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## Reproductive cycle of the sea urchin *Holopneustes purpurascens* (Temnopleuridae: Echinodermata)

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**Abstract** This study documents the annual reproductive cycle of the Temnopleurid echinoid *Holopneustes purpurascens* near Sydney. *H. purpurascens* reached sexual maturity between 11 and 26 mm diameter and was gonochoristic. The sequence of oogenesis was similar to previously described echinoids. However, the proportion of eggs at each stage showed no temporal pattern in abundance, suggesting that vitellogenesis is continuous throughout the year. Spermatogenesis was also similar to other echinoids and showed a significant increase in the proportion of partially spawned testes in spring, followed by a similar increase in the proportion of partially spawned testes in late spring and summer. Gonad indices showed a peak in weight from late winter to mid-spring ( $9.10 \pm 1.47$  for females;  $6.28 \pm 0.43$  for males; mean  $\pm$  SE), then a decline for both sexes. Although spawning was variable over time, there was a peak in the proportion of spawning from late winter to early summer that correlated with the observed peaks in gonad indices and gametogenic cycles. The demographic implications of this reproductive pattern are that new recruits can enter the population through synchronous spawning during the breeding period, and smaller haphazard spawning events at other times of the year.

densities of urchins often denude large areas of macroalgae and can even create “barrens” (Underwood et al. 1991), thus dramatically altering the community’s physical and biological structure (Leinaas and Christie 1995). Because of the density-dependent nature of the impact of urchins in temperate communities, knowledge of their life-history is important for understanding the ecology of these interactions.

Most urchins have defined annual reproductive cycles, with predictable cellular stages of gonadal development and spawning (Pearse and Cameron 1991), although the exact pattern of spawning for a species can vary among localities and years (Byrne et al. 1998). These reproductive cycles are influenced by a suite of exogenous and endogenous factors, including photoperiod (Walker and Lesser 1998), lunar cycles (Kennedy and Pearse 1975), temperature (Leahy et al. 1978), diet (Russell 1998), and density (Wahle and Peckham 1999). Reproduction in echinoids is generally well-documented due to the ease of obtaining mature gametes (Byrne 1989), and knowledge of their gametogenesis and spawning has played an integral role in a range of commercial applications, including the development of toxicity tests (Dinnel and Stober 1987) and echinoculture (Walker et al. 1998).

Despite the wealth of information on the reproductive periodicity of urchins in the families Diadematoidae, Toxopneustidae and Echinometridae in Australasia and worldwide, there are no studies of reproduction for on the family Temnopleuridae, even though this family occurs globally (Matsuoka and Inamori 1996) and includes approximately half the number of echinoid species in southern Australia (Edgar 1997). One Temnopleurid, *Holopneustes purpurascens*, commonly occurs in shallow subtidal waters in New South Wales, Australia. This species has a complex life cycle, with a highly derived mode of development, beginning with a planktonic apluteal larva (Morris 1995) that metamorphoses into a benthic stage that lives in the canopy of subtidal macroalgae (Steinberg 1995). Metamorphosis of the larvae occurs in response to a chemical cue

### Introduction

Sea urchins have a major impact on the structure and dynamics of shallow subtidal communities (reviewed by Harrold and Pearce 1987). In temperate regions, high

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isolated from one of its algal hosts (Williamson et al. 2000). This cue, and its effects on larval metamorphosis, appears to cause major differences in the size and distribution of the urchins on different host plants (Williamson et al. 2000, Williamson, unpublished).

To further understand the role of larval recruitment in the ecology of host-plant use by *H. purpurascens* – in particular patterns in the availability of larvae – it is essential to understand the reproductive cycle of these urchins. This study aimed to determine the variability in the pattern of gonadal growth and the timing of spawning activity. Histological examination of gonad development, the gonad index, and a population's readiness to spawn were documented, along with the minimum size of urchins at sexual maturity and the sex ratio of the urchins.

## Materials and methods

### Study site and collection of urchins

The reproductive cycle of *H. purpurascens* was studied at Bare Island (33° 59' 38"S, 151° 14' 00"E) at the north head of Botany Bay, Australia. Bare Island is a mosaic of habitats typical of shallow subtidal areas in temperate southeastern Australia. A more detailed description of such areas can be found in Underwood et al. (1991) and Steinberg (1995).

Larger and (putatively) reproductively mature *H. purpurascens* are most common on the kelp *Ecklonia radiata* (Steinberg 1995; Williamson et al. 2000). Since diet can alter both the size of urchin's gonads and their histological condition (Andrew 1986; Williamson 2001), this study, with the exception of the size of urchins at reproductive maturity, was restricted to *H. purpurascens* inhabiting *E. radiata*. Urchins occurred at high densities ( $26.0 \pm 2.76$  individuals per m<sup>2</sup>) at Bare Island (Williamson et al., unpublished) and were collected from the fronds of haphazardly selected *E. radiata* using SCUBA. *H. purpurascens* were taken back to the laboratory, measured and either dissected immediately or injected with potassium chloride (0.5 M) and later dissected. Since *H. purpurascens* elongate along the oral-aboral axis as they grow larger (Steinberg 1995), the size of each urchin was measured with Vernier calipers to the nearest millimeter as the maximum oral-aboral diameter. Animals were sorted into their sexes in the laboratory at each sampling time, resulting in an uneven number of males and females. Apart from the gonopore of males being slightly longer and more slender than those of females – an observation almost impossible to distinguish whilst diving unless the animal is about to spawn – there is no obvious external sexual dimorphism between males and females. Therefore, the sex of each urchin was determined by examining the colour of the gonads of the freshly dissected specimens and any gametes that had been released. No urchins with any visible injury were used in this study.

### Onset of sexual maturity

The size of *H. purpurascens* at the onset of reproductive maturity was determined by dissecting urchins and recording the presence or absence of gonads in specimens collected during this study. Data on smaller urchins collected at random on a range of macroalgae during 1997 were also included. If any gonad material was observed, regardless of the amount, the urchin was scored as having gonad present. This is an overestimate of maturity because the mere presence of gonads does not ensure that the animals are mature. However, an absence of gonads was taken to indicate immaturity. Since the only obvious trend in the data was between the presence of gonads and size, data for both sexes was pooled. Due to unequal

sample sizes, Cochran's tests could not be done. Therefore, prior to analysis, the assumptions of normality and homogeneity of variance were checked using a frequency histogram of the residuals, a plot of residual versus estimate of the means, and a normal probability distribution of the residuals. The pooled data were transformed into the double square-root. The size of immature and mature urchins was compared with a two-tailed unpaired *t*-test.

### Sex ratio

Biases in the ratio of sexes have been documented for a number of species of marine invertebrates (e.g. Pearse 1978; Otway 1994; Van Dover et al. 1999) and vertebrates (e.g. Sadovy and Shapiro 1987; Gillanders 1995). Such ratios have been used as broad scale diagnostic indicators of the mode of reproduction (gonochoristic or hermaphroditic) of a particular population, with hermaphroditic populations typically having uneven adult sex ratios (Gonor 1973a; Sadovy and Shapiro 1987). The sex ratio of *H. purpurascens* was determined using pooled data on the sexes obtained from the gonad index and the readiness to spawn studies (see Results). Chi-squared goodness-of-fit tests were used to see if the overall proportion of males and females deviated significantly from 1:1, and if the monthly proportion of males and females deviated significantly from this ratio over time.

### Histology of the gonads

Gonads collected from *H. purpurascens* monthly from August 1996 to July 1997 were processed for histological assessment of the cellular events of gametogenesis. At each sampling date, one gonad from each urchin was selected at random and preserved in Bouin's fixative for 24 h, then stored in 70% ethanol. Fixed gonads were dehydrated with graded ethanol (from 70 to 100%) using a Shandon Citadel 1000 automatic tissue processor and embedded in paraffin. After embedding, the wax blocks were trimmed and left on an ice slurry for approximately 10 min to facilitate sectioning. Three replicate sections of 5 µm thickness, approximately 100 µm distance from each other, were then cut from the middle portion of these blocks using a microtome (Microm HM 325). These sections were mounted on slides, dried overnight in a 37°C oven and stained with Mayer's haematoxylin and eosin (H+E). Coverslips were mounted with "DePeX" mountant and the slides were allowed to dry in a fume hood for at least 48 h before examination. The clearest of the three sections was analysed.

Since the ovaries appeared to be at a similar stage of maturity throughout the year, with oocytes at all stages of development present, ovary sections were classified according to changes in the relative abundance of the different cell types, as determined through assessment of 50 oocytes per individual. The different stages were: pre-vitellogenic oocytes, early vitellogenic oocytes, mid-vitellogenic oocytes, late vitellogenic oocytes, ova, and degenerating ova. The first 50 eggs to appear in the field of view that were sectioned at the nucleus or nucleolus were counted. Since the nuclei were not usually visible in mature or degenerating ova, these were classified according to their size and appearance. Cells in different stages of development were grouped into four categories for analyses: pre-vitellogenic oocytes; vitellogenic oocytes; ova; and degenerating ova. Each of the first three developmental groups was analysed separately. Degenerating ova were not analysed due to their low numbers.

A classification system for testes was based on the different stages used in Byrne (1990) and King et al. (1994). In this study testes were classified according to the change in relative abundance of the different stages of maturity of 30 testes per individual. Here, testes at different stages of maturity were placed into six categories: recovering testes; growing testes; premature testes; mature testes; partially spawned testes; and spent testes. For analyses the first three groups were combined into one category, "developing testes", and were analysed along with the mature testes and partially spawned testes.

A pilot study was done with samples collected on 21 August 1996 to determine whether all of the five gonads per individual and

different locations within each gonad were in a similar gametogenic state. Here, all gonads from three females and three males were preserved (see above), divided into three locations (top, middle and bottom), depending on the distance of the sample on the gonad from the gonopore, sectioned and analysed separately for each sex. Data on the two developmental groups that comprised the bulk of cells in the ovaries (i.e. vitellogenic oocytes – including early, mid, and late vitellogenic oocytes – and ova) and testes (i.e. developing testes and mature testes) were analysed. Prior to analyses, Cochran's tests (Winer et al. 1991) were used to examine homogeneity of variances ( $P > 0.05$ ). For each sex, the three locations within the gonads were compared separately with a two-factor nested analysis of variance, with urchin and location nested within urchin as random factors. If location was not significant in this analysis, it was pooled and data on the different replicates compared with a two-factor nested analysis of variance, with urchin and gonad nested within urchin as random factors. These analyses confirmed that gametogenesis is homogeneous throughout each gonad and among gonads of individual urchins (see Results). Consequently, only one section from the middle portion of one gonad per individual was examined thereafter.

The maturity stage of each gonad was compared using a one-factor analysis of variance to determine if the proportion of cells within the stages varied significantly over time. Due to unequal sample sizes within each developmental group, Cochran's tests could not be done and assumptions of normality and homogeneity of variance for each data set were checked using residuals as for the onset of sexual maturity and transformations made where appropriate. *A posteriori* pairwise comparisons (Tukey's test; Winer et al. 1991) tested at  $\alpha < 0.05$  were used to determine the location of differences among the treatment means after analysis of variance.

#### Gonad index

The gonad index (GI), which measures the relative changes in weight in the gonads over time, was used on urchins as early as 1934 (Moore 1934), and is based on the assumption that maturation and breeding coincide with maximum gonad weight. Since the size of urchins may influence the GI (Gonor 1972), the size range of urchins used was restricted to 30–60 mm. Urchins were dissected, their gonads removed, and the five gonads and the rest of the urchin were dried separately at 60°C to a constant weight for a minimum of 3 days. On several dates these urchins were also used for histological analyses. Thus, after the removal of one gonad, the remaining four were dried to a constant weight, and the gonad weight extrapolated to an approximation of the dry weight for each urchin. A pilot study showed that there was no significant difference between the dried weight of four gonads that had been extrapolated to include the weight of the fifth gonad ( $0.583 \pm 0.59$  g; mean  $\pm$  SE;  $n = 10$ ) and the dried weight of the five gonads ( $0.585 \pm 0.63$  g; mean  $\pm$  SE;  $n = 10$ ) for the same urchin (paired *t*-test,  $t = -0.324$ ,  $P = 0.754$ ; Cochran's test,  $C = 0.53$ ,  $P < 0.05$ ). After drying, the ratio of total gonad weight to total dry weight for each urchin was used to calculate the GI as:  $GI = (\text{gonad dry weight} / \text{total dry weight}) \times 100\%$ .

Raw data on dry weight for each sex (excluding the first sampling time due to the lack of replicates for females) was arcsine transformed and tested for homogeneity of variance using frequency histograms of the residuals, plots of the residuals versus estimates of the means, and normal probability distributions of the residuals. To see if there was a significant relationship between the sexes, the data were arcsine-transformed and tested with a Pearson rank correlation coefficient for a significant correlation. Since a significant correlation existed between the GI for males and females (see Results), data for each sex (including the first sampling time) were pooled for each month and tested for homogeneity of variance as above. Differences in GI for males, females, and pooled sexes over time were compared using one-factor analyses of variances. *A posteriori* pairwise comparisons (Tukey's test) were used to determine the factors contributing to differences among treatment means.

The pattern of gonad indices, and therefore reproductive cycles, for a species can vary between locations that are relatively close to each other, even if environmentally the locations appear similar (Ernest and Blake 1981; Byrne 1990; King et al. 1994). Therefore, to test the generality of these results, gonad indices were collected from urchins at three locations off the coast of Sydney (Bare Island, Long Bay and Shark Bay) on 23 January 2000. At each site 20 urchins were collected, the five gonads removed, and the gonad indices prepared and calculated as before. Differences in GI for males, females, and pooled sexes were compared using one-factor analyses of variances and Tukey's tests were used to determine differences among locations for significant results.

#### Readiness to spawn

Although an animal may be reproductively mature, unless it is actually shedding gametes, or spawning, it will have no consequence on the reproductive output of the population. An urchin's "readiness to spawn" can be directly measured using an injection of isotonic potassium chloride (KCl, 0.5 M), which mimics natural spawning of echinoderms when fully mature gametes are available (Tyler 1949; Pennington 1985; Hori et al. 1987; Levitan et al. 1992; King et al. 1994). Since immature urchins often shed infertile gametes (King et al. 1994), only urchins greater than 30 mm were used. At random sampling times urchins were collected, brought to the laboratory, and each urchin injected intracoelomically through the peristomal membrane with 3–5 ml of 0.5 M KCl, depending on size, and gently agitated. Gametes released within 15 min by each female were gently washed and placed in beakers of sterile seawater. Male gametes released within 15 min were collected and placed in Petri dishes on ice. Since mature urchins may also shed immature gametes by this method but these would not be fertilisable, eggs were placed in dishes of filtered seawater and dilutions of the sperm relative to the concentrations of eggs were added. Aliquots of sperm were mixed with eggs in filtered seawater and monitored and if fertilisation occurred, those urchins were scored as "spawned". After 30 min each urchin, whether it had spawned or not, was dissected and the sex scored. Male, female and the total number of urchins spawned at each sampling time were analysed separately using pairwise log-likelihood ratios (*G*-tests), with the Williams' correction (Williams 1976; Sokal and Rohlf 1981). Since more than one-fifth of the expected frequencies were less than five in data sets for the number of males spawned and the number of females spawned (which biases contingency analyses towards significance; Zar 1984) significance levels were taken at  $\alpha = 0.01$ . Data on spawning for each sex was arcsine transformed and tested with a Pearson rank correlation coefficient for a significant correlation to see if there was any relationship between the sexes. Since a significant correlation among the sexes was detected (see Results), data for both sexes were pooled and analysed over time in the same manner as males and females separately.

After several months it became apparent that the results from spawning were highly variable and it was proposed that spawning by *H. purpurascens* may be closely related to particular phases within the lunar cycle (M. Byrne, personal communication). Therefore, to determine if there was a lunar effect, the date of each sampling time was categorised into one of four arbitrary phases of the moon – new moon, first quarter, full moon, and last quarter – based on lunar data from Geodetic Operations, ACT. Categorised data were compared with one-factor analyses of variances for males, females, and all urchins separately. Due to unequal sample sizes within each category, assumptions of normality and homogeneity of variance were checked using residuals as for the gametogenic cycles.

## Results

### Onset of sexual maturity

Of the 697 urchins scored for the presence or absence of gonads, those without gonads ( $n = 34$ ) were significantly

smaller than those with gonads ( $n=663$ ) (unpaired  $t$ -test,  $t=-4.35$ ,  $P<0.001$ ). Gonads were not present until urchins were at least 11 mm in diameter, and all urchins over 26 mm contained gonads (Fig. 1). Therefore *H. purpurascens* reaches sexual maturity between 11 and 26 mm in diameter.

### Sex ratio

The overall sex ratio of *H. purpurascens* did not deviate significantly from 1:1 (344 males, 314 females;  $\chi^2=0.68$ , 1  $df$ ,  $P=0.408$ ). Similarly, there was no significant deviation from a sex ratio of 1:1 in monthly samples of urchins from August 1996 to July 1998 ( $\chi^2=19.14$ , 19  $df$ ,  $P=0.448$ ). No hermaphrodites were observed.

### Histology of the gonads

#### *Cellular events of gametogenesis*

For females, histological examination of the ovaries of *H. purpurascens* revealed that eggs in various stages of development were usually present (Fig. 2a). Unlike many other species of echinoids (King et al. 1994), there was no obvious pattern of early oocytes occurring at the periphery of an acinus and moving towards the centre of the lumen as they mature. However, pre-vitellogenic oocytes and early vitellogenic oocytes were often found clumped within the acinus. Late vitellogenic oocytes and ova were well distributed throughout the acini, and while it was common for ova to occur within the lumen, they

were occasionally located at the periphery. Nutritive phagocytes formed a layer around the germinal epithelium and occasionally extended into the lumen. Oogonia proliferated in areas where pre-vitellogenic oocytes and early vitellogenic oocytes occurred (Fig. 2b, c).

The pre-vitellogenic oocytes were basophilic, staining dark mauve, less than 25  $\mu\text{m}$  in diameter, and had a prominent spherical nucleus containing dispersed chromatin located near the centre of it (Fig. 2b). Vitellogenic oocytes were classified according to size, staining properties, and the structure of the nucleus and nucleolus. Early vitellogenic oocytes were 25–150  $\mu\text{m}$  in diameter, slightly eosinophilic, staining pink/purple, and contained a large spherical nucleolus (Fig. 2c, d). Mid-vitellogenic oocytes were 150–250  $\mu\text{m}$  in diameter, moderately eosinophilic, staining light pink, and were recognised by a large basophilic nucleolus (Fig. 2e). Late vitellogenic oocytes were 250–350  $\mu\text{m}$  in diameter, strongly eosinophilic and contained a prominent nucleus (Fig. 2f). Ova were structurally similar to late vitellogenic oocytes but larger in size (greater than 350  $\mu\text{m}$  in diameter) and had a reduced nucleus that was often not visible (Fig. 2g). Late vitellogenic oocytes and ova had abundant small yolk bodies (staining dark pink) interspersed with many large lipid droplets (transparent, i.e. not staining), representing storage of nutrients in the eggs (Fig. 2f, g). Degenerating ova were ova that had partly decomposed or had been incompletely cytosolled and were often surrounded by spaces in the lumen where ova may have previously been released (Fig. 2h).

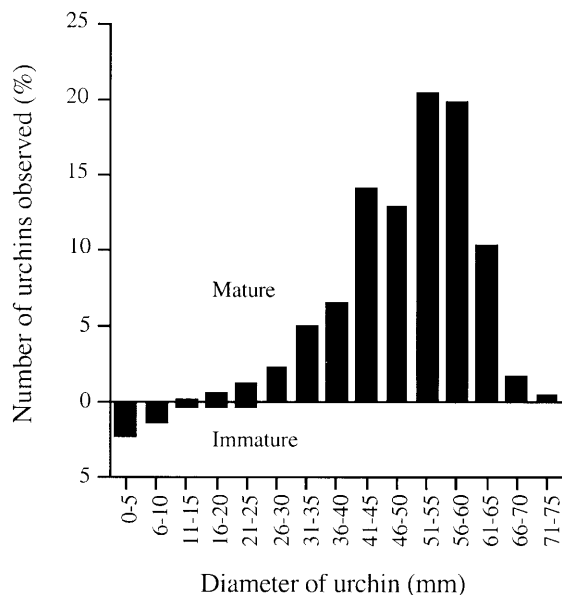
For males, testis development in *H. purpurascens* was divided into six stages (Fig. 3), based on the basophilia of the nucleus in developing stages, and the presence of spermatogenic columns and spermatozoa. These stages were used to describe the histology of spermatogenesis. Spermatids could not be discerned in any of the stages with the light microscope but were assumed to be present.

**Stage 1: recovering.** Recovering acini were small, with a basophilic layer of spermatogonia and primary spermatocytes lining the acinal wall (Fig. 3a). The lumen of the acini contained nutritive phagocytes and unspawned spermatozoa. The beginnings of spermatogenic columns were sometimes observed.

**Stage 2: growing.** Growing testis contained conspicuous columns of spermatocytes, projecting towards the lumen (Fig. 3b). These columns contained spermatogonia at the base, with primary and secondary spermatocytes and spermatozoa at the tips (Fig. 3c). At this stage the spermatocyte layer was fairly thick, with nutritive phagocytes still in the center of the lumen.

**Stage 3: premature.** This stage contained columns of developing spermatocytes that extended from the acinal wall into the lumen (Fig. 3d). Spermatozoa accumulated within the lumen among the few remaining nutritive phagocytes.

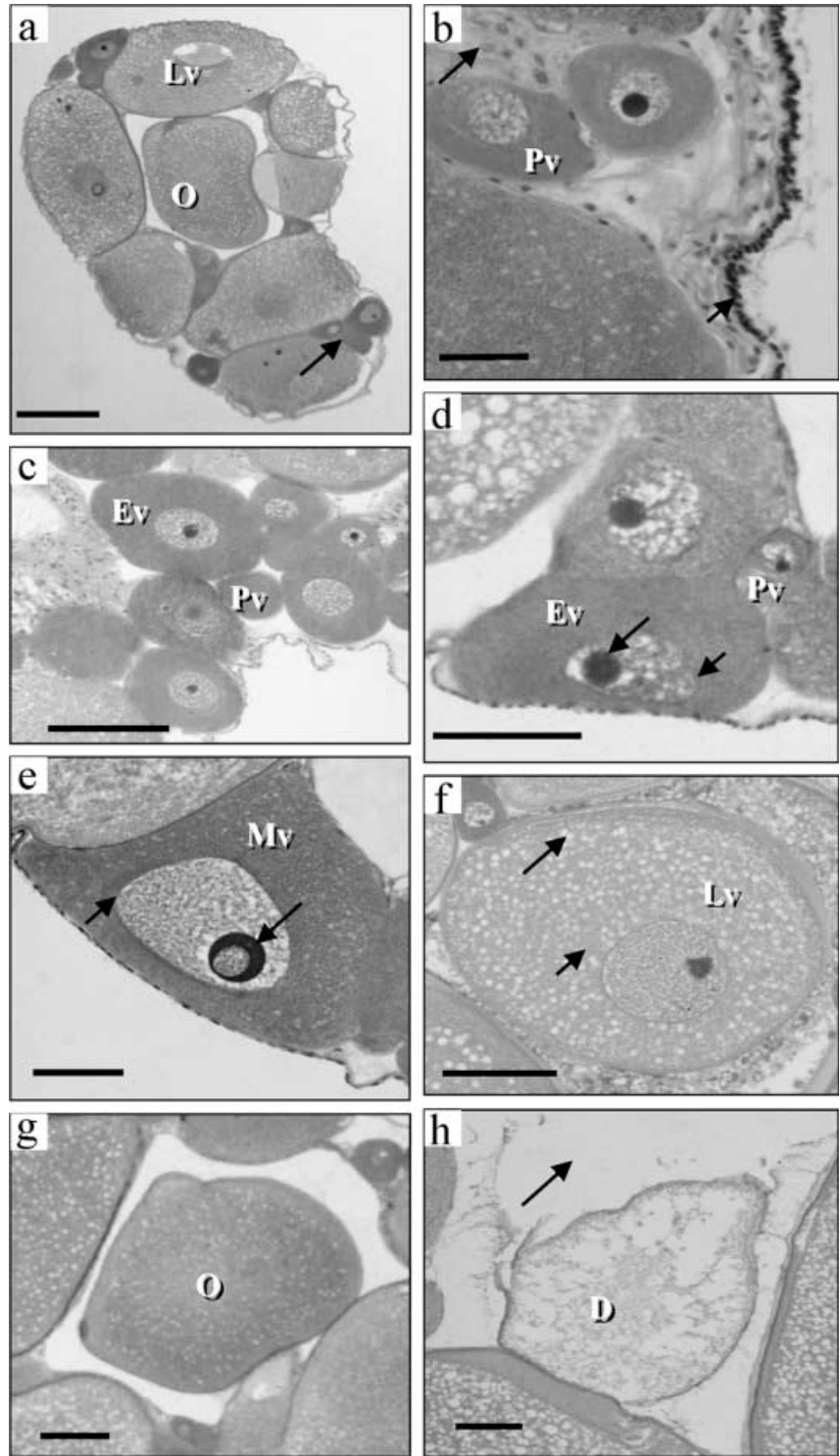
**Stage 4: mature.** Mature testes were filled with a dense mass of spermatozoa in the lumen (Fig. 3e). Columns of developing sperm were often absent, and nutritive



**Fig. 1** The size of *Holopneustes purpurascens* at sexual maturity as determined by the presence or absence of gonads. The size frequency of immature urchins is shown below zero and the size frequency of mature urchins above zero

**Fig. 2a–h** Histology of the ovaries of *H. purpurascens*.

**a** Cross-section through a typical acinus of an ovary showing late vitellogenic oocytes (*Lv*) and ova (*O*) next to pre-vitellogenic and early vitellogenic (*arrow*) stages. **b** Pre-vitellogenic oocytes (*Pv*) surrounded by oogonia (*large arrow*), and peritoneal cells (*small arrow*). **c** A cluster of pre-vitellogenic oocytes (*Pv*) and early vitellogenic oocytes (*Ev*). **d** Pre-vitellogenic oocytes (*Pv*) and early vitellogenic oocytes (*Ev*) showing their dense nucleoli (*large arrow*) within their nuclei (*small arrow*). **e** Mid-vitellogenic oocyte (*Mv*) with a large nucleolus (*large arrow*) within its nucleus (*small arrow*). **f** Late vitellogenic oocyte (*Lv*) with prominent nucleus. Lipid droplets (*large arrow*) are visible as clear circles within the cytoplasm, and although individual yolk bodies cannot be seen at this magnification, they constitute much of the darker, grainier areas within the cytoplasm (*small arrow*). **g** Ova (*O*). No nucleus is visible in this section. **h** Degenerating ova (*D*) next to space that may have held a previously spawned ova (*arrow*). Bars: **a** = 200  $\mu\text{m}$ ; **b** = 25  $\mu\text{m}$ ; **c–e** = 50  $\mu\text{m}$ , **f–h** = 100  $\mu\text{m}$



phagocytes were located towards the periphery along the germinal epithelium. Spermatozoa often appeared to “swarm” in strands (Fig. 3e).

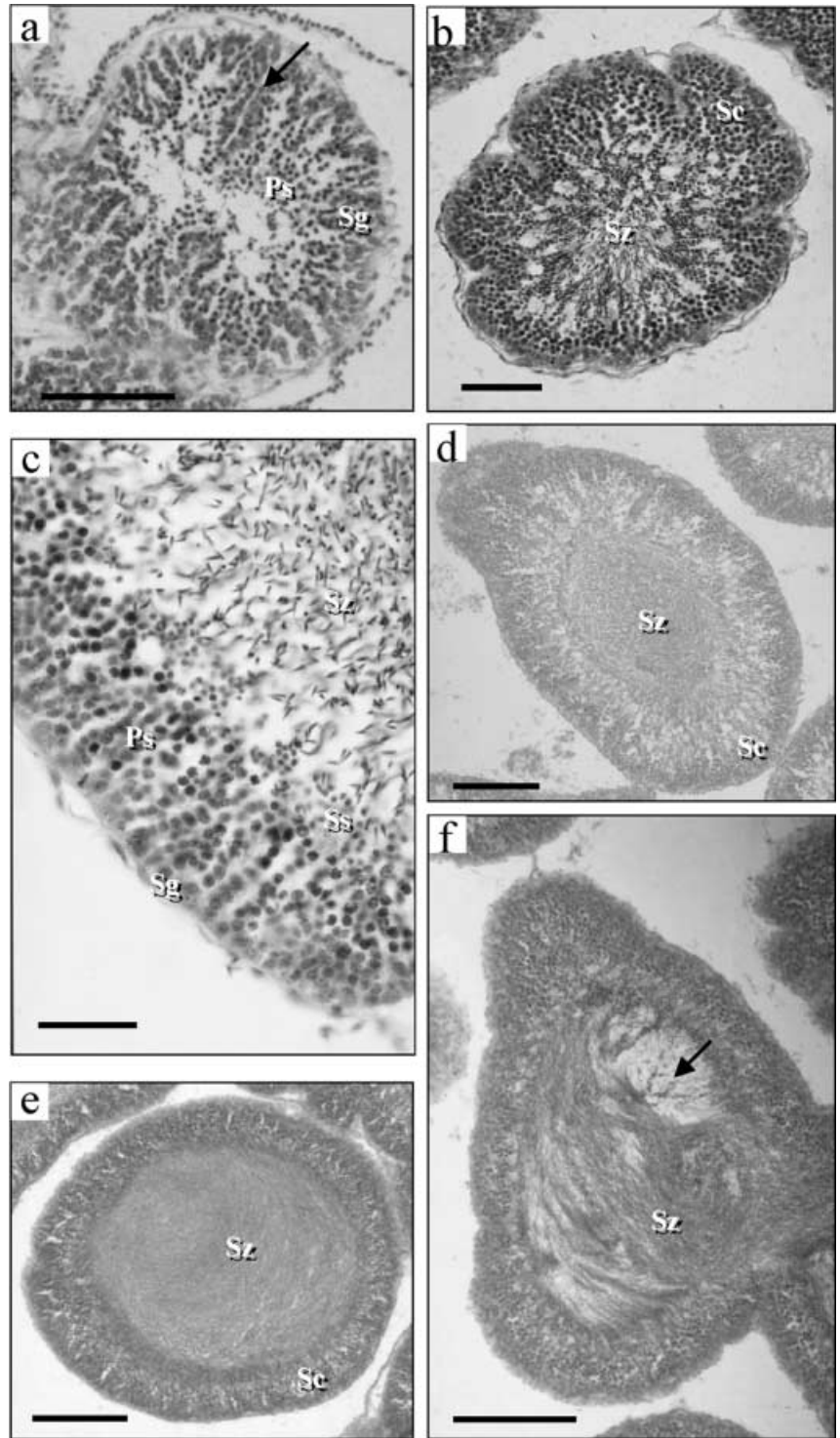
**Stage 5: partly spawned.** Partly spawned acini were similar to mature acini but the sperm was less concentrated, and spaces due to spawning were evident (Fig. 3f). Some of the acini appeared to have

spawned before others within a gonad and mature acini and partly spawned acini were often present next to each other. Swarming of spermatozoa were easily visible near spaces where spawning had occurred (Fig. 3f).

**Stage 6: spent.** No spent acini were observed in this study.

**Fig. 3a–f** Histology of the testes of *H. purpurascens*.

**a** Stage 1: recovering. A small acinus showing the location of spermatogonia (*Sg*), primary spermatocytes (*Ps*), and a relatively empty lumen. A haemal sinus (*arrow*) can be seen projecting into the acinus. **b** Stage 2: growing. Distinct spermatocyte columns (*Sc*) with spermatozoa (*Sz*) accumulating in the lumen. Nutritive phagocytes can be seen as eosinophilic dots in the centre. **c** Spermatocyte columns showing spermatogonia (*Sg*), primary spermatocytes (*Ps*), secondary spermatocytes (*Ss*), and spermatozoa with well developed flagella (*Sz*). **d** Stage 3: premature. Spermatocyte columns (*Sc*) and a central mass of spermatozoa (*Sz*). **e** Stage 4: mature. Spermatozoa (*Sz*) densely packed in the lumen and reduced spermatocyte columns (*Sc*). **(f)** Stage 5: partly spawned. Spermatozoa (*Sz*) in the lumen with spaces vacated by spawning (*arrow*). Note how the spermatozoa appear to “swarm” in strands. *Bars:* **a, b** = 100  $\mu\text{m}$ ; **c** = 20  $\mu\text{m}$ ; **d–f** = 200  $\mu\text{m}$



### Gametogenic cycles

The pilot study for females showed that the proportion of vitellogenic oocytes and ova were not significantly different throughout a single gonad (Table 1: a, b) and between the five gonads of a single urchin (Table 1: c, d), although the proportion of vitellogenic oocytes and ova were significantly different between individual urchins.

The homogeneous nature of developing cells throughout a single gonad and between the five gonads of each urchin validated our procedure of sectioning and analysing the middle portion of only one gonad per female urchin.

The oogenic cycle of *H. purpurascens* showed no annual temporal pattern (Fig. 4) at Bare Island. Gonads containing all stages of developing oocytes and ova were observed throughout the study (Fig. 4). There was no

**Table 1** Analyses of variances for the pilot study on gonadal developmental stages for females and males of *Holopneustes purpurascens*. (a), (b), (e), (f) ANOVAs comparing different locations

Females					Males				
Source	df	MS	F	P	Source	df	MS	F	P
(a) Vitellogenic oocytes [urchin×location(urchin)]					(e) Developing testes [urchin×location(urchin)]				
Urchin	2	21.356	889.830	0.000***	Urchin	2	762.956	4,964.300	0.000***
Location	6	0.024	0.003	1.000 NS	Location	6	0.154	0.038	0.998 NS
Residual	36	8.222			Residual	36	4.022		
(b) Ova [urchin×location(urchin)]					(f) Mature testes [urchin×location(urchin)]				
Urchin	2	15.556	862.240	0.000***	Urchin	2	596.289	12,213.00	0.000***
Location	6	0.0180	0.005	1.000 NS	Location	6	0.049	0.015	1.000 NS
Residual	36	3.933			Residual	36	3.233		
(c) Vitellogenic oocytes [urchin×gonad(urchin)]					(g) Developing testes [urchin×gonad(urchin)]				
Urchin	2	21.356	2.283	0.145 NS	Urchin	2	762.956	385.760	0.000***
Gonad	12	9.356	1.376	0.231 NS	Gonad	12	1.978	0.346	0.972 NS
Residual	30	6.800			Residual	30	5.711		
(d) Ova [urchin×gonad(urchin)]					(h) Mature testes [urchin×gonad(urchin)]				
Urchin	2	15.556	3.084	0.083 NS	Urchin	2	596.289	301.490	0.000***
Gonad	12	5.044	1.576	0.152 NS	Gonad	12	1.978	0.527	0.880 NS
Residual	30	3.200			Residual	30	3.756		

\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; NS not significant

significant difference in the proportion of either vitellogenic oocytes (one-factor ANOVA  $F_{11, 115} = 1.590$ ,  $P = 0.099$ ,  $\sqrt{x+1}$  transformed data) or ova (one-factor ANOVA  $F_{11, 115} = 1.843$ ,  $P = 0.054$ , untransformed data) within a gonad over time. Pre-vitellogenic oocytes showed a significant change in the proportion of cells over time (one-factor ANOVA  $F_{11, 115} = 3.376$ ,  $P < 0.001$ , untransformed data), however the Tukey's test showed that this was essentially due to two comparisons that were not temporally related: (1) significantly more pre-vitellogenic oocytes occurred in February 1997 than in August 1996; and (2) significantly more pre-vitellogenic oocytes occurred in September 1996 than in May 1997 (Fig. 4).

All analyses in the pilot study for males showed a significant difference in the proportion of developing testes and mature testes between individual urchins (Table 1: e–h), which may have been due to the low number of replicate urchins in the study. Although there was a significant interaction in the proportion of developing testes between urchin and location, location was not significant (Table 1: e), suggesting that testes were homogeneous throughout a gonad. Moreover, the proportion of mature testes throughout a gonad was not significantly different (Table 1: f). The proportion of both developing testes and mature testes were not significantly different between the five gonads within an individual (Table 1: g, h). Therefore, sectioning and analysing the middle portion of one gonad per male urchin gave a valid estimate of the gametogenic cycle at that time for an individual.

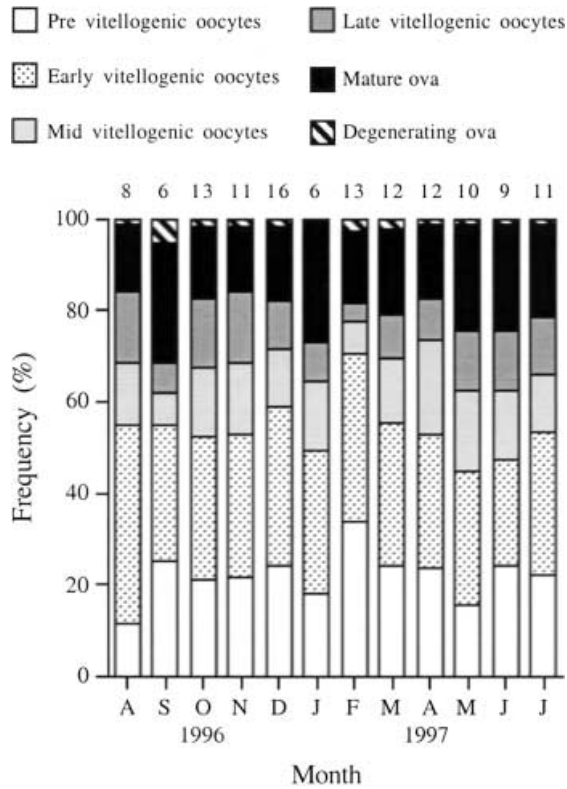
The spermatogenic cycle of *H. purpurascens* at Bare Island showed a distinct temporal pattern in the relative abundance of the different stages of testis maturity (Fig. 5). Growing testes, premature testes, mature testes,

within gonads; (c), (d), (g), (h) ANOVAs comparing different gonads within each urchin. Cochran's tests showed all data to be homogeneous, therefore all data were untransformed

and partially spawned testes were present throughout the year (Fig. 5). Recovering testes were present in low numbers for six months of the year (from August to February; Fig. 5), and no spent testes were observed in this study. There was a significant difference in the proportion of developing testes (i.e. recovering, growing, premature testis) throughout the year (one-factor ANOVA  $F_{11, 149} = 14.420$ ,  $P < 0.001$ , untransformed data) but there was no clear trend in these differences between months. Although present throughout the year, the proportion of mature testes significantly increased during spring (one-factor ANOVA  $F_{11, 149} = 11.572$ ,  $P < 0.001$ , untransformed data; Fig. 5). This, along with the significant increase in the proportion of partially spawned testes from September to January (one-factor ANOVA  $F_{11, 149} = 4.871$ ,  $P < 0.001$ ,  $\ln(x+1)$  transformed data; Fig. 5), indicates that spawning of males, although occurring throughout the year, is enhanced from spring to mid-summer.

#### Gonad index

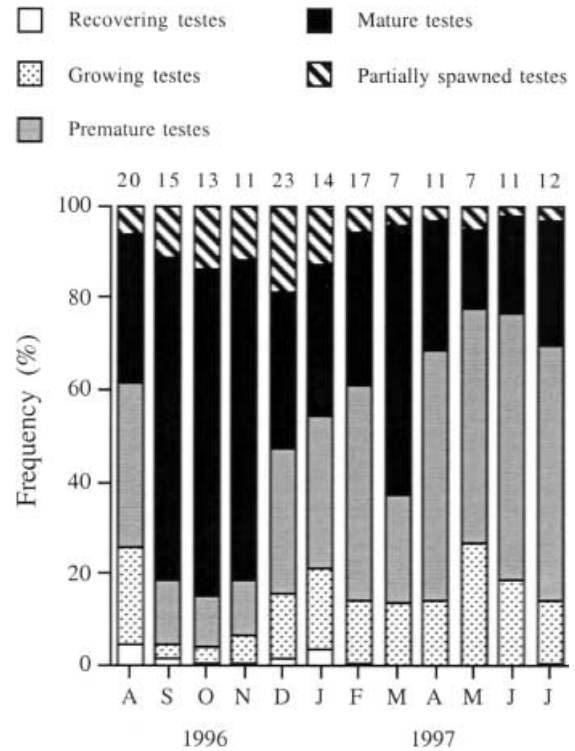
GI values of males at Bare Island revealed a strong temporal effect (one-factor ANOVA  $F_{23, 130} = 5.747$ ,  $P < 0.001$ ; Fig. 6 a). Although the a posteriori comparisons showed no clear trend between significant means, annual peaks in the male GI were observed in September/October in 1996 and from July to October in 1997 (Fig. 6a). These peaks, followed by a consistent drop in the index value, were indicative of large spawning events. The GI values for females (excluding the first sampling time) at Bare Island revealed a similar significant effect of time (one-factor ANOVA  $F_{23, 131} = 13.107$ ,  $P < 0.001$ ; Fig. 6b), although the values were consis-



**Fig. 4** Annual gametogenic cycle of female *H. purpurascens*. Histograms show the relative abundance of the different cell types within the ovaries in histological sections of gonads of urchins from Bare Island. Numbers above each column show the number of urchins from which the frequencies were estimated

tently higher and the variations more marked than those for males (compare Fig. 6a with Fig. 6b). Once again, a posteriori comparisons showed no clear trend between significant means; however, one large annual peak in the GI values occurring approximately 1 month after the start of the male peak (August to November 1997) was observed for female urchins (Fig. 6b). This suggests that the onset of spawning for females occurred approximately 1 month after the onset of spawning for males. The larger error bars, particularly in females, showed an intrapopulation variability in the development of the GI. Comparison of the GI values of both sexes showed a significant Pearson correlation coefficient ( $r_s = 0.77$ ,  $n = 24$ ,  $P < 0.01$ ), indicating some synchrony between the sexes. When sexes were pooled, values for the GI differed significantly among months but as with the analyses for males and females separately, the variations in the means that were significant showed no clear trends (one-factor ANOVA  $F_{24, 289} = 10.361$ ,  $P < 0.001$ ; Fig. 6c).

There were no significant differences among locations in the gonad indices of male urchins from Bare Island, Long Bay or Shark Bay (one-factor ANOVA  $F_{2, 31} = 2.290$ ,  $P = 0.118$ ). Gonad indices for females were significantly different at all locations (one-factor ANOVA  $F_{2, 23} = 6.346$ ,  $P = 0.006$ ; Tukey's Test), however, since the volume of urchins differed at each



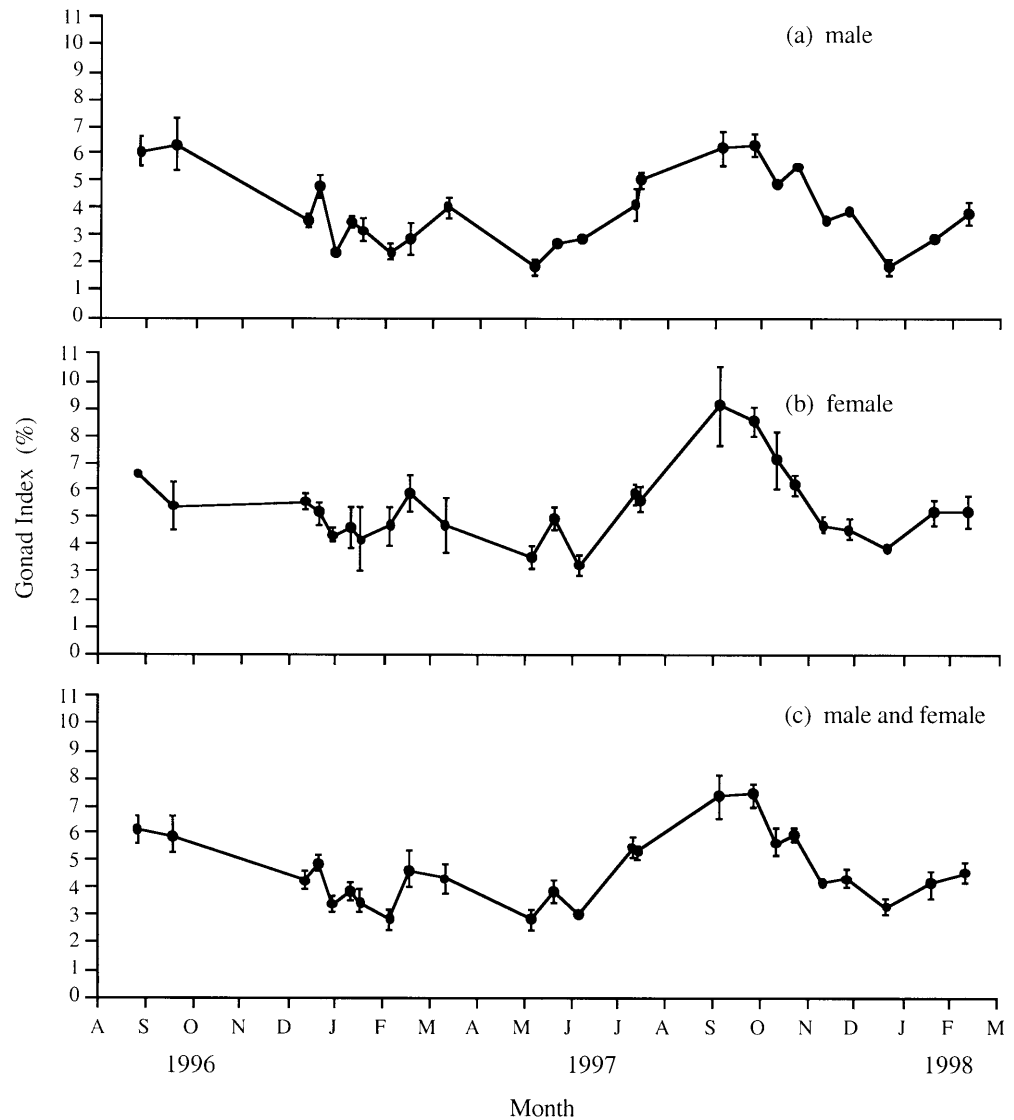
**Fig. 5** Annual gametogenic cycle of male *H. purpurascens*. Histograms show the relative frequencies of maturity stages of the testes in histological sections of gonads of urchins from Bare Island. Numbers above each column show the number of urchins from which the frequencies were estimated

location (mean  $\pm$  SE  $\text{cm}^2$ : Bare Island =  $76.16 \pm 7.52$ ; Long Bay =  $43.17 \pm 2.42$ ; Shark Bay =  $117.19 \pm 22.17$ ) and gonad indices are a function of size (Gonor 1972), it was impossible to distinguish whether this result was due to differences in location per se or if it was simply due to different sized urchins at each location (analysis by ANCOVA could not be done because the range of volumes at each location did not overlap). When sexes were pooled, there was a significant difference in gonad indices between locations (one-factor ANOVA  $F_{2, 57} = 7.052$ ,  $P = 0.001$ ), with indices at Long Bay being significantly larger than those at Shark Bay, but indices at Bare Island being not significantly different from the other two locations. It therefore appears that both the male and the total gonad indices, and perhaps the female indices, determined in this study at Bare Island are generally typical for *H. purpurascens* at different locations near Sydney.

#### Readiness to spawn

The proportion of males and females that could be induced to spawn by injection of KCl showed substantial variation over time (Fig. 7). For example, in February 1997, male urchins spawning on different days varied in their "readiness to spawn" by up to 75%, and females

**Fig. 6** Mean ( $\pm$ SE) gonad index for **a** male, **b** female, and **c** male and female *H. purpurascens* from Bare Island pooled over time. The number of replicates for each date and sex ranged from 3–12, with an average of 6



by up to 100% (Fig. 7). Neither the proportion of males ( $G_{(31)}=46.21$ ,  $P>0.01$ ; Fig. 7a) nor the proportion of females ( $G_{(31)}=33.22$ ,  $P>0.01$ ; Fig. 7b) that were induced to spawn changed significantly over time. However, since the mean proportion of males that were induced to spawn had a significant positive correlation with the mean number of females spawning ( $r_s=0.43$ ,  $n=32$ ,  $0.05>P>0.01$ ), data for both males and females were pooled to discern any overall trends in the data (Fig. 7c). There was significant temporal variation in the proportion of total individuals that were induced to spawn ( $G_{(31)}=58.12$ ,  $P<0.005$ ; Fig. 7c). There were two peaks in the pooled data: one in October–November 1996, and the other in August–December 1997 (Fig. 7c). These peaks in spawning followed the peaks in GI, with a lag of approximately 1–2 months (compare Fig. 6 with Fig. 7).

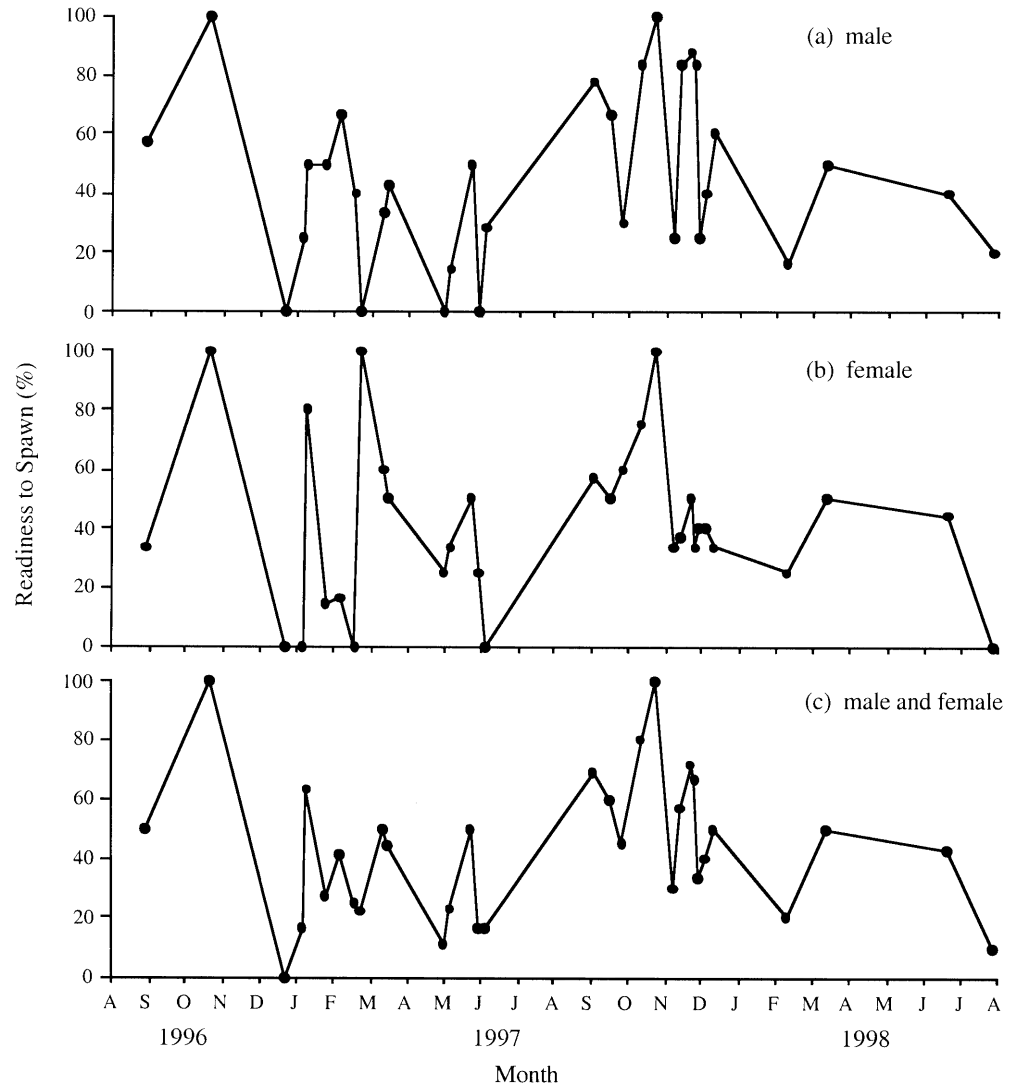
There was no significant difference in the proportion of either males (one-factor ANOVA  $F_{3, 28}=0.800$ ,  $P=0.504$ ), females (one-factor ANOVA  $F_{3, 28}=0.005$ ,  $P=0.999$ ), or all urchins (one-factor ANOVA  $F_{3, 28}=0.601$ ,

$P=0.620$ ) spawning during different phases of the moon, demonstrating that the lunar cycle was not a major factor inducing spawning in *H. purpurascens*.

## Discussion

*H. purpurascens* have a defined breeding period during spring and early summer, but are also capable of reproducing sporadically throughout the year. The continuous presence of mature gametes in both males and females, and the ability to reproduce at any time of the year, is atypical for the majority of echinoids (Byrne 1999). As adult *H. purpurascens* are patchily distributed amongst beds of the kelp *E. radiata* at Bare Island and more generally around Sydney (Williamson et al., unpublished), this flexibility in the timing of reproduction allows individuals that may have been isolated during the breeding period to release gametes when an appropriate cue is encountered. Therefore, new recruits may enter the population in two ways: (1) through synchro-

**Fig. 7** Percentage of *H. purpurascens* that were induced to spawn by potassium chloride injection over time for **a** male, **b** female, and **c** males and females pooled. The number of replicates for each date and sex ranged from 2 to 11, with an average of 6



nous spawning throughout the breeding season from the majority of adult urchins in *E. radiata* beds; and (2) through smaller haphazard reproduction events from encountering individuals at other times of the year.

Reproductive success for many marine invertebrates, including sea urchins, is greatly reduced where population size is small or intermediate (e.g. Levitan et al. 1992; Oliver and Babcock 1992; Babcock and Keesing 1999) due to the reduced chance of a sperm encountering a viable egg in time to successfully fertilise it, thus eventually decreasing the population over time (Allee 1931). Several studies on sea urchins have shown that dilution of sperm greatly reduces the percentages of fertilisation (reviewed in Levitan 1995), and *Strongylocentrotus purpuratus* in the field have less than a 10% chance for successful fertilisation when males are separated from females by as little as 1 m (Pennington 1985). Since clumping of individuals can increase reproductive success (Levitan et al. 1992), perhaps the patchy distribution of *H. purpurascens* (i.e. more than one urchin per plant) actually increases the

success of the smaller reproduction events outside the breeding season.

*H. purpurascens* reached sexual maturity at sizes between 11 and 26 mm in diameter, and was gonochoristic. Since the growth and maturation of echinoids is influenced by diet quality and quantity (Ebert 1968; Andrew 1986; Byrne 1990; Minor and Scheibling 1997; Barker et al. 1998; Byrne et al. 1998; Russell 1998; Meidel and Scheibling 1999; Williamson 2001), and urchins used to determine the onset of sexual maturity in this study were collected from several host plants of differing food quality, diet may be a causal factor in the range of maturation sizes observed here. The sex ratio of *H. purpurascens* was consistently 1:1, typical of gonochoristic echinoderms (Lawrence 1987).

The sequence of oogenesis in *H. purpurascens* was similar to those described for other echinoids: oogenesis started with primary germ cells that developed through several vitellogenic stages, and resulted in ova (Pearse and Cameron 1991). Pre-vitellogenic and early vitellogenic oocytes were consistently the most abundant types

of oocytes present, similar to echinoids such as *Stylocidaris affinis* and *Paracentrotus lividus* (Holland 1967; Byrne 1990), but contrasting with sea urchins with synchronous oogenesis, such as *Strongylocentrotus purpuratus* (Gonor 1973c) and *Centrostephanus rodgersii* (King et al. 1994). Late vitellogenic oocytes and ova of *H. purpurascens* were large and rich in lipid, characteristics that are consistent for other urchins with lecithotrophic larvae (Raff 1987; Emler 1995; Byrne et al. 1999). Unlike the majority of echinoids, however, the ovaries of *H. purpurascens* contained eggs at all stages of development. Since the standard method of classifying female histological sections is based on determining the overall stage of maturity of developing eggs within an acinus (Byrne 1990; King et al. 1994), another method had to be designed to describe the gametogenic cycle of these urchins. The method of determining the proportion of eggs in different stages of development used here proved to be a reliable descriptor of oogenesis for *H. purpurascens*. However, it may have overestimated the abundance of smaller eggs, such as pre-vitellogenic oocytes and early vitellogenic oocytes, due to the increased likelihood of encountering a smaller egg that has been sectioned through the nucleus than that of an advanced egg when counting.

The oogenic pattern was similar throughout the year, with all stages of developing oocytes and ova present each month. Moreover, the proportion of eggs at each stage showed no temporal pattern in abundance, suggesting that vitellogenesis is continuous in this species. The presence of ova throughout the year indicated that females of this species have the potential to spawn at any time of the year. This contrasts with the oogenic cycle of other Australian echinoids (e.g. Pearse and Phillips 1968; Williams and Anderson 1975; O'Connor et al. 1978; Laegdsgaard et al. 1991; King et al. 1994; Byrne et al. 1999), and the majority of echinoids worldwide (e.g. Bennett and Giese 1955; Fénaux 1968; Pearse 1969a, b, 1970; Dix 1970; Gonor 1973b; Pearse et al. 1986; Byrne 1990; Lozano et al. 1995s; Meidel and Scheibling 1998), where oogonial proliferation occurs during part of the year, followed by storage of ova for a few weeks to a couple of months prior to a discrete spawning period. Degenerating ova were observed in small numbers throughout the year in *H. purpurascens*, suggesting that not all ova were released when spawning occurred.

Alternatively, oogenesis in *H. purpurascens* may be a rapid process, where pre-vitellogenic oocytes develop into ova in one or a few weeks if conditions are favourable. Such rapid oogenesis has been observed for several other urchins, such as *Diadema setosum* (Pearse 1970; Lessios 1981) and *Centrostephanus rodgersii* (Byrne et al. 1998). If this was the case, monthly sampling may have obscured any specific pattern of oogenesis for *H. purpurascens*.

Spermatogenesis in *H. purpurascens* was similar to other species of echinoids (Pearse and Cameron 1991) and was classified into the maturity stages typical for echinoids. Most developmental stages of testes were

observed in all months. Unlike the oogenic cycle, however, the spermatogenic cycle showed a distinct temporal pattern in the relative proportion of the different maturity stages present. Although mature testes and partially spawned testes were present every month, indicating that gametes were released throughout the year, the significant increase in the proportion of mature testes in spring followed by a similar increase in the proportion of partially spawned testes in late spring and early summer, points to increased breeding activity during this time. The lack of any spent testes suggests that the production of new sperm in the germinal layer is continuous. Therefore, male *H. purpurascens* have the potential to be opportunistic spawners, responding to the presence of favourable exogenous factors. The temporal patterns of spawning observed (e.g. Fig. 7) suggest that favourable conditions for spawning occur during spring and early summer.

Consistently higher gonad indices were recorded for female *H. purpurascens* than for males, a pattern reported for some echinoids (e.g. Munk 1992; Jong-Westman et al. 1995; Meidel and Scheibling 1998) but not others (e.g. Bennett and Giese 1955). Also common in studies on other echinoids was the synchrony between the sexes observed here, where a gradual increase in gonad indices in winter, leading to a peak in late winter to mid-spring, occurred for both male and female urchins. Since gonad indices generally increase prior to a spawning event and then decrease during the event (Byrne 1999), this data is consistent with the pattern shown by the gametogenic data. Moreover, the seasonal timing of this peak in the reproductive cycle for male and female *H. purpurascens* was similar to many temperate echinoids, both in Australasia (e.g. Dix 1970; Walker 1982; King et al. 1994) and in the same season in the northern hemisphere (e.g. Keats et al. 1984; Meidel and Scheibling 1998).

Although reproductive cycles for some echinoids can differ between relatively close locations (Walker 1982; Byrne 1990; King et al. 1994, but see Byrne et al. 1998), the gonad indices for combined sexes and male *H. purpurascens* from three locations around Sydney were roughly synchronous. This suggests that the results presented here on the male reproductive cycle are representative of *H. purpurascens* in the Sydney region. It was impossible to directly determine if the female reproductive cycle studied at Bare Island was indicative of the region due to the variation of urchin sizes at the three locations studied. However, similarity in male indices at different locations, along with the lack of seasonality in the female spawning data and gametogenic cycle at Bare Island, suggests that there is unlikely to be a difference in the female reproductive cycle around Sydney.

Urchins typically allocate energy to the gonads just prior to and during gametogenesis (Pearse and Cameron 1991). The relationship with the GI and the gametogenic cycle indicates that *H. purpurascens* may also do this. Interestingly, when the GI increased in females the

proportion of oocytes in different stages of development remained unchanged, indicating that the ovaries were growing in bulk, but not changing in constitution. Therefore, although females may spawn episodically throughout the year, they are likely to release more ova during spring than at other times of the year due to an increase in the size of the gonads. As the GI for males increased, however, the proportion of growing and premature testes decreased and the proportion of mature and partially spawned testes increased. This suggests that, although the males appeared to be opportunistic spawners through the year, they increased the maturity of their testes in winter and had a large spawning event in spring, releasing proportionally more gametes than females during this time.

Unlike the gametogenic cycles and the gonad indices, the proportion of male and female *H. purpurascens* that could be induced to spawn was variable over time. When sexes were pooled, however, there was a peak in the proportion of spawning from late winter to early summer that correlated with the observed peak in the GI. This indicates that spawning occurred unpredictably throughout the year, but was highest during this time.

Variation in spawning throughout the year showed no relation to lunar phases, in contrast to reports of other urchins spawning in synchrony to this exogenous cue (Pearse 1968, 1970; Kennedy and Pearse 1975; Lessios 1984). Therefore, some other endogenous or exogenous cue may affect the spawning of the population. For example, the abundance of phytoplankton in the surrounding water may trigger the onset of spawning as in some echinoids (Himmelman 1975; Starr et al. 1990). Spawning may be an extremely localised phenomenon. Proximal cues may stimulate or stress a few individuals within a population to spawn and the gametes or pheromones released by these individuals in turn may initiate a broader spawning response. Such a model for inducing neighbouring conspecifics to spawn has been proposed for echinoids by a number of researchers (Rothschild and Swan 1951; Reese 1966; Kennedy and Pearse 1975; Pennington 1985).

In conclusion, *H. purpurascens* has a defined breeding season in spring and early summer, with the ability to reproduce throughout the year. While the majority of the population appear to be reproducing during this breeding season, reproduction at other times of the year is likely to occur on an individual basis in response to exogenous and/or endogenous factors.

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