REVERSAL AND MAINTENANCE OF CLAW ASYMMETRY IN THE SNAPPING SHRIMP *ALPHEUS HETEROCHELIS*

by

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Reversal and maintenance of claw asymmetry in the snapping shrimp *Apheus heterochelis*

Doctor of Philosophy, 1998, Arthur Thomas Read, Department of Zoology

The University of Toronto

ABSTRACT

The snapping shrimps *Alpheus heterochelis* have bilaterally asymmetrical claws. Loss of the snapper claw causes the contralateral pincer claw to transform into a snapper while a new pincer regenerates at the old snapper site resulting in asymmetry reversal. Paired simultaneous loss of the claws always results in them regenerating to their original configuration. No gross morphological differences were detected between early limb bud regeneration of the pincer and snapper, however the quantity of innervation remains higher on the snapper side, mimicking the pristine condition. Loss of first the pincer claw followed later by the snapper results in reversal of asymmetry if a stage 3 or higher stage limb bud is present at the original pincer site. Stage 3 limb buds have a well organized fibroblast network and show the beginning of segmentation. Loss of the snapper claw's dactyl may also induce pincer-to-snapper transformation occurring preferentially in males which have a more elaborate pincer claw and behaviourially are more defensive than females. Dactylotomy of the snapper claw is correlated to the loss of innervation to the snapper side which in males but not females now have fewer axons than the contralateral pincer (a condition not present prior to the manipulation), implying that it is the shift in axon numbers which triggers transformation. A manifestation of differences in innervation quantity between the claws may be a snapper-based inhibitory mechanism: a snapper or transforming pincer-to-snapper claw produces an inhibitory signal which limits the contralateral claw to a pincer. Damage to the snapper may induce pincer-to-snapper transformation but will not allow another snapper claw to regenerate at the pincer site whereas damage to a transforming claw often does result in the regeneration of a contralateral snapper. Thus less inhibition is required to restrict a newly regenerating claw to a pincer than it is to arrest an existing pincer claw. These results and the observation that a regenerating snapper claw passes through a distinct pincer-like stage suggests that the pincer is an immature snapper limited in its development by the inhibitory presence of a contralateral snapper.

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LIST OF FIGURES

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PUBLICATIONS FROM THESIS

The following parts of the thesis have been published or accepted for publication as scientific papers:

Chapter One: Govind, C.K. and Read, A.T. (1994) Regenerate limb bud sufficient for claw reversal in adult snapping shrimp. Biological Bulletin (Woods Hole), 186: 241-246.

Chapter Three: Read, A.T and Govind, C.K. (1997) Regeneration and sex-biased transformation of the sexually dimorphic pincer claw in adult snapping shrimps. Journal of Experimental Zoology, accepted May 15, 1997, 19pp.

Chapter Four: Read, A. T. and Govind, C. K. (1997) Claw transformation and regeneration in adult snapping shrimps: test of the inhibition hypothesis for maintaining bilateral asymmetry. Biological Bulletin, accepted August 19, 1997, 18 pp.

The idea for the first paper came from by C.K. Govind based upon some earlier experiments by Darby (1934). For each paper I was responsible for virtually every aspect of the study including design of the experiments, maintenance and preparation of the subjects, carrying out the procedures, collecting, analyzing and interpreting the data. I wrote the first draft of the papers which were revised by C.K. Govind, prepared all the figures and assisted in revising and correcting the subsequent versions.

We have obtained permission to reprint these papers for my thesis. .

GENERAL INTRODUCTION

Aristotle, in stating that it is only "natural for every animal to use its right hand in preference to its left" initiated a controversy that continues unabated. In Western societies, there is no longer any serious stigma associated with being a southpaw and indeed the present and preceding three out of four U.S. presidents are left-handed. Until quite recently however left-handed children were actively discouraged from preferentially using their left hand and studies continue to inform us of how relatively unfit left-handers are (Perelle and Ehrman, 1982; Halpern and Coren, 1991). The etiology of handedness remains elusive with theories ranging across the entire nature-nurture continuum.

In many animals the body is bilaterally symmetrical (divided into opposite and equal halves by a central longitudinal plane), while bilateral asymmetry or 'handedness' occurs when the opposite halves are in some way unequal. The latter condition was known to be present in organisms such as trilobites and/or their predators from the early Cambrian period (Babcock, 1993) however despite its antiquity and existence in such diverse groups, true bilateral asymmetry is, in fact, a rare and inconsistent phenomenon. In vertebrates it often occurs in a subtle manner. Humans, as alluded to, display functional preferences in the use of their left and right hands as well as anatomical asymmetries of some brain structures, most notably the planum temporale, a region thought to be important in the lateralization of language to the left hemisphere (Geschwind and Levitsky, 1968). The very existence of hemispheric asymmetries in non-human primates is more controversial, being denied by some (Hamilton, 1977) while accepted by others (eg. Dewson, 1977). On the other hand the right cerebral hemisphere of some non-primate mammals is known to be significantly larger than the left (Kolb, 1982) while song learning in canaries is asymmetrical in terms of neural mechanisms (Nottebohm, 1984).

Like the vertebrates, very few invertebrates display obvious bilateral asymmetry however, the decapod Crustacea is one group whose members illustrate a wide ranging degree and variety of this trait usually in the first paired thoracic chelipeds or claws. These appendages often consist of a

major and minor form; the major claw is larger and typically more elaborate in construction and may be specialized in function compared to the more generalized minor claw.

One of the most striking examples of claw bilateral asymmetry is seen in male fiddler crabs where the major claw or fiddle is unusually large, as much as 20-30 times larger than the minor claw and is specialized for highly ritualized territorial and courtship displays (Crane, 1975). The minor claw is used for gathering food and grooming. In females both claws are small and symmetrical, and similar in form and function to the minor claw of the male.

Lobsters also possess dimorphic claws however the difference is less dramatic. The crusher is stout and heavy with molar-like teeth along the closing edges, specialized to crack open the shells of bivalves, while the cutter is more slender and lighter with incisor-like teeth, used in territorial defense and prey capture (Scrivener, 1971; Govind and Lang, 1974).

Many other brachyurans exhibit asymmetry of the claws however the degree of specialization is much less obvious. For example in the blue crab the major and minor claw differ very little in outward appearance except for a slight difference in size (Govind and Blundon, 1985).

Claw asymmetry or handedness may occur in a fixed manner. In hermit crabs of the family Paguridae, the major claw appears only on the right side and its placement appears to be genetically fixed. Lateralization in these animals, adapted to living in discarded gastropod shells, consists of a larger right claw, decalcified abdomen spiralling to the right, reduced left abdominal flexor muscles and loss of pleopods on the right side. Asymmetry begins to show by the late larval stages, long before the crab inhabits a shell (Chapple, 1977a, b) and is the same regardless of whether the first shell occupied has a sinistral or dextral spiral (Chapple 1977a), emphasizing the lack of external influences on laterality.

In lobsters and male fiddler crabs handedness occurs in a random manner, that is the major claw may occur with equal probability on the left or right side. Once asymmetry has been

determined, it is permanently fixed. However, there occurs a critical period during ontogeny, long before handedness is manifested, when asymmetry is determined and may be manipulated (Yamaguchi, 1977; Govind and Pearce, 1989). In lobsters, the important factors in the determination of asymmetry are a minimal level of claw activity (Kent and Govind, 1982) and a difference in the activity between the two sides (Govind and Pearce, 1986), normally accomplished via manipulation of materials on the ocean floor. If both conditions are not achieved, two cutters usually develop. A double crusher never results, even if both sides have been exercised well above the threshold level.

In male fiddler crabs, the usual mode of asymmetry determination is by loss of one of its claws during the early critical period (Morgan, 1923) either through agonistic encounters or by manipulation of the substrate (Yamaguchi, 1977) with the remaining claw transforming into a major type. In contrast to lobsters, young fiddler crabs that do not experience claw loss during the critical period usually develop paired symmetrical major claws. This condition remains fixed for life, even with subsequent claw loss and regeneration (Yamaguchi, 1977). It appears therefore that in lobsters and fiddler crabs, the critical period is genetically fixed, but the determination of handedness is environmentally influenced (Govind, 1992).

In many other crabs such as blue crabs, stone crabs, shore crabs and velvet crabs, claw handedness appears to be fixed, since there exists a strong right-handed bias in populations of these animals. However, studies have shown that these crabs are capable of asymmetry reversal, even well into adulthood. While the process requires several lengthy moults (Hamilton et al., 1976; Simonson, 1985; Smith and Hines, 1991; Norman and Jones, 1991), it does indicate an ability to alter claw handedness based on external factors.

The renowned Danish physiologist August Krogh once expressed the idea that for many problems, there is an animal on which it can be most conveniently studied, almost as if that animal had been specifically created for the purpose (Krebs, 1975). The snapping shrimp *Alpheus heterochelis* (Say) is well suited to research in the areas of asymmetry and regeneration exhibiting

a dramatic form of bilateral asymmetry of the claws (Przibram, 1901). The large major or snapper claw is highly modified in form and function displaying extreme muscular hypertrophy and possessing on the movable dactyl a protruding plunger which fits into a complementary socket on the propodus. When forcefully closed the snapper produces a loud popping sound, accompanied by a jet of water which is used primarily in agonistic interactions such as territoriality and competition for mates (Hazlett and Winn, 1962; Nolan and Salmon, 1970) and, secondarily for subduing prey (pers. obs.). Contralateral to the snapper is a much smaller, relatively unmodified minor or pincer claw which is used for more general types of activities such as in burrowing, moving around bits of substrate and capturing and holding prey.

Snapping shrimp represent the other end of the nature-nurture continuum where intrinsic (genetic) factors appear to have little or no influence on the determination of asymmetry. In these shrimp, left and right-handed individuals occur with equal probability and like other crustaceans they are capable of quickly regenerating entire limbs, including the asymmetrical claws, which have been autotomized (or "thrown" by the animal, usually as a defensive ploy). More importantly they have the unusual and almost unique feature of being able to rapidly reverse their asymmetry throughout their lifespan, even well into adulthood. If both claws are simultaneously autotomized, or if the pincer alone is lost, the limbs regenerate in their original position. However if the snapper alone is thrown, the contralateral pincer transforms into a snapper while a new pincer regenerates at the old snapper site, hence the reversal of asymmetry (Wilson, 1903). Clearly, the pincer has the potential for further development but is inhibited by the presence of the contralateral snapper. Thus in this species we have a model that can be readily manipulated in an attempt to understand the factors responsible for determining handedness. Such insights may be extrapolated to other crustaceans and invertebrates and perhaps to vertebrates in light of recent research demonstrating our probable close affinities (Holley et al., 1995).

Wilson (1903) proposed the interesting but controversial idea that the pincer is simply an immature snapper repressed by the contralateral snapper. This was based upon his observations that a regenerating snapper appeared to pass through a distinct pincer-like phase before becoming a

snapper. Wilson's contention was not supported by Stephens and Mellon (1979) however who found that in terms of muscle fibre length and electrophysiological characteristics a newly regenerated snapper limb is more similar to a snapper than a pincer.

In snapping shrimp reversal of asymmetry involving plasticity of not only exoskeletal but neural, muscular and vascular tissue as well occurs throughout its lifespan (Mellon and Stephens, 1980; Govind and Pearce, 1988; Guchardi and Govind, 1990). To study such plasticity a hypothesis has been constructed which provides a conceptual framework on which my experiments were based. The relationship between the two claws may be envisioned as a see-saw (Govind, 1989) where normally one side is elevated (the pincer) and the other lowered (the snapper) because of the differences in neural input acting on the see-saw. Thus the snapper inhibits the pincer or if the pincer is lost the newly regenerating limb at the pincer site is limited to a pincer. When the snapper is lost, there is a sudden shift in the see-saw's equilibrium to favour the pincer side, the inhibition is removed and the pincer begins transforming into a snapper. The transforming pincer in turn inhibits the newly regenerating limb from becoming a snapper. When both limbs are lost, the "memory" of the differential input to the central nervous system causes the inhibitory signal to be preferentially channelled to the original pincer side allowing a snapper claw to regenerate at the original snapper side. In all cases bilateral asymmetry is maintained.

The see-saw hypothesis accounts for the various experimental manipulations to the snapper claw that trigger claw reversal apart from simple removal of the snapper claw. These manipulations to the snapper include cutting the nerve (Mellon and Stephens, 1978), tenotomy of the closer muscle (Govind et al., 1988), and cooling of the entire claw (Mellon and Cox, 1985). In each case there is a diminution of the neural input from the snapper claw and this serves to tip the see-saw so that the pincer side transforms into a snapper.

The model implies that greater neural input on one side tips the balance to favour that side allowing it to transform into a snapper but at the same time inhibiting the opposite side from regenerating into a snapper. That such inhibition is neurally based was shown when closer muscle

tenotomy or lesions to the nerve of a pincer undergoing transformation to a snapper allowed a snapper to regenerate at the old snapper site, the result being a symmetrical shrimp with double snappers (Young et al., 1994). These findings suggest that not only do neural factors trigger a pincer-to-snapper transformation but additionally, nerve mediated inhibitory factors from a pincer-to-snapper transforming claw limits the newly regenerating claw to a pincer type.

EXPERIMENTAL OBJECTIVES

The purpose of my investigation was to study claw asymmetry in terms of the phenomena of transformation, reversal and regeneration in snapping shrimp. I wanted to characterize the process itself, and to explore the proximate factors responsible for inducing the pincer to begin transforming into a snapper and how the contralateral regenerating limb is limited to a pincer. In addition, I wanted to study more thoroughly the morphological path that a snapper takes as it regenerates and examine the stability of the normal snapper/pincer equilibrium compared to the more atypical configurations occasionally seen such as snapper/snapper and pincer/pincer. The underlying goal in all of these experiments was to test the cross-inhibitory hypothesis associated with the see-saw model for maintaining claw bilateral asymmetry in adult snapping shrimps.

The specific objectives of my experiments are outlined below under four chapters.

Chapter One. Regenerate Limb Bud Sufficient for Claw Reversal

What are the minimum requirements for pincer-to-snapper transformation to occur at the pincer site after autotomy of the snapper and what minimal stage is capable of exerting an inhibitory influence on the contralateral old snapper site? To answer this it was first necessary to characterize the stages of limb bud regeneration. Additionally I also wanted to compare the regeneration rate of pincer and snapper limb buds in terms of growth and development.

Chapter Two. Cell types in regenerating claws

What is the fine structure of the regenerating claw and how is this correlated to the identifiable limb bud stages described above? Does this point to any clues regarding the factors responsible for asymmetry determination or the morphological features that impart competence in a limb bud?

Chapter Three. Regeneration and Sex-Biased Transformation of the Sexually Dimorphic Pincer Claw

What minimum damage to a snapper induces pincer to snapper transformation and does this occur in a similar manner in the sexually dimorphic claws? How is this correlated to the pattern of claw innervation of males and females and what does this tell us about the mechanism and factors responsible for asymmetry determination? Is there a behavioural correlation?

Chapter Four. Claw Transformation and Regeneration: Test of the Inhibition Hypothesis for Maintaining Bilateral Asymmetry

Is the inhibitory mechanism for pincer repression a valid one? This was tested by manipulating the claws in such a way as to interfere with the presumptive inhibitory signal and observing the resulting asymmetry.

CHAPTER ONE. REGENERATE LIMB BUD SUFFICIENT FOR CLAW REVERSAL

ABSTRACT

The paired, bilaterally asymmetric snapper and pincer claws in the adult snapping shrimp *Alpheus heterochelis* were simultaneously autotomized at the beginning of an intermoult, and the resulting growth of the limb buds was characterized into several stages. At the next moult the limb buds emerged as newly regenerated claws of the same morphotype as their predecessors. Next, the paired claws were autotomized sequentially, with the second autotomy timed to different stages of limb bud growth at the first autotomy site. When the snapper is autotomized and a limb bud varying from stages 1 to 5 is allowed to develop at this site before the pincer is removed, the paired claws regenerate in their previous configuration. Similarly, claw asymmetry is retained when the pincer claw is removed first and an early limb bud (stage 1-2) is allowed to form at this site before the snapper is autotomized. However, claw asymmetry is reversed if an advanced limb bud (stage 3-5) is allowed to form at the pincer site before the snapper claw is removed. Under these conditions a snapper regenerates at the pincer site and a pincer at the snapper site. Because the limb bud at this pincer site regenerates as a snapper rather than a pincer, claw transformation has occurred, with the stage 3-5 limb bud substituting for an intact pincer. Therefore, the minimal requirement for pincer-to-snapper transformation is a stage 3-5 limb bud. I postulate that the newly transforming snapper claw restricts regeneration at the contralateral old snapper site to a pincer, thereby ensuring that claw bilateral asymmetry is present, albeit reversed.

INTRODUCTION

The first pair of thoracic chelipeds, or claws, in adult snapping shrimps of the Alpheid family are much larger than the remaining thoracic limbs and are bilaterally asymmetric, consisting of a pincer and snapper claw. The snapper is a much hypertrophied structure almost half the size of the entire animal and is specialized into a powerful snapping tool; a hammer on the dactyl plunges into a matching socket on the pollex, resulting in a loud popping sound (hence the name snapping shrimps) and a jet stream of water (Hazlett and Winn, 1962). The snapping behaviour is used in agonistic encounters and also in crushing the shells of bivalves (McLaughlin, 1982). The contralateral pincer claw is smaller and used primarily in burrowing and feeding (Read et al., 1991).

Claw laterality, or handedness, is random in snapping shrimps, and the snapper appears with equal probability on the right or left side of the animal. However, handedness may be switched in adult shrimps. This happens when the snapper claw is removed at the beginning of an intermoult and in its place a new limb bud regenerates which at the next moult unfolds into a pincer claw, while the contralateral intact pincer claw is transformed into a snapper claw (Przibram, 1901; Wilson, 1903). When only the pincer claw is removed, a new pincer regenerates in its place; when both claws are removed, the regenerates appear in the same morphotype as their predecessors.

The latter procedure of removing both claws within an intermoult was used in an original and imaginative manner by Darby (1934), who varied the time interval between the two autotomies in the tropical shrimp *Alpheus armillatus*. These shrimps live off the coast of Bahama in ocean temperatures of 28-30^0 C and have an intermoult period of 10.5 days, or 252 h. His findings may be summarized as follows. In the experiment in which the snapper claw is removed first and then the pincer claw, despite varying the time interval between the two autotomies from 20 -120 h, the paired claws regenerated in their previous configuration. Autotomy of the pincer claw beyond 120 h after autotomy of the snapper did not allow for a sufficiently advanced limb bud to form on the pincer site, and a claw failed to regenerate at this site at the next moult. Thus, sequential removal of first the snapper and then the pincer within a single intermoult was similar to simultaneous removal

of both claws, as both procedures resulted in the regeneration of paired claws in their previous configuration (Wilson, 1903; Govind et al., 1986).

In another series of experiments Darby (1934) removed first the pincer claw, soon after a moult, and then at varying time intervals the snapper. If the snapper claw was removed up to 29 h after pincer autotomy, the paired claws regenerated in their original configuration. Later removal of the snapper claw led increasingly to a reversal of claw asymmetry; i.e., a pincer regenerated in place of the pristine snapper claw and a snapper regenerated in place of the pristine pincer claw. Thus, when the snapper claw was removed 33 h after pincer autotomy, reversal of asymmetry was seen in 50% of the animals; removal 40 h and 72 h after pincer autotomy produced reversal in 67% and 100% of the animals respectively. In these experiments in which the paired claws are autotomized sequentially, those with the shorter time interval between pincer and snapper autotomy are equivalent to removing both claws at the same time, because claw asymmetry is retained in the previous configuration, whereas those with the longer time intervals are equivalent to removing the snapper alone, because asymmetry is reversed. During these longer time intervals, what transpires that leads to reversal?

One of the events that transpires after a claw has been autotomized is the formation, at this site, of a limb bud that develops during the intermoult and emerges as a new claw at the next moult. Since reversal of claw asymmetry involves transformation of an existing pincer claw into a snapper, I considered the possibility that a limb bud may serve in place of an intact pincer as a suitable target for transformation. This would explain Darby's (1934) findings that claw asymmetry reverses at the longer time intervals between pincer and snapper autotomy, because a limb bud has had time to form at the pincer site. To test this hypothesis, I monitored the development of limb buds at the autotomy sites when both claws are removed simultaneously, producing a chart for limb bud growth. Using this growth chart, I repeated Darby's experiments but, rather than removing the second claw based on the time elapsed after removing the first claw, I removed the second claw at different stages of limb bud growth at the first autotomy site. I find

that presence of a sufficiently advanced limb bud at the pincer site when the snapper is autotomized leads to reversal of claw asymmetry, thereby explaining Darby's results. Additionally, our results demonstrate, for the first time, that the minimum requirement for pincer-to-snapper transformation is a limb bud.

MATERIALS AND METHODS

Adult snapping shrimps of the species, *Alpheus heterochelis* were collected from the tidal pools around Beaufort, North Carolina, and shipped to our laboratory in Scarborough, Ontario. The animals were held in 25 litre glass aquaria equipped with a bottom gravel filter and partitioned into 12 compartments with fibreglass screens (Govind et al., 1986). The aquaria were filled with artificial seawater that was kept at room temperature (22^0 C). A specially prepared diet - a blended mash of chicken livers and hearts, carrots and commercial cereal - was fed to the animals daily, and occasionally live food was provided in the form of *Tubifex* worms. The shrimps were sexed on arrival in the laboratory and their moult history during captivity was recorded.

Shrimps of both sexes were used, and only those with well-differentiated pincer and snapper claws were selected. These animals were allowed to moult twice before being used; this ensured that the claws were pristine, because earlier studies (Read and Govind, 1991) have shown that at least three intermoults are required for a newly regenerated limb to be fully differentiated. These claws are regarded as pristine in the present report (Fig. 1A) . In our laboratory, the intermoult period was between 14 and 30 days (an average of 23 days) at 22^0 C.

Removal of a claw was accomplished by gently pinching the limb near the base of the merus, thereby inducing the animal to autotomize the claw at a preformed fracture plane. The following experiments were performed using this procedure.

1. A day or two after a moult, the paired claws were removed within minutes of each other,

and this is regarded as simultaneous autotomy. The regenerating limb buds at both sites were monitored daily and sketches made that were subsequently categorized into a series of developmental stages. The regenerating limb buds were also measured, in blind observations, under a dissecting microscope with a calibrated eyepiece. To reduce the chance of damaging the very delicate limb buds by repeated handling, measurements were made at 3-4 day intervals. Even so, 11 of the 27 animals suffered damage to the buds and were excluded from the study. To compensate for differences in animal size, limb bud measurements were expressed in terms of a regeneration (R) value where R = (limb bud length / carapace length) x 100 (Bliss, 1960).

2. Within one or two days following a moult, the snapper claw was removed and the regenerating limb bud at this site was monitored daily. Next, the pincer claw was removed at varying stages of limb bud growth at the old snapper site. Of the 52 animals subjected to these sequential autotomies, 38 successfully regenerated paired claws at the next moult.

3. The experimental protocol was similar to that in experiment 2, except that the pincer claw was removed first and at various stages of its limb bud growth the snapper claw was removed. Twenty-four of 36 animals successfully regenerated paired claws at the next moult.

RESULTS

Stages of limb bud regeneration

Following autotomy of a claw, a new limb gradually regenerates at the base. The limb bud that forms is covered by a tough flexible cuticular coat that persists throughout the intermoult and is discarded only at the moult. Limb bud formation begins immediately as a small papilla (stage 1) that enlarges into an apical blastema (stage 2) (Fig. 2). The blastema elongates and acquires a club-

Figure 1. Representative pristine (A) and newly regenerated (B) pincer and snapper claws showing the hypertrophied snapper with the characteristic plunger on its dactyl (arrow). Pristine claws are asymmetric in size, whereas the newly regenerated ones are similar in size and substantially smaller. Scale 3 mm; magnification x5.5.

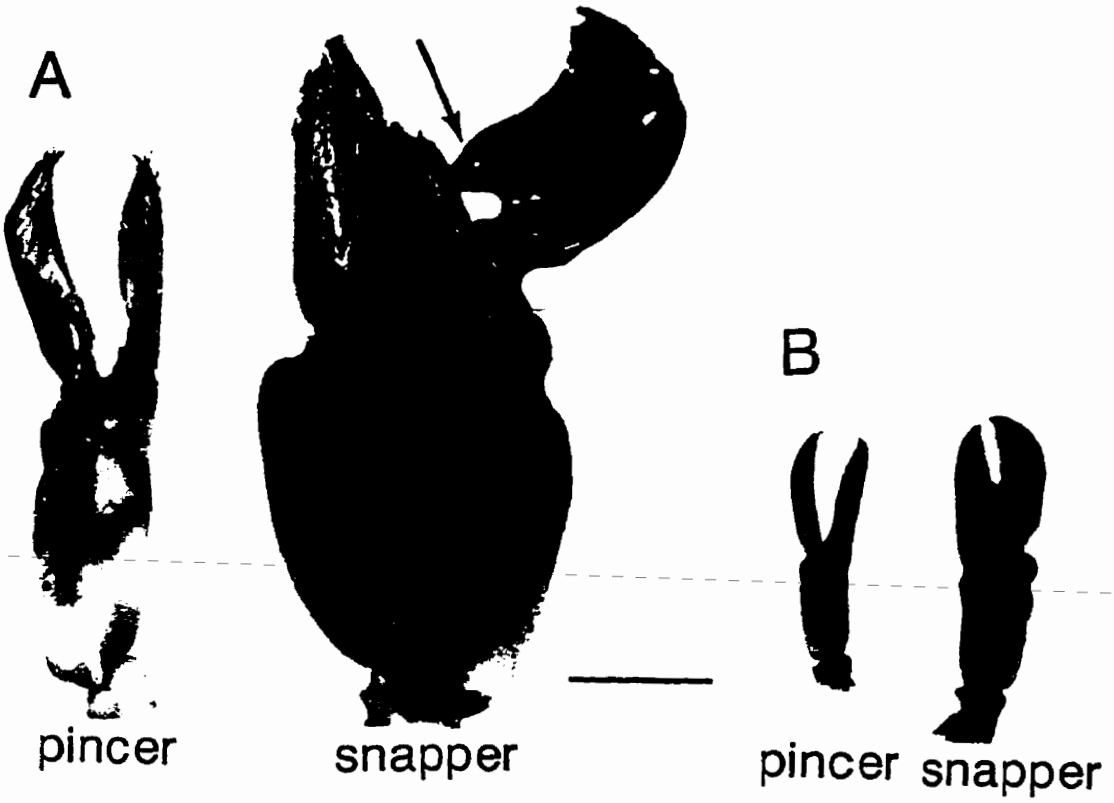
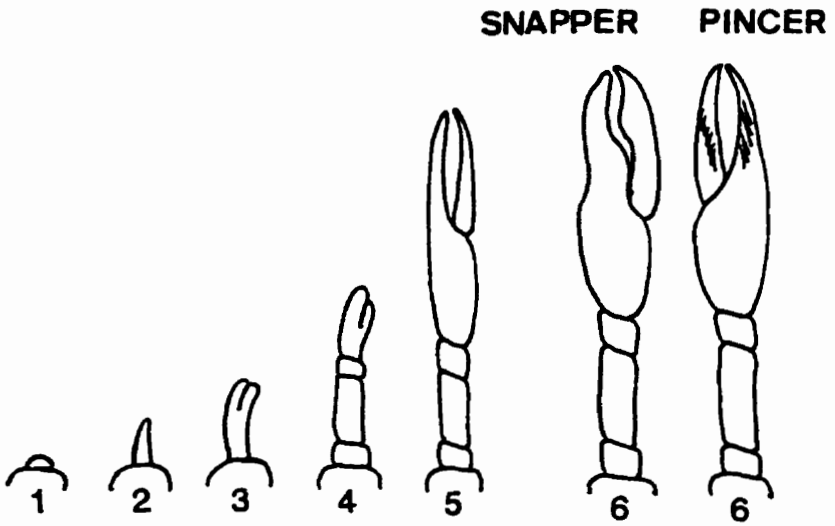

A
B
pincer
snapper
pincer
snapper

Figure 2. Stages in the regeneration of a limb bud at the site of an autotomized claw in adult snapping shrimps. The development of the limb bud, although a continuous process, has been categorized into six (1 - 6) separate stages based on external landmarks; at stage 6 the limb buds are differentiated into snapper and pincer types (see text for description of each stage).



LIMB BUD STAGES

like appearance distally. In the next stage (stage 3), a longitudinal furrow appears in the distal tip, marking the beginning of segmentation by dividing this region into the putative dactyl and propus segment. This is followed by the appearance of a series of transverse furrows along the length of the limb bud (stage 4). By stage 5, segmentation is complete and the limb has acquired typical pincer-like proportions. Further differentiation into a snapper-type claw is marked by the characteristic appearance of a plunger on the dactyl and a matching socket on the pollex (Stage 6) and, in the case of a pincer-type claw, the appearance of a fringe of hair on the propus and dactyl of male shrimps. These six stages represent the major external landmarks in the regeneration of a claw and were used as markers in the present study.

Simultaneous snapper and pincer autotomy

Although earlier experiments (Darby, 1934; Govind et al., 1986) had shown that simultaneous autotomy of both claws results in the regeneration of the paired claws in their previous configuration, these studies did not report on the growth of the limb buds. I repeated this experiment by autotomizing the paired claws within minutes of each other, one or two days after a shrimp had moulted, and monitoring limb bud growth in the ensuing intermoult. Limb bud growth was qualitatively similar on the two sides and followed the criteria listed above. Moreover, the regenerate limb buds of the two sides were also similar in length throughout the intermoult (Fig. 3). At specific times during the intermoult, either the snapper or pincer bud would be slightly more advanced, but there was no consistent pattern and frequently both buds were equal in size. When these limb buds unfolded as claws at the next moult (Fig. 1B), they were much smaller than their predecessors (Fig. 1A) but otherwise similar in morphotype. In all 16 of the 27 animals that successfully regenerated paired claws, the previous asymmetric configuration was retained (Fig. 4A).

Snapper autotomy followed by pincer autotomy

In this experiment, the snapper claw was autotomized one or two days after the shrimp had

Figure 3. Percent regeneration of snapper and pincer limb buds in adult shrimps following simultaneous autotomy of both claws. Percent regeneration was obtained as follows: (limb bud length / carapace length) X 100. A total of 128 limb buds were measured from 16 animals; each data point represents an average of 4-5 measurements.

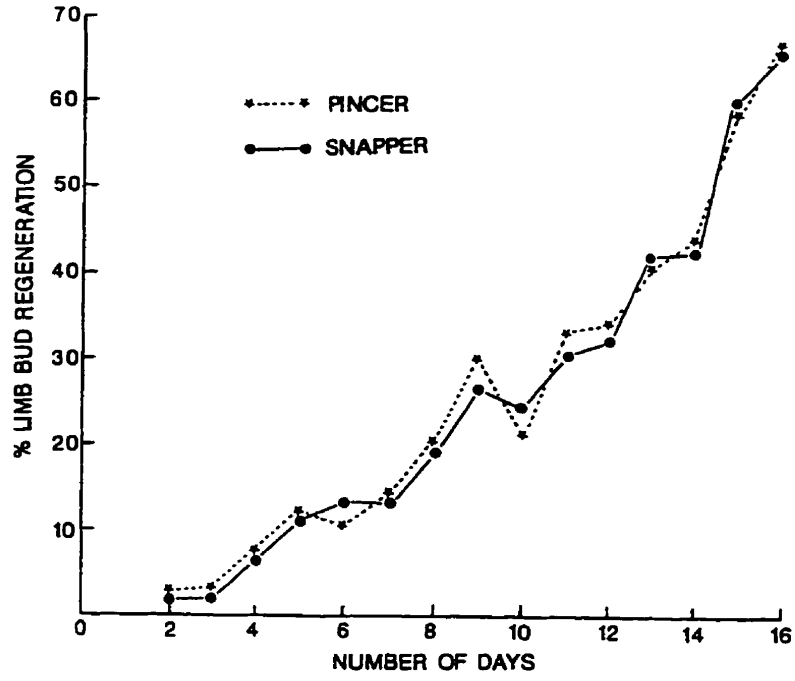
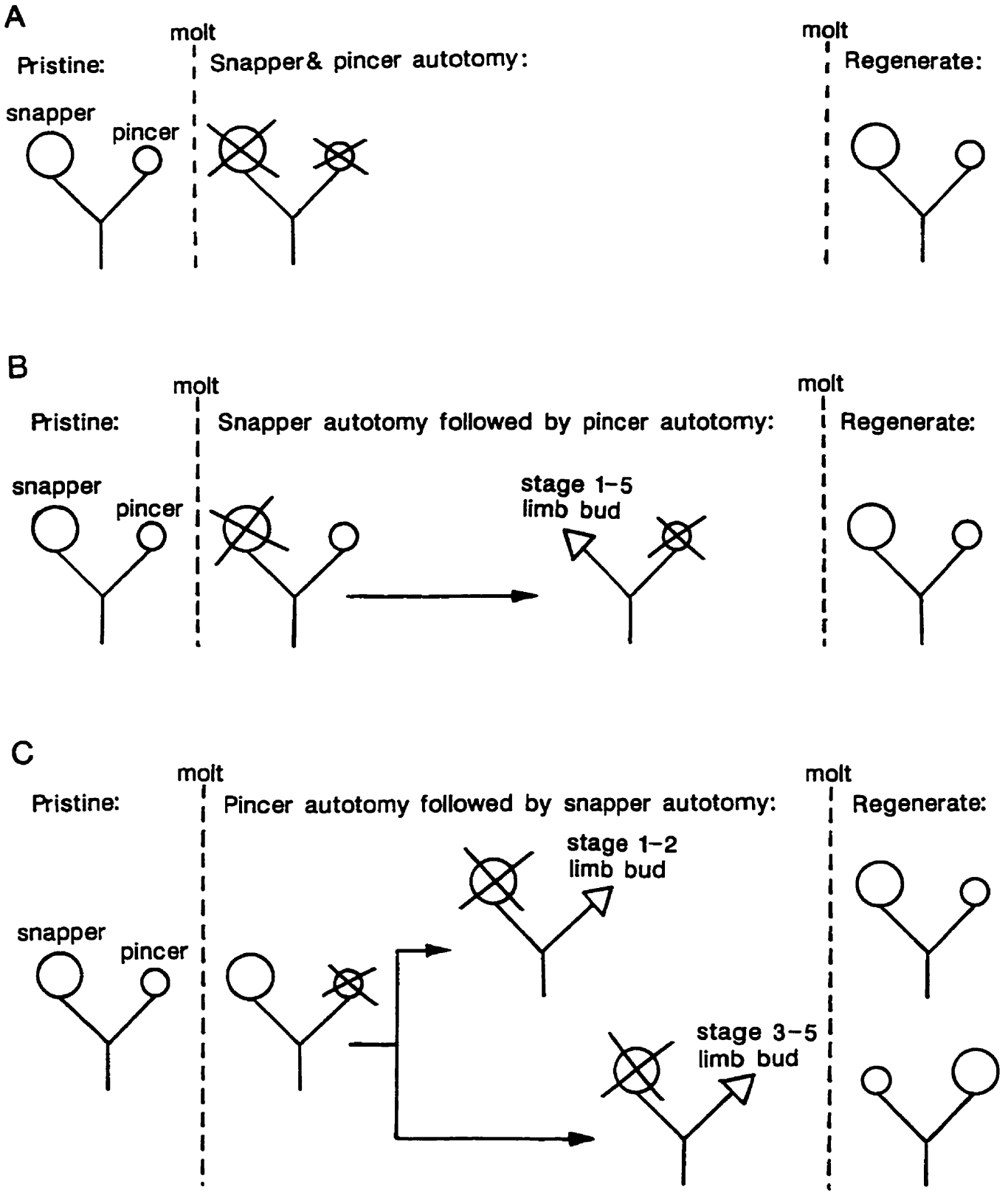

70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
% LIMB BUD REGENERATION
NUMBER OF DAYS
PINCER
SNAPPER

Figure 4. Pictorial representation of the configuration of the paired, asymmetric pincer and snapper claws of adult snapping shrimps in the following experiments: (A) Pincer and snapper claws autotomized simultaneously; regenerate claws appeared in the pristine configuration. (B) The snapper claw was autotomized and, at different limb bud stages at this site, the contralateral pincer claw was autotomized; the regenerate claws appeared in the pristine configuration. (C) The pincer claw was autotomized and, at different stages in the formation of a limb bud at this site, the snapper claw was autotomized; the regenerate claws appeared in the pristine configuration with stage 1-2 limb buds and in the reversed configuration with stage 3-5 limb buds.


A
molt
Pristine:
Snapper& pincer autotomy:
molt
Regenerate:
snapper
pincer
B
molt
Pristine:
Snapper autotomy followed by pincer autotomy:
molt
Regenerate:
snapper
pincer
stage 1-5
limb bud
C
molt
Pristine:
Pincer autotomy followed by snapper autotomy:
molt
Regenerate:
stage 1-2
limb bud
snapper
pincer
stage 3-5
limb bud

moulted, and the pincer claw was then autotomized at different stages of limb bud regeneration on the snapper side. Thus, the pincer was removed at each stage of limb bud growth classified as stages 1 to 5. Of a total of 33 animals in which the pincer claw was autotomized at different limb bud stages, 24 animals successfully regenerated both claws. In all cases, the paired claws mimicked their previous configuration.

In a few additional trials, the pincer claw was also removed when the limb bud at the snapper site was at stage 6 and already differentiated into a pincer claw. In these animals, a pincer appeared at the old snapper site, but a claw failed to form at the old pincer site because there was not enough time for limb regeneration.

Thus, allowing a limb bud to develop to an advanced stage at the snapper site before the pincer is removed leads to the regeneration of the paired claws in their previous configuration (Fig. 4B). In effect, this is similar to simultaneous autotomy of the paired claws, where the limb buds form at equivalent rates on the two sides and claw asymmetry is retained as described above.

Pincer autotomy followed by snapper autotomy

In this experiment, the snapper claw was autotomized at different stages of limb bud regeneration on the pincer side following autotomy of the pincer claw. At the next moult the morphotype of the newly regenerated paired claws was assessed in terms of retention or reversal of claw asymmetry. The results (Table I) show that with increasing time interval between removal of the paired claws, and hence increasing limb bud development, claw asymmetry was reversed. In other words, as the limb bud stage advances, the proportion of claw reversal increases until 100% reversal is reached at stage 3 - 5 limb buds.

Removal of the snapper claw when the limb bud is already sufficiently advanced to be discernible as a pincer type (stage 6) does not result in transformation; the limb bud at the pincer site unfolds as a pincer claw, whereas the snapper site lacks a newly regenerated limb or limb bud because of insufficient time for regeneration.

Table I. Configuration of claw asymmetry, whether retained in the previous configuration or reversed, following autotomy of first the pincer claw and then the snapper claw during a single intermoult in adult snapping shrimps. The pincer was removed first then, at various stages of its limb bud development, the snapper was removed. Number in brackets shows the number successful over the number of trials.

Limb bud stage	Asymmetry retained		Asymmetry reversed	
	number	%	number	%
<1.0 (7/10)	6	86	1	14
1.0-1.5 (10/12)	5	50	5	50
2.0-2.5 (7/10)	3	42	4	58
3.0-4.0 (5/8)	0	0	5	100
>4.5 (9/12)	0	0	9	100

The results of this series of experiment may be summarized as follows (Fig. 4C): removal of the snapper claw when a limb bud of stage 1 - 2 is present at the pincer site results in retention of claw asymmetry at the next moult in 50% or more of the experimental animals, but removal of the snapper claw when a limb bud of stage 3 - 5 is present at the pincer site results in reversal of claw asymmetry in all of the experimental animals.

DISCUSSION

When the paired claws were removed sequentially within an intermoult, they regenerated in their previous configuration if the snapper was autotomized first and a limb bud allowed to form at this site before the pincer was autotomized, providing there is enough time to regenerate a claw at the second site. This is the same as when the paired claws are autotomized simultaneously, the limb buds on the two sides regenerate at equivalent rates, and claw asymmetry is retained.

The paired claws also regenerate in their previous configuration when the pincer is removed first and an early limb bud (stage 1-2) is allowed to form before the snapper is removed in 50% or more of the animals. This is equivalent to removing both claws at the same time. However, paired claws regenerate in the reversed configuration in 100% of the animals when the pincer is removed and a more advanced limb bud (stage 3-5) is allowed to form at this site before the snapper is removed. This is equivalent to removing the snapper in the presence of an intact pincer, in which case the existing pincer claw transforms into a snapper at the next moult and a new pincer regenerates at the old snapper site. A stage 3-5 limb bud at the pincer site therefore acts like an intact, fully formed pincer claw in that they both transform into a snapper claw in response to removal of the contralateral snapper claw. In other words, a stage 3-5 limb bud is a suitable target for transformation.

Stage 3 limb buds are characterized by a longitudinal furrow marking the beginning of division of the two most distal segments, the dactyl and propus. The more proximal segments -

carpus, merus, and basi-ischium - are delineated in stage 4 buds, and segmentation is complete with final delineation of the dactyl and propus in stage 5 limb buds. Although the most advanced stage 4 and 5 limb buds resemble an intact pincer limb in possessing all of the segments, stage 3 buds with just the beginning of segmentation least resemble an intact limb; yet a stage 3 bud may be signalled into developing as a snapper. It would appear that the transforming signal released with autotomy of the snapper claw can influence the differentiation of tissue, not only in intact pincer claws, but also in a developing limb bud at the pincer site. Thus, for example, the intact pincer claw has a closer muscle whose mixture of fast and slow fibres is transformed into purely slow fibres of a snapper type by selective death of the fast fibres (Mearow and Govind, 1986) and transformation of the slow fibres from pincer to snapper type (Mellon and Stephens, 1980). In contrast, in a stage 3 limb bud with just the beginning of segmentation, a fully formed closer muscle is unlikely to be present, yet its subsequent development is directed toward snapper muscle rather than pincer muscle.

Shrimps in which the snapper is autotomized after a stage 3-5 limb bud has formed on the pincer site regenerate a new pincer at the snapper site, resulting in reversal of claw asymmetry. The regeneration of a pincer claw at a snapper site requires explanation. Both Wilson (1903) and Darby (1934) considered the possibility that the pincer claw represents a progressive stage in the development of a snapper claw and that inhibition from the contralateral snapper claw can arrest its development. Hence, when the inhibition is removed with snapper autotomy, the pincer continues its development into a snapper, which at the same time restricts claw regeneration to a pincer type on the opposite side. In this way, bilateral asymmetry of the paired claws is ensured. This hypothesis, involving a cross-inhibitory mechanism, would explain why a pincer regenerates at the old snapper site during claw reversal in the present experiments - transformation of the limb bud into a snapper would restrict regeneration to a pincer claw on the opposite side. The hypothesis would also be tenable in cases where only the pincer claw is autotomized, because the intact snapper claw would restrict regeneration to a pincer claw at the autotomy site. However, the hypothesis is insufficient to explain the case in which paired claws are autotomized simultaneously

and the regenerate claws appear in their previous configuration. With paired simultaneous autotomy, an additional mechanism would have to be invoked to allow the transforming signal to act at the old snapper site - either by preferentially channelling the signal to this site or by having receptors for the signal exclusively at this site.

CHAPTER TWO. CELL TYPES IN REGENERATING CLAWS

ABSTRACT

Cell types in the regenerating claws of adult snapping shrimps are described based on electron microscopy. Following autotomy of a limb, the coxal stump has numerous fibroblasts with long cytoplasmic processes that form small fluid compartments. These compartments provide structural framework and are inundated with mostly haemocytes and blood vessels. Agranular haemocytes were uncommon compared to granular ones which had prominent pseudopodia, vacuoles and lysosomes, features suggesting phagocytic function. Cytoplasmic network formed by fibroblasts persisted in the regenerating blastema and papilla as well as granular haemocytes and the appearance of blastemal cells. Close structural associations were observed amongst all three cell types. Regional proliferation of cells subdivides the distal tip of the papilla into the presumptive propus and dactyl and marks the beginning of segmentation proceeding in a distal to proximal direction. This is accompanied by the appearance of first afferent innervation proceeding also in a distal to proximal direction and multinucleate myoblasts identified by fragments of myofibrils, then efferent innervation and well organized muscle. A characteristic feature of the latter stages was prominent intercellular contacts between haemocytes and other cell types within the papilla. These junctions may serve for adhesion as well as for communication. The early and prevalent appearance of haemocytes in the regenerating limb bud, as well as their pluripotent nature in other regenerating tissues, implicates them as a potential source for the origin of blastemal cells.

INTRODUCTION

The ability of crustaceans to regrow lost limbs is legendary and as a result they have historically been favourite subjects for regeneration research (Skinner and Cook, 1991). Relative to vertebrates their tissue systems are less complicated and regeneration generally proceeds very rapidly. In the snapping shrimp *Alpheus heterochelis* for example an entire functional claw may regrow in less than three weeks.

Limb regeneration is epimorphic in crustaceans (Needham, 1965; Skinner, 1985) and in vertebrates capable of whole limb regeneration (Korneluk and Liversage, 1984; Kahn and Liversage, 1990) in that a blastema is first produced at the site of the lost limb and subsequent growth and differentiation of the blastema results in a newly formed limb. Consequently there is considerable interest in the ultrastructure of the blastema in which not only epithelial cells delimiting the blastema but also immigrant cells such as haemocytes provide cellular resources for the differentiation of limb tissues. For example, haemocytes in the crayfish *Asticus fluviatilis*, the larval mud crab *Rhithropanopeus harrisii* and the crab *Menippe rumphii* may transform into myogenic cells (Babu, 1987; Uhrik et al., 1989; Lumb et al., 1991) while in snapping shrimp they have been implicated in the recycling of muscle proteins and possibly acting as stem cells for new myofibrils (Govind and Pearce, 1994). In horseshoe crabs haemocytes in the form of plasmatocytes may give rise to muscle as well as to other tissue rendering them pluripotent (Clare et al., 1990). It therefore appeared profitable to conduct an electron microscopic study of limb regeneration in snapping shrimps to more clearly define the nature and distribution of cell types characteristic of the blastema when it first forms and when it subsequently differentiates into a limb. Such a study is facilitated by the fact that claw regeneration in snapping shrimp has been categorized based on external morphology into a series of six stages (Govind and Read, 1994). Limb bud stages 1 to 5 are similar between the paired regenerating claws which in snapping shrimps are highly dimorphic consisting of a large snapper claw and a much smaller pincer claw. The regenerating limb is recognizable as pincer or snapper at limb bud stage 6 just prior to

emergence of the newly regenerated claw.

In addition to having the ability to regenerate their paired asymmetric claws, snapping shrimp are capable of reversing their claw asymmetry following loss of the snapper claw (Wilson 1903); a pincer regenerates at the snapper site while the existing pincer transforms into a snapper. A reversal of claw asymmetry may also occur if first the pincer is removed then at a later time in the same intermoult, the snapper (Darby, 1934). It was subsequently determined that the critical period of snapper removal leading to asymmetry reversal was correlated with the presence of a stage 3 limb bud on the contralateral pincer side (Govind and Read, 1994). Thus I wished to determine in particular the ultrastructure of stage 3 limb buds in order to identify features that make this stage competent for transforming into a snapper.

I find that early stages of limb regeneration are characterized by several cell types; epithelial cells which circumscribe the blastema and give rise to the papilla, fibroblasts which with their elongated processes give rise to a scaffolding, and haemocytes which inundate the scaffolding. Proliferation of epithelial cells into the papilla to segment the distal tip of the papilla into a propus and dactyl marks the beginning of its differentiation into a limb and at this stage a pincer regenerate limb bud becomes competent to transform into a snapper claw.

MATERIALS AND METHODS

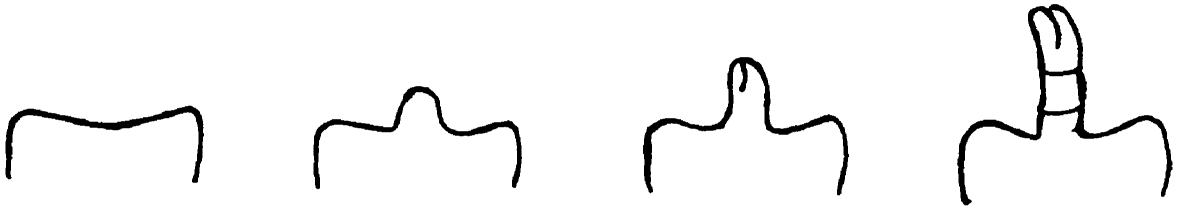
Snapping shrimp, *Alpheus heterochelis* were collected at low tide from intertidal zones along the coast near Beaufort, North Carolina by W. D. Kirby-Smith and shipped to Scarborough, Ontario. Upon their arrival in the laboratory they were sexed and kept in 20L aquaria, subdivided with plastic mesh screen into 12 compartments, each one housing a single shrimp. The animals were maintained at room temperature (22^{o}C) and fed twice weekly a diet consisting of a mash of beef heart, vegetables, eggs, algae, fish, pablum, flake fish food and tinned cat food all bound together with gelatin. On occasion they were fed live tubifex or small

compost worms.Under these conditions the shrimp had an intermoult period between 14 and 30 d depending upon their size, for an average of 23 d. To assure that the shrimp to be used experimentally were in pristine condition, they were allowed to moult at least twice (Read and Govind 1991) and a detailed daily moult history was kept for each animal.

Autotomy of the claws was carried out one or two days following ecdydsis. Pincer or snapper claws were removed by gently pinching the merus with forceps causing the animal to autotomize the claw at the preformed fracture plane. Thereafter the shrimp were examined on a daily basis to monitor limb regeneration which is divisible into six limb bud stages based on external morphology (Govind and Read, 1994). Selected limb bud stages were cut proximal to the autotomy plane and immersed in a marine fixative composed of 2.5% glutaraldehyde and 0.2% formaldehyde in 0.15M sodium cacodylate buffer at pH 7.4, containing sucrose, sodium chloride and calcium chloride (Govind and Pearce, 1985) for 1.5 - 2h. Regenerating limb buds, though soft were resistant to infiltration by the fixative due to a very tough, impervious protective capsule. In order for the fixative to gain access to the interior of more mature, longer limb buds, both ends of the limb were clipped off and the bud itself continuously injected with fixative using a 1 cc syringe. Additionally, by means of very fine insect pins, holes were created in the tough membrane covering the limb bud to allow fixative to enter. Following primary fixation, the tissue was next washed in the cacodylate buffer for 45 min and postfixed in 1% osmium tetroxide in buffer for 1.5 h. The tissue was dehydrated in an ethanol series and then infiltrated and embedded in epon-araldite resin and cured in a 60°C oven for 48 h. Both the infiltration and embedding were drawn out for a much longer period of time (2-4 d) part of which was carried out in a vacuum desiccator.

The limb buds were cut in cross and longitudinal section using a diamond knife mounted on an ultramicrotome. Thin (75 - 90 nm) sections were placed on formvar-coated single-slot grids, stained with uranyl acetate and lead citrate and examined with a Zeiss 9S and Siemens 102 electron microscope. The limb buds of 15 shrimp, covering stages 1 to 5 (Fig. 1) and ranging from 1.5 h to

Figure 1. Limb regeneration events based on an electron microscopic examination of limb bud stages 1 to 5. The coxal stump represents the post-autotomy period while blastema/papilla represents limb bud stages 1 and 2. Segmentation of the limb begins in stage 3 and is followed by tissue differentiation in limb bud stages 4 and 5.


coxal stump
blastema/papilla
segmentation
tissue formation

22 d post autotomy were successfully prepared for electron microscopy and examined. The regenerating limb buds of both snapper and pincer claws were examined however no difference was detected between them.

RESULTS

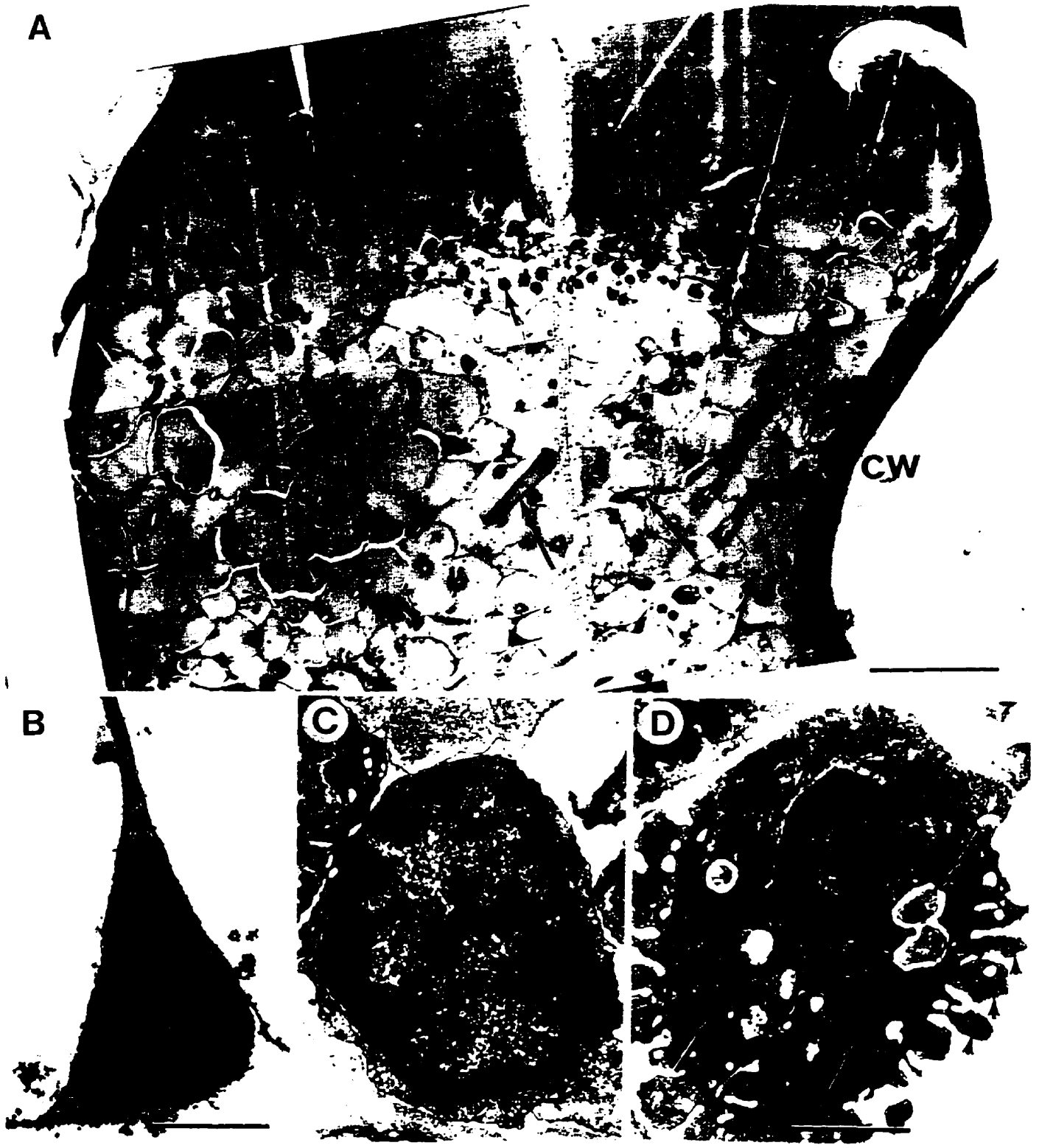
Figure 1 depicts limb bud stages of adult snapping shrimps examined in the present study beginning with the coxal stump, the emergence of the blastema/papilla, initial segmentation of the papilla and lastly, tissue differentiation specifically nerve and muscle.

Coxal stump

Immediately following removal of the limb an autotomy membrane expands across the stump covering the wound and reducing fluid loss to a minimum. As seen in longitudinal section (Fig 2A) the stump at the autotomy plane was covered by a very thin, dense membrane, lined below by a single layer of columnar epithelial cells. Subjacent to the autotomy membrane the stump was compartmentalized, each roughly spherical compartment being delineated by very thin strands composed of a bilayered membrane. The strands are extensions of the cytoplasm of stellate fibroblasts having very scanty cytoplasm and a relatively heterochromatic, multilobed nuclei (Fig 2B). The compartments were approximately equal in size and mostly empty, although some held concentrations of blood cells, especially those near the epidermis lining the cuticle along the sides of the stump (Fig 2A). Thus the fibroblasts form a scaffolding which provides a structural framework.

Within the scaffolding numerous small blood vessels were visible and many free cells, mostly haemocytes have gathered near the autotomy membrane (Fig 2A) . One type, relatively uncommon was agranular with scanty cytoplasm and had regular, oval shaped nuclei (Fig 2C).

Figure. 2. (A) Low magnification longitudinal-section through coxal stump showing compartmentalization (asterisks) of stump and haemocytes (small arrows) aggregating near autotomy membrane (am) and along coxal walls (cw). Large arrows indicate blood vessels. Cell types found within coxal stump include (B) fibroblasts with long thin cytoplasmic processes, (C) agranular haemocytes and (D) granular haemocyte with vacuolate pseudopodia (arrowheads) and cytoplasm containing dense granular inclusions (arrows) and phagosome (double arrow). A x220; B x5 000; C, D x10 400. Bars: A 100μm; B 1μm; C, D 2μm


A
CW
B
C
D

The other more common type (Fig 2D) was granular and had large amounts of cytoplasm containing many small, oval, electron dense, non-membrane bound inclusions with prominent cytoplasmic extensions (pseudopodia). In some of these granulocytes, likely amoebocytes, the pseudopodia were angular, highly vacuolated and numerous, characterized by the presence of heterogenous, membrane bound lysosomes including phagosomes and primary or possibly secondary lysosomes. Mitochondria were abundant and the nuclei of these granulocytes were irregular in shape and lobular.

Blastema - papilla (limb bud stages 1 and 2)

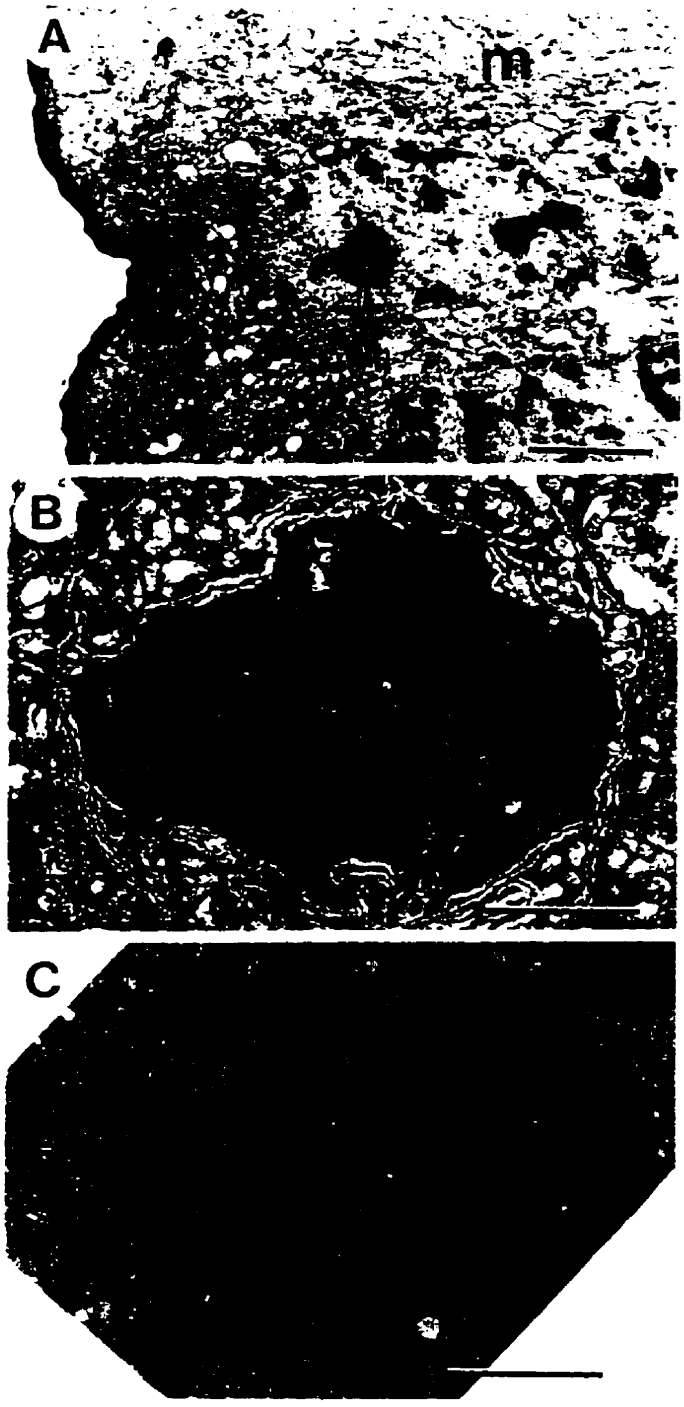
The initial gross sign of regeneration occurs as the blastema erupts from the coxal stump (stage 1 limb bud) followed by its elongation into a papilla (stage 2 limb bud) (Fig 1). The epithelium of the blastema and papilla was comprised of 2-3 layers of cells packed around the periphery of the blastema (Fig 3A). Epithelial cells had irregularly shaped, centrally positioned nuclei which were relatively euchromatic and possessed prominent nucleoli (Fig 4A). The abundant cytoplasm contained numerous mitochondria and was densely packed with ribosomes but had scanty endoplasmic reticuli and few Golgi bodies. Some of the epithelial cells were in active mitosis (Fig 4B).

The central region of the blastema subjacent to the epithelial cells was largely empty with a few isolated scattered cells (Fig 3A). Among these two distinctive cell types were the granular haemocytes and fusiform-shaped fibroblasts. The latter had large nuclei, abundant ribosomes, scanty cytoplasm with little rough endoplasmic reticulum and few mitochondria. They were also characterized by long, slender processes with some of the processes having thin filaments adhering to neighbouring blastema cells (Fig 3B). There appeared to be a close relationship between the haemocytes, fibroblasts and blastema cells. The blastema cells, representing the third cell type, were similar in appearance, being rather undifferentiated having large, euchromatic nuclei (Fig

Figure 3. A Low magnification longitudinal-section through limb bud (b) and coxal stump (cs) showing a proliferating epithelium and relative absence of cellular material within stage 2 limb bud. Below the papilla are fibroblasts (arrow) forming loose compartments and scattered haemocytes (arrowhead). The limb nerve (n) has retracted away from the autotomy plane and is enveloped by fusiform cells (double arrow). B Within the limb bud haemocytes (h) often appear closely associated with fibroblasts (f) which are characterized by thin, long cytoplasmic extensions (arrows). A x220; B x3 100. Bars: A 100μm; B 5μm


A
b
CS
B
h
f

Figure 4. A Epithelial cells with euchromatic nuclei, prominent nucleoli (arrow) and abundant mitochondria (m). B Cell in metaphase of mitosis with chromosomes aligned along the equatorial plate. C Undifferentiated blastema cells with highly euchromatic nuclei. A x3 100; B, C x3 900. Bar: 5μm


A
m
B
C

4C), indicative of rapidly growing cells with a high rate of protein synthesis. The cytoplasm had virtually no membranous organelles and was densely, though evenly packed with small electron dense granules, likely ribosomes occurring either singly or in rosettes (polysomes).

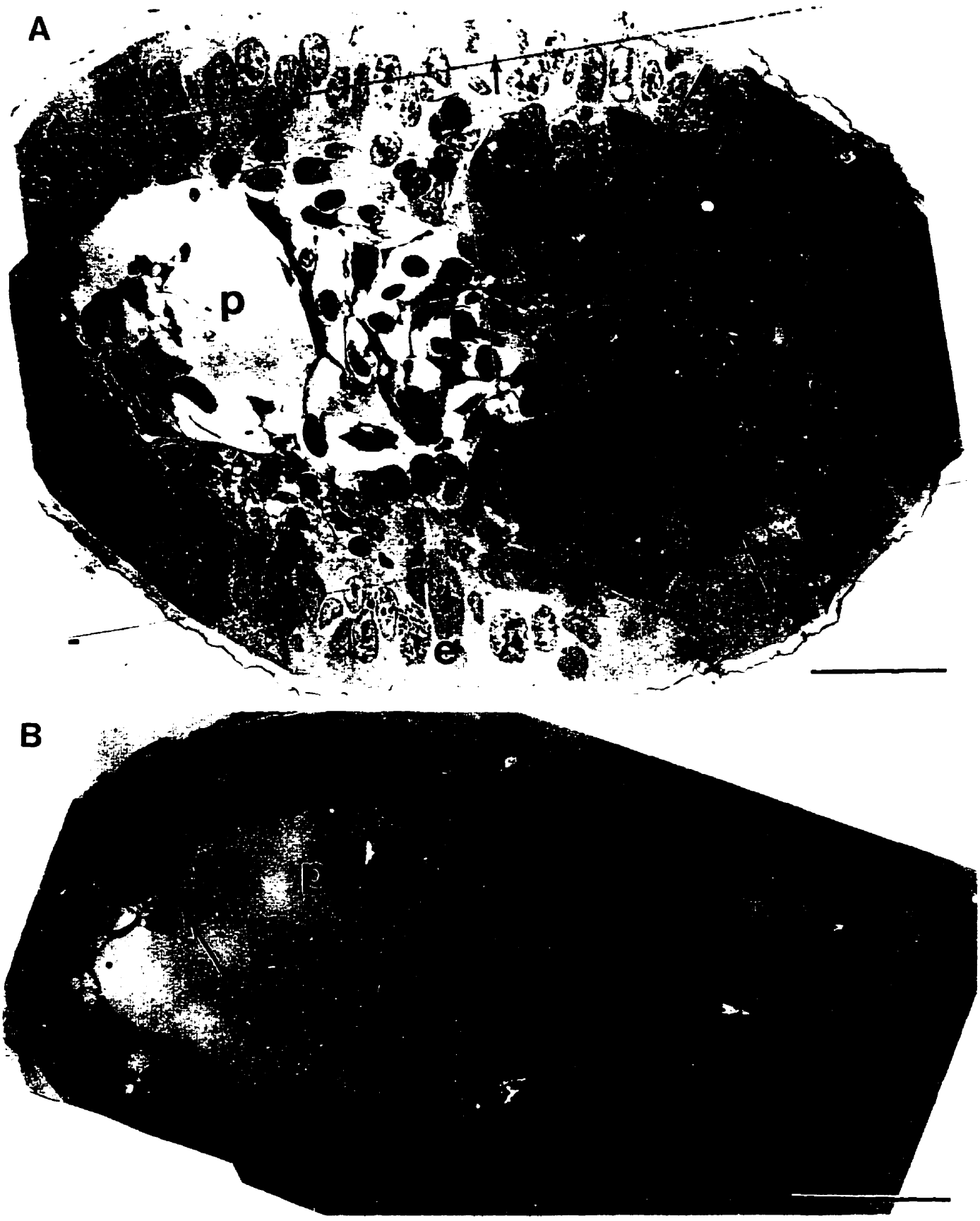
Below the papilla, proximal to the autotomy plane was the large pedal nerve (Fig 3A), many blood vessels and free cells including haemocytes, fibroblasts and blastema cells. These formed closely associated groups, contained within a network of cytoplasmic strands directly below the papilla. Around the perimeter of the nerve, within or perhaps subjacent to the perineurium were several large fusiform cells (Fig 3A).

Segmentation (limb bud stage 3)

A longitudinal furrow appears in the distal tip of the papilla marking the beginning of segmentation by dividing this region into the putative dactyl and propus segments (Fig 1). Opposing groups of proliferating cells, presumably epithelial in origin, have bulged inwards (Fig. 5A) to longitudinally differentiate the distal segments forming two distinct areas within that region of the limb bud: the small dactyl which at this point was empty and the much larger propus containing a number of different free cell types including granular and agranular haemocytes and fibroblasts with long and in some cases very irregular cytoplasmic extensions packed with mitochondria. No granular amoebocytes were observed.

Epithelial and free (blastemal) cells were observed in both early telophase and metaphase stages denoting active proliferation of these cells (Fig 5A). These cells also displayed granular cytoplasm with abundant free and attached ribosomes, polysomes and mitochondria as well as prominent rough endoplasmic reticulum and conspicuous Golgi bodies.

Figure 5. A Low magnification cross-section through distal region of stage 3 limb bud in which opposing groups of proliferating cells have bulged inwards (arrows) to begin segmentation into propus (p) and dactyl (d). Both epithelial (e) and free cells are in active mitosis (short arrows) B Segmentation is complete in stage 4 limb bud by a dividing wall of cuticle (c). Note also the large amount of empty space within the segments, the long cytoplasmic extensions between free cells (arrows) and the intimate association (double arrow)between different free cell types and epithelial (e) cells. A x480; B x570. Bar: 50μm


A
p
e
B
p

Tissue differentiation (limb bud stages 4 and 5)

In stage 4 limb buds a series of transverse furrows along the length of the limb bud appears representing division of the more proximal limb segments (Govind and Read 1994) (Fig 1). The distal region of the limb bud has clearly divided into a propus and dactyl with cuticular tissue between the respective segments (Fig 5B). Apart from the epithelial cells circumscribing the segments, the segments are still largely empty, however stellate and fusiform fibroblasts with long cytoplasmic processes form a network within the lumen. Some of the processes of the fibroblasts make contact with haemocytes and epithelial cells within the lumen (Fig 5B).

First appearance of muscle tissue was detected in cross-sections near the propus-dactyl joint (Fig. 6A, B). The tissue appears in the form of scattered fragments of striated myofibrils in two regions of the propus, probably indicative of the opener and closer muscles. The myoblasts possessed large euchromatic nuclei, prominent nucleoli and abundant ribosomes and mitochondria but virtually no Golgi bodies, endoplasmic or sarcoplasmic reticuli. No associated efferent innervation was observed. Also seen for the first time was sensory innervation in the form of outer dendritic segments surrounded by enveloping cells within the epithelial layer (Fig 6C). These were observed in the distal regions in early stage 4 limb buds and both distally and to a lesser extent proximally in later stage 4 buds, suggesting that afferent innervation regenerates in a distal to proximal direction.

Haemocytes and fibroblasts are still the predominant cell types in the lumen of the limb segment, often with contacts in the form of junctional complexes, between them (Fig 6D). Additionally, cells with lipid droplets in the cytoplasm were seen (not shown). These membrane-bound inclusions had a fine granular appearance but stained unevenly, were large and spherical and filled the cytoplasm. These cells had very prominent and densely stacked Golgi bodies, rough endplasmic reticuli and elongate, lobular nuclei.

Stage 5 limb buds though still small have acquired the typical pincer-like proportions and

Figure 6. A Myofibrillar fragments (arrows) observed in cytoplasm of intimately associated groups of myoblasts one of which is shown at higher magnification (double arrow) in B. B Myoblast displaying organization into sarcomeres delimited by Z-lines (z). Arrows show plasma membrane of myoblast which is still unfused at this stage. C Relationship between haemocyte (h), other cell types and cytoplasmic strands and filaments within the limb bud; note presence of presumptive innervation (arrow) within cytoplasm. Key features of these associations were junctional complexes (double arrow) shown at a higher magnification in D (arrows) extending from haemocyte to other cell types. A x2 900; B x26 400; C x4 400; D x16 800. Bars: A, C 5μm; B, D 1μm

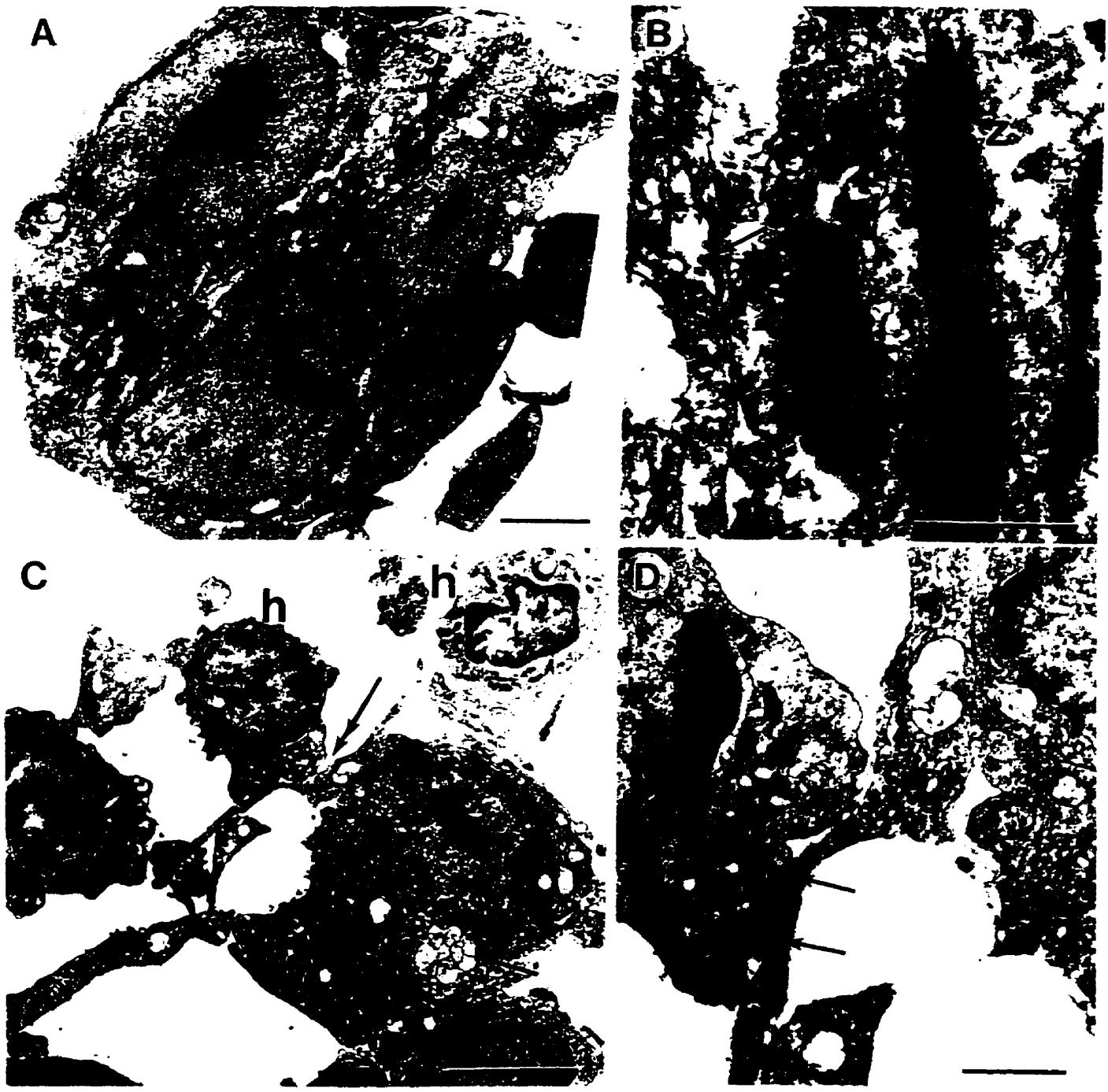
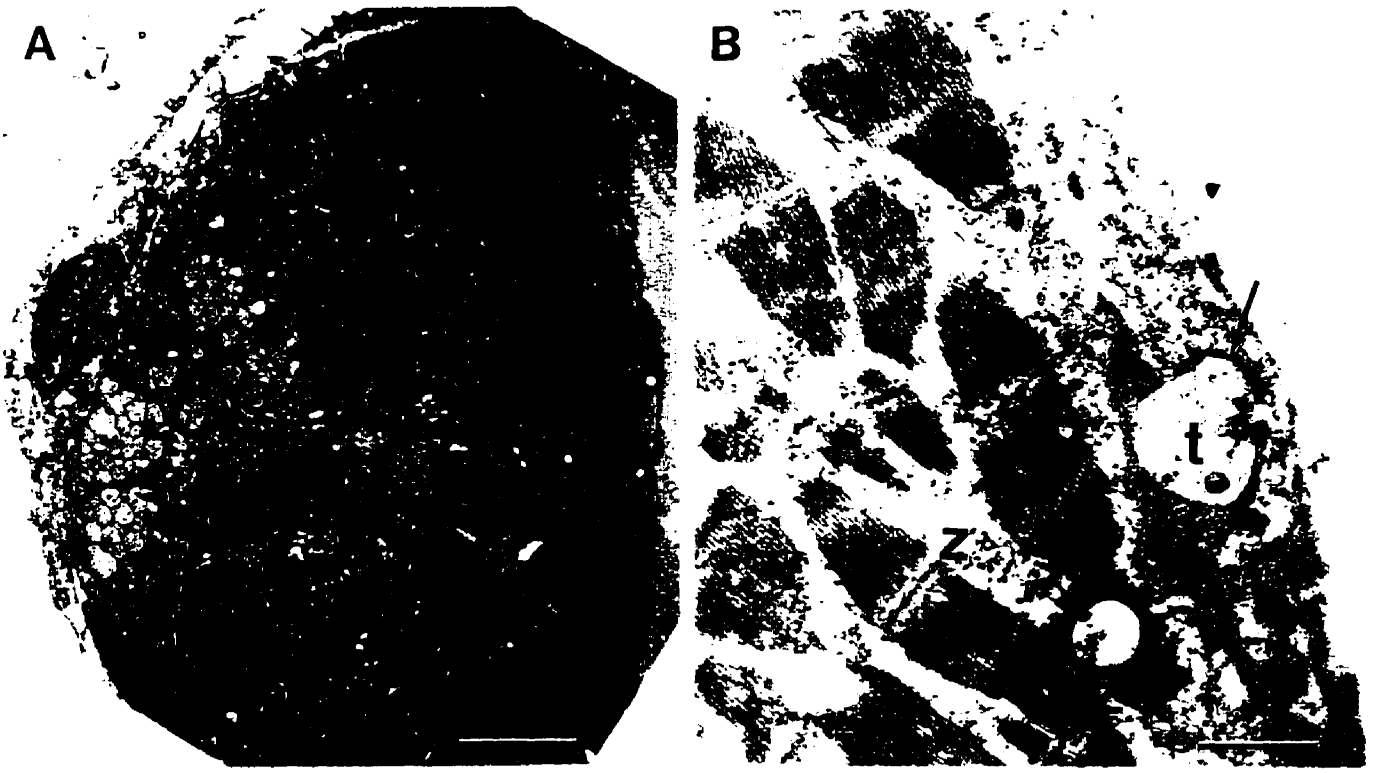

A
B
Z
C
h
h
D

Figure 7. A Well organized nerve with numerous bundles of unmyelinated axons (a) some in groups and glial nuclei (n) in a stage 5 limb bud. B Striated muscle with Z-lines (z) defining sarcomeres and a nerve terminal (t) containing clear synaptic vesicles and making synaptic contact (arrow) with muscle granular sarcoplasm in a stage 5 limb bud. A x3 100; B x15 200. Bars: A 1μm, B 5μm


A
B
t
z

were fully segmented. The segments possessed a well-organized nerve with unmyelinated axons and accompanying glial cells (Fig 7A) and well-defined muscle fibres with myofibrils and occasional synaptic terminals (Fig 7B).

DISCUSSION

One of the main findings from our study is the appearance of a scaffold formed by the interconnecting processes of fibroblasts in regenerating blastema. The long thin processes of the scaffolding likely provide minimal structural support most of which is probably supplied by the fluid within these compartments. However, the scaffold appears to provide a framework for the laying down of future limb tissue, the first of which to appear is the boundary between propus and dactyl. Further segmentation along the length of the papilla demarcates the entire limb and only after this does more specialized tissue appear such as nerve and muscle.

Subdivision of the distal tip of the papilla into the putative propus and dactyl is therefore the earliest indication that the regenerating blastema is destined to become a limb. This marks a stage 3 limb bud which is also the earliest time during regeneration of a pincer limb when removing the contralateral snapper claw will result in a reversal of claw bilateral asymmetry i.e. the regenerating pincer side will develop as a snapper and the snapper side will regenerate as a pincer (Govind and Read, 1994). Removal of the snapper claw before the pincer side has regenerated a stage 3 limb bud will result in the regeneration of the paired claws in their previous configuration and claw bilateral asymmetry is not reversed. Thus the transforming signal will only be effective on the regenerating tissue when it has committed to becoming a limb i.e. when it has begun segmentation. If the transforming signal is inhibition (Read and Govind, 1997) then it means that inhibition is applied early in development and regeneration, but can be lifted at almost any time as a fully formed pincer will transform into a snapper.

The other main finding is the identification of cell types in the blastema. Epithelial cells,

fibroblasts and haemocytes constitute the major types and all three appear to interact with each other via junctional complexes. A prominent feature of cells within the lumen of the limb bud particularly during stage 3 and 4 were the numerous intercellular contacts between different types of cells. These contacts usually took the form of slender, microvillus cytoplasmic extensions, sometimes flattened at the site of contact, similar to hemidesmosomes, although gap junctions were also observed. This was particularly evident between amoebocytic haemocytes and fibroblast-type cells responsible for the network of cytoplasmic extensions prominent within the luminal space of the papilla. In crayfish, similar types of junctional complexes were observed between granular haemocyte and glial cells of damaged ganglion nerve roots (Shivers, 1977). It was suggested that these contacts functioned as a means of identifying damaged tissue prior to ingesting it. Junctional complexes found between different cell types participating in muscle regeneration in crayfish were also thought to be involved in information exchange perhaps to synchronize the regeneration process amongst the different cell types (Uhrik et al., 1989). This seems a more likely function for the intercellular junctional complexes observed in the regenerating limbs of snapping shrimp, although the more obvious function of intercellular adhesion should also not be discounted.

Based on their structure, distribution and association with other tissue, we can begin to deduce the probable role of the identified cell types in regeneration. Possibly the most prevalent and prominent among the cell types are the haemocytes. Shortly after autotomy, they appear to converge at the wound site, especially concentrated beneath the autotomy membrane, but also alongside the coxal walls which implies that the latter is the migration route they take to the fracture plane. Most of these cells are granulated haemocytes, likely coagulocytes, degranulating in a process which may initiate scab formation (Hopkins, 1993). A number of studies have attempted to classify crustacean haemocytes (Sternshein and Burton, 1980; Martin and Graves, 1985; Hose et al., 1990), however the issue remains unclear in terms of terminology (amoebocytes, coagulocytes vs. hyaline cells, small/large granule haemocyte), function and relative abundance. This may simply be a reflection of how diverse haemocyte morphology is amongst different species and as Hose at al. (1990) emphasize, attempting to classify solely on the basis of

morphology without considering function is highly speculative.

Haemocytes are certainly critical to crustaceans successfully regenerating a limb bud. They appear to be involved in many aspects of limb regeneration including phagocytosis of necrotic material and micro-organisms, scab and blastema formation, acting as stem cells for the regeneration of other tissues and even recycling of proteins (Adiyodi, 1972; Uhrik et al., 1989; Hose et al., 1990; Govind and Pearce, 1994; this study). In snapping shrimp, granular haemocytes were the most abundant type, many involved in phagocytosis but others clearly serving other functions such as coagulation and possibly tissue differentiation. Small numbers of agranular haemocytes were also observed, but their function was unclear. Because tissue damage is minimal with autotomy, consisting primarily of blood vessels and degenerating nerve, this is likely the material which amoebocytic haemocytes were observed actively phagocytosing.

In crayfish, amoebocytic haemocytes were also found to be involved not only with the degeneration of damaged muscle, but under the influence of satellite cells were thought to transform into myogenic cells, possessing newly formed contractile filaments within the cytoplasm (Uhrik et al., 1989). Haemocytes were also closely associated with the degeneration of fast fibres and subsequent regeneration of slow fibres during pincer to snapper transformation in snapping shrimp (Govind and Pearce, 1994). In particular, amoebocytes were found to phagocytose fast fibres, transporting crystalline bodies into the new myotubes and possibly acting as stem cells for new myoblasts. In the present study of limb regeneration in snapping shrimp, first appearance of muscle was at stage 4, and involved cells of indeterminate origin coalesced (while remaining distinct) to form myotubes. In contrast, in *Paratelphusa* fibroblasts mobilize to create myoblasts, initially uni-, then multinucleate as they fuse and insert into their respective tendons and cuticle (Adiyodi, 1972). I am not sure whether the indeterminate cells seen in regenerating limbs of snapping shrimp were haemocytes or fibroblasts. Intimately associated with granular haemocytes were presumptive areas of innervation within the blastemal lumen. This leads me to speculate that either of these cell types (haemocytes or fibroblasts) or perhaps both may be involved in the

regeneration of afferent neurons.

How does whole limb or reparative regeneration compare in vertebrates and crustaceans? Epimorphosis, which requires that an apical epithelial cap form, dedifferentiated cells be produced and a critical mass of cells (the blastema) accumulate seems to occur in both groups. In vertebrates, this is exemplified by only a few species including the adult newt *Notophthalmus* (Korneluk and Liversage, 1984) and the early stage tadpole of the clawed frog *Xenopus* (Khan and Liversage, 1990), while in crustaceans, virtually all species including snapping shrimp are capable of regeneration. In effect, during epimorphic regeneration there is a recapitulation of the ontogenetic events that occur during embryonic limb bud development (Korneluk and Liversage, 1984). Heteromorphic regeneration, otherwise known as tissue regeneration, in which following injury there is an immediate attempt to reconstitute some tissues, seems to be restricted to vertebrates as exemplified by later stage *Xenopus* tadpoles (Khan and Liversage, 1990).

Perhaps the most controversial and unresolved issue concerns the origin of the blastema cells responsible for epimorphic regeneration. Four possibilities exist including: 1) haemocytes, 2) reserve cells, 3) wound epithelium or epidermal cells, and 4) dedifferentiation of stump tissues. In vertebrates it has been shown that although all four factors are necessary for limb regeneration to occur, the main and perhaps only source of progenitor cells is the dedifferentiation of mesodermal stump tissues (Liversage, 1991; Brockes, 1994) although there is evidence that reserve larval satellite cells may also act as a source for regenerating skeletal muscle in adult urodeles (Cameron et al., 1986). The situation in crustaceans is less clear cut. One school of thought is that, as with the vertebrates, the process is entirely local, due primarily to the dedifferentiation and redifferentiation of nearby epidermal (which are very proliferative), neural sheath or muscle cells (e.g. Needham, 1965; Adiyodi, 1972; Skinner, 1985), however there is little direct evidence for this contention. An epidermal origin for muscle, for example seems unlikely for at least two reasons. When epidermis retreats from the limb stump, the muscle insertions persist, an unexpected occurrence if both muscle and epidermis had a common origin. Also, regenerates

sometimes occur which consists of a permanently empty epidermal shell, suggesting that the epidermis is not able to give rise to other tissues or organs (Needham, 1965).

A second school of thought is that immigrant systemic cells play a vital role, particularly with respect to myogenesis. In addition to the evidence presented earlier (Uhrik et al., 1989; Govind and Pearce, 1994) and this study, Lumb et al. (1991), in documenting limb regeneration in larval mud crab showed that cells which gave rise to myoblasts were also found free in the hemolymph and appeared to be closely related to the haemocytes of other crabs. Haemocytes were also implicated by Babu (1987) who noted their migration to the cuticular shell where they differentiated into small patches of muscle. Plasmatocytes, possibly a distinct type of haemocyte of horseshoe crabs but similar to the fibroblasts described here, appear to serve as pluripotent cells, differentiating and giving rise to muscle, nerve and possibly blood vessels (Clare et al., 1990).

CHAPTER THREE. REGENERATION AND SEX-BIASED TRANSFORMATION OF THE SEXUALLY DIMORPHIC PINCER CLAW

ABSTRACT

Adult snapping shrimps *Alpheus heterochelis* have paired asymmetric claws, a large snapper which is similar in the sexes, and a much smaller pincer which is sexually dimorphic. The male pincer is slightly more hypertrophied compared to the female and therefore intermediate in size between the female pincer and the snapper. This has led to the suggestion that female pincer, male pincer and snapper represent a continuum in claw development. To test this possibility I examined regenerating snappers and found them to pass through a pincer-like stage in both sexes. In males they transiently possess the pincer-characteristic setose fringe. As well, the regenerating closer muscle shows a band of fast fibres reminiscent of the pincer closer muscle in both sexes. Since the male pincer more closely resembles the snapper in external and internal morphology than the female pincer, its transformation to a snapper may be more easily precipitated. To test this possibility I cut the snapper dactyl and this triggered transformation of the pincer to a snapper in 84% of male shrimps but only in 28% of female shrimps. During the intermoult in which the snapper dactyl is cut, axon numbers on the pincer side exceeded those on the snapper side in males but not in females. Since this shift in axon numbers favouring the pincer side occurs well before the pincer transforms to a snapper at the next moult it may signal transformation. These data suggest that a functional snapper may be more important to the male, perhaps a consequence of its heightened defensive role. This idea was tested by observing the behaviour of cohabiting heterosexual pairs when introduced to an intruder. I found that males were more likely than females to engage in agonistic interactions and took a more active role in guarding the burrow entrance.

INTRODUCTION

In the animal kingdom differences between the sexes are legion, extending from genes to behaviour and expressed in varying degrees. Some of the more noticeable differences are often expressed in bodily form and function. Male fiddler crabs, for instance, show a striking bilateral asymmetry of their paired claws: one is an enormously enlarged major claw for defence and courtship, and the other is a minor claw for feeding. Females, on the other hand, have paired minor claws (Crane, 1975). Both sexes begin development with similar paired claws, but in males the pair differentiates into major and minor types, while in females the pair differentiates into minor types, showing fundamental differences in genetic make-up and or expression between the sexes. Whether the major claw develops on the right or left side of the animal is a random event, based on the loss of a claw during a critical developmental period which triggers the intact claw into developing as a major. If claw loss is prevented during this period then both develop into major types, and if both claws are lost during this period then neither side develops major types (Yamaguchi, 1977). Beyond the critical period, claw type is fixed and cannot be altered.

More subtle differences in claw bilateral asymmetry appear between the sexes in the big-clawed snapping shrimp *Alpheus heterochelis* (family Alpheidae) (Fig. 1), where both males and females possess paired asymmetric claws consisting of a major or snapper claw and a minor or pincer claw (Przibram, 1901). The snapper claw is half as large as the entire animal and has a specialized hammer which snaps into a matching socket with sufficient force to eject a jet of water and make a loud popping sound; these behaviours are used in agonistic encounters during territorial disputes or competition for mates (Hazlett and Winn, 1962) and predation to crack open small bivalves or subdue small invertebrates (McLaughlin, 1982; pers obs). The pincer claw is less than half the size of the snapper, lacks the hammer and socket and is used in burrowing and feeding. While the snapper claw is similar between the sexes, the pincer claw is sexually dimorphic. The male pincer is larger and has a fringe of setae on its pollex and dactyl. The female claw is smaller and lacks the setal fringe (Fig. 1) (Wilson, 1903; Read and Govind, 1991).

Whether the snapper develops on the right or left side of the body appears to be determined by claw loss during the critical period (Young et al., 1994), much as in male fiddler crabs, with the intact claw developing into a snapper. However, unlike fiddler crabs, claw bilateral asymmetry once established in snapping shrimps, does not remain fixed in adult life as its placement can be reversed (Przibram, 1901).

Reversal is usually triggered when the snapper claw is lost and the intact pincer claw transforms into a snapper while a new pincer regenerates at the old snapper site. Transformation of the pincer to snapper claw is gradual (Wilson, 1903), so that initially the transformed claw has an intermediate appearance which is lost in subsequent moults as the snapper acquires its characteristic form. In keeping with the fact that the male pincer is larger and more robust than its female counterpart, transformation of the male pincer is more advanced than that of the female pincer after the first moult. However, in some male shrimps the newly transformed snapper claw still retains the characteristic pincer setose fringe on the pollex and dactyl. These observations led Wilson (1903) to suggest that the female pincer is the least differentiated form, resembling the larval form, while the slightly more differentiated male pincer represents an intermediate form, and the male and female snapper the final form. If the pincer claw represents stages in the development of the snapper based on external features I would expect corroborating evidence from internal morphology as well, such as in the muscle and innervation. I therefore studied regeneration of the snapper claw in males and females and found evidence in external and internal features for the hypothesis that the male pincer is much more differentiated towards a snapper than the female pincer. Because of its intermediate status I investigated whether the male pincer would transform more readily than its female counterpart in response to an identical trigger, i.e. snapper dactylotomy. Males were more sensitive than females to this procedure which resulted in more axon profiles on the pincer side. This shift in innervation between snapper and pincer side may trigger transformation.

That males have a relatively larger snapper claw than females (Schein, 1975) and possess a

pincer which is more snapper-like and more sensitive to transformation suggests that a functional snapper may be more critical to males than females. This may be a reflection of the dichotomous behavioral roles of the sexes, and is hinted at by studies showing that males tend to be more mobile than females (Nolan and Salmon, 1970; Knowlton, 1980). I found that when an established cohabiting heterosexual pair was introduced to an intruder, the male was much more likely to engage in agonistic interactions with the intruder than the female and that males took a more active role in guarding the burrow entrance than females.

MATERIALS AND METHODS

Adult snapping shrimps, *Alpheus heterochelis*, of both sexes were collected at low tides off the coast of Beaufort, North Carolina, and transported to Scarborough, Ontario, where they were held in the laboratory at room temperature, 23°C. The shrimps were housed individually in 25 litre glass aquaria, partitioned into 12 compartments with plastic screening (Young et al., 1994). They were fed at 2-3 day intervals with a specially prepared diet consisting of a mixture of fish, beef heart, carrots and commercial trout chow. Under these conditions the shrimps had an intermoult period between 19-26 days, for an average of 23 days. A detailed moult history was kept for each animal and in most cases experimental manipulations were carried out one or two days following ecdysis.

All animals were allowed to moult twice before being selected for study in order to ensure that the claws were fully differentiated (Read and Govind, 1991). To facilitate comparison, the control group of animals selected for study were approximately of the same size with a body length of 30 mm, a carapace length of 10 mm, and claw lengths of 16 and 10 mm for snapper and pincer claws respectively. The experimental group was more variable in size because of availability and number required (over 400) with ranges for body length of 25-36 mm, carapace length of 7.5-14 mm, snapper claw length of 11-20 mm and pincer claw length of 7-14.5 mm. Two types of

experimental manipulations were made. One type consisted of first anesthetizing the shrimp by cooling, then cutting the dactyl of the snapper claw close to its attachment to the claw so that most of it was removed. Snapper dactylotomy was performed usually 1-2 days after a moult and the experimental shrimps were observed over the next two intermoults. The cut end of the dactyl healed during the intermoult but the shrimp did not regenerate a dactyl over the next two intermoults which was the observation period. The other type of manipulation was to induce the animal to autotomize both its claws after ecdysis by gently pinching the limb with forceps just distal to the autotomy plane. In some cases, the shrimp were allowed to regenerate the limbs for one moult cycle then subjected to a second simultaneous autotomy of the pincer and snapper. The effect of this seemed to be to slow down the developmental program of the limbs so that the stages became more drawn out and thus more distinct. They were then studied by means of histochemistry, electron microscopy and simple observation.

Electron microscopy

I also prepared the nerves to the paired claws for electron microscopy by procedures outlined previously (Govind and Pearce, 1988). The shrimp was pinned, ventral side up, in a sylgard-lined dish, filled with marine crustacean saline, and the ganglion and attached nerves were exposed. The saline was replaced with primary fixative for 0.5 h, before the nerves to each side with their attached hemiganglia were dissected out of the animal, and placed in fresh fixative for a further 1 h. Following standard procedures for post-fixation, dehydration and embedding (Govind and Pearce, 1988), the nerves to each side were cut in cross section, as close to their exit from the ganglion, in order to capture all axons leading into and out of the ganglion. There are two principal nerves exiting from the ganglion to each claw, a small first nerve and a large second nerve (Govind and Pearce, 1988). Cross-sections of each nerve were photographed at x1 800, via a series of exposures and these were printed at a final magnification of x6 000, to make the smallest axon (<0.5 um diameter) in the nerves visible for counting. Photographic prints were assembled into a montage of the entire cross-section of the nerve and counts of the axons were made, without prior

knowledge of the experimental status of the shrimp.

I used the scanning electron microscope to compare the external morphology of the pincer claw of males and females. The claws were removed by pinching the propus with forceps thus inducing the animal to autotomize the limb. Routine methods of fixing, drying and coating were then used to prepare the claws (Read et al., 1991) prior to examining with a Hitachi S-530 scanning electron microscope.

Histochemistry

Shrimp intended for histochemical study were subjected to one and in some cases two consecutive simultaneous autotomies of the pincer and snapper. They were selected at various times during the first and second intermoult periods, usually when the regenerating limbs were still somewhat cryptic. Standard histochemical methods were used (Ogonowski and Lang, 1979). Limbs to be analyzed were autotomized, photographed, flash frozen by dipping in isopentane cooled with liquid nitrogen, and serial sectioned in a cryostat. The sections were affixed to coverslips, incubated in ATP medium and processed in calcium chloride, cobalt chloride, and ammonium sulphide to reveal differences in myofibrillar ATPase activity of the muscle: fast muscle fibres stained relatively darker than slow. The samples were secured to slides with permount, then photographed.

Behaviourial experiments

Males and females were matched according to size (carapace length: 10.5-11.5 mm; total length: 30.0-33.5 mm) and simultaneously introduced into 20L aerated observation tanks which were supplied with a gravel substrate to a height of 2 cm and filled with artificial seawater to a height of 7 cm. The temperature was held at 23°C and artificial light on a 12:12 h light:dark photoperiod was established. Animals were fed on a daily basis and a clear plastic tube, 2 cm in diameter and 15 cm long, open at both ends, was provided as a shelter. The shrimp were considered to be an established cohabiting pair if both were observed occupying the shelter in a

relatively tranquil manner within 2 d of being introduced and over a period of at least 5 d. The typical stance of an established pair was a tail-to-tail position within the shelter. Incompatible pairs were readily apparent, invariably resulting in one animal (usually the female) being evicted from the burrow or cannibalization of one shrimp by the other. Only established pairs were used in the study. Observations trials of 15min duration were carried out between 2 and 4 pm.

To establish a baseline for experiments to follow, general observations were conducted on undisturbed cohabiting pairs (hereafter referred to as the host male and female). Based upon a behavioural repertoire garnered from preliminary studies, the specific behaviours of each sex were recorded using a tape recorder, then quantified by tabulating and categorizing each occurrence of the behaviour. A total of 8 pairs were observed. Following this, the sex-related roles of these cohabiting pairs and six additional ones were assessed when the pair was subjected to a newly introduced male (hereafter referred to as the invader). In order to encourage greater interaction and prevent one sided contests, the invader was matched in size to the host male. Prior to the start of each trial, the invader was restrained for 10 min in one corner of the aquarium using a plastic mesh compartment (10 cm x 10 cm). During this period the host male and female remained in their shelter. The invader male was then released and observations recorded as before. Trials in which no interactions occurred were eliminated from the study. Each established pair and invader male were used only once with a total of 14 trials being recorded. In a follow-up experiment to assess which sex of the established pair guards the entrance, one end of the shelter was blocked and the relative positions of the male and female, while occupying the shelter were noted and recorded at hourly intervals. This was done for 5 different established pairs.

RESULTS

Comparison of claws between males and females

In addition to the differences between male and female pincer claws previously listed

(Wilson, 1903), there is also a prominent cuticular ridge on the mesial face of the propus in the male pincer similar to the snapper, that is lacking in the female pincer (Fig. 1). Differences in size of the pincer and snapper between males and females were determined by comparing the ratio of claw length to width and deriving a stoutness index; the stouter the claw the lower the index (Table 1). The data were analyzed by ANOVA and showed significant differences in stoutness index of different claw types ($F_{3,95} = 270.82$, $p < 0.0001$). Scheffè's multiple comparison test showed no significant differences in stoutness of the snapper claw between the sexes ($F_s = 0.125$, $p > 0.05$), however the male pincer claw was significantly stouter than its female counterpart ($F_s = 8.927$, $p < 0.05$). Thus in stoutness as well as in the presence of a cuticular ridge, the male pincer more closely resembles the snapper than the female pincer.

Innervation to the claws was assessed by counting axon profiles in the nerves as they exit the ganglion, thereby measuring total innervation. Two separate nerves originate from each hemi-ganglion serving the major and minor claws (Fig. 2); each is packed with myelinated and unmyelinated axons.

The majority of profiles within the nerves are sensory and the relatively few motoneurons are among a group of large myelinated axons in one quadrant of the nerve. Both nerves are qualitatively similar with a mixed population of sensory and motor axons. Counts of the total number of axons were made in several similar-sized male and female shrimps and showed that the snapper claw has many more axons than its counterpart pincer in both sexes (Table 2).

The difference in axon numbers between snapper and pincer sides however, is not as great in male compared to female shrimps as seen by the ratio of axon numbers between snapper and pincer sides (Table 2). The data was analyzed via a two factor ANOVA, the factors being claw type (snapper, pincer) and sex (male, female) coupled with a multiple comparisons test. There was no significant interaction effect ($F_{1,16} = 0.658$, $p = 0.4291$) nor was sex important ($F_{1,16} = 1.815$, $p = 0.1967$). Effect of claw type, however was significant ($F_{1,16} = 10.686$, $p = 0.0048$), and

Figure 1. Adult male (A) and female (B) snapping shrimps with paired asymmetric claws consisting of a large snapper claw and a small pincer claw. The snapper claw is similar in the sexes, whereas the pincer claw is sexually dimorphic. The male pincer is thicker, has a ridge on the propus (arrowhead) and has a prominent fringe of setae on its dactyl and propodus (insets of scanning electron micrographs). The female pincer is thinner and does not have the setose fringe. Scale bars, animals 10 mm; claws 1 mm.

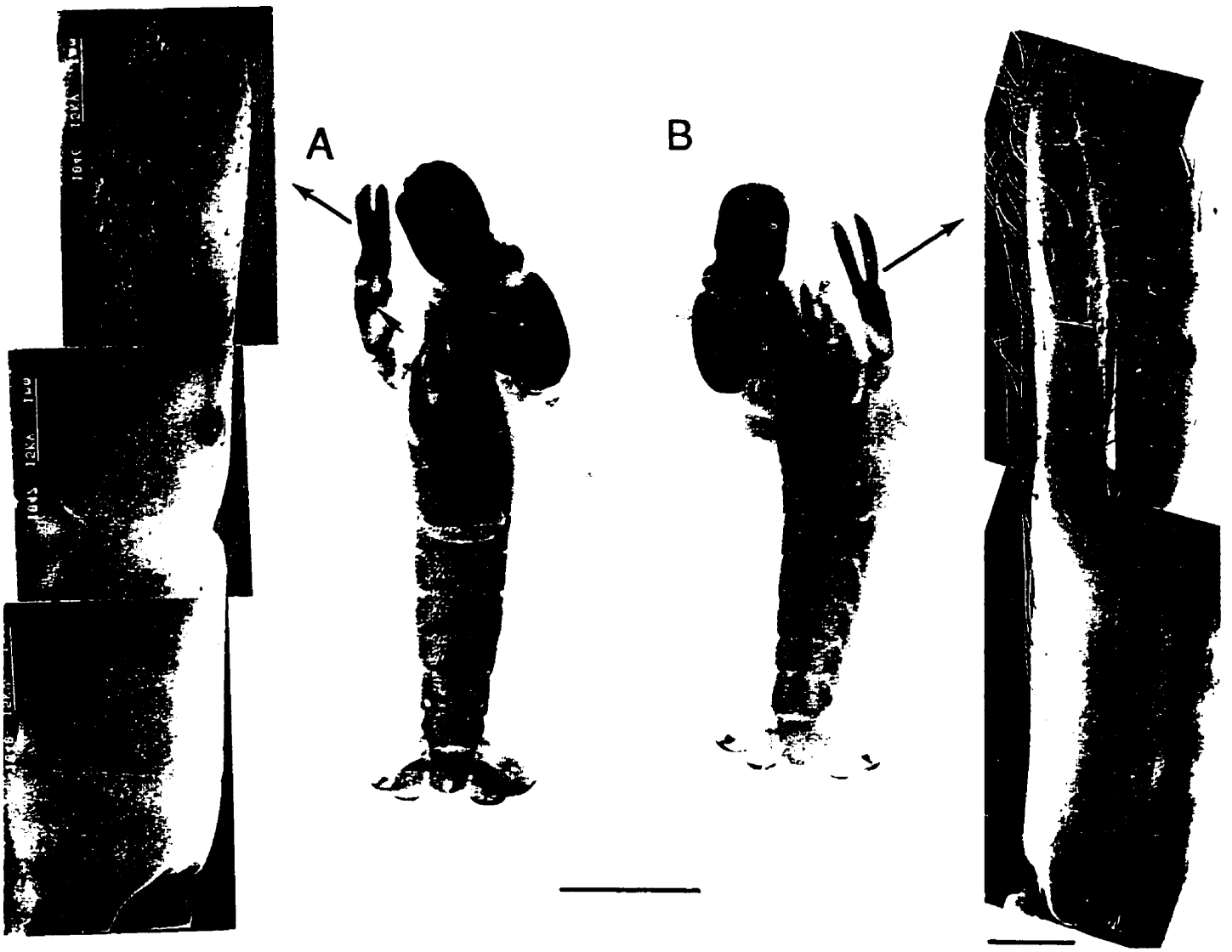

A
B

Table 1. Comparison of stoutness index (ratio of propus length to width) between paired snapper and pincer claws of adult male and female shrimps.

	Stoutness index (mean ± sem)	
	snapper	pincer
Males (n=25)	2.12 ±0.02	3.62 ±0.08
Females (n=24)	2.18 ±0.03	4.06 ±0.09

specifically Fisher's LSD test showed a significant difference between the number of axons innervating snapper and pincer of intact females (LSD=3412, $p<0.05$), but not of intact males (LSD=3412, $p>0.05$).

The male pincer (Mann-Whitney U-test, U=5.5, $p<0.05$) and snapper (U=9, $p,<0.05$) are innervated by a greater number of axons than the female counterparts however as Table 2 suggests the difference appears to be greater for the pincer claws. This sexual dimorphism in axon numbers is likely related to the fact that the male claw is more elaborate than its female homolog (Fig. 1).

Regeneration of the snapper claw

To examine the possibility that a putative snapper claw passes through a pincer-like stage I studied regeneration of the snapper claw by autotomizing both claws simultaneously. The claws regenerated to their previous configuration in over 100 shrimps of both sexes including 48 in which the regenerating limb buds had been autotomized a second time in order to slow regeneration. In 34% of shrimp subjected to two consecutive paired autotomies the limbs showed slight asymmetry into snapper/pincer, while in the remaining 66% it was observed that limbs on the snapper side, while regenerating seemed to pass through a distinct pincer stage. On both sides, the limbs initially were long, slender, featureless and quite weak in appearance, much like the pincer of a female (Fig. 3). The next feature to appear was a slight ridge on the mesial face of the propus in males, a feature also found in the male pincer. Also characteristic of the male pincer and appearing in the regenerating male snapper was the setose fringe on the dactyl and pollex which is lost with further development, but clearly shows the snapper claw passing through a pincer stage. Finally, a plunger and socket differentiate and mark the regenerating claw as a snapper. The claw continues to hypertrophy in the succeeding moults and acquires a pristine appearance. Thus a regenerating snapper in both sexes appears to pass through a pincer-like stage before acquiring its final form.

The above observations were strongly corroborated by histochemical evidence which determined the fibre composition of the closer muscle. In shrimps with pristine well differentiated

Figure 2. Cross-sections of first (A) and second (B) nerves to a male snapper claw in an adult shrimp showing tightly packed axons, most sensory, with a discrete group (arrow) of large axon profiles representing motoneurons. Numerous darkly stained structures are myelin nuclei. At higher magnification (C) the nerves are seen to be composed of unmyelinated (u) and myelinated (m) axons, the latter characterized by prominent darkly stained myelin sheath (arrow) and nuclei (double arrow). A, B, x370; C, x6,000. Scale bars, A, B, 50 μm; C, 5 μm

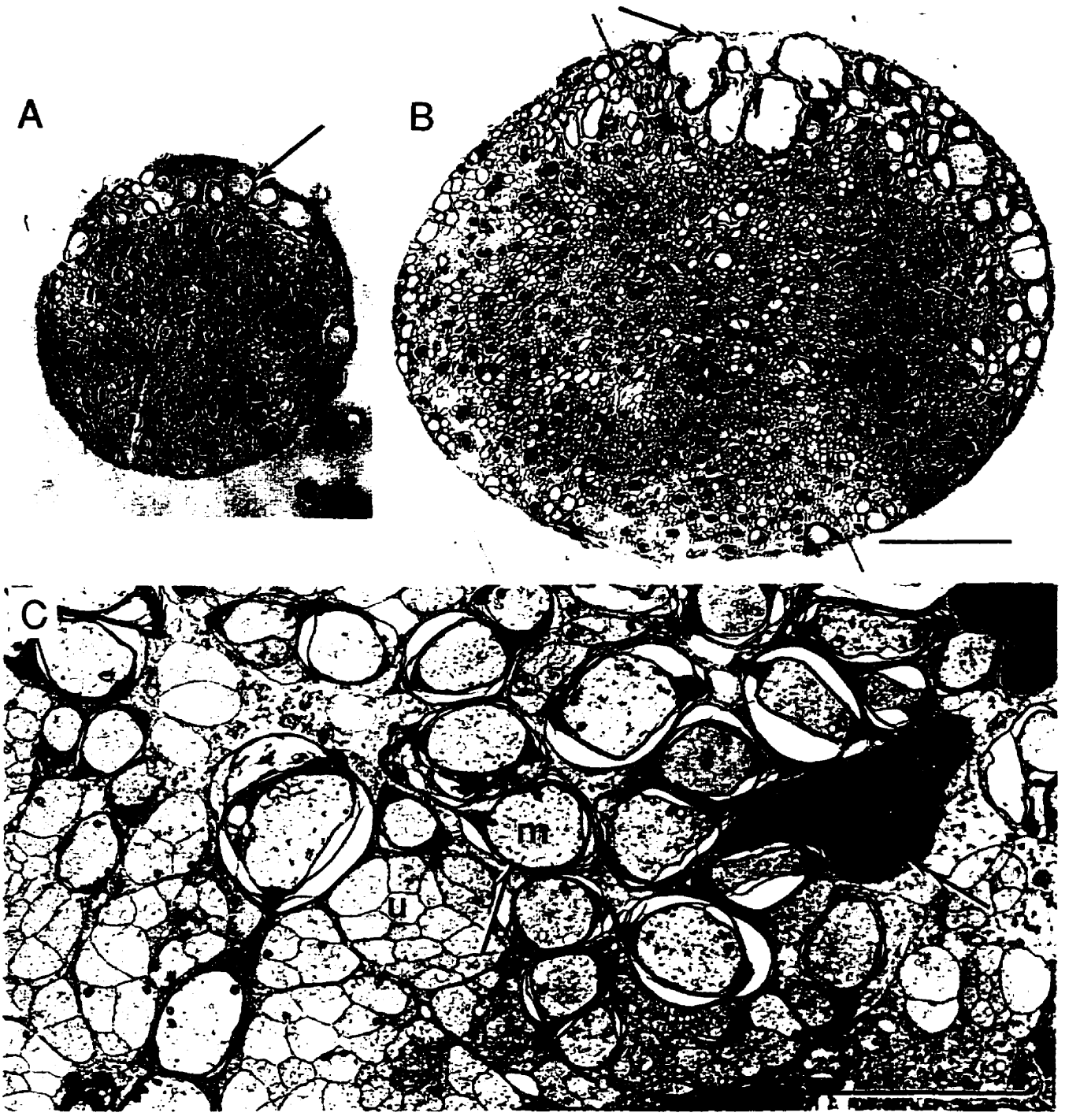
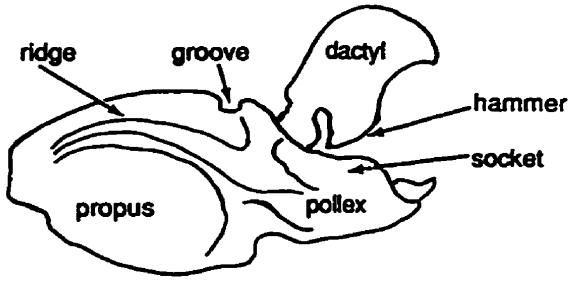

A
B
C
m
u

Table 2. Comparison of axons numbers between paired snapper and pincer sides of adult male and female shrimps with paired intact claws and snapper-dactylotomized claws.

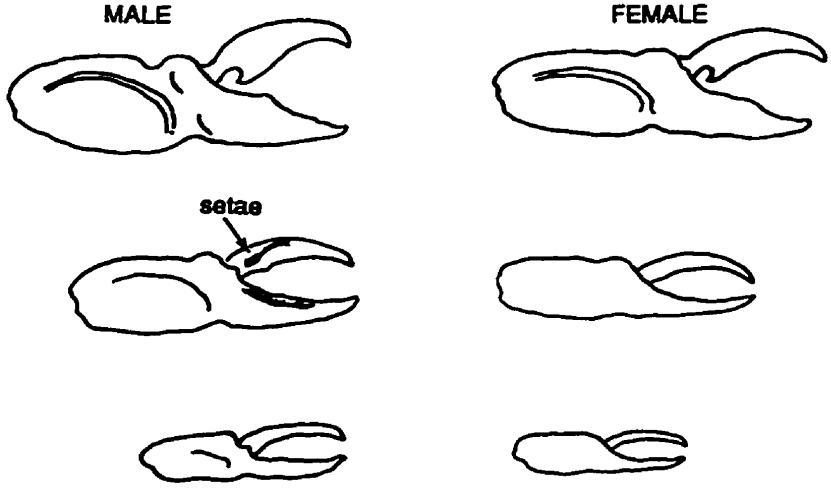
	Number of axons (mean ± sem)		
	snapper	pincer	snapper:pincer
Intact males (n=5)	16241 ±1491	13444 ±1030	54:46
Intact females (n=5)	15631 ±1143	10988 ±757	59:41
Snapper-dactylotomized males (n=3)	13122 ±2050	14450 ±2613	48:52
Snapper-dactylotomized females (n=3)	13323 ±412	11490 ±1051	54:46

Figure 3. A pristine snapper claw representative of both sexes, with characteristic hammer on the dactyl which fits into a matching socket on the pollex, a transverse groove across the propus and a heavy semi-circular ridge. Regenerating snapper depicts three stages in male and female shrimps; the two early stages resemble pincer claws including, in males, a fringe of setae on the pollex and dactyl.

SNAPPER



REGENERATING SNAPPER



claws, the pincer closer muscle has a prominent band of fast muscle sandwiched between slow, whereas the snapper closer muscle is composed only of slow fibres (Govind et al., 1986) (Fig. 4A, B). However, the regenerating limbs of animals subjected to paired autotomies did not initially show this pattern. In seven out of ten shrimps analyzed histochemically following paired autotomy, the regenerating limb buds on both the snapper and pincer sides possessed the fast band of fibres in the closer muscle (Fig. 4C, D). Indeed, the fast band was present in some animals in which the snapper side regenerate had already acquired the more overt snapper features. This was observed in both males and females. As regeneration proceeds, the fast muscle band seen in the immature snapper is gradually lost and replaced by slow muscle fibres, thus mimicking the pristine condition.

I also examined claw innervation following paired autotomy at different times during the intermoult (3, 6, 9 days) and into the next moult in a single trial. I found that there were more axons on the snapper side throughout the intermoult and into the next. Figure 5 shows the results of this experiment for males and females and, although, the actual numbers of axon profiles were considerably decreased because of degeneration, the snapper side had more axons than the pincer side at the time intervals chosen in the intermoult and into the next moult when the claws had regenerated.

Males are more sensitive to snapper dactylotomy than females

The male pincer claw compared to its female counterpart appears to be a more advanced stage in the differentiation of the snapper raising the possibility that it may transform more readily. To test this possibility I cut the dactyl of the snapper to act as a trigger which is milder than removal of the entire claw that invariably precipitated transformation in females (Govind et al., 1988) and males (unpublished data). Innervation of the dactyl makes up about one third of all the axons to the claw (unpublished data). Moreover, because the male and female snapper claws are similar, snapper dactylotomy would be equivalent between the sexes and provide a means for testing differences between the sexes. Experimental shrimps were considered to be those in which

Figure 4. Cross-sections of claws stained for myofibrillar ATPase activity showing fibre composition of the closer muscle in paired intact pincer (A) and snapper (B) claws of a male shrimp and in newly regenerating snapper claws of male (C) and female (D) shrimps where a small central band of fast fibres still persists, reminiscent of the pincer closer muscle. A, x16; B, x8; C, D, x40. Scale bar, 1 mm.

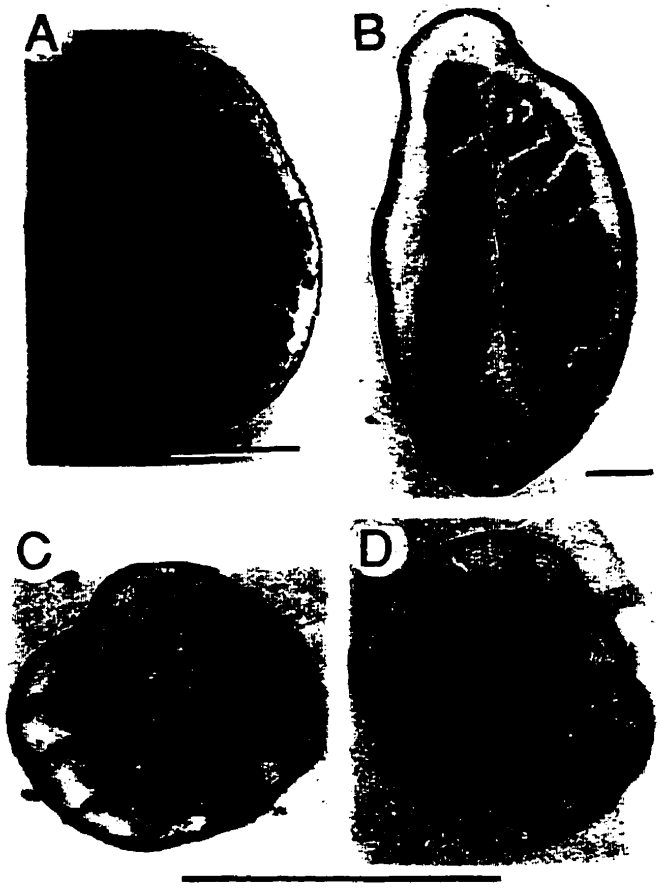
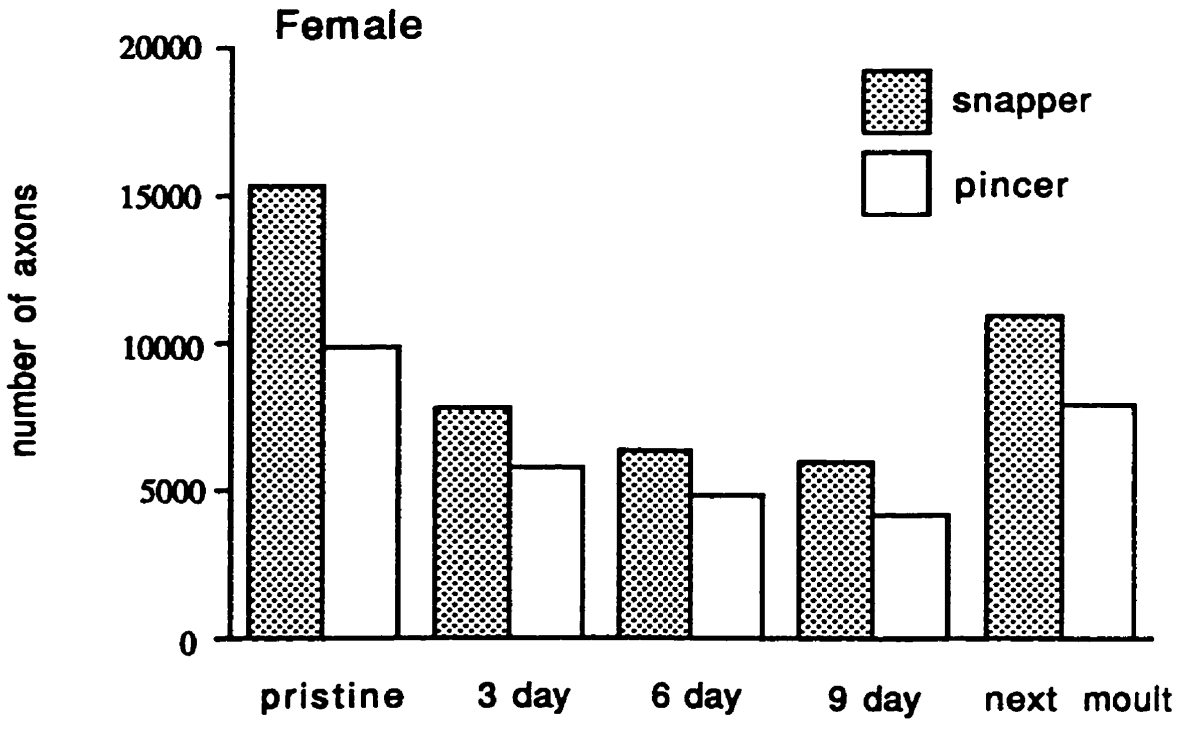
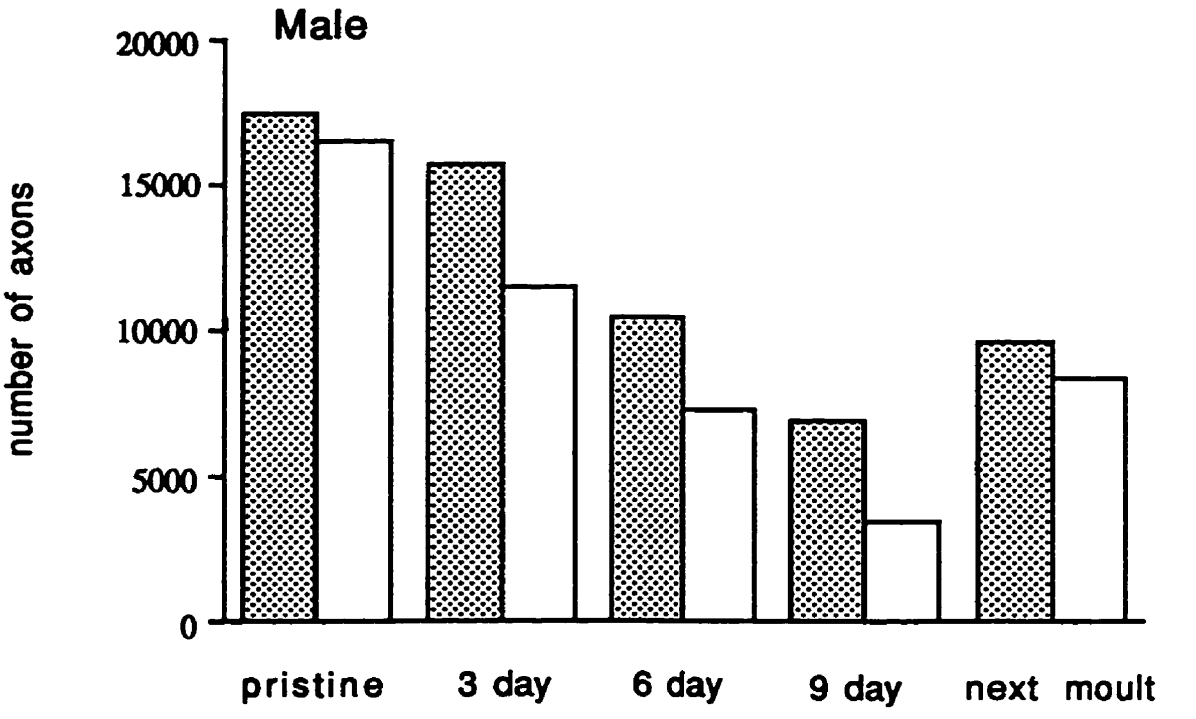

A
B
C
D

Figure 5. Histogram of total number of axons to paired snapper and pincer sides of male and female shrimps with pristine claws and following autotomy of the paired claws at several time intervals (3, 6, 9 days) during the intermoult and after the next moult. Greater axon numbers prevailed on the snapper side throughout this series. A single animal was used for each time period.


Female
20000
15000
10000
5000
0
number of axons
snapper
pincer
pristine
3 day
6 day
9 day
next moult


Male
20000
15000
10000
5000
0
number of axons
pristine
3 day
6 day
9 day
next moult

the snapper dactyl was removed and the shrimp passed through at least two successive moult cycles without autotomizing the dactylotomized limb. Dactylotomy was performed in over 300 shrimps of which more than 85% eventually threw the damaged snapper. Of the remaining shrimps, 43 were observed (Table 3) and most of these had the pincer transform into a snapper (Fig. 6). Regeneration of the lost dactyl did occur, but was an extremely protracted process requiring much longer than would be necessary to regrow the entire limb, likely because the propus-dactyl joint is not a natural autotomy plane. Rarely did the damaged limb autotomize after transformation of the pincer had occurred. Clearly, dactylotomy of the snapper is effective as a trigger for this process.

However, the effects of dactylotomy were more pronounced in male than in female shrimps (Table 3); 84% of male shrimps had their pincer transform into a snapper compared to 28% for females. The difference between the effect of snapper dactylotomy on males and females was significant (X^2=11.59, $p<0.001$), with males being more sensitive to the procedure than females

Based on the difference in the ratio of axon profiles between snapper and pincer sides between the sexes (Table 2), snapper dactylotomy might differently affect this ratio and might even reverse it in males, i.e. snapper dactylotomy in males might result in a greater number of axons in the transforming pincer-to-snapper claw compared to the existing snapper. In females the dactylotomized snapper would still possess more axons than the pincer. To test this possibility, the effect of snapper dactylotomy on the innervation was examined. Nerves to the claws were fixed 12-14 days after dactylotomizing the snapper since this would be the period of peak axon degeneration (which is what I was interested in) but prior to the regeneration of new axons that would follow. Thus it was not possible for me to know whether the animal being fixed would have undergone pincer transformation, only that it was very likely that females would not have transformed and males would have transformed.

Removal of the dactyl of the snapper claw had a dramatic effect on the limb's innervation.

Figure 6. A: An adult male snapping shrimp with paired snapper and pincer claws in which the snapper dactyl has been removed. B: The same animal, one moult later, in which the pincer has transformed into a snapper claw resulting in paired snapper claws; the newly transformed snapper is smaller than the original contralateral snapper. Scale bar, 10 mm.


B

Figure 7. Cross-sections of first (A) and second (B) nerves to a male snapper claw two weeks after the snapper dactyl had been removed showing axons not as tightly packed as in the intact claw nerves (compare Fig. 2), although a discrete group (arrow) of large presumably motor axon profiles are prominent. At higher magnification (C) there are clear signs of axon degeneration (arrows) including areas devoid of axons, as well as intact unmyelinated (u) and myelinated (m) axons. A, B, x370; C, x6,000. Scale bars, A, B, 50 μm; C, 5 μm.

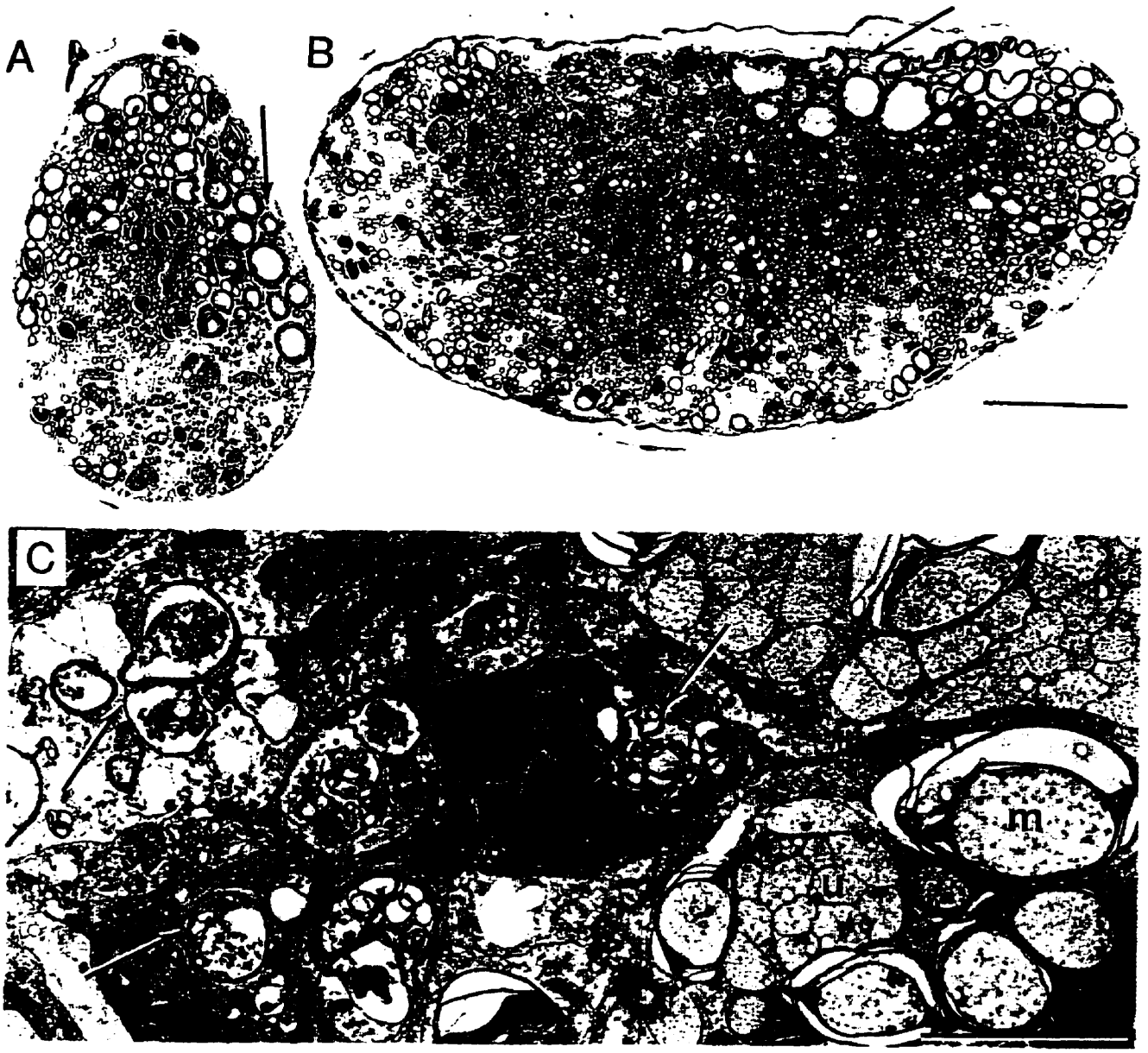

A
B
C
m
u

Table 3. Transformation of pincer to snapper claw in response to dactylotomy of the snapper in adult male and female shrimps

	Total	males	females
Number snapper dactylotomized	43	25	18
Number transforming pincer to snapper	26	21	5
% transforming pincer to snapper	61	84	28

Qualitatively, axon degeneration was indicated by the presence of electron-dense myelin figures and large intercellular spaces filled with a granular matrix (Fig. 7). Although degeneration of axons seemed to be quite extensive in both the first and second nerves, it was primarily confined to certain regions in each affected nerve where it involved both myelinated and unmyelinated axons. A small group of large myelinated axons, situated in one quadrant of both nerves and representing motor axons (Govind and Pearce, 1988), was unaffected. Thus dactylotomy of the snapper claw had a similar effect in both sexes, in that it resulted in the loss of both types of axons in its nerves.

To control for variations in size of experimental animals, the axon count data were transformed to snapper:pincer ratios and analyzed by two-factor ANOVA, the factors being claw type (snapper, pincer) and condition (intact males, intact females, snapper dactylotomized males, snapper dactylotomized females). Dactylotomy did significantly effect the ratios ($F_{3,24}$=14.29, p=0.0026) although there was no difference in its effect on males and females ($F_{3,24}$=0.073, p=0.7918). As Table 2 suggests, following a snapper dactylotomy the snapper/pincer ratio, for the first time, favoured the pincer in males. In females the snapper/pincer ratio still favoured the snapper, following the trend established for control shrimps of both sexes. The effect of snapper dactylotomy appears to be more pronounced in male shrimps than in females as the snapper has fewer axons than the pincer in males but not in females nor in control shrimps of both sexes.

Comparison of male and female behaviour

I analyzed the behaviours of already established cohabiting heterosexual pairs, rather than the initial male-female interactions which have been adequately described elsewhere (e.g. Nolan and Salmon, 1970). The behaviours of an established pair were categorized as follows: protruding (moves out of the shelter with claws and rostrum protruding then quickly retreats), exploring (leaves shelter and ambles about), digging (moves gravel using pleopods usually around the shelter), manipulating gravel (uses claws to move gravel about), snapping (forceful closing of snapper claw producing loud snap), fanning (rapid synchronous waving of pleopods), interacting (contact of any sort of one shrimp towards the other), grooming (cleans body using the second pair of thoracic limbs which are long, slender and extremely flexible) probing (uses flexible limbs to

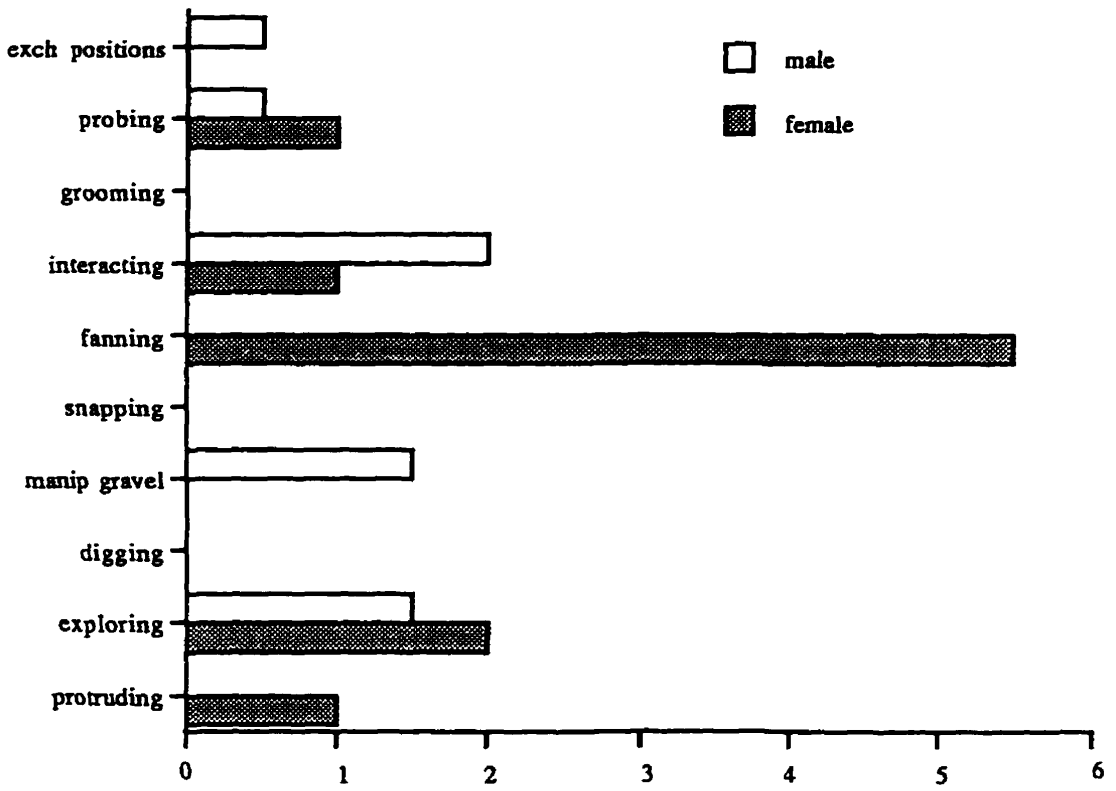
explore substrate), exchanging position (one shrimp pushes past the other while inside the tube).

As Fig. 8 shows the normal behaviour of males and females of an established cohabiting pair was essentially the same with two exceptions: 1) females were more often observed moving gravel from inside to outside the shelter, a behaviour perhaps related to her nurturing role and 2) females surprisingly, snapped far more frequently than males. Upon closer inspection the results for this behaviour were seen to be skewed by the frequent snapping of the female of one pair who subsequently moulted very soon after the observation period.

Having established a baseline for the behaviour of an established pair I was in a position to determine how the sexes would interact with an invader male. Thus the behaviours of the host male and female following introduction of the invader male were counted and placed into three categories for subsequent analysis: 1) general interactions which includes one shrimp lunging towards the other's head or tail, pursuit of one by the other, antenulation between two animals, and contact between the claws or tails; 2) snapping and 3) leaving the shelter (Table 4). Following the Bonferroni adjustment of the alpha error level it was found that host males interacted more frequently with the invader than host females ($t=3.03$, $p<0.005$), there was a larger number of snaps between the two males ($t=2.13$, $p<0.03$) and the host male left the shelter more often than did the host female when the invader was present ($t=2.35$, $p<0.02$). These data suggest that the male of a cohabiting heterosexual pair is more likely to engage in defensive behaviour than the female.

Finally when one entrance of the shelter was blocked and both shrimp were inside, the male of an established pair was more likely to be at the open entrance (55 occurrences) than the female (13 occurrences) ($X^2 = 44.92$, $p<0.0001$, $n=5$ pairs), suggesting that he takes a more active role in protecting the shelter.

Figure 8. Histogram comparing median of occurrence of different behaviours of established cohabiting paired (male and female) adult snapping shrimp (n=8); exch=exchanging; manip subst=manipulating substrate.



Median occurrences of behaviour

Table 4. Behaviour of host male and female snapping shrimps in the presence of an invader male

	Behaviour frequency (per trial, mean ±sem) of 14 trials	
	males	females
general interactions	7.1 ±1.2	2.5 ±0.7
snaps	4.1 ±0.7	1.9 ±0.8
leaves shelter	2.9 ±0.6	1.5 ±0.7

DISCUSSION

There is an obvious sexual dimorphism of the pincer claw in adult snapping shrimps: the male pincer is hypertrophied compared to the female and only it has a setose fringe on its dactyl and pollex (Wilson, 1903). The claws are dimorphic internally as well: the male pincer closer muscle has fewer fast fibres than the female (Govind et al., 1986) and the male pincer has significantly more axons than the female (Table 2). Altogether these differences point to the male pincer elaborating towards a snapper form. I find evidence for this as well, in that the regenerating snapper claw passes through a pincer-like stage in its external form and in the fibre composition of the closer muscle. Thus Wilson's (1903) initial hypothesis that the male pincer is intermediate between the female pincer and the snapper gains additional support.

However, another rigorous test of Wilson's hypothesis would be to discover if the more advanced male pincer claw transforms more readily to a snapper type than the less modified female pincer claw. This leads to the main finding of our study that when an equivalent stimulus for transformation is applied, such as snapper dactylotomy, males are more sensitive than females. Why this is the case is not known but it could well be that the advanced male pincer claw responds to a much weaker transforming signal, such as snapper dactylotomy, whereas the less advanced female pincer requires a much stronger signal, such as claw autotomy or nerve transection (Mellon and Stephens, 1978; Govind et al., 1988).

Wilson's (1903) hypothesis that the pincer represents an arrested stage in development of a snapper gains more currency with our finding that males are more sensitive to snapper dactylotomy than females. His hypothesis also provides a means for explaining claw bilateral asymmetry in adult snapping shrimps. When they first develop, the paired claws are symmetrical and pincer-like and in subsequent juveniles they differentiate into pincer and snapper types (Knowlton, 1973). Which one of the pair becomes the snapper appears to be determined by extrinsic factors such as differential use of the paired claws, with the claw that is used more becoming the snapper (Young et al., 1994). A similar mechanism operates in the determination of claw handedness in juvenile

lobsters (Govind and Pearce, 1986; Govind, 1992). The putative snapper may in turn inhibit the contralateral claw from further differentiation and arrest it at the pincer stage. Evidence for the existence of such an inhibitory influence has been uncovered in experiments in which the snapper claw is autotomized to trigger transformation of the pincer claw and neural pathways in this transforming claw are disrupted, either by removing the closer muscle or lesioning the large nerve (Young et al., 1994). While the pincer transforms into a snapper, rather than a pincer regenerating at the old snapper site, as happens usually, often a snapper claw regenerates. Presumably, reducing neural input removes an inhibitory signal from the transforming pincer-to-snapper claw that normally restricts regeneration of the contralateral claw to a pincer type.

Although the nature of the transforming signal is unknown, the present observations of the ratio of axon numbers between snapper and pincer side point to a neural influence. While this ratio favours the snapper side in shrimps with intact pristine claws, this is also the case early in the intermoult (3, 6, and 9 days) in which the paired claws are removed (Fig. 5). Therefore, bilateral differences in axon numbers are present before limb regeneration commences towards the end of the intermoult and continue in the premoult period (Govind and Read, 1994). In snapper dactylotomized shrimps however, axon counts also made in the intermoult period two weeks after surgery, showed bilateral differences in axon numbers favouring the pincer side in male shrimps (Table 2). Female shrimps continued to show more axons on the snapper side. With an average intermoult period of three weeks for adult shrimps in our laboratory, postmoult and intermoult periods occupy the first week while the premoult period begins early in the second week when the new exoskeleton for the transforming pincer-to-snapper claw is laid down (Govind and Read, 1994). Since bilateral differences in axon numbers precede pincer-to-snapper transformation, they are appropriately timed to trigger transformation. It is possible that such bilateral differences in axon numbers normally favouring the snapper side inhibit the opposite pincer side from continuing its development to a snapper. With reversal of these bilateral differences, as in the case of snapper dactylotomy in male shrimps, inhibition of the pincer claw is removed and it transforms into a snapper.

In the present study of snapper-dactylotomized shrimps bilateral changes in axon numbers were precipitated by removing the dactyl of the snapper claw with the result that these shrimps ended up with paired snapper claws. Had the snapper claw been removed completely, a pincer would have regenerated in its place and this would be in keeping with the lower number of axons on this side. Thus both steps of the asymmetry reversal phenomenon viz. transformation of the existing pincer into a snapper and regeneration of a new pincer at the old snapper site, may be controlled by a single neural-based inhibitory signal which originates from one claw, the snapper, and exerts its influence on the contralateral claw, restricting it to a pincer type.

The question we are left with now is why are males more sensitive than females to having their snapper claw damaged? It may be that a functional snapper is more important to males than females. Although these animals are not gregarious, they do tend to inhabit burrows in heterosexual, likely monogamous, pairs. I hypothesized that it is the male shrimps' responsibility to protect or defend the burrow, particularly during the period or season when the female is ovigerous and less mobile (Nolan and Salmon, 1970). Moreover, particularly in polygynous populations, males tend to be more mobile, shuttling back and forth between a few sedentary females (Knowlton, 1980). My behavioural experiments add further support for this contention, demonstrating that in a cohabiting heterosexual pair exposed to the threat of an intruder male, the host male was more likely than the female to leave the shelter and interact with the intruder. Indeed, the female seemed intent on avoiding any conflict. In these situations, a serviceable snapper would be critically important to the male. Since the female is in a protected burrow, defended primarily by her mate, it may not as important that she have a functional snapper. In reality, although an impaired snapper reduces success in intrasexual competition for shelters, it does not inhibit the shrimps' ability to pair with a member of the opposite sex (Conover and Miller, 1978).

Snapping shrimp use both the cocked claw (as a visual display) and the snap (as a tactile-acoustic display) as formalized threat displays (Schein, 1977). Clearly the former would be less energy consuming and may be preferred by females in which case loss of the snapper dactyl may

not be as debilitating as in males who may be more dependent on using the snap during agonistic interactions. The process of transforming a pincer to a snapper is certainly energy consuming, especially in females since their pincer is much less snapper-like to begin with than in the males. It may be more expedient for her energy stores to be used for egg production which is known to be correlated with larger body size (Knowlton, 1980) rather than the costly process of transforming a pincer into a snapper. The latter strategy would probably be redundant since, once paired she may rarely take part in agonistic interactions or burrow defence.

CHAPTER FOUR. CLAW TRANSFORMATION AND REGENERATION: TEST OF THE INHIBITION HYPOTHESIS FOR MAINTAINING BILATERAL ASYMMETRY

ABSTRACT

In the paired asymmetric claws of adult snapping shrimps, *Alpheus heterochelis*, the minor or pincer claw may transform into a major or snapper claw if the existing snapper claw is damaged or lost implying that an intact snapper claw normally inhibits the contralateral pincer claw from advancing to a snapper. I find that the pincer-to-snapper advancement in external form occurs almost immediately after the snapper is lost even as late as the premoult stage. The transforming claw in turn inhibits the newly regenerating pincer claw from becoming a snapper, but if the dactyl of the transforming claw is cut, then snapper-based inhibition is removed and the contralateral claw may regenerate as a snapper resulting in shrimps with paired snapper claws. However, damaging an established snapper claw will not allow another snapper claw to regenerate at the pincer site implying that less inhibition is required to restrict a newly regenerating claw to a pincer than it is to arrest an existing pincer claw. Inhibition may be manifested largely in terms of quantity of innervation and hence the greater innervation of the snapper side over the pincer side would inhibit the pincer side and this would account for the regeneration of paired claws in their previous configuration following loss of both claws. Loss of the paired claws in two consecutive moults retards their development and they often appear as pincers, but in succeeding moults one usually differentiates into a snapper and bilateral asymmetry is restored. However, shrimps with paired snapper claws retain this configuration over several moults unless one or both of the claws are lost in which case regeneration restores bilateral asymmetry. Thus, bilateral asymmetry of the paired claws of adult shrimps is governed by a strong intrinsic lateralizing mechanism in which the snapper claw inhibits the pincer from advancing to another snapper.

INTRODUCTION

Among crustaceans, bilateral asymmetry of the first pair of chelipeds is common, one of the paired claws is more enlarged and elaborate (major claw) than the other (minor claw). In snapping shrimps of the Alpheid family, the major or snapper claw is almost as large as the abdomen and has a hammer on the moveable dactyl that fits into a reciprocal socket on the fixed pollex (Fig. 1A, 2D) (Przibram, 1901). The closing action of the hammer into the socket is with such tremendous force that it is accompanied by a loud popping sound and a jet-expulsion of water, both of which are used in agonistic encounters (Hazlett and Winn, 1962; Ritzmann, 1974) or for crushing bivalve shells (McLaughlin, 1982). The minor or pincer claw is much smaller and used in burrowing and feeding.

An unusual feature of claw bilateral asymmetry in snapping shrimps is the ability to reverse its configuration; loss of the snapper early in an intermoult results in the transformation of the pincer to a snapper and the regeneration of a new pincer at the snapper site at the next moult (Przibram, 1901, Wilson, 1903). Rather than loss of the snapper claw, less drastic measures such as its denervation (Mellon and Stephens, 1978), dactylotomy (Read and Govind, 1997), or closer muscle tenotomy (Govind et al.,1988), are also sufficient to trigger transformation of the pincer into a snapper, with the result that these shrimps now possess paired snapper claws as the existing snapper repairs itself. Since these manipulations inducing pincer-to-snapper transformation involve damage to the nervous system of the snapper claw, it is likely that the transformation results from the loss of neural inhibition by the snapper claw which prevents the pincer claw from completing its development to a snapper (Wilson, 1903). This hypothesis is based on the observation that regenerating claws in adult shrimps pass through a distinct pincer-like stage before differentiating into a snapper claw (Wilson, 1903, Darby, 1934; Read and Govind, 1997). In this scheme, loss of the snapper claw removes its inhibition on the pincer which then advances to a snapper, and in turn inhibits the newly regenerating claw to a pincer.

Snapper-based inhibition of the pincer claw can also explain the fact that loss of the pincer

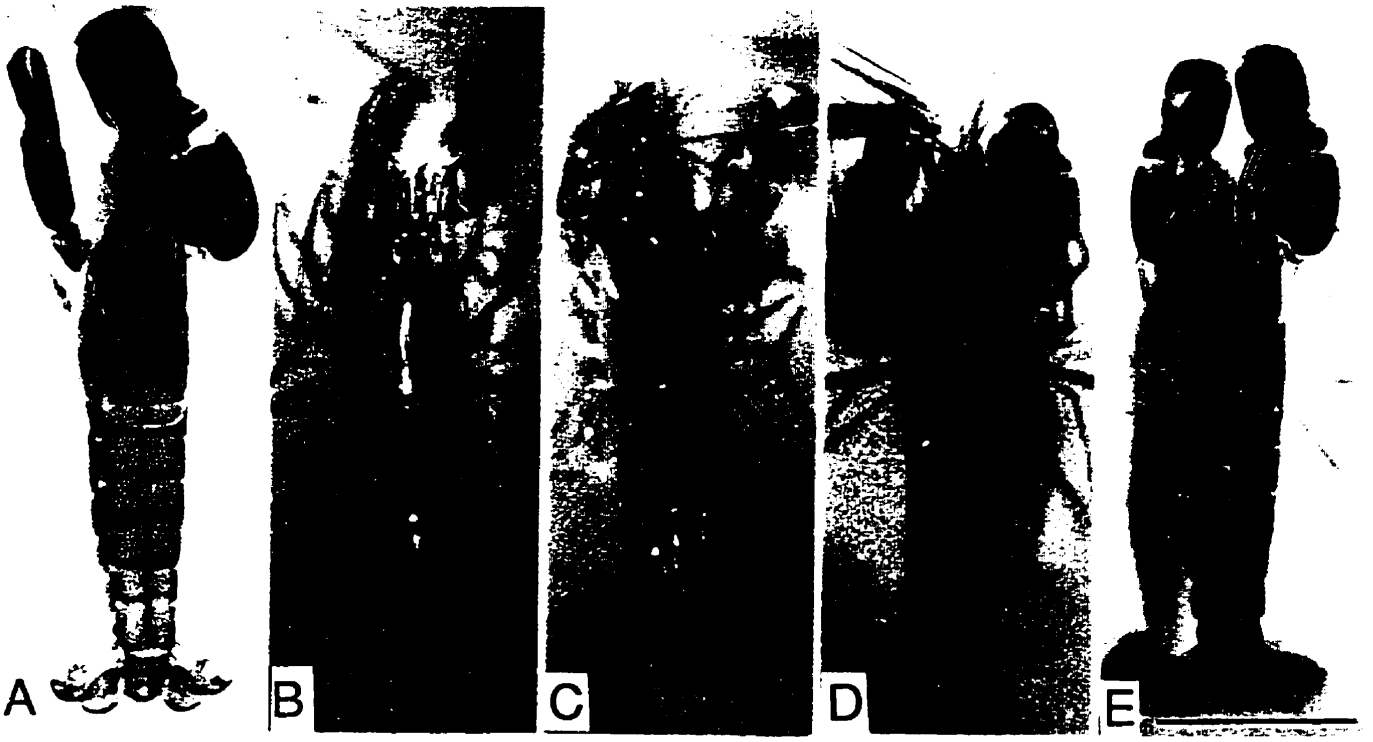
claw in adult shrimps results in the regeneration of another pincer (Wilson, 1903). To explain the fact that simultaneous loss of both claws results in claw regeneration in the same configuration it would have to be assumed that snapper-based inhibition prevails even in the absence of claws. Thus, no matter which claw is lost, inhibition by the snapper claw (or its site) on the pincer claw (or its site) ensures regeneration of bilateral asymmetry. Because the inhibitory signal has a neural basis it can be easily manipulated with minor surgery of the claws. Here I describe a number of experimental manipulations designed to explore some of the ramifications of the inhibition hypothesis for claw bilateral asymmetry in adult snapping shrimps. Our findings support the existence of a lateralizing mechanism based on inhibition from the snapper claw or from its site and its ability to switch from one side to the other.

MATERIALS AND METHODS

Adult snapping shrimps, *Alpheus heterochelis*, of both sexes were collected at low tides off the coast of Beaufort, North Carolina and transported to Scarborough, Ontario where they were held in the laboratory at room temperature, 23°C. The shrimps were housed individually in 25 litre glass aquaria, partitioned into 12 compartments with plastic screening (Young et al., 1994). They were fed at 2-3 day intervals with a specially prepared diet consisting of a mixture of fish, beef heart, carrots and commercial trout chow. Under these conditions the shrimps had an intermoult period between 19-26 days, for an average of 23 days. A detailed moult history was kept for each animal and in most cases experimental manipulations were carried out one or two days following ecdysis.

All animals were allowed to moult twice before being selected for study in order to ensure that the claws were fully differentiated (Read and Govind, 1991). Several types of experimental manipulations were made; the simplest one was to induce the animal to autotomize its claw after ecdysis by gently pinching the limb with forceps just distal to the autotomy plane. More complex

Figure 1. Adult snapping shrimps showing different configurations of their paired claws. (A) Pristine asymmetric configuration in which the snapper (right claw) is extremely hypertrophied with a pronounced hammer and socket and the pincer (left claw) is small, slender and lacks the snapping apparatus. (B) Newly regenerated paired pincer claws which are smaller than their pristine counterparts. (C) Paired snapper claws with newly regenerated snapper (right claw) and dactyl-less transformed pincer (left claw). (D) Newly regenerated paired snapper claws which are much smaller and not as highly differentiated as their pristine counterparts. (E) Pristine paired snapper claws in which the paired claws are similar in size and differentiation. Scale bar 10 mm. X 2.5


A
B
C
D
E

manipulations were done after first anesthetizing the shrimp by cooling, then cutting the dactyl of the snapper or pincer claw close to its attachment to the claw so that most of it was removed or sectioning the nerve in the snapper claw. The latter was accomplished by cutting a small flap of cuticle in the ventral side of the merus and pulling nerve 2 (the larger of two) so that it broke more proximally at the autotomy plane. In this way a substantial length of nerve 2 was removed and the cuticular flap was replaced and the shrimps were treated during the next two days with a wide spectrum antibiotic (Paragon). Limb immobilization was achieved by anesthetizing the shrimp, then applying cyanoacrylate glue to the thoroughly dried converging edges of the propus and dactyl. Experimental manipulations were usually performed 1-2 days after a moult and the experimental shrimps were observed over the next two to three intermoults.

Fibre composition of the claw closer muscle was undertaken by obtaining frozen cross-sections of the claws and staining these histochemically to detect myofibrillar ATPase activity by standard techniques (Ogonowski and Lang, 1979).

RESULTS

A simple and economical scheme for explaining how bilateral asymmetry is maintained in the face of claw loss and regeneration in adult snapping shrimps was that of Wilson's (1903) who regarded the pincer as an arrested snapper. Some of the ramifications associated with this scheme are explored in the experiments described below.

How late in the intermoult can inhibition to the pincer claw be removed?

In most previous studies the snapper is removed a day or two after the shrimp moults providing enough time for transformation and regeneration of claws so that at the next moult both claws appear at an advanced state. Can inhibition be removed later in the intermoult, even perhaps as late as the premoult stage? Snappers were autotomized at various stages of the moult cycle. The

latter was determined by measuring epidermal retraction and setal development in the pleopods (Aiken, 1973); pleopod stages may range from 0 (intermoult) to 5.5 (late premoult). Following ecdysis the contralateral claws were evaluated on their degree of transformation from pincer to snapper (Fig. 2) based on a number of morphological features, including a stoutness ratio (ratio of length to width), the presence and development of the transverse groove, tubercles, plumose setae and plunger and socket; all features unique to the snapper claw. Each transforming pincer claw was given a score out of 10 (pincer-to-snapper transformation index) with 0 being the equivalent of a pristine pincer and 10 a pristine snapper. The results indicated that the effect of a snapper autotomy on the contralateral pincer was not restricted to the intermoult stage or even early premoult stage. When done midway or even fairly late into the moult cycle i.e. as late as pleopod stage 4.0, the pincer in some cases showed clear signs of transformation (Fig. 3). There was a gradation in the effect related to when in premoult the snapper was autotomized and the interval between the snapper autotomy and the ensuing ecdysis.

In addition, for a number of animals (9 in all) the transforming pincer was selected for histochemical analysis, to characterize the closer muscle fibre type. In these shrimp, the snapper had been autotomized at pleopod stages ranging from 0 to 3.5 and the transforming pincers had a transformation index ranging from 2.5 to 6.0. Of the animals analyzed histochemically, all still retained the band of fast muscle, unique to the pincer (Govind et al., 1986), although in most animals it had begun to degenerate (Fig. 4). The main limiting factor seemed to be whether when the snapper was autotomized if there was sufficient time for any observable change to take place before ecdysis occurred. Clearly, snapper-based inhibition is removed almost immediately and is unrelated to the stage of the moult cycle. Moreover, the process is so dynamic, full advantage is taken of whatever time remains in the current moult period to get as well developed and functional a snapper as possible into use. This applies primarily to the external morphological features and in a very positive sense, in that new cuticle is being moulded and supplemented. A reversal of the muscle fibre types begins quite rapidly, but only in a negative sense with the degeneration of fast muscle in the existing pincer.

Figure 2. A pristine pincer claw (A) progressively developing via two selected stages (B, C) into a pristine snapper claw (D) claw which is characterized by a hammer (arrow) and socket (double arrow), a transverse groove and hypertrophy of the entire claw. The slender pristine pincer claw (A) acquires all the snapper features after the first moult (B), and in subsequent moults (C, D) becomes hypertrophied with further accentuation of the snapper features. Scale bar 3 mm. x6

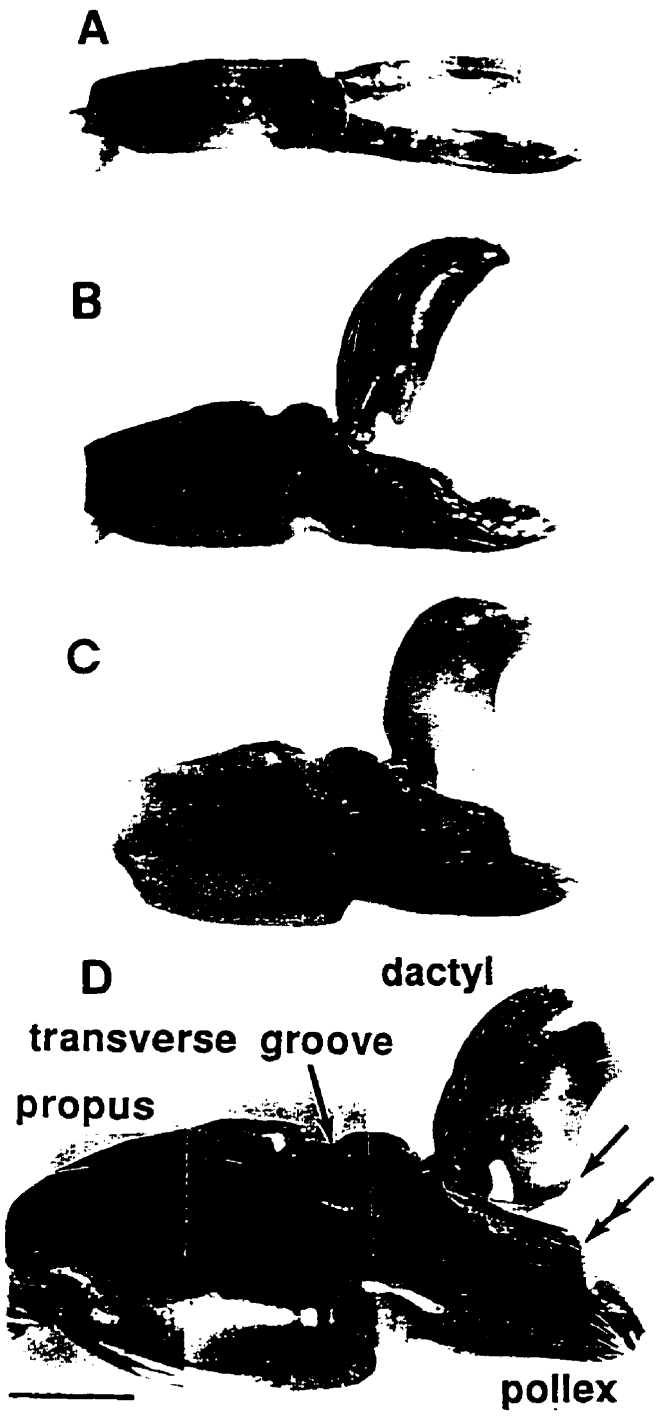

A
B
C
D
dactyl
transverse groove
propus
pollex

Figure 3. Relationship between pleopod stage in premoult shrimps subjected to snapper autotomy and pincer-to-snapper transformation index defined by 0 as pristine pincer and 10 as pristine snapper. The degree to which the pincer transforms is linearly related ($y=-1.162x + 6.179$) to the time when the snapper is removed in premoult shrimps. The transformation index was negatively correlated to the pleopod stage ($r^2=0.577$, $p=0.0001$). $n=37$.

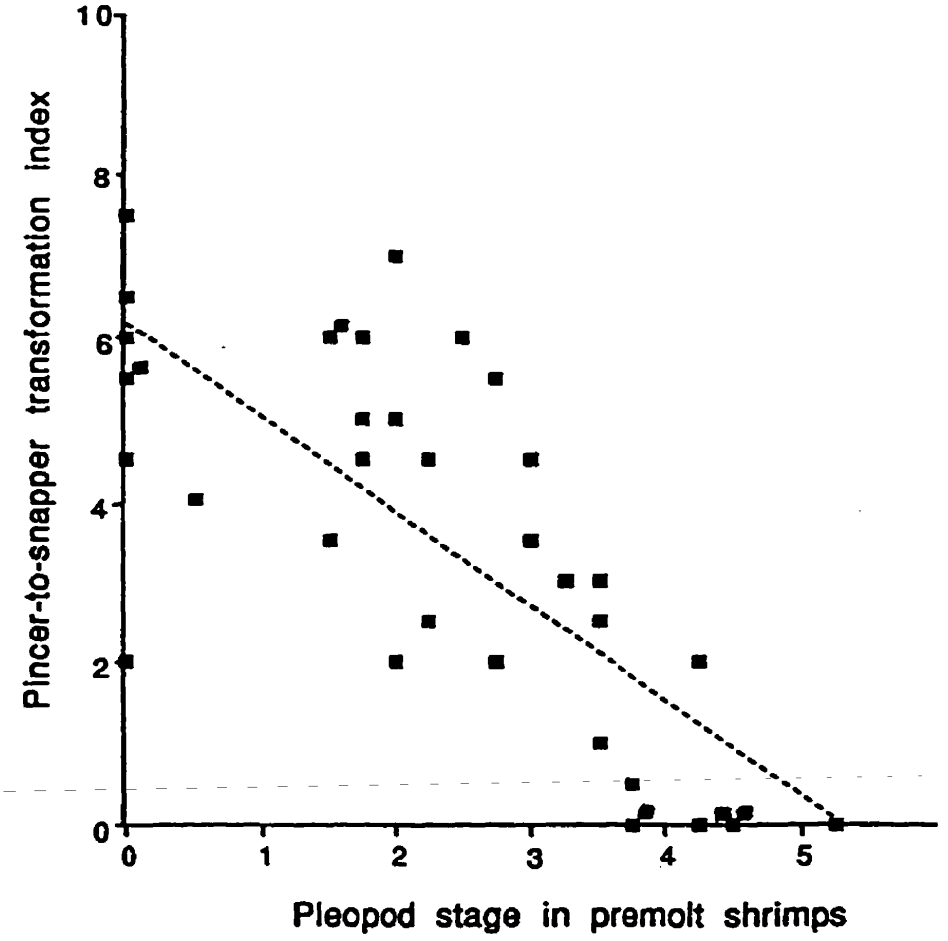
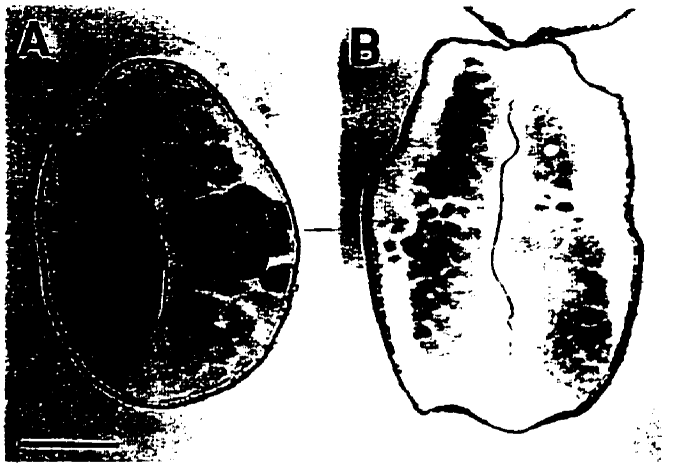

Pincer-to-snapper transformation index
10
8
6
4
2
0
0
1
2
3
4
5
Pleopod stage in premolt shrimps

Figure 4. (A) Cross-section of a pristine pincer claw in which the closer muscle, stained histochemically for myofibrillar ATPase, shows a characteristic central band of fast fibres (dark-staining) flanked by slow fibres (light-staining). (B) Cross-section of a pincer claw transforming to a snapper in which the central band of fast fibres (dark staining) have degenerated, while the flanking slow fibres (light staining) are intact. Scale bar 1 mm. x15


A
B

Is an intact transforming claw necessary to limit claw regeneration to a pincer at the contralateral snapper site?

It has been previously shown that denervating the transforming pincer claw will allow regeneration of a pincer or a snapper claw at the contralateral snapper site; the appearance of a snapper claw implying the loss of snapper-based inhibition (Young et al., 1994). To pursue this idea further, I wondered if lesser denervation to the transforming claw, such as that brought about by dactylotomy, might be equally effective. Ten animals were successfully manipulated so that their snapper was autotomized and their pincer dactylotomized. In all cases, the pincer transformed into a snapper, i.e. it became hypertrophied and developed a socket, despite lacking a dactyl (Table 1). In 7 of those 10 shrimp, a snapper regenerated on the contralateral side, resulting in a symmetrical animal, albeit one snapper lacking a dactyl (Fig. 1C). In addition, the regenerated snapper often did not grow to pristine proportions. In the other 3 shrimp a pincer regenerated at the snapper site, resulting in a reversal of asymmetry. Thus pincer dactylotomy, mimicking a mild form of denervation, appeared to be sufficient to remove snapper-based inhibition of the regenerating claw.

Can snapper-based inhibition of a regenerating pincer claw be removed?

Loss of the pincer claw results in the regeneration of another pincer claw presumably because the contralateral snapper restricts regeneration at this site to a pincer (Wilson, 1903). Is it possible to remove this snapper-based inhibition by damaging the snapper when regeneration is taking place at the pincer site? I tested this possibility by removing the pincer claw and at the same time cutting the snapper dactyl - procedures that were successfully accomplished in nine shrimp (Table 1). At the next moult, a normal appearing pincer regenerated at the original pincer site in these shrimps. On the contralateral side the intact snapper showed little regeneration of its cut dactyl for at least two subsequent moults. Snapper dactylotomy did not induce regeneration of a snapper claw at the pincer site.

Table 1. Configuration of paired claws in snapping shrimps following regeneration at one or both sites in response to various manipulations.

	Claw configuration at original			
	snapper site	pincer site	#	%
Pincer dactylotomy with snapper	pincer	snapper	3	30
dactylotomy	snapper	snapper	7	70
Pincer autotomy with snapper dactylotomy	snapper	pincer	9	100
Pincer autotomy with snapper denervation	snapper	pincer	15	100
Snapper autotomy at first moult;	snapper	pincer	33	70
paired autotomy at second moult	pincer	snapper	14	30
Snapper dactylotomy at first moult;	snapper	pincer	8	73
paired autotomy at second moult	snapper	snapper	3	27
Paired autotomy at first moult;	snapper	pincer	46	96
paired autotomy at second moult	pincer	snapper	1	2
	snapper	snapper	1	2
Paired autotomy at first moult; paired	snapper	pincer	22	79
autotomy at second moult and snapper-side	snapper	snapper	6	21
limb bud immobilized at third moult				

In an earlier experiment transecting nerve 2 in the transforming pincer claw permitted regeneration of a snapper claw at the old snapper site, implying the removal of snapper-based inhibition (Young et al., 1994). Therefore, nerve 2 was transected from the autotomy plane to mid-merus in the pristine snapper claw and the pincer claw was autotomized at the same time. The experiment was successfully accomplished in 15 shrimp (Table 1), and none of these animals regenerated a snapper: seven had regenerated a pincer by the end of the first moult cycle, twelve had a pincer by the end of the second moult cycle, and all had regenerated a pincer by the end of the third moult cycle. In 11 of these animals snapper function was restored, but to varying degrees: four could open and close the dactyl but not snap, five could snap weakly and three could snap with moderate force. In most cases, snapper function began returning near the end of the first moult cycle. Thus snapper denervation failed to bring about regeneration of another snapper claw at the pincer site.

Can snapper-side inhibition be weakened to permit regeneration of a snapper at the pincer site?

When both snapper and pincer claws are removed simultaneously regeneration at these sites is of a similar type claw (Przibram, 1901), suggesting that snapper-based inhibition is present even in the absence of the snapper claw. Can this inhibition present on the snapper side be weakened to allow regeneration of a second snapper on the contralateral side? I therefore tried removing both snapper and pincer claws at a time when possibly snapper-based inhibition was not well established. This would be the case immediately after reversal of asymmetry when the newly transformed snapper claw is not as elaborate nor hypertrophied as a pristine snapper, nor is the newly regenerated pincer claw as well differentiated as the pristine pincer (Przibram 1901; Wilson 1903). Forty-seven animals were successfully snapper autotomized and at the next moult, following a reversal of asymmetry, subjected to a paired autotomy. Relative to the most recent configuration, the location of the pincer and snapper was maintained in 33 and reversed in 14 animals (Table 1). Despite reversal of bilateral asymmetry snapper-based inhibition remained intact.

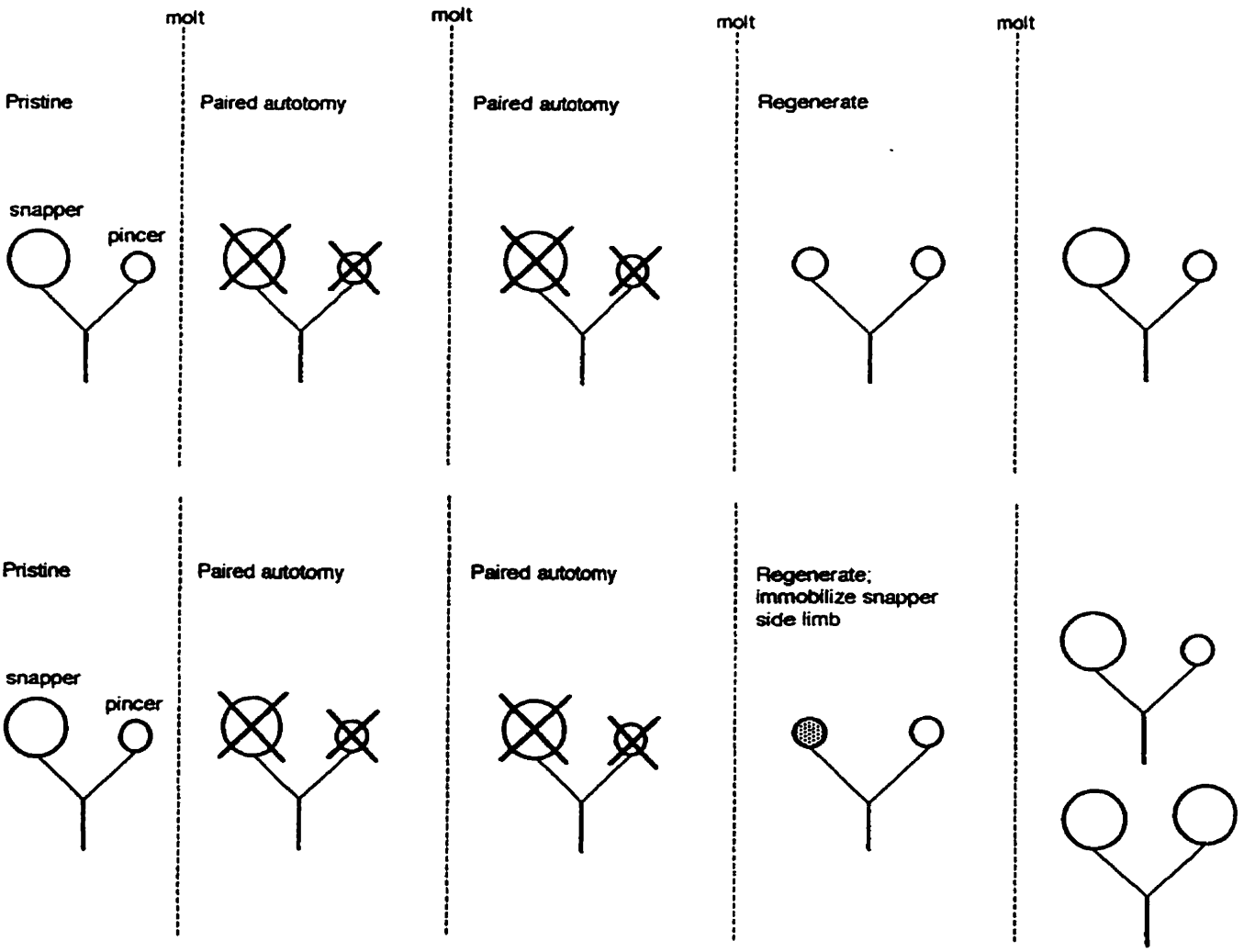
We next tried snapper dactylotomy as a means for inducing pincer transformation and out of 21 animals, 11 showed transformation of the pincer into a snapper while the damaged snapper repaired itself resulting in shrimps with paired snapper claws. Following paired autotomy in these 11 animals, 8 regenerated limbs to mimic the original asymmetric configuration while 3 regenerated double snappers (Table 1). Both the regenerated snapper claws were relatively small in comparison to the animal (Fig. 1D), although the limb on the original snapper side tended to be slightly larger than its counterpart. This was the first demonstration of a snapper claw regenerating at a pincer site with an extant snapper claw on the opposite side.

Can snapper-side inhibition survive successive claw loss?

A newly regenerated limb is usually smaller than its pristine condition signifying an immature state, a state which could probably be exaggerated with a second round of regeneration. Under these conditions does snapper-side inhibition still prevail? A group of 48 shrimp were successfully subjected to two consecutive paired autotomies of their claws and the returning asymmetry observed (Fig. 5) (Table 1). After the paired claws had regenerated for the first time, 75% were asymmetric although they were considerably smaller than the pristine, while the remainder were no more advanced than stage 5 limb buds or stage 6 pincers (Govind and Read, 1994). After they had regenerated for the second time they were even smaller with only 34% showing slight asymmetry into snapper/pincer, while 66% resembled stage 5 limb buds or stage 6 pincers (Fig. 1B). Over the next two moults, however, these pincer-symmetric shrimps reverted to the original snapper/pincer configuration. Forty six of 48 animals had their limbs regenerate to the original configuration, one experienced a reversal, and one regenerated paired snappers. These results show that snapper-based inhibition usually persisted with at least two successive paired autotomies, although occasionally it could be reversed or even absent.

As shown in the above experiment, after two successive paired autotomies the newly regenerated claws were small and the snapper was not highly differentiated, occasionally showing only the faintest trace of a snapper such as a poorly developed hammer; other than that the snapper

Figure 5. Pictorial representation of the paired asymmetric snapper (large circle) and pincer (small circle) claws of adult shrimps in two experiments. In the upper series the shrimps underwent two successive paired autotomies, regenerated paired pincer-like claws after the third moult and these claws differentiated into snapper/pincer claws in the pristine configuration after the fourth moult. This same experiment was repeated in the lower series but after the third moult the snapper-side limb was immobilized (shaded) and at the fourth moult paired claws appeared in the pristine snapper/pincer configuration but also in a snapper/snapper configuration.


molt
molt
molt
molt
Pristine
Paired autotomy
Paired autotomy
Regenerate
snapper
pincer
Pristine
Paired autotomy
Paired autotomy
Regenerate;
immobilize snapper
side limb
snapper
pincer

was more pincer-like in overall dimension. To test the possibility that even in this relatively immature condition manipulation of the snapper-side limb could reverse claw asymmetry, I performed the following experiment. A group of 28 shrimp were subjected to two consecutive paired autotomies, then the snapper-side limb bud was glued shut, effectively restricting the activity of this regenerating limb (Fig. 5) (Table 1). The majority (22) of these animals regenerated claws resembling the original configuration. However, six animals regenerated paired snappers showing that the limb bud at the pincer site was capable of developing into a snapper. When compared to the previous experiment in which shrimps were subjected to two successive paired autotomies with no limb immobilization, the number of snapper symmetric shrimps in the present experiment proved to be significant ($X^2 = 5.612$, $p < 0.02$).

Can snapper-symmetry be maintained following claw loss?

The generation of adult shrimps with paired snapper claws by regeneration of a second snapper raises questions about the stability of this unusual condition. These snapper-symmetric shrimps retained their symmetry following a subsequent moult at which time the second snapper assumed more pristine proportions (Fig. 1E). Symmetry was retained in a few shrimps which underwent three subsequent moults, showing that once snapper-symmetry is established it is retained through later moults. On the other hand, if the second snapper is removed as was done in three shrimps after they had moulted once, the shrimps regenerated a pincer in its place. Even if the second snapper was removed after three moults as was done in two shrimps, a pincer regenerated in its place. Finally, in three shrimps with similar-sized paired snapper claws, removal of both claws resulted in the regeneration of paired asymmetric claws in the original configuration. Clearly, the snapper-symmetric condition is relatively stable but not permanent as loss of one or both claws allows regeneration of asymmetric claws.

DISCUSSION

Our experiments gave paired regenerated claws in the usual asymmetric configuration of pincer /snapper as well as, in a few cases, in the unusual symmetric configurations of pincer/pincer or snapper/snapper. The pincer-symmetric condition appears to be ephemeral, because in succeeding moults, given adequate time for development, one of the claws becomes a snapper. In contrast, the snapper-symmetric condition once established can be maintained over several moults assuming a relatively permanent state. Indeed, snapper symmetric shrimps have been maintained for five moults providing there is no loss or damage to the claws (Pearce and Govind, 1987). Loss of one or both claws in these snapper-symmetric shrimps immediately restores the asymmetric configuration of the paired claws upon regeneration. These observations with symmetric-clawed shrimps viz. that the pincer-symmetric condition is ephemeral and the snapper-symmetric condition is more stable, tend to support the view that the pincer represents a stage in the development of the snapper with the final condition of claw regeneration being that of a snapper (Wilson, 1903, Darby, 1934).

With this developmental sequence in mind, lateralization of the paired claws may be easily achieved if the snapper claw or its putative site arrested the development of the contralateral claw to a pincer state. Although the nature of the inhibitory mechanism is not known there is considerable evidence to suggest that it has a neural basis and that it is removed most readily with loss of the entire claw but also with nerve transection (Mellon and Stephens, 1978), closer muscle tenotomy (Govind et al., 1988) or dactylotomy (Read and Govind, 1997), in other words, with some damage to the nervous system. One of the findings from the present study is the speed with which the pincer continues its development to a snapper once the inhibition is removed. Even late in the intermoult, as late as the premoult stage when the new exoskeleton is being laid down, loss of the snapper permits the pincer to immediately continue its development to a snapper. Indeed, one of the first changes is in the exoskeleton (present report) with changes in muscle composition and motor innervation (Stephens and Mellon, 1979; Quigley and Mellon, 1984; Mellon et al., 1981),

sensory innervation (Govind and Pearce, 1988), and vascularization (Guchardi and Govind, 1990) occurring later .

The next step in the lateralizing mechanism is for the claw advancing from the pincer to the snapper stage to exert an inhibitory influence on the regenerating contralateral claw and hold its development to the pincer stage. Elimination of the inhibitory influence should allow the regenerating claw to develop to a snapper and this was the case in shrimps in which removing the closer muscle in the transforming claw or transecting its nerve 2, allowed regeneration of a snapper claw on the opposite side (Young et al., 1994). I now report that simply cutting the dactyl of the transforming claw is sufficient to eliminate its inhibitory influence and permit regeneration of a snapper claw resulting in shrimps with paired snapper claws. The first snapper arises because the pincer, released from its inhibition by snapper autotomy, continues its development to a snapper. The second snapper arises because the newly regenerating claw is not restricted to a pincer stage because of dactylotomy of the transforming snapper.

Under this scheme the pincer would also advance to the snapper stage if the snapper-based inhibition was removed but without loss of the snapper claw. This happens readily when neural input is reduced in the snapper claw and the existing pincer continues its development to a snapper, resulting in shrimps with paired snapper claws (Mellon and Stephens, 1978; Govind et al., 1988). Along similar lines is our earlier finding that cutting nerve 2 of the transforming claw (Young et al., 1994) or our present finding that cutting off the dactyl of the transforming claw allows the regeneration of another snapper claw on the opposite side. But when these same surgeries were performed on a pristine snapper claw and at the same time the pincer claw was autotomized, another pincer regenerated in its place. In this case the presence of a pristine snapper although damaged was sufficient to inhibit regeneration to a pincer stage, but not when the damage was to a transforming snapper. Bearing in mind that the snapper claw has almost twice as many axons as its pincer counterpart in the pristine condition (Govind and Pearce, 1988), it is likely that dactylotomy of a transforming claw results in a much greater reduction of axons compared to the opposite side

than does dactylotomy of the pristine snapper claw. Moreover, because dactylotomy would affect similar structures in the transforming or pristine snapper claw it diminishes the possibility that qualitative aspects of the innervation are responsible for the different outcomes and points more to quantitative aspects. Limb regeneration in amphibians is dependent on a minimal amount of nerve in the blastema irrespective of the qualitative composition of the nerve, whether sensory or motor (Singer, 1978).

Quantitative differences in innervation between the two sides in adult snapping shrimps may also help explain the results of our experiments with paired autotomies; whether the paired autotomies were performed following reversal of asymmetry or in quick succession, the paired claws regenerated in an asymmetric configuration. The greater neural innervation to the snapper side would serve to inhibit the contralateral side even in the absence of the claws. The paired autotomy experiments point to the fact that claw lateralization in adult snapping shrimps appears to have a central locus, similar to that in juvenile lobsters where differential reflex activity from the paired claws lateralizes the ganglion into major and minor sides during a critical developmental period (Govind and Pearce, 1986). Once laterality is established in juvenile lobsters it remains fixed for its entire life, and claw loss results in the regeneration of a similar claw type. Conversely, in snapping shrimps claw laterality is not fixed and can be constantly reversed. The present experiments reveal a very strong intrinsic lateralizing mechanism in the form of snapper-based inhibition that resides centrally because it operates in the absence of the claws. The direction of the laterality can be readily changed via input from the claws in the form of the removal of the snapper-based inhibition of the contralateral pincer claw. A useful analogy for the lateralizing mechanism is that of a see-saw in which the beam, balanced on its fulcrum, can assume one of two inclined positions but rarely a horizontal position.

GENERAL DISCUSSION

Have I resolved the question of how claw asymmetry is maintained and reversed in adult snapping shrimp following loss of one or both claws? Recall the general hypothesis developed to explain the phenomenon of asymmetry reversal and maintenance: the relationship between the snapper and pincer is envisioned as a see-saw with, in a pristine animal, the snapper side depressed and maintaining an inhibitory effect on the elevated contralateral side, limiting it to a pincer. Loss of the snapper causes a sudden shift in the see-saw's equilibrium, now favouring the pincer side which released from the inhibitory effect of the snapper, itself begins transforming into a snapper. Meanwhile the transforming pincer becomes the new source of the inhibitory signal, limiting the contralateral regenerating limb to a pincer resulting in a reversal of asymmetry. According to the cross inhibitory hypothesis it follows that simultaneous loss of both claws has no important effect on the see-saw's equilibrium. As the limbs regenerate, the snapper side stays depressed, inhibiting the contralateral side, keeping it elevated and limiting it to a pincer as the original snapper side becomes a snapper. My experiments were thus designed with two primary goals in mind: 1) to test the see-saw and associated cross-inhibitory hypothesis and 2) to isolate the cue(s) responsible for maintaining the equilibrium of the see-saw and hence the maintenance or reversal of claw asymmetry.

I began by reviewing some of the older experiments related to the problem, repeating and subjecting them to more detailed scrutiny and following up on some of the more intriguing observations. In particular, I was interested by the studies of Darby (1934) who found that a reversal of asymmetry could be induced if the pincer was removed, waiting a specific period of time, then removing the snapper. I repeated this experiment but instead correlated removal of the snapper to specific limb bud stages on the pincer side finding that a reversal of asymmetry occurred with a stage 3 or higher limb bud. This led to the idea that perhaps the snapper side limb bud grows faster and achieves stage 3 more quickly at which point it begins inhibiting the contralateral side limiting it to a pincer. That was dispelled however by an experiment in which I observed that,

following a paired autotomy, both the snapper and pincer limb buds regenerate at the same rate, leaving the snapper regenerate with no advantage, at least over the first intermoult. Nevertheless, returning to our cross-inhibitory hypothesis, it appeared that once a regenerating limb bud reaches stage 3, it acquires the snapper-like capability of repressing the contralateral side, limiting it to a pincer.

Was there some subtle morphological feature of stage 3 limb buds making them competent, able to repress the contralateral regenerating limb? I investigated this question by subjecting the different limb bud stages to ultrastructural analysis, focusing in particular on the critical stage 3. Stage 3 is correlated by definition, with the beginning of distal segmentation, an observation confirmed at the ultrastructural level by the presence of opposing groups of proliferating cells, presumably responsible for the process. In addition, at stage 3, limb bud cells appear more differentiated and this stage just precedes the emergence of innervation, an important point to which I will return. Altogether, these factors may engender stage 3 limb buds with the competence if not the appearance of a mature claw.

What factor is responsible for setting up the see-saw's equilibrium, that is, what is it that causes one side of the see-saw to become depressed, develop into a snapper and begin sending inhibitory signals to repress the contralateral side? As mentioned, the first recognizable tissue to arise in a regenerating limb bud (aside from the cuticular epithelium) is the innervation. This observation, along with experiments in which the pincer was induced to transform into a snapper as a consequence of nerve-damaging manipulations performed on the snapper (Mellon and Stephens 1978; Govind et al., 1988; Read and Govind, 1997) led to the hypothesis that asymmetry is ultimately determined by quantitative differences in innervation. Indeed this is consistent with data showing that the snapper claw is innervated by many more axons than the pincer (Govind and Pearce, 1988), implying that this excess of axons is what sets up the inhibitory mechanism and keeps the see-saw depressed on the snapper side. From this it is obvious that loss of the snapper claw would cause a sudden shift in innervation (due to sensory axon degeneration), favouring the

pincer side and inducing it to transform into a snapper, a rationale borne out by quantitative axon studies (Govind and Pearce, 1988). But can differences in innervation and the see-saw model explain the results of paired autotomies, where the snapper and pincer always regenerate back to the original location (asymmetry maintained)? To answer this question I compared the innervation of regenerating snapper and pincer sides in a series of shrimp which had been subjected to paired autotomies. As it turned out, the number of axons remained higher on the snapper side throughout the period of regeneration, which was consistent with the see-saw hypothesis and the importance attributed to the relative innervation of the claws.

In considering the pronounced sexual dimorphism seen in the claws of snapping shrimp I realized that this might provide an alternate means of testing the cross-inhibitory hypothesis and innervation as a factor. The pincer of males is a much more elaborate structure than in females, being more hypertrophied and possessing a conspicuous ridge and fringe of setae on the dactyl and propus. I hypothesized that this dimorphism may be reflected in the innervation to the claws and if so, ablation of precise amounts of nerve on the snapper side may induce differential effects on males and females in terms of pincer-to-snapper transformation. The manipulation chosen was dactylotomy of the snapper since precise and significant amounts of nerve could be removed and I knew from previous trials that the procedure induced pincer-to-snapper transformation in some but, more importantly, not all animals. In fact a re-analysis of my data, along with additional trials proved that males were much more sensitive to dactylotomy of the snapper than females.

My explanation for the above result and how it fits in with the see-saw model is as follows: in females, the difference in the innervation between the pincer and snapper sides may be so large that the loss of innervation that would occur with a snapper dactylotomy may not be sufficient to cause a shift in innervation favouring the pincer side, thus maintaining the status quo. In males however, the difference in the innervation to the pincer and snapper may not be as large as occurs in females, a reasonable expectation since the pincer of males is more snapper-like than is the pincer of females. Thus loss of some innervation to the snapper of males due to a dactylotomy may

indeed cause a shift in innervation, now favouring the pincer side (similar to what occurs in a snapper autotomy), inducing it to begin developing into a snapper. I tested this hypothesis, again using numbers of axons as a means of quantifying innervation. As my results showed in both males and females there were relatively more axons innervating the snapper than the pincer. However, in males the difference was not as large thus loss of relatively similar numbers of axons in the snapper of both sexes could have quite different effects in terms of the distribution of innervation. And indeed, I found this to be the case after analyzing and comparing the claw innervation of dactylotomized males and females. In females, the sex in which snapper dactylotomy was much less likely to induce a pincer-to-snapper transformation, axon numbers still favoured the snapper side. In the male pincer, on the other hand, which likely would have gone on to transform into a snapper, there was an excess of axons compared to the snapper. In summary, these results lend support for the see-saw model in suggesting that the dominant snapper claw may be more likely to develop on the side with the greater quantity of innervation and further that the quantity of innervation may be an important factor in determining asymmetry in these animals.

In the final phase of the project a number of experimental manipulations were carried out to test the actual inhibitory hypothesis itself. To reiterate, in a shrimp with pristine claws the snapper inhibits the contralateral side, limiting it to a pincer. Loss of the snapper however removes that inhibition, allowing the pincer to begin transforming into a snapper which at the same time becomes the new source of inhibition, limiting the regenerating limb at the old snapper site to a pincer. Inhibition, likely using innervation as its basis, must also operate in the absence of claws in order to explain how a paired autotomy results in the regeneration of claws to their original configuration. Key to the inhibitory hypothesis is the notion, initially stated by Wilson (1903) that the pincer claw is merely an undeveloped snapper, inhibited from further differentiation by the presence of the contralateral snapper. This was based on the casual observation that a regenerating snapper claw seemed to pass through a pincer-like phase. I confirmed this rigorously showing that in the case of males, the regenerating snapper even acquires, then loses the distinctive setal fringe. However, I felt that this contention needed further corroboration and decided to investigate another

feature, namely the fast band of muscle unique to the pincer closer muscle. Using histochemical techniques, I found that as with the setal fringe, a regenerating snapper claw first acquires then loses the band of fast muscle in its closer muscle, providing additional support for the idea that the pincer is an undeveloped snapper. Also, in a number of cases my experiments gave unusual pincer symmetric and snapper symmetric configurations, however whereas the latter condition was quite stable, being maintained over many moult periods the former state was ephemeral, never lasting for more than one moult cycle, supporting the view that the pincer represents an earlier developmental stage of the snapper and in turn further buttressing our inhibitory hypothesis.

Interestingly, damaging a snapper by dactylotomy or denervation often allowed the pincer to transform into a snapper but these same manipulations did not allow a regenerating limb to become a snapper. This implies that less inhibition is necessary to limit a newly regenerating claw to a pincer than is required to arrest an existing pincer claw, a reasonable expectation since a pincer claw is not only much larger but developmentally much closer to being a snapper and so would require more repression than a newly regenerating claw. Along these same lines was the finding that damaging a transforming pincer allowed a snapper to regenerate on the contralateral side as opposed to the usual pincer. A transforming pincer might be expected to produce a relatively small amount of inhibition and normally as indicated above, that would be enough to limit a regenerating limb to a pincer, however damage to the transforming pincer removes even that minuscule degree of inhibition thus allowing the limb bud to develop into a snapper.

Immobilizing the snapper side limb bud following paired autotomies in some cases resulted in the pincer side limb bud developing into a snapper, a result also reconcilable with the inhibition hypothesis. Overt observational and axon count studies suggests that in animals subjected to multiple paired autotomies, the developmental program of both limbs is severely retarded and in particular the snapper side regresses to the point where its advantage over the pincer side is minimal and presumably so also its inhibitory influence, perhaps not unlike the situation during ontogeny when asymmetry is initially established. Under these conditions immobilization of its

dactyl (which may silence sensory axons effectively further reducing quantity of innervation) may remove any remaining inhibitory influence allowing the contralateral side which may incidentally, compensate by becoming more active, to also become a snapper.

From these experiments and observations and those of others a mechanism governing the determination of asymmetry in snapping shrimp can be conceived, a scenario which dictates both the initial asymmetric configuration in juveniles and that in adults. As a juvenile, the claws are symmetrical and pincer-like and presumably their innervation is also balanced (Young et al., 1994). Both claws manipulate objects on the ocean floor but at some point, for some reason there occurs a bias in limb activity favouring one side, similar to that in juvenile lobsters (Govind, 1992). At this stage growth and development is exceedingly rapid and the limbs very plastic (J. Pearce, personal communication) and likely exquisitely sensitive to sensory feedback. As the more active limb begins to hypertrophy, relatively more axons are added to it perhaps further increasing its activity in a cycle of positive feedback whereupon it starts inhibiting the contralateral side slowing down its maturation and ultimately halting its development beyond the pincer stage. Thus the initial see-saw and inhibitory paradigm is set up. This is similar to the mechanism envisioned for asymmetry determination in lobsters except that in shrimp, unlike lobsters beyond the sixth juvenile stage, asymmetry never becomes fixed and permanent and in this sense the neotenous shrimp are like lobsters that never grow up.

In adult shrimp many more axons innervate the snapper side keeping that end of the see-saw lowered. The important point here is that it is the excess number of axons, not the presence of the snapper per se which is responsible for the inhibitory influence that represses the contralateral side. Loss of the snapper causes a shift in neural balance now favouring the pincer side, which, released from the inhibition begins transforming into a snapper. Meanwhile the transforming pincer becomes the new source of inhibition, limiting the newly regenerating limb to a pincer and asymmetry is reversed. Loss of the pincer has no effect on the see-saw since the number of axons obviously remains higher on the snapper side, inhibition is preserved and a new pincer regenerates

maintaining the status quo. Loss of both limbs, though resulting in the degeneration of axons on both sides, again has no effect on overall equilibrium of the see-saw so that the snapper side, by virtue of its relatively greater number of axons (despite lacking a snapper claw) still maintains its inhibitory effect on the contralateral pincer side. Both the snapper and pincer claws regenerate to their initial location and the original asymmetrical configuration is maintained.

SUMMARY

One of the interesting aspects of adult snapping shrimps *Alpheus heterochelis* is the striking bilateral asymmetry of the first pair of thoracic chelipeds or claws; a much hypertrophied major or snapper claw which makes a loud popping sound when in it closes, and a much smaller minor or pincer claw (Prizibram 1901). A fascinating aspect of this claw bilateral asymmetry is that it can be readily reversed, thus loss of the snapper claw will result in the existing pincer claw transforming into a snapper at the next molt while a new pincer claw regenerates at the old snapper site (Przibram, 1901; Wilson, 1903). I have investigated factors which trigger the reversal of claw asymmetry as well as those maintaining bilateral asymmetry in adult male and female snapping shrimps. A brief summary of these specific investigations are presented below.

Claw regeneration in snapping shrimp was characterized into six distinct stages beginning with a small papilla (stage 1) which elongates (stage 2) and becomes distally segmented with a longitudinal furrow (stage 3). Transverse segmentations appear proximally (stage 4) followed by the acquisition of pincer-like proportions (stage 5) and finally differentiation of the snapper (stage 6). Following a paired autotomy, both the pincer and snapper limb buds regenerate in a qualitatively and quantitatively similar manner, always in their original configuration. A sequential autotomy in which the snapper is autotomized first then later the pincer correlated to different stages on the old snapper site results in the claws regenerating to their original configuration, similar to a paired autotomy. Removal of the pincer first followed by the snapper correlated to a limb bud stage 3 or greater at the old pincer site results in a reversal of asymmetry implying that a stage 3 limb bud is not only capable of being transformed into a snapper but is capable of repressing the contralateral regenerating limb.

The ultrastructure of the regenerating claws was correlated to different limb bud stages. Immediately after autotomy, the wound is covered by an epithelial lined membrane towards which free cells migrate. In stage 1-2 the blastema, now lined by a multilayered, relatively undifferentiated epithelial layer, erupts from the stump and becomes invaded by free cells including

haemocytes, fibroblasts and blastocytes. In stage 3 proliferating groups of cells subdivide the distal region into the presumptive propus and dactyl as the epithelial cells become more differentiated. Stage 4 is marked by the first appearance of afferent innervation which seems to develop in a distal to proximal manner within the epithelium and multinucleate myoblasts possessing fragments of myofibrils within the cytoplasm. Other cell types were also observed at this stage and intercellular contacts between haemocyte and other cell types become very prominent. During stage 5 myofibrils and associated nerve terminals make their first appearance.

Sexual dimorphism of the pincer claws is evident in several ways. Most obvious is the prominent ridge and fringe of setae on the propus and dactyl of males. In addition the male pincer is stouter and more snapper-like than is the female's. More subtly, the innervation differs in that the male pincer has relatively more axons and more importantly the difference in the quantity of innervation between the snapper and pincer is greater in females compared to males. Multiple paired autotomies slows down the developmental program of the regenerating limb, clearly demonstrating that the regenerating snapper claw passes through distinct pincer-like stages in terms of overt appearance (acquiring, then losing the ridge and setal fringe) and closer muscle fiber composition (acquiring then losing the band of fast muscle). Innervation however remains higher on the snapper side throughout the regeneration period. Dactylotomy of the snapper may induce a pincer-to-snapper transformation with males being significantly more sensitive to the procedure than were females, suggesting that a functional snapper is more important to males than females. Behaviourialy, this was found to be correlated with a heightened defensive role for males compared with females. In addition the increased propensity of male pincers to transform was correlated with axon count studies showing that in snapper-dactylotomized females, the snapper side retains a greater number of axons whereas in snapper-dactylotomized males the balance of innervation reverses in favour of the pincer. These results support the see-saw hypothesis and the theory that relative quantity of innervation is the asymmetry determining factor.

Pincer-to-snapper transformation, a process that occurs gradually, may be induced at any

time during the moult cycle, even well into pre-ecdysis, implying that snapper-based inhibition may also be removed at any time. Dactylotomy of the transforming pincer in most cases results in the regeneration of a snapper, suggesting that this manipulation removes the inhibition on the regenerating claw. Neither snapper dactylotomy nor denervation results in the regeneration of a snapper claw. Since these same operations may induce a pincer-to-snapper transformation the implication is that a regenerating claw requires less inhibition to be limited to a pincer. A paired autotomy preceded by a reversal of asymmetry induced by a snapper autotomy always results in bilaterally asymmetric claws albeit not always the same as in the original configuration. A paired autotomy preceded by a snapper-dactylotomy may result in the regeneration of symmetrical snapper claws. Thus the inhibition mechanism may be weakened by snapper dactylotomy but not snapper autotomy. Animals subjected to multiple paired autotomies nearly always regenerate asymmetric claws however in similarly treated animals in which the snapper-side claw was glued shut, symmetrical snappers sometimes result. This suggests that multiple paired autotomies will not erase the inhibition mechanism but may weaken it to the point where relatively minor procedures may override it. Cases of symmetrical snappers were observed to be relatively permanent whereas symmetrical pincer were not, supporting the view that the pincer is an undeveloped snapper and further bolstering the inhibition hypothesis.

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IMAGE EVALUATION
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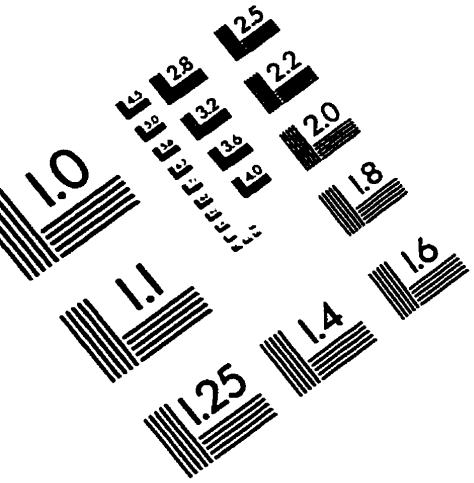

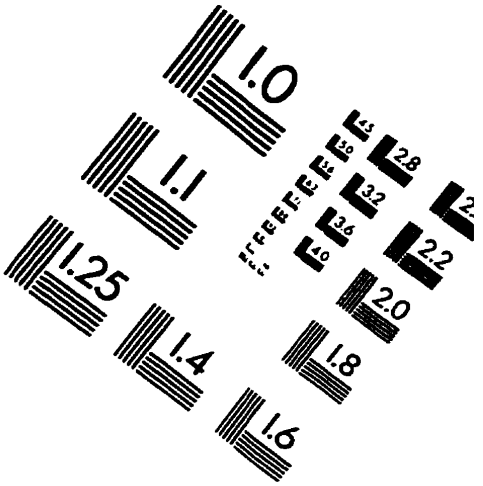

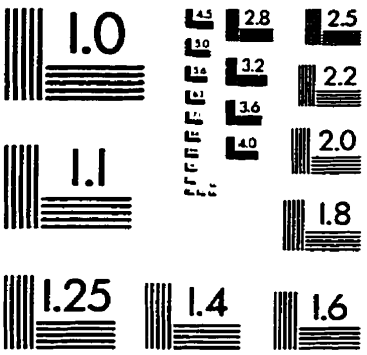

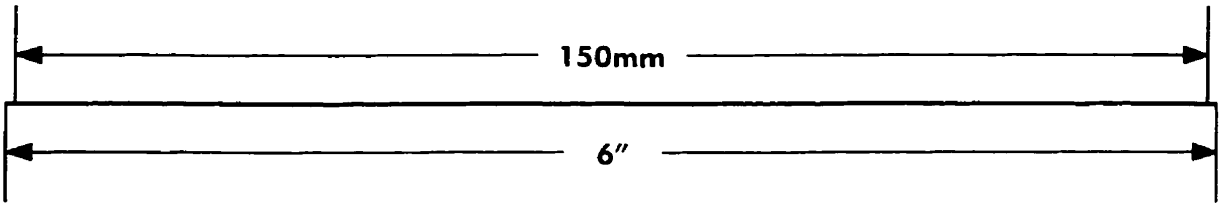

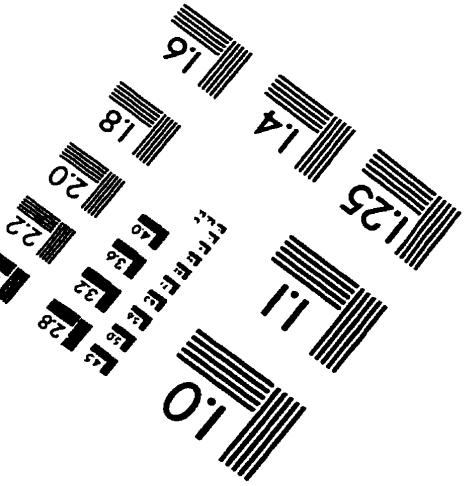

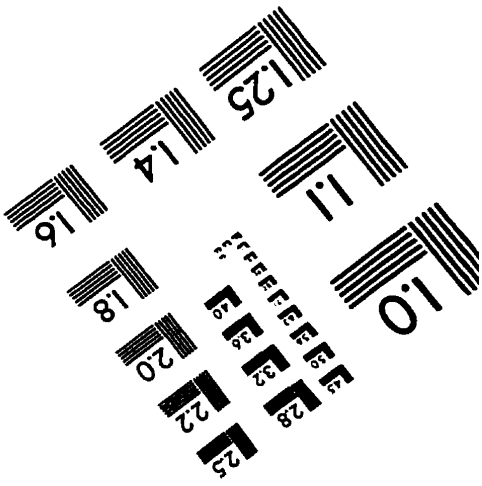

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