglobosa*

Nutrient (% of dry matter)	
Crude protein	40.02
Crude fat	7.49
Crude ash	7.44
Crude fibre	1.02
Nitrogen free extract	31.09
Calcium	1.50
Phosphorus	0.89
Amino acids (% of crude protein)	
Aspartic acid	8.34
Glutamic acid	17.00
Arginine	5.14
Threonine	3.59
Alanine	5.81
Proline	3.43
Serine	4.54
Histidine	3.75
Glycine	7.46
Valine	3.54
Methionine	2.20
Isoleucine	2.76
Leucine	7.28
Phenylalanine	4.18
Tyrosine	2.24
Lysine	3.78
Cysteine	0.90
Calculated parameters	
Calcium/phosphorus ratio (g/g)	1.69
Gross energy (MJ/kg)	18.03
Gross energy/crude protein (kJ/g)	45.07

Breeding

Mating behaviour and multiple copulation attempts were observed throughout the year. Females laid eggs up to six times a year (four times on average) from February to October, in 25–60-day intervals. The mean clutch size was 11 eggs (min. 3, max. 18). To provide conditions for proper nesting behaviour, each tank with mature females was equipped with a land area filled with a layer of quartz sand with a depth of at least 15 cm. Females laid double blunt-ended eggs with no diameter differentiation at the equator (Figure 4A). Freshly laid eggs were transferred to the incubator during morning controls. Incubation was carried out in plastic containers filled with vermiculite or sand and coconut fibre mixed with water in a 1:1 volume ratio and manually squeezed to remove excessive

water. Containers were placed in a Zoomed ReptiBator® with digital temperature regulation and humidity control. Throughout incubation, the substrate temperature was kept at 28°C and the relative humidity was >75%. In contrast to most other turtles, in which hatchling sex is determined by egg incubation temperature, in *E. subglobosa* sex is determined by chromosomes (Mazzoleni et al 2020), so no separate incubation protocols for males and females were applied. After the first 24 hours of incubation, eggs were examined for the presence of white patches (Figure 4A). From the fifth day of incubation, eggs were candled using a medical diagnostic LED flashlight. On day 7 embryos were visible (Figure 6B), and on day 14 the developing vein system could be seen around the shell surface. Between days 21 and 28, the heartbeat and first embryo movements were observed (Figure 6C). From the 40th day of incubation, embryo size made candling ineffective. Hatchlings emerged from eggs after 49–63 days, with a straight carapace length of 32–37 mm and body weight from 4 to 6 g (Figure 6D). For the first 48 hours, they were placed in separate containers in an incubator for yolk sac resorption. Further care was applied using the methodology described in the husbandry protocol section.

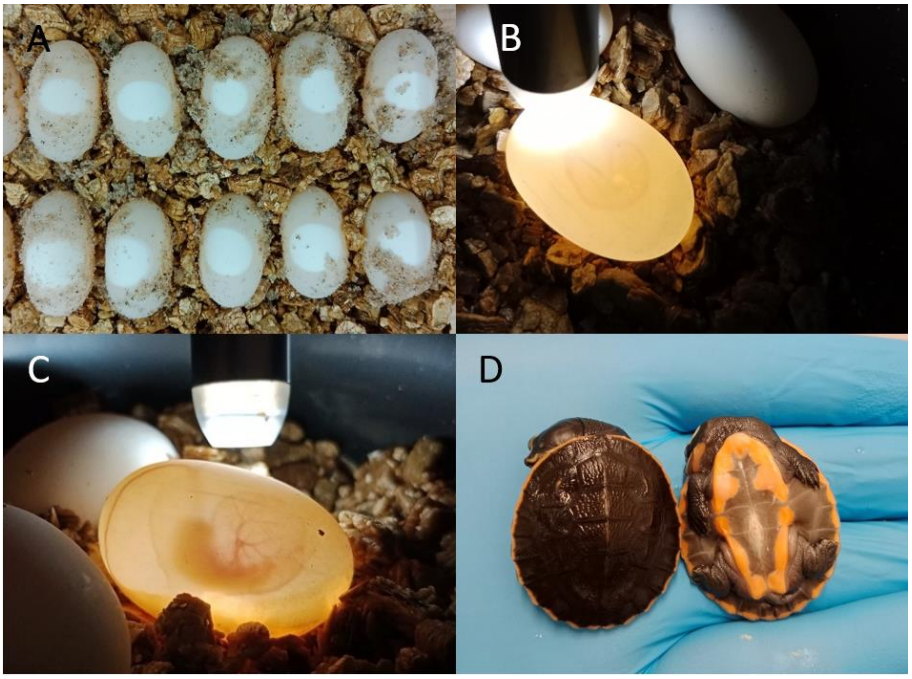

Figure 6. A) *Emydura subglobosa* eggs 24 h after laying, at the stage of white patch formation; B) 7-day-old embryo; C) 21-day-old embryo; D) 24 hour-old hatchlings.

CONCLUSIONS

To the best of the authors' knowledge, this is the first attempt to develop a freshwater turtle breeding line for scientific purposes. The objectives were met by the successful maintenance and breeding of two generations of *E. subglobosa* and the successful establishment of the Freshwater

Turtle Laboratory Line. Husbandry and breeding methodologies, as well as a dedicated basal diet, were also developed. Moreover, the value of the diet was verified in an independent nutritional trial. The extruded variant was used in a recently published study as a control feed resulting in a feed conversion ratio = 1.51 in juvenile *E. subglobosa* (Rawski et al. 2024). Further work is planned with the aim of obtaining subsequent generations and developing the genetic stock of the Freshwater Turtle Laboratory Line. The high availability of *Trachemys scripta* genetic lines, developed for the pet market in the USA and Asia, confirm that in the case of turtles, selective breeding may be an effective tool for obtaining a wide range of colour morphs and forms. In the case of the Chinese softshell turtle (*Pelodiscus sinensis*), multigenerational breeding was used to produce captive populations differing from wild-type animals in morphology, growth performance, and production efficiency (Zhou et al. 2013, Wang et al. 2022, Wang et al. 2024). Thus the selection of a model organism species, establishment of the FTLL, and development of its maintenance and breeding protocols and basal diets are important steps towards broadening possibilities of research on reptilian biology, genetics, physiology, immune development and evolution, as well as the use of turtles for educational purposes.

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