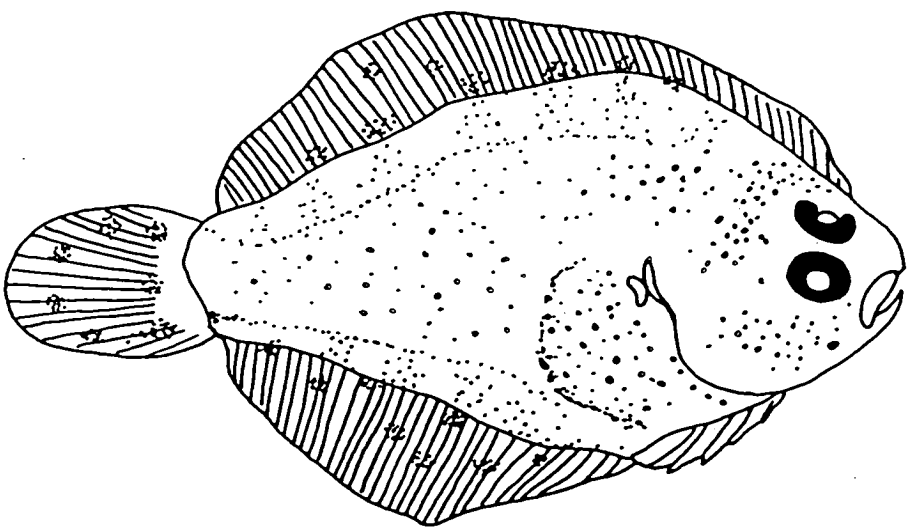
FEEDING BEHAVIOUR

OF

LARVAL GREENBACK FLOUNDER

RHOMBOSOLEA TAPIRINA



by

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Frontispiece: Greenback flounder (*Rhombosolea tapirina*) larva at 43 days post-hatching.

(Photograph courtesy of Tish Pankhurst)

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E.S. Cox
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March, 1997

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ABSTRACT

Understanding the feeding behaviour of larval fish is critical for determining optimal management protocols in aquaculture and understanding larval prey selectivity patterns of wild fish populations. This study examined the feeding performance of cultured greenback flounder larvae *Rhombosolea tapirina*, using the live feed organisms *Brachionus plicatilis* (rotifers), and *Artemia* sp., to determine the primary sensory modality involved in feeding, the relationship between mouth dimensions and prey size selected, and the effect of previous exposure to a prey species on subsequent prey selection. The proportion of larvae that fed on rotifers in the light (light intensity of 5-6 $\mu mol.m^{-2}.s^{-1}$), increased significantly from 66% to 96% from day 12 to day 27 post-hatching, respectively. In comparison, the proportion of larvae that fed on rotifers in total darkness (0 $\mu mol.m^{-2}.s^{-1}$) never exceeded 5% during the same period. This indicated that greenback flounder larvae were primarily dependent upon vision (a light dependent behaviour) to feed, with a lesser reliance upon non-visually mediated detection of prey. Internal horizontal dimensions of the mouth of greenback flounder larvae determined from serial histological sections, increased in a linear fashion with both increasing body size and age of larvae. Examination of the ratio of *Artemia* prey size (total length, carapace width, and carapace width with appendages) to larval mouth width, suggested that prey carapace width, not prey width with appendages, or prey length, limited the size of prey ingested, indicating that larvae must visually orient to ingest the prey head-on. When greenback flounder larvae were offered one of three discrete size fractions of *Artemia* prey, both prey size and larval age significantly effected larval feeding response. On days 12 - 17 post-hatching, larvae ingested *Artemia* prey of a single size range (100 - 200 μm screened size), which was considerably smaller than they were capable of ingesting. From 19 - 20 days of age and thereafter, there was a marked change in feeding performance, with larvae able to ingest all three *Artemia* prey size ranges offered (100 - 200 μm, 300-390 μm and 450 - 560 μm screened size, respectively), probably reflecting an ontogenetic increase in larval sensory capability, swimming speed and prey handling ability. Prior feeding experience of greenback flounder larvae to either rotifers only (Rotifer Treatment) or a mixed diet of *Artemia* and rotifers (*Artemia* and Rotifer Treatment), significantly effected subsequent

prey selection when larvae were offered a mixed diet of *Artemia* and rotifers, but did not effect the temporal onset of selection of the novel prey species (*Artemia*) by Rotifer Treatment larvae. Therefore the differences in prey selection by larvae with or without prior exposure to *Artemia* prey, was not due to the inability of larvae to handle and ingest a novel prey species, but reflects positive selection for familiar prey species. The latter indicates a learned component in the feeding behaviour of fish larvae. This has implications for the timing of the introduction of new live prey species during intensive culture of marine fish larvae.

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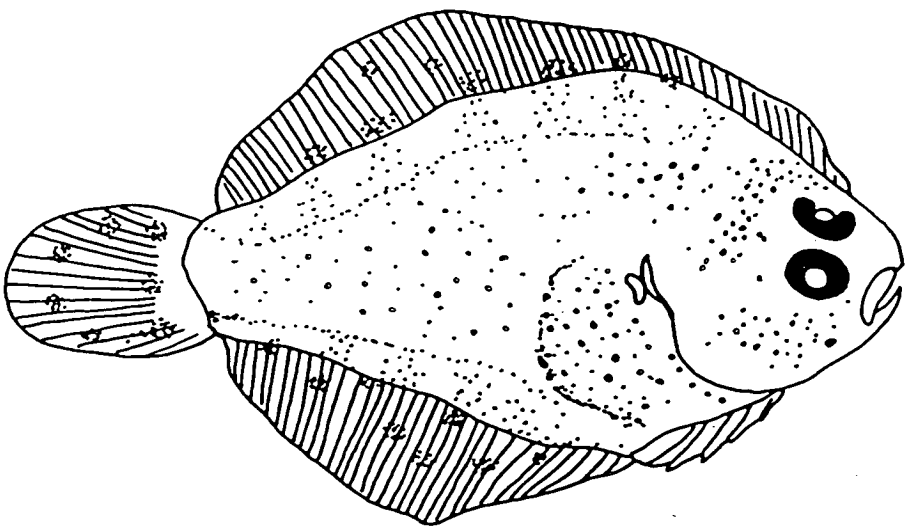
Scientific and common names of fish mentioned in the following text.

Scientific Name	Common Name
Achirus lineatus	Lined sole
Alosa pseudoharengus	Alewife
Ammotretis rostratus	Long-snouted flounder
Anchoa mitchilli	Bay anchovy
Aplodinotus grunniens	Freshwater drum
Archosargus rhomboidalis	Sea bream
Aristichthys nobilis	Bighead carp
Brevoortia tyrannus	Atlantic menhaden
Chanos chanos	Milkfish
Cichlasoma managuense	Central American cichlid
Clinocottus analis	Cottid
Clupea harengus	Atlantic herring
Coregonus clupeaformis	Whitefish
Coregonus artedii	Ciscoes
Cottus asper	
Cottus bairdii	Mottled sculpin
Cottus rhotheus	
Ctenopharyngodon idella	Grass carp
Dorosoma cepedianum	Gizzard shad
Dorosoma petenense	Threadfin shad
Embiotoca jacksoni	Black surfperch
Embiotoca lateralis	Striped surfperch
Engraulis mordax	Northern anchovy
Engraulis capensis	Cape anchovy
Esox lucius	Pike
Gambusia spp.	Mosquito fish
Hypoatherina tropicalis	
Hypophthalmichthys molitrix	Silver carp
Latris lineata	Striped trumpeter
Lepomis cyanellus	Green sunfish
Lepomis gibbosus	Pumpkinseed sunfish
Lepomis macrochirus	Bluegill sunfish
Leucaspius delineatus	Verkhovka
Lota lota	Burbot
Macruronus novaezelandiae	Hoki
Menidia audens	Mississippi silversides
Micropterus salmoides	Largemouth bass
Morone chrysops	White-bass
Morone saxatilis	Striped bass
Oncorhynchus gorbuscha	Pink salmon
Pagrus auratus	Snapper

Scientific Name	Common Name
Perca flavescens	Yellow perch
Pleuronectes platessa	Plaice
Pomoxis nigromaculatus	Black crappie
Rhombosolea tapirina	Greenback flounder
Salmo gairdneri	Rainbow trout
Sardinops sagax	Pacific sardine
Solea solea	Sole
Sparus aurata	Gilthead seabream
Spinachia spinachia	15-spined stickleback
Stizostedion vitreum	Walleye
Trachurus symmetricus	Jack mackeral

CHAPTER 1

INTRODUCTION



Larvae of most marine fishes are predatory planktivores, at least during the early life history stages (Hunter, 1981; Blaxter, 1986). The search, capture and ingestion of prey by planktivorous fish larvae involves a complex interaction of both environmental parameters external to the larval predator, and the physiological, morphological and behavioural characteristics of the individual (Bell, 1990). The ability of larvae to search for, perceive and then capture prey, is largely constrained by the genotypic and ontogenetic characteristics of the larva (e.g. sensory capabilities, locomotory ability, mouth dimensions and body size). The latter change rapidly during development, such that the ability of the fish to capture prey, and the size of prey able to be ingested, increases rapidly with increasing body size (Confer and Blades, 1975; Breck and Gitter, 1983; Blaxter, 1986; Noakes and Godin, 1988; Ghan and Sprules, 1993; Cook, 1996; Higgs and Fuiman, 1996). Within the constraints of the ingestible prey size range imposed by larval mouth dimensions, prey selectivity is strongly influenced by prior exposure to prey, and behaviours learned by the larvae (Ware, 1971; Vinyard, 1980; Checkley, 1982; Colgan *et al.*, 1986; Meyer, 1986). In addition, extrinsic environmental factors, including the spatial distribution and size spectrum of available prey in the environment, and the physical characteristics of the water column (light intensity, water turbidity and temperature), directly effect larval searching strategies (Bell, 1990). Intrinsic physiological needs of the larva (level of hunger, satiation, starvation), further modify searching behaviour (Blaxter and Ehrlich, 1974; Yin and Blaxter, 1987).

The ability of fish larvae to detect prey is central to the feeding process. Various sense organs have been implicated in prey perception by larval fish, including the eyes (vision), the mechanosensory lateral line and superficial neuromasts (mechanoreception), and organs of gustation and olfaction (chemoreception) (Dabrowski, 1982; Janssen, 1990; Batty and Hoyt, 1995; Higgs and Fuiman, 1996). However, the sense organs are initially, poorly developed (Blaxter and Jones, 1967; Hairston *et al.*, 1982; Appelbaum and Schemmel, 1983; Kawamura and Ishida, 1985; Blaxter, 1986; Harvey *et al.*, 1992; Walton *et al.*, 1994; Pankhurst and Butler, 1996), and this has implications for sensory functional capabilities. In larval fish which are primarily dependent upon vision in order to locate prey, visual resolution is initially severely constrained by fish eye/lens size and the stage of development of the retina, thereby imposing constraints upon prey detection by small fish (Hairston *et al.*, 1982; Breck and Gitter, 1983; Li *et al.*, 1985; Kotrschal, 1990; Pankhurst,

1993; Pankhurst, 1994). Visual acuity (the minimum angle that a stimulus can subtend at the eye and still be resolved) of larval fish is initially poor, with the optimization of photopic resolving power and visual sensitivity, being restricted by limited space within the retina (Kotrschal *et al.*, 1990). However, photopic visual acuity increases with increasing fish size (Blaxter and Jones, 1967; Johns and Easter, 1977; Li *et al.*, 1985; Pankhurst *et al.*, 1993; Pankhurst, 1994), as a function of increasing eye lens diameter and focal length of lens (Tamura and Wisby, 1963), with the consequence that search volumes and the distance at which larvae can detect a prey item (reactive distance), increases with increasing eye/fish size (Confer *et al.*, 1978; Breck and Gitter, 1983). Therefore, the enhanced visual resolution of the eyes of larger fish is likely to result in increased prey encounter rates, as fish grow. For larval species which shift from a pelagic to a demersal habitat later in ontogeny, the improved capacity of both the photopic and scotopic visual systems which occurs during ontogeny (Kotrschal *et al.*, 1990), may in part compensate for the corresponding decrease in light intensity associated with such a habitat shift (Wahl *et al.*, 1993).

Some species of larval fish have the capacity to detect prey using non-visual senses (mechanoreception and chemoreception), either in isolation (Holanov and Tash, 1978; Janssen, 1990; Batty and Hoyt, 1995), or in combination with vision (Batty and Hoyt, 1995; Higgs and Fuiman, 1996). The mechanosensory lateral line of fish larvae initially consists of a regular array of superficial neuromasts, some of which become enclosed within canals (canal neuromasts), usually later in larval life (Webb, 1989). Neuromasts respond to low frequency vibrations such as those produced by small scale water displacements (Webb, 1989). They play an important role in predator detection by fish larvae (Fuiman and Magurran, 1994), and have been implicated in the detection of live prey by fish larvae (Dabrowski, 1982; Hoekstra and Janssen, 1985; Blaxter and Fuiman, 1989, 1990; Janssen, 1990), presumably detecting water movements arising from prey locomotion. Functional responses of the mechanosensory lateral line, like the visual system, are likely to change during ontogeny, because of growth related changes in the neuromast array and lateral line canal formation. Blaxter and Fuiman (1989), determined that the superficial neuromasts of larval fish lie within a boundary layer of still water adjacent to the fishes body. They suggested that the neuromasts within this boundary layer act as distance receptors for low frequency water disturbances, until the ontogenetic

increase in larval swimming speed is sufficient to thin the boundary layer, at which stage the noise level from the fishes swimming movements would likely preclude this function. This is supported by the fact that lateral line canal formation occurs late in larval development, coinciding with the time when swimming speed of fish larvae increases significantly (Blaxter, 1986; Blaxter and Fuiman, 1989, 1990). As the lateral line system provides protection for the canal neuromasts, it is possible that the canal neuromasts act as distance receptors for predator/prey localisation in late larval stages, when boundary layer shear precludes the use of free neuromasts in this role during swimming (Blaxter and Fuiman, 1989). Jones and Janssen (1992), provided further support for the latter, because the ability of mottled sculpin (*Cottus bairdi*) to feed in the dark, in response to prey vibrations in the water column, decreased during the period when the superficial neuromasts were becoming enclosed within canals.

There is also strong evidence to support a prey perception role utilising chemical senses by larval fish (Dempsey, 1978; Appelbaum and Schemmel, 1983; Blaxter, 1986; Tanaka *et al.*, 1991). The detection of water-borne stimuli is achieved using the senses of olfaction (olfactory organs) and gustation (tastebuds). The use of chemoreception alone in the detection of prey has been demonstrated in juvenile sole *Solea solea* (Batty and Hoyt, 1995). Reliance upon chemoreception for prey detection has also been proposed for larval threadfin shad *Dorosoma petense* (Holanov and Tash, 1978), and larval and juvenile herring *Clupea harengus* (Dempsey, 1978; Batty *et al.*, 1986), which are thought to rely on olfaction in order to detect and remain within prey patches, while filter feeding prey from the water column.

The reactive distance of fish larvae to prey when non-visual senses are employed is considerably shorter compared to the reactive distance involving visual detection of prey, with the result that the search volume for non-visual feeding is relatively small (Higgs and Fuiman, 1986). This consequent decrease in search volume will possibly reduce the prey encounter rate, resulting in lower feeding responses of larvae reliant upon non-visual cues. However, it is unlikely that any one sensory cue will be used independent of other senses, and it has been suggested that poor development of one or more senses is compensated for by higher performance of another sense (Schellart, 1992). For example, the extent to which the lateral line is utilised in prey detection by fishes varies, with some species of fish

routinely relying on lateral line detection of prey, whilst others utilise lateral line cues only when necessary. However it is likely that the synergystic use of both lateral line and visual cues occurs whenever possible (Montgomery, 1989), as demonstrated for Atlantic menhaden larvae, *Brevoortia tyrannus*, in which optimal response to a probe stimulus was dependent upon combined use of both vision and mechanoreception (Higgs and Fuiman, 1996). Similarly, a dual reliance on vision and chemoreception in order to detect prey has been demonstrated for plaice *Pleuronectes platessa* (Batty and Hoyt, 1995).

The detection of prey is the first step in the feeding behavioural repertoire. The prey must then be captured and ingested. The ability of fish larvae to complete this prey capture sequence will change during the early developmental period as locomotor capacity and mouth dimensions change. Larval swimming behaviour is initially constrained by body morphology, most fish larvae having only a primordial finfold at the time of hatching, with median, lateral and caudal fins developing later. The hydrodynamic environment in which larvae exist is determined by their size and the speed at which they can move through the water, and by the physical properties of viscosity and density of the water (Webb and Weihs, 1986). These factors are used to determine the ratio of inertial and frictional forces imposed upon the swimming larva and are expressed as a non-dimensional Reynolds number, which changes in proportion with larval length. At low Reynolds numbers, body movements are counteracted by viscous forces which extend a large distance from the body. The most effective swimming mode at this stage is a serpentine or "anguilliform" motion involving an increasing amplitude of body wave travelling the entire length of the body, from anterior to posterior. At this stage, searching behaviour is usually conducted in a swim, stop and search sequence. As larvae grow, and the median and caudal fins develop, the Reynolds number approaches 200 (at this stage inertial forces become more important), and it is more efficient for larvae to switch to a sub-carangiform swimming mode, where the head is held relatively still and the amplitude of the body wave increases towards the tail (Batty, 1984; Blaxter, 1986; Webb and Weihs, 1986). At this time, it becomes energetically advantageous to swim using a continuous tail beat and glide motion, which may have implications for increased prey encounter and capture rates as a result of concomittant increased swimming speed (Drost, 1987).

For planktivorous larval fish that swallow their prey intact, some aspect of mouth dimension must limit the maximum size of prey that can be swallowed (Hunter, 1981), and this has led to the concept of "gape limitation" (Wong and Ward, 1972; Schmitt and Holbrook, 1984; Ghan and Sprules, 1993; Wahl *et al.*, 1993). It has been confirmed that during the first few weeks of larval life, when gape limitation imposes the most severe constraints upon the prey size range available to be ingested, larval fish actively select the smallest available zooplankton prey (Schmitt and Holbrook, 1984). However, prey size ingested is usually much smaller than maximum gape size indicates (Lemly and Dimmick, 1982; Ponton and Müller, 1990; Schael *et al.*, 1991; Shaw and Jenkins, 1992). For example, wild juvenile greenback flounder *Rhombosolea tapirina*, have been reported to consistently ingest prey only half the size of their mouth width (Jenkins, 1987; Shaw and Jenkins, 1992). Similarly, larval bluegill *Lepomis macrochirus*, and largemouth bass *Micropteris salmoides* (Lemly and Dimmick, 1982), the 15-spined stickleback *Spinachia spinachia* (Kislalioglu and Gibson, 1976), gilthead seabream *Sparus aurata* (Fernández-Diaz, 1994), and larval whitefish *Coregonus* sp. (Ponton and Müller, 1990), have all been shown to ingest prey sizes considerably smaller than their mouth dimensions.

Mouth gape increases with increasing body size of fish (Wong and Ward, 1972; Lemly and Dimmick, 1982; Dabrowski and Bardega, 1984; Schael *et al.*, 1991; Ghan and Sprules, 1993), and while the minimum prey size selected generally remains fairly constant during larval growth (Hansen and Wahl, 1981; Michaletz, *et al.*, 1987), the optimal prey size tends to be a constant function of mouth dimension (Werner, 1974). As a result, the prey size range available to be ingested increases during ontogeny and this has important energetic implications for the larvae (Hunter, 1981). There are many reports that confirm an increasing selectivity for larger prey with increasing size of fish larvae (Hartman, 1958 - rainbow trout *Salmo gairdneri*; Werner, 1974 - bluegill sunfish, and green sunfish *Lepomis cyanellus*; Lemly and Dimmick, 1982 - largemouth bass and bluegill sunfish; Hambright, 1991 - largemouth bass; Schael *et al.*, 1991 - yellow perch *Perca flavescens*, freshwater drum *Aplodinotus grunniens*, and black crappie *Pomoxis nigromaculatus*; Ghan and Sprules, 1993 - burbot *Lota lota*). The correlation between mouth dimensions and fish length is species specific and mouth dimensions per se do not necessarily provide an accurate estimate of optimum prey size selected by larval fish in the wild (Schael, *et al.*, 1991; Bremigan and Stein, 1994). This is probably because factors besides prey size, such

as prey visibility (Kislalioglu and Gibson, 1976; Zaret, 1980; Magnhagen, 1985), prey motility (Kislalioglu and Gibson, 1976; O'Brien *et al.*, 1976) and escape behaviour (Moore and Moore, 1976; Eggers, 1977; Meng and Orsi, 1991), and the density and spatial distribution of prey within the water column (Moore and Moore, 1976), all influence prey selection by larval fish.

As prey density within the environment increases, the larvae of some fish species have been shown to become more selective, opting for larger prey items (Werner and Hall, 1974; Rajasilta and Vuorinen, 1983; Magnhagen, 1985; Wetterer, 1989); however, prey size selection by other species has been shown to be independent of prey density (Mills *et al.*, 1986). Prey density has a strong influence upon the feeding behaviour of those fish larvae which are able to switch from particulate feeding, which requires larvae to be able to perceive individual prey, to filter feeding, which is non-selective in that perception of individual prey is not required (Batty *et al.*, 1986 - herring; Gibson and Ezzi, 1985 - herring; Holanov and Tash, 1978 - threadfin shad; James and Findlay, 1989 - Cape anchovy *Engraulis capensis*; Janssen, 1978 - alewife *Alosa pseudoharengus*; Janssen, 1980 - ciscoes *Coregonus artedii*; Drenner and McComas, 1980 - Mississippi silverside *Menidia audens*). For example, filter feeding in the light by ciscoes, herring and juvenile Cape anchovy is triggered by the presence of relatively high prey densities (Janssen, 1980; Gibson and Ezzi, 1985; James and Findlay, 1989), with the switch to particulate feeding occurring at lower prey densities. Similarly, in the dark, relatively dense patches of prey are required in order for filter feeding of herring larvae to be effective, and filter feeding generally ceases altogether at low prey densities in the dark (Batty *et al.*, 1986).

Within the constraints of larval size, developmental stage and prey availability, the ability of planktivorous fish larvae to search for and capture prey, is also effected by the physical properties of the environment. Visually mediated feeding is a light dependent behaviour which requires relatively high light intensity in early larval stages (Sbikin, 1974; Blaxter, 1968, 1986; Dayong *et al.*, 1994). The threshold light intensity for visually mediated feeding (light intensity below which feeding ceases) decreases with increasing age of larvae (Blaxter, 1968; Sbikin, 1974; Butler, 1995), reflecting an increase in sensitivity of the visual system. Optimal photopic visual resolution of fishes is achieved only in extremely clear waters which have low levels of dissolved and particulate substances (Lythgoe,

1980). It has been suggested that elevated levels of particulate substances (turbidity) alters the relative visibility of the prey (Werner and Hall, 1974), both by attenuation of light and because particulate scatter decreases visual contrast between the prey and background (Lythgoe, 1988), with the result that reactive distances of fishes are foreshortened (Vinyard and O'Brien, 1976; Confer *et al.*, 1978). This would ultimately decrease prey encounter rates and feeding success of fish larvae (Moore and Moore, 1976; Gardner, 1981; Mills *et al.*, 1986). However, Miner and Stein (1993), found that the feeding ability of larval bluegill sunfish was enhanced at low to mid turbidity levels. These investigators proposed that within the very short perceptive field of fish larvae, particulate light scatter may increase visual contrast of zooplankton prey which would be viewed against a bright, diffuse background.

Larval fish live in a dynamic environment in which feeding success, growth and survival hinges upon the ability to respond to change. Not surprisingly then, prior experience and learning plays an important role in prey selection by fish larvae (Beukema, 1968; Werner *et al.*, 1981; Bell, 1990), such that fish positively select for and are more effective at capturing prey which are familiar (Vinyard, 1980; Checkley, 1982). In contrast, feeding success on novel prey species is initially poor but increases rapidly after just a few episodes of exposure (Werner *et al.*, 1981; Colgan *et al.*, 1986; Meyer, 1986; Wahl *et al.*, 1995).

An understanding of the intrinsic and extrinsic factors which are involved in the search and capture of prey by planktivorous marine fish larvae is fundamental to the understanding of population dynamics in wild fish populations, but also for the provision of appropriate conditions for optimal feeding success in intensive culture situations. The development of new finfish species for intensive aquaculture is reliant upon the production of sufficient juveniles for ongrowing in land or seabased holding facilities. Early life history stages often represent one of the major bottlenecks in production, and optimization of larval feeding, growth and survival is central to overcoming this problem. The intensive culture of marine finfish larvae is still reliant upon the provision of live prey feeds for the early larval developmental phases, despite considerable work targetting the development of micro-encapsulated feeds for first feeding fish larvae (Person-Le Ruyet, 1990; Marte and Duray, 1991; Tandler and Kolkovski, 1991; Walford *et al.*, 1991). Without an understanding of the underlying processes and constraints involved in live prey perception

by planktivorous larval fish (i.e. sensory functions), it is not possible to accurately interpret larval feeding behaviour responses, to determine the optimal physical parameters for prey perception, or to develop microencapsulated diets which are likely to be acceptable prey targets for planktivorous fish larvae. To achieve optimal feeding responses in cultured larvae, an understanding of the species specific constraints imposed on prey size selection by the ontogenetic stage of larval development and body size, is paramount. Finally, because feeding regimes in intensive culture of marine finfish larvae involves the sequential provision of different prey of increasing size, often with quite different prey characteristics, then we must consider how prior experience of a prey species will influence larval prey selectivity patterns. The latter is essential if the culturist is to promote the successful transition to new live prey species. The present study aimed to lay the foundations for future provision of appropriate culture conditions, and feeding protocols, to optimise feeding of greenback flounder (*Rhombosolea tapirina*). The specific aims of this study were to:

- Determine the primary sensory modality involved in the feeding behaviour of greenback flounder larvae of increasing age,
- Assess prey size selection of greenback flounder larvae with increasing larval age and to correlate measurements of larval mouth width to prey size selected, and
- Assess the effect that previous exposure to a prey species had on subsequent prey selection in larvae of increasing age.

Greenback flounder were chosen as an experimental animal, both because this species is currently being examined as a candidate species for intensive aquaculture, and because it provides a model species for the examination of feeding behaviour of fish larvae in general. Greenback flounder, *Rhombosolea tapirina*, belong to the flatfish family, Pleuronectidae. They are found in temperate estuaries and coastal waters to depths of 55 m around Tasmania (Crawford, 1986), on the south coast of Australia, from southern New South Wales to southern Western Australia (Jenkins, 1987; Kuiter, 1993), and in New Zealand on the eastern coast of the South Island to depths of 100 m (Ayling and Cox, 1982). Adults of this species range from 25 cm to a maximum of 50 cm in total length, although they rarely attain the upper size limit (Kuiter, 1993).

There is very little literature available on greenback flounder, most of which has concentrated on descriptive biology. The growth and reproduction (Kurth, 1957) and local distribution and diet of Tasmanian populations have been assessed (Last, 1983). Mature but unfertilized ova were described by Kurth (1957), and late larval stages were described by Roper (1979). Crawford (1986), provided a detailed description of the developmental stages of both eggs and larvae of this species. More recently Jenkins (1987), examined composition of the diet and prey selection of greenback flounder larvae in Port Phillip Bay in southern Australia. A description of the development of the various sensory organs of greenback flounder larvae from day 1 post-hatching through to the early juvenile stage, was recently completed by Pankhurst and Butler (1996). Hart (1994), studied the effect of various external culture parameters, including salinity, temperature and photoperiod, on egg incubation and larval rearing and survival, in order to identify appropriate hatchery rearing conditions, for this species.

Experimental Design/Replication

All of the feeding behaviour experiments were conducted in a similar fashion in which larvae from a stock culture tank (and therefore a single cohort) were used to stock replicate test chambers on any one day. As a consequence, the experiments presented in this study represent a "simple pseudoreplicated" design as defined by Hurlbert (1984), in that larvae used for all replicate treatments were taken from a single experimental unit. This latter was a constraint imposed by the availability of culture tanks and the time required for one person to maintain the larvae and simultaneously conduct multiple feeding experiments. The feeding ability of larvae was assessed in terms of the percentage of fish feeding (criterion: presence or absence of food in the gut) after 1 h exposure to live feed organisms (either rotifers, *Artemia* sp., or a combination of both).

Mouth Gape Determination

An accurate measure of mouth size was required in this study for correlation with prey size selected by greenback flounder larvae. However, the definition of "mouth gape" varies between literature sources and the method by which the measurements have been obtained

(Table 1). Assessing gape using a gape micrometer device (developed by Arts and Evans, 1987) reflects the smallest internal mouth dimension of the fish larva, and this method is commonly used (Schael, et al., 1991; Ghan and Sprules, 1993; Bremigan and Stein, 1994). It involves inserting a series of cones into the mouth of a fish larva until resistance is felt. The geometric function of the cone is then used to convert cone diameter to mouth gape. Similar principles using graduated tapered cones or drills have been employed by Wong and Ward, (1972), and Kislalioglu and Gibson, (1976). Shirota, (1970, 1978) developed an equation ($D = \sqrt{2}(AB)$, where D = gape height, and AB = length of upper jaw), to estimate gape height based on measurements of the upper jaw length of the fish larva with the assumption that a 90° angle of jaw articulation represented the maximum gape height during feeding. Ponton and Muller (1990), estimated gape height of whitefish larvae using a microscope fitted with an ocular micrometer and measured the upper jaw length with jaws spread to inflection angles of 45° and 90°. These measurements were then substituted into the equation from Shirota (1970), in order to obtain an estimate of larval gape height. Dabrowski and Bardega (1984), also obtained gape height calculated from Shirota (1970), but used lower jaw length in their assessment. They assumed measurements taken with the jaws at an angle of 45° represented mean prey size, and measurements at 90° represented maximum prey size ingestible. Vernier calipers (Northcote, 1954), have also been used to measure larval mouth width or height, although usually this technique has been employed on larger larvae or juveniles, in which body length was ≥10 mm. Only gape height has been measured directly using an ocular micrometer fitted to a dissecting microscope (Hartman, 1958; Wankowski, 1979), but it is difficult to obtain accurate readings when manually spreading the jaws.

Whilst these various techniques have proved to be valuable tools for the measurement of jaw gape in larger larvae and juveniles, they do pose difficulties for the precise measurement of jaw gape in small larvae, in which mechanical devices may over-estimate jaw gape by stretching the jaws past the "normal" point of opening. In particular, when using an ocular micrometer or vernier calipers, it is not possible to effectively measure the gape of larvae less than 10 mm in total length (Arts and Evans, 1987). Due to constraints imposed by the small size of the early life history stages of greenback flounder (2 mm total length at hatching), we chose to use the horizontal internal dimension of the posterior buccal chamber (internal horizontal mouth width), as determined from serial histological

transverse sections, to estimate the upper size limit of prey that could be ingested (refer Pankhurst, 1994). The width of the buccal cavity is defined by rigid skeletal elements of the upper jaw and therefore represents the smallest dimension within the mouth cavity, through which the prey must pass. It has been suggested by other investigators that mouth width represents the best estimate of the upper limit of ingestible prey size by planktivorous larval fishes (Werner, 1979; Hunter, 1981).

Table 1 - Techniques used to measure mouthgape.

Author	Technique	Fish size (mm)	Species
Arts & Evans, 1987	Gape micrometer *[2]	7 - 31	Yellow perch, *Perca flavescens* Lake whitefish, *Coregonus clupeaformis* Lake herring, *Coregonus artedii*
Bence & Murdoch, 1986	Ocular micrometer - jaw height	10 - 50	Mosquito fish, *Gambusia* spp.
Bremigan & Stein, 1994	Gape micrometer *[2]	10 - 50	Bluegill, *Lepomis macrochirus* Gizzard shad, *Dorosoma cepedianum*
Cook, 1996	Vernier calipers.	15 - 42	Cottid, *Clinocottus analis*
Dabrowski & Bardega, 1984	Ocular micrometer - LJL*[1]	8 - 17 9 - 18 8 - 28	Silver carp, *Hypophthalmichthys molitrix* Grass carp, *Ctenopharyngodon idella* Bighead carp, *Aristichthys nobilis*
Fernandez-Diaz *et al.*, 1994	N/A	3.5 - 7	Gilthead seabream, *Sparus aurata*
Ghan & Sprules, 1993	Gape Micrometer	3.2 - 15	Burbot, *Lota lota*
Hambright, *et al.*, 1993	Gape micrometer	3.2 - 113	Largemouth bass, *Micropterus salmoides*
Hart & Hamrin, 1988	Calipers (0.01mm) - spread jaws, measured height	66.4 - 182.1	Pike, *Esox lucius*
Hartman, 1958	Ocular micrometer	N/A	Rainbow trout, *Salmo gairdneri*
Jenkins, 1987	Ocular micrometer	2 - 8	Greenback flounder, *Rhombosolea tapirina* Long-snouted flounder, *Ammotretis rostratus*
Kislalioglu & Gibson, 1976[b]	drills inserted into mouth	72 - 124	15-spined stickleback, *Spinachia spinachia*
Lemly & Dimmick, 1982	N/A	up to 15	Bluegill, *Lepomis macrochirus* Largemouth bass, *Micropterus salmoides*
Michaletz, *et al*, 1987	Mouthgape - vertical opening, no method given	8 - 40	Walleye, *Stizostedion vitreum* Whitebass, *Morone chrysops* Yellow perch, *Perca flavescens* Gizzard shad, *Dorosoma cepedianum*

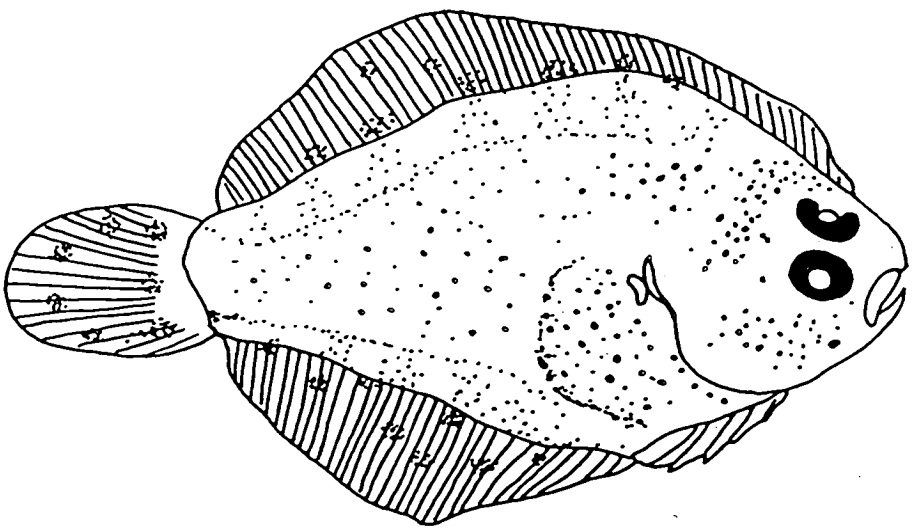
Author	Technique	Fish size (mm)	Species
Murdoch, 1990	Ocular micrometer-maxillary length *[1]	3.2 - 17.15	Hoki, *Macruronus novaezelandiae*
Northcote, 1954	Vernier caliper - Distance between lateral surfaces of mouth	35 - 80	*Cottus asper*, *Cottus rhotheus*
Ponton & Muller, 1990	Vernier caliper - Measured UJL *[1]	10 - 16	Whitefish, *Coregonus* sp.
Schael *et al.*, 1991	Gape micrometer *[2]	4.3 - 23.5	Freshwater drum, *Aplodinotus grunniens* Black crappie, *Pomoxis nigromaculatus* Yellow perch, *Perca flavescens*
Schmitt, 1986	Ocular micrometer - gape width at angle of 60°	5 - 17	*Hypoatherina tropicalis*
Schmitt & Holbrook, 1984	Mouth width & height. Method not stated	50 - 278	Black surfperch, *Embiotoca jacksoni* Striped surfperch, *Embiotoca lateralis*
Shaw & Jenkins, 1992	Ocular micrometer	5 - 15	Greenback flounder, *Rhombosolea tapirina*
Shirota, 1970	Ocular micrometer - UJL*[1]	2.6 - 25.1	Various species
Wankowski, 1979	Ocular micrometer	20 - 280	Atlantic salmon, *Salmo salar*
Werner, 1974	Ocular micrometer	33 - 124	Bluegill, *Lepomis macrochirus* Green sunfish, *Lepomis cyanellus*
Wong & Ward, 1972	Brass cones - 0.2mm increments (mouth width)	10 - 50	Yellow perch, *Perca flavescens*

*[1] UJL (upper jaw length) or LJL (lower jaw length) measurement substituted into equation to calculate mouth height, from Shirota (1970).

*[2] Gape micrometer as developed by Arts and Evans (1987).

CHAPTER 2

MATERIALS AND METHODS



2.1 Egg Production

2.1.1 Egg Production and Incubation (1995)

In July, 1995, fertilised eggs were provided by the Department of Primary Industries and Fisheries (DPIF) Marine Research Laboratories, Taroonah. Eggs were transported to the Department of Aquaculture the day after fertilisation, and were stocked immediately into each of 4 x 160 L black hemispherical larval culture tanks, at a density of approximately 50.L^{-1}, for incubation. The eggs were incubated at 12°C ± 1°C (temperature controlled environment) without aeration, and a flow-through recirculating seawater system exchanged 25% of the tank volume per day. A 63 µm drum outlet screen retained eggs in the larval rearing tank. Dead eggs were siphoned from the bottom of the tank daily.

2.1.2 Egg Production and Incubation (1996)

Sexually mature female broodstock were caught during June and August (1996), from Waubs Bay, Bicheno, by SCUBA divers using handnets. Sexually mature male broodstock (F1 cultured fish) were obtained from the DPIF Marine Research Laboratories, Taroonah. Broodstock were transported to the Department of Aquaculture Aquatic Centre, Launceston, where males and females were held separately (n = 10 - 15 per tank) in 1000 L recirculating seawater systems, at ambient winter temperature (10°C), and natural photoperiod. Each system had a reservoir (280 L) from which seawater was pumped into two, 1000 L culture tanks, before passing through a trickle filter, consisting of a sheet of dacron over seven boxes of bioballs. The tank surfaces were scoured and 50% of the tank volume was exchanged, three times per week. Broodstock were fed twice per day on 3 mm barramundi pellets (Gibsons).

Upon capture, wild-caught females were treated with an intraperitoneal injection of the Lutenising hormone-releasing hormone des-Gly^{10}, [D-Ala^{6}]- ethylamide (LHRHa), at a dosage of 100 µg.Kg^{-1} body weight. A second treatment was administered to unresponsive fish approximately one week later. Forty eight hours after injection, and daily thereafter, females were anaesthetised in a 0.02% solution of 2-phenoxyethanol, and were checked for the presence of fully hydrated, ovulated eggs using an ovarian biopsy.

Eggs from ovulated females were then stripped, by the application of gentle pressure to the abdominal area, and eggs from two or three females were collected in one container.

Male broodstock did not receive hormone treatment. Milt was readily expressed from spermeated males by the application of gentle pressure to the abdomen, and was collected in 5 ml syringes, taking care to avoid contamination with faeces, urine, or water. Eggs were fertilised by adding approximately 1 ml of milt per 100 ml of eggs and gently stirring for 10 seconds, after which time seawater was added and the eggs were transferred to a graduated cylinder, and left for 30 minutes to allow unfertilised eggs and debris to sink. Fertilised eggs remained buoyant and were decanted into a 20 L bucket which was transferred immediately into the marine fish hatchery (12 ± 1°C, 13 h light : 11 h dark). The eggs were left in a 20 L bucket for 1 h, at which time floating eggs were skimmed from the surface and transferred to a larval rearing tank at a stocking density of approximately $50.L^{-1}$. The eggs were incubated without aeration and a flow-through system exchanged 25% of the tank volume per day. A 250 μm drum outlet screen, retained eggs in the larval rearing tank. Dead eggs were siphoned from the bottom of the tank daily.

2.2 Larval Rearing - General

2.2.1 Larval Maintenance

In 1995, larvae (cohort 1 - see table 1) were reared at a density of approximately $50.L^{-1}$, in a recirculating seawater system which consisted of 4 x 160 L, black, hemispherical, fibreglass larval culture tanks, and a 200 L seawater reservoir. Seawater (12 ± 1°C) was pumped by an Onga FP10 submersible pump to the larval culture tanks, from the reservoir. Effluent water drained out of the larval culture tanks via 20 cm diameter drum-screens, before passing back to the reservoir via a trickle biofilter. The latter consisted of a layer of dacron over a substrate of bioballs (biofilter medium), with a submerged shell grit filter (pH buffering medium) beneath. Larval culture tank outlets were fitted with 63 μm mesh screens, from the time of hatching, to prevent loss of larvae. These were replaced with 250 μm outlet screens on day 6 post-hatching, when the larval locomotor capacity had

increased. The larvae were maintained in a photoperiod of 16 h light : 8 h dark. Overhead fluorescent strip lights (Thorn EMI 18 Watt) provided a light intensity of 4 $\mu mol.m^{-2} .s^{-1}$ (Li-Cor LI-250 light meter) at the water surface, during the photophase. Water exchange was maintained at 50% per day. Approximately 60% - 80% of the reservoir volume was exchanged with clean, 2 µm filtered seawater, twice weekly. Larval culture tank surfaces were cleaned with a scouring pad and siphoned daily, to remove dead larvae and debris.

In 1996, larvae (Cohorts 2 - 5) were reared in a recirculating seawater system consisting of 4 x 200 L reln tanks and a 300 L seawater reservoir (Figure 1). Seawater passed through each of a 25, 5 and 1 µm filter, before being stored in 2 x 225 L tanks, for equilibration to room temperature. This was used to exchange 80% of the recirculation reservoir volume per week. Seawater was pumped by an Eheim 240v submersible pump, from the reservoir to the larval culture tanks. Effluent water passed through a drum screen fitted to the tank outlet, before passing back to the reservoir, via spray booms situated above a trickle biofilter. The biofilter consisted of a layer of dacron matting over two, three-tier stacks of mesh-bottomed tote boxes. Two boxes in each filter stack contained scoria (biofilter medium) and the other contained shell grit. On the day of hatching (day 0), light aeration was introduced into the larval culture tanks and the water exchange rate was increased from 25% (incubation exchange rate), to 50% per day. A 250 µm drum screen placed over the tank outlet to retain larvae, was replaced with a 500 µm screen, on day 12 post-hatching. Water temperature was maintained at 12°C ± 1°C by a heating/cooling air conditioning unit in the hatchery. A photoperiod of 13 h light : 11 h dark, was provided by overhead fluorescent strip lights (Thorn EMI 18 Watt) providing a light intensity of 5 $\mu mol.m^{-2}. s^{-1}$ (Li-Cor LI-250 light meter), at the water surface, during the photophase. Seawater temperature and salinity were recorded daily, ammonia, pH and dissolved oxygen weekly, and adjustments were made to maintain acceptable levels of 12°C ± 1°C, 35 ppt salinity, 0 $mg.L^{-1}$ NH_3, pH - 8 ± 0.2, and dissolved oxygen >6 $mg.L^{-1}$, respectively.

Live feed (*Artemia* and rotifers) was introduced into the tanks when the larval eyes became pigmented (and were presumed to be functional) and the mouth opened (usually day four post-hatching). Maintenance feeding regimes were dependent upon subsequent

experimental protocols, and are described for each cohort of larvae in section 2.4.1, 2.4.2 and 2.4.3.

Once feeding commenced, effluent water from the culture tanks was passed over a 63 μm screen placed above the trickle filter, to prevent any prey from entering the recirculation system reservoir, and thus being re-introduced into the culture tanks. This was crucial, as *Artemia* had to be excluded from some of the rearing tanks used for those experiments assessing the effect of prior experience of a prey species upon subsequent prey selection by greenback flounder larvae (see section 2.4.3). In addition, the metabolic requirements of both *Artemia* and rotifers causes a rapid decrease in their enrichment levels over time, and therefore the removal of old food before the addition of freshly enriched prey, maximized the nutritive value of prey available to the larvae. Details of the cohorts of flounder larvae used for all experiments are provided in Table 2.

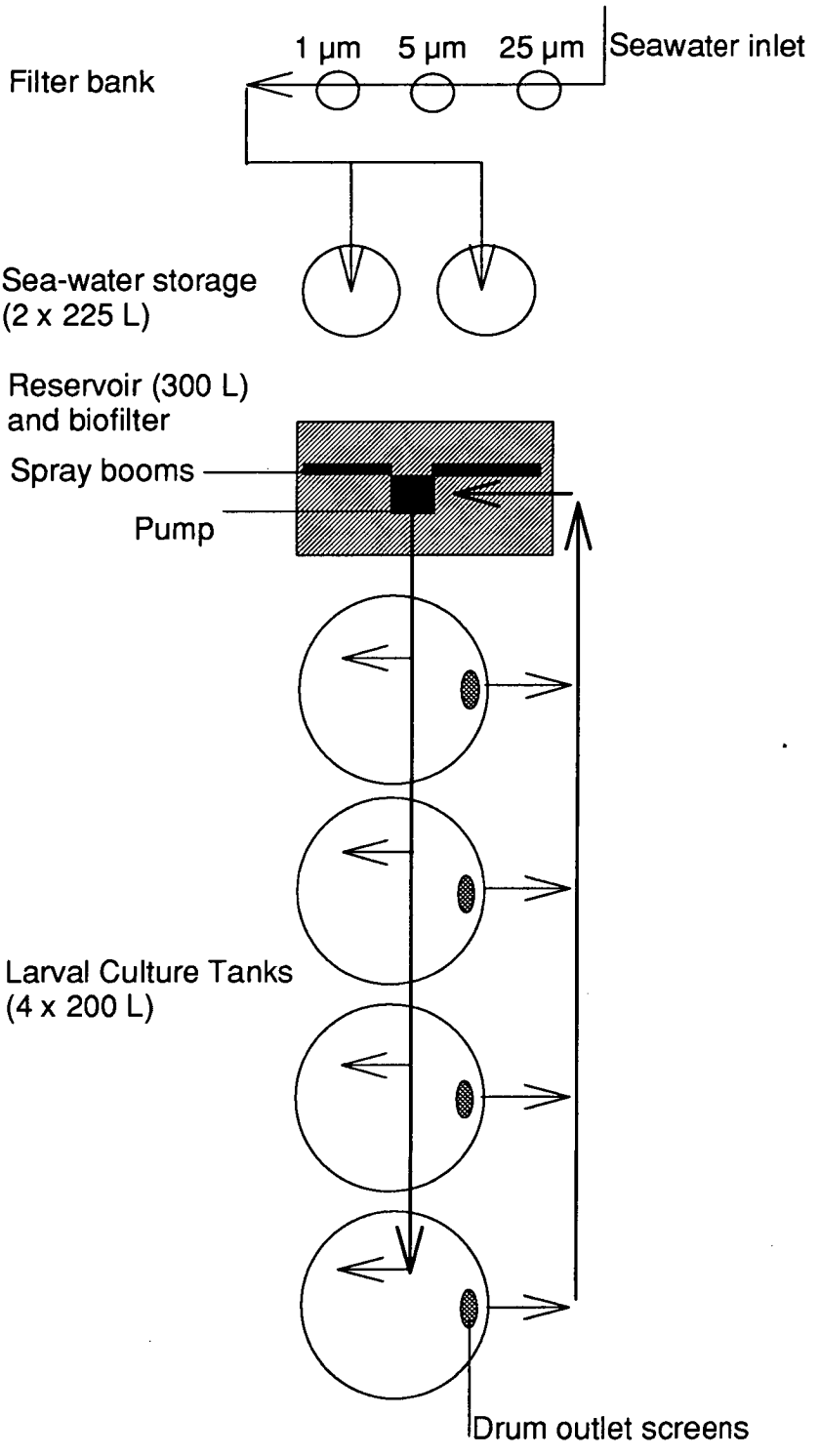


Figure 1 - Diagram of the recirculating seawater system used for culturing larval greenback flounder (cohorts 2 - 5), in 1996. The system comprised 4 x 200 L culture tanks, a reservoir with trickle biofilter and 2 x 225 L seawater storage tanks. Arrows indicate the direction of water flow from the reservoir into the culture tanks and effluent flow from the drum screens within the culture tanks, back to the reservoir via boom sprays suspended over a trickle filter.

Table 2 - Egg production details of larval greenback flounder, used in this study.

Larval Cohort	Egg Production	Hormone treatment (females)	Date Fertilised	Date Hatched
1 *[1]	DPI - Taroonah F1 males and females	LHRHa - intramuscular injection	12-7-95	15-7-95
2 *[2]	Wild-caught females. F1 males	LHRHa - intramuscular injection	27-6-96	1-7-96
3 *[3]	Wild-caught females. F1 males	LHRHa - intramuscular injection	2-7-96	6-7-96
4 *[4]	Wild-caught females. F1 males	LHRHa - intramuscular injection	1-9-96	5-9-96
5 *[5]	Wild-caught females. F1 males	LHRHa - intramuscular injection	2-9-96	6-9-96

*[1] Larvae used for histological determination of internal horizontal mouth dimension.

*[2] and *[3] Larvae used to examine prey size selection.

*[4] Larvae used to examine the effect of prior exposure to a prey species, on subsequent prey selection.

*[5] Larvae used to determine the primary sensory modality involved in feeding behaviour.

2.2.2 Live Feed Production

Large strain rotifers (*Brachionus plicatilis*), *Artemia* nauplii and metanauplii (INVE - *Artemia* Systems, Belgium), or a combination of both, were fed to greenback flounder larvae. Rotifers were obtained from 1000 L semi-continuous production culture tanks maintained in the Department of Aquaculture's Aquatic Centre. These were cultured on a combination diet of micro-algae (*Isochrysis galbana*, *Pavlova lutheri* and *Tetraselmis suecica*), and bakers yeast (0.45 g yeast per $1x10^6$ rotifers, administered twice daily). Rotifers were siphoned from the production tanks as required, collected on a submerged 63 µm screen, rinsed in fresh water and enriched at densities of 200 - 300.ml^{-1} with Nutripak^{Tm} (0.3 g.L^{-1} in seawater), for 6 h , at 22°C. Rotifers were then rinsed thoroughly with seawater, before being added to the larval rearing tanks at a density of 5.ml^{-1}, twice daily.

Artemia cysts were disinfected in a 20 L plastic cone with 200 ppm OCl^-, for 20 minutes, with vigorous aeration at a density of 1 - 2 g.L^{-1}. They were then drained onto a 100 µm screen and rinsed thoroughly before being resuspended in seawater in a hatching cone, and vigorously aerated at 26 - 28°C, for 22 - 24 h. *Artemia* nauplii were then harvested by turning the aeration off and allowing the buoyant cysts to float to the surface. The cysts and hatched *Artemia* nauplii were then separated by placing a black cover over the hatching cone and a light source at the base, so that the photopositive *Artemia* nauplii moved to the base of the cone, where they were drained onto a screen, leaving the buoyant cysts behind. *Artemia* nauplii were then rinsed and either fed out immediately after hatching, or metanauplii were enriched over a 24 h period with Nutripak™, at a density of 150 - 200.ml^{-1} and temperature of 25°C. Enrichment was achieved with two additions of Nutripak™ (each of 0.3 g.L^{-1} seawater, at time 0, and 12 h after commencement of enrichment). During the enrichment period, strong aeration was provided to maintain dissolved oxygen levels above 4 ppm. Enriched *Artemia* were harvested onto a 100 µm screen and were rinsed thoroughly in seawater to remove any residual enrichment media and metabolites, before being fed to the larval culture tanks at a density of 5.ml^{-1}. All *Artemia* were harvested in the morning and those to be kept for the afternoon feed were placed back into clean enrichment media until required.

On-grown *Artemia* were not used for routine larval culture but were used for some experimental protocols. These were obtained from 300 L semi-continuous culture bins, maintained at the Department of Aquaculture.

2.3 Histology

2.3.1 Fixation and Embedding

Five greenback flounder larvae were sampled randomly from larval cohort 1, (1995) on days 3, 4, 5, 6, 8, 9, 12, 13, 17, 20, 24, 26, 30, 34 and 38 post-hatching. Larvae were then terminally anaesthetised in 0.02%, 2-phenoxyethanol, pipetted into 2 ml vials with as little seawater as possible, and fixed overnight at 4°C in a solution of 2% glutaraldehyde in 0.1M phosphate buffer, to which 2 g sucrose was added per 100 ml buffer (method - appendix 1). Larvae were then washed three times in sucrose-0.1M phosphate buffer (10 minutes for each wash), and stored in 70% ethanol at 4°C, prior to morphometric measurement and embedding. Standard length (SL - distance from the rostral tip of the head to the caudal tip of the notochord), of whole fixed larvae was measured using a Zeiss Stemi 2000 dissecting microscope, fitted with an ocular micrometer. Stage of development was assessed according to Crawford (1986). Larvae were then dehydrated in an ethanol series and embedded in JB-4 (Polysciences kit) methyl-methacrylate resin (method: appendix 2). Embedded larvae were serially sectioned (2 μm) in the transverse plane, using a Microm (model HM 340) microtome. Sections were stained with a JB4-Polychrome stain (method - appendix 3), prior to mounting for histological examination.

An additional ten flounder larvae were sampled on each of the days listed above. Larvae were anaesthetised and SL was measured to allow comparison of lengths of fresh and fixed larvae.

Cohort 1 were reared on rotifers and *Artemia* nauplii. Rotifers were introduced twice daily from the time of first feeding, at a density of $5.ml^{-1}$. *Artemia* nauplii were added ($2.ml^{-1}$) along with rotifers, from day 24 post-hatching.

2.3.2 Mouth Gape Determination

Internal horizontal mouth width was measured from transverse histological sections of whole fish larvae (2.3.1), as the distance between Meckel's cartilage on either side of the upper jaw.

2.4 Feeding Behaviour Experiments

2.4.1 Determination of the primary sensory modality involved in the feeding behaviour of greenback flounder larvae.

Greenback flounder larvae from cohort 5, 1996 (see table 2) were reared in a 200 L larval culture tank (refer 2.2.1). Rotifers were introduced twice daily ($5.ml^{-1}$) from the time of first feeding (day 4 post-hatching), and *Artemia* nauplii were added once daily in addition to the rotifers, from day 12 post-hatching, at a density of 1 - $2.ml^{-1}$. Feeding behaviour trials were conducted under two test light regimes: 0 $\mu mol.m^{-2}.s^{-1}$ - absolute darkness; and 5 - 6 $\mu mol.m^{-2}.s^{-1}$ - light intensity at which larvae fed actively in the culture tank. Experiments were conducted in the constant temperature environment of the larval culture room (temperature range: 12°C ± 1°C), on days 12, 15, 18, 21, 24 and 27 post-hatching. The night before each experiment, 30 larvae were transferred from the 200 L larval culture tank, into each of 11, 2.5 L, black test chambers, where larvae were maintained in static seawater culture, for the duration of the experiment (Figure 2). Care was taken in the transfer of larvae to minimize both shock to the larvae, and the introduction of live feed into the test chambers. The chambers were then covered with black-out cloth secured with elastic, and an additional black-out cloth was placed over the top of all the chambers, which were then left undisturbed overnight. There were 5 replicate chambers for each of two "light intensity" treatments, plus an additional chamber, the "gut evacuation control chamber".

The next morning, larvae from the "gut evacuation" control chamber were sampled by quickly decanting the larvae into a light coloured tub in which larvae were easy to see.

Twenty larvae were immediately pipetted onto a glass histological slide and a squash preparation was made by lowering a glass coverslip onto the larvae. This procedure immediately killed the larvae. Larvae were examined under a dissecting microscope to determine whether the previous days food had been digested. When this was established, rotifers were washed through a 200 μm screen and collected on a 100 μm screen (= 100 - 200 μm prey size fraction). Rotifers were then added sequentially to each of the remaining ten chambers, at ten minute intervals, at a density of $2.ml^{-1}$. This resulted in a time delay of 90 minutes between food being added to the first and last test chamber. The order of addition of rotifers was randomly allocated between the treatments so that hunger of larvae did not confound the results. The covers on the 5 - 6 $\mu mol.m^{-2}.s^{-1}$ (light treatment) chambers were removed as feed was added. The 0 $\mu mol.m^{-2}.s^{-1}$ (dark treatment) chambers were left covered and rotifers were added by quickly sliding the cover back from the edge of each chamber, and pouring the food in. This was done under the cover of an additional black-out cloth so that the larvae were never exposed to light. The larvae were then left undisturbed for 1 h, before the larvae from each chamber were quickly decanted into a light coloured tub. Twenty larvae were immediately pipetted onto a glass slide, and a coverslip was lowered on top (squash preparation), allowing examination under a dissecting microscope, for the presence or absence of rotifers in the gut. The sampling procedure lasted approximately 30 seconds, and precluded visual feeding responses by the larvae during the sampling period. Prior to the feeding trials, rotifers were enriched with micro-algae to enhance visualisation of rotifers in the gut of the larvae. Presence/absence criteria were used to assess feeding behaviour because individual rotifers could not be distinguished.

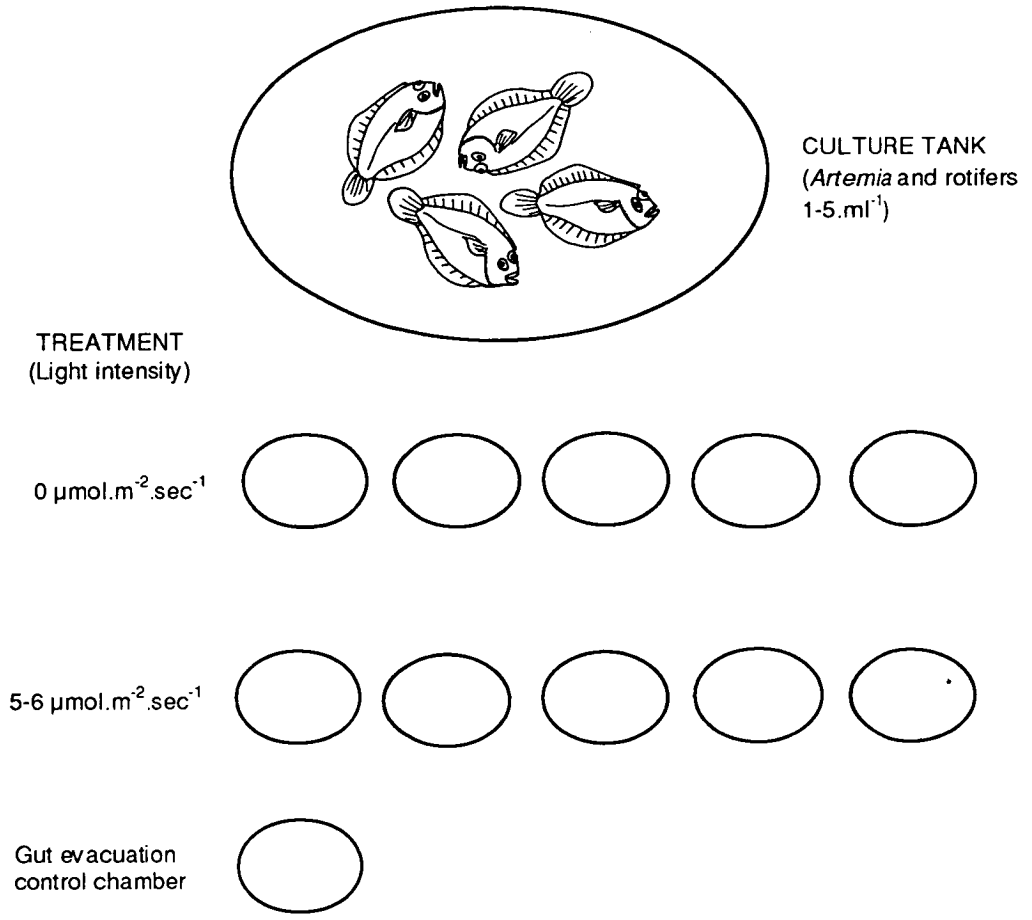


Figure 2 - Diagrammatic representation of the experimental design used to assess the primary sensory modality involved in the feeding behaviour of greenback flounder larvae. Larvae were cultured in a 200 L tank, on a mixed live feed diet of *Artemia* and rotifers. On the day prior to each experiment, 30 larvae were transferred into each of 10 test chambers and a "gut evacuation" control chamber, where they were left undisturbed overnight, in total darkness. On the day of the experiment, 20 larvae from the "gut evacuation" control chamber were sampled to confirm clearance of food from the gut, prior to commencement of the experiment. Rotifers (100-200 µm screened size fraction) were then added to the remaining 10 chambers, at a density of 1 - 2.ml^{-1}, and larvae were left to feed for 1 h, at which time they were examined under a dissecting microscope for presence or absence of food in the gut.

2.4.1.1 Statistical Analyses

A two-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparison of means test, were used to analyse the effect of increasing age on feeding response of larvae. Residual values (replicate means subtracted from treatment means) of arcsin$\sqrt{}$ transformed data were normally distributed (Shapiro-Wilk test, Prob<W = 0.139) for data from the light treatments, but data from the dark treatments were not normally distributed (Shapiro-Wilk test, Prob<W = 0.000). Cochran's test for homogeneity of variance was used to test that variances were equal. On days 21 and 24 post-hatching, *Artemia* were accidentally introduced into the dark treatment chambers. Whilst the difference in feeding response will be discussed, for the purpose of analysis, only data for rotifer consumption was used.

2.4.2 Determination of prey size selected with increasing age in greenback flounder larvae.

2.4.2.1 Cohort 2

Greenback flounder larvae from cohort 2, 1996 (see table 2) were reared in a 200 L larval culture tank (refer 2.2.1). Rotifers were introduced twice daily (5.ml^{-1}), and *Artemia* nauplii once daily (1 -2.ml^{-1}), from the time of first feeding (day 4 post-hatching). Feeding behaviour trials were conducted in the constant temperature environment of the larval culture room (temperature: 12°C ± 1°C; light intensity at the test chamber water surface: 5 - 6 µmol.s^{-1}.m^{-2}), using three screened size fractions of *Artemia* (100 - 200 µm, 300 - 390 µm and 450 - 560 µm), on days 11, 14, 17, 20, 23, 26 and 29 post-hatching. The night before each experiment, 30 larvae were transferred from the 200 L larval culture tank, into each of 16, 2.5 L black test chambers, where larvae were maintained in static seawater culture for the duration of the experiment. There were five replicate chambers for each of the three *Artemia* size fractions (treatments), and one additional chamber (the gut evacuation control chamber), was used to confirm larval gut clearance overnight (Figure 3). Care was taken when transferring larvae to minimize both shock to the larvae, and the introduction of live feed along with the larvae. The chambers were then covered with black-out cloth, and larvae were left undisturbed overnight. In the morning, larvae from the gut evacuation control chamber were quickly decanted into a light coloured tub, in

which the larvae were easy to see. Twenty larvae were then pipetted onto a glass histology slide and a coverslip was lowered onto them. Larvae were examined using a dissecting microscope, to confirm that the previous days food had been fully digested. When this was established, *Artemia* nauplii and on-grown *Artemia* (refer 2.2.2) were washed through 200, 390 and 560 µm screens, and collected on 100, 300 and 450 µm screens, respectively, to generate three discrete screened size fractions of prey (100 - 200 µm, 300 - 390 µm and 450 - 560 µm, respectively). The cloth covers were then removed as *Artemia* of the appropriate size fraction were sequentially introduced into the 15 remaining chambers, at ten minute intervals, at a density of $2.ml^{-1}$. This created an unavoidable delay of 2.5 h between addition of food to the first and last test chamber. The order of addition of *Artemia* between the various treatment test chambers was randomly allocated, so that hunger of larvae in any one treatment did not confound the results. The larvae were then left undisturbed, to feed for 1 h, at which time larvae from each chamber were quickly decanted into a light coloured tub. Twenty larvae were immediately pipetted onto a glass histology slide, and a coverslip was lowered on top (= a squash preparation), allowing immediate examination for the presence or absence of *Artemia* in the gastro-intestinal tract. Absolute numbers of *Artemia* ingested by individual larvae were not determined because in older larvae, *Artemia* were partially digested within the 1 h feeding period, thus precluding counts of individual prey items in the gut, across all ages examined. As a consequence, all feeding responses reported used a presence/absence criteria.

Fifty *Artemia* from each of the three screened size fractions were sampled and measured using a dissecting microscope fitted with an ocular micrometer. Total length (without spines), carapace width, and width with appendages, were recorded to establish absolute dimensions of the prey in each screened size fraction.

2.4.2.2 Cohort 3

Greenback flounder larvae from cohort 3, 1996 (see table 2) were used to repeat the prey size selection experiment described in 2.4.2.1. Larvae were reared in a 200 L larval culture tank using the same feeding protocols as described above, for larvae of cohort 2. Feeding behaviour trials were conducted in the constant temperature environment of the larval culture room (temperature: 12°C ± 1°C; light intensity at the test chamber water surface 5

- 6 $\mu mol.m^{-2}.s^{-1}$), on days 10, 13, 16, 19, 22 and 25 post-hatching. The experimental protocol was not altered from that used for cohort 2 (see Figure 3).

2.4.2.3 Statistical Analyses

Larval feeding responses were analysed using a two-way ANOVA to test for the interaction of prey size ingested and larval age, followed by a Tukey-Kramer means comparison test to analyse the change in feeding response with increasing larval age, for each prey size range. Alpha levels of $P<0.05$ were considered significant. Residual values (replicate means subtracted from treatment means) of arcsin$\sqrt{}$ transformed data were normally distributed (Shapiro-Wilk test, $p>0.05$). Cochran's test for homogeneity of variance was used to test that variances were equal. Data collected prior to days 19 and 20 post-hatching, when larvae started ingesting all three prey size ranges were not included for analysis.

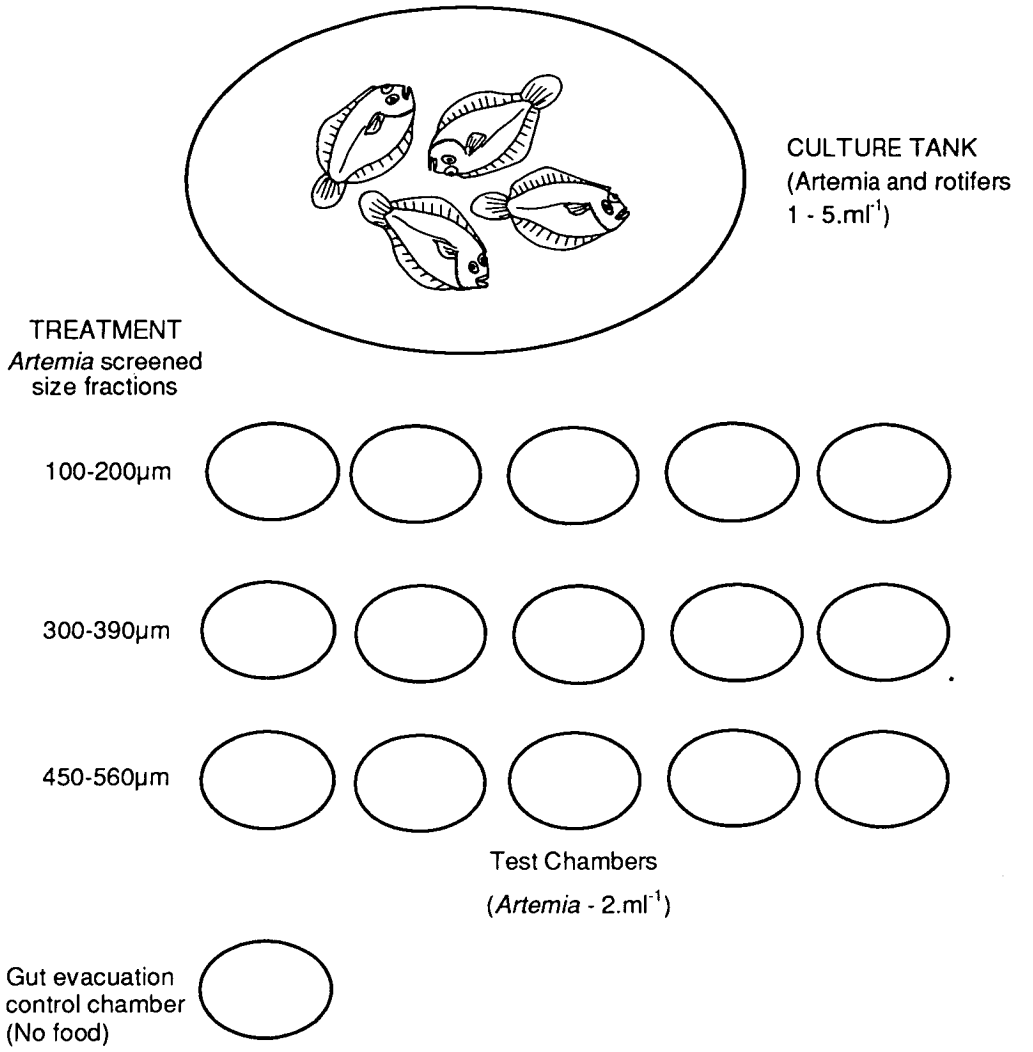


Figure 3 - Diagrammatic representation of the experimental design used to assess prey size selection by greenback flounder larvae. Larvae were reared in a 200 L tank on a mixed live feed diet of *Artemia* and rotifers. On the day prior to each experiment, 30 larvae were transferred into each of 15 test chambers, and a "gut evacuation" control chamber, where they were left undisturbed overnight, in total darkness. On the day of the experiment, 20 larvae from the gut evacuation control chamber were sampled to confirm clearance of food from the gut, prior to commencement of the experiment. *Artemia* (100-200 µm, 300 - 390 µm and 450 - 560 µm screened size fractions) were then added sequentially to the remaining 10 chambers, at a density of 1 - 2.ml^{-1}, and larvae were left to feed for 1 h, at which time they were examined under a dissecting microscope for presence or absence of food in the gut.

2.4.3 Determination of the effect that previous exposure to a prey species has on subsequent prey selection.

Greenback flounder larvae from cohort 4, 1996 (see table 2) were reared in 2 x 200 L larval culture tanks, to form two treatments; Treatment 1, in which larvae were exposed only to rotifers (5.ml^{-1}) from the onset of feeding, and Treatment 2, in which larvae were exposed to both rotifers (5.ml^{-1}) and *Artemia* (1 - 2.ml^{-1}) live feed, from the time of first feeding. Prey selection by larvae from treatment 1 (prior exposure to rotifers only) and treatment 2 (prior exposure to rotifers and *Artemia*), was then examined in feeding trials in which larvae were offered a mixed diet of both rotifers and *Artemia*. In this fashion, *Artemia* were a novel prey for larvae in treatment 1. Feeding behaviour trials were conducted in 2.5 L black test chambers, in the constant temperature environment of the larval culture room (temperature: 12°C ± 1°C; light intensity at the test chamber water surface: 5 - 6 μmol.m^{-2}.s^{-1}), on days 11, 14, 17, 20, 23, 26 and 29 post-hatching. There were five replicates per treatment, plus an additional chamber, the "gut evacuation" control chamber. The night before each experiment, thirty larvae from the appropriate 200 L larval culture tank were stocked into each of the ten test chambers, where larvae were maintained in static culture, for the duration of the experiment (Figure 4). The test chambers were covered with black-out cloth, secured with elastic, and were left undisturbed overnight. Care was taken during transfer of the larvae to minimise both shock to the larvae and introduction of live feed along with the larvae. The next morning, 20 larvae from the "gut evacuation" control chamber were examined using a dissecting microscope, to confirm that the previous days food had been fully digested. Rotifers and *Artemia,* of the same screened size fraction (100 - 200 μm), each at a density of 2.ml^{-1}, were then added sequentially into the test chambers, at ten minute intervals. This resulted in a time delay of 90 minutes between addition of food to the first and last test chamber. The order of addition of live feed between the treatments was randomly allocated between chambers, so that larval hunger in any one treatment did not confound the results. The larvae were then left undisturbed for 1 h before the larvae from each chamber were quickly decanted into a light coloured tub. Twenty larvae were then immediately pipetted onto a glass slide, and a coverslip was lowered onto the larvae (squash preparation), allowing examination for the presence of either rotifers, *Artemia*, or both rotifers and *Artemia*, in the gastro-intestinal tract. Absolute numbers of rotifers and *Artemia* ingested were not

determined, because *Artemia* were partially digested within the 1 h test period in older larvae, and individual rotifers were not resolvable under the light microscope. Therefore, all feeding responses were recorded using a presence or absence criterion. As for feeding experiment 2.4, rotifers were enriched with micro-algae prior to feeding out to enhance visualisation of this prey species within the gut of the larvae.

Fifty each of *Artemia* and rotifers from the 100 - 200 μm screened size fraction, were sampled and measured using a dissecting microscope fitted with an ocular micrometer. Total length (without spines for *Artemia*, with and without eggs for rotifers), carapace width of *Artemia*, lorica width of rotifers, and *Artemia* width with appendages were recorded to establish absolute dimensions of each prey species (see results - Table 6).

2.4.3.1 Statistical Analysis

Larvae were offered two prey species for this experiment which resulted in three possible feeding responses (selection of *Artemia* only, rotifers only, or both *Artemia* and rotifers). As a consequence, the assumption of independence required to run a 2-way ANOVA, was not satisfied. Therefore, data were analysed using a multiple analysis of variance (MANOVA), in conjunction with a canonical distribution analysis to test for the treatment affect on the three possible larval feeding responses, with increasing larval age.

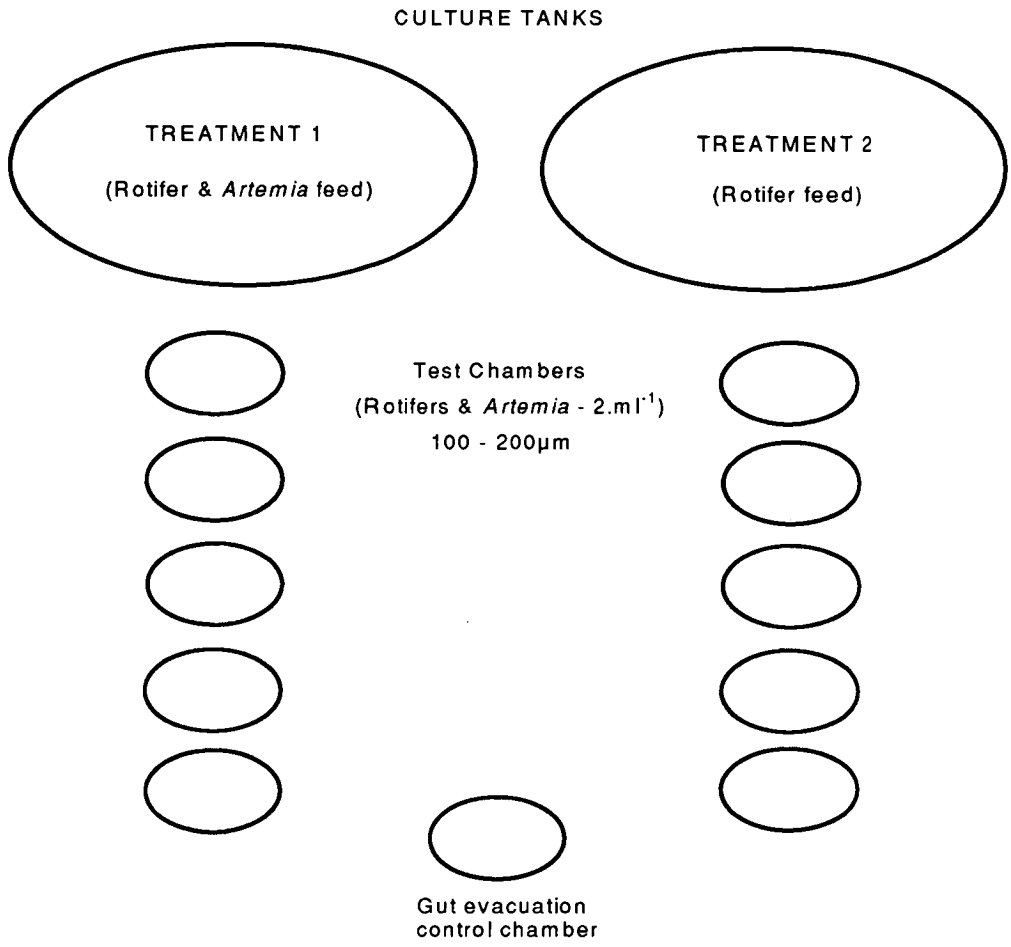


Figure 4 - Diagrammatic representation of the experimental design used to assess the effect of previous exposure to a prey species on the subsequent prey selection by greenback flounder larvae. Larvae were cultured in 2 x 200 L tanks on either rotifers, or a mixed diet of *Artemia* and rotifers. The evening prior to each experiment, 30 larvae were transferred into each of 10 test chambers and a "gut evacuation" control chamber, where they were left undisturbed, overnight, in total darkness. Prior to the experiment starting, 20 larvae from the "gut evacuation" chamber were sampled to confirm overnight clearance of food from the gut. A mixed prey of *Artemia* and rotifers (100 - 200 µm screened size fraction) were then added to the remaining 10 chambers, at a density of 1 - 2.ml^{-1}, and larvae were left to feed for 1 h, at which time they were examined under a dissecting microscope for the presence or absence of food in the gut.

2.6 Larval Growth and Stage of Development

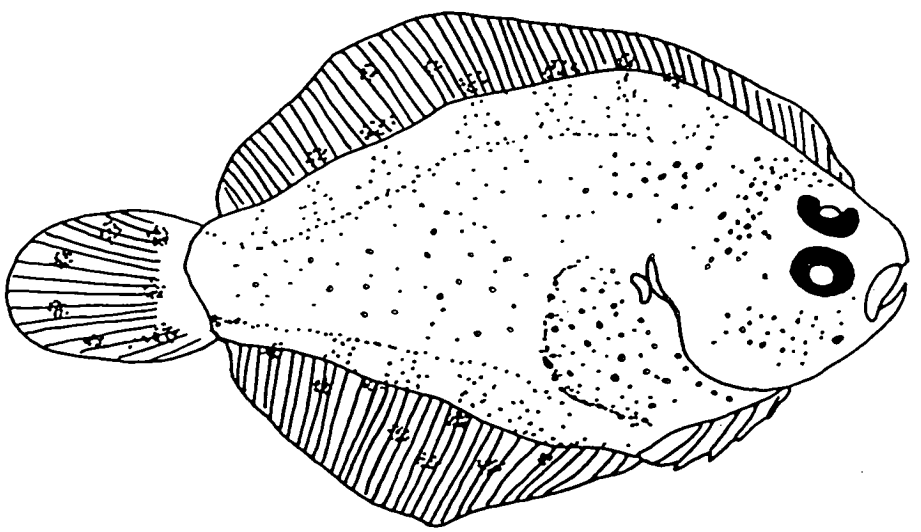
Ten greenback flounder larvae from the larval culture tanks of cohorts 1 - 5, were randomly sampled for morphometric measurements (usually on the day of feeding experiments - refer to Table 3). The larvae were then anaesthetised in 2-phenoxyethanol and examined using a Zeiss Stemi 2000 dissecting microscope, fitted with an ocular micrometer. Standard length (SL: distance from the rostral tip of the head to the caudal tip of the notochord) was measured and stage of development of each larva was assessed, according to the developmental staging system devised by Crawford (1986), for this species.

Table 3 - Ages at which SL was measured and developmental stages were assessed for larval cohorts 1 - 5.

Larval Cohort	Age at which larval length and developmental stage were assessed.
1	3, 4, 5, 6, 8, 9, 12, 13, 17, 21, 24, 26, 30, 34, 38
2	4, 8, 11, 14, 17, 20, 23, 26, 29
3	8, 10, 13, 16, 19, 22, 25
4	7, 11, 14, 17, 20, 23, 26, 29
5	6, 9, 12, 15, 18, 21, 24, 27

CHAPTER 3

RESULTS



3.1 Determination of the primary sensory modality involved in the feeding behaviour of greenback flounder larvae.

The ability of greenback flounder larvae to feed in the light differed markedly from the feeding ability of larvae maintained in the dark, with a consistently higher proportion of larvae feeding in the light on all days tested (Figure 5). There was a significant difference in the proportion of fish feeding in the light on day 12 post-hatching (66%), compared with the proportion of larvae feeding in the light on day 27 post-hatching (96%) (two-way ANOVA, df = 5, n = 30, Prob.>F = 0.0002), with a trend of increasing feeding performance from days 15 - 24 post-hatching. The proportion of larvae that fed in the dark on rotifers was consistently low, ranging from 2% on day 15, to a maximum of 5% on day 18 post-hatching and did not change significantly during ontogeny (two-way ANOVA, df = 5, n = 30, Prob.>F = 0.883) (not withstanding non-normal distribution of data). On days 21 and 24 post-hatching, 26% and 34% of larvae respectively, had fed on *Artemia* nauplii, when these prey items were inadvertently transferred into the test chambers, along with the larvae. The proportion of larvae feeding on *Artemia* in the dark on days 21 and 24 post-hatching, cannot however be compared with the proportion of larvae feeding on rotifers on these days, because larvae potentially had 16 h over night to feed on *Artemia*, compared with 1 h to feed on rotifers during the rotifer feeding trial.

Mean SL (± SE) of larvae from cohort 5 increased from 2.78 mm ± 0.03 on day 6 post-hatching to 5.4 mm ± 0.05 on day 27 post-hatching (Figure 6), as described by the regression equation, $y = 1.62 + 0.13*x$ ($r^2 = 0.95$). This represented a daily growth increment of 0.13 mm/day from 6 - 27 days of age.

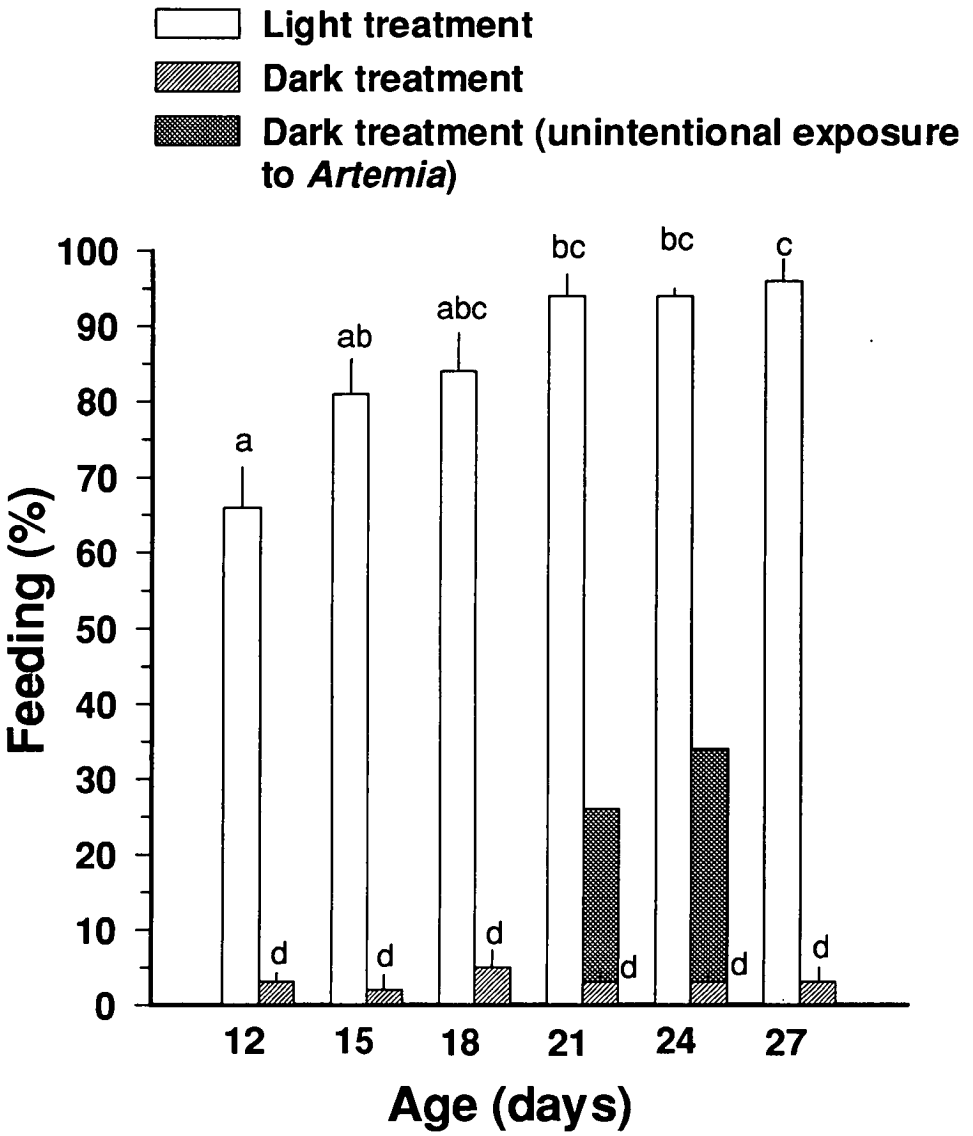


Figure 5 - Percentage of greenback flounder larvae feeding on rotifers at two light intensities; 0 $\mu mol.m^{-2}.s^{-1}$ (dark treatment - diagonal hatched bars), and 5-6 $\mu mol.m^{-2}.s^{-1}$ (light treatment - no fill bars), with increasing age of larvae. Dark cross-hatched bars (days 21 and 24 of age) indicate feeding incidence in the dark on *Artemia* nauplii, which were inadvertently transferred into the test chambers along with the larvae. Values are means (± SE) of five replicates (n = 100). Means sharing a common superscript are not significantly different.

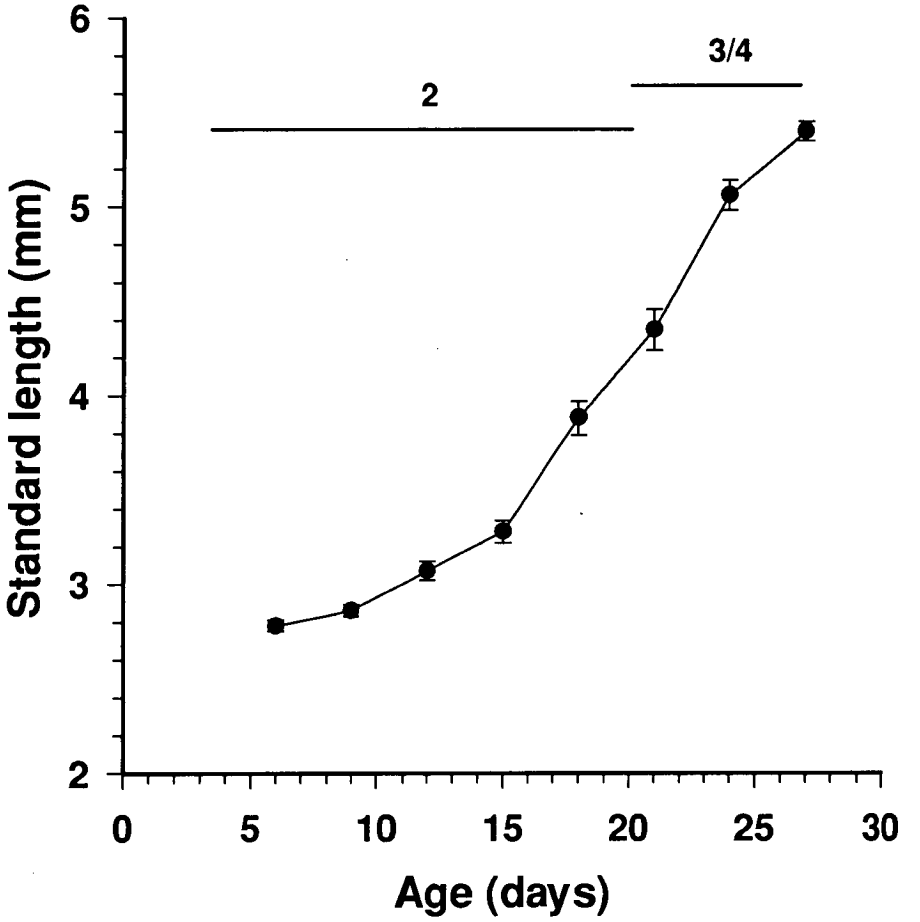


Figure 6 - Change in mean SL (± SE, n = 10) of greenback flounder larvae from cohort 5, with increasing age. Vertical bars denote SE and horizontal bars denote greenback flounder larvae developmental stage: 2 - yolk resorbed, notochord straight, 3 /4 - notochord flexion and migration of left eye (Crawford, 1986).

3.2 Assessment of prey size selection by greenback flounder larvae with increasing age.

3.2.1 Larval Feeding Responses

3.2.1.1 Cohort 2

On days 11, 14 and 17 post-hatching, a relatively low proportion of larvae consumed only the smallest screened *Artemia* prey size (100 - 200 μm). However, from day 20 onwards, larvae displayed a sudden increased ability to ingest all three *Artemia* size fractions presented (Figure 7). From 20 days of age, the interaction of prey size fraction and larval age, had a significant effect on the feeding response of larvae (two-way ANOVA, df = 6, n = 60, Prob>F = 0.035). Prey size, irrespective of larval age, significantly effected larval feeding success from 20 days of age (one-way ANOVA, df = 2, n = 60, Prob>F = 0.000), with the poorest feeding response observed for the largest prey size range, on all days. Within each screened prey size fraction, there was a significant effect of larval age on the feeding response of larvae (one-way ANOVA, df = 3, n = 60, Prob>F = 0.000). The proportion of larvae that consumed the two smaller prey size ranges (100 - 200 μm and 300 - 390 μm), increased significantly on day 26 post-hatching (Figure 8a and b). However, although the proportion of larvae able to ingest the largest prey size range (450 - 560 μm) increased with increasing larval age, the increase was not significant (Figure 8c).

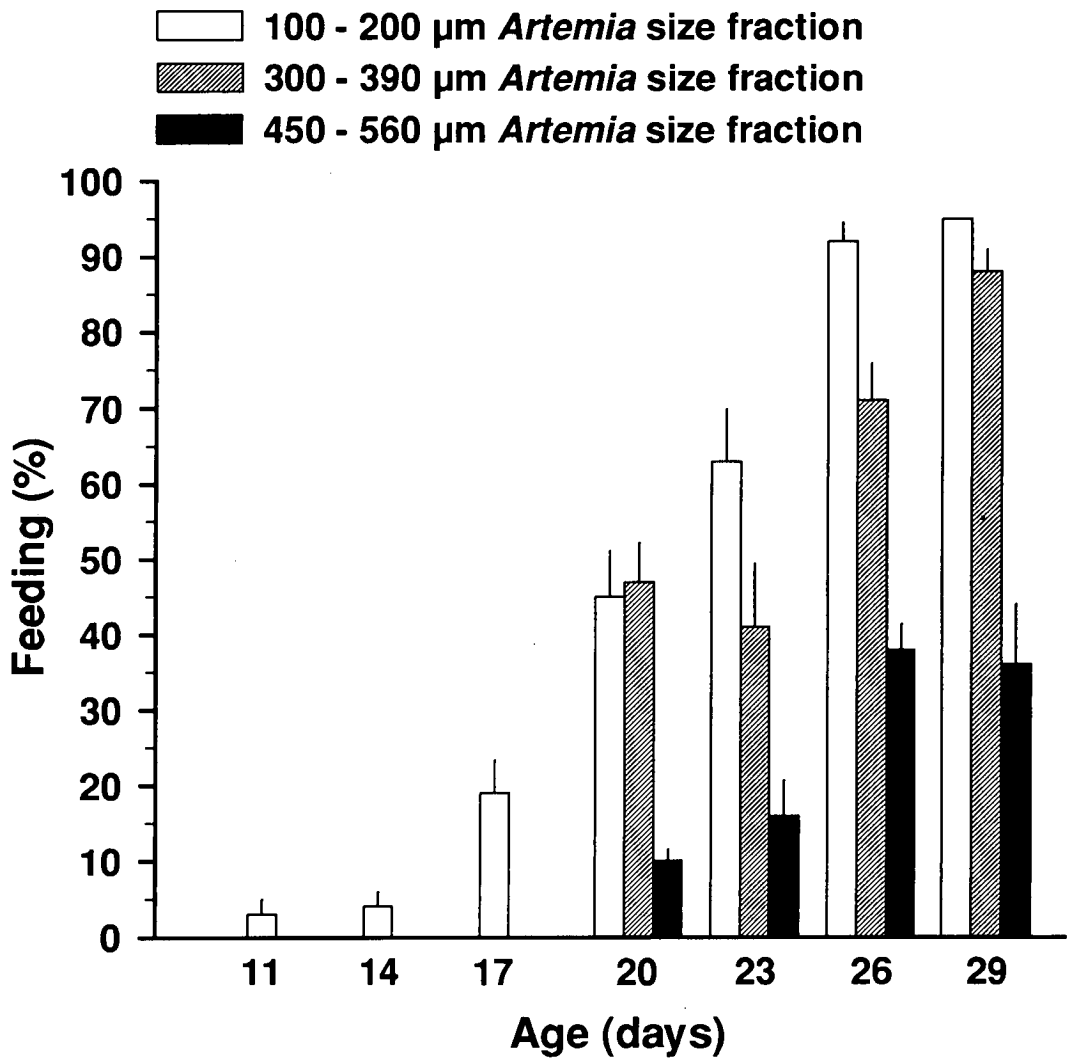
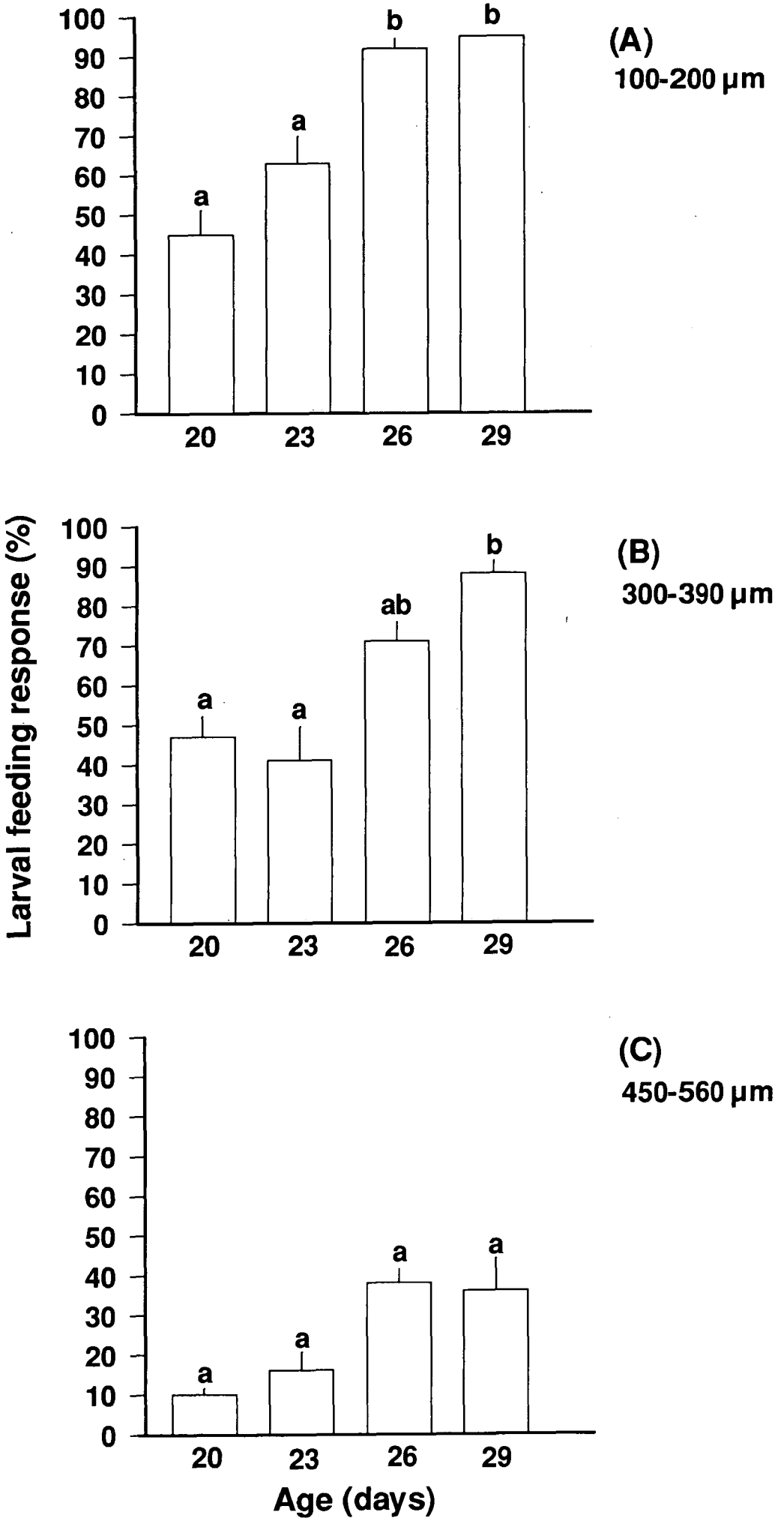


Figure 7 - Percentage of greenback flounder larvae feeding on each of three discrete size fractions of *Artemia* (100-200 µm, 300-390 µm, and 450-560 µm screen size), with increasing age of larvae. Values are means (± SE) of five replicates (n = 100).


(A)
100-200 µm
(B)
300-390 µm
(C)
450-560 µm
Larval feeding response (%)
Age (days)
100
90
80
70
60
50
40
30
20
10
0
20
23
26
29
a
a
b
b
a
a
ab
b
a
a
a
a

3.2.1.2 Cohort 3

The feeding response of greenback flounder larvae from cohort 3, when offered *Artemia* of three discrete size fractions, was similar to that observed for cohort 2. On days 13 and 16 post-hatching, relatively low proportions of larvae consumed only the smallest screened prey size range (100 - 200 μm) (Figure 9). However, from day 19 onwards, larvae displayed a sudden increased ability to ingest all three prey size ranges. From 19 days of age, the interaction of prey size fraction and larval age, had a significant effect on the feeding response of larvae (two-way ANOVA; df = 4, n = 45, Prob>F = 0.001). Prey size, irrespective of larval age, significantly influenced larval feeding success from day 19 onwards (one-way ANOVA, df = 2, n = 45, p = 0.000). The highest feeding response was observed for the smallest prey size range (100 - 200 μm), and the lowest feeding response was observed for the largest prey size range (450 - 560 μm) on all days. Within each screened prey size fraction, larval age had a significant effect on the feeding response of larvae (one-way ANOVA, df = 2, n = 45, Prob>F = 0.000). The latter reflects a general trend of increasing consumption of increasing prey size with increasing age of larvae. On day 19, less than 50% of larvae fed within each prey size range. There was a significant increase in the proportion of larvae able to ingest the 100 - 200 μm *Artemia* prey size fraction on day 22 post-hatching, and a significant increase in the proportion of larvae able to ingest the 300 - 390 μm prey size fraction on both days 22 and 25 post-hatching (Figure 10a, b). The proportion of larvae able to ingest the largest prey size range increased significantly only in 25 day old larvae (Figure 10c). By day 25, the feeding response for the two smallest prey size fractions approached 100%, whereas the feeding response for the largest prey size was 50%.

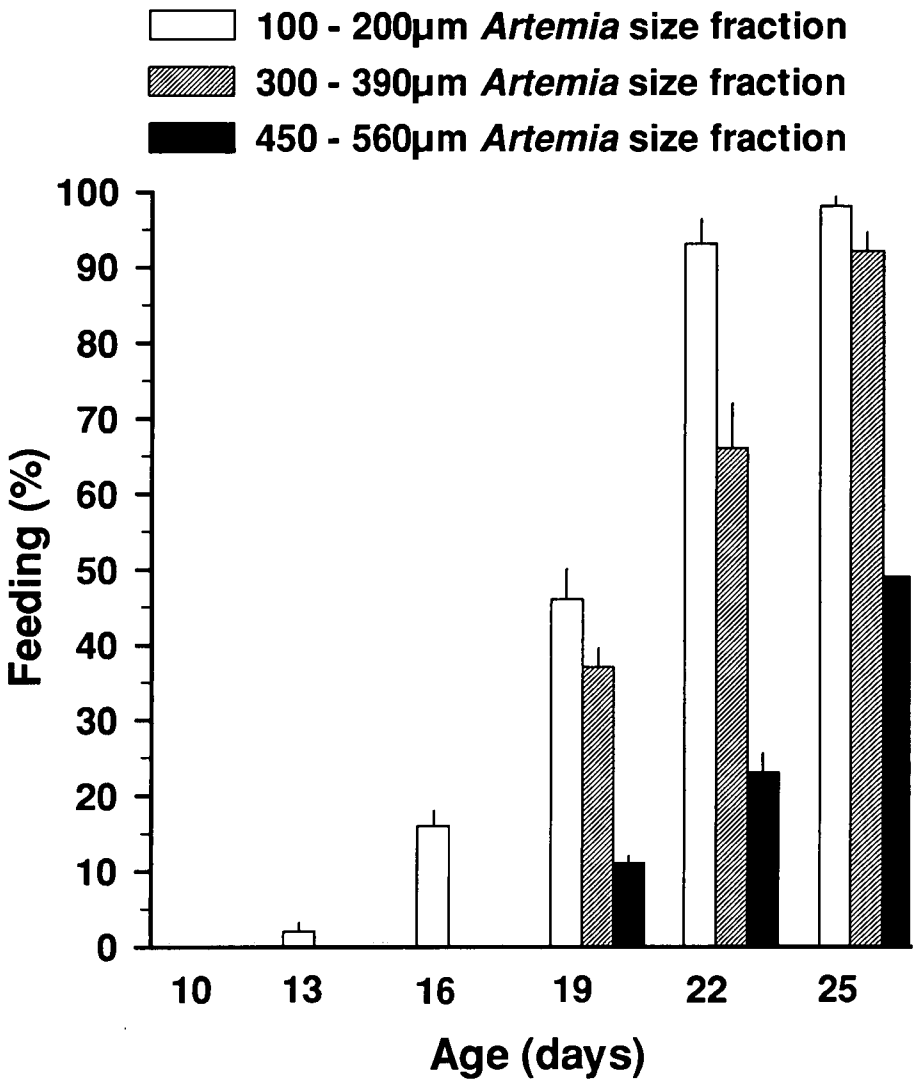
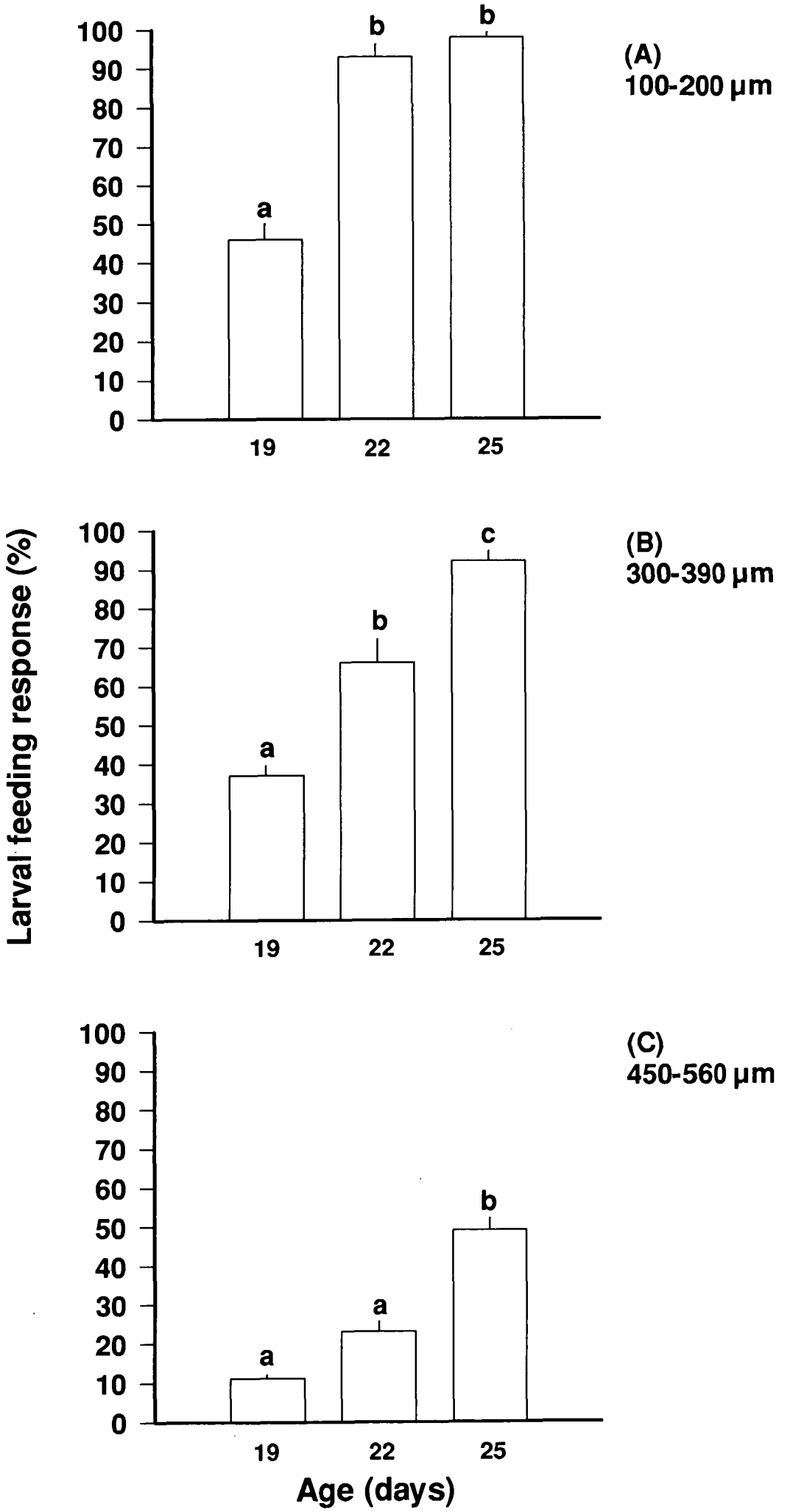


Figure 9 - Percentage of greenback flounder larvae feeding on each of three discrete size fractions of *Artemia* (100-200 µm, 300-390 µm, and 450-560 µm screen size) with increasing age of larvae. Values are means (± SE) of five replicates (n = 100).


Larval feeding response (%)
100
90
80
70
60
50
40
30
20
10
0
a
b
b
19
22
25
(A)
100-200 µm
a
b
c
(B)
300-390 µm
a
a
b
(C)
450-560 µm
Age (days)

3.2.2 Estimation of Larval Gape

Mean, internal horizontal mouth width (± SE) of larvae from cohort 1, determined from serial transverse histological sections (Figures 11a-d), increased from 236.25 μm ± 4.47 in 5 day old larvae, to 627.5 μm ± 31.9, in 38 day old larvae (Figure 12 and Table 4). The SL (mean ± SE) of live, anaesthetised larvae from cohort 1, increased from 2.43 mm ± 0.03 on day 3, to 6.94 mm ± 0.16, on day 38 post-hatching (Figure 13). No adjustment for fixation shrinkage was made to histologically derived morphometric measurements, because there was a close correlation between the mean SL of live and fixed larvae, from cohort 1 (Figure 14). Linear regressions and 95% confidence limits of the change in mean horizontal mouth width, with both increasing age and SL of larvae, are provided in Figure 15. The ratio of larval mouth width : SL of larvae, ranged from 0.087 in 5 and 38 day old larvae, to a maximum of 0.1 in 30 day old larvae (Table 4).

Table 4 - Mean horizontal mouth width (± SE, n=5) obtained from transverse histological sections of whole greenback flounder larvae (cohort 1), mean standard length (SL), and the ratio of larval mouth width : SL, with increasing age and developmental stage of larvae.

Age (days)	Mean SL (mm)	Developmental stage	Mouth width (μm) ± SE	Ratio of mouth width : SL
5	2.72	2	236.25 ± 4.47	0.087
8	2.8	2	267.5 ± 9.59	0.096
12	2.85	2	273.75 ± 5.97	0.096
17	3.28	3/4	317.51 ± 12.09	0.097
24	5.04	3/4	455 ± 37.52	0.09
30	5.1	3/4	512.5 ± 28.94	0.1
38	7.23	3/4	627.5 ± 31.9	0.087

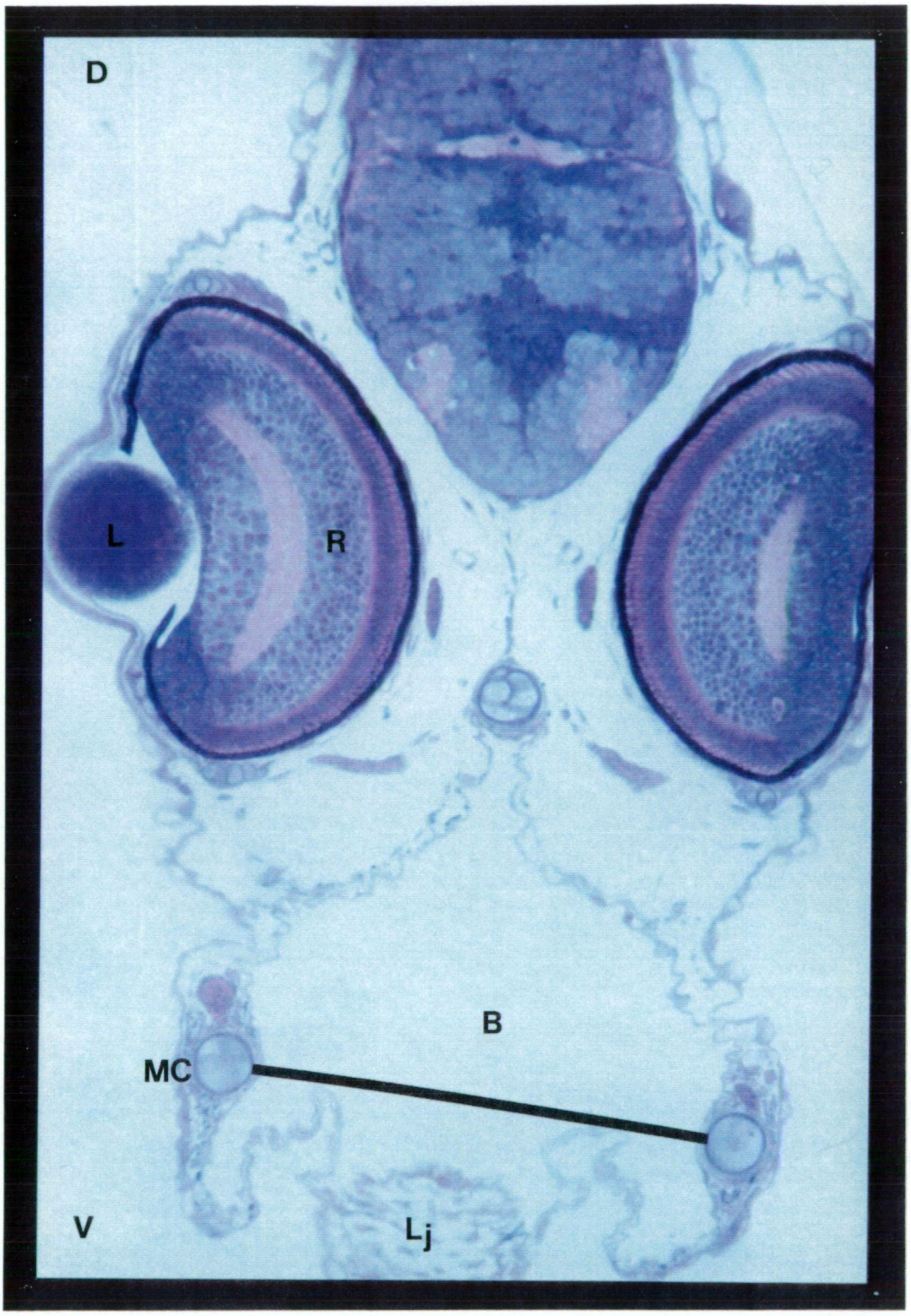


Figure 11a - Photomicrograph of a transverse histological section of a 5 day old (2.72 mm SL) stage 2 greenback flounder larva, indicating position of horizontal mouth width measurement (black bar = 180 µm) was taken as the distance between Meckel's Cartilage on either side of the upper jaw. D - Dorsal, B - buccal cavity, L - lens of right eye, Lj - lower jaw, MC - Meckel's Cartilage, R - retina of right eye, V - ventral.

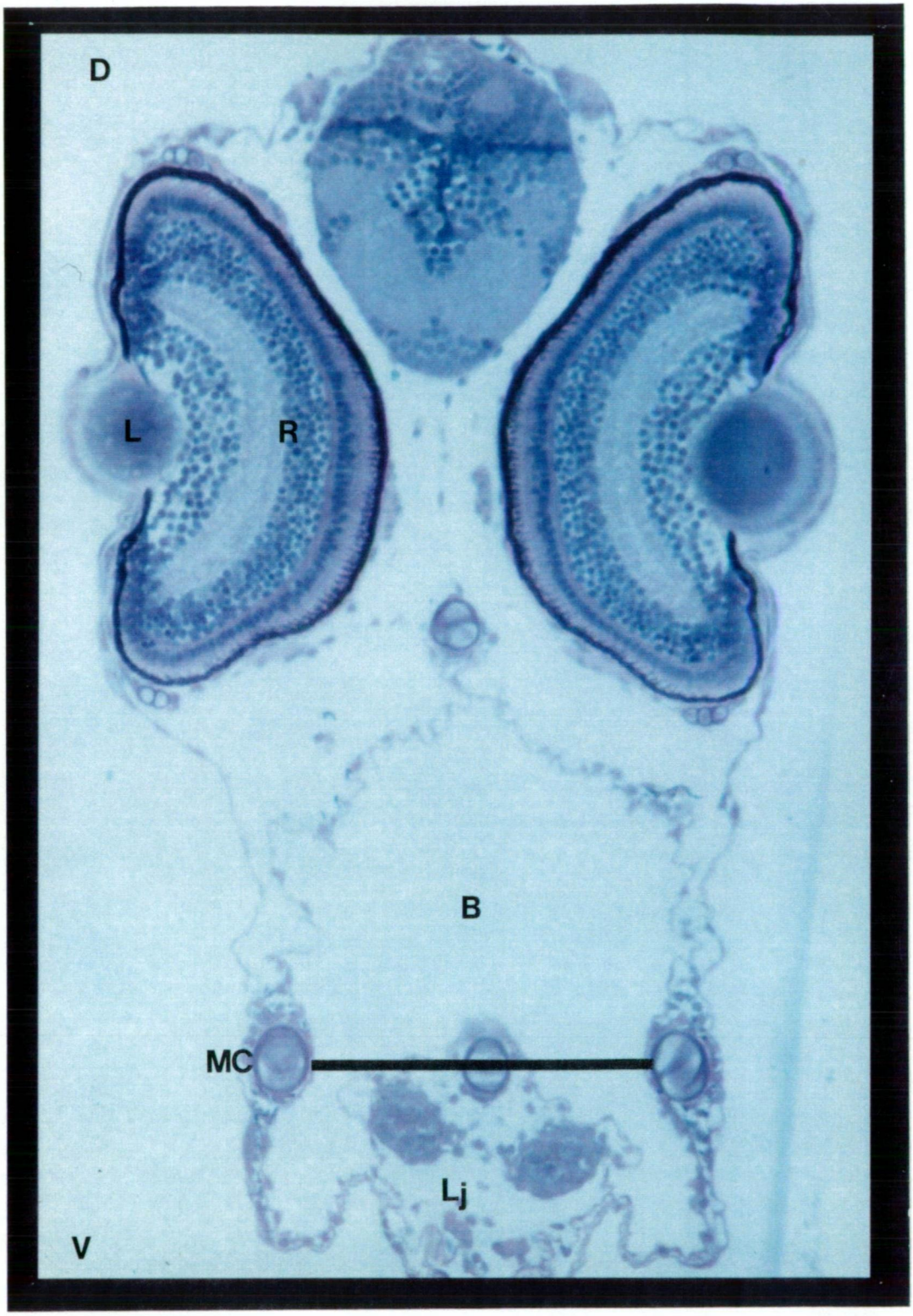


Figure 11b - Photomicrograph of a transverse histological section of a 12 day old (2.85 mm SL) stage 2 greenback flounder larva, indicating position of horizontal mouth width measurement (black bar = 138 μm) was taken as the distance between Meckel's Cartilage on either side of the upper jaw. D - Dorsal, B - buccal cavity, L - lens of right eye, Lj - lower jaw, MC - Meckel's Cartilage, R - retina of right eye, V - ventral.

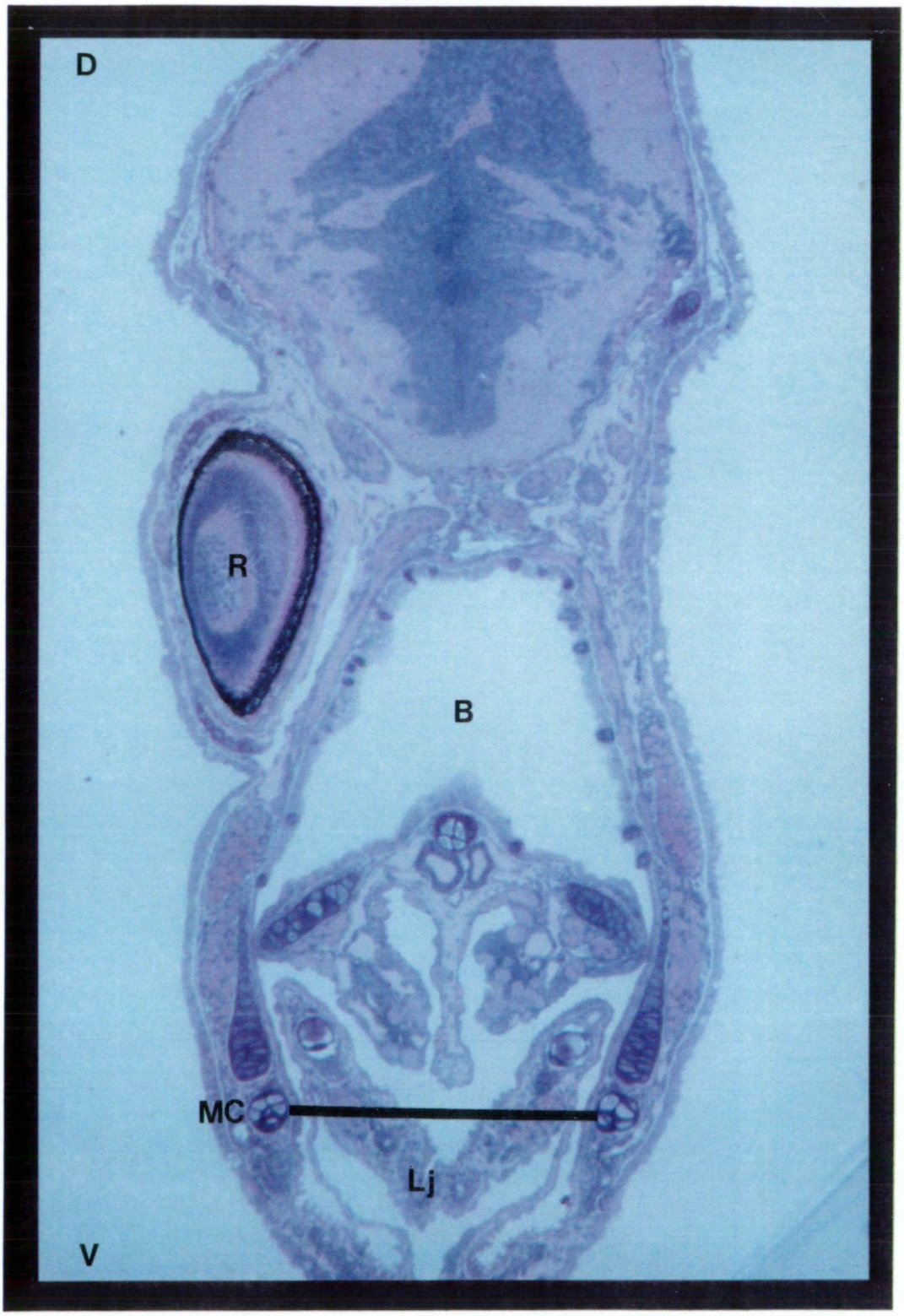


Figure 11c - Photomicrograph of a transverse histological section of a 24 day old (5.04 mm SL) stage 3/4 greenback flounder larva, indicating position of horizontal mouth width measurement (black bar = 270 μm) was taken as the distance between Meckel's Cartilage on either side of the upper jaw. D - Dorsal, B - buccal cavity, Lj - lower jaw, MC - Meckel's Cartilage, R - retina of right eye, V - ventral.

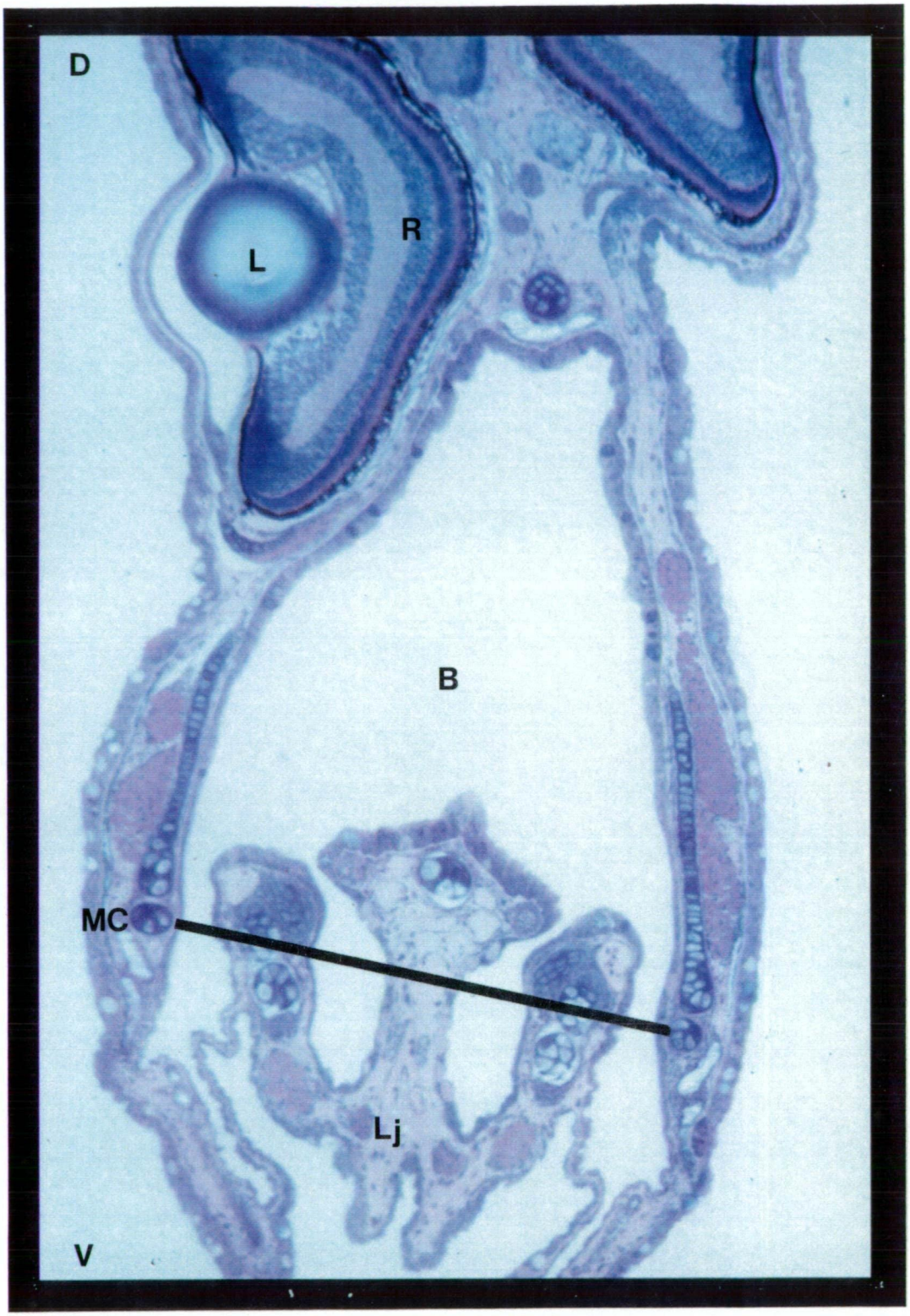


Figure 11d - Photomicrograph of a transverse histological section of a 30 day old (5.1 mm SL) stage 3/4 greenback flounder larva, indicating position of horizontal mouth width measurement (black bar = 395 μm) was taken as the distance between Meckel's Cartilage on either side of the upper jaw. D - Dorsal, B - buccal cavity, L - lens of right eye, Lj - lower jaw, MC - Meckel's Cartilage, R - retina of right eye, V - ventral.

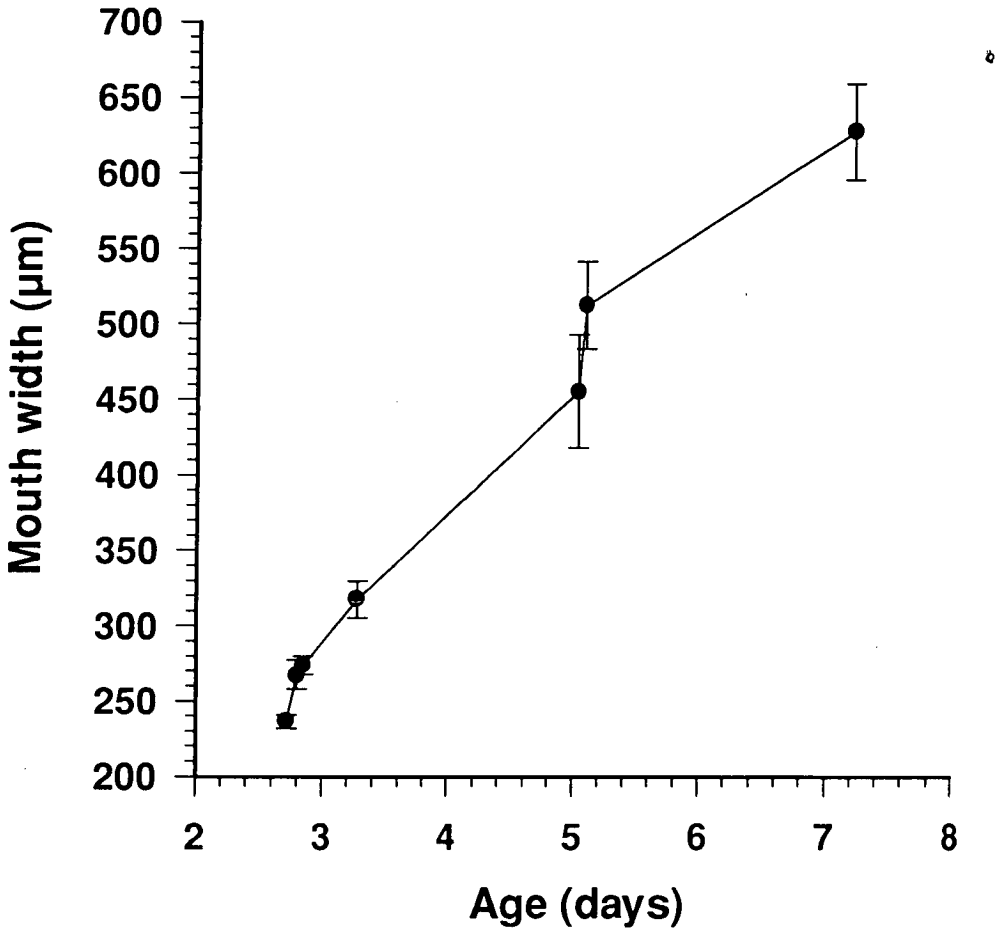


Figure 12 - Change in mean horizontal mouth width (± SE, n = 5), obtained from transverse histological sections of whole greenback flounder larvae, with increasing age of larvae from cohort 1. Vertical bars denote SE.

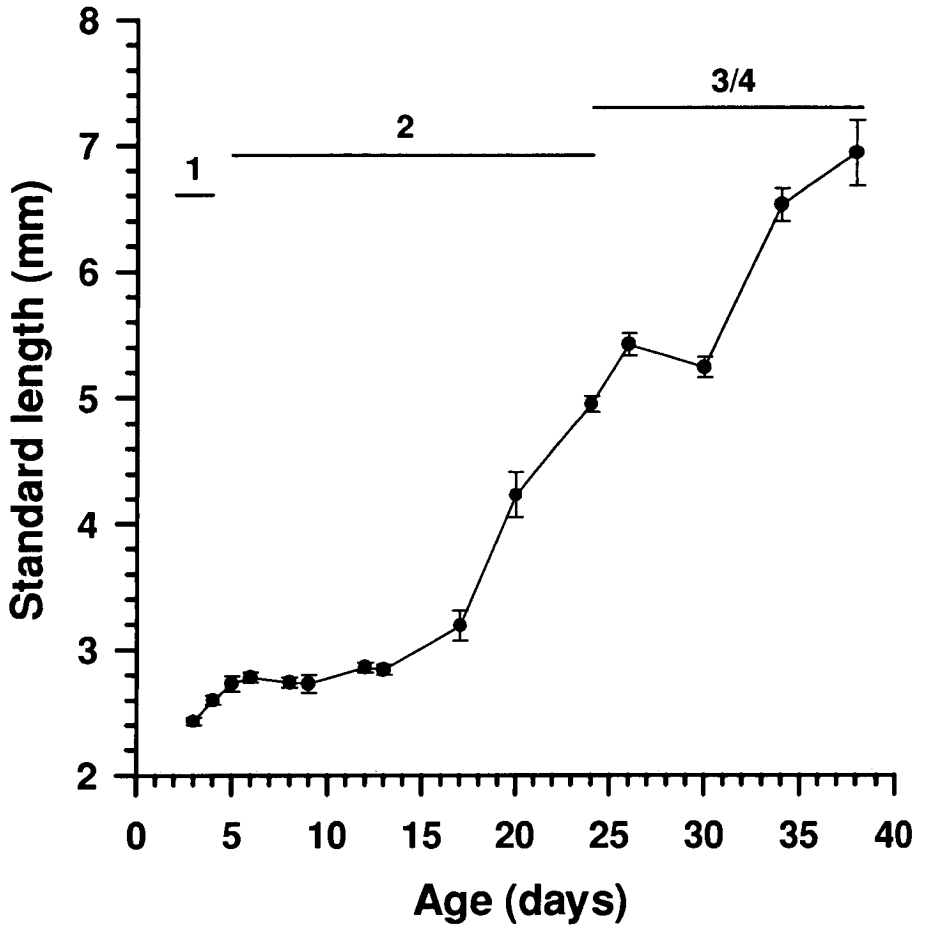


Figure 13 - Change in mean SL (± SE, n = 10) of greenback flounder larvae with increasing age, from cohort 1. Vertical bars denote SE and horizontal bars denote greenback flounder larvae developmental stage: 1 - yolk sac, 2 - yolk resorbed, notochord straight, 3/4 - notochord flexion and migration of left eye (Crawford, 1986).

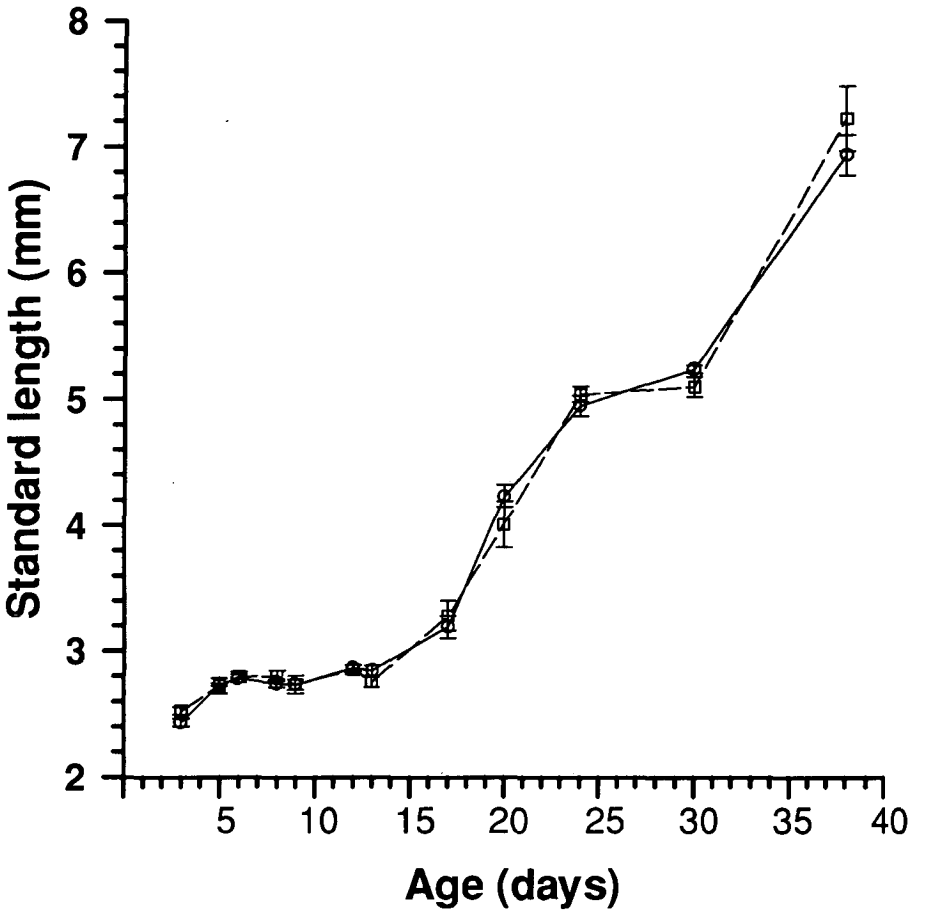


Figure 14 - Change in mean SL (± SE) of greenback flounder larvae from cohort 1, with increasing age of larvae. The solid line denotes SL of live, anaesthetised larvae (n = 10), and the broken line denotes SL of fixed larvae which were used for histological measurement of horizontal mouth width.

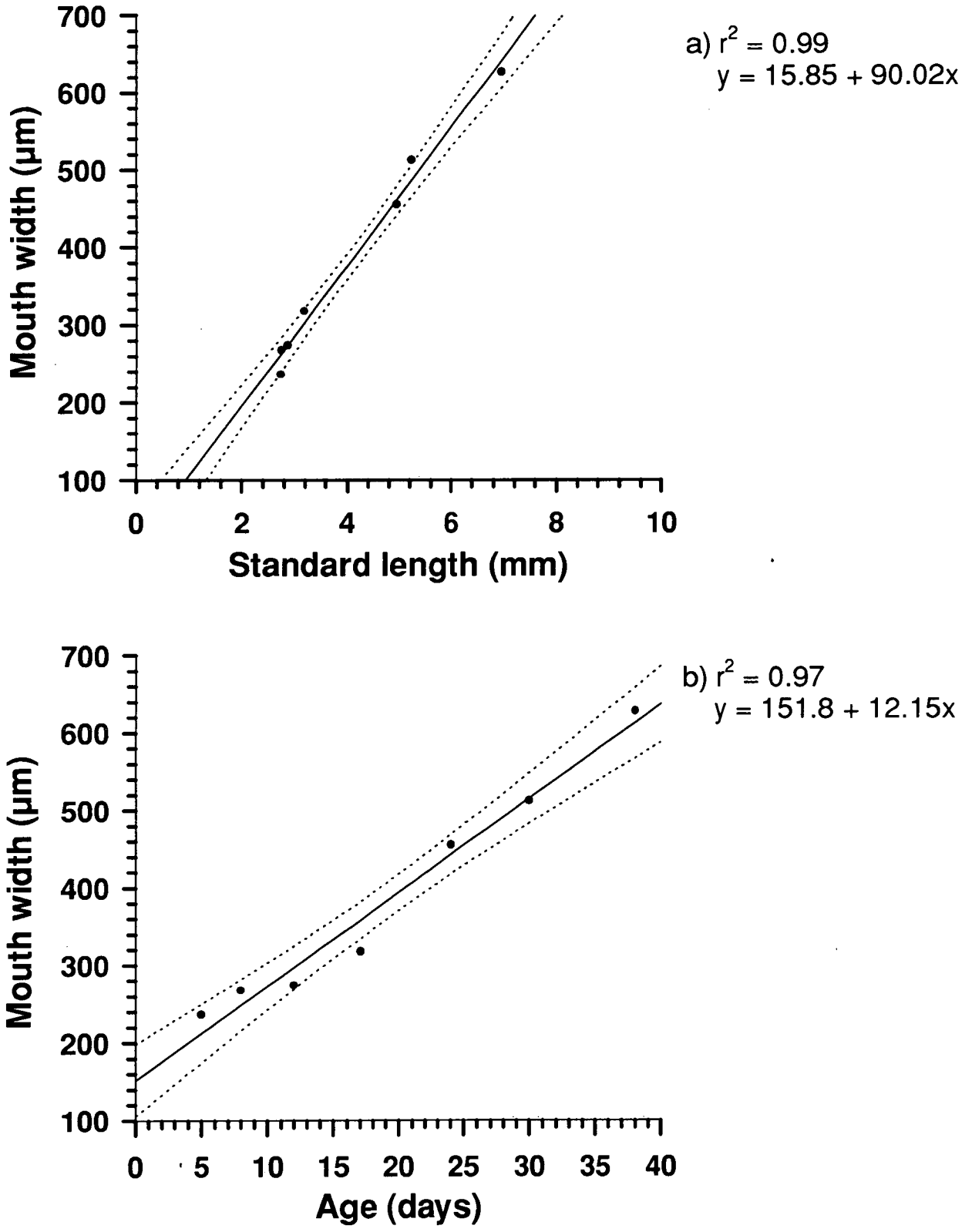


Figure 15 - Regressions of mean horizontal mouth width of larvae (n = 5) derived from serial transverse histological sections of greenback flounder larvae, with increasing standard length of larvae (a), and age of larvae (b), for cohort 1. Dotted lines denote 95% confidence limits for the regression lines.

3.2.3 Growth Profiles

SL (mean ± SE) of larvae from cohort 2 increased from 2.63 mm ± 0.03 on day 4, to 5.58 mm ± 0.07 on day 29 post-hatching (Figure 16a). Standard length (mean ± SE) of cohort 3 increased from 2.96 mm ± 0.03 on day 8, to 5.24 mm ± 0.11 on day 25 post-hatching (Figure 16b). Regression curve fits of change in SL with increasing age of live, anaesthetised larvae from cohorts 2 and 3 (larvae used for behavioural experiments to assess prey size selection by greenback flounder larvae), and cohort 1 (larvae for mouth width determination) are described by the equations; $y = 1.75 + 0.14*x$ ($r^2 = 0.97$), $y = 1.61 + 0.15*x$ ($r^2 = 0.96$), and $y = 1.72 + 0.13*x$ ($r^2 = 0.93$), respectively, representing growth increments of 0.14 mm, 0.13, and 0.13 mm/day, respectively (Figure 17).

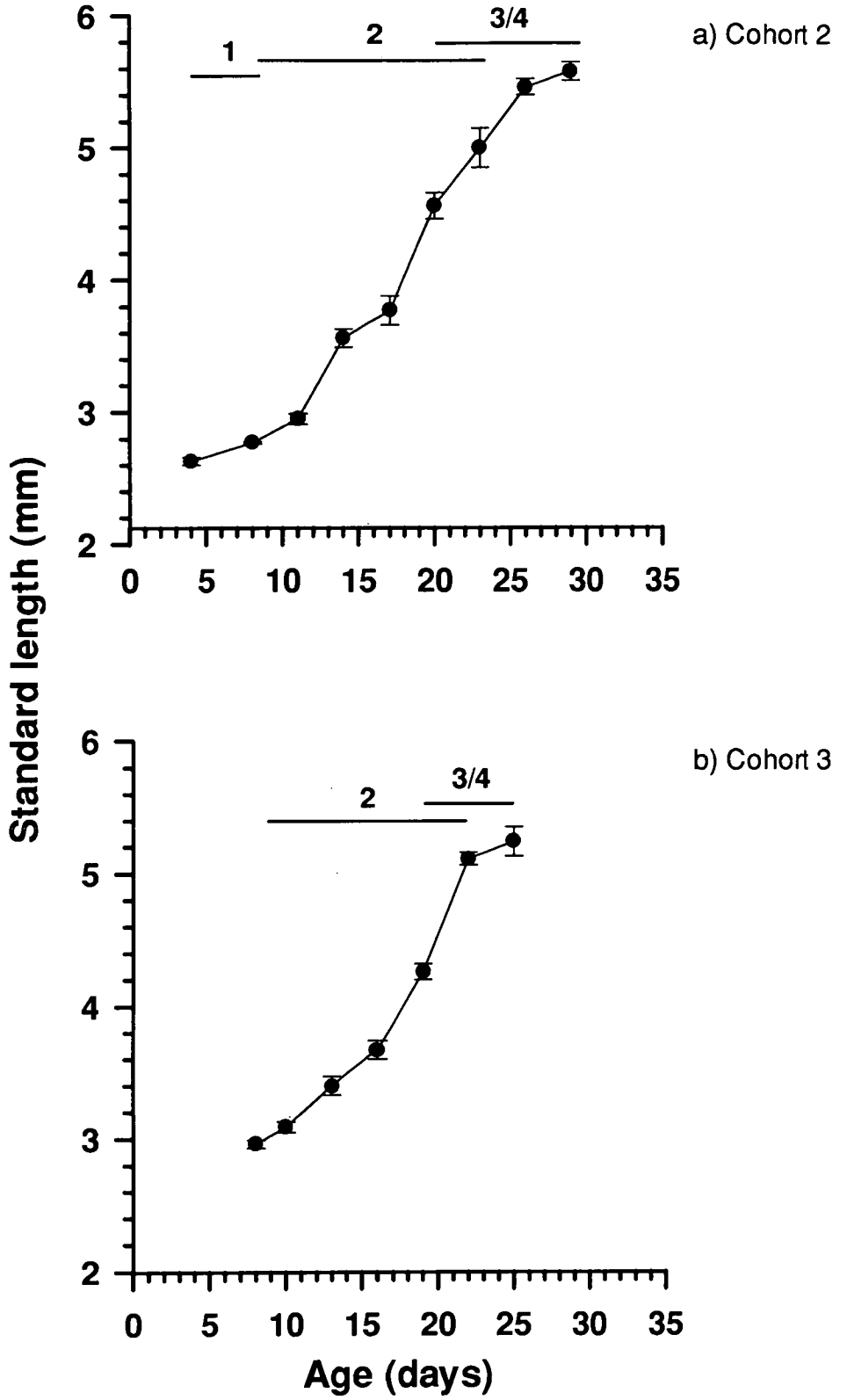


Figure 16 - Change in mean SL (± SE, n = 10) of greenback flounder larvae with increasing age in cohort 2 (a) and cohort 3 (b). Vertical bars denote SE and horizontal bars denote developmental stage of greenback flounder larvae, as described by Crawford (1986).

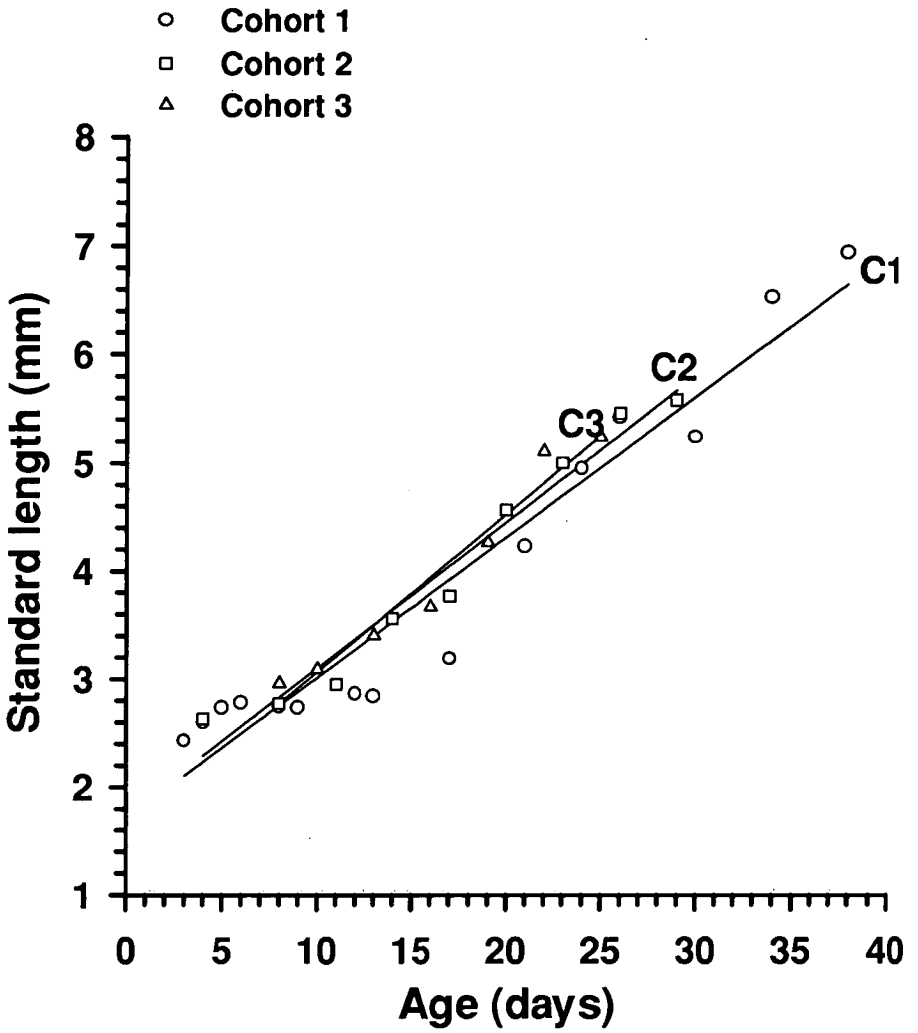


Figure 17 - Regression of SL (mm) against age of greenback flounder larvae for larval cohorts 1 (C1 - $y = 1.72 + 0.13x$, $r^2 = 0.93$), 2 (C2 - $y = 1.75 + 0.14x$, $r^2 = 0.97$), and 3 ($y = 1.61 + 0.15x$, $r^2 = 0.96$). Symbols are mean values: n = 15 for cohort 1, combining data for fresh and fixed larval lengths and n = 10 for cohorts 2 and 3.

3.2.4 Artemia Morphometrics

Morphology and absolute dimensions of *Artemia* used to examine prey size selection in greenback flounder larvae, changed considerably between the small and largest *Artemia* size fractions (Table 5 and Figure 18). Total length of *Artemia* increased from a mean of 470 μm (± 30) to 2250 μm (± 270), in the 100 - 200 and 450 - 560 μm screened size fractions, respectively. *Artemia* carapace width (without appendages), increased from a mean of 186 μm (± 20) to 360 μm (± 40), from the smallest size to the largest screened size fraction, whereas total width (with appendages) increased from 580 μm (± 50) to 1730 μm (± 200) from the smallest to the largest screened size fractions. Carapace width of *Artemia* provided the closest correlation with screen mesh sizes

Table 5 - Screened size fractions of live *Artemia* prey used to examine prey size selection in greenback flounder larvae, and corresponding mean (± SE, n = 50) dimensions (μm) of *Artemia* (total length without spines, carapace width and carapace width with appendages).

Screen mesh size (μm)	**Mean total length (μm)**	**Mean carapace width (μm)**	**Mean width with appendages (μm)**
100 - 200	470 ± 30	186 ± 20	580 ± 50
300 - 390	1430 ± 140	340 ± 30	1280 ± 220
450 - 560	2250 ± 270	360 ± 40	1730 ± 200

3.2.5 Ratio of Artemia Size : Larval Gape

The upper ninety-five percentile confidence limits from the regression describing the change in histologically determined mouth width with increasing SL of larvae from cohort 1 (Figure 15a), were used to determine the upper limit of horizontal mouth width for larvae of cohorts 2 and 3 (used for behavioural experiments which determined prey size selection with increasing age) (refer Appendix 4). The latter were used to determine the ratio of *Artemia* carapace width, TL, and width with appendages: larval mouth width for all ages of greenback flounder in cohorts 2 and 3 tested for prey size selection (Table 6). The ratio of *Artemia* carapace width : larval mouth width (CW:MW) was <1 for all ages of greenback flounder larvae, and all screened size fractions of *Artemia* which had been

consumed by larvae of cohorts 2 and 3 (Table 6). The ratio of *Artemia* carapace width with appendages : larval mouth width (CWA:MW) was consistently greater than unity for all ages of greenback flounder larvae and all screened size fractions of *Artemia* which had been consumed by larvae of cohorts 2 and 3 (Table 6). The ratio of *Artemia* total length : larval mouth width (TL:MW) was consistently >1 for *Artemia* size fractions 300 - 390 µm and 450 - 560 µm, for all ages of larvae in cohorts 2 and 3 which had consumed *Artemia* of these size fractions (Table 6). In those instances where larvae had ingested the 100 - 200 µm size fraction of *Artemia*, the ratio of *Artemia* TL:MW was >1 for cohort 2 larvae from 11 - 20 days of age and <1 in cohort 2 larvae from 23 - 29 days of age (Table 6a). In those instances where larvae had ingested the 100 - 200 µm size fraction of *Artemia*, t-the ratio of *Artemia* TL:MW was >1 for cohort 3 larvae from 10 - 19 days of age and <1 for cohort 3 larvae from 22 - 25 days of age (Table 6b).

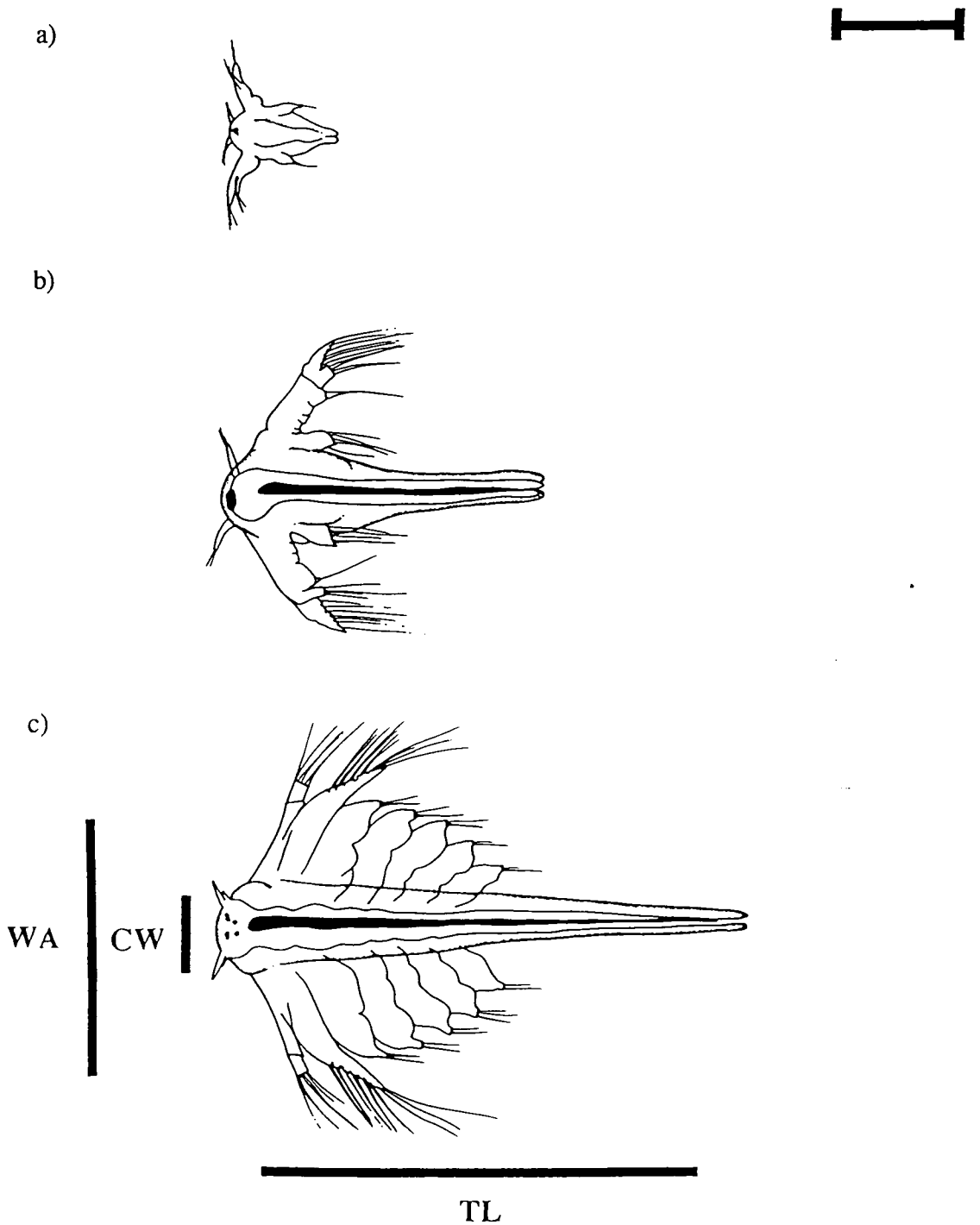


Figure 18 - Camera lucida diagrams of a representative *Artemia* from each of the three screened size fractions used to examine prey size selection of greenback flounder larvae: a) *Artemia* nauplii - 100 - 200 µm size fraction; b & c) ongrown *Artemia* instars - 300 - 390 µm and 450 - 560 µm, size fractions respectively. CW - carapace width, TL - total length, WA - width with appendages. Scale bar top right = 0.5 mm.

Table 6 - The ratio of *Artemia* prey size (TL - total length, CW - carapace width, CWA - carapace width with appendages) to larval mouth width, with increasing age of greenback flounder larvae from a) cohort 2 and b) cohort 3. Ratios not in bold text denote size fractions of *Artemia* which were ingested by larvae. Ratios in bold text denote size fractions of *Artemia* not ingested by greenback flounder larvae.

Table 6a - cohort 2

Age - days	***Artemia* size fractions**								
	100 - 200 µm			**300 - 390 µm**			**450 - 560 µm**		
	TL/MW	CW/MW	CWA/MW	TL/MW	CW/MW	CWA/MW	TL/MW	CW/MW	CWA/MW
11	1.57	0.6	1.93	**4.77**	**1.13**	**4.27**	**7.5**	**1.2**	**5.77**
14	1.35	0.55	1.67	**4.11**	**0.98**	**3.68**	**6.47**	**1.03**	**4.97**
17	1.26	0.5	1.55	**3.83**	**0.91**	**3.43**	**6.03**	**0.97**	**4.64**
20	1.06	0.45	1.31	3.22	0.81	2.88	5.07	0.86	3.9
23	0.97	0.42	1.19	2.94	0.75	2.63	4.62	0.79	3.55
26	0.89	0.38	1.10	2.7	0.69	2.42	4.25	0.73	3.27
29	0.87	0.36	1.08	2.66	0.64	2.38	4.18	0.68	3.22

Table 6b - Cohort 3.

Age - days	***Artemia* size fractions**								
	100 - 200 µm			**300 - 390 µm**			**450 - 560 µm**		
	TL/MW	CW/MW	CWA/MW	TL/MW	CW/MW	CWA/MW	TL/MW	CW/MW	CWA/MW
10	1.48	0.58	1.82	**4.5**	**1.07**	**4.03**	**7.08**	**1.13**	**5.44**
13	1.38	0.55	1.71	**4.21**	**1.0**	**3.76**	**6.62**	**1.06**	**5.09**
16	1.28	0.51	1.58	**3.90**	**0.93**	**3.49**	**6.13**	**0.98**	**4.71**
19	1.12	0.44	1.39	3.42	0.81	3.06	5.38	0.86	4.14
22	0.95	0.38	1.17	2.89	0.69	2.59	4.55	0.73	3.50
25	0.91	0.36	1.13	2.78	0.66	2.49	4.37	0.70	3.36

3.3 Determination of the effect that previous exposure to a prey species has on subsequent prey selection.

Greenback flounder larvae selected either rotifers only, *Artemia* only, or a mixture of rotifers and *Artemia*. Feeding responses of larvae which had previous experience with both rotifers and *Artemia* (A&R treatment) were markedly different from feeding responses of larvae which had previously experienced only rotifers (R treatment), and to which *Artemia* were a novel prey species (Figure 19). Prior feeding experience had a significant effect on subsequent larval prey selection (MANOVA: Pillai's trace statistic, df = 39, F = 5.9279, Prob. = 0.000). Results from a canonical discriminate analysis (CDA) confirmed a difference in prey selection of A&R treatment larvae, compared to prey selection by R treatment larvae (Figure 20). Canonical variate 1 explained 69% of the variation in larval feeding response, whereas canonical variate 2 explained 26.9% of the variation in larval feeding response. The variation between the proportion of larvae that fed in the A&R treatment and the R treatment along canonical variate 1, was largely due to the proportion of larvae that ingested *Artemia*. The variation in feeding response between the A&R treatment and the R treatment along canonical variate 2, was largely due to the proportion of larvae that ingested only rotifers. In addition, the variation between the two treatments along both canonical variate 1 and 2, was due to a lesser degree, to the proportion of larvae that ingested both *Artemia* and rotifers (CDA axes variation was attributed to the same factors in all CDA plots).

A high proportion of R treatment larvae (between 50% - 80%) consistently fed on rotifers only (Figure 19a). CDA confirmed the strong selection for rotifers by R treatment larvae (refer figure 20 - CDA reduced plot). No R treatment larvae fed on *Artemia* prior to 14 days-of-age, at which time 7% of larvae ingested both *Artemia* and rotifers. Fewer than 50% of R treatment larvae selected both *Artemia* and rotifers on any one day thereafter. On day 29 post-hatching there was little difference in the proportion of R treatment larvae selecting rotifers only, and those selecting both prey species. On only two occasions, days 20 and 26 post-hatching, did a small percentage of R treatment larvae select only *Artemia* (5 and 1%, respectively).

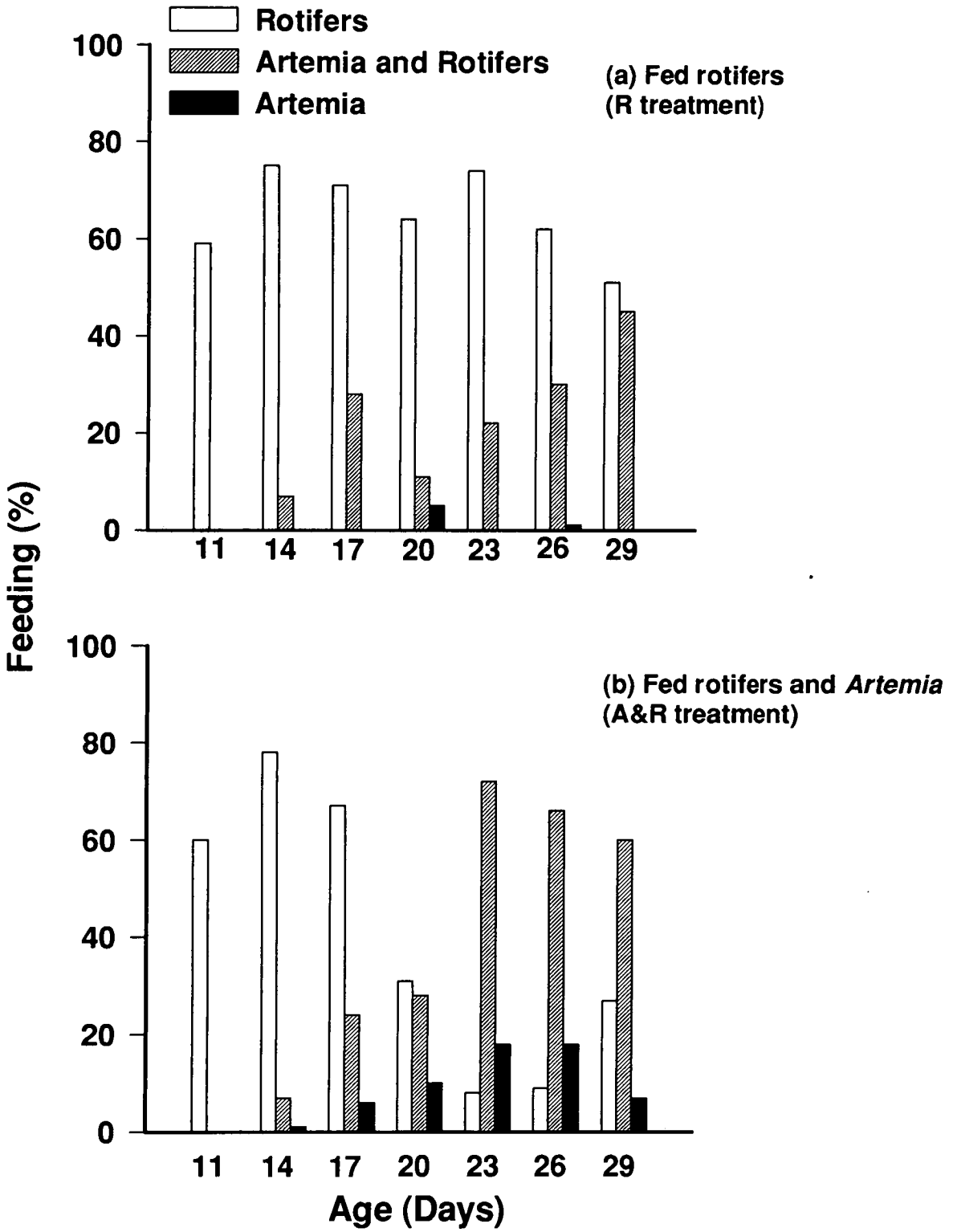


Figure 19 - Percentage of greenback flounder larvae feeding on rotifers only (no bar fill), rotifers and *Artemia* (cross-hatched bars), and *Artemia* only (black bars), in a) larvae which had previous exposure to only rotifer prey - R treatment, and b) larvae which had previous exposure to both rotifers and *Artemia* - A&R treatment, prior to feeding trials.

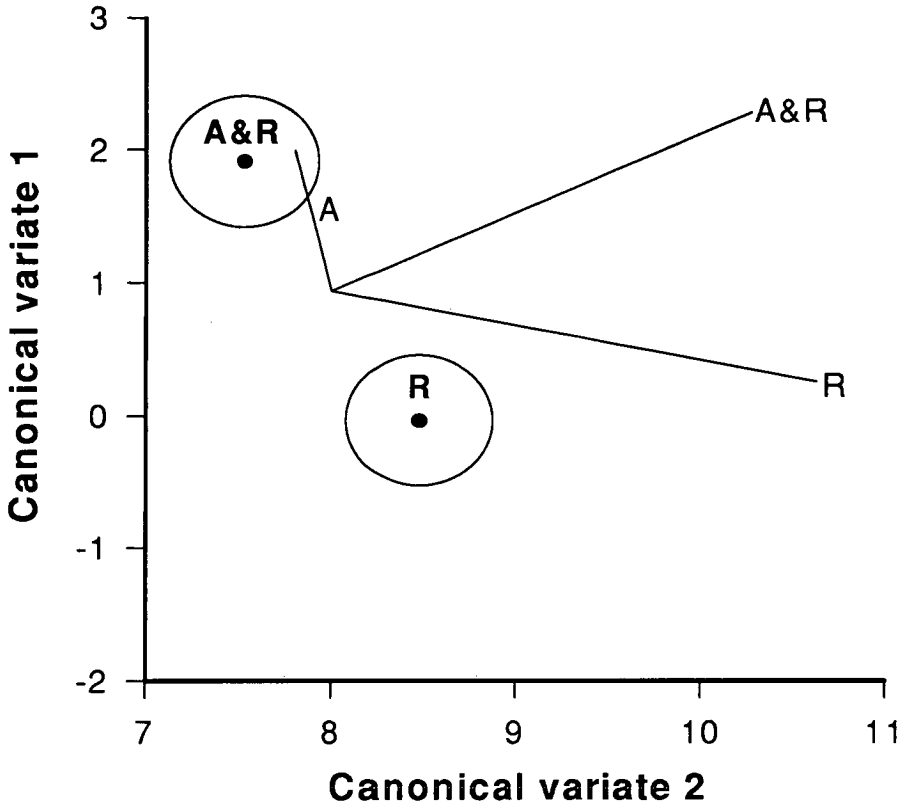


Figure 20 - Canonical distribution analysis reduced plot showing the effect of prior experience to *Artemia* and rotifers (A&R treatment) and only rotifers (R treatment), on subsequent prey selection with increasing age of greenback flounder larvae. Biplot ray A was most strongly associated with canonical variate 1 which explained 69% of the variation in larval feeding response between the two treatments. Biplot ray R was most strongly associated with canonical variate 2 which explained 26.5% of the variation in larval feeding response between the two treatments. Biplot ray A&R was associated with both canonical variates 1 and 2. Circles indicate 95% confidence ellipses for A&R and R treatment larvae. (Biplot rays: A - *Artemia*, R - rotifers, A&R - *Artemia* and rotifers).

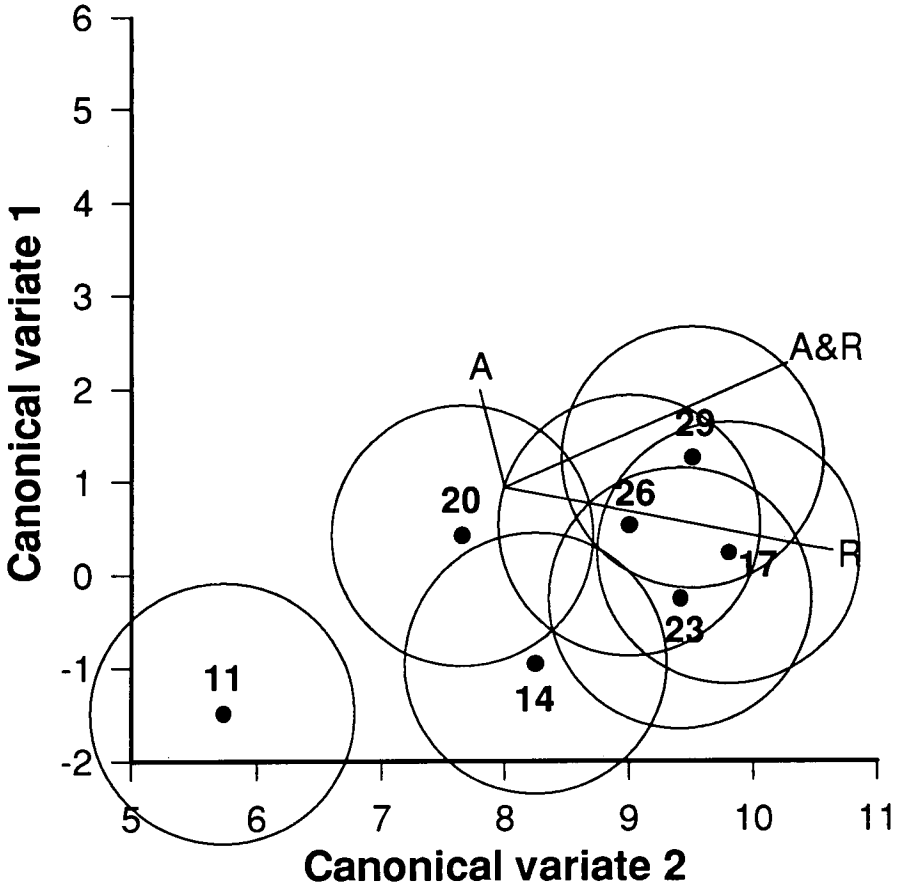


Figure 21 - Canonical distribution analysis reduced plot of the feeding response of larvae that had prior experience of rotifers, but to which *Artemia* were a novel prey. Biplot ray A was most strongly associated with canonical variate 1 which explained 69% of the variation in larval feeding response. Biplot ray R was most strongly associated with canonical variate 2 which explained 26.5% of the variation in larval feeding response. Biplot ray A&R was associated with both canonical variates 1 and 2. Circles indicate 95% confidence ellipses and numbers indicate larval age. (Biplot rays: A = *Artemia*, R = rotifers, A&R = *Artemia* and rotifers).

Eleven day old A&R treatment larvae, like R treatment larvae, selected only rotifers. From 11 - 17 days post-hatching, a high proportion of A&R treatment larvae (60 - 80%) selected rotifers only (Figure 19b). The proportion of A&R treatment larvae selecting only rotifers decreased thereafter, never exceeding 31% of larvae. On day 14 post-hatching, a small number of A&R treatment larvae (1%) selected only *Artemia*. The proportion of larvae selecting only *Artemia* increased thereafter to 18% in 26 day-old-larvae, but then decreased to 7% in 29 day old larvae. CDA which examined the effect of prior exposure to *Artemia* and rotifers on subsequent prey selection by larvae, confirmed an increasing preference by A&R treatment larvae for *Artemia* only, with increasing age of larvae (refer figure 22 - reduced CDA plot).

No larvae from the A&R, or R treatments, ingested *Artemia* prior to 14 days of age. Larvae started to ingest both rotifers and *Artemia* at the same age; however, a small number of A&R treatment larvae also started to select *Artemia* only. With the exception of 17 day old larvae in the R treatment group, a higher proportion of A&R treatment larvae selected both *Artemia* and rotifers, when compared to the feeding response of R treatment larvae.

3.3.1 Growth Profile

Standard length (mean ± SE) of larvae from cohort 4 increased from 2.81 mm ± 0.06 in 7 day old R and A&R treatment larvae to a maximum of 5.53 mm ± 0.12 and 5.69 mm ± 0.10 in 29 day old R and A&R treatment larvae, respectively (Figure 23). Regression curve fits of change in SL with increasing age of larvae are described by the equations; $y = 1.97 + 0.13x$ ($r^2 = 0.99$), and $y = 1.87 + 0.13x$ ($r^2 = 0.99$), respectively, representing an average daily growth increment of 0.13 mm/day for R treatment and A&R treatment larvae, respectively (Figure 24).

3.3.2. Prey Morphometrics

Mean lorica width of rotifers and mean carapace width of *Artemia* in the 100 - 200 µm screened size fraction, were similar (184 µm ± 2 and 186 µm ± 20, respectively); however, mean total length of rotifers was smaller (278 µm ± 3) than mean total length of *Artemia* (470 µm ± 30), even when eggs attached to rotifers were taken into account (380 µm ± 4) (Table 7).

Table 7 - Means (± SE, n = 50) of *Artemia* and rotifer total length, carapace width of *Artemia*, lorica width of rotifers, and carapace width plus appendages of *Artemia*, in 100 - 200 μm screened size fractions of *Artemia* and rotifers.

Screen mesh size (μm)	**Total length (μm)**	**Carapace width (*Artemia*) and lorica width (rotifers) (μm)**	**Width with appendages (μm)**
100 - 200, rotifers	278 ± 3 (380 ± 4 with eggs attached)	184 ± 2	n/a
100 - 200, *Artemia*	470 ± 0.03	186 ± 20	580 ± 50

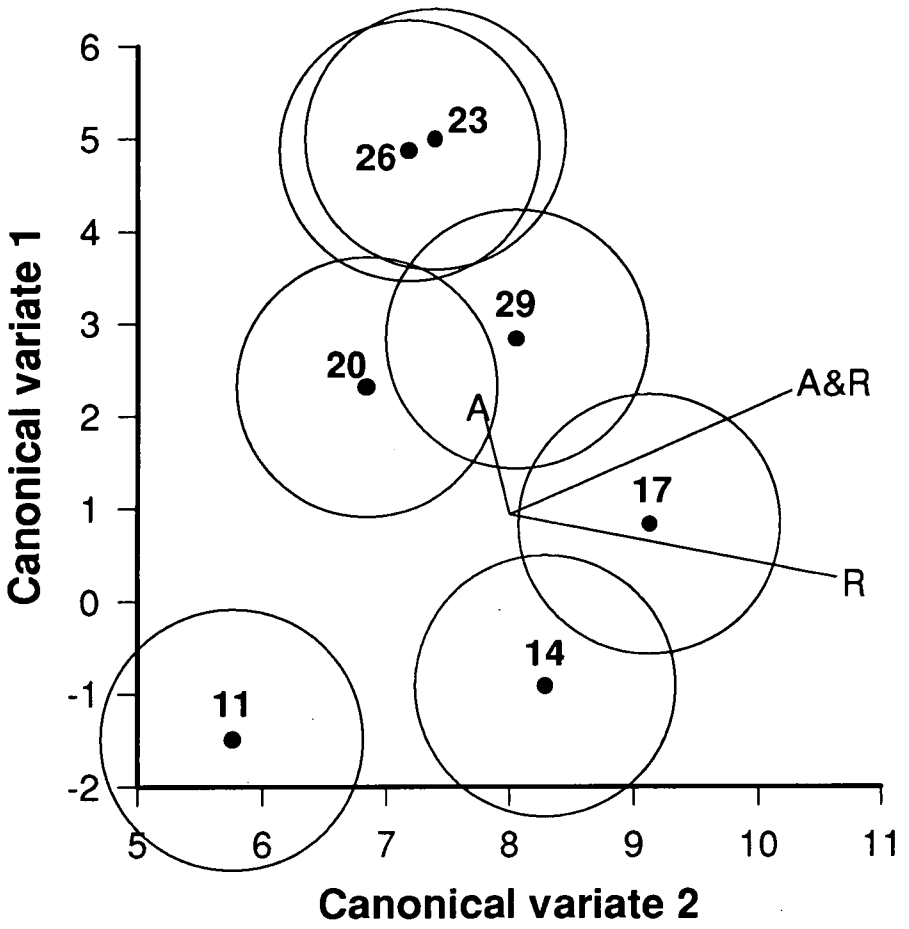


Figure 22 - Canonical distribution analysis reduced plot showing the feeding response of larvae that had prior experience to both *Artemia* and rotifers (A&R treatment). Biplot ray A was most strongly associated with canonical variate 1, which explained 69% of the variation in larval feeding response. Biplot ray R was most strongly associated with canonical variate 2 which explained 26.5% of the variation in larval feeding response. Biplot ray A&R was associated with both canonical variates 1 and 2. Circles indicate 95% confidence ellipses and numbers indicate larval age. (Biplot rays: A = *Artemia*, R = rotifers, A&R = *Artemia* and rotifers).

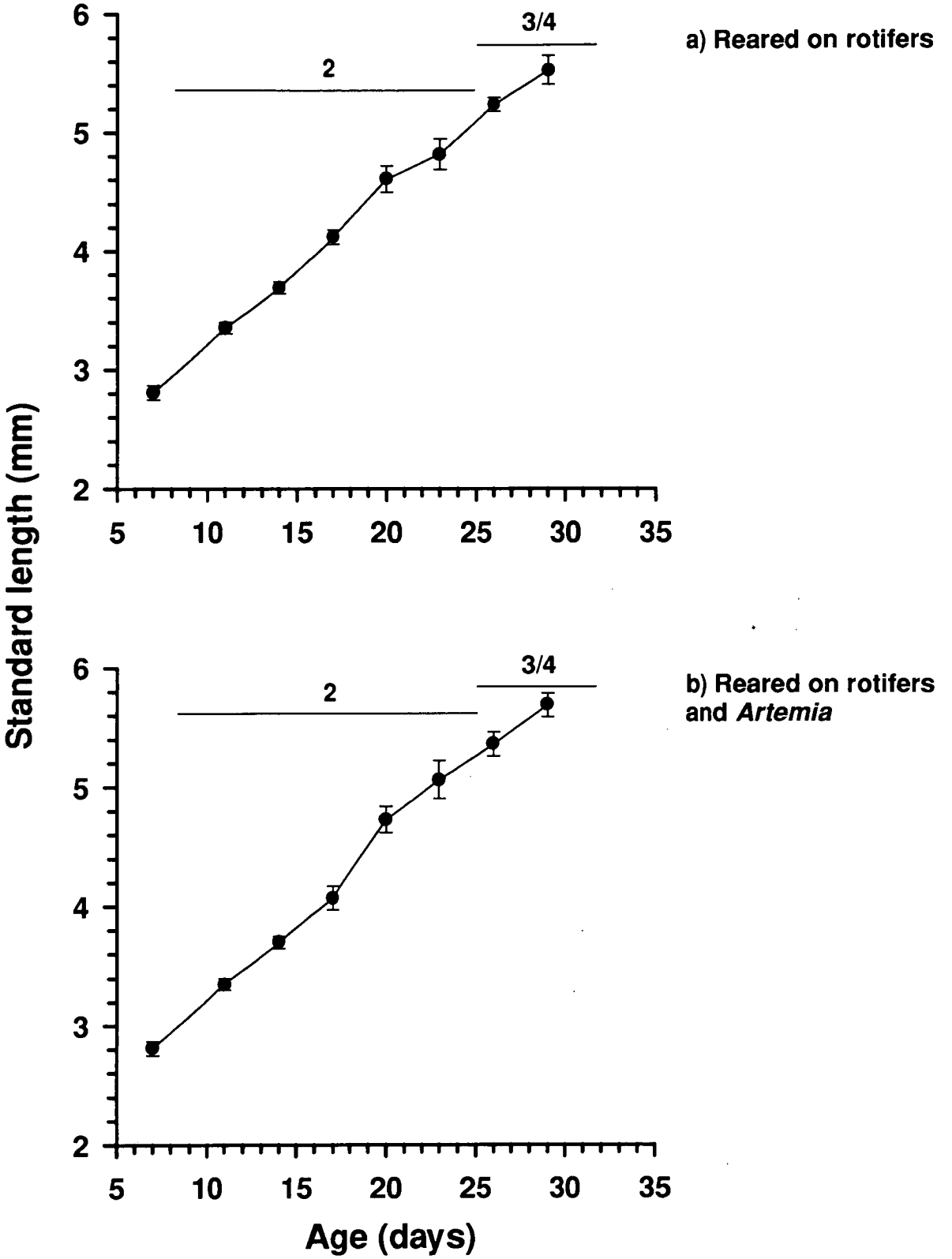


Figure 23 - Change in mean SL (± SE, n = 10) of greenback flounder larvae with increasing age in a) larvae which have had prior exposure to only rotifers (R treatment), and, b) larvae which had prior exposure to both rotifers and *Artemia* (A&R treatment). Horizontal bars denote developmental stage of greenback flounder larvae, as described by Crawford (1986).

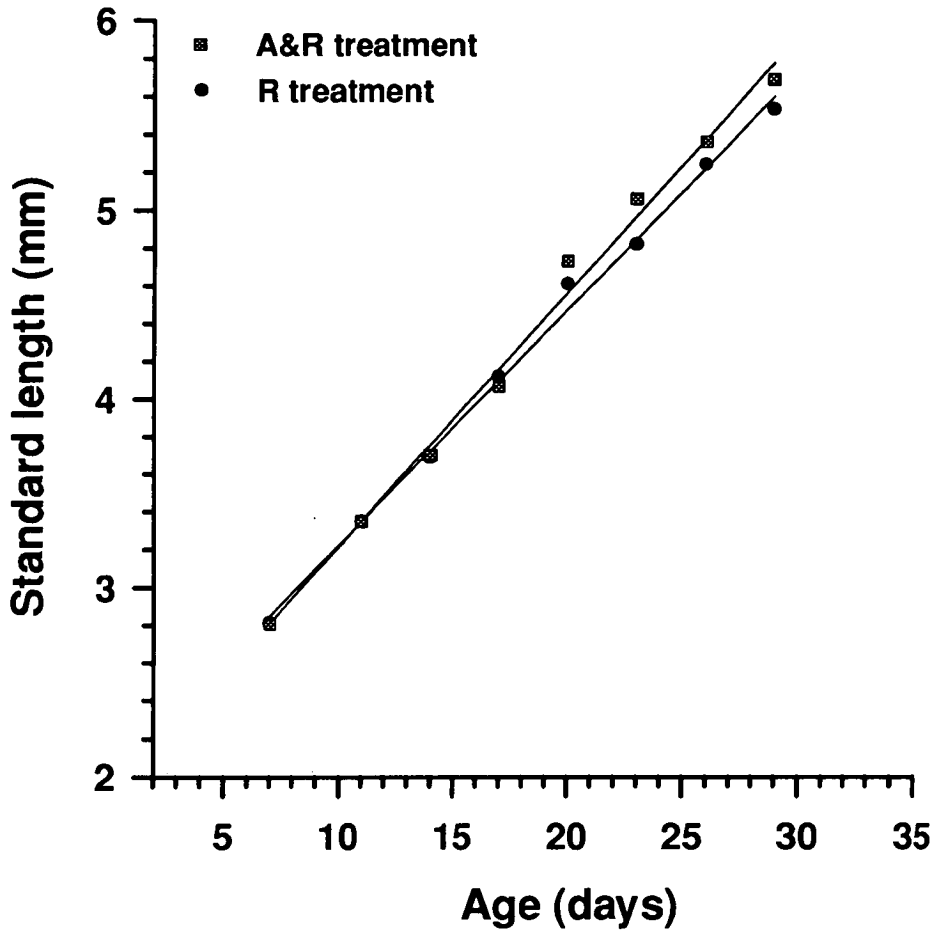
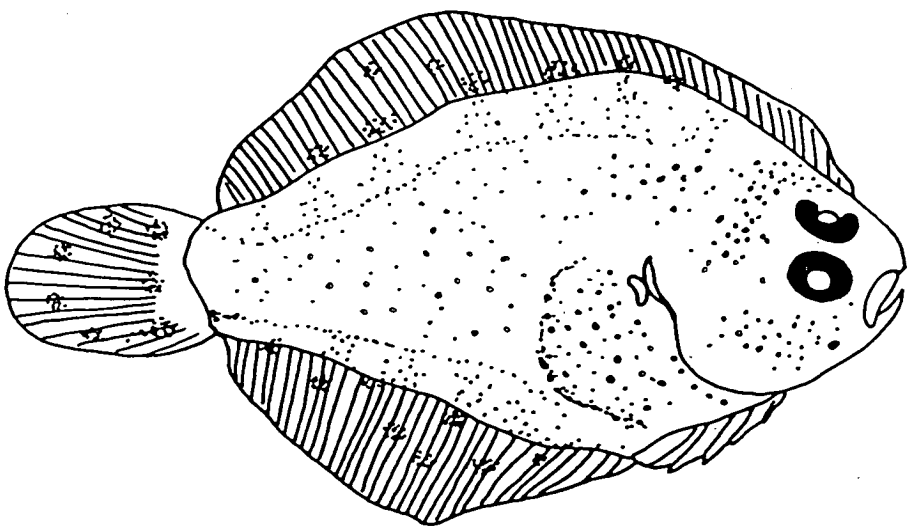


Figure 24 - Regression of SL (mm) against age of greenback flounder larvae for larval cohort 4; A&R treatment larvae - $y = 1.87 + 0.13x$, ($r^2 = 0.99$), and R treatment larvae - $y = 1.97 + 0.13x$, ($r^2 = 0.99$). Symbols are mean values, n = 10.

CHAPTER 4

DISCUSSION



4.1 Determination of the primary sensory modality involved in the feeding behaviour of greenback flounder larvae.

The proportion of greenback flounder larvae feeding in the light on day 12 post-hatching was significantly lower than the proportion of larvae feeding in the light at the completion of the feeding trial (27 days post-hatching). The general ontogenetic trend of increasing ability of greenback flounder larvae to capture prey (in the light), confirms earlier reports which indicate that feeding ability of larvae is initially poor, but increases with age of fish (Hunter, 1981; Mills *et al.*, 1984; Browman and O'Brien, 1992; Wahl *et al.*, 1993). For all life history stages of greenback flounder examined, a consistently high percentage (between 66 - 96% from days 12 - 27 post-hatching) fed in the light, whereas a relatively low proportion (2 - 5% from days 12 - 27, post-hatching) fed in the dark. There was no significant change in the proportion of greenback flounder larvae feeding in the dark on rotifers during ontogeny, and this, in conjunction with the high percentage of larvae feeding in the light, indicated that greenback flounder larvae are primarily dependent upon vision (a light dependent behaviour), to feed during the early life history stages.

Light intensity plays a critical role in visually mediated feeding of planktivorous fish larvae. The threshold light intensity for visually mediated planktivory of fish larvae, is initially relatively high (Blaxter, 1968; Sbikin, 1974; Dayong, *et al.*, 1994), being a function of the requirement for relatively bright light to bring about isomerisation of the photopigments within the very small cone photoreceptors found in the retinae of larval fish. Most marine fish larvae examined to date, have only single cone photoreceptors in the retina at the time of first feeding (Blaxter and Jones, 1967; Blaxter, 1968; Blaxter and Staines, 1970; Neave, 1984; Blaxter, 1986; Pankhurst, *et al.*, 1993; Pankhurst and Eagar, 1996), and this is also the situation in greenback flounder larvae (Pankhurst and Butler, 1996). Formation of both double cone and rod photoreceptors within the eyes of larval fish, occurs later in ontogeny (Blaxter, 1968; Neave, 1984b; Pankhurst *et al.*, 1993; Higgs and Fuiman, 1996; Pankhurst and Butler, 1996; Pankhurst and Eagar, 1996) and this, along with cone enlargement, provides increasing visual sensitivity (i.e. the ability to visually discriminate at light of low intensity) as the eyes grow (Kotrschal *et al.*, 1990). The diverse range of units used to report threshold light intensity for visual feeding of larval fish precludes meaningful

interspecific comparisons, but these investigations do show that the threshold light intensity for visually mediated feeding behaviour decreases during ontogeny. For example; the light intensity threshold at which visual feeding of plaice larvae ceased (criterion of <10% of fish feeding), was highly variable from year to year, but in general, was between 10 and 1 metre candles (m.c.) at first feeding, and by metamorphosis had dropped to 0.01 m.c. (Blaxter, 1968). Similarly, visual feeding of "verkhovka" (*Leucaspius delineatus*) ceased at 0.01 lux in larvae less than 10 mm in length, and at 0.001 lux in larvae 11 - 25 mm in length (Sbikin, 1974), also indicating an increase in visual sensitivity with increasing age, for this species. The light intensity threshold for feeding in striped trumpeter larvae (*Latris lineata*) (criterion of presence/absence of food in gut), decreased with increasing age, with the proportion of larvae feeding at a light intensity of 1 lux increasing from less than 10% between 15 - 19 days of age, to over 50% on day 28 post-hatching (Butler, 1995). This ontogenetic decrease in the threshold light intensity for visual feeding by fish larvae, probably reflects a change in cone photoreceptor function, because rods do not provide the acute visual resolution necessary for planktivory (Blaxter, 1969; Pankhurst and Butler, 1996). Increasing visual sensitivity also points to an expanding photic environment available to larval fish for visual prey searching in the wild, and it is not surprising that the end of the larval phase in some fish coincides with a significant shift in habitat, often to environments of lower light intensity (Dabrowki and Jewson, 1984; Kotrschal *et al.*, 1990).

Until quite recently, it was generally accepted that all marine fish larvae were obligate visual planktivores (Blaxter, 1969, 1986). However, recent studies have shown that some larvae have the capacity to feed in the dark, either by non-visually mediated sensory means (Blaxter, 1969; Sbikin, 1974; Dabrowski, 1982; Townsend and Risebrow, 1982; Batty and Hoyt, 1995), or they invoke non-selective filter feeding behaviour (Holanov and Tash, 1978; Janssen, 1980; Batty *et al.*, 1986). In the latter, reliance upon chemical detection of prey patches has been suggested (Holanov and Tash, 1978; Janssen, 1980; Batty *et al.*, 1986), presumably invoking search strategies involving detection of chemical concentration gradients emanating from prey organisms.

The senses implicated in non-visual feeding are chemoreception; involving the organs of gustation (taste) and olfaction (smell), and mechanoreception, in which the mechanosensory lateral line and superficial neuromasts are used in the detection of prey

(Blaxter, 1968; Dabrowski, 1982; Janssen, 1990; Higgs and Fuiman, 1996). In greenback flounder, these sense organs are poorly developed at first feeding (Pankhurst and Butler, 1996), but increase in complexity and presumably provide for greater non-visual sensory input as larvae grow. The present study suggests that greenback flounder larvae do have a non-visual feeding capability, but at a far lower level than their visual feeding capability. Non-visually mediated feeding has also been reported for other flatfish species. For example, sole larvae fed in the dark very effectively from early life history stages, whereas plaice larvae, like greenback flounder larvae, fed in the dark but at significantly reduced levels compared to the feeding response in the light (Blaxter, 1968; Batty and Hoyt, 1995). Non-visual feeding responses have also been reported in fishes besides Family Pleuronectidae. Pankhurst (1994), found during feeding trials that sparid larvae *Pagrus auratus*, which are pre-dominantly visual feeders, exhibited a low feeding incidence (10%) in the dark on just one occasion. This author proposed that the low level of feeding in the dark in this instance, may have been a result of involuntary prey ingestion in association with osmoregulatory drinking (Tytler and Blaxter, 1988). Northern anchovy (*Engraulis mordax*) larvae fed at low levels in the dark (10%), but only at high food densities ranging between 20 - 40 rotifers.ml^{-1} (Bagarinao and Hunter, 1983), whereas milkfish (*Chanos chanos*) larvae were unable to feed in the dark but juveniles displayed non-visual feeding responses, albeit at a lower feeding incidence than in the light (Kawamura and Hara, 1980).

Various studies have used ablation techniques in order to isolate the role of specific sense organs in the feeding behaviour of young fish. Batty and Hoyt (1995), examined the relative importance of visual, chemical and mechanosensory organs in the feeding behaviour of juvenile sole (20.8 mm mean TL) and plaice (19.4 mm mean TL). Feeding behaviour in the light and dark was recorded using an infra-red video imaging system. The role of mechanoreception in feeding was assessed using both intact fish, and those in which the neuromasts had been ablated with streptomycin sulphate. The role of chemoreception was determined by feeding dead prey to streptomycin sulphate treated fish in the dark, and vision was assessed by comparing feeding responses of fish in the light and dark. Both juvenile sole and plaice were able to feed on live prey in the dark, however plaice fed at significantly lower levels than they did in the light, indicating a strong reliance upon vision in the latter. Plaice feeding on live prey in the light were not effected by neuromast ablation, confirming a strong reliance on vision. Feeding ability of plaice in the dark on

live prey was markedly less than in the light, but was unaffected by neuromast ablation, indicating a reliance upon chemoreception for prey detection under these conditions. In comparison, the feeding response of juvenile sole in the dark was not significantly different from the feeding response in the light, suggesting that sole are mostly reliant upon non-visual senses for feeding. When streptomycin sulphate treated sole and plaice juveniles were offered dead prey in the dark, both were able to feed, although plaice did so at markedly reduced levels than when the same fish were offered dead prey in the light. Although plaice have a strong dependence upon vision for feeding, the latter indicates that sole especially, and to a lesser degree plaice, are able to detect prey using chemoreception in the dark (Batty and Hoyt (1995). In a similar study, the functional role of several sensory modes in Atlantic menhaden larvae (4 - 22 mm TL) was tested (Higgs and Fuiman, 1996). Larvae in which neuromasts were either intact, or ablated, were subjected to a manually advanced probe (dissecting pin attached to a glass pipette), under conditions of both light and dark. The response criteria used was whether larvae responded to the probe before, or after it touched, and in the former, the reactive distance to the probe was measured. Fully intact larvae displayed an ontogenetic increase in both reactive distance and response to the probe. However, in the absence of neuromasts, there was no ontogenetic change in either of these criteria, indicating the importance of neuromast proliferation in determining response and reactive distance to stimuli. In the absence of vision only (i.e. in the dark), there was a significant decrease in response to the probe, indicating the importance of vision in detection of the probe. Other investigators have also used ablation techniques to demonstrate mechanosensory mediated feeding in mottled sculpin juveniles which were unable to locate artificial stimuli, either in the water column or buried, after their superficial neuromasts had been ablated (Janssen, 1990). In another study, Hoekstra and Janssen (1986), found that juvenile mottled sculpin in which vision had been ablated, were still able to respond to moving prey, but ignored dead prey.

In those larval and juvenile fish species which swap from particulate to filter feeding modes in the dark (Holanov and Tash, 1978; Janssen, 1980; Batty *et al.*, 1986), chemical detection of prey has been implicated. Holanov and Tash (1978), suggested a reliance on chemoreception, not vision, by threadfin shad (TL: 7 - 14.1 cm), which were observed to filter feed in both light and dark conditions. A reliance on vision was discounted as a delay in feeding response of several minutes occurred at the start of experiments in both light and

dark conditions, and some fish were attracted to, and made filter feeding movements in patches of water previously inhabited by prey in light conditions. Similarly, gizzard shad (*Dorosoma cepedianum*) and Mississippi silversides were observed to filter feed in both light and dark conditions; however, the sensory basis of the dark feeding response was not determined (Drenner and McComas, 1980). Batty *et al.*, (1986), used an infra red video system to record the filter feeding behaviour of juvenile herring (TL: 145 - 165 mm), in the dark. Filter feeding occurred in both light and dark conditions. In the dark, filter feeding occurred at food densities of 70 to 80 prey ml^{-1}, but was not considered to be an effective mode of feeding unless prey were present in fairly dense patches (>200 prey ml^{-1}). In addition, the swimming mode changed from fish schooling in a relatively straight path in the light, to fish swimming individually in tight circles in the dark. The circling behaviour was thought to enable the exploitation of prey in the dark, with the fish relying on mechanoreception in order to detect and stay within food patches. Batty *et al.*, (1990), also found that juvenile herring filter fed in the dark, and continued to do so in the light at prey densities above 100 L^{-1}. At lower prey densites, fish changed from filter feeding to visual particulate feeding, presumably because this was more effective at low prey densities. In the latter, the threshold light intensity for visual particulate feeding was 0.001 lux.

The present study determined that greenback flounder larvae were pre-dominantly visual feeders, however, in a field study of the same species, Jenkins (1987), reported that over 90% of larvae (SL: 2 - 3.5 mm) had prey in their gastrointestinal tract throughout the day and night. In the latter study, larvae were sampled (n = 30) from the water column at a fixed station, six times between 1400 and 1000 hours, on one occasion on a moonless night, which presumably precluded visually mediated feeding behaviour . Larvae in the present study had a feeding duration of only 1 h, which may have under-estimated the feeding capacity of larvae in the dark, when compared to the feeding response observed by Jenkins (1987), for larvae that fed for approximately 10 - 12 h in the dark. It is also possible that prey type influenced night time feeding ability, as a considerable proportion of the night time diet consisted of bivalve veligers with poor escape ability, and non-motile invertebrate eggs (Jenkins, 1987). However, the mean number of prey ingested per larva in the field, did not vary significantly over the 24 h period examined. Gut clearance time of bivalve veligers, which formed a major component of the diet, was not assessed; however,

gut clearance time assessed on wild larvae captured and fed zooplankton ad-libitum, averaged 4 hours (Jenkins, 1987). It was unlikely therefore, that night time feeding levels in the field study were a result of long retention time of food in the gut. In addition, ingestion rates of non-motile invertebrate eggs increased during this time, implying a reliance upon chemosensory detection of prey. The prey density in the field study (Jenkins, 1987) was 30,000 - 90,000.m^{-3} (= 0.03 - 0.09.ml^{-1}), and in the present study was considerably higher being 2 x 10^6.m^{-3} (= 2.ml^{-1}). These two studies then appear to provide contradictory results. Larvae in the present study displayed a low feeding incidence at relatively high rotifer densities in the dark, whereas results from Jenkins' field study (1987), showed a high feeding incidence at relatively low prey densities during the night time. Clearly, this poses further questions and focusses future directions of study for this species. The involvement of individual sensory modalities in the feeding behaviour of greenback flounder larvae needs to be assessed further. In particular, attention needs to be directed at the relative contribution of those sense organs implicated in non-visual feeding responses, and also whether feeding strategies involve input from solitary sensory organs or combined input from a suite of sense organs. In addition, it is critical that the light intensity for threshold feeding behaviour is determined to define the duration of photopic feeding ability within the natural diurnal cycle of wild fish, but also to determine the optimal light intensity range for visual feeding of greenback flounder larvae in intensive culture. The latter is of particular importance for new species development because the few studies available to date indicate that photopic sensitivity, and therefore optimal light intensity range for feeding, changes in a species specific fashion during ontogeny.

4.2 Determination of prey size selection with increasing age in greenback flounder larvae.

Most marine larvae hatch at a relatively small size (2-5 mm TL, Blaxter, 1969, 1986). Prey size selection by planktivorous fish larvae is initially constrained by morphological and behavioural parameters (for example; mouth dimensions, locomotory capacity, sensory function), arising from size and developmental stage at hatching (Blaxter, 1969; Wong and Ward, 1972; Hunter, 1981; Hairston *et al.*, 1982; Blaxter and Fuiman, 1989; Bremigan and Stein, 1994; Higgs and Fuiman, 1996). As a result, in the early life history stages, fish larvae are able to ingest only a fraction of the prey spectrum available in the environment (Galbraith, 1967; Frank, 1988). Larval "gape" is likely to be one of the primary determinants of prey size selection by fish larvae, because planktivorous larvae ingest prey whole (Hunter, 1981; Dabrowski and Bardega, 1984; Ghan and Sprules, 1993; Bremigan and Stein, 1994). As a consequence, some critical mouth or "gape" dimension must ultimately determine the upper size limit of prey that can be ingested.

In the present study, it has been assumed that the internal horizontal dimension of the mouth, rather than the dorso-ventral mouth dimension, or an estimate of gape determined from some external morphological feature of the jaws or measure of jaw articulation, represented the absolute upper size limit of prey ingested, because it is the smallest dimension within the mouth cavity through which the prey must pass. This is not a novel concept. Werner (1979), Hunter (1981) and Pankhurst (1994), suggested that mouth width was a more accurate measure of larval swallowing capacity than mouth "gape". Furthermore, Hunter (1981), reported a close correlation between mouth width and the ability of larvae to capture prey. Internal horizontal mouth width of greenback flounder larvae determined histologically (cohort 1, figure 15), increased in a linear fashion with both age and standard length of larvae. Ninety five percent confidence limits of the regression of the change in internal horizontal mouth width with increasing SL of greenback flounder larvae (cohort 1, figure 15), were then used to extrapolate mouth dimensions of larvae with similar growth/age profiles, in subsequent prey size selection experiments (cohorts 2 and 3, appendix 4). This allowed examination of the ratio of morphological dimension of the prey ingested by larvae : horizontal mouth width (Table 6).

The latter suggested that prey carapace width rather than either prey width with appendages, or prey total length, was the morphological dimension of prey which determined larval prey size selection in greenback flounder, because in all cases in which larvae were able to ingest prey, this ratio was <1. This also suggests that the larvae must have been able to visually orientate towards the prey so that the prey were head on when ingested. Alternatively, it cannot be discounted that the prey were physically deformed during ingestion, although visual examination of the alignment of *Artemia* prey within the gut of greenback flounder larvae indicated that this prey species at least, was swallowed head on (pers. obsv.). In addition, other studies which examined both prey width and length in an effort to determine the prey dimension which imposed an upper limit on prey size ingested by fish larvae, also concluded that prey width not length, limited the prey size ingested (Govoni *et al.*, 1986; Swift, 1992; Ghan and Sprules, 1993). Ghan and Sprules (1993), established that the width of prey ingested by juvenile burbot, never exceeded maximum gape dimensions, whereas prey length frequently did, concluding that if the larvae orientated to take the prey head on, prey length was not a limiting factor. In contrast to the findings of the present study, Arthur (1976), found that maximum prey width with appendages was the critical dimension determining ingestible prey size by larvae of the Pacific sardine (*Sardinops sagax*), northern anchovy and jack mackeral.

Other investigators have examined the relationship of prey width : mouth gape in greenback flounder. Shaw and Jenkins (1992), and Jenkins (1987), examined gut contents of wild-caught greenback flounder and reported that both greenback flounder juveniles (Shaw and Jenkins, 1992) and larvae (Jenkins, 1987), ingested prey widths that were half the maximum mouth gape dimension or smaller. In the present study, the ratio of *Artemia* carapace width (CW) : larval mouth width (MW) for the smallest prey size fraction ingested, supports the latter field based study (ratio CW : MW = 0.36 - 0.6, refer table 6). However, the ratio of CW : MW for the two larger size fractions of *Artemia* ingested by greenback flounder larvae in the present study ranged between 0.66 and 0.86, indicating that larvae were capable of ingesting prey >0.5 of mouth dimension. This may simply reflect the different methods of "gape" determination employed in the field based studies compared with this laboratory based study. Shaw and Jenkins (1992), preserved whole juvenile flounder in 95% ethanol, and measured articulation of the jaw, using a dissecting microscope fitted with an ocular micrometer. In the earlier field investigation, (Jenkins,

1987), greenback flounder larvae were anaesthetised in benzocaine, fixed in 80% ethanol, the jaws were then manually opened, and the dorso-ventral mouth dimension (gape) was measured using a dissecting microscope fitted with an ocular micrometer. This method of gape measurement is prone to over estimation of mouth size in very small larvae, due to the fragile nature of the structures within the jaw which makes it difficult to estimate the point of natural jaw articulation (Arts and Evans, 1987).

For similar reasons, it is difficult to make meaningful comparisons between the results presented here and others which have determined"gape" using external morphological measures of the larvae, or those which measured the degree of jaw articulation of larvae. Hart (1994), substituted a measurement of lower jaw length into an equation from Shirota (1970), which was based upon an upper jaw dimension, to obtain an estimate of mouth gape (dorso-ventral height) for greenback flounder larvae. Even accounting for the difference in rearing temperatures (this study: 12°C; Hart: 15.5 - 16°C) by standardising fish ages to degree days, there was a marked difference in the mouth size measurements reported in the present study and data reported by Hart (1994), for the same species. The estimates of gape height from Hart (1994), were more than double the size of internal mouth width measurements determined in this study (Figure 25).

If larval mouth width was the only determinant of prey size selection by greenback flounder larvae, and if carapace width as we suggest, was the critical prey dimension defining the upper limit for prey size selection, then 14 - 17 day-old and 13 - 16 day-old larvae (cohorts 2 and 3, respectively), should have been physically capable of ingesting the 300 - 390 μm prey size fraction, and 17 and 16 day-old larvae (cohorts 2 and 3, respectively), should have been capable of ingesting the 450 - 560 μm prey size fraction (i.e. ratio of CW : MW ≤ 1, table 6). However, prior to 19 - 20 days of age, greenback flounder larvae selected only the smallest size fraction of *Artemia*, even though the ratio of prey size (carapace width) ingested : larval horizontal mouth width, indicated that larvae were physically capable of ingesting larger prey. This indicates that other factors besides mouth size were influencing prey size selection. At 19 - 20 days of age onwards, there was a significant shift in the feeding behaviour of greenback flounder larvae, with larvae ingesting all three size fractions of *Artemia* offered. In addition, prey size significantly effected larval feeding success, such that with a single exception (on day 20, cohort 2), the

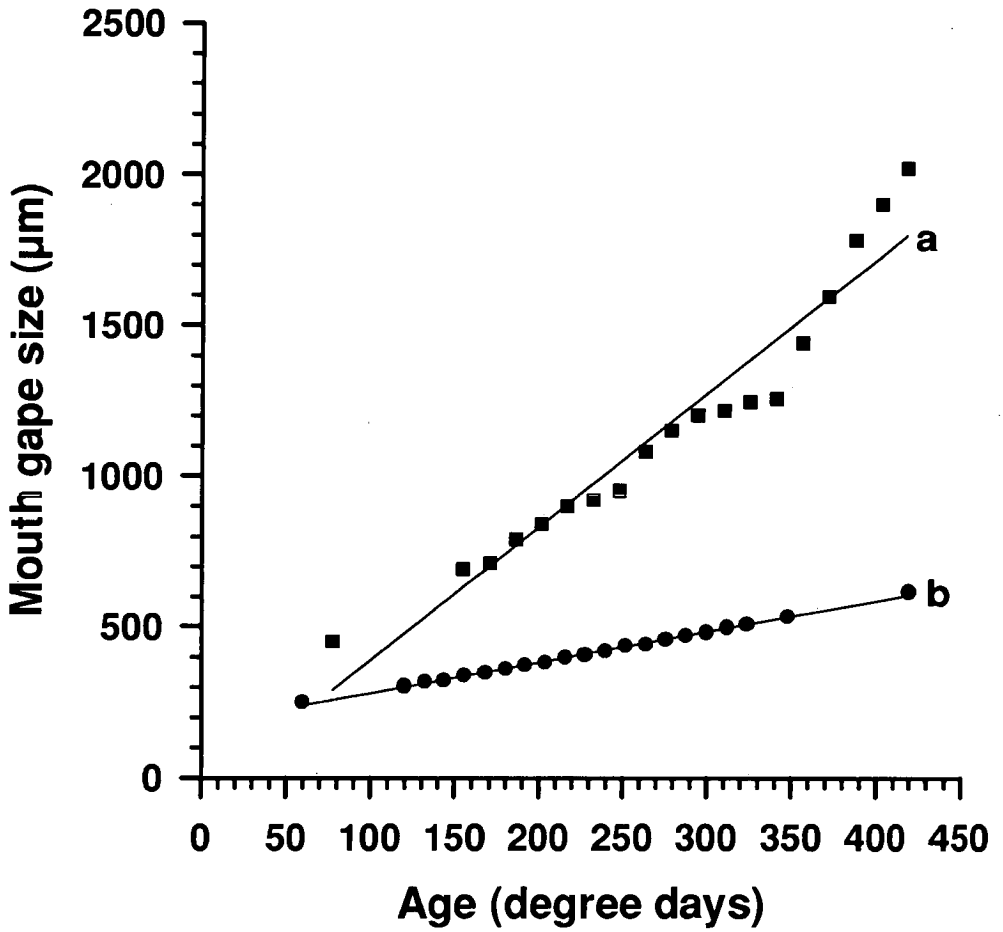


Figure 25 - Regression fits of change in a) mouth gape (µm), $y = -54.35 + 4.42x$, $r^2 = 0.93$ (from Hart, 1994), and b) internal horizontal mouth width determined from serial transverse sections of whole larvae, $y = 177.12 + 1.02x$, $r^2 = 0.99$ (present study), with increasing age (degree days) of greenback flounder larvae.

proportion of larvae feeding on each of the three size fractions of *Artemia*, on any given day, decreased in a stepwise fashion with increasing size of prey.

It appears then, that prey size selection by greenback flounder larvae was not solely determined by the ability of the larvae to physically ingest the prey. One possibility is that prey handling time varies both with larval age and prey size. Handling times of prey by largemouth bass larvae have been shown to increase rapidly with increasing prey size (Hoyle and Keast, 1987). The larger size fractions of *Artemia* used in this study represented a more difficult prey item for small larvae to capture, because the prey had more thoracic appendages, resulting in increased prey swimming speed. In addition, the prey length to width ratio increased considerably with increasing prey size, presumably requiring greater locomotory and orientation skills by the larvae in order to capture and ingest the prey. Despite the stepwise decrease in consumption with increasing prey size on any one day, the feeding incidence within any prey size fraction did increase with increasing larval age, indicating that the ability of larvae to handle larger prey sizes increased during ontogeny. Handling times of prey of increasing size have been shown to decrease with increasing larval age, for several species. For example, the handling efficiency of large prey (fathead minnows, *Pimephales promelas*) by juvenile yellow perch, was observed to improve as larval body length and gape size increased (Paskowski and Tonn, 1994). Similarly, whilst prey (zooplankton) handling times increased with increasing prey size, there was an ontogenetic decrease in handling times, for bay anchovy (*Anchoa mitchilli*), sea bream (*Archosargus rhomboidalis*) and lined sole (*Achirus lineatus*) (Houde and Schekter, 1980).

The ability of greenback flounder larvae to ingest all three size fractions of *Artemia* at 19 and 20 days of age, in cohorts 2 and 3, respectively, coincided with a shift in developmental profile from developmental stage 2, to developmental stage 3/4 (Figure 16). The transition from developmental stage 2 to stage 3/4 in greenback flounder larvae denotes the onset of notochord flexion and caudal fin development, which in Atlantic herring is characterised by a significant shift in swimming behaviour (Batty, 1984). Yolk sac and finfold larvae display bouts of very energetic swimming involving whole body 'serpentine'- like body undulations, interspersed with periods of rest (Hunter, 1981; Blaxter, 1986; Webb and Weihs, 1986). This swimming mode is energetically most

efficient for very small fish larvae because water viscosity is the dominant factor determining swimming mode at this time (a function of low Reynold's numbers) (Webb and Weihs, 1986). A suite of morphological changes, including yolk sac resorption, loss of the larval finfold, development of the caudal and median fins and increasing body size, coincides with an ontogenetic shift to a subcarangiform tail beat and glide swimming mode in fish larvae, which is more efficient as Reynold's numbers increase (Batty, 1984; Webb and Weihs, 1986). It is possible then that the sudden shift in feeding performance of greenback flounder larvae, which coincides with the onset of caudal fin development, reflects an increased capture or handling capacity in association with an ontogenetic shift in swimmimg mode.

Another possibility is that sensory functional capabilities shift significantly during this period (refer section 4.1, this study). Greenback flounder are primarily visual feeders during this early developmental period (this study). Stage 2 greenback flounder larvae have a single cone retina, which is characteristic of many first feeding fish larvae (Blaxter and Jones, 1967; Blaxter, 1968, 1986; Blaxter and Staines, 1970; Neave, 1984). During developmental stage 3/4 in greenback flounder, further photoreceptor types, double cones and rods, develop within the retina (Pankhurst and Butler, 1996). Cone photoreceptors provide the acute photopic vision required for planktivory and initially, visual acuity of fish larvae is poor, a constraint of small photoreceptor and small eye and lens size (Tamura and Wisby, 1963; Blaxter and Jones, 1967; Kotrschal *et al.*, 1990). However, an increase in eye and lens diameter associated with growth results in increasing photopic acuity because of increasing angular density of cones (Pankhurst and Butler, 1996). As a result, the greatest gains in photopic visual resolution of small fishes is likely to arise from an increase in eye/lens size (Kotrschal *et al.*, 1990). However, as in other fishes (Sadler, 1973; Breck and Gitter, 1983; Flamarique and Hawryshyn, 1996), eye and lens diameter of greenback flounder increases in a linear fashion with increasing fish age (Pankhurst and Butler, 1996) and would not, therefore provide a marked increase in visual resolution at 19 - 20 days of age, which might explain the significant shift in feeding behaviour thereafter. Moreover, the onset of developmental stage 3/4 denotes the onset of a period of dynamic change with respect to the visual field of flatfish larvae. Left eye migration occurs during developmental stage 3/4 and this has implications for the integration of visual input within the optic tectum. Visual feeding capacity as evidenced by feeding performance, does not

however, appear to be compromised during this period. This may reflect the increasing level of development of the optic tectum and integration capacity at this time. It is possible then that increasing visual functional and integration capabilities, in conjunction with a change in swimming behaviour, contribute to the sudden ability of greenback flounder larvae at 19 - 20 days of age, to ingest all three size fractions of *Artemia* prey.

4.3 The effect of previous exposure to a prey species on subsequent prey selection.

Previous exposure to a prey species significantly effected subsequent patterns of prey selection by greenback flounder larvae. Canonical distribution analysis determined that the differences in feeding responses observed in those larvae which had previously experienced both *Artemia* and rotifers (A&R treatment larvae), compared with those larvae which had previously experienced only rotifers (R treatment larvae), were largely attributed to differences in selection of either *Artemia* only, or rotifers only. For example, a high proportion of R treatment larvae continued to select only rotifers (51 - 75% of larvae in R treatment larvae from days 11 to 29 post-hatching) throughout the ontogenetic period examined. In contrast, A&R treatment larvae showed a trend of decreasing preference for rotifers during the same period. In addition, a higher proportion of A&R treatment larvae selected *Artemia* only, when compared with R treatment larvae. Growth rates of R and A&R treatment greenback flounder larvae were similar (0.12 and 0.13 mm/day, respectively). Because horizontal mouth dimensions of greenback flounder larvae increased in a linear fashion with age (Figure 15, section 3.2.2), then it is unlikely that there were significant differences in mouth dimensions of same age fish in the R and A&R treatment groups, which may otherwise have effected the prey selection patterns reported here. In addition, because both R and A&R treatment larvae were from a single cohort of eggs, and growth profiles were very similar, then the same visual constraints imposed by ontogenetic stage of development of the retina and small eye/lens size, applied to larvae in both treatments. Further to this, the differences in prey selection by R and A&R treatment greenback flounder larvae occurred despite the fact that larvae from both previous prey exposure treatments started to ingest *Artemia* (either in combination with rotifers, or alone), for the first time, at 14 days of age. This indicated that the temporal onset of *Artemia* selection/ingestion was not effected by prior prey exposure regimes, and that the differences in prey selection were not simply due to the inability of larvae in the R treatment group to handle and ingest the novel prey species. However, it is likely that capture and ingestion of *Artemia* (the novel prey) by R treatment larvae, involved increased handling times compared with experienced A&R treatment larvae, and this may in part explain the low proportion of R treatment larvae which selected only *Artemia* (5%

and 1% on days 20 and 26, post-hatching), or *Artemia* and rotifers (which never exceeded 50% during the period in question).

Several other studies have reported that prior exposure to a prey species influenced subsequent prey selection, and that prey handling times were implicated in feeding success when fish were offered a novel prey. Wahl, *et al.* (1995), found that although juvenile walleyes (*Stizostedion vitreum* - 100 mm mean TL) reared on pellets readily accepted live prey (golden shiners), of which the fish had had no previous exposure, they captured fewer prey than did experienced juveniles. After five days of exposure (each of 30 minutes) to the novel prey, the foraging success of inexperienced fish did not differ from that of experienced fish. The initial difference in feeding success was attributed to significantly poorer prey handling times for naive fish, which had to learn how to attack and capture live prey. Meyer (1986), also found that handling times and subsequent capture success of *Artemia* prey by Central American cichlid fry (*Cichlasoma managuense*, size range - 8.5 - 9.5 mm TL), with varying histories of prior experience of *Daphnia*, increased with experience. In the present study, although differential handling times for "novel" and "familiar" prey may explain reduced feeding rates on the novel prey, it does not explain why R treatment larvae selected only *Artemia* on just two of the days that larvae were tested (5% and 1% feeding incidence on days 20 and 26 post-hatching, respectively), whereas a proportion of A&R treatment larvae selected *Artemia* only, on every day tested, from 14 days of age. Other factors besides handling time of prey, must therefore be involved.

Prior experience of a prey impacts strongly upon feeding success and selection of prey by fish larvae and juveniles (Beukema, 1968; Hunter, 1972; Werner *et al.*, 1981; Bell, 1990). A learned response is implicit in the improved foraging success of largemouth bass (Colgan *et al.*,1986), and juvenile bluegill sunfish (Werner, *et al.*, 1981), after repeated periods of exposure to a novel prey. For example, largemouth bass (Colgan *et al.*,1986), and juvenile bluegill sunfish (Werner, *et al.*, 1981), were reared on artificial and live prey diets, and required between 4 - 8 prior exposure episodes with a novel live prey species, before maximum foraging efficiency on the new prey was observed. Similarly, Godin (1978), demonstrated that the latency time between visual fixation and attack by juvenile pink salmon (*Oncorhynchus gorbuscha*) feeding on a novel prey (*Artemia*), decreased 3.4

times, after 5 episodes of prior exposure indicating a learned component in recognition of novel prey types. It has been suggested that fish may learn to associate either negatively or positively, with certain morphologically distinct prey types (Vinyard, 1980). Therefore, prey characteristics such as size, visibility and motility, which have been reported to effect prey capture by fish larvae (Kislalioglu and Gibson, 1976; Vinyard, 1980; Zaret, 1980; Hunter, 1981; Checkley, 1982; Wright and O'Brien, 1982), are likely to form the basis of prey recognition. If learned prey preference is the basis of the differences in prey selection patterns observed in R and A&R treatment greenback flounder larvae, then prey characteristics which form the basis of prey recognition must be considered.

It is unlikely that prey size alone formed the basis of prey recognition and subsequent selection by greenback flounder larvae in the present study, because both prey types were screened to the same size fraction. Whilst mean total length of *Artemia* (470 μm) and mean total length of rotifers (278 μm without eggs and 380 μm with eggs) did not correspond closely, the respective prey width dimensions of *Artemia* and rotifers (carapace width and lorica width, respectively) were in close agreement (186 and 184 μm, respectively). If we accept that the critical prey dimension which determines prey size selection in greenback flounder is prey width without appendages, (refer section 3.2.5), then *Artemia* and rotifers of this screened size fraction represent very similar visual targets in terms of dimension alone. However, there are considerable differences in both morphology, visibility and patterns of motility of *Artemia* nauplii and rotifers. Rotifers are transparent zooplankton, in this case with a diffuse green colouration, resulting from enrichment with micro-algae prior to feeding to larvae. (In this study, micro-algae enrichment was undertaken in all feeding trials involving rotifers, to enhance subsequent detection of these prey within the gastro-intestinal tract of larvae, at completion of the feeding trials). *Artemia* nauplii on the other hand, are not transparent, and the exoskeleton has high spectral sensitivity in the yellow-orange waveband of 560 - 620 nm (Blaxter, 1975). In comparison with rotifers then, *Artemia* nauplii represent a high contrast visual target, either when viewed by larvae against the black wall of the test chamber, or when viewed by larvae from below, silhouetted against the bright background of downwelling room light. In addition, differences in locomotion are marked. Rotifers move in a relatively slow continuous spiralling motion, whereas *Artemia* nauplii have a rapid staggered motion, arising from coordinated beating of both the right and left antennae. It

can only be surmised that the differences in relative prey morphology, locomotion and visibility of *Artemia* nauplii and rotifers, are sufficient to provide the basis for recognition and then positive selection of familiar prey species versus novel prey species, which appear to be underlying the selection patterns observed here.

This study has established that prior experience to prey effected subsequent patterns of selection, such that greenback flounder larvae reared on rotifers only, continued to select for this familiar prey when offered a novel prey species in addition to the familiar prey. This has implications for the intensive culture of marine fish larvae which, because of mouth size constraints at first feeding, usually involves a sequential transfer of larvae to different prey species of increasing size, during ontogeny. This study indicates that prior exposure to a new prey species for an appropriate period of time is probably required if larvae are to effectively shift preference from one prey species to another. There are energetic/growth gains in larvae selecting larger prey items as body/mouth size increases (Polo *et* al., 1992; Bremigan and Stein, 1994; Paszkowski and Tonn, 1994) and further research is required to examine the time scale of exposure required to shift positive selection from a familiar to a novel prey species.

4.4 Summary

Examination of the primary sensory modality involved in feeding behaviour, indicated that greenback flounder larvae are primarily dependent upon vision in order to feed, at least in the early life history stages. In addition, the general ontogenetic trend of increasing ability of larvae feeding in the light, supports earlier reports that indicated that feeding ability of larvae was initially poor, but increased with age. Greenback flounder larvae were able to feed in the dark, although at a considerably lower level than in the light, supporting recent studies which have shown that some fish larvae have the ability to feed using non-visual senses. Although the feeding response of larvae in the dark treatment may have been due to the early onset of non-visually mediated feeding behaviour, there was no change in feeding response in the dark during ontogeny, and it was therefore, not possible to entirely discount that this feeding response may have been due to involuntary prey ingestion as a result of osmoregulatory drinking. The involvement of individual sensory modalities in the feeding behaviour of greenback flounder larvae needs to be assessed further. In particular, attention needs to be directed at the relative contribution of those sense organs implicated in non-visual feeding responses, and also whether feeding strategies involve input from individual sense organs, or combined input from a suite of sensory organs. In addition, it is critical that the light intensity for threshold feeding behaviour is determined in order to provide optimum light conditions for intensive culture of this species, and define the light intensity range for photopic visual feeding of greenback flounder larvae in the wild.

Prey size significantly effected the feeding response of larval greenback flounder. In addition, prey width, rather than either prey width with appendages, or prey total length, was the morphological dimension of prey which determined larval prey size ingested, indicating that larvae must have been able to visually orientate towards the prey so that the prey were head on, when ingested. A decrease during ontogeny in the proportion of greenback flounder larvae feeding with increasing size fraction of prey, indicated that the ability of larvae to physically ingest prey, was not the sole criterion that determined prey selection. A sudden marked increase in the ability of larvae to ingest all prey sizes offered, coincided with the onset of caudal fin development which may have reflected an increased prey capture or handling capacity by larvae, in association with a shift in swimmimg mode.

In addition, sensory functional capabilities increase with increasing body size in fish larvae, and it is possible that increasing integration of visual input, in conjunction with a change in swimming behaviour, contributed to the sudden ability of stage 3/4 greenback flounder larvae, to ingest all three size fractions of *Artemia* prey.

The selection of prey by greenback flounder larvae during ontogeny was found to be strongly influenced by prior exposure to prey species. The temporal onset of *Artemia* selection was not effected by prior prey exposure regimes and the differences in prey selection that were observed between larvae without prior exposure to *Artemia* (R treatment), and larvae with prior exposure to *Artemia* (A&R treatment), were not simply due to the inability of larvae to handle and ingest a novel prey species. The differences in prey characteristics between rotifer and *Artemia* prey may have been sufficient to allow recognition of, and positive selection for a familiar prey species, suggesting a learned component to feeding behaviour of fish larvae. Positive selection for familiar prey has implications for the intensive culture of marine fish larvae which introduces new and sequentially larger prey species during ontogeny. There is a need to determine the appropriate exposure time required to produce positive selection for new prey species, if effective transition to the new species is to be achieved.

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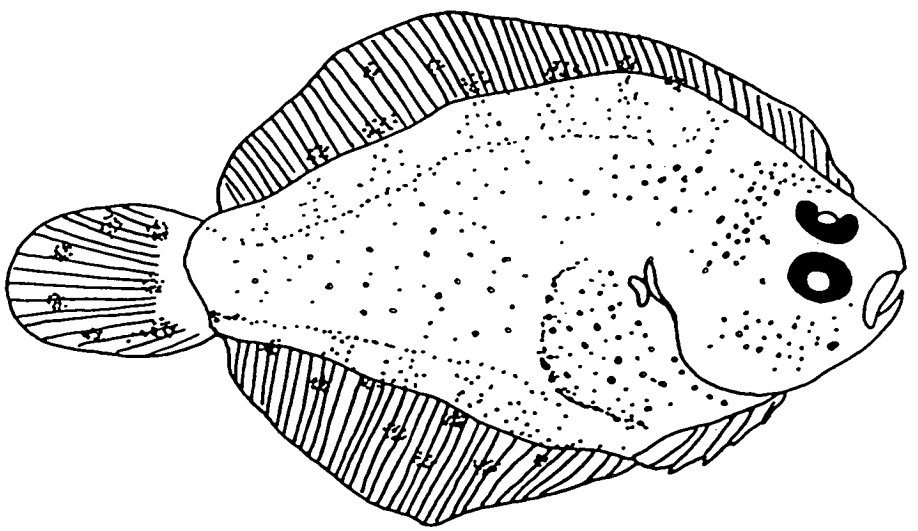

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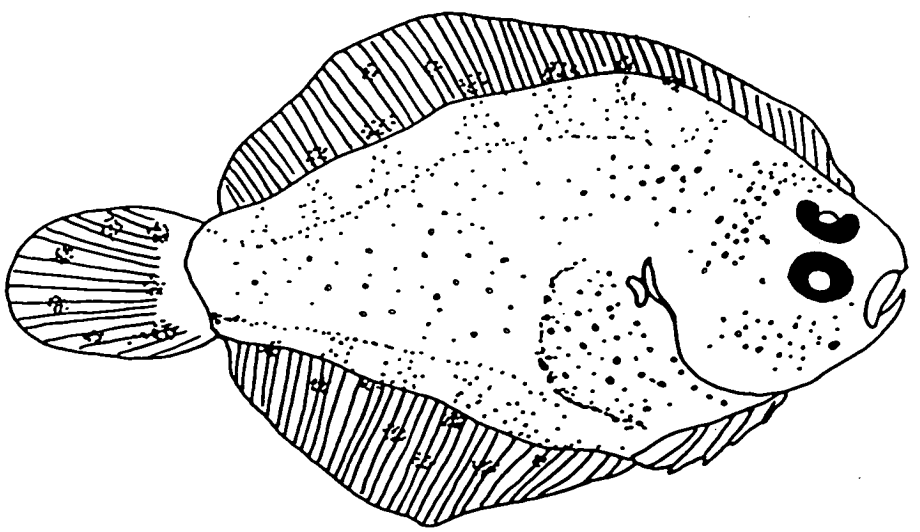
APPENDICES



Appendix 1

Glutaraldehyde fixative and phosphate buffer

Phosphate Buffer Recipe:

Stock 1: 0.1 M solution of $NaH_2PO_4.2H_2O$ 15.601 $g.L^{-1}$

Stock 2: 0.1 M solution of Na_2HPO_4 14.196 $g.L^{-1}$

Working Solution:

Mix the two solutions at an approximate ratio of 1:5, stock 1 : stock 2 to obtain a final pH of 7.4. Add 2 g of sucrose per 100 ml of buffer.

Glutaraldehyde Fixative:

Glutaraldehyde (25%)	5 ml
0.1 M phosphate buffer + sucrose, pH 7.4	20 ml

Appendix 2

Protocol for JB4 Methyl-methacrylate resin histology

Solutions:

Solution A

Solution B

Catalyst - Benzoyl peroxide

Method:

1. Fish were infiltrated for 24 h in Solution A (100ml) + catalyst (benzoyl peroxide - 0.9 g). One ml of this solution was added to each vial with one solution change after 3 h. The vials were placed on a rotary infiltrator to ensure even infiltration.
2. One part of Solution B was then added to 30 parts of Solution A + catalyst to make the final embedding resin.
3. Gelatin capsules (0.5 ml) were filled with the resin and one larva was placed into each capsule. The lid was then pressed on firmly to minimise the amount of oxygen present in the capsule. This was necessary because oxygen inhibits polymerisation of methyl-methacrylate resins.
4. Capsules were left overnight at room temperature to polymerise. Unpolymerised resin was then removed with 90% ethanol and the gelatin capsule was peeled off.
5. Blocks were stored in a dessicator, at room temperature, to prevent moisture uptake. Serial, transverse sections (2 μm) were cut from the rostral tip of the fishes jaw to the back of the eye using a Microm 340™ microtome, fitted with glass knives. Each section was lifted off the glass knife using a fine pair of forceps and placed onto a drop of distilled water, on a clean histological slide. The slides were dried on a slide warming tray at 37°C before being stained with a polychrome stain (Appendix 3), air dried and mounted in Shur/mount™.

Appendix 3

Polychrome staining method (Mackay and Mead, 1970. Modified by Griffin and Fahrenbach).

Polychrome I Stock:

Methylene blue	0.65g
Azure II	0.1g
Glycerol	50 ml
Methanol	50 ml
Distilled water	400 ml

Stir to dissolve, filter and use within 6 months.

Polychrome II Stock:

Stock solution:

0.2% aqueous basic fuchsin. Heat, stirring to dissolve, filter and use within 6 months.

Working solution:

1 part stock : 4 parts distilled water.

Stain 10 - 12 seconds in polychrome I, rinse in distilled water.

Stain 25 - 30 seconds in polychrome II, rinse in distilled water.

Air dry and mount.

Appendix 4

Mouth width measurements for greenback flounder larval cohorts 2 and 3, used to determine the ratio of prey size ingested : larval mouth width. Measurements were extrapolated from the upper 95% confidence limit of the regression of change in larval mouth width with standard length of larvae from cohort 1.

Cohort 2		Cohort 3	
Age (days)	**Mouth width (μm)**	**Age (days)**	**Mouth width (μm)**
11	300	10	318
14	348	13	340
17	373	16	367
20	444	19	418
23	487	22	494
26	529	25	515
29	538		