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HUSBANDRY REPORTS

Capture, Transport, and Husbandry of Elephant Sharks (*Callorhinchus milii*) Adults, Eggs, and Hatchlings for Research and Display

Catherine A. Boisvert,[1]* Camila Leite Martins,[2] Alison Grace Edmunds,[3] Julian Cocks,[1] and Peter Currie[1]

[1]ARMI, Monash University, Clayton, Victoria, Australia
[2]School of Biological Sciences, Faculty of Sciences, Monash University, Clayton, Victoria, Australia
[3]Melbourne Aquarium, Cnr King & Flinders St, Melbourne, Victoria, Australia

Elephant sharks (*Callorhinchus milii*) have the slowest evolving genome of all vertebrates and are an interesting model species for evolution research and a prized display animal. However, their deep water habitat, short breeding season, fragility, and susceptibility to stress-induced mortality have made them difficult animals to capture, keep in captivity, and obtain fertilized eggs from. Gravid females were captured by rod and reel from Western Port Bay, Australia and transferred to a 40 000 L closed aquaculture system to lay their eggs before being released. The water quality parameters, averaged over three seasons of 4–6 weeks (mean ± standard deviation) were: 16.8°C ± 2.31, salinity 37.1 ± 2.9 g/L, ammonia 0.137 ± 0.2 mg/L, nitrite levels 0.89 ± 0.9 mg/L, nitrate 66.8 ± 45.6 mg/L, pH 7.8 ± 0.18, dissolved oxygen levels 93.6 ± 5.3%, ORP 307 ± 63.3 mV. Eggs were incubated in purpose-built egg cages and embryos hatched after 143.6 days ± 1.3 at 16.9 ± 0.9°C of incubation. These procedures led to no adult mortality in the last 2 years and 620 eggs with known deposition date were collected over 4 years, of which 81.5% (±4.8) were viable. Collection of abundant embryological material with known deposition date is of paramount importance for evolutionary developmental research. We attribute this success to excellent water quality, maximum reduction of stress during capture, transport, handling, and captive care. Zoo Biol. 34:94–98, 2015.

Keywords: holocephali; EvoDevo; developmental series; husbandry; chondrichthyes

STATEMENT OF THE PROBLEM

The elephant shark (*Callorhinchus milii*) is one of the most basal member of the holocephali (Didier, 1995), an ancient lineage of cartilaginous fishes (Tozer and Didier Dagit, 2004; Maisey, 2012) who are the sister group to elasmobranchs (all modern sharks and rays) (Maisey, 2012). Their basal evolutionary position, staging table (Didier et al., 1998) and the fact that they have the slowest evolving genome of all vertebrates (Venkatesh et al., 2014) make them valuable models for evolutionary and developmental research (Cole et al., 2011; Gillis et al., 2011). Elephant sharks spend most of their lives at depths ranging from 200 to 500 m only migrating to shallow bays for a few weeks in summer to breed and lay eggs (Last and Stevens, 2009). They are difficult to catch and are very fragile animals, easily stressed during capture, handling, and transport (Martins et al., 2011), which sometimes leads to up to 50% of caught animals dying within the first few days post capture (Martins, unpublished data). Their smooth, nearly scale-less skin gets easily injured during rod and reel capture, which can lead to infection. Most animals do not eat readily once in captive care, and dietary

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*Correspondence to: Catherine A. Boisvert, ARMI, Monash University, Bldg 75, level 1, Wellington road, Clayton 3800, Victoria, Australia. E-mail: catherine.boisvert@monash.edu

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induced scoliosis begins to occur after 4 weeks of inadequate food supply or incorrect diet, which may lead to death. Embryological material has traditionally been collected from the oviducts of dead females (Ballard et al., 1993) or from hand collection using SCUBA (Didier et al., 1998; Gillis et al., 2011). However, an increase in ecological challenges has made collection of viable capsules more difficult via SCUBA in recent years. Since embryological staging cannot be estimated by "candling" as in other chondrichthyans (Payne and Rufo, 2012), it is best for embryological studies to use eggs laid in captivity and tagged at the date of deposition.

DESCRIPTION OF THE PROCESS

Capture, Handling, and Transport

One hundred and twenty impregnated adult elephant shark females were caught by rod and reel in Western Port bay, Victoria, Australia from 2010 and 2013, between February and May (Fishing permits DPI RP1000, RP1003, and RP1112). They were caught at an average depth of 4.6 m using squid (*Loligo opalescens*) and pilchards (*Sardinops sagax*) as bait. Gravid state was determined by season, location and presence of mating wounds. To prevent injury during capture, we covered the whole fishing line with aquarium airline and netted the animals with smooth plastic nets with small meshing (Environet^{TM}) (Fig. 1a). When necessary, the animals were handled by catching them by the tail and supporting their bodies behind the pectoral fins (Fig. 1b). Handling of elephant sharks must be minimized to reduce stress and prevent severe physiological changes (Martins et al., 2011), which can lead to mortality.

A maximum of eight animals were kept on the fishing boat in a 250 L circular tank with constantly flowing water, which maintains waste at a minimum and the dissolved oxygen (DO) levels close to 100%. The animals were transferred as soon as possible to a round and smooth transport tank (1 m radius) on a truck (Fig. 1c) oxygenated with pure oxygen fed through a ceramic airstone. Temperature, DO, and ammonia levels were monitored during transport and were kept below 19°C, 100%, and 0 mg/L respectively. In order to reduce negative effects of ammonia on the animals, it was sequestered with 0.26 ml/L of AMM-MASK (Baseline®). Maximum transport times were 40 min by boat plus 3 hr by truck.

Husbandry Conditions

Between 2011 and 2013, the animals were transferred to a purpose-build 40,000 L closed aquarium for 4 weeks to lay their eggs prior to being released or transferred to the Melbourne Aquarium. The system is composed of two 19,000 L circular (2.3 m radius and 1.2 m high) dark green fiberglass tanks, a 500 L sump, a sand filter, protein skimmer, a DLY-heat/chilling unit, fluidized bed biofilter, and a triogen ozone unit. *C. milii* has color vision (Davies et al., 2009, 2012) and animals housed in pale and dark blue, green and black tanks showed that color played an important role in their wellbeing. Animals in a dark color other than blue were more settled, had better survival and less injury from hitting the walls of the tanks (Fig. 1d). This research was conducted according to the Animal Ethics rules of Monash University Animal Services (Permit MAS/ARMI/2010/01).

Fig. 1. (a) Elephant shark caught using a fishing line with airline guard. (b) Handling of an elephant shark female. (c) Elephant shark in the transport tank. (d) Adult females in captivity. (e) Hatchlings in a recirculating system, (f) Hatchling (g) Female laying a pair of eggs.

Water quality parameters and feeding details were collected between one and four times a day and averaged over a 24 hr period before being averaged by season or over the three seasons period (mean ± standard deviation). Overall temperature was 16.8 ± 2.31°C, salinity was adjusted to be identical to the environment animals were caught the first year (42 g/L) at 41.6 ± 0.6 g/L in 2011 and to that of the environment they were released into (36 g/L) in 2012–2013 at 35.5 ± 0.8 g/L, ammonia 0.137 ± 0.2 mg/L, nitrite 0.89 ± 0.9 mg/L and nitrate 66.8 ± 45.6 mg/L (measured with colorimetric test kits). Nitrate and nitrite levels were controlled using Baseline® Nitrogen purge, which allows biofilter bacteria to convert aqueous nitrogen to gaseous nitrogen, therefore purging it from the system. Other parameters were kept as close to the capture environment as possible: pH 7.8 ± 0.18, dissolved oxygen levels 93.6 ± 5.3%. Elephant sharks are prone to infection in a closed system and the water was ozonated to levels between 300 and 350 mV (307 ± 63.3) of oxygen reduction potential (ORP) to reduce this risk. The animals were kept indoors without direct sunlight and natural photoperiod was followed with fluorescent lights turned on during the day to supplement a small skylight (Tozer and Didier Dagit, 2004).

It is important to entice the animals to eat live food as soon as possible after capture. The animals were fed, at the bottom of the tank, live mussel spat (around 5 cm in length) (*Mytilus galloprovincialis*) until satiated over a 12 hr period and as much frozen pipis (*Donax deltoids*) as they would eat within a 30 min period, morning and evening for a target of 10% of the fishes body mass. The amount of food needed is constant throughout the year and it is important to feed regularly and in large quantity because they can lose weight rapidly and develop scoliosis. Our females ate an average of 4.8 ± 4% body weight/day (BW/day) but as much as 18% BW/day.

Eggs and Hatchlings

Females store sperm in a *receptaculum seminis* (spermatheca) (Dean, 1906) after mating. Observation of females with specific markings show that they can lay one pair of eggs every 4 days (average 0.26 ± 0.19 egg/day/female) for a period of around 4 weeks (Fig. 1g). It takes around 24 hr to deposit a pair of eggs. The females eject the remaining sperm from the cloaca as a compact yellowish-white sperm plug once they have finished laying eggs for the season as described for *Callorhinchus capensis* (Freer and Griffiths, 1993).

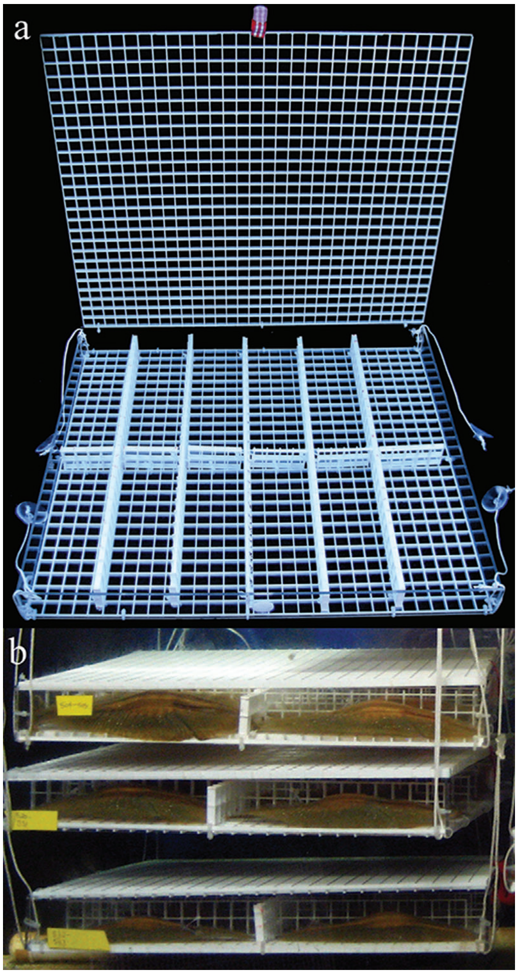

Fig. 2. (a) Egg cages designed to hold 12 elephant shark eggs and to allow for water flow, (b) containing eggs in the egg system.

A total of 620 eggs were collected from captive females over 4 years with an average of 187 (±45) per year from an average of 26 females. Eggs were tagged with a commercial clothes tagger and a plastic number tag referring to the date at which the eggs were laid. 81.5% (±4.8) were viable and were incubated in an 873 L artificial sea water closed system consisting of a sand biofilter, foam fractionator and a heater-chiller unit with UV radiation treatment. The parameters were (average ± SD): temperature 2010–2012, set at 16.5°C: 16.8 ± 0.9°C 2013, set at 12°C: 12.5 ± 1.58°C; salinity 2010 and 2013 set at 34.5 g/L: 35.8 ± 2.43 g/L, 2011 and 2012 set at 40 g/L: 39.4 ± 1.66; pH (all years) 8.08 ± 0.13; nitrate: 17.7 ± 28.1 mg/L, nitrite 1.0 ± 1.5 mg/L; ammonia 0.16 ± 1.38 mg/L. Egg cages were purpose-built in 2012 to keep the eggs flat and aerated during development (Fig. 2). The design was based on the observation (C. Boisvert during SCUBA collection and Lyon et al. (2011)) that egg capsules are deposited and buried flat and upright. We observed that eggs incubated in any other way quickly rotted. After 143.6 days ± 1.3 (4.7 months) at 16.9 ± 0.9°C of incubation, embryos exited through the weakened flap of the egg capsule (Freer and Griffiths, 1993; Lyon et al., 2011) and were kept in a circular black tank (Fig. 1e–f). The hatchlings and juveniles required even better water quality conditions than the adults and are more prone to infection (Caira, 2012), especially by monogenea. Some individuals were treated with praziquantel (3 ppm for 48 hr) in the tank water. They were fed chopped prawns (*Melicertus latisulcatus* and *M. plebeju*), calamari (*Sepioteuthis lessoniana* and *S. australis*) and whitebait (*Hyperlophus vittatus*) several times a day as they would only take fresh food. After 5 months, crushed pipis (*D. deltoids*) were introduced to their diet. Uneaten food was siphoned or netted 30 min after feeding. Using the husbandry methods described above, we were able to hatch and raise an embryo laid in captivity for over 3 years (Fig. 1f), a world's first.

DEMONSTRATION OF EFFICACY

Optimal Conditions for Adults

The procedures described above have led to 100% survival rate during capture and transport for all 3 years and the introduction of airline on the fishing line in the final year led to the complete absence of skin injury during capture. These procedures reduce the potential for infection but mostly, reduce

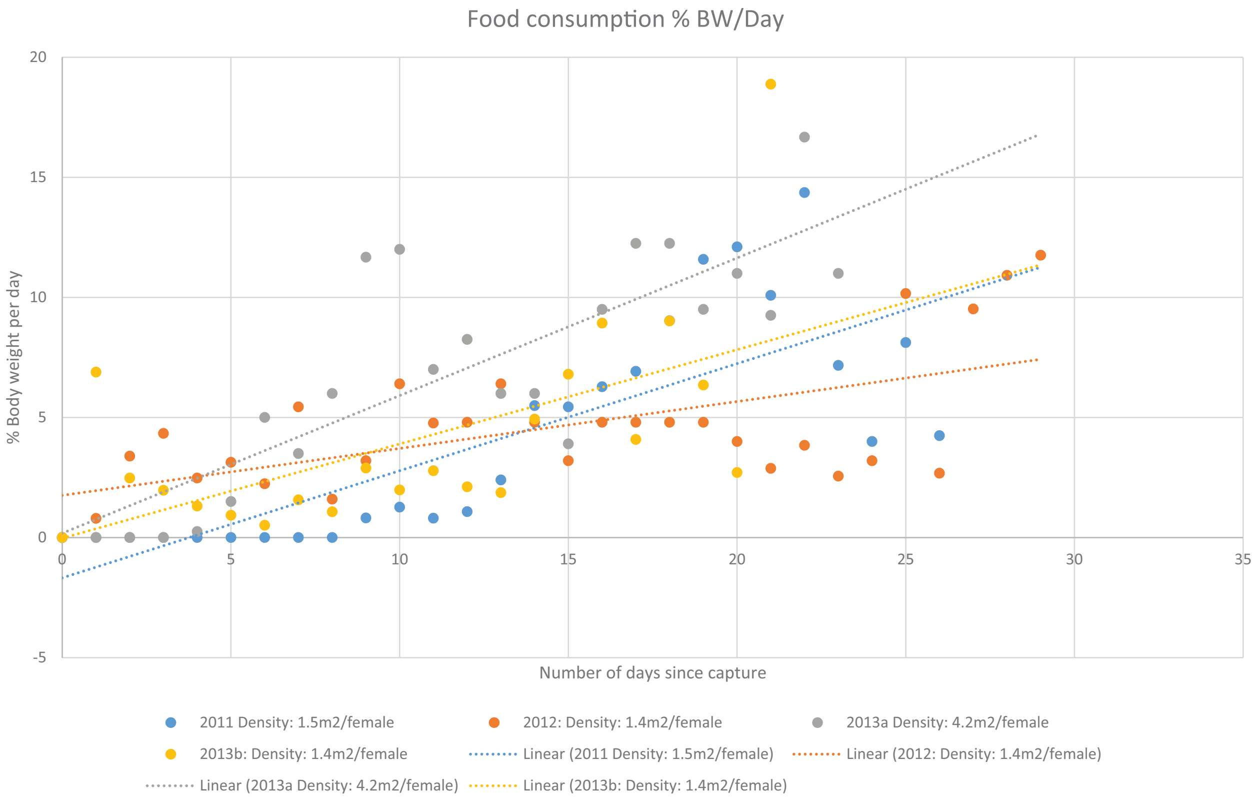

Fig. 3. Linear regression plots of food intake (% body weight per day) relative to stocking density (m^2 of tank area per female). In 2013, the facility was stocked twice (a) with eight females, five of which were released and three kept with group (b) with 22 newly caught females. The earliest onset of feeding was achieved in group 2013b where newly caught females were shown by other females what to eat.

stress. When stressed, elephant sharks turn silvery white, but regain their dark brown with gold and pink patterning colors when acclimatized. If the animals are very stressed, they may lie on their side for extended periods of time which is the major cause of "bloody eye," the result of a trauma to the efferent pseudo-branchial artery (Tozer and Didier Dagit, 2004) causing permanent blindness and which may lead to death. Keeping the animals below 19°C during transport and in captivity substantially decreased this behavior and the incidence of "bloody eye." Elephant sharks are gliders and require sufficient space to live long term in captivity. The reduction in mortality from 12% (three individuals) the first year to 0% the subsequent years could be attributed to a reduction in tank density and better tank oxygenation. In addition, there were no reports of scoliosis in any animals for the 3 years, which could be attributed to the animals feeding early on live food. The best feeding outcome was at the lowest density (Fig. 3); however, there is no statistical difference in the food consumed per individual for the 3 years (all in kg/individual/day: 2011: 0.1030 ± 0.1119; 2012: 0.1148 ± 0.0704, 2013 a first capture of eight individuals 0.1024 ± 0.1057; 2013b total 25 individuals 0.1024 ± 0.1057; P values for t tests 2011–12 = 0.64, 2011–2013a = 0.05, 2011–2013b = 0.98, 2012–13a = 0.06, 2012–2013b = 0.64). Because this closed system is used on a seasonal basis and stocked quickly, the amount of food offered must be limited and uneaten dead food removed quickly to keep the water parameters within acceptable ranges. For long term display, density should be kept around 6–8 m^2 of bottom area/animal in a tank that can be shallow (min 1 m deep) with a sand bottom. The animals should be fed a variety of fresh seafood (pilchards (*S. sagax*), prawns (*M. latisulcatus* and *M. plebeju*), calamari (*S. lessoniana* and *S. australis*), pipis (*D. deltoids*), whitebait (*H. vittatus*), squid (*L. opalescens*)) (Di Giacomo and Perier, 1996) several times a day, up to 10% BW/day, year round. Tank mates copy each other and the fastest onset of feeding was observed in 2013 when three individuals already feeding were kept with 22 newly caught females (Fig. 3).

Egg Production and Care

The procedure described here has yielded the highest number of eggs with known deposition date for this species to date. Eggs were incubated at 12°C in the final year to slow development down for research purposes. In order for the temperature to be constant for the entire developmental period, the temperature for the female tanks was lowered to 12°C but this led to a lowering in egg production and ejection of more sperm plugs. This suggests that a temperatures below 14°C would be an environmental trigger to signal the end of the reproductive season as the females would leave the warmer bays for the deep ocean. Hence, ideal temperature for egg productivity and animal welfare was 16°C.

The egg incubation system yielded egg viability levels much higher than reported for the bamboo and epaulette sharks (Harahush et al., 2007; Payne and Rufo, 2012) (2010: 76%, 2011: 79%, 2012: 85% and 2013: 86% vs. 33% for bamboo sharks, 48% for epaulette sharks) with the viability of eggs improving with the introduction of purpose built egg cages in 2012 (Fig. 2) leading to better aeration of the eggs during development. Eggs were monitored visually every other day and the underside was felt weekly. Rotten and slimy eggs were removed immediately as they resulted in increased nitrate and nitrite levels. Elevated nitrate and nitrite levels were dealt with water changes from 2010 to 2012 and a denitrifier (PhosBan© reactor 550^{TM} with NPX Bioplastics$^{®}$) in 2013.

CONCLUSIONS

The phylogenetic position and retention of primitive characteristics makes elephant sharks an ideal developmental model species for the study of the evolution of vertebrate characteristics. The methods described here to capture and care for females allow for a relatively large number of stageable eggs which can then be manipulated in ovo and used for developmental studies. Their use in recent studies (Cole et al., 2011; Gillis et al., 2011) has shown their importance in evolutionary research and the methods described here will allow for this model to be used on a larger scale with more flexibility, hopefully allowing us to better understand the mechanisms of jawed vertebrate evolution.

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