The use of a novel method of quantifying sperm to determine the potential for sperm depletion in male American lobsters *Homarus americanus* (H. Milne Edwards, 1837) (Decapoda: Astacidea: Nephropidae)

Benjamin C. Gutzler[1,2], Tracy L. Pugh[3] and Winsor H. Watson III[1]

[1]Department of Biological Sciences, University of New Hampshire, Durham, NH 03824, USA;

[2]Wells National Estuarine Research Reserve, Wells, ME 04090, USA; and

[3]Massachusetts Division of Marine Fisheries, New Bedford, MA 02744, USA

Correspondence: B. Gutzler; e-mail: bgutzler@gmail.com

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ABSTRACT

Sperm limitation is a concern for a number of heavily fished decapods; however, work to assess this concern is sometimes hampered by a lack of simple techniques to quantify sperm transferred during reproduction. Our primary goal was to determine if DNA measurements could be used to quantify the sperm content of spermatophores and thus facilitate investigations of sperm limitation in American lobsters (*Homarus americanus* H. Milne Edwards, 1837). This was achieved by measuring the amount of DNA in a sample and then calibrating those values by using flow cytometry to count the number of individual sperm present in the sample. Our results show that the DNA quantification technique provides a fast and accurate way to quantify sperm. We then demonstrated the utility of the method by using it to examine the rate at which males can produce sperm under simulated conditions of repeated mating events, a situation that might lead to a reduction in the number of sperm per spermatophore. While spermatophores obtained from male lobsters at three-day intervals varied substantially in the number of sperm they contained (range 427,090–5,028,996; mean 2,306,473), there was no clear decline in sperm count over time. These results suggest that male lobsters replenish their sperm supplies rapidly, and that sperm recharge rate is unlikely to be a factor that could lead to sperm limitation in American lobster populations.

Key Words: commercial fisheries, DNA quantification, electroejaculation, mating, sperm limitation, spermatophore

INTRODUCTION

Sperm limitation occurs when the number of eggs requiring fertilization exceeds the number of sperm available to fertilize them (Levitan & Petersen, 1995). This may occur if too few males are available as potential mates, the males present are incapable of producing sufficient quantities of sperm to fertilize the female's entire clutch of eggs, or the sperm passed are of poor quality (MacDiarmid & Butler, 1999). Fished decapods may be particularly vulnerable to sperm limitation because harvesting often leads to the removal of larger males that are capable of contributing the most sperm to the population (Sainte-Marie *et al.*, 2008; Sato, 2012; Pardo *et al.*, 2015; Ogburn, 2019).

Estimating the number of sperm that males in the population may be able to transfer is a key metric in determining whether sperm limitation is occurring, yet relatively little research has been done on this topic in decapods (Sainte-Marie, 2007; Ogburn, 2019), due in part to the technical challenges inherent in successfully extracting and enumerating sperm cells. Most previous efforts to quantify sperm in decapod ejaculates focused on manually counting them using hemocytometers or spermacytometers (Wolcott *et al.*, 2005; Butler *et al.*, 2011; Rains *et al.*, 2016). An alternative approach that was used by Pugh *et al.* (2015) involved measuring the size of individual sperm cells, using histological sections of spermatophores to estimate the total volume occupied by sperm, and extrapolating total counts from these volumes. Each of these methods requires subsampling of the spermatophore to make quantification manageable, and the whole process can be very time-consuming, especially when analyzing many samples. Another approach that has been used is counting sperm cells with flow cytometers, which provide both rapid and accurate counts of sperm cells by passing fluorescently stained sperm cells through a detector that measures fluorescence (Christensen *et al.*, 2004; Cournault & Aron, 2008; Dufresne

69 *et al.*, 2019). Unfortunately, flow cytometers are expensive and thus may not be readily available
70 to all researchers. We therefore set out to develop an improved method for quantifying the
71 number of sperm in a lobster's spermatophore in order to improve our ability to study the
72 reproductive capacity of species thought to be at-risk for sperm limitation.

73 Recent advances in genomics and molecular biology have now made it possible to
74 rapidly and inexpensively measure the quantity of DNA in a sample. Furthermore, because each
75 sperm cell contains a single copy of the genetic material of the father, it is possible to use the
76 amount of DNA in a sample to calculate the number of sperm present (Hines *et al.*, 2003; Doyle
77 *et al.*, 2011). An analogous approach has been used in lobsters to determine if early stage eggs,
78 obtained before they have started to divide, have been successfully fertilized (Johnson *et al.*,
79 2011). We sought to adapt DNA quantification to develop a fast and accurate method for
80 quantifying the number of sperm cells in the spermatophores of the American lobster *Homarus*
81 *americanus* (H. Milne Edwards, 1837).

82 *Homarus americanus* supports the most lucrative single-species fishery in the United
83 States (foss.nmfs.noaa.gov). While not a single-sex fishery, mature females are
84 disproportionately protected from harvest compared to males, which can result in conditions that
85 may put them at risk for sperm limitation (Cobb, 1995; Pugh, 2014; ASMFC, 2015; Jury *et al.*,
86 2019). The mating system is based on female choice, with a preference for a dominant male, and
87 males guard the female pre- and post-copulation for a period of several days (Atema, 1986;
88 Atema & Steinbach, 2007). Male lobsters can mate with several females sequentially (Waddy *et*
89 *al.*, 2017), however. the rate at which sperm can be produced may limit the amount transferred to
90 females later in the mating sequence. Consequently, female lobsters in heavily exploited
91 populations may be increasingly challenged to find a male capable of providing enough sperm to

fully fertilize their egg clutches (Gosselin *et al.*, 2003, 2005; Tang *et al.*, 2019). Situations of this type have been observed in other decapod species subject to sex-selective harvest (Hines *et al*., 2003; Carver *et al.*, 2004; Sato *et al.,* 2005; Sato, 2012; Pardo *et al.,* 2015, 2017). Although lobster resource managers often focus on conserving females and boosting egg output (see for example ASMFC American Lobster Board proceedings August 2017; http://www.asmfc.org/uploads/file/59f0fb52AmLobsterBoardProceedings_Aug2017.pdf), the efficacy of such measures would be limited if a sperm-limited situation exists. An improved ability to directly measure the number of sperm produced by male lobsters would provide an important tool to better understand the reproductive capacity of a population, and assess whether sperm limitation may be occurring.

The ejaculate of *H. americanus* is morphologically typical of nephropid lobsters, consisting of a single, tubular spermatophore made up of the tightly packed sperm mass surrounded by acellular layers that provide protection and energy reserves for the sperm (Fig. 1; Kooda-Cisco & Talbot, 1982; Subramoniam, 1993; Comeau & Benhalima, 2018). For clarity, we refer to this entire ejaculated structure as the spermatophore. During mating, the spermatophore is transferred to the female's seminal receptacle, where it is stored for several months until it is used to externally fertilize eggs that are subsequently attached to the female's abdomen (Aiken *et al.*, 2004). The internal storage of the spermatophore within the female's seminal receptacle makes it very difficult to estimate the amount of sperm passed during mating. Histological examination of the seminal receptacle's contents can yield an estimate of sperm volume (Pugh, 2014), but a simpler approach to assessing the number of sperm males can produce is to procure spermatophores directly from the male via electroejaculation (Kooda-Cisco & Talbot, 1983; Pugh *et al.*, 2015). Spermatophores obtained this way are morphologically normal and can be

used for artificial insemination, suggesting little difference between those obtained "on demand" and those extruded during mating (Aiken *et al.*, 1984). Because lobsters produce sperm continually and are capable of mating year-round, electroejaculation can provide viable sperm samples throughout the year (Aiken & Waddy, 1986; Waddy *et al.*, 1995; Comeau & Benhalima, 2018).

We first developed and tested a DNA-based method for estimating the number of sperm within a spermatophore. We then used this method to estimate the number of sperm typically contained within a given volume of sperm mass, which then provided us with a tool to calibrate previous, and future, volume-based estimates of male fecundity. We subsequently demonstrated the utility of this method with an experiment designed to determine if sperm reserves can be depleted by repeated simulated mating events (electroejaculations) under controlled conditions in the laboratory.

MATERIALS AND METHODS

Sperm quantification technique

Male lobsters ($N = 10$, carapace length (CL) 73–93 mm) were trapped off the coast of New Hampshire and Massachusetts, US by personnel from the University of New Hampshire (UNH), New Hampshire Fish & Game, and/or Massachusetts Division of Marine Fisheries, and held in a recirculating tank on the UNH main campus (Durham, NH, USA) from May through September 2018. Lobster health was assessed visually to ensure no shell diseased males were included. Spermatophores were obtained by electroejaculation (Fig. 1) following the methods of Kooda-Cisco & Talbot (1983), and immediately transferred to 1 ml of 0.33 M $MgCl_2$ prior to processing. The $MgCl_2$ was used to reduce the incidence of spontaneous acrosomal reactions caused by the

presence of calcium in seawater (Talbot & Chanmanon, 1980a), and to inhibit the textural changes of the spermatophore's outer plug material, which commonly occur following contact with seawater (Subramoniam, 1993; Waddy *et al.*, 2017).

A total of 11 spermatophores were collected from the 10 lobsters (one lobster was sampled a second time, two weeks after the first sampling). Only one spermatophore was collected per sampling event; if spermatophores were produced from both gonopores, one was randomly selected for processing and the other was discarded. Forceps were used under a dissecting microscope while the spermatophore was in the $MgCl_2$ solution to remove as much of the acellular plug material as possible from each spermatophore to ensure the sperm were free to disperse in the solution. The remaining sperm mass, diluted in 0.33M $MgCl_2$, formed the stock solution, from which we created a series of dilutions to provide a range of concentrations of sperm. The stock solution was vortexed for several seconds then split into either four concentrations (100%, 75%, 50%, and 25%), or three concentrations (100%, 67%, and 33%), by diluting samples of the stock solution with 0.33M $MgCl_2$ to a final volume of 240 µl. This process yielded a total of 42 distinct subsamples that were used to construct a calibration curve of the relationship between the amount of DNA in a sample and the number of sperm cells present. From each of these diluted subsamples, 150 µl was used for DNA quantification, and 72 µl was used to count the number of sperm cells present using flow cytometry. The remainder of the subsample was held in reserve to allow samples to be rerun or examined microscopically. The cell counts from the flow cytometry served as the "real" sperm counts against which we calibrated the DNA measurements (see below).

To quantify the DNA present, each 150 µl subsample was sonicated for ~10 seconds to break up any small remaining portions of the sperm plug, further diluted 1:1 with deionized

161 water and vortexed for ~5 seconds. The osmotic shock from the addition of the deionized water
162 served to lyse the sperm cells and enhance DNA yield during the extraction process. Following
163 dilution with deionized water, a GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-
164 Aldrich; St. Louis, MO, USA) was used to extract DNA from 150 µl of each sample, following
165 the kit protocol for "fresh whole blood." This extracted DNA was then immediately quantified
166 using a Qubit 2.0 fluorometer with the Qubit dsDNA HS (high sensitivity) assay (Thermo Fisher;
167 Waltham, MA, USA).

168 A 72 µl subsample was used for flow cytometry to generate sperm cell counts. The
169 sample was stained with 8 µl of acridine orange (1:10,000 acridine orange in 1% Tween-80
170 (Thompson & Hunt, 1966)), which binds with the DNA present in the cell. A volume of 40 µl of
171 the stained sample was further diluted in 960 µl of 0.33M $MgCl_2$ and run through a Becton-
172 Dickinson FACScalibur flow cytometer (BD Biosciences; San Jose, CA, USA) at a flow rate of
173 60 µl min^{-1}. The cytometer used a 488 nm laser to excite fluorescence, which was measured
174 between 515 and 545 nm, allowing the cytometer to count the stained sperm cells as they passed
175 through the detector. The amount of time taken to reach 10,000 total detections was multiplied
176 by the flow rate to yield the volume of sample measured. The output from the flow cytometer
177 was analyzed in Flowing Software 2.5.1 (Perttu Terho, http://flowingsoftware.btk.fi) using
178 fluorescence intensity, forward scatter, and side scatter parameters to exclude detections of
179 cellular debris or plug material remaining in the sample. The number of sperm cells detected was
180 then divided by the volume of sample analyzed to yield the concentration of sperm cells in the
181 sample.

182 We used linear regression through the origin (i.e. intercept term set to 0) to determine the
183 relationship between the concentration of sperm cells (determined by the flow cytometer) and the

concentration of DNA present (determined by fluorometry). The resulting equation could then be used to predict the number of sperm present in a sample containing a given amount of DNA. Regression through the origin was chosen as the most appropriate measure of the relationship as the sperm cells themselves are the only cellular material in a spermatophore, and thus there should be no DNA present beyond that contained within the sperm cells (Kooda-Cisco & Talbot, 1982; Eisenhauer, 2003).

All statistical analyses were conducted in SPSS 26 (IBM; Armonk, NY, USA), unless otherwise specified. Residual plots were inspected for all parametric tests to ensure that the data satisfied the assumptions of normality and homoscedasticity.

Estimation of the number of sperm per volume of sperm mass

Spermatophores were obtained via electroejaculation from seven additional lobsters (captured as described previously; CL 72–92 mm) and immediately cut into transverse sections 1–2 mm long ($N = 25$ segments), using dissecting scissors under a dissecting microscope. Each section was illuminated using transmitted light and photographed using an Olympus DP21 digital camera (Olympus; Tokyo, Japan) mounted on the microscope, then transferred to $MgCl_2$ for DNA extraction and quantification as described above. The photographs were analyzed using the measurement tools of Fiji (Schindelin *et al.*, 2012) to model the volume of the sperm mass within each section as a series of truncated cones (Supplementary material Fig. S1). Although this approach assumes a circular cross-section for the packed sperm rather than the trefoil section actually present (Kooda-Cisco & Talbot, 1982), it allows for changes in the width of the sperm mass along the length of the spermatophore section and can be estimated from a single image. At the end of this process, for each of the 25 sections of sperm mass, we knew the volume, as

calculated from the photographs (Supplementary material Fig. S1), and the number of sperm, as calculated using the DNA quantification method described above. A linear regression was then applied to these data to allow us to determine the number of sperm in a given volume of the sperm mass portion of a spermatophore. Prior to pooling data from different individuals for this regression, an ANCOVA was conducted to ensure that the relationship between sperm count and sperm mass volume was consistent between individual males.

Test of sperm depletion

The new quantification technique described above was then used to test the potential for sperm limitation due to repeated matings by using repetitive electroejaculations to simulate a series of mating events. We hypothesized that the number of sperm present in each spermatophore would decline over the course of several sampling times due to the need to produce new sperm to replenish the sperm passed during mating. During June 2019, early in the typical mating season, male lobsters were collected from New Hampshire waters as described previously. These lobsters (N = 12, 72–82 mm CL) were held in flow-through seawater tanks at the UNH Coastal Marine Laboratory (New Castle, NH, USA) for one week prior to beginning the experiment to ensure no mating activity had occurred immediately prior to the experiment. Water temperatures during this period ranged 9–16 ºC. Lobsters were fed herring (*Clupea harengus* Linnaeus, 1758) *ad libitum* every other day throughout the experiment. Lobsters were stimulated to produce a spermatophore on day 0, and then again after 3, 6, and 12 d. The three- day interval was chosen because male lobsters commonly cohabitate with a female for several days before and after mating (Atema, 1986), and thus intervals of several days between mating events are common (Waddy *et al.*, 2017). Both the left and right gonopores of each lobster were shocked at each time

period, but for consistency, only spermatophores produced by the left gonopore were collected for analysis. The whole spermatophore was sonicated and used for DNA extraction. Samples were processed in triplicate to produce three separate measurements of DNA content, allowing failed extractions to be excluded from the analyses. The average amount of DNA present across the triplicate samples was used in the regression developed in the previous experiments to calculate the number of sperm present within each spermatophore. The number of sperm present per spermatophore at each time interval was analyzed in R 3.5.2 (R Core Team, 2018) and RStudio 1.1.463 (R Studio Team, 2016) using a Friedman test, blocking by individual lobster.

RESULTS

Sperm quantification technique

A linear regression of cell counts from the flow cytometer against the quantity of DNA measured by the Qubit showed a significant linear relationship between the two measures in the 42 subsamples used to construct the calibration curve ($r^2 = 0.852$; $F_{1,41} = 235.588$, $P < 0.001$; Fig. 2). The regression yielded the following equation: no. of sperm = 800,795.574 × μg DNA. This equation was used to convert the quantity of DNA present in a sample to the number of sperm present for all subsequent analyses.

Quantifying the DNA content at different sample dilutions also provided a way to determine the reliability of the sperm quantification technique. When the DNA concentrations of the diluted samples were scaled up to reflect the original volume of the sample and compared with the undiluted samples (stock solution with a concentration of 100%), 10 of 26 (38%) of the diluted samples were within ± 10% of their respective 100% concentration DNA values, and 18

of 26 (69%) were within ± 20% of their respective 100% concentration DNA values. A paired t-test found no significant difference between the calculated original concentration of the diluted samples and their respective 100% concentration samples ($t = 1.082$, $df = 27$, $P = 0.289$). The DNA quantification technique thus appears to be reliable and insensitive to the dilution process.

Estimation of the number of sperm per volume of sperm mass

The ANCOVA testing whether the number of sperm varied consistently with the volume of 24 sperm mass segments collected from seven male lobsters found that, while the intercept terms for individual males differed, there were no differences in the slopes of the relationships (Table 1). The 24 segments of spermatophore were therefore pooled for inclusion in a single regression. There was a significant linear relationship between the volume of the sperm mass within discrete spermatophore sections (as determined by image analysis) and the number of sperm present, as determined by DNA quantification ($r^2 = 0.844$; $F_{1,23} = 124.014$, $P < 0.001$; Fig. 3). The equation for this regression was: no. of sperm = 462,474 × mm^3 volume of sperm mass + 88,049. Consequently, the estimated volume occupied by a single sperm cell in these samples was 2.162×10^{-6} mm^3.

Test of sperm depletion

Of the 12 lobsters tested, 11 produced spermatophores at all four sampling intervals and were included in the analyses. No obvious trends were found in the number of sperm produced in the spermatophores obtained in the repeated samples (Fig. 4A). There were no significant differences in the number of sperm per spermatophore produced over time (Friedman Chi square = 2.89, $df = 3$, $P = 0.41$, Fig. 4B). The number of sperm per spermatophore varied widely

between lobsters, from a minimum estimated value of 427,090 to a maximum estimated value of 5,028,996 sperm within a single spermatophore, and even identically sized individuals produced spermatophores with dramatically different sperm counts (Fig. 4A, starred markers). Overall, the mean number of sperm per spermatophore was 2,306,473 ± 1,245,315 (mean ± SD, $N = 44$).

DISCUSSION

The use of DNA to quantify lobster sperm

Using measurements of DNA to quantify the number of sperm cells present in a whole spermatophore, or a portion of one, appears to be an improvement over other techniques that have been used for the same purpose. It is faster and less expensive than using a handheld hemocytometer (G. Gnanalingam, personal communication), an expensive flow cytometer, or analysis of histological sections (TP and BG, personal observations). The cost to analyze each sperm sample using the method presented here is under $5 (USD), after acquiring the required instrument (Qubit fluorometer). Although there is preparation time associated with the DNA extractions, many samples can generally be run in parallel and thus there is little additional time investment associated with processing more samples. It is also likely that the DNA method is more accurate, because it makes it possible to directly measure all sperm contained within a sample, rather than extrapolating the total amount of sperm present in a spermatophore based on measurements obtained from multiple subsamples.

While the DNA quantification method we outline proved to have many advantages and provide accurate values, it could probably be improved with some minor modifications. While the extraction process is simple and involves a commercially available kit, it may not be thoroughly optimized for this application. The diploid genome size for *H. americanus* has been

299 measured to be 9.49 ± 0.2 pg of DNA (Deiana *et al.*, 1999). The amount of DNA per sperm
300 estimated from the data presented here, however, is markedly less, at 1.25 pg, corresponding to a
301 diploid genome size of 2.50 pg of DNA. This may be the result of poor extraction efficiency. The
302 "sticky" spermatophore of *Homarus* presents a challenge when attempting to isolate sperm cells.
303 Further efforts to improve extraction efficiency are warranted, perhaps including investigating
304 the use of the NaOH solutions that have been used previously to help dissolve anomuran crab
305 spermatophores (Sato *et al.*, 2008).
306
307 *Comparisons with previous estimations of sperm in American lobsters*
308 We were able to determine the number of sperm in a given volume of the sperm mass of a
309 spermatophore by combining image analysis of a section of a spermatophore with a
310 measurement of the number of sperm contained within the portion using the DNA quantification
311 technique. This procedure made it possible to then calculate the total amount of sperm in a whole
312 spermatophore and then compare these values with previous estimates. Pugh *et al.* (2015)
313 measured the volume of sperm masses within 48 spermatophores obtained from American
314 lobsters as equal to 60–100 mm CL, and reported volumes ranging from 0–20 mm^3. They then
315 calculated, by dividing the total volume of the sperm mass by the volume of an individual sperm,
316 that those spermatophores (not including those with no sperm) contained anywhere from
317 1,002,181 to 107,364,726 sperm. A hypothetical 5 mm^3 sperm mass would therefore contain
318 26,315,789 sperm. By comparison, our results (Fig. 3) suggest that a 5 mm^3 sperm mass would
319 contain 2,400,419 sperm. This difference might be due to variability in estimates of the space
320 occupied by a single sperm cell. We estimated the volume occupied by a single sperm cell, using
321 the regression shown in Fig. 3, to be 8.649×10^{-6} mm^3. This is larger than the value of 1.34 ×

322 10^{-7} mm^3 that can be calculated for a sperm with a cylindrical body 20 µm long and a diameter
323 of 3 µm (Talbot & Chanmanon, 1980b), or the volume of 1.9×10^{-7} mm^3 estimated by Pugh *et al.*
324 (2020). It should be taken into account, however, that each sperm also possesses three spikes that
325 extend out almost 40 µm, and the sperm are oriented randomly within the sperm mass rather than
326 packed in a maximally efficient manner (Kooda-Cisco & Talbot, 1982). While the volume
327 occupied by the body of the sperm might be close to the values estimated by Talbot &
328 Chanmanon (1980b) and Pugh *et al.* (2015), the total space occupied by an individual sperm cell
329 is likely to be larger than would be expected based on the measurements of the sperm body alone.
330 Given the random packing of sperm and the difficulty inherent in trying to estimate the volume
331 occupied by both the sperm cell and its extensions, we thus believe the DNA-based approach is
332 probably the most accurate method to estimate the number of sperm cells in a spermatophore.
333 Furthermore, preliminary tests suggest that this method may also be a good way to quantify the
334 number of sperm contained within a seminal receptacle (BG, unpublished data).
335
336 *Comparisons of sperm output between American lobsters and other decapods*
337 These new estimates suggest that the number of sperm per spermatophore produced by *H.*
338 *americanus* is lower than in some other decapod species (reviewed in Sainte-Marie, 2007).
339 Although the smaller adult male *H. americanus* tested here may only produce 2–3 million sperm
340 per spermatophore, this greatly exceeds estimates of the number of eggs produced by females
341 each time they spawn, typically ~ 10,000 for females of an equivalent size to the males used, but
342 can be up to nearly 100,000 in larger females (Estrella & Cadrin, 1995). This produces a
343 sperm:egg ratio of ~ 200-300:1 for lobsters of the size tested here, which exceeds the sperm:egg
344 ratios reported for some other decapods (Sainte-Marie, 2007; Butler *et al.*, 2011), assuming the

spermatophore is used for only one clutch of eggs. Female American lobsters, however, have been known to use a single spermatophore to fertilize more than one clutch of eggs (Waddy & Aiken, 1986), unlike spiny or rock lobsters, which have no long term spermatophore storage capacity. This high sperm:egg ratio in American lobsters is consistent with other decapod species that use stored sperm to fertilize multiple clutches (Carver *et al.*, 2004; Rodgers *et al.*, 2011). Furthermore, the lack of sperm depletion between successive extrusions of spermatophores (Waddy *et al.*, 2017; herein) suggests male *H. americanus* may be capable of greater reproductive output during a mating season than other decapods that may take weeks to months to recover sperm stores following mating (Kendall *et al.*, 2002; Sato *et al.*, 2005; Butler *et al.*, 2015; Pretterebner *et al.*, 2019).

Sperm depletion and implications for mating in the American lobster

Although there was no significant effect of sampling time across all lobsters in the test of sperm depletion, individual lobsters varied widely in the number of sperm produced per spermatophore (Fig. 4A). Of the 11 lobsters that produced spermatophores at all four sampling times, four produced more sperm in the last spermatophore than in the first, whereas two produced final spermatophores containing less than 75% of the sperm in the first one they produced. Even identically sized lobsters produced a range of sperm per spermatophore. For example, three 80 mm CL lobsters produced averages of 3.2×10^6, 1.62×10^6, and 1.56×10^6 sperm per spermatophore, respectively (Fig. 4A, starred markers). All the lobsters tested were collected on the same day, from traps within a few miles of each other, and were held in the same flow-through ambient seawater system, and there were no obvious visible differences between the individuals that might explain these discrepancies. While it is clear that male lobsters produce

spermatophores that vary widely in terms of their size and in the number of sperm cells (consistent with the findings of Pugh *et al*., 2015), the factors underlying these differences in spermatophore contents remain unclear, and this remains an important topic for future research.

Because only spermatophores from the left gonopore, and not the theoretically independent right gonopore, were collected throughout the experiment, depletion on one side would not necessarily prevent mating. It is unknown whether male lobsters use one gonopore preferentially, or use the two gonopores sequentially in successive matings. Regardless of whether the absence of sperm depletion across successive spermatophores is due to rapid recharge of sperm reserves, or reflects stored sperm in the vas deferens, these data suggest that male American lobsters are likely capable of inseminating at least eight females within the space of two weeks with little to no sperm depletion. The high potential reproductive output of male lobsters found here is consistent with previous reports suggesting that male lobsters are capable of mating with many females within a relatively short time window (Pugh, 2014; Waddy *et al.*, 2017). The rate-limiting factor for reproduction is therefore probably the typical period of cohabitation with a female post-copulation. If multiple females undergo simultaneous molts, a single male may not mate with all the potentially receptive females present (Pugh, 2014; Waddy *et al.*, 2017; BG, unpublished data), despite having sufficient sperm to do so.

Even if male lobsters do not experience reductions in the number of sperm cells they are able to pass during mating, there may be reductions in the amount of seminal fluid or other accessory components of the spermatophore. These provide paternity assurance and protection, in the case of the sperm plug (Pugh *et al*., 2015), and help maintain the viability of the sperm for the months between insemination and fertilization of eggs (Subramoniam, 1993; Comeau & Benhalima, 2018). Although we did not measure the amount of accessory materials present in

each spermatophore, others have indicated that reserves of sperm and accessory materials may regenerate at different rates (Kendall *et al.*, 2001; Sato *et al.*, 2005; Butler *et al.* 2015). The relative importance of sufficient accessory materials, compared with sperm count alone, in fertilization success for American lobsters remains unclear (Pugh *et al.*, 2015).

In a limited preliminary trial during the winter of 2018, three lobsters were sampled three times each over the course of 4 d (baseline, 2 d later, and 4 d later), and each experienced a nearly 50% decline in sperm count at each sampling (BG, unpublished data). Male lobsters produce sperm throughout the year, suggesting that seasonal variation is likely not the cause of this discrepancy (Kooda-Cisco & Talbot, 1983; Aiken & Waddy, 1986; Comeau & Benhalima, 2018). It may instead be the result of a nutritional requirement that was not met for those animals because they were held in the laboratory for some time prior to the trial (Talbot *et al.*, 1983; Aiken *et al.*, 1984). Caution should therefore be taken if conducting studies of reproductive systems during times of the year when lobsters are not typically reproductively active and in an optimal nutritional state.

The apparent lack of sperm depletion in *H. americanus* stands in stark contrast to what is seen in some other decapod species, and deserves further study. Improved techniques, such as the one presented here, will fortunately make future studies of lobster reproductive physiology faster and easier and thus accessible to a broader group of scientists.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Crustacean Biology* online.

S1 Figure. Process used to model volume of sperm mass from photographs of spermatophores.

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594

595 **List of tables**

596 **Table 1.** Results of a preliminary ANCOVA to determine whether regression coefficients
597 differed significantly between different males ($N = 7$) when comparing the volume of the sperm
598 mass in a section of spermatophore with the number of sperm present, as determined by DNA
599 quantification. Though individual lobsters had significantly different intercepts, due to the
600 differences in sizes of the spermatophore segments used, there were no significant differences in
601 coefficients between males.

602

Source	**df**	**MS**	**F**	***P***
Volume	1	5.573×10^{11}	108.06	<0.001
Lobster	6	4.145×10^{10}	8.04	0.001
Volume × Lobster	5	9.003×10^{9}	1.75	0.199
Error	12	5.157×10^{9}		

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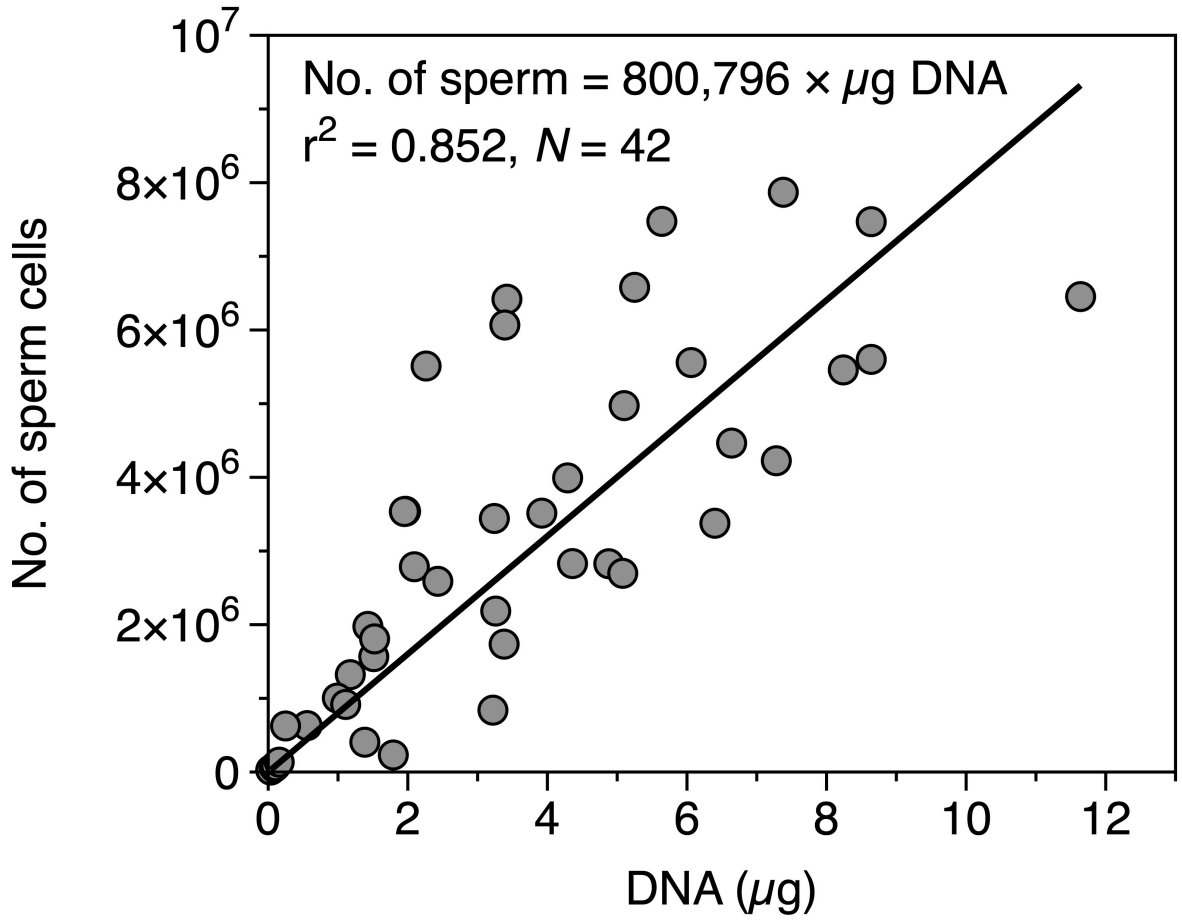
608 **List of figures**

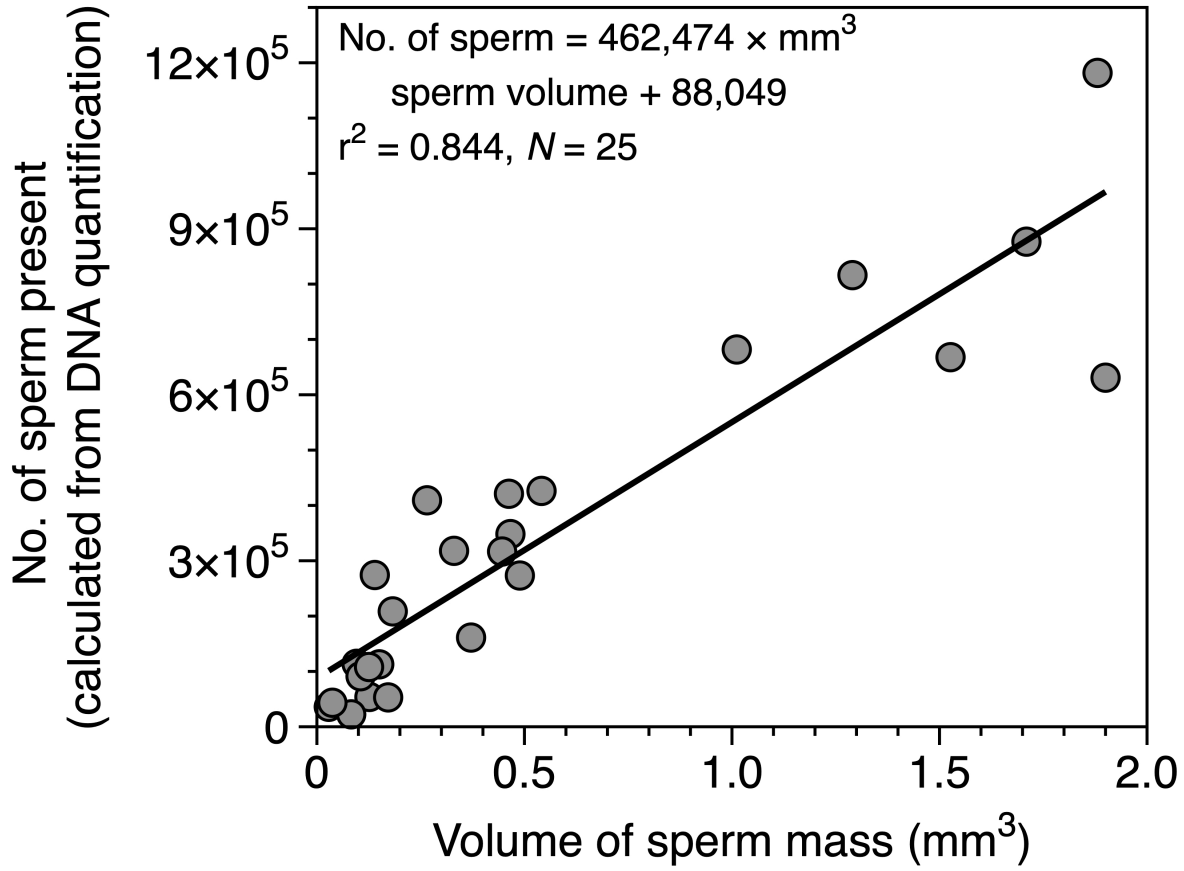
609

610 **Figure 1.** Spermatophores produced by two different male American lobsters *Homarus*
611 *americanus*, showing the differences in shape and composition possible between spermatophores.
612 Both lobsters were ~ 90 mm CL. The white material is the sperm, which is surrounded by layers
613 of acellular material. Scale bars = 2 mm.



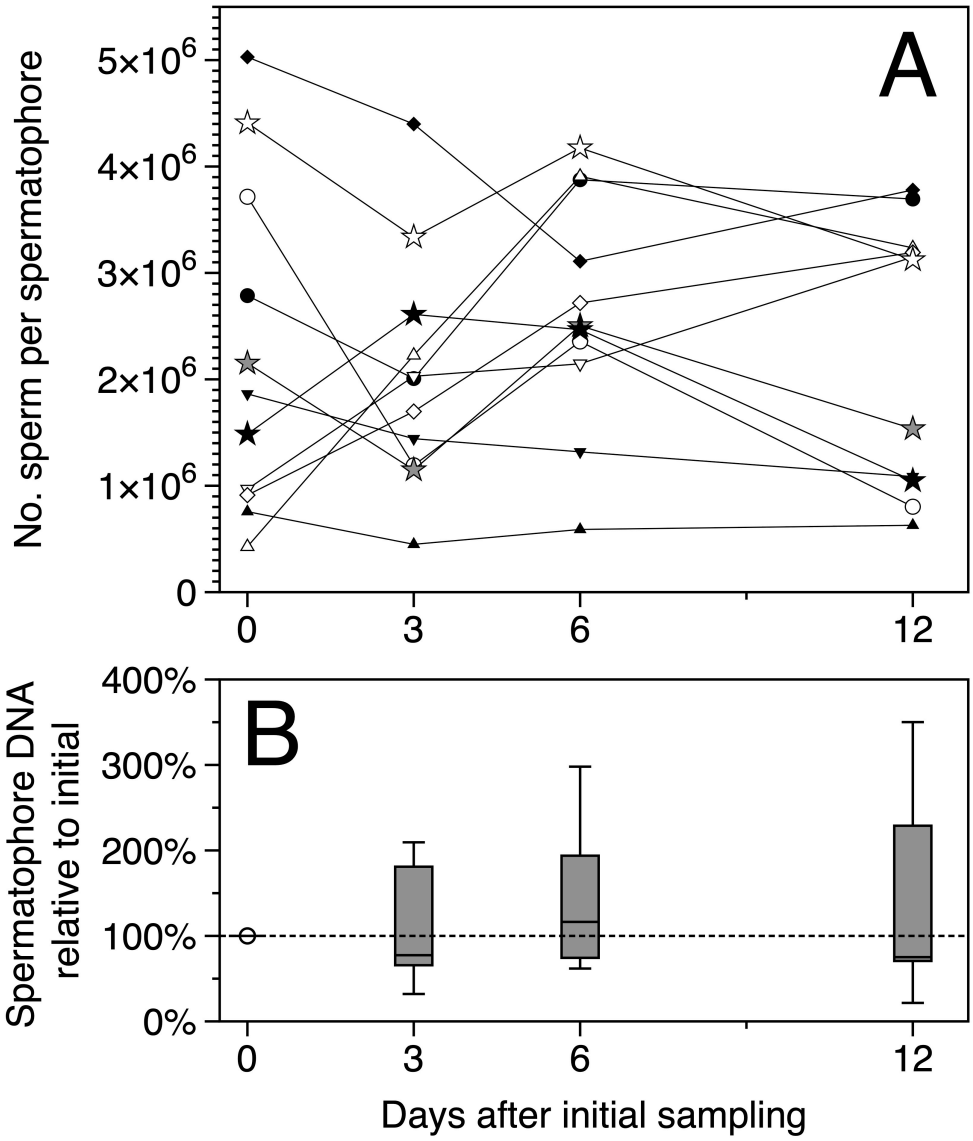
614

615 **Figure 2.** The relationship between the amount of DNA (µg), measured by the Qubit system, and
616 the number of sperm cells present, as measured by flow cytometry, in 42 subsamples taken from
617 11 spermatophores.



618

619 **Figure 3.** The relationship between sperm mass volume (determined by image analysis) and the
620 number of sperm contained within the mass (determined by DNA quantification and the
621 regression developed previously (see Fig. 2)).



622

623 **Figure 4.** Results of the test of sperm depletion, showing no significant differences between time
624 points in the number of sperm per spermatophore (N = 11 lobsters; Friedman Chi square = 2.89,
625 df = 3, P = 0.41). Scatter plot depicting variations in sperm counts between individual lobsters,
626 with each line representing a different lobster. Each point is the average of the triplicate DNA
627 measurements from the spermatophore produced at each time point. Star-shaped markers
628 indicate the results from three 80 mm CL lobsters, showing disparities in sperm count between
629 identically sized males (**A**). Box and whisker plot showing changes in DNA content (and thus
630 sperm) per spermatophore during the test of sperm depletion. Data were normalized to the initial
631 spermatophore DNA content for each lobster. Whiskers represent the extent of data within 1.5
632 IQR and the dashed line represents 100% of the initial concentration (no change) (**B**).
633

Figure S1. Demonstration of the process used to model sperm mass volume from photographs of spermatophores. A photograph of a spermatophore, showing the central sperm mass surrounded by acellular plug material. Red lines are 1 mm increments, used to calibrate measurements. Note that the original photograph from which this was taken depicts the whole spermatophore (Fig. 1) and this image has been cropped to better show details (**A**). The central sperm mass is extracted from the photograph and divided into a series of isosceles trapezoids (**B**). For each trapezoidal segment, measurements of three dimensions are made using Fiji (Schindelin *et al.*, 2012). Bottom radius R_1, top radius R_2, and height H are used to calculate the sperm volume as a truncated cone, using the equation $\text{Volume} = \pi \times H \times ((R_1^2 + R_2^2 + R_1 \times R_2) / 3)$ (**C**).