Reproductive strategy and spawning activity of sand flathead, *Platycephalus bassensis* (Platycephalidae)

by

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ABSTRACT. - The aim of this study was to describe and determine the reproductive and spawning strategy of the sand flathead, *Platycephalus bassensis* Cuvier, 1829 based on histological analysis of ovary and testis. The presence of multiple group synchronous gamete development in both sexes within a reproductive season confirmed the hypothesis that sand flathead is a serial spawner and is reproductively active from October to March. Spawning frequency estimated from a combination of information acquired from field sampling and results from a laboratory experiment, indicated that sand flathead spawned on average once every 4-5.3 days during the spawning season. A clear pattern of diel periodicity in spawning was apparent in sand flathead with the peak of spawning activity during the day. Results of this study showed that estimating spawning periodicity from the presence, and age of postovulatory follicles is more reliable than using the presence of hydrated oocytes. This study highlights the details of reproductive biology of a serial spawner and its potential for variability, which can have implications for recruitment and adult population dynamics.

RÉSUMÉ. - Stratégies de reproduction et de ponte de *Platycephalus bassensis*.

Le but de la présente étude était de décrire et de définir les stratégies de reproduction et de ponte de *Platycephalus bassensis* Cuvier, 1829, en se basant sur une analyse histologique ovarienne et testiculaire. Chez les animaux des deux sexes, la présence, au cours d'un cycle saisonnier, de gamètes à des stades multiples de développement confirme l'hypothèse que ce poisson présente des pontes multiples d'octobre à mars. Pendant cette période, des informations obtenues à partir de données obtenues sur le terrain, d'une part, et expérimentalement, d'autre part, indiquent que *P. bassensis* pond en moyenne tous les 4-5,3 jours. Un cycle journalier a été mis en évidence avec un pic de ponte pendant la journée. L'estimation de cette périodicité est plus fiable en prenant comme critères la présence et l'âge des follicules post-ovulatoires au lieu de la présence d'ovocytes hydratés. Cette étude met donc en évidence la biologie reproductive d'un poisson à pontes multiples et sa variabilité potentielle, ce qui peut avoir des implications dans la dynamique de recrutement de la population adulte.

Key words. - Platycephalidae - *Platycephalus bassensis* - Australia - Tasmania - Gonad development - Atresia - Postovulatory follicle - Spawning periodicity.

Fish are generally defined as either having synchronous ovarian development where oocytes develop synchronously and are released in a single spawning event, or as multiple spawners, where several batches of oocytes are produced and released at different times during the spawning season (Wallace *et al*., 1987; Pankhurst, 1998). This latter group is sometimes described as showing multiple group synchrony where clear oocyte clutches are identifiable, or as being asynchronous where there is frequent ovulation of smaller clutches, and a wide range of oocyte stages is present in the ovary (Tyler and Sumpter, 1996). A multiple spawning strategy is typically seen in species that are taking advantage of favourable environmental conditions for larval survival and growth over a prolonged period (Hunter and Macewicz, 2003). However, variability in batch size and the frequency, time, and duration of egg release add a level of complexity when determining the effect of reproductive activity on population dynamics (DeVlaming, 1983). Given that many multiple spawners have extended spawning seasons, this can result in recruitment of juveniles occurring over an extended period. Consequently individuals from the same year-class, but which recruited at different times within this year-class, may show substantial variation in size. This is to some extent is due to the differential growth pattern of different size classes but also variability in growth rates due to environmental conditions (Luo and Musick, 1991).

Such variation in size within a year class, especially early in the reproductive lifetime, has the potential to affect reproductive life-history characteristics (e.g. age at maturity) among individuals within the year class, with some individuals delaying maturity and therefore, having a different reproductive investment strategy (Luo and Musick, 1991). Multiple spawning may also cause differential adult survivorship due to variability in age at which fish reach a minimum size

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threshold for survival (Law and Grey, 1989). All these factors affect the size and age of the breeding population and potentially result in population-specific life history characteristics.

Sand flathead (*Platycephalus bassensis)* is a temperate benthic species that lives in coastal sandflat habitats down to 100 m. Populations of sand flathead in Tasmania (south-eastern Australia) are reproductively active for six months, October-March, suggesting that individuals have a multiple spawning strategy (Jordan, 2001). However, it is also possible that a protracted spawning season can result from different individuals in the population producing and releasing a batch of eggs at different times (Bye, 1984). Therefore, spawning strategies of individuals can only be determined by looking at gonadal development patterns and examining gonads histologically (Hunter and Macewicz, 1985a).

Multiple spawners exhibit diversity in temporal patterns of egg release including daily, semi-lunar, or lunar (Robertson, 1991; Scott *et al*., 1993), while some species show no such spawning patterns (Robertson *et al*., 1990). Timing of spawning is thought to maximize successful fertilisation and minimise the risk of predation on newly released eggs. Species that release eggs daily may do so throughout daylight hours or may restrict spawning activity to dawn or dusk (Ferraro, 1980; Pankhurst and Fitzgibbon, 2006). Determining the timing of spawning can be achieved by monitoring ovarian dynamics (Scott *et al*., 1993), or by direct observation of spawning activity (Pankhurst and Fitzgibbon, 2006).

The best indirect estimate of spawning frequency is determined from the percentage of mature females with ovaries containing recent post-ovulatory follicles (POFs) (Hunter and Macewicz, 1985a), or hydrated oocytes (Brown-Peterson *et al*., 2001), indicative of recent, and imminent ovulation, respectively. POFs are normally identifiable 1-3 days after spawning in temperate environments (Hunter and Macewicz, 2003), during which time POFs do not fragment, but retain their integrity (DeMartini and Fountain, 1981; Hunter and Macewicz, 1985a). The relationship between the age of POFs and their histological appearance can be determined by either collecting wild fish in a time-series relative to a known spawning time, or to induce ovulation in fish in the laboratory and examine the POFs at known times after spawning (Hunter and Macewicz, 2003).

Another complexity associated with multiple spawners is that not all vitellogenic oocytes may be spawned during the spawning season. Some oocytes will undergo atresia and subsequent resorption (Hunter and Goldberg, 1980). Suboptimal environmental conditions such as low water temperature, variation in nutritional state, inappropriate photoperiod, and stress may all cause oocyte atresia (Hunter and Macewicz, 1985b; Clearwater and Pankhurst, 1997; Simonsen and Gundersen, 2005). In many fish species, the prevalence of atretic oocytes varies during the spawning season, but is highest towards the end of the spawning season (Kurita *et al*., 2003). The presence of atretic oocytes is often an indicator of the imminent cessation of spawning and can be used to distinguish those females in post-spawning condition from immature females (Marshall *et al*., 1993; DeMartini *et al*., 2000). The capacity to identify atresia is also required to age POFs accurately, because some stages of atresia can be very similar in appearance to late stage POFs.

Theoretically, differences in the pattern of energy investment among the individuals of a population may be seen in variation in growth rates and consequently in reproductive investment. Small individuals would not be able to invest significantly in egg production, as fecundity is proportional to the size of fish (Bagenal, 1966; Kjesbu *et al*., 1998). However, the part of variance in individual fecundity explained by female size is species-dependent (Kamler, 2005). In addition, the egg size may differ in relation to the size of adult individuals (Trippel, 1998). There is likelihood of a shorter spawning season for small individuals. Shorter spawning periods for serial spawners lead to fewer batches of eggs being released during the spawning season (Trippel *et al*., 1997; Brown-Peterson *et al*., 2001). Therefore, the estimation of spawning periodicity among different size classes of fish improves the precision of estimates of energy investment in reproduction by a species. In the present study, we used histological examination of ovaries of fish obtained from the wild, and hormone-induced ovulation in captive fish to estimate spawning periodicity of the sand

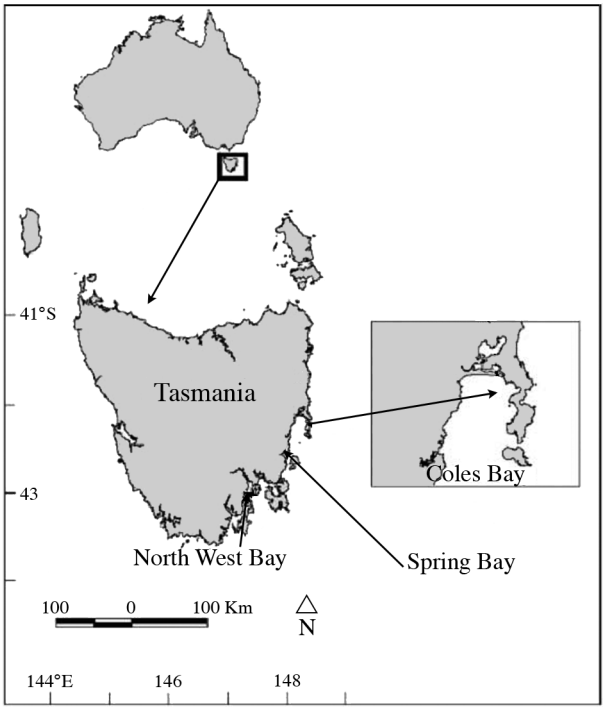

Figure 1. - Map showing sampling locations for sand flathead in southeastern Tasmania.

flathead to allow more accurate assessment of seasonal reproductive output. Additionally, patterns of gonadal development, diel timing of spawning, and occurrence of atresia were assessed to document more thoroughly the reproductive dynamics of sand flathead in coastal waters of eastern Tasmania.

MATERIALS AND METHODS

Field sampling

Sampling to determine the patterns of maturation and gonad development was undertaken seasonally from the inshore region of Coles Bay, Tasmania (Fig. 1) between March 2001 and February 2002, using hook and line (Tab. I). Sand flathead were also sampled over 24 hours, weekly, and monthly in Coles Bay between October 2002 and May 2003 for estimating spawning frequency and diel timing of spawning. Fish were sampled in the morning (05:30-10:00), midday (10:00-15:00), and afternoon (15:00-21:00) on 2nd, 10th, 17th, and 24th October 2002. Due to difficulties in collecting animals from Coles Bay, sampling was extended to Spring Bay (55 km south of Coles Bay) and North-West Bay (130 km south of Coles Bay) only, on the 10th and 24th October, respectively. Fish could not be caught between dusk and dawn using either gill-net or hook and line, therefore no night-time samples were obtained. Hook and line is generally less selective than other fishing methods and is thought to give the best representative sample of the wild population (King 2007). Samples collected in this study give a representative sample of the size distribution of the wild sand flathead population (Bani, 2005). All specimens were collected and sacrificed according to the ethical guidelines of University of Tasmania Animal Ethics Committee (Permit #A0007564).

Laboratory processing and histological analysis

To prevent rapid degeneration of POFs, all fish were dissected either on board or within three hours of capture. Fish were classified as male, female or immature, measured to the nearest millimetre total length, and weighed to the nearest gram. Visceral (viscera plus gonads) weight for each fish was recorded and somatic weight was calculated as total body weight minus visceral weight. To assess liver condition, liver was removed and weighed to the nearest 0.1 mg. Gonads were removed, weighed, macroscopically staged (Tabs II, III), and preserved in formalin-acetic acid- calcium chloride (FAACC).

Histological analysis was used to verify macroscopic staging, determine age of POFs (see below), and occurrence of atresia in ovaries. Preserved gonads were dehydrated in an ethanol series, embedded in paraffin, and sectioned at 6 μm, before staining with Mayer's Haematoxylin and Eosin. Usually there are no significant differences in maturation and oocyte frequency distribution between right and left ovaries (Laroche and Richardson, 1980; DeMartini and Fountain, 1981; West, 1990), therefore, in this study, the left ovary was used for all histological analysis. To minimize possible variation in the developmental stage of oocytes due to their position in the ovary (e.g. Forberg, 1982; Gooley *et al*., 1995) a longitudinal section and 6-8 transverse sections from left ovary were used for histology (as per. Hunter and Macewicz, 1985a).

The size frequency distribution of oocytes within the intact ovaries of 10 females at each stage of maturity was determined macroscopically. Sections 1-2 mm thick were cut from the middle of left ovary, the oocytes teased apart with hypodermic needles, and further separated by immersion in an ultrasound bath for 3-5 mins (as per. Lowerre-Barbieri and Barbieri, 1993). The maximum diameter of approximately 300 randomly-selected oocytes was measured under

Table I. - Sampling schedule and exact date and time of *Platycephalus bassensis* collection in Coles Bay (CB), North-West Bay (NWB), and Spring Bay (SB). *: Mid-point of 4-5 hours sampling time.

Year	Location	Sampling schedule	Date and time*	Objective of sampling
2001/02	CB	Seasonally	Mar-Jun-Oct-Feb	Pattern of gonad development
2002/03	CB	24 h (and for weekly)	2nd and 3rd Oct- 12:00, 17:30, and 6:00	Determination of spawning periodicity and occurrence of atresia
	SB	24 h (and for weekly)	10th and 11th Oct- 13:00, 19:00, and 5:30	
	CB	24 h (and for weekly)	17th Oct- 12:00	
	NWB	24 h (and for weekly)	24th and 25th Oct- 11:30, 18:30, and 6:30	
	CB	Monthly	18th Nov- 9:00	
	CB	Monthly	17th Dec- 7:30	
	CB	Monthly	17th Jan- 12:00	
	CB	Monthly	4th Feb- 16:00	
	CB	Monthly	6th Mar- 13:00	
	CB	Monthly	6th May- 13:30	

Table II. - Microscopic, macroscopic, and histological staging criteria used for sand flathead ovaries. *: Adapted from West (1990). #: Adapted from Jordan (2001). ¶: Adapted from Davis and West (1993).

Maturity stage	Category	Microscopic histology*	Macroscopic ovary#	Macroscopic whole oocyte¶
1	Immature	*Chromatin nucleolar*: Very small oocytes. Clear, spherical nucleus surrounded by a thin layer of purple-stained cytoplasm. No nucleolus visible.	Small trap with rounded edge, pale pinkish colour.	*Unyolked:* Spherical transparent bodies.
2	Previtellogenic	*Perinucleolar:* Oocyte size increases because of thick cytoplasm around a light nucleus, containing few to many peripheral nucleoli.	Ovaries reddish-pink and translucent. Virgin: Ovary wall thin and transparent. Recovering: Ovaries flaccid and ovary wall thick and opaque.	*Unyolked:* Spherical transparent bodies. Nucleus clear and brownish in colour.
3	Early vitellogenesis	*Cortical alveoli:* Appearance of yolk vesicles in cytoplasm, thick &pink-stained zona radiata distinguishable.	Almost length of body cavity. Ova not visible.	*Partially yolked:* Some yolk granules appear, becoming darker with increased size.
4	Late vitellogenesis	*Vitellogenic yolk:* Marked increase in oocyte size. Cytoplasm filled with yolk granules, oil vesicles and yolk vesicles. Peripheral nucleolus around the nuclear membrane.	Full length of body cavity. Ova visible.	*Yolked:* Oocytes completely opaque except for the translucent perivitelline border.
5	Final oocyte maturation	*Nuclear migration:* Migration of nucleus to periphery of cytoplasm, fusion of oil vesicles into the oil droplet, coalescence of yolk granules to form uniform plate.	Ovaries occupy all available space of body. Transparent oocytes visible in partly or not emptied ovaries with developing oocytes for the next spawning.	*Nuclear migration:* Parts of oocytes become translucent as yolk coalesced.
6	Ovulated	*Hydration:* Yolk granules fused into a few plates. Thecal cells appear like a string.	Large hydrated oocytes easily expressed with slight pressure. Ovaries pinkish and granular.	*Hydration:* Whole oocytes is translucent, expect for the oil droplet.
7	Spent	Spent: Postovulatory follicles present.	Ovaries flaccid and bloodshot with thick wall.	

a stereo-microscope using transmitted light and bright-field illumination. Stage of whole-oocyte development was assessed from macroscopic appearance (Tab. II) of each oocyte (Davis and West, 1993).

Macroscopic staging of the gonad was validated histologically, in which ovaries were staged based on the presence of the most advanced type of oocyte (Wallace *et al*., 1987; West, 1990). Histological classification of oocytes (Tab. II) was assigned, based on terminology defined by Yamamoto (1956) and staging criteria from West (1990). Females were considered sexually mature if they were classified as stage 3 or higher. Females larger than size at maturity (≥ 24.7 cm) in Coles Bay (Bani and Moltschaniwskyj, 2008) were used for the estimation of proportion of mature fish present during the spawning season. Females that were preovulatory were identified by the presence of oocytes displaying germinal vesicle migration or hydrated oocytes, and recently ovulated fish by the presence of POFs, (Hunter and Macewicz, 1985a). Females that had ceased spawning activity were identified by an absence of hydrated oocytes or POFs and the presence of high proportions of atretic vitellogenic follicles (Hunter and Macewicz, 1985b; Marshall *et al*., 1993). The atretic stage of ovaries was based on the presence of different types of atretic oocytes (Tab. IV). In sand flathead the structure of delta (δ) stage atretic oocytes was almost indistinguishable from highly degenerated POFs, therefore the presence of alpha (α), beta (β), and gamma (γ) stages of atretic oocytes in ovaries was used. The total percentage of the three stages of atretic oocytes in each ovary was determined by counting 100 randomly-selected oocytes from ovarian histology.

To determine the presence of spermatozoa in testis lobules and the gonadal stage of males, a longitudinal section (5 µm) of each testis near the posterior tip and a transverse sec-

Table III. - Macroscopic and histological staging criteria used for sand flathead testis. *: Adapted from Jordan (2001). #: Adapted from Pankhurst *et al*. (1987) and Takashima and Hibiya (1995).

Maturity stage	Category	Microscopic histology[#]	Macroscopic testis[*]
1	Immature	Immature: Abundance of spermatogonia, some primary spermatocytes.	Small, white and threadlike testis, occupy ¼ length of body cavity.
2	Early spermatogenic	Primary spermatocytes predominate, presence of secondary spermatocytes and spermatids.	Flattened white tube, occupy more than ¼ length of body cavity.
3	Spermatogenic	Increasing number of secondary spermatocytes, presence of spermatids and spermatozoa.	Becoming large. No sperm expelled when testis cut.
4	Partially spermiated	Predominance of spermatids and spermatozoa.	Almost length of body cavity and large. Some sperm expelled when testis cut.
5	Fully spermiated	Spermatozoa predominate, mature sperm present in spermatic ducts.	Full length of body cavity and swollen. Sperm runs freely with slight pressure on belly.
6	Spent	Spent: Residual spermatozoa. Spermatogonia present towards testis margin.	Testis broad, flaccid and bloodshot. No milt expressible.

Table IV. - Histological staging criteria of atresia in sand flathead ovary. Atresia stages, adapted from Hunter and Macewicz (1985*b*); (*) *Alpha stage*: Granular, dark, and basophilic staining of cytoplasm. Disintegrated nucleus. Slightly dissolved zona radiata accompanied by loss of striation and uneven diameter. (#) *Beta stage*: Much smaller than the original oocyte. Numerous disorganized granulosa cells. One or more large intracellular vacuoles. (†) Gamma stage: Extra or intercellular flocculent materials encapsulated by a layer of granulosa and thecal cells. In sand flathead elongated with one or two vacuoles.

Atretic states	Histological characteristics
0	Absence of α^* stage of atresia. Possible present of insignificant $\beta^\#$ stage of atretic yolked oocytes.
1	Less than 50% of yolked oocytes are affected by atresia.
2	More than 50% of yolked oocytes are atretic. $\gamma^\dagger$ atretic oocytes may be present.
3	Almost all (yolked) oocytes affected by atresia. β stage is dominant.

tion of the mid-section of the testis were examined. The relative proportion of gamete stages in each testis was calculated by scoring the stage present under intersections of an ocular grid with 100 intersections. In the transverse section, three grids were placed from the outer edge to the centre of the testis, while another two grids were haphazardly placed on the longitudinal section. The histological stage of testis maturation was determined using the criteria described in table III, outlined in Pankhurst *et al*. (1987) and Takashima and Hibiya (1995).

Age determination of Post Ovulatory Follicles

In order to estimate the age of POFs for the purpose of determining spawning frequency, the sequence of histological changes that occur during the deterioration and resorptive process of the follicles was described from the ovaries of females for which the time of ovulation and egg release was known. To obtain ovaries containing known age POFs, 10 sexually mature females were captured by hook and line from Coles Bay in late October. Females were immediately anaesthetized with benzocaine (50 mg L^{-1}); three females were injected intra-peritoneally with luteinizing hormone releasing hormone analogue (LHRHa (Sigma); 50 μg kg^{-1} body weight) and seven females with human chorionic gonadotropin (hCG (Sigma); 500 U kg^{-1} body weight), fin clipped, and placed into a 300-L fibreglass tank with oxygenation for transportation to the University of Tasmania's aquaculture facility in Launceston, Tasmania, within three hours, where they were placed in 1000-L, temperature-controlled tanks supplied with recirculating seawater. Water temperature was maintained at equivalent ambient temperature (15°C) for both hormonal treatments since temperature is the major determinant of time of ovulation, irrespective of the nature of hormone treatment (Pankhurst and Poortenaar, 2000).

Twelve hours after hormone injection, females were anaesthetized (as before) and examined for ovulation. All fish were ovulated and stripped of eggs using light abdominal pressure. Stripped fish were then killed < 6, 12, 24, 36, 48, and 72 h post-stripping. Two females were killed at each time point, except at 12 and 36 h, when only one fish was killed. Ovaries from sacrificed fish were removed for histological examination (as above) and description of postovulatory follicle structure. The subsequent description of POF deterioration over 72 h allowed an estimation of the age of POFs from freshly captured wild fish based on the size and appearance of the follicles (from large and folded to small and V-shaped), alignment of granulosa cells (from continuous arrangement to collapsed features), and lumen shape (from open to not discernible) (Hunter and Goldberg, 1980).

Spawning frequency (percentage of mature females spawning per day) was estimated as the percentage of mature females with POFs < 48 h old (Hunter and Macewicz, 1985a). As only small percent (< 2%) of mature females had hydrated oocytes in the ovary, an estimate of ovulatory frequency using the presence of hydrated oocytes was not attempted. Assessment using only POFs that were < 48 h old avoided confusion of POFs with other structures, such as atretic oocytes. Spawning frequency was estimated for mature females (≥ 24.7 cm) and also for large females (> 30 cm) where 100% of females attained sexual maturity (Bani and Moltschaniwskyj, 2008). Spawning frequency was estimated by dividing 100 (representing the total population of females) by the percentage of females with POFs in the ovaries (DeMartini and Fountain, 1981). This assessment assumes that the interovulatory period is constant among individuals within the population.

An indirect assessment of the spawning activity of males at different times of the day was made by classifying the level of spermiation in males captured during the peak spawning period in October 2003. Males were classified as non-spermiated (no milt expressible), partially spermiated (viscous milt expressible), or fully spermiated (fluid milt easily expressible).

Statistical analysis

The difference in somatic condition between atretic and non-atretic females was assessed using an analysis of covariance (ANCOVA), with somatic weight (body weight minus visceral weight) as the response variable, and total length as the covariate. A similar analysis was performed for liver condition using liver weight as the response and somatic weight as the covariate. This analysis provides a size-independent measure of somatic and liver condition (Jakob *et al*., 1996; Hayes and Shonkwiler, 2001). A χ^2 test of independence was used to determine differences in spawning frequency of females among months, the proportion of mature fish (female and male) at different times during the day, and changes in proportions of gamete type among macroscopic state of testes.

RESULTS

Gonad development

Stage 1 and 2 ovaries had no vitellogenic oocytes, oocytes in Stage 3 ovaries had increased in size (approx. 0.2 mm) and contained yolk vesicles. Stage 4 ovaries were marked by the appearance of yolk granules in the oocyte cytoplasm (Tab. II). This was accompanied by increase in the thickness of the zona radiata. Migration of the nucleus towards the periphery in stage 5 signalled the onset of final oocyte maturation, when the yolk and lipid material in the

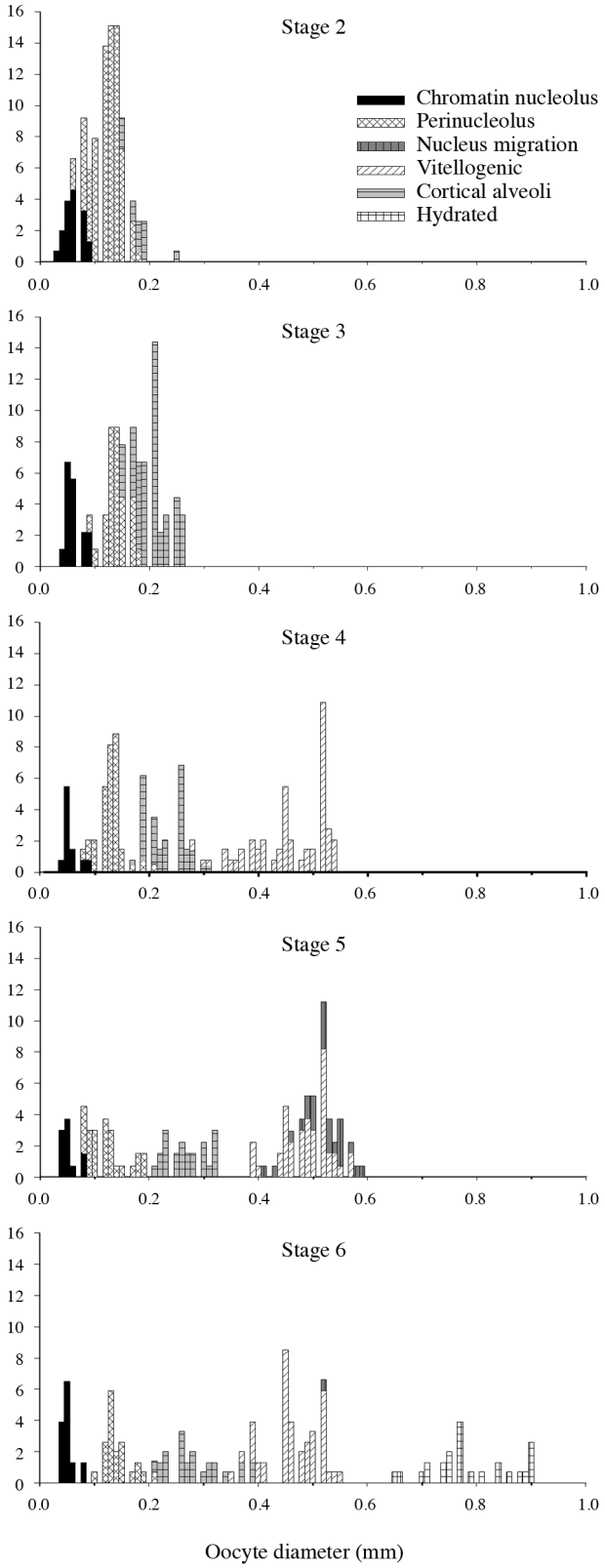

Figure 2. - Oocyte size frequency distribution in sand flathead at different stages of gonadal maturation. n = 10 females for each maturity stage.

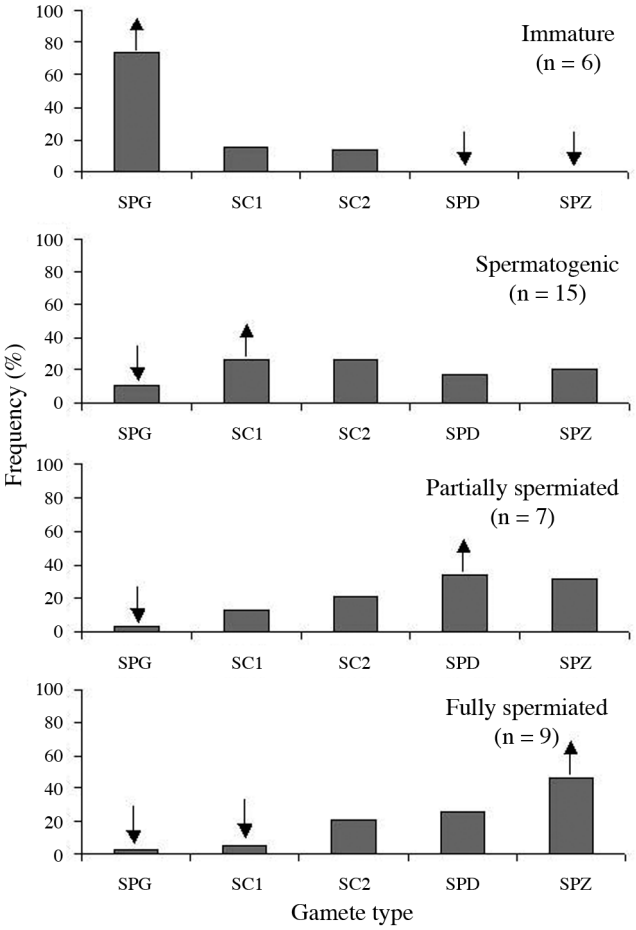

Figure 3. - Frequency of different gamete stages, including spermatogonia (SPG), primary (SC1) and secondary (SC2) spermatocytes, spermatids (SPD) and spermatozoa (SPZ), in histological sections from each macroscopic testicular stage of sand flathead. The arrows indicate the direction in which the observed frequencies differed from expected frequencies, generated under the assumption that gamete type is independent of testis stage. n = number of individuals, with 500 observations per testis of each individual.

Table V. - Percentage of mature and atretic female sand flathead during the spawning season in Coles Bay. Percentage of atresia includes ovaries that are classified in states 2 and 3 of atresia. n = number of sexually mature sized females (≥ 24.7 cm) in each month.

	Month					
	Oct	Nov	Dec	Jan	Mar	May
Mature %	94.9	100	100	67.4	89.9	12
Atresia %	2.6	0	0	23.9	0	68
n	39	30	30	46	28	25

oocyte cytoplasm coalesced during hydration (Stage 6 ovaries). After spawning, females generally had a following cohort of oocytes undergoing final maturation (germinal vesicle migration) and these individuals were classified as stage 5. Spent (Stage 7) ovaries contained chromatin nucleolar and perinucleolar oocytes, alongside some atretic oocytes, but all other oocyte types were absent.

Mature ovaries had polymodal size distribution of oocytes, with considerable overlap in the sizes of different stage oocytes (Fig. 2). In previtellogenic and early vitellogenesis stages, all oocyte diameters were < 0.26 mm. Oocyte diameters increased to a maximum size of 0.54 and 0.59 mm in Stage 4 and 5 ovaries, respectively. In Stage 5, there was an overlap in the size of vitellogenic oocytes and oocytes undergoing germinal vesicle migration. Oocyte hydration was accompanied by enlargement of oocytes (to ca. 0.9 mm diameter). Smaller, mostly vitellogenic oocytes were present in all females that had hydrated oocytes. The presence of a range of oocytes stages in ovulated females (stage ≥ 4), together with several modes in size-frequency distribution of oocytes is consistent with asynchronous oocyte development and multiple episodes of ovulation during the spawning season.

Changes in macroscopic states of testes were characterised by significant changes in proportions of gamete type ($\chi^2 = 255.2$, df 12, $p < 0.001$). Immature testes contained significantly more spermatogonia (SPG) than any other testis stage (Fig. 3). A few primary (SC1) and secondary (SC2) spermatocytes were also present, but no spermatids (SPD) or spermatozoa (SPZ) were recorded in immature testes. Testes undergoing spermatogenesis contained all gamete types, but with a higher proportion of SC1 (Fig. 3). Spermatids were more common in partially spermiated testes than in any other testis stage. Fully spermiated testes were characterised by high proportions of SPZ in the tubule lumens. The presence of all gamete types in spermiated testes (partially and fully) indicates continuous gamete maturation.

Reproduction and spawning strategy

From October to December, almost all mature sized females (≥ 24.7 cm) were at stage 3 or higher (Tab. V) while there was no or little evidence of atresia (State 2 and/or 3). However, the proportion of mature females decreased by 30% in January compared to December. Sexually mature females exhibited degree of atresia in January. In March, ~90% of females were mature, with no evidence of occurrence of atresia (Tab. V). By May, atresia was common (68%) in potentially reproductively active females in Coles Bay.

Mid-way during the spawning season (January) the somatic condition of atretic and non-atretic females was similar ($F = 0.03$, df 1,44, $p = 0.849$). However the liver condition of non-atretic females was significantly higher than that of fish showing atresia ($F = 8.20$, df 1,44, $p < 0.005$); atretic females had a mean liver weight (1.04 ± 0.04), adjusted for somatic weight, 20% less than that of non-atretic females (1.31 ± 0.05). By May, there was no significant difference in either somatic weight (2.40 ± 0.01) ($F = 0.14$, df 1,23, $p = 0.714$), or liver weight (1.46 ± 0.08) ($F = 0.29$, df 1,23, $p = 0.591$) between atretic and non-atretic females.

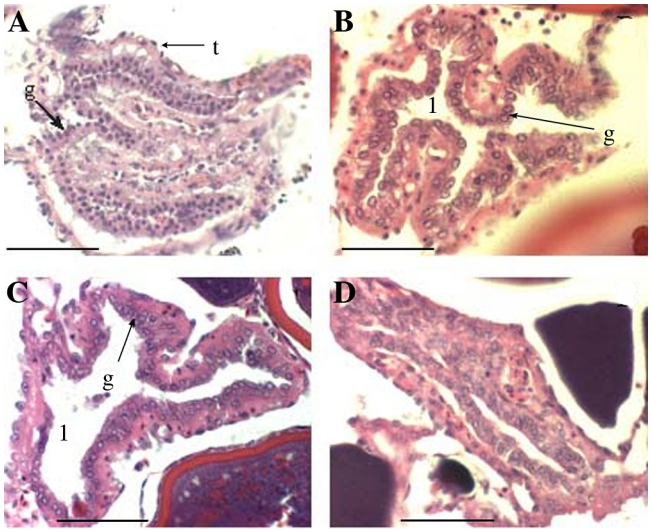

Figure 4. - Characteristics of sand flathead POFs A) < 6 h, B) 24 h, C) 48 h, and D) 72 h post-spawning. g = granulosa cell, l = lumen, t = thecal cell. Scale bars 50 µm.

Spawning periodicity

Post-ovulatory follicles were readily distinguishable from atretic follicles for up to 48 h post-ovulation. Early stage POFs (< 6 h post-ovulation) appeared as a series of folded loops (Fig. 4A). The nucleus was located at the apex of the cuboidal or columnar granulosa cells, which were arranged in an orderly way along the edge of the lumen. Deformation of POFs appears to start 6 h post-ovulation since 12 h POFs had intermediary structure between 6 and 24 h POFs. Twenty-four hours post-ovulation the POFs had fewer folds and granulosa cells did not show alignment (Fig. 4B). Vacuoles were seen in the nucleus of some granulosa cells. The underlying thecal cells were present, although less distinct than in early stage POFs. At 48 h post-ovulation, POFs displayed a closed lumen and diffused thecal cells layer into the granulosa cells layer (Fig. 4C). Beyond 48 h, POFs were compressed, with degeneration of nuclei of granulosa and thecal cells and similar in appearance to γ stage atretic follicles (Fig. 4D).

Table VI. - Monthly spawning frequency determined for sand flathead from the proportion of ovaries of mature females (≥ 24.7 cm) with POFs < 48 h old. Values in brackets are the number of mature females. Spawning frequency was estimated by dividing 100 by the percentage of females with POFs < 48 h old in the ovaries (DeMartini and Fountain, 1981).

Month	% ovaries with POFs < 48 h	Spawning frequency (days)
October	17.8 (101)	5.6
November	23.3 (30)	4.3
December	26.7 (30)	3.7
January	15.2 (45)	6.6
February	Not enough data	Not enough data
March	16.7 (26)	6

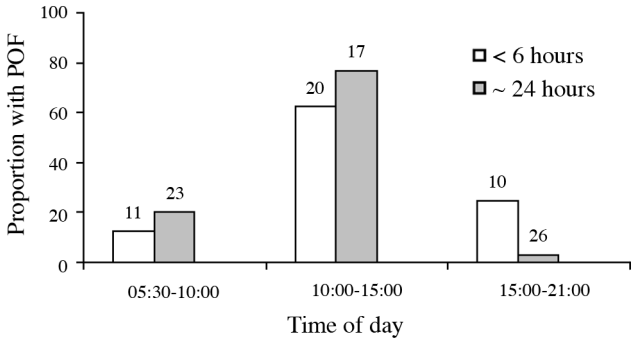

Figure 5. - Proportion of mature female sand flathead with new (< 6 h) and ~ 24 h POF at different times of the day during spawning season. Values above bars are numbers of mature females.

The proportion of mature females with or without hydrated oocytes did not differ at different times during daylight hours ($\chi^2 = 1.6$, df 2, $p = 0.442$). In contrast, the proportion of mature females with new (< 6 h) and ~ 24 h POFs in samples taken at different times throughout the day was significantly different ($\chi^2 = 6.3$, df 2, $p = 0.043$), indicating a diel cycle in spawning activity (Fig. 5). From 10:00-15:00 h, 62% and 77% of females had POFs < 6 h and ~ 24 h, respectively (Fig. 5), indicating that spawning occurred mostly early in the day. This is supported by this fact that 12 h POFs were not observed in ovaries of wild fish obtained between 05:30 and 15:00. Males, in contrast, did not show a clear diel cycle in reproductive activity during the day ($\chi^2 = 0.9$, df 2, $p = 0.639$). The majority of males had fully spermiated testis throughout the day, ranging from 66% of individuals (before 10:00 h) to 80% (between 10:00 and 15:00), suggesting were ready to spawn at any time of the day during the spawning season.

Spawning frequency of mature females (≥ 24.7 cm) did not significantly differ ($\chi^2 = 2.1$, df 4, $p = 0.720$) over the months of spawning. Females spawned at 3.7-6.6 day intervals in different months of spawning period (Tab. VI). During all spawning months, an average of 18.8% of mature females (≥ 24.7 cm) and 25.1% of large females (> 30 cm) had POFs < 48 h old in the ovaries. Therefore, it appears that mature females (≥ 24.7 cm) spawn approximately every 5.3 days during October-March, and potentially could be releasing a batch of eggs up to 34 times each year. Large females (> 30 cm) spawn every 4 days and therefore may release 45 batches of eggs every year. The total number of released eggs may increase if the spawning period extends to April.

DISCUSSION

Sand flathead displayed asynchronous oocyte development, with continuous development of batches of oocytes, as evidenced by the lack of modal peaks in the size-frequency

distribution of oocytes in sexually mature fish. The implication is that individual fish spawn multiple times within a reproductive season, and this is supported by estimates of ovulatory frequency of 4-5.3 days. Male sand flathead also exhibited asynchronous gamete development, and this would be predicted from the need for gamete development in males to be synchronised with female activity at the population level (Pankhurst, 1998). The presence of all gamete stages in partially and fully spermiated males, suggests recruitment and subsequent maturation of immature gamete types for multiple spawning episodes. This confirms that sand flathead is a serial spawner, and is capable of spawning multiple times during the spawning season.

The duration of the spawning period of sand flathead throughout eastern and southern Tasmania is estimated to be six months (Jordan, 2001), generally consistent with the outcomes of the present study. Mature females had POFs present from October to March, suggesting that the population was reproductively active throughout this time, resulting in an extended spawning season. Since samples were not collected in April, spawning in this month can not be excluded and therefore spawning season of sand flathead may even extend to April. The extended spawning in sand flathead is consistent with length of spawning periods in other Australasian temperate species (Davis and West, 1993; Hesp *et al*., 2004), suggesting that an extended spawning period may be attributed to a moderate water temperature in temperate regions. In multiple spawners, spawning can be protracted to take advantage of extended periods of favourable environmental conditions (DeVlaming, 1983).

The occurrence of atresia in the ovaries of mature females (≥ 24.7 cm) in October was mostly related to the presence of alpha and beta atresia of unyolked oocytes. This does not appear be related to the cessation of spawning activity as yolked oocytes in the ovary remained intact. Occurrence of atresia in the early vitellogenic stage has been recognized as a natural process regulating the surplus of oocytes in the early vitellogenic stages recruited into successive stages of development (Tyler and Sumpter, 1996). Mature females showed atresia in January, but not in March, corresponding with low liver condition indices in January. It is difficult to explain why atresia did not occur in March, although the absence of atresia in the middle of the spawning season is not unusual. The presence of atresia in January suggests that ovarian resorption may have been occurring due to poor nutritional condition that was in turn, compromising reproductive activity (Hunter and Macewicz, 1985b; Hay and Brett, 1988; Lambert *et al*., 2000). Liver indices support the possibility that reproductive regression in January was related to nutritional status. Changes in water temperature in Coles Bay were minimal between December and March (16-17°C) (Bani, 2005), suggesting that the reduced proportion of mature females, and higher levels of atresia in January could not be attributed to short term fluctuations in water temperature. As there is little likelihood of spawning by individuals with high levels of atresia i.e. states 2 and 3 (Hunter and Macewicz, 1985a), seasonal reproductive output of mature females would have been substantially reduced due to high levels of atresia in mid-spawning season (January). Given the small percentage of mature females and a high level of atresia by May, it is likely that almost all females were reproductively inactive by this time.

Male sand flathead are probably capable of spawning throughout the day as the percentage of males with partially or fully spermiated testes was not different during the day, a pattern seen in males of other fish species (Grier and Taylor, 1998; Yoneda *et al*., 1998). This was in contrast to the pattern of diurnal changes in the activity of the testis in some fish species (Pankhurst and Poortenaar, 2000). Female sand flathead appear to have a peak in spawning activity at first light. However, it was only possible to collect fish from 05:30-21:00h, despite line-fishing and gill-netting between 21:00-05:30h, therefore it is not possible to discount the possibility of night-time spawning. Daytime spawning is unusual, as most fishes with diel spawning patterns typically spawn around dusk (Ferraro, 1980; Colin and Clavijo, 1988) to reduce visual predation on the eggs (Robertson, 1991). This suggests that there might be a small risk associated with releasing eggs at dawn for sand flathead. Determining the precise time of spawning will only be possible by catching greater numbers of ovulated females from the wild or by observing spawning behaviour of captive fish.

In the present study two hormones (LHRHa and hCG) were used to maximize the likelihood of stimulating ovulation as there was no information on hormone efficacy in stimulating ovulation of sand flathead. It has been demonstrated that LHRHa and hCG do not act similarly in ovulation and a daily fluctuation is possible either in pituitary response to LHRHa or in gonad response to hCG, or both (Matsuyama *et al*., 1998). The timing of ovulation is also dependent on the timing of GnRHa or hCG injection, but apparently not on circadian rhythms (Shiraishi *et al*., 2006). Nevertheless, it is unlikely a difference in the kinetics of the response will have influenced the ageing of POFs (Pankhurst and Poortenaar, 2000). It would be of interest to further explore the effect of hormone type on the ageing of POFs and POFs' structure.

Estimates of spawning frequency in the present study are the first to be presented for sand flathead and are believed to be representative of the spawning population, with the caveat that they assume a constant interovulatory period for all individuals. POFs of sand flathead were still fully distinguishable two days after spawning giving an estimate of spawning frequency of ~5.3d. Although there are numerous species showing daily, lunar and semi-lunar spawning cycles (reviewed in Robertson, 1991; Scott *et al*.,

1993) intermediate periods appear to be less common. However, they have been reported in cobia *Rachycentron canadum*; spawning every 5-12 days (Brown-Peterson *et al*., 2001), Mediterranean sardine *Sardina pilchardus sardina*; spawning every 11-12 days (Ganias *et al*., 2003), spotted seatrout *Cynoscion nebulosus*; spawning every 4.4 days (Roumillat and Brouwer, 2004), Spanish mackerel *Scomberomorus commerson*; spawning every 5.9 days (Mackie *et al*., 2005) and European hake *Merluccius merluccius*; spawning every 5-12 days (Murua and Motos, 2006). In sand flathead, larger females showed greater spawning frequency than smaller fish. A similar increase in spawning frequency with increasing fish size has also been reported in the Mediterranean sardine, *Sardina pilchardus sardine*, (Ganias *et al*., 2003) and carpenter seabream, *Argyrozona argyrozona*, (Brouwer and Griffiths, 2005). Variability in spawning frequency among different-sized females in a population suggests that the effect of body-size of reproductively active females should be factored in to estimates of spawning frequency, and subsequent estimates of egg production in multiple spawners. Furthermore, estimates of egg production based on spawning frequency should be treated with caution because it is possible that the number of eggs released may differ from one spawn to the next.

The results of the present study show that occurrence of high levels of atresia, especially in larger individuals, can have a marked impact on egg production. The effect of increasing occurrence of atresia on reducing egg production towards the end of the spawning season is well recognized and generally considered in estimates of stock reproduction (Hunter and Macewicz, 2003; Santos *et al*., 2005). However, less attention has been given to the occurrence of atresia in mid-spawning season, largely because the extent and timing is difficult to predict. It is unclear whether the mid-season effect reported here is a regular seasonal event or not but it does suggest that constant egg production levels over spawning months may not always be attained due to the occurrence of atresia. This may result in over-estimation of reproductive output if constant egg production over the spawning season is assumed.

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