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Maturity, fecundity, and reproductive cycle of the spotted ratfish, *Hydrolagus collieri*

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Abstract Size at maturity, fecundity, and reproductive periodicity were estimated for the spotted ratfish, *Hydrolagus collieri* (Lay and Bennett, 1839), off the coast of California, Oregon, and Washington. Maximum body size and size at median maturity were greater for females than males. Skeletal muscle concentrations of the steroid hormones testosterone (T) and estradiol (E₂) predicted similar, but slightly smaller sizes at maturity than the morphological criterion. Stage of maturity for males was estimated identically using internal organs or external secondary sexual characters, thus allowing non-lethal maturity assessments. Size at median maturity was greater north of Point Conception for females, and north of Cape Mendocino for males. Peak parturition occurred from May to October, with

increased concentrations of E₂ in skeletal muscle of females correlating with ovarian recrudescence during November to February. No significant seasonal trends in female T were apparent, but mean female 11-ketotestosterone (11KT) was 300% greater in April than any other month during the parturition season. There was a marginal evidence for increased number and size of ova with maternal size. Extrapolation of the hypothesized 6 to 8-month egg-laying season to observed mean parturition rates of captive specimens yielded an estimated annual fecundity of 19.5–28.9 egg cases. Differences in fecundity among higher taxonomic classifications of chondrichthyans were detected with chimaeriform fishes more fecund than lamniform, myliobatiform, squaliform, and rhinobatiform fishes, and less fecund than rajiform fishes.

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Introduction

Hydrolagus collieri is a member of the monophyletic class Chondrichthyes (Schaeffer 1981; Maisey 1984, 1986; Didier 1995; Grogan and Lund 2004), which includes the subclasses Holocephali (chimaeras or ratfishes) and Elasmobranchii (sharks and rays). Holocephalans are differentiated from elasmobranchs by numerous morphological characters, most notably a palatoquadrate fused to the neurocranium and non-replaceable teeth fused into three pairs of hypermineralized tooth plates (Maisey 1986; Didier 1995; Lund and Grogan 1997). Holocephalans are evolutionarily significant with ancestors originating at least 300 million years ago (Grogan and Lund 2004).

Holocephalans occur in marine environments worldwide except polar seas. There are 37 species in the single order Chimaeriformes (Compagno 2005; Moura et al. 2005; Barnett et al. 2006; Quaranta et al. 2006) with approximately

ten new species awaiting formal description (Dominique Didier, Millersville University, personal communication). Members of the order Chimaeriformes are commonly called chimaeroids or chimaeras; however, only the former term will be used in this paper, as the latter refers only to members of the genus *Chimaera*.

Hydrolagus colliei occurs from southeast Alaska (Wilimovsky 1954) to the tip of Baja and within the northern Gulf of California (Grinols 1965). Its bathymetric distribution is quite broad, along the shelf and slope from the intertidal (Dean 1906; Cross 1981) to 913 m depth (Alverson et al. 1964). Although there is not currently a directed fishery for *Hydrolagus colliei*, they are captured and discarded by recreational fishermen, as well as commercial bottom trawl and longline fisheries.

The impetus for studying the life history of chondrichthyans has been well documented during the past 3 decades. The majority of chondrichthyans have k-selected life-history characteristics such as lesser growth rate, greater longevity, later age at first maturation, and lesser reproductive output than most teleosts (Holden 1974; review in Cailliet and Goldman 2004). These biological characteristics, combined with their tendency to aggregate in large groups (Steven 1933; Strasburg 1958; Springer 1967; Klimley 1987) make chondrichthyans more susceptible to overfishing than most teleosts (Holden 1973; Cailliet 1990; Hoenig and Gruber 1990; Bonfil 1994; Walker 1998; Stevens et al. 2000). Fishes that inhabit the deep waters of the continental slope or beyond may exhibit k-selected life-history characteristics that are more extreme than their shallow-dwelling relatives, potentially making these species even more vulnerable to overexploitation (Anon 1997; Gordon 1999; Cailliet et al. 2001; Roberts 2002; Clarke et al. 2003). These obstacles to sustainable harvest are compounded by vast under-reporting of chondrichthyan catch (Bonfil 1994), and misidentification and intentional combination of taxonomic categories in catch statistics (Dulvy et al. 2000).

Chimaeroids are oviparous, forming egg cases that encapsulate individual embryos (Dean 1906). One or two egg cases are extruded onto the seafloor during parturition (Veronica Franklin, Monterey Bay Aquarium, personal communication). Gestation is estimated at 9–12 months for *Hydrolagus colliei* (Dean 1906) and 5–12 months for the elephantfish, *Callorhynchus milii* (Gorman 1963; Didier et al. 1998), both with similar stages of embryological development. Development of *Hydrolagus colliei* embryos collected by Dean (1906) off California indicated that egg case deposition occurred year-round with a maximum occurring approximately July to September. Sathyanesan (1966) found a similar pattern off northwestern Washington, where gravid females were present in July, August, and February, yet mature ova were most abundant and in utero egg cases most prevalent in females during July and

August. Lack of clear seasonality of reproduction also was evident in *Hydrolagus barbouri*, which produces eggs throughout the year and displays no distinct spawning season (Kokuho et al. 2003).

Estimation of fecundity may be difficult because offspring production in many chimaeroids probably is continuous (Dean 1906; Sathyanesan 1966; Kokuho et al. 2003). Chimaeroids are serial indeterminant spawners, making it difficult to determine duration of spawning season, because vitellogenic oocytes are found in the ovary in various stages of development for protracted and often poorly defined time periods. Spawning frequency is also particularly difficult to determine, because collecting enough fresh specimens for histological analysis of post-ovulatory follicles is difficult because of their offshore distribution and the use of fishery-dependent sampling methods. Sperm can be stored in the chimaeroid oviducal gland (Smith et al. 2004), indicating that timing of mating is not necessarily coincident with timing of parturition. To obtain a better resolution of the seasonal reproductive cycle, concentrations of the steroid hormones testosterone (T), 11-ketotestosterone (11KT), and estradiol (E₂) were analyzed in adult females. These are representatives of the primary hormones involved in the reproduction of teleosts (Borg 1994; Pankhurst 2008) and elasmobranchs (Callard et al. 1991, 1993; Koob and Callard 1999). 11-Ketotestosterone is not as commonly assayed as T and E₂ in females, but is of interest in this study because it is correlated with implantation in elasmobranchs (Manire et al. 1999), and onset of spawning period (Kime and Manning 1982; FitzPatrick et al. 1986; Mayer et al. 1992) in teleosts. Concentrations of T and E₂ were analyzed in females and males from all stages of reproductive development to determine whether these hormones can predict size at maturity. This is the first study to assess steroid hormone concentrations for a species in the order Chimaeriformes.

The purpose of this project is to assess the reproductive biology of the spotted ratfish, *Hydrolagus colliei*, as it relates to life-history evolution and present and potential direct or indirect harvest of the species. This study provides a baseline of life-history information for chimaeroids. These data are used to quantitatively test the hypotheses that fecundity is greater in oviparous than viviparous chondrichthyan lineages and that duration of reproductive season increases with increasing midpoint of depth range.

Materials and methods

Hydrolagus colliei (Lay and Bennett, 1839) were collected from the continental slope and shelf of California, Oregon, and Washington with the greatest number of samples off Monterey Bay, California (Appendix 1). Monterey Bay

samples were collected from monthly trawl and long-line surveys from October, 2003 to April, 2005 (conducted by NOAA NMFS, Southwest Fisheries Science Center, Santa Cruz Laboratory). Trawls conducted by the Northwest Fishery Science Center West Coast Groundfish Survey between May and October from 2004 to 2007 provided specimens from numerous locations off the coast of California, Oregon, and Washington.

To define the stage of reproductive development we measured various morphometrics. Lengths were measured to the nearest millimeter and mass to the nearest gram. External measurements of precaudal length (PCL), snout-vent length (SVL), and inner clasper length were recorded following Didier and Rosenberger (2002). We measured total mass, liver mass, gonad mass, oviducal gland width, uterus width, seminal vesicle mass, testes length, and testes width. The number of mature ova (those >6 mm diameter with yellow coloration; Stanley 1961), fully developed ova (those >20 mm diameter; Stanley 1961), and diameter of the largest ovum were recorded for each ovary.

Blood was extracted into lithium heparinized microcentrifuge tubes from a subset of females and males via cardiac puncture shortly after capture. Blood samples were put on ice for 1–3 h, then centrifuged at approximately 1,300g for 10 min. Plasma was pipetted off into microcentrifuge tubes and stored frozen at -18°C for later analysis. Skeletal muscle tissue was excised from the dorsum, just posterior to the uterine openings, and frozen at -18°C for later analysis. Plasma was not used for all hormone assays because the authors could not be at sea during all months of the year to collect live specimens. Skeletal muscle was more easily acquired than plasma by collaborating collectors and was used for the majority of hormone assays.

Before hormone assay, plasma samples were thawed, centrifuged at 14,000g for 5 min, and 500 μl of plasma was transferred to $12 \times 75\text{-mm}$ borosilicate vials. Ether extractions were conducted by adding diethyl ether (2 ml) to each borosilicate vial and vortexing for 4 min on a multi-tube vortexer. The ether and aqueous phases were allowed to separate for 3 min, and the aqueous phase was then fast-frozen in a methanol-dry ice bath for 2 min before decanting the ether phase into a new $12 \times 75\text{-mm}$ borosilicate vial. The procedure was repeated on the remaining aqueous phase, and the second ether sample was decanted into the same vial as the first. Ether was evaporated under a gentle stream of nitrogen in a 37°C water bath. Extracts were resuspended in 2 ml 0.1 M phosphate buffer for assay (1:4 dilution).

Two muscle samples of approximately 500 mg were used for T/11KT (mean \pm SE: 517 ± 1 mg) and E_2 assays (mean \pm SE: 515 ± 2 mg). These muscle samples were excised from a large piece of tissue on dry ice, weighed, transferred directly to 1–2 ml borate buffer in a $12 \times 75\text{-mm}$

borosilicate vial, and homogenized for 30 s. Homogenate was transferred to a $16 \times 125\text{-mm}$ borosilicate vial and 4 ml diethyl ether was added to the sample followed by vortexing for 4 min on a multi-tube vortexer. The ether and aqueous phases were allowed to separate for 2 min, followed by centrifugation at 5,600g for 1 min at 4°C to pellet lipids in the tissue mass, and fast-freezing of the aqueous layer in a methanol-dry ice bath for 2 min before decanting the ether phase. As with the plasma, this procedure was repeated, ether layers were combined, and ether was evaporated. Hormone pellets were stored at -20°C until reconstitution in 250 μl 0.1 M phosphate buffer and assay. Reconstitution volume was determined from pilot assays, which we used to evaluate the volume necessary to fall within the linear range of the T/11KT and E_2 standard curves.

Plasma and muscle samples from females were assayed for T, 11KT, and E_2 , and samples from males were assayed for T and 11KT. Samples were analyzed for E_2 using double antibody radioimmunoassay kits (Siemens Medical Solutions Diagnostics, Los Angeles, California). Samples were analyzed for T with double antibody radioimmunoassay kits from Diagnostic Systems Laboratories (Webster, Texas), and for 11KT with enzyme-immunoassay kits (Cayman Chemicals, Ann Arbor, Michigan). Manufacturer's protocols were strictly followed. A control sample was generated from 20, 500-mg muscle samples processed in the same manner as described above, resuspended in 300 μl 0.1 M phosphate buffer, and pooled (6 ml). Aliquots (6, 1-ml samples) were used for intra- and inter-assay controls. Testosterone was quantified in two separate assays with intra-assay coefficients of variation (CVs) of 2.86 and 3.56%, and inter-assay CV of 4.21%. 11-Ketotestosterone was assayed on 7, 96-well plates with intra-assay CVs of 9.22, 20.4, 12.9, 11.9, 15.5, and 15.7%, and an inter-assay CV of 16.7%. Estradiol was quantified in two separate assays with intra-assay CVs of 10.3 and 4.5%, and inter-assay CV of 8.3%. Each kit was validated for *Hydrolagus colliei* muscle steroids by generating serial dilutions of the control sample (see previous paragraph) and assessing parallelism with the standard curve using a *t* test modified to compare slopes (Zar 1996, p. 355). Significant parallelism was achieved for all hormones (11KT: $t = 0.025$, $df = 8$, $P = 0.98$; T: $t = 1.614$, $df = 6$, $P = 0.2$; E_2 : $t = 1.95$, $df = 6$, $P = 0.1$). Recovery was assessed by spiking the control sample with each kit standard, and by determining the relationship between expected (based on known control concentration) and observed concentrations. Slopes of all observed versus expected regressions approximated 1.0, indicating good recovery (11KT: $F = 80.63$, $df = 1, 7$, $P < 0.001$, $\beta_1 = 1.05$, minimum recovery = 69%; T: $F = 5928.7$, $df = 1, 6$, $P < 0.001$, $\beta_1 = 1.26$, minimum recovery = 96%; E_2 : $F = 15.06$, $df = 1, 6$, $P = 0.012$, $\beta_1 = 0.75$,

minimum recovery = 111%). Sensitivities of the assays were 1.3 pg ml^{-1} for 11KT, 0.05 ng ml^{-1} for T, and 1.4 pg ml^{-1} for E_2 .

Weight-at-length data were analyzed with both sexes combined, because there were no sex-specific trends. A non-linear regression was fit using the model that produced the greatest adjusted R^2 value, a two-parameter power function: $W = aL^b$, where W is weight in kg, L is snout-vent length in mm, a and b are estimated iteratively.

Reproductive status

Stage of reproductive development was determined based on macroscopic assessment of development of the ovaries, oviducal gland, thickness of the uterine wall, epididymis, and claspers (after Stanley 1961; Gorman 1963; Ebert 1996; Walmsley-Hart et al. 1999). We refer to this as the morphological criterion. The stages are defined for females as follows: (1) embryo: developing within egg case, (2) juvenile: uterus narrow with thin epithelial wall; oviducal gland marked by a minor widening of the oviduct; oocytes barely visible and whitish, (3) adolescent: oviducal gland slightly swollen and differentiated from uterus but without visibly contrasting tissue zones; uterine wall thin; oocytes small ($\leq 6 \text{ mm}$ diameter) and whitish, (4) adult: oviducal gland fully developed with bulbous bullet-shape and sharply contrasting tissue zones and whitish; uterine wall thick, especially proximal to uterine openings where it is muscular and resistant to compression; oocytes large ($> 6 \text{ mm}$ diameter), yellow, and vascularized, (5) gravid adult: fully or partially developed egg case present in uteri. The stages are defined for males as follows: (1) embryo: developing within egg case, (2) juvenile: no coiling of epididymis; post-pelvic claspers extremely short, uncalcified, and soft, (3) adolescent: epididymis enlarged, with few coils; post-pelvic claspers beginning to elongate but not completely calcified, (4) adult: epididymis with many, tight coils; post-pelvic claspers elongated, completely calcified and rigid.

In assessing the maturity of males using the morphological criterion, a correspondence was observed between the stages of maturity defined above, and distinct stages of frontal tenaculum development defined as: (1) juvenile: frontal tenaculum not yet erupted, (2) adolescent: frontal tenaculum erupted, yet not fully developed, with hooks uncalcified or not present, (3) adult: frontal tenaculum fully developed with calcified hooks. This correspondence was tested by regressing maturity stages estimated using the frontal tenaculum criterion on stages estimated using the morphological criterion, using a t test to test the hypothesis that $\beta_1 = 1$ (1:1 agreement of maturity stage).

To verify the maturity status in males, the ratio of inner clasper length to body length was plotted against body

length. For females, the ratio of oviducal gland width to body length was plotted against body length. An abrupt change in the proportion of these ratios to body length was assumed to represent onset of maturity. Testis length was plotted against inner clasper length to test whether growth of internal and external reproductive organs was collinear and whether abrupt increases in size, associated with maturation were concurrent.

Length at 50% maturity was estimated using a logistic regression equation (Roa et al. 1999; Mollet et al. 2000; Neer and Cailliet 2001). To test for the differences of length at maturity between sexes and geographic regions, these factors were included as main effects in a binomial logistic regression. Geographic regions were defined as north or south of Point Conception or Cape Mendocino, two coastal promontories that create oceanographic anomalies. Planned pairwise comparisons among regions were performed with an experiment-wise error rate of $\alpha = 0.0125$. To identify the temporal relationship of steroid hormone production and onset of maturity, estimates of size at median maturity derived from the morphological criterion were compared with estimates derived from the concentration of E_2 in the skeletal muscle of females and T from males. To create a steroid hormone criterion, maturity status was assigned to individuals based on a threshold concentration, assumed to indicate the onset of maturity. Threshold concentrations were estimated by identifying an abrupt increase of steroid hormone concentration with snout-vent length. Analysis of residual sums of squares (ARSS) was used to determine whether the logistic regression equations used to predict length at maturity differed between morphological and hormone maturity criteria (Chen et al. 1992).

Reproductive seasonality

Wet weights of liver, gonads and oviducal glands were measured to the nearest gram, and expressed as a ratio of total mass as the hepatosomatic (HSI), gonadosomatic (GSI) and oviducalsomatic indices (OGI), respectively. These indices, calculated from a sample representative of all seasons, were plotted against month of capture to determine whether temporal parturition patterns occurred, thus increasing precision of fecundity estimates. Such indices alone, however, may not provide enough evidence to estimate periodicity of parturition, as for batoids (Maruska et al. 1996). Steroid hormone analysis, therefore, was used to verify reproductive seasonality in females.

Temporal trends in steroid hormone concentrations of plasma and skeletal muscle were compared with a series of t tests, using an experiment-wise error rate of $\alpha = 0.05$. During two surveys off the southern Oregon coast in early June and early September, 2006, paired samples of plasma and skeletal muscle were sampled from each individual.

Temporal differences in levels of each hormone were tested separately within plasma and muscle, to verify that trends were similar between the two, as they are in the teleost fish *Mycteroperca microlepis* (Heppell and Sullivan 2000). Among individuals from both sampling periods, correlation between plasma and muscle hormone concentration was tested for each hormone separately.

A pilot test was used to determine whether the variable delay of sampling or freezing of specimens subsequent to capture was a source of measurement error in the greater study. For a subset of individuals, muscle was sampled immediately after capture, and again after specimens remained at ambient sea surface temperature for 3 h. Paired *t* tests were performed separately for each hormone to detect whether hormone concentrations were affected by the length of time between capture and sampling or freezing of specimens.

Seasonal changes in OGI, the number of mature ova per female, and muscle concentrations of steroid hormones were tested using ANOVA. Assumptions of normality were tested with Kolmogorov–Smirnov tests, and homoscedacity with Levene’s tests. Post hoc multiple comparison tests were performed using Hotchberg’s GT2 test, because sample sizes varied greatly. Kruskal–Wallis tests were used to compare seasonal changes in the number of fully developed ova per female, HSI, and GSI, because of heteroscedacity. For these non-parametric statistics, post hoc multiple comparisons tests were performed using the Nemenyi–Damico–Wolfe–Dunn test (Hollander and Wolfe 1999; Hothorn et al. 2006). Least-squares regression analyses were used to test the hypothesis that duration of spawning season of demersal chondrichthyan species increases with increasing midpoint of bathymetric range (Appendix 2; data for chimaeroid species from multiple ocean basins and elasmobranchs from the eastern North Pacific).

To estimate a maximum annual fecundity (the number of offspring produced per year), the observed rate of parturition (egg cases deposited per week) from each of two captive *Hydrolagus colliciei* at the Monterey Bay Aquarium was multiplied by the number of weeks in a year. To estimate a realized annual fecundity, the maximum annual fecundity was multiplied by the estimate of the proportion of the year the wild adult population is spawning, as determined with steroid hormones, indices of organ weights, and presence of in utero egg cases and mature ova in adult females. Descriptions of mating and egg-laying of captive *Hydrolagus colliciei* were provided from personal observations of Gilbert Van Dykhuizen, of the Monterey Bay Aquarium.

Fecundity of chimaeroid species ($n = 2$) was quantitatively compared with that of other chondrichthyans (Appendix 3) with a series of randomization tests ($\alpha = 0.05$). Mean fecundity was compared between viviparous species and oviparous species with a *t* test. Fecundity

for each species was estimated as the midpoint of the range reported in the literature, or the average if available. Before testing, least-squares linear regression analyses were used to test for increasing fecundity with increasing maximum size (TL, or DW when TL not available) among species within each species group. Groups with significant effects of size on fecundity were split into separate groups by a size threshold that minimized within-group variance in fecundity. The randomization test solved the problem of heteroscedacity by comparing the observed mean differences among species groups to a null distribution of 100,000 mean differences, each generated by bootstrapping two samples from the pooled fecundities and calculating the difference between means. The latter analysis was performed in the statistical computing environment R, version 2.4.1 (R Development Core Team 2007). All comparisons among taxa were based on the classification system of Compagno (1999). The two rajiforms with multiple oviparity, *Raja binoculata* and *R. pulchra*, were considered outliers because of their potential to produce many more offspring than other rajiforms (Ebert and Davis 2007), and therefore were excluded from analyses.

Differences between oocyte size distribution of left and right ovaries of mature females were tested with a Kolmogorov–Smirnov test. The number of mature oocytes, and maximum ovum size per adult female were tested for potential increase with body length, using least-squares regression.

Results

The relationship between *Hydrolagus colliciei* body weight and length was described by the equation $W(\text{kg}) = 2.459 \times 10^{-7}(\text{SVL})^{2.755}$ (Appendix 4). Snout-vent length was not measured for all individuals. A linear regression of SVL on PCL, however, provided a good fit [females: $n = 474$, $\text{SVL} = 0.576(\text{PCL}) - 4.097$, $R^2 = 0.988$; males: $n = 237$, $\text{SVL} = 0.556(\text{PCL}) - 1.358$, $R^2 = 0.987$], therefore, the resulting sex-specific linear equations were used to predict the missing SVL values.

Maturity

An abrupt increase in the ratio of oviducal gland width to snout-vent length with increasing snout-vent length corresponded well with onset of female maturity, as determined by the morphological criterion ($n = 476$; Fig. 1). Uterus width also increased with maturity, but is not displayed. A large, gelatinous mass was found in the accessory genital gland (also termed “receptaculum seminis” or “digitiform gland”) of 89% of adult females ($n = 198$) and only 5% of sub-adult females ($n = 91$).

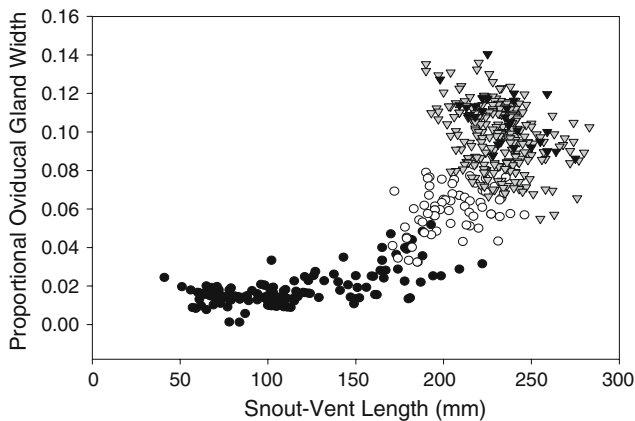


Fig. 1 Ratio of oviducal gland width to snout-vent length of *H. coliei*, displayed as a function of snout-vent length for individuals defined by the morphological criterion as juveniles (filled circles), adolescents (open circles), adults (gray triangles), and gravid adults (black triangles)

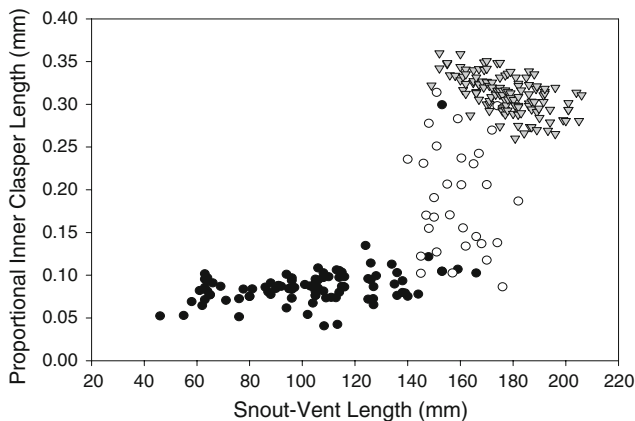


Fig. 2 Ratio of inner clasper length to snout-vent length of *H. coliei*, displayed as a function of snout-vent length for individuals defined by the morphological criterion as juveniles (filled circles), adolescents (open circles), and adults (gray triangles)

An abrupt increase in the ratio of inner clasper length to snout-vent length at 140 mm SVL corresponded well with onset of maturity ($n = 252$; Fig. 2), with few exceptions. The timing and scale of the abrupt increase in inner clasper length were similar to those of testis length ($t = 63.062$, $df = 213$, $P < 0.001$; Appendix 5). Testis length was a suitable proxy for testicular growth, as testes grew isometrically in length and width ($t = 63.208$, $df = 222$, $P < 0.001$; Appendix 6). Male maturity assessments using the frontal tenaculum development criterion were not significantly different from assessments using the morphological criterion ($t = 1.450$, $df = 213$, $P = 0.2$; Fig. 3). These results indicate that accurate estimates of maturity can be determined from male external morphology, and therefore do not necessarily require lethal sampling techniques.

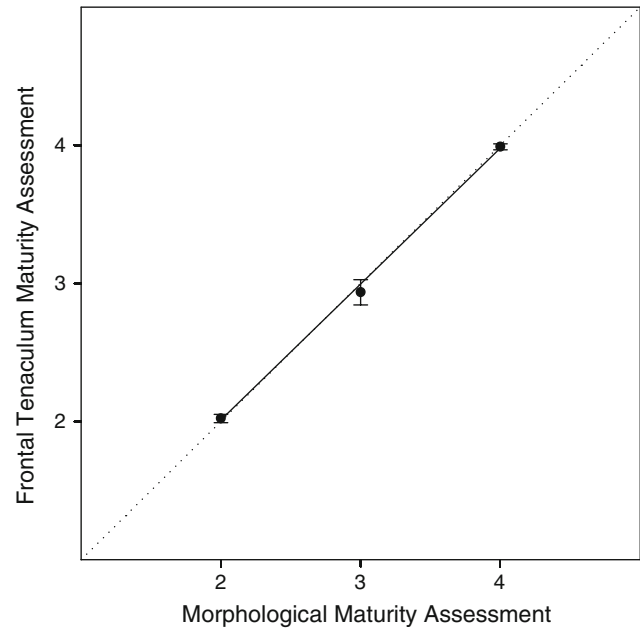
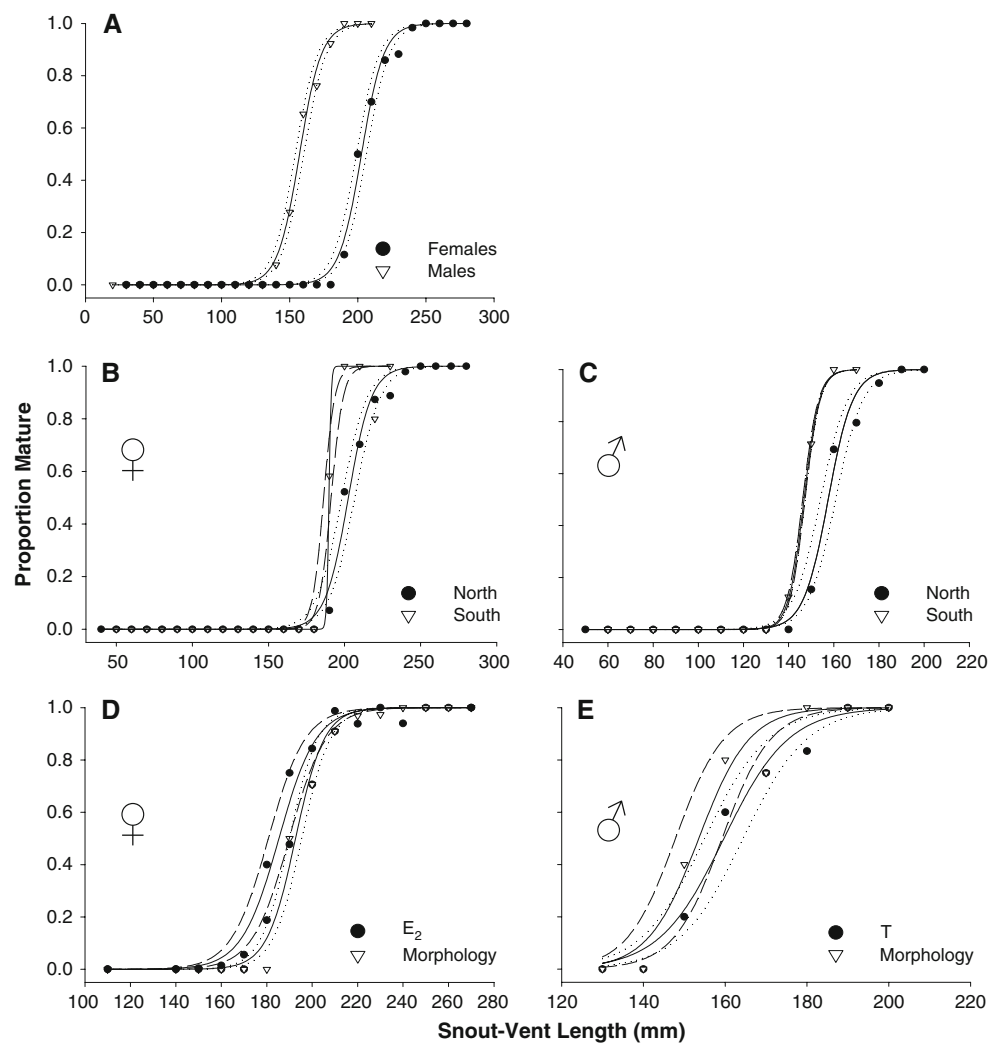


Fig. 3 Comparison of the *H. coliei* maturity estimates made from the morphological criterion and the frontal tenaculum criterion ($R^2 = 0.973$). Error bars represent 95% confidence intervals. A dotted line with a slope of one and intercept of zero is shown for reference

The purpose of the frontal tenaculum and prepelvic claspers was revealed during two observations of mating at the Monterey Bay Aquarium (Gilbert Van Dykhuizen, personal communication). Males used their frontal tenaculum to grapple the pectoral fin of the female, and subsequently the prepelvic clasper was attached to the female's ventrum, corroborating previous observations (Didier and Rosenberger 2002). A single bifid clasper was inserted into the female with each terminal lobe swelling to 2 cm in diameter after insertion into one of the two uterine openings. The mating pairs swam around the tank in a normal swimming posture, side by side, while copulating for approximately 37–120 min. During parturition, egg cases took 18–30 h to completely emerge from the uterine openings, then remained attached to the uterus by a thin filament for 3–6 days before deposition.

Length at maturity varied significantly between sexes ($\chi^2 = 219.098$, $df = 1$, $P < 0.001$) and regions ($\chi^2 = 25.296$, $df = 2$, $P < 0.001$). For individuals from the entire survey area, size at 50% maturity (Fig. 4a) was 202.8 mm SVL for females (95% CI: 199.0–206.4, $n = 530$) and 157.2 mm SVL for males (95% CI: 154.1–160.4, $n = 278$). Post hoc sex-specific comparisons indicated that female size at maturity differed between the region south of Point Conception and the two regions to the north (south vs. central: $\chi^2 = 6.650$, $df = 1$, $P = 0.010$; south vs. north: $\chi^2 = 6.937$, $df = 1$, $p = 0.008$), whereas male size at maturity differed between the region north of Cape Mendocino and the two

Fig. 4 Maturity ogives **a** for male ($R^2 = 0.995$) and female ($R^2 = 0.995$) *H. collieri*, based on the morphological criterion, **b** for females north and south of Point Conception, based on the morphological criterion, **c** for males north and south of Cape Mendocino, based on the morphological criterion, **d** for females using the morphological criterion and estradiol concentration, **e** for males using the morphological criterion and testosterone concentration. Broken lines are 95% confidence bands



regions to the south (north vs. central: $\chi^2 = 15.175$, $df = 1$, $P < 0.001$; north vs. south: $\chi^2 = 21.922$, $df = 1$, $P < 0.001$). Separate logistic curves were fit for each sex and region for which length at maturity varied. Size at 50% maturity was greater for females captured north of Point Conception (202.5 mm SVL; 95% CI: 198.2–206.7) than south (189.8 mm SVL; 95% CI: 186.4–191.3; Fig. 4b). Size at 50% maturity was greater for males captured north of Cape Mendocino (163.1 mm SVL; 95% CI: 157.8–168.2) than south (147.3 mm SVL; 95% CI: 145.4–148.8; Fig. 4c).

Length at maturity was significantly different between maturity criteria within the subset of females assayed for estradiol ($F = 5.518$, $df = 3, 24$, $P = 0.01$), but did not differ within the subset of males assayed for testosterone ($F = 1.774$, $df = 3, 10$, $P = 0.2$). Female size at maturity indicated by estradiol concentration (185.3 mm SVL; 95% CI: 180.7, 189.9) was lesser than indicated by the morphological criterion (192.7 mm SVL; 95% CI: 190.0, 195.5; Fig. 4d). There was a trend of smaller size at maturity estimated by testosterone concentration (153.5 mm SVL; 95%

CI: 147.9, 159.3) than the morphological criterion (159.7 mm SVL; 95% CI: 155.0, 164.3; Fig. 4e).

Smallest mature specimens were 190 mm SVL for females and 149 mm SVL for males. Maximum and minimum sizes for each sex were north of Cape Mendocino. Minimum size (female: 41 mm SVL; male: 46 mm SVL) is believed to be near size at birth. Maximum size for females was 283 mm SVL (633 mm TL) and 208 mm SVL (496 mm TL) for males. Maximum size previously was reported as 965 mm TL (Miller and Lea 1972), suggesting this population has decreased dramatically in body size, that original records were overestimated, or simply that the record represents an anomalous individual.

Effects of differing sample collection methods

Temporal trends were similar between plasma and muscle for all hormones (Appendix 7); however, none of the trends were significant (Appendix 8). There was a significant positive correlation of hormone concentration between plasma

and muscle samples for T ($\chi^2 = 9.626$, $r = 0.65$, $P = 0.002$) and 11KT ($\chi^2 = 6.673$, $r = 0.563$, $P = 0.01$), but not for E₂ ($\chi^2 = 1.516$, $r = 0.261$, $P = 0.2$). Concentrations were greater in plasma than muscle. Hormone concentrations of muscle samples collected immediately after capture were not significantly different from samples collected 3 h later from the same individuals (Appendix 9); however, there was a slight trend of decreased concentration with increased time before sampling for E₂ (Appendix 10).

Reproductive seasonality

Gravid females were detected in January, and May through October (Fig. 5a). The size-frequency distribution of oocytes did not differ between left and right ovaries (Kolmogorov–Smirnov: $Z = 0.955$, $P = 0.3$), therefore, total number of ova per female was used for analyses. The number of mature ova per female varied among months (ANOVA: $F = 4.543$, $df = 7$, 167 , $P < 0.001$; Fig. 5b); significantly greater in August and September than all other time periods (Hochberg GT2: $df = 167$, $P \leq 0.034$). The number of fully developed ova, 20 mm diameter or greater (Stanley 1961), varied dramatically among months (Kruskal–Wallis: $\chi^2 = 20.322$, $df = 10$, $P = 0.026$; Fig. 5c). The seasonal presence of fully developed ova and in utero egg cases indicated a parturition season of 6–8 months per year.

Concentration of 11KT in muscle of females varied among months (ANOVA: $F = 6.404$, $df = 6$, 110 , $P < 0.001$; Fig. 6a); significantly greater in April than any other month (Hochberg GT2: $df = 110$, $P \leq 0.001$). Concentration of T in muscle of females did not vary significantly among months (ANOVA: $F = 2.042$, $df = 7$, 120 , $P = 0.055$; Fig. 6b), but there was a slight trend of greater concentrations May to August than September and October. Concentration of E₂ in muscle of females varied among months (ANOVA: $F = 2.732$, $df = 10$, 140 , $P = 0.004$; Fig. 6c); significantly greater in November than March, May, and September (Hochberg GT2: $df = 140$, $P \leq 0.017$).

Mean OGI varied among months (ANOVA: $F = 2.958$, $df = 7$, 156 , $P = 0.006$; Fig. 7a); significantly lesser in January than July, August, September and October (Hochberg GT2: $df = 156$, $P \leq 0.045$). Mean GSI varied among months for females (Kruskal–Wallis: $\chi^2 = 32.523$, $df = 10$, $P < 0.001$; Fig. 7b); significantly greater in October than in February, March, or November (Nemenyi–Damico–Wolfe–Dunn test: $P \leq 0.019$). Mean GSI also varied among months for males (Kruskal–Wallis: $\chi^2 = 39.279$, $df = 9$, $P < 0.001$; Fig. 7c), greatest in January to March, and least in May, June and July (Nemenyi–Damico–Wolfe–Dunn test: $P \leq 0.016$). Mean HSI did not vary among months for females (Kruskal–Wallis: $\chi^2 = 16.205$, $df = 10$, $P = 0.094$) or males (Kruskal–Wallis: $\chi^2 = 11.534$, $df = 9$, $P = 0.241$).

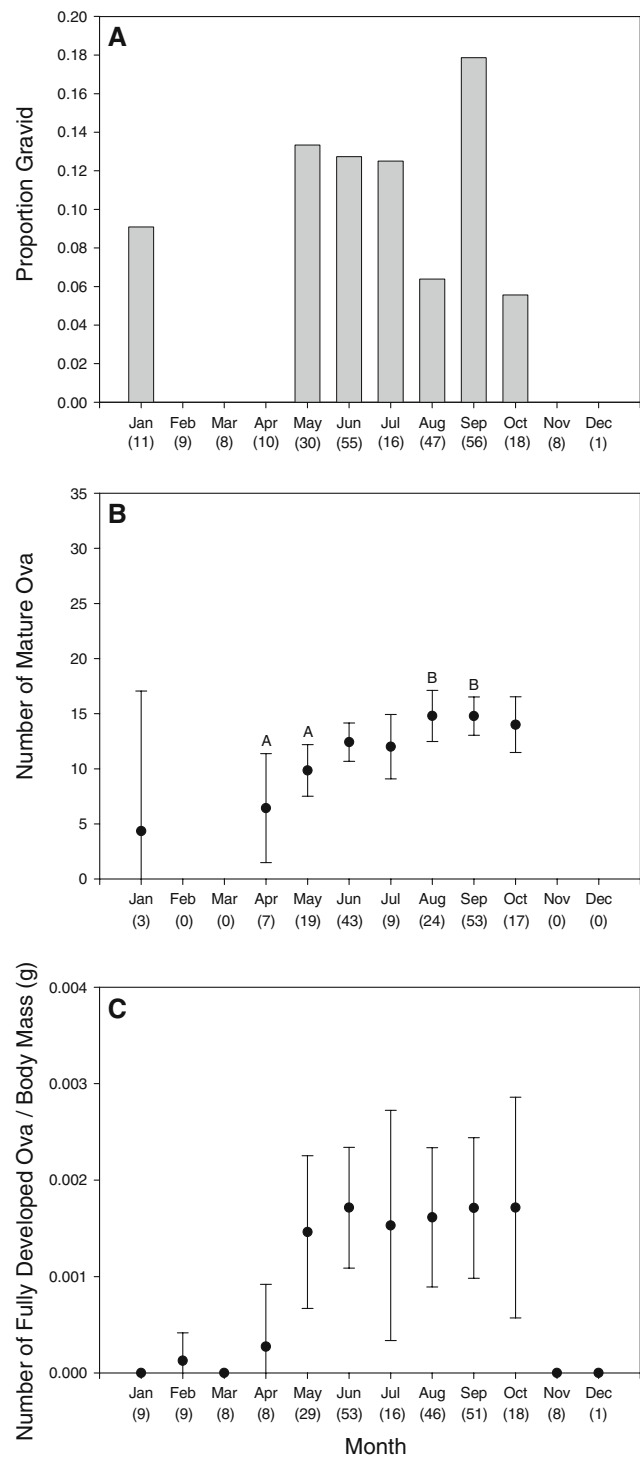


Fig. 5 **a** Proportion of adult female *H. coliei* in gravid reproductive state, **b** mean number of mature ova per female, and **c** mean number of fully developed ova per female (standardized by total body mass), by month. Sample sizes are in parentheses. Letters indicate significant differences among months

Duration of spawning season increased with depth distribution for all chondrichthyan groups combined ($t = 3.262$, $df = 24$, $R^2 = 0.278$, $P = 0.003$). This relationship was not

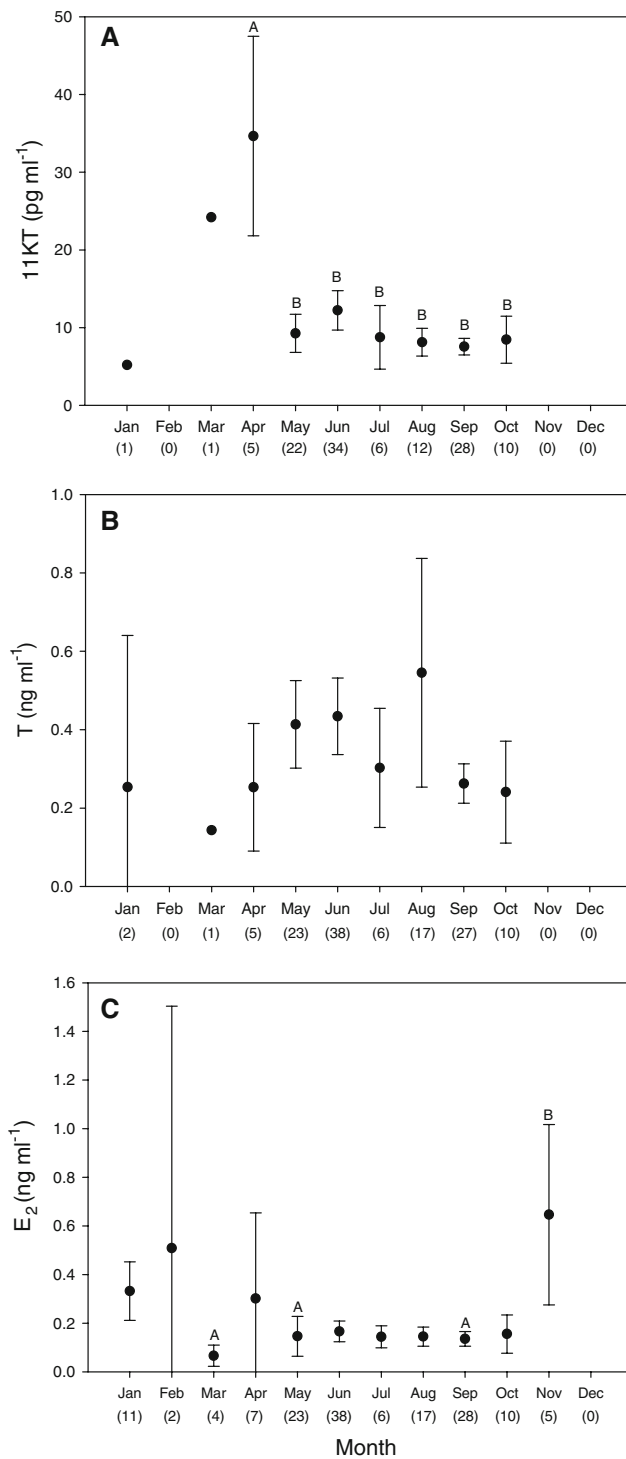


Fig. 6 Female *H. colliei* **a** 11KT, **b** T, and **c** E₂ skeletal muscle concentration by month. Error bars represent 2 SE. Sample sizes are in parentheses. Letters indicate significant differences among months

present within viviparous ($t = 0.651$, $df = 14$, $P = 0.5$) and oviparous ($t = 1.352$, $df = 10$, $P = 0.2$) groups, however, nor within specific chondrichthyan taxonomic orders (chimaeroids: $t = 0.464$, $df = 4$, $P = 0.7$; carcharhinoids: $t = 1.579$, $df = 6$, $P = 0.2$).

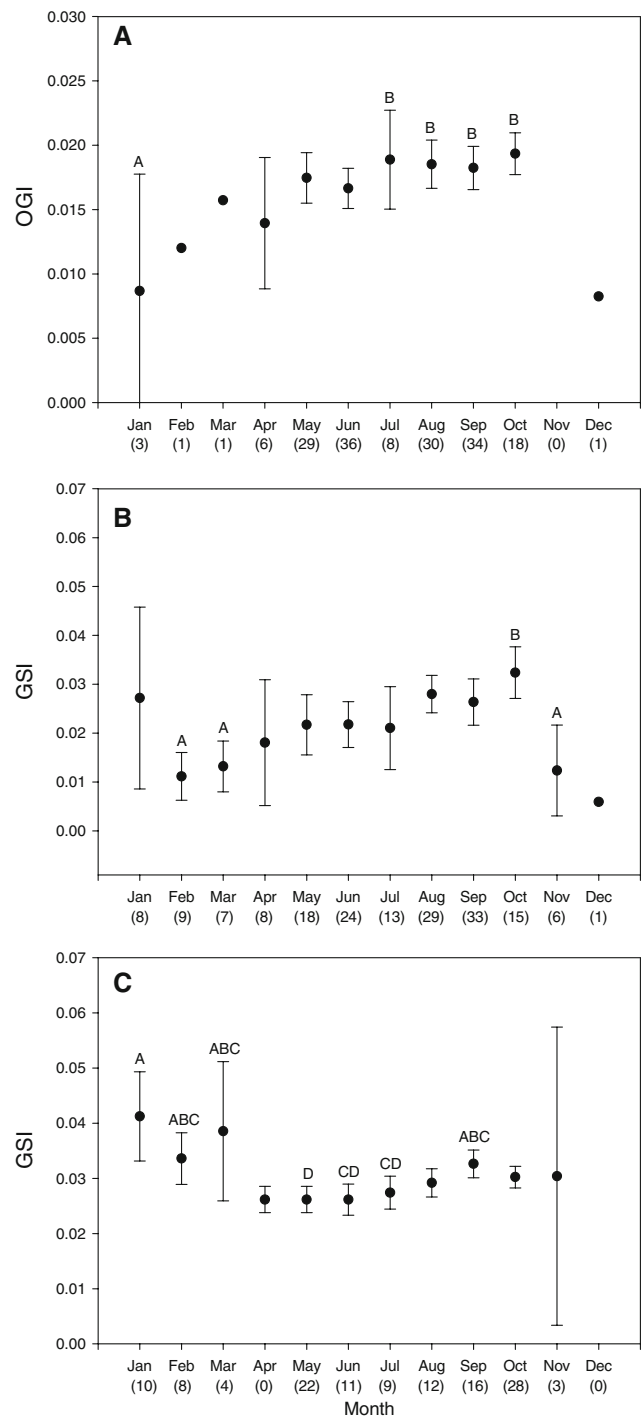


Fig. 7 **a** Mean female *H. colliei* oviducal gland index; mean **b** female, and **c** male gonadosomatic index by month. Error bars represent 95% confidence intervals. Sample sizes are in parentheses. Letters indicate significant differences among months

Fecundity

Two captive *Hydrolagus colliei*, taken from Puget Sound, Washington, deposited 18 and 20 egg cases, respectively, while monitored for 24 consecutive weeks from December

1996 to June 1997 at the Monterey Bay Aquarium (Gilbert Van Dykhuizen, personal communication). Annual fecundity was 19.5–28.9. The number of fully developed ova, 20 mm diameter or greater (Stanley 1961), increased with adult female somatic weight ($r = 0.394$, $df = 54$, $P = 0.003$; Appendix 11). For reproductively active females, the maximum ovum diameter increased with snout-vent length ($t = 3.833$, $df = 159$, $P < 0.001$). For the latter analysis, females were considered reproductively active if their ovaries contained at least one follicle of 16 mm diameter or greater, a threshold that became apparent when maximum ovum size was compared with snout-vent length (ANCOVA: interaction term = maximum ovum size \times snout-vent length; $F = 7.734$, $df = 1$, 203, $P = 0.006$; Fig. 8).

Fecundities differed between oviparous and viviparous species ($t = 10.433$, $df = 83$, $P < 0.001$); mean fecundity of viviparous species was lesser (8.1; SE 0.94) than mean fecundity of oviparous species (50.1; SE 6.2). Fecundity decreased with size within the order Squaliformes ($t = 2.312$, $df = 12$, $P = 0.039$), so species were grouped into large (TL > 60 cm) and small (TL < 60 cm) categories. There was no significant relationship between fecundity and body size of other taxonomic orders. When these same species were grouped by higher taxonomic level, the oviparous chimaeriform fishes had greater fecundity than the viviparous lamniform ($P = 0.013$), myliobatiform ($P = 0.002$), rhinobatiform ($P = 0.005$), and squaliform fishes (small: $P = 0.012$; large: $P = 0.004$), lesser fecundity than the oviparous rajiforms ($P = 0.050$), and similar fecundity to the carcharhiniforms ($P > 0.9$), with both reproductive modes represented.

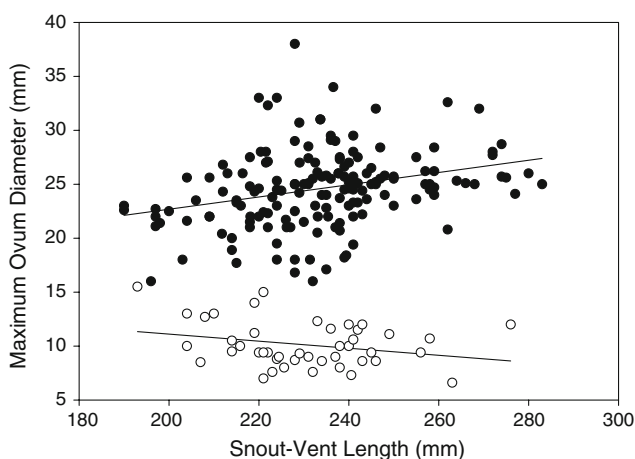


Fig. 8 Maximum ovum diameter per adult female *H. colliei* as a function of snout-vent length. Filled circles represent reproductively active individuals (those with maximum ovum diameter of 16 mm or greater; $R^2 = 0.079$), and open circles represent inactive individuals (those with maximum ovum diameter of <16 mm)

Discussion

We found *Hydrolagus colliei* had a discrete reproductive cycle, contradicting previous reports indicating the occurrence of spawning during all seasons (Dean 1906; Sathyanesan 1966). In utero egg cases and fully developed oocytes were most prevalent May to October, indicating parturition is occurring only during this time period. The seasonal cycle was corroborated by adult female GSI and OGI. Annual fecundity range was estimated to be 19.5–28.9, based on extrapolation of the observed mean parturition rate to the hypothesized 6 to 8-month egg-laying season. Determination of the timing and duration of the parturition season with morphological data enabled interpretation of steroid hormone roles in the adult female reproductive cycle.

Fluctuations of T, 11KT and E_2 in plasma are related to many important reproductive events in oviparous fishes. The observed correlation and similar temporal changes between hormone concentrations of plasma and skeletal muscle indicates that the timing of specific reproductive events can also be interpreted from fluctuations of these hormones in skeletal muscle. This correspondence between plasma and muscle is an important result, indicating that muscle biopsy may be an easy, non-lethal method of sampling hormones of large sharks, from which plasma is logistically difficult to collect.

11-Ketotestosterone is correlated with oviposition (Manire et al. 1999) in female elasmobranchs, pre-vitellogenic oocyte growth (Matsubara et al. 2003), onset of spawning period (Kime and Manning 1982; FitzPatrick et al. 1986; Mayer et al. 1992), ovulation (Slater et al. 1994; Semenkov et al. 2006), and oocyte maturation (Semenkov et al. 2006) in female teleosts, and sexual behavior in male teleosts (Borg 1987; Kindler et al. 1991). Greater concentration in April is consistent with the apparent function of 11KT in pre-parturition stages of reproduction. If the correlation with sexual behavior is consistent for females, the observed peak may also be related to mating activity. Although there was no clear physical evidence of mating activity, Dean (1903) suggested that mating occurs shortly before parturition based on the presence of fresh mating scars on gravid females.

Testosterone is correlated with ovarian recrudescence (Sumpter and Dodd 1979), oocyte maturation (Semenkov et al. 2006), oviposition (Sumpter and Dodd 1979), and encapsulation (Rasmussen et al. 1999). Testosterone is synthesized by developing follicles and luteal tissue, whereas estradiol is primarily synthesized by developing follicles (Callard et al. 1993). The observed seasonal fluctuation of T was not very distinct, but the increase during May to August may be consistent with its role in oviposition and encapsulation.

Estradiol is correlated with ovarian recrudescence (Sumpter and Dodd 1979; Sulikowski et al. 2004), increased oviducal gland weight (Sulikowski et al. 2004), follicle size (Sulikowski et al. 2004), elevated plasma vitellogenin (Woodhead 1969; Craik 1978; 1979; Ho et al. 1980; Takemura and Kim 2001), oviposition (Sumpter and Dodd 1979) and induction and maintenance of structural proteins and enzymes in the oviducal glands (Dodd and Goddard 1961; Koob et al. 1986). Relative levels of E_2 to progesterone (P_4) influence oviduct contraction with P_4 acting as an inhibitor (Abrams-Motz and Callard 1989). Estradiol may enhance the sensitivity to relaxin, which may facilitate oviposition, gestation and parturition by allowing increased dilation of the reproductive tract (Koob et al. 1984) and perhaps controlling smooth muscle action required for these processes (Callard et al. 1993). In *Hydrolagus colliei*, E_2 was greatest in November, and least from March to October, indicating that its primary role may be ovarian recrudescence.

Steroid hormone cycles of oviparous chondrichthyans have been generalized as a process wherein E_2 increases during follicular growth (preovulation) and decreases during luteal development (Callard et al. 1991). This pattern was evident for *Hydrolagus colliei*; however, maximum E_2 concentration preceded maximum ovulatory activity by as long as 5 months, a much greater duration than anticipated. This discrepancy may represent the difference between population and individual hormone cycles.

Hydrolagus colliei has either a constant or endogenous reproductive cycle (sensu Whittier and Crews 1987). In the former situation, the gonads are always prepared for reproduction, but sexual behavior is cued by environmental conditions, and in the latter situation, reproductive activity is seasonal, but has minimal environmental regulation. Under these criteria, *Hydrolagus colliei* has a constant cycle, because onset of parturition of the wild population coincided with dramatic annual environmental changes in thermocline depth and primary productivity associated with coastal upwelling. Captive *Hydrolagus colliei* under constant conditions in the Monterey Bay Aquarium extruded egg cases in all seasons, adding evidence of environmental regulation. Alternatively, reproductive activity could be regulated by social cues with captive individuals remaining constantly reproductively active because of the perpetual proximity of males, a situation that is not probable in some wild chondrichthyan populations (Economakis and Lobel 1998). Further evidence for an endogenous cycle regulated by social cues is provided by Bell (2003), in which captive female *Callorhynchus milii*, in the absence of males, retained their annual reproductive cycle while temperature and photoperiod were kept relatively stable.

The current paradigm regarding reproductive seasonality in the marine environment is that animals that inhabit

deeper depths are less likely to have seasonal cycles of reproductive output (Wourms 1977; Parsons and Grier 1992). Although this relationship was present when analyzing all chondrichthyans combined, it was not apparent when oviparous and viviparous species were analyzed separately, indicating an association with reproductive mode. The effect was also not significant within the chimaeroids or carcharhinoids. It is not surprising that this generalization is faulty. Pankhurst et al. (1987) established that three species of mid-slope teleosts, inhabiting depths from 600 to 1,300 m, had short duration, discrete spawning cycles with consistent periodicity among years. Recently, researchers have shown that productivity from coastal upwelling off California can have a major influence on benthic communities as deep as 4,100 m (Ruhl and Smith 2004). These insights indicate that the evolution of discrete versus continuous reproductive cycles is a much more complex process than previously thought.

Researchers have suggested that assays of the steroid hormones T and E_2 in plasma serve as an accurate, non-lethal method of determining size at maturity for chondrichthyans (Sulikowski et al. 2004; Awruch et al. 2008). In this study, however, the steroid hormone maturity criterion predicted significantly smaller size at maturity than the morphological criterion for females, and a similar, yet insignificant trend for males. The differences were not great, but the results indicate that hormone data sometimes identified somewhat smaller individuals as already mature. In the teleost fish *Mycteroperca microlepis* neither E_2 nor T from plasma or muscle were consistent indicators of maturity (Heppell and Sullivan 2000).

It is logical that hormones related to maturity should increase before morphological maturity, as T is correlated with development of intromittent organs (Schreibman et al. 1986), and 11KT is correlated with expression of secondary sexual physiological characters in teleost fishes (Idler et al. 1961; Hishida and Kawamoto 1970; de Ruiter and Mein 1982; Pottinger and Pickering 1985; Borg 1987; Borg et al. 1993). The synchronous development of the frontal tenaculum and internal reproductive organs was also observed, albeit qualitatively, in the elephant fish *Callorhynchus milii* (Gorman 1963). Gorman (1963), however, also reported that prepelvic claspers were only present in adult males, but they were omnipresent in *Hydrolagus colliei* males. The gelatinous mass of the accessory genital gland, which contains desquamated epithelial cells (Stanley 1963) was present in 89% of mature females. The function of this secretion is unknown, but also was present in nearly all mature females in a previous study (Stanley 1963).

Size at maturity and maximum size of *Hydrolagus colliei* were greater in northern than southern regions. This latitudinal pattern is commonly observed in fishes (Parsons 1993; Carlson and Parsons 1997; Yamaguchi et al. 2000;

Lombardi-Carlson et al. 2003; Heibo et al. 2005). Spatial differences in size at maturity often are caused by temperature (Parsons 1993; Yamaguchi et al. 2000; Walker 2007), photoperiod (Parsons 1993) and prey abundance (Yamaguchi et al. 2000). Such variance can also result from differential fishing intensity with greater exploitation rates causing smaller size at maturity through density-dependent compensation or simply artificial selection for smaller sizes (Stearns 1992; Conover and Munch 2002; Roff 2002; Olsen et al. 2004; Sosebee 2005; Edeline et al. 2007). Unfortunately, catch of *Hydrolagus colliei* is typically not reported, so it is not possible to test the latter hypothesis. However, there are greater temperatures and lesser nutrients (perhaps creating a lesser abundance of the benthic epifauna and infauna that *Hydrolagus colliei* prey upon; Johnson and Horton 1972) on the seafloor of the shelf and upper slope south of Point Conception than north, and to a lesser extent, south of Cape Mendocino than north (Tolimieri 2007). Differential temperature and food availability, therefore, likely are the primary factors causing a latitudinal gradient in size at maturity of *Hydrolagus colliei*. It is also possible that differences in size may be the product of multiple discrete breeding populations (or metapopulations), as suggested for the Port Jackson Shark, *Heterodontus portusjacksoni* (Tovar-Ávila et al. 2007). There is insufficient evidence, however, to evaluate the latter hypothesis.

The simple method of fecundity estimation by extrapolation of observed parturition rates was used instead of methods in Holden (1975) because assumptions of Holden's methods likely were violated. Holden (1975) used the proportion of gravid individuals sampled from the wild population as a proxy for the proportion of the population depositing egg cases during a given period of time. A primary assumption of this method is that the gravid population is sampled appropriately. This assumption is likely not met for *Hydrolagus colliei*, as parturition in this species occurs primarily on or around seamounts. These regions are poorly represented in surveys because they comprise an exceptionally small area of the seafloor and their rocky, rugose surface cannot be sampled with trawl gear. Holden's (1975) alternative method of estimating fecundity relies upon the following assumptions: (1) all mature ova produced by an adult female during a given year are formed before the onset of parturition, and therefore are simultaneously present in the ovary, (2) this group of mature ova will all be placed in egg cases and extruded during the current spawning season and (3) no ovum resorption occurs. It is doubtful that these assumptions are met for *Hydrolagus colliei*, in which ovum resorption and/or follicle atresia was commonly observed. The number of mature and fully developed ova in females and female GSI did not decrease gradually toward the end of the spawning season

(Figs. 5bc, 7b), as would be expected if the former two assumptions were met.

Fecundity of *Hydrolagus colliei* is similar to that estimated for *Callorhynchus milii* (16–24; Bell 2003), but much greater than that estimated for *Chimaera monstrosa* (mean 6; Moura et al. 2004). The latter difference, however, could be caused in part by the method of fecundity estimation. Moura et al. (2004) estimated fecundity by calculating the mean number of fully developed ova adult per female. Similarly, Tovar-Ávila et al. (2007) estimated fecundity for *Heterodontus portjacksoni*, a shark with single oviparous reproductive mode, as the mean number of oocytes $\geq 75\%$ of ovulatory size in adult female sharks in the months before the parturition season. The latter method provided similar estimates to that derived by extrapolation of captive parturition rates (Tovar-Ávila 2006). In *Hydrolagus colliei*, however, the mean number of oocytes greater than 10 mm diameter ($\geq \sim 35$ –50% of ovulatory size) per female captured before the egg-laying season was only 10 (range 1–36; $\sigma = 9.04$; $n = 32$), lesser than half the mean fecundity estimated from captive parturition rates. This discrepancy indicates that the method of simply enumerating maturing ova may be inadequate for chimaeroids, and other chondrichthyans with single oviparity that display rapid vitellogenesis (Holden et al. 1971) and protracted production of mature ova.

The number of fully developed ova increased with adult female somatic weight, providing marginally significant evidence for increased fecundity with size. This effect occurs in another chimaeroid, *Callorhynchus milii* (Bell 2003), and other organisms, including skates (Matta 2006), sharks (Simpfendorfer 1992; Yano 1995), and rockfishes (Boehlert et al. 1982; Bobko and Berkeley 2004; Cooper et al. 2005). There is also some evidence for increasing maximum ovum size with maternal body size. Larger females may provide more nutrients to their young, potentially increasing offspring fitness (Berkeley et al. 2004a). Increased embryo, egg, egg case or yolk sac size and/or nutrient content with larger maternal size occurs in many fishes, including skates (Hoff 2007) and rockfish (Berkeley et al. 2004a).

Although there are many chondrichthyans with one of the two maternal size effects observed in *Hydrolagus colliei*, there are few species that exhibit both (Cortés 2000). There seems to be a tradeoff between the number of offspring and offspring size within and among chondrichthyan populations (review in Cortés 2000), indicating the existence of a specific ratio of the two that would maximize fitness (Roff 2002). Increased number and fitness of offspring with increased maternal size is an important biological component in conservation planning (Berkeley et al. 2004b).

Oviparity was previously considered the plesiomorphic state of chondrichthyans (Wourms 1977; Compagno 1990; Wourms and Lombardi 1992; Dulvy and Reynolds 1997), but a recent review indicated that viviparity is the ancestral state (Musick and Ellis 2005). Musick and Ellis (2005) revisited the ideas of Holden (1973) and hypothesized that oviparity evolved to increase fecundity in small-bodied species, which have too little coelomic space to produce many live offspring per litter. The latter hypothesis is supported by the trend for greater fecundity in oviparous than viviparous species among chondrichthyans with small body size, a phenomenon previously disputed by Wourms and Lombardi (1992). In the present study, fecundity was quantitatively compared among chondrichthyan lineages to provide more explanatory power for these hypotheses by incorporating interpretation of fecundity evolution into the evolution of reproductive modes. The results of the present study indicate that the oviparous Chimaeriformes evolved greater fecundity than their viviparous elasmobranch relatives, but lesser fecundity than the more derived oviparous species within the order Rajiformes. With the Chimaeriformes added into the analysis of evolution of fecundity, more evidence is provided that supports the hypothesis of viviparous plesiomorphy in chondrichthyans, as argued by Musick and Ellis (2005).

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