J. exp. Biol. **156**, 387–398 (1991)
Printed in Great Britain © *The Company of Biologists Limited 1991*

ONTOGENY OF HEART FUNCTION IN THE LITTLE SKATE *RAJA ERINACEA*

BY BERND PELSTER* AND WILLIAM E. BEMIS
Department of Zoology, University of Massachusetts, Amherst, MA 01003, USA

Accepted 3 October 1990

Summary

The development of the cardiovascular system has been analysed in embryos of the little skate, *Raja erinacea*, ranging in age from 27 to 144 days after spawning. Circulation starts at the end of the first month. At that time, the heart is S-shaped, there is no differentiation between ventricle and conus arteriosus and no valve formation is detectable. Complete differentiation of the central circulatory system and its valves was observed at about 40 days after spawning, although there are changes in proportion that occur before hatching.

In the smallest embryos used for physiological studies, 27 days post-spawning (0.01–0.03 g body mass), circulation of blood was observed and heart rate was 35–40 beats min^{-1}. Heart rate increased with development, reaching a maximum of 65–68 beats min^{-1} at a body mass of 0.2 g (60 days post-spawning) and then slowly decreased until just prior to hatching.

Ventricular diastolic pressure remained below 0.1 kPa throughout development whereas ventricular systolic pressure increased significantly with increasing body mass. In small embryos (<0.05 g) the conus arteriosus collapsed at the end of each heart beat, completely occluding its lumen and separating the ventral aorta from the diastolic heart. In larger animals (>0.1 g) serial flap valves in the conus separated the ventral aorta from the diastolic ventricle, and the conus supported ventral arterial blood pressure because of its elastic properties.

Introduction

With increasing body mass, developing vertebrate embryos soon reach the state where a circulatory system is necessary to supply the growing tissue with nutrients and to remove metabolic waste products. Accordingly, cardiac development usually precedes that of the other organs.

The morphology of heart development in fishes has attracted attention for a long time (Weber, 1908; Scammon, 1911; Ballard, 1973). Most observations on the development of heart function in teleosts have been made in the course of describing developmental series (cf. Paine and Balon, 1984; Cunningham and

* Present address: Institut für Physiologie, Ruhr-Universität Bochum, Universitätsstrasse 150, D-4630 Bochum, Germany.

Key words: circulation, blood pressure, ontogeny, embryo, elasmobranchs, *Raja erinacea*.

Balon, 1986). Such studies report a rapid increase in heart rate within the first few days after the first heart beat was noticed. Further studies on the physiology of fish embryos, however, have usually been restricted to gas exchange and metabolism (for a review, see Rombough, 1988).

Because the embryos are large and easily studied, the morphological and physiological development of the chicken heart has been closely followed (for reviews, see Romanoff, 1960; Clark, 1984). A slow heart rhythm is initially established by the anterior portion of the cardiac tube and, as development proceeds, heart rate comes under the control of more posterior regions of the heart tube and shows a rapid acceleration to about 180 beats min^{-1} by day 4 (Clark and Hu, 1982). Meanwhile, development of fully functional valves and completion of heart folding occurs. After the initial rise, heart rate remains relatively constant until just prior to hatching (Clark, 1984). The chicken circulatory system initially operates at a very low pressure of below 0.133 kPa which gradually increases during development (Clark and Hu, 1982). Nevertheless, this pressure must be sufficient to ensure circulation of the blood.

As in birds, elasmobranch embryos offer an excellent opportunity for the study and description of early cardiovascular haemodynamics. The initial stages in development of avian and elasmobranch hearts are remarkably similar; later in development, avian and elasmobranch hearts begin to diverge both anatomically and functionally. A notable difference between the two circulatory systems is the early development of functional gills in elasmobranchs. The present study focuses on blood pressures measured in the central cardiovascular system of embryos of the little skate, *Raja erinacea* Mitchill, from the initial stages of circulation until just before hatching. The changes observed are correlated with morphological development of the central circulatory organs.

Materials and methods

Animals and physiological measurements

Egg cases of *Raja erinacea* were obtained from our breeding colony at the National Marine Fisheries Service aquarium in Woods Hole using methods generally similar to those of Luer and Gilbert (1985). Egg cases were collected from November 1989 to June 1990 within 24–48 h after spawning and kept in a tank with running sea water (17–18 °C). A few days before experimentation the egg cases were transferred to the Zoology Department in Amherst and kept in an aquarium with well-aerated sea water at 16–18 °C.

Experiments were performed with embryos ranging from 27 to 144 days post-spawning with a body mass of 0.01–4.35 g. The embryos, with the yolk sack still attached, were removed from the egg cases and incubated in a small experimental chamber containing about 20 ml of well-aerated sea water (18±1 °C) with 0.1 g l^{-1} neutralized MS 222. Heart and conus arteriosus were exposed by a small incision in the ventral body wall. In small embryos with a body mass below 0.1 g it was

possible to penetrate the body wall with the electrode and to measure blood pressures without dissection.

Blood pressure was recorded with a servo-null micro-pressure system (Modell 900, World Precision Instruments, New Haven, Connecticut, USA), as used by Clark and Hu (1982). The system has an accuracy of ±0.5 % at full scale (13.3 kPa) and a risetime of 20 ms for 10–90 % response, which overcomes the problem of signal damping observed in some other systems (Clark and Hu, 1982). Microelectrodes with a tip diameter of 2–5 μm were filled with 3 mol l^{-1} NaCl and mounted on a micromanipulator. Under microscopic control a microelectrode was placed beside the ventricle at the depth of the later location inside the organ and the pressure was set to zero. The electrode was then inserted into the ventricle for continuous recording. Preparations were usually stable for at least 1 h with decreases in cardiac frequency and blood pressure of less than 10 %. During this period it was sometimes necessary to reposition the electrode; however, this did not change the results. Even when a broken electrode had to be replaced identical readings were obtained at a single recording site. To obtain a good recording it was imperative to locate the tip of the electrode freely in the bloodstream as contact between the electrode tip and a membrane or valve caused significant noise in the signal. In some cases pressure was also recorded in the conus arteriosus and the ventral aorta.

In some preparations arterial blood velocity was also measured using a pulsed Doppler flowmeter (Bioengineering, Iowa, USA). The 1 mm crystal was placed above the anterior end of the conus arteriosus. All signals were continuously recorded on a Physiograph chart recorder (Narco Bio-Systems, Houston, Texas, USA).

Morphological studies

After physiological recording, the embryos were fixed in 4 % formaldehyde in sea water. A parallel series of embryos (ranging from day 20 to day 150) was also collected for dissection and histological study. Two specimens [27 days, 0.01 g, 18 mm total length (TL) and 107 days, 1.98 g, 91 mm TL] were cleared and mounted whole in Eukit (Calibrated Instruments, Ardsley, New York, USA). A series of embryos and isolated hearts of larger specimens was dehydrated in alcohol, embedded in methacrylate (Historesin, LKB, Bromma, Sweden), and sectioned (3 μm) in transverse or frontal planes with a rotary retracting microtome (HM 330, Microm, Heidelberg, Germany). Sections were stained with Toluidine Blue and Acid Fuchsin. Whole embryos sectioned especially for studying early heart development were taken at 34 days (approx. 0.04 g, 31 mm TL), 39 days (approx. 0.07 g, 34 mm TL) and 50 days (0.13 g, 41 mm TL).

Results

During the 5–6 months of development in the egg case, the embryo of the little

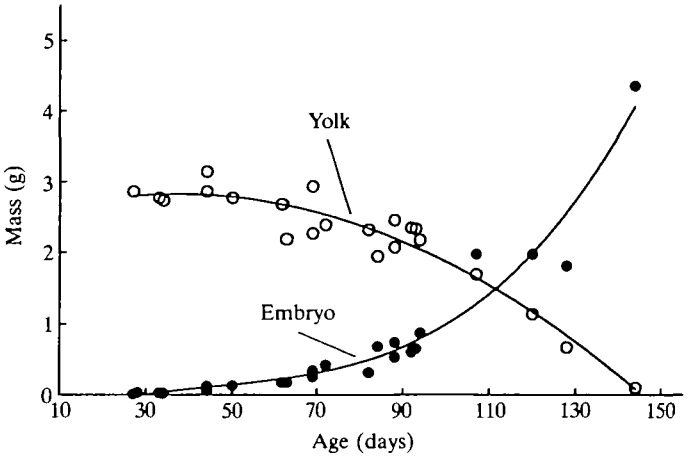

Fig. 1. Body mass and yolk mass of skate embryos plotted against days of incubation after spawning.

skate grows exponentially, gaining 70–80 % of its body mass within the last two or three months (Fig. 1).

In the smallest embryo studied physiologically, (27 days post-spawning, 0.01 g, 18 mm TL) the heart tube is already folded into the S-shape characteristic of early heart development in vertebrates (Fig. 2A) and the atrium already lies dorsal to the ventricle and conus arteriosus. In embryos of this age, the separate heart chambers are only poorly demarcated, and the myocardium is uniform in thickness along its length. There is no trace of valves separating the presumptive chambers, although small constrictions are apparent at the atrio-ventricular and ventro-conal boundaries.

At the opposite extreme in this study are embryos in which the heart is almost fully differentiated (Fig. 2B–E), although subject to allometric changes before hatching. Fig. 2B shows the heart and arterial tree of an older embryo (107 days, 1.98 g, 91 mm TL). Fig. 2C–E shows sections through the heart of a 50-day embryo (0.13 g, 41 mm TL) that demonstrate how complete the major features of heart development are by this time. The thin walls of the sinus venosus open into the atrium *via* a small orifice (Fig. 2C). The atrio-ventricular valve is present on the left side of the heart (Fig. 2B,D). By day 50, the wall of the ventricle has developed trabeculae (Fig. 2D), and some cells of the myocardium are starting to show a striated appearance. Within the conus arteriosus is a series of three flap or pocket valves (Fig. 2B). Each conal valve has three leaflets projecting into the lumen (Fig. 2C). Fig. 2C–E also documents the characteristic appearance and distribution of endocardial cushion tissue in the atrio-ventricular region and conus arteriosus. The endocardial cushion tissue forms valves and other connective tissue specialisations.

Embryos of intermediate ages (e.g. 34 days, 31 mm TL; and 39 days, 34 mm TL) show that development of the heart is extremely rapid. By day 34, the atrio-

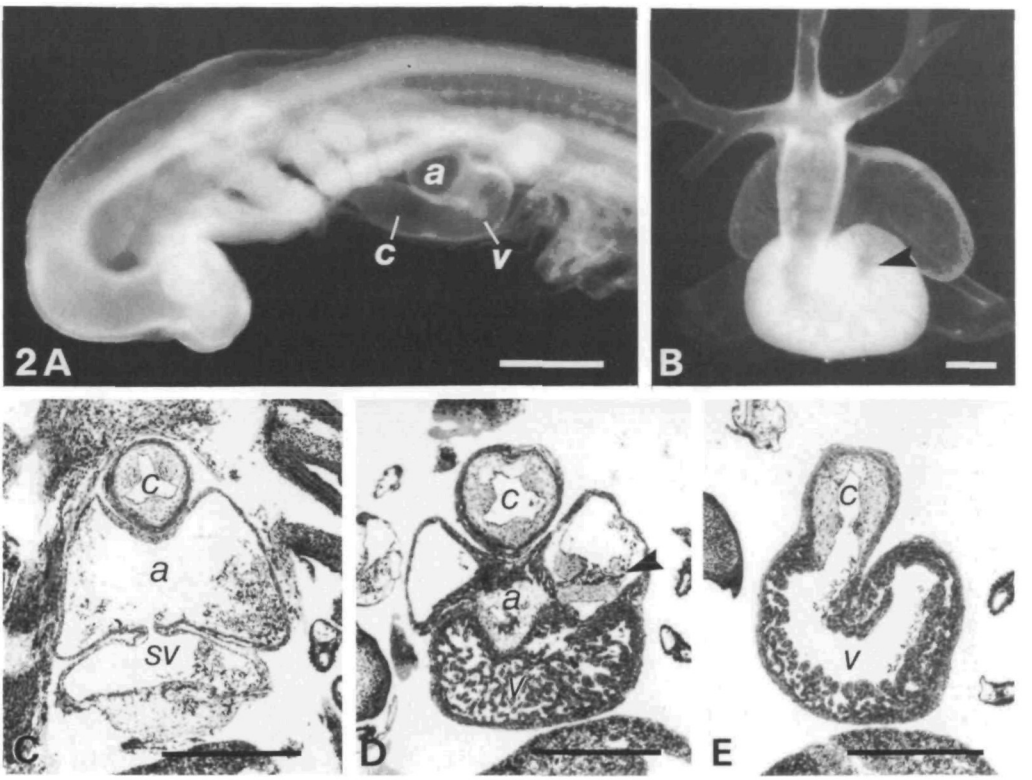

Fig. 2. Developmental anatomy of the skate heart. (A) Left lateral view of head and pharynx of embryo at 27 days post-spawning (0.01 g, 18 mm total length). (B) Ventral view of isolated heart of embryo at 107 days post-spawning (1.98 g, 91 mm total length). (C, D, E) Frontal sections through the central cardiovascular system of a 50-day embryo (0.13 g, 41 mm total length) in ventral view, showing the sinus venosus (*sv*), atrium (*a*) and conus arteriosus (*c*) in C, the atrium, atrio-ventricular valve (arrowhead), ventricle (*v*) and conus arteriosus in D, and the ventricle and conus arteriosus in E. *a*, atrium; *c*, conus arteriosus; *sv*, sinus venosus; *v*, ventricle. The arrowheads indicate the atrio-ventricular boundary or valve. Scale bars, 0.5 mm.

ventricular valve is present, and separate leaflets of the conal valves are apparent in an embryo of 39 days.

Fig. 3 shows an example of recordings of blood velocity and ventricular pressure for a 0.12 g animal. During diastole, two phases can be separated in the pressure trace. After systole, diastolic ventricular pressure was initially only about 0.03 kPa. This was followed by a slight increase in pressure to 0.08 kPa, representing the atrial pressure generated by atrial contraction in order to fill the ventricle. During the subsequent systole the pressure increased to 0.42 kPa. The simultaneous velocity signal recorded in the conus arteriosus shows one clearly defined velocity peak.

In the youngest embryos of 27–45 days (0.01–0.03 g), the heart rate was low (35–40 beats min^{-1}; Fig. 4). With further development, heart rate nearly doubled

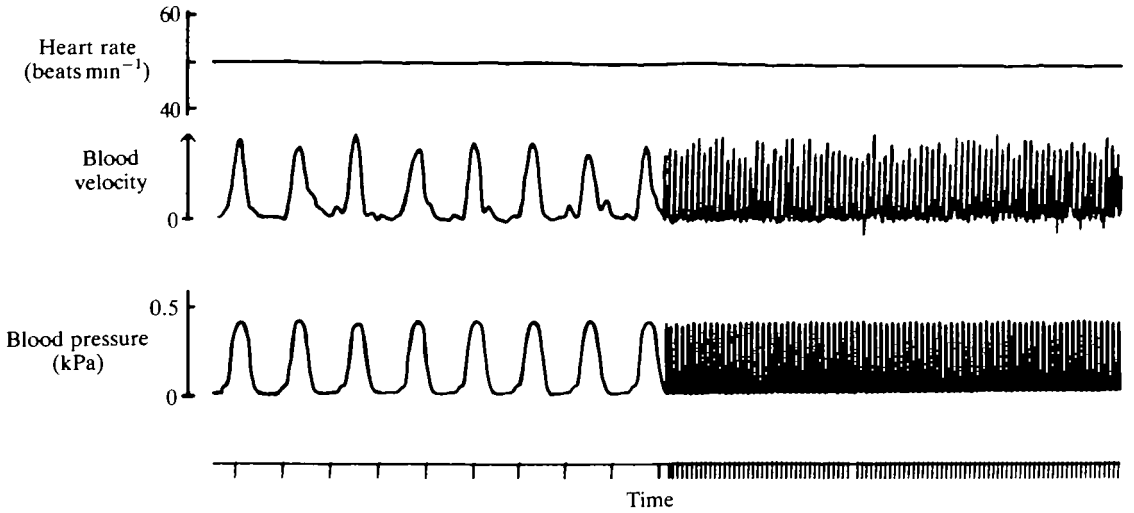

Fig. 3. Original recordings of blood velocity in the conus arteriosus and of ventricular blood pressure in an embryo 44 days after spawning (0.12 g). Each tic on the time scale marks 1 s.

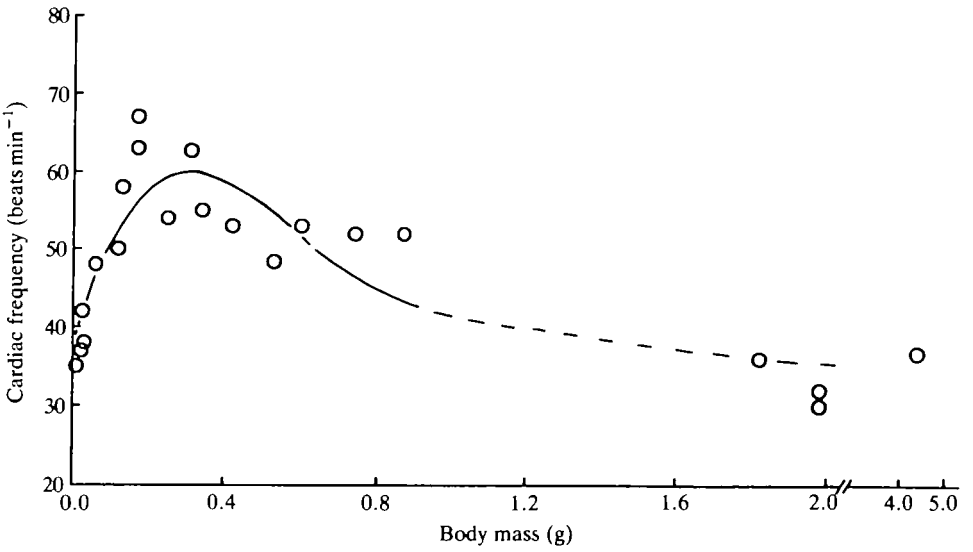

Fig. 4. Changes in heart rate with increasing body mass.

to about 60–68 beats min^{-1} in a 0.2 g animal. Heart rate then slowly decreased until just prior to hatching.

Atrial, initial diastolic and peak systolic pressures are plotted against body mass in Fig. 5A. Atrial and diastolic pressures remained very low throughout development, but systolic pressure increased significantly. On a logarithmic scale, the

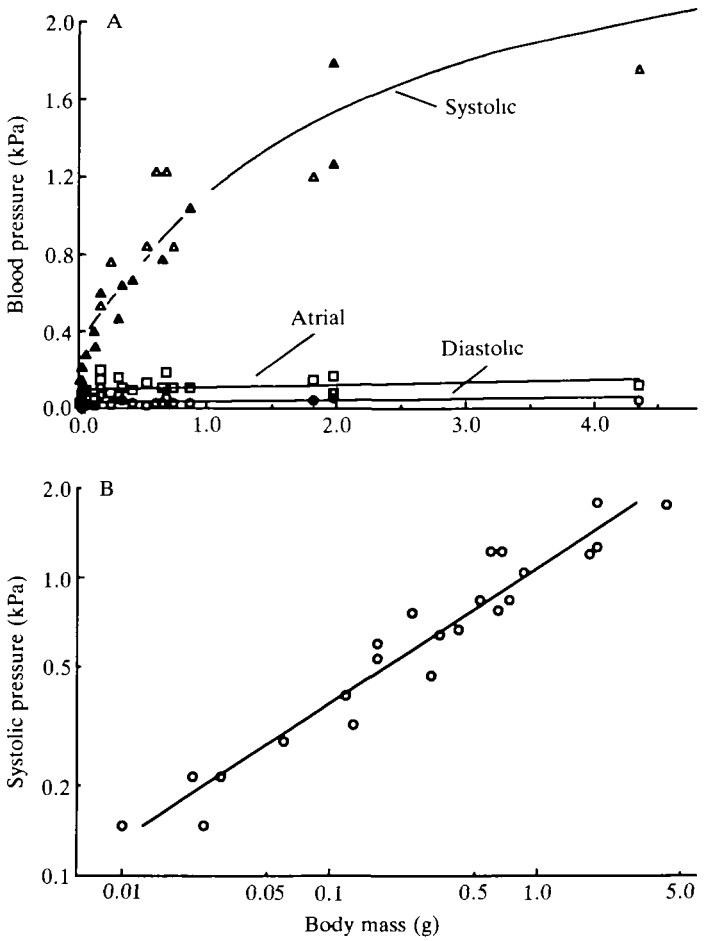

Fig. 5. (A) Changes in atrial (□), initial diastolic (○) and systolic (△) blood pressure with increasing body mass. Pressures were recorded in the ventricle. (B) Systolic ventricular pressure *versus* body mass plotted on a logarithmic scale.

relationship between systolic ventricular pressure and body mass was linear (Fig. 5B; systolic pressure$=1.07B^{0.45}$, where B is body mass, $P<0.01$).

In small embryos (body mass<0.05 g) the conus usually collapsed completely during systole, making it impossible to record conal blood pressure. Fig. 6A shows a sample recording from a 0.06 g animal, which was the smallest embryo studied for which both conal and ventricular pressures could be reliably recorded. The overlay of these traces reveals that conal pressure followed ventricular pressure during ventricular systole, but that conal pressure remained elevated compared to that of the ventricle during ventricular diastole. In recordings from a 0.74 g animal, systolic pressures in the ventricle and the conus arteriosus were again very similar (Fig. 6B, solid and dashed lines). During ventricular diastole, conal pressure initially far exceeded ventricular pressure and decreased only just before the onset

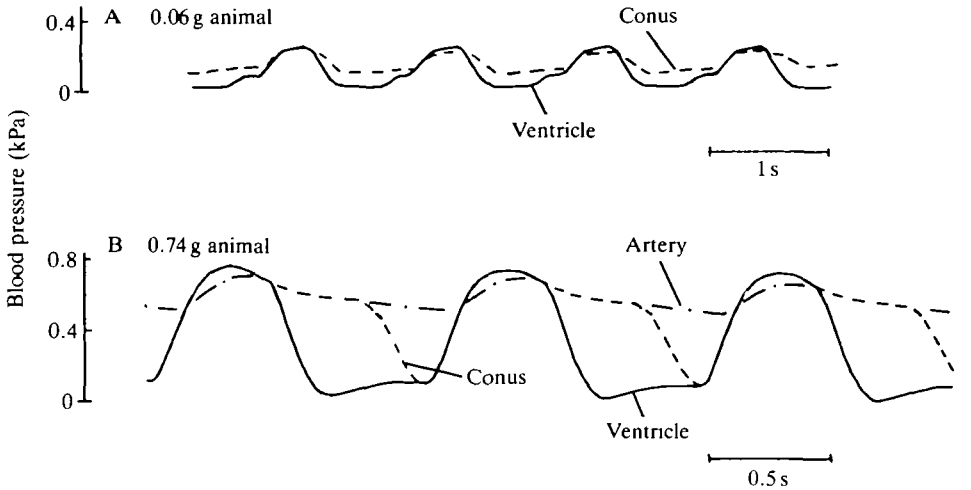

Fig. 6. Serial overlays of pressure recordings obtained from the ventricle, the conus arteriosus and the ventral aorta of two skate embryos (A) 44 days (0.06 g) and (B) 88 days (0.74 g) after spawning.

of the following contraction of the ventricle. Systolic blood pressure recorded in the ventral aorta (Fig. 6B, dashed and dotted line) generally followed systolic pressures in the ventricle and conus arteriosus. Significantly, however, pressure in the ventral aorta remained high during relaxation of the conus. Thus, the ventral aorta and afferent vessels of the gills experience only small systolic–diastolic pressure variations compared to those in the conus arteriosus and the ventricle.

Discussion

Critique of methods

In fish, MS 222 has been shown to reduce heart rate by about 25 % after an initial increase during the first minutes of exposure (Randall *et al.* 1965; Houston *et al.* 1971), but the intense movements of the embryos made recordings without sedation impossible. In some animals heart rate was counted before anaesthesia and dissection. A comparison of these values with those recorded in the anaesthetized state did not indicate a significant decrease in heart rate, especially as the heart rate was not constant with time. Nevertheless, we cannot exclude a slight chronotropic effect of MS 222. A possible inotropic effect could not be assessed. Similarly, a decrease in systolic pressure has been observed in adult fish during exposure to MS 222, although the effect is less pronounced than the effect on heart rate (Randall *et al.* 1965).

The small size of the blood vessels, even compared to the 1 mm Doppler crystal, made it impossible to localize exactly the site from which the signal arose. This, together with the changing diameter of the conus arteriosus along its length during

systole, prevented a calibration of the velocity signal and thus made quantification of blood flow impossible.

Comparative developmental morphology

The basic features of the development of the cardiovascular systems of elasmobranchs have been known for more than a century (Balfour, 1876, 1878); however, no previous study of elasmobranchs has attempted to correlate developmental morphology with heart function. The most detailed embryological studies of elasmobranchs are available for *Squalus acanthias* (Scammon, 1911), which is an excellent species for phylogenetic comparison with the skate. There are difficulties in deriving an accurate table for comparing the embryonic stages of various species of elasmobranchs (Scammon, 1911, p.78) that result from differences in reproductive biology and rate of development. Nevertheless, our day 27 embryo of *Raja erinacea* (in which little differentiation of the heart was present; Fig. 2A) closely resembles Scammon's stages 23 and 24 of *Squalus acanthias* (*Squalus* embryos 9 mm and 11.5 mm TL, respectively; see Scammon, 1911, p. 30).

Subsequent differentiation of the heart in *Raja* occurs very rapidly, so that, within about 2 weeks, most features of the adult heart are present. This is indicative of the generally rapid rate of differentiation of the circulatory system, which achieves its function earlier than most other organ systems. After about day 50, further development of the heart in *Raja* is largely allometric (e.g. differential thickening of the myocardium of the various chambers). These patterns appear to hold true for *Squalus* as well (Scammon, 1911).

Heart frequency

In fish embryos, as in bird embryos (Clark and Hu, 1982), the heart starts contracting shortly before circulation of blood can be observed (McElman and Balon, 1979, 1980; Cunningham and Balon, 1986). Blood flow was observed in all our preparations, although in the smallest embryos the haematocrit appeared to be very low, as estimated by the colour of the blood and the ease with which single erythrocytes could be seen.

The initial increase in cardiac frequency with development observed in our study is similar to those reported for teleosts (Paine and Balon, 1984: Cunningham and Balon, 1986). The heart rate in embryonic skates, however, is only half, or even less, that reported for teleosts, probably because of differences in body size (teleost embryos are usually smaller than those of elasmobranchs).

By analogy with chick embryos (Clark and Hu, 1982), it is tempting to assume that the increase in heart frequency in skates reflects the transition from an intrinsic rhythm of the conus and ventricle to pacing by the sinus venosus. So far, however, we have no proof of this transition and, even if it occurs, it does not explain the subsequent slowing down of the heart as development proceeds.

Blood pressure

The increase in blood pressure during ontogeny of the little skate is closely correlated to the increase in body mass, as has also been observed for bird embryos (Tazawa, 1981; Clark and Hu, 1982) and for larvae of the frogs *Pseudis paradoxa* (W. Burggren, personal communication) and *Rana catesbeiana* (Pelster and Burggren, 1991). Both body mass and heart muscle mass increase with development (see Fig. 2) and the increase in arterial blood pressure might also be correlated to heart growth. Our observations, however, show that older embryos not only have more cardiac muscle cells but also have higher concentrations of contractile proteins per cardiac muscle cell, as shown by the development of regular striated patterns. Thus, the force developed per cell may also increase over the period of development.

The systolic ventricular pressures of about 0.2 kPa recorded in the youngest skate embryos (approximately 30 days after spawning) were very close to the ones reported for bird embryos of a similar developmental stage (Clark and Hu, 1982). Within the next 10–20 days of development, the embryo develops increasingly lengthy external gills, which by day 80–90 are being resorbed and replaced by the internal gills. In spite of these significant morphological changes in the circulatory system, the ventricular systolic pressure shows an uninterrupted, and remarkably good, correlation to body mass, as is also the case with the bird embryo, which does not encounter these changes in the respiratory organs.

The observed logarithmic–linear relationship between systolic blood pressure and body mass in the embryos does not extrapolate to the adult; there must be a point in development at which blood pressure stabilizes or the increase is at least very much reduced. Johansen *et al.* (1966) reported a ventral aortic pressure of 4.9 kPa for a 20 kg *Raja binoculata*, and in dogfishes weighing 1–2 kg pressures of 3–5 kPa have been measured (Piiper *et al.* 1970; Butler and Taylor, 1971). Our results show that when the embryo of the little skate leaves the egg case the ventral aortic pressure is already very close to the final value to be expected in the fully grown adult.

Function of the conus arteriosus

A report on the functional morphology of the heart in adult big skates (*Raja binoculata*, Johansen *et al.* 1966) illustrates a pressure profile consistent with contraction of the conus arteriosus. In contrast, Satchell and Jones (1967) did not observe any secondary rise in pressure due to conal contraction in the Port Jackson shark (*Heterodontus portusjacksoni*). In none of our preparations did we observe a conal pressure peak greater than that of the ventricle during systole, although the pressure in the conus remained elevated during the initial ventricular diastole. This can either be attributed to muscular activity or to a slow relaxation of elastic tissue. Thus, the main functin of the conus is to prolong the period of high pressure, which may explain why its activity is not very obvious in the velocity recordings, as the highest velocity is recorded at the onset of ventricular

contraction. Histological study confirms that there are cardiac muscle fibres in the wall of the conus of elasmobranchs, although this muscular layer is much thinner than that of the ventricle (Yamauchi, 1980).

In contrast to amphibians, the conus arteriosus of the little skate and other elasmobranchs has no spiral valve but is characterized by the presence of serial flap valves. These valves appear early in embryonic development as shown in this study, and are important for the separation of the ventral aorta from the ventricle during diastole (Satchell and Jones, 1967). Circulation of blood starts before these conal valves are formed. Backflow of blood from the ventral aorta into the ventricle during diastole is initially prevented by a complete diastolic collapse of the presumptive conus arteriosus. The ventricle is thus exclusively filled from the atrium. About 39 days after spawning the conal valves are present and prevent regurgitation of blood into the ventricle during diastole.

We wish to thank Mrs G. Ryfa and Mr D. Fahey for expert technical assistance and Dr W. Burggren for providing research facilities and for critical comments on the manuscript. Financial support by the Deutsche Forschungsgemeinschaft (DFG – Pe 389/2–1) to BP and of UMASS/NOAA Cooperative Marine Education and Research Program and NFS BSR-8806539 to WEB are gratefully acknowledged.

References

BALFOUR, F. M. (1876). The development of the elasmobranch fishes. From B to G. *J. Anat. Physiol.* **10**, 672–688.

BALFOUR, F. M. (1878). *A Monograph on the Development of Elasmobranch Fishes*. London: Macmillan and Co.

BALLARD, W. W. (1973). Normal embryonic stages for salmonid fishes, based on *Salmo gairdneri* Richardson and *Salvelinus fontinalis* (Mitchill). *J. exp. Zool.* **184**, 7–26.

BUTLER, P. J. AND TAYLOR, E. W. (1971). Response of the dogfish (*Scyliorhinus canicula* L.) to slowly induced and rapidly induced hypoxia. *Comp. Biochem. Physiol.* **39**A, 307–323.

CLARK, E. B. (1984). Functional aspects of cardiac development. In *Growth of the Heart in Health and Disease* (ed. R. Zak), pp. 81–103. New York: Raven Press.

CLARK, E. B. AND HU, N. (1982). Developmental hemodynamic changes in the chick embryo from stage 18 to 27. *Circulation Res.* **51**, 810–815.

CUNNINGHAM, J. E. R. AND BALON, E. K. (1986). Early ontogeny of *Adinia xenica* (Pisces, Cyprinodontiformes). III. Comparison and evolutionary significance of some patterns in epigenesis of egg-scattering, hiding and bearing cyprinodontiforms. *Env. Biol. Fish.* **15**, 91–105.

HOUSTON, A. H., MADDEN, J. A., WOODS, R. J. AND MILES, H. M. (1971). Some physiological effects of handling and tricaine methanosulfonate anesthetization upon the brook trout (*Salvelinus fontinalis*). *J. Fish. Res. Bd Can.* **28**, 625–633.

JOHANSEN, K., FRANKLIN, D. L. AND VAN CITTERS, R. L. (1966). Aortic blood flow in free-swimming elasmobranchs. *Comp. Biochem. Physiol.* **19**, 151–160.

LUER, C. A. AND GILBERT, P. W. (1985). Mating behavior, egg deposition, incubation period, and hatching in the clearnose skate, *Raja eglanteria*. *Env. Biol. Fish.* **3**, 161–171.

MCELMAN, J. F. AND BALON, E. K. (1979). Early ontogeny of walleye, *Stizostedion vitreum*, with steps of saltatory development. *Env. Biol. Fish.* **4**, 309–348.

MCELMAN, J. F. AND BALON, E. K. (1980). Early ontogeny of white sucker, *Catostomus commersoni*, with steps of saltatory development. *Env. Biol. Fish.* **5**, 191–224.

PAINE, M. D. AND BALON, E. K. (1984). Early development of the northern logperch, *Percina caprodes semifasciata*, according to the theory of saltatory ontogeny. *Env. Biol. Fish.* **11**, 173–190.

PELSTER, B. AND BURGGREN, W. W. (1991). Central arterial hemodynamics in larval bullfrogs (*Rana catesbeiana*): Developmental and seasonal influences. *Am. J. Physiol.* (in press).

PIIPER, J., BAUMGARTEN, D. AND MEYER, M. (1970). Effects of hypoxia upon respiration and circulation in the dogfish *Scyliorhinus stellaris*. *Comp. Biochem. Physiol.* **36**, 513–520.

RANDALL, D. J., SMITH, L. S. AND BRETT, J. R. (1965). Dorsal aortic blood pressures recorded from the rainbow trout (*Salmo gairdneri*). *Can. J. Zool.* **43**, 863–872.

ROMANOFF, A. L. (1960). *The Avian Embryo: Structure and Functional Development*. New York: Macmillan.

ROMBOUGH, P. J. (1988). Respiratory gas exchange, aerobic metabolism, and effects of hypoxia during early life. In *Fish Physiology*, vol. XI (ed. W. S. Hoar and D. J. Randall), pp. 59–161. New York, London: Academic Press.

SATCHELL, G. H. AND JONES, M. P. (1967). The function of the conus arteriosus in the Port Jackson shark, *Heterodontus portusjacksoni*. *J. exp. Biol.* **46**, 373–382.

SCAMMON, R. E. (1911). Normal plates of the development of *Squalus acanthias*. In *Normentafeln zur Entwicklungsgeschichte der Wirbeltiere*, Bd. 12, (ed. F. Keibel), pp. 1–140. Jena: Gustav Fischer.

TAZAWA, H. (1981). Measurement of blood pressure of chick embryo with an implanted needle catheter. *J. appl. Physiol.* **51**, 1023–1026.

WEBER, A. (1908). Recherches sur quelques stades du développement de coeur de la raie. *C. R. Ass. anat., Paris* **10**, 10–14.

YAMAUCHI, A. (1980). Fine structure of the fish heart. In *Hearts and Heart-like Organs*, vol. 1 (ed. G. H. Bourne), pp. 119–148. New York, London: Academic Press.