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Late maturity and evidence for female biennial spawning in the sea pen *Pennatula aculeata* (Anthozoa, Pennatulacea) in eastern Canada

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Abstract

Little is known about reproduction of the sea pen *Pennatula aculeata*, which is found at high densities and constitutes vulnerable habitat in the Gulf of St. Lawrence, Canada. Sex ratio, size at maturity, and reproductive status were investigated for colonies collected in May, August, and October 2015 along the Gaspé Peninsula. Reproductive status was assessed by histological determination of gamete sex and maturity and by stereomicroscope measurement of the diameters of the leading cohort of oocytes (LCO) or sperm cysts of histologically mature colonies. Colonies were gonochoric, with a sex ratio of 1:1. Based on the distribution of gamete diameters in May, three groups of female and a single group of male colonies were identified. Female colonies classified in the group of LCO with the smallest diameter (0.10–0.39 mm) were shorter in total length (TL < 130 mm) than females in the other two groups of LCOs with larger diameters, and very few spawned from May to October. A major reduction in the proportion of large female colonies (TL ≥ 130 mm) belonging to the group with the largest diameter (≥0.55 mm) and of mature male colonies between May and August indicated one annual spawning event. Persistence of histologically mature (vitellogenic) female colonies classified in the two smaller oocyte diameter groups after spawning is indicative of prolonged oogenesis (at least 24 months). The fact that only approximately half of the large female colonies had ripe oocytes (diameter ≥0.55 mm) in May prior to spawning, while the other half had vitellogenic oocytes of much smaller diameter which persisted after the spawning period, suggests that they reproduce only every other year. Estimated size and age at which 50% of colonies spawned were greater in females (148 mm TL, 11–14 years) than in males (101 mm TL, 8–11 years). These reproductive characteristics may reduce resilience of *P. aculeata* to various anthropic stressors.

KEYWORDS
gametogenesis, reproductive cycle, reproductive timing, sex ratio, size at maturity

1 | INTRODUCTION

Sea pens (Pennatulacea) are colonial octocorals that cover extensive areas on soft bottoms of the ocean worldwide, from intertidal to abyssal depths (Williams, 2011). At sufficiently high density (e.g., Ashford et al., 2019), sea pens provide structural habitat for other invertebrate and vertebrate organisms (e.g., Baillon et al., 2012; Baillon, Hamel, & Mercier, 2014; Miller et al., 2012; Tissot et al., 2006). Sea pen colonies are slow-growing, long-lived, and have limited or no mobility, and therefore, they are highly vulnerable to adverse impacts of bottom fishing (DFO, 2010; Murillo et al., 2018). Dense aggregations of sea pens are considered vulnerable marine ecosystems (VMEs) in the Northwest Atlantic (FAO, 2009; NAFO, 2014). In Canada, regional habitats containing dense sea pen fields have been delineated and identified as sea pen Significant Benthic Areas (DFO, 2017; Kenchington et al., 2016). Their overlap with historic (2005–2014) bottom fishing footprints has been measured to implement appropriate management actions for their protection against proposed or ongoing fishing activities (Koen-Alonso et al., 2018).

One important concern about possible impacts of intense fishing activity on sea pens is potential reproductive harm that could affect the recovery of, and the connectivity among, fields. Determination of reproductive harm from physical disturbance requires prior knowledge of reproduction and has been attempted only in a few soft or horny corals (Alcyonacea; e.g, Henry et al., 2003; Linares et al., 2008; Tsounis et al., 2012) but not in sea pens. Pennatulaceans are generally gonochoric, with mature colonies exhibiting exclusively either male or female gonads (reviewed by Kahng et al., 2011 and Lopes et al., 2012). Most of the studied species reproduce by broadcast spawning and produce lipid-rich, positively buoyant, lecithotrophic larvae. Sufficient densities of mature colonies and synchrony of spawning are two factors that favor high gamete concentrations and successful fertilization in broadcast spawners under suitable environmental conditions (Baillon, Hamel, Wareham, et al., 2014; Langton et al., 1990; Oliver & Babcock, 1992; Servetto & Sahade, 2016). Among the relatively small number of pennatulacean species studied for their reproductive behavior, different patterns of spawning have been described, varying from one single seasonal spawning event in the year, most frequently observed in shallow-water species, to continuous year-round spawning observed in some deep-water species (reviewed by Baillon, Hamel, Wareham, et al., 2014; Lopes et al., 2012; Servetto et al., 2013).

Our study focusses on *Pennatula aculeata* Danielssen 1860, which is widespread at depths ranging 110–550 m (depth range extending to 2300 m) along the eastern coast of North America from Nova Scotia to southern New England (Langton et al., 1990; WoRMS Editorial Board, 2021). In the lower estuary and gulf of the St. Lawrence River, eastern Canada, this species forms dense fields in areas located in the Laurentian, Esquiman, and Anticosti channels (Bourdages et al., 2018; DFO, 2017). One study has been conducted on this species' reproduction, and it consisted of examining gonad morphology and gametogenesis in 22 colonies (33.5- to 235-mm height; likely equivalent to total length, TL, as described in methods below) caught at three sites (113- to 231-m depth) in the Gulf of Maine in August 1993 (Eckelbarger et al., 1998). This study indicated that *P. aculeata* is a gonochoric, nonbrooding, broadcast spawner. Multiple gametogenic stages were found within individual female and male polyps, suggesting no distinct seasonal reproductive period. Aquarium observations reported in Eckelbarger et al. (1998) also revealed that male colonies expel intact sperm cysts (containing spermatozoids) at spawning, possibly to prevent dilution of sperm. In the only other study of reproduction in the genus *Pennatula*, Edwards and Moore (2008) sampled year-round and observed synchronous maturation of oocytes, from which they inferred a brief July or August spawning period in *Pennatula phosphorea* at 18- to 20-m depth off the west coast of Scotland.

The general objective of our study was to obtain additional knowledge on the reproductive biology of *P. aculeata* to support risk analysis for bottom-contact fishing or scientific surveying and the development of appropriate fish habitat management and conservation plans. More specifically, we examined the sex ratio, size at maturity, and temporal variation in reproductive status of *P. aculeata* from the Gulf of St. Lawrence, eastern Canada.

2 | METHODS

2.1 | Field sampling

The study area was located in the northwest Gulf of St. Lawrence, along the northern coast of the Gaspé Peninsula, Quebec, Canada (latitudinal and longitudinal boundaries in Table 1; mapped by asterisks in 2018: Figure 1). This area is at the southern border of a sea pen Significant Benthic Area, located in the Laurentian Channel (DFO, 2017). A 3-m wide beam trawl equipped with heavy tickler chains and 17-mm mesh in the cod end was used to collect colonies of *P. aculeata*. At each station, 10-min tows over a median distance of

TABLE 1 Sampling date, number of tows per sampling date, corresponding ranges of latitude and longitude, depth range, and bottom temperature range for collection of colonies of *Pennatula aculeata* in the Gulf of St. Lawrence

Year	Month	Days	Tows (*n*)	Latitude	Longitude	Depth (m)	Temperature (°C)
2014	July	21	3	49.117–49.141	−64.457 – −64.387	262.0–290.0	5.10–5.84
2015	May	10–11	5	49.116–49.141	−64.458 – −64.386	250.5–301.5	5.37–5.62
2015	August	6	3	49.139–49.141	−64.452 – −64.452	282.0–288.5	5.70–5.71
2015	October	20	3	49.141–49.148	−64.445 – −64.463	277.0–286.5	5.68–5.71

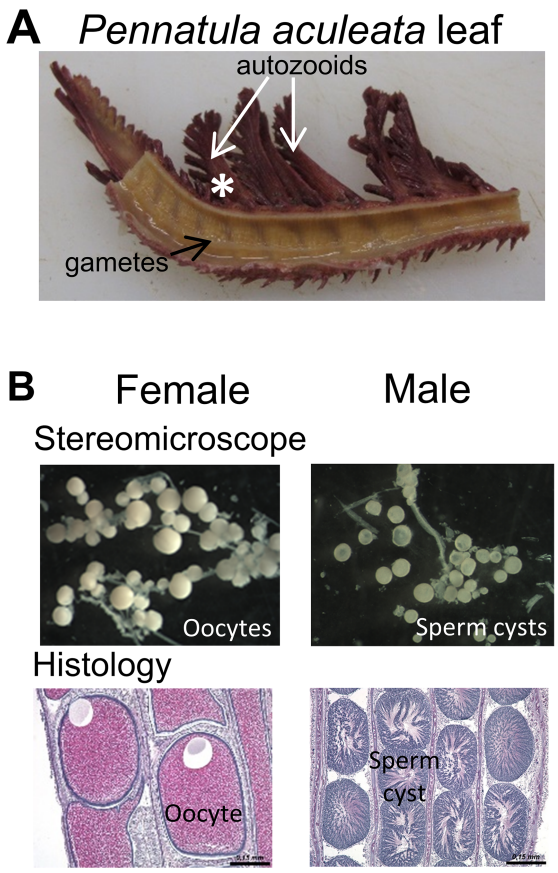


FIGURE 1 Methods for sex identification of colonies of *Pennatula aculeata*. **A.** Region of the leaf where gametes were observed and measured using a stereomicroscope; the gastrovascular cavity at the base of the autozooids is indicated by an asterisk. **B.** Appearance of male and female gametes observed under a stereomicroscope or on a histological section

720 m were made along lines parallel to isobaths, at depths ranging 250–301 m (Table 1). Colonies were sampled on four dates: July 2014 and May, August, and October 2015 (Table 1). In 2014, a total of 45 colonies of *P. aculeata* >70 mm in colony TL (see below for method of measurement), a size range previously reported to be reproductively active (Eckelbarger et al., 1998), were collected for development of methods to assess sex and maturity. In 2015, random sampling of catches of *P. aculeata*, stratified by TL, was conducted. We set an objective of at least five colonies of *P. aculeata* for each 20-mm size bin of TL, ranging 45–225 mm, on each of the three sampling dates, to assess sex ratio, size at maturity, and temporal changes in reproductive status. Sample size in 2015 varied from 80 to 150 per sampling date.

2.2 | Measurement and dissection

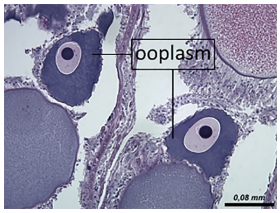
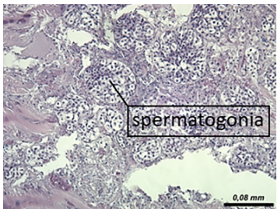
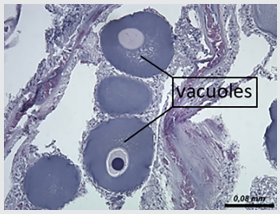
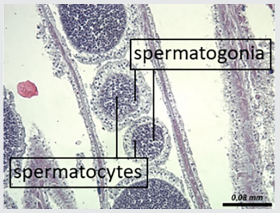
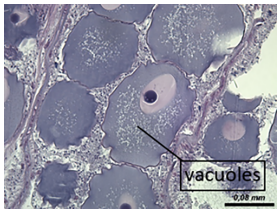
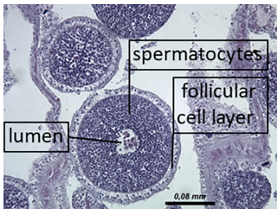
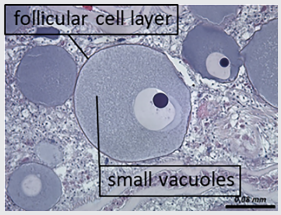
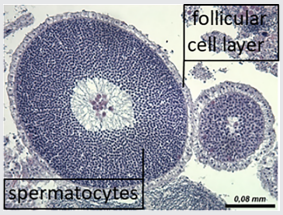
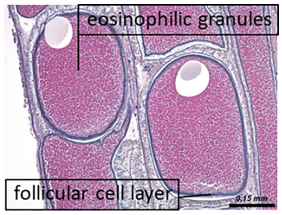
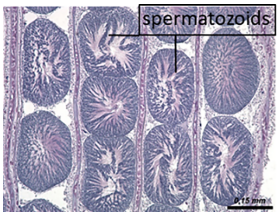
A colony of *P. aculeata* is composed of a primary (axial) polyp with a peduncle anchored in soft sediment and an exposed erect distal portion, the rachis, from which pairs of opposed polyp-bearing leaves branch laterally. Colony TL was measured to the nearest millimeter along the rachis, from peduncle tip to distal leaf apex, with the leaves aligned along the longitudinal axis. Each colony was partitioned into basal, central, and apical leaf zones by counting the total number of leaves and dividing by 3 (modified from Baillon, Hamel, Wareham, et al., 2014; Edwards & Moore, 2008; Pires et al., 2009; Soong, 2005). Each leaf consists of up to 12 partially fused secondary polyps, called autozooids, which have both feeding and reproductive functions (Figure 1A). Each autozooid is subdivided internally by eight mesenteries. The gametes develop along the edge of mesenterial filaments which extend into the gastrovascular cavity of the autozooid (Eckelbarger et al., 1998).

Colonies collected in 2014 and 2015 were rinsed in seawater immediately after capture. In 2014, colonies were then fully immersed in 4% phosphate-buffered formaldehyde (Roberts, 2012) and 6 months later were removed from formalin, rinsed with seawater, measured in TL, and one pair of opposed leaves was excised from the middle of each of the three leaf zones. Sex and maturity were assessed by histology on one leaf and gamete morphology by stereomicroscopy on the other leaf of a pair. In 2015, fresh colonies were measured in TL, and based on results of 2014 and literature, one pair of opposed leaves was excised only from the middle of the central leaf zone, fixed in formaldehyde, and processed 3–9 months later. According to Edwards and Moore (2009), characteristics of oocytes from the central leaf zone accurately represent colony oogenic cycle and maturity because this zone lies between the basal zone, which contains a larger proportion of smaller oocytes, and the apical zone, which produces a greater number of larger oocytes. In 2015, sex and maturity were assessed by histology on one leaf and by stereomicroscopy on the other leaf of a pair.

2.3 | Histological assessment of sex and maturity

The proximate part of formalin-fixed leaves, including the gastrovascular cavity at the base of the autozooids, where most gametes were observed (Figure 1A), was dehydrated and embedded in paraffin wax for preparation of 6-μm-thick histological sections which were stained with hematoxylin and eosin (Luna, 1968). On one section each from the basal, central, and apical leaf of each colony in 2014, or from the central leaf of each colony in 2015, sex was determined based on the presence of oocytes in females or sperm cells in males (Figure 1B). For a minority of specimens, no gametes were found in the histological section, likely due to inadequate positioning of the section plan through the reproductive tissue. Consequently, sex could not be identified for this section and so the specimen was categorized as undetermined. Maturity of male and female gametes was graded from I to V using morphological criteria (Table 2) based on descriptions of maturation in other octocoral species (Baillon, Hamel, Wareham, et al., 2014; Beazley & Kenchington, 2012). For each colony, the most advanced stage of gamete maturity was determined and the predominant maturity stage (>50% of gametes) was assessed in the area of the

TABLE 2 Criteria for histological assessment of gamete maturity stages in colonies of *Pennatula aculeata*

	Females		Males	
Stage	**Description**	**Illustration**	**Description**	**Illustration**
I	The first gametogenic stages (oogonia) are embedded in the mesenteric mesoglea. Oogonia have a highly basophilic ooplasm without vacuoles and a high nucleus:cytoplasm ratio.		The first gametogenic stages (spermatogonia) are embedded in the mesenteric mesoglea. Spermatogonia have clear nuclei and are loosely packed.	
II	Previtellogenic oocytes appear. They are characterized by a moderately to highly basophilic ooplasm with clear and dispersed vacuoles occupying <50% of the cytoplasm. A thin follicular cell layer may be present.		Aggregations of loosely packed spermatocytes with condensed dark nuclei and occasional spermatogonia are present. A thin follicular cell layer may be present.	
III	Previtellogenic oocytes with >50% of their ooplasm packed with clear vacuoles are present. They are no longer fully embedded in the mesentery. A thin to thick follicular cell layer is present.		Sperm cysts contain a thick layer of spermatocytes with dark nuclei but occasionally surrounded by a clear halo. A small lumen, often filled with cellular debris, begins to appear in the center of sperm cysts. Cysts are either embedded in the mesentery or floating freely in the gastrovascular cavity. A thin to thick follicular cell layer is present.	
IV	Late previtellogenic oocytes with 100% of their lightly basophilic ooplasm packed with clear vacuoles are present. There are no eosinophilic droplets. Stage IV oocytes are larger than stage III oocytes and have a thick follicular cell layer.		Sperm cysts are detached from the mesentery and float freely in the gastrovascular cavity. They contain a thick layer of darkly stained spermatocytes arranged around a distinct lumen and are surrounded by a thick follicular cell layer.	
V	Vitellogenic oocytes are present. They are characterized by a granular ooplasm with eosinophilic granules (vitellus) at the periphery and eventually filling the whole ooplasm. A thick follicular cell layer is present.		Mature sperm cysts are present. They are characterized by the presence, in addition to spermatocytes and spermatids, of spermatozoids with their tails projecting toward the center of the lumen and gradually filling the whole cyst. A thick follicular cell layer is present.	

histological section where the most advanced gametes were found. Hereafter, colony reproductive status was identified either by the most advanced gamete stage present (Stage I, II, III, IV, or V colonies) or by the combination of most advanced and predominant gamete stages present (e.g., Stage V-I, V-II, V-III, or V-IV colonies), with Stage V-V colonies being in an advanced prespawn condition.

2.4 | Stereomicroscope assessment of sex and maturity

A rapid stereomicroscopic method was developed to assess sex and maturity of colonies of *P. aculeata* and was validated by histological examination described above, so that large samples could easily be processed for determination of size at maturity. This method was

derived from an indicator of ovarian development in fish (Kennedy et al., 2011; Kjesbu, 1994), based on measurement of the diameter of the largest oocytes in each specimen, to identify the leading cohort of oocytes (LCO). One basal, central, and apical leaf of each colony in 2014, or one central leaf of each colony in 2015, was dissected to expose the reproductive tissue located in the gastrovascular cavity at the base of the autozooids, where the largest oocytes or sperm cysts were observed (Figure 1A). Photographs were taken using a digital Moticam 5.0 camera (Motic Instruments Inc., Viking Way, Richmond, BC, Canada) mounted on a Leica MZ 7.5 stereomicroscope (Leica Microsystems Inc., Concord, ON, Canada). The maximum horizontal and vertical Feret diameters of the five largest oocytes or sperm cysts were measured on the numeric photographs using the Image-Pro Premier 9.2 image analysis software (Media Cybernetics, Inc., Rockville, MD, USA).

Criteria to classify reproductive tissue as male or female based on stereomicroscopy were developed by examination of photographs of reproductive tissue of colonies of known sex (based on histology). This method was validated in 2015 by comparing the two methods of sex assessment by histology and stereomicroscopy. Sperm cysts had well-defined contours and were initially translucid (Figure 1B). The most advanced sperm cyst stages were more opaque but were smaller and more blueish or grayish compared with female gametes of similar stage. Female gametes had an irregular contour. They had an opaque center and were generally whitish when small and were fully opaque and cream colored at more advanced stages (Figure 1B).

2.5 | Data analysis

Normality (Shapiro–Wilk test) and homoscedasticity of the data set were tested. Nonparametric tests were used because the data set did not meet assumptions of normality and homogeneity of variance. The significance level was fixed at 0.05. Percentage of overall agreement between the two methods of sex identification, by histology or by stereomicroscopy, was determined for each sampling date in 2015. To assess whether method performance varied as a function of reproductive status, percentage of agreement between methods was compared among sampling dates using the chi-square test. To compare maturity among apical, central, and basal leaf zones within colonies sampled in 2014, oocyte or sperm cyst diameter (calculated as the mean of maximal horizontal and vertical Feret diameters) measured by stereomicroscope was compared between colony leaf zones using a paired Wilcoxon signed-rank test. Proportions of male and female sea pens with gametes at different most advanced histological stages of maturity were compared between colony leaf zones using the same test. For sea pens sampled in 2015, oocyte or sperm cyst diameters were compared among colonies at different stages of histological maturity using a Kruskal–Wallis test (KW) followed by the Dunn's multiple comparisons test. A chi-square test was used to test whether sex ratio deviated significantly from 1:1.

Visual examination of histograms of gamete diameter for Stage V colonies in May 2015 revealed a unimodal distribution for sperm cysts and a trimodal distribution for oocytes. Three oocyte diameter groups (I–III) were defined based on the histogram (see results below). The percentage of Stage V-V colonies was compared across the three oocyte diameter groups using Fisher's exact test. Female TL was compared among oocyte diameter groups using a KW.

To assess temporal variation in reproductive status, proportions of male and female colonies with Stage V gametes in the central leaf zone were compared among sampling dates using a chi-square test. TL of Stage V-V colonies was compared among sampling dates using a KW. The relation between colony TL and gamete diameter of the leading cohort was explored for each sex and sampling date using correlation analysis and graphical examination. We used chi-square tests to compare the frequency of colonies at different stages of histological maturity or in different oocyte diameter groups. Analyses of female sea pens were further refined by defining two TL classes of colonies (small or large), based on oocyte maturity stage and diameter group, and considering them separately within and across sampling dates.

Size at sexual maturity was assessed in colonies collected in May 2015, prior to the spawning period, when the largest oocytes and sperm cysts were observed (see results below). Histologically, a colony was considered sexually mature if it was at Stage V-V. After evaluation of temporal variation in female reproductive status, a second approach based on stereomicroscopy was used to assess maturity of female colonies: Those belonging to oocyte diameter Group III in May were considered mature, with the potential to spawn in the spring or early summer. For further comparison with the histological method, size at maturity was also calculated using a more inclusive maturity criterion, which included oocytes in Groups II and III. Percentages of mature colonies were calculated for 20-mm TL classes to achieve a minimum sample size of five colonies per TL class. Colony TL at maturity (TL_{50}) was defined as the TL at which 50% of colonies were sexually mature. The colony TL at which 25% (TL_{25}) or 90% (TL_{90}) of colonies were mature was also estimated. We estimated TL_{25}, TL_{50}, and TL_{90} from the observed proportions of mature colonies in each TL class using a PROBIT procedure (SAS Institute Inc., 2008).

3 | RESULTS

3.1 | Protocol validation, sex ratio, and gamete size

The vast majority of colonies of *P. aculeata* collected in 2014 (95.6%; 43 of 45) had gametes of same sex in the apical, central, and basal leaf zones. In two colonies, one that appeared to be all male (103-mm TL) and one all female (83-mm TL), sex could not be determined in the basal zone because no gamete was found on the histological section. In female colonies, a greater proportion of more mature gametes was observed in the apical and central than in the basal leaf zones ($\chi^2 = 23.9$, $df = 2$, $p < 0.0001$; Figure 2). The same pattern was observed in males but was not statistically significant ($\chi^2 = 3.9$, $df = 2$, $p = 0.15$; Figure 2).

Sex could be assigned by histology in 84.8% of the 375 colonies sampled in 2015. The overall agreement between histological and

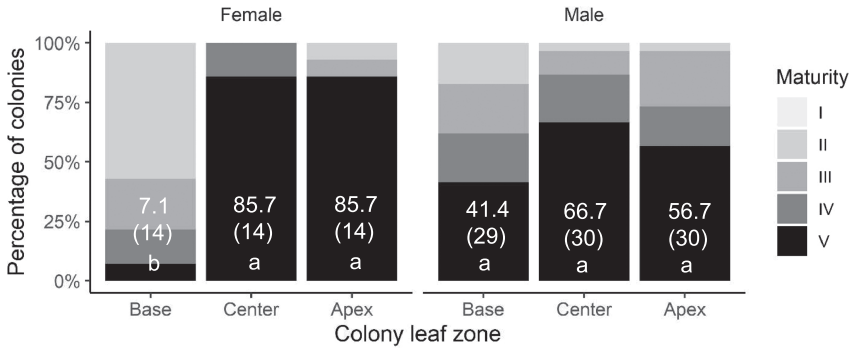


FIGURE 2 Variation in histological maturity among three leaf zones of female and male colonies of *Pennatula aculeata* collected in the Gulf of St. Lawrence in July 2014. For each leaf zone, the most advanced gamete maturity stage present was assessed, and the percentage of colonies at each of the five stages of maturity (I–V) is shown in different shades. Percentage of colonies at Stage V and the sample size (in parentheses) is indicated on each bar; different letters indicate significant differences in percentage of colonies in Stage V among leaf zones within sex

TABLE 3 Percentages (and counts) of male and female colonies identified by histological and stereomicroscopic methods for sex assignment in *Pennatula aculeata* collected in 2015 in the Gulf of St. Lawrence

Sampling month	Histology	Stereomicroscopy			Overall agreement
		Male	Female	Undetermined	
May ($n = 145$)	Male	44.1% (64)	0.0% (0)	4.1% (6)	91.7% (133)
	Female	0.0% (0)	42.8% (62)	2.8% (4)	
	Undetermined	0.7% (1)	0.7% (1)	4.8% (7)	
August ($n = 150$)	Male	41.3% (62)	0.0% (0)	5.3% (8)	86.0% (129)
	Female	0.0% (0)	42.7% (64)	4.7% (7)	
	Undetermined	2.7% (4)	1.3% (2)	2.0% (3)	
October ($n = 80$)	Male	33.8% (27)	3.8% (3)	3.8% (3)	86.3% (69)
	Female	0.0% (0)	48.8% (39)	3.8% (3)	
	Undetermined	0.0% (0)	2.5% (2)	3.8% (3)	

Note: Cells along each diagonal indicate consistent sex assignment by histology and stereomicroscopy, expressed as a percentage of monthly sample size; other cells indicate inconsistencies between the two methods. Overall agreement is the sum of values in cells on each diagonal by month. Number of colonies in parentheses.

stereomicroscope methods of sex identification was ≥86% by sampling date (Table 3), with no significant differences over time ($\chi^2 = 2.73$, $df = 2$, $p = 0.26$). The proportion of colonies whose sex was identified by histology but not by stereomicroscopy did not differ among sampling dates ($\chi^2 = 1.02$, $df = 2$, $p = 0.60$) and was always ≤10.0%. The proportion of colonies whose sex was identified by stereomicroscopy but not by histology was smaller (≤4.0%) and also did not differ among sampling dates ($\chi^2 = 1.96$, $df = 2$, $p = 0.38$). In October only, three colonies were identified as males by histology but as females by stereomicroscopy. Two of these colonies (100- and 145-mm TL) were immature histologically (Stages II-II and II-III, respectively), and the third (111 mm) was at Stage V-IV of maturity.

For all sampling dates in 2015, the observed sex ratio based on histology did not differ significantly from the expected 1:1 ratio (Table 4).

In 2015, the diameter of the largest oocytes or sperm cysts measured by stereomicroscopy increased with colony histological stage of maturity and was highest in colonies with Stage V gametes (Females, KW, $\chi^2 = 61.7$, $df = 3$, $p < 0.0001$; Males, KW, $\chi^2 = 35.0$, $df = 3$, $p < 0.0001$; Figure 3). In these colonies, diameter (median, first–third quartiles) was significantly greater in females (0.46 mm, 0.36–0.54 mm) than in males (0.30 mm, 0.27–0.33 mm; Wilcoxon, $\chi^2 = 84.1$, $df = 1$, $p < 0.0001$). Oocyte diameter at Stage V was much more variable than oocyte diameter at earlier stages and more variable than Stage V sperm cyst diameter (Figure 3).

3.2 | Seasonal variation in reproductive condition

The proportion of male colonies sampled in 2015 that were at Stage V of histological maturity declined from nearly 90% in May, to ~50% in August, and further declined to ~20% in October ($\chi^2 = 49.6$, $df = 3$, $p < 0.0001$; Figure 4A). Among these colonies, those at the most advanced maturity stage (V-V) predominated in May and dropped to nearly 0% in August and October (Figure 4B). During the same time period, there was a progressive increase in the proportion of Stage IV male colonies, from <5% in May to >60% in October (Figure 4B). No significant temporal change in TL could be detected in Stage V-V male colonies (KW, $\chi^2 = 2.61$, $df = 1$, $p = 0.11$), but sample size for this category was only 2 in August and 0 in October (Figure 5). In Stage V colonies, sperm cyst diameter was positively—but not always significantly—correlated with colony TL (May, $r = 0.23$, $n = 61$, $p = 0.07$; August, $r = 0.28$, $n = 35$, $p = 0.01$; October, $r = 0.28$, $n = 7$, $p = 0.054$; Figure 6). In May, a left-skewed unimodal distribution of sperm cyst diameters was observed in Stage V colonies, with >90% of diameters ranging 0.25–0.4 mm (Figure 7A). The vast majority of the largest sperm cysts (≥0.29-mm diameter) were found in Stage V-V colonies ≥95-mm TL sampled in May (Figure 6).

By contrast, >80% of female colonies were at Stage V in May and August 2015, and this proportion declined to just over 50% in October 2015 ($\chi^2 = 13.4$, $df = 3$, $p = 0.006$; Figure 4A). In May 2015, Stage V-V female colonies largely predominated (Figure 4B). In August 2015, the proportion of Stage V-V colonies declined while the proportion of Stage V-II colonies, likely postpawn, increased substantially (Figure 4B). Relative proportions of Stage V-V and Stage V-II colonies were similar in August and October 2015. Median TL of Stage V-V colonies decreased from 142 mm in May to 109 mm in October (KW, $\chi^2 = 10.3$, $df = 2$, $p = 0.006$; Figure 5). Diameter of oocytes in the leading cohort was positively correlated with colony TL (May, $r = 0.43$, $n = 53$, $p = 0.001$; August, $r = 0.64$, $n = 57$, $p < 0.0001$; October, $r = 0.64$, $n = 23$, $p = 0.0009$). Largest oocytes (diameter >0.55 mm) were observed in Stage V-V colonies ≥118-mm TL collected in May (Figure 6).

Stage V female colonies collected in May 2015 separated into three groups based on the diameter of oocytes in the leading cohort: Group I, 0.27 ± 0.04 mm (mean ± standard deviation); Group II, 0.48 ± 0.04 mm; and Group III, 0.66 ± 0.05 mm (Figure 7A). The percentage of Stage V-V sea pens was smaller in colonies with Group I oocytes than in colonies with Group II or III oocytes (Fisher's exact test, $p \leq 0.05$; Figure 7B). TL (medians, first–third quartiles) was smaller in colonies with Group I oocytes (115 mm, 107–130 mm) than in colonies with Group II or III oocytes (146 mm, 130–161 mm) (KW, $\chi^2 = 12.1$, $df = 2$, $p = 0.002$; Figure 7C). Based on this analysis, female sea pens were divided into small (TL < 130 mm) or large (TL ≥ 130 mm) colonies to further explore temporal variation in reproductive status.

Among small female sea pens, the proportion of Stage V colonies tended to decline over time in 2015 (Figure 8A) but this trend was not statistically significant ($\chi^2 = 5.4$, $df = 2$, $p = 0.06$). There was no significant temporal variation in the percentage of colonies at Stage V-II ($\chi^2 = 1.7$, $df = 2$, $p = 0.43$) or Stage V-V ($\chi^2 = 0.38$, $df = 2$, $p = 0.83$; Figure 8B). Small colonies were predominantly classified in oocyte diameter Group I in May and in Group II in August and October ($\chi^2 = 13.3$, $df = 4$, $p = 0.01$; Table 5). A minority of small colonies were classified in Group III (Table 5), but these were the largest of small colonies (118- to 130-mm TL; also see Figure 6) and their proportion declined from 22% in May to 0% in August (Table 5).

Among large female sea pens, the proportion of Stage V colonies was similar in May and August 2015 (100% and 89%, respectively; Fisher's exact test, $p > 0.23$; Figure 8A). The proportion of large

TABLE 4 Variation in sex ratio (identified by histological examination) and test of the hypothesis of 1:1 sex ratio for colonies of *Pennatula aculeata* collected in the Gulf of St. Lawrence in 2015

Sampling month	Females	Males	χ^2	*p*
May	62	64	0.016	0.90
August	64	62	0.016	0.90
October	39	27	1.1	0.29

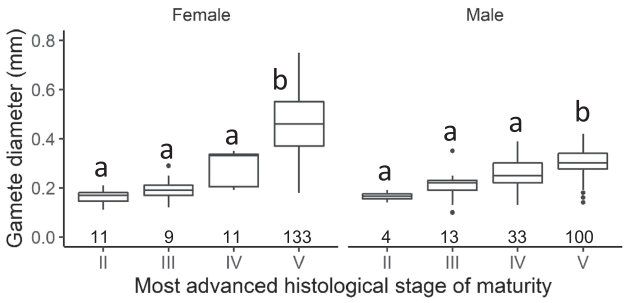


FIGURE 3 Boxplots of gamete diameter in female and male colonies of *Pennatula aculeata* at different most advanced stages of histological maturity in colonies collected in the Gulf of St. Lawrence from May to October 2015. Diameters of the largest oocytes or sperm cysts (in the leading cohort of gametes) in the central leaf zone are presented. The line in the middle of the box corresponds to the median; the top and bottom of the box are the 75th and 25th percentiles determining the interquartile range (IQR); whiskers extend outside of the box to a distance of 1.5 × IQR, and outlier values are black circles. Different letters indicate significant differences in gamete diameter among stages of maturity within sex. Sample sizes are indicated below each box

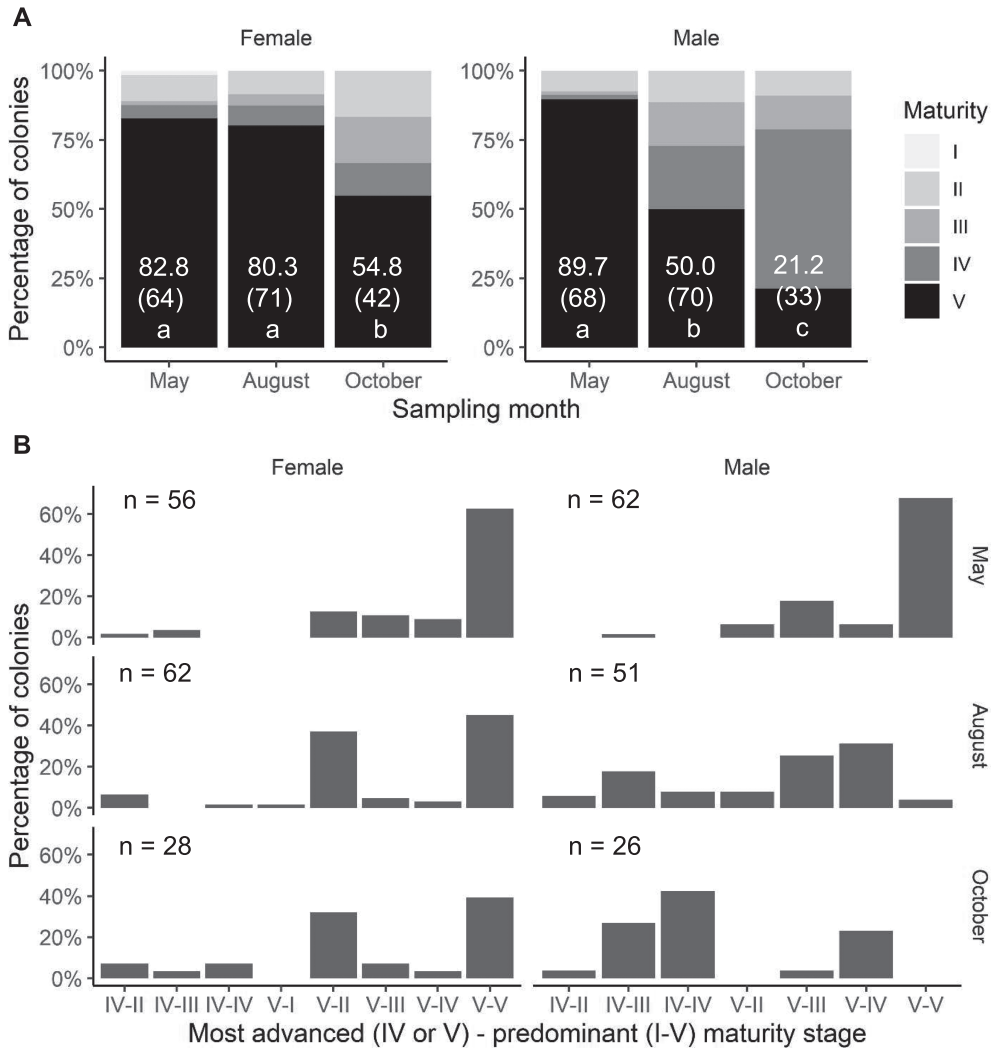


FIGURE 4 Temporal variation of histological maturity stage in the central leaf zone of female and male colonies of *Pennatula aculeata* collected in the Gulf of St. Lawrence in 2015. **A.** Percentage of colonies at different most advanced stages of maturity in each month, with the percentage of colonies in Stage V and sample size (in parentheses) indicated on each bar; different letters indicate significant differences in percentage of colonies in Stage V among sampling months within sex. **B.** Percentage of Stage IV and V colonies with different combinations of the most advanced stage present (first Roman numeral; Stage IV or V) and predominant histological stage of maturity (second Roman numeral, connected by a hyphen; Stage I, II, III, IV, or V), with sample size indicated on each figure panel

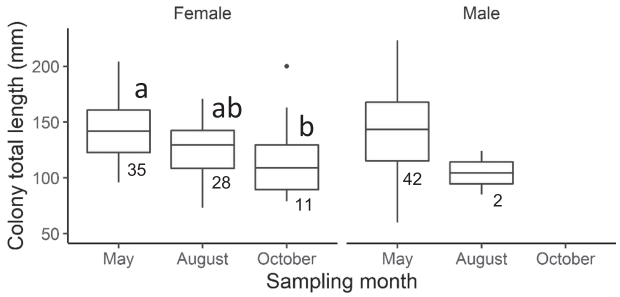


FIGURE 5 Boxplot of seasonal variation in total length of female and male colonies of *Pennatula aculeata* at the most advanced maturity stage (histological Stage V-V) in the Gulf of St. Lawrence in 2015. Numbers in graphs are sample size, and different letters indicate significant differences among sampling months in female colonies. See Figure 3 for description of boxplot features

colonies at Stage V-V declined from May to October 2015 ($\chi^2 = 6.5$, $df = 2$, $p = 0.038$), while the proportion of Stage V-II colonies increased markedly ($\chi^2 = 14.5$, $df = 2$, $p = 0.0007$; Figure 8B). Stage V-II colonies observed in August and October were very likely in a postspawning state, with residual Stage V oocytes left over from spawning. There was also temporal variation in the distribution of large colonies among the three oocyte diameter groups ($\chi^2 = 10.9$, $df = 4$, $p = 0.03$). Colonies with Group III oocytes predominated in May, and those with Group II oocytes predominated in August (Table 5). In May and August, combined percentages of colonies classified in Groups II and III were similar (83.3% and 84.0%, respectively; Table 5). This similarity reflects the replacement of high proportions of Stage V-V colonies with oocyte diameter ≥0.55 mm in May by high proportions of Stage V-II colonies with oocyte diameter <0.55 mm in August, as well as a stable proportion of Stage V-V colonies with oocyte diameter <0.55 mm (Figure 6). In October, there was a clear tendency for large female colonies classified in Group III to increase in proportion, but this change was not significant probably because of the small sample size (Table 5). In each month, the proportion of colonies classified in the different oocyte diameter groups differed significantly between small and large colonies (May, $\chi^2 = 11.4$, $df = 2$, $p = 0.003$; August, $\chi^2 = 10.0$, $df = 2$, $p = 0.007$; October, $\chi^2 = 7.1$, $df = 2$, $p = 0.03$), with higher percentages of Group I and lower percentages of Group III oocytes in small colonies (Table 5).

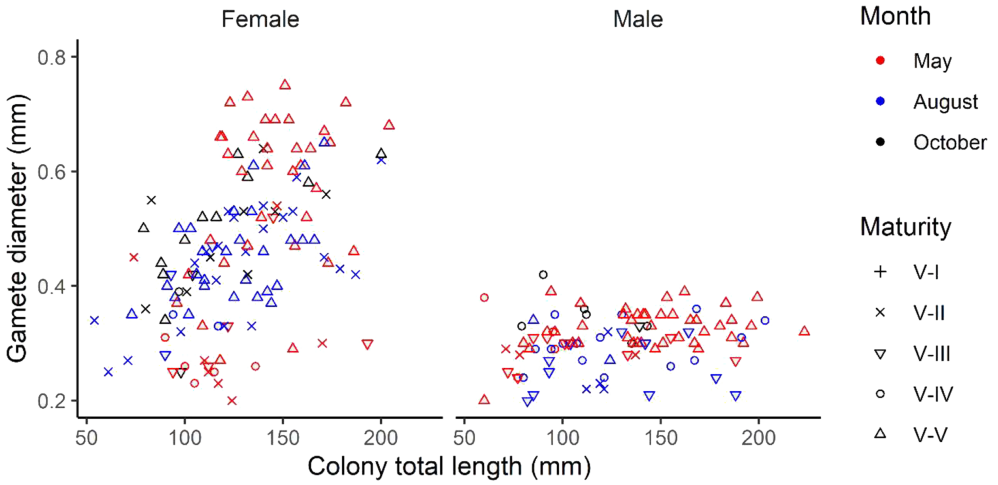


FIGURE 6 Seasonal variation in the relationship between the diameter of the largest oocytes or sperm cysts and total length and maturity of Stage V male and female colonies of *Pennatula aculeata* sampled in the Gulf of St. Lawrence in 2015 and whose sex was identified by stereomicroscope examination. Colony maturity is defined by the combination of the most advanced stage present (Stage V in all cases) and the predominant histological stage of maturity (Stage I, II, III, IV, or V)

3.3 | Size at maturity

Female and male colonies used for determination of size at maturity in May 2015 had similar ranges of TL (Figures 6 and 9A). Based on histology, the proportion of mature (Stage V-V) male colonies increased with size, with a TL_{50} of 101 mm (Figure 9A) and a TL_{90} of 151 mm (lower and upper fiducial limits, 132–198 mm). The smallest mature male colony had a TL of 60 mm. In female colonies, TL_{50} determined by histology and by stereomicroscopy (with the criterion of oocyte diameter ≥0.4 mm) were very similar, 119 mm and 114 mm, respectively (Figure 9A,B). In female colonies, as determined by histology, TL_{25} and TL_{90} were 102 mm (77–113 mm) and 152 mm (139–185 mm), respectively, compared with 89 mm (47–105 mm) and 166 mm (146–220 mm), respectively, by stereomicroscopy. The smallest mature female colony was 96-mm TL as determined by histology and 74-mm TL by stereomicroscopy. Using the more stringent stereomicroscopic maturity criterion of oocyte diameter ≥0.55 mm, the smallest mature female colony was 118-mm TL; TL_{25} of female colonies was 121 mm (88–136 mm), and TL_{50} was 148 mm (133–176 mm) (Figure 9B). Mature female colonies defined by this criterion represented 50% of colonies in the 140-mm TL class, 70% in the 160-mm TL class, and again 50% in the 180-mm TL class.

4 | DISCUSSION

4.1 | Protocol validation, sex ratio, and colony maturity

As in other octocoral species, colonies of *P. aculeata* have no externally visible sexual traits (Kahng et al., 2011). By combining histology and stereomicroscopy, we have developed and validated morphological criteria to distinguish male and female reproductive products of *P. aculeata* based on stereomicroscope examination alone. Opacity (opaque oocytes in females and translucent sperm cysts in males) proved to be a reliable criterion, as described for other octocoral species (Hamel et al., 2020; Kahng et al., 2011). The smoother contour of sperm cysts compared with oocytes was an additional criterion. Moreover, in *P. aculeata*, gamete diameters ≥0.42 mm were observed only in females, as noted previously by Eckelbarger et al. (1998). However, in our study, mature oocytes were not yellow or orange, as previous studies have reported in *P. aculeata* (Eckelbarger et al., 1998) and in other pennatulaceans including *P. phosphorea* (Edwards & Moore, 2008), *Funiculina quadrangularis* (Edwards & Moore, 2009) and *Halipteris finmarchica* (Baillon et al., 2015). Possible influences on oocyte color such as timing of sampling relative to spawning, preservation method, or food sources are unknown.

P. aculeata is gonochoric, as reported by Eckelbarger et al. (1998), and sex can be identified by examining reproductive products at a single location along the rachis. The sex ratio was 1:1, as reported for several species of octocorals including pennatulaceans (reviewed by: Baillon et al., 2015; Hamel et al., 2020; Kahng et al., 2011). A balanced sex ratio is considered optimal for populations of gonochoric species with random mating (Leigh et al., 1985; Mari et al., 2008).

In female colonies of *P. aculeata*, gamete maturity was less advanced in the basal than in the upper leaves, as reported for females of other pennatulaceans including *F. quadrangularis* (Edwards & Moore, 2009), *Anthoptilum murrayi* (Pires et al., 2009), *Anthoptilum grandiflorum* (Baillon, Hamel, Wareham, et al., 2014), and *H. finmarchica* (Baillon et al., 2015). One proposed explanation for this vertical differentiation is a development and growth gradient, with new leaves and polyps being generated only at the base of the colony and therefore maturing at a later colony age than the older polyps at the center or apex of the colony (Baillon, 2014; Baillon, Hamel, Wareham, et al., 2014; Soong, 2005). Other contributing factors could be an environmental gradient, with more food available for autozooid maturation (Soong, 2005) or fewer stressful interactions (competition, predation) at the top than at the base of the colony. In male colonies of *P. aculeata*, there was no significant difference in gamete maturity among the different leaf zones, as reported by Baillon, Hamel, Wareham, et al. (2014) for *A. grandiflorum*. These observations are consistent with sperm cyst development being faster (see below) and presumably energetically less costly than oocyte development.

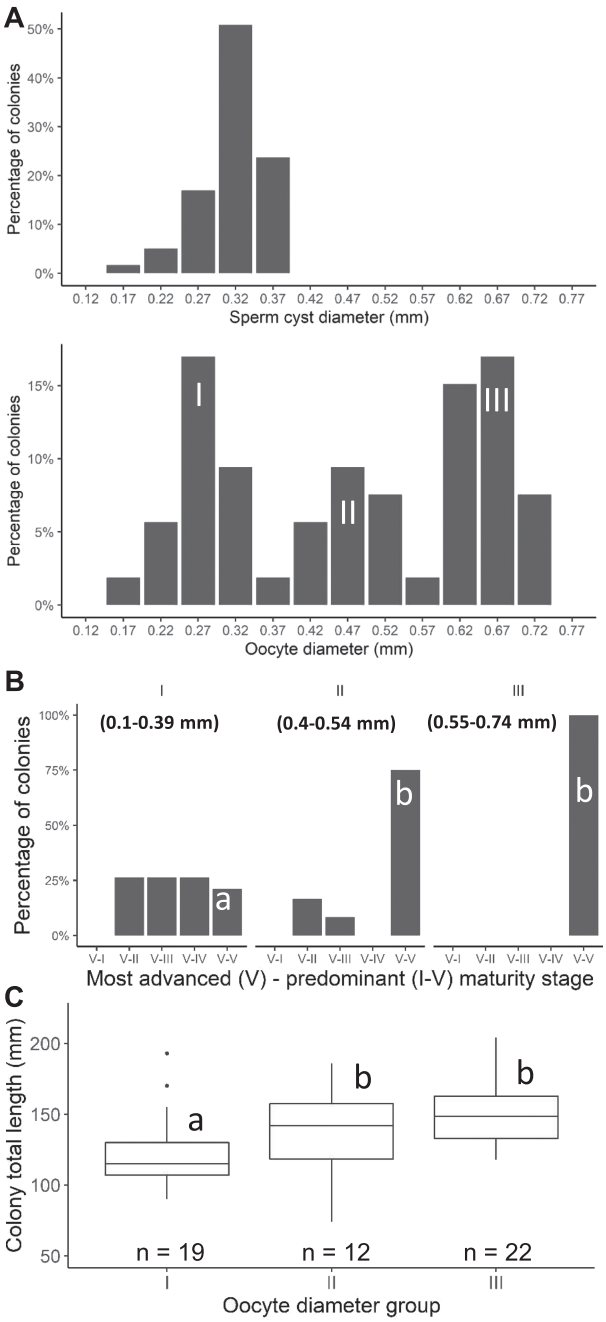


FIGURE 7 Characterization of different groups of Stage V male and female colonies of *Pennatula aculeata* collected in the Gulf of St. Lawrence in May 2015, based on the diameter of the largest oocytes or sperm cysts (i.e., leading cohort). **A.** Histograms showing a single mode for sperm cysts and three modes for oocytes (Groups I, II, and III). **B.** Percentage of female colonies at different stages of colony maturity, which is defined by the combination of the most advanced stage present (Stage V in all cases) and the predominant histological stage of maturity (Stage I, II, III, IV, or V) and grouped by oocyte diameter (range for each group in parentheses); different letters indicate significant differences among groups. **C.** Boxplot of total length of female colonies in the three oocyte diameter groups, with significant differences among groups indicated by different letters and sample size (*n*) shown for each group. See Figure 3 for description of boxplot features

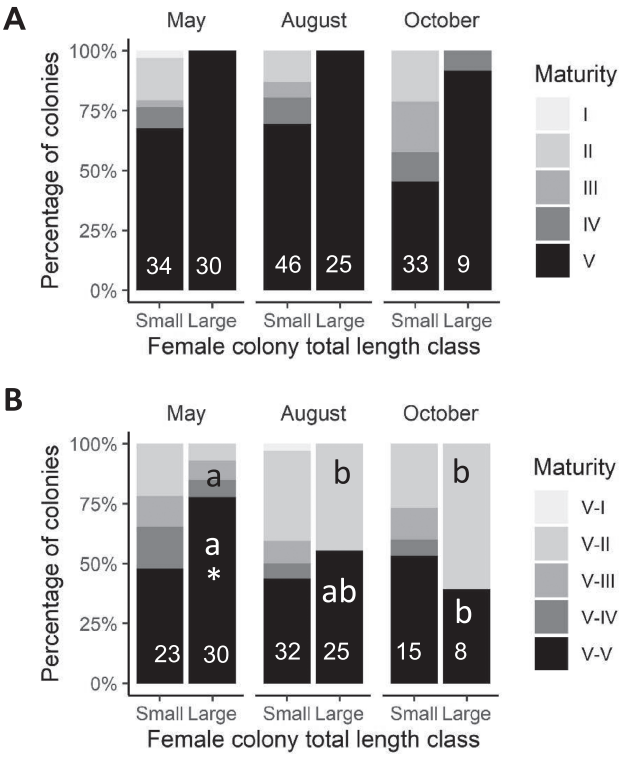


FIGURE 8 Temporal variation in histological maturity of female colonies of *Pennatula aculeata*, categorized as small (<130 mm total length, TL) or large (≥130-mm TL), collected in the Gulf of St. Lawrence in 2015. **A.** Percentage of colonies at different most advanced stages of histological maturity. **B.** Percentage of Stage V colonies with different predominant stages of maturity (indicated by the second Roman numeral after the hyphen). There were significant differences in the proportion of prespawn (V-V) and of postspawn (V-II) colonies among sampling months within colony TL class (indicated by different white and black letters, respectively) and in proportion of prespawn colonies between colony TL classes in May (indicated by an asterisk)

Further studies are needed to assess whether oocytes and sperm cysts are fewer per polyp in the basal than in the higher leaf zones of colonies of *P. aculeata*, as shown for other pennatulaceans (Baillon et al., 2015; Servetto et al., 2013; Soong, 2005).

4.2 | Reproductive cycle

To investigate the reproductive cycle of pennatulaceans, several previous studies examined the size distribution of oocytes or sperm cysts within individual colonies collected up to several times a year, a labor-intensive method usually performed on small samples (reviewed by Baillon et al., 2015). In the present study, we assessed histological maturity and the diameter of only the largest, most developed oocytes (the LCO) or sperm cysts to represent the leading cohort of gametes, which allowed us to process a greater number and size range of colonies at each sampling date. Using this approach revealed that Stage V

TABLE 5 Temporal variation in the proportion of female colonies of *Pennatula aculeata* in gamete maturity Stage V with the largest oocytes (i.e., leading cohort) of different diameter (Groups I–III), for small (<130-mm total length, TL) and large (≥130-mm TL) colonies sampled in the Gulf of St. Lawrence in 2015

Colony size	Month	% of colonies in each oocyte diameter group			Post hoc comparisons	*n*	Median TL (first-third quartiles)
		I (0.1–0.39 mm)	II (0.4–0.54 mm)	III (0.55–0.74 mm)			
Small							
	May	60.9	17.4	21.7	A	23	113 (104–120)
	August	43.8	56.3	0.0	B	32	106 (94–117)
	October	33.3	53.3	13.3	AB	15	98 (89–107)
Large							
	May	16.7	26.7	56.7	C	30	155 (142–169)
	August	16.0	64.0	20.0	D	25	147 (137–161)
	October	0.0	37.5	62.5	CD	8	143 (132–165)

Note: Different capital letters indicate significant differences between months within each of the two colony size categories.

female colonies fell into three discrete groups based on the diameter of oocytes. The difference in mean oocyte diameter between Groups I and II and between Groups II and III was about 0.2 mm, similar to the ~0.25- to 0.3-mm annual oocyte growth observed in colonies of *P. phosphorea* living in warmer and shallower water (see Edwards & Moore, 2008: Figure 1). The LCO represents only a small proportion of the overall pool of oocytes in a female colony, and it develops with increasing synchronicity prior to spawning (Baillon et al., 2015; Edwards & Moore, 2008). Diameters of Group III oocytes fell in the range of maximum oocyte diameters previously reported in *P. aculeata* and *P. phosphorea* (Eckelbarger et al., 1998; Edwards & Moore, 2008) and in other pennatulaceans (reviewed by Baillon et al., 2015), and as such this group is considered to be in prespawning condition. Large oocytes with abundant nutrient stores are common in octocoral species with nonfeeding lecithotrophic larvae, a reproductive strategy that likely improves larval survival (Chia & Crawford, 1973; Edwards & Moore, 2009).

At our study site in the Gulf of St. Lawrence, colonies of *P. aculeata* appear to spawn once annually in late spring or early summer. Indeed, from May to August 2015, there was a drastic reduction in the proportion of male colonies at the most advanced maturity stage (V-V) and a coincident marked decline in the proportion of female Stage V-V colonies with oocyte diameter ≥0.55 mm, indicating release of ripe gametes by both sexes sometime between the two months. Note that some oocyte growth may have occurred between May and the actual spawning event, resulting in spawned oocytes being larger than observed in May. Another spawning event in the fall is highly unlikely because no male Stage V-V colonies were observed in October. Our observations are thus consistent with previous reports of a single annual spawning period, usually in spring or summer, in other North Atlantic pennatulaceans including *P. phosphorea* (reviewed by Baillon et al., 2015). Spawning in these species is seemingly associated with phytoplankton bloom and downward flux of phytodetritus (Baillon et al., 2015; Hamel et al., 2020). Eckelbarger et al. (1998: p. 687) suggested that *P. aculeata* has "no distinct seasonal reproductive period" in the Gulf of Maine based on the co-occurrence of multiple oocyte maturity stages in individual polyps of colonies collected only in August but recognized that seasonal sampling was needed to confirm this interpretation. Although the present study corroborates the co-occurrence of different oocyte maturity stages (and size classes of Stage V oocytes) in individual polyps of *P. aculeata*, this reflects prolonged oogenesis rather than more or less continuous spawning through the year.

Our results indicate that oogenesis lasts at least 24 months in *P. aculeata*. Evidence for this interpretation is the division in May 2015 of Stage V female colonies among three discrete oocyte diameter groups (I–III), of which only females in Group III spawned between May and August 2015, whereas colonies of Groups I and II persisted and continued to grow their oocytes through August and October 2015. Additionally, in May there was a positive relationship between female colony TL and oocyte diameter, and colonies of Group I were smaller than colonies of Groups II and III. Thus, reproductive status of females was followed over time separately for small (TL < 130 mm) and large (TL ≥ 130 mm) Stage V colonies. Among small females, in May only some of the largest colonies (118- to 129-mm TL) belonged to Group III, whereas most others belonged to Group I. It is proposed that these Group I females would have progressed to Group II in May 2016 (some progression from Group I to Group II was indeed observed from May to October 2015) and to Group III in May 2017 and would have spawned in spring or summer 2017. Many of these initially small colonies would also have grown to become large

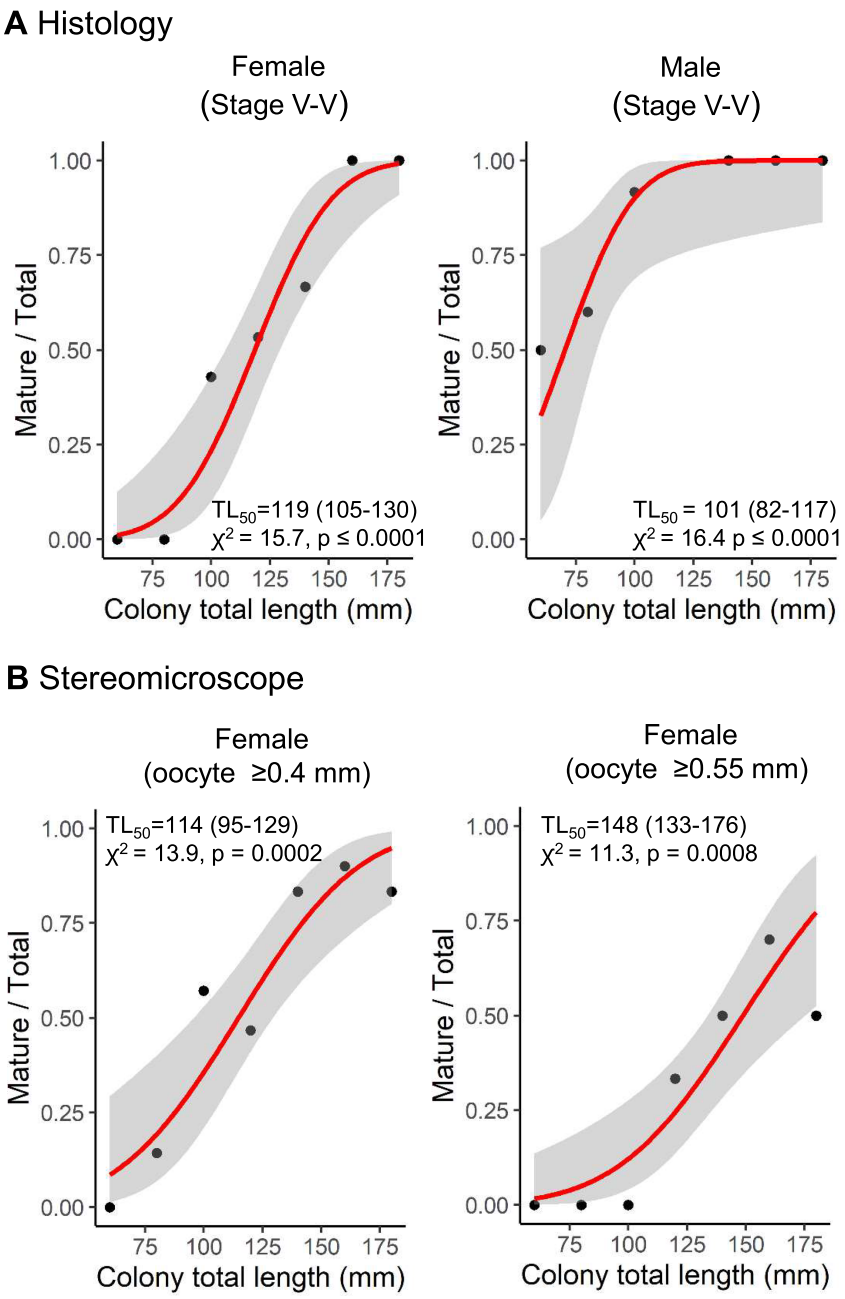


FIGURE 9 Relationship between colony total length (TL) and proportion of mature female and male colonies of *Pennatula aculeata* sampled in the Gulf of St. Lawrence in May 2015, based on two methods of maturity assessment. **A.** Proportion of colonies in gamete maturity Stage V-V (oocytes in Stage V were present and predominant), assessed by histology. **B.** Proportion of female colonies with mean diameter of leading oocyte cohort ≥0.40 or ≥0.55 mm, with maturity assessed by stereomicroscopy. Size at maturity (TL_{50}) is indicated with lower and upper 95% fiducial limits (shaded region), and χ^2 and *p* values from PROBIT analysis are provided

colonies over a period of 2 years, based on the general growth curve (not sex- or site-specific) for *P. aculeata* in the Gulf of St. Lawrence (Murillo et al., 2018: Figure 7). As for female colonies that were already large in May 2015, those belonging to Group II would have progressed to Group III during fall and winter and would have spawned in 2016. As our interpretation of oogenesis progression pertains to Stage V female colonies only (i.e., those with vitellogenic oocytes), total oogenesis including previtellogenesis is certainly longer than 24 months. Thus, *P. aculeata* is similar to other pennatulacean species in having protracted oogenesis (reviewed by Baillon et al., 2015), a mechanism which may allow mature oocyte production to be adjusted later in development rather than at the time of primary oocyte appearance (Brazeau & Lasker, 1990).

Intriguingly, only about half of the large Stage V female colonies belonged to Group III in May and appeared to spawn in 2015. This strongly suggests that, uniquely among pennatulaceans examined so far, female spawning may be biennial. In August and October, large colonies in postspawning condition (Stage V-II) had residual Stage V oocytes as their most advanced histological maturity stage, indicating they had spawned, but it is the predominant Stage II oocytes (of about 0.15- to 0.2-mm diameter) that formed the true leading cohort that would progress to Stage V over the next 2 years. Thus, most large female Stage V-V colonies classified as Group III in May 2015 would have spawned the same year, be classified as Group II in May 2016, and have spawned again in 2017. However, more frequent sampling throughout the year, repeated over several years with

sequential observations of successive cohorts of known age, combined with complete colony oocyte inventory, is needed to refine our understanding of the progression of oogenesis and pace of spawning in large female colonies.

In contrast to female colonies, only one group of Stage V male colonies of *P. aculeata* was identified based on the diameter of the leading cohort of mature sperm cysts, indicating that spermatogenesis was faster than oogenesis, as is typical in octocoral species (Kahng et al., 2011). In October, advanced spermatogenesis (Stage IV) was apparent in most male colonies of *P. aculeata* and it is likely that these colonies would have reached Stage V and spawned the following spring or summer. This is similar to *H. finmarchica*, in which the duration of spermatogenesis was estimated to be 8–10 months (Baillon et al., 2015).

4.3 | Size at maturity

The proportion of mature female (oocyte diameter ≥0.55 mm) colonies of *P. aculeata* increased with TL, as observed in other octocoral species, indicating greater allocation of energy to somatic growth than to reproduction in early life, possibly to minimize size-dependent mortality (Watling et al., 2011). The smallest mature female and male colonies measured 118- and 60-mm TL, respectively, and would be ~10–13 years and ~6–7 years of age, respectively, based on the growth curve for *P. aculeata* in the Gulf of St. Lawrence (Murillo et al., 2018). The estimates of TL_{50} for *P. aculeata* (148 mm, ~11–14 years old in females; 101 mm, ~8–11 years old in males) correspond to 49% and 33%, respectively, of the maximum TL observed for this species in the Gulf of St. Lawrence (305 mm, Murillo et al., 2018). These relative TL_{50} values are higher than those reported for a bathyal pennatulacean in the Canadian Arctic, *Umbellula encrinus* (23%–24% for females and 7%–14% for males), in which male colonies also matured at a smaller size and younger age than female colonies (Hamel et al., 2020). Estimated size at first maturity in pennatulaceans ranges from 12% (females and males) of maximum colony length in the deep-sea *H. finmarchica* in eastern Canada to 35–65% (females) in the shallow-water *P. phosphorea* off Scotland (Edwards & Moore, 2008; reviewed by Baillon et al., 2015).

4.4 | Reproductive resilience to fishing

This study has revealed aspects of the reproductive biology of *P. aculeata* that may reduce resilience to frequent stresses from bottom trawling and other mobile or fixed-bottom fishing gear. Late maturity (>5 years old) decreases the probability that colonies of *P. aculeata* can reproduce before being caught or damaged (de Juan et al., 2020). Further, maturity is later in females than in males and is compounded by protracted oogenesis, with reproductive output possibly constrained by biennial spawning. Selective removal or destruction of colonies by fishing could affect the ability of populations to reproduce by reducing density of mature colonies, decreasing colony fecundity by injury, or altering the operational sex ratio if females are more likely than males to be removed, reproductively harmed, or require more time to recover from injury (e.g., Linares et al., 2008; Tsounis et al., 2012). Sufficient densities of mature colonies of both sexes and synchronized spawning are two factors contributing to high gamete concentrations and successful fertilization in broadcast spawners (Baillon, Hamel, Wareham, et al., 2014; Langton et al., 1990; Lasker et al., 1996; Oliver & Babcock, 1992; Servetto et al., 2013). Finally, a single annual spawning event limits the ability of the population to reproduce successfully if necessary conditions are disrupted during this critical time period and, therefore, it increases vulnerability to stress (Hare et al., 2016).

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