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THE EFFECT OF AN ARTIFICIAL INCUBATION ENVIRONMENT ON HATCHLING SIZE AND BEHAVIOR IN THE CUTTLEFISH, *SEPIA OFFICINALIS*

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EMBRYONIC EXPERIENCE
BEHAVIORAL PLASTICITY
BODY PATTERNING
PREDATION
CUTTLEFISH CULTURE

A great deal is known about development in *Sepia officinalis*; however, much of this knowledge comes from animals incubated in laboratory conditions. Since cuttlefish are behaviorally plastic and known as embryos to perceive environmental stimuli from within the egg, we wondered if they are affected by incubation environment and thus whether laboratory-incubated cuttlefish exhibit natural behavior. We investigated the effects of incubation environment on hatchling size, defense and predation behavior in *Sepia officinalis* by comparing artificially-incubated hatchlings to naturally-incubated ones. Contrary to our expectations, no significant differences were apparent in hatchling size, disruptive body patterning and predation. A significant difference did exist between groups in one type of body patterning: Artificially-incubated hatchlings appeared to be better at producing a uniform body pattern than naturally-incubated individuals, possibly due to their prenatal experience with a homogeneous artificial substrate. This difference suggests some caution when interpreting experiments utilizing laboratory-incubated hatchlings, but overall, the limited effect of artificial incubation conditions demonstrated in this experiment bolsters confidence in previous behavioral results. These results are also promising for cuttlefish culture; eggs that would otherwise be lost as bycatch could instead be cultured artificially

INTRODUCTION

Sepia officinalis (Linnaeus, 1758) rely heavily on both body patterning and predation skills for survival and growth. Cuttlefish skin has both chromatic and textural components that allow it to change appearance and effect crypsis. Cryptic strategies include background matching (general color resemblance or variable color resemblance), disruptive coloration and deceptive resemblance (Cott 1940, Hanlon & Messenger 1996). Though a wide range of body patterns are possible, most work has focused on the two extremes of the spectrum: the uniform pattern, in which homogeneous color and brightness are present over the entire body, and the disruptive pattern, in which the cuttlefish projects many small, irregular, disparate pieces in a way that breaks up the outline of the body (Cott 1940, Boycott 1965). On uniform substrates such as sand or mud, the uniform pattern is generally thought to be employed in background matching, while the disruptive pattern can be used for both background matching (variable color resemblance) on a variegated substrate or as a way to occlude body shape. (However, these patterns may also function in deceptive resemblance, a possibility which will be explored in the discussion.) Cuttlefish are also voracious hunters of shrimp, using vision to target their prey (Wells 1958). They have two methods of attack: a rapid tentacular strike for shrimp, fish and small crabs and a pouncing maneuver for large crabs (Messenger 1968, Duval *et al*. 1984, Chichery & Chichery 1988). While effective prey capture is essential for obtaining food, foraging and prey capture can potentially put a cuttlefish at risk of detection by predators. Thus, both body patterning and predation may be subject to behavioral plasticity.

Of the 30 stages of development currently defined in cuttlefish embryos (Lemaire 1970), response to touch and odors is possible by the 23rd (Romagny *et al*. 2012). Response to visual information is made possible by the 25th stage via early maturity of the visual system and increased transparency of the egg membrane due to osmotic swelling (Paulij *et al*. 1991, Darmaillacq *et al*. 2008, Romagny *et al*. 2012). Thus, during the last weeks of embryonic development, cuttlefish embryos are able to perceive the outside world, and may learn and modify their behavior in response. As one example, stage 30 embryos exposed to bright light six times for three minutes at a time over the course of a few hours decreased their behavioral response (measured by mantle contrac-

tion) over time, demonstrating habituation to the stimulus (Romagny *et al*. 2012). Similarly, cuttlefish exposed to a non-preferred prey species (crabs) during the last week of development showed a preference for this prey item after hatching (Darmaillacq *et al*. 2008, Guibé *et al*. 2010). Finally, prenatal cues from potential predators influence the strength of brain lateralization in cuttlefish hatchlings (Jozet-Alves & Hébert 2013), a trait associated with more efficient information-processing in vertebrates (see Jozet-Alves *et al*. 2012 for discussion). These learning abilities and behavioral plasticity have tremendous potential advantages for young cuttlefish by allowing embryos to adapt to their post-hatching environment before eclosion. For instance, knowledge of the presence of a predator with a particular predation strategy could allow cuttlefish to prioritize the development of suitable defense strategies. Indeed, older cuttlefish discriminate between predators and employ targeted defensive strategies in response to different species (Langridge 2009).

Young cuttlefish are behaviorally plastic. Previous experiments with hatchlings (up to one week old) and juveniles (up to 17 weeks) (Hanlon & Messenger 1988) have demonstrated behavioral plasticity in response to limited enrichment of a laboratory setting with sensory stimuli during the post-embryonic period. For instance, sensory enrichment of a bare tank with a sandy substrate, rocks, shells, artificial algae and the presence of conspecifics is associated with faster growth and memory maturation during the first three months of life (Dickel *et al*. 2000). Likewise, experience with a sandy substrate soon after hatching results in young that are better at burying in the following days (Poirier *et al*. 2004). It is also worth noting that the brightness of artificial rearing tanks has been shown to affect growth, with a dark (black) background associated with the highest growth (Sykes *et al*. 2011). Extending the logic of this work to the pre-hatching period, we hypothesized that experiencing an artificial environment during the prenatal period could affect hatchling behavior as well.

With a few exceptions, knowledge about early behavior in cuttlefish comes from experiments on animals hatched in the laboratory (*e.g*. Wells 1958, Messenger 1968, Dickel *et al*. 2000, Poirier *et al*. 2004, Chiao *et al*. 2005, Poirier *et al*. 2005, Jozet-Alves & Hébert 2013), a fact not always indicated by the authors. However, little research attempting to quantify the effects of artificial incubation on behavior in *S. officinalis* exists. This lack of knowledge regarding the effects of artificial incubation is problematic for our ability to generalize conclusions based on the results from artificially-incubated individuals to "natural" behavior. Research with other species and with *Sepia* suggest that a (post-embryonic) laboratory situation can profoundly affect behavioral traits and that behavioral results often diverge between different laboratories due to variance in environmental parameters and experimenters (Dickel *et al*. 2000, Chesler *et al*. 2002, Poirier *et al*. 2004, Lewejohann *et al*. 2006, Sykes *et al*. 2011). This can result directly from differences in experimental protocol or genetics, but also via an interaction between genotype and laboratory environment (Chesler *et al*. 2002).

Given the highly-developed brain and behavioral plasticity of cuttlefish (*e.g*. Dickel *et al*. 2000, Poirier *et al*. 2004, Agin *et al*. 2006), coupled with the embryo's ability to perceive, and be influenced by, the world outside the egg (Romagny *et al*. 2012), it seems likely that artificial conditions during embryonic development may affect the behavior of hatchling *S. officinalis*. If so, it could cause their behavior to differ from animals developed under natural conditions. In that case, experiments on cuttlefish reared in an artificial environment might yield unrealistic behavioral results. The natural environment provides numerous sources of sensory stimulation not present in most laboratory settings. For cuttlefish, these could include natural light, a natural light cycle, water-borne odors from numerous other organisms, water movement (currents, tidal rhythm, etc.) and fluctuations in temperature. In contrast, cuttlefish eggs in most artificial situations are exposed to more stable physical parameters in their environment such as temperature and light, a lack of certain natural stimuli (including currents and organisms such as epibionts and plankton), but a surfeit of unnatural ones (including unnatural color schemes and anthropic handling). No study has directly addressed whether incubation in a standard laboratory setting would have a detectable impact on basic hatchling behavior.

In addition to corroborating or casting doubt on existing experimental results, this question has implications for cuttlefish culture. Female *S. officinalis* lay their eggs on vertical objects on the seabed including seagrasses and algae (Basuyaux & Legrand 2013), as well as basket traps set by commercial fishermen to capture adult cuttlefish. Each year, thousands of cuttlefish eggs are laid on such traps in the English Channel, and when these traps are retrieved, the eggs are discarded, damaged or destroyed (Blanc & Daguzan 1998). Potentially, these eggs could be saved and cultured artificially. Indeed, several authors (*e.g*. Pascual 1978, Forsythe *et al*. 1994, Domingues *et al*. 2002) have already managed to culture multiple generations in the laboratory. This raises the possibility of redirecting a normally squandered resource for later release, harvest or experimentation. An assessment of the effects of an artificial incubation environment on some basic behaviors necessary for growth and survival is a critical first step in the pursuit of this possibility.

Our experiments aimed to evaluate the reliability of existing results obtained with cuttlefish incubated *ex situ* and their chances of survival in the first week by quantifying the effect of laboratory incubation on the subsequent hatchling behavior of this species. In order to assess the effect of an artificial incubation environment, we tested hatchling size (one measure), body patterning behavior

(three measures) and predatory behavior (five measures) of hatchlings incubated in the sea and in the lab. These nine measures represent only a small fraction of the behavioral tests possible (other potential assays include prey preference trials, reaction to predator odor and the Prawn-in-a-Tube procedure) but our goal was to assess behavior directly relevant to hatchling survival. These data will help us to assess the validity of earlier experimental results obtained from hatchlings incubated in the laboratory and add to our knowledge about cuttlefish culture.

METHODS

Egg collection and treatment: Sepia officinalis eggs (135) were recovered on May 16, 2014 by a professional diver (OB) from a pre-placed tether at a depth of 3m in the area of Pointe d'Agon (48°59.547N-1°38.671W, English Channel) and taken to Synergie Mer et Littoral (SMEL), a marine research facility in Blainville-sur-Mer, France. These eggs were in the very early stages of embryonic development (< 15 days old) and were designated as the artificially-incubated treatment group ("Lab"). At the same time, 150 eggs from the same location were left *in situ* and designated as the naturally-incubated treatment group ("Sea"). "Lab" eggs were placed in a polyethylene basket (29 × 19 × 14.5 cm) and immersed in an aerated 200 L tank matched to local sea surface temperature (14.5-18.9 °C). Temperature affects the rate of embryonic growth and yolk consumption, as well as the ultimate size of the hatchling (Boletzky 1983, Bouchaud 1991). (Though regional surface sea temperature was monitored throughout the experiment, no local temperature data are available for the eggs left *in-situ*.) Eggs were exposed to artificial illumination from 08 h 00 to 18 h 00.

As the estimated date of hatching approached (based on the technique of accumulated degree days from Basuyaux (2011)), "Sea" eggs were monitored by a professional diver before being collected June 26, 2014 and transported to the laboratory, 7-12 days before the dates of hatching. "Sea" embryos were estimated (Romagny *et al*. 2012) to be between stages 26 and 30 when collected. After "Sea" egg collection, both "Lab" and "Sea" eggs were placed in separate hatching tanks (90 L, 720 × 470 × 360 mm, opaque white) with 100 % daily water renewal (18.9-19.2 °C).

Hatching occurs mostly at night (Paulij *et al*. 1991), and hatchlings were collected at 08 h 00 each morning. (The morning after hatching was designated as "Day 1".) Hatchlings were placed in individually-labeled vials (diameter = 4.5 cm, height = 6.4 cm) with numerous perforations for water flow. Cuttlefish remained in these floating tubes in their natal treatment tank until testing. Individual identity was maintained throughout the study. Experiments did not begin on a treatment group until at least three hatchings had occurred in a single night. All experiments were conducted using water (19.2 °C) from hatchlings' natal tank. In total, 47 individuals from the "Sea" group (hatched July 4-7) and 51 from the "Lab" group (hatched July 7-10) were tested.

Mantle Length: Immediately prior to their first encounter with prey, Mantle Length (ML) was estimated in order to compare growth between the two incubation conditions. Day 4 was chosen rather than Day 1 as it reflects the consumption of most or all of the embryonic yolk reserve prior to any prey ingestion (Boletzky 1983). To estimate cuttlefish ML, two photographs were taken in which the cuttlefish was lying flat on the bottom and not moving. Using the image analysis software ImageJ, the distance between the tip of the mantle and a point midway between the eyes was measured. Eyes were used instead of the mantle edge since they were easily identifiable in photographs. The two measurements were averaged, unless they deviated by more than 0.2 cm from each other, in which case a third photograph and measurement were made. The most disparate of the three values was eliminated and the resulting pair of values was averaged.

Behavioral tests

Uniform Background Test

Testing: On the morning after hatching (Day 1), hatchlings were selected in a randomized order for testing. Between 9 h 00-10 h 30, six cuttlefish were placed individually in small (diameter = 100 cm, height = 1 cm) uniform medium gray (Mean Gray Value (MGV) = 101 +/- 3.9) arenas and filmed concurrently for 22 min. This was repeated until all of the day's hatchlings had been tested. The arenas were lit indirectly with two Zenitech 0221A 500W lights (540 lux) mounted on tripods and filmed with a Panasonic HDC-SEM60 camera. After filming, cuttlefish were returned to their individually-labeled tubes in their natal tanks.

Video analysis: Using VLC Media Player, two snapshots were captured of each individual at 11 and 21 min, allowing the cuttlefish the time to habituate to the experimental arena. Snapshots were only taken during moments after cuttlefish had settled and were motionless. Following the method developed by Di Poi *et al*. (2013), we used ImageJ to select the outline of the cuttlefish mantle and measure the Heterogeneity Index (HI). This value was calculated in ImageJ by an equation using the deviation of the MGV of each individual pixel (x) from the MGV of the whole cuttlefish (x), and the total number of pixels selected (N): HI = 1N(x-x)2 (Di Poi *et al*. 2013). This value measures body pattern uniformity (higher HI = less uniform). The two values were averaged for each individual.

We also used ImageJ to compare the MGV of the cuttlefish to the MGV of the substrate. After selecting the outline of and measuring the MGV of the cuttlefish mantle, we then measured an equivalently-sized portion of the adjacent substrate. By dividing the mantle MGV by the substrate MGV, we were able to calculate a ratio from 0 to 1 expressing the degree of match between the mantle and the substrate (0 = no match, 1 = perfect match). The two values were averaged for each individual and group medians calculated. A low HI and high MGV match indicate a good match between mantle appearance and the surrounding substrate.

Disruptive Background Test

Testing: Using the same set-up and procedure as for the Uniform Background Test, cuttlefish were filmed individually on Day 2 for 25 min against a "checkered" background (squares = 3 × 3 mm) between 10 h 30 and 12 h 00. After filming, cuttlefish were returned to their individually-labeled tubes in their natal tanks.

Video analysis: Using VLC Media Player, two snapshots were taken of each individual at 15 and 22 min. In order to assess disruptiveness, we employed the procedure developed by Barbosa *et al*. (2007), and graded 11 components of body pattern on a scale of 0-3 based on relative strength of expression. This resulted in a "Disruptive Score" that ranged from 0 to 33 (higher score = more disruptive). The two values were averaged for each individual.

Initial Prey Encounter

Shrimp collection: Shrimp (*Crangon crangon* and *Palaemonetes* sp.) were collected as needed in the vicinity of Blainville-sur-Mer using hand nets in small pools during low tide. They were maintained in aquaria with well-oxygenated water for one to five days prior to testing. Only shrimp between 0.7 cm and 1.4 cm were selected for predation experiments. One to four hours before testing, shrimp were collected from the larger aquaria and placed in smaller individual containers until the moment of testing.

Testing: Cuttlefish were tested four days after their date of hatching (between 12 h 00 and 18 h 00), from July 7 to 13, 2014 and were not fed prior to testing. Four cuttlefish were tested at a time in individual containers (10.5 × 12 × 5.4 cm) filmed by a Canon IXUS camera positioned 40-43 cm away and lit indirectly with a Zenitech 0221A max 500 W (500 Lux, light Meter Testo). Testing occurred in 140 mL of water from each cuttlefish's respective hatching tank at temperatures equal or slightly above hatching tank temperature (18.3-19.5 °C). Cuttlefish were gently removed from their hatching tank in their vials, transported to the testing arena and transferred into their respective container using a spoon. They were allowed to acclimate in the arena for five minutes before testing. At that point, video recording commenced, and shrimp were poured from small tubes into the testing arena. Video recording was stopped after five minutes, at which point both shrimp and cuttlefish were removed from the arena and cuttlefish were transferred back into their respective vials and transported back to their hatching tank.

Video analysis: Videos were analyzed using VLC Media Player and ImageJ software. Several variables were recorded: Attempted Capture Rate, Capture Rate, Success Rate, Latency to Capture and Distance of Detection. Capture Rate measured the percent of cuttlefish from each group that captured their shrimp. Success Rate was calculated as the percentage of total capture attempts that resulted in a successful capture for each group (failed attempts at capture were defined as tentacle extension without successfully subduing the shrimp). In instances in which the shrimp was successfully captured, we calculated Latency to Capture as the time between shrimp detection [defined as the moment that the cuttlefish oriented towards the shrimp (Messenger 1968)] and successful capture (defined as the moment the cuttlefish's tentacles touched the shrimp and successfully subdued it) for each group. Finally, for Distance of Detection, a snapshot was taken (using VLC Media Player) at the moment of detection and the distance between the shrimp and nearest cuttlefish eye was measured with ImageJ software. Together, these five variables are indirect measures of feeding motivation, visual acuity, cautiousness and overall predation ability.

Statistical analysis

R and StatXact®7 (Cytel Studio©) were used to conduct all statistical analyses. Data analyzed were ML, HI, Disruptive Score, Percentage of Cuttlefish that Captured Shrimp, Success Rate (total number successful captures/total number of attempted captures), Latency to Capture and Distance of Detection. The ML met parametric assumptions (Shapiro Wilk, $\alpha = 0.05$, $p > 0.05$), so data are reported as means +/- SEM and a t-test was used to compare the two groups. All other data failed to meet parametric assumptions (Shapiro Wilk, $\alpha = 0.05$, $p < .0001$), so data are reported as medians +/- SEM and non-parametric Fisher Exact tests and permutation tests were employed.

Ethical considerations

All procedures were carried out in accordance with the Directive of the European Parliament and of the Council of the European Union (2010/63/UE) regarding the care and use of animals for experimental procedures, and approved by the regional ethical committee (Comité d'Ethique NOrmandie en Matière d'EXpérimentation Animale, CENOMEXA; agreement number 54) (cuttlefish: project authorization on September 25, 2014). Experiments were supervised by several individuals (ASD, CJA, LD and OB) certified to work with cephalopods.

RESULTS

Mean Mantle Length on Day 4

Mean ML on Day 4 of "Sea" cuttlefish (n = 47) was 1.04 cm (+/- 0.01 cm SEM) and 1.03 cm (+/- 0.01 cm SEM) for "Lab" cuttlefish (n = 51) with no significant difference between groups (t-test, $\alpha = 0.05$, $p = 0.28$).

Uniform Background Test

Heterogeneity Index

Median HI was 7.12 (+/- 0.22 SEM) for "Sea" (n = 51) and 8.21 (+/- 0.30 SEM) for "Lab" (n = 47) (Fig. 1A). A higher HI indicated a less uniform body pattern. A permutation test showed that "Sea"-incubated cuttlefish had significantly higher HI (less uniform body pattern) than

"Lab"-incubated cuttlefish (permutation test, $\alpha = 0.05$, $p = 0.02$).

Mean Gray Value Match

Median MGV match was 0.79 (+/- 0.06 SEM) for "Sea" (n = 51) and 0.78 (+/- 0.06 SEM) for "Lab" (n = 47) (Fig. 1B). A higher MGV match indicated better correspondence to substrate color. A permutation test showed no significant difference between groups (permutation test, $\alpha = 0.05$, $p = 0.95$).

Disruptive Background Test

Disruptive Score

Median Disruptive Score (out of 33) was 9.50 (+/- 0.90 SEM) for "Sea" (n = 51) and 8.37 (+/- 0.74 SEM) for "Lab" (n = 47) (Fig. 2). A higher Disruptive Score indicated a more disruptive body pattern. A permutation test showed no significant difference between groups (permutation test, $\alpha = 0.05$, $p = 0.77$).

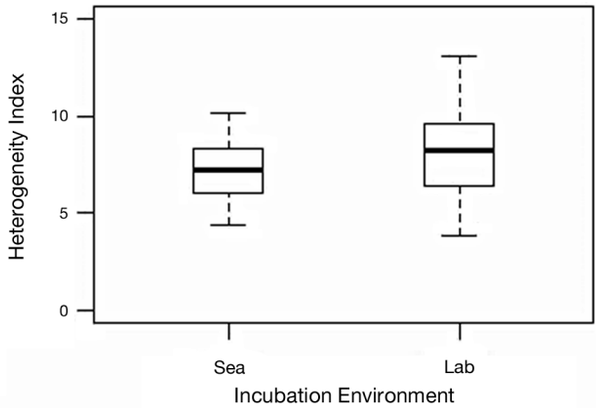

Fig. 1A. – Heterogeneity Index (HI) against a uniform background on Day 1 reflects the ability to produce a uniform body pattern. Data are displayed as median (bars), inter-quartiles (boxes) and minimum/maximum values (whiskers). Lab-incubated cuttlefish show more uniformity (lower HI) than Sea-incubated (permutation test, n = 47, 51, $\alpha = 0.05$, $p = 0.02$).

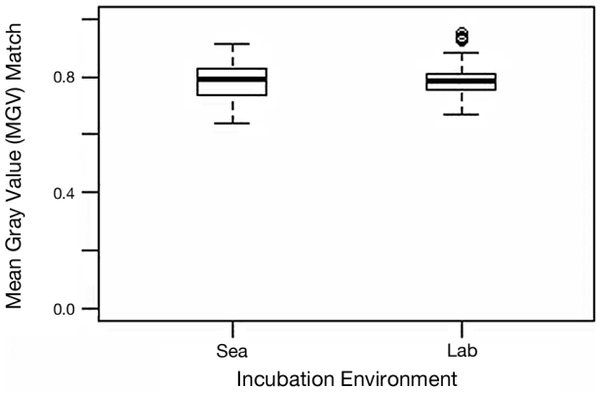

Fig. 1B. – Mean Gray Value (MGV) match to a uniform background on Day 1 reflects the ability to produce general color resemblance. Data are displayed as median (bars), inter-quartiles (boxes), minimum/maximum values (whiskers) and outliers (circles). There is no significant difference between groups (permutation test, n = 47, 51, $\alpha = 0.05$, $p = 0.95$).

Initial Prey Encounter

Attempted Capture Rate

Thirty-one of 47 (66 %) of "Sea"-incubated hatchlings and 37 of 51 (73 %) "Lab"-incubated hatchlings attempted shrimp capture with no significant difference between groups (Fisher Exact test, $\alpha = 0.05$, $p = 0.52$) (Table I).

Capture Rate

Twenty-nine of 47 (61.7 %) of "Sea"-incubated hatchlings and 36 of 51 (70.6 %) "Lab"-incubated hatchlings captured their shrimp during the first five minutes of their initial prey encounter with no significant difference between groups (Fisher Exact test, $\alpha = 0.05$, $p = 0.40$) (Table I).

Success Rate

"Sea"-incubated cuttlefish (n = 29) made a total of 33 attempts to capture shrimp, of which 29 (87.9 %) were successful. "Lab"-incubated cuttlefish (n = 36) made a total of 41 attempts to capture shrimp, of which 36 (90 %) were successful, with no significant difference between treatment groups (Fisher Exact test, $\alpha = 0.05$, $p > 0.99$) (Table I).

Latency to Capture

An extreme outlier (> 120 sec) was removed from each group prior to analysis. The median latency between detection and attack was 5.5 sec (+/- 2.41 sec SEM) for

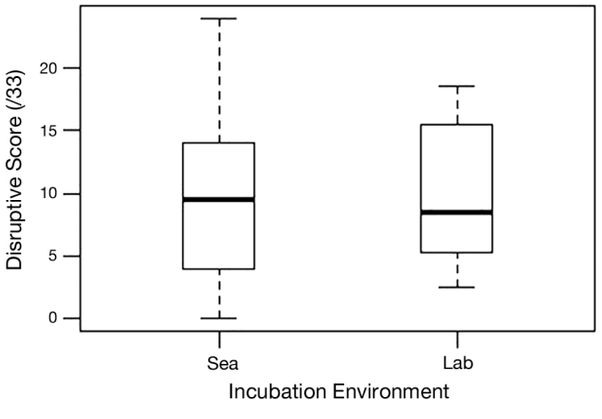

Fig. 2. – Disruptive Score (/33) against a checkered background over time on Day 2 reflects disruptive body-patterning ability. Data are displayed as median (bars), inter-quartiles (boxes) and minimum/maximum values (whiskers). There is no significant difference between groups (permutation test, n = 47, 51, $\alpha = 0.05$, $p = 0.77$).

Table I. – Attempted Capture Rate, Capture Rate and Success Rate during the initial encounter with prey on Day 4. No significant difference exists between treatment groups in these tests (Fisher Exact test, n = 47, 51, $\alpha = 0.05$).

	Definition	Sea	Lab	p
Attempted Capture Rate	percentage of cuttlefish that attempted captured	66.00 %	73.00 %	0.52
Capture Rate	percentage of cuttlefish that captured shrimp	61.70 %	70.60 %	0.40
Success Rate	the percentage of successful captures	87.90 %	90.00 %	0.99

"Sea" (n = 28) and 5 sec (+/- 1.36 sec SEM) for "Lab" (n = 35) with no significant difference between groups (permutation test, $\alpha = 0.05$, p = 0.08) (Fig. 3A).

Distance of Detection

The median latency between detection and attack was 4.31 cm (+/- 0.50 cm SEM) for "Sea" (n = 29) and 3.24 cm (+/- 0.49cm SEM) for "Lab" (n = 36) with no significant difference between groups (permutation test, $\alpha = 0.05$, p = 0.65) (Fig. 3B).

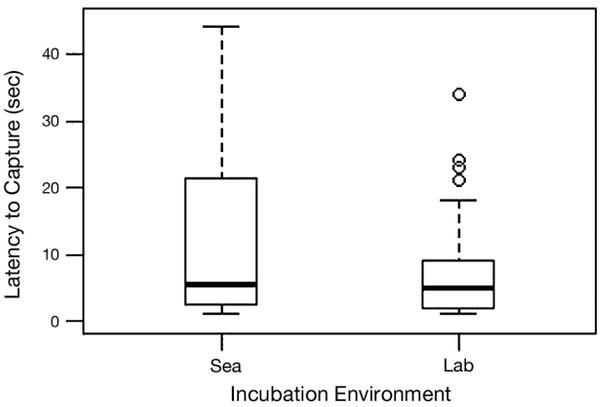

Fig. 3A. – Latency to Capture (sec) is the time between detection and capture on Day 4. One extreme outlier was eliminated from each group before analysis. Data are displayed as median (bars), inter-quartiles (boxes), minimum/maximum values (whiskers) and outliers (circles). No significant difference exists between treatment groups (permutation test, n = 28, 35, $\alpha = 0.05$, p = 0.08).

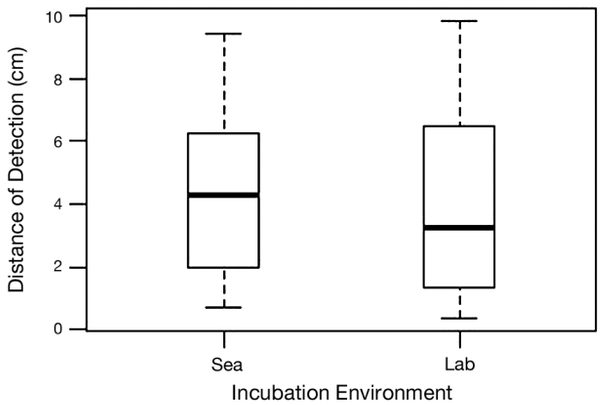

Fig. 3B. – Distance of detection (cm) on Day 4. Data are displayed as median (bars), inter-quartiles (boxes) and minimum/maximum values (whiskers). No significant difference exists between treatment groups (permutation test, n = 29, 36, $\alpha = 0.05$, p = 0.65).

DISCUSSION

We investigated the effects that an artificial incubation environment has on subsequent hatchling size and behavior. Contrary to our expectations, we saw only one difference between "Sea" and "Lab" groups in nine measures of size, body patterning and predation, suggesting that artificial incubation conditions had little effect on rates of development and subsequent hatchling behavior.

Mantle Length estimated on Day 4 represents embryonic and hatchling growth determined exclusively by yolk reserves. Water temperature can have dramatic effects on the pace and duration of embryonic growth, which in turn affects the rate of yolk consumption, feeding motivation and predatory behavior. Higher temperatures accelerate growth and yolk absorption, resulting in shorter development time (which can vary as much as 40 to 90 days) but also in smaller hatchlings (Boletzky 1983, Bouchaud 1991). Temperature is also associated with differences in the emergence of hatchling pursuit behavior (Dickel *et al*. 1997). While the "Lab" cuttlefish in our experiment (and in typical artificial environments) experienced steady water temperatures, it is likely that the thermal regimes experienced by the "Sea" group included frequent fluctuations due to currents and tides. Because of this more complex temperature regime, we had expected that "Sea" hatchlings would differ in size from "Lab" cuttlefish maintained at the same mean temperature. In contrast, ML was similar between the two treatment groups, suggesting that the overall thermal mean is more relevant to embryonic growth than any thermal fluctuations experienced. This possibility deserves further exploration, as no *in situ* temperature measurements were made during this experiment, and prior investigations of temperature and embryo growth have involved steady thermal regimes (*e.g*. Boletzky 1983, Bouchard 1991).

In addition to size, we measured two body patterns. These tests have been used extensively in the literature (*e.g*. Chiao *et al*. 2005, Barbosa *et al*. 2007, Barbosa *et al*. 2008, Di Poi *et al*. 2013) to measure defensive ability, since body patterning is the primary means of defense in cuttlefish (Hanlon & Messenger 1996). Heterogeneity Index (HI) measures the overall uniformity of pattern and a hatchling with low HI is considered to be well-camouflaged on a uniform background. Hatchlings

are notoriously poor at producing uniform body patterns (Hanlon & Messenger 1988, Dickel *et al*. 2006), and we hypothesized that animals enriched by the natural environment would be better at this task. Results show some evidence that this is indeed the case. Heterogeneity Index against a uniform background ranged between 3.87 and 13.06 and "Lab" hatchlings showed a significantly lower HI (less disruptive) than "Sea" hatchlings. The fact that "Lab" incubated cuttlefish are slightly better at producing uniform body patterns is perhaps due to fact that they experienced an artificially homogeneous substrate (PVC plastic) during development and deserves further inquiry. This potential difference should also be considered when interpreting past and future behavioral results.

In contrast, while "Lab" cuttlefish showed stronger uniform pattern-matching in this test, the same was not true of their ability to match the color of the background. Mean Gray Value match assessed the correspondence between the overall color of the mantle to that of the immediately adjacent substrate, and values closer to 1 indicate a high degree of correspondence. Mean Gray Value match ranged between 0.64 and 0.95, and there was no significant difference between the MGV match of "Sea" and "Lab", suggesting that unlike HI, the color-matching abilities are not affected by incubation environment.

Body patterning against a checkered background was also tested and the overall "disruptiveness" evaluated. Disruptive Score allowed us to obtain an overall sense of difference in the disruptive pattern between incubation groups. Disruptive Scores ranged from 8 to 10 (out of 33) and did not significantly differ between groups. We had expected "Sea" cuttlefish to be more efficient in their cryptic abilities due to their prenatal exposure to a variegated natural background. In contrast, the lack of difference between the "Sea"- and "Lab"-incubated hatchlings in Disruptive Score suggests that this feature of body patterning behavior is not affected by incubation environment.

While we have followed traditional body pattern interpretations and concluded that a less disruptive (lower HI) and better color match (higher MGV match) on a uniform substrate and that a higher Disruptive Score on a checkered substrate are most adaptive, it is not entirely clear whether this is the case. Cuttlefish have multiple strategies for creating crypsis (background matching, disruptive coloration and deceptive resemblance). Any particular body pattern may function in multiple cryptic strategies and two different patterns may be equally effective in certain circumstances (Hanlon & Messenger 1988). For instance, a cuttlefish displaying a disruptive body pattern on a uniform background may not be attempting background matching, but rather deceptive resemblance of stone or shell fragments. This possibility is supported by the fact that hatchling cuttlefish often display seemingly "inappropriate" body patterns (*i.e*. a disruptive body pattern on a uniform substrate) for the first two to three days (Hanlon & Messenger 1988). Perhaps we have misinterpreted the "ideal" cryptic strategy. It seems instead that the proper strategy is determined by size and thus changes throughout the lifetime of an individual (Hanlon & Messenger 1988). Further investigation is necessary to resolve this question, and the interpretation of our camouflage results may change as we learn more about strategies for different size classes on a variety of substrates.

Finally, we measured five aspects of predatory behavior during hatchlings' first encounter with their preferred prey, shrimp (Wells 1958): Attempted Capture Rate, Capture Rate, Success Rate, Latency to Capture and Distance of Detection. Successful predation is critical at this time, since hatchlings' yolk reserves are nearly depleted and they are at their most vulnerable (Wells 1958). While many factors influence these variables, making it impossible to impute a single cause to each measure, together they give us an overall sense of motivation and predation efficiency in hatchling cuttlefish and allow us to identify differences due to incubation environment. We hypothesized that the enrichment present in the natural incubation environment would stimulate development and result in more adept hatchlings with higher Capture Rate and visual skills. We also reasoned that hatchlings incubated in the natural environment would show more cautiousness due to their experience with visual and odor cues from other organisms and differences in feeding motivation due to their exposure to a more variable temperature regime. In contrast, none of these variables differed significantly between groups and Capture Rate was very high (between 85 % and 90 % in accordance with Messenger 1968). This suggests that lab-raised hatchlings would be equally capable of feeding during this critical period of growth.

The literature suggests a high degree of behavioral plasticity in hatchling and juvenile cuttlefish. In terms of growth, enrichment and environmental factors such as dark tank color promote growth (Dickel *et al*. 2000, Sykes *et al*. 2011), while experience with external stimuli such as a natural substrate or conspecifics alter behavior (Poirier *et al*. 2004, Poirier *et al*. 2005). Given the cuttlefish's high plasticity in other areas, the strong evolutionary pressure on the tested behaviors and the embryo's ability to perceive beyond the egg, it seemed logical that prenatal environment would have dramatic effects on size and behavior. This was not supported by our data. One potential reason for this unexpected outcome is that our experiment focused on hatchlings fewer than five days old, and it is possible that differences due to incubation environment would appear later in development or in some unmeasured aspect of post-predatory growth or behavior. Behavioral plasticity probably requires a fair amount of brain development, especially of such crucial structures in the supraesophageal mass as the vertical lobe. These structures are still developing during the first few months (Dickel *et al*. 1997, Dickel *et al*. 1998, Dickel *et al*. 2013), so perhaps behavioral plasticity only

develops later. Alternatively, it may simply be that these particular aspects of behavior are highly pre-programmed and not subject to plasticity.

There were some significant limitations to the data we were able to collect during this experiment. Logistical constraints necessitated that "Sea" eggs be removed from the wild 7 to 12 days prior to the majority of eclosions (stages 26-30) when embryos were most sensitive to external stimulation. It is also possible that the laying site from which the eggs were collected was atypically barren of stimulation and enrichment, which would limit the effect of natural incubation. This could be addressed in the future by collecting eggs from multiple laying sites and via censuses of the marine life that typically occurs at these sites. Most importantly, we were only able to investigate a limited range of behaviors in our experiment. It is possible that plasticity is not manifest in hatchling growth, body patterning or predation, but is present in other aspects of behavior, such as learning, memory or activity.

Despite the limitations in experimental design which constrain our conclusions from this experiment, the lack of strong differences between groups is manifest. The overall lack of difference between incubation groups bolsters confidence in existing behavioral findings and has practical implications for cuttlefish conservation. These results, added to the success of several authors (*e.g.* Pascual 1978, Forsythe *et al.* 1994, Domingues *et al.* 2002) in culturing multiple generations in the laboratory, suggest that artificial incubation does not alter natural hatchling behavior. This implies, but in no way guarantees, that the survival, at least of hatchlings, will not be compromised, although survival was not tested directly in our experiment. In the future, cuttlefish eggs laid on basket traps normally lost during the harvest of adults could instead be incubated and repurposed for conservation, research or as an additional source of product. Indeed, larger-scale trials examining the feasibility of egg and hatchling incubation in artificial tanks are already in progress.

The obvious next step in the characterization of the effects of artificial incubation is to repeat these experiments with cuttlefish collected from the wild at hatching and compare them to eggs spawned by captive females. This may allow us to gauge more fully the entire course of embryonic development as well as the contribution that a laboratory setting can have on the embryo indirectly through any maternal transmission of hormones (as seen in birds-Groothuis & Schwabl 2008). In addition, direct observation of eggs and hatchlings in the field would give a clearer picture of the stimuli experienced by embryos in the wild and the natural behavior of hatchlings. Despite the difficulties, field observations (via SCUBA, remote sensing or photography) are certainly possible with today's technology and would do much to elucidate the effect of prenatal stimulation on subsequent behavior. For example, measurements of physiological parameters such as turbidity would allow us to estimate the visual field of cuttlefish embryos and censuses of marine fauna at laying sites would provide an idea of what odor cues might be sensed. Finally, behavioral experiments conducted in the natural environment would be the ultimate assessment of the effect of incubation environment.

CONCLUSION

This experiment provides evidence that incubation in a laboratory environment does not strongly affect three fundamental aspects of behavior and survival in cuttlefish hatchlings: embryonic growth, body patterning and predation ability. The one difference identified in uniform body patterning urges further investigation as well as caution when interpreting results from body pattern experiments with artificially-incubated hatchlings. (We may also need to reevaluate "conventional" interpretations of what constitutes the best cryptic strategy in a particular situation.) The overall lack of differences between naturally- and artificially-incubated hatchlings bolsters confidence in existing experimental data. Our results are also encouraging from a conservation perspective; artificially-incubated cuttlefish could augment fishery stocks or replace wild-caught cuttlefish in certain situations. Future work on this question should strive to test cuttlefish spawned from captive females and collected directly from the wild at the moment of hatching and to test a broader range of behaviors for the entire juvenile period.

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