Reproduction in five sympatric batoid species
(Family Urolophidae) from south-eastern
Australia

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I am the author of the thesis entitled

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General Abstract

Urolophid species form a major component of bycatch in trawl and seine fisheries within southern Australia and like many chondrichthyan species of low biological productivity compared with teleost fishes are highly susceptible to the effects of fishing, yet little is known about their population dynamics and life history characteristics. The aim of the present study was to determine the reproductive parameters required for use in fisheries stock assessments, ecological risk assessments, and species risk of extinction assessments of five species within two genera of the family Urolophidae and to provide some insights into the diversification of the reproductive strategies within these five species. All five species were within a common geographical area and subjected to similar levels of fishing pressure.

Common across the family Urolophidae is viviparity with lipid histotroph (uterine milk) supplied to the embryos during gestation. However, two distinct evolutionary reproductive strategies were found specific to each genera. The genera *Trygonoptera* develop large follicles, which supply most of the nutrients for embryo development, and exhibit only moderate matrotrophy (~1000% increase in mean wet mass from egg to full-term) (nevertheless highly matrotrophic compared with most viviparous shark species). The genera *Urolophus* develop small follicles, which supply only a small amount of nutrients for embryonic development, and exhibit extensive matrotrophy (2500–7200% mean wet mass gain) and among the highest matrotrophic contributions recorded for any chondrichthyan species.

These distinct reproductive strategies and the amounts of maternal contributions were found to have some correlation with the periodicity of the reproductive cycles among the five urolophid species. Moderate matrotrophic contribution corresponds to *Trygonoptera* species having short periods of embryonic growth (5–7 months), whereas the extensive matrotrophic contribution corresponds to *Urolophus* species having lengthened periods of embryonic growth (10–19 months). In theory, if the matrotrophic contribution or gain in wet mass from egg to full-term embryo is above ~5000%, then it is likely that the maternal demand is so great that the ‘period of embryonic growth’ lengthens to more than 12 months.
In all urolophid species, follicle development occurs synchronously with embryonic growth which has led to the two genera having different strategies involving the eggs in utero. Trygonoptera species develop their embryos and large follicles during the short period, then encase their eggs in utero in a transient brown envelop where the eggs remain in a dormant-like state for an extended period (5–7 months) before embryonic growth progresses. Urolophus species develop their embryos and small follicles during longer periods, but have minimal or no egg encapsulation but allows for the transition from egg to embryo to be comparatively short (1–3 months).

With follicle growth occurring synchronously with embryonic growth, the periodicities of their reproductive cycles were able to be determined through the ‘periodicity of the eggs in utero’, ‘periodicity of the embryos in utero’, and the ‘periodicity of the parturition’. Reproductive cycles range from annual (T. imitata, U. viridis, and U. paucimaculatus) to biennial (U. bucculentus and U. cruciatus) and are among the first batoids found to be biennial.

Stress-induced aborting caused by capture and handling, however, was common for the present study. Aborting eggs and embryos made determination of the periodicity of the reproductive cycle difficult, particularly for, U. cruciatus and U. paucimaculatus where large numbers of animals were sampled. For Urolophus cruciatus in particular, this required a two step approach to reduce ambiguity in interpretation of available data. The first step required stepwise sequential questioning from analysis of standard reproductive data associated with population biology, and the second step required testing for redistribution of females aborting or of uncertain reproductive condition according to alternative hypothesised reproductive cycles. This allowed for the biennial reproductive cycle to be determined in U. cruciatus. However, the first approach of stepwise sequential questioning was designed so that it can be used for all reproductive studies of viviparous species with or without the occurrence of aborted eggs and embryos and was applied to U. paucimaculatus and U. viridis as further cases.

For stock assessments, ecological risk assessments, and species risk of extinction assessments it is important to note that the reproductive biology varied among
sampling regions for *Urolophus paucimaculatus* and *U. viridis*, and is possibly for *Trygonoptera imitata*, *U. bucculentus*, and *U. cruciatus* although each region could not be sampled sufficiently to provide much evidence of spatial differences. Growth rates between populations from eastern and western Victoria were found to differ with smaller size-at-birth, litter size, length-at-maturity and length-at-maternity found in Lakes Entrance than in Port Phillip Bay and Western Bass Strait. In *T. imitata* and *U. paucimaculatus* inshore areas appear to have more suitable environments for females in breeding condition than offshore areas and could be occurring in other urolophid species.

Viviparity in elasmobranches allows females to produce larger sizes-at-birth with a higher rate of survival through advanced feeding, digestion, movement, and behaviour (Goodwin *et al.* 2002); however, in urolophid species, this is offset by their low biological productivity with maximum litter size across all five urolophid species of only 2–7 and reproductive cycles of 1–2 years. This suggests these urolophid species are vulnerable to the effects of fishing and has contributed to several species within the family Urolophidae being classified as ‘Threatened’ or ‘Near Threatened’. Low biological productivity, such as small litters and long reproductive cycles, regionality, population segregation, and nursery areas for breeding females should be considered possible for many, if not all, urolophid species.
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Most importantly I would like to thank my mum. Thank you
Preface

The material presented in five chapters of the present study is published in two journal papers and three submitted manuscripts for journal publication. To acknowledge the work of co-authors to several chapters within this thesis the following describes the publication citations.

Chapter 1 (General introduction). Provides the information for each of the introductions within the published journal papers (Trinnie et al. 2009; Trinnie et al. 2012) and the submitted papers (Trinnie et al. 2012 submitted-a; Trinnie et al. 2012 submitted-b; Trinnie et al. 2012 submitted-c).


Chapter 3 (*Trygonoptera imitata*). Work within chapter 3 has been published as a journal article (Trinnie et al. 2009).


Chapter 4 (*Urolophus bucculentus*). Work within chapter 4 has been published as a journal article (Trinnie et al. 2012).


Chapter 5 (*Urolophus cruciatus*). Work within chapter 5 has been submitted for publication (Trinnie et al. 2012 submitted-a).

Chapter 6 (*Urolophus paucimaculatus*). Work within chapter 6 has been submitted for publication (Trinnie et al. 2012 submitted-b).


Chapter 7 (*Urolophus viridis*). Work within chapter 7 has been submitted for publication (Trinnie et al. 2012 submitted-c).

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Chapter 1 – General Introduction

Commercial significance and impacts

The family Urolophidae is a highly diverse family, known as stingarees in Australia and round rays in other parts of the Indo-Pacific region. Of the 28 known species within the two genera *Trygonoptera* and *Urolophus*, more than half are found in Australian waters (Last and Stevens 2009) where they inhabit most substrates from estuaries and shallow embayment’s including sandy, muddy, rocky reef, and seagrass beds to the continental shelf and slope. Most species are found in high abundances throughout the continental shelf region with only several species on the continental slope to depths of at least 420 m.

Southern Australia provides most of Australia’s urolophid species diversity. In south-eastern Australia; from New South Wales to the Great Australian Bight, there are as many as 12 urolophid species within this broad area (Last and Stevens 2009); most being rated as common to abundant, and several being rated as sparse or localised (Walker and Gason 2007) and, in south-western Australia; southern coast of Western Australia to the Great Australian Bight, as many as 9 urolophid species are found in this common area (Last and Stevens 2009). Several urolophid species have been found to resource partition their diets and habitats (White *et al.* 2004) which have allowed for overlapping distributions.

This often means that within an area, several urolophid species are susceptible to the effects of fishing, particularly when targeting benthic teleost or crustaceans. Within south-eastern Australia, urolophid species constitute a major component of the bycatch of chondrichthyan species caught by commercial fishing of predominantly demersal trawl and Danish seine, with less caught by gillnets and longlines within the Southern and Eastern Scalefish and Shark Fishery (SESSF). A mean annual catch of ~1140 tonnes was discarded, often dead, because they have little commercial value, during 2000–06 (Walker and Gason 2007). In south-western Australia, 67% of the total trawl catches of elasmobranches when targeting prawns or scallops consisted of urolophids (Jones *et al.* 2010). Though urolophids have little or no commercial value,
retention of abundant urolophid species might provide saleable products in the future, given their palatability (Last and Stevens 2009; Stroud 1975).

Several urolophid species within the SESSF exhibit declining abundances due to effects of commercial fishing. Comparison of catch rates between studies in 1976–77 and 1996–97 shows an overall decline in catch rates of 65%–80% in several localised areas for *Urolophus bucculentus, U. viridis, U. cruciatus* and the less common *U. sufflavus* (Graham et al. 2001). A separate study, while examining catches and catch rates in the SESSF during 2000–06 using low, medium, and high ‘catch susceptibility’ grading evaluated *U. viridis, U. paucimaculatus* and *U. bucculentus* at medium risks of further decline (Walker and Gason 2007). Within Port Phillip Bay, Victoria, during 1970–91 a brief increase in abundances was observed for both *Trygonoptera imitata* and *U. paucimaculatus*, a possible response to commercial and recreational fishing of their competitors, and the optimisation of food resources from a then scallop dredge fishery (Hobday et al. 1999). However, a more recent study found that abundances have since declined throughout the bay (Parry et al. 2004).

In 2004, most of Australia’s Urolophidae species were assessed by a committee for the International Union for Conservation of Nature (IUCN) (Kyne and Cavanagh 2004). Many south-eastern urolophid species became listed as threatened because of the intensity of fishing in the SESSF: *Urolophus bucculentus, U. sufflavus*, and *U. viridis* were listed as ‘Vulnerable’ and *Trygonoptera imitata* as ‘Near Threatened’ on the IUCN red list and only recently through decreased fishing activity in the SESSF was the ‘Endangered’ listing not be implemented for at least *U. viridis. Urolophus orarius* was listed as ‘Endangered’ because of its low abundance and narrow distribution rather than from reduced abundances from the effect of fishing pressure. In south-western Australia, no urolophid species were listed as ‘Threatened’ because of the low intensity of fishing pressure and relatively stable or negligible catch trends (Laurenson et al. 1993). For the majority of urolophid species however, there are little or no data reported for the reproductive biology or other life history characteristics, or accurate historical catch surveying required for in depth evaluations. Of the few species in areas of high commercial fishing, marked declines in abundance occurred in most of them and suggest urolophids are susceptible to high levels of fishing pressure despite being bycatch.
Since the IUCN assessment of Australia’s urolophid species, an Ecological Risk Assessment (ERA) has been established on all chondrichthyan and other fish species: to quantitatively assess the biological productivity and catch susceptibility of each species. An approach was first applied to bycatch elasmobranch species in northern Australia (Stobutzki et al. 2002) and then applied in south-eastern Australia assessment to all chondrichthyan species, which also included holocephalans (Walker et al. 2008). A total of 121 chondrichthyan species (77 shark, 36 ray, and 8 holocephalan species) found distributed within the range of the SESSF were subject to the ERA (Walker et al. 2008), Most of these chondrichthyan species are non-targeted species taken as byproduct and bycatch. The five urolophid species of the present study are included in this ERA and the reproductive biology parameters are essential.

Reproduction within chondrichthyan species

Chondrichthyan species are unique, and differ from teleost fishes by their variety of reproductive modes, ranging from egg laying species (oviparity) to live-bearing species (viviparity). Viviparity is further categorised into lecithotrophy and matrotrophy (Hamlett et al. 2005a) based on the amount of maternal contribution given to the internally developing embryos. In lecithotrophic viviparity, the fertilized egg or yolk sac provides the embryos nutrients during the entire development. Lecithotrophic species generally show a 20% decline in dry weight (or organic matter) from egg to embryo. Matrotrophic viviparity supplements yolk from other sources such as a placenta, the ingestion of histotroph or uterine milk, ingestion of unfertilized ova, and, in the extreme case, cannibalism of siblings in utero. Matrotrophic species generally shows a dry weight gain during embryonic development that can be minimal (any loss greater than -20%) to extensive. In viviparous species, female body size is often larger than in oviparous species to accommodate the embryos in utero and can result in the production of a smaller number of larger offspring (Goodwin et al. 2002).

The female reproductive system generally begin development as paired structures (Wourms 1977) and comprises ovaries, oviducts, ostia, oviducal glands and uteri (Hamlett and Koob 1999), however, adaptation for viviparity have a profound effect...
on the organization of the reproductive organs and can often become asymmetrical in adults (Wourms 1977). Within the ovaries of juveniles, follicles are generally either non-existent or small with no yolk and begin to enlarge through a process of folliculogenesis in maturing animals. At the onset of maturity, follicles continue to enlarge but accumulate yolk through the process of vitellogenesis where ripe follicles are produced for ovulation. The ripe follicles pass through the ovary wall to discharge ova into the oviduct. The ostia act as a funnel which serves to transport the ovulated eggs through the oviduct.

From the ostia, the eggs travel through the oviducal gland. The oviducal gland is the site of egg encapsulation, sperm storage and egg fertilization. In several shark species, sperm can be stored in the females oviducal glands for several months prior to ovulation (Storrie 2004). The oviducal gland contains four zones; club, papillary, baffle and terminal. The club and papillary zone produce the various types of egg jelly that initially surrounds the egg, the baffle zone produces the tertiary egg case, and the terminal zone is the site of sperm storage and produces the hairs that adorn the exterior of the egg case that helps attach the egg case to the surrounding habitat (Hamlett et al. 2005b). Oviducal glands can be more predominant in oviparous (Wourms 1977) or viviparous species that produce an egg case, candle case or egg envelop (Hamlett et al. 2005b).

Post fertilization and egg capsulation, the egg leaves the oviducal gland and is deposited into the uterus. In oviparous species, the uterus normally serves as only a passageway for the eggs prior to external deposition, whereas for viviparous species becomes highly developed for the various reproductive modes during embryo development (Wourms 1977).

The male reproductive system is also typically paired organs; internally these include the testes, genital ducts, Leydig glands, ductus deferens, seminal vesicles, and externally, the copulatory appendages called claspers at the posterior bases of the pelvic fins which calcify in mature animals (Hamlett and Koob 1999). The testis is the sight of initial sperm production; spermatocyst development commences from single Sertoli cells and terminates at sperm release. Primary spermatocytes undergo 7 stages of development prior to becoming secondary spermatocytes, these then develop into
spermatotids with emerging flagella, and then into tightly bundled mature spermazoa. Spermatozoa occur throughout the ductus deferens and seminal vesicles. The process of spermiogenesis, where spermatozoa mature into motile sperm occurs in the genital ducts. The male reproductive cycle is linked to the female reproductive cycle by providing sperm for fertilization at or before ovulation.

By understanding the periodicity of the female and male reproductive systems and determining female length-at-maturity and length-at-maternity ogives, litter size, sex ratio of embryos, and by combining this information with time series of catch and indices of abundance then estimates of population biomass and recruitment trends can be determined through fisheries modelling. However, the female reproductive cycle is highly complex and in viviparous chondrichthyan species determining its periodicity requires determining the ‘period of eggs in utero’, ‘period of embryonic growth’, and hence the ‘period of pregnancy’, ‘period of ovarian cycle’ and the synchronisation among these cycles and among the pregnant females in the population. The periodicity of the reproductive cycles in female chondrichthyan species can range from biannual, through annual, biennial, to triennial or possibly longer (Tanaka et al. 1990). Although cycles of biennial or longer are generally rarer, cycles longer than triennial has been disputed (Compagno et al. 2005).

Determining female maturity (the proportion of the population in mature condition) and maternity (the proportion of the population contributing to recruitment into the population each year) is important for population dynamic studies and fisheries modelling. In most studies, maturity is determined through macroscopic observations associated with the development of the follicles or uterus and the presence or absence of eggs or embryos. Field based macroscopic observations require internal examination, while several new approaches such as blood hormone testing (Awruch et al. 2008) are being tested to reduce the need for animals to die, however these are not yet widely circulated and cover only several species. The onset of female maturity through macroscopic observation is best characterised as the onset of vitellogenesis (Braccini et al. 2006; Huveneers et al. 2007; Walker 2007), the process of the follicles enlarging and accumulating yolk prior to ovulation. Maternity is best characterised from pregnant (with eggs or embryos in utero) and post-partum females found in the population (Walker 2005).
Several approaches have also been taken to determine the onset of maturity of males in chondrichthyan species. As in females, studies adopt a variety of reproductive indices based on condition of the reproductive organs (testes, vas deferens or seminal vesicles), but the most common approach is to adopt calcification of the male claspers (Hazin et al. 2001; Simpfendorfer 1992). These become calcified and rigid in mature animals, and are easily classed externally, making for easy accessibility, whereas the testes, vas deferens or seminal vesicles require internal examination. It is difficult to determine which character best defines maturity and each index should be considered separately (Walker 2005; Walker 2007).

Batoid ancestral lineage and evolution of reproduction strategies in urolophid species

Trends in chondrichthyan assemblages are towards greater species diversity with increased niche partitioning (Underwood 2006). The evolution of chondrichthyan species, their ancestral lineage, and the development of their many reproductive modes receive ongoing conjecture. Much of the past conjecture has been based on fossil records, morphology, reproductive mode, and dentition, but more recently, modern genetic techniques such as DNA sequencing provide new insights. From early evolutionary studies, it was generally agreed that oviparity is the plesiomorphic reproductive strategy for chondrichthyan species (Compagno 1990; Dulvy and Reynolds 1997; Wourms 1977), but this is being questioned and it is suggested that yolk-sac viviparity is the ancestral strategy (Lund 1980; Musick and Ellis 2005).

Through molecular analyses, the batoids (Batoidae) is thought to have evolved separately from shark species and they are a sister-group to the sharks. Estimates suggest that the batoids diverged from other shark species by the early Jurassic if not earlier (200–300 million years ago) (Aschliman et al. 2012; Musick and Ellis 2005; Underwood 2006). The first step towards species diversity of batoids is thought to have began 150–190 millions years ago, with the early clades of skates (Rajiformes) diverging from the other batoids ~190 million years ago, and fanrays (Platyrhinoidei) (now removed from Myliobatiformes by Achliman Nishida et al 2012) and the electric rays (Torpediniformes) diverging ~180 million years ago. Among these other batoids, the guitarfish (Rhinobatiformes) and sawfishes (Pristioformes) diverged ~150
million years ago and then the panrays (Zanobatoidei) and their close relatives
(Myliobatiformes) (which includes Urolophidae and now excludes Platyrhinoidei)
diverged from Rhinobatiformes and Pristioformes, and the Myliobatidei
(Myliobatiformes without Platyrhinoidei) then diverging from the Zanobatoidei ~140
million years ago. However, the comparatively high diversity of Myliobatidei species
found today accentuated within a relatively short time period (70–90 million years

Yolk-sac viviparity is thought to be the plesiomorphic state in all orders of Batoidae
(Musick and Ellis 2005) and from these ancestral batoid species, the Rajiformes is the
only clade to have evolved oviparity. The Torpediniformes, Platyrhinoidei,
Rhinobatiformes, Pristioformes, and Zanobatoidei retained yolk-sac viviparity. The
Myliobatidei evolved lipid histotroph viviparity (although thought to be limited to
only Myliobatidei) (Musick and Ellis 2005).

The current theory that all Myliobatidei evolved lipid histotroph viviparity is
consistent with all observation reported for species in the family Urolophidae of
maternal contribution through trophonemata and uterine histotroph (Last and Stevens
1994). The developing embryo is initially nourished by an external yolk sac before the
uterus develops vascularised appendages, or trophonemata, that secrete histotroph
organically rich in lipid and protein, ingested by the embryo or absorbed by external
gill filaments as it grows to full term (Hamlett et al. 1996). This allows urolophid
species to produce few, but relatively large offspring (Hamlett and Koob 1999).

A common evolutionary adaptation within the family Urolophidae that may separate
this family from its ancestor and from other families within the Myliobatidei is the
degree of atrophy in the female reproductive tract. In all studied Australian urolophid
species, the right reproductive tract has become non-functional including the right
uterus (Edwards 1980). The family originally included the genera Urobatis and
Urotrygon of the Americas, and several species were originally classified as
Urolophus, now form there own family Urotrygonidae following DNA sequencing.
Whether Urolophidae and Urotrygonidae share the same ancestor is unknown;
Urobatis jamaicensis has retained both functional reproductive tracts, except the right
side is less fecund than the left side (Fahy et al. 2007). In Urobatis halleri, the right
ovary and oviduct are small and non-functional, but the right uterus remains functional (Babel 1967).

Urolophidae and Urotrygonidae share the same reproductive strategy and body shapes, particularly the lanceolated tail, ovarian follicle cell morphology, the oviducal gland morphology, and the development of the embryo *in utero* (Babel 1967); however, embryo size-at-birth, follicle size at ovulation, and amount of maternal contribution are likely to vary among species, genera, and families within the Myliobatidei.

*The history of research in urolophid reproductive biology*

The first known quantitative study on the reproductive biology of a urolophid species worldwide was on *Urobatis halleri*, then known as *Urolophus halleri*, in the USA (Babel 1967). This study found that sexual maturity was attained at a relatively small size compared with other batoid species, maximum litter size was six, embryonic growth took ~3 months, and the reproductive cycle was likely to be annual, though several females were found to reproduce biannually and were out of phase with the rest of the population.

Formerly the common stingaree *Trygonoptera testaceus* and then changed to the eastern shovelnose stingaree *T. sp B*, and then to *T. imitata*, and the sparsely-spotted stingaree *U. paucimaculatus* in Port Phillip Bay (PPB), Victoria, were the first urolophid species studied in Australia (Stroud 1975). This study provided a descriptive overview of litter sizes within Australian urolophid species which ranged 2–6 and 2–5 for *T. imitata* and *U. paucimaculatus*, respectively. However, the gestation periods or reproductive periodicity of either species was not determined. This was shortly followed by a further study on *U. paucimaculatus* from PPB (Edwards 1980), which focussed more on diet and age, but found the smallest female to give birth was 340 mm TL and litters of 2–6 offspring were again recorded. However, as for Stroud (1975), because sampling occurred only during March–April and August–September, the reproductive cycle could not be reliably determined, and Edwards (1980) assumed a biannual cycle, as found by Babel (1967) for *U. halleri*. 

Chapter 1 – General Introduction
which is no longer part of Urolophidae, and determined the reproductive output of 6–12 pups per year for *U. paucimaculatus*.

With the lack of accurate information on the periodicity of urolophid reproductive cycles and the inconsistency in litter size within Australia, a generalised statement that the family Urolophidae have a 3 month gestation and 2–4 offspring per pregnancy (Last and Stevens 1994) is seemingly based on Babel (1967), because Stroud (1975) and Edwards (1980) research were not cited. A preliminary study on *U. cruciatus* and *U. expansus* on the reproduction, age and growth (Treloar 2001) provides further evidence that reproduction within the family should not be generalised, but could not reject the generalised statement of 3-month gestation and litters of 2–4.

The first study to determine conclusively the reproductive periodicity in Australian urolophid species was undertaken on four sympatric urolophid species *T. mucosa*, *T. personata*, *U. lobatus*, and *U. paucimaculatus* in south-western Australia (White 1998; White *et al.* 2002; White *et al.* 2001; White and Potter 2005). All four species were found to have annual reproductive cycles and litters sizes of 1–2. White and Potter (2005) then disagreed with Edwards (1980) that litter sizes of 6 and biannual cycles were possible in *Urolophus paucimaculatus*.

Of the few studies that have been undertaken on Australian urolophid species, the most extensively studied urolophid species is *Urolophus paucimaculatus* in Port Phillip Bay, Victoria (Edwards 1980; Officer and Parry 1995; Stroud 1975) and off the lower west coast of Western Australia (Plattell *et al.* 1998; White and Potter 2005) where the period of the reproductive cycle remains unresolved; at least in south-eastern Australia. However, the periods of the reproductive cycles of most urolophid species are yet to be determined. Of the 22 species found in Australian waters, fewer than 10 species have had reproductive biology assessed, age or diet analysed; and these are only the most abundant, shallower water species. Many are sympatric species, and their response to both environmental conditions and competition for resources would suggest that separate strategies are likely to have evolved to sustain this degree of diversity.
As stated, the family Urolophidae no longer has species found in the Americas; all American species were reclassified to the genus *Urobatis* within the family Urotrygonidae. However, their morphologies remain similar and are useful for comparative purposes. Recent information on Urotrygonidae species comes from a study of *Urobatis jamaicensis* (Fahy et al. 2007) which found that litter sizes range 4–7 and the reproductive cycle is biannual. A study comparable to Babel (1967) on *Urobatis halleri* found the female reproductive cycle to be annual (Chris Mulls pers comm.) and the male breeding cycle to be annual (Mull et al. 2008). The most recent study on *Urotrygon rogersi* found a possible triannual cycle (Mejia-Falla et al. 2012) which suggests that reproductive cycles for species in the Americas are shorter than species in Australia. However, further studies are required to determine how similar the families of Urolophidae and Urotrygonidae are for comparative studies and evolutionary traits.

**Aims and objectives of the present study and thesis outline**

The overall aim of the present study was to provide estimates of the reproductive parameters for contribution to fisheries stock assessments, ecological risk assessments, species extinction risk assessments, and species reproductive ancestry traits of five urolophid species within the genera *Trygonoptera* and *Urolophus* found in south-eastern Australia through macroscopic observation of the reproductive biology. Hence, the present study determines key reproductive parameters including the sex ratio at birth, the litter-size–maternal-length relationship, the proportion of the population at any length in mature condition, the proportion of the female population at any length in maternal condition, and the periodicity of the breeding cycle.

Initially there were two key objectives.

1. To determine through quantitative analysis the reproductive parameters of *Trygonoptera imitata* (Chapter 3), *Urolophus bucculentus* (Chapter 4), *U. cruciatus* (Chapter 5), *U. paucimaculatus* (Chapter 6), and *U. viridis* (Chapter 7).

2. To explore spatial variation in the reproductive parameters of *U. paucimaculatus* and *U. viridis* (Chapters 6 and 7).
During the course of this study, it became evident that the methodological approach used within the literature to determine the reproductive biology of viviparous chondrichthyan species could not help explain certain aspects found within several of the studied urolophid species. Few studies provide an explicit process for determining the periodicity of the reproductive cycle, where for many chondrichthyan species the data require careful interpretation and evaluation of uncertainty associated with estimates of periodicity. Viviparous species where asynchronous breeding or long reproductive cycles can complicate the determination of trends, and small sample sizes across broad spatial or temporal scales can often provide misleading results. Pregnant females aborting eggs and embryos can be a common occurrence within yolk-sac viviparous species as the cloaca can often become flaccid, and without any attachment the embryos can easily be ejected from the uteri. Distinguishing between whether a female has aborted eggs or embryos or has just given birth is often difficult.

In *Trygonoptera imitata* (Chapter 3) and *Urolophus bucculentus* (Chapter 4) the periods of gestation and periods of ovarian cycles and hence the reproductive cycles were relatively clear and thus required only a simple approach to the determination of the reproductive biology. However, several complicating issues such as stress-induced aborting and extended periods of eggs *in utero* found for *U. cruciatus* (Chapter 5) made it necessary to adopt methods involving stepwise hypothesis testing to determine the period of the reproductive cycle and other aspects of its reproductive biology. Instead of the simple approach applied for *T. imitata* and *U. bucculentus* in chapters 3 and 4, these methods were more appropriate for *U. cruciatus*, *U. paucimaculatus*, and *U. viridis* in chapters 5, 6 and 7. Determining reproductive cycles in chondrichthyan species needs approaches that make uncertainty explicit in the way that comparisons can be made among studies and species.

A third objective was then added to the earlier two objectives.

1. Develop a research approach that overcomes methodological problems such as pregnant females aborting eggs and embryos upon capture and handling and consequentially large numbers of post partum females, asynchronous breeding, and long reproductive cycles that can be used for comparisons across all species (Chapter 2).
Further objectives of the present study were to explore evolutionary adaptations within the family Urolophidae to demonstrate reproductive traits that urolophid species share and the traits that differ within each genus of the family and to the common ancestral species within the family Urotrygonidae. During analysis, it became apparent that each studied species had different reproductive traits. To highlight these unique traits, each chapter within the present study, individually discusses, and then compares, these unique traits. These reproductive traits are then discussed at a family level and are used to broaden our knowledge of batoid reproduction and evolution.

Fourth and fifth objectives involving interpretation of results from the present study and other studies were added.

1. Contribute to placing reproductive traits of each species into an evolutionary perspective, by comparisons within each genus, among genera, within the family Urolophidae, and between the families Urolophidae and Urotrygonidae (Chapters 4–7).

2. Discuss the implication of the present study for urolophid management and conservation and to broadening our knowledge of their evolution through the reproductive biology (General Discussion. Chapter 8)
Chapter 2 – Materials and Methods

List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ANCOVA</td>
<td>Analysis of Covariance</td>
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<tr>
<td>CI</td>
<td>Corner Inlet</td>
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<td>C</td>
<td>Clasper index</td>
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<tr>
<td>ETL</td>
<td>Embryo Total Length</td>
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<td>ETW</td>
<td>Embryo Total Weight</td>
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<td>G</td>
<td>Testis index</td>
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<td>GLM</td>
<td>General Linear Model</td>
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<td>GSI</td>
<td>Gonadosomatic Index</td>
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<td>HSI</td>
<td>Hepatosomatic Index</td>
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<tr>
<td>LE</td>
<td>Lakes Entrance</td>
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<tr>
<td>LFD</td>
<td>Largest Follicle Diameter</td>
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<td>L_{50}</td>
<td>Length at 50% proportion</td>
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<td>L_{95}</td>
<td>Length at 95% proportion</td>
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<td>P_{max}</td>
<td>Maximum Proportion</td>
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<tr>
<td>PPB</td>
<td>Port Phillip Bay</td>
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<tr>
<td>SAS</td>
<td>Statistical Analysis Software</td>
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<tr>
<td>SBC</td>
<td>Sex Breeding Condition</td>
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<td>TL</td>
<td>Total Length</td>
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<td>TW</td>
<td>Total Weight</td>
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<td>U</td>
<td>Uterus index</td>
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<td>V</td>
<td>Seminal vesicle index</td>
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<td>WBS</td>
<td>Western Bass Strait</td>
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Sampling

Samples of all specimens for each of the five urolophid species were collected by commercial fishers during August 2002–March 2003 and March 2004–October 2006 from Port Phillip Bay (PPB) using beach seine, western Bass Strait (WBS) using Danish seine, Corner Inlet (CI) using ring seine, and Lakes Entrance (LE) using Danish seine and demersal otter trawl. *Trygonoptera imitata* (Chapter 3) and *U. paucimaculatus* (Chapter 6) were found in all four regions (Fig. 2.1), whereas *U. bucculentus* (Chapter 4), *U. cruciatus* (Chapter 5), and *U. viridis* (Chapter 7) were found only in WBS and LE (Fig. 2.2).

All animals caught by the fishers were retained and processed in the laboratory. Each animal was sexed, measured for total length (TL) (distance from the tip of the snout to the distal edge of the caudal fin), disc length (DL) (distance from the tip of the snout...
to the distal end of the pectoral fin) and disc width (DW) (distance across the pectoral fins), and weighed for total body mass (TW). All measurements were recorded to the nearest millimetre and mass were recorded to the nearest gram.

For males, the liver was weighed and the claspers were measured from the ventral surface skin flap joining the pelvic fins to the distal tip of the clasper. The paired testes (together with the epigonal gland) were weighed and the seminal vesicles were removed to determine their fullness (Table 2.1).

For females, the reproductive tract exhibits atrophy of the right organs. Thus, only the left ovary and left uterus were measured and weighed and the largest follicle diameter (LFD) of the left ovary was measured. If present, the eggs and embryos *in utero*, and any embryos found aborted upon capture were counted, and for each embryo TL, total mass (with and without yolk-sac) and sex were recorded. Neonates and aborted embryos were differentiated from each other through examination of stomach contents: aborted embryos contained a greenish concretion waste material within the spiral value dissolved shortly after birth (Babel 1967) and neonates contained diets external to the mother’s influence.

*Allometric and isometric relationships for total body mass and various size measurements*

For all five urolophid species, the TW (*w*)–TL (*l*) relationship was determined using the model *w* = *aclb*, where *a* and *b* are parameters estimated by linear regression of \( \ln(w) = \ln(a) + b \ln(l) \) and *c* is a factor correcting biases caused by natural logarithmic transformation (Beauchamp and Olsen 1973). For differences between males and females, the slopes and elevations of the TW–TL linear relationships were tested by analysis of covariance (ANCOVA) using the generalised linear modelling procedure (‘Proc GLM’) of the computer statistical package SAS (Version 8.02 SAS Institute, NC, USA) (Walker 2007). Where possible, the TW–TL relationships were also tested for four sex-breeding conditions (SBC) among males, females non-pregnant, females pregnant with eggs *in utero*, and females pregnant with embryos *in utero* because pregnant females were expected to be heavier than non-pregnant females. ANCOVA (with the terms SBC, covariate TL and SBC x TL) was adopted to test for the effects
of factor SBC on the TW–TL linear relationship and statistically compared using the least-square means test in SAS (Walker 2007). Isometric relationships were determined using the linear regression of \( l_1 = a' + b'l_2 \), where \( a' \) and \( b' \) are parameters estimated by the model and \( l_1 \) and \( l_2 \) represent isometric measurements among pairs of TL, DL, and DW.

**Growth of embryos and period of embryonic growth**

Aborted embryos collected during sampling were included in all analyses where maternal length was not required. The embryo mass (w)–embryo TL (ETL) (l) relationship was determined using the model \( w = acl^b \), where \( a \) and \( b \) are parameters estimated by linear regression of \( \ln(w) = \ln(a) + b \ln(l) \) and \( c \) is a factor correcting biases.

For *T. imitata* (Chapter 3) and *U. cruciatus* (Chapter 5), the ‘period of embryonic growth’ and seasonal patterns of embryonic growth were determined by plotting mean TL of embryos and mean mass of embryos from all U=5 females and the aborted embryos against month over the calendar year. For *U. bucculentus* (Chapter 4), *U. paucimaculatus* (Chapter 6), and *U. viridis* (Chapter 7) by plotting embryos against day of year (day one = January 1). Specific to *U. bucculentus* (Chapter 4), the ‘period of embryonic growth’ was examined by linear regression in SAS, where \( a \) and \( b \) are parameters and \( y \) and \( x \) represent the relationship between embryo length (y) and day of year (x). Specific to *U. paucimaculatus* (Chapter 6), the ‘period of embryonic growth’ was examined by logarithmic regression in Microsoft Excel, where \( a \) and \( b \) are parameters and \( y \) and \( \log(x) \) represent the relationship between embryo length (y) and day of year (x) and linear regression in SAS, where \( a \) and \( b \) are parameters and \( y \) and \( x \) represent the relationship between embryo mass (y) and day of year (x).

The stage of gestation when the yolk sac is totally absorbed was determined by plotting yolk sac mass / (embryo mass + yolk sac mass) against embryo length (Walker 2005; Walker 2007) to illustrate the transition from a lecithotrophic to a matrotrophic source of nutrition for embryos in utero. Mean egg mass for pregnant females with eggs in utero only (U=4) and mean embryo mass were used to determine the mass gain from egg to full-term embryo (Walker 2005; Walker 2007).
**Period of ovarian cycle**

The synchrony between the ‘period of embryonic growth’ and the ‘period of the ovarian cycle’ was examined as the first step towards determining the reproductive cycle. The relationship between LFD and embryo length for U=5 females were determined by linear regression in SAS, where \(a\) and \(b\) are parameters and \(y\) and \(x\) represent LFD \((y)\) and embryo TL \((x)\).

In addition, the LFD for each female was used to determine the ‘period of the ovarian cycle’. LFD plotted against day of year when pooled across all uterus conditions for mature and maturing females provides ambiguous information, which is consistent with reports for shark species (Walker 2005; Walker 2007). Hence, the seasonal ovarian cycle was investigated by plotting LFD against day of year (day one = January 1) for each uterus condition separately. U=4 females contained follicles with only small LFD and therefore provided no information on seasonal follicle growth, but they did provide information about the timing of ovulation. U=5 females provided the most reliable representation of the seasonal growth pattern of follicles and the relationship between LFD and day of year was used to describe the annual growth rate of the follicles (Walker 2005; Walker 2007) and examined by linear relationship where \(a\) and \(b\) are parameters and \(y\) and \(x\) represent LFD \((y)\) and day of year \((x)\). The regression line and the 95% confidence limits for U=5 females were superimposed onto the scattergrams of LFD against day of year for U=3 and U=6 females for comparison with the ovarian cycle of the maturing and post-partum population (Walker 2005; Walker 2007). Specific to *U. bucculentus* (Chapter 4), the regression line with 95% confidence limits for U=5 females was extended to demonstrate the ~24-months cycle. Specific to *U. paucimaculatus* (Chapter 6), growth of follicles of U=6 females was assumed to mirror U=5 females however, the U=5 female linear growth regression line (mean with 95% prediction intervals) when superimposed onto the U=6 scattergram showed differences. The LFD against day of year relationship for U=6 females during January–June was determined separately through linear regression in SAS.

**Reproductive cycle in males**
For all five urolophid species in the males, mean gonadosomatic index (GSI) and hepatosomatic index (HSI) against month for mature males were examined for seasonal synchrony in breeding condition (Maruska et al. 1996; Rossouw 1987). GSI was calculated as 100 x gonad mass / TW, and HSI as 100 x liver mass / TW for testis development conditions. The seminal vesicle fullness indices 0, 1, 2, 3 and 4 were converted to decimal values of 0.10, 0.25, 0.50, 0.75 and 1.00, respectively, and the decimal values \( Y \) were transformed to \( \sqrt{Y} \) for plotting seminal vesicle fullness against month (Walker 2007). Specific to \( U. bucculentus \) (Chapter 4), seminal vesicle fullness were tested for temporal variation using General Linear Regression in GenStat with total length as a covariate and month as a factor.

Reproductive cycles of females

For female urolophid species \( T. imitata \) (Chapter 3) and \( U. bucculentus \) (Chapter 4) through evidence of seasonal synchrony in the female reproductive biology from among individual females the ‘period of eggs ‘in utero’, ‘period of embryonic growth’ and hence the ‘period of pregnancy’, and ‘period of ovarian cycle’ was used to determine the periodicity of the reproductive cycle (Braccini et al. 2006; Huveneers et al. 2007; Walker 2005; Walker 2007).

This method was then applied to \( U. cruciatus \) (Chapter 5). Unlike \( T. imitata \) (Chapter 3) and \( U. bucculentus \) (Chapter 4) which have clear-cut reproductive cycles, the study on \( U. cruciatus \) required a clear unambiguous process to resolve uncertainty in the periodicity of the reproductive cycle. For \( U. cruciatus \), uncertainty was caused by stress-induced aborting during capture and handling of pregnant females, together with the presence of eggs in utero for a major proportion of the pregnant females all year (a condition expected in the populations of species with asynchronous gestation) and a short highly synchronous ‘period of embryonic growth’ (an indicator of synchronous reproductive cycles). This created the need to demonstrate which reproductive parameters can be determined through analysis of systematic trends across a number of animals (step 1), and which parameters are assumed by the author without any real evidence, which are inferred from other species, or which require
further testing such as testing a set of hypotheses (step 2). This stepwise approach and the order within the steps adopted are designed to be applicable for determining the reproductive cycle of any viviparous chondrichthyan species. The approach makes explicit any assumptions made or any uncertainty associated with a hypothesis.

**Determination of periodicity of female reproductive cycle within *U. cruciatus***

Applying the two-step approach to determining the period of the reproductive cycle of females required considering each of the ovarian cycle and uterine cycle separately. For determining the ovarian cycle, seasonal growth of oocytes was considered for each of six indices for uterus condition (Table 2.1) associated with uterine development (non-breeding condition where U=1, U=2 or U=3) and uterine cycle (alternatively referred to as the ‘pregnancy cycle’) (breeding condition where U=4, U=5, or U=6). For females having reached breeding condition, the pregnancy cycle can be viewed as consisting of six periods that vary widely among females within a population, a species, and different species (Fig. 2.3).

For our purposes, the ‘pregnancy cycle’ of a female was considered as consisting of five ‘pregnancy periods’ and one ‘inter-pregnancy period’ based on macroscopic inspection of the uteri. The five ‘pregnancy periods’ can be simplified to two phases. The ‘eggs in utero phase’ includes two periods: the ‘period of ovulation’ (period from when first ovulated follicle is present as an egg in utero to when last ovulated follicle is present as an egg in utero) and the ‘period of eggs in utero only’. The ‘embryos in utero phase’ includes three periods: the ‘period of mix of eggs in utero and embryos in utero’, the ‘period of embryos in utero only’, and the ‘period of parturition’ (period from birth of the first embryo to birth of the last embryo). Seasonal growth of embryos during the ‘embryos in utero phase’ provides key information on synchrony among breeding females during the pregnancy cycle and embryonic growth. The ‘inter-pregnancy phase’ consists only of the ‘period of inter-pregnancy’.

Where the ‘pregnancy cycle’ exceeds one year and is less than two years, the population has two breeding components where at any time their ‘pregnancy cycles’ are at two different stages out of phase by one year, such as *Urolophus bucculentus* with an biennial cycle (Trinnie et al. 2012), where pregnant females could be carrying
either small embryos <150 mm or large embryos ≥200 mm TL at the same time of year. Similarly, where the ‘pregnancy cycle’ is up to three years, the population has three breeding components where at any time their ‘pregnancy cycles’ are at three different stages out of phase by one and two years such as for Galeorhinus galeus (Walker 2005), and so on. Although it does not appear to be an issue for U. cruciatus, there can be the further complication of ‘pregnancy cycles’ varying annually and biennially in separate regions as demonstrated for Mustelus antarcticus (Walker 2007) and M. manazo (Yamaguchi et al. 2000).

Determining the reproductive cycle of U. cruciatus involved two steps. The first step required answering yes-or-no answers to six sequentially asked questions about the ‘pregnancy cycle’ and the ‘ovarian cycle’ (Fig. 2.4; also see results). The second step required testing three alternative hypotheses based on four separate sets of assumptions on the effects of aborted eggs and embryos on available data.

For U. cruciatus, a further difficulty arose in determining the reproductive cycle because of pregnant females aborting eggs or embryos during capture and handling indicated by the presence of aborted eggs or embryos in collecting containers provided by commercial fishers. Hence, the proportion of apparent post-partum females in the population (those recorded in U=6 condition) was inevitably higher than the actual proportion of post-partum females.

Initial inspection of the data for U. cruciatus indicated that an exceptionally high proportion of the population’s mature females were in the U=4 condition and they occurred during every month of the year, whereas U=5 females occurred only during the 6-month period Dec–May. This enabled dividing the sampling year into two six-month periods: Dec–May and Jun–Nov. The relative proportions of U=4, U=5 and U=6 females sampled during Dec–May provided a basis for addressing uncertainty of the reproductive cycle of U. cruciatus caused by pregnant females aborting from stress induced by capture and handling, which resulted in U=4 and U=5 females being observed as U=6 females.

If the reproductive cycle is annual or shorter, embryonic growth in the whole population would have to be synchronised during Dec–May, and U=4 females would
not have been found during Jan–Apr (the month of December being the transition period from egg to embryo and the month of May being the transition from parturition to ovulation). A hypothesised annual cycle with synchronous embryonic growth and eggs in utero is shown (Fig. 2.5a), but the data from the present study are not consistent with an annual or shorter reproductive cycle; the data are more consistent with either a biennial (Fig. 2.5b) or a triennial cycle without a resting period (Fig. 2.5c) or a triennial cycle with a resting period (Fig. 2.5d). Longer cycles are possible, but unlikely and therefore not considered here. Thus, for the present study, alternative expected ratios of $U=4$, $U=5$, and $U=6$ females during each of the two 6-month periods of Dec–May and Jun–Nov were explored for each of the hypothesised biennial cycle (Hypothesis 1), triennial cycle without resting year (Hypothesis 2), and triennial cycle with resting year (Hypothesis 3) (Table 2.5).

To further explore these alternative hypotheses for describing the reproductive cycle (biennial, triennial with resting year, and triennial without resting year), the $U=6$ females required redistribution of all $U=6$ females into the possible alternative uterus conditions (i.e. $U=4$, $U=5$, and $U=6$) according to four alternative sets of assumptions covering several possibilities for the seasonality of post-partum females in the population. The redistributed observations were then compared with expected numbers for each of three possible reproductive cycles. However, to make the comparisons it was necessary to adjust the numbers of $U=4$, $U=5$ and $U=6$ females such that their sum for each of the Dec–May and Jun–Nov periods were the same. For this purpose, each of the adjusted sums was put equal to 100 and the adjusted numbers of $U=4$, $U=5$ and $U=6$ females expressed as percentages. For Hypothesis 1 and 2, during Dec–May all four sets of assumptions were applied on the basis that there were no $U=6$ females, during Jun–Nov, $U=6$ females were assumed aborted $U=4$ females and reassigned as $U=4$ females accordingly. For Hypothesis 3, a proportion of $U=6$ females were to remain $U=6$, the number of $U=6$ females which equalled 33% were left as $U=6$, the remained of animals were redistributed based on the following four assumptions.

*Assumption 1:* $U=6$ females were aborted $U=5$ females.
*Assumption 2:* $U=6$ females were aborted $U=4$ and $U=5$ females in equal numbers.
Assumption 3: 6 females were aborted 4 and 5 females in the ratio of 4 and 5 females observed (~7:1).
Assumption 4: 6 females were aborted 4 and 5 females such that 52 aborted embryos found accounted for 26 recorded 6 females as 5 females, based on a mean litter size of two, with the remaining 6 females being 4 females.

The observed and the expected data for each of three hypotheses for each 6-month period were compared using a $\chi^2$ test.

**Determination of the periodicity of the reproductive cycle in U. paucimaculatus and U. viridis**

The stepwise hypothesis testing approach devised for *U. cruciatus* (Chapter 5) (Figure 2.4) was then applied to female urolophid species *U. paucimaculatus* (Chapter 6) and *U. viridis* (Chapter 7) to determine their reproductive cycles. For *U. paucimaculatus*, the approach used for *T. imitata* (Chapter 3) and *U. bucculentus* (Chapter 4) does not address the potential biasing effects of stress-induced aborting during capture and handling. For *U. viridis*, because the ‘period of eggs in utero’, ‘period of embryonic growth’ and hence ‘period of pregnancy’, and ‘period of ovarian cycle’ were asynchronous and indeterminable; further comparisons against similar urolophid species, in particular *U. paucimaculatus* because it is found in similar locations, reach similar sizes and have several common reproductive traits, were made to justify any conclusions made in determining the periodicity of the reproductive cycle.

**Litter size and sex ratio of embryos**

To describe the relationship between litter size and maternal TL for all five urolophid species, the number of embryos in utero were plotted against maternal TL for 5 pregnant females. Parameters $a$ and $b$ for the litter size ($y$)–maternal TL ($x$) relationship were determined by linear regression using SAS. To test the hypothesis of a 1:1 sex ratio a Chi-squared test with Yates’ continuity correction was applied to all of the embryos in utero pooled from the 5 pregnant females (Braccini et al. 2006). Specific to *U. cruciatus* (Chapter 5), the number of egg in utero were plotted
against maternal TL for U=4 pregnant females to demonstrate the significant amount of U=4 females in the population.

_Female and male length-at-maturity and length-at-maternity_

Female urolophid species _T. imitata_ (Chapter 3) and _U. bucculentus_ (Chapter 4) were defined as mature at the presumed macroscopic onset of vitellogenesis where LFD >3 mm (Table 2.1), or, if the follicles were too damaged to measure, where uterus condition was U=2, 3, 4, 5 or 6; otherwise, the female was classed as immature at the time of capture.

Female urolophid species _U. cruciatus_ (Chapter 5), _U. paucimaculatus_ (Chapter 6), and _U. viridis_ (Chapter 7) were defined as mature at the presumed macroscopically determined onset of vitellogenesis where LFD >1 mm (Table 2.1), or, if the follicles were too damaged to measure, where uterus condition was U=2, 3, 4, 5 or 6; otherwise, the female was classed as immature at the time of capture.

For _T. imitata_ (Chapter 3) and _U. viridis_ (Chapter 7), with annual reproductive cycles, a female was defined as maternal if, at the time of capture, it would have contributed to recruitment for the next recruitment season had it survived (Walker 2005; Walker 2007). A female was classified as in maternal condition if U=4, 5 or 6 at the time of capture, because recruitment is annual and the reproductive cycle continuous; hence, once a female is recruited into the population it will continue to recruit each year if it survives.

For _U. bucculentus_ (Chapter 4), with a biennial reproductive cycle, a female was classified as in maternal condition if U=5 with embryos in their second year of gestation, based on embryos larger than 160 mm TL at the time of capture, had it survived, it would have given birth before June 1; hence, once a female is recruited into the population it will continue to recruit every second year if it survives.

For _U. cruciatus_ (Chapter 5), with a biennial reproductive cycle, because of the difficulty of interpreting the cycle in each U=4 and U=6 individual, all U=4, U=5 and U=6 females are classed as maternal and all U=1, U=2, and U=3 females classed as
non maternal based on a one year reproductive cycle. The maternity ogive was then adjusted for $P_{\text{max}}$ to equal 0.5 to concur with a biennial cycle.

For *U. paucimaculatus* (Chapter 6), a female was classed as in maternal condition if in $U=4$ or $U=5$ condition at the time of capture; there was, however, uncertainty about $U=6$ females being maternal. Because of the effects of potential misclassification from stress-induced aborting from capture and handling, $U=6$ females had to be considered as part of the hypothesis-testing approach.

For males of all five urolophid species, three independent indices were used to determine maturity: testis development (G), seminal vesicle condition (V), and clasper calcification (C) (Table 2.1). Males were classed as immature for $G=1$ or $G=2$ and mature for $G=3$ testis development, as immature for $V=1$ and mature for $V=2$ or $V=3$ seminal vesicle fullness, and immature for $C=1$ or $C=2$ and mature for $C=3$ clasper calcification. Specific to *T. imitata* (Chapter 3), the sigmoid relationship between clasper length and TL was used to compare the results of maturity based on logistic regression with those of maturity based on the growth of the clasper.

Logistic regression was used to determine the length-at-maturity ogives of males and females using the various maturity indices and the length-at-maternity ogive of females. The proportion of males or females in the population mature or proportion of females maternal was expressed as a function of TL and the parameter values and statistical diagnostics were estimated by logistic regression in SAS. To provide a dichotomous variable for logistic regression, a value of 1 was assigned to mature animals and maternal females and a value of 0 to immature animals and non-maternal females. The logistic equation was reformulated for biological interpretation using SAS probit analysis, based on the parameters $P_{\text{max}}$, $l_{50}$ and $l_{95}$, where $P_{\text{max}}$ is the maximum proportion in mature and maternal condition, and $l_{50}$ and $l_{95}$ are lengths at which 50% and 95% of the population are in mature or maternal condition, respectively (Walker 2005; Walker 2007).

An additional step for the maternity ogives of *U. bucculentus* (Chapter 4) and *U. cruciatus* (Chapter 5) were needed because the initial conditions of the logistic regression requires $P_{\text{max}}$ to equal 1.0 which signifies parturition to occur annually.
The parturition frequency for maternity analysis of *U. bucculentus* and *U. cruciatus* requires $P_{\text{max}}$ to equal 0.5 for a biennial cycle (Walker 2005; Walker 2007) to demonstrate that only 50% of the population gave birth annually.

For *U. bucculentus*, this was undertaken by classing the females into 100 mm TL size classes and the ratio of number of females in maternal condition to total number of females observed was altered where appropriate to represent 0.5 in each 100 mm size classes (Walker 2007).

For *U. cruciatus*, which had much uncertainty in the reproductive cycle (see above), this was undertaken simply by a maternity ogive where $P_{\text{max}}$ equals 1.00, for each size class where the ratio of number of females in maternal condition to total number of females observed exceeded 0.5, then the number of maternal animals was adjusted down to give a ratio of precisely 0.5 (Walker 2007).

An additional step for the maternity ogives of *U. paucimaculatus* (Chapter 6) was needed because for a pregnancy cycle less than one year, the reproductive cycle could be considered as annual ($P_{\text{max}} = 1.0$ for the maternal ogive), but given the large number of U=6 females observed in the population, the reproductive cycle could be considered biennial ($P_{\text{max}} = 0.5$). The large number of females exhibiting stress-induced aborting from capture and handling creates a need to redistribute some of the females classed U=6 into females classed U=4 and U=5. However, because of the uncertainty distinguishing actual resting and post-partum females from aborted females, the U=6 females were redistributed into U=4 and U=5 so that $P_{\text{max}}$ was varied to represent 100%, 90%, 80%, 70% and 60% of the population of large females contributing to recruitment each year. The SAS probit procedure was applied for logistic regression using the proportion of maternal animals in each 25-mm size-class. Where the number of maternal females in any particular size class exceeded the assumed proportion $P_{\text{max}}$ for each of a range of hypothesized $P_{\text{max}}$ values (i.e. $P_{\text{max}} = 1.00$ for 100%, 0.90 for 90%, 0.80 for 80% and so on), then the number of U=6 females was adjusted so that the ratio of the number maternal and total number was adjusted to be consistent with the required $P_{\text{max}}$. For input into the SAS probit procedure for each hypothesized $P_{\text{max}}$ to ensure the proportion then reached 1.00, a weighted statement (i.e. $x 1/ P_{\text{max}}$) was applied to the number of maternal females in
each 25-mm size-class. The resultant ogive from logistic regression was then un-weighted (i.e. x P_max) to represent the required P_max.

Statistical testing for spatiality and temporality

Despite samples being collected of all species during 2002 to 2006, no temporal analysis was undertaken because of the limited sample sizes when broken into a yearly regime: all years were therefore pooled. For statistical analysis of T. imitata (Chapter 3), U. bucculentus (Chapter 4) and U. cruciatus (Chapter 5), the data were pooled across all regions because there were insufficient data to make spatial comparisons. For U. paucimaculatus (Chapter 6) and U. viridis (Chapter 7) each region was separately analysed and compared.

Allometric and isometric relationships for total body mass and various size measurements

For U. paucimaculatus (Chapter 6) and U. viridis (Chapter 7), ANCOVA (terms region, covariate TL, and interaction region x TL) was adopted to test for effects of region on the male and female TW–TL linear relationships and were statistically compared using the least-squares means tests using ‘Proc GLM’ in SAS. Starting with the interaction terms, if the P-value (P>0.05) was statistically non-significant the interaction term was eliminated so that the P-values of the region and covariate remained (step-wise backward elimination).

Growth of embryos and the pregnancy cycle

For U. paucimaculatus (Chapter 6) and U. viridis (Chapter 7), ANCOVA (with terms region, covariate ETL, and region x ETL) was adopted to test for regional effect on the ETL–embryo mass relationship and were statistically compared using the least-squares means tests using ‘Proc GLM’ in SAS. Starting with the interaction terms, if the P-value (P>0.05) was statistically non-significant the interaction term was eliminated so that the P-values of the region and covariate remained (step-wise backward elimination).
Period of ovarian cycle

For *U. paucimaculatus* (Chapter 6), ANCOVA (with terms region, covariate ETL, and region x ETL) was adopted to test for regional effects on the LFD–Embryo length relationship and ANCOVA (with terms region, covariate day of year, and region x day of year) was adopted to test for regional effects on the LFD–day of year relationship in *U*=5 females using ‘Proc GLM’ in SAS. For *U. viridis* (Chapter 7), the ovarian cycle was indeterminable and could not be tested for region.

Litter size and sex ratio of embryos

For *U. paucimaculatus* (Chapter 6), ANCOVA (with terms region, covariate maternal TL, and region x maternal TL) was adopted to test for regional effect on the litter size–maternal TL relationships using ‘Proc GLM’ in SAS. For *U. viridis* (Chapter 7), the litter size was similar and was not tested for region.

Female and male length-at-maturity and length-at-maternity

For *U. paucimaculatus* (Chapter 6), the relationship between the proportion of animals mature or maternal and TL were tested for the effects of region by a step-wise backward elimination procedure through the Wald $x^2$ likelihood-ratio test using ‘Proc Logistic’ in SAS (Walker 2005; Walker 2007). For *U. viridis* (Chapter 7), despite the regional differences in other reproductive parameter for male and female *U. viridis*, regional comparisons of maturity and maternity ogives could not be made because of the lack of immature animal when split into each region.
### Table 2.1. Index used for various reproductive organs to indicate sexual development

The assumption of the maturity of each indice is also listed (modified from Walker 2005)

<table>
<thead>
<tr>
<th>Organ or tissue</th>
<th>Index</th>
<th>Description</th>
<th>Maturity assumption</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>U. paucimaculatus, U. cruciatus, U.viridis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovary</td>
<td>GI=1</td>
<td>Largest follicle diameter &lt;1 mm; follicle not yolked</td>
<td>Immature</td>
</tr>
<tr>
<td></td>
<td>GI=2</td>
<td>Largest follicle diameter 1 mm; follicles yolking</td>
<td>Maturing</td>
</tr>
<tr>
<td></td>
<td>GI=3</td>
<td>Largest follicle diameter &gt;1 mm; follicles yolked</td>
<td>Mature</td>
</tr>
<tr>
<td><strong>T. imitata, U. bucculentus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovary</td>
<td>GI=1</td>
<td>Largest follicle diameter &lt;2 mm; follicle not yolked</td>
<td>Immature</td>
</tr>
<tr>
<td></td>
<td>GI=2</td>
<td>Largest follicle diameter 2–3 mm; follicles yolking</td>
<td>Maturing</td>
</tr>
<tr>
<td></td>
<td>GI=3</td>
<td>Largest follicle diameter &gt;3 mm; follicles yolked</td>
<td>Mature</td>
</tr>
<tr>
<td>Uterus</td>
<td>U=1</td>
<td>Uniformly thin tubular structure</td>
<td>Immature</td>
</tr>
<tr>
<td></td>
<td>U=2</td>
<td>Thin tubular structure partly enlarged posteriorly</td>
<td>Maturing</td>
</tr>
<tr>
<td></td>
<td>U=3</td>
<td>Uniformly enlarged tubular structure; pre ovulatory and pre-maternal animal</td>
<td>Mature</td>
</tr>
<tr>
<td></td>
<td>U=4</td>
<td>Pregnant; <em>eggs in utero</em> present without macroscopically visible embryos; post ovulatory</td>
<td>Mature</td>
</tr>
<tr>
<td></td>
<td>U=5</td>
<td>Pregnant; <em>embryos in utero</em> macroscopically visible</td>
<td>Mature</td>
</tr>
<tr>
<td></td>
<td>U=6</td>
<td>Enlarged tubular structure distended, post partum animal or possible aborted pregnancy</td>
<td>Mature</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Testis</strong></td>
<td>GI=1</td>
<td>Thin with epigonal gland dominant</td>
<td>Immature</td>
</tr>
<tr>
<td></td>
<td>GI=2</td>
<td>Thickened and elongated</td>
<td>Immature</td>
</tr>
<tr>
<td></td>
<td>GI=3</td>
<td>Enlarged and predominant with testicular lobules present</td>
<td>Mature</td>
</tr>
<tr>
<td>Seminal vesicle</td>
<td>VI=1</td>
<td>Thin translucent walls and seminal fluids absent</td>
<td>Immature</td>
</tr>
<tr>
<td></td>
<td>VI=2</td>
<td>Thickened opaque walls and seminal fluids present</td>
<td>Mature</td>
</tr>
<tr>
<td></td>
<td>VI=3</td>
<td>Thickened opaque walls and seminal fluids absent</td>
<td>Mature</td>
</tr>
<tr>
<td>Seminal vesicle fullness</td>
<td>0</td>
<td>empty</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>up to 25% full</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26–50% full</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>51–75% full</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>76–100% full</td>
<td></td>
</tr>
<tr>
<td>Clasper</td>
<td>CI=0</td>
<td>Pliable with no calcification</td>
<td>Immature</td>
</tr>
<tr>
<td></td>
<td>CI=1</td>
<td>Partly calcified</td>
<td>Immature</td>
</tr>
<tr>
<td></td>
<td>CI=2</td>
<td>Rigid and fully calcified</td>
<td>Mature</td>
</tr>
</tbody>
</table>
Fig. 2.1. Sampling locations for collection of *Trygonoptera imitata* and *Urolophus paucimaculatus* in south-eastern Australia. Shaded areas are sampling locations: Western Bass Strait, Port Phillip Bay, Corner Inlet and Lakes Entrance.
Fig. 2.2. Sampling locations for collection of *Urolophus bucculentus*, *U. cruciatus*, and *U. viridis* in south-eastern Australia. Shaded areas are sampling locations; Western Bass Strait and Lakes Entrance.
Fig. 2.3. Six periods of pregnancy cycle for viviparous species.

The six periods are shown to be of equal length here, but the periods vary widely among separate species.
Q1: Is embryonic growth synchronous among females in each breeding component of the population?

Embryonic growth occurs synchronously (seasonally) among females in each breeding component of population; hence, duration of embryonic growth and 'period of parturition' determinable.

Q2: Are 'period of ovulation' and 'period of eggs in utero' synchronous among females in each breeding component of population?

'Period of ovulation' and 'period of eggs in utero' synchronous and determinable.

Q3: From answers to Q1 and Q2, can periodicity of 'pregnancy cycle' be determined?

Duration of 'pregnancy cycle' determinable.
Fig. 2.4. Sequence for stepwise sequential questioning for determining duration of reproductive cycle.

Alternative answers are shown in pairs of shaded boxes to each question in shaded diamond shape. ETL, embryo TL; LFD, largest follicle diamet
Fig 2.5. Annual, biennial, and triennial reproductive cycles as alternative hypotheses for *U. cruciatus*.

The embryos *in utero* phase (U=4) is shown as 6 month preceded by the eggs *in utero* phase for 6 months (annual reproductive cycle) (a), 18 months (biennial reproductive cycle) (b), and 30 months (triennial reproductive cycle) (c). Each 12-month period is divided into the two 6-month periods of Dec–May and Jun–Nov; eggs *in utero* are represented by embryo lengths of zero; most likely the inter-pregnancy phase forms part of embryos *in utero* phase for *U. cruciatus*. 
Chapter 3 – *Trygonoptera imitata* (Eastern Shovelnose stingaree)

Abstract

In applying a quantitative macroscopic approach to the reproduction of *Trygonoptera imitata*, the present study contributes to understanding the wide diversity in the reproductive biology of the family Urolophidae and provides insights to help determine phylogenetic relationships. This localised species is taken as bycatch in several inshore fisheries and potentially impacted by a range of other anthropogenic pressures, including introduced species, particularly in shallow-water pupping areas. *T. imitata* can be characterised as a species of comparatively low matrotrophic histotrophy with an extended period of relatively large eggs *in utero* (5–8 months) followed by rapid growth of the embryos (4–6 months). The reproductive cycle is annual with parturition occurring during late-February–April, followed immediately by ovulation. Mean size-at-birth is ~225 mm total length and there is a ~1000% gain in mean wet mass from egg (15 g) to full-term embryo *in utero* (150 g), the lowest reported for any viviparous batoid with lipid histotroph. Litter size increases with maternal length, reaching a maximum of seven, and sex ratio of embryos is 1:1. Maximum length and estimates of the maturity-ogive parameters $l_{50}$ and $l_{95}$ are similar for females and males.
Results

Composition of samples

Males (212–734 mm TL; 98–4700 g TW) and females (200–760 mm TL; 83–5200 g TW) grew to similar size (Fig. 3.1). About half the samples came from Port Phillip Bay and most U=5 pregnant females were found only in shallow waters of Port Phillip Bay and Corner Inlet (Table 3.1).

Allometric and isometric relationships for total body mass and various size measurements

The effect of sex on the body mass–TL relationship (Fig. 3.2) was significant (ANCOVA: $F_{1, 591} = 26.06$, $P<0.0001$). A least squares means test indicated that males and females were highly significantly different ($P<0.0001$). The effect of sex and female breeding condition on body mass–TL relationships among 282 males, 229 non-pregnant females (U=1–3 and U=6 females), 22 pregnant females with eggs (U=4), and 28 pregnant females with embryos (U=5) was highly significant (ANCOVA: $F_{3,560} = 11.57$, $P<0.0001$) and the covariate ln(l) was highly significant (ANCOVA: $F_{3,560} = 36766.7$, $P<0.0001$). Females and males were significantly different among the large-TL classes. Least squares means indicated that U=4 pregnant females were not significantly different from U=5 pregnant females ($P=0.4363$), so the data for all pregnant females (U=4 and U=5) were pooled. These pregnant females pooled were highly significantly different from the non-pregnant females ($P=0.0062$) and from males ($P<0.0001$); however, males were not significantly different from the non-pregnant females ($P=0.2544$).
A least squares means test indicated that DL–TL \((P<0.6442)\) and DW–TL \((P<0.2357)\) morphometric relationships were not significantly different between males and females. The variables for each morphometric relationship (sex combined) are given by the equations

\[
DL = -9.364 + 0.598 \text{ TL}, \quad \text{where } P=0.001; \quad r^2 = 0.991; \quad n = 388, \quad \text{and}
\]

\[
DW = 4.163 + 0.634 \text{ TL}, \quad \text{where } P=0.001; \quad r^2 = 0.989; \quad n = 388.
\]

**Reproductive cycles of females and males**

The females have a continuous, annual reproductive cycle. Analysis of data for females indicated that the period-of-pregnancy was \(~12\) months, ovulation occurred during late-February–April, and eggs *in utero* occur for a period of 5–8 months until commencement of the embryonic gestation during November–December. The embryos reach full term by the following late-February–April, the time of birth after a period of 4–6 months. The LFD remained relatively constant with little development during the \(U=4\) period but when the females reached the \(U=5\) condition the ovarian cycle was synchronous with the embryonic gestation period and LFD increased from 10–15 mm to a maximum of 43 mm during the 4–6 months.

Seasonal trends in the male breeding cycle were determined for mature males from GSI, HSI and seminal vesicle fullness means plotted against month. Mean GSI increases during October–December and then decreases through the months January–September (Fig. 3.3a). Mean HSI (Fig. 3.3b) exhibits a similar seasonal pattern; it increases during August–November to peak in February before then decreasing to a minimum by August. The seminal vesicle fullness increased from empty in all animals during July–December to peak at \(~75\)% fullness by April (Fig. 3.3c), indicating there was only a short period when males can inseminate females, a period coinciding with the peak ‘period of ovulation’.

**Growth of embryos and period of embryonic growth**

The growth of the embryos during gestation and timing of parturition was determined from 115 embryos collected (106 embryos from 28 pregnant females and 9 aborted
embryos from unknown pregnant females). Embryo mass plotted against embryo total length (ETL) (Fig. 3.4a) is curvilinear which was modelled with the power curve. The largest embryo was 250 mm ETL and 243 g wet mass, whereas the smallest neonate collected was 200 mm TL and 83 g wet mass, demonstrating that all stages of embryonic development had been collected so that the size-at-birth and ‘period of embryonic growth’ could be reliably determined.

Mean mass of ovarian follicles for females with LFD >35mm at 14.7 g (s.e. 1.2 g; range 11.5–18.1 g) was similar to mean mass of eggs in utero at 15.1 g (s.e. 1.0 g, range 11.0–18.5 g). The presence of eggs in utero for U=4 females (n=23) were found throughout most of the year with the exception of February and June–August when sampling was minimal. During embryonic development the egg forms the external and internal yolk sacs and the external yolk sac was consumed by the time the embryos reached 200–250 mm mean ETL or 75–243 g mean mass (Fig. 3.4b). Histotroph was supplied to the embryo during the latter part of the ‘period of embryonic growth’, although the exact timing, either before or after external yolk sac absorption, was not determined. Data for determining patterns of consumption of the internal yolk sac during gestation were not collected, but neonates were found with internal yolk sacs weighing up to 0.2 g.

Scattergrams of mean ETL (Fig. 3.5a) and mean embryo mass (Fig. 3.5b) against month for U=5 females indicate that embryos are present from 5 November to 21 April and that parturition in the population is complete by the end of April. Size of full-term embryos ranged 200–250 mm ETL and 83–253 g mass, indicating wide variation in the population. Parturition occurs most likely during late-February–April suggesting the ‘period of embryonic growth’ is 4–6 months. Whether size-at-birth of embryos varied with maternal TL of the females could not be determined from the present study; however, embryo TL and mass for small (<650 mm TL) and large (>700 mm TL) maternal females were similar during late-February–April, indicating that maternal TL may not influence size-at-birth or parturition date. Full-term embryos reached 225 mm mean ETL and 150 g mean mass during April and are indicative of the presumptive average size-at-birth. Based on this value, both females and males were born at ~30% of their maximum TL (l_{max}). The increase from mean egg mass in utero (15.1 g) to mean full-term embryo mass (150 g) indicates ~1000%
increase in wet mass gain during development, but this varies 550–1600% between the lowest and highest birth masses of 83 and 243 g, respectively.

*Period of ovarian cycle*

The ‘period of embryonic growth’ was synchronous with the ovarian cycle for U=5 pregnant females, as indicated by the linear relationship between LFD and embryo length (Fig. 3.6) \( (r^2=0.86; \ P<0.001) \). LFD for 307 females ranged 1–43 mm across all uterus conditions (U=1–6), but ranged 1–7 mm for U=1 and U=2 (Fig 3.7bc), 4–29 mm for U=3 (Fig 3.7d), 4–25 mm for U=4 (Fig 3.7e), 13–43 mm for U=5 (Fig 3.7a), and 4–35 mm for U=6 females (Fig 3.7f). Vitellogenesis had commenced in only a small number of U=1 and U=2 females, but had commenced for all U=3 females.

The U=5 scattergram and linear regression of LFD against day of year (Fig. 3.7a), used to depict follicle growth during embryonic growth, indicated that LFD increased from 10–15 mm during November to a maximum of 43 mm by April the following year. These linear regressions indicated that the U=5 females were ready to ovulate immediately after parturition in late-February–April. However, the females were in the U=5 condition for only <6 months of the year and consequently the actual ovulation date and subsequent annual periodicity of the reproductive cycle could not be determined from the U=5 females alone.

The timing of ovulation can be determined from the presence and periodicity of U=4 pregnant females in the population, females in the U=3 condition with large LFD, the presence of U=6 post partum females with large LFD, and from observing females in the process of ovulating as indicated by the presence of yolk in the oviduct or oviducal glands. Although no ovulating females were captured, U=4 females \( (n=19) \) were found during March–May (days 75–136) and during September–January (days 248–006) which gave no indication of when ovulation occurred. The presence of U=6 females was expected to confirm when ovulation occurred, but, because of the high aborting rate of females upon capture, uncertainty occurred in distinguishing U=6 females from U=4 females or from U=5 females, which might have aborted their eggs or embryos and been misclassified as a U=6 female. In addition, U=6 females might have been misclassified as U=3 females because of the tendency for the U=6
condition to revert to the U=3 condition after parturition as the distended walls of the uteri gradually contract. Hence, the reproductive condition of each female classed as U=6 was reinterpreted as U=4, U=5 or U=6 depending upon the most parsimonious hypothesis on the timing of annual parturition.

Based on the assumption that parturition occurs during late-February–April (inferred from the size of embryos in the preceding section), no female prior to late-February could be in U=6 condition because, if a female was mature during this period, it is expected to be pregnant. Thus, the U=6 females (Fig. 3.7f) found during January–early February (LFD 13–25 mm) are likely to have been U=5 females (circled in Fig. 3.7f) that have aborted their embryos upon capture, and thus have been reclassified accordingly. Females with LFD <9 mm during March–May are likely to have been U=4 females (circled in Fig. 3.7f) that had aborted their eggs, because both U=5 and U=6 females are expected to have only large follicles (LFD >25 mm) that are ready to be ovulated during this period. On the other hand, females with LFD >25 mm during February–May were likely to be a mixture of U=5 females having aborted their embryos (i.e. apparent U=6 females) and of actual U=6 post partum females (i.e. LFD >30 mm). Females found during the second half of the year were likely to be a mixture of all U=3, 4, 5 and 6 conditions and, because there was no basis for separating them, they therefore could not be included in further ovarian cycle analysis (circled in Fig. 3.7f).

Parturition occurring during late-February–April, the presence of U=4 females during March–May and U=6 females with large LFD values during late-February–May, and the absence of U=6 females with large LFD during June–December support our conjecture that ovulation occurred soon after, parturition. Despite the lack of sampling during June–early-September, U=4 females continued to be found during late-September–January (248–006 days of year) (Fig. 3.7e), which indicated that, after ovulation during late-February–April, the eggs in utero are found for 5–8 months before the beginning of gestation during November (days 300–330). The scattergram of LFD against day of year for the U=4 condition (Fig. 3.7e) shows LFD increasing gradually from 5–8 mm in May (~day 120) to 8–25 mm during October–December (days 240–355). Follicles of two U=4 females with LFD of 20 mm and 25 mm found during October appear to be going through atresia.
Seasonal growth of follicles in U=3 females was evident throughout the year as they approached first ovulation (Fig. 3.7d). Comparison between U=3 and U=5 animals shows that some U=3 females display early growth and were ready to ovulate by April. Predominantly though, animals with LFD <10 mm were likely to have only recently progressed to U=3 and would remain thus until November, after which follicles would grow in readiness for ovulation and first pregnancy.

*Litter size and sex ratio of embryos*

Linear relationships were fitted between the number of eggs *in utero* and maternal TL ($r^2 = 0.33; P=0.001$) and between the number of embryos *in utero* and maternal TL ($r^2 = 0.28; P=0.001$) (Fig 3.8ab). The smallest recorded U=4 (n=19) and U=5 (n=28) pregnant females were 515 mm and 610 mm TL, respectively. A maximum litter size of seven was found in each of the U=4 and U=5 pregnant females of 720 mm and 760 mm TL, respectively.

A total of 106 embryos from 28 pregnant females were sexed based on macroscopic inspection from the presence or absence of claspers of which 43 (40%) were females, 40 (38%) were males, and 23 (22%) were of unknown sex due to their early stage of development. Based on the male to female ratio of embryos *in utero*, the sex ratio was 1:1 ($\chi^2 = 0.048$, d.f. = 1, $P=0.826$).

*Female and male length-at-maturity and length-at-maternity*

As to be expected, the parameter estimates of $l_{50}$ and at $l_{95}$ were greater for length-at-maternity (labelled (b) in Fig. 3.9) than for the length-at-maturity of females (labelled (a) in Fig. 3.9) with an estimated growth during this transition of 64 mm and 115 mm TL, respectively. Females and males reached maturity at a similar size at $l_{50}$ and $P_{max}$ equals 1 for each of the female and male maturity ogives and the female maternity ogive. The parameters for the length-at-maturity ogive for females were determined from 145 immature and 152 mature animals, of which the largest immature female observed was 482 mm TL and the smallest mature female observed was 446 mm TL.
The parameter values (with 95% confidence limits) for females was 475 (465, 488 mm TL) at $l_{50}$ and 505 (492, 534 mm TL) at $l_{95}$, where $l_{50}$ was ~63% and $l_{95}$ was ~66% of $l_{\text{max}}$. The parameters for the length-at-maternity ogive were determined from 162 non-maternal and 132 maternal females based on a one-year parturition cycle (i.e. $P_{\text{max}}=1$). The smallest animal in maternal condition was 524 mm TL and the largest animal in non-maternal condition was 665 mm TL. The parameter values of females in maternal condition was 539 (525, 552 mm TL) at $l_{50}$ and 620 (604, 642 mm TL) at $l_{95}$, where $l_{50}$ was ~71% and $l_{95}$ was ~81% of $l_{\text{max}}$.

For males, analysis of indices for the three criteria defining maturity all gave similar estimates for each of $l_{50}$ and $l_{95}$. The parameter values were 483 (473, 494) mm for testis development (Fig. 3.10a), 481 (472, 490) mm for seminal vesicle fullness (Fig. 3.10b), and 476 (468, 483) mm for clasper calcification (Fig. 3.10c) at $l_{50}$. Graphical comparisons of the three maturity methods (Fig. 3.10d) indicate the similarity between methods. $l_{50}$ was reached at a mean value of ~65% of $l_{\text{max}}$ for the three methods.

Clasper length (CL) showed a sigmoid relationship with TL. Claspers were <20 mm CL in animals <420 mm TL; growth increased rapidly from 20–55 mm CL at 420–500 mm TL, and calcification was complete in most males >500 mm TL. The exception was one male, which was had 36 mm CL at 537 mm TL. Gradual clasper growth continued in the animals >500 mm TL to a maximum of 80 mm CL. There is reasonable agreement between the growth of the clasper and the 476 mm and 518 mm TL values at $l_{50}$ and $l_{95}$ for clasper maturity, respectively.

**Discussion**

The collection of animals represented all size classes from neonates to large mature animals of both sexes caught throughout Victorian and adjacent waters. Most pregnant females were found only in the shallow waters of Port Phillip Bay and Corner Inlet, whereas small immature and maturing females were found mostly in the waters off Lakes Entrance and in Bass Strait. This suggests the importance of the shallower more protected waters to the pregnant *T. imitata* and the possibility that
these areas are where females give birth (Heupel et al. 2007). Port Phillip Bay, for example, is a semi-enclosed embayment with high potential to be affected by environmental change and anthropological pressure, and exhibits recent localised decline in numbers of *T. imitata* (Hobday et al. 1999) However, further information is needed on the population movement patterns and the importance of specific regions such as Port Phillip Bay to determine whether declines in abundance continue into the future.

*Total body mass and total length*

The maximum TL (760 mm) recorded for females in the present study is smaller than reported previously (800 mm TL) (Last and Stevens 1994). In all other studied Australian urolophid species, sexual dimorphism occurs where maximum size and length-at-maturity is greater in females than males (see Chapters 4–7; White and Potter 2005). However, this is not the case for *T. imitata*: the two sexes mature at similar TLs. Females reached a slightly larger *L*ₘₐₓ than males, but the difference in mass between the sexes in large animals occurred only because the females were pregnant. Sexual dimorphism occurs for most species of chondrichthyans (Last and Stevens 1994), particularly among viviparous and ovoviviparous species, presumably because of selection pressure for a larger body size to meet the higher energy demands of embryo development and nourishment (Sims 2003).

*Reproductive cycles of females and males*

The reproductive cycle that is annual appears to be the most common among urolophid species: i.e. *Trygonoptera imitata* (Present chapter), *Urolophus paucimaculatus* (Chapter 6), *U. viridis* (Chapter 7), *U. lobatus* (White and Potter 2005), *T. personata* (White and Potter 2005), and *T. mucosa* (White and Potter 2005). *Urolophus bucculentus* (Chapter 4) and *U. cruciatu* (Chapter 5) from south-eastern Australia are the only species reported to have biennial reproductive cycles and *Urolophus halleri* from California, USA, is reported to have a biannual reproductive cycle (Babel 1967). However, the results presented for *U. halleri* can be better interpreted as annual than biannual. Data presented for *U. halleri* in Figures 12 and 27 of that study, along with the information on the ‘period of embryonic growth’
(embryos are found only during June–September), the ovarian follicles take one full year to develop, the peak ovulation period is June, and the peak male breeding condition in May–July all suggest an annual cycle. The possibly of a biannual cycle comes from a small percentage of the population that appears ready to ovulate about December, but there is no evidence to confirm that they did ovulate and begin gestation; it is more likely that the follicles of these females will undergo atresia. Hence there can be a tendency to mistake an annual reproductive cycle as a biannual cycle.

Within the predominant pattern of annual and biennial reproductive cycles for urolophid species, the ‘period of eggs in utero’ and the ‘period of embryonic growth’ vary markedly. The annual reproductive cycle of T. imitata has an extended ‘period of eggs in utero’ (5–8 months) and a shorter ‘period of embryonic growth’ (4–6 months); similar periods of eggs in utero and embryos in utero are also found in T. personata (White et al. 2002) and T. mucosa (White et al. 2002), which also have annual reproductive cycles. Most species of Urolophus with annual cycles, on the other hand, have eggs in utero for only a short period (1–2 months) and embryos in utero for a longer period (10–12 months) (see Chapters 4–7; White and Potter 2005). With the biennial cycle of U. bucculentus (Chapter 4), the eggs in utero period is also short (2–3 months), but the ‘period of embryonic growth’ is exceptionally long (15–19 months). The only exception is the biennial cycle of U. cruciatus (Chapter 5); this also has an extended ‘period of eggs in utero’ (~18 months) before the ‘period of embryonic growth’ (4–6 months). The extended ‘period of eggs in utero’ as observed in other species is referred to as delayed development (Marshall et al. 2007) or embryonic diapause (Renfree and Shaw 2000; Simpfendorfer 1992); however, in the present study for T. imitata no cellular morphology was undertaken to distinguish the extended ‘period of eggs in utero’ between delayed development and embryonic diapause.

Chondrichthyan species provide varying lecithotrophic and matrotrophic nutrition contributions for embryonic development and there appears to be an evolutionary trend away from lecithotrophic nutrition to matrotrophic nutrition (Wourms 1977). Oviparous and the more primitive viviparous chondrichthyan groups producing large ovarian follicles, such as squalidae sharks (Braccini et al. 2006) and phosphoridae
(Hudson et al. 2005), tend to be highly lecithotrophic with minimal or no matrotrophic contribution. Batoid and recent shark species, on the other hand, tend to be more matrotrophic, but the degree of matrotrophy varies wide among the chondrichthyan groups and this is evident among the genera of Urolophidae. The matrotrophic contribution in T. imitata of ~1000% increase in mean wet mass from egg to embryo, the lowest reported for any batoid species (present study); most other lipid-histotroph viviparous batoid species are regarded as highly matrotrophic, with histotroph contributions of 1680–6000% (Musick and Ellis 2005). Comparatively, Urolophus species from Australia range 2500–4500%, with the exception of U. bucculentus at 7200% (Chapters 3–7). In Urolophus species, eggs in utero are small and the initial lecithotrophic stage is short; the embryos are mainly dependent on histotroph for growth. However, in T. imitata, the eggs in utero are much larger and the lecithotrophic ‘period of embryo growth’ is substantially longer; the matrotrophic histotroph is introduced only late in the gestation period. This is indicative of the genus Trygonoptera being more primitive than the genus Urolophus.

The process of encapsulation of eggs appears to be degenerating with evolution among the genera of the family Urolophidae. The eggs in utero of T. imitata are encased in a transient brown egg envelop through what appear to be vestigial processes during early gestation. The egg envelop of T. imitata is similar to the delicate brown membranes described for T. mucosa and T. personata and assumed to be impermeable to sperm (White et al. 2002), whereas Urolophus species have minimal egg encapsulation in only a translucent membrane. This may also be influencing the ‘period of eggs in utero’ with Trygonoptera species demonstrating an extended period of ‘eggs in utero’, whereas comparatively Urolophus species have only a short ‘period of eggs in utero’ before embryonic gestation. The oviducal gland where egg encapsulation occurs is likely to be different between Trygonoptera species and Urolophus species. Urolophus species are demonstrating an evolutionary change and lack the egg case producing zones within the oviducal gland; seen in two American Urobatis species—U. halleri and U. jamaicensis—which have lost of the club and papillary zones within the oviducal glands and lack discrete baffle zones required for producing an egg envelop (Hamlett et al. 2005b). Histological study of the eggs in utero may provide a clearer understanding of the purpose of this extended
‘period of eggs in utero’ and whether delayed development or embryonic diapause occurs.

Fertilisation of the *T. imitata* egg can only occur prior to encapsulation and probably occurs within the oviducal glands in conjunction with sperm storage, as observed in other chondrichthyan species (Hamlett *et al.* 2005b; Storrie 2004). Mating and sperm storage in *T. imitata* must occur before or during ovulation. This is consistent with the male seasonal patterns observed in seminal vesicle fullness, GSI and HSI.

It is suggested maximum LFD within a species depends on the period of the reproductive cycle; i.e. <30 mm maximum LFD in annual reproductive species and >40 mm maximum LFD in biennial or triennial species (Walker 2007). This generalisation for sharks, however, does not apply to urolophid or other batoid species. Several urolophid species have LFD values that are relatively small (12–30 mm) and reproduction is annual (present study)(White *et al.* 2002; White and Potter 2005), whereas *T. imitata* has 43 mm maximum LFD with an annual reproductive cycle, and *U. cruciatus* and *U. bucculentus* have 12 mm and 24 mm maximum LFD, respectively, with biennial reproductive cycles. Rhinobatids can also be found with large ~50 mm LFD with annual reproductive cycles (Marshall *et al.* 2007). The period of the reproductive cycle cannot be predicted from any single reproductive variable; factors such as maximum LFD, ‘period of ovarian cycle’, matrotrophy versus lecithotrophy, ambient conditions, and size of full-term embryo compared with L_{max} all need to be considered together. Each chondrichthyan species needs to be assessed independently to determine its reproductive periodicity.

The ovarian cycle of *T. imitata* is highly synchronised with the period-of-pregnancy. The LFD during the 5–8 months of the extended ‘period of eggs in utero’ increase only marginally from the post-ovulation size until gestation begins. The LFD for the U=5 condition increases as the embryos increase in size, and at ovulation all pregnant females carry large follicles. Although the minimum size for ovulated follicles was not determined in the present study, >30 mm LFD seems likely. This suggests that once maternal, they continually reproduce throughout life without rest periods between pregnancies. However, there are exceptions; in the present study two U=4 females with large follicles (20 and 25 mm LFD, respectively) were out of synchrony
with the rest of the population. These two females found during October are likely to be going through atresia because they also had eggs in utero and their largest were too small for ovulation. In addition, one U=4 female found during January, with only a maximum ~3 months available for gestation, suggests that its eggs were not fertilised during the mating season (January–June); alternatively, the eggs remain in diapause for another year because of unfavourable conditions for this particular female. The embryos found during December averaged 79 mm ETL and similarly, the follicles of this U=4 female remained small late into the season. No U=4 females were found during February, which suggests that further delayed development is unlikely.

Similar to *Trygonoptera imitata*, *T. mucosa* and *T. personata* from south-western Australia (White *et al.* 2002) have parturition dates of June and April, respectively. Parturition dates vary among the urolophids with *Urolophus paucimaculatus* and *U. lobatus* giving birth during October–December (White and Potter 2005). Although it is uncertain why parturition dates vary among urolophid species, White and Potter (2005) proposed that a diet of polychaetes by *Trygonoptera* species, unlike that of *Urolophus* species which prefer crustaceans, might be the reason why *Trygonoptera* species give birth during winter. *Trygonoptera imitata* has the largest eggs in utero among the Australian urolophids (Edwards 1980; Treloar 2001; White 1998) and is the only reported urolophid to have both an external yolk sac (utilised during gestation) and an internal yolk sac present at birth. Although, the internal yolk sac is only (0.2 g) at birth and the extent of its contribution to embryo development is unknown, it may allow for greater survival of neonates during the winter months because feeding may not be of primary importance.

*Litter size and sex ratio of embryos*

Determining litter size as a measure of recruitment is problematic in chondrichthyan species because of stress-induced aborting during capture and handling, especially in yolk-sac or lipid histotroph viviparous species lacking distinct uterine compartments. This is prevalent among many batoids species and several shark species such as *Galeorhinus galeus* (Walker 2005) and *Squalus megalops* (Braccini *et al.* 2006), where their uteri and cloaca dilate causing the embryos to dropout, whereas other species such as *Mustelus antarcticus* (Walker 2007) have uterine compartments and are
less likely to abort. Although not reported for any other species, a study in south-western Australia of four urolophid species (White and Potter 2005) assumes aborting of embryos occurs naturally during pregnancy which progressively reduces the number of embryos *in utero* as gestation progresses. This raises questions about whether counts of early-term litters and full-term litters provide an equally representative indication of litter size.

The assumption for the south-western Australian urolophid species that early-term litters are not an accurate measure of full-term litter size creates a large magnitude of difference in full-term litter sizes between south-eastern Australia and south-western Australia. In south-eastern Australia, maximum litter sizes found at full term are 13 for *Urolophus gigas* (Dagley et al. 2012 in prep), 7 for *T. imitata* (Present chapter), 6 for *U. paucimaculatus* (Chapter 6), 4 for each of *U. bucculentus* (Chapter 4) and *U. cruciatus* (Chapter 5), and 3 for *U. viridis* (Chapter 7). Similarly, in California, USA, maximum litter size of 6 is found for *U. halleri* (Babel 1967). Conversely, in south-western Australia, maximum litter sizes found at full term are 1–2 for *U. lobatus*, *U. paucimaculatus*, *T. mucosa* and *T. personata* (White and Potter 2005).

The studies in south-eastern Australia indicate that natural aborting throughout pregnancy is not a major factor in biasing measures of litters. In *T. imitata*, for example, large maternal females with litter sizes of 5–7 were observed in the field and in experimental tanks (Trinnie. personal obs) during early-term pregnancy and at full-term (April). In a comprehensive study of *U. halleri* embryos (Babel 1967), natural aborting was not reported to occur. Stress-induced aborting due to capture, on the other hand, does occur in urolophid species. In the present study, pregnant *T. imitata* females were observed to aborted their eggs and embryos in fishing nets and onboard commercial vessels. Following death the cloaca of maternal females appears to relax and can cause the release of the embryos and hence bias measures of litter size, growth of the embryos, and the gestation period. Results from the present study indicate that the litter size–maternal TL relationship may be influenced by females aborting their embryos. However, this level of stress-induced aborting through capture does not appear to have the same magnitude of effect on the litter size–maternal TL relationship as that reported for possible natural aborting in south-western urolophid species.
In south-western Australian urolophid species, the low litter sizes at full-term might be a consequence of relatively large sizes-at-birth compared with those for south-eastern Australian urolophid species. A difference can be seen between the two regions in the mean size-at-birth/L\textsubscript{max} ratio. In south-eastern Australian urolophid species, females are born at 25–32% and males at 30–39% of their L\textsubscript{max}, whereas south-western Australia urolophid species females are born at 35–49% and males at 42–52% of their L\textsubscript{max}. This suggests that as maternal TL increases, urolophid species have larger litters in south-eastern Australia than in south-western Australia. In south-eastern Australia, embryo size at full-term remains constant irrespective of maternal TL, whereas in south-western Australia urolophid species are possibly naturally aborting their young as pregnancy progresses to incorporate the larger embryo sizes, but litter size is small at parturition.

_Female and male length-at-maturity and length-at-maternity_

Determination of the relationship between the proportion of the population mature or maternal at TL is essential where fishery stock assessments provides predictions of mature and maternal population numbers and biomass (Walker 2007). Maturity based the onset of vitellogenesis (i.e., process of yolk accumulation) is less prone to observer bias (Braccini _et al._ 2006; Walker 2007) than definitions based on uterus condition which can be subjective, particularly in chondrichthyan species because the U=6 condition immediately follows pregnancy and before ovulation can resemble the U=3 condition (Walker 2007). The onset of vitellogenesis is species-specific and defining when it occurs is clearly essential. For example, _Squalus megalops_ follicles begin to yolk at >15 mm LFD (Braccini _et al._ 2006), _Orectolobus ornatus_ at >20 mm LFD (Huveneers _et al._ 2007), whereas _Galeorhinus galeus, U. bucculentus_ (Chapter 4) and _U. gigas_ yolk at >3 mm (Walker 2007), which is similar to _T imitata_. The variation that occurs among urolophid species is seen in the smaller species _U. paucimaculatus, U. viridis_ and _U. cruciatus_ (Chapters 5, 6, and 7) which all yolk at >1 mm (present study).

The present study shows that most of the pregnant female population is likely to contribute to annual recruitment each year and is represented by the annual maternity
ogive (labelled (b) in Fig. 3.9). However, large mature females were also found throughout the year with empty uteri (U=6 females) which could be indicative of a rest period. These females might be unable to breed in a particular year due to unfavourable environmental conditions, malnourishment or metabolic deficiencies and require a rest period. However, upon close examination 40 of the 78 U=6 females with empty uteri may have been U=4 or U=5 females misclassified as U=6 because they showed signs of having aborted their eggs or embryos upon capture. Indications of aborting of eggs or embryos were large follicles in the ovaries during January–March, the presence of in utero histotroph and aborted eggs or embryos present in the field collecting containers. Many of the eggs and embryos found could be matched to a particular female and thereby enable more accurate allocation to the appropriate U=4 or U=5 pregnant female condition. Careful consideration was taken when making these adjustments because U=6 females are pivotal in determining the period between pregnancies or frequency of recruitment and incorrect reallocation of these animals bias the life history parameters estimates. The small numbers of females that were not reallocated might have been resting animals, although they were still included within the maternity ogive and $P_{\text{max}}$ equals 1. There is the possibility for other chondrichthyan species that have annual reproductive cycles to have a higher number of resting animals and considerations should be made on whether $P_{\text{max}}$ equals 1 for the population.

The growth in female TL from when reaching maturity $L_{50}$ to when reaching maternity $L_{50}$ is ~64 mm TL. This represents the transition in the reproductive cycle from early maturation of the ovary, through the first ovarian cycle and first ovulation, to the first ‘period of embryonic growth’ and finally the birth of the new recruits. Once the female reaches maternal condition, recruitment will be annual thereafter as the ovarian cycle and gestation periods become synchronous. For annual species such as $T.\ imitata$ this transition size is believed to represent approximately one year of growth in TL. Separating the maturity and maternity ogives is essential because chondrichthyan species with greater than one year reproductive cycles, e.g. triennial species like school shark $Galeorhinus\ galeus$ (Walker 2005), could concede up to 3 years in growth from first reaching the onset of maturity to becoming maternal. From a reproductive perspective it is also important to represent growth in TL as well in terms of age and growth. Age and growth in any chondrichthyan species can be used...
to define the age-at-maturity, the age-at-maternity and the maximum age to establish
the biological productivity of the species over the course of its maternal life. The age
and growth could also help confirm the periodicity of the reproductive cycle if there is
some conjecture associated with the outcomes.

The development of the seminal vesicles, claspers and testes all occur at a relatively
similar time in *T. imitata* males. This occurrence is thought to be uncommon in
chondrichthyans, where internal sexual development usually occurs before the sexual
maturity of the claspers (Braccini *et al.* 2006; Neer and Thompson 2005; Walker
2007). Based on the present study, any of the three maturity condition indices
describe maturity in male *T. imitata*, although clasper condition can be performed on
live animals.

In conclusion, the present study provides the parameters required for a separate
assessment of *T. imitata*, which is necessary because all species within the family
Urolophidae demonstrate differences in many of the reproductive attributes. These
include litter sizes, sizes-at-birth, attainments of maturity and maternity, matrotrophic
versus lecithotrophic contributions, and reproductive periodicity. The annual
reproductive cycle, large neonates, and a maximum litter size of 6–7 makes *T. imitata*
one of the most reproductively robust urolophid species, yet they are highly
susceptible to environmental and anthropogenic pressures. The family Urolophidae is
found throughout the world and several species are already listed under the IUCN
redlist. Further studies are needed of all urolophid species where fishing impacts on
their abundances and to provide better insights into their phylogenetic relations.
Table 3.1. Collection of samples from each region with females shown in their reproductive conditions

<table>
<thead>
<tr>
<th>Region</th>
<th>No. females in each uterus condition</th>
<th>No. Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U=1</td>
<td>U=2</td>
</tr>
<tr>
<td>Corner Inlet</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Lakes Entrance</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>Port Phillip Bay</td>
<td>94</td>
<td>3</td>
</tr>
<tr>
<td>Western Bass Strait</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>141</strong></td>
<td><strong>7</strong></td>
</tr>
</tbody>
</table>
Fig. 3.1. Length-frequency composition of samples for (a) females (b) males.
Fig. 3.2. Relationships between total body mass and total length.

Plots of mean total mass against TL (——), with 95% confidence limits (— — —) and 95% prediction limits (- - -), for females total (U=1–6) (a), and males (b) and the comparison of the mean for females total (— — —) and males (——) (c). Values for parameters and statistical quantities from linear regression analysis to derive the equation $w=ac^l$ are given in the following tabulation:

<table>
<thead>
<tr>
<th>Category</th>
<th>$a$  (s.e. range) x $10^{-6}$</th>
<th>$b$ (se)</th>
<th>n</th>
<th>$r^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female total</td>
<td>3.13 (2.75–3.52)</td>
<td>3.201 (0.020)</td>
<td>310</td>
<td>0.989</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Males</td>
<td>3.90 (3.37–4.46)</td>
<td>3.157 (0.023)</td>
<td>280</td>
<td>0.985</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pregnant females (U=5)</td>
<td>7.56 (3.68–15.50)</td>
<td>3.069 (0.111)</td>
<td>50</td>
<td>0.940</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

where $w$ is total body mass, $l$ is total length, $a$ and $b$ are parameters; $a$ has been adjusted for the Beauchamp and Olson (1973) correction factor, $n$ is sample size, $r^2$ is square of correlation coefficient, and $P$ is the probability of the statistical significance.
Fig. 3.3. Various breeding conditions against month for mature males.

Trends in gonadosomatic index (GSI) for mature males determined from testis development (GI) (a), and HSI (b), and seminal vesicle fullness (c). • mean monthly value; bars, standard error for monthly value; number above bar is monthly sample size.
Fig. 3.4. Mass of embryo (a) and yolk sac proportion (b) against embryo length.

(a) Each data point is derived from the embryo mass and embryo length with 95% confidence limits (– – –) determined from U=5 pregnant animals and aborted embryos. (b) Yolk mass proportion is yolk sac mass/(embryo mass + yolk sac mass). Values for parameters and statistical quantities from linear regression analysis to derive the equation $w = acl^b$ are given in the following tabulation:

<table>
<thead>
<tr>
<th>$a$ (s.e. range) x $10^{-7}$</th>
<th>$b$ (se)</th>
<th>n</th>
<th>$r^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.03 (5.38–9.12)</td>
<td>3.474 (0.054)</td>
<td>107</td>
<td>0.976</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

where w is embryo mass, l is embryo length, a and b are parameters; a has been adjusted for c Beauchamp and Olson (1973) correction factor, n is sample size, $r^2$ is square of correlation coefficient, and P is the probability of the statistical significance. Raw data (●).
Fig. 3.5. Mean embryo length and mean embryo mass against month.

Derived from the mean embryo length (a) and mean embryo mass (b) of the litter from each of pregnant animals with macroscopically visible embryos (U=5); ●, overall mean; bars, standard deviation, ○ macroscopically visible eggs for U=4 females, n number of pregnant animals.
**Fig. 3.6.** Relationship between largest follicle diameter (LFD) and mean embryos length.

LFD are plotted against mean embryo length from pregnant females with macroscopically visible embryos (U=5). Values for parameters and statistical quantities from linear regression analysis are given in the following tabulation:

<table>
<thead>
<tr>
<th>a (±s.e.)</th>
<th>b (±s.e.)</th>
<th>n</th>
<th>$r^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.332 (±1.354)</td>
<td>0.11269 (±0.00879)</td>
<td>27</td>
<td>0.86</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

$a$ and $b$ are parameters, $n$ is sample size, $r^2$ is square of correlation coefficient, and $P$ is the probability of statistical significance; raw data (*).
Largest follicle diameter (LFD) against day of year for uterus conditions U=1–6.

LFD against day of year for females for each of the uterus conditions. Mean follicle diameter (—) with 95% confidence limits (———) are presented for pregnant females with in utero embryos (U=5) (a), immature females (U=1) (b), non-pregnant maturing females (U=2) (c), non-pregnant mature females (U=3) with the super imposed U=5 linear regression (d), pregnant females with in utero eggs (U=4) (e), and post partum or females that have aborted upon capture (U=6) (f). Values for parameters and statistical quantities from linear regression analysis for U=5 females are given in the following tabulation:

<table>
<thead>
<tr>
<th>U</th>
<th>( a ) (±se)</th>
<th>( b ) (±se)</th>
<th>n</th>
<th>( r^2 )</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>-28.41 (5.23)</td>
<td>0.132 (±0.013)</td>
<td>51</td>
<td>0.782</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\( a \) and \( b \) are parameters, n is sample size; \( r^2 \) is square of regression correlation coefficient, and P is probability of statistical significance. Day of year (Day 1 = January 1)
Fig. 3.8. Number of eggs and embryos in utero against maternal total length.

Number of eggs in utero (a) and embryos in utero (b) are plotted against maternal TL; mean number (——) with 95% confidence limits (---) and 95% prediction limits (- - - -). Values for parameters and statistical quantities from linear regression analysis of U=5 females are given in the following tabulation:

<table>
<thead>
<tr>
<th></th>
<th>a (±s.e.)</th>
<th>b (±s.e.)</th>
<th>n</th>
<th>r²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eggs in utero</td>
<td>-4.354 (±3.667)</td>
<td>0.013 (±0.006)</td>
<td>11</td>
<td>0.33</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Embryos in utero</td>
<td>-11.249 (±4.662)</td>
<td>0.022 (±0.007)</td>
<td>25</td>
<td>0.28</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

a and b are parameters, n is sample size, r² is square of correlation coefficient, and P is the probability of statistical significance. Raw data (*)
Fig. 3.9. Comparisons of length-at-maturity and length-at-maternity ogives.  
Proportion of female population in mature condition (---), with 95% confidence intervals (---) (a), and maternal condition (---) (b), with 95% confidence intervals (---) against total length. Values of parameters and statistical quantities for the equation $P_l = \frac{P_{\text{max}(l)}}{1 + e^{-\ln(190) \cdot l_{95}/l_{50} - l_{50}}}^{-1}$ determined from probit analysis are given in the following tabulation:

<table>
<thead>
<tr>
<th>Condition</th>
<th>$l_{50}$ (CI)</th>
<th>$l_{95}$ (CI)</th>
<th>$P_{\text{max}(l)}$</th>
<th>n</th>
<th>N</th>
<th>ML</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature</td>
<td>475 (465, 488)</td>
<td>505 (492, 534)</td>
<td>1.00</td>
<td>152</td>
<td>297</td>
<td>-14.041</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Maternal</td>
<td>539 (525, 552)</td>
<td>620 (604, 642)</td>
<td>1.00</td>
<td>188</td>
<td>294</td>
<td>-103.769</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

where $l$ is total length measured in millimetres, $P_l$ is proportion of animals at TL $l$, $l_{50}$ and $l_{95}$ are parameters, $P_{\text{max}(l)}$ is an asymptotic constant, n is the total number of animals classed as being mature or maternal, and N is the total number of animals examined for maturity or maternity, ML is maximum likelihood, and P is probability of statistical significance.
Fig. 3.10. Maturity of males based on three separate conditions.

Proportion of population mature against TL (——) with 95% confidence intervals (- - - - -) for males determined from testis development (a), seminal vesicle fullness (b), and clasper calcification (c) and comparison between each condition; (——) GI, (- - - - -) VI, and (– – –)CI (d). Males were classed as immature for G=1 or G=2 and mature for G=3 testis development, as immature for V=1 and mature for V=2 or V=3 seminal vesicle fullness, and immature for C=1 or C=2 and mature for C=3 clasper calcification. Values of parameters and statistical quantities for the equation $P = P_{max}(1 + e^{-\ln(19)(1-0.50/1.50})^{-1}$ determined from probit analysis are given in the following tabulation:

<table>
<thead>
<tr>
<th>Method</th>
<th>$l_{50}$ (CI)</th>
<th>$l_{95}$ (CI)</th>
<th>$P_{max}$</th>
<th>n</th>
<th>N</th>
<th>ML</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis development</td>
<td>483 (473–494)</td>
<td>570 (552–594)</td>
<td>1.000</td>
<td>97</td>
<td>277</td>
<td>–101.631</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Seminal vesicle fullness</td>
<td>481 (472–490)</td>
<td>548 (534–569)</td>
<td>1.000</td>
<td>103</td>
<td>279</td>
<td>–82.229</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Clasper calcification</td>
<td>476 (468–483)</td>
<td>518 (508–535)</td>
<td>1.000</td>
<td>109</td>
<td>281</td>
<td>–53.488</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

where $l$ is total length measured in millimetres, $P$ is proportion of animals at TL $l$, $l_{50}$ and $l_{95}$ are parameters, $P_{max}$ is an asymptotic constant, $n$ is the total number of animals classed as mature, and $N$ is the total number of animals selected in statistical procedure, ML is maximum likelihood, and $P$ is probability of statistical significance.
Chapter 4 – *Urolophus bucculentus* (Sandyback stingaree)

Abstract

*Urolophus bucculentus*, the largest urolophid species found in southern Australia, exhibits a biennial reproductive cycle. Ovulation occurs during Oct–Jan followed by a 15–19-month ‘period of embryonic growth’ followed by parturition during Apr–May and a short rest period while the ovarian follicles continue to develop for subsequent ovulation. Male breeding condition peaks during Apr–Jun to coincide with the ‘period of parturition’. *Urolophus bucculentus* has the highest matrotrophic contribution reported for any urolophid species, with a mean wet mass gain from egg *in utero* (4 g) to full term embryo *in utero* (250 g) of ~6250% (maximum ~7200%), and perhaps explains the biennial female reproductive cycle where 50% of females contribute to each year’s recruitment. Litter size (1–5) increases with total length (TL). Females reach a longer maximum length (*l*<sub>max</sub>) than do males (885 mm versus 660 mm TL). Length-at-maturity for males and females at *L*<sub>50</sub> (length at 50% mature) is ~414 mm TL (63% of *l*<sub>max</sub>) for males and ~502 mm TL (57% of *l*<sub>max</sub>) for females, length-at-maternity indicates that recruitment production occurs later in life at ~632 mm TL (71% of *L*<sub>max</sub>).
Results

Composition of samples

Sexual dimorphism was observed within the population of *U. bucculentus* as females (273–885 mm TL; 263–9500 g TW, n=162) reached larger sizes than the males (265–672 mm TL; 242–4092 g, n=95) (Fig. 4.1).

Allometric and isometric relationships for total body mass and various size measurements

The effect of sex on the body mass–TL relationship was not significant (ANCOVA: $F_{1, 234} = 1.81; P>0.05$); however, because the females grew larger than the males, the relationships for females and males are presented separately (Fig. 4.2ab)

- Females total: $TW=0.00000498*1.009* TL^{3.147}$ (n=95; $r^2=0.964$; rmse=0.129)
- Males: $TW=0.00000378*1.005* TL^{3.195}$ (n=95; $r^2=0.964$; rmse=0.129)
- Pregnant females (U=5): $TW=0.00000059*1.009* TL^{3.507}$ (n=95; $r^2=0.964$; rmse=0.129).

The effect of sex and female breeding condition on body mass–TL relationships among 95 males, 111 non-pregnant females (U=1–3 and U=6 females), 6 pregnant females with eggs *in utero* (U=4), and 45 pregnant females with embryos *in utero* (U=5) was highly significant (ANOVA: $F_{3,234} = 4.30; P<0.05$) and the covariate ln(l) was highly significant (ANCOVA: $F_{1,234} = 8574.78; P<0.001$). Least squares means indicated that males were not significantly different from non-pregnant females ($P=0.4791$) or from U=4 pregnant females ($P>0.05$) and so the data for these females (U=1–3, U=4 and U=6 females) were pooled. The pooled data were significantly different from U=5 pregnant females ($P<0.05$). Hence, at any TL, U=5 females are heavier when pregnant than non-pregnant and U=4 females combined (Fig. 4.2c).

A least squares means test indicated that DL–TL ($P>0.05$) and DW–TL ($P>0.05$) isometric relationships were not significantly different between males and females.
The variables for each isometric relationship (sexes combined) are given by the equations

\[
DL = -14.93 + 0.664 \, \text{TL} \quad (P<0.001; \, r^2 = 0.985; \, n = 84), \quad \text{and} \\
DW = -4.82 + 0.800 \, \text{TL} \quad (P<0.001; \, r^2 = 0.987; \, n = 83).
\]

Reproductive cycles of females and males

Seasonal trends in the male breeding cycle were determined for mature males from mean GSI, HSI and transformed seminal vesicle fullness plotted against month. The general linear regression indicated that mean GSI remained relatively constant throughout the year as the effects of month was not significant \((P>0.05)\); however, the covariate TL was highly significant \((P<0.001)\) indicating the gonads are proportionally heavier as the animals grow (Fig. 4.3a). The effect of month was highly significant for mean HSI \((P<0.01)\) with livers proportionally heavier during March, April, May and September than October and November (Fig. 4.3b). The effects of month on transformed seminal vesicle fullness was highly significant \((P<0.001)\); some seminal vesicle fullness was present throughout the year but it peaked during April–July (Fig. 4.3c).

The periodicity of the female reproductive cycle was based on the information determined within the ‘period of eggs in utero’, ‘period of embryonic growth’, and the ‘period of ovarian cycle’.

Growth of embryos and period of embryonic growth

The timing of parturition and growth of the embryos during gestation was determined from collection of 113 embryos (98 embryos from 45 pregnant females and 15 aborted embryos from unknown pregnant females). Embryo mass plotted against embryo TL (Fig. 4.4a) is curvilinear which was modelled with the power curve

\[
\text{Embryo mass} = 0.00000778 \times 1.006 \times \text{ETL}^{3.071} \quad (n=109; \, r^2=0.978; \, \text{rmse}=0.247).
\]
The largest embryo was 265 mm ETL and 285 g wet mass, whereas the smallest neonate collected was 265 mm TL and 242 g wet mass, demonstrating that all stages of embryonic development had been collected and the size-at-birth and ‘period of embryonic growth’ could be reliably determined.

Mean ovarian follicle mass for females with LFD >20 mm was 3.7 g (s.e. 0.30 g; range 2.4–4.8 g) was similar to mean mass of eggs in utero at 4.0 g (s.e. 0.18 g; range 3.8–4.4 g). Eggs in utero for U=4 females (n=6) were found during Oct–Jan in any given sampling year. During embryonic development, the egg formed the external yolk sac and was consumed by the time the embryos reached 180 mm mean ETL and 55 g mean wet mass (Fig. 4.4b). Histotroph was supplied to the embryo during the ‘period of embryonic growth’, although the exact timing, either before or after external yolk sac absorption, was not determined.

The scattergrams of mean ETL (Fig. 4.5a) and mean embryo mass (Fig. 4.5b) versus day of year indicated that at any particular sampling time females carried two different size groups of embryos; e.g. during Jan–May (day-of-year 0–150) females carried either small embryos (0–161 mm ETL; 0–34 g) or large embryos (220–265 mm ETL; 145–275 g). Small embryos in utero were first observed during October and large embryos in utero were observed until May, 18 months later. The ETL scattergram and linear regression of ETL against day of year (Fig. 4.5a) indicates a two-year ‘period of embryonic growth’ by much tighter clustering of data points around a two-year regression line than a one-year regression line ETL = -76.611+day-of-year*0.416 (n=62; r^2=0.888; rmse=22.067).

Day of year was adjusted by adding 365 days for embryos 56–147 mm ETL during day of year 0–150 and for embryos 124–260 mm ETL during day of year 277–365, or by adding 730 days for embryos 220–264 mm ETL during day of year 0–150. Embryonic gestation started as early as October and as late as January and the ‘period of embryonic growth’ ranged 15–19 months depending on the pregnant female within the population. The full-term embryos reached 250 mm mean ETL during Apr–May indicative of average size-at-birth. Based on this value, females and males were born at ~30% and ~39%, respectively, of their maximum TL (Lmax). The increase from mean egg mass in utero (4.0 g) to mean full-term embryo mass (250 g) indicates an
~6250% increase in mean wet mass gain during development, with an ~7200% (285 g) maximum wet mass gain.

Period of ovarian cycle

The ‘period of embryonic growth’ was synchronous with the ‘period of ovarian cycle’ for U=5 pregnant females, as determined by the positive correlation between LFD and embryo length, with a rapid increase in LFD shortly before parturition (Fig. 4.6) ($r^2=0.6574$, d.f.=38). LFD for 151 females ranged 1–24 mm across all uterus conditions (U=1–6), but ranged 1–19 mm LFD for U=1 (Fig. 4.7b) and U=2 (Fig. 4.7c), 5–24 mm for U=3 (Fig. 4.7d), 3–8 mm for U=4 (Fig. 4.7e), 2–24 mm for U=5 (Fig. 4.7a), and 1–22 mm for U=6 (Fig. 4.7f). Vitellogenesis had commenced for most U=2 females and for all U=3 females.

The U=5 scattergram and linear regression of LFD against day-of-year [Fig. 8(a)] used to depict follicle growth during embryonic growth, indicated that LFD increased from 2 mm during November to a maximum of 24 mm by April, ~17 months later. Two distinct clusters were observed for LFD (<6 mm and 6–10 mm) when day-of-year was >180 days, and clusters (<10 mm and 10–24 mm) when day-of-year was <180 days. A third possible cluster of two U=5 females (circled in Fig. 4.7a) is present with the follicles >16 mm when day-of-year was >240; however, we interpret these two females as outliers where their ovarian follicles have grown faster than those in the rest of the population. The linear regression LFD = -6.079+day-of-year*0.026 (n=62; $r^2=0.794$; rmse=2.931) indicated a mean annual growth in the first year of 8 mm y$^{-1}$. The mean LFD then increased from 8 mm y$^{-1}$ to 17 mm y$^{-1}$ during the second year. The females with full-term embryos in the second year gestation were in the U=5 condition only during Jan–May and the timing of ovulation and annual periodicity of the reproductive cycle could not be determined from the U=5 pregnant females alone.

The timing of ovulation was determined from the presence and periodicity of U=4 females in the population, females in the U=3 condition with large LFD, the presence of U=6 post partum females with large LFD, and from observing females in the process of ovulating as indicated by the presence of yolk in the oviduct or oviducal
glands. Although no ovulating females were captured, \( U=4 \) females (n=6) were found during Oct–Jan with small follicles <10 mm indicating recent ovulation and suggesting this is the ‘period of ovulation’. The presence of \( U=6 \) females alone were expected to confirm when ovulation occurred; however, it was the presence of \( U=6 \) females (Fig. 4.7f) with large LFD during May–Dec (n=4), together with the presence of \( U=3 \) females with large LFD during May–Dec, and the presence of \( U=4 \) females during Oct–Dec which indicated that once parturition occurred the follicles remain in the ovary until Oct–Dec before ovulation. The scattergram of LFD against day-of-year for \( U=3 \) females (Fig. 4.7d) shows the growth of follicles in \( U=3 \) virgin females throughout the year. \( U=3 \) (Fig. 4.7d) and \( U=6 \) (Fig. 4.7f) female plots with the (\( U=5 \)) linear regression (from Fig. 4.7a), extended to include the period May–Oct, shows that follicles continue to grow until ovulation occurs and that the species has a two-year ovarian cycle.

With parturition occurring during Apr–May and all \( U=5 \) females LFD reaching a mean of 17 mm during pregnancy, post-partum \( U=6 \) females should not be found with small LFD values during May–Dec if all pregnant females are on a continuous reproductive cycle, and all \( U=6 \) females should be ready to ovulate and begin the next reproductive cycle. Hence, our observations of \( U=6 \) females with small LFD values are possibly due to aborting upon capture or handling by \( U=4 \) or \( U=5 \) females, of eggs or embryos respectively, so the \( U=6 \) females with small LFD values were excluded from the ovarian cycle analysis. Nevertheless, given the uncertainties associated with these \( U=6 \) females, there remains the possibility that at least a small proportion of the population is not on a continuous reproductive cycle, but is resting for a substantial period.

In summary, the data indicate that the ‘period of embryonic growth’ is 18–22 months; ovulation occurs during Oct–Jan before commencement of embryonic growth, the embryos reach full term during Apr–May, when parturition occurs. The ‘period of ovarian cycle’ is synchronous with the ‘period of embryonic growth’ where the LFD increases from 2 mm to 24 mm during the \( U=5 \) condition, and a short rest period (4–6 months) occurs after parturition, while the follicles continue to grow or remain stable until the subsequent ‘period of ovulation’ during Oct–Jan.
Litter size and sex ratio of embryos

The linear relationship indicated a gradual increase in the number of embryos in utero with increasing maternal TL. Number of embryos in utero =0.000121*1.031* TL\(^{1.520}\) (n=45; \(r^2=0.265\); rmse=0.245) (Fig. 4.8). A maximum litter size of four and five was found in the U=4 and U=5 pregnant females of 710 mm and 725 mm TL, respectively. The smallest recorded U=4 (n=6) and U=5 (n=45) pregnant females were 625 mm and 580 mm TL, respectively.

A total of 98 embryos from 45 pregnant females were sexed based on macroscopic inspection for the presence or absence of claspers of which 44 (45%) were females, 39 (40%) were males, and 15 (15%) were of unknown sex due to their early stage of development. Based on the male to female ratio of embryos in utero, the sex ratio was 1:1 (\(\chi^2 = 0.193, \text{ d.f.} = 1, P>0.05\)).

Female and male length-at-maturity and length-at-maternity

As expected, the parameter estimates of \(L_{50}\) and at \(L_{95}\) were longer for length-at-maternity (labelled (b) in Fig. 4.9) than for the length-at-maturity of females (labelled (a) in Fig. 4.9) with differences during this transition of 130 mm and 121 mm TL, respectively. Females reached maturity at a larger size for \(L_{50}\) than do males. \(P_{\text{max}}\) equals 1.0 for each of the female and male maturity ogives and \(P_{\text{max}}\) equals 0.5 for the female maternity ogive. The parameters for the length-at-maturity ogive for females were determined from 38 immature and 107 mature females. The largest immature female observed was 675 mm TL and the smallest mature female observed was 450 mm TL. The parameter values (with 95% confidence limits) for females was \(502\) (478, 522) mm TL for \(L_{50}\) and 594 (570, 629) mm TL for \(L_{95}\); \(L_{50}\) was \(\sim 57\%\) and \(L_{95}\) was \(\sim 67\%\) of \(L_{\text{max}}\) (Maximum likelihood = –34.479; P<0.001). The parameters for the length-at-maternity ogive for females were determined from 26 maternal and 99 non-maternal females based on a two-year cycle. Females were classed in maternal or non-maternal condition for each 100-mm- TL size-class, which indicated that half of the females were in maternal condition at large sizes. For a biennial reproductive cycle \(P_{\text{max(l)}}=0.50\) to fit the two-year cycle, the number of maternal animals in the length
class of 700–799 mm TL were adjusted from 15 to 14.5, and those in the length class of 800–899 mm TL were adjusted from 3 to 2.5. The smallest female in maternal condition was 595 mm TL and the largest female in non-maternal condition was 840 mm TL. The parameter values of females in maternal condition was 632 (624, 639) mm TL for $L_{50}$ and 715 (703, 732) mm TL for $L_{95}$; $L_{50}$ was ~70% and $L_{95}$ was ~81% of $L_{\text{max}}$ (Maximum likelihood = –246.58; P<0.001).

For males, analysis of indices for the three criteria defining maturity all gave similar estimates for each of $L_{50}$ and $L_{95}$. The parameter values for $L_{50}$ based on 19 immature and 58 mature were 411 (343, 445) mm TL for testis development (Fig. 4.10a) (Maximum likelihood = –47.884; P<0.001), and based on 15 immature and 63 mature were 417 (393, 444) mm TL for clasper calcification (Fig. 4.10b) (Maximum likelihood = –4.7; P<0.001). Graphical comparisons of the two maturity methods (Fig. 4.10c) indicate similar ogives for the two methods. The ogive based on seminal vesicles fullness could not be generated because insufficient immature animals were sampled; however, the histogram of frequency of immature and mature males in 50-mm size classes demonstrates that males >400 mm TL are all mature (Fig. 4.10d). $L_{50}$ was reached at a mean value for the two methods generating maturity ogives where $L_{50}$ was ~62% of $L_{\text{max}}$.

**Discussion**

The maximum TL (885 mm) recorded for females (Present study) exceeds the previous recorded maximum TL (800 mm) (Last and Stevens 1994) making *U. bucculentus* the largest urolophid species found in southern Australia. Sexual dimorphism expressed as larger maximum sizes for females than males in *U. bucculentus* is common among urolophid species (see Chapters 5, 6, 7; White and Potter 2005), with the only exception being *Trygonoptera imitata* (Chapter 3). As expected, *U. bucculentus* pregnant with embryos are significantly heavier than non-pregnant females, which is also common among viviparous chondrichthyan species (Walker 2007).
Reproduction cycles of females and males

The evolutionary adaptation in batoid species to viviparity with lipid histotroph might be expected to facilitate reproductive cycles of relatively short duration; typical within the family Urolophidae with the majority of urolophid species having annual reproductive cycles, e.g. Trygonoptera imitata, Urolophus paucimaculatus, U. viridis, U. lobatus, T. personata, and T. mucosa (Table 4.1), and Urobatis halleri (Chris Mull, personal communications) or with a biannual reproductive cycle, e.g. Urobatis jamaicensis (Fahy et al. 2007). Biennial reproductive cycles are generally unexpected for batoid species, but the present study and two similar studies on U. cruciatus and U. gigas (Table 4.1) demonstrate the occurrence of biennial reproductive cycles in batoid species and highlight the need for further investigation of batoid reproduction within Australia and worldwide.

Whereas the majority of viviparous batoid species reproduce annually, the biennial reproductive cycle for U. bucculentus appears to be correlated with its extensive matrotrophy. Matrotrophy reaches a zenith in batoid species with reported increases in mass from egg to full-term embryos of 1000–4900% (Musick and Ellis 2005) e.g. Daysatis centroura with ~3000% and annual reproduction (Capape 1993). Batoid species are regarded as extensively matrotrophic among the chondrichthians as are viviparous and ovoviviparous sharks (Musick and Ellis 2005), with a maximum matrotrophic contribution for U. bucculentus (7200% wet mass increase) found as part of the present study being exceptional. Comparatively, within the family Urolophidae, Urolophus paucimaculatus, U. cruciatus, and U. viridis have matrotrophic contributions of ~2550–5400% (Table 4.1), Urobatis jamaicensis has 4600% (Fahy et al. 2007), and Trygonoptera imitata has 1000–1600% (Table 4.1).

Viviparity in batoid species reflects the basic selection for increased efficiency in maternal-fetal maintenance, resulting in a larger size-at-birth and a reduction of egg size (Wourms 1977), where the growth of the embryos is more reliant on histotroph than on the initial egg. But, how much matrotrophic contribution can a batoid species provide without limiting its rate of reproduction? This becomes evident among urolophid species, e.g. U. paucimaculatus and U. viridis (3000%–5400% matrotrophic contributions). Both these species have annual reproductive cycles and
both are adapted to producing relatively small follicles (14–16 mm LFD), which take the entire 12 months to develop, suggestive of competing energetic demands between supplying histotroph and growing follicles. For *U. bucculentus* with higher energetic demands during pregnancy (7200% matrotrophy contribution) and the production of larger follicles (24 mm LFD maximum) than *U. paucimaculatus* and *U. viridis*, the energetic demands may be too great to allow for an annual reproductive cycle and the ‘period of ovarian cycle’ has extended to 24 months. This suggests a limit below the level supplied by *U. bucculentus* where contribution is sufficient to allow annual reproduction in urolophid species and possibly other viviparous chondrichthyans. There is seemingly a trade off between lecithotrophy and matrotrophy; with decreasing the follicle size and increasing matrotrophic contribution, which may not be the best solution for improved reproductive output and species survival. There are however, environmental conditions to consider e.g. warm versus cold water. The gummy shark *Mustelus antarcticus* can reproduce annually in the warmer waters of South Australia whereas in the colder waters of Victoria they can only produce biennial (Walker 2007). Similar environmental factors could also be affect *U. bucculentus* and other chondrichthyan species from Victorian waters and cannot be discounted.

Matrotrophy in *U. bucculentus* (Present study) was determined from the increase in wet mass gain from egg to full-term embryo, accounting for the entire water content, and the inorganic and organic material in the yolk and histotroph supplied by the maternal female. Although not undertaken as part of the present study, dry mass and ash mass of the egg and embryo allow differentiation of the mass of inorganic and organic material from the mass of water and better differentiates the matrotrophic contribution (assuming none of the inorganic and organic materials are volatile not driven off through the drying process). The organic composition of histotroph within batoid species varies in the richness of protein, lipid and fatty acid compositions; e.g. *Dasyatis americana* has more lipids than *Urobatis halleri* (Hamlett et al. 2005b). Both species have the same wet mass matrotrophic contribution of ~3000%. Further investigation of the composition of dried eggs and embryos could provide a greater understanding of the energy requirements for matrotrophy in *U. bucculentus* and the variability in histotroph material among urolophid species and may provide further comparisons among species (Huveneers et al. 2011).
Most *U. bucculentus* females have a continuous reproductive cycle. All U=5 females are capable of ovulating for the subsequent reproductive cycle; several U=6 females with large follicles were found during December, and U=4 females were found during Oct–Jan. However, many U=6 females were also found with small follicles (<10mm) throughout the year which do not conform to a continuous reproductive cycle, despite all U=5 females having large follicles at parturition. For the follicles in U=6 females to be small, several U=5 females must have undergone follicular atresia and are resting between pregnancies for a considerable period of time. A more in-depth study of the follicular development and atresia in U=6 females applying histological observations (not undertaken in the present study) may provide a better understanding of the proportion of the population that miss a reproductive cycle, which potentially affects the estimates of the maternity ogive parameters.

For the genuine U=6 *U. bucculentus* females, the follicles remain large for 4–6 months after parturition. Rest periods between pregnancies where the follicles continues to grow while the female is in the U=6 condition is common among chondrichthyan species with biennial or triennial reproductive cycles, as the follicles generally require a longer period of growth than the embryos, such as for the wobbegong *Orectolobus ornatus* (Huveneers *et al.* 2007) and the school shark *Galeorhinus galeus* (Walker 2005) and is likely a product of the ‘period of embryonic growth’ being shorter than the full two years in *Urolophus bucculentus*. However, all other Urolophidae species studied so far have been found to ovulate directly after parturition; it seems that urolophid species prefer to have extended periods of eggs in utero rather than have rest periods like in *U. bucculentus* (see Chapters 3, 5, 6, 7; White, Hall *et al* 2002; White, Platell *et al*., 2001; White and Potter 2005).

In chondrichthyan species, fertilization occurs within the terminal zone of the oviducal gland (Hamlett *et al.* 2005b) and requires mating to precede ovulation. In *U. bucculentus* (present study) the male breeding season coincides with the period immediately following parturition. Male seminal vesicle fullness peaks during Apr–Jul, although some males appear capable of breeding all year round. This suggests that sperm storage must occur in *U. bucculentus* U=6 females for ovulation to occur during Oct–Jan and the eggs to be fertile. Sperm storage is found in chondrichthyan
species (Hamlett et al. 2005b; Storrie 2004), but no urolophid species has been found
to store sperm (Babel 1967). The present study did not focus on this aspect of the
reproductive biology, so further studies and the mating period of males and the
histology of the female reproductive oviducal gland may determine whether or not
sperm storage occurs in urolophid species. Due to the lack of genuine U=6 females
during May–Sep in the present study, further sampling is needed to confirm whether
ovulation could occur before September and whether or not the resting period
between pregnancies is shorter than 4–6 months.

Seasonal differences in the timing of parturition occur among species within the
family Urolophidae. In south-eastern Australia, *U. bucculentus* (Present study) and *U.
cruciatu*s give birth during Apr–May and *Trygonoptera imitata* during Mar–Apr,
mucosa* and *T. personata* give birth during May–Jun and Apr–May, respectively, and
*U. paucimaculatus* and *U. lobatus* species give birth during Nov–Dec and Oct–Nov,
respectively (Table 4.1). In south-western Australia, competition among species is
thought to have resulted in the partitioning of resources and prey items (White and
Potter 2005). By partitioning the small invertebrates among predators, e.g. *Trygonoptera*
species feeding on polychaete worms and *Urolophus* species feeding on
small epibenthic crustaceans, competition for the resources can be reduced; however,
the abundances of many small invertebrate species can fluctuate throughout the year.
Thus, parturition occurs when one or several prey species are most abundant, allowing
neonates to utilize the abundance of available food sources during early development.
Changes of diets are also observed among the sympatric species found within south-
eastern Australia; *Urolophus paucimaculatus* are feeding mainly on small epibenthic
crustaceans (amphipods and shrimps) (Officer and Parry 1995), *T. imitata* on
polychaete worms (Officer and Parry 1995) and *U. bucculentus* on the wrinkled
swimmer crab *Liocarcinus corrugatus* (unpubl. data) and suggests that diet of
neonates are also affecting the timing of parturition of south-eastern Australian
urolophid species.

In determining the biennial reproductive cycle for *Urolophus bucculentus*, the present
study demonstrates the need to examine closely several components of the
reproductive biology. In addition, the present study, in conjunction with studies of
other closely related urolophid species, demonstrates that it is inadvisable to infer reproductive periodicity in a species from its taxonomic family, unless absolutely necessary and supported by some commonality in the collected data, such as, similar maximum total size, litter sizes, maximum embryo sizes, or LFD. All Australian urolophids were at one time referred as having litters of 2–4 that take about 3 months to gestate (Last and Stevens 1994).

Obtaining sufficient sample sizes for both the males and females in the present study was difficult and required several years of sampling. Even then, seminal vesicle maturity ogive for the males could not be produced because of insufficient data. Further sampling is required for males to reduce the uncertainties in GSI, HSI, seminal vesicle fullness, and maturity estimates, as well as female maturity and maternity. However, the females collected included all uterus conditions and of eggs and embryos in utero from all seasons. With *U. bucculentus* being distributed throughout south-eastern Australia, further studies are required to investigate spatial variation in the value of reproductive parameters.

*Litter size and sex ratio of embryos*

The family Urolophidae exhibit a diversity of litter sizes as occurs among batoid species in general. Species found in south-eastern Australia vary considerably with maximum litter sizes of 4–5 in *U. bucculentus* (Present study), 5–6 in *U. paucimaculatus*, 6–7 in *T. imitata*, 13 in *U. gigas*, 3–4 in *U. cruciatus*, and 2–3 in *U. viridis* (Table 4.1). Similarly, maximum litters of 7 in *Urobatis jamaicensis* (Fahy et al. 2007) and 6 in *Urobatis halleri* (Babel 1967) are found in species from the USA. In south-western Australia, *U. paucimaculatus*, *U. lobatus*, *T. mucosa*, and *T. personata*, however, are reported with litters of only 1–2 (Table 4.1).

The differences of litter sizes between south-eastern Australia and south-western Australia appear to be representing different reproductive patterns. South-eastern Australian urolophid species produce relatively small embryos at birth with mean size-at-birth-to-$L_{\text{max}}$ ratios of 28–32% for females and 30–39% for males compared with south-western Australian species with ratios of 35–46% for females and 42–49% for males (Table 4.1). To reduce predation and thereby increase survival, south-
eastern Australian species may have evolved to reduce full-term embryo size and increase litter size, whereas south-western Australian species have evolved to increase full-term embryo size and reduce litter size.

Litter size increases with maternal TL in *U. bucculentus*, a strategy commonly occurring amongst chondrichthyan species (Walker 2007): presumably, the female body cavity enlarges with TL allowing for an increased capacity for more embryos. When comparing fecundity among species, the reproductive cycle needs to be considered. For example, over a 2-year period, *U. bucculentus* gives birth to a maximum of 3–5 neonates which is comparable to the 1–2 neonates produced annually in south-western Australian species and *U. viridis* in south-eastern Australia, whereas most annual species, e.g. *U. paucimaculatus* and *T. imitata* found in south-eastern Australia give birth to a maximum of 5–7 neonates which equates to 10–14 over two years.

**Female and male length-at-maturity and length-at-maternity**

The present study follows the trend initiated by Walker (2005) defining female maturity as the onset of vitellogenesis based on LFD, rather than a combination of *L*<sub>FD</sub> and uterus condition because it reduces biases caused by observer differences in staging uterus condition (Walker 2005). This allows for the separation of the maturity and maternity ogives used to more clearly differentiate when maturity occurs in the population and when females contribute recruits to the population. Vitellogenesis occurs at varying follicle size associated with yolkling within chondrichthyans: *Squalus megalops* yolk at >15 mm (Braccini et al. 2006), *Orectolobus ornatus* at >20 mm (Huveneers et al. 2007), and *Mustelus antarcticus* yolk at >3 mm (Walker 2007). Similar to *Mustelus antarticus*, within the urolophid family vitellogenesis occurs in relatively small follicles: *U. bucculentus* >3mm (present study), *Trygonoptera imitata* >3 mm, *U. cruciatus*, *U. paucimaculatus* and *U. viridis* >1mm (Table 4.1). This assumes that vitellogenesis is correlated with LFD, but species where follicle size remains large, e.g. *S. megalops* and *O. ornatus*, take longer to undergo vitellogenesis than species that have smaller maximum size of follicles, e.g. the family Urolophidae. Because of the variability among chondrichthyans, each species needs to be individually assessed for vitellogenesis.
The determination of a continuous biennial reproductive cycle was essential for defining maternal condition in *U. bucculentus* and providing a maternity ogive with $P_{\text{max}}$ equal to 0.5 where only half the population contributes to recruitment into the population each year. Most other urolophid species have annual reproductive cycles and will therefore have $P_{\text{max}}$ equal to 1.0 for the maternity ogive, where the whole population for these species is breeding each year once the first pregnancy is reached. There were, however, the small minority of *U. bucculentus* that were unable to continuously reproduce and were resting between cycles, but the present study could not confidently distinguish the resting animals from the non-resting animals.

Most chondrichthyan species are described as having late attainment of maturity. It has been generalized that maturity is attained at about 60% of $L_{\text{max}}$ (Holden 1974) as cited in (Neer and Thompson 2005); although this was based on the combination of $L_{\text{FD}}$ and uterus condition, and maternity was not distinguished. The present study shows that on average *U. bucculentus* attains maturity based on $l_{50}$ at ~57% of $L_{\text{max}}$; however, maternity is not reached until a later stage in life at ~71%. This is also consistent with *T. imitata* maturing at ~63% and maternal at ~71%. *Urolophus paucimaculatus* is the only urolophid species consistent with the Holden (1974) generalization maturing at 48% and maternal at ~62% (Table 4.1). It is evident that females of several urolophid species are not contributing recruits into the population until much later in life. For biennial species such as *U. bucculentus* the size increase from maturity to maternity can represent at least two years of growth from the first onset of maturity to maternity.

Testis development and clasper calcification give similar estimates for male maturity in the present study, but due to insufficient immature animals, the seminal vesicle ogive could not be determined. However, the seminal vesicle histogram is consistent with these maturity ogives. This suggests that any of the applied indices could be used to determine maturity for *U. bucculentus*. The advantage of clasper calcification is that it allows for the animals to live after classification. All males in the genus *Urolophus* mature at a smaller size than the females (Table 4.1), which is consistent with *U. bucculentus* in the present study; however, the males mature at a similar stage.
of life to the females with the mean length-at-maturity in *U. bucculentus* males of ~62% of $L_{\text{max}}$ compared to females at ~57% of $L_{\text{max}}$.

The reproductive cycle of *U. bucculentus* differs from the annual and biannual reproductive cycles previously found in urolophid species and other viviparous batoid species, and thus demonstrating that the periodicity of the reproductive cycle within batoid species is less conclusive than previously thought. Litter size also varying among batoid species highlights the need to ensure that each species is individually assessed for biological productivity and its susceptibility to commercial fishing or other anthropogenic pressures. Producing a maximum of 4–5 pups biennially suggests low biological productivity in *U. bucculentus*, but there is a need for additional information on longevity and post-capture survival to undertake an ecological risk assessment for this species.
<table>
<thead>
<tr>
<th>Urolophid species</th>
<th>Region</th>
<th>Reproductive Periodicity</th>
<th>Matrotrophic Size of LFD at the onset of vitellogenesis</th>
<th>Length at Maturity</th>
<th>Maternity</th>
<th>Maximum Litter size</th>
<th>Size at birth</th>
<th>Timing of Reference Parturition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mm</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Urolophus bucculentus</td>
<td>SE</td>
<td>Biennial</td>
<td>6250–7200</td>
<td>57</td>
<td>62</td>
<td>71</td>
<td>4-5</td>
<td>Apr–May Present study</td>
</tr>
<tr>
<td>U. paucimaculatus</td>
<td>SE</td>
<td>Annual</td>
<td>2550–3000</td>
<td>48</td>
<td>70</td>
<td>62</td>
<td>6-7</td>
<td>Aug–Dec Edwards 1980; Chapter 6</td>
</tr>
<tr>
<td>U. cruciatus</td>
<td>SE</td>
<td>Biennial</td>
<td>4000–4375</td>
<td>44</td>
<td>61</td>
<td>56</td>
<td>3-4</td>
<td>Apr–May Chapter 5</td>
</tr>
<tr>
<td>U. viridis</td>
<td>SE</td>
<td>Annual</td>
<td>3800–5400</td>
<td>56</td>
<td>68</td>
<td>67</td>
<td>2-3</td>
<td>All year Chapter 7</td>
</tr>
<tr>
<td>Trygonoptera imitata</td>
<td>SE</td>
<td>Annual</td>
<td>1000–1600</td>
<td>63</td>
<td>65</td>
<td>71</td>
<td>6-7</td>
<td>Mar–Apr Trinnie et al 2009</td>
</tr>
<tr>
<td>U. paucimaculatus</td>
<td>SW</td>
<td>Annual</td>
<td>i.d.</td>
<td>#</td>
<td>80</td>
<td>81*</td>
<td>1-2</td>
<td>Nov–Dec White and Potter 2005</td>
</tr>
<tr>
<td>U. lobatus</td>
<td>SW</td>
<td>Annual</td>
<td>i.d.</td>
<td>#</td>
<td>68</td>
<td>72*</td>
<td>1-2</td>
<td>Oct–Nov White and Potter 2005</td>
</tr>
<tr>
<td>T. mucosa</td>
<td>SW</td>
<td>Annual</td>
<td>i.d.</td>
<td>#</td>
<td>78</td>
<td>69*</td>
<td>1-2</td>
<td>May–Jun White and Potter 2005</td>
</tr>
<tr>
<td>T. personata</td>
<td>SW</td>
<td>Annual</td>
<td>i.d.</td>
<td>#</td>
<td>82</td>
<td>73*</td>
<td>1-2</td>
<td>Apr–May White and Potter 2005</td>
</tr>
</tbody>
</table>

*White and Potter 2005 defines this as maturity but is equivalent to maternity in the present study; i.d. information deficient
Fig. 4.1. Length-frequency composition of samples for (a) females and (b) males.
Fig. 4.2. Relationships between total body mass and total length.

Plots of mean total mass against TL (-----), with 95% confidence limits (--- - - -) and 95% prediction limits (- - - -), for females total (U=1–6) (a), and males (b) and the comparison of the mean for females total (--- - - -) and males (-----) and pregnant females (- - - -)(c). Values for parameters and statistical quantities from linear regression analysis to derive the equation $w=ac^l$ are given in the following tabulation:

<table>
<thead>
<tr>
<th>Shark category</th>
<th>$a$ (s.e. range) x 10^{-6}</th>
<th>$b$ (se)</th>
<th>$c$</th>
<th>$n$</th>
<th>$r^2$</th>
<th>rmse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female total</td>
<td>4.98 (3.28–7.55)</td>
<td>3.147 (0.067)</td>
<td>1.009</td>
<td>95</td>
<td>0.964</td>
<td>0.129</td>
</tr>
<tr>
<td>Males</td>
<td>3.78 (3.07–4.66)</td>
<td>3.195 (0.033)</td>
<td>1.005</td>
<td>165</td>
<td>0.988</td>
<td>0.110</td>
</tr>
<tr>
<td>Pregnant females (U=5)</td>
<td>0.59 (0.22–1.16)</td>
<td>3.507 (0.125)</td>
<td>1.009</td>
<td>45</td>
<td>0.948</td>
<td>0.097</td>
</tr>
</tbody>
</table>

where $w$ is total body mass, $l$ is total length, $a$ and $b$ are parameters, $c$ is the Beauchamp and Olson (1973) correction factor, $n$ is sample size, $r^2$ is square of correlation coefficient, and rmse is root mean square error for this regression.
Fig 4.3. Various breeding conditions against month for mature males.

Trends in gonadosomatic index (GSI) for mature males determined from testis development (GI) (a), and HSI (b), and seminal vesicle fullness (c). • mean monthly value; bars, standard error for monthly value; number above bar is monthly sample size.
**Fig. 4.4.** Mass of embryo (a) and yolk sac as a proportion (b) against embryo length.

(a) Each data point is derived from the embryo mass and embryo length with 95% confidence limits (— — —) determined from U=5 pregnant animals and aborted embryos. (b) Yolk mass proportion is yolk sac mass/(embryo mass + yolk sac mass). Values for parameters and statistical quantities from linear regression analysis to derive the equation $w=acl^b$ are given in the following tabulation:

<table>
<thead>
<tr>
<th>a (s.e. range) x 10^-6</th>
<th>b(se)</th>
<th>c</th>
<th>n</th>
<th>$r^2$</th>
<th>rmse</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.78 (6.16–9.83)</td>
<td>3.071 (0.045)</td>
<td>1.006</td>
<td>109</td>
<td>0.978</td>
<td>0.247</td>
</tr>
</tbody>
</table>

where $w$ is total body mass, $l$ is total length, $a$ and $b$ are parameters, $c$ is the Beauchamp and Olson (1973) correction factor, $n$ is sample size, $r^2$ is square of correlation coefficient, and rmse is root mean square error for this regression. Raw data (●).
Fig. 4.5. Mean embryo length and mean embryo mass against day of year.

Derived from the mean embryo length (a) and mean embryo mass (b) of the litter from each of pregnant female with macroscopically visible embryos \((U=5)\). Values for parameters and statistical quantities from linear regression analysis are given in the following tabulation:

\[
\begin{array}{cccccc}
\text{Parameter} & \text{Value} & \text{SE} & n & r^2 & \text{rmse} & P \\
-76.611 & 12.483 & 62 & 0.888 & 22.067 & <0.001 \\
\end{array}
\]

a and b are parameters, \(n\) is sample size, \(r^2\) is square of regression correlation coefficient, and rmse is root mean square error for the regression, and \(P\) is probability of statistical significance; raw data (*).
Fig 4.6. Relationship between largest follicle diameters (LFD) and embryos length.

LFD are plotted against embryo length from pregnant females with macroscopically visible embryos (U=5). raw data (•). The relationship is positively correlated ($r^2 =0.6574$; df 38).
Fig. 4.7. Largest follicle diameter (LFD) against day of year for uterus conditions U=1–6.

LFD against day of year for females for each of the five uterus conditions. Mean follicle diameter (-----) with 95% confidence limits (---) are presented for pregnant females with embryos in utero (U=5) (a), immature females (U=1) (b), maturing females (U=2) (c), non-pregnant females (U=3) with the super imposed U=5 linear regression (d), pregnant females with eggs in utero (U=4) (e), and U=6 females with the super imposed U=5 linear regression (f). Values for parameters and statistical quantities from linear regression analysis for U=5 females are given in the following tabulation:

<table>
<thead>
<tr>
<th>U</th>
<th>$a'$ (±se)</th>
<th>$b'$ (±se)</th>
<th>n</th>
<th>$r^2$</th>
<th>rmse</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>-6.079 (1.415)</td>
<td>0.026 (0.002)</td>
<td>45</td>
<td>0.794</td>
<td>2.931</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

$a$ and $b$ are parameters, $n$ is sample size, $r^2$ is square of regression correlation coefficient, and rmse is root mean square error for the regression, and $P$ is probability of statistical significance.
Fig. 4.8. Number of embryos *in utero* against maternal total length.

Mean number of embryos (——), 95% confidence limits (– – –), and raw data (*) are plotted against maternal total length of pregnant females with macroscopically visible embryos (U=5). Values for parameters and statistical quantities from linear regression analysis to derive the equation \( w = acl^b \) are given in the following tabulation:

<table>
<thead>
<tr>
<th>a (s.e. range) x 10^{-4}</th>
<th>b (se)</th>
<th>c</th>
<th>n</th>
<th>( r^2 )</th>
<th>rmse</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.21 (0.0745–19.60)</td>
<td>1.520 (0.425)</td>
<td>1.031</td>
<td>45</td>
<td>0.265</td>
<td>0.245</td>
</tr>
</tbody>
</table>

where \( w \) is number of embryos, \( l \) is total length, \( a \) and \( b \) are parameters, \( c \) is the Beauchamp and Olson (1973) correction factor, \( n \) is sample size, \( r^2 \) is square of correlation coefficient, and \( rmse \) is root mean square error for this regression.
**Fig. 4.9.** Comparisons of length-at-maturity and length-at-maternity ogives.

Proportion of female population in mature condition (---), with 95% confidence intervals (- - -) (a) and maternal condition (---), with 95% confidence intervals (- - -) (b) against total length. Values of parameters and statistical quantities for the equation $P = \frac{P_{\text{max}}}{1 + e^{-\ln(19)(1 - \frac{l}{95 - l_{50}})}}$ determined from probit analysis are given in the following tabulation:

<table>
<thead>
<tr>
<th>Condition</th>
<th>$l_{50}$ (CI)</th>
<th>$l_{95}$ (CI)</th>
<th>$P_{\text{max}}$</th>
<th>n</th>
<th>N</th>
<th>ML</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature</td>
<td>502 (478, 522)</td>
<td>594 (570, 629)</td>
<td>1.00</td>
<td>107</td>
<td>145</td>
<td>-34.479</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Maternal</td>
<td>632 (624, 639)</td>
<td>715 (703, 732)</td>
<td>0.50</td>
<td>26</td>
<td>125</td>
<td>-246.58</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

where $l$ is total length measured in millimetres, $P$ is proportion of animals at TL $l$, $l_{50}$ and $l_{95}$ are parameters, $P_{\text{max}}$ is an asymptotic constant, $n$ is the total number of animals classed as being mature or maternal, and $N$ is the total number of animals examined for maturity or maternity, ML is maximum likelihood, and $P$ is probability of statistical significance.
Fig. 4.10. Maturity of males based on three separate conditions.

Proportion of population mature against TL (——) with 95% confidence intervals (- - - - -) for males determined from testis development (a), and clasper calcification (b) and comparison between each condition; (——) Gl, and (- - - - CI (c). Seminal vesicle fullness histogram (d). Males were classed as immature for GI=1 or GI=2 and mature for GI=3 testis development, as immature for VI=1 and mature for VI=2 or VI=3 seminal vesicle fullness, and immature for CI=1 or CI=2 and mature for CI=3 clasper calcification. Values of parameters and statistical quantities for the equation $P = P_{\text{max}}(1 + e^{-\ln(19)(1-l_{50}/l_{95})})^{-1}$ determined from probit analysis are given in the following tabulation:

<table>
<thead>
<tr>
<th>Method</th>
<th>$l_{50}$ (CI)</th>
<th>$l_{95}$ (CI)</th>
<th>$P_{\text{max}}$</th>
<th>n</th>
<th>N</th>
<th>ML</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis development</td>
<td>411 (343–445)</td>
<td>592 (547–698)</td>
<td>1.000</td>
<td>58</td>
<td>77</td>
<td>–47.884</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Clasper calcification</td>
<td>417 (393–444)</td>
<td>446 (427–543)</td>
<td>1.000</td>
<td>63</td>
<td>78</td>
<td>–4.700</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

where $l$ is total length measured in millimetres, $P$ is proportion of animals at TL $l$, $l_{50}$ and $l_{95}$ are parameters, $P_{\text{max}}$ is an asymptotic constant, $n$ is the total number of animals classed as mature, and $N$ is the total number of animals selected in statistical procedure, ML is maximum likelihood, and $P$ is probability of statistical significance.
Chapter 5 – *Urolophus cruciatus* (Banded stingaree)

Abstract

Determination of the reproductive cycle of *Urolophus cruciatus* was uncertain because a large proportion of eggs and embryos in utero were lost from aborting pregnant females due to stress induced by capture and handling and required a two step approach to reduce ambiguity in interpretation of available data. The first step required yes-or-no answers to six sequentially-asked questions from analysis of standard reproductive data associated with population biology, and the second step required testing three alternative hypotheses for the reproductive cycle based on four separate assumptions on the patterns of aborted eggs and embryos. The approach enabled rejection of annual and shorter reproductive cycles with high certainty, but the triennial cycle (with or without a resting period) could only be rejected on the basis of its rarity among chondrichthyan species. Hence, the reproductive cycle is most likely biennial where ovarian follicle enlargement is synchronous with embryonic growth (4–6 months), but there is little or no follicle enlargement during the ‘period of eggs in utero’ (12–18 months). Parturition (May–June) and ovulation (May–November) are seasonal and a wet mass gain from egg in utero to full-term embryo of ~4000% indicates the species is highly matrotrophic.
Results

Allometric and isometric relationships for total body mass and various size measurements

Females (132–442 mm TL; 30–1000 g TW; n=448) attained greater size and mass than males (140–351 mm TL; 34–600 g TW; n=316) (Fig. 5.1). The effect of sex on the $\ln(TW)–\ln(TL)$ relationship was not significant (ANOVA: $F_{df=1, 741} = 0.71$, $P=0.3991$). For any TL, the males and females weighed the same, but because the females grow larger than the males, results for the two sexes are presented separately.

Females: $TW = 0.0000155 \times 1.006 \times TL^{2.977}$ ($P<0.001$; $r^2 = 0.961$; $n = 435$), and

Males: $TW = 0.0000110 \times 1.004 \times TL^{3.034}$ ($P<0.001$; $r^2 = 0.953$; $n = 309$)

A least squares means test indicated that DL–TL ($P=0.4860$) and DW–TL ($P=0.3938$) isometric relationships were not significantly different between males and females, but the regressions were highly significant when the data for the two sexes were pooled. The variables for each isometric relationship (sex combined) are given by the equations

$$DL = 21.02 + 1.526 \times TL$$ ($P<0.001$; $r^2 = 0.958$; $n = 282$), and

$$DW = 0.29 + 1.520 \times TL$$ ($P<0.001$; $r^2 = 0.954$; $n = 282$).

Growth of embryos and the pregnancy cycle

The ‘period of embryos in utero’ and ‘period of parturition’ were determined from 94 embryos collected (43 embryos came from known pregnant females and 51 were aborted embryos from unknown pregnant females). Embryo length against mass showed a curvilinear growth pattern. The largest embryo was 130 mm ETL and 35 g wet total mass whereas the smallest neonate collected was 132 mm TL and 30 g wet total mass, and the study can demonstrate that embryos of all stages of development had been collected. Full-term embryos ranged 120–130 mm ETL and 31–35 g mass with an average size-at-birth of 125 mm and a mean mass of 32 g.
Mean mass of ovarian follicles for females with LFD >11 mm was 0.9 g (s.e. 0.1 g; range 0.5–1.2 g) which was similar to mean mass of eggs in utero at 0.8 g (s.e. 0.1 g, range 0.5–0.8 g). The presence of eggs in utero for U=4 females (n=153) were found during every month of the year with the exceptions of June and July when sampling was minimal. During embryonic development, the egg was consumed by the time the embryos reached 100–110 mm ETL or 10–17 g mass (Fig. 5.2a) with all embryo yolk sacs consumed by the end of March. Histotroph was supplied to the embryo during the latter part of the period of embryonic growth, although the exact timing, either before or after external yolk sac absorption, was not determined.

Scattergrams of mean ETL with standard deviation (Fig. 5.2b) and mean embryo mass (Fig. 5.2c) against month for U=5 females indicate that embryos are present from December to May; parturition being completed by the end of May. With only one U=5 female found during December and parturition most likely occurring during April–May, the period of embryonic growth was 4–6 months. Based on the mean ETL-at-birth, the males and females were born at 37% and 29%, respectively, of their maximum TL (Lmax). Comparing mean egg in utero mass (0.8 g) with mean full-term embryo total body mass (32 g) and maximum full-term total body mass (35 g) indicates 4000–4375% increase in wet total mass gain.

Period of ovarian cycle

LFD for 439 females ranged 1–15 mm across all uterus conditions (U=1–6), but ranged 0–2 mm for U=1, 0–10 mm for U=2, 2–12 mm for U=3, 1–14 mm for U=4, 4–14 mm for U=5, and 1–15 mm for U=6 females. Vitellogenesis had commenced for all U=3 females and most U=2 females, but in only a small number of U=1 females.

The period of embryos in utero was synchronous with the ovarian cycle for U=5 pregnant females, as indicated by the linear relationship between LFD and embryo length.

$$LFD = 1.846 + ETL \times 0.07861 \ (P<0.001; \ r^2 = 0.83; \ n=20)$$

One pregnant female was removed from the regression because its single embryo and corresponding LFD was an outlier compared with the rest of the population; the
embryo was under-developed and possibly dead and affecting LFD. The U=5 scattergram and linear regression of LFD against day of year (DOY) ($r^2=0.57$; $P=0.001$) (Fig. 5.3a), used to depict follicle enlargement during embryonic growth, indicated that LFD increased from 5 mm at the start of December (DOY 340) to a maximum of 14 mm by the end of May (DOY 147) of the following year. These linear regressions indicated that the U=5 females were ready to ovulate immediately after parturition in April–May (DOY 120–180). However, the females were in the U=5 condition for only <6 months of the year and consequently the actual ovulation date and subsequent annual periodicity of the reproductive cycle could not be determined from the U=5 females alone. Females in the U=1, U=2 and U=3 condition (Fig. 5.3bcd) indicate when the onset of vitellogenesis occurs, but provides no indication on reproductive periodicity.

Five U=4 pregnant females were observed in the process of ovulating, one during May and four during September–November (Fig. 5.3e). This suggests that ovulation can occur concurrently with parturition during May but can also occur over the protracted period of May–November. However, a large proportion of the mature females was recorded as U=4, thus indicating no obvious seasonality in the ovarian cycle. Of the 153 U=4 females, 120 were found with small follicles (<7 mm) throughout the year, which is indicative of the post-ovulation size.

The presence of U=6 females was expected to confirm when ovulation occurred (Fig. 5.3f). However, the large number of U=6 females with large and small LFD and high rate of stress induced aborting from capture and handling, distinguishing U=6 females from U=4 females aborting eggs and from U=5 females aborting embryos was unreliable. In addition, some U=6 females might have been misclassified as U=3 females because of the tendency for the U=6 condition to revert and resemble the U=3 condition as the distended walls of the uteri gradually contract after parturition, consequently, neither the U=3 nor U=6 females could provide evidence for the timing of parturition or ovulation.

**Periodicity of the female reproductive cycle**
The answer to the first of six sequentially asked questions (Q1) (Fig. 2.4) was ‘yes’; hence, the present study demonstrates that the ‘period of embryos in utero only’ was synchronous among the U=5 females and the ‘period of parturition’ was determinable. Macroscopically visible embryos were observed in utero in U=5 females throughout December–May and exhibited a trend of seasonal growth (Fig. 5.2bc). Q2 could not be fully answered with a ‘yes’ or ‘no’, apart from a ‘yes’ for the ‘period of ovulation’ (May–Nov). The ‘period of eggs in utero only’ was initially unresolved because eggs in utero were throughout the year (Fig. 5.3e) implying more than one possible ‘period of eggs in utero only’ (i.e. 18 and 30 months). Q3 could not be fully answered with a ‘yes’ or ‘no’, because of the uncertainty associated with the answer to Q2. Hence, answers to Q1 and Q2 together, implied more than one possible periodicity for the ‘pregnancy cycle’ (e.g. 24 and 36 months) (see Fig. 2.5). The answer to Q4 was ‘yes’; LFD correlated positively with ETL for U=5 females and their ovarian cycles were synchronous and continuous during the ‘period of embryos in utero’. The answer to Q5 was a tentative no; because the LFD patterns of U=3, U=4, and U=6 females only shared some consistency with the pattern for U=5 females. However, the large numbers of U=3, U=6 and U=4 females found throughout the year provided little insight into when parturition or ovulation occurred particularly given a high variation in LFD. The answer to Q6 was ‘no’; the reproductive cycle was inconsistent with an annual or shorter cycle.

In summary, it was concluded from the stepwise sequential questioning that the reproductive cycle was synchronous and seasonal among breeding females, and was longer than annual. The ‘period of eggs in utero’ was unusually long but in indeterminate, the ‘period of embryos in utero’ was 4–6 months, parturition occurred during Apr–May, and ovulation occurred during May–Nov. However, the ‘period of pregnancy’, ‘period of eggs in utero’, and identity of U=6 females and therefore ‘period of reproductive cycle’ remained uncertain.

The chi-squared testing on the observed versus hypothesised expected numbers of U=4, U=5 and U=6 females for each of the two 6-month periods of Dec–May and Jun–Nov periods, found that the observed data was highly significantly different to the expected data; biennial reproductive cycle with a resting year (Hypothesis 1: \( \chi^2 = \))
57.04; P<0.01), triennial cycle with no resting year (Hypothesis 2: $\chi^2 = 49.05$; P<0.01), triennial reproductive cycle with a resting year (Hypothesis 3: $\chi^2 = 37.56$; P<0.01) (Table 5.1). However, after adjustment for aborted eggs and embryos using the four assumptions, the data showed some agreement between the observed and the expected; biennial cycle without a resting year (Hypothesis 1, Assumption 1; $\chi^2 = 1.44$; P=0.230) or a triennial cycle without a resting year (Hypothesis 2, Assumption 2; $\chi^2 = 0.06$; P=0.806). Hypothesis 3 became the least consistent after U=6 adjustments (Hypothesis 3, Assumption 4; $\chi^2 = 4.7$; P=0.095) (Table 5.2).

Given the comparatively low $\chi^2$ value for the biennial cycle without a resting year (Table 5.2) and comparisons among other urolophid and sympatric batoid species (Table 5.3) (see also Discussion), it is most likely that the periodicity of the reproductive cycle was biennial without a resting year. For a biennial reproductive cycle, the ‘pregnancy cycle’ would be 18–24 months comprising a ‘period of eggs in utero’ of 12–18 months followed by a ‘period of embryos in utero’ of 4–6 months, and the ovarian cycle would synchronous with the ‘period of embryos in utero’ with little or no growth during the long ‘period of eggs in utero’.

The ovarian cycles for U=4 and U=6 females were consistent with a biennial reproductive cycle. The scattergram of LFD against DOY for U=4 females (Fig. 5.4a) indicated that post ovulation the LFD remain small, generally <7-mm during the entire period of eggs in utero. During the Jun–Nov period, several females with 7–9 mm LFD probably had the largest LFD values prior to gestation. During Dec–May, the ovarian follicles of 7–11 mm LFD were probably atretic. The scattergram of LFD against DOY for U=6 females (Fig. 5.4b) indicated a small number of genuine U=6 females. Hence, some of the females aborting upon capture and handling could be reclassed. U=6 females with 7–14 mm LFD during Dec–May were probably aborted U=5 females and all other females with <7-mm LFD were probably aborted U=4 females.

Reproductive cycles of males
GSI, HSI, and seminal vesicle fullness all indicated seasonal trends in the male breeding cycle. Mean GSI gradually increased from a minimum during April to peak during Oct–Jan and then rapidly decreased during subsequent months until April (Fig. 5.5a). Mean HSI (Fig. 5.5b) also showed a distinct seasonal pattern with a minimum in July, increasing from Aug–Dec to then peak during Mar–Apr before decreasing rapidly. Seminal vesicle fullness peaked close to 100% full during Mar–Aug before decreasing to a mean fullness of 50–75% (Fig. 5.5c), indicating a period when males are most likely breeding.

*Litter size and sex ratio of embryos*

The smallest recorded U=4 and U=5 females were 212 mm and 251 mm TL, respectively. A maximum litter size and a number of eggs *in utero* of four occurred in each of the U=4 and U=5 females of 265 mm and 317 mm TL, respectively. No attempt was made to correlate the number of eggs *in utero* with maternal TL (Fig. 5.6a) because the eggs were too delicate to handle. The power curve model was fitted between the number of embryos *in utero* and maternal TL ($r^2 = 0.44$; $P=0.001$) (Fig. 5.6b). A U=5 female (highlighted) of 412 mm TL was removed because it was suspected of aborting several embryos upon capture. There were few pregnant females in the population >350 mm TL (nine U=4 and one U=5 pregnant females).

A total of 43 embryos from 22 pregnant females of which 15 (35%) were females, 21 (49%) were males, and 7 (16%) were of unknown sex due to their early stage of development. Based on the male to female ratio of embryos *in utero*, the sex ratio was 1:1 ($\chi^2 = 0.69$, d.f. = 1, $P = 0.4062$).

*Female and male length-at-maturity and length-at-maternity*

As expected, estimates of parameters $l_{50}$ and $l_{95}$ (with their 95% confidence limits) were greater for length-at-maternity than for the length-at-maturity of females (Fig. 5.7). Based on these ogives females mature at a larger size than males. $P_{\text{max}}$ equals 1.0 for each of the female and male maturity and to be consistent with a biennial reproductive cycle $P_{\text{max}}$ equals 0.5 for the female maternity. The parameters of the length-at-maturity ogive for females were determined from 19 immature and 401
mature animals. The largest immature female observed was 235 mm TL and the smallest mature female was 190 mm TL. The female maturity parameter estimate (with 95% confidence intervals) was 193 (188, 198 mm TL) for $l_{50}$ and 239 (238, 240 mm TL) for $l_{95}$ (Fig 5.7a), where $l_{50}$ was ~44% and $l_{95}$ was ~54% of $l_{\text{max}}$.

The parameters for the length-at-maternity ogive for females were determined from 353 maternal and 84 non-maternal animals based on a hypothesized two-year cycle. Half of the females able to reproduce were in maternal condition and the total number of animals was adjusted by a factor $P_{\text{max}}(l) = 0.50$ to fit the hypothesized two-year cycle. The smallest animal in maternal condition was 211 mm TL and the largest animal in non-maternal condition was 300 mm TL. The maternity parameter estimates was 246 (242, 250 mm TL) for $l_{50}$ and 294 (290, 299 mm TL) for $l_{95}$ (Fig 5.7b); $l_{50}$ was ~56% and $l_{95}$ was ~67% of $l_{\text{max}}$.

For males, analysis of indices for the three criteria defining maturity all gave similar estimates for each of $l_{50}$ and $l_{95}$. The maturity parameter estimates were 209 (197, 217) mm for $l_{50}$ and 271 (266, 278) for $l_{95}$ for testis development (Fig. 5.8a), 212 (203, 218) mm for $l_{50}$ and 253 (249, 259) for $l_{95}$ for seminal vesicle development (Fig. 5.8b), and 217 (209, 223) mm for $l_{50}$ and 261 (257, 266) for $l_{95}$ for clasper calcification (Fig 5.8c). Graphical comparisons of the three maturity methods (Fig. 12d) indicate the similarity between methods. $L_{50}$ was ~60% and $L_{95}$ was ~74% of $L_{\text{max}}$ for the three methods.

**Discussion**

*Reproductive cycle of females and males*

From our two-step approach of answering six sequentially asked questions and redistribution of U=6 females on the basis of assumed patterns of aborting eggs and embryos, the presence of eggs *in utero* all year is inconsistent with an annual or shorter reproductive cycle. If the effects of capture and handling on U=6 females aborting eggs and embryos are ignored, a triennial cycle with a resting year would be the most parsimonious hypothesis. However, if the assumptions accounts for these
effects explicitly, both a triennial cycle without a resting period and a biennial cycle are equally consistent with the data.

Within the family urolophidae most species are found to reproduce annually (Table 5.3), and only one species has been found to reproduce, biennially, i.e., *U. bucculentus* (Trinnie *et al.* 2012), a species sympatric to *U. cruciatus*. A triennial reproductive cycle has not yet been reported for any urolophid or in turn, batoid species and would suggest that a biennial cycle is more likely than a triennial cycle.

Periods of embryonic growth vary throughout the family urolophidae, but there appears to be separate patterns between the two genera *Trygonoptera* and *Urolophus*. Short embryonic growth periods of only 4–8 months occur for *T. imitata*, *T. mucosa* and *T. personata*, similar to *Trygonorrhina fasciata* (Marshall *et al.* 2007). Much longer periods of embryonic growth occur for *U. paucimaculatus*, *U. viridis* and *U. lobatus* (10–12 months) (Trinnie, unpublished data; White *et al.* 2001) and for *U. bucculentus* (15–19 months) with its biennial cycle (Trinnie *et al.* 2012). The short period of embryonic growth and long period of eggs in utero for *U. cruciatus* is an exception to the pattern for the genus *Urolophus*.

Extended ‘period of eggs in utero’—referred to embryonic diapause (Simpfendorfer 1992) or delayed development (Marshall *et al.* 2007) in other chondrichthyan species; however, egg cellular development was not investigated as part of the present study—is possible for *U. cruciatus* and has been observed in other urolophid species, however, only within the *Trygonoptera* species, never within the *Urolophus* species, and not longer than 5–7 months (Table 5.3). No *Urolophus* species has been observed with longer than 1–3 months for eggs in utero (Table 5.3). Period of eggs in utero of up to 9 months has been observed in other viviparous chondrichthyan species (Marshall *et al.* 2007; Simpfendorfer 1992) for which it is argued to allow for optimal breeding conditions when food availability for energy is at its highest (Marshall *et al.* 2007). Having the ‘period of eggs in utero’ extended beyond 12 months suggests factors other than food availability provide adaptive advantage in retaining the eggs. If the period of eggs in utero is 12–18 months in *U. cruciatus*, this becomes the longest ‘period of eggs in utero’ reported for any viviparous chondrichthyan species.
and a biennial reproductive cycle, if it extended beyond 18 months it become a triennial reproductive cycle.

In all urolophid species and many viviparous batoid species so far studied, ovarian follicle development is synchronous with embryonic growth: as the embryos develop, the mother expends energy in the growth of the follicles in preparation for ovulation immediately after parturition. However, during the extended ‘period of eggs in utero’, the rhinobatid rays (Marshall et al. 2007; Simpfendorfer 1992), Trygonoptera sp, and U. cruciatus (present study) show little or no follicle growth. Their reproductive strategies of the Trygonorrhina fasciata and Trygonoptera sp, which differ from that of U. cruciatus and other Urolophus sp, involves developing relatively large follicles (30–50 mm LFD) during a 3–8 month period concurrently with a period of embryo growth followed by an extended rest ‘period of eggs in utero’ of 4–9 months because of the high energy requirements of developing both large follicles and large embryos in a relatively short period. Urolophus cruciatus, on the other hand, develop relatively small follicles (14 mm LFD) during the 4–6 months period of embryo growth, but require an extended period of eggs in utero, of up to at least 12 months despite the seemingly low energy requirements during pregnancy.

This raises the question of why U. cruciatus has such an extended ‘period of eggs in utero’ which has effectively lengthened the periodicity of the reproductive cycle beyond an annual cycle. One explanation is matrotrophic contribution to embryonic growth. By producing large follicles during pregnancy, Trygonorrhina fasciata and the Trygonoptera sp. rely on relatively low matrotrophic contributions; e.g. T. imitata embryos have only ~1000% in wet mass gain from egg to full-term embryo. All species of Urolophus, on the other hand, produce small follicles for ovulation and matrotrophic contributions are much higher; e.g. U. bucculentus (Trinnie et al. 2012) has the highest wet mass gain of any urolophid species of ~7000% followed by the longest recorded period of embryonic growth of 15–19 months. Urolophus paucimaculatus (Trinnie, unpublished data) and U. viridis (Trinnie, unpublished data) have gains of ~4900% and ~4700%, respectively, which is comparable with U. cruciatus of ~4375%. However, U. paucimaculatus and U. viridis have 10–12-month ‘periods of embryo growth’, whereas U. cruciatus is much shorter at only 4–6 months. This demonstrates that as matrotrophic contribution increases, the ‘period of
embryonic growth’ seemingly increases and *Trygonoptera sp.* and *Trygonorrhina fasciata* have a more energy efficient strategy of reproduction (Table 5.3).

Thus, with follicle and embryo development occurring in half the time of other *Urolophus sp.*, but matrotrophic contribution remaining high and thereby markedly increasing energy requirements of *U. cruciatus* during pregnancy. The consequence might be that *U. cruciatus* requires an extended period of time before going through the next cycle of pregnancy.

Aborting upon capture and handling was found to be substantial and the reassignment of *U=6* females to *U=4* and *U=5* females based on logical alternative assumptions became essential to the determination of the periodicity of the reproductive cycle. But, there remains an under representation of *U=5* females during Dec–May. If more *U=5* females were actually observed in the population during Dec–May rather than assuming they were there, then the biennial reproductive cycle is the most likely conclusion. The lower than expected proportion of *U=5* females could be explained by reproductive segregation within the population, similar to what has been found for several shark species (Castro 1993; Salomon-Aguilar *et al.* 2009; Simpfendorfen and Milward 1993), with the *U=5* females migrating to areas outside of the sampling regions of the present study. This would explain why only a small number of *U=5* females caught were >350 mm TL; the present study has likely under sampled the larger reproductive females.

Other urolophid species have been found to migrate to shallow waters for parturition; e.g., *Trygonoptera imitata* was found to have either nursery or pupping grounds within shallow bays and inlets, but not in the waters of Bass Strait (Trinnie *et al.* 2009), *U. cruciatus* are found two shallow bays of Corner Inlet and Westernport Bay, and may require further sampling to determine whether the *U=5* females are migrating to these areas.

Age and growth studies indicate a maximum age of only 10+ years for *U. cruciatus* females (Treloar 2001), which is comparable with *U. paucimaculatus* and *U. viridis* (Trinnie, unpublished data) and suggests *U. cruciatus* has a medium biological productivity (Walker *et al.* 2008). However, based on the late attainment of maternity,
Triennial or biennial reproductive cycles indicate *U. cruciatus* may only reproduce two or three times during its life cycle and suggests a low biological productivity.

**Litter size and sex ratio of embryos**

The similar results for litter size from numbers of eggs and embryos *in utero* indicate litter size increases with maternal TL to a maximum of four for *U. cruciatus*. However, the litter size was affected by stress-induced aborting from capture and handling, but the entire litter was generally aborted in U=5 females, whereas the eggs tended to fuse in U=4 females because of the delicate nature of the enveloped eggs. Hence, the number of eggs *in utero* is a less reliable indicator of the relationship of litter size against maternal TL for *U. cruciatus*.

Maximum litter sizes vary widely among urolophid species. Maximum litters which are generally larger in south-eastern Australia (2–13) (including *U. cruciatus* with 4) (Trinnie *et al* 2012) than species in south-western Australia (1–2) (White *et al*. 2002; White *et al*. 2001; White and Potter 2005). The mean size-at-birth to L_max ratio for *Urolophus cruciatus* is consistent with that for other south-eastern Australian urolophid species at ~29% for females and 37% for males which is smaller than the mean size-at-birth to L_max ratio for south-western Australian urolophid species.

**Female and male length-at-maturity and length-at-maternity**

In south-eastern Australia, *Urolophus cruciatus* has the smallest L_max, size-at-birth, size-at-maturity and size-at-maternity of any urolophid species. Chondrichthyan species generally attain late sexual maturity at ≥60% of L_max (Holden 1974) whereas female *U. cruciatus* in comparison at L50 are ~44% of L_max for maturity and ~57% of L_max for maternity. Other urolophid species in south-eastern Australia have higher L50 values of 50–63% of L_max and 62–74% for maternity (Trinnie, unpublished data).

Irrespective of the maturity indices used for males, clasper calcification, testis development and seminal vesicle development showed similar results at L50. This means that determining maturity through external features of the claspers could be...
used without the need for internal investigations. Male *U. cruciatus* matured at a larger size than the females, consistent with most urolophid species.
Table 5.1. Comparison of observed and expected numbers of U=4, U=5 and U=6 females for hypothesised biennial and triennial reproductive cycles

Observed numbers for each uterus condition were adjusted to percentage of total number for each of the two 6-month periods of Dec–May and Jun–Nov. $\chi^2$ value are sum of $(\text{Obs}–\text{Exp})^2 / \text{Exp}$ values across each uterus condition for the two 6-month periods.

<table>
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<th>Six-month period</th>
<th>Total numbers</th>
<th>Observed or expected number expressed as percentage for each uterus condition</th>
<th>(Obs–Exp)$^2 / \text{Exp}$ values for each uterus condition</th>
<th>$\chi^2$ value</th>
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<td></td>
<td></td>
<td>U=4</td>
<td>U=5</td>
<td>U=6</td>
</tr>
<tr>
<td><strong>Observed numbers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dec–May</td>
<td>168</td>
<td>44.0</td>
<td>13.1</td>
<td>42.9</td>
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<tr>
<td>Jun–Nov</td>
<td>178</td>
<td>46.1</td>
<td>0.0</td>
<td>53.9</td>
</tr>
<tr>
<td><strong>Expected numbers for three alternative hypotheses</strong></td>
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<td></td>
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<tr>
<td><strong>Hypothesis 1: Biennial cycle with no resting year</strong></td>
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<tr>
<td>Dec–May</td>
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<tr>
<td>Jun–Nov</td>
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<td>100.0</td>
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<td>Jun–Nov</td>
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<td>100.0</td>
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<td><strong>Hypothesis 3: Triennial cycle with resting year</strong></td>
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<tr>
<td>Dec–May</td>
<td>33.3</td>
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<td>Jun–Nov</td>
<td>66.7</td>
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</tbody>
</table>

**Adjusted observed numbers after reassigning U=6 females as aborting U=4 or U=5 females based on four alternative assumptions**

<table>
<thead>
<tr>
<th>Total numbers</th>
<th>Hypothesis 1 and Hypothesis 2</th>
<th>Hypothesis 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dec–May</td>
<td>168</td>
<td>44.0</td>
</tr>
<tr>
<td>Jun–Nov</td>
<td>178</td>
<td>66.9</td>
</tr>
<tr>
<td><strong>Assumption 2:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dec–May</td>
<td>168</td>
<td>65.5</td>
</tr>
<tr>
<td>Jun–Nov</td>
<td>178</td>
<td>66.9</td>
</tr>
<tr>
<td><strong>Assumption 3:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dec–May</td>
<td>168</td>
<td>77.1</td>
</tr>
<tr>
<td>Jun–Nov</td>
<td>178</td>
<td>66.9</td>
</tr>
<tr>
<td><strong>Assumption 4:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dec–May</td>
<td>168</td>
<td>59.5</td>
</tr>
<tr>
<td>Jun–Nov</td>
<td>178</td>
<td>66.9</td>
</tr>
</tbody>
</table>
Table 5.2. Chi-squared values for hypothesised biennial and triennial reproductive cycles after assumed U=6 adjustments.

$\chi^2$ values are sum of $\frac{(\text{Obs} - \text{Exp})^2}{\text{Exp}}$ values across each uterus condition for the two 6-month periods (Dec–May) and (Jun–Nov). Probability of statistical significance (*P<0.05; **P<0.01).

<table>
<thead>
<tr>
<th>Assumption</th>
<th>Hypothesis 1</th>
<th>Hypothesis 2</th>
<th>Hypothesis 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df 1</td>
<td>df 1</td>
<td>df 2</td>
</tr>
<tr>
<td>Assumption 1</td>
<td>1.44 (P=0.230)</td>
<td>20.77 **</td>
<td>7.68 *</td>
</tr>
<tr>
<td>Assumption 2</td>
<td>10.6 **</td>
<td>0.06 (P=0.806)</td>
<td>18.32 **</td>
</tr>
<tr>
<td>Assumption 3</td>
<td>41.52 **</td>
<td>6.14 *</td>
<td>32.32 **</td>
</tr>
<tr>
<td>Assumption 4</td>
<td>3.76 (P=0.052)</td>
<td>2.12 (P=0.145)</td>
<td>4.7 (P=0.095)</td>
</tr>
</tbody>
</table>
Table 5.3. Summary data for several sympatric urolophid species and a rhinobatid ray

LFD, largest follicle diameter.

<table>
<thead>
<tr>
<th>Species</th>
<th>Periodicity of reproductive cycle</th>
<th>LFD (mm)</th>
<th>'Embryos in utero' phase’ months</th>
<th>'Eggs in utero' phase’ months</th>
<th>Matrotrophic contribution</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trygonoptera imitata</td>
<td>Annual</td>
<td>43</td>
<td>5–7</td>
<td>5–7</td>
<td>100%</td>
<td>Trinnie et al 2009</td>
</tr>
<tr>
<td>Urolophus paucimaculatus</td>
<td>Annual</td>
<td>16</td>
<td>10–12</td>
<td>1–2</td>
<td>4700%</td>
<td>Trinnie unpublished data</td>
</tr>
<tr>
<td>U. viridis</td>
<td>Annual</td>
<td>16</td>
<td>10–12</td>
<td>1–2</td>
<td>4900%</td>
<td>Trinnie unpublished data</td>
</tr>
<tr>
<td>U. bucculentus</td>
<td>Biennial</td>
<td>24</td>
<td>14–19</td>
<td>2–3</td>
<td>7000%</td>
<td>Trinnie et al 2012</td>
</tr>
<tr>
<td>U. cruciatus</td>
<td>Biennial</td>
<td>14</td>
<td>4–6</td>
<td>12–18</td>
<td>4325%</td>
<td>Present study</td>
</tr>
</tbody>
</table>
Fig. 5.1. Length-frequency composition of samples for females (black) and males (grey).
Fig. 5.2. Yolk mass proportion and mean embryo length and mean embryo mass against month.

Each data point is derived from the embryo mass, mean yolk mass, and embryo length determined for the litter of each of 22 pregnant animals with macroscopically visible embryos. Yolk mass proportion against embryo length (a) is yolk sac mass/ (embryo mass + yolk sac mass). Mass of the egg in utero (b). Mean embryo length (b) and mean embryo mass (c) of the litter from each of pregnant animals with macroscopically visible embryos, overall mean; bars, standard deviation; number above bar is number of embryos collected; ◦, eggs in utero; number above is number of maternal females.
Fig. 5.3. Ovarian largest follicle diameter against day of year for uterus conditions $U=1-6$.

Largest follicle diameter against day of year for females for each of the of five uterus conditions. Mean follicle diameter (——) with 95% confidence limits (— — — —) and 95% prediction intervals (- - - - -) are presented for pregnant females with embryos in utero ($U=5$) (a), immature females ($U=1$) (b), non-pregnant females ($U=2$) (c) and ($U=3$) (d), pregnant females with eggs in utero ($U=4$); o open circle are females in the process of ovulating (e), and postpartum females ($U=6$) (f). Values of parameters and statistical quantities for the regression equation $o=a'+b't$ for pregnant females with embryos in utero ($U=5$) are given in the following tabulation:

<table>
<thead>
<tr>
<th>$U$</th>
<th>$a'$ (±se)</th>
<th>$b'(±se)$</th>
<th>$n$</th>
<th>$r^2$</th>
<th>rmse</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>-5.33 (3.20)</td>
<td>0.03523 (0.00725)</td>
<td>21</td>
<td>0.568</td>
<td>1.36</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

where $t$ is day of year, $o$ is follicle diameter, $a'$ and $b'$ are parameters, $n$ is sample size, $r^2$ is square of regression correlation coefficient, and rmse is root mean square error for the regression, and $P$ is probability of statistical significance.
Fig. 5.4. Ovarian follicle diameter against day of year for revised uterus conditions $U = 4$ and $U = 6$

Follicle diameter against day of year for females in $U = 4$ (a) and $U = 6$ (b) conditions and the possible characteristics when fit to a biennial reproductive cycle.
Fig. 5.5. Various breeding conditions against month for mature males.

Trends in gonadosomatic index (GSI) for mature males determined from testis development (GI) (a), and HSI (b), and seminal vesicle fullness (c). • mean monthly value; bars, standard error for monthly value; number above bar is monthly sample size.
Fig. 5.6. Number of eggs and embryos \textit{in utero} against maternal total length

Number of eggs \textit{in utero} (a) and embryos \textit{in utero} (——) with 95\% confidence limits (– – –) (b) are plotted against maternal TL. Highlighted females in (a) with only one egg \textit{in utero} are unknown because when the eggs combined \textit{in utero} they were recorded as at least one (remove from graph but reference in paper). Highlight female in (b) is believe to have aborted upon capture and was removed from analysis. Values for parameters and statistical quantities from curvilinear regression analysis to derive the equation \( w = acl^b \) are given in the following tabulation:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>a (s.e.)</td>
<td>1.42 (0.0026–88.6)</td>
</tr>
<tr>
<td>b (s.e.)</td>
<td>2.108 (0.721)</td>
</tr>
<tr>
<td>c</td>
<td>1.024</td>
</tr>
<tr>
<td>n</td>
<td>22</td>
</tr>
<tr>
<td>( r^2 )</td>
<td>0.437</td>
</tr>
<tr>
<td>rmse</td>
<td>0.217</td>
</tr>
</tbody>
</table>

where \( w \) is number of embryos, \( l \) is total length, \( a \) and \( b \) are parameters, \( c \) is the Beauchamp and Olson (1973) correction factor, \( n \) is sample size, \( r^2 \) is square of correlation coefficient, and rmse is root mean square error for this regression. Raw data (*)
Population of female population in mature condition (——), with 95% confidence intervals (- - -) (a), and maternal condition (——) (b), with 95% confidence intervals (- - -) against total length. Values of parameters and statistical quantities for the equation $P_l = P_{\text{max}}(1 + e^{3\ln(19)(1-l/l_50-l_95)})^{-1}$ determined from probit analysis are given in the following tabulation:

<table>
<thead>
<tr>
<th></th>
<th>$l_{50}$ (CI)</th>
<th>$l_{95}$ (CI)</th>
<th>$P_{\text{max}}(l)$</th>
<th>n</th>
<th>N</th>
<th>ML</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature</td>
<td>193 (188, 198)</td>
<td>239 (238, 240)</td>
<td>1.0</td>
<td>401</td>
<td>420</td>
<td>-39.047</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Maternal</td>
<td>246 (242, 250)</td>
<td>294 (290, 299)</td>
<td>0.5</td>
<td>353</td>
<td>437</td>
<td>-360.57</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

where $l$ is total length measured in millimetres, $P_l$ is proportion of animals at TL $l$, $l_{50}$ and $l_{95}$ are parameters, $P_{\text{max}}(l)$ is an asymptotic constant, $n$ is the total number of animals classed as being mature or maternal, and $N$ is the total number of animals examined for maturity or maternity, ML is maximum likelihood, and $P$ is probability of statistical significance.
Fig. 5.8. Maturity of males based on three separate conditions.

Proportion of population mature against TL (——) with 95% confidence intervals (- - - -) for males determined from testis condition (a), seminal vesicle condition (b), and clasper condition (c) and comparison between each condition; (——) GI, (- - -) VI, and (– – –) CI (d). Males were classed as immature for GI=1 or GI=2 and mature for GI=3 testis condition, as immature for VI=1 and mature for VI=2 or VI=3 seminal vesicle condition, and immature for CI=0 or CI=1 and mature for CI=2 clasper condition. Values of parameters and statistical quantities for the equation $P=P_{\text{max}}(1+e^{-\ln(19)(1-l_{50}/l_{95}-l_{50})})^{-1}$ determined from probit analysis are given in the following tabulation:

<table>
<thead>
<tr>
<th>Method</th>
<th>$l_{50}$ (CI)</th>
<th>$l_{95}$ (CI)</th>
<th>$P_{\text{max}}$</th>
<th>n</th>
<th>N</th>
<th>ML</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis condition</td>
<td>209 (197–217)</td>
<td>271 (266–278)</td>
<td>1.000</td>
<td>263</td>
<td>304</td>
<td>–277.808</td>
<td>***</td>
</tr>
<tr>
<td>Seminal vesicle</td>
<td>212 (203–218)</td>
<td>253 (249–259)</td>
<td>1.000</td>
<td>282</td>
<td>307</td>
<td>–153.201</td>
<td>***</td>
</tr>
<tr>
<td>Clasper condition</td>
<td>217 (209–223)</td>
<td>261 (257–266)</td>
<td>1.000</td>
<td>275</td>
<td>307</td>
<td>–207.015</td>
<td>***</td>
</tr>
</tbody>
</table>

where $l$ is total length measured in millimetres, $P$ is proportion of animals at TL $l$, $l_{50}$ and $l_{95}$ are parameters, $P_{\text{max}}$ is an asymptotic constant, $n$ is the total number of animals classed as mature, and $N$ is the total number of animals selected in statistical procedure, ML is maximum likelihood, and $P$ is probability of statistical significance (*$P<0.1$; **$P<0.01$; ***$P<0.001$).
Chapter 6 – *Urolophus paucimaculatus* (Sparsely-spotted stingaree)

Abstract

The periods of ‘eggs in utero’, ‘embryos in utero’ and ‘ovarian cycle’, and mean size-at-birth, and sex ratio were similar among four separate regions across south-eastern Australia during 2002–06, but the population of animals in Port Phillip Bay (PPB) has larger maximum TL, size-at-maturity and size-at-maternity than do the populations in each of Lakes Entrance (LE) and Corner Inlet (CI); size-at-maturity and size-at-maternity in western Bass Strait (WBS) could not be statistically compared. Parturition and ovulation occurred during Aug–Oct in PPB and Sep–Dec in LE. Maximum litter size varied with maximum TL observed among the regions (six in PPB, five in each of LE and CI, and four in WBS). There was uncertainty in classifying females for maternal condition as the reproductive cycle appears to range from a continuous annual cycle to a non-continuous biennial cycle. Much of the uncertainty arises from the ambiguity of observation of non-pregnant mature females, which have either aborted from stress induced through capture and handling or are in a ‘resting year’ between pregnancies. Most likely they are reproducing annually with a small proportion of females non-continuous and resting between pregnancies.
Results

Allometric and isometric relationships for total body mass and various size measurements

A total of 1,628 animals of both sexes were sampled across the four regions (Table 6.1). The effects of region on the \( \ln(TW) - \ln(TL) \) relationship for males (ANCOVA: \( F_{df=3, 666} = 3.63, P=0.127 \)) and females (ANCOVA: \( F_{df=3, 956} =0.88, P=0.449 \)) was not significant and the data were pooled for each sex. A larger size and greater mass were attained by females (500 mm TL; 1438 g TW) than the males (401 mm TL; 624 g TW) (Table 6.1; Fig. 6.1). The effect of sex on the \( \ln(TW) - \ln(TL) \) relationship was not significant (ANCOVA: \( F_{df=1, 1623} = 0.49, P=0.484 \)) and at the same TL, males and females were of similar mass. The effects of sex and female breeding condition on body mass–TL relationships among 667 males, 538 non-pregnant females (U=1–3 and U=6 females), 58 pregnant females with eggs in utero (U=4 females), and 196 pregnant females with embryos in utero (U=5 females) were highly significant (ANCOVA: \( F_{df=3,1458} = 9758.79, P<0.001 \)). Least squares means indicated that U=4 pregnant females were significantly different from U=5 pregnant females (\( P=0.003 \)). U=4 pregnant females were not significantly different from non-pregnant females (\( P=0.385 \)). U=5 pregnant females were significantly different from the non-pregnant females (\( P<0.001 \)) and males (\( P<0.001 \)). The males were not significantly different from the non-pregnant females (\( P=0.520 \)) or from U=4 females (\( P=0.326 \)).

Females total: \( TW = 0.0000113 \times 1.007 \times TL^{2.972} \) (\( P<0.001; r^2 = 0.967; n = 957 \)),

Males: \( TW = 0.0000127 \times 1.007 \times TL^{2.948} \) (\( P<0.001; r^2 = 0.944; n = 667 \))

U=5 pregnant females: \( TW = 0.0000116 \times 1.007 \times TL^{2.977} \) (\( P<0.001; r^2 = 0.917; n = 195 \)),

A least squares means test indicated that DL–TL (\( P=0.315 \)) and DW–TL (\( P=0.125 \)) isometric relationships between males and females were not significantly different. The variables for each morphometric relationship (sexes combined) are given by the equations

\[
DL = -4.628 + 0.581 \times TL \quad (P<0.001; r^2 = 0.971; n = 961), \\
DW = 17.937 + 0.589 \times TL \quad (P<0.001; r^2 = 0.970; n = 961).
\]
Growth of embryos and the pregnancy cycle

The ‘period of embryos in utero’ and ‘period of parturition’ were determined from 553 embryos collected (447 embryos were from known pregnant females and 106 were aborted embryos from unknown pregnant females). For the relationship between ETL and ETW, regions were tested and showed no significant difference (ANCOVA: $F_{df=3, 96} = 125.46, P=0.253$) and thus were subsequently pooled. Embryo mass plotted against ETL is a curvilinear growth pattern.

$$ETW = 0.00000134 \times 1.005 \times ETL^{3.470} \quad (P<0.001; r^2 = 849; n=88)$$

The largest embryo was 185 mm ETL and 86 g wet total mass whereas the smallest neonate collected was 132 mm TL and 30 g wet total mass; however, this maximum size occurred only in PPB, six of the 195 pregnant females contained only one large embryo in utero which had grown larger than the mean size-at-birth. Excluding these six pregnant females, the mean embryo length indicates that birth would occur at 155 mm TL and 37 g mean total mass.

Mean mass of ovarian follicles for females with LFD >9 mm was 0.79 g (s.e. 0.01 g; range 0.35–1.4 g) which was similar to mean mass of eggs in utero at 0.75 g (s.e. 0.14 g; range 0.72–0.96 g). The presence of eggs in utero for U=4 females (n=57) were found in every month of the year; however, the comparatively few eggs in utero found (DOY <180) (n=9) were interpreted as infertile (see section Ovarian cycle). During embryonic development the external yolk sac was consumed by the time the embryos reached a mean embryo TL of ~110 mm and a mean mass of ~10 g (Fig. 6.2a). The depletion of the yolk sac corresponded to the initial rapid growth in embryo TL and most of the mass gained was after the yolk sac was completely absorbed.

Scattergrams of mean ETL (Fig. 6.2b) and mean ETW (Fig. 6.2c) against DOY indicated the ‘period of embryos in utero’ of ~10 months in all regions. Embryos first became visible during Nov–Dec (DOY 305–365) in the regions of PPB, CI and WBS and reached the mean size-at-birth by the following Sep–Oct (DOY 244–304) with the parturition period completed by the end of October (Fig. 6.2bc). However, LE differed from the other regions with the timing of parturition as late as December (DOY >335) and embryos first becoming visible in January (highlighted) (DOY <31).
In all regions, the periods of eggs *in utero* is likely to occur for only a short period (1–2 months) before embryonic growth commences; hence, the ‘period of pregnancy cycle’ was ~12 months. Comparing mean mass of eggs *in utero* (0.75 g) to mean mass of full-term embryos (37 g) indicates a ~4900% wet mass gain.

**Ovarian cycle**

LFD for 961 females ranged 1–16 mm across all uterus conditions (U=1–6), but ranged 1–2 mm for U=1 females; with the exception of one female which had a 10 mm LFD, 1–14 mm for U=2, 1–14 mm for U=3, 1–15 mm for U=4, 1–16 mm for U=5, and 1–16 mm for U=6 females. Vitellogenesis had commenced in only three U=1 females, but had commenced in most of the U=2 and U=3 females.

For the relationship between LFD and embryo length, the effects of region was tested and showed no significant difference (ANCOVA: $F_{df=3, 181} = 0.68$, P=0.567) and thus the data were subsequently pooled across regions. The ‘period of embryos *in utero*’ was synchronous with the ‘period of ovarian cycle’ for U=5 pregnant females, as indicated by the linear relationship between LFD and embryo length

$$LFD = -2.379 + ETL \times 0.097 \quad (P<0.001; r^2 = 0.57; n=182).$$

Linear regression was fitted to the U=5 scattergram with 95% prediction intervals to demonstrate the ovarian cycle in pregnant females (Fig. 6.3a). LFD increased from 1–4 mm to a maximum 16 mm over a period of ~10 months during pregnancy and U=5 females were ready to ovulate immediately after parturition. Regional differences occurred as females from PPB, WBS and CI, showed LFD reached a maximum during Aug–Oct (DOY 213–304), whereas those from LE reached a maximum during Nov–Dec (DOY 305–365) (highlighted).

Little growth of the follicles occurred during the U=1 condition (Fig. 6.3b). Many U=2 females appear synchronised with the U=5 ovarian cycle and developed their uteri and ovarian follicles concurrently (Fig. 6.3c). Most U=3 females with their developed uteri were also consistent with the period of ovulation in U=5 females (Fig. 6.3d); however, several females had developed uteri, but the follicles remained small and not ready for ovulation.
The pattern of LFD in U=4 females provided a reliable representation of the timing of ovulation (Fig. 6.3e). Animals from PPB (n=2) were observed with yolk in the oviducal gland (i.e. ovum passing through) during August (DOY 213–243) and from LE (n=1) during November (DOY 305–334); consistent with the U=5 females, ovulation occurred earlier in PPB, CI and WBS than in LE. These three U=4 animals demonstrate that all developed oocytes in the ovary were ovulated and the post-ovulation LFD size class is 2–5 mm. The rest of the U=4 animals, which had been observed with eggs in utero had either one of two distinct LFD size classes (1–5 mm) (ovulation complete) and (8–15 mm) (ovulation in progress) (Fig. 6.3e). During Feb–May (DOY 32–151), several animals were observed with eggs in utero; these are thought to have been unfertilized because their growth was not synchronous with the rest of the population.

The pattern of LFD in the U=6 females was expected to confirm that the periods of parturition and periods of ovulation occurred during Aug–Dec, but U=6 females were present all year in large numbers and with a wide range in LFD (Fig. 6.3f). The U=6 females with large LFD during Aug–Dec support the conjecture that ovulation occurred soon after parturition. However, the range of U=6 LFD throughout the year, suggests that some animals have aborted from stress during capture and handling and some animals are likely to be in a resting year between pregnancies.

If the population had a continuous reproductive cycle, U=6 females should not be observed during Jan–Jul and U=6 females should not be found during Aug–Dec with small LFD; these are not expected to be ready for ovulation that year. Thus, the interpretation of the U=6 females found during Jan–Jul and small (<8 mm LFD) in Aug–Dec demonstrate that some females are not reproducing each year.

Four alternative interpretations are made for the U=6 females. Interpretation 1: U=6 females found during Jan–Mar (DOY 1–90) (circled and labelled A in Fig. 6.4a) with the large follicles are interpreted as resting. All of these females had more than one enlarged ovarian follicle that appeared ready to ovulate; however, these are outside of the population’s normal ovulation period (Aug–Nov) and not expected to be ovulating until the next ovulation period (Aug–Nov) and the oocytes are likely to undergo
ataresia. If ovulated, their eggs *in utero* are likely to be unfertilized. Interpretation 2: U=6 females found during Jan–Jul (DOY 1–181) (circled and labelled B in Fig. 6.4a) are interpreted as a combination of U=5 females aborting and U=6 females that are resting. The period of females in U=4 condition is short and occurs mainly during Jul–Dec and U=4 females aborting are unlikely to be found during this period.

Interpretation 3: U=6 females found during Jul–Dec (DOY 182–365) (circled and labelled C in Fig. 6.4a) fit with the timing of parturition and ovulation in the population and many are likely to be genuine U=6 post partum females. However, a number of U=5 females observed aborting indicates that these U=6 females are a mix of aborting U=5 females and genuine U=6 post-partum females. Interpretation 4: U=6 females found during Jul–Dec (DOY 182–365) (circled and labelled D in Fig. 6.4a) are a mix of U=4 females observed aborting their eggs and U=5 females aborting small embryos, and because of the large number of females in this category, U=6 females are resting for a year.

The U=6 females (circled and labelled B in Fig. 6.4a) demonstrate synchronous growth of follicles during Jan–Jul (DOY 1–181). Although there is a mixture of U=5 females aborting their embryos and non-pregnant U=6 females resting, this growth is most likely representing the follicle growth of non-pregnant resting females. The growth of follicles for non-pregnant resting females was found to be faster than when a female is in the U=5 condition where it has to grow both follicles and embryos synchronously (Fig. 6.4c). The possibility of having females in a non-continuous cycle is also corroborated by the U=4 females with unfertilized eggs during Jan–Jul.

*Reproductive cycles of females*

From the stepwise sequential questioning (methods chapter Fig. 2.4), the answer to Q1 was ‘yes’; the present study can demonstrate that embryonic growth was synchronous within the population and the ‘period of embryos *in utero*’ and the ‘period of parturition’ occur seasonally (Fig. 6.2bc). The answer to Q2 was ‘yes’; the ‘period of eggs *in utero*’ and the ‘period of ovulation’ were synchronous and seasonality determinable (Fig. 6.3e). The answer to Q3 was ‘yes’; the duration of the ‘pregnancy cycle’ was determinable from the ‘period of embryos *in utero*’ and ‘period
of parturition’, from the ‘period of eggs in utero’, and from the ‘period of ovulation. The answer to Q4 was ‘yes’; LFD was synchronous with ETL (results section Ovarian cycle). The answer to Q5 was ‘no’; the pattern of growth of LFD against DOY in U=5 females was determinable (Fig 6.3a), but not consistent with the U=3, U=4, or U=6 females (Fig 6.4c), and the answer to Q6 was initially ‘no’; reproduction is not consistent with an annual or shorter cycle.

From the data collected as part of the present study, it can be concluded that the reproductive cycle was synchronous across the population, the period of embryos in utero was ~10 months, the period of parturition was 1–2 months, the ‘period of ovulation’ and ‘period of eggs in utero’ was each 1–2 months; hence, the period of the pregnancy cycle was 12 months, the oocytes grew synchronously with embryos, and the period of ovarian cycle was 10–12 months. However, the large number of U=6 females in the population compared with U=4 and U=5 females, the different average growth rates of oocytes between U=5 and U=6 females, and the U=4 females with suspected unfertilized eggs in utero (see discussion) suggests that there are a number of females resting between pregnancies. The number of U=4–5 females (n=255) compared to U=6 females (n=346) suggests this proportion is more comparable to a biennial reproductive cycle than an annual cycle as more annuals are resting than are reproducing. On the other hand, the number of observed females aborting and number of aborted embryos (n=106) and eggs found suggest that fewer animals than observed were actually resting. However, the true proportion of resting animals is indeterminable due to the unknown quantity of aborted eggs and embryos during capture. The answer to Q6 was therefore a tentative ‘yes’; the reproductive cycle is more likely to be annual than biennial.

Reproductive cycles of males

Seasonal trends in male breeding cycle were determined for mature males from GSI, HSI, and seminal vesicle fullness means plotted against month. The pattern of mean GSI against month (Fig. 6.5a) showed a peak during Jan–Mar (months 1–3) before decreasing in April (month 4) and remaining low through the rest of the year. The mean HSI (Fig. 6.5b) also showed a seasonal pattern with a marked peak in April, one month after the GSI peaked before decreasing to its minimum through to December
(month 12) and early the following year. The mean seminal vesicle fullness (Fig. 6.5c) decreased in fullness during Jan–Feb before increasing to nearly 100% full for the rest of the year. Males are capable to mating throughout most of the year including the extended period of ovulation for the females across all regions.

**Litter size and sex ratio of embryos**

The effect of region on the relationships between litter size and maternal TL for U=5 females was not significant (ANCOVA: $F_{df=3, 194} = 2.03$, $P = 0.111$) and linear regression was fitted for data pooled across the region (Fig. 6.6). However, the maximum litter size recorded for all U=5 females (n=195) was six found in an animal >400 mm TL from PPB compared with the lowest maximum of four in WBS, therefore the regions were presented separately. The smallest recorded U=5 female was 278 mm TL from LE. A total of 58 pregnant U=4 females with eggs *in utero* were recorded, but the eggs were too delicate to count precisely *in utero*.

A total of 433 embryos sexed from 195 pregnant females had a sex ratio of 210 (48%) females, 187 (43%) males, and 36 (8%) of unknown sex due to their early stage of development. Based on the male to female ratio of embryos *in utero*, the sex ratio was 1:1 ($X^2 = 0.133$, d.f. = 1, $P = 0.248$).

**Female and male size-at-maturity and size-at-maternity**

Female size-at-maturity or size-at-maternity ogives and associated parameters from WBS could not be estimated and compared with other regions and had to be excluded from the analysis. Comparison for regional differences in size-at-maturity ogives using Wald $X^2$ likelihood ratio test indicated no significant difference between LE and CI for maturity (Wald: $X^2_{1,301} = 0.644$, $P=0.422$) and length (Wald: $X^2_{1,301} = 15.20$, $P<0.001$) but LE and CI pooled, was significantly different from PPB (Wald $X^2_{1,955} = 43.07$, $P<0.001$) and length (Wald $X^2_{1,955} = 206.40$, $P<0.001$). Due to the uncertainty of $P_{max}$ in maternity, the maternity ogives were not tested for region, however graphically they followed similar trends to the maturities. LE and CI females mature smaller and reach maternal condition at a size smaller than those for PPB females. As
expected, the parameter estimates for $l_{50}$ and $l_{95}$ were higher for the length-at-maternity ogives than for the size-at-maturity ogives.

The female size-at-maturity ogive and its parameters for LE and CI pooled were determined from 41 immature and 340 mature animals where the largest immature female observed was 241 mm TL and the smallest mature female observed was 196 mm TL (Fig. 6.7a). For females, size-at-maturity $l_{50}$ was ~51% and $l_{95}$ was ~54% of $l_{\text{max}}$. The female size-at-maturity ogive for PPB was determined from 54 immature and 421 mature animals (Fig. 6.7b). For females, size-at-maturity $l_{50}$ was ~50% and $l_{95}$ was ~55% of $l_{\text{max}}$. The regional comparison between size-at-maturity from LE and CI pooled and PPB is presented (Fig. 6.7c).

The periodicity of the reproductive cycle for *U. paucimaculatus*, however, remains uncertain, which is required for maternity analysis and $P_{\text{max}}$. Based on the assumption that all U=6 females are accounted for as genuine post-partum or U=4–5 females having aborted, then the reproductive cycle would be continuous and annual. The size-at-maternity ogive and its parameters for LE and CI pooled were determined from 132 non-maternal and 249 maternal animals based on a one-year cycle ($P_{\text{max}}$ equals 1), where the smallest animal in maternal condition from LE was 260 mm TL (Fig. 6.7d). For size-at-maternity, $l_{50}$ was ~64% and $l_{95}$ was ~74% of $l_{\text{max}}$. The size-at-maternity ogive for PPB was determined from 192 non-maternal and 283 maternal animals, where the smallest animal in maternal condition was 268 mm TL (Fig. 6.7e). For size-at-maternity, $l_{50}$ was ~64% and $l_{95}$ was ~78% of $l_{\text{max}}$. The regional comparison of size-at-maternity ogives between LE and CI pooled and PPB is presented (Fig. 6.7f).

However, the large number of U=6 animals found does not justify redistributing all unknown U=6 females as U=4–5 pregnant females. Pooled across the year, the current proportion of readily identifiable pregnant females (U=4–5; n=255) compared with apparent U=6 post-partum females (n=346) is less than 50%, which is more consistent with a biennial than an annual reproductive cycle. But, the number of aborted embryos (n=106) which could be attributed to at least (n=30) U=5 females having aborted should be considered. Along with all genuine post-partum females (U=6 females with LFD >8 during DOY >180) this would increase the proportion of
maternal females to non-maternal females to greater than 50%. Furthermore, anecdotal evidence suggests that females aborting eggs could be consistent with the rate of U=5 females aborting embryos which would increase this maternal percentage. There is a need to also account for aborting during capture and handling in the nets. However, this would indicate that the actual percentage of the population breeding each year could not be determined from the present study. Hence, a family of length-at-maternity ogives for LE and CI and for PPB have been developed to demonstrate $P_{\text{max}}$ equal to 1.0, 0.9, 0.8, 0.7, and 0.6, which represents 100%, 90%, 80%, 70% or 60% of the population of large females breeding each year for the LE and CI and PPB regions (Fig. 6.7gh).

Male maturity ogives were determined from each of seminal vesicle fullness, clasper calcification, and testis development. Comparison for regional differences in size-at-maturity ogives using Wald $x^2$ likelihood ratio test for seminal vesicle fullness was significantly different for region between LE and PPB (Wald: $x^2_{1,501} = 63.76$, $P<0.001$) and length (Wald: $x^2_{1,501} = 249.19$, $P<0.001$), for clasper calcification was significant different for region between LE and PPB (Wald: $x^2_{1,501} = 63.76$, $P<0.001$) and length (Wald: $x^2_{1,501} = 296.64$, $P<0.001$), and for testis development was significant different for region between LE and PPB (Wald: $x^2_{1,501} = 7.07$, $P=0.008$) and length (Wald: $x^2_{1,501} = 246.13$, $P<0.001$). LE males mature smaller than those for PPB males. As with the females, few male samples came from CI or WBS and were not statistically tested. All three methods provided similar results for maturity with a slightly larger $l_{50}$ for clasper calcification (Fig. 6.8). For LE, the percentage of $l_{50}$ and $l_{95}$ of $l_{\text{max}}$ based on seminal vesicle fullness was ~72% and ~80%, respectively, based on clasper calcification was ~72% and ~81%, respectively, and based on testis development was ~71% and ~86%, respectively. For PPB, the percentage of $l_{50}$ and $l_{95}$ of $l_{\text{max}}$ based on seminal vesicle fullness was ~69% and ~76%, respectively, based on clasper calcification was ~70% and ~79%, respectively, and based on testis development was ~69% and ~86%, respectively.

**Discussion**

*Spatial differences within south-eastern Australia*
Despite intense sampling during the present study, none of the animals collected reached the maximum sizes of 573 mm TL for females and 440 mm TL for males reported previously for this species from PPB (Edwards 1980). The present study is more consistent with findings from an annual trawl survey of PPB (1990–2010) which indicate that maximum TL has reduced to ~500 mm TL (Hirst et al. 2010; Parry et al. 2002). Over the past 20 years, several changes to PPB have occurred. In particular, the cessation of the commercial scallop dredging which was thought to have released nutrients and food from the sediments into the ecosystem, while several environmental changes have affected water temperature, freshwater runoff, and salinity. Many teleost and invertebrate species have shown significant productivity changes in PPB during this period; e.g., the population size of sand flathead (Platycephalus bassensis) has reduced to ~10% over the past decade (Hirst, Heislers et al. 2010).

Irrespective, of whether maximum size has changed, both male and female *U. paucimaculatus* in the inshore regions of PPB and CI grow larger than those in the offshore regions of WBS and LE, which suggests inshore habitats are more suitable for growth to larger sizes than offshore habitats. The larger size-at-birth, size-at-maturity and maternity, and larger litter size are consistent with growth in PPB than other regions for this species.

Port Phillip Bay is a broad shallow basin largely protected from the Bass Strait currents and experiences higher average temperatures and evaporation, while being nutrient rich from freshwater runoff and sewage treatment (Murray and Parslow 1999; Walker 1999), which are probable all important factors contributing to suitable conditions for *U. paucimaculatus*. Conversely, common among all urolophid species in Victorian waters of south-eastern Australia is the sparse distribution in WBS (pers. obs). Western Bass Strait dominated by rocky reef seems relatively unsuitable for these benthic urolophid species compared with the sandy areas in PPB, CI, and LE.

To further understand the growth among each of the studied areas a study on age and growth is required. *Urolophus paucimaculatus* as with most urolophid species have low fecundity, late attainment of maturity and maternity, and medium biological
productivities (Walker and Gason 2007) which makes them susceptible to the effects of high fishing pressures. For LE in particular, commercial fishing pressure is much higher than in the other areas in the present study, and the higher fishing mortality could be causing the population in at least LE to change its strategy to reproduce at a smaller size and earlier in life.

Spatial differences between south-eastern Australia and south-western Australia

Litter sizes of 1–6 and increasing with maternal TL (present study), did not differ from the previous range of 2–6 found in *U. paucimaculatus* from SE Australia (Edwards 1980). However, PPB was the only region where litters of 6 were observed and was directly related to the larger TL. Given *U. paucimaculatus* is a widely distributed species, maximum litter size of 6 is not typical for this species when most other regions have less, mostly 4–5. Interestingly, in PPB, the opposite also occurred with several U=5 females having only one extremely large embryo *in utero* and several other U=4 females had only one developed LFD, suggesting some of the population has particularly low fecundity.

Maximum litter sizes among urolophid species vary widely (Table 6.2) among different regions tend to be larger in SE Australia than SW Australia. Particularly noticeable is the larger maximum litter size of *U. paucimaculatus* in SE Australia than in SW Australia. These differences between SE and SW Australia led White (2005) to question whether SE Australian urolophid species actually have these larger maximum litter sizes. It was observed in all urolophid species from SW Australia, despite up to a maximum of 6 embryos observed in early term litters, that they abort during the early stages of pregnancy to give birth naturally to a maximum of only 1–2, termed ‘naturally aborting during pregnancy’ (Chapter 3). Observation of large numbers of aborted embryos during capture and handling is common amongst *U. paucimaculatus* (present study; White and Potter 2005) and may affect reported relationships between litter size and maternal TL, but the high levels of natural aborting females reported for SW Australia has not been observed in SE Australia. The present study, along with studies of other urolophid species from SE Australia, all provide evidence for similar maximum litter sizes during early-term pregnancy and during late-term pregnancy.
Aborting naturally during early stages of pregnancy in SW Australia might also explain the larger sizes-at-birth in SW Australia than in SE Australian urolophid species. For example, in U. paucimaculatus, the sizes-at-birth in SE Australia are at 31% and 34% of maximum TL for females and at 38% and 42% for males of maximum TL in PPB and LE, respectively (present study), compared with higher ~46% of maximum TL for females and ~49% for males in SW Australia (White and Potter 2005). This also occurs for the other urolophid species. The size-at-birth for most SE Australian urolophid species are generally 25–34% of maximum TL for females and 30–42% for males, whereas size-at-birth for SW Australia urolophid species is comparatively larger at 35–46% of maximum TL for females and 42–49% for males (Table 6.2). However, there is the exception of U. viridis found in SE Australia which has a small litter size and large size-at-birth and therefore comparable to species in SW Australia.

The smaller sizes-at-birth coincides with the smaller sizes-at-maturity and size-at-maternity in SE Australia than in SW Australia (Table 6.2). In SE Australia, for both PPB and LE regions, female U. paucimaculatus in the respective regions attain maturity at ~50% and maternity at ~64% of \( l_{max} \), whereas in SW Australia females mature (equivalent to maternity in the present study) at ~81% of \( l_{max} \). Males also mature at smaller sizes in SE Australia (70–72% of \( l_{max} \)) than in SW Australia (80%).

A generalisation of smaller size-at-birth equates to smaller size-at-maturity and size-at-maternity does not apply to all urolophid species, although it appears that maximum TL can have an effect on the onset of maturity and maternity. The species of U. cruciatus and U. viridis (\( l_{max} \) of 500 mm TL) in SE Australia are also consistent with U. paucimaculatus by having smaller sizes-at-birth and smaller sizes-at-maturity and size-at-maternity. Whereas, the two larger species T. imitata and U. bucculentus (\( l_{max} \) of 800 and 900 mm TL, respectively) retain the smaller sizes-at-birth and larger litter sizes, but they mature and become maternal at a larger size which is similar to SW Australia urolophid species.

*Female reproductive cycle periodicity*
Regardless of the differences between SE Australia and SW Australia, both the present study and White and Potter 2005 refute the earlier conclusion of a biannual reproductive cycle for *U. paucimaculatus* (Edwards 1980). Edwards (1980) determination of the biannual reproductive cycle was based on data collected from only two sampling periods of Mar–Apr and Aug–Sep, and because of the lack of periodical sampling, no direct determination of the periodicity of the reproductive cycle could be made. It appears that the biannual periodicity found earlier in *Urolophus halleri* (Babel 1967) was assumed; however, *Urolophus halleri*, now referred to as *Urobatis halleri*, no longer resides within the family Urolophidae and recent findings suggest *Urobatis halleri* also reproduces annually (Chris Mull. pers comm.). Embryo length versus month in the present study shows that maximum ETL is reached early during gestation, or more precisely, the length of the body and tail, though slender, develop to almost full length by Mar–Apr, but embryo mass is relatively low at that time. Embryo mass increases at a more constant rate throughout the period of embryonic growth and parturition occurs when mass reaches a certain level. The embryos appearing close to full size in TL during Mar–Apr might also explain the earlier conclusion *U. paucimaculatus* reproduced biannually (Edwards 1980). Embryonic growth is relatively slow and ETL may have been similar during Aug–Sep as during Mar–Apr. The earlier conclusion highlights how easy it is to draw wrong conclusions from small data sets.

The periodicity of the reproductive cycle in female *U. paucimaculatus* in SE Australia (present study) is mostly annual with a proportion of the population unlikely to be reproducing continuously and is resting. Whereas, a continuous annual reproductive cycle for all *U. paucimaculatus* is assumed in SW Australia (White and Potter 2005). To make direct comparisons between regions, maternity in the present study was made with $P_{\text{max}}$ equal to 1.0 (Table 6.3) with the implicit assumption of an annual reproductive cycle.

The large number of non-pregnant females within the present study, however, suggested a non-continuous reproductive cycle. Unless non-maternal U=6 females are reassigned to being maternal, *U. paucimaculatus* could only be recruiting every second year; however, this is improbable because of the large number of U=4 and U=5 females found aborting their eggs and embryos during capture and handling.
This excessive rate of aborting thus undermined the determination of the proportion in maternal condition.

Without acknowledging the possibility of a resting period occurring between pregnancies, the most likely hypotheses that could have been made was that all U=6 females are redefined as pregnant and maternal and contributing to recruitment each year. However, this is also improbable because of the observed U=4 females with unfertilized eggs *in utero* and the faster follicle growth in U=6 females than in U=5 females, suggesting that at least some U=6 females are resting. Therefore, several hypothesised maternity ogives with a range of $P_{\text{max}}$ values of 1.00, 0.90, 0.80, 0.70 and 0.60 were determined in the present study to express the uncertainty associated with the identification of U=6 non-pregnant females. The most conservative interpretation is that no unobserved females aborting during capture and handling which would mean ~60% are breeding each year. Alternative interpretations where most U=6 females are reassigned as aborting U=5 or U=4 females would put $P_{\text{max}}$ closer to 1.0 (80–90%). This hypothesis would include the possibility that a number of females aborted embryos and eggs in the sampling gear but were not readily observed. Whichever hypothesis is more correct, it raises the question of whether any population of chondrichthyan species has 100% of the large females breeding continuously when they are dependent on finding a mate and favourable environmental conditions. Hence determination of $P_{\text{max}}$ and whether it is 1.00 for chondrichthyan species requires further study as well as using sampling methods that can retain all aborted embryos and eggs, whether in the water or on a vessel.

The results of the present study provide an understanding of the reproductive biology of *U. paucimaculatus* from SE Australia and demonstrate spatial variation in their reproduction. The SE Australian population is at least reproductively isolated, if not a separate species, from the SW Australian population (White and Potter 2005) (Table 6.3). This is further supported by their different morphological features; i.e. *U. paucimaculatus* in SE Australia has spots in most of the population (Stroud 1975) whereas in SW Australian species is spotless (White and Potter 2005). Further study, particularly genetic studies are required to resolve the degree of separation.
Table 6.1. Sample size and maximum total length and mass from each collected region for males and females (with their reproductive conditions)

<table>
<thead>
<tr>
<th>Region</th>
<th>Sample size</th>
<th></th>
<th>Male total</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U=1 U=2 U=3 U=4 U=5 U=6 Total</td>
<td>For each female uterus conditions</td>
<td>Females</td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>Port Phillip Bay</td>
<td>56 66 70 35 76 173 476</td>
<td>299 500 401 1438 624</td>
<td>114</td>
<td>112</td>
<td>134</td>
</tr>
<tr>
<td>Corner Inlet</td>
<td>29 10 12 4 23 71 149</td>
<td>97 480 480 508</td>
<td>112</td>
<td>112</td>
<td>112</td>
</tr>
<tr>
<td>Lakes Entrance</td>
<td>15 31 38 14 68 71 237</td>
<td>204 450 365 777 484</td>
<td>134</td>
<td>134</td>
<td>134</td>
</tr>
<tr>
<td>WBS</td>
<td>14 5 14 5 30 31 99</td>
<td>67 430 360 760 421</td>
<td>58</td>
<td>58</td>
<td>58</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>114 112 134 58 197 346 961 667</strong></td>
<td></td>
<td><strong>667</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urolophid species</td>
<td>Region</td>
<td>Reproductive cycle periodicity</td>
<td>Matrotrophic contribution</td>
<td>Size of LFD at the onset of vitellogenesis (mm)</td>
<td>Size (TL) at l50 relative to lmax Maturity (%)</td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------</td>
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<td>---------------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td><em>U. paucimaculatus</em></td>
<td>SE (PPB)</td>
<td>Annual</td>
<td>&gt;1</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td><em>U. paucimaculatus</em></td>
<td>SE (LE)</td>
<td>Annual</td>
<td>&gt;1</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td><em>Urolophus bucculentus</em></td>
<td>SE</td>
<td>Biennial</td>
<td>&gt;3</td>
<td>57</td>
<td>44</td>
</tr>
<tr>
<td><em>U. cruciatus</em></td>
<td>SE</td>
<td>Biennial</td>
<td>&gt;3</td>
<td>57</td>
<td>44</td>
</tr>
<tr>
<td><em>U. viridis</em></td>
<td>SE</td>
<td>Annual</td>
<td>&gt;1</td>
<td>56</td>
<td>56</td>
</tr>
<tr>
<td><em>Trygonoptera imitata</em></td>
<td>SE</td>
<td>Annual</td>
<td>&gt;3</td>
<td>63</td>
<td>63</td>
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<td><em>U. paucimaculatus</em></td>
<td>SW</td>
<td>Annual</td>
<td>i.d.</td>
<td>#</td>
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<td><em>U. lobatus</em></td>
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<td>Annual</td>
<td>i.d.</td>
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<tr>
<td><em>T. mucosa</em></td>
<td>SW</td>
<td>Annual</td>
<td>i.d.</td>
<td>#</td>
<td>78</td>
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<tr>
<td><em>T. personata</em></td>
<td>SW</td>
<td>Annual</td>
<td>i.d.</td>
<td>#</td>
<td>82</td>
</tr>
</tbody>
</table>

*White and Potter 2005 defines this as maturity but is equivalent to maternity in the present study; i.d. information deficient.
### Table 6.3. Comparisons between the present study and previous studies on *Urolophus paucimaculatus*

Disc widths (DW) from Stroud (1977) and White *et al* (2005) have been converted to TL for comparative purposes and all values relating to size are in millimetres. In White *et al* (2005), their maturity index is more comparable to the maternity index in the present study and has been used to compare size at maternity.

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Region</td>
<td>LE</td>
<td>PPB</td>
<td>PPB</td>
<td>PPB</td>
</tr>
<tr>
<td><strong>Maximum size</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females TL (mm)</td>
<td>480</td>
<td>500</td>
<td>573</td>
<td>487*</td>
</tr>
<tr>
<td>Males TL (mm)</td>
<td>380</td>
<td>401</td>
<td>428</td>
<td>382*</td>
</tr>
<tr>
<td><strong>Female maturity and maternity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maturity at l₅₀ (mm) (% of lₘ₅₀)</td>
<td>229 (51%)</td>
<td>248 (50%)</td>
<td>smallest mature = 289 (&gt;60%)</td>
<td></td>
</tr>
<tr>
<td>Maternity at l₉₀ (mm) (% of lₘ₅₀)</td>
<td>289 (60%)</td>
<td>322 (64%)</td>
<td>340 (59%)</td>
<td>?</td>
</tr>
<tr>
<td><strong>Male maturity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maturity at l₅₀ (mm) (% of lₘ₅₀)</td>
<td>~263 (72%)#</td>
<td>~280 (70%)#</td>
<td>smallest mature = 281 (&gt;79%)</td>
<td>325 (80%)***</td>
</tr>
<tr>
<td>Gestation period (months)</td>
<td>10–12</td>
<td>10–12</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Ovulation period (months)</td>
<td>10–12</td>
<td>10–12</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>MOD (mm)</td>
<td>16</td>
<td>16</td>
<td>13±2</td>
<td></td>
</tr>
<tr>
<td>Litter size</td>
<td>1–5</td>
<td>1–6</td>
<td>2–6</td>
<td>2–6</td>
</tr>
<tr>
<td>Mean size at birth (mm)</td>
<td>150–160</td>
<td>150–160</td>
<td>76–109</td>
<td>192**</td>
</tr>
<tr>
<td>Time of parturition</td>
<td>Sep–Dec</td>
<td>Aug–Oct</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6.1. Length-frequency composition of samples for females and males from Lakes Entrance (LE) (a), Port Phillip Bay (PPB) (b), Corner Inlet (CI) (c) and Western Bass Strait (WBS) (d).
Fig. 6.2. Yolk sac proportion, embryo total length and mean embryo mass against day of year.

Yolk sac proportion against embryo total length (a), and day of year against embryo total length (b) and embryo mass (c) from all pregnant females and aborted embryos. Highlighted animals were found in Lakes Entrance only. The logarithmic equation for (b) is $ETL=20.371 \ln(\text{DOY})+20.385$; $r^2=0.4824$ and linear equation for (c) is $ETW=0.0833\text{DOY}+4.405$; $r^2=0.4788$. Yolk mass proportion is yolk sac mass/(embryo mass + yolk sac mass). ETL, embryo total length; ETW, embryo total mass; DOY, day of year.
Fig. 6.3. Ovarian follicle diameter against day of year for females uterus conditions U=1–6.

Largest follicle diameter against day of year for females for each of the six uterus conditions. Mean oocyte diameter (——) with 95% confidence limits (– – – –) and 95% prediction intervals (- - - - -) are presented for pregnant females with in utero embryos (U=5) (a), non-pregnant animals developing females (U=2) (b), non-pregnant virgin females (U=3) (c), pregnant animals with in utero eggs (U=4) (d), and postpartum females (U=6) (e). Values of parameters and statistical quantities for the regression equation $o=a'+b't$ for pregnant females with in utero embryos (U=5) are given in the following tabulation:

<table>
<thead>
<tr>
<th>U</th>
<th>$a'$ (±se)</th>
<th>$b'$ (±se)</th>
<th>n</th>
<th>$r^2$</th>
<th>rmse</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2.242 (0.739)</td>
<td>0.035 (±0.003)</td>
<td>31</td>
<td>0.823</td>
<td>1.684</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

where $t$ is day of year, $o$ is follicle diameter, $a'$ and $b'$ are parameters, $n$ is sample size, $r^2$ is square of regression correlation coefficient, and rmse is root mean square error for the regression, and $P$ is probability of statistical significance.
Fig. 6.4. Hypothesised descriptions of various U=6 females allocations.

Description of the various hypotheses for the U=6 females (a), linear regression of U=6 females in the possible resting period (b), and the comparison between U=6 and U=5 females largest follicle growth when non-pregnant vs pregnant (c).
Fig. 6.5. Various breeding conditions against month for mature males.

Trends in gonadosomatic index (GSI) for mature males determined from testis development (GI) (a), and HSI (b), and seminal vesicle fullness (c). • mean monthly value; bars, standard error for monthly value; number above bar is monthly sample size.
Fig. 6.6. Number of embryos in utero against maternal total length.

Mean number of embryos (---), 95% confidence limits (-----), and raw data (*) are plotted against maternal total length of (U=5) pregnant females. Lakes Entrance (a), Port Phillip Bay (b), Corner Inlet (c), and Western Bass Strait (d). Values for parameters and statistical quantities from linear regression analysis to derive the equation $y=a+bx$ are given in the following tabulation:

<table>
<thead>
<tr>
<th>$a'$ (±s.e.)</th>
<th>$b'$ (±s.e.)</th>
<th>n</th>
<th>$r^2$</th>
<th>rmse</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>−3.121 (±0.599)</td>
<td>0.01475 (±0.00164)</td>
<td>195</td>
<td>0.29</td>
<td>1.096</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

where $y$ is dependent variable, $x$ is independent variable, $a'$ and $b'$ are parameters, $n$ is sample size, $r^2$ is square of correlation coefficient, and rmse is root mean square error for this regression, and P is the probability of statistical significance.
Fig. 6.7. Female length-at-maturity and length-at-maternity ogives. Population status at maturity against TL, (—), with 95% confidence intervals (— — —), for \textit{U. paucimaculatus} females. Females were classed as mature if the largest ovarian follicle diameter >1 mm and maternal if U=4, U=5 or U=6. Values of parameters and statistical quantities for the equation \(P_l=P_{\text{max}}(1+e^{-ln(19)(1-l_{50}/l_{95}-l_{50})})\) determined from probit analysis are given in the following tabulation:

<table>
<thead>
<tr>
<th>Species</th>
<th>Condition</th>
<th>Region</th>
<th>(l_{50}) (mm)</th>
<th>(l_{95}) (mm)</th>
<th>Probit</th>
<th>(z)</th>
<th>ML</th>
<th>(P^{*})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>Maturity</td>
<td>LE+CI</td>
<td>229 (225, 233)</td>
<td>295 (286, 297)</td>
<td>1.80</td>
<td>140</td>
<td>181</td>
<td>-15.828</td>
</tr>
<tr>
<td>(b)</td>
<td>Maturity</td>
<td>PPB</td>
<td>240 (231, 251)</td>
<td>278 (274, 280)</td>
<td>1.80</td>
<td>141</td>
<td>182</td>
<td>-167.242</td>
</tr>
<tr>
<td>(c)</td>
<td>Comparison of maturity between regions</td>
<td>LE+CI</td>
<td>229 (225, 233)</td>
<td>295 (286, 297)</td>
<td>1.80</td>
<td>140</td>
<td>181</td>
<td>-15.828</td>
</tr>
<tr>
<td>(d)</td>
<td>Maturity</td>
<td>LE+CI</td>
<td>256 (250, 263)</td>
<td>314 (312, 318)</td>
<td>1.80</td>
<td>248</td>
<td>381</td>
<td>-3422.778</td>
</tr>
<tr>
<td>(e)</td>
<td>Maturity</td>
<td>PPB</td>
<td>278 (274, 282)</td>
<td>310 (307, 313)</td>
<td>1.80</td>
<td>241</td>
<td>378</td>
<td>-2334.276</td>
</tr>
<tr>
<td>(f)</td>
<td>Maturity</td>
<td>LE+CI</td>
<td>285 (281, 297)</td>
<td>322 (322, 326)</td>
<td>1.80</td>
<td>306.5</td>
<td>381</td>
<td>-1668.669</td>
</tr>
<tr>
<td>(g)</td>
<td>Maturity</td>
<td>LE+CI</td>
<td>279 (276, 282)</td>
<td>312 (312, 316)</td>
<td>1.80</td>
<td>257</td>
<td>381</td>
<td>-1668.276</td>
</tr>
<tr>
<td>(h)</td>
<td>Maturity</td>
<td>LE+CI</td>
<td>276 (272, 277)</td>
<td>318 (316, 320)</td>
<td>1.80</td>
<td>215</td>
<td>381</td>
<td>-1132.087</td>
</tr>
<tr>
<td>(i)</td>
<td>Maturity</td>
<td>PPB</td>
<td>322 (312, 322)</td>
<td>379 (376, 384)</td>
<td>1.80</td>
<td>243</td>
<td>381</td>
<td>-3422.778</td>
</tr>
<tr>
<td>(j)</td>
<td>Maturity</td>
<td>PPB</td>
<td>312 (312, 312)</td>
<td>377 (374, 379)</td>
<td>1.80</td>
<td>257</td>
<td>381</td>
<td>-2334.276</td>
</tr>
<tr>
<td>(k)</td>
<td>Maturity</td>
<td>PPB</td>
<td>287 (280, 288)</td>
<td>334 (332, 336)</td>
<td>1.80</td>
<td>306.5</td>
<td>381</td>
<td>-1668.669</td>
</tr>
<tr>
<td>(l)</td>
<td>Maturity</td>
<td>PPB</td>
<td>288 (287, 289)</td>
<td>325 (323, 327)</td>
<td>1.80</td>
<td>248</td>
<td>381</td>
<td>-1132.087</td>
</tr>
</tbody>
</table>

where \(l\) is total length measured in millimetres, \(P_l\) is proportion of animals at TL \(l\); \(l_{50}\) and \(l_{95}\) are parameters, \(P_{\text{max}}\) is an asymptotic constant, \(n\) is the total number of animals classed as being mature or maternal (adjusted in parentheses), and \(N\) is the total number of animals examined for maturity or maternity. ML is maximum likelihood, and \(P^{*}\) is probability of statistical significance (*P<0.1; **P<0.01; ***P<0.001).
Fig. 6.8: Male length-at-maturity based on seminal vesicle condition, clasper calcification, and testis development.

Proportion of population mature against TL (——) with 95% confidence intervals (- - - - -) for *U. paucimaculatus* males determined from seminal vesicle condition, clasper condition, testis condition and comparison between all three methods. Males were classed as immature for V=1 and mature for V=2 or V=3 seminal vesicle condition, as immature for C=1 or C=2 and mature for C=3 clasper condition, and as immature for G=1 or G=2 and mature for G=3 testis condition. Values of parameters and statistical quantities for the equation \( P = P_{\text{max}} \left( 1 + e^{-\ln(19)(1-l50/l95-l50)} \right)^{-1} \) determined from probit analysis are given in the following tabulation:

<table>
<thead>
<tr>
<th>Region</th>
<th>Method</th>
<th>( l50 ) (CI)</th>
<th>( l95 ) (CI)</th>
<th>( P_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>LE</td>
<td>Seminal vesicle condition</td>
<td>261 (254–266)</td>
<td>292 (288–298)</td>
<td>1.000</td>
</tr>
<tr>
<td>PPB</td>
<td>Seminal vesicle condition</td>
<td>263 (257–270)</td>
<td>301 (294–308)</td>
<td>1.000</td>
</tr>
<tr>
<td>LE</td>
<td>Clasper condition</td>
<td>265 (259–269)</td>
<td>296 (292–301)</td>
<td>1.000</td>
</tr>
<tr>
<td>PPB</td>
<td>Clasper condition</td>
<td>267 (261–271)</td>
<td>300 (293–307)</td>
<td>1.000</td>
</tr>
<tr>
<td>LE</td>
<td>Testis condition</td>
<td>263 (259–269)</td>
<td>314 (307–352)</td>
<td>1.000</td>
</tr>
<tr>
<td>PPB</td>
<td>Testis condition</td>
<td>265 (259–271)</td>
<td>314 (307–352)</td>
<td>1.000</td>
</tr>
</tbody>
</table>

where \( l50 \) is total length measured in millimetres, \( P \) is proportion of animals at TL \( l \), \( l50 \) and \( l95 \) are parameters, \( P_{\text{max}} \) is an asymptotic constant, \( N \) is the total number of animals classed as mature, and \( V \) is the total number of animals selected in statistical procedure, ML is maximum likelihood, and \( P \) is a probability of statistical significance (*P*<0.1; **P**<0.01; ***P***<0.001).
Chapter 7 – *Urolophus viridis* (Greenback stingaree)

Abstract

The female reproductive cycle was asynchronous as ovulation and parturition occurred throughout the year in each of the two separate regions of Lakes Entrance (LE) and Western Bass Strait (WBS) sampled during December 2002–October 2006. The period of embryonic growth was synchronous with the period of the ovarian cycle within a pregnant female but asynchrony among pregnant females. The reproductive cycle for this species is most likely annual. Litter size (1–3) increased with maternal total length (TL). Maximum TL and mass for females (494 mm TL and 1091 g) and males (384 mm TL and 520 g), mean size-at-birth of 160 TL and mean mass 46 g, and female size-at-maturity of 259 mm TL and size-at-maternity of 314 mm TL at $l_{50}$ (length at 50% mature or maternal) in LE were markedly smaller than the maximum TL and mass for females (508 mm TL and 1220 g) and males (428 mm TL and 708 g), mean size-at-birth at 185 TL and 85 g, and female size-at-maturity and size-at-maternity at 314 mm TL and 343 mm TL at $l_{50}$ in WBS. Males in both regions matured at 270–280 mm TL based on three separate criteria.
Results

*Allometric and isometric relationships for total body mass and various size measurements*

A total of 421 animals across both sexes and the two regions were sampled; females (range 188–494 mm TL; 60–1091 g TM; n=146) and males (range 163–384 mm TL; 45–520 g TM; n=80) from LE were smaller than the females (range 195–508 mm TL; 87–1220 g TM; n=66) and males (range 188–428 mm TL; 64–708 g TM; n=129) from WBS. Sexual dimorphism was apparent in both regions with females growing to larger TL and TM than the males.

The effect of region on the ln (TM)–ln (TL) relationship for females (ANCOVA: \( F_{df=1,197} = 21.44 \), P<0.001) and males (ANCOVA: \( F_{df=1,200} = 20.26 \), P<0.001) was statistically significant, so data from the two regions were treated separately. The mean mass of each of females and males at any given TL was lower in LE than WBS. The effect of sex on the ln (TM)–ln (TL) relationship was then tested, but it was not statistically significant for sex (ANCOVA: \( F_{df=1, 205} = 1.36 \), P=0.246) in LE or WBS (ANCOVA: \( F_{df=1, 192} = 2.13 \), P = 0.146). The mean mass of females and males were similar within each region at any TL where TL is expressed in mm and TM in grams.

LE

- Females: TM = 0.0000177 x 1.013 x TL\(^{2.890} \) (P<0.001; \( r^2 = 0.876 \); n = 134),
- Males: TM = 0.0000308 x 1.008 x TL\(^{2.792} \) (P<0.001; \( r^2 = 0.946 \); n = 72)

WBS

- Females: TM = 0.0000247 x 1.002 x TL\(^{2.846} \) (P<0.001; \( r^2 = 0.960 \); n = 64),
- Males: TM = 0.0000575 x 1.004 x TL\(^{2.969} \) (P<0.001; \( r^2 = 0.925 \); n = 129)

A least squares means test indicated that DL–TL (P=0.110) and DW–TL (P=0.113) isometric relationships were not significantly different between males and females. The variables for each morphometric relationship (sexes combined) are given by the equations

\[
DL = 0.984 + 0.559 \text{ TL} \quad (P<0.001; \ r^2 = 0.949; \ n = 169), \text{ and}
\]

\[
DW = 27.392 + 0.572 \text{ TL} \quad (P<0.001; \ r^2 = 0.922; \ n = 169).
\]
Growth of embryos and period of embryonic growth

The timing of parturition and growth of the embryos during gestation were determined from 83 embryos collected (n=53 in LE and n=30 in WBS) of which 77 embryos came from the 42 pregnant females in LE and 20 pregnant females in WBS and 6 were aborted embryos (n=4 in LE, n=2 in WBS) from unknown pregnant females. The effect of region on the relationship between ETL and ETM was statistically significant (ANCOVA: $F_{df=1,80} = 306.81$, $P<0.001$). ETM plotted against ETL was presented separately for each region to show the variation in embryo growth in utero (Fig. 7.1ac). Both regions show curvilinear growth in ETL, where the maximum embryo size at full term was 170 mm ETL and 69 g mass for LE and 205 mm ETL and 124 g mass for WBS. The smallest free-swimming neonates found in each region (163 mm TL and 45 g in LE and 188 mm TL and 64 g in WBS) demonstrate that full-term embryos were collected, and therefore the predicted mean ETL and predicted mean mass of embryos-at-birth were 160 mm and 46 g, respectively, for LE and 185 mm and 85 g, respectively, for WBS. The mean mass of embryos at any given TL was lower in LE than WBS.

Mean ovarian follicle mass for females from each region with LFD >11 mm was 1.8 g (s.e. 0.30 g; range 1.2–2.4 g); differences between the regions were not detected. The mass of the eggs in utero could not be determined, so mean oocyte is used in the present study for further analysis. The presence of U=4 females (n=9) were found during October in LE (n=3) and during April and July in WBS (n=6). During embryonic development, the egg formed the external yolk sac and was absorbed by the time the embryos reached ~120 mm ETL in LE (Fig. 7.1b) and ~140 mm ETL in WBS (Fig. 7.1d). Histotroph was supplied to the embryos during the period of gestation, but the exact starting time relative to external yolk sac absorption was not determined.

Scattergrams of ETL and embryo mass for each region against day of year (Fig. 7.2a-d) provided no information on embryo growth rate. Based on the mean size-at-birth for each region, full-term embryos were found during all months of collection for both LE and WBS. Embryonic development and the parturition period among pregnant females in each region within the population are asynchronous.
Comparing mean follicle mass (1.8 g) with mean full-term embryo mass (46 g in LE and 85 g in WBS) indicates a wet mass gain of ~2550% in LE embryos and ~4700% in WBS. Mean-ETL-at-birth expressed as a percentage of their maximum TL ($l_{\text{max}}$), females and males were 31% and 37%, respectively, for LE, and 37% and 44%, respectively, for WBS.

**Period of ovarian cycle**

LFD for 212 females ranged 1–17 mm across all uterus conditions (U=1–6 females), but ranged 0–1 mm for U=1, 1–14 mm for U=2, 2–15 mm for U=3, 1–12 mm for U=4, 2–16 mm for U=5, and 2–17 mm for U=6 females. Vitellogenesis had commenced in all except one U=3 female and most U=2 females, but not for any U=1 females. Differences were found in the periodicity of LFD development between regions and are therefore presented separately, but size of LFD for the various uterus conditions did not vary between the regions.

Strong positive correlations between LFD and embryo length for U=5 females in LE ($r^2 = 0.829$; df = 36) and in WBS ($r^2 = 0.894$; df = 13) indicate growth of oocytes and embryos are synchronous within an individual pregnant female and ovulation can immediately follow parturition. Plots of LFD against day of year (DOY) for U=5 females showed all size classes of LFD present throughout the year in both regions, which is indicative of a lack of synchrony and seasonality among the pregnant females in the periodicity of follicle development (Fig. 7.3ab).

Several U=4 females provided some information about the timing of ovulation. The period of ovulation and period of eggs *in utero* were also asynchronous. One U=4 female was found during October (DOY 289) with eggs *in utero* in LE (Fig. 7.3e), and two U=4 females were found during April (DOYs 116 and 117) and three in July (DOY 210) in WBS (Fig. 7.3f). Most U=4 females had finished ovulating based on the smallest LFD for U=5 females, but a female with 12 mm LFD in July (DOY 210) was likely to have been in the process of ovulating. The period of eggs *in utero* is likely to be very short (1–2 months) given the lack of U=4 females in the population. Few U=3 females (Fig. 7.3cd) were sampled and U=6 females (Fig. 7.3gh) were
Reproductive cycles of females

From the stepwise sequential questioning (Fig. 2.4), the answer to Q1 (Is embryonic growth synchronous among females in each breeding component of the population?) was ‘no’. This demonstrates that embryonic growth was asynchronous and there is neither a clear ‘period of embryonic growth’ nor ‘period of parturition’ among pregnant females in the population (Fig. 7.2). The answer to Q2 (Are ‘period of ovulation’ and ‘period of eggs in utero’ synchronous among females in each breeding component of the population?) was ‘no’. Hence because only a small number of eggs were found in utero, the ‘period of eggs in utero’ and seasonality is indeterminable (Fig. 7.2.), but it can be concluded that the ‘period of ovulation’ was very short, most likely only 1–2 months among pregnant females. The answer to Q3 (from answers to Q1 and Q2, Can periodicity of pregnancy be determined?) was ‘no’. Just as ‘the period of embryonic growth’, ‘period of parturition’, ‘period of eggs in utero’, and ‘period of ovulation’ were indeterminable because of asynchrony among pregnant females; the duration of the ‘pregnancy cycle’ was indeterminable because of asynchrony. The answer to Q4 (Is ovarian follicle growth (LFD) synchronous with embryo growth?) was ‘yes’. LFD is synchronous with ETL (see subsection Ovarian cycle). The answer to Q5 (Is LFD against day of year trend for U=5 females consistent with U=3, U=4 and U=6 females?) was ‘no’. Because the reproductive cycle was asynchronous, the period of ovarian cycle through the various uterus conditions was also indeterminable (Fig. 7.3.). The answer to Q6 (Is the reproductive cycle consistent with an annual or a shorter period?) could not be made directly from the data collected in the present study. Only one conclusion could be made from the data: the reproductive cycle was non-seasonal and asynchronous among pregnant females, but growth of embryos were synchronous with growth of oocytes in individual pregnant females, which is consistent with ongoing reproductive cycling where ovulation follows soon after parturition.
The only approach to better understand the periodicity of the reproductive cycle from the data collected by the present study is to consider information from other urolophid species (see discussion). Comparisons of \( l_{\text{max}} \), LFD, size-at-birth, size-at-maturity and size-at-maternity, litter size and matrotrophic contribution among species of the genus *Urolophus* provides information on the likely duration of the reproductive cycle of *U. viridis*. Given that most urolophid species of similar maximum TL, LFD have annual reproductive cycles, the most likely cycle for *U. viridis* is annual; although for a species exhibiting non-seasonality; adaptation might have resulted in cycles longer or shorter than one year depending on prevailing conditions. Hence, (Q6) (Is the reproductive cycle consistent with an annual or a shorter period?) was answered with a tentative ‘yes’. *Urolophus viridis* is hypothesised to have a continuous annual asynchronous reproductive cycle. Given the reproductive cycle and the ovarian cycle are annual and synchronous with embryonic growth, it is hypothesised that the ‘period of pregnancy’ is likely to be \(~12\) months with a ‘period of embryonic growth’ of \(~10\) months and a ‘period of eggs in utero’ of 1–2 months and the ‘period of ovarian cycle’ 10–12 months (Fig. 7.4).

*Reproductive cycles of males*

Seasonal trends in the male breeding cycle were determined for mature males from GSI, HSI, and seminal vesicle fullness. The regions were compared graphically and showed no distinct difference in the seasonal patterns or monthly variation. Mean GSI gradually decreased during March–July before increasing during August–January (Fig. 7.5a). Mean HSI peaked during April–October, and decreased during November–February (Fig. 7.5b). Seminal vesicle fullness showed that the males always contain some seminal fluid in the seminal vesicles throughout the entire year (Fig. 7.5c) with peak fullness during April–July, which is consistent with an asynchronous female reproductive cycle.

*Litter size and sex ratio of embryos*

The maximum litter size recorded from 62 \( U=5 \) pregnant females with embryos *in utero* was three in an animal of 430 mm TL from WBS. The effect of region on the linear relationship of litter size against maternal TL was not statistically significant.
(ANCOVA: $F_{df=1,59} = 20.68, P = 0.103$); nevertheless, the regions were presented separately because the smallest recorded $U=5$ female was 303 mm TL in LE (Fig. 7.6a) and 368 mm TL in WBS (Fig. 7.6b). WBS had only one female (430 mm TL) with three embryos, whereas several females (>430 mm TL) from LE had only one embryo in utero. These large animals may have aborted one or more embryos during capture and handling; however, females that may have aborted embryos could not be distinguished from non-aborted females and were included in the analysis. A maximum of 2 eggs found in $U=4$ pregnant females was observed in utero in animals >370 mm TL. The smallest $U=4$ pregnant female in LE was 320 mm TL and the smallest $U=4$ in WBS was 352 mm TL.

A total of 77 embryos from 62 pregnant females were sexed from the presence or absence of claspers of which 31 (40%) were females, 41 (53%) were males, and 5 (7%) were unknown sex due to their early stage of development. The Chi-square tests with Yates’ continuity correction showed no significant difference in sex ratio from 1:1 ($\chi^2 = 1.299$, d.f. = 1, $P = 0.25$).

**Female and male length-at-maturity and length-at-maternity**

The length-at-maturity ogives are presented separately by region for females, but region could not be tested statistically because of the low number of animals, particularly among small animals, in WBS. LE had 4 immature and 141 mature females based on the onset of vitellogenesis as the criterion for maturity; the largest immature female observed was 295 mm TL and the smallest mature female observed was 264 mm TL. Estimates of values with 95% confidence intervals for $l_{50}$ and $l_{95}$ in LE were 259 (255, 263 mm TL) and 316 (313, 319 mm TL), respectively (Fig. 7.7a); $l_{50}$ was reached at ~52% of $l_{\text{max}}$. WBS had 10 immature and 56 mature animals, with the largest immature female observed was 318 mm TL and the smallest mature female observed was 315 mm TL. Due to small sample size in WBS, the logistic regression could generate only the mean $l_{50}$ value for females of 314 mm TL without the 95% confidence intervals (Fig. 7.7b); $l_{50}$ was reached at ~62% of $l_{\text{max}}$. A comparison for size-at-maturity between regions is shown (Fig. 7.7c).
The length-at-maternity ogives were determined from 29 non-maternal and 116 maternal females in LE and 18 non-maternal and 48 maternal females in WBS based on the hypothesized one-year cycle (i.e. $P_{max} = 1.00$) where all U=4 females, U=5 females and U=6 females were assumed to produce offspring contributing to recruitment. The smallest females in maternal condition were 302 mm TL in LE and 332 mm TL in WBS. Estimates of $l_{50}$ and $l_{95}$ for the maternity ogive were 314 (305, 320 mm TL) and 363 (353, 379 mm TL) respectively, in LE, (Fig. 7.7d), and 343 (331, 357 mm TL) and 363 (351, 415 mm TL) respectively, in WBS (Fig. 7.7e). Both size-at-maturity and size-at-maternity were larger in WBS than LE (Fig. 7.7f) and as expected, the parameter estimates for $l_{50}$ and $l_{95}$ were higher for the length-at-maternity ogives than for the length-at-maturity ogives in both LE (Fig. 7.7g) and WBS (Fig. 7.7h). Females at $l_{50}$ in maternal condition were at ~64% and ~68% of $l_{max}$ in LE and WBS, respectively.

Male size-at-maturity ogive was not tested for the effect of region on any of the three applied sets of indices for testis development, seminal vesicle condition, and clasper calcification because of the low number of animals when split between the two regions. Inspection of preliminary estimates of separate ogives by region had wide 95% confidence intervals; hence, the data were pooled across the two regions. $L_{50}$ estimates were 281 (265, 290 mm TL) for testis development (Fig. 7.8a), 270 (252, 280 mm TL) for seminal vesicle condition (Fig. 7.8b), and 276 (260, 285 mm TL) for the clasper calcification (Fig. 7.8c) were similar (Fig. 13d). $L_{50}$ in males (based on mean between each method 276 mm TL) was ~72% of $l_{max}$ in LE and ~64% of $l_{max}$ in WBS.

**Discussion**

*Spatial variation in reproduction of U. viridis within south-eastern Australia*

The present study found spatial differences in the reproductive biology of *U. viridis*. Females from LE are born at a smaller size (TL and body mass), reach maturity and maternity at smaller TL, and have smaller $l_{max}$ (TL and body mass) than their counterparts from WBS. The males are born smaller and reach smaller total lengths in
LE than in WBS, but because of insufficient data, size-at-maturity could not be tested for differences.

Four possible explanations are given for the differences between the two regions. (1) WBS is the further most westerly part of the distribution of *U. viridis* suggesting WBS has lower habitat suitability which might have affected their growth. (2) Commercial fishing is much less in WBS than in LE (Walker and Gason 2009) and the higher fishing mortality on *U. viridis* in LE might have led to the smaller \( l_{\text{max}} \) and smaller apparent size-at-maturity and size-at-maternity. (3) Different environmental conditions are found between these two regions, as they are in two separate biogeographical provinces. During the recent ice ages, eastern Victoria was periodically separated from western Victoria by a land bridge through Bass Strait creating separate environmental conditions between the regions. Eastern Victoria is mostly influenced by the warm East Australian Current, whereas western Victoria is mostly influenced by cooler deep water of the southern ocean from summer upwelling and warmer water from the Leeuwin Current at other times of the year and have been a source of speculation for regional differences for several chondrichthyan species found within Victoria waters (Tovar-Avila *et al.* 2007; Walker 2007). (4) Despite relatively large sample sizes, the size ranges for both males and females were insufficient in WBS to determine robust length-at-maturity and length-at-maternity ogives.

Whether regional differences are real or apparent requires further investigation. Most important for further study, is whether these populations, particularly LE are comparable to the populations within New South Wales where they are most abundant and have had constant and intense fishing pressure during the past 30 years. There is also a ‘closely-related species’ (IUCN 2013) to *U. viridis* occurring off north-western Tasmania, raising the question of whether all specimens collected during the present study were *U. viridis*. During sampling, there was no indication that WBS animals were different from the LE animals in morphology or reproduction, but genetic studies such as those of *Mustelus* species comparing differences between the Indo-Pacific and Australasia (Boomer *et al.* 2012) maybe required to answer this question.

*Spatial differences in reproduction of urolophid species between south-eastern Australia and south-western Australian*
Two types of reproductive strategy are evident when comparing reproductive traits of urolophid species of similar \( \text{l}_{\text{max}} \) between south-eastern (SE) Australia (Dagley et al. 2013 *in prep*; Trinnie et al. 2009; Trinnie et al. 2012; Trinnie et al. 2013 submitted-a; Trinnie et al. 2013 submitted-b) and south-western (SW) Australia (White et al. 2002; White et al. 2001; White and Potter 2005). Urolophid species in SE Australian produce much larger litter sizes and smaller embryo sizes-at-birth than in SW Australia. They also reach maturity and maternity at smaller sizes in SE Australia than in SW Australia. Small size-at-birth together with large litter size presumably increases reproductive output more in SE Australia than in SW Australia.

In SE Australia, the maximum litter size varies widely across urolophid species (1–13), but is commonly 4–6, whereas in SW Australia litter sizes of 1–2 only are found. The size-at-birth relative to \( \text{l}_{\text{max}} \) is smaller in SE Australian urolophid species for females (range 25–32%) and males (30–39%), than in SW Australian urolophid species for females (35–49%) and males (42–52%). Female size-at-maturity (44–62%) and size-at-maternity (56–68%) in SE Australian urolophid species are smaller than female size-at-maturity (the equivalent to size-at-maternity in the present study) in SW Australian urolophid species (69–81%). Male urolophid species mature at smaller sizes in SE Australia (61–72%) than in SW Australia (68–82%) (Table 7.1).

*Urolophus viridis* with its small litter size (1–3), falls between the large size-at-birth–small litter-size strategy of SW Australia and the small size-at-birth–large litter-size strategy of SE Australia. In LE, the small mean size-at-birth, small size-at-maturity and small size-at-maternity are consistent with the SE Australian strategy, whereas, in WBS, the large mean size-at-birth; large size-at-maturity and size-at-maternity are more consistent with the SW Australia strategy. Animals in LE are likely to have lower neonate survival because of smaller size, but females have higher reproductive output. Conversely, animals in WBS have higher neonate survival because of larger size, but lower female reproductive output. This makes LE neonates more susceptible to the effects of competition and predation than WBS neonates. Whether females from LE have more reproductive cycles within their life time than those from WBS is unknown; it might be that animals in WBS grow faster than those in LE, but this cannot be resolved without age and growth data.
This pattern of two alternative reproductive strategies is not so distinct for every urolophid species and may change depending on $l_{\text{max}}$. For example, *T. imitata* (Trinnie *et al.* 2009) and *U. bucculentus* (Trinnie *et al.* 2012) in SE Australia produce large litters and have small size-at-birth, but reach maturity and maternity at larger sizes when expressed as a percentage of $l_{\text{max}}$, making them more comparable to SW Australia urolophid species. Having much larger $l_{\text{max}}$ may allow these species to vary between these strategies because they might be less affected by predation and competition, even at the neonate size.

*Asynchronous reproductive cycle*

Female *U. viridis* appear to have an asynchronous non-seasonal reproductive cycle, which is consistent with an asynchronous breeding cycle in the males. However, because of the lack of synchrony and seasonality, small sample size of pregnant females and sporadic nature of sampling, any difference in the periodicity of the reproductive cycle between the regions off Victoria could not distinguished. Hence, to determine the periodicity of the reproductive cycle, individual *U. viridis* in the populations of the two regions are assumed to have the same period.

Comparisons then had to be made to other, closely related urolophid species so as to hypothesis the reproductive cycle. When separating the genera *Urolophus* and *Trygonoptera* within the family Urolophidae, trends become apparent in the reproductive parameters of $l_{\text{max}}$, litter size, ‘period of eggs in utero’ and ‘period of embryonic growth’, LFD vs ETL, maximum LFD, ETM versus ETL, and the amount of matrotrophic contribution within each genus.

$L_{\text{max}}$ recorded for *U. viridis* (present study) exceeds the previous recorded $l_{\text{max}}$ (Last and Stevens 1994), such that *U. viridis* reaches an $l_{\text{max}}$ comparable to those of the sympatric urolophid species *U. paucimaculatus* and *U. cruciatus* (~500 mm TL) (Last and Stevens 1994). This separates the urolophids into two $l_{\text{max}}$ size classes within SE Australia as *U. gigas*, *U. bucculentus* and *T. imitata* all reach an $l_{\text{max}}$ of 800–890 mm TL. In SW Australia, all four urolophid species of *U. paucimaculatus*, *T. mucosa*, *T. personata* and *U. lobatus* (based on DW to TL conversion) reach an $l_{\text{max}}$ similar to
that of *U. viridis*. However, when comparisons are made within the family Urolophidae, $l_{max}$ is seemingly unrelated to periodicity of the reproductive cycle.

*Urolophus viridis* is the only known urolophid species with asynchronous embryonic growth within the family Urolophidae; all other species studied so far are highly synchronous. Periods of embryonic gestation can be generalized by genus with short periods of embryonic growth of 4–8 months within *Trygonoptera* and longer periods of 10–19 months for *Urolophus* (Table 7.1); *U. cruciatus* is an anomaly and is discussed below. The present study can only hypothesise the period of embryonic growth for *U. viridis*, but it is likely follows the same pattern of most other *Urolophus* species with relatively long embryonic growth periods (except *U. cruciatus*).

The ‘period of eggs in utero’ within the genus *Urolophus* is short for most *Urolophus* species (1–3 months) (*U. cruciatus* again excluded), whereas all *Trygonoptera* species so far investigated exhibit extended ‘periods of eggs in utero’ of about 4–7 months commonly referred to as embryonic diapause (Waltrick *et al.* 2012) or delayed development (Marshall *et al.* 2007) in which the eggs in utero are encased in brown transient capsules. The small number of U=4 females found for *U. viridis* in the present study indicates that the ‘period of eggs in utero’ (transition stage from egg to embryo) is short as is common for most species of *Urolophus* (*U. cruciatus* again excluded). *Urolophus cruciatus* is an exception with an extended period of eggs in utero of 12–18 months, and is unique among all urolophid species as the majority of mature females were in the U=4 condition throughout the entire year and unlike any other *Urolophus* species, the period of embryonic growth is only 4–6 months. The trade off for *U. cruciatus* to shorten its period of embryonic growth is to extend the period of eggs in utero. With the few U=4 females found in the present study, there is no indication that this occurs in *U. viridis*.

All studied urolophid species, irrespective of genus show synchrony between growth in embryo and growth of oocytes regardless of the periodicity of the reproductive cycle in the U=5 females. The only difference between *Trygonoptera* and *Urolophus* species is the size of LFD; the *Trygonoptera* species grow their follicles to much larger sizes (up to 45 mm LFD) than the *Urolophus* species (10–24 mm LFD). The size of LFD in *U. viridis* has a maximum of 16 mm and consistent with the genus.
**Urolophus.** Within an individual *U. viridis* female, the reproductive cycle is continuous and the cycle of parturition can be followed by immediate ovulation and fertilization.

In general, the ‘period of pregnancy’ for most species of *Urolophus* consists of a short period of eggs *in utero*, long ‘period of embryonic growth’, concurrent growth of embryos and oocytes, and ovulation occurring shortly after parturition. The ‘period of pregnancy’ for species of *Trygonoptera*, on the other hand, consists of an extended ‘period of eggs *in utero*’, short ‘period of embryonic growth’, concurrent growth of embryos and oocytes, and ovulation occurs shortly after parturition, except for a period of several months of no growth of oocytes. In *U. viridis*, this could not be determined precisely because of the asynchrony in the population, but in terms of $L_{\text{max}}$, LFD, ‘period of eggs *in utero*’ and ‘period of embryonic growth’; they are likely to be similar to other *Urolophus* species. The period of pregnancy in *Urolophus* species is commonly 10–12 months, with the only two exceptions of *Urolophus bucculentus* and *U. cruciatus* which have biennial reproductive cycles (Table 7.1).

Trinnie *et al* (2012) suggests that the amount of matrotrophic contribution could affect the period of embryonic growth in urolophid species. *Urolophus viridis* has comparable matrotrophic contributions to *U. paucimaculatus* and *U. cruciatus*, which both have periods of embryonic growth less than 12 months, whereas *U. bucculentus* has a much higher matrotrophic contribution and a period of embryonic growth longer than 12 months. For *Trygonoptera* species the amount of matrotrophic contribution is far less than any *Urolophus* species and is a direct result of the larger LFD and has allowed for the short periods of embryonic growth. Despite the regional differences, the matrotrophic contribution in *U. viridis* is thought to be consistent with other *Urolophus* species and allow for embryonic growth of less than 12 months.

To conclude that *U. viridis* has a biannual reproductive cycle, the most obvious observation would be to see two synchronous <6 month periods of embryonic growth occurring within the year, as seen in the biannual reproduction of *Urobatis jamaicensis* (Fahy *et al.* 2007). This is not observed in *U. viridis* and compared with other Australian urolophid species, such an exceptionally fast rate of reproduction is yet to be found in the family Urolophidae. If *U. viridis* was to have a biennial or
longer reproductive cycle, two distinct size classes of oocytes in the ovaries at any
time would have been observed, as seen for the biennial reproductive cycle of
*Urolophus bucculentus* (Trinnie *et al*. 2012). Embryonic growth in *U. viridis* would
have to be exceptionally slow and the females would be putting very little energy into
reproduction since they have only small litter sizes hence, the most parsimonious
hypothesis is an annual reproductive cycle for *U. viridis*.

Together, the maximum litter size of three and hypothesised annual reproductive
cycle suggest that the reproductive biology of *U. viridis* provides for low annual
population recruitment, which potentially makes the species vulnerable to impacts
from the effects of commercial fishing as demonstrated in NSW by up to 80% decline
in numbers (Graham *et al*. 2001). Further collection of females with eggs and
embryos *in utero* and immature and mature males and females are needed to clarify
the asynchrony within the reproductive cycle, the duration of the period of gestation,
and the extent of the differences in size-at-maturity and size-at-maternity. The present
study also indicates the importance for studies of chondrichthyan species to collect
samples from separate regions and if attempting to test for regional effects, the
necessity of adequate sample size.
Table 7.1. Comparison of reproductive parameters between south-eastern and south-western Australian urolophid species

<table>
<thead>
<tr>
<th>Urolophid species</th>
<th>Region</th>
<th>Reproductive periodicity</th>
<th>Period of embryonic growth</th>
<th>Matrotrophic contribution</th>
<th>Size of LFD at vitellogenesis</th>
<th>Size relative to l&lt;sub&gt;max&lt;/sub&gt;</th>
<th>Maximum Litter size</th>
<th>Size at birth relative to l&lt;sub&gt;max&lt;/sub&gt;</th>
<th>Timing of parturition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urolophus viridis</td>
<td>SE (WBS)</td>
<td>Annual</td>
<td>~10</td>
<td>~4700</td>
<td>&gt;1</td>
<td>62</td>
<td>34</td>
<td>44</td>
<td>All year</td>
<td>Present study</td>
</tr>
<tr>
<td>U. cruciatus</td>
<td>SE</td>
<td>Biennial</td>
<td>6</td>
<td>4000–4375</td>
<td>&gt;1</td>
<td>44</td>
<td>31</td>
<td>42</td>
<td>Sep–Dec</td>
<td>Trinnie et al 2013 submitted-b</td>
</tr>
<tr>
<td>U. bucculentus</td>
<td>SE</td>
<td>Biennial</td>
<td>15–19</td>
<td>6250–7200</td>
<td>&gt;3</td>
<td>57</td>
<td>29</td>
<td>37</td>
<td>Apr–May</td>
<td>Trinnie et al 2013 submitted-a</td>
</tr>
<tr>
<td>T. mucosa</td>
<td>SW</td>
<td>Annual</td>
<td>~8</td>
<td>i.d.</td>
<td>i.d.</td>
<td>57</td>
<td>33</td>
<td>34</td>
<td>Jun–Jul</td>
<td>Dagley et al 2013</td>
</tr>
<tr>
<td>T. personata</td>
<td>SW</td>
<td>Annual</td>
<td>5</td>
<td>i.d.</td>
<td>i.d.</td>
<td>57</td>
<td>33</td>
<td>34</td>
<td>Apr–May</td>
<td>White, Hall et al 2002</td>
</tr>
</tbody>
</table>

F, Females; M, Males; LFD, largest follicle diameter; PPB, Port Phillip Bay; LE, Lakes Entrance; WBS, Western Bass Strait; l<sub>max</sub>, maximum total length

- White et al defines this as maturity but is equivalent to maternity in the present study; i.d. information deficient

Chapter 7 – Greenback stingray
Fig. 7.1. Mass of embryos and yolk sac as a proportion against embryo length

Plots of embryo mass against embryo length for Lakes Entrance (LE) (a) and Western Bas Strait (WBS) (c) and yolk mass proportion against embryo length for LE (b) and WBS (d) determined for the litter of each of the pregnant animals with macroscopically visible embryos. Yolk mass proportion is yolk sac mass/(embryo mass + yolk sac mass). Values for parameters and statistical quantities from linear regression analysis to derive the equation \( w = acl^b \) are given in the following tabulation:

<table>
<thead>
<tr>
<th>Region</th>
<th>( a ) (s.e. range) ( \times 10^{-7} )</th>
<th>( b(se) )</th>
<th>c</th>
<th>n</th>
<th>( r^2 )</th>
<th>rmse</th>
</tr>
</thead>
<tbody>
<tr>
<td>LE</td>
<td>1.06 (0.66–1.69)</td>
<td>3.445 (0.099)</td>
<td>1.003</td>
<td>52</td>
<td>0.960</td>
<td>0.176</td>
</tr>
<tr>
<td>WBS</td>
<td>3.11 (2.19–4.41)</td>
<td>3.277 (0.070)</td>
<td>1.001</td>
<td>29</td>
<td>0.988</td>
<td>0.100</td>
</tr>
</tbody>
</table>

where \( w \) is total body mass, \( l \) is total length, \( a \) and \( b \) are parameters, \( c \) is the Beauchamp and Olson (1973) correction factor, \( n \) is sample size, \( r^2 \) is square of correlation coefficient, and rmse is root mean square error for this regression.
Fig. 7.2. Embryo length and embryo mass against day of year.

The embryo length (a) and embryo mass (b) of all pregnant animals and aborted embryos with macroscopically visible embryos from Lakes Entrance and embryo length (c) and embryo mass (d) from Western Bass Strait against day of year. O, eggs in utero
Chapter 7 – Greenback stingaree

Fig. 7.3. Ovarian follicle diameter against day of year for uterus conditions $U=3–6$.

Follicle diameter against day of year for each of the females in uterus conditions $U=3–6$. Pregnant females with in utero embryos ($U=5$) (a) LE and (b) WBS, non-pregnant animals ($U=3$) (c) LE and (d) WBS, pregnant animals with in utero eggs ($U=4$) (e) LE and (f) WBS, highlighted is a female in the process of ovulating, and postpartum females ($U=6$) (g) LE and (h) WBS. WBS, Western Bass Strait; LE, Lakes Entrance
Fig. 7.4. Hypothesised periodicity of gestation and the ovarian cycle.

The gestation period of eggs in utero for 1–2 months and 10–12 months of embryos in utero (a) and the ovarian cycle over 2 years (b) for WBS and LE.
(o) WBS (●) LE. WBS, Western Bass Strait; LE, Lakes Entrance
Fig. 7.5. Various breeding conditions against month for mature males.

Trends in gonadosomatic index (GSI) for mature males determined from testis development (GI) (a), and HSI (b), and seminal vesicle fullness (c). • mean monthly value; bars, standard error for monthly value; number above bar is monthly sample size.
Fig. 7.6. Number of embryos \textit{in utero} against maternal total length.

Mean number of embryos (——), 95% confidence limits (— —), 95% prediction limits (— — —), and raw data (•) are plotted against maternal total length of pregnant females with macroscopically visible embryos (U=5) from Lakes Entrance (a) and Western Bass Strait (b). Values for parameters and statistical quantities from linear regression analysis to derive the equation $y=a+bx$ are given in the following tabulation:

<table>
<thead>
<tr>
<th>Region</th>
<th>$a'$ (±s.e.)</th>
<th>$b'$ (±s.e.)</th>
<th>n</th>
<th>$r^2$</th>
<th>rmse</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lakes Entrance</td>
<td>$-0.552$ (±0.468)</td>
<td>$0.005$ (±0.001)</td>
<td>43</td>
<td>0.265</td>
<td>0.3850</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Western Bass Strait</td>
<td>$-1.335$ (±1.295)</td>
<td>$0.007$ (±0.003)</td>
<td>19</td>
<td>0.212</td>
<td>0.4750</td>
<td>$&lt;0.001$</td>
</tr>
</tbody>
</table>

where $y$ is dependant variable, $x$ is independant variable, $a$ and $b$ are parameters, $n$ is sample size, $r^2$ is square of correlation coefficient, and rmse is root mean square error for this regression, and $P$ is the probability of statistical significance.
Fig. 7.7. Female length-at-maturity and length-at-maternity ogives in each of two regions.

Population mature and maternal against TL (——), with 95% confidence intervals (———), for females from regions LE and WBS. Maturity ogive (a), maternity ogive (b), and the comparison between maturity and maternity for LE females (c). Maturity ogive (d), maternity ogive (e), and the comparison between maturity and maternity for WBS females (f). Comparison between maturity ogives of LE and WBS (g) and comparison between maternity ogives of LE and WBS (h). Animals were classed as mature if the largest ovarian follicle diameter >1 mm.

Values of parameters and statistical quantities for the equation $P_l = P_{\text{max}} (1 + e^{-\ln(19)(1-l/l_{95}-l/l_{50})})$ determined from probit analysis are given in the following tabulation:

<table>
<thead>
<tr>
<th>Region</th>
<th>$l_{50}$ (CI)</th>
<th>$l_{95}$ (CI)</th>
<th>$P_{\text{max}}$</th>
<th>n</th>
<th>N</th>
<th>ML</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maturity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LE</td>
<td>259 (255, 263)</td>
<td>316 (313, 319)</td>
<td>1.00</td>
<td>141</td>
<td>146</td>
<td>-18.7829</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WBS</td>
<td>314</td>
<td>326</td>
<td>1.00</td>
<td>56</td>
<td>66</td>
<td>-4.0701</td>
<td>0.231</td>
</tr>
<tr>
<td>Maternity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LE</td>
<td>313 (255, 263)</td>
<td>316 (313, 319)</td>
<td>1.00</td>
<td>116</td>
<td>145</td>
<td>-76.6428</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WBS</td>
<td>342 (331, 357)</td>
<td>363 (351, 415)</td>
<td>1.00</td>
<td>48</td>
<td>66</td>
<td>-7.1544</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

where $l$ is total length measured in millimetres, $P_l$ is proportion of animals at TL, $l_{50}$ and $l_{95}$ are parameters, $P_{\text{max}}$ is an asymptotic constant, $n$ is the total number of animals classed as being mature or maternal, and $N$ is the total number of animals examined for maturity or maternity, ML is maximum likelihood, and $P$ is probability of statistical significance. WBS, Western Bass Strait; LE, Lakes Entrance. A female at 377 mm TL from LE was removed from the maturity analysis because it was thought to have been misidentified as immature.
Maturity of males based on three separate conditions in the two regions pooled.

Proportion of population mature against TL (—) with 95% confidence intervals (-----) for males determined from testis condition (a), seminal vesicle condition (b), and clasper condition (c) and comparison between each condition; (-----) GI, (-----) VI, and (-----) CI (d). Males were classed as immature for G=1 or G=2 and mature for G=3 testis condition, as immature for V=1 and mature for V=2 or V=3 seminal vesicle condition, and immature for C=1 or C=2 and mature for C=3 clasper condition. Values of parameters and statistical quantities for the equation \( P = P_{\text{max}} \left(1 + e^{-\ln(19)(1-l50/l95-l50)}\right)^{-1} \) determined from probit analysis are given in the following tabulation:

<table>
<thead>
<tr>
<th>Method</th>
<th>( l_{50} ) (CI)</th>
<th>( l_{95} ) (CI)</th>
<th>( P_{\text{max}} )</th>
<th>n</th>
<th>N</th>
<th>ML</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis condition</td>
<td>281 (265–290)</td>
<td>328 (321–338)</td>
<td>1.000</td>
<td>179</td>
<td>199</td>
<td>-80.731</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Seminal vesicle condition</td>
<td>270 (252–280)</td>
<td>302 (293–314)</td>
<td>1.000</td>
<td>189</td>
<td>199</td>
<td>-22.749</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Clasper condition</td>
<td>276 (260–285)</td>
<td>305 (297–316)</td>
<td>1.000</td>
<td>191</td>
<td>203</td>
<td>-24.548</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

where \( l \) is total length measured in millimetres, \( P \) is proportion of animals at TL \( l \), \( l_{50} \) and \( l_{95} \) are parameters, \( P_{\text{max}} \) is an asymptotic constant, \( n \) is the total number of animals classed as mature, and \( N \) is the total number of animals selected in statistical procedure, ML is maximum likelihood, and \( P \) is probability of statistical significance.
Chapter 8 – General Discussion

The family Urolophidae have numerous sympatric species occupying most niches across southern Australia’s continental shelf. Understanding the extent of this diversification is essential to understanding each species role in the ecosystem and their susceptibility to any potential anthropogenic or environmental effects. The present study demonstrates that urolophid species studied from south-eastern Australia in fact, do not have similar reproductive strategies (Last and Stevens 1994) but have highly diverse strategies with parturition frequencies ranging from annual to biennial, maximum litter size varying from as low as 1–2 to as high as 13 (Dagley et al. 2012 in prep), different sizes-at-birth, different size-at-onset of maturity and length-at-maternity, and significant differences in the seasonal timing of parturition. Combined with prey and resource partitioning (Parry et al. 1995; Walker et al. 2008), this has allowed for this diversity of species to occur in the south-eastern Australian ecosystem.

The degree of species diversification among Australian Urolophidae species becomes most apparent in the spatial separation between groups of urolophids from south-eastern Australia and from south-western Australia: the two regions being completely separated by a large distance. Most evident is the reproductive adaptation demonstrated within *U. paucimaculatus*, which is distributed across the entire continental shelf of southern Australia, with spatial differences in all reproductive parameters between the two regions. It is also evident that the south-western Australia population of *U. paucimaculatus*, and possibly other south-western Australian urolophid species (White and Potter 2005), once had the ability to produce larger litters: the existence of up to 6 eggs in utero occur but, they can produce a maximum of only two full-term embryos: which suggests they have adapted, or are currently adapting, to producing smaller litters and larger sizes-at-birth. We could be observing the loss of an ancestral trait once shared between the two spatially separated urolophid communities. When comparing urolophid species of similar maximum TL, urolophids from south-eastern Australia generally have larger litter sizes (with the exception of
U. viridis), give birth to smaller young, and become mature and maternal at smaller sizes than urolophids from south-western Australia.

Spatial differences and diversification in the reproductive biology of several species found by the present study produces insights into evolutionary trends and ancestral traits of urolophids at the genus level. Australian Urolophidae species either produce large follicles, which then require only moderate matrotrophy for embryonic growth (~1000%) (referred to as high matrotrophy for matrotrophic sharks species), or produce small follicles, which then require extensive matrotrophy for embryonic growth (~2500–7000%). All Trygonoptera species studied—T. imitata (present study), T. mucosa and T. personata (White et al. 2002)—produce relatively large follicles and therefore moderate matrotrophy with eggs in utero retained for extended periods encased in transient brown egg envelops before losing the egg-casing when embryonic growth begins, whereas the Urolophus species (present study) and Urolophus lobatus (White et al. 2001) produce only small follicles and therefore have extensive matrotrophy with minimal egg encapsulation.

The strategy of extensive matrotrophy has shaped the genus Urolophus into one of the most matrotrophic genera within the order Batoidae, and for the exception of several oophagous shark species, among all sharks and rays (Musick and Ellis 2005). However, maternal investment in chondrichthyan species and its effect on the periodicity of the reproductive cycle has received little attention. Within urolophid species there is compelling evidence that matrotrophic contribution and their ‘period of embryonic growth’ are related: the higher the matrotrophy, the longer the ‘period of embryonic growth’. For example, Trygonoptera imitata has a matrotrophic contribution from egg to full-term embryo of ~1000%, and the embryos take only 5–7 months for development to full-term, Urolophus paucimaculatus and U. viridis have a maximum of ~2500–4000% increase from egg to full-term embryo, take 10–12 months for development, and all three species complete their reproductive cycles within an annual period. However, Urolophus bucculentus has a maximum of ~7000% increase from egg to full-term embryo, takes 14–19 months for development to full-term and can only reproduce biennially. If this pattern is correct, the higher the matrotrophic contribution the longer the ‘period of embryonic growth’. This rule, however, does not apply for all species; U. cruciatus is an exception, and has
shortened its ‘period of embryonic growth’ to only 4–6 months, but maintains extensive matrotrophic contribution (~4000%).

Through understanding the demands of matrotrophy on urolophid reproduction, the present study demonstrates the effect of *U. cruciatus* adapting to have the shortest ‘period of embryonic growth’ within the genus *Urolophus*, while retaining extensive matrotrophy: but losing the ability to reproduce annually. The species has extended the ‘period of eggs in utero’ by up to 18 months, which restricts them to reproducing biennially. However, in what state have we found them in their evolutionary development? Did their ancestor reproduce annually or biennially? These and many questions remain unanswered. *Urolophus cruciatus* is also not the only south-eastern Australian urolophid species to show this reproductive strategy, *U. gigas* has also been found to have and extended period of eggs in utero (Dagley et al. 2012 in prep), suggesting that a possible correlation between these two species and within the genus *Urolophus*.

Why extensive matrotrophy is so demanding in these urolophid species, by creating longer periods from egg to full-term embryo, is unknown. Environmental cues such as lower waters temperature might slow the embryonic growth and raises the questions of whether urolophid species from tropical waters off Northern Australia have shorter embryonic growth and reproductive cycles; or whether it is the nutritional value of the histotroph produced by each species or genus that is the limiting factor. Urotrygonidae species from America have seemingly shorter reproductive cycles than the Australian Urolophidae species. But is it due to their different diets, degrees of matrotrophy, environmental cues, organic histotroph, or other family traits?

On an evolutionary scale, the various alternative reproductive cycles in chondrichthyan species and the evolution of viviparity remains unclear, although DNA sequencing is consistent with yolk-sac viviparity as the ancestral trait in batoids, along with lipid histotroph viviparity and matrotrophy within the Myliobatidei (Aschliman et al. 2012). Results from the present study are consistent with this theory as all urolophid species were found to be highly or extensively matrotrophic. However, the present study also found that urolophid species have different
reproductive strategies at the genus level, which may help further our understanding of batoid reproductive evolution.

If yolk-sac viviparity is the ancestral reproductive trait in batoid species, then the genus *Trygonoptera* seemingly retains some variant of this primitive ancestral reproductive strategy whereby they produce large follicles and encase the egg within an egg envelop *in utero* although this is discarded prior to embryo development. *Urolophus* is further from being yolk-sac viviparity as it produces small oocytes and minimal or no egg encapsulation. The extensive matrotrophy in *Urolophus* species provides more matrotrophic contribution than in *Trygonoptera* species.

Among the Myliobatidei and to some extent all batoids, there are many variations of reproductive strategies among extant species today. Similar to the reproductive strategies found in *Trygonoptera* species can also be found in the common eagle ray *Myliobatis Aquila*, which also produces large ovulatory follicles of similar size to *T. imitata* (present study), encases the egg in a diaphanous capsule, but supplies lipid histotroph and matrotrophy during pregnancy (Capape *et al.* 2007). If this strategy is the more primitive, then they might be seen as being similar to the rhinobatoid species and might help explain the common ancestral trait; e.g. the fiddler ray *Trygonorrhina fasciata* (Marshall *et al.* 2007) which also produces large ovulatory follicles of size similar to that of *T. imitata* and encases the egg within an egg envelop *in utero*. However, the fiddler ray retains the embryo within this envelop during the entire ‘period of embryonic growth’. *Urolophus* species are more consistent with the dasyatid species; e.g. *Dasyatis sabina* (Johnson and Snelson Jr 1996) and the urotrygonid species *Urobatis halleri* (Babel 1967). *Urolophus* species produce relatively small follicles, produce extensive matrotrophy, and have little or no egg encapsulation, and all have similar development of the trophonemata.

The findings of the present study, particularly for the genus *Trygonoptera*, show that batoid reproductive biology is not fully understood and genetic study including further DNA sequencing are required. The oviducal gland within *Urobatis halleri* was found to have lost the ability to produce an egg envelop (Hamlett *et al.* 2005b), but within *Trygonoptera*, the oviducal gland should have retained traits of egg encapsulation and needs to be resolved. Lipid histotroph is highly variable in
Myliobatidei with seemingly several strategies occurring. Reproductive studies determining the reproductive cycle and reproductive variables such as LFD (mm and g), mass of egg *in utero*, full-term embryo mass, matrotrophic contribution and development of trophonemata are needed so that these strategies and their familial relationships can be better understood. Further studies of the lipid and organic material *in utero* will also contribute to resolving some of this uncertainty (Hamlett *et al.* 2005b).

To conclude, the reproductive parameters of five urolophid species from south-eastern Australia have been determined and are available for fisheries stock assessment, ecological risk and species extinction risk assessments. Two chapters of this thesis have been published in international reviewed journals and are available to a wide audience and the other three chapters are currently submitted for peer review. Thus the aim of the present thesis was achieved successfully. The results have already been used for an ecological risk assessment, with most urolophid species classified as medium to medium-high risk to the effects of fishing. This indicates that as bycatch, byproduct, or targeted species, based on their large distributions and ‘medium biological productivity’, urolophids have the potential to be managed sustainably (Walker and Gason 2007). This is, however, of little consequence to pre-existing fishing practices, with historic fishing levels causing several species to decline dramatically over a 20-year period (Graham *et al.* 2001; Hobday *et al.* 1999). Species extinction risk assessments for *U. viridis* and *U. bucculentus* produce listings of ‘Vulnerable’ and *Trygonoptera imitata* a listing of ‘Near Threatened’ under the IUCN red list evaluation checklist (IUCN 2008). The reproductive biology of all presently studied urolophid species show high vulnerability to the effects of fishing because of their relatively low litter sizes, long reproductive cycles, and late attainments of maturity and maternity. Despite relatively large distributions, there is potential for localised depletions for all species, and species such as *Urolophus cruciatus* and *U. paucimaculatus* may need further investigations by the IUCN because of declining catch trends in selected regions. There is evidence enough to suggest that threatened or extinction risk assessments may need to be regionalised for urolophid species, not singularly across their distributions. On an evolutionary scale, the genera within the family Urolophidae demonstrate two separate reproductive strategies, and these strategies affect the reproductive output for each species. Niche partitioning and
species diversification has also led urolophid species from south-eastern Australia to have large litters and small size-at-birth compared to south-western Australia with small litters and large size-at-birth. A comprehensive Australian sharks and rays guide (Last and Stevens 2009) has also been updated to include species specific biological information for each of the urolophid species investigated by the present study, and to remove the generalisation of reproduction across the family Urolophidae of 3-month gestations and 2–4 litter sizes and now provides individual species details on reproductive cycles.
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