

**Does multiple paternity increase with female body size in the common  
map turtle (*Graptemys geographica*)?**

by

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Supervisor's signature

## ABSTRACT

In the common map turtle, females are much larger than males. In general, larger females can produce more and/or bigger offspring. It has also been shown that larger offspring survive better. Therefore, larger female map turtles should be more attractive to males. If males incur non-trivial costs of mating, such as missed opportunities or sperm limitation, they should mate preferentially with larger females. Accordingly, multiple paternity should be more common in larger females. To test this prediction, we captured 34 gravid females spanning the full size range of reproductive females and induced egg laying with oxytocin. We then collected blood samples from 338 hatchlings for paternity analyses. The number of sires was deducted from the paternal alleles at 3 microsatellite loci. Due to laboratory setbacks, only eight of the 34 clutches were analyzed. A third paternal allele was found only once, for one hatchling, at one locus. The fact that no strong evidence of multiple paternity was found is inconclusive. Low sample size, sub sampling the clutches and the low variability of the three microsatellite loci reduce the ability to detect multiples sires in a clutch. The question of multiple paternity augmenting with female body size in the common map turtle is left unanswered, but might soon come as the molecular laboratory work is almost sorted out: DNA extraction and PCRs are troubleshooted, and fragment analysis is soon to follow.

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## INTRODUCTION

Animals exploit two types of mating systems : combat and scramble. In the combat mating system, individuals fight each other to gain access to sexually receptive members of the opposite sex. Males are usually bigger than females in this case since a larger body size tends to give a competitive advantage that translates into better mating success (Alcock, 2001; Gans & Huey, 1988; Shuster & Wade, 2003). In a scramble mating system, individuals race to reach receptive members of the opposite sex first. More competitive individuals have a higher searching endurance, perseverance and perceptiveness, not aggression (Alcock, 2001). Females are usually bigger than males in this case (Shuster & Wade, 2003).

In birds and mammals, males are usually larger than females (Kreb & Davies, 1997). Sexual size dimorphism, with females larger than males, is the norm for most freshwater turtles (Ernst *et al.*, 1994). However, sexual size dimorphism like the one found in the common map turtle (*Graptemys geographica*) is rarely this extreme, with no overlap between sexes: male carapace length is between 102 and 149 mm at maturity, while female carapace length is between 205 and 290 mm (Figure 1). Males thus measure half the length of females and average 20% of their mass (Vogt, 1980). Very little is known about the map turtle's reproduction. This gregarious and wary species is very hard to approach. Nesting females less than 190mm in carapace length have not been caught (Newman, 1906). Courtship and mating apparently occur both in spring and autumn when the turtles are still aggregated at the hibernacula (Vogt, 1980). During both seasons pairs of *G. geographica* have been observed walking at the bottom of the lake, a male following a female as if to mate (Ernst *et al.*, 1994). However, since males and

females are both able to store sperm (Kuchling, 1999), the actual time of mating is unknown and could occur all year long (Gist *et al.*, 2001). Courtship under the form of males stroking ('titillating') their forefoot claws on the female's orbital region has been documented in other species of *Graptemys* such as *G. ouachitensis* and *G. pseudogeographica*, but not in *G. geographica*. Elongated fore claws are not present in this species like they are in the other two (Vogt, 1980). A male may mount a female after cloacal contact, or swim to her head and, after making snout to snout contact, rapidly bob his head up and down. Coitus can last 15 seconds to over 4 hours, during which time the male stays at a 45° angle above the female carapace and remains motionless, with his forelimbs usually hanging over his head (Vogt, 1980). We strongly suspect map turtles use a scramble mating system, because the female is so much bigger than the male, and because males show no signs of aggression.

This project studies sexual selection in the common map turtle. Sexual selection occurs when individuals differ in their ability to conquer mates. As mentioned earlier, males may compete directly (male-male competition), or indirectly by trying to appeal to member of the opposite sex (female choice) (Alcock, 2001; Krebs & Davies, 1997). Multiple paternity, which arises when more than one father sires a clutch, can occur in both the combat and the scramble mating system, due to direct and genetic benefits that are not mutually exclusive.

It is interesting for females to mate with more than one male if they receive direct benefits in return. Such direct benefits are nuptial gifts or access to resource-rich territories that will help them produce healthier offspring. Protection from other sexually

harassing males can be offered through mate-guarding, before or after copulation. Parental care is another important direct benefit females can derive, when the male helps care for the offspring (Alcock, 2001; Krebs & Davies, 1997). Male *G. geographica* being so small compared to females suggests that they cannot force copulations and that mating would follow female choice. Because a single copulation is sufficient to fertilize all of a female's eggs (Pearse *et al.*, 2002) and because female turtles do not receive direct benefits such as nuptial gifts or parental care in exchange for copulation (Pearse & Avise, 2001; Kuchling, 1999), female *G. geographica* should not be expected to engage in multiple mating. However, precisely since there is no parental care for the offspring, that nests are subject to heavy predation, and that emergent hatchling survival is very low (Gans & Huey, 1988; Kuchling, 1999), it is in the female's top priority to acquire the best genetic benefits for her offspring. Several hypotheses suggest that females gain from polyandry. Such genetic benefits of multiple matings are indemnifying the female against the possibility that one of her mates is not fertile (Reynolds, 1996). It could avoid genetic incompatibility between male and female genotypes (Zeh & Zeh, 1996), avoid inbreeding and genetic defects resulting from stored sperm (Reynolds, 1996). It can promote the gain of 'good genes' for survival and sexual attractiveness, and increase genetic diversity among offspring (Pearse & Avise, 2001). Stored sperm provides an opportunity for multiple paternity as a result of sperm competition or cryptic female choice (Pearse & Avise, 2001).

In reproduction, individuals of both sexes should be picky when it comes to mate choice in order to maximize their own reproductive output. From the female's point of

view, eggs are very costly to produce/incubate. The only way for a female to augment her fitness is by rearing more offspring (Alcock, 2001; Krebs & Davies, 1997). This translates in a bigger body size in turtles (Iverson, 1992; Kuchling, 1999; Vogt, 1980). First off, the hard shell of turtles limits the interior volume available for food intake, breathing volume, energy and water storage, and reproductive output (Gans & Huey, 1988; Kuchling, 1999). Secondly, pelvic opening structure constrains egg size and offspring size in smaller bodied species (Congdon & Whitfield, 1987). Larger turtles can therefore produce more eggs, since they can accumulate more resources, and/or bigger eggs, because of their larger pelvic opening (Gans & Huey, 1988). Egg size is positively correlated to body size of hatchlings and larger hatchlings have a higher survival rate (Packard & Packard, 1988). A larger body size is also valuable by decreasing the risk of predation when nesting (Kuchling, 1999). Furthermore, larger females have survived longer (Shine, 2005), and viability should be an attractive trait amongst long lived species (Blouin-Demers *et al.*, 2005). Male preference for larger females has been documented in other reptiles, for example the garter snake (Shine *et al.*, 2001). For these reasons, we hypothesize that bigger females should be more attractive to males.

Males can sire offspring more quickly than females can produce them, and so their reproductive success is limited by access to receptive females (Krebs & Davies, 1997; Alcock, 2001). Even if there is no combat or courtship in map turtles, sperm itself might not be so cheap to produce. Olsson *et al.* (1997) demonstrated that spermatogenesis might entail a major part of reproductive costs of male adders, reducing the number of times they can remate successfully. Spermatogenesis is an episodic event in temperate turtles: it begins in early summer and sperm leaves the testes to be stored in

the epididymys in autumn. If mating is indeed promiscuous and occurs all year long, it is advantageous for males to conserve sperm and aliquot it amongst receptive females (Gist *et al.*, 2001). Mating can be time consuming; coitus itself can last up to four hours (Vogt, 1980). Thus, if males incur non-trivial costs of mating, such as missed opportunities or sperm limitation, they should mate preferentially with larger females. Therefore, we think that multiple paternity should be more common in clutches of larger female map turtles.

Multiple paternity has been documented in marine turtles (Kichler *et al.*, 1999; Moore, 2001; Crim *et al.*, 2002; Hoekert *et al.*, 2002) and in every freshwater species studied to date: painted turtles (*Chrysemys picta*; Pearse *et al.* 2002), snapping turtles (*Chelydra serpentina*; Galbraith, 1991), wood turtles (*Clemmys insculpta*; Galbraith, 1991 in Galbraith *et al.*, 1993), and giant Amazon side-neck turtles (*Podocnemis expansa*; Valenzuela 2000). In most cases, a high percentage of clutches was multiply sired.

In this study, microsatellite analysis will be used to determine if the occurrence of multiple paternity in a natural population of the common map turtle (*Graptemys geographica*) is correlated to female body size.



## **METHODOLOGY**

### ***3.1 Field work:***

Between June 7<sup>th</sup> and August 11<sup>th</sup> of the 2005 nesting season, 34 gravid females spanning the full size range of reproductive females were captured in Lake Opinicon (47° 37'N, 76° 13'W, Figure 2). Most females were hand caught while nesting on land on sandy Hump Island (n= 25) or by snorkelling around the island (3). One nesting female was caught on Dr Weatherhead's grassy property. Five other females were also caught in basking traps: 2 in Brooks Bay and 3 in Telephone Bay. Based at the Queen's University Biology Station, we would venture out to Hump Island around 9 am in the morning or around 7 pm in the evening. (Note: snorkelling is fruitless in the evenings because the sun is too low on the horizon to see anything in the water; algal blooms also decrease visibility during the day). Gravid females were recognized by feeling their abdomen for eggs with our fingers through the opening of the turtles' hind legs. Females were identified numerically in the order they were caught (#1-36), as well as alphanumerically by a unique marginal scute code. (Due to confusion, there never was a female #8, and female #13 escaped). Females were measured with callipers and weighed, and blood samples (0.03-0.05 ml) were drawn from the coccygeal vein. Egg laying was induced by an intramuscular injection of oxytocin (20 USP units/ml, 0.5 ml/kg) (Ewert and Legler 1978). Females were placed in a water bath in a dark room where they released their eggs. Females shot in the morning who didn't lay eggs by noon were given a second identical injection of oxytocin. After egg laying we felt the females' abdomen to verify if they were still retaining eggs (which never seemed to be the case). Females were set free at their capture site in the following days. Two females were caught a second time as

they were gravid with their second clutch of the season: females # 4 and #32. Female #4 was caught 24 days after her first capture, and laid only a single egg after one oxytocin injection. We were afraid her eggs perhaps weren't fully formed, and so we chose to release her instead of forcing her to release her eggs. Clutches were incubated in moist vermiculite (1:1 m/m) at 29°C. Water was added every third day to compensate evaporation. The hatchlings emerged 55-65 days later (mean = 60.23, SE = 0.63). Hatchlings were processed and identified numerically in the order they emerged (#1-338). Some eggs took a few days longer to hatch. Hatchlings were weighed and measured. Blood (0.03-0.05 ml) was drawn from all 338 surfacing hatchlings, either from the coccygeal vein (Figure 3) or subcarapacial vein using a 0.5ml insulin syringe fitted with a 28½ gage needle (Bulté *et al.*, 2005). Dead eggs (n = 67) were dissected to acquire tissue samples. All blood and tissue samples were stored in 70% ethanol at 4°C. Shortly after being processed, hatchlings were released at the site where their mother was captured.

### **3.2 Lab work**

Molecular genetic techniques able to identify single individuals are providing answers to a variety of questions concerning mating systems and kinship (Moon *et al.*, 2006). Microsatellites are stretches of DNA consisting of short tandem repeats of nucleotides in a non-coding region. Their often numerous alleles differ in the number of these repeats. Individuals seldom inherit the same copy from their mother and from their father, and so two unrelated individuals rarely have the same pair of sequences (Alberts

*et al.*, 2002; Queller & Strassmann, 1993). This makes microsatellites a great tool for assessing multiple paternity (Alberts *et al.*, 2002; Moon *et al.*, 2006).

Turtles are diploid: one DNA copy coming from the mother, the other from the father (Ernst *et al.*, 1994). Knowing the mother's genotype, one can subtract it from the hatchling's genotype and deduce the father's allelic contribution. After doing this for all the siblings in the clutch, if more than two father alleles are present for more than one hatchling at more than one locus, we can incur that more than one father sired the clutch (Lee & Hays, 2004). To achieve these results, three steps are necessary for every individual: extraction of the DNA from the blood sample, Polymerase Chain Reaction (PCR) of the desired microsatellite loci, and automated Fragment Analysis (FA) revealing allele sizes.

Unless otherwise specified, all manipulations were performed wearing gloves and with sterile autoclaved pipette tips and tubes in CAREG's common molecular laboratory at the University of Ottawa. Two different sets of pipettes were used to avoid contamination: Dr Blouin-Demers' for DNA extractions and a second set borrowed from Dr Guy Drouin for PCRs and FAs.

### 3.2.1 DNA extraction

DNA was isolated from all hatchlings' and mothers' blood samples (n=374) using a salt extraction method, slightly modified from De Souza's (2001) protocol. Because the ethanol in which samples were stored inhibits the activity of proteinase used in this technique, the alcohol had to be evaporated before proceeding with the extraction.

### *3.2.1.1 Initial evaporation step (and spectrophotometry of extracted samples)*

Different techniques were tried in attempt to standardize the final quantity of purified DNA obtained from raw samples. We tried either shaking or not the raw samples prior to pipetting to homogenize the blood in the solution, and varied the initial volumes pipetted for the evaporation step. After the DNA extraction of the first few samples was completed, their concentration was assayed with one of two different spectrophotometers. We wanted to figure out the optimal volume to pipett from the raw blood samples in the initial evaporation step that would give desirable DNA concentrations at the end of the extraction process.

The DNA samples were diluted in water (1/50) and their absorbance was read at 260nm (for DNA quantity) and at 280nm (to verify the purity of the sample). The first spectrophotometer used, the BioMate 3 Thermospectronic in CAREG's common molecular lab, analyzes one sample at a time with quartz cuvettes borrowed from Dr Marc Ekker's lab. The initial reading of a blank (consisting of the same water used to dilute the extracted samples) need not be repeated between each subsequent sample. The second spectrophotometer used is the Synergy HT from Bio-tek Instruments Inc. situated in the BioSciences building, and was utilized with the kind help of Dr Alp Oran. This spectrophotometer and the KC4 program version 3.4 read 96 DNA samples at once on a Corning Inc Costar 96 well UV flat bottom plate (courtesy of Dr Oran). At least 300µl are needed to fill the quartz cuvette, but the plate's wells cannot hold more than 200µl. With the Synergy HT spectrophotometer three blanks per plate were used and positive controls consisted of previously analyzed DNA samples on the BioMate. A sample's

reading at 260nm was always corrected by subtracting the reading of the blanks. This is done automatically and a receipt is printed on the BioMate spectrophotometer, and the KC4 program on the Bio-tek spectrophotometer can export the corrected 260nm readings to an Excel file. The following formula was used to infer the sample's DNA concentration in (ng/μl):

$$\begin{aligned} \text{DNA concentration (ng/}\mu\text{l)} &= \text{corrected absorbance at 260nm} \times \text{dilution factor} \times \text{DNA constant} \\ &= 0.abc \times 50 \times 50 \end{aligned}$$

Sample purity was also examined by dividing the absorbance at 260nm by the one at 280nm; if the ratio falls between 1.8 and 2.0, the sample is of good quality (which was usually the case with our samples).

After analyzing the first extracted samples, we found the following recipe for the evaporation step gave the most desirable DNA concentration at the end of the extraction: first shaking the raw blood sample and pipetting 125μl into a 1.5ml Eppendorf tube. Tubes were left uncapped, covered with a layer of Kimwipes (taped in place over the tube rack to prevent debris from falling in the samples) and placed to dry in a fume hood. Evaporation lasted 1-2 days, until all the ethanol was gone and the blood was a little dry dark clump. The blood disintegrates in the ethanol over time (after 4 months or so), and so it is possible to pipette cells with a p200 Gilson pipette without clogging the tip. 125μl sampled enough blood to allow us to see a DNA pellet at the end of the extraction, thus making the process easier, and gave DNA concentrations ranging from 10-125 ng/μl.

DNA samples with concentrations higher than this range (for example samples isolated previously with different initial evaporation volumes) were diluted and placed in 0.6ml Eppendorf tubes in the working solutions boxes, while the original DNA sample was saved and placed elsewhere in stock boxes.

#### *3.2.1.2 Lysis step*

Once the blood was dry and had the aspect of a little dark clump, the DNA extraction could continue. 600µl of cell lysis buffer and ~15µl of Proteinase-K were added to the samples, which were placed in a 55°C water bath until the blood cells ruptured (usually 1-3 days). Samples were flicked with fingers, vortexed, and more Proteinase-K was added if necessary when the clumps of blood weren't disintegrating. Samples didn't spend more than 24h at a time at 55°C; the temperature was lowered to 40°C overnight. When the samples transformed into a homogenous yellowish solution, De Souza's protocol (2001) was followed for the next extraction steps, and 12 samples took ~1.5 hours to complete.

#### *3.2.1.3 Final extraction step*

200µl of Protein precipitation solution (7.5 M Ammonium Acetate) was added, samples were vortexed for 15 seconds and centrifuged at maximum speed for five minutes. The top aqueous phase was removed and placed in a new autoclaved 1.5ml Eppendorf tube. 600µl of isopropanol was added, causing the DNA to precipitate in a white stringy mass floating freely in the solution. Samples were centrifuged at maximum speed for five minutes to collect the DNA in a small pellet. The supernatant was

removed and the pellet was washed with 200µl of 70% ethanol. Samples were centrifuged again at maximum speed for three minutes. The ethanol was removed and the pellet left to dry for ~20 minutes, after which it was resuspended in TE (10 mM Tris-HCl, 0.2 mM EDTA, pH 7.5). Samples were then incubated at 37°C until homogenous in the solution (usually overnight). If a pellet was still visible, tubes flicked with fingers or vortexed for a few seconds, then centrifuged and placed again at 37°C until the pellet dissolved. Samples were then stored at 4°C.

### 3.2.2 Polymerase chain reaction

PCRs took a month to troubleshoot (once we actually obtained bands) because no one at the University of Ottawa ever used such a small reaction volume. We used the microsatellite primers (Invitrogen) TerpSH 2U (5'-3': TGG CCA GCA GGA GTA ATG), TerpSH 2L (CTA TTA GGG CAG AGA CGA), TerpSH 5U (TTG CTG CTA TAT GCT TAA T), TerpSH 5L (CCT CCC TGC CTA TTG A), TerpSH 7U (CAC ACA CAC TGT ATT TTG ATA) and TerpSH 7L (CTA TGC CCT TTC TAG TTT G) developed for the *Malaclemys terrapin* (Hauswaldt & Glenn 2003) and used successfully by Freedberg *et al.* (2005) on another species of map turtles, *Graptemys khonii*. PCR amplification were optimized with unlabeled primers and tested on 2% agarose gels. A gradient PCR was performed to find the optimal annealing temperatures of the 3 sets of primers. PCRs were performed in 10µl volumes consisting of 1X buffer, 0.2mM dNTPs (both reagents domestically made in Dr Marc Ekker's lab), 4 pmole forward primer, 4 pmole reverse primer, 0.5 Units *Taq* DNA polymerase (Invitrogen), 6.1µl of water and 1µl of DNA template (concentration ranged 40ng/µl-100ng/µl). Three master mixes, one

for every pair of primers, were performed with all reagents except the DNA to minimize pipetting and omission errors. Thus, to know each individual's genotype, three PCR reactions were necessary. All reagents (including the DNA) were homogenized prior to pipetting. PCR manipulations were performed on ice. PCR cycles were performed on Eppendorf Mastercycler® egradient machines. For primers TerpSH 5 and 7, an initial amplification cycle (2 min at 94°C) was followed by 30 cycles of 1min at 94°C, 1 min at 50°C, 1 min at 72°C, and a final extension of 1 min at 72°C. For primer TerpSH 2, the annealing temperature was increased to 55°C. These cycles last approximately two hours. See Annex B. We were in communication with Dr Travis Glenn, who developed the SH primers used in this study, in order to obtain a copy of his clone to use as a positive control during our PCRs. Those efforts were unsuccessful and our PCRs were never done with positive control.

Depending on the number of samples analyzed, PCR reactions could be placed in 0.2ml Eppendorf tubes, or in a 96 well plate (ABgene Thermo-fast®). Since primers SH 5 and 7 have the same annealing temperature, they can share the same thermocycler, which means no more that 48 samples can be processed at once, unless they each have their own cyler. In CAREG's common molecular facility, five PCR cyclers are available and must be reserved before use.

### *3.2.2.1 PCRs with tubes*

When tubes are used, it saves time not to identify them prior to starting the experiment. A schematic can be drawn of where the PCR sample should go in the 96 well thermal cyler on an excel sheet (see appendix A). Three PCR racks are gathered



and each is identified SH2 or 5 or 7. Then three empty 0.2ml tubes are picked up and 1 $\mu$ l of DNA of the desired sample is pipetted in each tube. Tubes are placed down in the following order: one on the PCR rack corresponding to the SH2 reaction, the other of the 2<sup>nd</sup> PCR rack corresponding to the SH5 reaction, and the last tube on the 3<sup>rd</sup> PCR rack corresponding to the SH7 reaction. The same is repeated with all the DNA samples. 9 $\mu$ l of the related master mix is then aliquoted to each of the tubes on the matching PCR rack (i.e.: taken together are the SH2 master mix and the SH2 PCR rack occupied with tubes filled with DNA: these DNA tubes are filled with 9 $\mu$ l of master mix to complete the 10 $\mu$ l PCR reaction). Tubes are closed once this is done. Tubes are then identified with number on the caps (#1-x); add the same numbers manually to the Excel sheet. Tubes are spun for a few seconds to insure the DNA and the master mix are in contact before being placed on the thermal cyclers. Note: the manipulator must be very careful not to move the tubes out of the PCR racks before they are identified.

#### *3.2.2.2 PCRs with plates*

Plates are easier and faster to use since no caps need to be opened and closed and samples don't need to be manually identified. They are also great since the adhesive plastic cover can be peeled off one row/column at a time, making the transfer of PCR products to the fragment analysis plate less confusing. However, errors are easier to make, such as mismatching wells.

The same protocol described above can be used with plates and with tubes, except with a plate the master mix is distributed before the DNA. Plates can't be spun as well as tubes can, therefore the DNA is added second (following the Excel sheet diagram) and

placed at the bottom of the well directly in contact with the master mix. The 10 $\mu$ l are then mixed by pipetting up and down a few times. The plastic cover tapes the plate shut; care must be taken to insure the tape sticks properly the first time, because unsticking it can cause the solution in the wells to explode up and stick to the plastic cover, resulting in contamination or loss of reaction volume. The plate can be spun on the IEC-MultiRF Thermo IEC plate centrifuge to insure the DNA and the master mix are in contact.

### 3.2.3 *Fragment analysis*

I didn't have time to completely troubleshoot the fragment analysis, and attempted doing so until April 21<sup>st</sup> 2006. Genotyping was done on Beckman Coulter's CEQ 8000 Genetic Analysis System, which no one at the University of Ottawa knew how to use when I started my honors project in September 2005. To reveal allele sizes, PCRs were performed with fluorescent primers that label amplification products, which were then recognized and sized by the CEQ. These labeled products deteriorate quickly and so the PCRs needed for the Fragment Analysis were performed on that same day. They can be kept wrapped in foil at 4°C for a few days (up to a week), but more PCR product needs to be included in the fragment analysis mix.

The following WellRED dyes (Sigma-Proligo) were used to label the forward primers: D2 for SH2, D3 for SH5, and D4 for SH7. To save money and maintain balance in the PCR reaction, we used 0.2 pmole of labeled forward primer and 0.2 pmoles of *un*labeled forward primer. The remaining PCR recipe stayed the same. One fragment analysis for each individual was performed by combining (poolplexing) the PCR

products from all three PCR reactions together in one well. (We wanted to do poolplexing as opposed to single plexing because reagent costs are decreased by 2/3 this way). A 40µl poolplexing reaction consisted of 0.5µl of Size Standart 600 (Beckman Coulter), 27.5µl of Sample Loading Solution (Beckman-Coulter), and the following amounts of PCR products: 6µl SH2, 4µl of SH5, and 2µl of SH7. (Less SH7 products are used because the D4 dye is very intense). Samples were transferred directly to a CEQ sample plate with a 2-20µl multichannel pipettor borrowed from Dr Ekker's lab. The Size Standard and the SLS were 'master mixed' together, transferred to eight 0.6ml Eppendorf tubes and subsequently aliquoted to the sample plate's wells using a 10-100µl multichannel pipettor borrowed from Dr Ekker's lab. The plate was spun on a IEC-MultiRF Thermo IEC plate centrifuge to make sure i) no bubbles were present at the bottom of the wells, and ii) all the poolplexed solutions were in contact with one another (no Size Standart means no fragments will be sized). A drop of mineral oil (Beckman-Coulter) was added over the samples to prevent evaporation. A buffer plate loaded with 10 drops of Separation Buffer (Beckman Coulter) was run simultaneously with the sample plate. The data collected from each sample plate was saved in the computer on the C drive in the "Carine Fragment Analysis" folder. Two things were included in the folder name: the analyzed clutches' mother number and the date the plate was run on.

I didn't have time to learn how to use the CEQ's software to analyze the data. Instead I looked at each poolplexed sample's results graph, and manually entered the size of the peaks in an excel spread sheet, as well as the date the sample was run. This way, if some peaks seem ambiguous, all the original information about that sample can be retraced in the CEQ program by localizing the plate it was run on by clutch name and by

date. Some clutches were run on more than one plate to attempt to see the peaks that did not appear in the poolplexed samples on the first try.

I assumed that clutches with more than two paternal alleles were fathered by more than one male. A third allele appearing in only one offspring was classified as the result of an allelic mutation (Roques *et al.*, 2006). When the hatchling's alleles didn't match either of its mothers', these peaks were considered possibly inaccurate. To try to solve this problem, these samples were reanalyzed (a new PCR and fragment analysis were performed) to see if they gave different peaks. Ambiguous samples that could not be retested were not considered for paternity analyses. When a sample gave different results on different plates, I kept the ones that matched the mother's genotype for paternity analyses. One example of such ambiguous peaks is the occasional appearance of a high peak before the first peak on the Size Standard, occurring at all loci but more often for locus 5. This peak wouldn't appear or even give a hint of a peak when the same sample was run on other plates. Since these results weren't clear cut, and that these ambiguous peaks appeared at more than one locus, I arbitrarily chose not to include them in my analyses. Another example of ambiguous peaks, for locus 5, is a peak at 75 nucleotides. Sometimes there would only be a hint of a peak, slightly higher than the background noise and much lower than another common peak at 135 nucleotides. Since it was the only bump higher than the background noise for this locus, and that this locus has low heterozygosity (see discussion), I chose to keep it my analyses.

#### 3.2.4 *Statistical analysis*

Statistical analyses were performed in Excel and S-plus.

Data from female #4's second clutch was not included in the calculation on mean clutch size for the first linear regression ('Total eggs laid' vs 'Maternal carapace length') since the female did not lay her full clutch. Data from both clutches of female #32 were included in all analyses. In all analyses, I assumed that the females laid all their eggs.

## RESULTS

### 4.1 Clutch data

Instead of using carapace length to characterize female body size as it is currently done in the literature, I thought at first that an estimate of her abdominal volume might be more accurate. I arbitrarily used the formula to calculate a cylinder's volume to define female abdominal volume:

$$V = \frac{1}{4} \cdot \pi \cdot d^2 \cdot h$$

where “d” represents the mean of half of both carapace length and carapace width [ $(\frac{1}{2} CL + \frac{1}{2} CW) / 2$ ], and “h” is the carapace height. However, correlation between this new variable and carapace length, carapace width, plastron width, and carapace height all had a very high  $R^2$  of 0.98, 0.97, 0.99, and 0.80, respectively. These correlations were all highly significant with a p-value of 0. I therefore defined female body size as carapace length in the following analyses.

The smallest captured female measured 204mm in carapace length, and the largest female 274mm. The mean clutch size was 11.16 (SE=0.49) eggs, and ranged from 6-15 eggs. Unhatched eggs were present in 77% of clutches. Hatchling success (number of emergent hatchlings/number of eggs in the clutch) ranged from 0 to 100%. Larger females did not lay more eggs than smaller females (log10 transformation of ‘Number of eggs in clutch’:  $R^2=0.007$ ,  $p=0.64$ ,  $N=35$ ; Figure 4), but laid heavier eggs ( $R^2=0.26$ ,  $p=0.013$ ,  $N=23$ ) from which larger offspring hatched ( $R^2=0.29$ ,  $p<0.001$ ,  $N=35$ ; Figure 5). The first clutches laid were not weighed, which is why the sample size is lower for that statistic. There was no relation between female body size and hatching success, quantified as the number of emerging hatchling/number of incubated eggs (Squared

transformation of ‘Percentage of hatching success’:  $R^2=0.04$ ,  $p=0.26$ ,  $N=34$ ; Figure 6).

Conditions of normality were tested with a Komolgorov-Smirnov GOF test and homocedasticity was inspected visually for these analyses.

#### *4.2 PCRs and fragment analysis*

Genotypes were collected for a fraction of eight clutches out of a possible 35 clutches. A mean of 40.97% (SE=6.39%) of those eight clutches was successfully genotyped at locus SH2, 49.31% (SE=6.12) at locus SH5, and 40.2% (SE=6.75) at locus SH7. Since we always tried to genotype full clutches in one shot, unsuccessful results were the remaining percentages. These unsuccessful results yielded ambiguous peaks or no peaks at all. Fragment analysis was attempted for 110 out of a possible 338 hatchlings. A total of 36 were genotyped successfully at loci SH2 and SH7, and 43 hatchlings at loci SH5 (Table I); this represents 11% and 13%. Only three mothers were genotyped completely at all three loci. A total of 4 (perhaps 5) different alleles were found at locus SH2, 2 alleles were found at locus SH5, and 5 (perhaps 6) alleles were found at locus SH7 (Table I). The word ‘perhaps’ is used when the new allele was observed only once.

More than two paternal alleles were found only once, for hatchling #53 in clutch of female #1 at locus SH7. Other than this case, multiple paternity was never detected (Table II). Never were there clear results (unambiguous peaks) of a hatchling’s genotype not matching his mother’s.

## DISCUSSION

### 5.1 Discussion of methodology

#### 5.1.1 Field sampling

Since we captured four females the first night we went ‘turtle hunting’, and never caught that many on subsequent occasions, I believe we missed the beginning of the 2005 nesting season. Sampling before June 7<sup>th</sup> might have given us more females. This was impossible for me however, since I only started my stage at QUBS on June 7<sup>th</sup> 2005.

#### 5.1.2 Blood sampling

We developed a new technique to bleed hatchling emydid turtles. Published studies by Bennett (1986) and Wibbels et al. (1998) provided methodologies for bleeding hatchling sea turtles in the subcarapacial vein of the neck. Weighing roughly 20g, sea turtles are twice the size of hatchling freshwater turtles, thus making the extraction process easier. We succeeded in taking blood samples from the coccygeal vein, which runs along the dorsal midline of the tail (Figure 3). Our success at obtaining blood from the coccygeal vein was independent of the size of the hatchling (logistic regression with carapace length as the independent variable:  $X^2 = 0.0012$ ,  $P = 0.97$ ). This suggests that this technique could be practicable on hatchlings of smaller species, such as painted turtles (*Chrysemys picta*) or spotted turtles (*Clemmys guttata*) that overlap in size with the smaller common map turtle hatchlings (Ernst 1994). We believe that the coccygeal vein should be preferred over the subcarapacial vein for venipuncture in hatchlings for three reasons. First, bleeding never occurred when we sampled from the coccygeal vein, whereas occasional bleeding occurred when we took blood from the subcarapacial vein. Second, we obtained undesirable extracellular fluid more often and in greater amounts when we used the



subcarapacial vein. Third, vital organs are less likely to be damaged when blood is taken from the dorsal side of the tail than from the head and neck regions. We wrote an article on this technique that is now in press in *Herpetological Review*: see Bulté *et al.* (2006).

### 5.1.3 Blood sample storage

The initial evaporation step of the DNA isolation process was very time consuming. Even though we were pipetting 125 $\mu$ l of every sample (blood mixed with ethanol) for evaporation, varying quantities of pure blood were collected, due to the varying amount of blood in the initial sample (0.03-0.05ml) and if the blood was pure or mixed with lymphatic fluids when it was taken from the specimens. The volume of alcohol in the raw samples wasn't controlled either. More blood present in the evaporation tube meant more Proteinase-K and more time spent in the water bath were needed for the cells to lyse completely. In order to skip this evaporation step, samples should be stored in Queens Lysis Buffer in a 1:8 ratio of blood to buffer instead of 70% ethanol, as suggested by De Souza (2001). Since this is a lysis buffer, blood cells will have already started to disintegrate, shortening the time needed for the lysis step.

### 5.1.4 Polymerase chain reaction

When troubleshooting the PCRs, we were originally using Dr Ekker's domestically made *Taq* but it wasn't performing enough in a 10 $\mu$ l volume. We then switched to commercial *Taq* (Invitrogen). We started using 0.1 $\mu$ l of Invitrogen's *Taq* compared to the 0.4 $\mu$ l we were using with Dr Ekker's *Taq*. I forgot to adjust the water volume of the PCR reaction when we switched reagents, making final reaction volumes

9.7µl instead of 10µl. I realized my mistake on April 21<sup>st</sup> 2006. PCRs still work in a 9.7µl volume.

#### *5.1.5 Fragment analysis*

Some authors (Hauswaldt & Glenn, 2003; Freedberg *et al.*, 2005) use 1/10 labeled and unlabelled primers when performing fragment analysis. This could be attempted if money is a concern.

##### *5.1.5.1 Fragment analysis: missing peaks*

Missing peaks, when no peaks appear on the fragment analysis sample's results graph, could be due to the DNA. The concentration of that sample was perhaps too high or too low, or alternatively the DNA could have been altogether omitted from the PCR tube. The first kind of error would be plausible if no peaks were observed at all three loci since 1µl of DNA from the same working solution tube is used in all three PCR reactions. This was minimized by assaying sample DNA concentration via spectrophotometry and diluting the appropriate samples to make suitable working solutions.

Errors could also occur from the PCR mix. 10µl is a small volume for a PCR reaction (pers. comm.: Gary Hatch, Dr John Basso). By bad luck, perhaps not enough primers or *Taq* were present in that particular sample's aliquot, and so the desired locus was not amplified at all, or at least not in satisfying concentrations to be detectable by the CEQ during the fragment analysis. If pipetting errors diminished the final volume in the PCR tube, the reagents' proportions are changed which can offset amplification. One can

suspect this kind of error when visualizing the poolplexed sample's graph and seeing background noise from that primer's color but no peaks.

Errors could occur again when preparing samples for the fragment analysis. The first analyses were performed without a multichannel pipettor, and so omitting to put one of the three PCR reactions in the poolplexing tube is possible, which would result in no peaks for that locus. One can suspect this kind of error when visualizing the poolplexed sample's graph and seeing no background noise from that primer's color. Errors can occur also when using a multichannel pipettor. It is difficult to pipet the same amount of solution in all 8 tips, especially when volumes are small like the ones desired for loci 7 (2 $\mu$ l) and 5 (4 $\mu$ l). Perhaps less PCR product was contributed to some poolplexed samples which diminished the intensity of those peaks beyond background noise level.

#### *5.1.5.2 Fragment analysis: ambiguous peaks*

Compared to single plexing, poolplexing the three PCR reactions in one well for the fragment analysis dilutes the labelled products disproportionately and causes crosstalk between different dyes, resulting in ambiguous peaks (Beckman Coulter, 2004). Clear peaks should rise above the Size Standard, and there should be no more than two per loci. Unspecific amplification can cause other peaks, but they should not be as omnipresent as the 'real' peaks once the PCRs are optimized, which we think we achieved. If peaks are too low, one can augment the amount of PCR product in the FA mix. A second solution is performing an ethanol precipitation. This purification step removes excess anions in the PCR products that interfere with the Sample Loading Solution (Beckman Coulter, ?). Contact the common molecular lab technician Philip Pelletier for method.

Since locus SH5 only had two alleles total (see section 3.2), including the sometimes ambiguous peak at 75 nucleotides in paternity analyses when there was only a ‘hint’ of a peak did not affect the estimate of multiple paternity. This unique hint of a peak at 75 nucleotides occurred in different individuals, and other hatchlings had clear cut peaks at 75 nucleotides. This leads me to believe that perhaps this ‘hint’ of a peak was caused by a mutation in the flanking sequence of the particular individual’s microsatellite locus that hampered this second allele from amplifying properly with SH2 primers.

## ***5.2 Discussion of results***

### *5.2.1 Clutch data*

We captured females spanning the full range of reproductive females. Clutch sizes ranging from 6-15 eggs are comparable to what is found in the literature (Ernst, 1994), and the fact that larger females are more lay larger clutches is concordant as well (Iverson, 1992; Shine, 2005).

### *5.2.2 Paternity analysis*

#### *5.2.2.1 Undetectable multiple paternity?*

To increase our ability of detecting multiple paternity we originally wanted to genotype all the offspring in each clutch at five microsatellite loci. Due to incessant labwork setbacks (see appendix C), only three microsatellite loci were optimized, only 8 clutches were attempted, and only 40.2% (SE=6.75) to 49.31% (SE=6.12) of these

clutches' offspring were successfully genotyped. The eight clutches chosen for analysis span the full range of reproductive females' body size.

Since a third paternal allele was discovered in one hatchling at one locus in one clutch only, this third allele could be the result of an allelic mutation and not be caused by multiple paternity (Rocques *et al.*, 2006). However, since the clutches were sub sampled (40-49%), perhaps we missed the other siblings possibly fathered by this second sire.

The fact that no strong evidence of multiple paternity was found in these eight clutches of the common map turtle is inconclusive. Because the fragment analysis wasn't troubleshooted and often gave ambiguous graphs, I was very conservative when including peaks for the paternity analyses, and might have undervalued multiple paternity. Moreover, sub sampling the clutch diminishes the power to detect additional fathers, especially if the ratio of fathers is highly skewed (Pearse *et al.*, 2002); this might be the case in our clutch #1. Not having the mother's genotype (which occurred in 5 out of 8 clutches), hence not knowing if she is homo or heterozygous, diminishes the power to detect multiple sires as well. Multiple paternity could also be underestimated whenever two paternal alleles are observed at one locus: I have no way of determining if one heterozygous male or two homozygous males fathered the clutch. I therefore conservatively assume it was a heterozygous male. Underestimating the number of sires this way would be infrequent if i) the microsatellite loci were highly variable and ii) if numerous loci were genotyped. Evidently two males might share the same alleles at one locus but not at another. Conversely, my analysis shows that the three microsatellite loci

used in this study have very low heterozygosity: 4 (maybe 5) different alleles were found in 36 hatchlings at locus SH2, 2 alleles in 43 offsprings at locus SH5, and 5 (maybe 6) alleles in 36 individuals at locus SH7. Many hatchlings share the exact genotype as their mother, and the same alleles are present in many clutches. It is thus impossible to guess the number of sires per clutch if the father alleles are unidentifiable. With such low heterozygosity at all three loci, two males sharing an identical genotype at these loci could sire the same clutch and this would be undetected. My data set is disappointing since Freedberg *et al.* (2005) who had used these loci on another species of map turtles observed that SH2, 5 and 7 yielded 14, 11 and 11 alleles, respectively in 209 turtles. My low sample size and the low variability of the three microsatellite loci thus reduce my ability to detect multiples sires in a clutch.

Other than the microsatellite loci not being variable, another cause to the neutral genetic information collected in this study could be an inbred population. Inbreeding is defined as the mating of two related individuals (full sibs, cousins, etc.) (Roff, 2002). Since related individuals share the same alleles, inbreeding leads to loss of heterozygosity, gene depression, and loss genetic diversity in a population. This could explain why the same alleles were present in different clutches and why the microsatellite loci had so little variance. The heavy predation on nests could perhaps cause inbreeding if the surviving offspring at any given year are from the same clutch (Galbraith *et al.*, 1993), or if a particular mother's nesting site is protected and her offspring survive every year. However, Lake Opinicon's population is unlikely to be one big family reproducing

solely amongst each other, since this lake is part of the Rideau Canal Waterway and is connected to other lakes from which immigration and emigration can occur.

A third reason could explain the apparent low genetic variability of our turtle population: that it is situated at the northern limit of this species' distribution. *G. geographica* has an extensive range : from Quebec and northwestern Vermont, it extends west through the Great Lakes into southern Wisconsin and eastern Minnesota and then south into Kansas, northeastern Oklahoma, Arkansas, Tennessee, Alabama and northwestern Georgia (Ernst *et al.*, 1994). Outlying populations are often founded by fewer individuals which can result in a significant reduction in multilocus heterozygosity and allelic variation. Reduced gene flow, small population size, and founder effects will all promote genetic drift and result in reduced genetic variation in peripheral populations (Lesica & Allendorf, 1995).

#### 5.2.2.2 *Multiply sired clutches*

For the reasons stated in the previous section, multiple paternity in clutches of *G. geographica* has possibly been underestimated in this study. I would have expected to see multiple paternity for several reasons. First, multiple paternity has been found in numerous animal taxa (Reynolds, 1996). It is a common phenomenon in both marine and freshwater turtles, and has been documented in every freshwater species studied to date (Pearse & Avise, 2001). Second, turtles don't form pair bonds (Kuchling, 1999), and so are free to mate repeatedly. Third, map turtles are gregarious (Vogt, 1980), so it is

doubtful females are male limited. Fourth, no mechanisms preventing female insemination by more than a single male have been found (Galbraith *et al.*, 1993). Fifth: female turtles can store viable sperm for up to four years (Pearse *et al.*, 2001). How the sperm from different males is stored in the sperm storage tubules (sequentially layered or mixed) is unknown (Gist & Congdon, 1998). Therefore, even if females mate only once a year prior to the mating season, it is feasible that sperm from previous copulations could be mixed in with this newly acquired sperm before fertilization occurs, resulting in multiply sired clutches. Furthermore, stored sperm comes at no extra cost to the female in painted turtles: in Pearse & Avise's study (2001), hatchlings fathered by the same male's sperm in consecutive years had the same hatching success as offspring sired by different males.

Several hypotheses suggest that females gain from polyandry. Females might be picky when it comes to mate choice; alternatively, multiple matings and multiple paternity could simply be the result of females conceding to mate as a tactic to avoid male harassment (Lee & Hays, 2004). Proposed genetic benefits of multiple matings are: indemnifying the female against the possibility that one of her mates is not fertile (Reynolds, 1996); avoiding genetic incompatibility (Zeh & Zeh, 1996), inbreeding and genetic defects resulting from stored sperm (Reynolds, 1996); promoting the gain of 'good genes' and increasing genetic diversity among offspring (Pearse & Avise, 2001). Stored sperm provides an opportunity for multiple paternity as a result of sperm competition or cryptic female choice (Pearse & Avise, 2001). Thus, if females can detect variation in male genetic quality or compatibility, they could adjust their mating behaviour and sperm storage accordingly, and play an active role in enhancing their



overall genetic fitness (Pearse *et al.*, 2002). Therefore, the more sperm a female accumulates by mating with different males, the more choice she has to fertilize her eggs. Furthermore, by storing sperm, females might enjoy the benefits of multiple paternity over a long period of time (Roques *et al.*, 2006).

A few studies have suggested a significant biological advantage to multiple paternity. Pearse *et al.* (2002) demonstrated that more eggs are laid in multiply clutches of painted turtles. Madsen & Olsson (1998) demonstrated multiply sired broods of adders (*V. berus*) and sand lizards (*L. agilis*) have higher embryonic survival, fewer deformities and, in *L. agilis*, the offspring are heavier and survive better during their first year of life. Furthermore, normal looking young with malformed sibling (or half-siblings) in this natural population survive less well than young with no deformed siblings. Conversely, Lee & Hays (2004) suggest that the environment (predation, temperature, flooding) plays a much stronger role in determining the success of clutches, than whether paternity has been single or multiple. In our study most females were caught nesting at the same place, on Hump Island, so environment possibly plays a more subtle role.

If multiple paternity was discovered, it would be interesting to see if it is related to female body size. If a positive trend is observed, it would suggest that males are more attracted to larger females, as we predicted. Pearse *et al.* (2002) found no significant relationship between female carapace length and single versus multiple paternity of clutches of painted turtles. However, females that laid at least one multiply sired clutch were on average larger than females that laid only single-paternity clutches. Their results suggested a potential male preference for larger female as mates.

If there was no trend to multiple paternity and female body size, it is possible males don't select who they mate with. Perhaps because there are no costs to spermatogenesis; we never tested this aspect of our hypothesis. Or perhaps because larger hatchlings don't enjoy a higher survival rate (Gibbons *et al.*, 1999). We gathered data on hatching success but this does not necessarily equal hatchling survival to age of reproduction. Pearse *et al.*'s (2002) study revealed that more eggs were present in multiply sired clutches of painted turtles. Larger turtles laid more eggs in that study, but this is not the case for this study of common map turtles. My results show that the amount of eggs laid doesn't increase with female body size ( $R^2=0.007$ ,  $p=0.64$ ), but that larger females lay bigger eggs ( $R^2=0.26$ ,  $p=0.013$ ). If bigger hatchlings issued from those bigger eggs don't survive better than smaller hatchlings, small and large mothers enjoy the same fitness, and should be equally attractive to males.

#### 5.2.2.3 *Singly sired clutches*

It is also possible that female *G. geographica* sire their clutches with only one male. Several reasons could perhaps explain this trend. Maybe the genetic advantages to polyandry stated previously are not biologically important enough. Secondly, mating might entail some heavy costs. Very little is known about the map turtle's reproduction; the fact that no courtship has ever been documented doesn't mean it is inexistent. Vogt (1980) reported that copulation can last a long time, up to four hours. Injuries or parasite transmission could also be suffered during mating (Krebs & Davies, 1997), which would discourage females from seeking additional mates.

Female map turtles could avoid multiple matings because they are so much bigger and stronger than males, that forced copulation by these conspecifics would not be an issue. Storing viable sperm from years past like some turtles do (Kuchling, 1999) would also nullify the need to seek additional matings.

Female map turtles might avoid multiple paternity by several mechanisms even though multiple matings arise. First, even if there is sperm storage, sperm depletion can occur. The fertility and/or hatching success of across year clutches is contradictory, with no changes in some species, like the previously mentioned painted turtle (Pearse & Avise, 2001), but a decline in others, like in the European pond turtle (*Emys orbicularis*; Roques *et al.*, 2006) and the promiscuous green turtle (*Chelonia mydas*; Fitzsimmons, 1998). Second, (as mentioned above) we don't know how the sperm is stored in the storage tubules or how it leaves the storage tubules to fertilize the clutches (Gist & Congdon, 1998). Male precedence is feasible if the different sperms are layered on top of each other and don't mix. Third, through cryptic choice, females might choose to use sperm (Olsson *et al.*, 1997) from only one male to fertilize the whole clutch. Fourth, through sperm competition, one male's sperm might be a better competitor and 'win' all the ova (Olsson & Madsen, 1998).

#### 5.2.2.4 Other turtles species

In bottom-walking and terrestrial species, like the snapping turtle and the wood turtle, males are larger than females and can force copulation (Ernst *et al.*, 1994).

Male snapping turtles defend home ranges through combat (Pearse & Avise, 2001).

In wood turtles, male dominance hierarchies exist, and paternity analyses have shown that superior males fathered a significantly higher proportion of clutches compared to low ranking males (Galbraith 1991, *in* Pearse & Avise, 2001). Multiple paternity is expected to occur when females are forced to suffer several matings.

In aquatic species like the painted turtle, males are smaller than females but can overlap in size with them (Ernst *et al.*, 1994). Berry and Shine (1980, *in* Pearse *et al.*, 2002) believe forced copulation is unlikely in aquatic species. Male painted turtles engage in an elaborate courtship to seduce a conspecific female, during which time the female can judge male quality (Pearse *et al.*, 2002). Perhaps multiple paternity evolved following female choice to mate several times in order to gain genetic benefits to her offspring, as discussed earlier.

In map turtles, females are much bigger than males and no courtship has been documented. How females judge male quality is unknown. If multiple paternity occurs is also unknown.

## FUTURE WORK

Future work should finish troubleshooting the fragment analysis on the CEQ so clear graphs like the one in Figure 7 can be obtained reliably. Then the sample size in this study should be enhanced, and other/more microsatellite loci used, hoping they are highly variable on the common map turtle. The new genotypes should be combined to the ones acquired in the present study. Hauswaldt & Glenn's (2003) other loci (SH1, SH3, SH8) could be attempted. Since SH2, 5 and 7 amplified effectively in this study and since SH1 and 8 amplified more often than SH7 when tested across seven turtle species by the authors, perhaps SH1 and 8 are good candidates for *G. geographica*. In another study, King & Julian (2004) developed 26 microsatellite loci for the bog turtle and tested them on 13 species of emydidae, including *G. geographica*. Though only four map turtles were tested, some loci demonstrated 4 or 5 alleles, which suggest a high degree of polymorphism.

If multiple paternity was detected in our clutches of common map turtles, interesting questions could be asked. Do the fathers sire the same proportion of the clutch? Does multiple paternity increase hatching success? And offspring size at hatching? Are there more deformities in singly sired clutches than multiply sired ones? Analyzing dead eggs to see if they are full sibs or half sibs to live hatchlings would be interesting. And perhaps the most interesting question in light of this study: does multiple paternity increase with female body size?

Male costs of reproduction and hatchling survival could be analyzed as well, to give our hypothesis more weight.

## CONCLUSIONS

Multiple paternity is a common phenomenon in both marine and freshwater turtles, and has been documented in every freshwater species studied to date. Potential advantages in terms of genetic advantages leading to higher hatchling survival have been discussed. Female map turtle being so large compared to males suggests that she can avoid forced copulations and choose mating partners. We think that larger females should be more attractive to males since they are more fecund, and that multiple paternity should accordingly be more common in their clutches. Larger female do not lay more eggs than smaller females but their eggs are significantly heavier, which may lead to higher hatchling survival.

No strong evidence of multiple paternity was detected in this project. Persistent laboratory setbacks resulted in low sample size, sub sampling of clutches and low variability of the three microsatellite loci. This severely reduced our ability to detect multiples sires in a clutch and rendered the results inconclusive. The question of multiple paternity augmenting with female body size in the common map turtle is left unanswered. However, the answer might come soon, as DNA extraction and PCRs have been troubleshooted, and fragment analysis will soon follow with the help of CAREG's common molecular facility's technician, Philip Pelletier.

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## REFERENCES

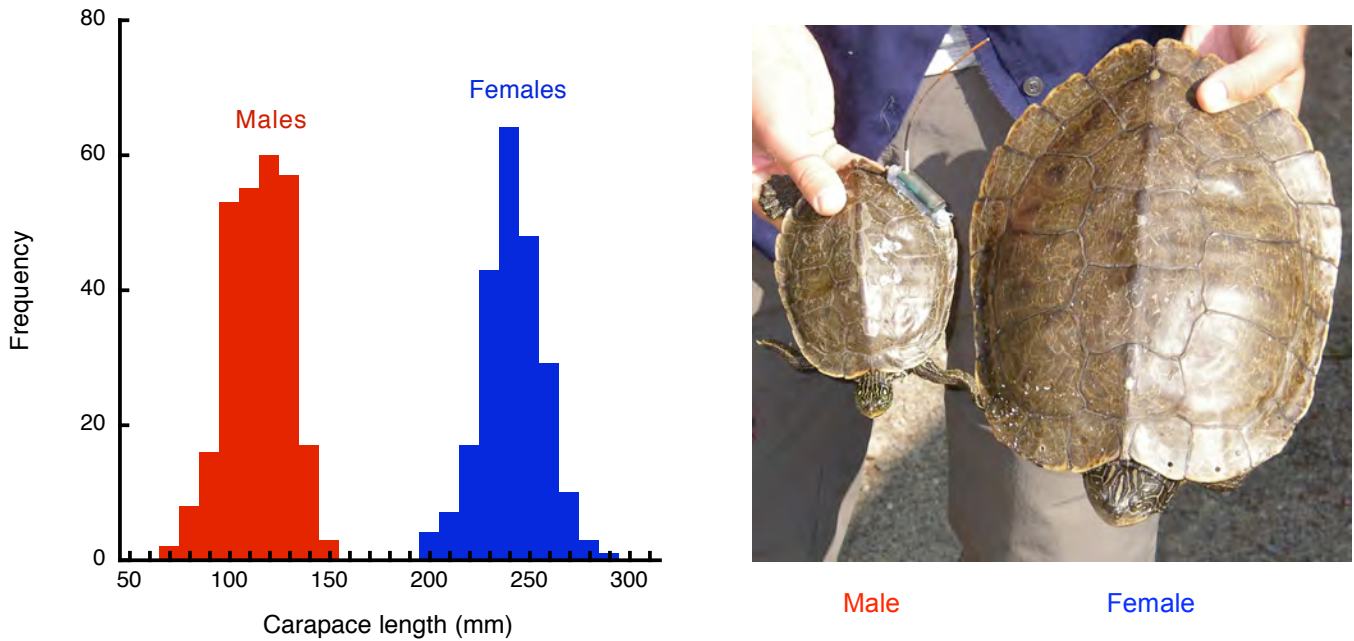
1. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002). Molecular biology of the cell. Fourth edition. Garland Science, New York, New York. p.511.
2. Alcock J (2001). Animal Behavior, An Evolutionary Approach. Sinauer associates, inc., 7th edition, Sunderland, Massachusetts. 543 pages.
3. Blouin-Demers G, Gibbs HL, Weaterhead PJ (2005). Genetic evidence for sexual selection in black ratsnakes, *Elaphe obsoleta*. Animal Behavior 69: 225-234.
4. Congdon JD, Gibbons JW (1990). Morphological constraint on egg size: a challenge to optimal egg size theory? Ecology 84: 4145-4147.
5. Congdon JD, Nagle RD, Dunham AE, Beck CW, Kinney OM, Yeoman RS (1999). The relationship of body size to survivorship of hatchling snapping turtles (*Chelydra serpentina*): an evaluation of the “bigger is better” hypothesis. Oecologia 121: 224-235.
6. Crim JL, Spotila LD, Spotila JR, O’Connor M, Reina R, Williams CJ, Pladinos FV (2002). The leatherback turtle, *Dermochelys coriacea*, exhibits both polyandry and polygyny. Molecular Ecology 11: 2097-2106.
7. De Souza L (2001). DNA extraction protocols. In: Gibbs HL (2001). Lab protocols. Laboratory of Molecular Ornithology and Herpetology. Ohio State University.
8. Eberhard WG (1998). Female role in sperm competition. In: Sperm competition and sexual selection (Birkhead TR and Moller AP, eds). San Diego: Academic Press; 91-116.
9. Ernst CH, Lovich JE, Barbour, RW (1994). Turtles of the United States and Canada. Smithsonian Institute Press, Washington. 578p.
10. Fitzsimmons NN (1998). Single paternity of clutches and sperm storage in the promiscuous green turtle (*Chelonia mydas*). Molecular Ecology 7: 575.
11. Freedberg S, Ewert MA, Ridenhour BJ, Neiman M, Nelson CE (2005). Nesting fidelity and molecular evidence for natal homing in the freshwater turtle, *Graptemys konhii*. Proceedings of the royal society B 272, 1345-1350.
12. Galbraith DA (1991). Studies of mating systems in wood turtles (*Clemmys insculpta*) and snapping turtles (*Chelydra serpentina*) using DNA fingerprinting. PhD dissertation, Department of Biology, Queen’s University, Kingston, Canada. In: Galbraith DA, White BN, Brooks RJ, Boag PT (1993). Multiple paternity in clutches of snapping turtles (*Chelydra serpentina*) detected using DNA fingerprints. Canadian Journal of Zoology 71:318-324.
13. Galbraith DA, White BN, Brooks RJ, Boag PT (1993). Multiple paternity in clutches of snapping turtles (*Chelydra serpentina*) detected using DNA fingerprints. Canadian Journal of Zoology 71:318-324.
14. Gans C and Huey RB (1988). Biology of the reptilia, volume 16, Ecology B, Defense and life history. Alan R. Liss Inc., New York. pp.387-440.
15. Gist DH, Dawes SM, Turner TW, Sheldon S, Congdon JD (2001). Sperm storage in turtles: a male perspective. Journal of experimental zoology 292: 180-186.
16. Gist DH and Jones, JM (1989). Sperm storage within the oviduct of turtles. Journal of morphology 199: 379-384.
17. Gist DH and Congdon JD (1998). Oviductal sperm storage as a reproductive tactic of turtles. Journal of experimental zoology 282: 526-534.



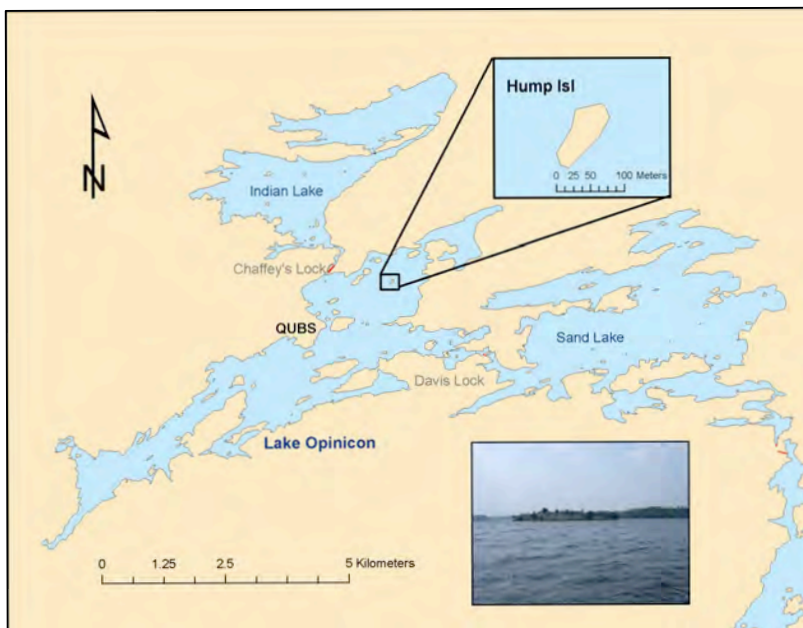
18. Hauswaldt SJ and Glenn TC (2003). Microsatellite DNA loci from the Diamondback terrapin (*Malaclemys terrapin*). *Molecular Ecology Notes* 3: 174-176.
19. Hoekert WEJ, Neuféglise H, Schouten AD, Menken SBJ (2002). Multiple paternity and female-biased mutation at a microsatellite locus in the olive ridley sea turtle (*Lepidochelys olivacea*). *Heredity* 89: 107-113.
20. Iverson JB (1992). Correlates of reproductive output in turtles (order testudines). *Herpetological monographs* 6: 25-42.
21. Keller L, Reeve HK (1995). Why do females mate with multiple males-the sexually selected sperm hypothesis. *Advances in the Study of Behavior* 24:291-315.
22. Kichler K, Holder MT, Davis SK, Marquez-M R, Owens DW. Detection of multiple paternity in the Kemp's ridley sea turtle with limited sampling. *Molecular Ecology* 8: 819-830.
23. King TL, Julian SE (2004). Conservation of microsatellite DNA flanking sequence across 13 Emydid genera assayed with novel bog turtle (*Glyptemys muhlenbergii*) loci. *Conservation Genetics* 5:719-725.
24. Krebs JR, Davies NB (1997). *Behavioral Ecology An Evolutionary Approach*. 4th edition, Blackwell Science, University Press, Cambridge.
25. Kuchling, G (1999). *The Reproductive Biology of the Chelonia*. Springer-Verlag Berlin Heidelberg, Germany.
26. Lee PLM & Hays GC (2004). Polyandry in a marine turtle: Females make the best of a bad job. *PNAS* 101: 6530-6353.
27. Lesica P. and Allendorf FW (1995). When Are Peripheral Populations Valuable for Conservation? *Conservation Biology* 9: 753-760.
28. Moore MK, Ball Jr RM (2002). Multiple paternity in loggerhead turtle (*Caretta caretta*) nests on Melbourne Beach, Florida: a microsatellite analysis. *Molecular Ecology* 11:281-288.
29. Newman HH (1906). The habits of certain tortoises. *Contributions from the Zoological Laboratory of the University of Michigan*, No. 104.
30. Olsson M & Madsen T (1998). Sexual selection and sperm competition in reptiles. *In* Birkhead TR & Moller AP (1998). *Sperm competition and sexual selection*. Academic Press. San Diego, California, p.502-570.
31. Olsson M, Shine R, Madsen T, Gullberg A, Tegelstrom H (1996). Sperm selection by females. *Nature* 383: 585.
32. Packard G.C., M.J. Packard. 1988. Physiological ecology of reptilian eggs and embryos. Pp 523-605 *In* C. Gans and R. B. Huey (Eds.), *Biology of the Reptilia*, Vol. 16. Alan R. Liss, New York, New York, U.S.A.
33. Pearse DE, Avise JC (2001). Turtle mating systems, behavior, sperm storage and genetic paternity. *The American Genetic Association* 92:206-211.
34. Pearse DE, Janzen FJ, Avise JC (2002). Multiple paternity, sperm storage, and reproductive success of female and male painted turtles (*Chrysemys picta*) in nature. *Behavioral Ecology and Sociobiology* 51:164-171.
35. Queller DC, Strassmann JE (1993). Microsatellites and Kinship. *TREE* 8: 285-288.
36. Reynolds JD (1996). Animal breeding systems. *Trends in Ecology and Evolution* 11: 68-72.
37. Roff DA (2002). *Life history evolution*. Sinauer Associates, Inc. Sunderland, Massachusetts, USA. p.39.

38. Roques S, Diaz-Paniagua C, Porthault A, Perez-Santiagosa N, Hidalgo-Vila J (2006). Sperm storage and low incidence of multiple paternity in the European pond turtle, *Emys orbicularis*: A secure but costly strategy? *Biological conservation* 129: 236-243.
39. Shine R (2005). Life-history evolution in reptiles. *Annu. Rev. Ecol. Evol. Syst.* 36: 23-46.
40. Shine R, O'Connor D, Lemaster MP, Mason RT (2001). Pick on someone your own size: ontogenetic shifts in mate choice by large male garter snakes result in size-assortative mating. *Animal Behavior* 61: 1133-1141.
41. Shuster S, Wade MJ (2003). *Mating Systems and Strategies*. Princeton University Press, Princeton, N.J. p.241
42. Valenzuela N (2000). Multiple paternity in side-neck turtles *Podocnemis expansa*: evidence from microsatellite DNA data. *Molecular Ecology* 9: 99-105.
43. Vogt RC (1980). Natural history of the map turtles *Graptemys pseudogeographica* and *G. ouachitensis* in Wisconsin. *Tulane Studies in Zoology and Botany* 22: 17-48.
44. Zeh JA & Zeh DW (1996). The evolution of polyandry I: intragenomic conflict and genetic incompatibility. *Proceedings of the Royal Society of London. Series B, Biological Sciences* 263: 1711-1717.
45. Personal communications:
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  - b. Gary Hatch. Technician, Dr Marc Ekker's molecular laboratory. Department of Biology, University of Ottawa.

**FIGURES AND TABLES**



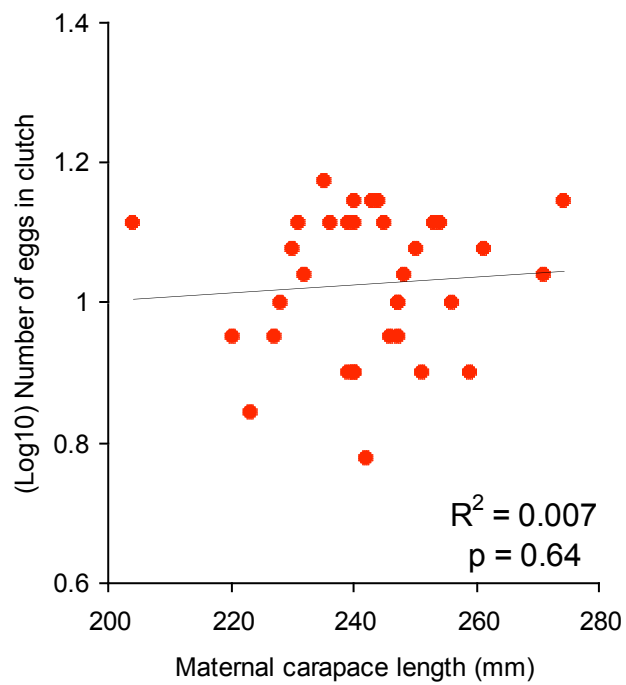
**Figure 1.** *Left* At maturity, females *G. geographica* (N=226) are larger than males (N = 271). *Right* Fully mature male next to a fully mature female. Data from Lake Opinicon's population.



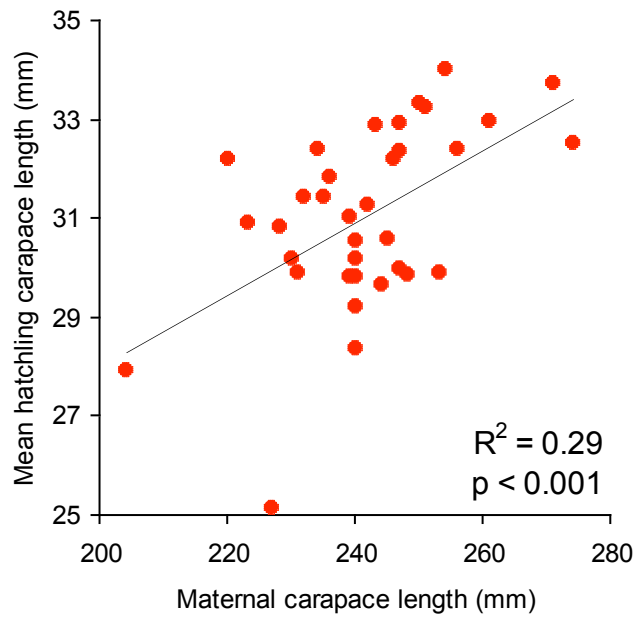
**Figure 2.** Study site: Lake Opinicon, in the Rideau Canal Waterway. Both enlargements are Hump Island, a popular nesting site.



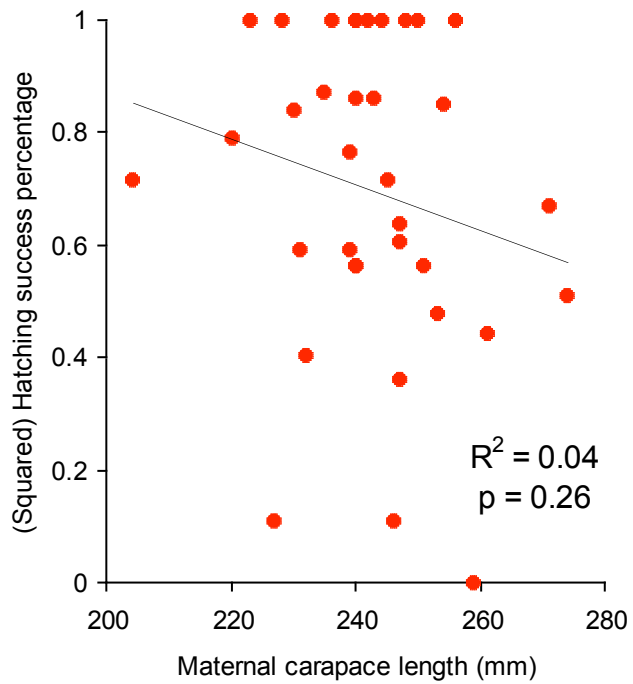
**Figure 3.** New technique developed for bleeding hatchling emydid turtles in the tail.



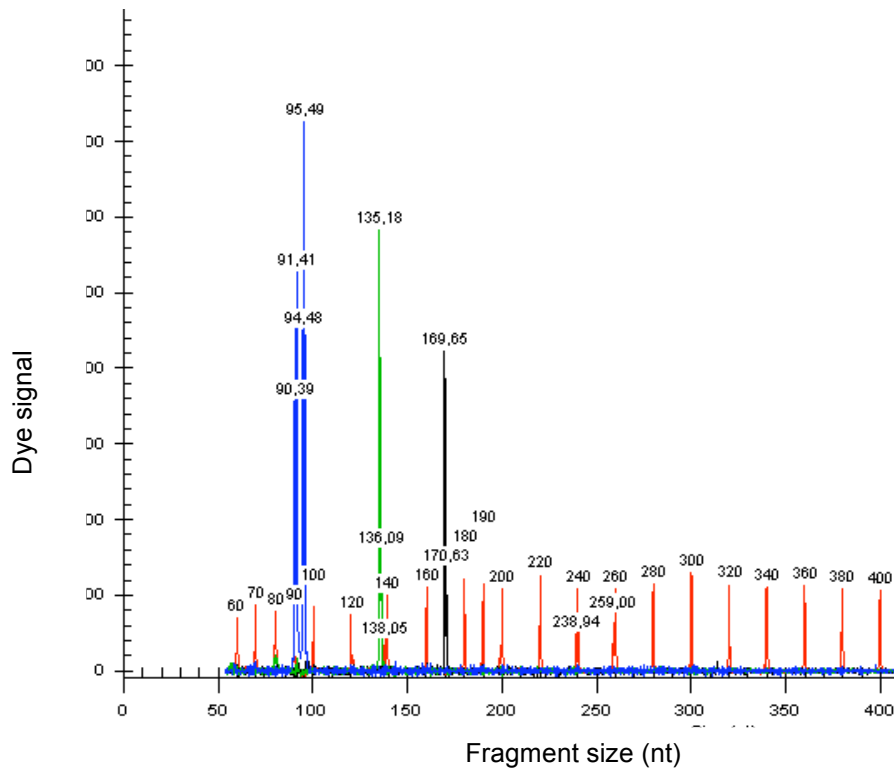
**Figure 4.** Larger females do not lay more eggs than smaller females (N = 34 clutches).



**Figure 5.** Hatchling body size increases with female body size (N = 336 hatchlings from 35 clutches). Each data point represents the mean carapace length (mm) per clutch.



**Figure 6.** There is no relation between hatching success and female body size (N = 35 clutches).



**Figure 7.** Example of a perfect poolplexed graph during fragment analysis with the CEQ. Data from hatching #207.

**Table I.** Total alleles detected in eight clutches of Common map turtles at loci 2, 5, and 7 (SH2, 5 and 7) by fragment analysis. M: Mother identification number; A: number of alleles found at this locus; N: number of hatchlings genotyped; %: percentage of clutch genotyped. Gray rows indicate that the mother's genotype was not obtained.

M	Loci 2	A	N	%	Loci 5	A	N	%	Loci 7	A	N	%
1	170, 178	2	9	69.23	75, 135	2	7	53.85	90, 94 <sup>a</sup> , 110, 115	4	7	53.85
2	99, 170, 178	3	5	55.56	75, 135	2	6	66.67	90, 94, 110, 118	4	4	44.44
5	170, 194	2	2	15.38	135	1	7	53.85	90, 94, 118	3	8	61.54
17	170, 178, 194	3	5	35.71	135	1	3	21.43			0	0
20	170, 178, 194	3	3	25	135	1	6	50	90, 110, 118	3	6	50
22	178	1	4	36.36	75, 135	2	3	27.27	90, 110, 137 <sup>a</sup>	2, 3 <sup>b</sup>	4	36.36
23	162 <sup>a</sup> , 170, 194	2, 3 <sup>b</sup>	4	33.33	135	1	6	50	90, 94	2	5	41.67
31	170, 178	2	4	57.14	75, 135	2	5	71.43	90, 118	2	3	42.86

<sup>a</sup> Indicates that this allele was found in only one individual.

<sup>b</sup> Based on that one extra allele found in only one individual.

**Table II.** Probable father alleles detected in eight clutches of Common map turtles at loci 2, 5, and 7 (SH2, 5 and 7) by fragment analysis. M: Mother identification number; A: number of alleles found at this locus; S: number of inferred sires in clutch. Gray rows indicate that the mother's genotype was not obtained.

M	Loci 2	A	S	Loci 5	A	S	Loci 7	A	S
1	170,178	2	1	75, 135	2	1	90, 94 <sup>a</sup> ,115	2, 3 <sup>b</sup>	1, 2 <sup>b</sup>
2	(99 or 170), 178	1 or 2	1	75, 135	2	1	90, (94 or 118)	2	1
5	170, 194	2	1	135	1	1	94, 118	2	1
17	(170 or 178), 194	2	1	135	1	1			
20	(170 or 194), 178	2	1	135	1	1	90, 118	2	1
22	178	1	1	75, 135	2	1	90, 137 <sup>a</sup>	2	1
23	170, (162 <sup>b</sup> or 194)	2	1	135	1	1	90, 94	2	1
31	170, 178	2	1	75, 135	2	1	90, 118	2	1

<sup>a</sup> Indicates that this allele was found in only one individual.

<sup>b</sup> Based on that one extra allele found in only one individual.

## ANNEX C

### *Laboratory setbacks incurred during the fragment analysis*

- No one at the University of Ottawa was trained to use Beckman Coulter's CEQ genetic system when I started my honors project in September.
- There are gaps in the User's Guides and Training manuals: information on how to transfer PCR products to the CEQ for fragment analysis (PCR volumes, special purification treatments) is not available. When I called Beckman Coulter's customer service to get this information, they told me to wait for a representative to show me how to do this.
- PCRs had to be optimized before starting the fragment analysis, and we aimed to start this last step in January. Effectively, PCRs were optimized on the 13<sup>th</sup> of January 2006.
- Although we requested the fragment analysis training in mid-December of 2005, the meeting was set for February 17<sup>th</sup>, and was postponed one more week due to an ice storm that day prevented the Beckman representative from driving from Montreal. On February 24<sup>th</sup> the training was cut early (only after being pushy was I shown how to visualize my data) and the representative assured us he would come back two weeks later. On that day he arrived at 14:30 instead of 10:00 like scheduled, and we never got around to being shown how to use the CEQ program treat data (at that time I didn't know it would be important).
- On March 29<sup>th</sup> 95 viruses were detected on the computer that controlled the CEQ. It took a week to clean it up, during which time I couldn't perform fragment analyses. (It turns out the computer was hooked up to the internet without having an anti-virus installed).
- The next two weeks of analysis were not conclusive, giving results worst than before. We finally figured out the capillary array was expired after spending 6 weeks at room temperature (the limit is four). A new set took a week to arrive (Beckman won't ship reagents kept at 4°C later than Tuesday, in case the package gets lost during a week end). The new capillaries arrived on the 12<sup>th</sup> of April, and were defective (capillary D was too long). We attempted to run a plate anyway, by not placing any samples in row D, but machine won't take it. If one capillary is out, they are all out. Beckman sent us a new array that arrived on April 18<sup>th</sup>. As one last push to obtain results, (for my thesis due a week later), I ran a 96 well plate on the CEQ on the 21<sup>st</sup> of April, and obtained zero results. Obviously troubleshooting the fragment analysis needs more work. This is when I gave up on lab work for this project.

