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Evidence for multiple year classes of the giant Australian cuttlefish *Sepia apama* in northern Spencer Gulf, South Australia

Karina C. Hall · Anthony J. Fowler · Michael C. Geddes

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Abstract Giant Australian cuttlefish form a mass spawning aggregation at a single site in northern Spencer Gulf (NSG) in South Australia every austral winter. Samples of cuttlefish were collected from this region over three consecutive years. Analysis of regular growth increments in the cuttlebones of these individuals, revealed a polymorphism in growth pattern for both sexes. Three distinct ''bone patterns'' were identified based on the variation in increment widths over the lengths of the bones. All bones analysed conformed to one of the three bone patterns, and the increment width patterns were consistent between years. Interpretation of the patterns, suggested that *Sepia apama* have two alternative life cycles. The first involves rapid juvenile growth during the first summer after hatching, with maturity reached within 7–8 months. These individuals return to spawn in their first year as small individuals. The second life cycle involves much slower juvenile growth during the first summer, with maturity deferred until their second year, when they return to spawn as much larger individuals. Thus, the age compositions of populations of *S. apama* in the NSG appear to consist of two year classes for both sexes.

Keywords Cuttlefish · *Sepia apama* · Life history · Ageing · Cuttlebone · Growth increments · Phenotypic plasticity

K. C. Hall (✉) · A. J. Fowler
South Australian Research and Development Institute, PO Box 120, Henley Beach, Adelaide, SA 5022, Australia
e-mail: karinacuttle@hotmail.com

K. C. Hall · M. C. Geddes
Department of Environmental Biology, University of Adelaide, Adelaide, SA 5005, Australia

Introduction

Historically, age-based population parameters such as growth rate and lifespan were estimated indirectly for cephalopods through length-based methods (Voss 1983). However, recent age estimates from direct ageing methods and the culture of known-age individuals in captivity suggest that length-based methods may overestimate ages for cephalopods (Caddy 1991; Jackson et al. 2000). The protracted spawning period, seasonal plasticity in growth rates and migratory life cycle of many species can produce ''micro-cohorts'' of different sizes in a single year or area that can be incorrectly interpreted as separate-year classes (Hatfield and Rodhouse 1994; Pierce and Guerra 1994; Lipinski 1998). Therefore, it is preferable to develop a method of direct age estimation for cephalopod species.

Direct age estimation involves the identification and interpretation of periodic growth

increments deposited in hard tissues over the lifetime of an individual (Campana and Thorrold 2001). Four criteria must be fulfilled for a structure to be appropriate for age estimation (Beamish and McFarlane 1983): (1) that increments are sufficiently clear for accurate interpretation; (2) that increments can be correlated with a regular and measurable time scale; (3) that increments form throughout life; and (4) that once formed increments are permanent and not resorbed or remobilised during periods of negative growth. Growth increments in fish otoliths have been effectively used for this purpose for many years (Pannella 1971; Campana and Neilson 1985) and techniques to visualise and accurately interpret increments are well established (Campana 2001).

Attempts to develop similar methods for cephalopod species are more recent. Regular growth increments have been identified in the statoliths, gladii, stylets, cuttlebones, beaks and eye lenses of many species (Rodhouse and Hatfield 1990). Statoliths are functionally and structurally analogous to fish otoliths and have thus been the focus of most attention to date, with promising results obtained for many squid species (Jackson et al. 2000). Attempts to age cuttlefish from statoliths have been less successful due to the difficulty in interpretation of growth increments. The irregular and concentric deposition of aragonite crystals in cuttlefish statoliths, result in a strong radial appearance, and the low percentage of organic matter make opaque zones indistinct (Bettencourt and Guerra 2000). To date only one cuttlefish species, *Sepia officinalis*, has been aged successfully via statoliths, and the daily periodicity of increment formation was only validated for juveniles and individuals up to 240 days old (Bettencourt and Guerra 2001; Challier et al. 2002; Challier et al. 2005). The age structures of adult populations are yet to be verified by direct age estimates.

Most attempts to age cuttlefish have concentrated on the cuttlebone. This structure functions as a dorsal backbone providing both support and buoyancy control (Fig. 1). It consists of a thin, hard, calcified, dorsal shield and a ventral porous phragmocene comprised of numerous narrow chambers, delineated by chitinous septa (Bandel and von Boletzky 1979). The cuttlefish controls its buoyancy by moving gas or liquid into or out of the chambers as required (Denton and Gilpin-Brown 1961). As the cuttlefish grows, further septa are laid down at the anterior end (Fig. 1). Early studies concluded that the periodicity of chamber formation was daily (Choe 1963; Packard 1972), however, recent studies have shown that periodicity is related to growth rate rather than chronological age (Richard 1969; von Boletzky 1974; Ré and Narciso 1994; Bettencourt and Guerra 2001).

The growth rate of cephalopods is strongly influenced by temperature and food availability and thus subject to seasonal fluctuations. The width of individual chambers in the phragmocene also vary with growth rate (Hewitt and Stait 1988), which allowed *S. officinalis* from Southern Brittany to be separated into two age groups based on the seasonal variation in chamber widths (Le Goff et al. 1998). Analysis of patterns in cuttlebone micro-structure also separated two different stocks of *S. esculenta* in Korean waters (Kim and Hong 1991).

Sepia apama is the largest cuttlefish species in the world, with a maximum recorded size of 520 mm mantle length (ML) (Gales et al. 1993) and over 12 kg weight (K. S. Kassahn, unpublished data). It is widely distributed across temperate southern Australia from Moreton Bay in southern Queensland to Point Coates in Western Australia (Lu 1998). A dense spawning aggregation of *S. apama* occurs every austral winter over a restricted area of rocky reef in the northern Spencer Gulf (NSG) of South Australia (Fig. 2). A small hand-jig fishery that targets the aggregation underwent rapid expansion between 1993 and 1998 (Hall and Fowler 2003). An understanding of the life history of the species and age structure of populations in the NSG was required to facilitate appropriate management of the fishery.

Analysis of length–frequency data from the aggregation area suggested the presence of multiple year classes in the population (Hall and Fowler 2003). However, given the doubt surrounding age estimates derived from length-based methods for cephalopods, the possibility of a direct ageing method was also investigated (Hall and Fowler 2003). Regular growth increments

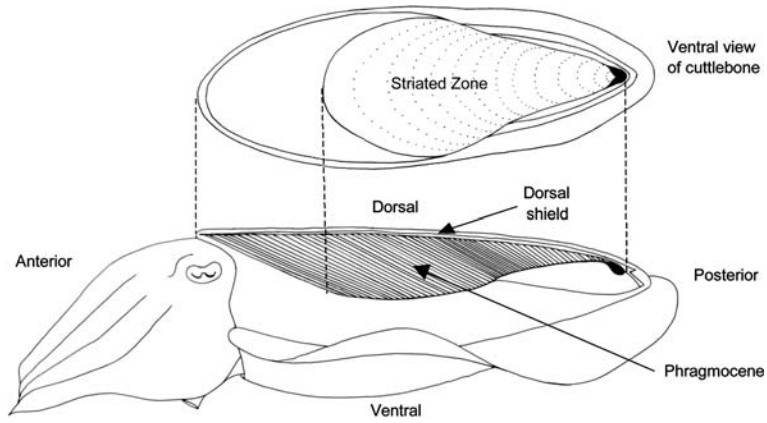

Fig. 1 Diagram illustrating the location and orientation of the cuttlebone within the dorsal mantle of cuttlefish

were identified in the cuttlebone, statoliths, beaks and eye lenses of *S. apama*; but only in the cuttlebone were increments visible over the entire length of the growth axis (Hall and Fowler 2003). The main aims of this study were: (1) to determine if seasonal variations in the widths of increments in the cuttlebones of *S. apama* could be used to estimate the age of individuals; and if so, (2) to determine the age structure of populations in the NSG to gain a better understanding of the life history of the species.

Methods

Sample collection

Spencer Gulf is a shallow hydrological inverse estuary with little freshwater inflow (Nunes Vaz et al. 1990). In the upper reaches, water temperatures vary considerably with averages from 12°C in mid-winter to 28°C in mid-summer (Nunes and Lennon 1986). *S. apama* aggregate along approximately 8 km of coastline (with a subtidal reef area of 0.64 km^2) over hard substrate, in 2–8 m depth of water. A sample of approximately 30 cuttlefish was collected from a random location at the aggregation area at the start (May) and towards the end (August) of each spawning season from 1998 to 2000 (Table 1). Individuals within a 20 m radius of the boat anchor were captured with a hand net while snorkelling. Cuttlefish were stored on ice for transport back to the laboratory and dissected the following day. A sample of hatchlings was also collected from the aggregation area in September or October each year (Table 1). Hatchlings were captured with a small hand net as soon as they emerged from egg capsules and preserved in 70% ethanol.

Outside of the spawning season, i.e. from September to March, few cuttlefish were present at the aggregation area. To provide a more comprehensive analysis of the life history, samples from other areas of NSG (north of latitude 33°55′S; Fig. 2) were collected during this period. Samples were obtained, as by-catch, from commercial prawn trawlers (Carrick 1997) in November and April of 1998–2001. The prawn trawlers used standard paired otter trawl equipment with a cod-end mesh size of 45 mm. Samples were also obtained in February and/or April of 1999–2001 from a systematic trawl survey completed by SARDI Aquatic Sciences in the NSG. The survey was designed to document the recruitment of 0+ snapper using a purpose-built fine mesh net with a cod-end mesh size of 12 mm deployed with a small otter trawl. Sampling stations covered most channel areas of NSG but exact locations varied between sampling trips. Overall, a total of 488 cuttlefish were sampled for the study; 207 were female, 253 were male and 28 were hatchlings (Table 1).

Cuttlebone analysis

The dorsal ML and weight of each cuttlefish was recorded before the cuttlebone was extracted via a longitudinal incision along the mid-line of the

Table 1 Samples of *S. apama* collected from the main aggregation area (aggregation) and wider northern Spencer Gulf (NSG) for age estimation by analysis of cuttlebone microstructure

Date	Sample location	Sample method	Number male:female	Hatchlings ML range (mm)	Males ML range (mm)	Females ML range (mm)
19 May 1998	Aggregation	Hand net	17:13		147–280	135–233
4 August 1998	Aggregation	Hand net	14:10		140–275	137–210
17 September 1998	Aggregation	Hand net	10	12.3–13.6		
15 November 1998	NSG	Prawn trawl	15:16		88–170	100–155
2 February 1999	NSG	Snapper trawl	6:4		69–107	74–97
14 April 1999	NSG	Prawn trawl	22:21		85–265	67–233
28 May 1999	Aggregation	Hand net	23:13		133–330	163–247
29 July 1999	Aggregation	Hand net	19:11		152–300	147–215
23 September 1999	Aggregation	Hand net	6	12.4–14.2		
7 November 1999	NSG	Prawn trawl	2:2		140–150	103–140
20 February 2000	NSG	Snapper trawl	28:14		53–236	92–192
8 April 2000	NSG	Snapper and prawn trawl	26:20		78–235	78–196
19 May 2000	Aggregation	Hand net	14:15		170–305	172–248
3 August 2000	Aggregation	Hand net	15:19		153–302	138–189
20 October 2000	Aggregation	Hand net	12	10.7–13.7		
4 April 2001	NSG	Snapper trawl	52:51		68–273	47–225
Total			253:207	10.7–14.2	53–330	47–248

dorsal mantle. Cuttlebones were measured (bone length, BL) and dried at room temperature. To reveal the internal micro-structure, the soft ventral phragmocene of the cuttlebone was sliced along the longitudinal axis with a scalpel, until the surface of the hypostracum (ventral surface of the dorsal shield) was reached. The phragmocene was carefully cut and scraped away from the surface of the hypostracum on one half of the bone (Fig. 3a). Removed septa left a distinct line on the surface of the hypostracum (Fig. 3b). A growth increment was considered to be the width of a complete chamber, i.e. the distance between two consecutive septa (Fig. 3d, e).

Digital images were taken along the length of the hypostracum from the posterior forked region to the anterior rim. Images were taken via a stereo dissecting microscope, fitted with a digital video camera that was connected directly to a computer terminal. Increment widths (IncWi) were measured from saved images to the nearest 0.01 mm with SigmaScan Pro© image analysis software and the total number of increments calculated for each bone (IncNo). For hatchling bones, growth increments could be seen through the dorsal surface of the cuttlebone without preparation under a dissecting microscope with transmitted light (Fig. 3c). Digital images of the whole bones were taken and IncWi and IncNo measured as above.

Data analysis

The increments along each bone were numbered from the anterior (most recently formed) to the posterior end. This allowed the patterns of IncWi from different bones of the same sample date to be plotted on one graph, aligned to the date of capture. Three consistent patterns of IncWi were observed, and the bones from each sample were divided into three ''bone pattern'' groups. For each combination of bone pattern and sex within a sample, the mean IncWi of every increment was calculated and plotted against increment number from the anterior end, to provide an average pattern of IncWi. Since the pre-hatch increments of different individuals were rarely aligned exactly, mean IncWi was only calculated to the hatching mark (as indicated by a short section of very narrow increments; see Sect. ''Results'' for more detail). The mean IncWi of the last ten anterior increments (AvIncWi) up to and including the penultimate increment was determined for each individual, to indicate growth rate just

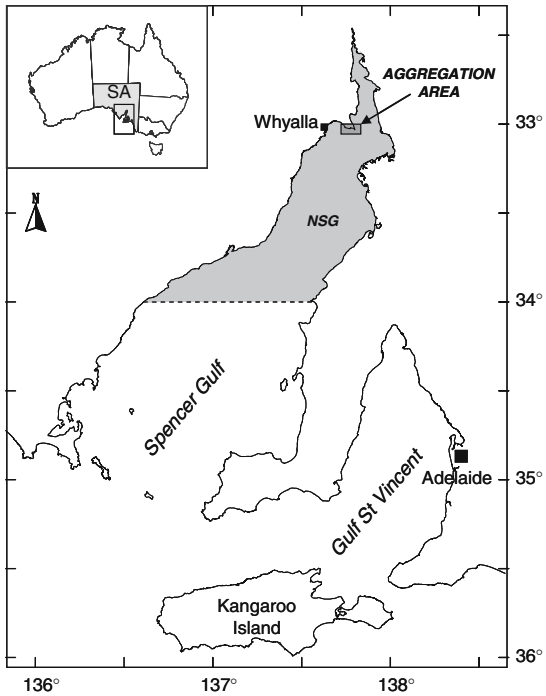

Fig. 2 Map of the South Australian Gulf system showing the shape and orientation of Spencer Gulf and the location of the aggregation area and NSG

prior to capture. The last increment was excluded in case of partial formation at the time of capture.

The mean BL, IncNo and AvIncWi were calculated for each bone pattern and sex combination in each sample. No statistical between-sample date comparisons were attempted due to the unbalanced distribution of bone patterns amongst samples and some small sample sizes. For six samples of adequate size, a 2×2 factorial multivariate analysis of variance (MANOVA) was used to test for differences between the two independent variables of bone pattern and sex, for the three attributes of BL, IncNo and AvIncWi as dependent variables. Wilk's criterion was used in the presentation of results, but four different criteria produced similar results. For most samples only two of three bone patterns were present in sufficient numbers for statistical comparison, and only for April 2001 were all three bone patterns tested simultaneously. Sample sizes in combination cells were usually unequal. To investigate the impact of each main effect and interaction on the individual variables, a 2×2 univariate ANOVA was completed post hoc on each variable at each sample date, using Bonferroni adjusted alpha values of 0.015 to guard against inflated type I errors (Tabachnick and Fidell 2001). A discriminant function analysis (DFA) was also completed on the whole data set for each sex (149 males and 122 females) to assess whether group membership of the three bone patterns could be reliably predicted by the three variables, regardless of sample date.

The multivariate statistical assumptions of homoscedasticity, linearity and normality were evaluated by visual assessment of residual plots, bivariate scatterplots, frequency histograms and normal probability plots of appropriately grouped data. Both BL and IncNo required logarithmic transformation to correct for heteroscedasticity (Zar 1999). As both MANOVA and DFA tests are particularly susceptible to the influence of outliers, the Mahalonobis distance of each case from the overall group centroid was checked to ensure it did not exceed the critical χ^2 value of 16.3 ($df = 3$; $p < 0.01$) (as per Tabachnick and Fidell 2001). No outliers were identified based on this criterion. All statistical analyses were completed using SPSS Version 10.0 for Windows or JMP in Student Version 4.0 and assessed at the $p = 0.05$ significance level unless otherwise stated.

Results

There was a strong linear relationship between BL and ML for both sexes (males: $r^2 = 0.99$, $n = 247$; females $r^2 = 0.99$, $n = 205$). This relationship was significantly different from isometry in both cases [paired t-test (two-tailed); males $t = -26.4$, $df = 246$, $p < 0.01$; females $t = -29.8$, $df = 204$, $p < 0.01$] with the difference between the two variables increasing in larger individuals.

Growth increments, as delineated by the remnants of removed septa, were highly visible on the surface of the hypostracum and were easily measured and counted (Fig. 3). Few bones were rejected for analysis ($n = 10$; 2.2% of bones examined); only those with severe injuries that resulted in excessive calcification on the dorsal

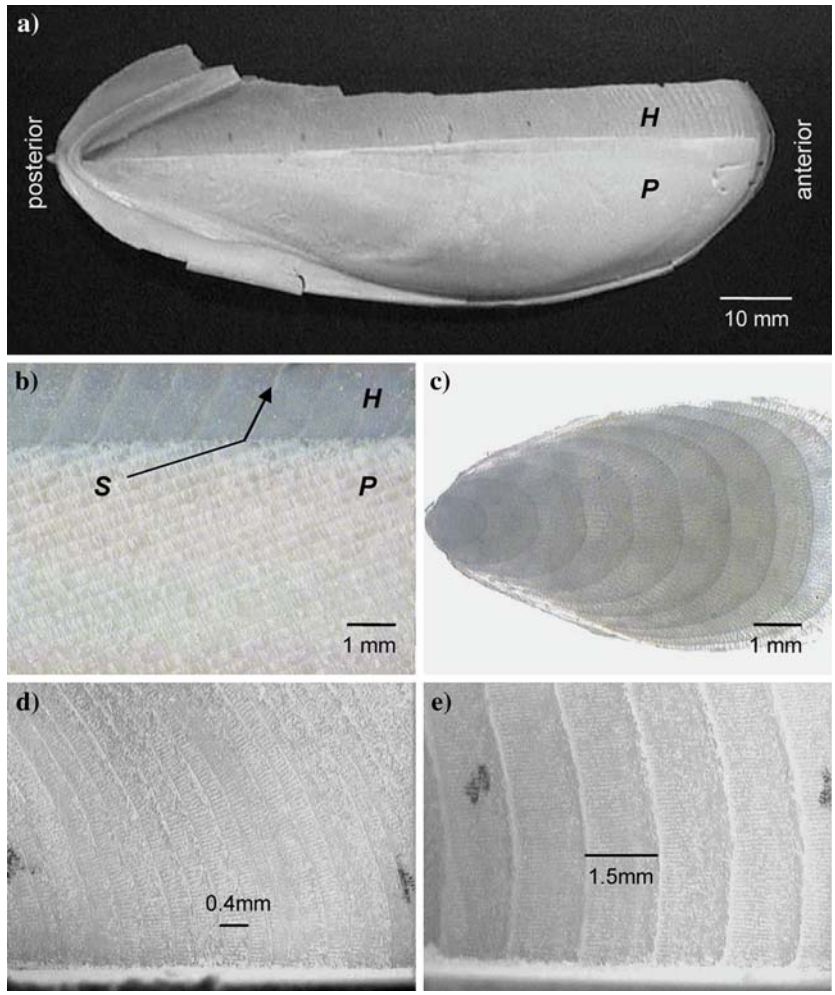

Fig. 3 (**a**) Cuttlebone of *S. apama* prepared for viewing the internal increment structure on the ventral surface of the dorsal shield (hypostracum); **(b)** junction between the internal chambers of the phragmocene (*P*) and the surface of the hypostracum (*H*) showing the pattern formed by the remnants of the septa (*S*) after the phragmocene was removed; (**c**) cuttlebone of *S. apama* hatchling with fully formed internal microstructure; (**d**) example of the narrow increments and (**e**) wide increments found in a single bone

shield that obscured growth increments. For all other bones, growth increments could be confidently interpreted along the entire length of the bone, including the pre-hatch region in the fork at the posterior end. IncWi varied considerably over the length of a single bone (Fig. 3d, e) and a number of consistent patterns in this variation were discernible (Fig. 4).

Description of increment width patterns

Hatchlings emerged from egg capsules in September and October, with fully formed cuttlebones of between 10 and 13 mm BL and 10–13 IncNo (Figs. 3c, 4a). IncWi became progressively narrower towards the anterior end of the bone, the section deposited just prior to hatching. In November, NSG samples contained small adults of both sexes with bones of between 100 and 120 mm BL and only one distinctive pattern of IncWi (Fig. 4b). The posterior end of the bones showed a similar pattern to that of hatchling bones, followed by a short section of very narrow increments. This was interpreted as the ''hatching mark'' (indicated by arrows in Fig. 4). IncWi then increased to form a section of wider increments, followed by a second section of narrow IncWi, before another section of rapidly increasing IncWi just prior to capture (Fig. 4b).

For NSG samples taken in February and April, there were three distinct patterns of IncWi in bones (Fig. 4c–e; only those for February are shown). Bone pattern 1 was characterised by a single section of wide IncWi following hatching,

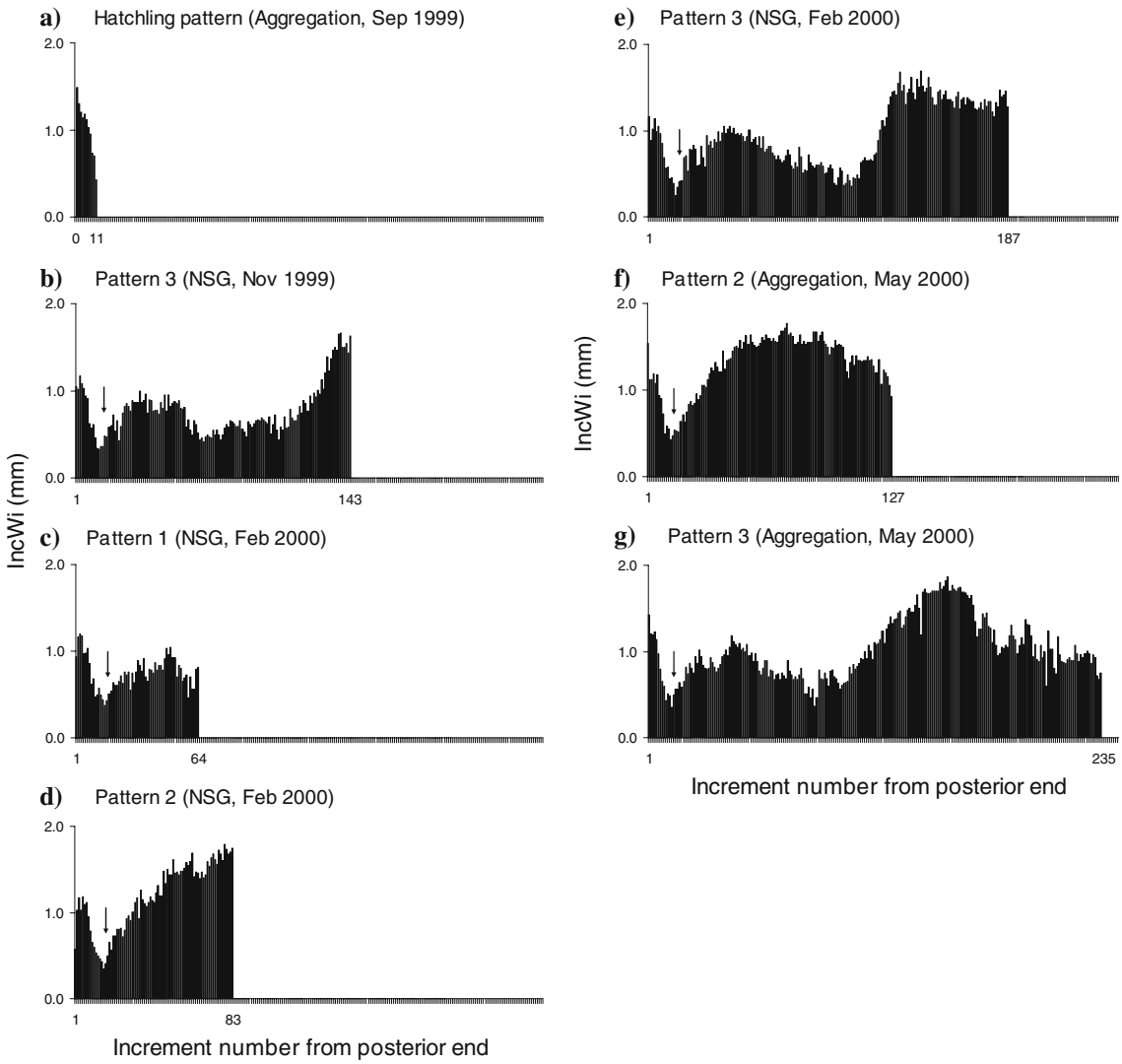

Fig. 4 Representative examples of the different IncWi patterns of cuttlebones of *S. apama* collected in different months (September 1999–May 2000). The hatching mark of each bone pattern is indicated by an *arrow*

with a slight decrease in IncWi prior to capture and was generally found in the smallest animals (50–60 mm BL) (Fig. 4c). Bone pattern 2 was characterised by a rapid increase in IncWi after hatching with no subsequent decline in IncWi prior to capture and was generally found in larger animals (90–100 mm BL) (Fig. 4d). Bone pattern 3 showed two sections of increased IncWi post-hatching and was found in the largest animals (>170 mm BL) (Fig. 4e). This pattern appeared to be an extension of the bone pattern found in November (Fig. 4b). Thus, the bones from individuals captured in November were also designated as bone pattern 3.

All cuttlefish sampled at the aggregation area in May, regardless of sex or size, had bones with either of two distinct patterns of IncWi. The patterns in bones of smaller males and females showed only one section of wider IncWi after hatching (Fig. 4f) and appeared to be an extension of bone pattern 2 found in the February samples. The patterns in the bones of larger adults of both sexes had two regions of wide IncWi after hatching (Fig. 4g) and appeared to be

an extension of bone pattern 3 found in the February samples. All of the individuals collected from the aggregation area were sexually mature.

Variation in increment width patterns

Within a sample, the individual patterns within each bone pattern group were relatively consistent (Fig. 5a, b), as indicated by the small error bars associated with mean IncWi patterns (Fig. 5c). Large deviations in patterns were usually related to prior injuries of cuttlebones, which could be identified by a calcified aberration on the dorsal shield or a black line in the striated zone (von Boletzky and Overath 1991). Injuries were often followed by a short section of very narrow IncWi (Fig. 5a) and the incidence of prior injury to cuttlebones was quite high ($n = 58$, 12% of bones analysed). The sections that showed the least variation tended to be those with either increasing or decreasing IncWi (Fig. 5b). The synchronous nature of these events suggests that their timing may be controlled by external factors that affect all individuals. It also implies that the non-alignment of the pre-hatch sections of different individual patterns (Fig. 5b) may represent differences in hatch date.

Mean IncWi for each bone pattern at each sample date were consistent across all 3 years of sampling for both sexes (Figs. 6, 7). For example, bone patterns 2 and 3 in May 1998 (Figs. 6a, 7a), May 1999 (Figs. 6b, 7b) and May 2000 (Figs. 6c, 7c) were quite similar. Furthermore, there was little difference in bone patterns 2 and 3 between May and August samples in a given year.

Variation in characteristics of each bone pattern

Bones of the three pattern groups differed significantly in BL, IncNo and AvIncWi ($p \leq 0.01$; MANOVA; Table 2). Pattern 3 bones were the largest in all samples, followed by pattern 2 bones, and then pattern 1 bones (Fig. 8a). Mean BL also differed significantly between the two sexes for some samples ($p < 0.01$; ANOVA; Table 3), particularly for pattern 3 bones leading up to and during the spawning season in April, May and August each year, when males tended to have much larger bones than females (Fig. 8a). There was a general increase in mean BL for all bone patterns from November to the following May each year (Fig. 8a). Similarly, mean IncNo also increased for each bone pattern over the same period (Fig. 8b). The difference in mean IncNo between bone patterns 2 and 3 was very pronounced in all samples (Fig. 8b; $p < 0.01$; ANOVA; Table 3). Pattern 3 bones had approximately twice the number of increments as pattern 2 bones. In contrast, there was less difference in mean IncNo between pattern 1 and 2 bones and between the two sexes for any given bone pattern (Fig. 8b; ANOVA; Table 3).

The trends in variation of mean BL and IncNo suggested that animals with pattern 1 and 2 bones may be of similar age (similar mean IncNo) but that pattern 2 individuals reach a larger mean BL for the same time period, possibly through higher growth rates. This hypothesis was supported by the trends in the variation of mean AvIncWi between bone patterns (Fig. 8c). This attribute was assumed to reflect the growth rate of the individual just prior to capture. Pattern 2 bones had a higher mean AvIncWi than pattern 1 bones in all samples, which suggested that they maintained higher growth rates. Mean AvIncWi was also high for pattern 3 bones in November, but subsequently declined through February to May. There was little variation in AvIncWi between the sexes for any given bone pattern, particularly when compared with the degree of difference between different bone patterns (ANOVA; Table 3).

Membership of the three bone pattern groups was reliably predicted using the three variables log BL, log IncNo and AvIncWi (100% correct classification; DFA). Two discriminant functions were described with a combined χ^2 value of 543.99 ($df = 6$; $p < 0.01$; DFA). After removal of the first discriminant function there was still a strong association between the groups and predictors ($\chi^2 = 164.65$; $df = 2$; $p < 0.01$). The two functions accounted for 85.7 and 14.3%, respectively, of the between-group variability for males and 89.1 and 10.9%, respectively, for females. For both sexes, the first function maximally separated pattern 3 bones from the other two groups, while the second function discriminated pattern 1 bones

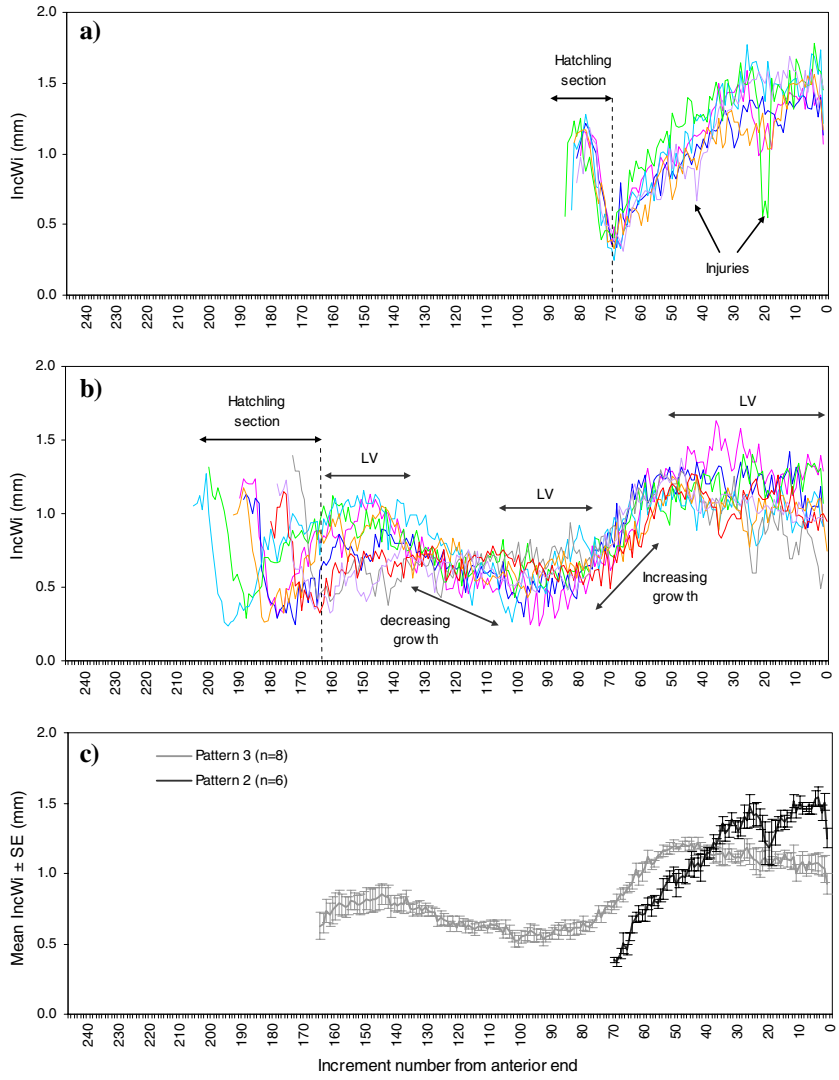

Fig. 5 Individual IncWi patterns for pattern 2 (**a**) and pattern 3 (**b**) bones of female *S. apama*, sampled from the NSG in February 2000 (aligned to the date of capture). (**c**) Mean IncWi patterns averaged for each bone pattern with *standard error bars* indicating level of variation between individuals. *LV* indicates sections with relatively large variation between individual patterns

from pattern 2 bones (Fig. 9). The loading matrix of correlations between predictors and discriminant functions (Table 4) indicate that the best predictor for distinguishing pattern 3 bones from the other two patterns was log IncNo (Function 1), whilst AvIncWi and log BL were the best predictors for separating between pattern 1 and 2 bones (Function 2).

Periodicity of increment formation

To assess the possible periodicity of increment formation, the difference in mean IncNo of each bone pattern between successive sample dates was divided by the number of elapsed days in the time interval (Table 5). As the exact hatch date could not be determined from analysis of the cuttlebone micro-structure a range of possible values for hatch dates between 1 September and 31 October (based on hatching times observed in the field; Hall and Fowler 2003) were used to calculate the rates for the first growth period. These initial rates of increment formation were higher for pattern 2 bones (1.6–2.4 days per increment) than pattern 1 bones (2.0–3.0 days per increment) (Table 5). During the second growth period, however, from February to April, this was reversed and pattern 1 bones had a very rapid

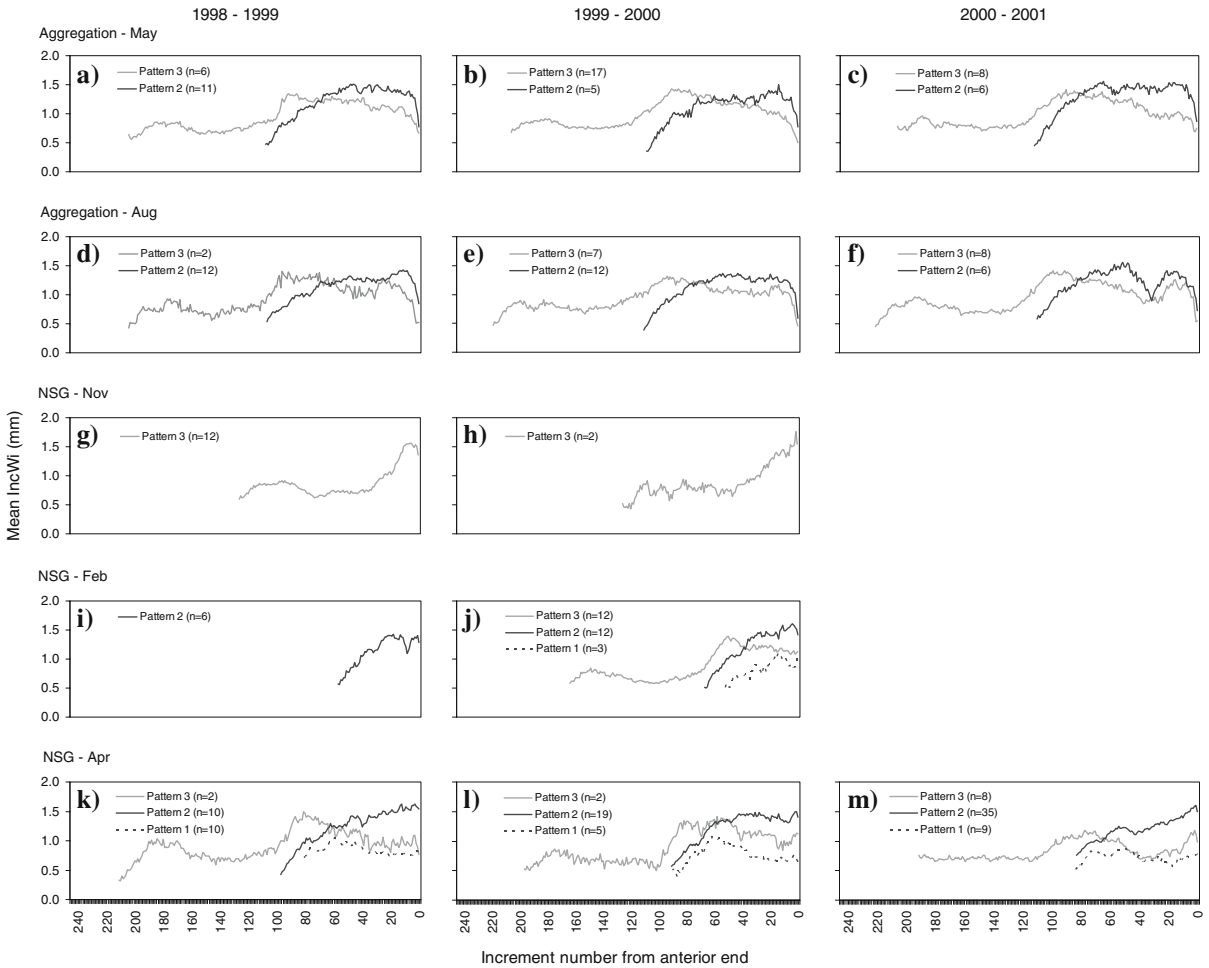

Fig. 6 Mean IncWi of each bone pattern for male *S. apama* sampled from the aggregation area in May (**a–c**) and August (**d–f**), and from the NSG in November (**g–h**), February (**i–j**) and April (**k–m**) each year (May 1998 to April 2001)

rate of increment formation (1.4 days per increment). These increments were narrow in comparison to those from pattern 2 bones.

Data for pattern 1 bones were combined with that of pattern 3 bones to investigate the potential connection between the two. The similarity in the IncWi pattern of the early part of the pattern 3 bone with that of the pattern 1 bones, suggests that the pattern 1 bones continue to grow and become pattern 3 bones in the subsequent year. No pattern 1 bones were found in samples from the aggregation area in winter but quite advanced pattern 3 bones were found in November, not long after the hatching season. If these were pattern 1 bones from the previous April, a much slower rate of increment deposition must occur during the intervening period (almost 6 days per increment) (Table 5). For all growth periods after November, the rate of increment formation for pattern 3 bones was around 2 days per increment.

''Age'' structure of populations

To investigate the possible age structure of populations of *S. apama*, individuals of two samples were categorised according to bone pattern, size and sex (Fig. 10). The first sample consisted of cuttlefish recorded on transects at the spawning aggregation area in May 2000 (Fig. 10-a, b). Females conformed to a unimodal size

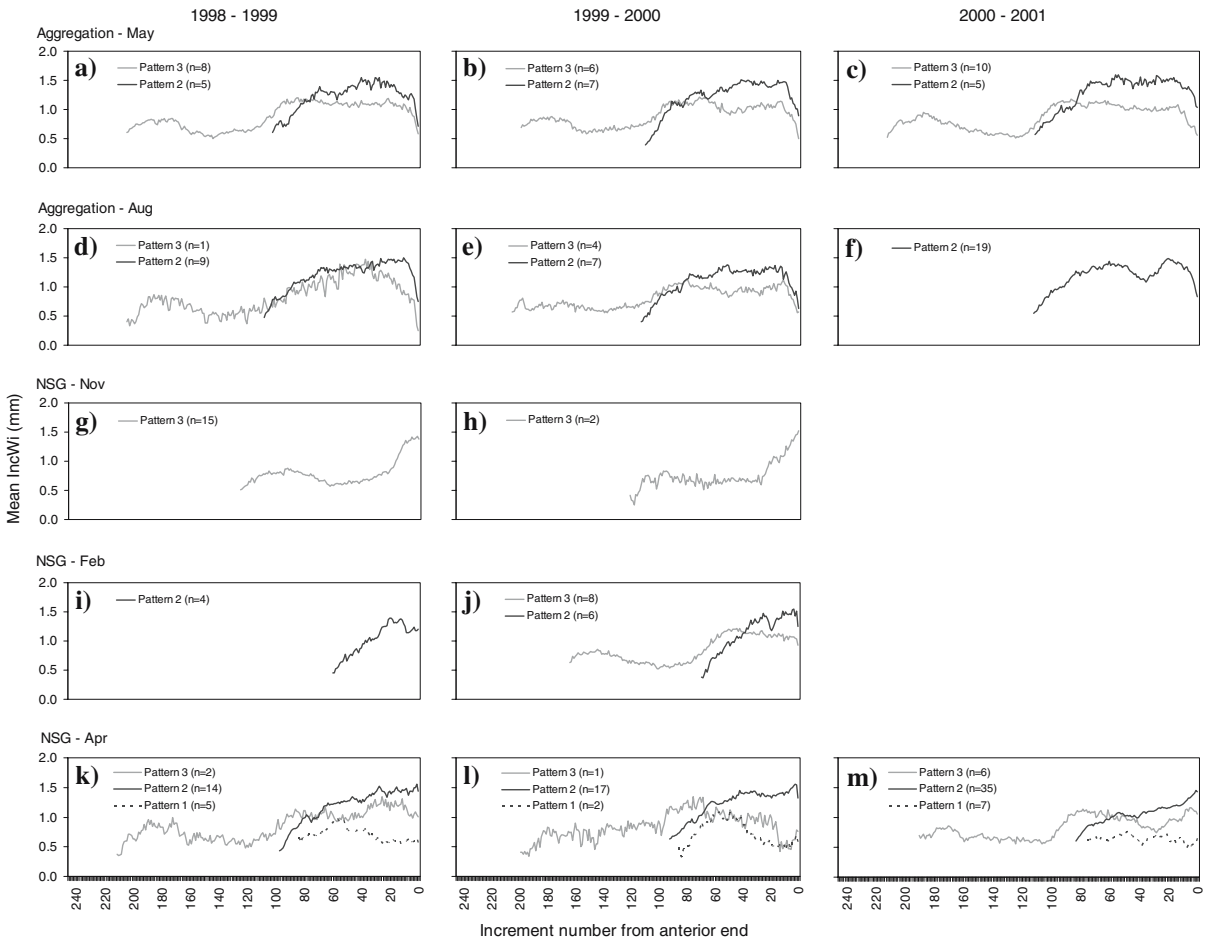

Fig. 7 Mean IncWi of each bone pattern for female *S. apama* sampled from the aggregation area in May (**a–c**) and August (**d–f**), and from the NSG in November (**g–h**), February (**i–j**) and April (**k–m**) each year (May 1998 to April 2001)

distribution that was divisible into two groups according to bone pattern (Fig. 10b). Individuals with pattern 2 bones accounted for 34% of females, whilst a larger percentage (66%) had pattern 3 bones. The size distribution of males was also unimodal, but was skewed to the left by a small number of much larger individuals (Fig. 10a). Males were also divisible into two groups according to bone pattern, with males with pattern 2 bones contributing a slightly larger percentage (58%) to the total than those with pattern 3 bones (42%). No individuals with pattern 1 bones were found at the aggregation area in any year.

The second sample was collected broadly from throughout the NSG in April 2001. This demonstrated a wide size distribution for both sexes (Fig. 10c, d). The female size distribution was again unimodal but consisted of individuals of all three bone patterns (Fig. 10d). Individuals with pattern 2 bones accounted for 80% of females, whilst pattern 1 and 3 bones accounted for only 10% each. The male size distribution showed three size modes, which corresponded with the division of individuals into three groups according to bone pattern (Fig. 10c). Most males (69%) had pattern 2 bones, whilst only 18 and 13% of males had pattern 1 and 3 bones, respectively.

Discussion

The analysis of regular growth increments in the cuttlebones of *S. apama* from NSG revealed a

Table 2 Results of MANOVA between different bone patterns and sexes using three variables (log BL, log IncNo and AvIncWi) simultaneously

Sample	*n*	Sources of variance	Wilks' Λ	*F*	*df*	*P* > *F*
May 1998	30	Pattern (2, 3)	0.1521	69.67	2,25	<0.0001*
		Sex	0.8138	2.86	2,25	0.0761
		Pattern × sex	0.9400	0.80	2,25	0.4612
April 1999	39[a]	Pattern (1, 2)[a]	0.1030	148.00	2,34	<0.0001*
		Sex	0.7255	6.43	2,34	0.0043*
		Pattern × sex	0.9391	1.10	2,34	0.3435
May 1999	35	Pattern (2, 3)	0.2206	53.00	2,30	<0.0001*
		Sex	0.9231	1.25	2,30	0.3009
		Pattern × sex	0.7519	4.95	2,30	0.0139*
February 2000	38[b]	Pattern (2, 3)[b]	0.1267	113.74	2,33	<0.0001*
		Sex	0.8734	2.39	2,33	0.0257*
		Pattern × sex	0.9986	0.02	2,33	0.1643
May 2000	29	Pattern (2, 3)	0.0673	166.42	2,24	<0.0001*
		Sex	0.6159	7.48	2,24	0.0030*
		Pattern × sex	0.7791	3.40	2,24	0.0500*
April 2001	100	Pattern (1, 2, 3)	0.1866	61.14	4,186	<0.0001*
		Sex	0.9112	4.53	2,93	0.0132*
		Pattern × sex	0.9452	1.33	4,186	0.2604

Different samples were tested separately

* Significant at the $p = 0.05$ significance level

[a] Pattern 3 bones were removed from April 1999 sample for analysis due to small sample size ($n = 4$)

[b] Pattern 1 bones were removed from February 2000 sample for analysis due to small sample size ($n = 3$)

polymorphism in growth pattern for both sexes. Three distinct bone patterns were identified based on the variation in increments widths over the lengths of the bones. All bones conformed to one of the three bone patterns, and the increment width patterns were consistent between years. The three bone patterns differed significantly in BL, IncNo and AvIncWi.

Assuming that all individuals hatch in spring from September to October, this polymorphism in growth pattern of cuttlebones suggests the existence of two alternative life cycles for *S. apama*. The first, represented by pattern 2 bones, involves rapid juvenile growth during the first summer after hatching, with maturity reached within 7–8 months. These individuals return to spawn in their first year as small individuals. The second life cycle, represented by the pattern 1 and 3 bones, involves much slower juvenile growth during the first summer, with maturity deferred until their second year, when they return to spawn as much larger individuals. This interpretation implies that the age compositions of the population consists of two year classes for both sexes. This explanation adequately accounts for the wide size distribution of males, but was a surprising result for females, which have a narrower unimodal size distribution.

Based on the different early growth patterns of the cuttlebone micro-structure of each year class, neither appears to return to the spawning aggregation in the following year to spawn a second time. There were no pattern 2 bones found with a second region of wide increments, that would be indicative of smaller animals returning to spawn the following year; and there were no bones with three sections of wide increments, which would be expected if the larger animals survived to spawn again. All pattern 2 and 3 bones disappeared from samples after the end of the spawning season in August, with a new cohort of pattern 3 bones found in November. Whether this means all animals from the aggregation area die after spawning or move elsewhere is not known, but they do not reappear at the aggregation area, nor are they found more widely in NSG during the subsequent summer. These observations point to *S. apama* being semelparous irrespective of life cycle type or sex.

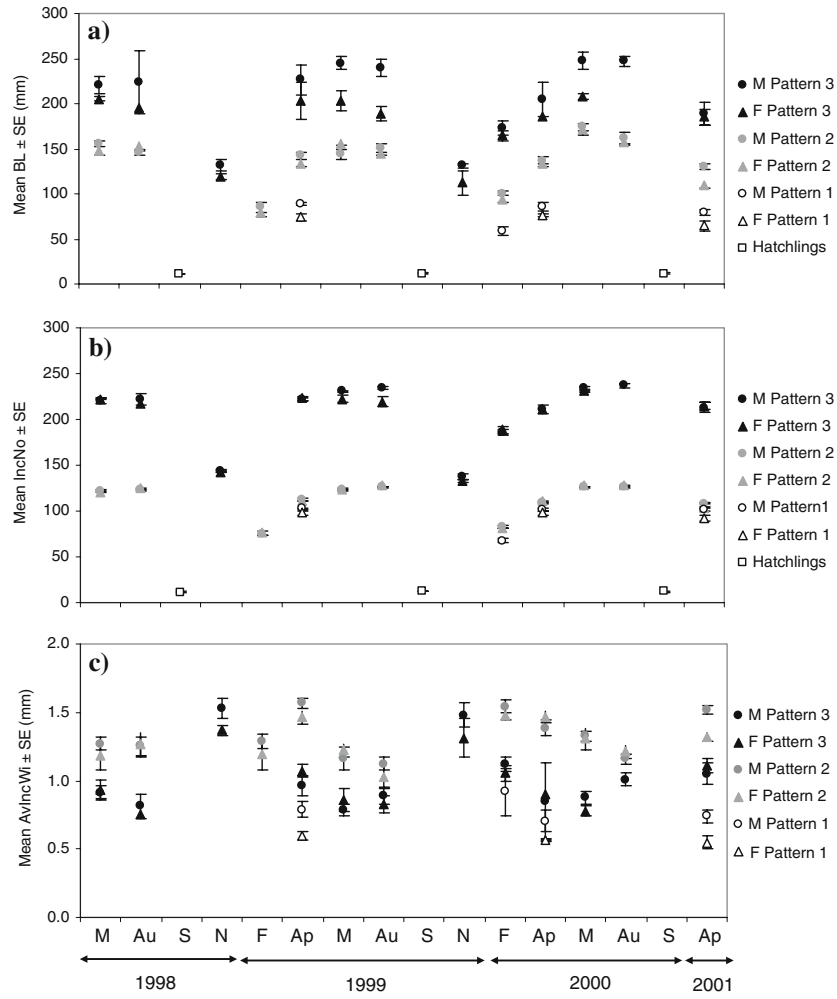

Fig. 8 Mean BL (**a**), IncNo (**b**) and AvIncWi (**c**) of each bone pattern and sex combination in samples collected from the aggregation area and NSG in May 1998 to April 2001

The above interpretation of results relies on a number of assumptions: (1) that increment width in cuttlebones is related to growth rate, which is in turn related to seasonal fluctuations in water temperature; (2) that pattern 1 bones in NSG samples collected during February and April, undergo very slow growth over winter and become pattern 3 bones the following November; (3) that all individuals in the northern Gulf are spawned during the winter (May to August) and hatch during the spring (September to October); and (4) that the consecutive samples from the wild populations are not confounded by migration of different cohorts into or out of the area. The feasibility of each of these assumptions in relation to the biology of *S. apama* and that of other *Sepia* species is considered below.

Relationship between increment width and growth rate

Marginal increment analysis of the variation in AvIncWi of bones from different sampling dates, provided evidence that width of increments is related to water temperature. Bones collected during the summer months of November and February had much wider AvIncWi than those collected during the cooler months of April, May and August. A similar result was found for cuttlebones of *S. officinalis* collected from the English Channel (Hewitt and Stait 1988; Le Goff et al. 1998) at regular intervals over 2 years. These studies also noted two characteristic patterns in the distribution of increment widths along the length of the bone, and distinct zones of narrow

Table 3 Results of two-way univariate ANOVA between different bone patterns and sexes for variables log BL, log IncNo and AvIncWi

Sample date	*n*	Sources of variance	*df*	Log BL		Log IncNo		AvIncWi	
				F	*P* > *F*	*F*	*P* > *F*	*F*	*P* > *F*
May 1998	30	Pattern (2, 3)	1	147.29	<0.0001*	6301.62	<0.0001*	21.14	<0.0001*
		Sex	1	4.71	0.0392	0.00	0.9859	0.18	0.6708
		Pattern × sex	1	0.12	0.7360	1.86	0.1842	0.72	0.4024
April 1999	39[a]	Pattern (1, 2)[a]	1	322.00	<0.0001*	104.86	<0.0001*	223.24	<0.0001*
		Sex	1	16.23	0.0003*	7.02	0.0120*	7.14	0.0114*
		Pattern × sex	1	3.93	0.0553	4.23	0.0472	0.63	0.4339
May 1999	35	Pattern (2, 3)	1	91.57	<0.0001*	3297.62	<0.0001*	29.22	<0.0001*
		Sex	1	1.66	0.2068	1.79	0.1906	0.92	0.3454
		Pattern × sex	1	10.65	0.0027*	4.22	0.0484	0.01	0.9056
February 2000	38[b]	Pattern (2, 3)[b]	1	207.71	<0.0001*	2700.77	<0.0001*	62.28	<0.0001*
		Sex	1	2.25	0.1429	0.02	0.8780	1.35	0.2537
		Pattern × sex	1	0.13	0.7181	0.49	0.4901	0.00	0.9811
May 2000	29	Pattern (2, 3)	1	81.92	<0.0001*	3600.71	<0.0001*	98.49	<0.0001*
		Sex	1	10.01	0.0039*	0.00	0.9860	1.45	0.2397
		Pattern × sex	1	6.33	0.0187*	1.21	0.2824	0.55	0.4655
April 2001	100	Pattern (1, 2, 3)	2	139.651	<0.0001*	629.11	<0.0001*	121.13	<0.0001*
		Sex	1	1.02	0.0013*	7.14	0.0089*	5.44	0.0218*
		Pattern × sex	2	1.95	0.1483	2.65	0.0758	2.93	0.0582

Different samples were tested separately

* Significant at the $p = 0.05$ significance level; total $p = 0.044$

[a] Pattern 3 bones were removed from April 1999 sample for analysis due to small sample size ($n = 4$)

[b] Pattern 1 bones were removed from February 2000 sample for analysis due to small sample size ($n = 3$)

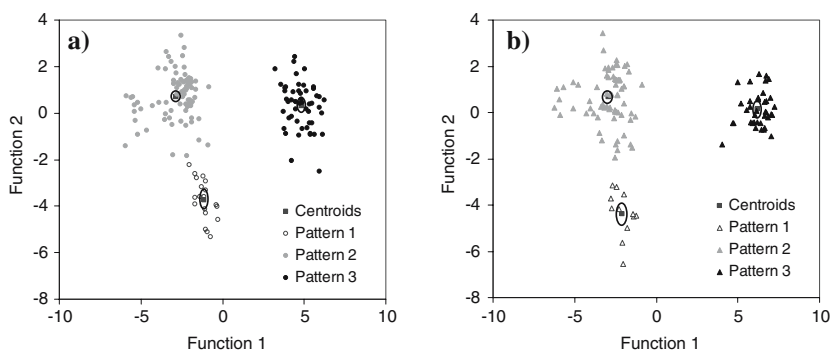

Fig. 9 Scatter plots of first two discriminant function scores for male (**a**) and female (**b**) *S. apama* derived using three cuttlebone variables log IncNo, log BL and AvIncWi for each bone pattern. Group *centroids* and 95% CI (*circles* around *centroids*) are also indicated

Table 4 Pooled within-groups correlations between discriminating variables and standardised canonical discriminant functions

Sex	Variable	Function 1	Function 2
Males	Log IncNo	0.796[a]	0.415
	Log BL	0.369	0.711[a]
	AvIncWi	– 0.293	0.630[a]
Females	Log IncNo	0.726[a]	0.369
	AvIncWi	– 0.193	0.796[a]
	Log BL	0.302	0.668[a]

[a] Largest absolute correlation between each variable and a discriminant function

increments, which corresponded to winter periods.

Unfortunately, previous experimental studies that investigated the effects of water temperature on cuttlebone growth of *Sepia* reared in captivity were primarily concerned with the rate of increment formation, and made only casual reference to variation in increment width. Nevertheless, all observed ''narrow tightly spaced'' increments deposited in cuttlebones during exposure to colder water temperatures (e.g. Richard 1969). This was explored for *S. apama* through aquarium

Table 5 Mean IncNo of pattern 2 and 3 bones at successive sample dates, differences in the number of days between sample dates and the mean IncNo over that time period, and corresponding number of days per increment

Bone pattern	Sample date	Mean IncNo	No. days difference	IncNo difference	No. days/increment
Hatchling	1 September to 31 October[a]	11			
Pattern 2	20 February	82	172–112[a]	71	2.4–1.6[a]
	8 April	110	47	28	1.7
	19 May	128	35	18	2.3
Hatchling	1 September to 31 October[a]	11			
Pattern 1	20 February	67	172–112[a]	56	3.1–2.0[a]
	8 April	101	47	34	1.4
Pattern 3	7 November	137	213	36	5.9
	20 February	186	105	49	2.1
	8 April	211	47	25	1.9
	19 May	233	41	22	1.9

Pattern 1 bones were included in the pattern 3 analysis

[a] Potential range of values created by the variation in hatch date from 1 September to 31 October

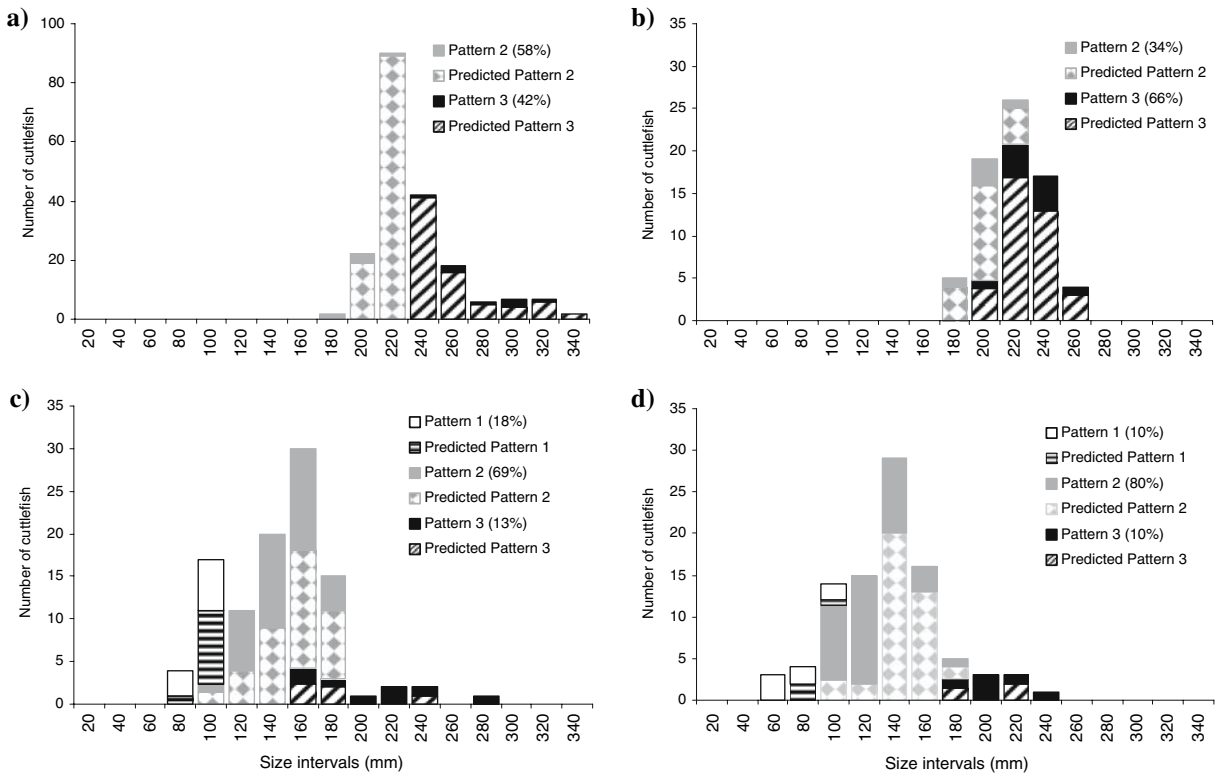

Fig. 10 Length frequency histograms of male (**a**, **c**) and female (**b**, **d**) *S. apama*, sampled in transects at the aggregation area in May 2000 (**a**, **b**) and from the NSG in April 2001 (**c**, **d**). Proportions of different bone patterns in each size interval are indicated. *Note:* bone patterns of non-aged individuals were predicted based on the frequencies of analysed bone patterns in each size class

experiments in which juveniles were subjected to different regimes of water temperature and food availability (Hall and Fowler 2003). Juveniles maintained on higher food rations had much higher growth rates and wider and more numerous increments in the cuttlebone. Temperature

also had a significant effect on cuttlebone growth but accounted for a much smaller proportion of the overall variance. There was a strong correlation between average increment width and mean growth rate (Pearson correlation coefficient = 0.831; $p < 0.01$; $n = 75$), which suggested that the increment widths in cuttlebones varied according to growth rate (Hall and Fowler 2003).

Relationship between pattern 1 and 3 bones

The similarity in patterns of increment width between pattern 1 bones and the early section of pattern 3 bones, suggests that they constitute parts of the same life cycle 12 months apart. This interpretation forms the basis of the predicted age composition of two year classes. For this to occur, the rate of increment formation must decrease concurrently with a decrease in IncWi. This was the case for *S. officinalis* experimentally reared in captivity at a number of different water temperatures (Richard 1969; Ré and Narciso 1994; Bettencourt and Guerra 2001). Richard (1969) found the rate of increment formation decreased with decreasing temperature. At 25°C one increment was deposited every 1.6 days, which then decreased at each successive drop in temperature to one increment every 2.6 days at 20°C, 4.3 days at 15°C and 5.4 days at 13°C. There was also a decrease in periodicity of increment formation from 1 every 3 days in summer to 1 every 12 days during winter for wild populations of *S. officinalis* (Hewitt and Stait 1988). Water temperatures in the NSG decrease to 11–12°C during mid-winter; therefore the predicted rate of 5.8 days per increment, necessary for pattern 1 bones in April to become pattern 3 bones in November, is considered likely. The rate of increment formation in the cuttlebones of juvenile *S. apama* reared in aquaria also indicated that at lower temperatures the rate of increment formation decreased (Hall and Fowler 2003).

Other factors have also been related to changes in either the rate of formation or width of increments in cuttlebones of other *Sepia* species. For example, malnutrition resulted in deposition of narrow increments at a slower rate in the cuttlebones of *S. officinalis* reared under suboptimal feeding regimes in captivity (von Boletzky 1974). For juvenile *S. apama* reared in aquaria, low food availability accounted for a larger proportion of the total variation in increment number than temperature (Hall and Fowler 2003). Thus, fluctuations in food availability not coincidental with seasonal variation in water temperature may confound the interpretation of cuttlebone increment widths.

Migration may also reduce the rate of increment formation in the cuttlebone irrespective of water temperature. Natsukari et al. (1991) observed a decrease in rate of increment formation in the cuttlebones of *S. esculenta* due to migration from a semi-closed inlet to offshore waters, despite insignificant differences in summer temperatures between the two areas. However, as with most studies of wild populations, the potential complication of successive sampling of different cohorts could not be ruled out as a possible explanation for the differences observed in that study.

Synchronous spawning and hatching

An alternative hypothesis for polymorphism in bone patterns is that the pattern 3 bones from November derived from individuals that were spawned and hatched elsewhere at a different time, presumably during the summer or autumn, and were thus aged less than 12 months. Multiple spawning periods or hatch dates have been used to explain the variation in growth patterns observed for many squid species, even in the absence of empirical evidence for multiple spawning periods (Macy III 1995; Brodziak and Macy 1996; Macy and Brodziak 2001). There is no evidence to support this for *S. apama* in the NSG. Most individuals collected during summer were immature or maturing (Hall and Fowler 2003). All eggs monitored in situ at the aggregation area hatched over a relatively short hatching period, between September and October (Hall and Fowler 2003). Thus, the evidence to date supports the hypothesis that all individuals in NSG are spawned during winter and hatch in spring.

Migration and mixing of cohorts

Adequate information is not available to make similar conclusions for other areas of the Gulf or

State waters. Therefore, it is possible that spawning may occur at other times of the year elsewhere, and individuals may migrate into the northern Gulf and join this population. If this was the case the polymorphism in patterns of growth increments would result from the mixing of different stocks rather than year classes. However, the consistency in the patterns across all individuals of each bone pattern at each sampling time suggests that they simultaneously experience similar environmental conditions, and that migration would need to be synchronised en masse to produce such consistency. One approach to address this question is through an analysis of the chemical composition of the statoliths or cuttlebones. Comparison of the composition of pre-hatch or juvenile portions to adult sections, or between different bone patterns, may provide information on the stock structure of *S. apama* in NSG and elucidate potential migration patterns of different life cycle types (Campana 1999). Until such information becomes available, the possibility that the pattern 3 bones found in November are less than 12 months old cannot be ruled out.

Conclusion

Overall, the available evidence suggests that the variation in bone structure is produced by alternative life cycle types of varying length, rather than the mixing of different micro-cohorts or stocks. Thus, the most coherent view of the results is that the population of *S. apama* in the NSG consists of two distinct year classes that differ significantly in juvenile growth pattern. Similar alternative life cycles have been described for a temperate cuttlefish species, *S. officinalis*, from the Northern Hemisphere (von Boletzky 1983; Le Goff et al. 1998). The potential mechanisms that produce these alternative life cycles, however, are completely unknown and represent an exciting avenue for further research.

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