Twins! Microsatellite analysis of two embryos within one egg case in oviparous elasmobranchs

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Abstract

Elasmobranchs display various reproductive modes, which have been key to their evolutionary success. In recent decades there has been a rise in the number of reported cases of foetal abnormalities including fertilised, double-embryos held within one egg capsule, hereafter referred to as twins. Previously, the occurrences of twin egg cases have been reported in two batoid and one shark species. We report the first cases of twins in three species of oviparous elasmobranchs: the undulate ray (Raja undulata), the nursehound (Scyliorhinus stellaris), and the small-spotted catshark (Scyliorhinus canicula). We investigated the genetic relationships between the twins in S. stellaris and S. canicula using microsatellite markers. Whilst the S. stellaris twins displayed the same genotypes, we found that the S. canicula twin individuals arose through heteropaternal superfecundation. This is the first reported incidence of such a paternity in elasmobranchs. The relationship between environmental change and reproductive strategy in elasmobranchs is unclear and further research is needed to determine its effect on the prevalence and mechanisms of formation of elasmobranch twins.

Introduction

Elasmobranchs comprise almost 1,200 species [1,2] of sharks and batoids (guitarfishes, sawfishes, skates and rays) [3] that display complex reproductive modes, characterised by low numbers of offspring that are born, or hatched, as active, fully-formed individuals [4]. All extant elasmobranchs employ internal fertilisation, with unique organ systems that increase the efficiency and likelihood of fertilisation, whilst minimising sperm wastage and the predation of unfertilised eggs [4,5]. Their diversity of reproductive traits is suggested to be a major selective advantage that has contributed to the group’s success [6]. However, these
reproductive traits, coupled with slow growth, long gestation times, and late sexual maturity, have also increased the susceptibility of elasmobranchs to extinction in the current era of over-exploitation and climate change [7].

Elasmobranchs display distinct reproductive modes: oviparity (egg-laying), and viviparity (yolk sac, histotrophic, oophagic, placental viviparity)[4,8]. Approximately 43% of elasmobranchs, predominantly the skates and benthic sharks, are oviparous [9,10]. The female reproductive organs of nearly all oviparous elasmobranchs consists of paired ovaries that each secrete oocytes into individual reproductive tracts (uteri) [11–14]. Each uterus comprises of oviducal (shelling) glands and muscular regions, before joining to form one lower uterus to release the fully formed eggs into the environment via the cloaca [11–14]. Typically, a single embryo is found within each egg case. Twin egg cases are rare, being reported in the oviparous skates Leucoraja erinacea [15], Sympterygia bonapartii [16] and one viviparous (yolk sac) shark species Mustelus asterias [17]. Double vitellogenic oocytes have also been observed in Sympterygia acuta [18]. Twin egg cases are only a common feature in the oviparous batoid species Beringraja binoculata [19], and Beringraja pulchra [20–23]. For this reason, Ishihara et al., (2012) proposed a new genus for these species, “Beringraja” [24].

Here we report two individuals formed in the same egg capsule in the undulate ray (Raja undulata), and fertilized double-embryo egg cases in the nursehound (Scyliorhinus stellaris) and the small-spotted catshark (Scyliorhinus canicula), here after referring to fraternal double-embryos as twins. Uniquely, our study employed microsatellite analysis to understand the reproductive origins of the double-embryos in S. stellaris and S. canicula.

Methods

Sample collection

On the 6th of September 2013 an egg case containing two embryos from R. undulata was laid by a wild-caught mother, within a clutch of unknown size, at the SEA LIFE aquarium Weymouth, UK. The S. stellaris egg cases were laid in captivity by a wild-caught population at the Native Marine Centre (Portland, UK). The source population of S. stellaris individuals had either deposited eggs in captivity after copulation in the wild, or after copulation in the captive environment with other wild-caught individuals. The egg cases from S. canicula were from a captive breeding population held at the Deutsches Meeresmuseum (Stralsund, Germany), made up of a source population of both captive and wild individuals.

The egg cases from both shark species were sent to the University of Manchester, UK, at approximately 4 weeks, and 1 week, post-laying, for S. canicula and S. stellaris, respectively [25,26]. In Manchester, the embryos were held in 55L seawater tanks at 15˚C, dissolved oxygen > 95%, and 35ppt salinity, in a 12 hour light-dark cycle, until hatching. To ensure the nitrogenous waste contents was maintained at safe levels for the developing sharks, nitrate, nitrite and ammonia were routinely monitored, and water changes were carried out three times a week. The S. stellaris and S. canicula egg cases were photographed alongside a ruler, using a Canon PowerShot G16 camera, and the size of the egg cases, embryos, and external yolk sacs were measured using ImageJ [27]. The volumes of the external yolk sacs were calculated using the formula for an ellipsoid.

Ethical approval for work was granted from the Animal Welfare Ethical Review Board of the University of Manchester.

DNA extraction, amplification and analysis

The S. stellaris and S. canicula embryos were fin-clipped post-hatch and the tissues stored in 98% ethanol for DNA extraction. A further 6 captive offspring S. stellaris samples were added
to the dataset to investigate polymorphisms within the species. In *S. canicula*, the potential parents (fathers = 7, mothers = 11), 60 potential siblings, and the twin individuals from the captive breeding program were fin clipped to analyse parentage (in total n = 80). Samples were extracted using the Bioline Isolate II Genomic kit [28] with an extended digestion time of 10 minutes to maximise the genomic DNA yield. Genomic DNA (20-70ng/μl), was amplified with one primer cocktail containing 5mM of the three tail dyes (FAM, VIC and NED), 5mM of each forward microsatellite marker, and 10mM of each reverse microsatellite loci [29]. The 11 microsatellite primers and thermal cycling conditions were selected from Griffiths et al. [20]: Scan02, Scan03, Scan04, Scan05, Scan06, Scan09, Scan10, Scan12, Scan12, Scan15 and Scan16. PCR reactions consisted of 1μl of genomic DNA, 1μl of the primer cocktail, 3μl of ddH2O, and 5μl of QIAGEN Multiplex PCR Kit [30]. The products were genotyped using an ABI sequencer with GeneScan™ 500 LIZ™ dye Size Standard and scored using GeneMapper v.4.0 (Applied Biosystems). Allele scores were checked for user error in Microchecker [31].

GenePop (v 4.2) [32, 33] was used to calculate observed heterozygosity (*H*<sub>O</sub>), expected heterozygosity (*H*<sub>E</sub>) and number of alleles per locus (*N*<sub>A</sub>). Cervus [34] was used to calculate polymorphism information content (PIC) and frequency of null alleles F(Null) [29]. Parentage analysis for all offspring of *S. canicula* was determined using a full-likelihood and pair-likelihood-score combined (FPLS) method in Colony [35] and using a parent-pair log-likelihood ratio (LOD) analysis in Cervus [34]. Colony analysis was conducted under the assumption of female and male polygamy without inbreeding or clones. The simulation program within Cervus was used to produce 10,000 offspring and parental genotypes from allele frequencies taken from the North-Atlantic sampled by Gubili et al. [36] to generate statistically significant LOD scores at a strict confidence level of 95%. Microsatellite markers for both *S. stellaris* and *S. canicula* that displayed PIC values ≥0.500 were displayed for the twins and six individuals to visually highlight similarities and differences in the genotypes.

**Results**

**Undulate ray, *Raja undulata***

The *R. undulata* twin embryo egg case length and width (excluding horns) was 58mm and 35mm respectively. While there was no reported difference at the time, these measurements show the egg case length to be slightly shorter when compared to other egg cases in the same clutch and those typical for the species (80.4 ± 4.4mm)[37–39]. During incubation the egg case was kept with others of the same clutch in 2500L natural seawater and maintained at 16.5˚C ± 1.8˚C with a dissolved oxygen of >95% and a salinity of 35ppt. Appropriate life support systems were also in place to ensure the nitrogenous waste contents were maintained at safe levels for the developing egg cases. On the 23<sup>rd</sup> of April 2014 the egg case displayed signs of being unviable and so was opened, revealing two small dead juveniles (Fig 1A1). One juvenile was smaller and exhibited the early signs of decay with no evidence of a yolk sack while the larger juvenile was in the final stages of yolk sack absorption. It is unknown if the individuals were attached to a single yolk, or whether the egg consisted of two separate yolks. The disc width of the larger individual within the twin egg case was 4 cm (Fig 1A2 and 1A3), whereas a fully developed, healthy individual which hatched 8 days later from the same clutch had a disc width of 9 cm (not shown).

**Nursehound, *Scyliorhinus stellaris***

The *S. stellaris* twin egg case was larger than its paired egg case (i.e. the case laid at the same time as the twin egg case from the other oviduct, Fig 1B1). The twin egg case, excluding the tendrils, was 12.25 cm long, 5.65 cm wide and 3.70 cm deep. The average size of *S. stellaris* egg...
cases from the same cohort was 11.58 ± 0.1 cm, 4.32 ± 0.05 cm, 2.88 ± 0.06 cm in length (excluding the tendrils), width, and depth respectively (mean ± SEM, n = 12). At 12 weeks of development the external yolk sacs of the S. stellaris twins had a combined volume of 37.17 cm³ (Fig 1B2), more than twice that of a single yolk sac from a typical S. stellaris embryo reared under the same conditions (15.91 ± 0.93 cm³, mean ± SEM, n = 12). The twins survived for 12 weeks and developed to stage 20 defined by Ballard et al. [26], and stage 3 defined by Musa et al. [25], with total body lengths of 0.70 cm and 0.80 cm.

Genetic analysis of the S. stellaris twins revealed identical genotypic fingerprints on all 9 successfully amplified loci; individuals did not amplify with Scan06 and Scan16. The PIC was ≥0.500 for 4 of the 11 microsatellites for all samples (Table 1). As the individuals were developed from two yolks, rather than being monovular (i.e. two individuals with a single yolk), the monozygosity in the genotypes probably emanates from a lack of species-specific loci, and therefore a loss of interrogated diversity between the twins. Of the markers used Scan02, Scan09, Scan10, Scan13, and Scan15 displayed the highest $H_o$ and $H_e$ levels for the greatest number of individuals (87.5% to 100% of the sample size) and $N_a$ for each was equal to or above 4 (Table 2). Overall, average genetic diversity for all eleven markers was $H_o = 0.427$ and $H_e = 0.413$ (Table 2).

Small-spotted catshark, Scyliorhinus canicula

The twin S. canicula egg case (Fig 1C1) was 6.52 cm in length (excluding the tendrils), 2.21 cm in width, and 1.57 cm in depth, making it slightly larger than the single embryo egg cases from the same clutch (6.33 ± 0.04 cm, 2.18 ± 0.05 cm, 1.36 ± 0.02 cm in length, width and depth respectively, mean ± SEM, n = 11). The lengths of the S. canicula twins at approximately 9 weeks post-laying were 4.67 cm and 4.69 cm, whilst the external yolk sac volumes measured 2.63 cm³ and 2.75 cm³. The lengths and key morphological features suggest that the twins reached somewhere between stages 28 and 32 of the Ballard et al. (1993) [26] developmental scale, and stage 4 of the Musa et al. (2018) [25] developmental scale. Due to concern for their well-being, the egg case containing the S. canicula twins was opened and the embryos (Fig 1C2) were transferred to individual artificial egg cases with larger dimensions and continued their development at 15°C. Both animals survived with good health to hatch.
The average genetic diversity for all microsatellites was $H_o = 0.524$ and $H_e = 0.577$ (Table 3). The PIC was $\geq 0.500$ on 6 of the 11 microsatellites (Table 1). Parentage analysis suggested that the twins derived from different paternities. Cervus parent pair non-exclusion probabilities all equal to or less than 1.30E-03 and Colony probability index of parent pairs were between 0.512 and 1.000 accurate (Table 2). Cervus gave more conclusive results in parentage due to the simulations for the log-likelihood ratio. These results suggest heteropaternal superfecundation (individuals from separate paternities, and therefore products of two distinct copulatory events) for the twins (DY1 and DY2) in *S. canicula* (Table 3).

**Table 1.** Genotypic information gathered from each microsatellite locus for the twin individuals (DY1, DY2) and a further randomly selected 6 possible siblings (SIB1-SIB6) of *S. stellaris* and *S. canicula* to display the genotype variance. Microsatellites with a polymorphism information content (PIC) value equal to or higher than 0.500 were used to display genotypes.

| S. stellaris | ID  | Scan02 | Scan 09 | Scan10 | Scan15 |
|--------------|-----|--------|---------|--------|--------|
| DY1          | 135 | 137    | 131     | 133    | 268    | 276    | 250    | 252    |
| DY2          | 135 | 137    | 131     | 133    | 268    | 276    | 250    | 252    |
| SIB1         | 125 | 137    | -       | -      | 268    | 274    | 248    | 250    |
| SIB2         | 125 | 139    | 133     | 133    | 274    | 274    | 248    | 250    |
| SIB3         | 125 | 139    | 133     | 133    | 266    | 274    | 250    | 250    |
| SIB4         | 137 | 139    | 133     | 133    | 268    | 268    | 248    | 250    |
| SIB5         | 123 | 137    | 131     | 133    | 268    | 276    | 250    | 252    |
| SIB6         | 133 | 141    | 129     | 131    | 274    | 276    | 258    | 260    |

| S. canicula | ID  | Scan02 | Scan04 | Scan06 | Scan12 | Scan15 | Scan16 |
|-------------|-----|--------|--------|--------|--------|--------|--------|
| DY1         | 132 | 136    | 257    | 265    | 233    | 237    | 119    | 121    | 254    | 256    | 283    | 285    |
| DY2         | 136 | 142    | 257    | 257    | 227    | 237    | 119    | 121    | 254    | 256    | 283    | 285    |
| SIB1        | 132 | 134    | 257    | 267    | 233    | 237    | 119    | 121    | 254    | 256    | 283    | 285    |
| SIB2        | 136 | 140    | 257    | 265    | 227    | 227    | 117    | 119    | 258    | 260    | 281    | 287    |
| SIB3        | 132 | 144    | 257    | 265    | 227    | 233    | 119    | 121    | 256    | 260    | 279    | 281    |
| SIB4        | 132 | 132    | 257    | 265    | 229    | 237    | 117    | 119    | 256    | 258    | 283    | 285    |
| SIB5        | 132 | 132    | 265    | 265    | 227    | 229    | 117    | 119    | 254    | 258    | 283    | 285    |
| SIB6        | 132 | 134    | 257    | 263    | 229    | 237    | 119    | 119    | 256    | 258    | 283    | 285    |

The average genetic diversity for all microsatellites was $H_o = 0.524$ and $H_e = 0.577$ (Table 3). The PIC was $\geq 0.500$ on 6 of the 11 microsatellites (Table 1). Parentage analysis suggested that the twins derived from different paternities. Cervus parent pair non-exclusion probabilities all equal to or less than 1.30E-03 and Colony probability index of parent pairs were between 0.512 and 1.000 accurate (Table 2). Cervus gave more conclusive results in parentage due to the simulations for the log-likelihood ratio. These results suggest heteropaternal superfecundation (individuals from separate paternities, and therefore products of two distinct copulatory events) for the twins (DY1 and DY2) in *S. canicula* (Table 3).

**Table 2.** Microsatellite information gathered from each locus for the entire population studied (including the twins) for *S. stellaris* and *S. canicula*. N% = Percentage of individuals scored, $N_a =$ numbers of alleles, $H_e =$ expected heterozygosity, $H_o =$ observed heterozygosity.

| Loci information | S. stellaris | S. canicula |
|------------------|--------------|-------------|
| Locus            | $N_a$ | $H_e$ | $H_o$ | $N_a$ | $H_e$ | $H_o$ |
| Scan 02          | NED  | 100 | 7    | 1.000 | 0.858 | 100 | 6    | 0.754 | 0.738 |
| Scan 03          | FAM  | 63  | 3    | 0.200 | 0.378 | 96  | 4    | 0.513 | 0.182 |
| Scan 04          | VIC  | 50  | 3    | 0.250 | 0.607 | 98  | 5    | 0.689 | 0.474 |
| Scan 05          | NED  | 100 | 2    | 0.125 | 0.125 | 100 | 4    | 0.543 | 0.450 |
| Scan 06          | FAM  | 38  | 1    | 0.000 | 0.000 | 100 | 9    | 0.730 | 0.713 |
| Scan 09          | VIC  | 88  | 4    | 0.714 | 0.626 | 100 | 2    | 0.025 | 0.000 |
| Scan 10          | NED  | 100 | 4    | 0.750 | 0.742 | 98  | 5    | 0.585 | 0.526 |
| Scan 12          | FAM  | 100 | 2    | 0.125 | 0.125 | 100 | 6    | 0.666 | 0.638 |
| Scan 13          | VIC  | 100 | 4    | 0.500 | 0.517 | 96  | 4    | 0.382 | 0.429 |
| Scan 15          | FAM  | 100 | 5    | 0.875 | 0.717 | 98  | 5    | 0.739 | 0.859 |
| Scan 16          | VIC  | 38  | 1    | 0.000 | 0.000 | 98  | 6    | 0.730 | 0.756 |
Here we report the first incidence of an egg case containing two embryos in the oviparous Raja elasmobranch, the undulate ray (R. undulata). We also add two new species of oviparous benthic sharks (S. stellaris and S. canicula) to the list of elasmobranchii twin eggs, and provide the first genetic evidence of heteropaternal superfecundation in S. canicula. 

The S. canicula and S. stellaris eggs all had two yolk sacs, indicating that two oocytes were released into the same oviducal gland for shelling in a single egg case. Genetic analysis revealed that the S. canicula twins were from heteropaternal superfecundation, meaning that each oocyte was fertilized by a different male, and thus suggesting sperm storage within the oviducal gland. Previous findings showed that females isolated from males for up to two years can produce fertile eggs [11], displaying longevity of the sperm and sperm storage which could account for the heteropaternal superfecundation reported here, if the female only mated with one individual during ovulation.

The mechanisms of double-embryo formation in the three oviparous elasmobranch species cannot be fully elucidated until development is tracked from ovary secretion, through the oviducal gland, to deposition. However, our findings are the first reported cases of shark twins in captive environments and provide the first evidence of heteropaternal superfecundation in a species of oviparous elasmobranch. The evolution of twin egg cases as a method of reproductive biology may have implications on the population persistence, if such individuals are unlikely to survive. However, if viable, increasing the number of individuals per reproductive output by producing twin egg cases would be advantageous. Overall there are an increasing number of reports on the occurrence of reproductive mutations such as double-embryo egg cases and conjoined individuals. The captive species which produce twin egg cases usually display high reproductive performance and plasticity [40], although without human input, twin egg cases typically do not succeed to hatch [18]. Considering the significant stress on wild populations of sharks and rays, further research is needed to understand and identify the mechanisms producing, and consequences of, elasmobranch twins.

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References

1. Naylor GJP, Caira JN, Jensen K, Rosana KAM, White WT, Last PR. A DNA sequence–based approach to the identification of shark and ray species and its implications for global elasmobranch diversity and parasitology. Bull Am Museum Nat Hist. 2012; 367(367):1–262.

2. Compagno LJ V. Checklist of living Chondrichthyes. Reprod Biol phylogeny Chondrichthyes sharks, batoids chimaeras. 2005; 503–48.

3. Naylor G, Fedrigo O, Andrés López J. Phylogenetic Relationships among the Major Lineages of Modern Elasmobranchs. 2005

4. Carrier J, Musick J, Heithaus M. Biology of Sharks and Biology of Marine Birds. 2004. 487–521 p.

5. Henningsen AD, Smale M, Garner R, Kinnunen N. Reproduction, Embryonic Development, and Reproductive Physiology of Elasmobranchs. In: Elasmobranch Husbandry Manual: Captive Care of Sharks, Rays, and their Relatives. Ohio Biological Survey; 2004. p. 227–36.

6. Carrier JC, Musick JA, Heithaus MR. Biology of sharks and their relatives. Taylor & Francis Group; 2012. 633 p.

7. Dulvy NK, Simpfendorfer CA, Davidson LNK, Fordham S V., Bräutigam A, Sant G, et al. Challenges and Priorities in Shark and Ray Conservation. Curr Biol. 2017; 27(11):R565–72. https://doi.org/10.1016/j.cub.2017.04.038 PMID: 28586694

8. Hamlett W, Knigth DP, Pereira F, Steele J, Sever M. Oviducal glands in chondrichthians. In: Reproductive biology and phylogeny of chondrichthyes: sharks, batoid and chimaeras. Enfield: Science Publishers Inc; 2005. p. 301–36.

9. Last P, White W, de Carvalho M, Séret B, Stehmann M, Naylor G. Rays of the World. 1st Edition. Australia: CSIRO Publishing; 2016. 790 p.

10. Compagno LJ V. Alternative life-history styles of cartilaginous fishes in time and space. Environ Biol Fishes. 1990; 28(1–4):33–75.

11. Dodd JM. Reproduction in Cartilaginous Fishes (Chondrichthyes). Vol. 9, Fish Physiology. Academic Press; 1983

12. Ellis JR, Shackley SE. The reproductive biology of Scyliorhinus canicula in the Bristol Channel, U.K. J Fish Biol. 1997; 51(2):361–72.

13. Coelho R, Erzini K. Reproductive aspects of the undulate ray, Raja undulata, from the south coast of Portugal. Fish Res. 2006; 81(1):80–5.
14. Serra-Pereira B, Figueiredo I, Leonel & Gordo S. Maturation of the Gonads and Reproductive Tracts of the Thornback Ray *Raja clavata*, with Comments on the Development of a Standardized Reproductive Terminology for Oviparous Elasmobranchs. Ivone Figueiredo & Leonel Serrano Gordo. 2011; 3 (1):160–75.

15. Richards S., Merriman D., Calhoun L. The biology of the little skate. *Raja erinacea* Mitchell. Stud Mar Resour South New England IX Bull Bingham Oceanogr Collect. 1963; 18(3):5–67.

16. Jañez JA, Suedo MC. Scientific Note Oviposition rate of the fanskate *Sympterygia bonapartii* (Elasmobranchii, Rajidae) (Miûller & Henle, 1841) held in captivity. Vol. 4, Pan-American Journal of Aquatic Sciences. 2009.

17. Farrell ED, Mariani S, Clarke MW. Reproductive biology of the starry smooth-hound shark *Mustelus asterias*: geographic variation and implications for sustainable exploitation. *J Fish Biol.* 2010; 77 (7):1505–25. https://doi.org/10.1111/j.1095-8649.2010.02771.x PMID: 21078015

18. Mabragaña E, Lucifora LO, Corbo M de L, Díaz de Astarloa JM. Seasonal Reproductive Biology of the Bignose Fanskate *Sympterygia acuta* (Chondrichthyes, Rajidae). Estuaries and Coasts. 2015; 38 (5):1466–76.

19. Ebert DA, Davis CD. Descriptions of skate egg cases (Chondrichthyes: Rajiformes: Rajoidei) from the eastern North Pacific. *Zootaxa.* 2007; 1393:1–18.

20. Ishiyama R. Studies on the rajid fishes (Rajidae) found in the waters around Japan. *J Shimonoseki Coll Fish.* 1958; 7:191–394.

21. Hitz CR. Observations on Egg Cases of the Big Skate (*Raja binoculata* Girard) Found in Oregon Coastal Waters. *J Fish Res Board Canada.* 1964; 21(4):851–4.

22. Kang H-W, Jo Y-R, Kang D-Y, Jeong G-S, Jo H-S. Spawning Characteristics and Artificial Hatching of Female Mottled Skate, *Beringraja pulchra* in the West Coast of Korea. Dev Reprod. 2013; 17(3):247–55. https://doi.org/10.12177/DR.2013.17.3.247 PMID: 25949140

23. Howard M. Fecundity, egg capsule size and neonate morphometrics of big skate, *Beringraja binoculata* (Girard, 1855). In: The Elasmobranch Husbandry Manual II: Recent Advances in the Care of Sharks, Rays and their Relatives Special Publication of the Ohio Biological Survey. Ohio Biological Survey; 2017. p. 451.

24. Jeong C-H, Ishihara H, Treloar M, Bor PH, Senou H, Jeong CH. The Comparative Morphology of Skate Egg Capsules (Chondrichthyes: Elasmobranchii: Rajiformes). Bull Kanagawa prefect Mus Nat Sci. 2012;(41):17–33.

25. Musa SM, Czachur M V, Shielis HA. Oviparous elasmobranch development inside the egg case in 7 key stages. *PloS One.* 2018; 13(11):e0206984. https://doi.org/10.1371/journal.pone.0206984 PMID: 30399186

26. Ballard WW, Mellinger J, Lechenault H. A series of normal stages for development of *Scyliorhinus canicula*, the lesser spotted dogfish (Chondrichthyes: Scyliorhinidae). *J Exp Zool.* 1993; 267(3):318–36.

27. Schneider CA, Rasband WS and Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012. pp. 671. https://doi.org/10.1038/nmeth.2089 PMID: 22930834

28. ISOLATE II Genomic DNA Kit Product Manual. 2017. Available from: www.bioline.com/isolate

29. Griffiths AM, Casane D, McHugh M, Wearmouth VJ, Sims DW, Genner MJ. Characterisation of polymorphic microsatellite loci in the small-spotted catshark (*Scyliorhinus canicula* L.). *Conserv Genet Resour.* 2011; 3(4):9–3.

30. QiAGEN Multiplex PCR Kit. 2019. Available from: https://www.qiagen.com/gb/shop/pcr/end-point-pcr-enzymes-and-kits/regular-pcr/qiagen-multiplex-pcr-kit/

31. van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Mol Ecol Notes.* 2004; 4(3):353–8.

32. Raymond M, Rousset F. An exact test for population differentiation. *Evolution (N Y).* 1995; 49(6):1280–3.

33. Raymond M, Rousset F. GENEPOP (version 1.2): population genetics software for exact tests and eucenicism. *J Hered.* 1995; 86:248–9.

34. Marshall TC, Slate J, Kruuk LEB, Pemberton JM. Statistical confidence for likelihood-based paternity inference in natural populations. *Mol Ecol.* 1998; 7(5):639–55 https://doi.org/10.1046/j.1365-294x.1998.00374.x PMID: 9633105

35. Jones OR, Wang J. COLONY: a program for parentage and sibship inference from multilocus genotype data. *Mol Ecol Resour.* 2010; 10(3):551–5. https://doi.org/10.1111/j.1755-0998.2009.02787.x PMID: 21565056
36. Gubili C, Sims DW, Verissimo A, Domenici P, Ellis J, Grigoriu P, et al. A tale of two seas: contrasting patterns of population structure in the small-spotted catshark across Europe. R Soc Open Sci. 2014; 1 (3):140175. https://doi.org/10.1098/rsos.140175 PMID: 26064555

37. Gordon CA, Hood AR, Ellis JR. Descriptions and revised key to the eggcases of the skates (Rajiformes: Rajidae) and catsharks (Carcharhiniformes: Scyliorhinidae) of the British Isles. Zootaxa. 2016; 4150 (3):255–80. https://doi.org/10.11646/zootaxa.4150.3.2 PMID: 27515657

38. Luer CA, Walsh CJ, Bodine AB, Wyffels JT. Normal embryonic development in the clearnose skate, Raja eglanteria, with experimental observations on artificial insemination. In: Biology of Skates. Dordrecht: Springer Netherlands; 2007. p. 133–49.

39. Caldeira BF. Morfologia e biometria do desenvolvimento embrionário da raia Sympterygia acuta Garman, 1877 (Elasmobranhii; Rajidae). 2006

40. Jañez JA, Meijide FJ, Lucifora LO, Abraham C, Argemi F. Growth and reproduction in captivity unveils remarkable life-history plasticity in the smallnose fanskate, Sympterygia bonapartii (Chondrichthyes: Rajiformes). Neotrop Ichthyol. 2018; 16(4).