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# Measuring the costs of alternative reproductive tactics in horseshoe crabs, *Limulus polyphemus*

Matthew Denman Smith*, Hunter E. Schrank, H. Jane Brockmann

*University of Florida, Department of Biology, Gainesville, FL, U.S.A.*

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Trade-offs are inherent to alternative reproductive tactics (ARTs), and identifying the costs and benefits of tactics is essential to understanding their evolution and maintenance within a population. Male horseshoe crabs exhibit two condition-dependent ARTs: males that are in better condition arrive on spawning beaches attached to a female, while males in poorer condition join spawning pairs as satellites and engage in sperm competition. Previous research has identified several benefits to the attached tactic, but the costs are less well understood. We examined a previously uninvestigated potential cost to the attached male tactic: nutritional stress caused by a restricted ability to feed. We found that field-caught attached males produced 57% less faeces in a 12 h period than satellite males, and had 2.5 times emptier digestive tracts than satellite males. We further examined this cost using stable isotopes because nutritionally stressed animals are predicted to have higher $\delta^{15}N$ levels. We found that field-caught attached males had higher $\delta^{15}N$ values than satellite males. However, higher $\delta^{15}N$ values could result from nutritional stress or from feeding on higher trophic levels. We tested this experimentally and found that starved animals had higher post-treatment $\delta^{15}N$ values compared to animals that were fed. Furthermore, the digestive tracts of field-caught attached males contained three times more sea grass (lower tropic levels have lower $\delta^{15}N$ values) than satellite males. These findings mean that the higher $\delta^{15}N$ values of field-caught attached males likely result from fasting rather than differences in diet. Taken together, our results indicate that a period of nutritional stress caused by reduced food consumption is a novel cost of the attached tactic. This study provides a key piece of information to explain why ARTs in horseshoe crabs take the form they do and provides a novel method for studying costs associated with ARTs in other species.


Alternative reproductive tactics (ARTs) by males are common in competitive mating systems (Taborsky et al. 2008). For example, in some anurans, 'sneaker' males sit silently near larger vocalizing males and either intercept females that are attracted to the callers, or wait to take over a resident male's calling site or territory (Wells 1977a, b; Robertson 1986a, b). The evolution and maintenance of such discrete alternative phenotypes is puzzling because we generally expect that if one phenotype is only slightly less successful than the other, it would be eliminated by selection (Brockmann 2001). In some cases, ARTs are maintained as a genetic polymorphism, but in most cases they depend on the individual's phenotype (e.g. body size, condition) and the circumstances in which they live (e.g. population density, sex ratio; Gross 1996; Zamudio & Chan 2008). In order for alternative tactics to be maintained at high frequencies, conditions must exist under which each tactic is more successful than the other (i.e. fitness curves must cross; Brockmann & Taborsky 2008). This means that trade-offs are inherent to ARTs. For example, an animal simply cannot simultaneously call and sneak, or maintain a territory and disperse widely in search of females. As a consequence, the phenotypes that maximize fitness for one tactic are different from those that maximize fitness for the other tactic. Therefore, the decision about which tactic to follow is based not only on an individual's phenotype and the circumstances in which it lives, but also on the costs and benefits of the alternative tactics. Understanding the nature of the trade-offs for each tactic is vital to understanding why particular tactics take the form they do, and to understanding the evolution and maintenance of alternative mating tactics within populations (Brockmann et al. 2008). In this study, we examine the trade-offs associated with ARTs of male horseshoe crabs.

Horseshoe crabs have a highly competitive, explosive mating system (Brockmann 1990) in which males show two condition-dependent, alternative phenotypes (Brockmann & Penn 1992; Brockmann 2002). Younger males in better condition (based on visual inspection of the carapace) attach to females at sea and arrive on spawning beaches paired in amplexus with females. The attached

* Correspondence: M. D. Smith, University of Florida, Department of Biology, 316 Bartram Hall, P.O. Box 118525, Gainesville, FL 32611, U.S.A.
*E-mail address:* madsmith@ufl.edu (M. D. Smith).

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male remains with the female until she has completed egg laying for the season, and then detaches and seeks another female. Unattached, older males in poorer condition roam the shoreline, join spawning pairs as satellites on the beach, and engage in sperm competition with attached males and other satellite males (Brockmann & Penn 1992; Brockmann et al. 1994; Brockmann 1996, 2002). These behavioural differences are not just a consequence of a male being unable to locate or hold onto a female, but instead result from an evolved decision rule based on age or condition (Brockmann 2002). That is, individuals maximize fitness by switching tactics at a given age or condition (i.e. fitness curves of the two tactics cross with condition).

Several trade-offs involving differences in paternity and righting behaviour have been identified for each tactic. During each 1-week spawning cycle (Cohen & Brockmann 1983; Barlow et al. 1986; Sekiguchi 1988; Smith et al. 2002), attached males normally mate with only one female, whereas satellite males may join several pairs. During each mating bout in a spawning cycle, satellite males have similar paternity success compared to attached males (Brockmann et al. 2000), but attached males do not always compete for paternity with satellites (depending on the number of unattached males present). In contrast, satellite males must always engage in sperm competition (Brockmann et al. 1994, 2000). Satellites appear at the beach to mate more often than attached males (Brockmann & Penn 1992), but they do not always find a mating pair. Lastly, horseshoe crabs are often overturned on the beach, leaving them vulnerable to desiccation and predation (Botton & Loveland 1989, 1993). When this happens, attached males are better able to right themselves than satellite males because righting ability is related to condition (Penn & Brockmann 1995). But also, the risk of coming ashore may be greater for unattached males because they may overturn more easily than attached males since they do not have a large female to act as an 'anchor' and stabilize them against wave action. Taken together, attached males have higher mating success overall (Brockmann et al. 2000) and are less likely to become stranded than unattached (satellite) males. However, if an age or condition threshold for switching tactics has evolved that maximizes fitness, then we would expect some compensating costs associated with the attached tactic. In this study we investigate a previously unexplored, potential cost to the attached male tactic: nutritional stress caused by a reduced ability to feed while attached to a female (Brockmann 2003).

Adult horseshoe crabs feed on a variety of items (e.g. bivalves, polychaetes, crustaceans) by digging into the substrate, stirring up sediment with their walking legs, and grasping food and directing it to their ventral mouth with their chelae and chelicerae. Gnathobases (leg bases) that surround the mouth macerate the food and also help to manipulate food into the mouth where it is then drawn into the esophagus (Manton 1964; Wyse & Dwyer 1973; Botton 1984; Botton et al. 2003). During the breeding season, attached males hold onto the posterior opisthosomal spines of the female using a modified pair of pedipalps. As a result of amplexus, the attached male's mouth is dorsal to and covered by the female's telson (Brockmann 2003); moreover, attached males cannot bury themselves in the substrate to feed in their normal manner. Thus, our first hypothesis is that the attached tactic inhibits feeding ('reduced feeding hypothesis'). In Florida, males typically remain attached for a 1-week spawning cycle (mean $\pm$ SD length of attachment is $3.7 \pm 6.1$ days; Brockmann & Penn 1992), but occasionally may stay attached up to 51 days (Brockmann 2003). In other populations, they can remain attached for much longer (Shuster 1954); for example, in New England, attached pairs have been observed overwintering together (Barlow et al. 1987; Moore 2004). If attaching to females inhibits feeding, then our second hypothesis is that fasting is costly; that is, it results in a period of nutritional stress for males adopting the attached tactic ('nutritional stress hypothesis'). Since attached males do not spend energy locating spawning pairs as satellite males do, and since attached males are 'carried' along by females (females are the ones exerting energy in locomotion and digging while spawning and during circatidal movements to and from the beach), it is possible that fasting is not nutritionally stressful for attached males.

### *Reduced Feeding Hypothesis*

The first prediction of this hypothesis is that if the attached tactic inhibits feeding, then attached males will not defecate at all, or will produce less faecal material than satellite males (Table 1). We tested this prediction with a waste production experiment that compared the amount of faeces produced by attached and satellite males. Alternatively, if physical condition affects assimilation efficiency and subsequently the amount of faeces defecated, then differences in waste production may be due to the differences in condition between attached and satellite males. We tested this possibility by analysing the relationship between condition and waste production in both males and females. Additionally, our ability to detect differences in waste production assumes that food transit times (the latency for an indigestible marker to first appear in faeces; Karasov & del Rio 2007; Barboza et al. 2009) are the same for attached males and satellite males. Transit time can be influenced by the size of the animal's digestive tract, by the amount, type and quality of food intake (Barboza et al. 2009), and possibly by an animal's condition. Thus, differences in waste production between attached and satellite males may be due to differences in transit time. We tested this with an experiment by hand-feeding crabs with an indigestible marker to determine food passage time (Table 1). This experiment also allowed us to test a second

**Table 1**
Summary of the hypotheses, predictions and assumptions regarding a cost to feeding for the attached male reproductive tactic in horseshoe crabs, and the methods we used to test them

| Hypothesis | Prediction | Method |
|---|---|---|
| Reduced feeding | (1) Attached males defecate less than satellite males | Measure waste production over 12 h in wild animals |
| | (a) Assumes condition does not influence defecation | Correlate condition and faeces in males and females |
| | (b) Assumes equal gut transit times | Measure gut transit times in hand-fed, wild animals |
| | (2) Attached males more motivated to feed than satellites | Measure food consumption during hand feeding |
| | (3) Attached males eat less food than satellite males | Examine gut fullness of wild animals |
| Nutritional stress | (4) Higher $\delta^{15}$N values in attached males | Measure $\delta^{15}$N values of faeces of wild animals |
| | (c) Assumes starved animals will have higher $\delta^{15}$N | Experimentally starve animals, measure $\delta^{15}$N of faeces |
| | (d) Assumes no differences in diet between tactics, or, if differences exist, attached males are feeding at a lower tropic level (lower $\delta^{15}$N) | Examine gut contents of wild animals |

The reduced feeding hypothesis addresses whether attached males eat less than satellite males; the nutritional stress hypothesis addresses whether this reduction in feeding is costly.

prediction of the reduced feeding hypothesis: if the attached tactic inhibits feeding, then attached males should be more motivated to eat than satellite males (Table 1). A third, more direct prediction is that if the attached tactic inhibits feeding, then the amount of food in the digestive tract should be lower for attached males compared to satellite males (Table 1). We tested this by examining the gut fullness of attached and satellite males.

*Nutritional Stress Hypothesis*

Nutritional stress due to a period of fasting (i.e. when feeding is forgone in favour of other activities; McCue 2010) or starvation (i.e. when feeding is prevented due to some extrinsic limitation; McCue 2010) can be inferred from stable isotope values of animal tissues (Hobson et al. 1993; Gannes et al. 1997, 1998; del Rio & Wolf 2005; Castillo & Hatch 2007; McCue 2007; McCue & Pollock 2008; del Rio et al. 2009). If an animal is in a negative energetic balance, $^{15}N$ is preferentially retained, while $^{14}N$ is excreted. As a result, $\delta^{15}N$ values increase in tissues over time as the animals 'feed on themselves' (McCue & Pollock 2008). Increased $\delta^{15}N$ values during fasting or starvation occurs in a wide variety of taxa and tissue types (reviewed in McCue & Pollock 2008), including in the excreta of lizards (Castillo & Hatch 2007) and rattlesnakes (McCue 2007). Thus, the first prediction of the nutritional stress hypothesis is that values of $\delta^{15}N$ from faeces of attached males will be higher compared to satellite males and females (Table 1). We tested this prediction by examining the stable isotope signatures of faeces from attached males, satellite males and females.

Alternatively, variation in $\delta^{15}N$ values can reflect differences in the trophic level at which animals are feeding: as animals feed at higher trophic levels, the value of $\delta^{15}N$ in their tissues increases (Deniro & Epstein 1981; Michener & Schell 1994). Hence, changes in $\delta^{15}N$ values may reflect either nutritional stress or differences in diet. Inferring that differences in $\delta^{15}N$ values are due to nutritional stress, rather than differences in diet, requires demonstrating that: (1) fasting or starving does indeed cause an increase in the $\delta^{15}N$ values of faeces in horseshoe crabs, and (2) any differences in diet between attached and satellite males cannot explain the differences in $\delta^{15}N$ values. We tested whether differences in $\delta^{15}N$ values were due to fasting in two ways. First, we conducted a 4-week experiment in which we starved some animals, but fed others. If differences in $\delta^{15}N$ values are due to nutritional stress rather than differences in diet, then $\delta^{15}N$ values should be higher in animals that were starved compared to those that were fed (Table 1). Second, we compared the gut contents of attached and satellite males. If gut contents analysis revealed that attached males fed at a higher tropic level than satellite males, then we would not be able to tell whether high $\delta^{15}N$ values were due to nutritional stress or diet. However, if gut content analysis revealed that diets of attached males were at a lower trophic level than those of satellite males, then high $\delta^{15}N$ values would be due to nutritional stress.

## METHODS

We conducted this study during 2008–2011 at the University of Florida Seahorse Key Marine Laboratory. Seahorse Key is a 67-ha island that is part of the Cedar Keys National Wildlife Refuge along the northwestern Gulf coast of Florida. We collected adult horseshoe crabs as they initially came to the beach to spawn during evening high tides. Each animal was marked uniquely with a numbered thumb tack, placed immediately into a clean bucket, transported to the laboratory on the island, and placed into separate, randomly assigned holding tanks (one crab per tank). The holding tanks were $61 \times 61 \times 20$ cm deep, and fed by a flow-through, running sea water system. For all animals used in our experiments, we measured their body size (maximum carapace width in cm) and assessed their condition using an index based on visual inspection of their carapace. Each individual was assigned a condition score based on (1) carapace colour, which darkens as the carapace erodes, (2) the amount of mucus present, which deters fouling organisms, and (3) the degree of pitting of the carapace, which is caused by chitinoclastic bacteria (modified from previous studies; for complete methods see Brockmann & Penn 1992; Brockmann 1996; Brockmann 2002). Each of the three criteria had a maximum score of 5 points, thus a maximum of 15 points was possible and represented the highest condition. Previous studies have shown that attached males are in better condition than satellite males, but there are no differences in carapace width (CW) between males that show different mating status (attached or satellite; Brockmann & Penn 1992; Brockmann et al. 1994; Brockmann 1996, 2002); these patterns were also supported for the animals used in this study.

*Measuring Waste Production*

During 4–17 October 2008 and 10–14 March 2009, we conducted an experiment to test whether attached males produced less faecal waste than satellite males (Table 1: prediction 1). Each experimental replicate consisted of three animals collected from the beach at the same time: a female, her attached male, and one satellite male associated with this pair ($N = 27$ replicates and 81 individual horseshoe crabs). In the running sea water system where we conducted this experiment, sea water is pumped into a large holding tank before entering the individual tanks, and water runs out of each individual tank through an overflow pipe. Hence, the relatively heavy packet of waste produced by horseshoe crabs did not flow out of the individual tanks. However, some debris did flow in, which could affect our measure of waste produced. So, in addition to the three tanks that each housed an attached male, a satellite male and a female, we added a fourth empty tank as a control for each replicate. We left all individuals in their individual holding tanks without food for 12 h before collecting waste (i.e. attached males and females were not coupled during the experiment). The average food passage time of horseshoe crabs was not known, and so we chose a 12 h time period (a priori) based on our best estimate of what it might be; this time period turned out to be reasonable because all animals had produced waste within 12 h. After the crabs had been returned to the ocean, we siphoned out all visible waste and debris from each holding tank through a fine-mesh plastic filter. We then rinsed off this plastic filter and collected the sample remaining on a piece of filter paper. Each sample was dried for 4 h in an oven at 60 °C and weighed (in g, minus the weight of the filter paper).

Body size was positively correlated with the amount of faeces produced (linear regression: $r^2 = 0.25$, $F_{1,78} = 26.0$, $P < 0.0001$). Therefore, we applied a size correction to our measure of waste production ((log waste − control)/log CW × 100) and compared the amount of waste (minus the amount of debris found in the control tanks) that was produced among the groups using paired $t$ tests. If differences in waste production were due to the differences in physical condition between attached and satellite males (Table 1: assumption a), then we would expect a relationship between condition and the amount of faeces produced for both males and females. We analysed the influence of condition on the amount of faeces produced with two ANOVAs: one for males and a second for females.

*Measuring Food Transit Time*

We tested the assumption of equal transit times (Table 1: assumption b) by conducting an experiment during 28–30 March

and 12–27 April 2010 that compared transit time between male tactics (females were also tested for comparison). We collected animals for this experiment ($N = 16$ for attached males, 15 for satellite males, and 14 for females), and then left them in the holding tanks for 12 h before the experiment began so that all had defecated prior to the start of the experiment. We cut a large, fresh shrimp into 10 equal pieces ($1.07 \pm 0.08$ g) and soaked it for 10–30 min in a solution of 2.1 g carmine red dye (an inert digestion marker) and 1–2 ml of water. We fed each animal by taking it out of the holding tank, turning it ventral side up on a table and placing individual pieces of shrimp in its mouth, ad libitum for 20 min. We then checked the animals every 3 h until we observed the red dye in their faeces. We chose this 3 h interval based on observations from the waste production experiment.

We wanted to compare the transit time among our three groups, but we also wanted to know whether the amount of shrimp consumed or the animal's physical condition influenced transit time. Therefore, we conducted an ANCOVA with transit time as the response variable, status (attached, satellite or female) as the explanatory variable, and the amount of shrimp consumed and the crab's physical condition as covariates. Additionally, the reduced feeding hypothesis predicts that attached males will eat more than satellite males when given an opportunity to feed (Table 1: prediction 2). We compared the amount of shrimp eaten among the three groups with an ANOVA, and then conducted least squares means contrasts to identify specific differences between groups.

### *Stable Isotope Analysis of Faeces*

To test whether values of $\delta^{15}$N of faeces were higher for attached males compared to satellite males (Table 1: prediction 4), we collected faeces from the waste production experiment (spring 2008 samples only, $N = 19$ for each group). We removed any sand present in the samples, and then each faecal sample was ground to a homogenous, fine powder using a mortar and pestle. All samples were analysed by the Stable Isotope Mass Spectrometry Lab in the Department of Geological Sciences at the University of Florida to determine values of $\delta^{15}$N (‰ normalized to air). We compared stable isotope values among the three groups with an ANOVA, and then conducted least squares means contrasts to identify specific differences between groups.

### *Effect of Starvation on Faecal Stable Isotope Values*

We first tested whether differences in $\delta^{15}$N values between attached and satellite males are due to nutritional stress (Table 1: assumption c) by conducting an experiment where we fed some crabs and starved others. On 27 March 2011, we collected 20 satellite males from Seahorse Key and brought them to the lab at the University of Florida in Gainesville. Crabs were kept on a 12:12 h light:dark cycle in a 1360-litre tank of filtered sea water with a salinity of 28–30 ‰, at room temperature (approximately 21 °C). Partial water changes were conducted every third day to alleviate nitrate build-up. We hand-fed all crabs a diet of freshly frozen bay scallops (*Chlamys patagonica*) that were purchased from a local grocery store (Publix brand). Every other day for 4 weeks, each crab was fed ad libitum for 15 min. After this acclimation period, we placed each crab in a 22-litre container with an oxygen bubbler for 48 h. We checked the containers every 3 h and collected any faeces that were produced. The faeces collected at this time were used to obtain the pretreatment $\delta^{15}$N values. We then randomly chose 10 crabs to receive a feeding treatment, and another 10 crabs for a starving treatment. For animals in the feeding treatment, we continued the same feeding schedule as before. Animals in the starving treatment received no food, but were handled exactly as in the feeding treatment to simulate the feeding process. The experiment ran for 4 weeks, and on the last day we feed all crabs once ad libitum for 15 min. We then placed animals back into individual containers for 48 h and collected all faeces to obtain post-treatment $\delta^{15}$N values. Each faecal sample was placed onto a coffee filter and dried for 4 h in an oven at 60 °C. The samples were then removed from the filter, ground to a homogenous powder, and analysed at the University of Florida Stable Isotope Mass Spectrometry Lab. All horseshoe crabs were then fed ad libitum for 1 week and returned to the beach from which they had been collected.

We conducted paired *t* tests on the feeding and starving groups separately to test whether the mean difference between pre- and post-treatment values was different from zero. We then conducted a matched-pairs analysis of grouped data with 'treatment' (feeding or starving) as a grouping variable. This analysis performs two *F* tests that evaluate whether the values across treatment groups differ: (1) the 'mean difference' tested whether the change across the pair of responses (pre- and post-treatment values) differed in the feeding and starving groups; (2) and the 'mean mean' tested whether the average response for a subject differed in the feeding and starving groups (SAS Institute 2007).

### *Gut Contents Analysis*

The above experiments reflect our best efforts to measure the feeding habits of attached and satellite males without sacrificing animals. While these experiments can show support (or not) for the reduced feeding and nutritional stress hypotheses, they are indirect. Therefore, a measure of gut contents was needed to confirm the waste production experiment and to fully interpret the isotope results. We attempted to use a nonlethal lavage technique, but this failed. Thus, in order to measure directly what and how much males were eating, we decided to sacrifice a limited number of animals and examine their gut contents. We collected 10 attached males and 10 satellite males on 20 April 2011 while they were spawning on the evening tide. We collected these animals on the first day of that particular week-long spawning cycle to maximize the likelihood that attached males would have some food in their gut. This also allowed us to compare directly (and conservatively) the amount of food in the gut between attached and satellite males (Table 1: prediction 3). The animals were euthanized by immediately placing them in a freezer for 24 h (Botton & Ropes 1989). The euthanized crabs were fixed in 10% formalin for 4–5 days; we then dissected out the digestive tract and stored it in 90% ethanol for 2 weeks (Botton & Ropes 1989). We first cut open the digestive tract (esophagus, proventriculus, mid gut and hindgut) to estimate gut fullness (e.g. a score of 100% was assigned if the entire length and width of the gut was filled). Gut contents were then removed by hand and placed in vials with 90% ethanol (Botton & Ropes 1989).

During the removal of gut contents, we specifically separated sea grass that was found in the esophagus and proventriculus (but not the lower digestive tract) from other materials because it represents a low trophic-level food source. Therefore, differences in sea grass consumption between attached and satellite males may inform whether any differences in $\delta^{15}$N values are the result of nutritional stress, or of feeding on different trophic levels (Table 1: assumption d). All sea grass was then dried in an oven at 60 °C for 4 h and weighed (mg). We used *t* tests to compare (1) gut fullness and (2) amount of grass in the esophagus and proventriculus between attached and satellite males.

To meet the assumptions of normality and homogeneity of variance, we log-transformed the values of (1) the amount of faeces defecated, (2) transit times, (3) weight of shrimp consumed and (4)

**Table 2**
Values of various measures for three groups of horseshoe crabs (attached males, satellite males, and females) collected from Seahorse Key, FL, U.S.A., in 2008–2010

| | Attached males | | | Satellite males | | | Females | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean±SD | *N* | 95% CI | Mean±SD | *N* | 95% CI | Mean±SD | *N* | 95% CI |
| Carapace width (cm) | 16.2±0.85 | 43 | 16–16.5 | 16.1±1.0 | 43 | 15.8–16.4 | 21.4±2.1 | 43 | 20.8–22.1 |
| Defecation (g, dry weight) * | 0.79±1.1 | 27 | 0.34–1.24 | 1.24±1.4 | 27 | 0.70–1.78 | 3.8±3 | 27 | 2.61–4.94 |
| Shrimp consumed (g, wet weight) | 0.48±0.3 | 16 | 0.31–0.65 | 0.44±0.3 | 16 | 0.28–0.59 | 0.29±0.2 | 16 | 0.16–0.42 |
| Transit time (h) | 18.9±9.1 | 16 | 14.0–24.0 | 17.5±6.7 | 15 | 13.8–21.2 | 22.8±10.7 | 14 | 16.3–29.0 |
| Faeces $\delta^{15}$N (‰) | 5.0±1.3 | 19 | 4.4–5.6 | 4.2±1.1 | 19 | 3.7–4.7 | 4.0±1.6 | 19 | 3.3–4.8 |
| Gut fullness (%) | 12.4 ± 1.2 | 10 | 8.9–17.4 | 31.0±1.2 | 10 | 22.2–43.4 | — | — | — |
| Foregut sea grass (mg) | 9.4 ± 2.3 | 10 | 4.2–14.5 | 3.1±1.4 | 10 | 0–6.3 | — | — | — |
| Median condition† | 8 | 43 | 7–10‡ | 7 | 43 | 5–9‡ | 7 | 43 | 6–9‡ |

* Waste minus debris in control tanks. Control tanks had an average of 0.13 ± 0.3 g of material present (95% CI = 0.01–0.26).
† Based on carapace colour, amount of mucus present and degree of pitting of the carapace (5 points each, 15 points denotes best possible condition).
‡ 25% and 75% quartiles.

sea grass weight in the gut prior to all analyses. All tests were two tailed, and all variation is reported as standard error, except where noted. Statistical tests were performed with JMP v.8 (SAS Institute, Inc., Cary, NC, U.S.A.), and all figures were created using Sigma Plot (SYSTAT Software 2008) and Adobe Illustrator (Adobe Systems 2007).

## RESULTS

### *Waste Production*

The mean amount of waste produced (controlled for body size) differed between the three groups (Table 2, Fig. 1). In support of prediction 1, satellite males produced 57% more waste than attached males (paired *t* test: $t_{25} = 2.7$, $P = 0.012$); females also produced more waste than either attached males ($t_{25} = 6.1$, $P < 0.0001$) or satellite males ($t_{25} = 5.4$, $P < 0.0001$). Condition was not related to the amount of faeces produced for males (ANOVA: $F_{7,\ 45} = 0.8$, $P = 0.569$) or females ($F_{7,\ 19} = 0.6$, $P = 0.722$), which verifies assumption a.

### *Transit Time*

We found verification for assumption b: the whole model ANCOVA was not significant ($F_{10,\ 34} = 1.0$, $P = 0.440$), and transit time was not influenced by mating status ($F_2 = 1.3$, $P = 0.284$), carapace condition ($F_7 = 1.0$, $P = 0.421$), or the amount of food consumed ($F_1 = 0.2$, $P = 0.669$; Table 2, Fig. 1). In contrast to prediction 2, attached and satellite males did not differ in the amount of food eaten during the 20 min feeding period (contrasts: $F_{1,\ 45} = 0.2$, $P = 0.679$); however, females ate more (contrasts: $F_{1,\ 45} = 4.1$, $P = 0.048$) than both attached and satellite males (Table 2).

### *Stable Isotope Analysis of Faeces*

In accordance with prediction 4, the mean $\delta^{15}$N values for attached males were slightly higher than those for satellite males (difference = 0.82 ± 0.4‰; contrasts: $F_{1,\ 54} = 3.6$, $P = 0.063$) and females (difference = 0.99 ± 0.4‰; contrasts: $F_{2,\ 54} = 3.1$, $P = 0.024$; Table 2, Fig. 2). We found no difference between satellite males and females (difference = 0.18 ± 0.4‰; contrasts: $F_{1,\ 54} = 0.2$, $P = 0.677$; Table 2, Fig. 2).

### *Experimental Starvation*

The mean difference between pre- and post-treatment was not greater than zero for the fed group (mean = −0.54 ± 0.4‰; paired *t* test: $t_8 = -1.4$, $P = 0.208$), but was greater than zero for the starved group (mean = 1.06 ± 0.2‰; paired *t* test: $t_9 = 6.3$, $P = 0.0001$), confirming assumption c. Analysis of grouped data showed

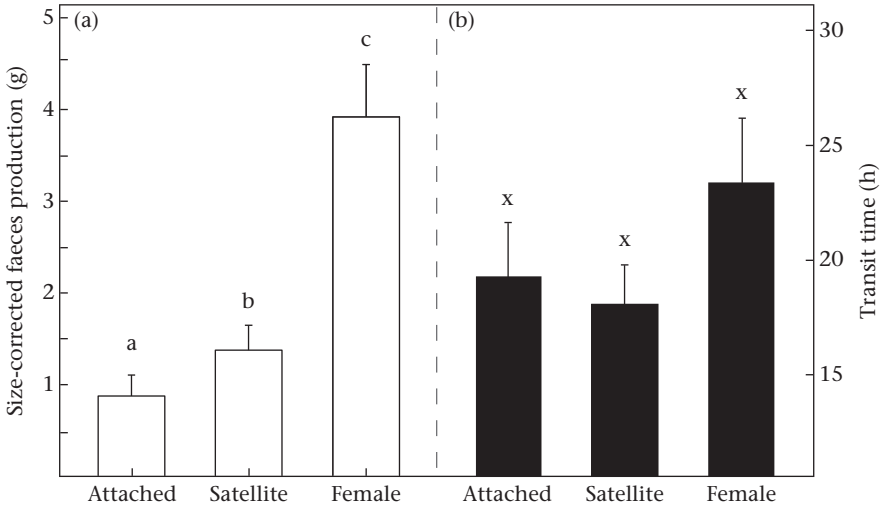


**Figure 1.** (a) Mean ± SE faecal mass produced in 12 h by wild-caught horseshoe crabs ($N = 19$/group), corrected for the amount of waste found in control tanks and the body size (carapace width, CW) of animals: ((waste − control)/CW × 100). (b) Mean ± SE transit time of food through the gut in horseshoe crabs that were experimentally fed carmine-red-dyed shrimp ($N = 16$ attached males, $N = 15$ satellite males, $N = 14$ females). All individuals from the three groups were collected from Seahorse Key, FL, U.S.A. Different letters above bars denote significant differences ($P = 0.05$) between groups based on least squares means contrasts.

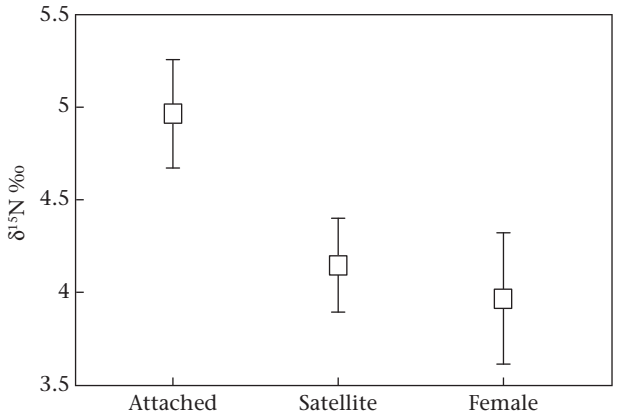


**Figure 2.** Mean ± SE values of $\delta^{15}N$ stable isotopes in faeces produced by three groups of horseshoe crabs ($N = 19$ each for each group) collected from Seahorse Key, FL, U.S.A. Values for attached males were higher than those of satellite males ($P = 0.063$) and females ($P = 0.024$).

differences in the response (pre- and post-treatment) of $\delta^{15}N$ values across the two treatment groups (fed and starved) for both the among-pairs 'mean mean' (matched pairs: $F = 9.1$, $P = 0.008$) and the within-pairs 'mean difference' (matched pairs: $F = 14.9$, $P = 0.001$; Fig. 3).

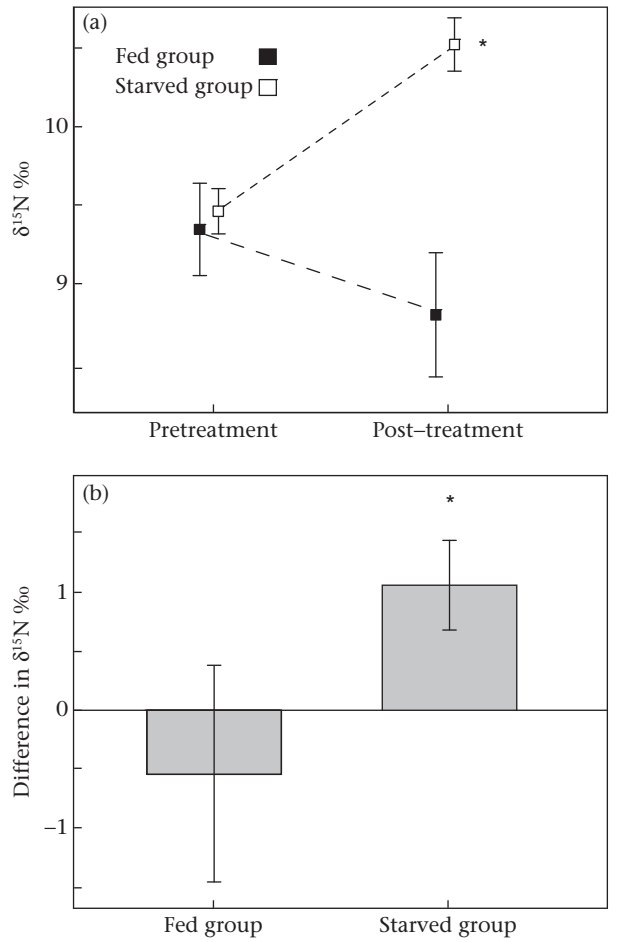


**Figure 3.** (a) Mean ± SE pre- and post-treatment values of $\delta^{15}N$ from two groups of satellite male horseshoe crabs: one group was starved for 4 weeks and the other group was fed scallops ad libitum for 4 weeks ($N = 10$ each). (b) Mean ± 95% CI difference in $\delta^{15}N$ values for the two experimental groups (post-treatment minus pretreatment). An asterisk indicates a significant difference ($P \leq 0.001$) between pre- and post-treatment values.

*Gut Contents*

In accordance with prediction 3, gut fullness (all contents) was 150% greater ($t$ test: $t_{18} = 4.0$, $P = 0.0008$) for satellite males compared to attached males (Table 2, Fig. 4). Additionally, we found confirmation for assumption d: attached males had 200% more sea grass in the esophagus and proventriculus ($t$ test: $t_{15} = 2.3$, $P = 0.035$) than did satellite males (Table 2, Fig. 4).

## DISCUSSION

The maintenance of condition-dependent ARTs in a population depends on there being conditions under which each tactic is more successful (Gross 1996; Brockmann & Taborsky 2008). Until this study, the trade-offs for the attached tactic in male horseshoe crabs have not been obvious. Satellite males produced more faeces than attached males, had higher gut fullness and had slightly lower $\delta^{15}N$ values than attached males. Thus, our results support the hypothesis that advantages of the attached tactic come at a cost of reduced feeding and nutritional stress.

Food transit times (range 6–39 h) were well within the time that most males remain attached to females (mean ± SD = 3.7 ± 6.1 days; Brockmann & Penn 1992). Coupled with the fact that attached males produced some faeces during the waste production experiment, it appears that males and females probably feed while paired. However, attached males only produced approximately half as much waste on average as satellite males. In addition, there was no difference in transit time between attached and satellite males, and condition did not influence the amount of faeces produced. Thus, the results from the waste production experiment demonstrate support for the reduced feeding hypothesis. The gut content analysis showing that the guts of satellite males were 2.5 times fuller than those of attached males also strongly supports this hypothesis.

Lower faecal production and an emptier gut could have been due to attached males being less motivated to feed, as opposed to being due to the physical constraint of being attached. But, this appears to be unlikely because the amount of food eaten by attached and satellite males in the transit time experiment did not differ. In fact, the reduced feeding hypothesis makes the opposite prediction: that attached males should be more motivated to eat. We found no support for this prediction, although our measure of consumption might not accurately reflect motivation to feed due to the rather artificial feeding conditions. Alternatively, our results may have been due to satellite males eating more recently. For example, perhaps attached males do not feed at all after attaching, and that the waste we saw was what remained of their intake prior to attaching. Nevertheless, both possibilities suggest reduced food consumption for attached males in this population. Furthermore, we show that this reduced feeding is costly for males that are using the attached tactic, even though attached males may have lower energetic requirements than satellite males, because the female 'carries' them and because they are not required to spend energy locating multiple mating groups on each tide. Additionally, given the trade-off between food passage time and digestive efficiency (Penry 1993), males may slow their food passage time to increase assimilation efficiency when attached, thereby compensating for not eating as much as the satellite males. Our study was not designed to test this, but passage times did not differ for attached and satellite males, and evidence of increased $\delta^{15}N$ values for attached males refutes this hypothesis and demonstrates nutritional stress in attached males.

A model proposed by del Rio & Wolf (2005) predicts that $\delta^{15}N$ values will increase with fasting time. It seems paradoxical that $\delta^{15}N$ should increase in excreta of starving animals, even though $^{15}N$ is preferentially retained and $^{14}N$ is excreted. However, the increase in

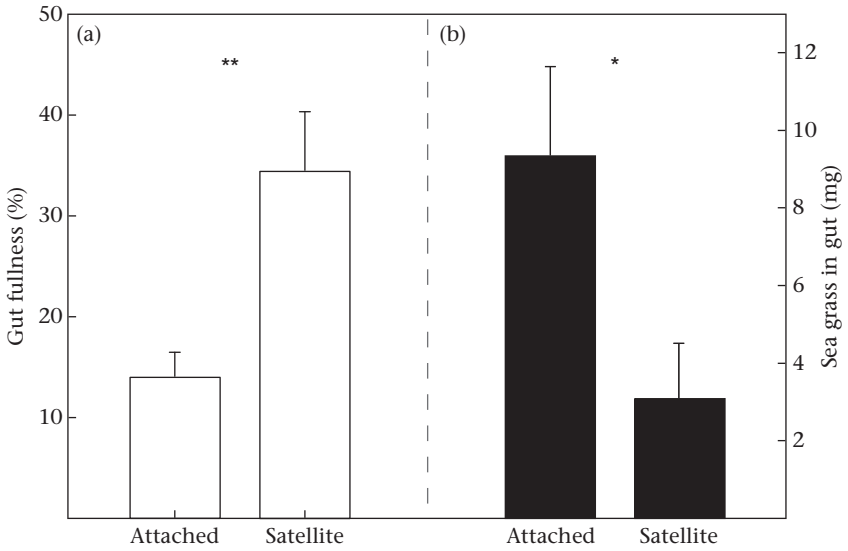


**Figure 4.** Mean ± SE (a) percentage of gut fullness and (b) mass of sea grass found in the gut of attached and satellite male horseshoe crabs ($N = 10$ each). Asterisks indicate significant differences ($^{*}P \leq 0.05$; $^{**}P \leq 0.001$) between attached and satellite males.

$\delta^{15}$N values of excreta during starvation is thought to result from the breakdown of structural proteins (that tend to have higher $\delta^{15}$N values) that progressively contribute to the pool of labile proteins (i.e. those most readily metabolized to nitrogenous waste), thus becoming the primary source of nitrogen in the excreta (Castillo & Hatch 2007; McCue 2007). Additionally, in horseshoe crabs, nitrogenous waste is converted to ammonia and dumped via their book gills and coxal gland (Towle et al. 1982) rather than in their faeces.

Our observations of horseshoe crabs showed that the faeces of attached males had $\delta^{15}$N values that were 0.82 ± 0.4‰ higher than satellite males. While this difference was not statistically significant, the effect size was nearly 20% higher for attached males, and lack of significance may be due to our relatively small sample sizes. In order to attribute this difference to nutritional stress we first had to show that starvation produces an increase in $\delta^{15}$N values in faeces. Experimentally, we found that a 4-week starvation increased $\delta^{15}$N values by 1.06 ± 0.2‰ from the initial measurement, whereas the values did not change for animals that were fed. The degree of enrichment that we found observationally and experimentally is comparable to the results from other studies. For example, in quail that were fed a reduced food intake, blood $\delta^{15}$N values increased by 0.8‰ compared to controls (Hobson et al. 1993); in lizards, uric acid $\delta^{15}$N values increased by 2.2‰ after 14 days of starving (Castillo & Hatch 2007); in *Daphnia*, whole body tissue $\delta^{15}$N values increased by 0.4‰ after 5 days of starving compared with controls (Adams & Sterner 2000); and in spider hatchlings, whole body tissue $\delta^{15}$N values increased by 1.3‰ after 12 days of starving compared with initial values (Oelbermann & Scheu 2002).

We found little evidence that differences in $\delta^{15}$N values between attached and satellite males reflects a difference in the trophic level at which they are feeding or a difference in their diet. Horseshoe crabs create a slurry of sediment and food when they feed (Botton et al. 2003), and it seems likely that attached males are able to grab food particles missed by the females. However, we found that attached males are less similar to females in $\delta^{15}$N values than are satellite males. Because attached males are physically associated with females, we would have expected the opposite outcome. Perhaps attached males selectively feed on animal tissue (which has higher $\delta^{15}$N values) rather than on plant matter and detritus (which is a typical food source that has low $\delta^{15}$N values; Carmichael et al. 2004). If attached males ate at a higher trophic level (e.g. ate less organic material) than satellite males or females, it could explain why attached males had higher $\delta^{15}$N values, and also why they differed more from females than from satellite males. However, we found the opposite pattern: attached males actually consumed three times more plant material than did satellite males. This result, along with the finding that experimental starving increased $\delta^{15}$N values, indicates that the increase in $\delta^{15}$N values of naturally occurring attached males are the result of a period of fasting, as opposed to differences in diet.

The pattern of more plant material in the diets of attached males may further explain how amplexus disrupts feeding. In addition to blocking the mouth and preventing attached males from burying into the substrate, amplexus may also interfere with the processing of food. Horseshoe crabs grab food items using terminal pincers located on their chelicerae, pedipalps and prosomal legs 2–4 (sensu Botton et al. 2003), which then direct the food towards the mouth for processing (Manton 1964; Wyse & Dwyer 1973; Botton 1984; Botton et al. 2003). Like many Chelicerata, horseshoe crabs use pairs of biting or chewing coxal gnathobases to process food (Manton 1964). Coxal feeding is performed by repeated transverse abduction and adduction of the proximal segments of the walking legs (i.e. gnathobases and coxa). Spines on each gnathobase are directed inward and serve to hold and macerate food. During the rhythmic movements of feeding, successive pairs of legs move out of phase (phase difference = 0.5 s), resulting in food being partially shredded and pushed towards the mouth (Manton 1964; Wyse & Dwyer 1973). Once drawn into the mouth, food passes through the esophagus into the proventriculus, which is a muscular organ that further fractures food into a pulp by muscle action (Botton et al. 2003).

Larger food items are processed by gnathobases of legs 3 and 4, which shred out a strand of tissue and draw it forward towards the mouth. Hard food items are gripped between the chilaria (highly reduced, spine-covered appendages) and are frequently held in position by extending the genital operculum 90° from the normally flat position. Once in position, hard food items are cracked by the 6th coxae before being passed to gnathobases of legs 2–4. In contrast, soft food is manipulated by limbs 1–4 and can be placed directly into the mouth (Manton 1964). Amplexus may inhibit the grasping of food with chelicerae (as they are in close contact with

a female's opisthosoma when attached) or interfere with the rhythmic movements of coxal feeding that are needed to process larger, tougher food items, and may also prevent attached males from extending their genital operculum to hold harder food items in place. Thus, attached males may be restricted to feeding on soft food items that can be grasped by the pincers of their more posterior legs and placed directly in the mouth, and then macerated by the proventriculus. Sea grass may be an easily accessible food item that could fulfil such requirements.

Taken together, our results demonstrate that reduced consumption of food and a period of nutritional stress are costs of the attached tactic not previously considered. There is evidence that male horseshoe crabs in other populations remain attached longer than those in the Seahorse Key population used in our study (Shuster 1954; Barlow et al. 1987; Moore 2004), and that males in other horseshoe crab species are more firmly attached and remain attached for longer periods (Botton et al. 1996; Brockmann & Smith 2009), suggesting even greater costs of attaching in those populations and species.

In conclusion, this is one of the first studies to use stable isotopes to investigate a predicted period of nutritional stress in a natural population of animals. Moreover, this study furthers our understanding of the trade-offs in this system and provides a key piece of information that may potentially explain why these alternative tactics in horseshoe crabs take the form that they do. Our findings show that the satellite tactic has specific benefits in that these males are able to feed, whereas feeding is restricted for attached males. Low-energy alternative phenotypes or behaviours often evolve as a release from the energetic demands of 'preferred' phenotypes (Taborsky 1998; Widemo 1998; Cummings & Gelineau-Kattner 2009), and in some systems, a male's success for a given tactic may partially depend on energy reserves (McCauley et al. 2000). Therefore, older males in poorer condition may not be able to afford the cost of reproduction (i.e. periodic fasting) that accompanies being attached to a female during breeding. Consequently, the satellite tactic may allow males to maintain (or regain) a positive energy balance while still obtaining reproductive success. Investigating this hypothesis is the next step to fully understanding the evolution and maintenance of alternative tactics in horseshoe crabs. Finally, the feeding costs associated with reproduction that we found, and the use of stable isotopes techniques to measure such costs, has implications in other systems with similar ARTs (Wells 1977a, b; Robertson 1986a, b) and in systems with extended periods of mate guarding (Alberts et al. 1996; Sparkes et al. 1996; Saeki et al. 2005).

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