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Individual phenotype, conspecific interactions and access to reproduction in an iteparous cryptic poikilotherm : the European Pond Turtle *Emys orbicularis*

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List of abbreviations

ANT – Animal Network Toolkit

BCI – Body condition index

BM – Body mass

E. orbicularis – *Emys orbicularis*

GoG – Gambit of the group

MS - Microsatellite

PCA – *Petite Camargue Alsacienne*

PrDM – Probability of detecting multiple paternity

SV – Shell volume

Introduction

I. Phenotype and fitness

According to sexual selection theory (Bateman 1948), males' fitness (i.e. individual's genetic contribution to future generations through reproductive success, namely breeding success, offspring number and survival) is limited by the quantity of mates. Accordingly, males do compete for access to the females (Trivers 1972; Rowe et al. 1994). Males can maximize their fitness either by mating with the largest number of females (usually without providing any parental care to their offspring) or by selecting a single female while contributing to parental care (Clutton-Brock 1989). In both cases, fitness can be related to several properties of the phenotype called individual quality (Wilson and Nussey 2010).

Mating and parental care are well known in birds, fish and mammals (Andersson 1994; Birkhead 1995). Yet, this is far less described in turtles, whereas in these species' indirect fitness benefits due to genetic diversity are more important (Andersson 1994; Birkhead 1995). Indeed, clutch size, nesting frequency, quality of nesting sites and sperm storage have been reported to influence female turtles' fitness (Pearse and Avise 2001; Pearse et al. 2001; McGuire et al. 2011). Body size appears to contribute to individual quality as an advantage for competing for receptive females in males (Darwin 1871; Andersson 1994). In females, body size is positively associated with egg number (Zuffi et al. 2006). Body colour pattern can also contribute to individual quality in reptiles, as it is related to individual physiological (Teyssier et al. 2015), nutritional (Brenes-Soto et al. 2017) and immune status (Ibáñez et al. 2014), but also to individual age through melanin deposit over the years (Cao et al. 2019). Finally, body colour pattern may play a role in individual attractiveness and mate choice in turtles since they can identify yellow and red wavelengths (Passos et al. 2014).

II. Reproduction and social network

Beside individual phenotype, sexual selection may also depend on inter-individual, i.e. group or social context. Social networks consist of three components, which are the above-mentioned mating system, but also the social structure and social organization (Kappeler et al., 2013). Social organizations have been demonstrated in several species of freshwater turtles, including the gopher turtle *Gopherus agassizii* (MacCrae et al. 1981), the snapping turtles *Chelydra serpentina* (Galbraith et al. 1987, 1993; MacCrae et al. 1981) and the European Pond Turtle *Emys orbicularis* (Masin et al. 2020). The dominance hierarchies influence the access to mates in the wood

turtle *Clemmys insculpta* (MacCrae et al. 1981), high-ranked males having a better reproductive success than low-ranked ones (Galbraith 1991; Kaufmann 1992). Individual body size, sexual maturity and aggressiveness can determine social hierarchies, permitting males to access to prolonged copulations with females (Kaufmann 1992). However, post-copulatory sperm competition and/or selection also affects males' reproductive success (Pizzari and Birkhead 2002), so the number of copulation events of one male does not always correlate with the number of offspring sired by this male. Moreover, male fertilization success is skewed in the first or last male to mate with the female (Birkhead and Møller 1998). Frequent interactions with the same mates commonly generate dominance hierarchies where the status of individuals is associated with significant differences in access to resources, fertility, and rearing success (Clutton-Brock et al. 1982a, 1984; Walters and Seyfarth 1986; Holekamp and Swale 2000).

Social networks are commonly assessed based on the gambit of the group (GoG) hypothesis for collecting and analysing data (Whitehead and Dufault 1999; Sosa et al. 2018). This hypothesis is part of graph theory, used to study animal association models (Franks et al. 2009). It assumes that when animals are grouped together spatially or temporally, they interact with each other (Whitehead and Dufault 1999). Membership of the same group is used to define the association. This makes it possible to calculate measures of association and to analyse social structure. From interactions and associations, the relationships between individuals can be deduced.

III. Mating system investigated with genetic analyses

In all species including turtles where females have a limited number of gametes (i.e. eggs), individual fitness is expected to be less impacted by multiple mating in females than in males. Yet multiple mating can improve females' fitness, as it is favourable for females to mate with several viable males, rather than with one single not fully fertile male (Olsson et al. 1996; Sheldon 1994). In addition, in turtles where sperm storage occurs, multiple mating enables females to fill their sperm reservoir with sperm from competing sires (Parker 1970; Birkhead and Møller 1998; Simmons 2001), to select sperm amongst mates (Olsson et al. 1996), to reduce the frequency of still-born offspring (Madsen et al. 1992; Olsson et al. 1994, 1996) and thus, to increase their reproductive success (Keller and Reeve 1995; Madsen et al. 1992; Jennions and Petrie 2000). From a genetic point of view, multiple mating and polyandry prevents loss of allelic diversity, maintain heterozygosity levels and genetic diversity in a population and ultimately increase population size (Zane et al. 1999).

According to Mendelian laws, every offspring inherits genetic material from their parents. In diploids, each offspring inherits one full copy of genetic material from its mother and one from its father. Thus, it is genetically possible to determine parent-offspring relationships (Thompson 1975; Thompson and Meagher 1987). This analysis requires several co-dominantly inherited, neutral and polymorphic markers, i.e. markers containing two or more alleles per locus (Flanagan and Jones 2018), such as microsatellites. Microsatellites are non-coding genomic sequences consisting of tandem repeats and exhibiting high mutation rates (Jehle and Arntzen 2002).

Several investigations in freshwater turtles have reported multiple paternity cases (Galbraith et al. 1993; Galbraith 1991; McTaggart 2000; Valenzuela 2000), usually in a high percentage of broods. These studies show that multiple paternity is common, and that females can mate with several males and produce clutches fertilized by more than one male. Yet, none of these genetic-based studies addressed the potential link with individual phenotype or social organisation of the study population.

IV. Hypotheses and framework

In order to tackle this lack of knowledge, we studied the European Pond Turtle *Emys orbicularis*, which shows sexual dimorphism (Kaviani et al. 2015, Rovero et al. 1999) and multiple paternity (Roques et al. 2006, Dux et al. 2017). This study thus proposes to test the following hypotheses:

1) *The multiple paternity reported in Emys orbicularis suggests that females may be more central than males in the population, allowing females to access many males; in other words, multiple paternity may be driven by female's centrality.*
2) *If Hyp1 holds, considering holding a central position in the group may induce extra costs, central females may be larger and multiple paternity improve their fitness; in other words, females being larger and/or using multiple paternity may produce more and/or bigger eggs.*
3) *Opposite, when single paternity occurs, mate choice may be involved, females selecting one single male based on his phenotype; in other words, male's fitness may be driven by male phenotype.*

To address these hypotheses, direct observations of a captive population of *E. orbicularis* have been analysed to date and count copulation and oviposition events to be related to above-mentioned social network. Clutches have been collected (2012-2020) to assess paternity through genetics analyses, run in collaboration with Dr Katrin Theissinger, University of Landau, Germany. Finally, body images have been

processed in 2020 to assess the colour patterns to be related to the above-mentioned social organisation and reproductive outputs.

This project is part of a reintroduction program of the European Pond Turtle in Alsace. In 2021, the captive breeding facility will be closed after the nesting season and individual released in the wild where they will be monitored by CNRS-IPHC. Knowing the social organisation of the captive breeding stock prior release will permit (a) in the short term, identifying individuals to be released together to increase the probability of effective reproduction in the wild and (b) in a longer term, assessing the potential changes in the social structure of the population once in a wild natural area.

Materials and Methods

I. Study site

This study was held at the conservatory husbandry of European pond turtles hosted by the research station of the Petite Camargue Alsacienne (PCA) national nature reserve (Saint-Louis, France; Figure 1). Since 2005, this captive facility hosts 23 adults European pond turtles dedicated to produce young individuals for a program of reintroduction of the species in Lauterbourg led by the local council Conseil Départemental du Bas Rhin. At PCA, turtles live freely in an artificial open-air enclosure (30x40 m^2) including a pond connected to the table water. Since 2012, the zootechnical monitoring of this captive population is ensured by CNRS-IPHC (Strasbourg) and the zoological and botanical park of Mulhouse. Since 2018, the University of Koblenz Landau (Germany) oversees genetic monitoring.

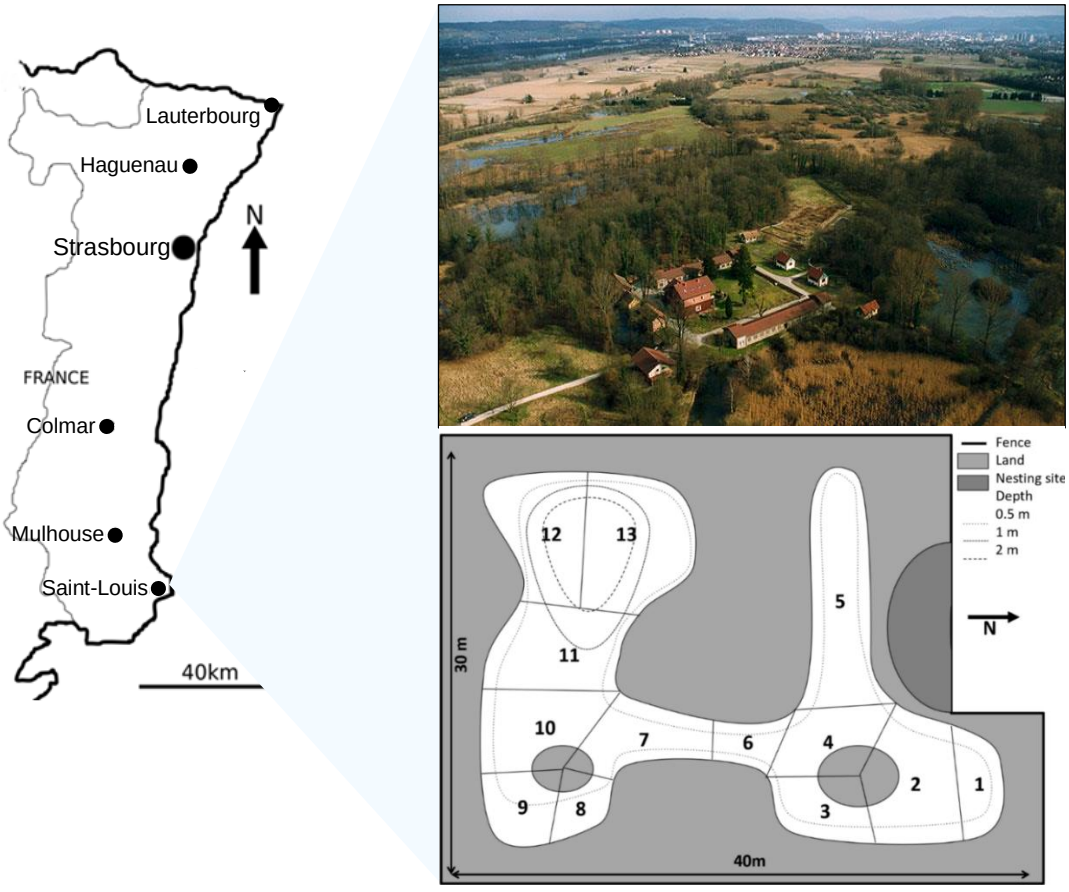

Figure 1. Localisation of PCA in Saint-Louis (Alsace, NE of France), PCA skyview and sectorized captive facility of the conservatory husbandry of European Pond Turtles used in the present study

II. Study species

The European Pond Turtle *Emys orbicularis* (Linnaeus 1758) is a native freshwater turtle that inhabits ponds and swamps in central and southern Europe, the Near East and North Africa (Bensettiti and Gaudillat 2004). Adults (Figure 2.a) can reach 20 cm body length for up to 1 kg body mass with an estimated lifespan of 80 years. Its carapace is black to brown with yellow streaks, the plastron can be from yellow light to black, and the scales on the head, neck, limbs and tail are adorned with bright yellow to dark dots. The legs are webbed and have claws, the tail is long and slender. There is a sexual dimorphism in this species, females being larger with a rounder carapace and a flatter plastron compared to males. Sexual maturity is reached after 8-10 years, earlier in males than in females (Bury et al. 2012).

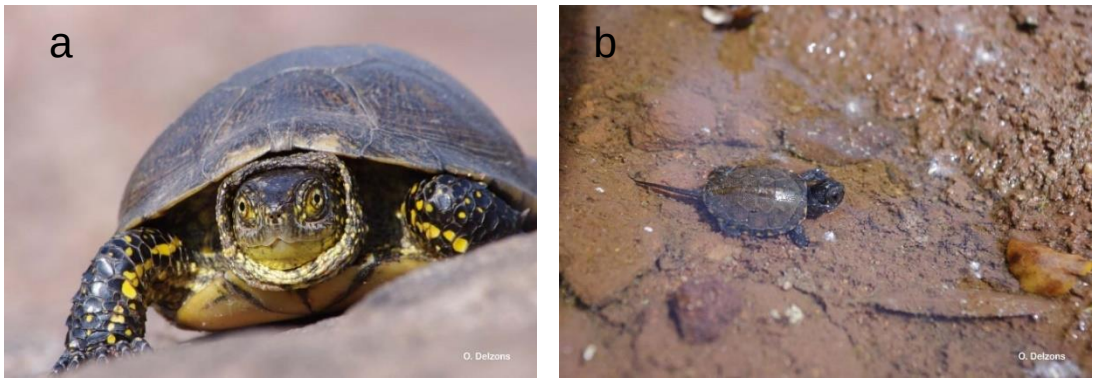

Figure 2. The European Pond Turtle (Delzons O. MNHN)

Reproduction is annual and highly dependent on weather conditions (Figure 3). From October to March, European Pond Turtle overwinter in the mud or in a burrow dug in a bank. At spring turtles come back to activity. Breeding season consists in two major periods: by March mating takes place, and from May to July females deposit their eggs in a nest they dig on emerged places slightly elevated compared to water level (Bensettiti and Gaudillat 2004). Places with soft, warm and south exposed ground are commonly used by females for oviposition. Oviposition consists in females digging a 20 cm deep dip where they lay 3 to 14 eggs they protect afterward with excavated soil. The species is highly dependent on temperature conditions that affect the number of nests laid per female per year (from one to three nests per year, Joos et al. 2017), the timing of hatchling (in fall or in subsequent spring), and the sex of the offspring, temperatures warmer than 29°C producing statistically more females than males (Bensettiti and Gaudillat 2004).

The European Pond Turtle is mostly diurnal, feeding underwater on insects, molluscs, crustaceans and their larvae, but also on dead fish and amphibians, and on

their eggs, and spending most of its time sun basking during daytime. In the wild, *E. orbicularis* can move 40 to 80 m per day and may migrate if disturbed. The species has not been reported as territorial nor as showing any social organisation, but males often compete during the breeding season (Bensettiti et Gaudillat 2004).

E. orbicularis is classified as Near Threatened in the Red List of the International Union for Conservation of Nature, and this species is protected in Europe since it is *mentioned* in the Annex II of the Convention on Wildlife and the Natural Environment of Europe (Bern, 1979). It is also mentioned in appendices II and IV of the European directive Habitats-Faune-Flore since 1992.

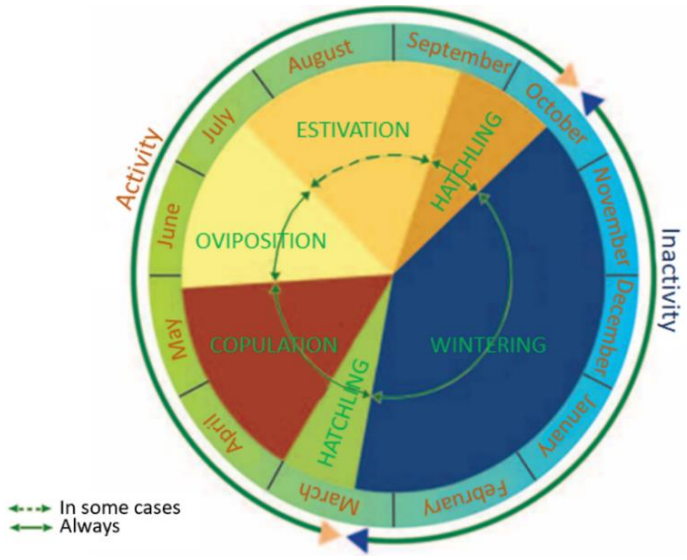

Figure 3. European Pond Turtle life cycle (adapted from Priol 2009)

III. Field protocols

This study is based on 3 different approaches merged to test the above-mentioned hypotheses.

a. Adult marking and biometrics

The breeding group of the PCA consists in 23 (15 females and eight males) adult individuals of unknown age formerly captured in 2004 in a wild population of Brenne, France. Each animal is identified permanently (subcutaneous chip) and temporarily (white paint on the back) for easing ID reading during field observations.

Every year since 2012, at beginning (early April) and end (mid-July) of the breeding season, all animals were captured for individual paint remarking and biometric monitoring. Paint marking is temporary but lasts over one single season. Paint marks consisted in individual alphanumerical codes set and dried on the shell.

During paint drying, turtles were measured using an electronic calliper (±0.01mm, Twincal) and weighed with an electronic spring scale (±0.1g, Kern). Body measurements were: straight length of the shell (midline between distal edges of the nuchal and supracaudal scutes) and of the plastron, width of the shell (highest value), width of the plastron before and after the bridge suturing the shell and the plastron, body height (highest value) and plastron-to-cloacae distance.

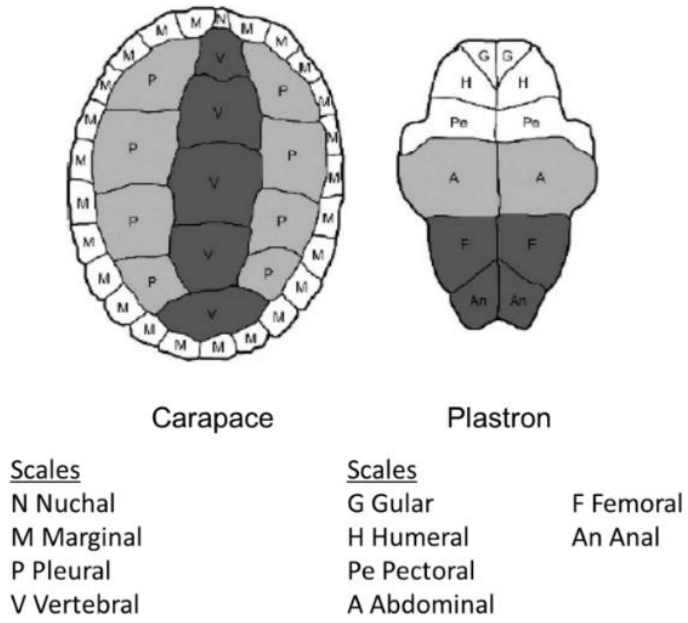

Figure 4. Shell and plastron of E. orbicularis (adapted from Hernandez-Divers et al. 2009)

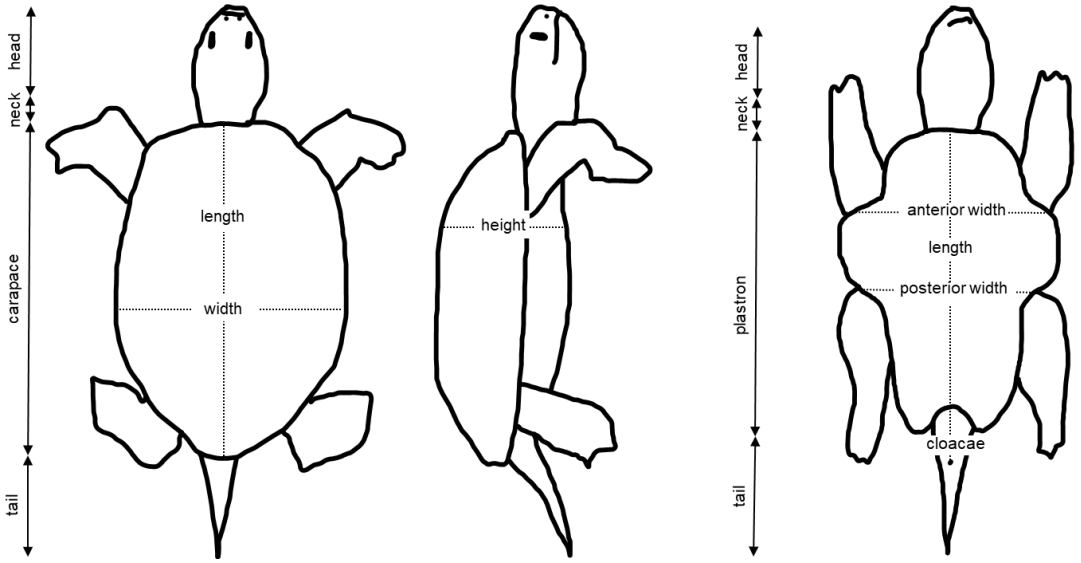

Figure 5. Biometrics of E. orbicularis

Body condition index BCI is commonly used as a proxy of fitness (Peig and Green 2010; Rohr and Palmer 2013). Due to sexual dimorphism and seasonal changes in body mass, BCI was calculated for each sex using biometry measured after winter following Loehr et al. (2007): *BCI = BM/SV where* BM is body mass (in g) and SV is the shell volume (*SV = Pi * shell length(mm) * shell height(mm) * shell width(mm) / 6000,* in cm^3). ANOVA and post hoc tests were used for comparing sexes and individuals.

b. Egg marking and biometry

Since 2012, from mid-May to mid-July, the potential nesting sites located within the enclosure were monitored daily by a field observer from 6pm to 11pm, in order to collect clutches for conservatory purpose. When a female was observed digging her nest, turtle's ID, location and time were recorded. After egg deposition and nest cavity refilling, the female was captured by hand before it reached water and weighed (hereafter referred as post-oviposition body mass, since 2018). Its nest was then excavated in order to collect eggs. Nest excavation consisted in careful digging with spoons and brushes for preventing eggs to break. Collected eggs were placed in a box with a layer of sand for preventing eggs to roll during the transportation in a technical building next to the enclosure. Eggs were then marked and measured: egg marking consisted in writing with a soft pencil on the eggshell egg's ID (namely female's ID + incremental numbers). Eggs were measured (standard length and width, ±1mm) with an electronic capillary and weighed with an electronic spring scale (±0.1g) before being placed in dedicated plastic boxes containing vermiculite. Boxes were then placed artificial incubators to prevent natural predation on eggs, control incubation temperature and humidity and monitor oogenesis.

Fitness were compared amongst females (clutch and egg sizes) using female ID, SV and year as explanatory variables.

c. Adult body pattern

A customized apparatus was designed to assess individual body colour pattern in all 23 individuals (Figure 6.a). This apparatus consisted in a 40x30x30 cm aquarium full of water with a white background and a graduated ruler in order to make standard underwater pictures. Turtles were individually placed by hand in the apparatus where different body profiles were photographed from the two polar views (head/neck, legs and tail from top/below), and from the two sides (shoulder, leg, tail from left/right). Photo sessions required between 20 and 30 minutes per individual before it was released.

Photo raw colours were first homogenised by correcting the white balance using Adobe Photoshop (v21.0.1.47). Images were then analysed following a customised protocol developed under ImageJ (v1.8.0). Image analyses consisted in (1) 8-bit conversion for converting the image to grayscale, which is necessary to use a threshold, (2) Huang threshold treatment for separating image into two colours, corresponding to black skin or background and yellow dots, (3) Nuclei Watershed Separation (https://imagej.net/Nuclei_Watershed_Separation) for separating touching and noisy objects using dots' size range [0.001-Infinity cm²] (Figure 6.b). Image analyses were run on the epidermis parts only, because of the individual white painted mark on the carapace would have biased results. This permitted to assess (1) the number of individual yellow dots, (2) the mean metrics (surface area, shape, centroid, perimeter) of yellow dots and (3) the proportion of epidermis covered by yellow dots, (hereafter referred as skin *yellowness*) (Table 1).

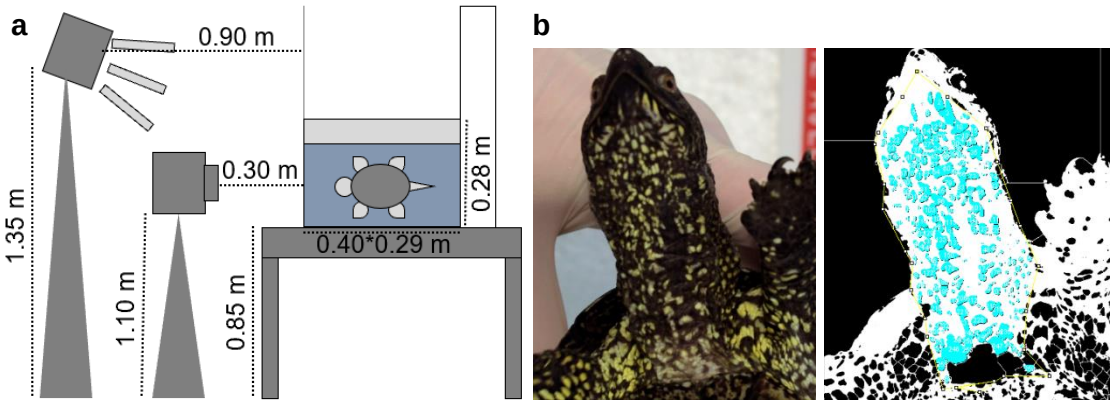

Figure 6. a. Custom apparatus b. Photography before and after analysis on ImageJ

Table 1. Example of raw results after image analysis on ImageJ

Neck								
Slice	Count	Total Area	Average Size	% Area	Perimeter	Circularity		
NECKidBOB	265	4.265	0.016	27.604	0.474	0.784		
	Label	Area	X	Y	Perimeter	Circularity	AR	Round
1	NECKidBOB	0.015	2.499	1.194	0.603	0.533	2.948	0.339
2	NECKidBOB	0.058	2.693	1.287	1.05	0.663	2.266	0.441
3	NECKidBOB	0.091	2.891	1.532	1.627	0.43	1.889	0.529
263	NECKidBOB	0.089	4.185	7.162	1.441	0.537	1.187	0.843
264	NECKidBOB	0.013	4.691	7.137	0.539	0.565	2.52	0.397
265	NECKidBOB	0.061	3.453	7.305	1.082	0.654	1.454	0.688

Yellowness amongst individuals were compared considering ID, sex and profile view as explanatory variables

d. Social network

Social network (individual associations and interactions) was studied using the nearest neighbour approach that consists in identifying pairs of closest individuals in random conditions of time and space. In 2018 and 2019, direct observations were made by focusing successively on each area of the pond (Figure 1), at the rate of three daily sessions of 20 minutes: the date-time, location, behaviour and neighbourhood (nearest neighbour) of each observed turtle were recorded using CyberTracker software (V 3.514, CyberTracker Conservation). At least once a day all individuals were observed. In 2018, 1928 observations were recorded within 20 days, all after the egg laying season (July-August). In 2019, 3349 observations were done in 55 days, including 2254 before the egg laying season (April-June) and 1095 during the egg laying season (June-July). For each day, three classes were defined : « earlyday » from 9:00 to 11:59, « midday » from 12:00 to 15:59, « dayend » from 16:00 to 18:59.

Data were analysed using the software R (R-3.6.2, R Studio Team, 2020) and the package ANT “Animal Network Toolkit software” (Sosa et al., 2018). This package is useful to measure network metrics, perform data randomization and statistical permutation tests. In this part, we expected to generate the centrality (ineigenvector centrality) of each individual (from 0 to 1) to discriminate the central individuals from the peripheral ones, in order to (1) verify if the population is socially structured and (2) test which metrics drives centrality. The different chosen explanatory variables were sex, length, yellowness (in %), season (2019 before oviposition = 2019bo, 2019 during oviposition = 2019o, 2018 after oviposition = 2018ao), and ID was chosen as a random variable. To control for the day, time and location of individual associations, a group by individual matrix (gbi) was created. From the gbi, individuals’ simple ratio index of associations was computed, which allows to correct for the sampling effort for each pair of individuals. This index depends on the frequency of the association of the two targeted individuals, per location and time of day. To control for data dependency of associations, data stream permutations were performed with the function "perm.ds.grp". This approach allows to swap individuals within observations that occurred at the same location and time. The “Eigenvector Centrality” network metrics were computed with the function met.eigen.

To test if the population is socially structured (Whitehead, 1999), centralities’ standard deviations of both real and permuted networks were compared. Random networks should show lower standard deviations than non-random networks, as links are homogeneously distributed in random network.

To test the effect of the explanatory variables on the centrality, the function ”stat.glmm” was used on the following model: **ineigen ~ sex + length + yellowness + season + (1|ID)**. By comparing the regression coefficients of the link between predictive variables and explanatory variables of the model on real data with the regression coefficients of permuted data obtained using data stream permutations, we assessed whether the relationship between predictive variables and explanatory variables are significantly different from randomness. To this end, the function “ant(test)” was used to compute permuted p-values. For each model, the dispersion of the residuals returned by the function “ant” in the object $model.diagnostic was also assessed. A visual representation of the permuted regression coefficients posterior distribution was also available in the object $post.dist returned by the function “ant”.

Results were presented as reader-friendly graphs based on Gephi software (V 0.9.2) that was used to model and visualize social networks. The association indices generated on ANT were used to populate the "weight" column as edges file.

e. Genetic analyses

DNA isolation and microsatellites genotyping were performed as follow: Blood samples collected from adults and offspring were placed on Whatman FTA cards before being extracted according to the protocol described in Johanson et al. (2009). Seven variable microsatellite loci (Table 2, Pedall et al. 2009) were amplified in two multiplex PCR batches using the QIAGEN Type-it Microsatellite PCR Kit (Hilden, Germany). A Primus 96 Cycler (PEQLAB Biotechnologie GmbH, Erlangen, Germany) was used for template amplification. The PCR program was initiated with denaturing at 95°C for 5 min, then 30 cycles with a denaturing at 94°C for 30 sec, followed by annealing at 55°C for 90 sec and elongation at 72°C for 60 sec, and a final elongation at 60°C for 30 min. The fragment analysis was carried out using the CEQ 8000 Sequencer (Beckman Coulter, Krefeld, Germany).

The number of alleles, presence of linkage disequilibrium and observed / expected heterozygosities (H_o/H_e) per locus were computed with GENEPOP 4.2 (Raymond and Rousset 1995) for the breeding group. The presence of null alleles per locus was tested with Micro-Checker (van Oosterhout et al. 2004). A genotyping error rate of 0.05 was calculated by blindly repeating 10% of the samples.

Table 2. Used microsatellite primers from Pedall et al. (2009) for genetic fingerprinting of Emys orbicularis individuals

Loci	Sequence 5'-3'	Repeat motive	Batch	Product size (bp)
msEo25/a	F:GTGACGTGTGTAACCAATGTG R:TAGAGAATGTCTGCCTGTCC	(CA)34	1	238-280
msEo29	F:ACTTCATCGGATGCATGAAG R:ACTTTTGGACTACTGCAGCC	(CT)14	1	318-320
msEo32	F:CGAGTCTTTGGATTACACCG R:GTTGAGGTGACTGTGATTGC	(TG)4 (CA)12	1	162-176
msEo41	F:ATAGCTTCAGCCTTAACTGTG R:AGCCAGAACTATGGGGGTG	(ATCT) 17	1	146-168
msEo21	F:GTAGTAACCCACTTGATGAG R:TTACCTGGCAATTACCTGGC	(GA)11	2	161-175
msEo7	F:AAGTGACCATAGCTGTCAGG R:AGAGCCCTTGATTTAGGCTC	(CAAA) 5 (CA)14	2	258-304
msEo2	F:TTCAAACCAATCCGATGAGG R:GCCTTTCTATGAAATGCTACATG	(CA)15	2	132

Maternal assignment and sibship inference were performed using multi-locus genotypes of candidate mothers and offspring per year. To define the clutches and assign a mother to each clutch, the software COLONY 2.0 was used. It is used to study polyploid species, and assigns each offspring to a mother, based on multi-locus genotype data. The full-pedigree likelihood approach was used, considering the likelihood of the entire structure of the pedigree and jointly infers parentage and sibships. The selected parameters were by default: medium run, full-likelihood analysis, without inbreeding nor clones. Only maternal assignments with greater than 95% probabilities were considered.

To determine paternal assignment, i.e. the minimum number of fathers of each clutch, the software Gerud2.0 was used (Jones, 2005) which analyses the multi-locus genotypes of the offspring and their mothers based on the allele frequencies per clutch. The software suggests possible paternal genotypes explaining the genetic composition of the clutch, which were ranked by likelihood. These theoretical genotypes were compared with the genotypes of each of the eight candidate fathers, calculating an allelic pool match score. For assigning a father we tolerated an allele

mismatch between offspring and father in two out of seven loci, accounting for potential microsatellite mutation events. Since Gerud2.0 does not allow missing data, the number of loci studied was reduced to 5 for certain clutches (Appendix II). All females but A and W were analysed at least once, as the minimum number of eggs required for paternity analyses was three, and it was not available for any year for these females.

The software PrDM (Neff and Pitcher 2002) was used to evaluate the probability of detecting multiple paternity (PrDM). The data used were the number of alleles per locus and their frequency, the number of samples and fathers' contribution. Eleven different scenarios were used to represent situations of equal males' contribution (50:50, 25:25:25:25, 33.3:33.3:33.3, 20:20:20:20) and moderate skewed contribution (75:25, 50:33.5:16.5, 60:30:20, 75:12.5:12.5, 40:20:20:20, 33.5:33.5:16.5:16.5, 30:22.5:22.5:15.10). These contributions were based on recommendations by Neff and Pitcher (2002) and on available paternity data on EPT (Roques et al. 2006, Dux et al. 2017, Refsnider et al. 2009). The average number of offspring per clutch was used for the simulations. The resulting values are the power of the applied genetic markers, with higher values indicating higher probabilities to detect multiple paternity in the defined scenario.

All analyses were conducted in R V.3.6.2. For all tests, alpha = 5%. The package ggplot2 has been used to design the plots, and the package ANTs was used to analyse the social network. All residuals were acceptable (if not mentioned) and are presented in the appendices.

f. Ethics

Animal handling has been approved by the French Ministry for National Education, Higher Education and Research and by the Ethical Committee for Animal Experimentation (CREMEAS, CEEA 35, Strasbourg, APAFIS#649-201505121120811_v1).

Results

I. Biometry

a. Adult body condition

Body mass was positively correlated to body volume (Figure 7), in both females (R^2=0.96; p<2e-16; Appendix I) and males (R^2=0.92; p=0.001). Females were larger than males (females: 657±130; N=15; males: 545±101; N=8; t=2.07; p=0.05), the largest females (Z, O and H) being twice as large and heavy than smaller males (Bob, 2 and K).

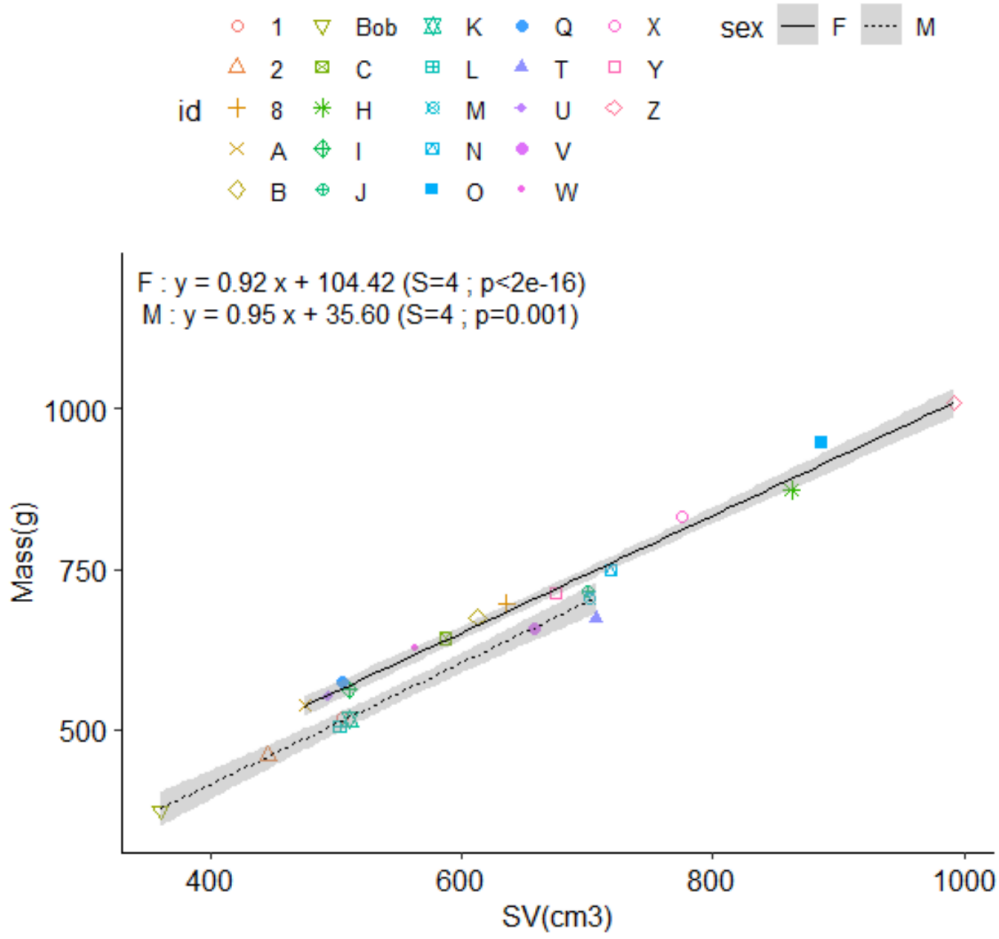

Figure 7. Relationship between body mass and body volume in the 23 adult Emys orbicularis monitored at PCA from 2012 to 2020

Due to sexual dimorphisms, BCI was calculated for each sex separately. Mean BCI was 1.08±0.04 and 1.02±0.02 in females and males, respectively. For both females and males, BCI differed significantly between individuals (females: F=9.90; p=1.6e-13; Figure 8; males: F=3.7; p=0.003; Figure 9), ID accounting for 58% and 35% of BCI variance in females and males, respectively. Females, M, H, Z, J have a

lower BCI than A, B, C and I ($p<0.05$) whereas males T has the lowest BCI opposite to Y ($p=0.001$).

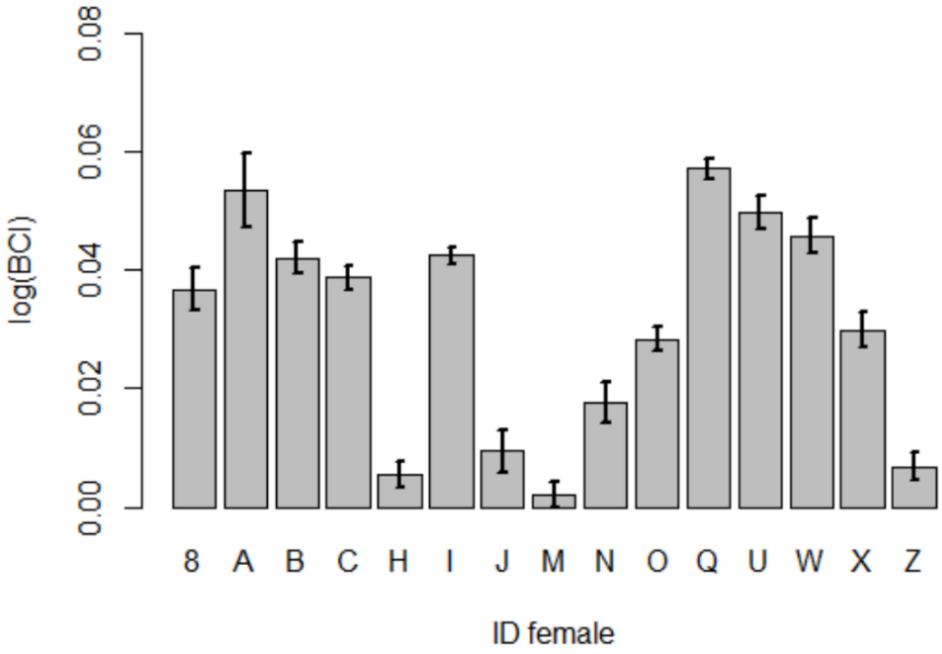

Figure 8. Variation of log(BCI) in females Emys orbicularis monitored at PCA from 2012 to 2020

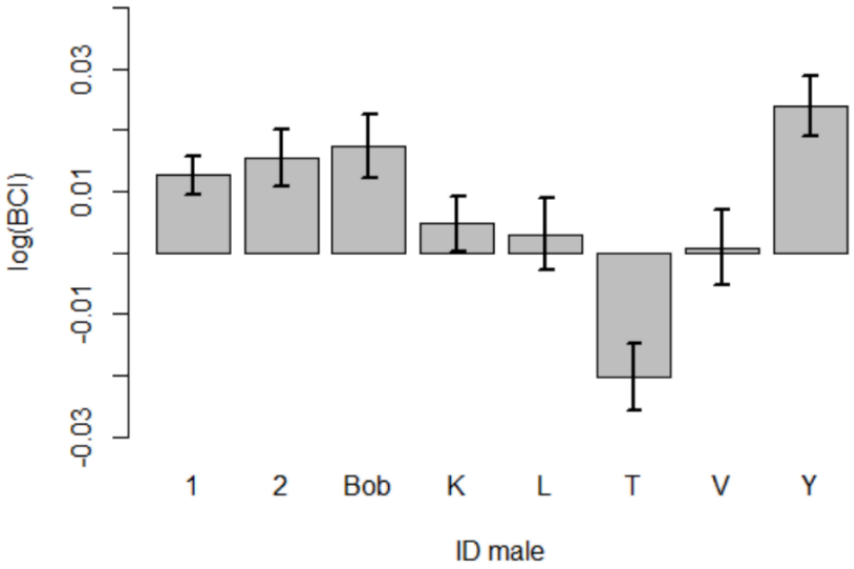

Figure 9. Variation of log(BCI) in males Emys orbicularis monitored at PCA from 2012 to 2020

b. Female reproductive effort

From 2012 to 2020, 112 clutches have been collected, accounting for 1060 eggs in total. On average, females produced 1 clutch of 8 eggs for a total clutch mass of 78±20g. There were significant differences in clutch size amongst females ($F=5.23$;

p=7.25e-07), females O and M laying bigger clutches than other females (p<0.05), and A tinier ones (p<0.05; Figure 10). However, neither SV (F=0.15; p=0.70) nor year (F=0.58; p=0.45) explained the variance of the clutch size.

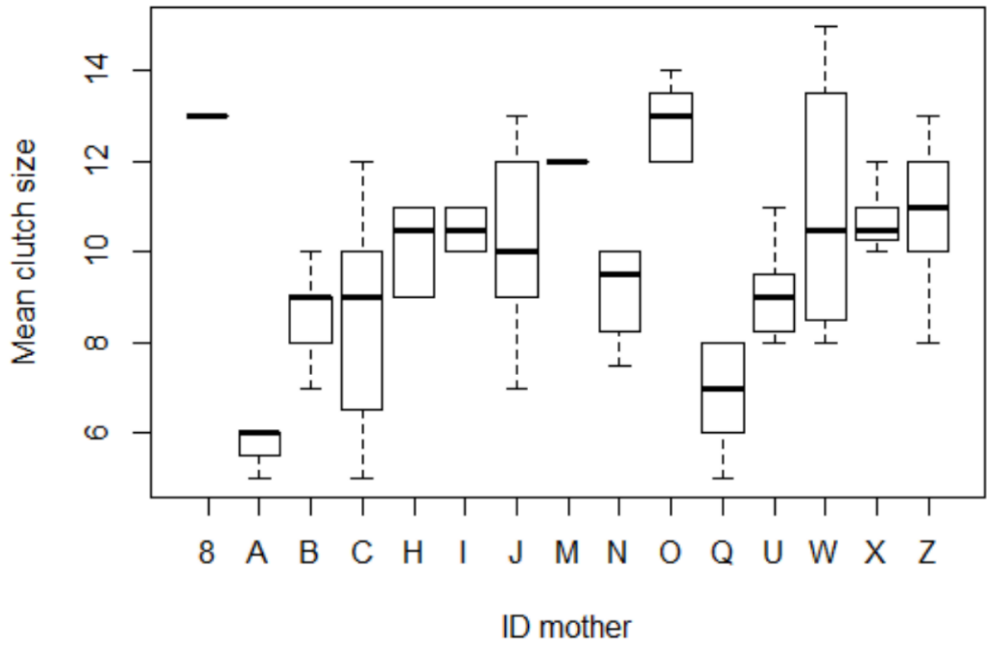

Figure 10. Mean clutch size in European Pond Turtles among nine years (2012-2020)

Similarly, there were significant differences in clutch mass amongst females (F=6.29; p=3.10e-08), female O laying heavier clutches than other females, and A lighter ones (p<0.05; Figure 11). Neither SV (F=0.03; p=0.86) nor year (F=0.90; p=0.35) explained the variance of the clutch mass.

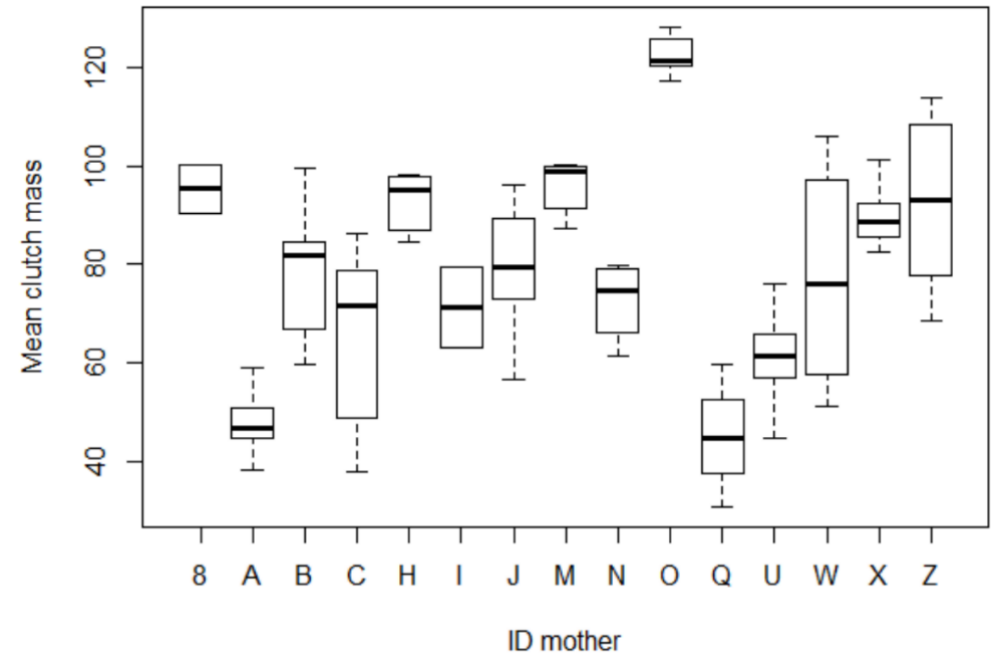

Figure 11. Mean mass of the produced clutches among nine years (2012-2020)

c. Adult body colour pattern: yellowness

There were significant differences in body yellowness amongst individuals (Figure 12), ID explaining 23% of yellowness variance, Bob being more yellow than K (p=0.01) and W (p=0.03). Besides, profile view had major effects on yellowness variance, neck and shoulders being more yellow than the other profiles (p<0.05).

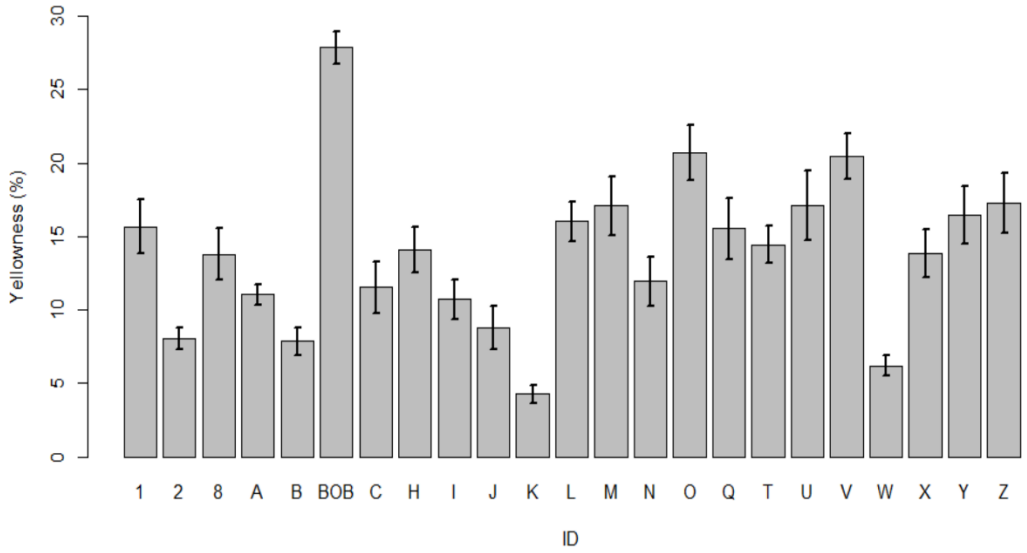

Figure 12. body yellowness in adults Emys orbicularis monitored at PCA from 2012 to 2020

II. Social structure of the population

Individual centralities averaged before oviposition in 2019 0.74±0.11 (from 0.43 (male Bob) to 1 (female 8), during oviposition in 2019 0.66±0.15 (from 0.25 (male K) to 1 (male L) and after oviposition in 2018 0.76±0.14 (from 0.52 (female B) to 1 (female J). The standard deviations of the observed centralities were always higher than the random centralities ones (p=0.002 for “2019 before oviposition”, 0.02 for “2019 during oviposition” andp<1.10-3 for “2018 after oviposition”; Figure 13), indicating that the studied population was socially structured with central and peripheral individuals (Figure 14).

There were no significant effects of shell length (p=0.95), sex (p=0.63), yellowness (p=0.20) or season (p2019bo=0.91; p2018ao=0.29) on centrality.

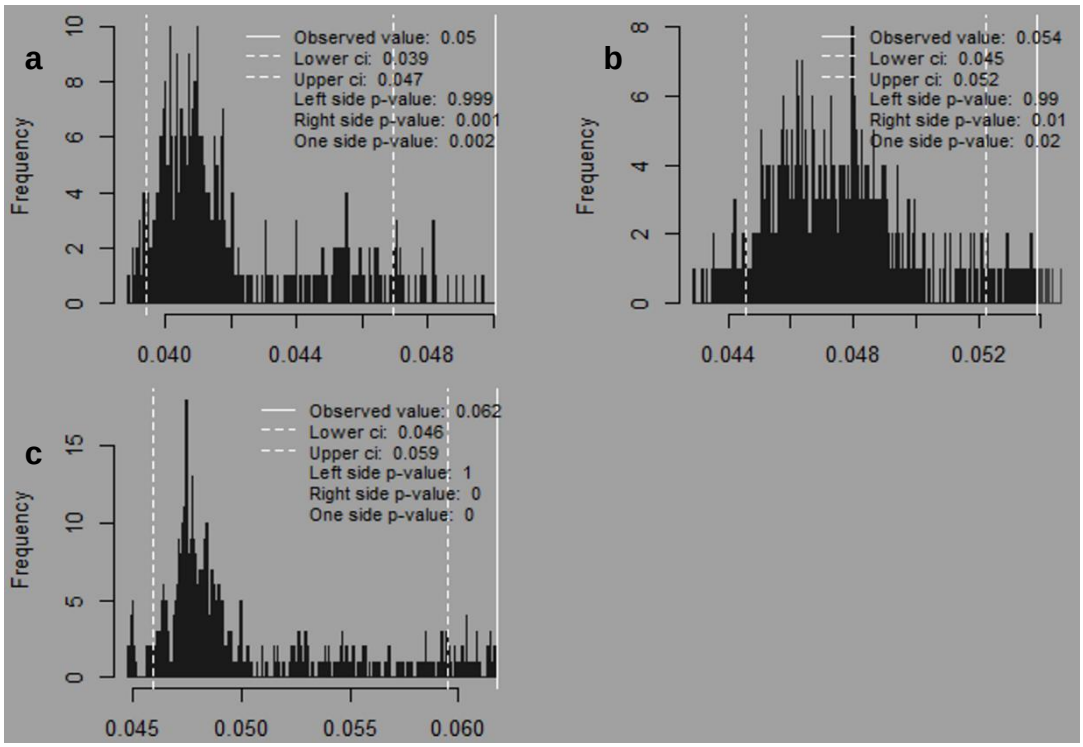

Figure 13. Standard deviations of observed (white line) and random (black lines) centralities before oviposition in 2019 (a), during oviposition in 2019 (b) and after oviposition in 2018 (c) in adults Emys orbicularis monitored at PCA

The social networks (Figure 14) are composed of individuals (nodes), males and females (nodes labels' colour), who have different sizes (nodes' colour) and different centralities (nodes' size). These individuals interact together (each line between two nodes). The strength of each interaction corresponds to one association index. The colour of the line between two individuals becomes darker when the association index increases.

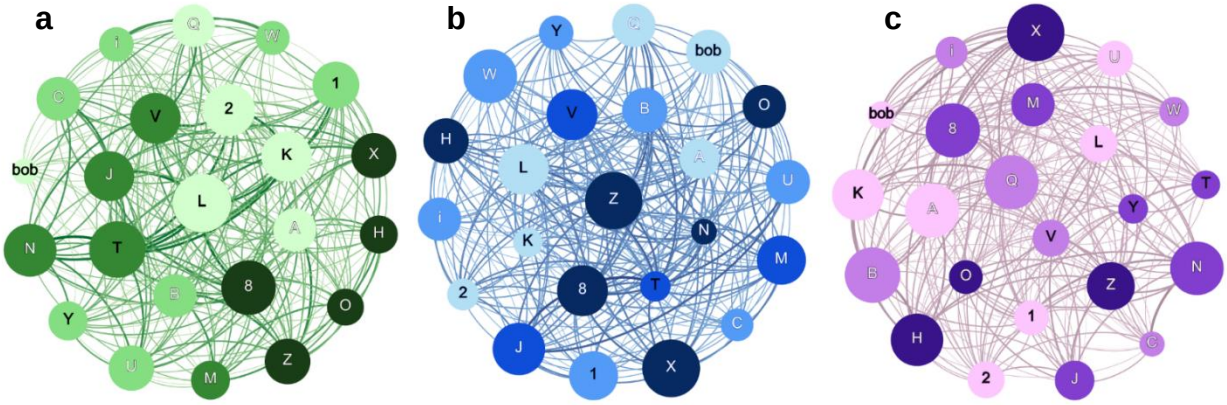

Figure 14. Social networks before oviposition in 2019 (a), during oviposition in 2019 (b) and after oviposition in 2018 (c) in adults Emys orbicularis monitored at PCA

III. Genetic analyses

From 2012 to 2019, genotype of 217 juveniles from 40 clutches belonging to 13 females were analysed (Appendix II).

a. Linkage disequilibrium

No combination of genes linked in non-random proportions among any locus pair was detected (i.e. no linkage disequilibrium), and no evidence of null alleles was found, hence the alleles selected are suitable for studying paternity. The number of alleles per locus (NA) ranged from 4 to 11 (Table 3). Expected (HE) and observed (HO) heterozygosities ranged from 0.48 to 0.86 and from 0.43 to 0.88, respectively (Table 2). As expected, the low difference between HE and observed HO underlines the absence of inbreeding. However, loci msEo2, msEo29, msEo32, and msEo2 show low heterozygosities, which means that their genetic variabilities are low. Because some alleles selected to describe the genotypes show little variability, it is thus difficult to discriminate parents based on their genotypes, specially fathers. Consequently, nine offspring instead of eight were attributed to mother Z in 2016, and in some cases (represented by “male1|male2”), we were not able to discriminate two potential fathers based on their genotypes (Appendix II).

Table 3. Number of alleles, expected and observed heterozygosity per locus

Locus	N_A	H_E	H_O
msEo21	5	0.69	0.61
msEo2	5	0.66	0.57
msEo7	4	0.61	0.43
msEo29	5	0.48	0.48
msEo32	4	0.55	0.52
msEo25/a	6	0.77	0.87
msEo41	11	0.86	0.88

b. Multiple paternity detection

The mother does not make much of a difference in detecting multiple paternity (Appendix I), however the probability increases with the number of offspring (Table 4). The most problematic results are for the clutches with three or four offspring for which multiple paternity was not detected.

Table 4. PrDM outputs (mean PrDM per number of analysed offspring)

Number of fathers	Fathers' contributions	3	4	5	6	8	9	11
2	50:50	42%	66%	79%	88%	93%	96%	97%
	75:25	32%	51%	63%	72%	81%	86%	90%
3	33,3:33,3:33,3	56%	80%	91%	96%	99%	99%	100%
	50:33,5:16,5	51%	75%	86%	93%	97%	98%	99%
	60:30:20	46%	68%	81%	88%	94%	96%	98%
	75:12,5:12,5	34%	53%	64%	76%	83%	87%	91%
4	25:25:25:25	63%	86%	95%	98%	99%	100%	100%
	40:20:20:20	60%	83%	93%	97%	99%	100%	100%
	33,5:33,5:16,5:16,5	60%	83%	93%	97%	99%	100%	100%
5	20:20:20:20:20	67%	88%	96%	99%	100%	100%	100%
	30:22,5:22,5:15:10	65%	87%	95%	98%	100%	100%	100%

c. Multiple paternity

Multiple paternity was detected in 15 of 40 clutches (38%) (Annex II), with two (N=14 clutches) to three (N=1 clutch) fathers being involved in one given clutch. The proportion of clutches with multiple paternity laid by a given female ranged from 0 (no multiple paternity for females 8, C and J) to 100% (for female U) (Figure 15).

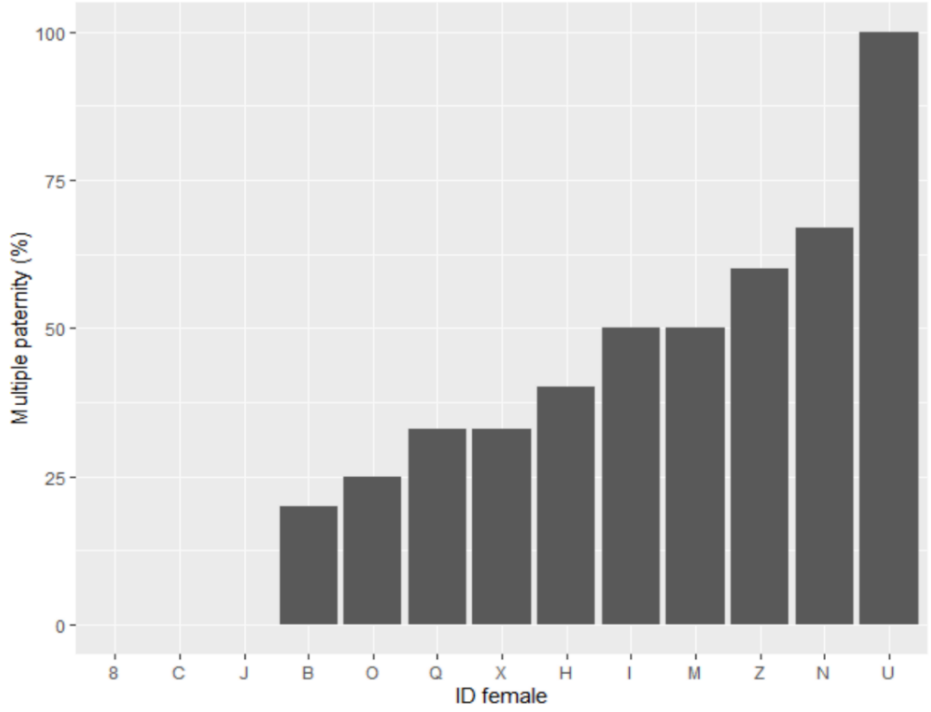

Figure 15. Occurrence of multiple paternity (in %) in clutches in Emys orbicularis monitored at PCA from 2012 to 2020

Polyandry occurred for other females but one (the single clutch analysed from female 8 being associated to male L, Table 5). Each female was fertilised by 1 to 4 different males (on average, 3±1 males). Similarly, polygyny occurred in all males except male T being associated to female N (Table 5). Each male fertilised one to 11 different females (on average, 5±2 females). Each male contributed to the fertilisation of minimum one clutch. Each male fertilised 5%-65% of the analysed clutches (on average, 23±13%). Males who fertilised most of the clutches are 1 (65%), V (33%) and L (28%).

d. Males' phenotype and paternity

To test if male's phenotype (colour, SV) could explain single paternity, two spearman tests (N=8) were realised: (1) Male's colour is correlated to single paternity (Rs=0.81; p=0.01; Figure 16), therefore yellower males are more often involved in single paternity. (2) Male's SV is not correlated to single paternity (Rs=-0.60; p=0.12).

To test if male's phenotype (colour, volume) could explain total contribution in clutches' fertilisation, two spearman tests (N=8) were realised: Neither (1) the male's colour (Rs=0.31; p=0.45), (2) nor the male's SV (Rs=-0.23; p=0.59) is correlated to the percentage of fertilized clutches.

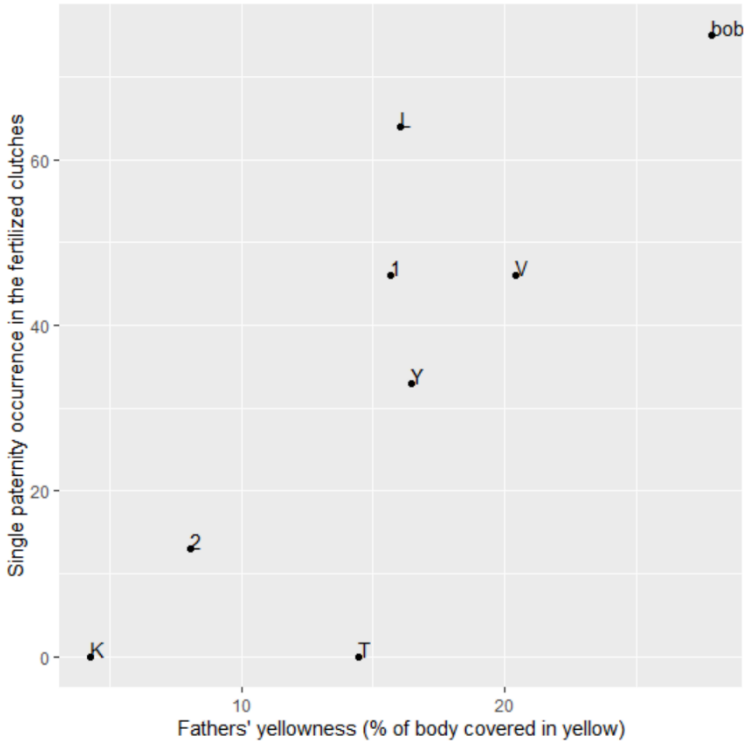

Figure 16. Effect of the father's yellowness on the occurrence of single paternity in the fertilized clutches

Table 5. Clutch (C) and eggs (E) production per pairs, and occurrence of multiple paternity.

	1	2	bob	K	L	T	V	Y	Occurrence of multiple paternity in clutches	Males who fertilized	Main mate(s) (occurrence in analysed clutches)	Analysed clutches/total
8					1C (11E)				0%	13%	L (100%)	3%
B	2C (6E)		2C (9E)	1C (3E)			2C (7E)		20%	50%	1, V, bob (40%)	13%
C	1C (3E)				1C (4E)		1C (3E)		0%	38%	L, 1, V (50%)	5%
H	3C (10E)				4C (18E)		3C (10E)		40%	38%	L (80%)	13%
I					1C (3E)			2C (10E)	50%	25%	Y (100%)	5%
J	1C (7E)				2C (12E)		1C (7E)		0%	38%	L (67%)	8%
M	1C (2E)							2C (8E)	50%	25%	Y (67%)	5%
N	2C (5E)	2C (7E)	2C (8E)			2C (6E)			67%	50%	bob, 1, T, 2 (67%)	8%
O	3C (11E)				2C (5E)		2C (6E)	1C (2E)	25%	50%	1 (75%)	10%
Q	3C (10E)	2C (6E)					1C (4E)		33%	38%	1 (100%)	8%
U	2C (11E)	1C (2E)		1C (1E)			2C (11E)		100%	50%	1, V (100%)	5%
X	3C (11E)	1C (2E)							33%	25%	1 (100%)	8%
Z	5C (40E)	2C (17E)					1C (10E)	1C (6E)	60%	50%	1 (100%)	13%
Unique father of the fertilised clutch	46%	13%	75%	0%	64%	0%	46%	33%				
Fertilized females	85%	38%	15%	15%	46%	8%	62%	31%				
Main mate(s) (occurrence in analysed clutches)	Z, X, U, Q (100%)	N, Q (67%)	N (67%)	U (50%)	8 (100%)	N (67%)	U (100%)	I, M (100%)				
Fertilised clutches/total	65%	20%	10%	5%	28%	5%	33%	15%				

Discussion

This study combined individual (biometry) and populational (genetic and sociality) to assess how individual phenotype and group structure may act on access to reproduction in the European Pond Turtle. We showed that the study population was socially structured with central and peripheral individuals, presented significant cases of multiple paternity (38% of the clutches analysed) whereas cases of single paternity was associated with males showing most pronounced body colour patterns.

I. Mating system and social structure

In order to determine the occurrence of multiple paternity in our population, we studied the genomes of 23 adults and offspring from 40 clutches, and multiple paternity was detected in 38% of the analysed clutches (Appendix II). It has already been observed that *E. orbicularis* females can mate with several males during the same reproductive season (Rovero et al. 1999; Dux 2017), which can result in multiple paternity (Roques et al. 2006). However, the occurrence of multiple paternity in the study of Roques et al. (2006) is lower, as the multiple paternity concerned only 2 clutches out of 20 analysed clutches (i.e. 10%).

In order to test our first hypothesis, which is that multiple paternity occurs due to the centrality of females, the centrality of individuals during three distinct seasons was studied. Thus, we found that the standard deviation of centralities within the population differs significantly from randomness (Figure 13), in line with the literature which observed socially structured populations in captive *E. orbicularis* (Masin et al. 2020). The effect of different metrics (including sex) on centrality was also tested. However, none of the chosen metrics significantly impact the centrality of individuals. It has already been observed that in *E.* orbicularis, social interactions and ranks are independent of both the size (Masin et al. 2020) and the mass (Rovero et al. 1999), in line with our results. Finally, centrality does not depend on the sex of individuals, so we rejected our first hypothesis, i.e. the females are not more central than males, and this does not explain the occurrence of multiple paternity.

II. Phenotype and males' fitness

Across the study of the adults' phenotypes, a sexual dimorphism was observed, the largest females being twice as large and heavy than smaller males (Figure 7). Sexual dimorphism was expected, as it has already been reported that in *E. orbicularis*, females are significantly larger than males (Rovero et al. 1999). Within males, different values of body yellowness were observed, with an individual

significantly more yellow than others (bob) and an individual significantly more melanistic than others (K) (Figure 12). This yellowness was positively correlated with the occurrence of single paternity in the fertilised clutches (Figure 16), i.e. the yellower males are more often involved in single paternity, according to our second hypothesis. The yellower males could be more visually more attractive (Passos et al. 2014) for the females than the melanistic ones, and thus have an exclusive access to some females during the reproductive season, resulting in single paternal clutches.

Additionally, bob is an individual who was sired by two adults from the breeding station, so he is the youngest male. His low melanism (in opposition to his high yellowness) is therefore consistent with the literature reporting body melanisation through life (Cao et al. 2019). In addition, he fertilized four clutches in 2018 and 2019 (13% of the 2018-2019 clutches) and he was the single father of three of them. Age could therefore impact the reproductive success of individuals, as younger males may have more viable sperm than older ones.

In addition, males were differentiated according to their BCI, as T had a significantly lower BCI than other males, and Y had the highest BCI among males (Figure 9). T only fertilized one of 40 clutches analysed (Appendix II), so low BCI of T could indicate that this individual is weaker than other males, which may prevent him from accessing females during the breeding season (reference males fight and access females). However, the size of the males does not significantly impact their exclusive access to clutches, nor their reproductive success in terms of offspring.

III. Phenotype and females' fitness

Differences in body size were observed amongst females, but body volume was correlated neither with clutch size (Figure 10) nor with the average mass of the clutch (Figure 11), so we refuted our third hypothesis. This was unexpected regarding to previous studies, which found that egg number was positively associated with female body size (Zuffi et al. 2006). Perhaps there are too few females (N=15) in the studied population to detect a significant difference in the egg number between large and small females.

Studying body colour, we observed that female W was significantly more melanistic than the other adults (Figure 12). This female produces clutches containing 10 eggs on average, and weighing 75g on average, so she does not differ significantly from other females in terms of clutch size or clutch weight. Finally, in females any correlation was found between body yellowness and fitness.

IV. Error discussion

a. Biometrics

In terms of data collection, some egg laying events may not have been observed (early morning egg laying), in which case not all eggs laid were collected or measured. Besides, to select the mass at the end of winter can bias the calculation of the body condition index, because the animals may not have recovered their weight lost during the wintering period. Finally, the low number of individuals (N=23) may affect the power of some tests, and consequently reduce the significance of some effects (global and not global). For example, BCI calculation show that larger individuals have lowest BCI, in both males and females.

b. Genetic analysis

Four of the seven selected loci show low heterozygosities (H_O<0.60) are not polymorphic enough in the study population, which is not ideal for detecting multiple paternity and for identifying fathers. For example, genotypes 1 and V are 71% identical on the chosen loci, and these two males are often found associated together (impossible to determine which of the two is the father) in the paternity analyses results. Other consequence, there was one error in eggs attribution because mother Z laid 8 eggs in 2016, and the software colony attributed 9 offspring to this mother in 2016. To address these issues, the number of loci could be increased to increase genetic resolution of these closely related individuals, or other loci could be analysed.

In addition, the male bob was sired by two adults from the study population, which reduced heterozygosity in the breeding population. If further paternity analyses are carried out in the future, he should therefore be withdrawn from the population.

c. Social network

Different observers have done 2018 and 2019 proximity observations, which could bias the data collection and consequently, the following analyses. Besides, the closed restricted area could force promiscuity, and this can skew social interactions, especially in terms of access to mates. Such promiscuity may have biased genetics results. No relation has been established between the centrality, ID, colour, sex and the season of observation. An additional metric that could impact centrality is age, but this is not available for the individuals studied. Finally, the number of observations in each season is not enough, so the effort could be increased to increase the power of the tests.

Perspectives

A long-term perspective could be to test the survival of offspring, which could be related to parents' phenotype, to the clutch size, or to the size of the egg. The reproductive strategy of the mother (single paternity, multiple paternity) could also impact the viability of the offspring. Moreover, an additional colorimetric approach can be considered, using a spectrophotometer to study the colour (HSV system) of the yellow spots.

Finally, all our analyses were carried out on individuals bred in captivity, which can skew their social interactions, and therefore the occurrence of multiple paternity within the analysed clutches. As the 23 adults studied will be released to an open environment in Lauterbourg in 2021, it would be interesting to study the structure of the population, the social interactions and the occurrence of multiple paternity after release. Thus, we could test whether the high occurrence (38%) of multiple paternity within the analysed clutches is or is not due to significant promiscuity within the closed pond.

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Appendices

Appendix I. PrDM results per female

		Mothers ID															
		B	C	H			I	J	M	N	O	Q	U	X	Z		8
Number of fathers	**Fathers' contributions**	**4**	**4**	**3**	**5**	**9**	**8**	**6**	**5**	**6**	**4**	**4**	**6**	**4**	**5**	**9**	**11**
2	50:50	66%	63%	42%	81%	96%	93%	88%	80%	88%	65%	68%	87%	67%	77%	95%	97%
	75:25	50%	48%	32%	64%	87%	81%	73%	64%	73%	50%	53%	71%	51%	61%	84%	90%
3	33,3:33,3:33,3	80%	77%	56%	92%	100%	99%	96%	91%	96%	79%	82%	95%	81%	88%	99%	100%
	50:33,5:16,5	75%	72%	51%	88%	99%	97%	93%	87%	93%	74%	77%	92%	75%	84%	98%	99%
	60:30:20	68%	66%	46%	82%	97%	94%	89%	81%	89%	68%	70%	87%	70%	78%	96%	98%
	75:12,5:12,5	52%	50%	34%	66%	88%	83%	74%	65%	74%	52%	55%	79%	53%	62%	86%	91%
4	25:25:25:25	86%	84%	63%	96%	100%	99%	98%	95%	98%	85%	88%	98%	87%	93%	100%	100%
	40:20:20:20	83%	81%	60%	94%	100%	99%	97%	93%	97%	83%	85%	97%	84%	91%	99%	100%
	33,5:33,5:16,5:16,5	83%	81%	60%	94%	100%	99%	97%	93%	97%	83%	85%	97%	84%	91%	100%	100%
5	20:20:20:20:20	89%	87%	67%	97%	100%	100%	99%	97%	99%	84%	91%	99%	90%	95%	100%	100%
	30:22,5:22,5:15:10	87%	85%	65%	96%	100%	100%	98%	96%	99%	87%	89%	98%	88%	94%	100%	100%

Appendix II. Results summary of genetic analyses. The maximum number of eggs produced per male is between brackets.

Mother	No. of analysed clutches (2012-2019)	No. of analysed offspring per clutch (mean ± SE)	Year	Total no. of eggs	Total no. of offspring analysed	No. of loci analysed	Minimum no. Of fathers	Male 1	Male 2	Male 3
B	5	5 ± 3	2012	6	4	7	2	1|K(3)	V(2)	
			2016	9	3	7	1	1(3)		
			2017	8	5	7	1	V(5)		
			2018	9	5	5	1	bob(5)		
			2019	10	4	5	1	bob(4)		
C	2	4 ± 1	2018	11	4	6	1	L(4)		
			2019	9	3	7	1	1|V(3)		
H	5	5 ± 2	2012	9	9	7	2	L(7)	1|V(4)	
			2015	9	3	6	1	L(3)		
			2018 (1st clutch)	12	5	7	1	L(5)		
			2018 (2nd clutch)	10	5	7	2	L|1(3)	1|V(3)	
			2019	11	3	7	1	1|V(3)		
I	2	7 ± 2	2018	10	5	6	2	L(3)	Y(2)	
			2019	11	8	7	1	Y(8)		
J	3	6 ± 1	2012	9	7	7	1	V|1(7)		
			2016	7	5	7	1	L(5)		
			2017	12	7	7	1	L(7)		
M	2	5 ± 1	2013	12	4	7	1	Y(4)		
			2016	14	6	7	2	Y(4)	1(2)	
N	3	6 ± 0	2017	9	6	7	2	T|2(4)	1(2)	
			2018 (1st clutch)	11	5	7	2	1|2(3)	bob|T(2)	
			2019	10	6	7	1	bob(6)		
O	4	4 ± 1	2013	12	5	7	3	L(2)	Y(2)	1|V(2)
			2014	12	3	7	1	L(3)		
			2015	12	5	7	1	1(5)		
			2017	13	4	7	1	1|V(4)		
Q	3	4 ± 0	2016	8	4	7	1	1|2(4)		
			2018 (2nd clutch)	15	4	7	2	1(2)	2(2)	
			2019	8	4	7	1	1|V(4)		
U	2	6 ± 4	2016	10	8	7	2	1|V(7)	K(1)	
			2018 (2nd clutch)	10	3	6	2	1|2|V(2)	1|V(2)	
X	3	4 ± 2	2012	10	4	7	1	1(4)		
			2015	11	6	7	2	1(4)	2(2)	
			2018 (1st clutch)	13	3	7	1	1(3)		
Z	5	8 ± 3	2012	13	5	7	1	1(5)		
			2013	11	6	6	2	2(7)	1(2)	
			2016	8	9	7	2	1|2(7)	1|2(3)	
			2017	13	13	7	1	1(13)		
			2018	12	8	6	2	1|V|Y(6)	1|V(4)	
8	1	11	2017	13	11	7	1	L(11)		
13 mothers	40 clutches	5 ± 2 offspring per clutch	8 years	422	217	5 to 7 loci	38% of multiple paternity	40 clutches	15 clutches	1 clutch

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Titre français : Phénotype individuel, interactions conspécifiques et accès à la reproduction chez un poïkilotherme cryptique : la Cistude d'Europe *Emys orbicularis*

Titre anglais : Individual phenotype, conspecific interactions and access to reproduction in an iteparous cryptic poikilotherm : the European Pond Turtle *Emys orbicularis*

Résumé : La théorie de l'évolution prédit que le succès reproducteur d'un organisme, c'est-à-dire le nombre de descendants, dépend de ses traits phénotypiques et des interactions conspécifiques. Chez les espèces ovipares où les femelles fournissent la plupart des coûts de reproduction, on prévoit que les femelles plus grosses produiront de plus grosses couvées. Aussi, lorsque les femelles s'accouplent avec plus d'un seul mâle pendant la saison de reproduction, les interactions conspécifiques devraient être centrées autour des femelles. Ces deux prédictions ont été testées chez une espèce dimorphique, la Cistude d'Europe *Emys orbicularis*, où des cas de paternité multiple ont été observés. Nous avons étudié la biométrie individuelle et les interactions conspécifiques chez 23 adultes, ainsi que la paternité (simple ou multiple) au sein de pontes collectées de 2012 et 2020 dans l'élevage conservatoire de la Petite Camargue Alsacienne, à Saint-Louis (France). Parmi les 112 couvées collectées, la paternité multiple concernait 38% des couvées, avec 2 (35%) à 3 (3%) pères par couvée. Les mâles présentant davantage de tâches jaunes sur leur corps sont plus souvent impliqués dans la paternité simple. Cependant, la position de la femelle au sein de la population n'a pas permis de prédire la stratégie de reproduction privilégiée. Puisque la reproduction et potentiellement la dynamique de la population dépendent principalement des traits maternels, les femelles doivent faire l'objet d'une attention particulière, en particulier chez les espèces dont la conservation est préoccupante.

Abstract : The theory of evolution predicts that the reproductive success of an organism, i.e. the number of offspring, depends on its phenotypic traits and conspecific interactions. In oviparous species where females provide most of the reproductive costs, larger females are predicted to produce larger clutches. Additionally, when females mate with more than one single male during the breeding season, conspecific interactions are predicted to be centred around females. These two predictions were tested in a female-biased sexual size dimorphic species, the European Pond Turtle *Emys orbicularis*, where multiple paternity has been reported but whose social interactions are unknown. We investigated individual biometry and conspecific interactions in 23 adults and genetic-inferred paternity of their offspring born between 2012 and 2020 in the conservatory facility of Petite Camargue Alsacienne, Saint-Louis, France. For the 112 collected clutches, multiple paternity occurrence frequency was 38%, with 2 (35%) to 3 (3%) fathers per clutch. Fathers' body colour has been correlated with single paternity. However, female position in the social network could not predict individual mating (single versus multiple paternity) strategy. When reproduction and potentially population dynamics mainly depend on maternal traits, females have to be highly considered, particularly in species of conservation concern.

Mots-clés : système d'accouplement; plasticité phénotypique; paternité multiple; microsatellites; réseau social

Key Words: mating system; phenotypic plasticity; multiple paternity; microsatellites; social network