

Single and multiple mating reduces longevity of female dumpling squid (*Euprymna tasmanica*)

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Keywords:

cephalopod;
cost;
life span;
mating;
survival;
trade-off.

Abstract

For many species, mating is a necessary yet costly activity. The costs involved can have an important influence on the evolution of life histories and senescence. Females of many species mate multiply, and this behaviour can inflict a longevity cost. Most studies investigating the effects of multiple mating on female survival have been conducted on insects, and the effects in other taxa are largely unknown. We investigate the effects of both a single mating and a second mating on longevity in female dumpling squid (*Euprymna tasmanica*), a species in which both sexes mate multiply. Through comparing the longevity of virgin, once-mated and twice-mated females, we found that a single mating reduced female life span by 15 days on average. A second mating resulted in an additional 8 day (on average) longevity cost, despite no difference in total clutch mass, number of clutches, single egg mass or number of eggs per clutch between once-mated and twice-mated females. This demonstrates a cost to multiple mating which may be independent of the cost of egg production. Furthermore, total clutch mass and female life span were positively correlated, whereas female life span decreased with increasing average water temperature. The presence of an additive effect of reproduction on longevity suggests that multiple mating in cephalopods may have benefits that outweigh these costs, or that there is a conflict in optimal mating frequency between males and females.

Introduction

Organisms allocate resources to both survival and reproduction throughout their lifetime to maximize fitness (Williams, 1957; Partridge & Harvey, 1988; Barnes & Partridge, 2003). However, there is generally a trade-off between survival and reproduction such that it is not possible to maximize both (Reznick, 1992). Assessing this trade-off is crucial to understand the evolution of life-history strategies, ageing and reproductive behaviours. Many studies demonstrate that life span is decreased when individuals invest in activities related to reproduction (Chapman *et al.*, 1995; Andrade, 1996; Gems & Riddle, 1996; Golet *et al.*, 1998). A classic example is Clutton-Brock *et al.*'s (1989) research

demonstrating that lactation has a negative effect on overwinter survival of female red deer (Golet *et al.*, 1998). In addition to parental care, other reproductive behaviours can decrease life span, including mate search (Andrade, 2003), courtship (Cordts & Partridge, 1996; Clutton-Brock & Langley, 1997) and mating (Kimura & Chiba, 2015). Currently, the majority of research into some of these costs, for example mating costs, has been conducted in insects (Chapman *et al.*, 1998; Vahed, 1998; Martin & Hosken, 2004). This limits our ability to make generalizations regarding the evolution of life-history strategies.

Mating is an essential component of reproduction for many species, yet there are a variety of costs associated with mating for both males and females. These costs could be inflicted through disease transfer (Knell & Webberley, 2004), energy allocation (Franklin *et al.*, 2012) and injury (Stutt & Siva-Jothy, 2001). In extreme cases, one individual in the pair may even die (Andrade, 1996). These costs can differ between the

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sexes and cause a conflict in optimal mating frequency. There are several examples of mating costs differentially affecting males and females: female praying mantids (*Pseudomantis albofimbriata*) can cannibalize males (Barry *et al.*, 2008), male bed bugs (*Cimex lectularius*) traumatically inseminate females (Morrow & Arnqvist, 2003) and male crickets (*Gryllus campestris*) are more likely to be predated upon than females (Rodríguez-Muñoz *et al.*, 2011). In many cases, it is necessary for the female to live longer after copulation so that she has time to produce offspring. Thus, mating costs may be particularly important in shaping the optimal reproductive strategy for females and could help us to understand conflicts between the sexes in mating frequency and the evolution of polygamy.

Mating costs in females can be difficult to assess because mating is generally tied to egg or offspring production. As such, there are only a handful of studies investigating the cost of mating independently of the costs of egg/offspring production (Gems & Riddle, 1996; Chapman *et al.*, 1998; Schrempf *et al.*, 2005; Bateman *et al.*, 2006). These studies tend to assess the cost of mating by comparing single-mated and multiple-mated females. The results vary, from a decrease in longevity (Chapman *et al.*, 1995; Gems & Riddle, 1996; Bateman *et al.*, 2006), to no effect detected (Kotiaho & Simmons, 2003; Martin & Hosken, 2004) or even an increase in longevity (Vahed, 1998; Wagner & Harper, 2003; Schrempf *et al.*, 2005). Whilst there can be many benefits of multiple mating for females (Svärd & McNeil, 1994; Arnqvist & Nilsson, 2000; Squires *et al.*, 2012), the optimal reproductive strategy is likely to be a balance of these benefits against costs, such as a longevity cost.

Cephalopods are an ideal group to research mating costs because they mate multiply (Hanlon *et al.*, 1999; Emery *et al.*, 2001; Squires *et al.*, 2014), are generally short-lived and perform copulatory behaviours which may be costly to females. These behaviours include traumatic insemination in argonauts (Wells & Wells, 1977), prolonged copulations in dumpling squid (Franklin *et al.*, 2012) and continuation of copulation in the presence of predators (Franklin *et al.*, 2014). Here, we use dumpling squid, *Euprymna tasmanica* (Pfeffer, 1884), to assess the effects of single and multiple mating on female longevity. Dumpling squid are a short-lived (5–8 months; Sinn & Moltschanivskyj, 2005), small (4–7 cm) squid species found in shallow waters off the southern coast of Australia. Mating is energetically costly (Franklin *et al.*, 2012) and can last up to 3 h (mean \pm SEM: 86.1 ± 7.6 min; range: 48.3–184.2 min; Franklin *et al.*, 2012). Females tend to lay multiple clutches of eggs for several days after mating (Squires *et al.*, 2013), but generally do not provide care for the offspring. Both sexes mate multiply; in the wild, a single clutch of eggs can have up to four sires (Squires *et al.*, 2014). Females that mate multiply

produce larger hatchlings relative to egg mass and lay clutches at a faster rate (although they lay the same total number of eggs; Squires *et al.*, 2012). By comparing the adult life span of virgin, once-mated and twice-mated female dumpling squid, we investigated the hypotheses that once-mated females will experience a longevity cost and that twice-mated females will experience an additional reduction in longevity.

Materials and methods

Squid culture

We collected juvenile female and adult male *E. tasmanica* from December 2010 until February 2011 during multiple shallow (< 5 m) night SCUBA dives at Clifton Springs (38°09'18S, 144°34'03E) and St Leonards (38°10'13S, 144°43'11E) in Port Phillip Bay, Victoria, Australia. Upon capture, squid were transferred to facilities at the Victorian Marine Science Consortium (VMSC) in Queenscliff. Here, we housed juvenile females individually in round plastic buckets (diameter \times height: 20 \times 19 cm, volume = 6.0 L) until they reached sexual maturity and were then relocated to larger glass tanks (length \times width \times height: 24 \times 24 \times 24 cm, volume = 13.8 L). Males were housed individually in glass tanks. Each holding aquarium contained a layer of sand substrate and a short (diameter \times length: 5.5 \times 6.5 cm) length of PVC pipe for shelter and in which females could lay eggs. Aquarium lights provided a reverse 12 : 12-h day/night cycle, and all aquaria received a constant flow of aerated, ambient temperature (13–21 °C) seawater pumped directly from Port Phillip Bay. We fed squid *Palaemon* shrimp *ad libitum*, checked them and recorded the water temperature every second day.

Experimental protocols

We investigated the effect of single and multiple mating on adult life span (sexual maturity until death) because all female squid were captured as juveniles. To determine the onset of sexual maturity, juvenile females were monitored every day for the appearance of the accessory nidamental gland. This gland forms part of the female reproductive tract and is responsible for providing a protective outer coating to the egg prior to laying (Norman & Lu, 1997). During sexual maturity, the gland develops a coral red colour (Bloodgood, 1977) that can be observed through the translucent ventral wall (Fig. 1). The day the gland was first visible was recorded as day one of the squid's adult life span. On this day, squid were blotted and weighed and moved to a larger tank. To ensure females had completed sexual maturation, we allowed enough time for them to reach adult size (45 days) before administering a mating treatment.



Fig. 1 Ventral image of adult female *Euprymna tasmanica* depicting the coral red accessory nidamental gland. Initial appearance of this gland was used as a marker for sexual maturity.

On day 45, females were randomly allocated to one of three treatment groups; virgin ($n = 11$), once-mated ($n = 10$) or twice-mated ($n = 10$). Each female was blotted and weighed before being placed in the mating chamber ($10 \times 10 \times 11.5$ cm, volume = 1.2 L) and allowed 10 min to acclimate. This chamber had constant flowing seawater. For the once-mated and twice-mated treatments, a wild-caught, adult, male squid was added to the mating chamber and squid were left to mate. If mating had not commenced after 10 min, the female was gently disturbed to allow the male to initiate copulation. On 15 occasions, squid would not mate for unknown reasons. If this occurred, we removed the male and 10 min later added a different male. Squid were monitored every 15 min until the conclusion of mating and then moved back to their holding tanks. Two hours after this, they were returned to the mating chamber and the same procedure was followed. However, this time, only females in the twice-mated treatment were paired with a male. All females allocated to the once-mated treatment mated with a male and all those allocated to the twice-mated treatment mated with the second male. Mating durations were similar to previous experiments (range: 48.3–184.2 min; Franklin *et al.*, 2012). Males were not mated more than once per fortnight to allow for spermatophore replenishment. After treatment, we monitored squid every second day for eggs and to check survival. Every clutch was weighed, and clutch masses were summed for each squid to calculate total clutch mass across life span. We also counted number of eggs in each clutch and weighed ten randomly selected eggs to calculate average single egg mass. Eggs from virgins were kept in brooding chambers to confirm they were infertile. Two ‘virgin’ females laid multiple fertile clutches indicating they had mated in the wild before sexual maturity and

stored the sperm. These females were excluded from the analysis. Final sample sizes were virgin = 9, once-mated = 10 and twice-mated = 10.

Statistical analysis

All statistical analyses were conducted in R v. 3.1.1 (R Core Team, 2014). We used a negative binomial generalized linear model (GLM; MASS package; Venables & Ripley, 2002) to assess the effect of treatment (virgin, once-mated, twice-mated) on adult life span. To investigate factors affecting adult life span, we included treatment, total clutch mass and average water temperature in the laboratory (i.e. throughout adult life span) as fixed effects. We also investigated whether the proportion of dumpling squid that laid eggs differed between treatment groups, and, for the dumpling squid that laid eggs, we determined if total clutch mass, number of clutches, number of eggs per clutch or single egg mass differed between treatment groups. To investigate whether proportion of dumpling squid laying eggs, total clutch mass or number of eggs per clutch varied between the treatment groups, we ran GLMs with treatment as the independent variable. Generalized linear mixed models (GLMM) were used to assess the effect of treatment on number of clutches and single egg mass. Squid ID was included as a random effect for ‘number of clutches’ to account for overdispersion and for ‘single egg mass’ because single egg mass was calculated for each clutch and 13 squid laid more than one clutch. Probability error distributions used were binomial (logit link) for proportion of squid laying eggs, gamma (log link) for total clutch mass and single egg mass, Gaussian (identity link) for number of eggs per clutch and Poisson (log link) for number of clutches. We also ran a Gaussian GLM (identity link) to determine whether weight at maturity differed between treatment groups. For all statistical analyses, we assessed the significance of each term in the models using Wald χ^2 tests (R: ANOVA). Significant terms were investigated further using z -tests (adult life span, proportion that laid eggs, number of clutches) or t -tests (single egg mass) to determine which groups differed from one another. Final model fit was checked using residual plots.

This study was carried out with approval from the University of Melbourne Animal Ethics Committee (ID: 0810874.3) and followed recommendations for the ethical use of cephalopods in scientific experiments outlined in Moltschaniwskyj *et al.* (2007). All animals were collected under Fisheries Victoria collecting permits (permit number: RP962).

Results

Female life span was significantly affected by treatment (virgin, once-mated or twice-mated; $\chi^2 = 24.5$, d.f. = 2,

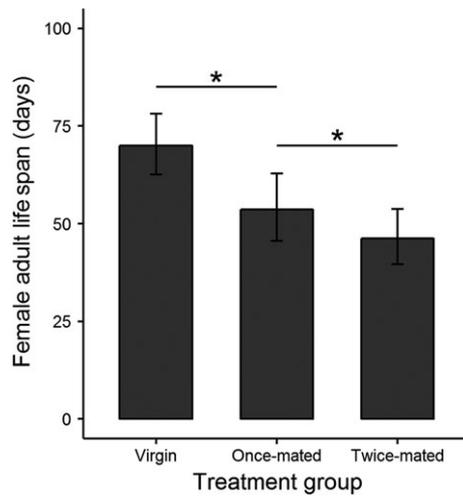


Fig. 2 The effect of mating treatment (virgin, once-mated or twice-mated) on female *Euprymna tasmanica* adult life span. Graph shows expected values (MLE, 95% CIs) when total clutch mass and average adult temperature are held constant [total clutch mass = 0 g; average temperature = 17.2 °C (average value across all squid)]. Adult life span was calculated from sexual maturity until death. Asterisks signify significantly different groups.

$P < 0.001$), total clutch mass ($\chi^2 = 47.8$, d.f. = 1, $P < 0.001$) and average temperature ($\chi^2 = 6.7$, d.f. = 1, $P = 0.01$). Further investigation revealed that virgin females lived for 15 days longer on average than once-mated females ($z_{24} = 3.12$, $P = 0.002$), and once-mated females lived 8 days longer on average than twice-mated females ($z_{24} = 2.06$, $P = 0.040$; Fig. 2). Female life span increased with total clutch mass (Fig. 3a), whereas average temperature was weakly negatively correlated with life span (Fig. 3b).

There was a difference in the proportion of squid that laid eggs across treatment groups ($\chi^2 = 6.69$, d.f. = 2, $P = 0.035$); fewer virgin females laid eggs than once-mated females ($z_{26} = 1.94$, $P = 0.05$) and there was no difference between once-mated and twice-mated females ($z_{26} = 0.00$, $P = 1.00$). For the squid that laid eggs, we detected a difference between the treatment groups in number of clutches laid ($\chi^2 = 13.60$, d.f. = 2, $P = 0.001$) and single egg mass ($\chi^2 = 14.36$, d.f. = 2, $P < 0.001$). Virgins laid fewer clutches and had lower single egg mass than once-mated females (number of clutches: $z_{25} = 3.68$, $P < 0.001$; single egg mass: $t_{67} = 2.70$, $P = 0.007$). However, there was no significant difference in number of clutches or single egg mass between once-mated and twice-mated females (number of clutches: $z_{25} = 1.01$, $P = 0.31$; single egg mass: $t_{67} = 1.63$, $P = 0.10$). There was no effect of treatment on total clutch mass ($\chi^2 = 4.51$, d.f. = 2, $P = 0.10$), number of eggs per clutch ($\chi^2 = 4.71$, d.f. = 2, $P = 0.09$) and no difference between the treatment groups in weight at maturity ($\chi^2 = 0.53$, d.f. = 2, $P = 0.60$; Table 1).

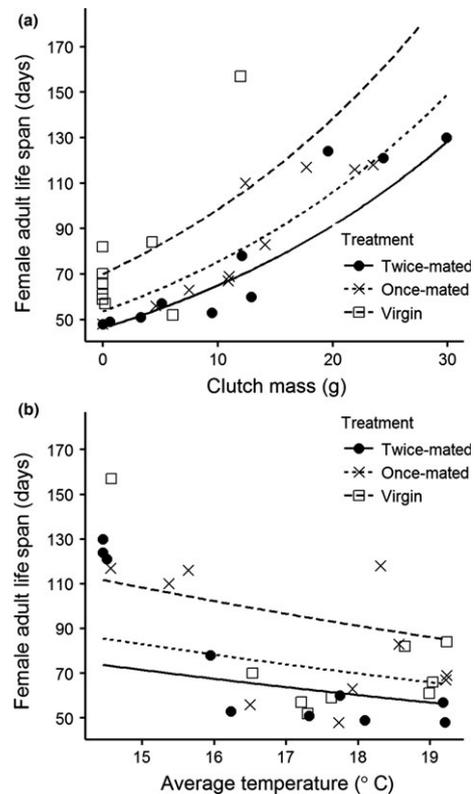


Fig. 3 The correlations between female *Euprymna tasmanica* adult life span and (a) total clutch mass (grams); (b) average water temperature across a female's life span (°C). (a) Average water temperature was held constant (at 17.2 °C, average value across all squid), and (b) total clutch mass was held constant (at 9.1 g, average value across all squid). Adult life span was calculated from sexual maturity until death. Slopes were significantly different from zero.

Discussion

Our results demonstrate that there is a longevity cost for once-mated females and an additional longevity cost for twice-mated females. There was no increase in total clutch mass, number of clutches, number of eggs per clutch or single egg mass between once-mated and twice-mated females, suggesting increased investment in eggs is not responsible for the additional longevity cost. We also detected a positive correlation between female life span and total clutch mass and a weak negative correlation between female life span and average water temperature. Single and multiple mating may have similar longevity costs for females in other cephalopod species because many cephalopods mate multiply (Hanlon *et al.*, 1999; Emery *et al.*, 2001; Squires *et al.*, 2014) and perform costly copulatory behaviours (Wells & Wells, 1977; Franklin *et al.*, 2012, 2014). Although mating multiply could provide benefits to females (Squires *et al.*, 2012), a longevity cost could lead to a

Table 1 Mean (\pm SE) parameters for each treatment group.

Parameter	Virgin	Once-mated	Twice-mated	Statistical test
Weight at maturity (g)	2.46 (\pm 0.22)	2.38 (\pm 0.18)	2.67 (\pm 0.23)	$\chi^2 = 0.53$, d.f. = 2, $P = 0.60$
Proportion that laid eggs	0.44	0.90	0.90	$\chi^2 = 6.69$, d.f. = 2, $P = \mathbf{0.035}$
Total clutch mass (g)*	5.65 (\pm 2.45)	13.73 (\pm 2.10)	13.04 (\pm 3.30)	$\chi^2 = 4.51$, d.f. = 2, $P = 0.10$
Number of clutches*	1.5 (\pm 0.3)	5.0 (\pm 1.0)	3.6 (\pm 0.9)	$\chi^2 = 13.60$, d.f. = 2, $P = \mathbf{0.001}$
Eggs per clutch*	96.3 (\pm 9.6)	52.9 (\pm 4.1)	60.8 (\pm 4.2)	$\chi^2 = 4.71$, d.f. = 2, $P = 0.09$
Single egg mass (mg)*	34.3 (\pm 0.9)	51.5 (\pm 1.4)	59.8 (\pm 1.3)	$\chi^2 = 14.36$, d.f. = 2, $P < \mathbf{0.001}$

Bold indicates $P < 0.05$.

*Only for squid that laid clutches.

conflict between males and females in optimal mating frequency.

Singly mated females lived, on average, 15 days less than virgin females. This reduction in longevity could be related to energy expenditure, disease, injury, physiological changes or egg production costs. Dumpling squid experience an energetic cost of copulation (Franklin *et al.*, 2012); but they recover from this cost within 30 min. Therefore, it seems unlikely that this energetic cost would cause a 15 day decrease in longevity. Injuries have been reported in other cephalopods and may occur in *E. tasmanica*. Males insert their hectocotylus into the female's mantle cavity and leave it there for the duration of mating. Upon conclusion, males roughly remove the hectocotylus with several forceful movements (Squires *et al.*, 2013). These could cause internal damage to the females, particularly because the suckers on the distal portion of the hectocotylus have a toothed sucker ring (Norman & Lu, 1997). Disease is also possible because the females were raised individually in the laboratory, albeit from wild-caught juvenile stage, whereas the males were wild caught as adults. Males may have carried pathogens into the laboratory that females had not encountered before (Padros *et al.*, 2001; Kik *et al.*, 2011). Investment into immune function could also be affected and may increase or decrease with reproduction (Nunn *et al.*, 2000; Rolff & Siva-Jothy, 2002). This could increase metabolic costs or susceptibility to diseases, respectively. Mating can cause other physiological changes, such as endocrine changes (Fernández-Guasti *et al.*, 2010; Shi & Murphy, 2014) and alterations to gene expression (McGraw *et al.*, 2008; Dalton *et al.*, 2010). These changes are likely to redirect investment from survival and growth to processes relating to reproduction. Physiological changes related to egg production may occur after a single mating in dumpling squid. We observed more single-mated dumpling squid laid eggs than virgins and single egg mass was greater in once-mated females compared to virgin females. Whilst we did take total clutch mass into account in our statistical model, egg production may still influence longevity. This could occur if non-fertilized and fertilized eggs inflict differing costs on females (David, 1963) or if egg production has a

greater effect than accounted for in our model (Reznick, 1992). In our experiment, virgin females produced eggs of smaller mass, which may suggest differing costs between fertilized and nonfertilized eggs. Further investigation into physiological costs of egg production would provide insight into the longevity cost of a single mating.

Multiple mating also negatively influences longevity in dumpling squid. Once-mated females lived longer, on average, than twice-mated females, despite no difference in total clutch mass. This result aligns with preliminary trials where females that were mated three times (in 3 days) would often die after the third mating. However, studies conducted in the laboratory may exacerbate costs associated with multiple mating because females do not have the ability to escape males. Even so, our results indicate that one or several factors can have an additive impact on female survival. Factors that could have a cumulative effect with multiple copulations include injury, seminal toxins, disease, metabolic costs or egg production. Chapman *et al.* (1995) demonstrated in *Drosophila melanogaster* that male seminal fluid is responsible for a reduction in female life span and that increasing exposure had an additive effect. In cephalopods, males transfer large bundles of spermatophores to females. These may contain products that are detrimental to female life span; however, this has never been assessed. As mentioned above, injuries are possible in *E. tasmanica*. Injury could have a cumulative effect if additional damage is caused during the second mating (Blanckenhorn *et al.*, 2002). Disease could also have a cumulative effect on life span if not all males in the population carry a pathogen. In this case, mating more than once increases the likelihood that a female will mate with an infected male or be infected with more than one type of pathogen (Roberts *et al.*, 2015). It is possible that egg production costs differ between once-mated and twice-mated females. However, we found no difference in total clutch mass, number of clutches, single egg mass or number of eggs per clutch between once-mated and twice-mated females. Additionally, a previous study in dumpling squid found no difference in proportion of eggs hatched between once-mated and twice-mated females, suggesting similar fertilization success (Squires

et al., 2012). Therefore, we suggest that the reduction in longevity associated with a second mating is unlikely due to egg production.

In our study, female longevity was related to other factors besides mating. Longer female life span was weakly associated with cooler average water temperatures over their adult life and increased total clutch mass. Cooler temperatures can reduce metabolic rate (Brown *et al.*, 2004), thus reducing growth and delaying the onset of sexual maturity (Sudo, 2003). This decrease in metabolism can prolong life span, which could explain why female dumpling squid raised in cooler temperatures may live longer. However, this relationship was quite weak and should be interpreted carefully. The positive correlation between longevity and total clutch mass was stronger. This association may be because females that live longer lay eggs over a longer period or it may indicate that higher quality females live longer and lay more eggs.

The major finding of this experiment, that both once-mated and twice-mated females experience a reduction in longevity, suggests that multiple mating is costly for female dumpling squid. This could indicate a conflict between males and females in optimal mating frequency. It is likely that a female obtains enough sperm from a single copulation to fertilize all her eggs because there is no difference in hatching success, the number of clutches or total number of eggs produced by dumpling squid females mated once or twice in the laboratory (Squires *et al.*, 2012). However, females that mate multiply produce eggs faster and have larger hatchlings relative to egg mass (Squires *et al.*, 2012). Larger hatchling size could increase offspring survival, improving fitness of multiply mated females (Fox & Czesak, 2000; Tamada & Iwata, 2005), despite the longevity cost. The mechanisms responsible for the potential fitness benefits of multiple mating in *E. tasmanica* remain unclear. For example, fertilization may be biased towards males with compatible genes ('genetic compatibility' hypothesis; for reviews, see Simmons, 2005; Tregenza & Wedell, 2000), intrinsically good genes ('good genes' hypothesis; for reviews see Jennions & Petrie, 2000; Yasui, 1997) or both. Further investigation into dumpling squid survival in the wild is needed to determine whether laying eggs faster and producing larger hatchlings translates into increased fitness. Nonetheless, the results reported here, and previous research demonstrating an energetic cost to copulation (Franklin *et al.*, 2012), clearly demonstrate that single and multiple mating is costly for female dumpling squid.

Acknowledgments

We thank Z. Squires, B. Wegener and R. Watson for help with animal collection and C. Monahan, G. Gully and A. Alcaro for help with animal husbandry. We are

grateful for the constructive comments from two anonymous reviewers.

Funding

This work was supported by The Hermon Slade Foundation (HSF 08/2).

Competing interests

We have no competing interests.

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Data deposited at Dryad: doi: 10.5061/dryad.65079

Received 27 November 2016; accepted 27 February 2017