Optimising the hatching success of artificially incubated eggs for use in a conservation program for the western saw-shelled turtle (*Myuchelys bellii*)

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ABSTRACT

Artificial incubation of eggs and the release of hatchlings into the wild is a common conservation intervention designed to augment threatened turtle populations. We investigate a range of incubation temperatures to establish an optimal temperature for maximum hatching success of western saw-shelled turtle (*Myuchelys bellii*) eggs. We report on the influence of incubation temperature on incubation duration and hatching success and describe two experimental incubation methods which, for the same incubation temperature (27°C), resulted in 77% and 97% hatching success, respectively. Eggs were incubated at constant temperatures (27°C, 28°C and 29°C) to determine the influence of temperature on incubation period, hatchling morphology and external residual yolk. Incubation duration was negatively correlated with incubation temperature. We report on the morphology of eggs and hatchlings and show that their dimensions are positively correlated with maternal adult size and mass. A constant incubation temperature of 27°C produced the highest hatching success and smallest external residual yolk on hatching and is therefore recommended for incubation of eggs for population reinforcement programs. Our study is the first to optimise artificial incubation procedures for *M. bellii* and will be a valuable resource for *M. bellii* and other threatened freshwater turtle conservation initiatives.

Keywords: artificial incubation, Bell's turtle, conservation, endangered species, freshwater turtle, head-starting, population augmentation, population reinforcement, wildlife management.

Introduction

Almost half of Australian freshwater turtle species are under threat, with 11 of the 27 species listed as vulnerable, endangered, or critically endangered (Van Dyke *et al.* 2018; Turtle Taxonomy Working Group 2021). Predation of nests by the introduced European red fox (*Vulpes vulpes*) decreases juvenile recruitment and has been suggested as a predominant driver of decline in many Australian freshwater turtle populations (Thompson 1983; Spencer *et al.* 2017; Van Dyke *et al.* 2019). The artificial incubation of turtle eggs and release of neonate hatchlings into the wild is an important conservation strategy designed to avoid threats to the most vulnerable stage in turtle life history, and to increase recruitment of juveniles in threatened turtle populations. This strategy is particularly beneficial where the direct management of threats to turtle populations is difficult or unachievable.

Incubation temperature does not influence the sex of Australian chelid turtles (Mazzoleni *et al.* 2020). Nevertheless, temperature has a profound influence on incubation duration, hatching success, hatchling condition, phenotype, and posthatching growth, fitness and survival (Micheli-Campbell *et al.* 2011; Noble *et al.* 2018). Turtle eggs have a narrow range of incubation temperatures conducive to a high proportion of

viable hatchlings, and temperatures outside of this viable incubation range reduce embryo survival (Birchard 2004). It is therefore imperative that artificial incubation methods use temperature regimes that are appropriate to each species.

The western saw-shelled turtle (*Myuchelys bellii*) is an endangered Australian freshwater turtle (IUCN 2021). Also known as Bell's turtle, *M. bellii* is endemic to the high-elevation rivers and streams of the Namoi River, Gwydir River and Border Rivers basins (Chessman 2015). Populations predominantly comprise mature individuals, and juvenile recruitment is low, possibly due to high levels of nest predation by the introduced European red fox (Fielder *et al.* 2014). Hence, artificial egg incubation may be a valuable conservation strategy for *M. bellii*. The optimal techniques for incubating *M. bellii* eggs are unknown so a better understanding of the factors affecting hatching success will improve conservation prospects and outcomes for future population reinforcement programs. Our study aimed to investigate the influence of incubation temperature on incubation duration, identify an optimal constant incubation temperature and hydric conditions, and establish an incubation method to maximise hatching success for head-starting *M. bellii*. This method should help secure viable populations of the species as part of a New South Wales Government conservation program entitled 'Turtles Forever: Securing Australia's Wild Populations of Bell's Turtle'.

Methods

Our study was conducted over two breeding seasons (breeding season 1: November 2017 to March 2018; and breeding season 2: November 2018 to March 2019). Turtles were captured in the Macdonald River within 20 km of Bendemeer, New South Wales (30.870°S, 151.151°E) Australia. Field capture methods were identical to those reported by Fielder *et al.* (2014). Adult females were palpated in the rear leg inguinal pockets to detect hard-shelled eggs, and gravid females were transported to the University of New England. Gravid female turtles were weighed (±0.01 g), and egg-laying was induced by intramuscular injection of 2.2 mL kg⁻¹ of Ilium Syntocin (Troy Animal Healthcare, Sydney), active constituent 10 IU mL⁻¹ oxytocin, into the rear thigh (Fielder *et al.* 2014). Following injection, females were placed unrestrained into individual opaque plastic tubs (650 × 400 × 300 mm) containing 10 L of aged water. The tubs were covered with a dark cloth and the females were monitored throughout oviposition. Over the two breeding seasons, a total of 27 separate turtles were induced and each laid a single clutch of 11–30 eggs. In breeding season 1, 13 turtles were induced and in breeding season 2, 14 turtles were induced. Following egg-laying, female turtles were weighed and later released at their point of capture.

As each egg was deposited, the time was recorded, and eggs were immediately weighed (±0.001 g) and measured using Vernier callipers (±0.1 mm). Eggs were handled gently, and care was taken to maintain egg orientation (Limpus *et al.* 1979). Eggs were placed into clear plastic containers, directly onto a bed of moist vermiculite (1.1:1 parts water to vermiculite by weight) (Fielder *et al.* 2014) such that approximately one-third of the shell was in contact with the vermiculite. The eggs were placed in two rows and spaced at least 20 mm apart. To account for clutch effects, in breeding season 1, a split clutch design was used in which eggs from each female were systematically divided into three groups that were each assigned to one of three constant incubation temperatures: 27°C (*n* = 83 eggs), 28°C (*n* = 82 eggs) or 29°C (*n* = 80 eggs). Eggs were incubated in Panasonic MIR-554 406L incubators (±0.5°C). In breeding season 2, all eggs (*n* = 279) were incubated in a single incubator at a constant 27°C.

In breeding season 1, eggs were placed into plastic incubation containers (110 mm × 170 mm × 70 mm) and covered with loose-fitting plastic lids to allow airflow, and vermiculite moisture was replenished every 2–3 days. In breeding season 2, the plastic incubation containers were larger (150 mm × 220 mm × 70 mm) and were sealed with plastic film (Glad® Wrap, Clorox Australia Pty Ltd) held in place with a rubber band and a tight-fitting clip-on lid. The lid had a 146 mm × 216 mm area cut out of the centre leaving a 20 mm border. The containers were weighed fortnightly to track moisture loss. As necessary, a syringe was used to replenish water directly into the vermiculite, and care was taken to avoid water directly contacting the eggs. Eggs were checked daily and the date of hatching for each egg was recorded as the day on which the hatchling had completely emerged from the egg.

Upon hatching, the length and width of the external portion of yolk sac was measured using a ruler (±0.5 mm) to approximate the two-dimensional area (mm²) of yolk that had not been internalised. The total amount of residual yolk, including the proportion of yolk that had been internalised, was not recorded. In both breeding seasons, hatchlings were placed back into their incubator until external yolk was internalised, usually within 24–48 h, although some hatchlings took up to 72 h for complete internalisation of yolk. We used containers with 3–5 mm depth of aged water to individually house hatchlings in the incubator. Once yolk sacs had been internalised, hatchlings were weighed (±0.001 g), and Vernier callipers (±0.1 mm) were used to measure straight carapace length, carapace width, midline plastron length and plastron width (just posterior to the bridge). To derive a proxy for body condition index, the area of the plastron was calculated using the formula for finding the area of an ellipse. Body condition index = body mass/((plastron length/2) × (plastron width/2) × π). After being weighed and measured the hatchlings were placed into plastic holding tubs

(320 mm × 570 mm × 380 mm) with an overhead UV-B light source, a basking platform, and 50 mm depth of aged water maintained at 22°C using a submersible aquarium heater. Hatchlings were released at their mother's point of capture 3–4 weeks after hatching.

To identify the genotypic sex of a sample of hatchlings from breeding season 2, blood samples were collected from 73 hatchlings using QIAcard FTA Elute Micro cards (QIAGEN, Melbourne) and analysed using a PCR sex test (Dissanayake *et al.* 2022).

Statistical analyses

We conducted tests in R (ver. 3.5.0; R Core Team 2018) to examine the effects of female body size on clutch size and mass. Parametric statistics were used for analyses when assumptions of the tests were met (Pearson's), otherwise non-parametric Spearman rank correlations were used. We tested the effect of female mass on clutch size (number of eggs), clutch mass, time lag from injection with oxytocin to commencement of oviposition, and duration of oviposition. We also tested the effect of female carapace length on clutch size and clutch mass. Finally, we tested the influence of clutch size on duration of oviposition after injection.

To examine factors influencing individual eggs (rather than entire clutches), we constructed mixed-effects models in R (ver. 3.5.0; R Core Team 2018) using the package lme4 (ver. 1.1-15; Bates *et al.* 2015). To control for female (and clutch) effects and avoid pseudoreplication, we included female ID as a random effect for all mixed-effects models. We assessed the significance of comparisons using the Anova function in the car package (ver. 2.1-6; Fox and Weisberg 2011) with Type II sums of squares and the Chi-square test statistic. We used quantile–quantile plots (qqp) and residual plots to check model fit. When necessary, the qqp function in the car package and the fitdistr function in the MASS package (ver. 7.3-51.4; Venables and Ripley 2002) were used to determine the appropriate model distribution. All models were linear models without data transformations unless otherwise specified.

We used these models to conduct the following tests: the effect of female mass on egg mass, hatchling mass, and hatchling carapace length; the effect of female carapace length on egg mass and hatchling mass; the effect of egg mass on hatchling mass; the effect of incubation temperature on incubation duration (after accounting for egg mass), hatching success (glmer) (Binomial distribution), residual yolk sac area (linear model on $\log_{10}$-transformed data), hatchling mass, hatchling straight carapace length, and hatchling body condition index; the effect of egg mass on incubation duration; and the effect of the incubation method applied during each breeding season on hatching success of eggs incubated at 27°C. For tests using incubation temperature, only data from breeding season 1 (during which eggs were randomised among three temperature

treatments) were used. The influence of different incubation temperatures on hatching success, external yolk sac area, and on body condition index was tested with *post hoc* Tukey tests using the glht function in the multcomp package (Hothorn *et al.* 2008). We tested whether the sex ratio of a sample of hatchlings differed from parity using an exact binomial test.

Results

In this study we developed an egg incubation protocol that resulted in a high hatching success rate for *M. bellii*. Eggs incubated at 27°C in breeding season 2 had a significantly higher hatching success rate (97%) (261 hatched/269 fertile eggs) than eggs incubated at 27°C in breeding season 1 (77%) (64 hatched/83 fertile eggs) ($\chi^2 = 17.59$, $P < 0.0001$, d.f. = 1).

For the 27 female *M. bellii* induced in breeding seasons 1 and 2, the time interval between injection of synthetic oxytocin and the commencement of oviposition, the duration of oviposition, and clutch size varied between individual turtles (Table 1). Adult female mass was not correlated with either the duration of oviposition ($r_s = -0.30$, $P = 0.123$) or the interval between the injection of oxytocin and the onset of oviposition ($r_s = 0.01$, $P = 0.955$). Clutch size was not correlated with the interval between the injection of oxytocin and the completion of oviposition ($r_s = 0.05$, $P = 0.816$).

Over breeding seasons 1 and 2, a total of 27 separate turtles were induced and each laid a single clutch of eggs. In breeding season 1, 13 turtles were induced and in breeding season 2, 14 turtles were induced. A total of 25 eggs failed to develop a white patch during incubation and were considered infertile (Thompson 1985; Fielder *et al.* 2014).

Egg dimensions were similar between breeding seasons 1 and 2 and were similar to those reported by Fielder *et al.* (2014) (Table 2). Mass of adult females was positively correlated with clutch size ($r_s = 0.50$, $P = 0.008$, d.f. = 25) (Fig. 1), clutch mass ($r_s = 0.72$, $P < 0.0001$, d.f. = 25), egg mass ($\chi^2 = 24.43$, $P < 0.0001$, d.f. = 1), hatchling mass ($\chi^2 = 24.86$, $P < 0.0001$, d.f. = 1), and hatchling carapace length ($\chi^2 = 21.19$, $P < 0.0001$, d.f. = 1). Straight carapace length of adult females was positively correlated with clutch size ($r_s = 0.45$, $P = 0.020$, d.f. = 25), clutch mass ($r_s = 0.56$, $P = 0.002$, d.f. = 25), egg mass ($\chi^2 = 10.46$, $P = 0.001$, d.f. = 1), and hatchling mass ($\chi^2 = 8.54$, $P = 0.004$, d.f. = 1). There was a strong positive correlation between egg mass and hatchling mass ($\chi^2 = 249.72$, $P < 0.0001$, d.f. = 1).

Incubation temperature was negatively correlated with incubation duration ($\chi^2 = 496.00$, $P < 0.0001$, d.f. = 2) (Fig. 2). In breeding season 1, eggs incubated at 27°C had an incubation duration of 59.5 ± 1.3 days (range 57–64 days), eggs incubated at 28°C had an incubation duration of 54.1 ± 1.8 days (range 48–60 days), and eggs incubated at
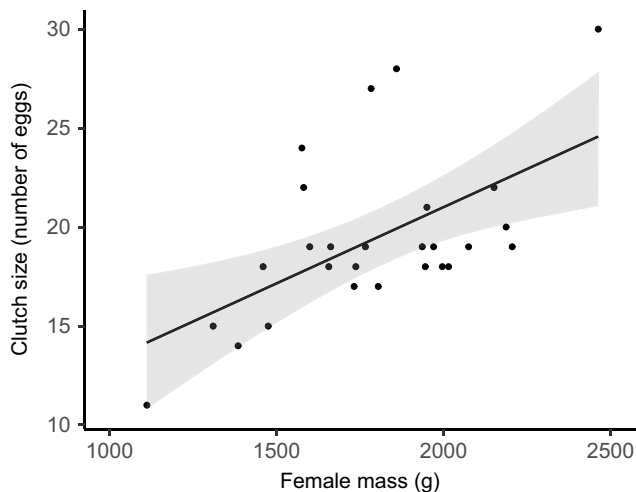
Table 1. Hormonal induction of egg-laying in 27 gravid female *M. bellii* in breeding seasons 1 and 2.

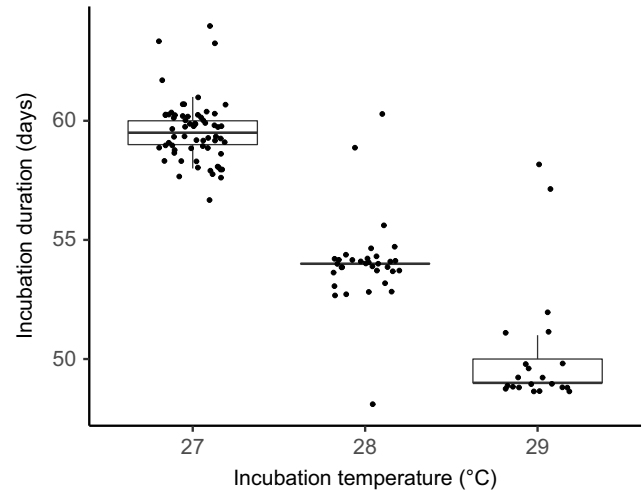|	Female mass prior to induction (g)	Female mass after induction (g)	Time interval to first egg (min)	Laying duration (min)	Clutch size (eggs)
Mean ± s.d.	1932.7 ± 341.2	1792.3 ± 305.4	35.6 ± 13.7	73.7 ± 55.3	19.4 ± 4.1
Range	1167–2743	1111–2462	16–77	16–212	11–30

Table 2. Mean egg dimensions of 524 eggs from 27 *M. bellii* clutches, and of 35 eggs from two *M. bellii* clutches reported by Fielder *et al.* (2014).

	Egg length (mm)	Egg width (mm)	Egg mass (g)
This study	30.1 ± 1.7 (25.9–35.3)	21.9 ± 1.3 (18.1–25.3)	8.4 ± 1.3 (5.0–11.8)
Fielder *et al.* (2014)	30.6 ± 0.1	21.9 ± 0.1	8.6 ± 0.1

Values for egg dimensions are mean ± s.d. (range).

Fig. 1. *M. bellii* clutch size was positively correlated with adult female mass ($r_s = 0.50$, $P = 0.008$, d.f. = 25). The shaded area represents 95% confidence interval for the prediction of clutch size from maternal mass.

Fig. 2. Influence of three constant incubation temperatures on incubation duration of *M. bellii* eggs. Incubation temperature was negatively correlated with incubation duration ($\chi^2 = 496.00$, $P < 0.0001$, d.f. = 2). The thick lines represent median values, lower and upper box boundaries represent 25th and 75th percentiles respectively, while the limits of the whiskers denote the 1.5-fold IQRs. Data points are jittered.

29°C had an incubation duration of 50.3 ± 2.6 days (range 49–58 days). In breeding season 2, eggs incubated at 27°C had an incubation duration of 60.3 ± 0.9 days (range 58–64 days). The incubation duration for eggs incubated at 27°C was significantly different between breeding seasons 1 and 2 ($\chi^2 = 0.011$, $P < 0.011$, d.f. = 1); however, incubation duration was recorded in whole days and the difference in mean incubation duration of less than one day may be an artefact of variability in the time of day at which daily checks for hatched eggs were conducted. Egg mass did not influence incubation duration ($\chi^2 = 0.65$, $P = 0.420$, d.f. = 1). Incubation temperature strongly influenced hatching success ($\chi^2 = 36.59$, $P < 0.0001$, d.f. = 2). In breeding season 1, eggs incubated at 27°C had a higher hatching success rate (77.1%) than eggs incubated at 28°C (40.2%) or 29°C (29.6%) (Tukey's

post hoc tests: $P < 0.0001$ for both comparisons); however, hatching successes of eggs incubated at 28°C or 29°C were not significantly different from each other (Tukey's *post hoc* test, $P = 0.135$).

In breeding season 1, hatchlings from eggs incubated at 27°C had a significantly smaller external residual yolk sac area than hatchlings from eggs incubated at 28°C and 29°C (Tukey's *post hoc* tests: $P < 0.05$ and $P < 0.001$ respectively); however, the external residual yolk sac areas of hatchlings from eggs incubated at 28°C or 29°C were not significantly different from each other (Tukey's *post hoc* test, $P = 0.162$) (Fig. 3).

Incubation temperature within the range 27–29°C did not influence hatchling mass ($\chi^2 = 0.07$, $P = 0.967$, d.f. = 2) or carapace length ($\chi^2 = 3.07$, $P = 0.216$, d.f. = 2) but there was mixed evidence of its influencing the body condition index of hatchlings. Eggs incubated at 27°C had significantly higher body condition index than eggs incubated at 28°C (Tukey's *post hoc* test, $P = 0.035$) but not eggs incubated at 29°C (Tukey's *post hoc* test, $P = 0.866$), and eggs incubated at 28°C and 29°C were not significantly different (Tukey's *post hoc* test, $P = 0.261$). Average hatchling dimensions, mass
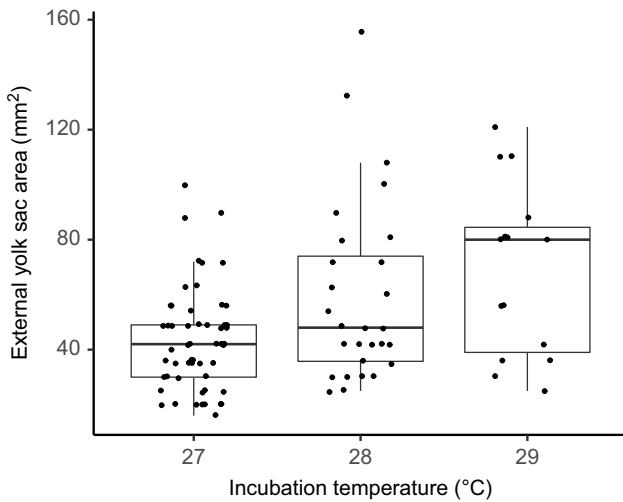
Fig. 3. Influence of three constant incubation temperatures on the external residual yolk sac area of *M. bellii* hatchlings at time of hatching. Box boundaries represent 25th and 75th percentiles, thick lines represent median values. Data points are jittered.

and body condition were similar between breeding seasons (Table 3). Two hatchlings (siblings incubated at 27°C in breeding season 2) had developmental anomalies: both were missing a right eye and both had a lower mandible protruding to the left side.

The sex ratio of 73 hatchlings from eggs incubated at constant 27°C in breeding season 2 did not significantly differ from parity (29 males, 44 females, exact binomial test, 95% CI = 0.481–0.715, $P = 0.101$).

Discussion

Incubation temperature influenced hatching success, with the lowest temperature (27°C) maximising hatching success rate. Hatching success at 27°C was 77% in breeding season 1 and 97% in breeding season 2. In breeding season 2 we modified our incubation method with the aim of improving hatching success. The key improvements employed were the use of plastic film to seal egg containers, weighing of egg containers at fortnightly intervals to quantify water loss, replenishing lost water using a syringe to place water directly into the vermiculite bedding and avoid any direct wetting of eggs. We hypothesise that this method reduced the occurrence of fungal or bacterial infections and improved

control of hydric conditions. Although beyond the scope of this study, it is worth mentioning that this improved method was applied in two subsequent breeding seasons, resulting in 100% (141 hatched/141 fertile eggs) and 99% (294 hatched/295 fertile eggs) hatching success rates. The mean hatching success rate over breeding seasons 2, 3 and 4 was 99%. The consistent 97–100% hatching success rates suggest that 27°C may be close to the optimal incubation temperature for *M. bellii*, and that our incubation method used in season 2 is highly effective. We recommend the incubation method we describe for breeding season 2 for future *M. bellii* egg incubation. Future refinements to our method could include testing fluctuating incubation temperature regimes, cooler temperatures, and measuring posthatching fitness. There is a body of evidence that fluctuating incubation temperature regimes more closely resembling natural nest conditions are beneficial for both hatching success and posthatching fitness (Niehaus *et al.* 2012; Noble *et al.* 2018).

Six studies that report hatching success rates greater than 90% for Australian freshwater turtles applied constant incubation temperatures between 26°C and 32°C and provide data on hatching success for *Chelodina expansa* (Booth 2002), *Chelodina longicollis* (Georges 1988; Booth 2014), *Emydura macquarii* (Thompson 1988; Booth and Yu 2009) and *Elusor macrurus* (Georges and McInnes 1998). Most of these studies achieved hatching success rates greater than 90% by incubating eggs at a constant temperature of 28°C or higher. In contrast, our highest hatching success rate for *M. bellii* was achieved at 27°C. The distribution of *M. bellii* is restricted to high altitudes (600–1100 m) with a temperate climate (Fielder *et al.* 2015). Our comparatively low optimal incubation temperature may reflect the likely cooler natural incubation conditions within this turtle's geographic distribution. However, egg incubation studies for Australian freshwater turtles have employed a range of incubation methods with various hydric and temperature regimes and varying substrate media. This variation in methods means that reported egg hatching success rates may not be directly comparable between studies.

Our results for incubation duration align with other published data for turtles, in which incubation duration is shorter at higher temperatures (Lawson and Rollinson 2021). Our results for eggs incubated at 29°C align with the results of Fielder *et al.* (2014) in which *M. bellii* eggs from two clutches incubated at a constant 29°C had durations of 49 and 51 days. However, our result of 60 days for eggs ($n = 325$) incubated at 27°C differs from the 80-day duration reported by

Table 3. Dimensions and mass (means $\pm$ s.d.) of 379 hatchlings from breeding seasons 1 and 2 combined.

	Straight carapace length (mm)	Straight carapace width (mm)	Plastron length (mm)	Plastron width (mm)	Hatchling mass (g)
Mean ± s.d.	26.9 ± 1.6	25.4 ± 1.9	23.7 ± 1.6	13.6 ± 1.3	5.2 ± 0.9
Range	20.4–30	16–29.9	17–31.3	9–20.4	2.9–7.2

Cann (1998) for *M. bellii* eggs incubated at approximately 27°C. The reason for this difference is unknown, but we suggest it is likely that the approximate incubation temperature reported by Cann (1998) was either lower or more variable than our incubation regime.

In many turtle species, lower incubation temperatures can result in more yolk being converted into hatchling body tissue (Booth 2006, 2018). In our study, hatchlings from eggs incubated at lower temperatures tended to internalise a greater proportion of yolk prior to hatching and so hatchlings incubated at 27°C had comparatively smaller external residual yolk sac areas upon hatching. Shorter incubation duration resulting in a lesser amount of yolk converted to body tissues has been reported for other freshwater turtles including *Chelydra serpentina* (Packard *et al.* 1987) and *C. expansa* (Booth 2000). An exception is reported by Booth (1998) for *E. macquarii* where residual yolk mass was not affected by incubation temperature in eggs incubated at a range of constant temperatures between 24°C and 31°C. Comparatively larger amounts of residual yolk have also been reported for hatchling turtles incubated under drier conditions (Packard *et al.* 1987; Booth 2002) and under more saline conditions (Bower *et al.* 2013). It should be noted that these studies euthanised the hatchlings and dissected out total residual yolk for measurement, whereas our study measured only the external portion of the residual yolk of live hatchlings. In our study, incubation temperature had no significant effect on hatchling carapace length, which may suggest that thermal effects on the conversion of yolk to tissue may not be readily differentiated within the thermal range of 27–29°C. In breeding season 1 of our study, hydric conditions were not precisely maintained, so it is unknown whether incubation temperature and/or hydric condition influenced external residual yolk sac area.

Moisture interacts with temperature to influence hatching success, incubation duration, and yolk absorption (e.g. Packard *et al.* 1987). This interaction is less pronounced in turtle species with rigid-shelled eggs compared with those with flexible-shelled eggs that exchange water more readily with the environment (Packard *et al.* 1981). *M. bellii* has rigid-shelled eggs. Booth (2002) and Booth and Yu (2009) found that relative to flexible-shelled species, hydric incubation conditions had less of an influence on the embryonic development and hatchling fitness in two other chelid species with rigid-shelled eggs (*C. expansa* and *E. macquarii*). In breeding season 2 we controlled the hydric environment within the egg incubation containers. We sealed the egg containers with plastic film, monitored egg container weight fortnightly to track moisture loss, we used a syringe to add measured quantities of water to the vermiculite (as required), and took care to ensure that added water did not come into direct contact with eggs. This process entailed less handling, less variation in moisture levels, reduced risk of exposure of eggs to pathogens, and, at the

incubation temperature of 27°C, resulted in a higher hatching success rate than in breeding season 1.

Maternal influences on neonate size have been reported across all groups of reptiles (e.g. Packard *et al.* 1987; Janzen 1993; Congdon *et al.* 1995; Reichling and Gutzke 1996; Nelson *et al.* 2004). Our study found a positive correlation between adult female size and clutch size, egg mass, hatchling mass and hatchling size. This aligns with other studies (Congdon and Gibbons 1987; McKnight *et al.* 2018; Iverson *et al.* 2019). Furthermore, we found hatchling mass was positively correlated with egg mass but was not influenced by incubation temperature. This finding is consistent with the reviews of Booth (2006, 2018) that found overall hatchling mass is not influenced by incubation temperature, but the fractions of mass contained as hatchling tissue and as residual yolk is influenced by incubation temperature – hatchlings incubated at cooler temperatures have proportionally more tissue and less residual yolk.

Genetic sex determination is believed to be ubiquitous in the family Chelidae (Martinez *et al.* 2008; Warner 2011; Mazzoleni *et al.* 2020) and therefore we hypothesised that incubation temperature would not influence the sex of *M. bellii* hatchlings. Incubation of eggs at a constant 27°C produced both male and female hatchlings and the sex ratio did not significantly differ from 1:1. Although these results are likely indicative of genetic sex determination in *M. bellii*, they do not definitively rule out temperature-dependent sex determination, as 27°C could be the pivotal incubation temperature that produces a 1:1 sex ratio in *M. bellii*.

Our study draws on a robust sample size of 27 clutches and 524 eggs to report on *M. bellii* egg dimensions and mass, clutch size, and hatchling dimensions and mass. Information on Australian freshwater turtle egg dimensions, clutch size and female size is not often reported, yet this information may be valuable for distinguishing between eggs of species and subspecies (McKnight *et al.* 2018) and may also be useful for identifying the nests (intact or predated) of sympatric species. However, the sympatric eastern long-necked turtle (*C. longicollis*) has eggs that are about 20 mm wide and 30 mm long with a mass of 6–7 g (Kennett *et al.* 2009) and therefore the eggs or eggshells of *M. bellii* and *C. longicollis* cannot be reliably distinguished based on length or width.

Although the average clutch size of 19.4 eggs that we report here for *M. bellii* is larger than the average clutch size of 14 eggs reported for *C. longicollis* by Kennett *et al.* (2009), the range of clutch sizes has considerable overlap between the two species and therefore clutch size cannot be reliably used to distinguish between the two species. The clutch size, egg dimensions and egg mass we report for *M. bellii* are similar to data presented for two *M. bellii* clutches reported by Fielder *et al.* (2014) (Table 2), and hatchling dimensions are also similar.

Fielder *et al.* (2014) report that *M. bellii* has one of the lowest reproductive outputs of any Australian chelid, with

annual fecundity estimated at 14.3 eggs per adult female. This assertion was based on their reported average clutch size of 18.3 eggs (derived from a sample of three clutches with a total of 55 eggs), their reported proportion of 78% of mature females that are productive in any one season (based on a sample of 84 mature females), and their finding that individual females lay only one clutch per breeding season (based on data from seven consecutive breeding seasons). Our study reports an average clutch size of 19.4 eggs based on 27 clutches (524 eggs), and therefore our data suggest that annual fecundity (calculated on the basis of clutch size × clutches per season × average proportion of mature females breeding annually) is 15.1 eggs per female. This value is only slightly higher than the annual fecundity of 14.3 eggs per female reported by Fielder *et al.* (2014) and therefore we support their conclusion that *M. bellii* has low annual fecundity relative to values reported for many other Australian freshwater turtles.

Head-starting is conducted as part of many turtle conservation programs (Burke 2015) and aims to increase juvenile recruitment by artificially improving hatching success (Eiby and Booth 2011). The release of head-started hatchlings produced by the artificial incubation of eggs aims to bypass threats to embryo survival in the wild. Our study suggests that artificial incubation is a strategy that could successfully be employed to boost hatchling numbers in wild *M. bellii* populations. Neonate turtles tend to have low survival rates (Heppell *et al.* 1996) and therefore large quantities of hatchlings need to be produced (whether naturally or through artificial incubation) to ensure that some reach maturity and are recruited into the adult breeding population. In the case of *M. bellii*, artificial incubation is an efficient and productive augmentation strategy, as adult turtles are locally abundant, gravid females can be readily captured for induction, and our described incubation method results in high hatching success rates. The occurrence of nesting behaviour several weeks after the harvesting of all eggs via hormonal induction has been reported in *E. macquarii* and *C. expansa* (McCosker 2002). It is unknown whether *M. bellii* carry out nesting behaviour once released into the wild following the harvesting of eggs via hormonal induction but, if so, hormonal induction would not reduce the risk of predation of nesting turtles by terrestrial predators. Releasing artificially incubated neonates soon after hatching, rather than raising them in captivity, has the advantage of avoiding any risk of hatchlings becoming habituated to captivity and failing to develop appropriate behaviours for predator avoidance, foraging and habitat selection (Bennett *et al.* 2017; Santori *et al.* 2021).

Some authors warn that artificial incubation and/or head-starting programs may be ineffective relative to the benefit of conserving adult turtles (e.g. Heppell *et al.* 1996; Seigel and Dodd 2000; Páez *et al.* 2015). However, in the case of *M. bellii*, nest depredation by a novel invasive predator, the

European red fox, is potentially so high (>95%) that the breeding effort of wild adult turtles is effectively negated, and juvenile recruitment may be insufficient to replenish wild populations (Streeting *et al.* 2021). Other benefits associated with head-starting programs include opportunities to engage the community by using hatchling turtles as ambassadors to generate public interest in conservation (Burke 2015). The survival, growth, and movement of released hatchlings from this study are the subject of ongoing research, as are other conservation strategies for *M. bellii* including the *in situ* protection of wild nests (Streeting *et al* 2021). Population reinforcement programs require long-term monitoring to assess their effectiveness (Carstairs *et al.* 2019). The hormonal induction of wild-caught gravid females and the incubation of eggs at 27°C proved to be an efficient and successful way of harvesting *M. bellii* eggs and producing hatchlings for release into the wild. We recommend these methods to support population augmentation programs for the western saw-shelled turtle.

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